Chapter - 2

Theoretical Background

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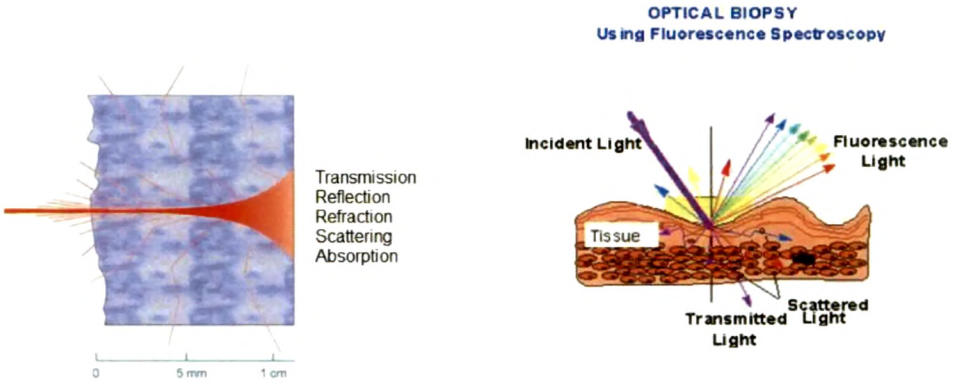
From the last several years, fluorescence spectroscopy has become a sensitive optical tool and has emerged as an important optical diagnostic technique for early and non-invasive diagnosis of diseases. In optical diagnostics, one of the primary goals is to obtain information about tissue morphology and bio-chemical composition, through the measurement of both reflected (1; 2) and fluorescence light (3). The ability to infer cellular information from these indirect measurements relies on an understanding of the interaction of light with tissue structures on the cellular and sub-cellular levels. Due to the complicated physical composition of cells, it has been difficult to obtain such an understanding, in a comprehensive manner, for which a detailed knowledge of tissue optics is essential.

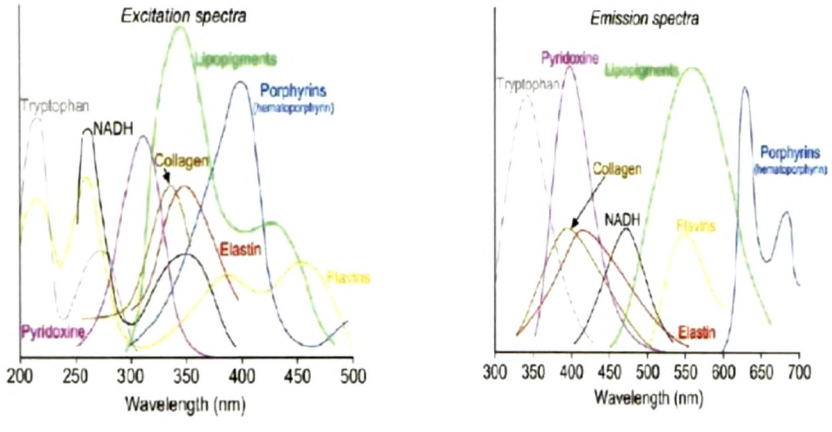
2.1 Tissue optics

Tissue optics deals with light tissue interaction, in the course of light propagation in the tissue medium. Light interacting with tissue can be transmitted, reflected, refracted, scattered and/or absorbed. Absorbed light can cause heat, chemical/conformational change, fluorescence, phosphorescence etc. (Fig.2.1). In complex materials, any combination of interactions are possible. The exact nature of each process depends on the physical and chemical structure of the biomaterial. Cells contain molecules, which become fluorescent, when excited by UV/Vis radiation of suitable wavelength. This fluorescence emission, arising from endogenous fluorophores, is an intrinsic property of cells and is called auto-fluorescence.



(a) (b)

Figure 2.1: (a)Interaction of light with tissues and (b) Tissue Simplistic view: Highly scattering and absorbing medium



(a) (b)

