ORIGINAL RESEARCH ARTICLE

Discriminating Benign and Malignant Brain Tumors By Spectral Analysis of Cerebrospinal Fluid

ABSTRACT

The preliminary study is concerned with an innovative technique for the discrimination of malignant brain tumor from benign with reasonable sensitivity and specificity. The entire study is based on the relative fluorescence intensity and life-time of bio-fluorophores like tryptophan and tyrosine which are then validated as a set of ratio parameters. Resultantly, it was seen that malignancy was characterized by three-fold enhancement of tyrosine. The picosecond-time resolved decay time measurement was done, which exhibited two-fold decrease in decay time for malignant sample. The improved accessibility, with minimal invasiveness to the patient, and lower cost are encouraging aspects of this technique.

Key words: Cerebrospinal fluid (CSF), fluorescence spectra, time resolved spectra, malignant brain tumor, benign brain tumor.

INTRODUCTION

Tumors in the central nervous system and the brain represent approximately 1.8% of all newly diagnosed cancers in the world [1]. There are more than hundred different histologic sub-forms of brain neoplasms that present themselves in very diverse forms: either as a primary tumor, within the brain parenchyma or secondary brain tumor due to metastasis from other primary sources [2]. Brain tumors, belong to the category of most aggressive tumors and it is also the most common solid tumors in childhood brain tumors. Despite the fact that leukemia and brain tumor together account for almost more than half of all the pediatric malignancies and cancer deaths, recent data suggests that brain tumors are mostly underdiagnosed especially in low- and middle-income countries. As a result, the incidence of brain tumor tends to increase progressively, whereas the survival does not improve as much as the other cancers[3]. Furthermore, the nonspecific

pathognomonic features of pediatric brain tumors involving symptoms such as nausea, behavioral changes, head-ache, and seizures are often

misdiagnosed to be common pediatric conditions, hence leading to delayed diagnosis of the tumor[4–6].The clinical presentation varies based on age and tumor location, and due to the cellular heterogeneity in pediatric brain tumors, their classification becomes challenging.[7–9]Currently the pre-operative and post-operative diagnosis of brain tumor are performed by neuroimaging methods such as magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET), followed by tissue biopsy which is considered to be the "golden standard" method for brain tumor diagnosis as well as prognosis.[10]

Since some tumors have the tendency to infiltrate the tissue, recurrence even after surgical excision is possible and also pseudo-progression can occur due to treatment related necrosis. This makes the diagnosis and prognosis by classic anatomical imaging quite complicated. Since, MRI is suitable to monitor the size and recurrence of tumor but it is not feasible to follow-up with the molecular

discrepancies during tumor evolution. Despite the advances in neuroimaging, the conventional techniques such as infusion or diffusion imaging show unreliable accuracy in their performance and interpretation. Similarly, tissue biopsies can only reveal the molecular composition of the primary tumor obtained from the initial tumor resection, and prognosis remains unknown.

Since the brain biopsy cannot be performed frequently, efficient follow-up becomes certainly demanding.[11]These factors tend to complicate the precise diagnosis and longitudinal monitoring of brain tumor, hence leading to irreversible neurological abnormalities in children such as ataxia, epilepsy and endocrine dysfunction, despite complete cancer remission.[12,13]Hence, there is an indispensable need for a minimally invasive tumor molecular profiling that can be performed on a frequent basis. The implementation of liquid biopsies

for clinical diagnosis and longitudinal monitoring of pediatric brain tumor evolution will be a feasible solution, since, molecular studies

show remarkable specificity, rather than morphological studies.[11,14] Principally, proliferating tumor tend to shed tumoral content such as circulating tumor cells, proteins, amino-acids, cell-free nucleic acids, etc., the biomolecules are dispersed in a series of body fluids ranging from Cerebrospinal fluid (CSF), Blood and Urine.[15,16]

The CSF serves as the most suitable biological medium to detect any molecular anomalies in the central nervous system pathology. Since, the cerebrospinal fluid is produced in the intra-ventricular region of brain, it is simply a continuous extension of the Central Nervous System (CNS); hence it is the most proximal fluid to the intracranial tumor pathology.

