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Pathological fingerprinting of tissue observed through the window of fluorescence spectroscopy

Shaiju S Nazeer and Ramapurath S Jayasree

Biophotonics and Imaging Lab, Biomedical Technology Wing

Sree Chitra Tirunal Institute for Medical Sciences & Technology,

Poojappura, Thiruvananthapuram - 695 012, India.

There is a high demand for non invasive, non hazardous and fast techniques for the disease diagnose at its early onset which are affordable to common man. The background of the demand is the fact that an early stage diagnosis helps the physician or surgeon to intervene with effective therapeutic strategies to reduce morbidity and mortality. When considering the already prevailing techniques, each modality has its own merits and demerits. The advancement in the properties of light sources and supporting technologies and the potential of optical based techniques give a strong indication that optical pathology and non invasive optical diagnostics will replace the conventional techniques in next generation diagnostics. Spectral information are capable of providing biochemical and metabolic fingerprinting which is very essential for an early stage diagnosis, but lacks among the currently available techniques. Most useful spectroscopic techniques which have proven to be useful in diseases diagnosis are Raman spectroscopy, Infrared spectroscopy, Magnetic resonance spectroscopy and fluorescence spectroscopy. This review focuses on the fluorescence spectroscopy and the supporting statistical tools, the most economic and fast technique from the above list. Details on the instrumentation for *in vitro* and *in vivo* studies have also been reviewed. Case studies based on oral cavity disorders, brain tumor and liver abnormalities have been discussed in detail.

Keywords: Fluorophores, Pathalogy, Non invasive, Fluorescence spectroscopy, Raman spectroscopy.

1 Introduction

This review provides the potential of a most widely known technique viz. fluorescence spectroscopy for the early diagnosis of various tissue pathologies. Considering the beginners in this field, the basics of fluorescence phenomenon, introduction to the instrumentation and the detectable fluorophores from tissue along with their excitation-emission profile have been included. Various spectral analysis techniques that may be adopted during the diagnosis and the role of statistical analysis to support this technique to improve the reliability and accuracy have also been covered in this review article. An overview of the dreaded disease, cancer along with the currently adopted techniques for its diagnosis and the limitations are also given here.

2 Cancer

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in economically developing countries. Choice of unhealthy lifestyles like smoking, alcoholism, physical inactivity and unhealthy diet are considered as the major cause for increased burden of cancer in developing countries. As a result, the demand for early and accurate diagnosis and treatment of cancer has increased tremendously in modern medical field [1].

Cancer of a specific organ which result as the abnormal growth or division of cells eventually results in an abnormal mass of tissues. Rapid and uncontrolled cell division and growth are prime characteristics of cancer cells. Cells undergo an unusual pattern of growth named neoplasia which is followed by irreversible changes like activation of oncogenes or disappearance or inactivation of tumor suppressor genes called as dysplasia. These earlier physical and morphological changes to the cells are of diagnostic and therapeutic significance and are designated as precancerous changes [2].

Corresponding author :
e-mail: jayasree@sctimst.ac.in (Ramapurath S Jayasree)

Cancer is one of the leading cause of disease associated deaths in India. Among men, lung, esophagus, stomach, oral and pharyngeal cancers are the leading cause and among women, cancers of cervix and breast are predominant forms followed by those of stomach and esophagus. Cancer incidence is higher in females compared to males. The age related incidence rate varies from 44 to 122 per 100,000 population in males and 52 to 128 per 100,000 females [3, 4]. The incidences in rural areas are quite low compared to urban counterparts. It is estimated that nearly one million new cancer cases are being detected annually in the country and cancer mortality rates are under-reported due to poor or incomplete diagnosis [5].

Metaplastic or dysplastic stages of cancers are treatable with proper and timely medical intervention. This emphasises the need and importance of early and exact diagnosis of the disease when it is in an early and manageable stage. Optical spectroscopic techniques such as fluorescence spectroscopy, diffuse reflectance spectroscopy, infrared spectroscopy and Raman spectroscopy have proven as potential diagnostic tools [6-19]. In this review, we are discussing the advantages of optical diagnosis, specifically fluorescence spectroscopic diagnosis over currently available diagnostic techniques. Instrumentation and different modalities of fluorescence spectroscopy and the methodology for spectral acquisition and data analysis usually adopted in pre-clinical/clinical autofluorescence spectroscopy are also discussed.

2 Currently adopted techniques for cancer detection and its limitations

2.1 Biopsy-Histopathology

Biopsy is a medical procedure performed by a clinician involving removal of tissues for examining the presence or extent of a disease. This tissue is later examined under a microscope by a pathologist, and can also be analyzed chemically. Depending on the procedures involved, biopsy is mainly divided into three namely, excisional biopsy, incisional or core biopsy, and needle aspiration biopsy. The procedure involving removal of entire suspicious area is called excisional biopsy. Removal of a tiny sample of tissue from a suspicious area without damaging the histological architecture of the tissue's cells is termed as incisional or core biopsy. Removal of tissue or fluid from a suspicious area using a needle without maintaining the histological architecture of the tissue specimen is called a needle aspiration biopsy [20-23].

Histopathology is the microscopic examination of a biopsy or a surgical specimen by an expertise to study the pathological details of the tissue. For this, a series of long and tedious procedures are involved. Initially, the specimen must be placed in a fixative (usually, 10% formaldehyde in water) that stabilizes the tissue and prevents from further decay. This tissue has to be embedded, sectioned and stained before evaluation under a microscope by a pathologist. Combination of hematoxylin and eosin (H&E) are the commonly used stain in histopathology for the clear visualization of pathological structures. Nuclei will be stained blue using hematoxylin and cytoplasm and the extracellular connective tissue matrix will be stained pink using eosin. There are several other techniques which have been used to selectively stain cells with varying biochemical or morphological pattern. Masson's trichrome and Sirius red staining are used to study variation in collagen within tissue. Oil red O and Sudan black staining are used to detect lipids within tissues. Perls' Prussian blue staining is used to evaluate iron deposition in tissues [24-27].