Figure 2.2: Fluorescence Spectra of Endogenous Tissue Fluorophores

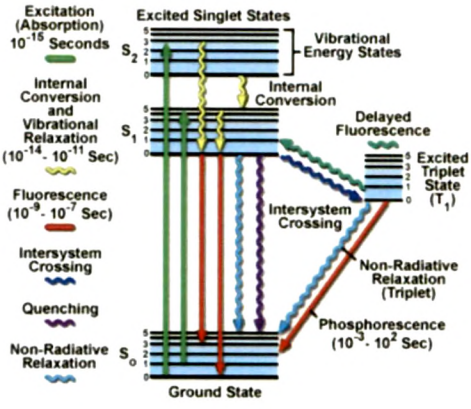


Figure 2.3: Jablonski Energy diagram

Obtained intensities depend on optical properties of tissue, such as reflectivity, scattering coefficient, particle size, optical homogeneity, absorption coefficient etc. A number of fluorophores, ranging from structural proteins to various enzymes and coenzymes, some of which participate in the cellular oxidation-reduction processes, are present in the human tissue which can reflect the bio-chemical and morphological changes taking place in cancerous and other diseased tissues (5; 6; 7; 8; 9). In case of cancer, the primary focus of study here, the endogenous fluorophores of interest are, pyridinic (NADPH), tryptophan, collagen, FAD (Flavin Adenine Dinucleotide), its derivatives, vitamins, lipids and porphyrins . These are particularly useful as fluorescent markers, since they fluoresce in the higher wavelength visible region, when excited by lower wavelength visible light, thereby avoiding the potentially harmful ultraviolet radiation. Changes occurring in the cell and tissue state during physiological and/or pathological processes result in modifications of the amount and distribution of endogenous fluorophores and chemical-physical properties of their microenvironment. Therefore, analytical techniques based on auto-fluorescence monitoring can be utilized in order to obtain information about morphological and physiological state of cells and tissues. Moreover, auto-fluorescence analysis can be performed in real time because it does not require any treatment of fixing or staining of the specimens. In the past few years spectroscopic and imaging techniques have been developed for many different applications both in basic research and diagnostics.(4) Table:1

and Fig.2.2 show the excitation and emission maxima of endogenous fluorophores in human biological tissue. Fluorescence activity can be schematically illustrated with the classical Jablonski diagram (Fig.2.3).

Jablonski Energy diagram

Jablonski energy diagram describes the absorption and emission of light. Figure 2.3 illustrates singlet ground electronic state S_0, singlet first S_1, singlet second S_2 and triplet first T_1 electronic excited states. At each energy level, fluorophores can exist in a number of vibrational energy levels, which are represented by the multiple lines in each electronic state. Once a fluorophore has absorbed energy (green line in the diagram) in the form of electromagnetic radiation, there are number of routes of emissions by which it can return to ground state.

If the photon emission occurs between states of the same spin state (e.g. $S_1 \rightarrow S_0$) this is termed fluorescence (red colour in the diagram). If the spin state of the initial and final energy levels are different (e.g. $T_1 \rightarrow S_0$), the emission is called phosphorescence. Three nonradiative deactivation processes are also significant here: internal conversion (IC), intersystem crossing (ISC) and vibrational relaxation (VR). Internal conversion is the radiationless transition between energy states of the same spin state (compare with fluorescence-a radiative process). Intersystem crossing is a radiationless transition between different spin states (compare to phosphorescence). Vibrational relaxation, the most common of the three, for most molecules, occurs very quickly ($< 1 \times 10^{-12} seconds$) and is enhanced by physical contact of an excited molecule with other particles with which energy, in the form of vibrations and rotations, can be transferred through collisions.

The differences in the concentrations, quantum efficiency or in the binding sites and environment of the endogenous fluorophores of diseased and normal tissues get reflected in their fluorescence properties giving rise to a contrast in fluorescence between the normal and the diseased tissue sites. Therefore several studies have exploited the observed difference in intensity or line shape of autofluorescence between diseased and normal tissues for the diagnosis of cancer of

Table 1: Excitation and Emission Maxima of Endogenous Fluorophores.

Endogenous fluorophores	Excitation maxima (nm)	Emission maxima (nm)
Amino acids		
Tryptopham	280	350
Tyrosine	275	300
Phenylalamino	260	280
Structural proteins		
Collagen	325, 360	400, 405
Elastin	290, 325	340, 400
Enzymes and coenzymes		
FAD, Flavins	450	535
NADH	290, 351	440, 460
NADPH	336	464
Vitamins		
Vitamin A	327	510
Vitamin K	335	480
Vitamin D	390	480
Vitamin B_6 compounds		
Pyridoxine	332, 340	400
Pyridoxamine	335	400
Pyridoxal	330	385
Pyridoxic acid	315	425
Pyridoxal 5′- phosphate	330	400
Vitamin B_{12}	275	305
Lipids		
Phospholipids	436	540, 560
Lipofuscin	340-395	540, 430-460
Ceriod	340-395	430-460, 540
Porphyrins	400-450	630, 690