Any alterations to the brain tumor and its microenvironment are directly comprehended by assaying the CSF proteome[17–19.] Considering its tendency to remain unexposed to the systemic circulation, and its lower volume compared to other bio-fluids, CSF remains as the optimum reporter of pathological and physiological changes within the

CNS. Also, the possibility of diagnosing brain tumor simply by CSF protein profiling even before neuroimaging was evidently demonstrated in a pre-symptomatic glioma induced rodent model. [20]Usually, cerebrospinal fluid is obtained by a physician while performing a bed-side procedure called lumbar puncture which involves removal of CSF sample from the sub-arachnoid space of the spine; this procedure is minimally invasive when compared to the tumor resection method for performing assays.[21,22]

The in vivo detection of cancer by analyzing CSF can be performed by understanding the tumor metabolomics of low-molecular weight metabolites and intermediate molecules which reflect the response to perturbations in the tissue pathophysiology. Such metabolomic profiling can overcome the inability to detect the entirety of an

invasive brain tumor, and hence elucidate the biochemical approach adapted in order to screen, and monitor the tumor progression.

Cancer cells usually exist within a complex micro-environment where there are several alterations in cell-surface structure and function due to the increased cellular proliferation. Such deviations are reflected by change in uptake of certain nutrients like amino acids, considering their vital role in energy generation, maintaining cellular redox homeostasis and most importantly they are significant for sustaining the proliferative drive of cancer cells.[23–25]Regulation of amino acid transport is thus important to sustain proper availability of nutrients, and cancer cells have to maintain adequate amino acid supply to put up with the proliferative drive.[24,26] In case of malignancy, there is a huge demand for amino acids to carry out cell division, protein synthesis and energy generation therefore the uptake and transport of certain amino acids increase in malignant transformation. Hence, amino acids are potential neuro-oncological biomarkers; this has been supported by a number of studies that employ radio-labelled amino acid tracers in PET scans, to diagnose brain malignancies with high intracellular amino acid metabolism; where the tumor grade is proportional to the tracer

uptake rate.[27,28] But due to lack of consistency of data and its inability to demonstrate cost-efficiency, this method is not widely used in clinical settings. [29]

Taking these detection deficiencies into account, high proficiency optical diagnostic methods are used to detect changes in biochemical and biophysical properties of tissues and determine its diseased state. Optical diagnosis has been performed by adopting a broad range of spectroscopic methods, wherein the specific optical spectrum of the sample tissue depicts the molecular and biophysical profile of the affected tissue. This biochemical information can be derived by Raman Scattering signals, Absorption or Fluorescence spectral signals.[30,31]

In this current study the essence of optical biopsy revolves around laser induced fluorescence spectroscopy, where a quantitative measure of the fluorescence intensity of the endogenous fluorophores aid in disclosing the pathological state of the affected tissue. Fluorescence is attained when an electron in ground state absorbs a photon of the appropriate

wavelength in the UV-Visible region and the electron then gets excited to a higher vibrational state; the electron then returns to the ground state accompanied by a spontaneous emission of radiation with retention of spin multiplicity; this radiation or luminescence is called fluorescence.

The mean time interval between absorption of a photon and the emission of fluorescence by the electron is called the fluorescence life time or decay time (τ).[32,33]Since, all biological tissues including brain and CSF contain endogenous fluorophores such as Tryptophan, Tyrosine, NADH, Flavins etc., when irradiated with light of appropriate wavelength, native fluorescence is exhibited. Quantification of the fluorescence intensity and the fluorescence decay kinetics, explicates the variations in electronic energy states of molecules within the tissue, and hence provides the biochemical evidence behind the cancer diagnosis.[34,35] Several researches have been performed to distinguish spectral features of the malignant tissues (Breast/Prostate/Lungs/Colon/Esophagus/Blood) from benign and

normal ones based on their excited state decay time of some important fluorophores in terms of emission, Stokes' shift and time resolved spectroscopy (TRS).[35–40] Laser induced fluorescence spectroscopy has even been used for a wide range of neuro-diagnostic applications such as amino acid detection in Huntington's disease but it has been neglected in brain cancer diagnosis.[41]

During malignancy the biological fluids present in the human body is sensitive to the biochemical or physiological differences in any tissue located within, hence alteration of biomolecules like amino acids in

CSF occurs much before patent manifestation in the brain tissue. In that case, laser induced fluorescence spectroscopy of CSF based endogenous fluorophores can serve as a tool of early diagnosis and prognosis.