2.2 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a noninvasive tool to investigate the internal anatomy and physiology of living subjects. This technique works on the principle of nuclear magnetic resonance (MR), which works on the principle that atomic nuclei in a strong magnetic field absorb and re-emit electromagnetic waves at a characteristic or 'resonant' frequency, which falls in the radio frequency (RF) range. This technique is considered to be safe for humans and animals because neither the strong magnetic fields nor the radio waves have any known adverse effects on them. The wealth of information contained in the signal makes this technique extremely versatile, in terms of gross structural properties of the tissue and its biochemistry. The techniques used to elicit and analyze the signal can be readily tailored to amplify the factors of interest,

providing high-resolution images of specific structures, such as arteries, lesions, and white matter tracts [28]. Advanced MRI techniques are suitable to acquire information on the biochemical and functional aspects of the region of interest.

2.3 Computed tomography imaging

Computed tomographic (CT) imaging produces tomographic images of specific areas of an object noninvasively using x-rays. A volume of data is produced that can be manipulated in order to demonstrate various bodily structures based on their ability to block or transmit the X-ray beam. Generally, the images generated are in the axial or transverse plane, perpendicular to the long axis of the body. But modern scanners are capable of reformatting these data in various planes or even as volumetric representation of structures. Since its introduction in the 1970s, CT has become an important tool in medical imaging to supplement x-rays radiography and medical ultrasonography. It has more recently been used for preventive medicine and/or screening of diseases. The main advantages of CT over traditional 2D medical radiography are CT completely eliminates the superimposition of images of structures outside the area of interest and, differences between tissues that differ in physical density by less than 1% can be distinguished using CT due to the inherent high-contrast resolution. Moreover, data from a single CT imaging consisting of either multiple contiguous or one helical scan can be viewed as images in the axial, coronal, or sagittal planes, depending on the diagnostic task. This is referred to as multiplanar reformatted imaging [29, 30].

2.4 Limitations of the currently available diagnostic tools

Currently, biopsy followed by histopathology is considered as the gold standard for cancer screening. But this technique highly depends on tissue staining and morphological pattern recognition, and the methodology followed in the process has remained largely unchanged for nearly fifteen decades. Moreover, this technique is time consuming and has limited statistical confidence level due to inherent operator variability [31]. Therefore, more sensitive screening methodologies are necessary to overcome the limited molecular detection capabilities of the available immunohistochemical method.

Morphometric details of malignancy associated tissue changes can be evaluated using techniques like MR and CT Imaging. But both these techniques have their own limitations like high cost and non availability and accessibility to common man. Moreover, these techniques require administration of an external contrast agent for high resolution images [32-34]. Additionally, these techniques lack detailed molecular information of tissue transformation even though they are sufficient to provide high resolution images [25, 26]. The ionizing property of the X-ray radiation used in CT imaging is also harmful to the cells and tissue, which includes chances of mutation leading to cancer in organs like breast, lungs, thyroid and stomach [35]. A dedicated medical and paramedical team is required for the acquisition and interpretation of the results. The patients, in most cases need extensive pre-scan preparations, like fasting in the case of CT and should be devoid of any metallic implants in the case of MRI. Compared to these techniques the diagnosis based on optical methods are easier, cost effective and are capable of providing biochemical information.

2.5 Advantages of optical diagnostic tools

Biological tissues are complex and inhomogeneous in nature. Therefore, interaction of light with tissue depends on the specific composition and morphology of the tissue and the physical nature of light. Due to microscopic differences in the index of refraction within the tissue, the incident light may be scattered multiple times either elastically or inelastically. This light can be absorbed by endogenous tissue flurophores (e.g. extra cellular collagen, phospholipids and intracellular NADH, FAD) which may then release their excess energy by radiative decay, producing fluorescence and by non radiatively absorbed chromophores (e.g. hemoglobin and melanin) producing diffuse reflectance signal. Variation in these enzymes, proteins or biochemicals results in the change in fluorescence or reflectance signal. The variations in the fluorescence or reflectance spectra can be utilized to study the morphological and biochemical profile of the tissue.

3 Interaction of light with biological tissues

Understanding of the light-tissue interactions and the light propagation within the tissue both *ex vivo* and *in vivo* is the fundamental step in determining optimum light- based technologies for diagnosis and treatment. Therefore, the interpretation of a diagnostic optical measurement and the therapeutic intervention requires the clear understanding of optical properties of tissue and light-tissue interactions.

Interaction of light with tissue mostly involves scattering and absorption. When light is incident on the tissue surface, a part of it is absorbed by the tissue and the non absorbed part undergoes multiple elastic scattering. This will ultimately emerge from the tissue surface as diffuse reflectance signal that carries both quantitative and qualitative information about tissue biochemistry and structure. A certain fraction of this emerging light is collected by the detector and the remaining part escapes undetected. The amount of light collected mainly depends on the optical properties of tissue such as scattering and absorption coefficients. In the case of fiber optic probe assisted system, the amount of light collected also depends on the properties of the probe like radius, number of excitations , collection fibers used etc. The absorption coefficient reflects information about the concentration of physiologically relevant absorbers in the tissue like hemoglobin and melanin. The scattering coefficient is directly related to the size and density of scattering centers in tissue, such as cells and nuclei. Malignancy associated changes in tissues will result variation in these absorbers and scattering centers that causing spectral variations. These variations are utilized for tissue characterization and disease diagnosis.