various human organs, such as breast, lung, bronchus, cervix, oral cavity, and the gastrointestinal tract. Fluorescence emission can differ significantly in normal and cancerous tissues due to the differences in concentration of absorbers (10; 11) and scatterers, as also the scatterer sizes (12; 13). The absorption in the visible range occurs primarily due to the presence of blood, whose amounts vary in various tissue types (14). The presence of scatterers leads to randomization of light, thereby generating a depolarized component in the fluorescence spectra, indicating that polarized fluorescence spectroscopy can be useful in isolating the characteristic spectral features from the diffuse background. The fact that cancerous tissues are much more inhomogeneous with irregular nuclear size distribution and tighter packing, indicates that the corresponding spectral fluctuations will be more prone to randomization. It should be noted here that multiple scattering effects at the excitation and emission wavelengths is not the sole contributor to depolarization of fluorescence. Even in absence of multiple scattering, fluorescence can be depolarized due to intrinsic causes, such as random orientation of the fluorophore molecules, rotational diffusion of fluorophore, radiationless energy transfer etc. [Lakowicz]. However, it has been shown previously that in a turbid medium like tissue, depolarization of fluorescence due to multiple scattering dominates over the other causes of depolarization of fluorescence (15; 16; 17; 18).

The effect of scattering and absorption makes a tissue optically turbid. The fluorescence spectra from such a turbid medium suffer from distortions due to scatterers and absorbers. Role of absorbers and scatterers can result in severe distortion, not only in the spectral line shape, but also in the intensity information. Fluorescence spectra of many biological chromophores overlap one another (19). Hence, it is very difficult to find out the individual contribution of each fluorophore into the fluorescence spectra. To add to that, there is masking of this information by absorbers and scatterers. Therefore, it is important to extract the intrinsic fluorescence from the measured (bulk) fluorescence, from which we should be able to recover the fluorescence line shape and the concentration of fluorophores present in the tissue.

Intrinsic fluorescence is defined as the fluorescence that is only due to fluorophores without the interference of absorbers and scatterers. It provides direct

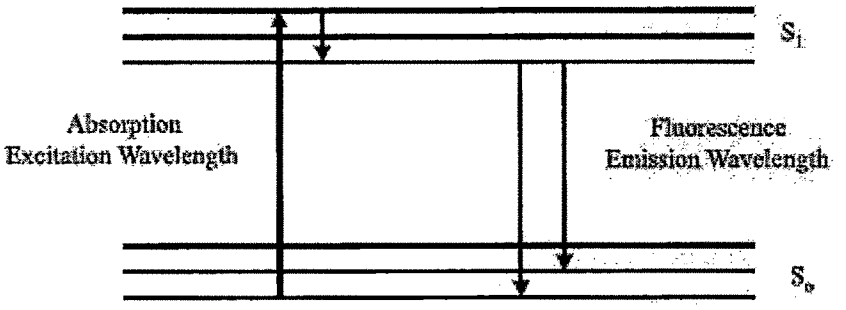


Figure 2.4: Energy level diagram illustrating the phenomena of absorption and fluorescence

biochemical information and the concentration of the tissue fluorophores. Intrinsic fluorescence spectroscopy (IFS) is not only important for diagnosis of tumor but also for surgery.

When a biological molecule is illuminated with specific wavelengths of ultraviolet (UV) or visible (VIS) light (excitation), which lies within the absorption spectrum of that molecule, fluorescent biological molecule (fluorophores) will absorb this energy and be activated from its ground state (state of lowest energy; S_0) to an excited state (state of higher energy; S_1). The molecule can then relax back from the excited state to the ground state by generating energy in the form of fluorescence, at emission wavelengths, which are longer than that of the excitation wavelengths (20). This is illustrated schematically in the Fig.2.4. Furthermore, there are non-fluorescent light absorbers (such as haemoglobin) and scatterers (cell and sub-cellular organelles) in tissues that modulate the tissue fluorescence intensity at the excitation and emission wavelengths.

The phenomenon of fluorescence displays several general characteristics for a particular biologic molecule (20). First, due to losses in energy between absorption and emission, fluorescence occurs at emission wavelengths, which are always red-shifted, relative to the excitation wavelength. Secondly, the emission wavelengths of fluorescence are independent of the excitation wavelength. Third, the fluorescence spectrum of a biological molecule is generally a mirror image of its

absorption spectrum.

2.2 Previous studies on breast tissues

Alfano et al measured the fluorescence spectra of normal and neoplastic breast tissue in vitro from two patients at 488nm and 457.9nm excitation(5). Normal and malignant tissues showed a main peak at about 515nm. The normal tissues showed two additional peaks at 556nm and 592nm. They reported that some normal tissues exhibited Raman peaks at 1037, 1243, 1588, 2761 and 2963 cm^{-1} (5).