In the current study, the diagnosis of brain tumor by distinguishing malignant from benign and normal, is performed on the Cerebrospinal Fluid, since it circulates via every portion of the brain, even in case of

focal malignancies and complex tumor location. Fluorescence spectroscopy and time resolved fluorescence spectroscopy measurements of two main fluorophores i.e., tryptophan and tyrosine were obtained since these fluorophores undergo significant changes with transformation into malignancy[27,42–45]. This study proves to be the beginning of new technique which might supplement the other available imaging techniques and gold standard histopathology, since it is less invasive, and it overcomes the shortcomings of pseudo progression, focal malignancies and delayed prognosis. And the entire process can be done within 60 minutes, unlike the other procedure.

METHODS AND MATERIALS

Patients and normal controls

Brain tumor patients and non -tumor patients were selected for this study from the Neurology department of Madras Medical College, Chennai, India. Before any procedure, the approval to perform the study had been obtained from the institutional ethics committee of Madras Medical College and the patients were informed about the investigation and formal informed consents were obtained.

CSF from the control, benign and malignant tumor cases were obtained by lumbar puncture; for the control group samples were obtained from cases that required spinal tap as a part of the procedure for other complications such as accidental head injury.

Instrumentation and analysis

The analysis was performed in a conventional Fluoromax-4 spectrophotometer (Horiba Jobin Yvon) with capability to obtain synchronous fluorescence emission spectra (SFES) or Stoke’sspectra

as propounded by Alfano, in the range of 200-600 nmof plain Cerebrospinal fluid (CSF) taken in a quartz cuvette.
Three types of spectra are common in the field of fluorescence spectroscopy. In fluorescence emission spectra (FES), one particular wavelength is selected for the excitation of a molecule, and the fluorescence emission spectrum is obtained by rotating the emission grating over a predetermined range. The reverse is true of fluorescence excitation spectra (FXS), in which the peak emission band of a

molecule is selected, and the excitation grating is rotated to scan the excitation spectra. Such FES and FXS are very useful for understanding the absorption and emission characterizing of a particular molecule.

In contrast, for getting the information about the relative concentration about a set of molecules in a composite mixture like blood, urine or CSF, synchronous spectra (or Stokes Spectra) is more reliable[48]. In synchronous fluorescence spectra (SFS), both gratings are synchronously rotated with a suitable offset of wave length to obtain the fluorescence band of every molecule in the predetermined range. The wavelength offset and scan range are not unique; they are determined empirically by trial and error for a given set of experimental protocols.
Fluorescence spectroscopy was performed on spectral domain (FSD) and time domain (FTD)for 12 samples of CSF each of 1ml by volume, obtained from 12 subjects. FSD was exclusively by synchronous fluorescence spectra of samples, employing HORIBA

spectrofluorometer and FTD with femtosecond (500fs) ultra violet laser pulses at 266 nm and other detection optics frequently used in ultrafast kinetic labs. The instrument response function for the TCSPC system was ~ 1 ns. The data analysis was carried out by using software provided by IBH (DAS-6), which is based on a

deconvolution technique using nonlinear least-squares methods. The quality of fit was evaluated by the reduced $\chi 2$, weighed residual and autocorrelation of the residuals.

RESULTS

1. **Results for Fluorescence Spectral domain (FSD)data**

Out of 12 samples one was from a subject (of age 23M) who was a normal control, who came to surgical ward due to accidental head injury from whom CSF was collected for other analyses and shown in Fig1.It may be considered to be the representative FSD of the control.It hasonly one peak at 360nm; which is due to the essential amino-acid tryptophan. Note the spectral width (full width at half maximum (FWHM) is 25 nm)

Then the **Fig 2** shows the typical spectral features (FSD) of 4 cases of hydrocephalus. It can be seen that there is a main peak at 360nm (like that of the control, due to tryptophan), 375nm due to an unidentified fluorophore (probably a fatty enzyme) and 450nm due to NADH . The FWHM for this spectra is = 50 nm.

Fig 3 shows the representative FSD of CSF of three patients with benign tumor (craniopharyngioma).The FDS of benign tumor shows features more similar to that of the control. That is, for the CSF of benign cases too, the main spectral peak is at 360nm and FWHM-= 35nm; in addition there is a weak secondary peak at 450nm (due to NADH)

Fig 4 represents the FSD of CSF of three malignant cases. It can be easily seen that in all the cases of malignancy there is a shoulder or a peak at 327nm. In fig 4 (a) there is a shoulder at 327nm with a ratio of intensity R= I327/I360≈0.7.