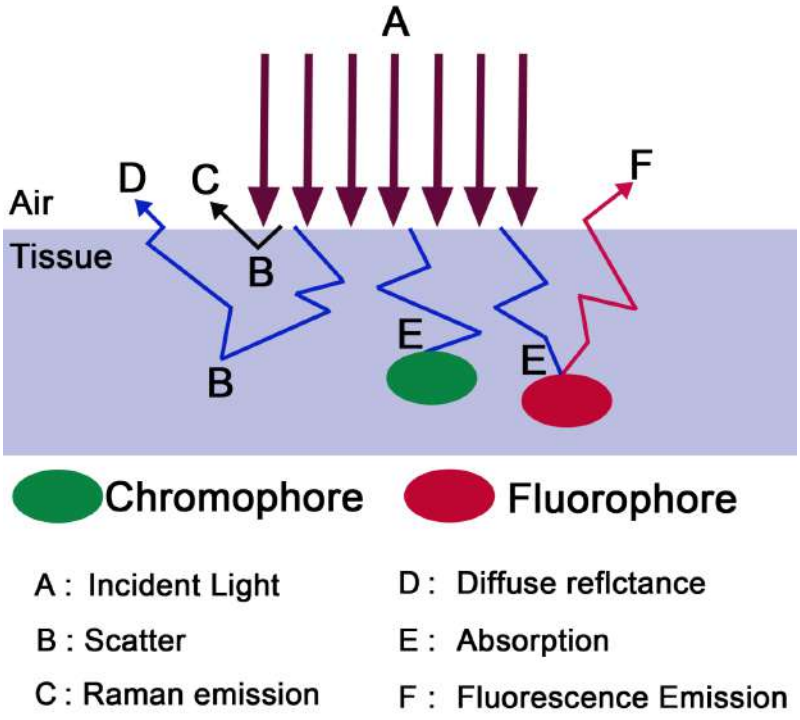

Fig 1. Variety of light-tissue interactions

Figure 1 represents the various processes that occur when an optical photon “A” from air medium is incident on the tissue surface. Green and red dots represent intrinsic tissue chromophores and flurophores. “B” represents the intrinsic scatters within tissues. “C” represents Raman scattering from the tissue surface

which is an inelastic process where the energy of scattered photon changes. “D” stands for diffuse reflectance from cellular and structural components of the tissue where majority of photons undergo numerous scattering and absorption events before being emitted. Absorption of photon by various components of tissue is another possibility shown in "E". Photons with varying energy/wavelength are absorbed and show less scattering that facilitate measurement of chromophores. Fluorescence scattering is another important inelastic process shown in “F”, where the energy and wavelength of the re-emitted photon changes due to the presence of intrinsic flurophores within the tissue

Likewise, structural and biochemical information of tissue can be obtained by measuring absorption, diffuse reflectance, fluorescence and Raman scattering. These spectroscopic methods have different physical basis and all have the potential to become an alternative method to overcome the limitations of conventional diagnostic methods.

4 Phenomenon of fluorescence

Luminescence is the emission of light form any substance and occurs from electronically excited states. The nature of the excited state differentiates luminescence into two categories, fluorescence and phosphorescence. In excited singlet states, the electron in the spin of the excited orbital is paired (of opposite direction) to the second electron in the ground state orbital. Consequently, return to the ground state follows an allowed spin state and occurs rapidly by emissions of a photon. The emission rates of fluorescence are typically 10^{-8} s, so that a typical fluorescence lifetime is nearly 10 ns. Phosphorescence is emission of light from triplet excited states, where the electron in the excited orbital has the same spin orientation as the ground state electron. Transitions to the ground state are forbidden and hence the system follows an interstate crossing before relaxing back to the ground state and hence the emission rates are slow (10^{-3} to 100 s), so that phosphorescence lifetimes are typically milliseconds to seconds [36].

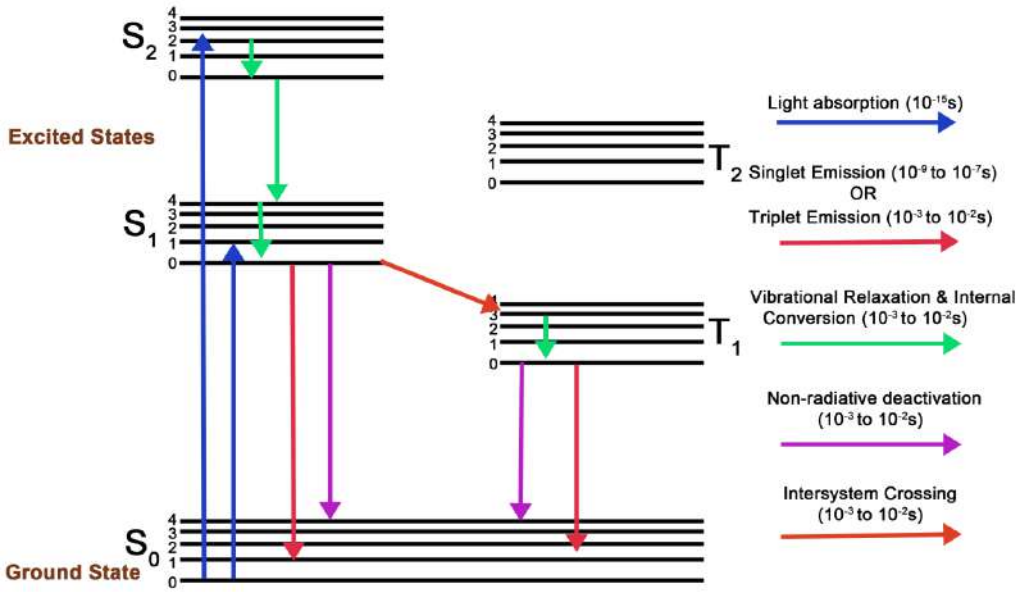

Fig 2. Jablonski diagram showing the transitions between different electronic states

A typical Jablonski diagram is shown in Fig 2. The singlet ground, the first and second electronic states are depicted by S_0, S_1 and S2, respectively. At each of these electronic energy levels, the fluorophores

can exist in a number of vibrational energy levels, denoted by 0, 1, 2, etc. The transitions between the states are depicted as vertical lines to illustrate the instantaneous nature of light absorption [36].

Absorption of light is usually followed by several processes. A fluorophore is usually excited to high vibrational level S_1 or S_2. With rare exceptions, molecules in -condensed phase rapidly relax to the lowest vibrational level of S_1. This process is called internal conversion and generally occurs in a time span of 10-12 s or less. Since fluorescence lifetimes are typically of the order of 10^{-8} s, internal conversion is generally complete prior to emission. Hence fluorescence emission generally results from a thermally stable excited state, that is, the lowest-energy vibrational state S_1. Decay to the ground state typically occurs to a higher vibrational ground state, which then quickly relaxes to the lower ground state and attains thermal equilibrium [36].