Glassmann et al (21) investigated the fluorescence emission spectra at 353nm excitation of normal and malignant breast cell lines and attributed the peak observed at 450nm to reduced pyridine nucleotide fluorescence and the shoulder at 525nm to flavin fluorescence. Lohmann et al (22) found that the fluorescence of breast tissue at 340nm to 380nm excitation was associated with connective tissue fibres in between lactiferous ducts and lobular complexes. They also found that in the malignant tissues, the fluorescence of healthy adjacent connective tissue is even higher than that observed in normal breast.

Majumdar et al (23) studied the UV excited autofluorescence of human breast tissues. They suggest that the concentrations of collagen, NADH and tryptophan are different in normal, benign and cancerous tissues, which are responsible for better discrimination among these tissues, when excited with 337nm wavelength. With 310nm excitation (24), malignant and normal human breast tissues could be distinguished with a sensitivity and specificity value approaching 100%. Here the authors used the spectrally integrated fluorescence intensity as the discriminating parameter. Steady-state measurements on the anisotropy of fluorescence from malignant and normal breast tissues have been reported by Mohanty et al (17). They developed a model to simulate the dependence of anisotropy on the tissue thickness. The optical transport properties of human breast tissues have been studied by several authors (25; 26; 27; 28; 29). These works are based on reflectance measurements and frequency domain photon migration. Detailed investigations have been undertaken by Pradhan and co workers to identify cut-off

spectral parameters for breast tissue diagnosis (30), as also Cervical tissues. Depolarization studies have been undertaken for understanding light propagation in the tissue, a highly turbid medium. The spectral fluctuation and wavelet domain studies have been initiated by the above group for identifying finer distinction between tissue types (31; 32). Intrinsic fluorescence has also been extracted for characterizing dysplastic state of human breast tissues (33).

It is worth mentioning that, fluorescence depolarization is a well known technique for studying molecular aspects of tissue materials (34). The use of polarized light scattering for biological-cell differentiation was first demonstrated by Bickel et al (35). Alfano et al (36) found that in the case of fluorophores in low or moderate viscosities at room temperature, Brownian rotational motion is the main cause of depolarization.

2.3 Fluorophores present in breast Tissue

Many types of fluorophores are present in breast tissue. Fluorophore like tryptophan and tyrosine are amino acids, which are the building blocks of proteins. Tryptophan is responsible for about 90% of the intrinsic fluorescence of proteins (37). It is sometimes inserted into proteins, acting as a sensitive marker, whose fluorescence properties change with the state of the proteins. Collagen is a tough structural protein that one finds abundantly in mammals. The pyridine nucleotides NADH and NAD(P)H fluoresce strongly in at 440-460nm (nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, respectively). NADH is an important player in forming energy-rich ATP (adenosine triphosphate) and is therefore an indication of cellular metabolism. Flavins (FAD) are better known by generic name of Vitamins B_2 , and the different varieties of flavins have very different quantum yields. The strongest of the lot are flavin mononucleotide (FMN) and riboflavin (38). Flavins can also bind to proteins (i.e., flavoproteins), but flavoproteins are usually weak in fluorescence. Porphyrins are intermediate products in the biosynthesis of heme. Normal accumulations of porphyrins are low in tissues, but abnormalities in heme synthesis can cause a high accumulation of metal-free porphyrins such as protoporphyrins IX (PP IX). Abnormal accumulations of porphyrins occur in some haemolytic

disease, anaemia and alcoholism (39). Porphyrins attract large attention because of their role in the human body, ability to accumulate in many kinds of cancer cells, as well as magnetic and optical properties. These features make them useful in cancer medicine and photodynamic therapy (40; 41; 42).

2.4 Previous studies on cervical tissues

Fluorescence spectroscopy has the potential to improve the efficacy of cervical precancerous detection. Several studies have been performed to elucidate the relationship between fluorescence spectra and the underlying tissue biochemistry and morphology (3; 22; 43). For example, Ramanujam et al. (3), used a model of turbid tissue fluorescence to describe spectral data acquired from the cervix at 337 nm excitation (3). Results showed that the contribution of collagen fluorescence decreases and the contributions of reduced nicotinamide adenine dinucleotide (NADH) fluorescence and haemoglobin absorption increase as the tissue progresses from normal to dysplastic. These findings are consistent with histopathologic changes associated with cervical dysplasia. The epithelial thickening, which is commonly seen in dysplastic cervical tissues, could explain the decreased contribution of the collagen fibres that are present in the underlying stroma. It is also hypothesized that abnormal tissues have an increased metabolic rate, and therefore an increased concentration of the reduced electron carriers, NADH and flavin adenine dinucleotide ($FADH_2$), and a decreased concentration of the oxidized electron carriers, NAD^+ and FAD. This difference in metabolic rate could contribute to the spectral differences observed between normal and abnormal tissues in vivo. An increased contribution of haemoglobin is consistent with the increased vascularization seen in severely dysplastic tissues. One limitation of this study is that it assumed the tissue is a homogeneous layer with a constant scattering coefficient. Durkin et al. showed that chromospheres concentrations are not reliable when predicted with this type of simplified tissue model (29). A model was developed by Zonios et al. to predict the fluorescence spectra of normal and neoplastic colonic tissue at 370nm excitation (44). Monte Carlo techniques were used to predict the propagation of photons at the excitation and emission wavelengths. The distribution of fluorophores was measured from