What is all the more conspicuous is the FWHM is 84 nm, indicating the merger of a hidden band with peak at 327nm. This hidden band becomes manifested in the next malignant sample Fig 4(b).In 4b,there is a distinct sharp peak at 327nm with R1 as 0.4 This is more or less true for another case of malignancy as shown in 4(c).Here R1= 0.6.

Here too there is a peak at 385 nm (unidentified) and a prominent one at 450 nm due to NADH.

The basic idea behind this line of investigation is that the biomolecules of any tissue go out of proportion when normal tissue becomes diseased. This is true for the conventional blood chemistry analysis of plasma of patients of diabetic mellitus or fluorescence spectral analysis of blood or urine components of cancers[37,39]

1 **Spectral domain results**

Considering all the above figures, a deduction could be made that malignancy is manifested by a new peak at 327 nm with an average

value of R_1=0.54 for malignant tumor cases; and R_1<0.2 for benign cases and controls. It is important mention that the peak at 327nm is most likely due to the amino acid tyrosine which is well documented to have fluorescence peak at 327[40].Hence the presence of malignancy could be detected by the enhancement of tyrosine in CSF about three times higher in comparison with the normal control or benign tumors.

It is important to note that tryptophan and tyrosine are two closely related biomolecules present in any cell or even in the plasma. In normal plasma, the ratio between tyrosine and tryptophan is 0.7 only. But, for acute lymphoblastic leukemia which is another most common pediatric malignancy[40] this ratio becomes 1.5. That is, tyrosine is enhanced about twice more in proportion to tryptophan as shown in fig 5. Note that the peak at 327 in this figure in the UV region is due to tyrosine; it is 1.5 times higher than that at 360 nm due to tryptophan. Spectra beyond 400 nm must be ignored: they are due to NADH and FAD present in abundance in blood plasma and almost absent in CSF.

It is important to draw attention to the fact that the regulation of amino acid transport is important to sustain proper availability of nutrients; and cancer cells have to maintain adequate amino acid supply to put up with the proliferative drive.[24,26] In case of malignancy, there is a huge demand for amino acids to carry out cell division, protein synthesis and energy generation therefore the uptake

and transport of certain amino acids increase in malignant transformation[42]. For any protein requirement tyrosine is the essential amino acid, a building block and also aromatic and hence becomes detectable in spectroscopies. It appears the secret of abnormal elevation of tyrosine as a malignancy biomarker lies here. In fact, in chemotherapy suitable formulation is administered to suppress tyrosine since the latter helps cell proliferation. [49,50]

2 Results of Fluorescence Time Domain data (FTDS)

With reference to the Table 1, it can be seen that the decay time has two components τ_2 and τ_3, with approximately equal amplitude ($B_2$and B_3). Attention is drawn to the $\tau_2$values :all the 8 cases out of 11 have a lifetime of 2.3±0.2 ns.In contrast, τ_2 for malignant case is 1.84, i.e., 21% smaller decay time. Focusing our attention toτ_2 alone, it can be seen that there is a definite decrease in lifetime of the excited state molecule, when malignancy occurs.

DISCUSSION

When a suitable photon interacts with a biomolecule, it absorbs the photon energy and goes to excited state (S1). From there it emits a photon of lower energy or longer wavelength (fluorescence spectrum) and then goes to the ground state (S0). The time it spends in the excited state is characteristics of the molecule and environment, as much as the fluorescence spectrum**.** Fluorescence spectral studies have two distinct windows of observation, namely emission spectra and life time of the excited states. The latter strongly depends upon the environment. When the excited molecule undergoes stronger solute – solute or solute- environmental interactions the life time decreases.

Such a decrease in the decay time is characteristic of malignancy in any tumors because overcrowding is the indication of malignancy[46]. For example, in the research paper on prostate cancer, the decay time constant, done with femtosecond laser pulse gave for (τ2) 124 ps for control,60 ps for benign and 42 ps for malignant prostate cancer[51,52] ;

for (τ3) 1134 ps for control,1103 ps for benign and 580 ps for malignant breast cancer[46].Unfortunately, such data are not available for CSF anywhere else for any comparison. In any case, significant reduction in lifetime of fluorophores may be characteristic of malignancy since it is indicated by the overcrowded growth of cells. Hence the biomolecules will easily get de-excited by stronger interactions among themselves and also by collisions, particularly so in a liquid medium such as the CSF.