Molecules in the S_1 state can also undergo a spin conversion to the first triplet state T_1. Emission from T_1 is termed as phosphorescence and is generally shifted to longer wavelengths relative to fluorescence. Conversion from S_1 to T_1 is called intersystem crossing. Transition from T_1 to singlet ground state is forbidden, and, as a result, rate constants for triplet emission are several orders of magnitude smaller than those for fluorescence [36].

5 Optical spectroscopy in medical diagnostics

The emergence of spectroscopy and spectral imaging shows the enormous potential of these tools in the field of medical diagnostics. Spectroscopic techniques such as Fluorescence, diffuse reflectance, Raman and Infrared, and their mapping/imaging modalities provide a means of visualizing molecular structure spatially in 2- and 3-dimensions. These techniques provide detailed biochemical information on lipids, proteins, extracellular matrix proteins (elastin and collagen), carbohydrates, cellular nucleic acids, glycogen etc., in a qualitative and quantitative manner [31, 37-39]. These tools have been used to diagnose a range of different pathologies especially in the field of cancer. They have the advantage of providing a biochemical fingerprint of the constituents whether originating from simple molecular systems to more complex ones like cells, tissues or biofluids. Further, they are rapid, non-contact, non-destructive, and reagent and label-free methods. These techniques can overcome the limitations often possessed by conventional diagnosis methods. Spectroscopic techniques have the ability to provide tissue characteristics at the molecular level accurately, quickly, and in an objective manner. The techniques applied to disease diagnosis have been collectively described as either “spectral histopathology” or “spectral cytology” depending on whether tissue or cells are being investigated.

6 Autofluorescence Spectroscopy

Fluorescence spectroscopy, an optical spectroscopic method based on the excitation-emission phenomena of light, has been widely used for quantitative and qualitative analysis of biological molecules and tissues. Fluorescence spectroscopy assisted by organic/inorganic chemical systems is used to monitor biologically relevant metallic ions, carbohydrates and proteins [40-42]. But the label free method of fluorescence spectroscopy which is well known as autofluorescence spectroscopy is used to analyze endogenous flurophores within biological tissues/fluids for the diagnosis of various diseases [43, 44]. Biological molecules that exhibit endogenous fluorescence with their excitation and emission properties are given in Table 1. Additionally, parameters like hemoglobin concentration and redox ratio which helps in the diagnosis of many diseases can be derived from the fluorescence properties of these inherent fluorophores. In optical diagnosis, autofluorescence spectroscopy and its laser induced analogue with favorable features like economic affordability, compatibility and rapidity is used as a diagnostic aid.

Table 1. Excitation and emission maxima of biological molecules that exhibit endogenous fluorescence

Endogenous fluorophores	Excitation Maxima (nm)	Emission Maxima (nm)
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Structural proteins		
Collagen	325	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
Ceroid	340–395	430–460, 540
Porphyrins		
Protoporphyrin IX	400–450	630,695
Corpoporphyrin	400–450	670

6.1 Fluorescence spectroscopic techniques in cancer diagnosis

In 1924, autofluorescence from malignant tumors was first observed by Policard [45]. He observed red fluorescence from hematoporphyrin in the sarcomas of laboratory rats using ultraviolet radiation excitation [45]. For the last three decades, several researchers all over the world have been utilizing fluorescence spectroscopy as a diagnostic aid for malignancy associated changes in tissues from different parts of the human body. Alfano and his team have successfully demonstrated that variation in autofluorescence from normal and cancerous tissues can be effectively utilized for colon, - gynecologic and lung cancer diagnosis [46-48]. Autofluorescence from lesions were further utilized by various groups for diagnosis of malignancy associated with organs like brain, breast, cervix, colon, liver, prostate and oral cavity [12, 43, 49-68]. All these studies used various approaches in terms of wavelengths of excitation, diagnostic algorithms, and data analysis for differentiating between normal and diseased tissues.

7 Instrumentation and analytical techniques

Recent advancement in optical technologies and instrumentation have become a boon in diagnostic, therapeutic and basic science applications of spectroscopic techniques in medicine. Optical spectroscopic techniques and their imaging and mapping modalities are being developed for a variety of biomedical applications like *in vivo*, minimally invasive or *ex vivo* tissue diagnostics, including sensing molecular concentrations of delivered pharmaceutical or contrast agents, probing tissue physiologic status, and detecting early stages of disease.

7.1 Point Monitoring

The last decade has seen a great momentum in research and productivity in the field of optical tissue diagnostics. Often, these optical biopsy/pathology systems are based on point monitoring and imaging/mapping. An appropriate electromagnetic radiation will be directed on a small point or an area of the sample to perform spectral measurement either directly or using a fiber optic probe. Fiber optic probes consisting of excitation and pick up fibers to guide and to collect light are useful for *in vivo* spectral acquisition. The collected signals from the samples are usually very weak. Measurement of such weak signals can be achieved by detection systems coupled with a sensitive photomultiplier tube or a charge coupled device camera and proper optical filtering systems.

7.2 Bench top autofluorescence spectrometer

The optical spectroscopic measurements can be carried out using spectroflurometers. The instrument consists of a source, an excitation and emission monochromator and a photomultiplier tube. The experimental setup of typical spectroflurometer (Fluorolog-III-Jobin Yvon Inc., Edison, New Jersey) is illustrated in Fig 3.