fluorescence images of 5μm− thick transverse frozen−thawed sections, and absorption and scattering coefficients were calculated from in vitro measurements of diffuse reflectance and transmission. The predicted spectra exhibited the characteristic differences of normal and abnormal tissues measured in vivo. Fluorescence microscopy and spectroscopy have also been used to examine cervical tissue autofluorescence in 4μm thick frozen-thawed sections from biopsies. When exciting at 337, 380 and 460nm, Mahadevan found that epithelial fluorescence was seen only in the most superficial layers, with brighter, more widespread fluorescence present in the stroma (43). Lohmann et al. reported similar cervical autofluorescence patterns for frozen-thawed tissue sections at 365nm excitation (22). A major limitation of the studies described above is that they were performed using frozen-thawed tissue. Because the metabolic indicators NADH and FAD are potentially important fluorophores, it is important to measure fluorescence from tissue which is in as close a metabolic state to the in vivo situation as possible. Schomaker et al. showed that the fluorescence of NADH decreases exponentially in the 2 h following tissue removal, as NADH present in the tissue is oxidized to NAD^+, which is nonfluorescent (45). While fast freezing a tissue biopsy can preserve the metabolic state, oxygenation occurs as the sectioned tissue thaws prior to microscopic examination. Therefore, measurements made from frozen-thawed sections are not expected to accurately reflect the autofluorescence present in situ. Imaging the autofluorescence of metabolically active tissue would be much more informative. A microtome that can rapidly prepare fresh tissue slices (46; 47; 48) was developed by Krumdieck and colleagues. Using this device, it is possible to produce fresh tissue slices of a consistent thickness with minimal tissue trauma. The optimal thickness of a slice suitable for culture lies between the lower limit of 50−100μm, where a large percentage of the cells are destroyed at the cut surfaces, and the upper limit of 400−500μm, where the cells in the centre undergo necrosis due to oxygen and nutrient deprivation. The functional integrity of liver slices obtained with this microtome has been studied and tissue is viable in culture for up to 20 h with minimal loss of biochemical function (48).

2.5 Fluorophores present in cervical Tissue

A number of naturally occurring fluorophores are present in cervical tissue. This includes NADH (present in the epithelial layer of cervical tissue), FAD, collagen (present in the stromal layer of cervical tissue) and elastin. The pyridine nucleotides and the flavins have an important role in cellular energy metabolism (49). Nicotinamide adenine dinucleotide is the major electron acceptor; its reduced form is NADH, and the reduced nicotinamide ring is fluorescent (50). Flavin adenine dinucleotide is the other major electron acceptor; the oxidized form, FAD, is fluorescent, while the reduced form, $FADH_2$, is not (51). Several investigators have shown that the endogenous fluorescence near 500 nm, when excited in the near-UV region is weaker in tumor tissue relative to normal surrounding tissues (3; 45). The differences may lie in the decrease in the oxidized forms of flavins and the relative amount of NADH in malignant tissues. The fluorophore NADH is present in the epithelial layer of cervical tissue.

The aromatic amino acids tryptophan, tyrosine, and phenylalanine contribute to protein fluorescence (52). Typically tryptophan accounts for the majority of protein fluorescence, and its emission is sensitive to the polarity of the environment. At excitation wavelengths above 295nm, only tryptophan is excited. From 280 to 295nm, both tyrosine and tryptophan are excited; however, energy transfer from tyrosine to tryptophan is quite common, and the emission may be dominated by that of tryptophan. Below 280nm, all three aromatic amino acids can be excited; however, the quantum yield of phenylalamine is relatively low in comparison with that of tryptophan and tyrosine.

Autofluorescence has been noted in the structural proteins collagen (53), and elastin autofluorescence is also suspected to be associated with cross-links, which are autofluorescent with an excitation maximum at 325nm and an emission maximum at 400nm (53).

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