CONCLUSION

In this short, preliminary investigation, done as a proof of concept, it has been shown that the malignancy of brain tumor could be detected by the spectral analysis of CSF itself. Since the CSF, circulates through the central nervous system, it gains close contact with the tumor tissue, and carries the biomolecules of interest. The fluorescence spectra and time decay data of the CSF could provide a wealth of information on the diseased condition of the tissue. It could become as diagnostic practice similar to monitoring of jaundice by colorimetric analysis of blood and urine of the patient

The fluorescent spectral analysis of CSF will open up new window of valuable information about the variety of brain diseases such encephalitis and malignancy. What has been seen so far is only the tip of an iceberg and a lot more needs to be done for detailed understanding. In any case, CSF now becomes a secret agent of important messages, from an impregnable Fort Knox!.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table1 shows an important set of data: the excited state lifetime as measured under femto-second pulsed laser excitation at 266nm.

CRITERIA	AGE/ GENDER	CONDITION	□2	□3	B2	B3
CONTROL	7 months/F	C.Hydrocephalus				
	2 months/F	C.Hydrocephalus	2.76	6.27	44.47	42.47
	2Y/M	O.Hydrocephalus	2.6	6.01	47.44	38.34
	1Y/F	Hydrocephalus	1.28	4.8	34.92	46.34
	8 months/M	Hydrocephalus	1.46	4.77	36.77	39.47
	23Y/M	Frontal bone fracture	2.44	5.84	45.34	38.36
BENIGN	51Y/M	Cranio -phayngioma	2.38	5.89	43.9	45.21
	48Y/M	Neurocytoma		5.15	34.75	45.94
	48Y/M	Pituitary adenoma	2.25	6.02	50.92	36
MALIGNANT	2Y/F	Medullary grade 4 embryonal tumor	1.84	5.5	43	44
	32Y/F	Medulloblastoma	-	-	-	-
	4Y/F	High grade glioma	1.7	5.8	58	42

FIGURE CAPTIONS

Fig 1 shows the typical spectral features (FDS) of CSF of a normal control

Fig 2 shows the typical spectral features (FDS) of CSF of 4 cases of hydrocephalus.

Fig 3 shows the representative FDS of CSF of three patients with benign tumors

Fig 4 (a), (b), (c) shows the FDS of CSF of three patients with malignant tumors

Fig 5 shows the representative FDS of plasma of patient of ALL taken from reference [53]