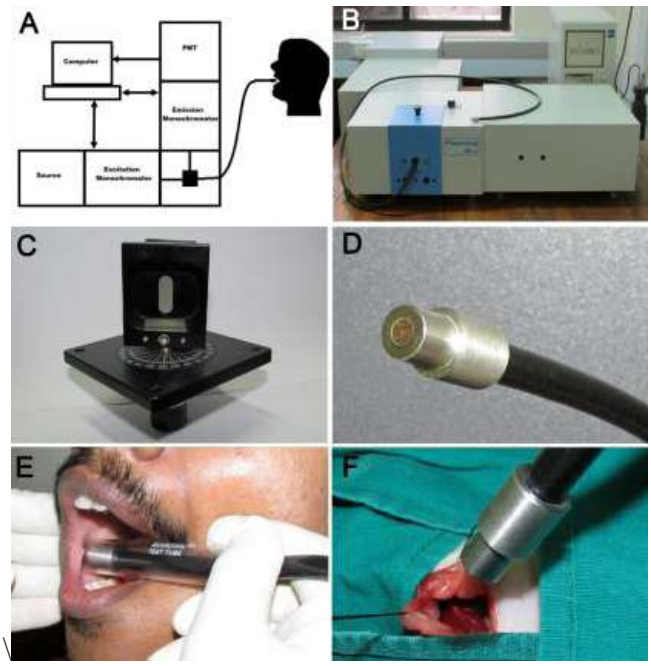

Fig 3. (A) Schematic representation of *in vivo* fluorescence measurement setup. (B) Spectrofluorometer with the fiber-optic probe. (C) Solid state sample stage for *ex vivo* analysis (D) Fiber-optic probe bundle for *in vivo* spectral acquisition. (E) *In vivo* spectral acquisition from oral cavity using a fiber-optic probe (F) minimally invasive spectral acquisition from liver.

Special sample holder as shown in Fig 3C facilitates the recording of fluorescence spectra from solid samples including *ex vivo* tissue samples. Using this, light ray of desired excitation wavelengths can be allowed to fall perpendicularly on the tissue surface with appropriate spot size. The emission from the sample can also be collected with respect to the excitation beam.

A multimode fiber-optic probe consisting of illumination fibers and collection fibers enable *in vivo*/ minimally invasive measurements (Fig 3D-F). This is usually a bifurcated Y type fiber-optic probe which is coupled with the sample compartment. One arm of the Y type fiber-optic probe is connected to the light source. The desired excitation wavelength can be selected and the light is transmitted to the tissue site through this arm. The received fluorescence signal will be directed back to the spectrometer through the other arm. A transparent covering at the tip of the fiber can be used to protect the distal end of the Y type fiber optic probe in order to avoid contamination. To compensate for this protective cover, a correction factor has to be uniformly applied to all spectra.

7.3 Laser induced fluorescence spectrometer

Schematic diagram of the laser induced fluorescence spectrometer used for *in vivo* measurements is shown in Fig 4. Laser wavelengths of 337 and 405 nm are generally used as the excitation beam for laser induced fluorescence studies. Laser beam is guided through a bifurcated optical fiber to the fiber-optic probe in a stainless steel ferrule of approximately 5-8 mm diameter. This probe consists of illumination and collection fibers. Laser of appropriate wavelength is directed to the tissue site using the illumination fiber and emitted signal from the tissue is collected using collection fibers. The probe will direct the emitted radiation from the sample through a filter, which avoids the back scattered radiation and is detected at the spectrometer.

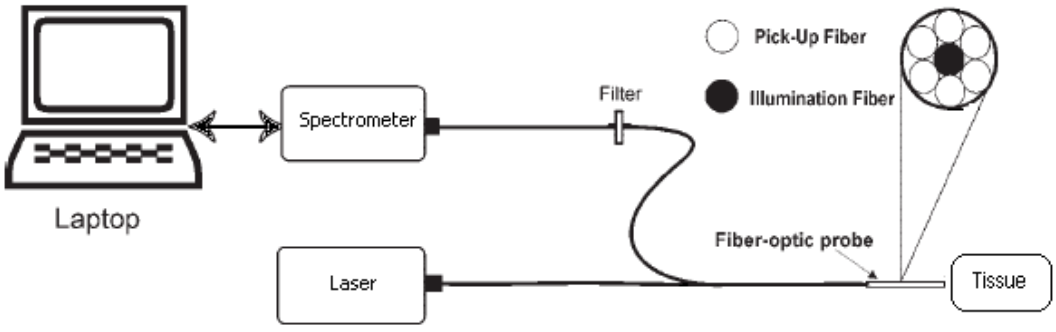

Fig 4. Schematic representation of laser induced autofluorescence spectroscopy mesurements

8 Processing of spectral data and analysis

All the collected spectra have to be baseline corrected and then normalized. Even though there are several methods to do this, the commonly used method for normalization is with respect to the maximum intensity of a specific flurophore. From the normalized data, peak intensity corresponding to the major peaks can be extracted. Using appropriate statistical software, variations in the prominent fluorescence peak intensity between the different tissue types can be evaluated.

8.1 Area under a specific peak

Gaussian curve fitting analysis can be performed to obtain area under a specific autofluorescence peak. This can be done using an in house developed program which works on a MATLAB platform or software like Origin. Gaussian fitting program enables us to understand the spectral variation during tissue

transformation by precisely determining the peak position and bandwidth using Gaussian spectral functions. This fitting program uses the Marquardt–Levenberg algorithm by an iterative process that finds the true absolute minimum value of the sum of squared deviations. Pictorial representation of curve fitting analysis carried out on fluorescence emission spectra is given in Fig 5.

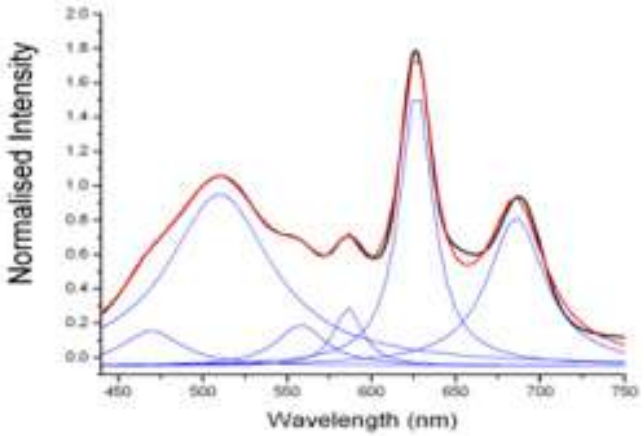

Fig 5. Illustration of curve-fitting of the normalised autofluorescence spectrum of brain tissues showing constituent bands.