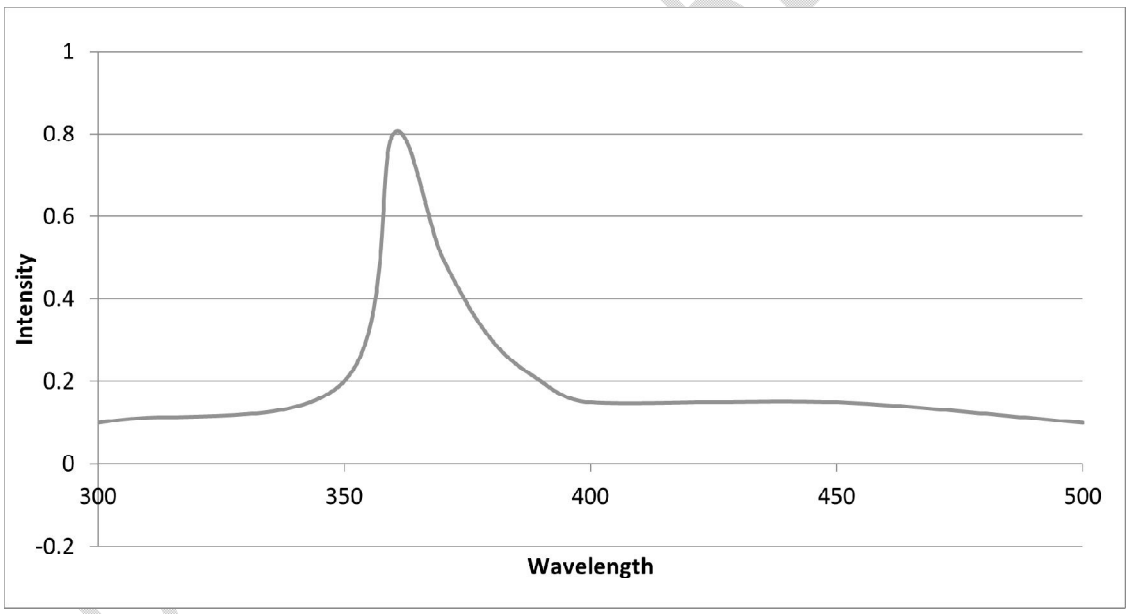

Figure 1

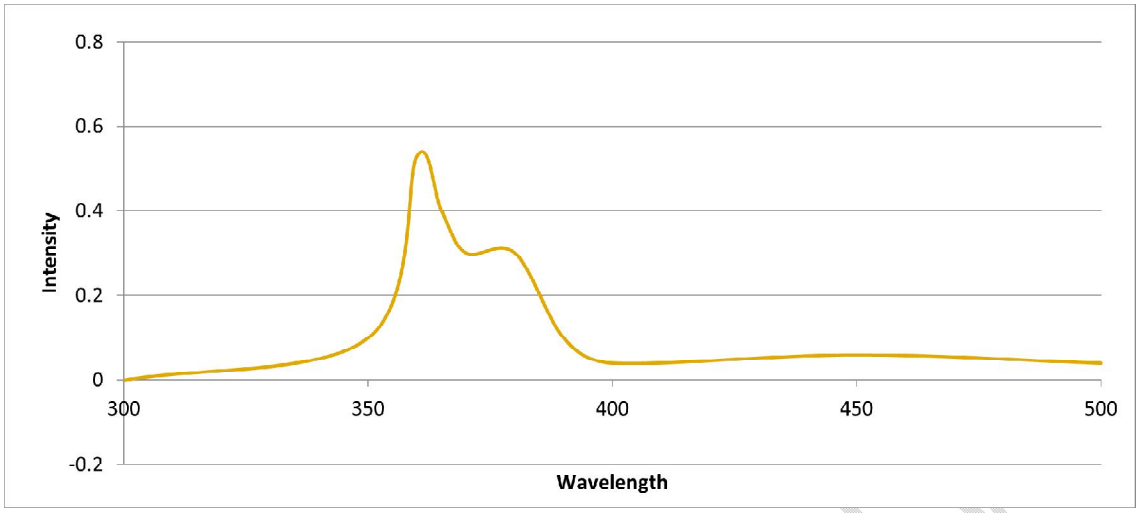

Figure 2

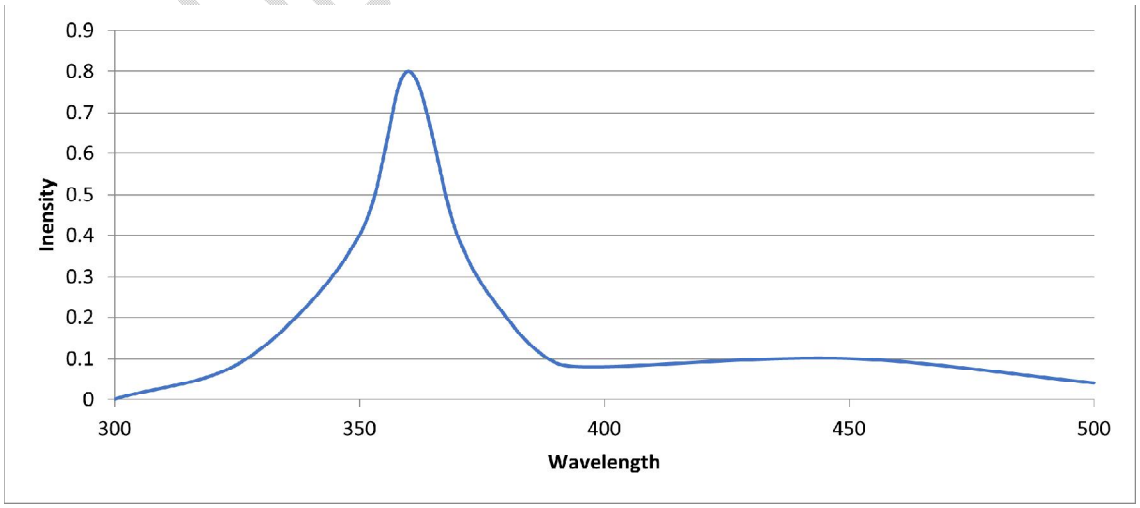

Figure 3

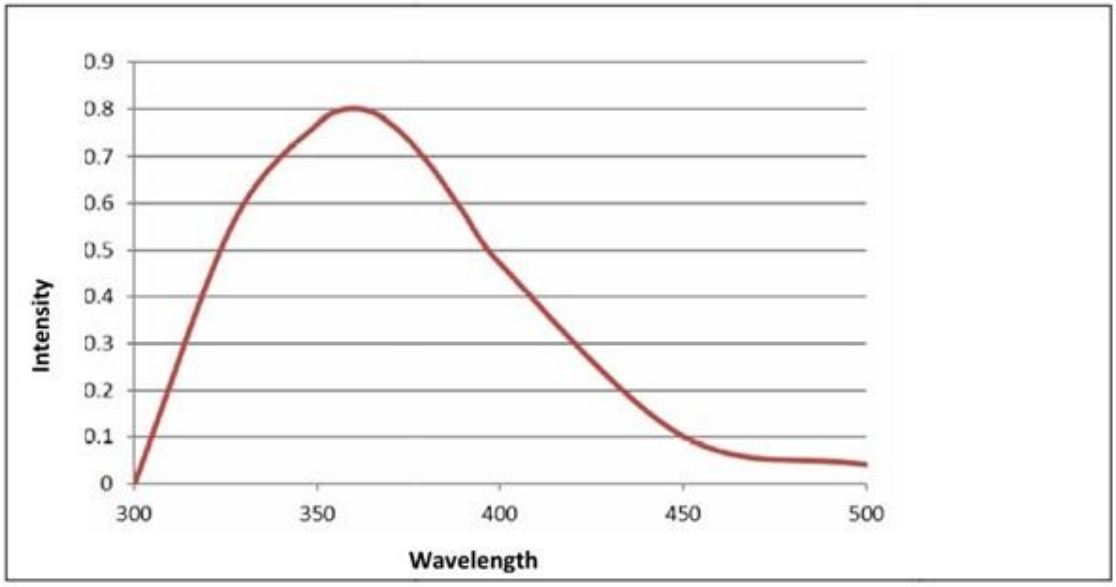
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Intensity
300
350
400
450
500
Wavelength

Figure 4 (a)

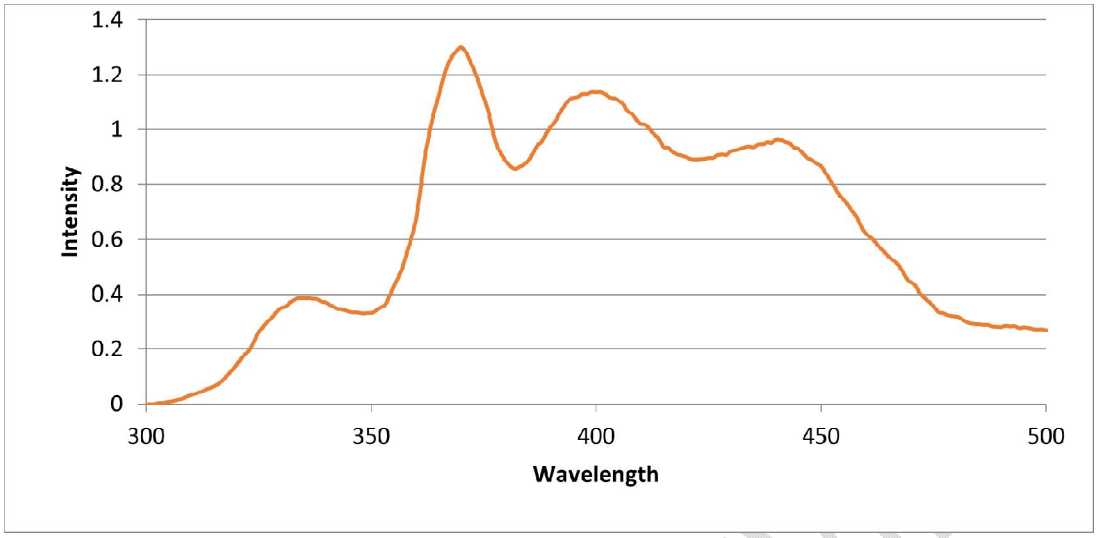

Figure 4 (b)