8.2 Spectral intensity ratio

Normalized intensity ratio of the emissions from two different fluorophores can be utilised to get a rough idea about over all tissue biochemistry. Usually ratio between normalised intensity of collagen and NADH or FAD and protoporphyrin is used to evaluate variation in tissue biochemistry. These spectral intensity ratios can be plotted together and 'cut off' lines for different groups can be assigned according to the pathological grading of the corresponding group of tissues. Using the position of discrimination line, binary evaluation of the diagnostic methodology using fluorescence spectroscopy can be evaluated.

8.3 Principal component analysis

High dimensional spectral data space often increases the complexity of data analysis and results in inefficient execution of conventional statistical analysis and clustering algorithms (e.g. soft independent modeling of class analogy, linear discriminant analysis (LDA), etc.). Principal component analysis (PCA) is the best method usually adopted to reduce these spectral data to smaller manageable components without losing the useful information. PCA simplifies the complex multidimensional dataset and extract the key variables within the dataset as loadings and scores. These loading vectors and PC scores extracted are mutually related to the original spectrum. ANOVA is then used to identify the most significant PCs ($p < 0.05$) for differentiation between different tissue types. These PC scores are fed to the development of LDA algorithms for multiclass classification [9,69,70].

8.4 Linear discriminant analysis

Discriminant analysis is a classification technique to convert a set of observations into predefined classes. The purpose is to determine the class of an observation based on a set of variables known as predictors or input variables. Based on a set observations for which the classes are known, the classification model has been built. This set of observations is referred as the training set. On the basis of this training set, the classification technique constructs a set of linear functions of the predictors, known as discriminant functions, such that: $L = b_1x_1 + b_2x_2 + \ldots + b_nx_n + c$, where the b's are discriminant coefficients, the x's are the input variables or predictors and c is a constant.

LDA determines the discriminant function that maximizes the variances between groups while minimizing the variances between members of the same group [9]. Performance of the diagnostic algorithms provided by discriminant analysis models to predict the tissue groups are estimated in an unbiased manner using leave-one tissue site-out, cross-validation method. This method of cross validation produces a confusion matrix that compares predicted versus actual group membership [69]. The common objectives of discriminant analysis are (i) to investigate differences between groups; (ii) to discriminate the groups effectively; (iii) to identify important discriminating variables; (iv) to perform hypothesis testing on the differences between the expected groupings; and (v) to classify new observations into pre-existing groups [12-14].

8.5 Diagnostic Accuracies

8.5.1 Sensitivity and Specificity

Evaluation of diagnostic performance of a methodology in clinical/animal studies on tissue discrimination is distinctively evaluated using binary calculation results, sensitivity and specificity.

Sensitivity (Se) = true positive / (true positive + false negative)

Specificity (Sp) = true negative / (true negative + false positive)

Sensitivity is defined as the fraction of diseased sample correctly classified and the specificity is defined as the fraction of non-diseased tissues correctly classified [12]. When these results of diagnostic discrimination are compared with histopathological evaluation or visual inspection by clinician, in any case in the clinical scenario there could be four possibilities. When a diagnostic result indicates that the person has disease and this evaluation is confirmed by the gold standard, then this diagnosis is assigned as True Positive (TP). If the diagnostic result indicate that the person has disease, but gold standard indicates that the disease is absent then this diagnosis is assigned as False Positive (FP). In agreement with the gold standard, if the diagnostic result indicates that the person has no disease then it is assigned as True Negative (TN). If the procedure results indicate that the person has no disease which is not in agreement with the gold standard, then the diagnostic result is assigned as False Negative (FN).

Hence, a diagnostic discrimination result with 100% sensitivity indicates that every diseased individual is correctly identified and a specificity of 100% indicates that every disease-free individual is correctly identified. An ideal diagnosing system should be always highly sensitive and specific. But for many diagnostic procedures, these two are inversely related and increase in one is often related with the decrease of the other.

8.5.2 ROC curve analysis

The performance of classifiers can be assessed by comparing their average misclassification rate. Receiver operating characteristic (ROC) curve is a graphical way of illustrating the performance of a binary classifier system by changing the discrimination threshold. This has been carried out by plotting the fraction of true positives out of the total actual positives versus the fraction of false positives out of the total actual negatives at various threshold settings. For this, Signal Intensity Ratio (SIR) and PC-LDA scores obtained for different groups are fed into the statistical classifier. Area under the ROC curve (AUC) is an index of overall discriminative ability of the specific classification model. The AUC summarizes the ROC and provides a single measure of the performance of a classifier and the ability of the model to discriminate. If AUC is greater than 0.60, then the classification is considered as good [9, 71].

8.6 Redox ratio evaluation

The electron transport chain is the most efficient means of energy production in cells. The electron transport chain produces energy in the form of Adenosine triphosphate by transferring electrons to molecular oxygen. The metabolic coenzymes FAD and NADH are the primary electron acceptor and donor, respectively,

in oxidative phosphorylation. Optical redox ratio is the ratio of fluorescence intensity of these electron acceptor and donor. This optical redox ratio provides relative changes in the oxidation reduction state in the cell. The redox ratio is sensitive to changes in the cellular metabolic rate and vascular oxygen supply [72, 73].

The redox ratio is computed on the basis of NADH and FAD related signals using the following equation

$$\text{Redox ratio} = \frac{\text{FAD intensity}}{\text{FAD intensity} + \text{NADH intensity}}$$

where, FAD intensity and NADH intensity are the emission intensities of these fluorophores at 520 and 460 nm, respectively [16, 74].

8.7 Total hemoglobin concentration estimation

Hemoglobin is a nonfluorescent metalloprotein [44, 75]. Hemoglobin peak in the autofluorescence spectra of tissues has been considered as an anomaly by majority of the researchers [76-78]. In order to correct these artifacts in fluorescence spectra due to blood absorption, many research groups have adopted mathematical correction factors assisted by the results of diffuse reflectance spectroscopic data [76, 78, 79]. The reports suggest a first-order approximation method that involves division of fluorescence spectral data with the diffuse reflectance data from the same tissue site to remove the hemoglobin absorption artifacts. Thus, the possibilities of using fluorescence spectroscopy for evaluating the changes in the hemoglobin concentration have not been exploited in detail. In 2009, Liu and Vo-Dinh have proposed a ratio-metric method based on Spectral filtering modulation (SFM) for the estimation of total hemoglobin and oxygen saturation using synthetic tissue phantom models [80].