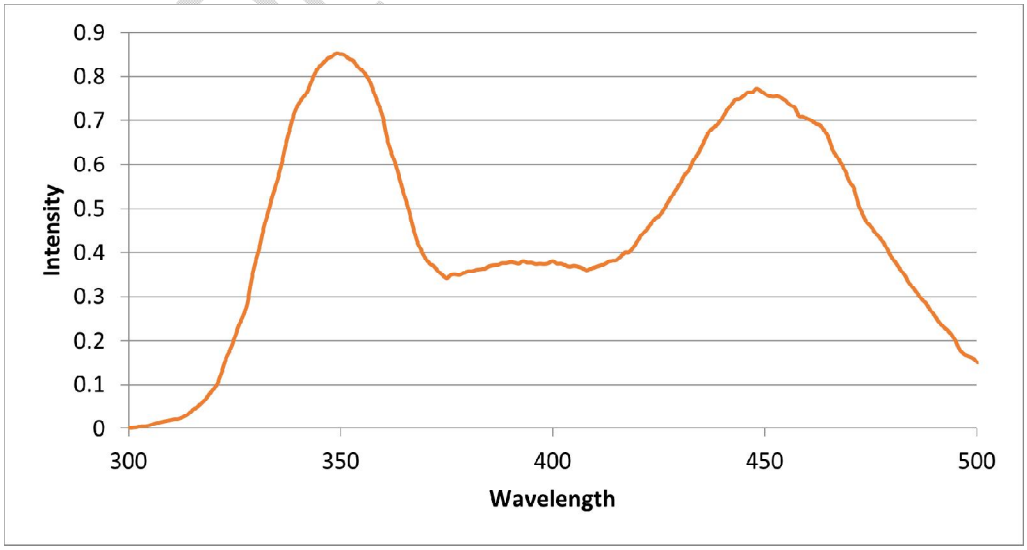

Figure 4(c)

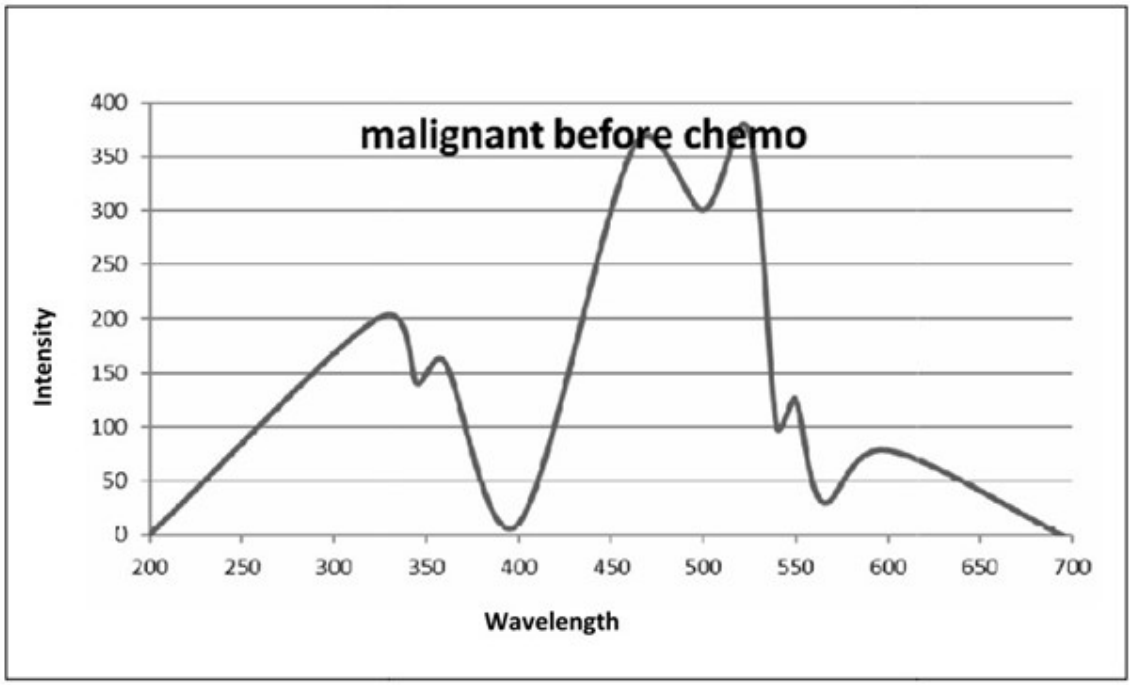
malignant before chemo
Intensity
Wavelength
400
350
300
250
200
150
100
50
0
200
250
300
350
400
450
500
550
600
650
700

Figure 5