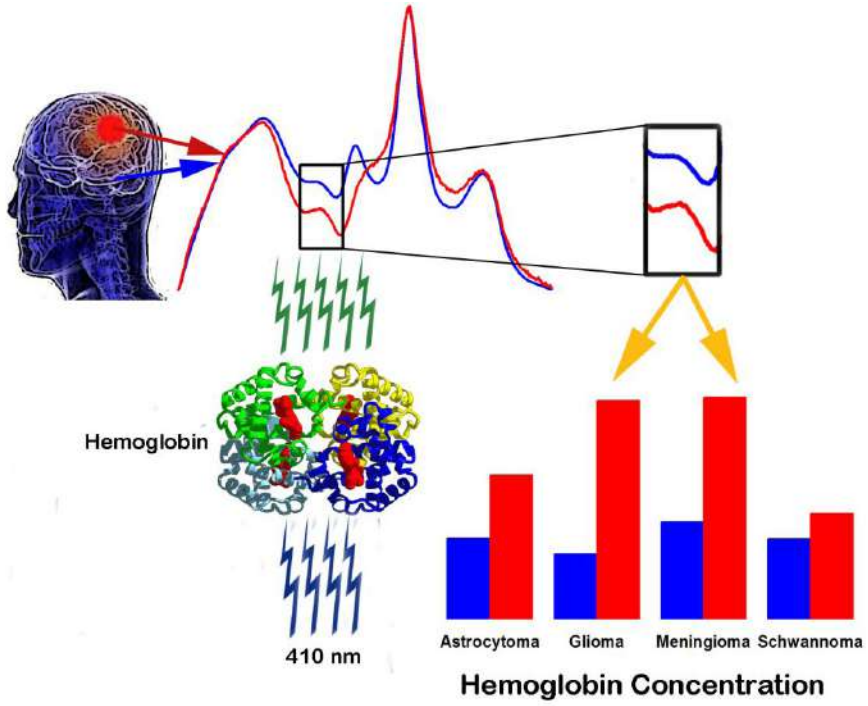

Fig 6. Schematic representation of spectral filtering modulation effect on autofluorescence spectra due to hemoglobin absorption in brain tissue [Reproduced from ref 63].

Schematic representation of spectral filtering modulation effect on autofluorescence spectra due to hemoglobin absorption from brain tumor tissues is given in Fig 6. The total hemoglobin concentration can be determined by considering the ratio of fluorescence emission intensities at 500 and 570 nm of the 410 nm excited spectra, as per the report of Liu and Vo-Dinh [80]. The extinction coefficients of oxygenated and deoxygenated hemoglobin at these wavelengths are equal. Hence the ratio between these provides the total concentration of hemoglobin.

9 Fluorescence diagnosis in preclinical and clinical scenario

For many years, application of fluorescence spectroscopy has become an area of interest among many clinical and oncological research groups. In the following sections we are discussing preclinical and clinical trials using fluorescence spectroscopy on malignant changes in oral cavity, liver and brain tissues reported by various research groups in the world.

9.1 Oral cancer diagnosis

In vivo and *ex vivo* use of autofluorescence spectroscopy have been successfully established by many research groups all over the world for oral cancer diagnosis. Several pilot studies to distinguish oral lesions from normal mucosa with good sensitivity and specificity have been carried out by various research groups [77, 81-86]. Ebenezar *et al*, carried out an *in vivo* fluorescence excitation study using 635 nm and characterised variation in porphyrin excitation from normal volunteers and oral cancer patients [82]. From the excitation spectra, they have calculated three potential ratios (I410/I505, I410/I540, and I410/I580) and input these variables for a stepwise linear discriminant analysis. They obtained an overall sensitivity and specificity of 100% in differentiating between normal and cancer. Gillenwater *et al*, successfully carried out an autofluorescence emission study to differentiate between healthy volunteers and patients with premalignant or malignant oral cavity lesions [83]. They have used excitation wavelengths 337, 365 and 410 nm in the emission range of 350 to 700 nm and found out variation in endogenous flurophores NADH and Porphyrin in healthy and cancerous tissues. Autofluorescence spectroscopy is also utilized to differentiate potentially malignant oral cavity disorders like oral submucous fibrosis (OSF), leukoplakia, ertyhroplakia and lichen planus from normal oral cavity [11,14,87]. Haris *et al*, differentiated oral mucosa of healthy volunteers and patients with oral submucous fibrosis using *in vivo* modality of autofluroscence spectroscopy [11]. They have used 320 nm excitation wavelength in order to evaluate variation in endogenous flurophores such as collagen and NADH in the oral cavity. In another study, Venugopal *et al*, carried out autofluorescence differentiation between oral mucosa of healthy volunteers and patients with leukoplakia using 410 nm excitaion wavelength [87]. Using SIR and discriminant analysis they have obtained a sensitivity of 96 and 100% and overall specificity of 100 in differentiating between normal and leukoplakia. Recent studies have shown that autofluorescence spectroscopy can provide good diagnostic efficiency to discriminate between oral cavity cancers with varying pathological grading. Using 405nm laser excitation, Subhash and his group evaluated the efficacy of fluorescence spectroscopy to differentiate between different oral cavity tissues, specifically, normal, hyperplasia, dysplasia and squamous cell carcinoma using porphyrin emission peaks [12, 56]. Further using SIR and discriminant analysis they have evaluated the sensitivity and specificity values in a clinical trial. Sivabalan *et al* have reported the use of native fluorescence spectroscopy technique to characterize normal and oral submucous fibrosis from the oral cavity of pre and post treated patients [16]. Early tissue alterations in oral cavity by lifestyle habits like tobacco chewing/smoking, arecanut chewing and alcoholism can also be evaluated using fluorescence spectroscopy. In our earlier studies, we have carried out autofluorescence spectral differentiation between volunteers with habits like arecanut chewing and tobacco chewing/smoking with that of healthy volunteers without any such habits [62, 64]. We have observed variation in collagen and flavin level in the case of arecanut chewing habitues and hemoglobin

and porphyrin level in the case of tobacco chewing/smoking habitues in comparison with habitues and non-habitues.

9.2 Liver cancer diagnosis

Optical diagnosis of liver associated malignancies using autofluorescence spectroscopy has not yet been studied elaborately. In a study using portable fiber-optic system for fluorescence spectral acquisition, Fabila *et al*, carried out an *ex vivo* study on liver tissue biopsy from liver fibrosis patients [53]. They have acquired fluorescence spectra from tissue biopsies using a portable fiber-optic system and empirical discrimination algorithms based on fluorescence intensity ratio at 500 nm and 680 nm was developed. They have obtained a sensitivity and specificity of around 80% and 85% , respectively in this trial. In a study using laser induced fluorescence spectroscopy, Li *et al*, evaluated the differences in serum spectra between normal people and liver cancer patients [54]. Croce *et al*, carried out autofluorescence emission study on cryostatic tissue sections obtained from fibrotic and steatotic liver patients [50]. They have used excitation wavelength of 366 nm to evaluate variation in endogenous flurophores, NADH, collagen, and vitamin A using fluorescence imaging and microspectrofluorometric techniques. Sauvage *et al*, spectroscopically evaluated liver tissue from normal and non-alcoholic fatty liver disease induced mice using a fibre probe assisted laser induced fluorescence system with two laser diodes that provided excitation light at 375 and 405 nm [88]. First report on *in vivo* assessment of liver fibrosis using diffuse reflectance and fluorescence spectroscopy system was carried out by Fabila *et al* [52]. Male wistar rats were differentially exposed to chronic administration with carbon tetrachloride (CCl_4) and *in vivo* spectral measurement from the liver surface of each animal was carried out by a minimal invasive laparoscopic procedure. Fluorescence spectral acquisition was carried out from animals grouped as control healthy, mild fibrosis, moderate fibrosis, and advanced fibrosis. Using fluorescence intensity, they have obtained a sensitivity of 73% and specificity of 94% for the recognition of hepatic fibrosis

9.3 Brain tumor diagnosis

Studies relating the fluorescence behaviour of the fluorophores of brain tumor tissues are limited. Saraswathy *et al*, carried out *ex vivo* autofluorescence monitoring of different human brain tumor samples [15]. The autofluorescence measurements at four different excitation wavelengths 320, 370, 410, and 470 nm, were carried out for five different brain tumor types namely, glioma, astrocytoma, meningioma, pituitary adenoma, and schwannoma. Variation in endogenous flurophores such as collagen, NADH, flavin and porphyrin was observed between adjacent normal and tumor tissues. Sensitivity of 86 to 100% and specificity of 66 to 100% is observed in differentiation between adjacent normal and tumor tissues using multivariate analysis. Lin *et al*, carried out a clinical trial that provides an effective way of separation of brain tumors and infiltrating tumor margins from normal brain tissues, *in vivo* using combined autofluorescence and diffuse-reflectance spectroscopy [55]. They have developed a two-step empirical discrimination algorithm based on autofluorescence and diffuse reflectance at 460 and 625 nm. Using this algorithm, they have obtained a sensitivity and specificity of 100 and 76% in differentiating infiltrating tumor margins from normal brain tissues. Many other reports on brain tumor diagnosis using autofluorescence spectroscopy are centered on 5-aminolevunic acid (ALA) induced protoporphyrin synthesis in the brain tumor [49, 59]. In our earlier studies, we have carried out fluorescence spectroscopic study on surgically removed fresh and formalin fixed brain tissues identified pathologically as astrocytoma, glioma, meningioma, and schwannoma [63, 66]. Adjacent normal and tumor tissue were differentiated using the emissions from fluorophores collagen, flavin adenine dinucleotide, phospholipids, and porphyrin which were analyzed using 320 and 410 nm excitations. Using SFM effect, total hemoglobin concentration within the adjacent normal and tumor tissues was also evaluated. We have obtained a sensitivity of 70 to 100% and specificity of 75 to 100 % in differentiation

between adjacent normal and brain tumor tissues using 320 nm excited spectral dataset. Also we have obtained a sensitivity of 37 to 100% and specificity of 66 to 100 % in differentiation using 410 nm excited spectral dataset.

10 Conclusions

Fluorescence spectroscopy can be used for the fingerprinting of various fluorophores associated with different diseases. This helps to monitor the changes in the fluorophores and hence is an efficient tool for early diagnosis of a variety of diseases. The fluorophore associated with the particular disease may be identified and studied for most efficient results. For example, when change in collagen emission is important for the diagnosis of OSF, it is the change in porphyrin which is more important for the diagnosis of oral leukoplakia. Additionally, the metabolic details like redox ration and the hemoglobin concentration in a particular tissue can also be derived from this technique which adds on to its beauty of having multiple parametric approaches, increasing the accuracy of diagnosis. This is a very easy and low cost technique which could be used as the initial screening in health care centers for checking oral cavity disorders and cervical cancer using a fiber optic probe assisted acquisition of spectral data. Mathematical algorithms will further support the original spectral findings.

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Shaiju S Nazeer completed his Ph D from Kerala University in 2014 and is pursuing his post doctoral fellowship in Monash University, Melbourne, Australia under the Endeavour Research Fellowship. He received his M Sc and M Phil in Physics with Spectroscopy as specilaised subject in 2006 and 2008, respectively from Annamalai University, Chidambaram. He also has received CSIR Senior Research Fellowship during his Ph D program to work in Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. His areas of research include biospectroscopy and nano biophotonics.

Ramapurath S Jayasree is the Head of Biophotonics and Imaging Laboratory of Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum since 2010. She gained her post graduate degree in Physics from Cochin University of Science and Technology and doctoral degree in Vibrational Spectroscopic techniques from the University of Kerala. Her research interests are nano biophotonics, Biospectroscopy and Bioimaging. She is the recipient of PSN National award for excellent R & D in rural technology based on her works on therapeutic application of lasers in rural area. She is also the recipient of the Endeavour Research Fellowship of Australian Government in 2010.