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Optical Biopsy of Benign and Malignant Tissue by Time Resolved Spectroscopy

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Pathological condition of malignant tissue could be analyzed by spectral domain or time domain spectroscopy, the two being the complementary to each other in optical biopsy (OB) of cancer. This paper reports results of time resolved emission spectroscopy (TRS) of 24 excised tissue samples of breast and prostate (normal control = 12; benign = 4; malignant = 8), employing a 390 nm, 100 fs, Ti-Sapphire laser pulses.The fluorescence decay times were measured using streak camera and the resultant data were fitted for single and bi-exponential decays with reliability of 97%. Our results show the distinct difference between normal, benign and malignant tissues mostly due to the emission spectra of Nicotinamide Adenine Dinucleotide (NADH), Flavin Mononucleotide (FAD) and also due to the heterogeneity of micro environments associated with the diseased tissues. In this short report, fit is also shown that TRS of breast tissues are similar to those of prostate tissues.

Key words: Optical biopsy; Time resolved emission spectra; Malignant breast or prostate tissue; NADH and Flavin.

Introduction

Optical biopsy (OB) is a newly emerging technique to assess the different pathological conditions particularly of cancer, with tissue (1) or blood as sample targets (2). It is well known that histopathology of the excised malignant tissue is the ultimate decision maker. Yet it is quite invasive and stressful, particularly, when vital internal organs have become malignant. It is in this context the sophisticated imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI) are employed for diagnosis, or more often for follow up monitoring of cancer treated cases. Compared to the conventional excised tissue biopsy OB is non invasive as the probe laser beam can reach the target location directly as in the case of oral cancer, or through optical fiber, for example for lung cancer. Compared to CT or PET, the patients are not exposed to non ionizing radiation in OB. Another important advantage is OB can give information about the status of the lesion in real time with minimal stress as in the case of fluorescence endoscope for monitoring neoplastic Barrett's esophagus (3).

Optical biopsy has a broad scope , such as fluorescence spectroscopy (4), Optical coherence tomography (5), elastic and inelastic scattering spectroscopy (6), though only the first one has reached the clinical stage (7). Here again there

Abbreviation: NADH: Nicotinamide Adenine Dinucleotide; FAD: Flavin Mononucleotide; CCD: Charge-coupled Device; MRI: Magnetic Resonance Imaging; CT: Computed Tomography; OB: Optical Biopsy; TRS: Time Resolved Spectroscopy.

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are spectral domain and time domain investigations with one photon or two photon excitation (8). Another major step forward is the fluorescence imaging which has the potential clinical applications (9).

A number of research groups have reported their results done clinical settings with different approaches. Some of the most promising one are: time resolved mammography of 150 subjects (10), fluorescence imaging for prostate cancer diagnosis (11); differentiation between adenomatous and non adenomatous polyps (12); a recent paper by Meier *et al*. combining spectral and time domain decay data of head and neck cancer tissue (13). Employing N2 laser and dye laser as excitation sources, even high grade dysplasia, low risk tissue and carcinoma could be classified in Barrett's esophagus (14). They were discriminated in spectral and also in time domain.

When tissue goes out of control as in the case of cancer, the biochemistry and biophysics of the cells undergo significant changes. Some of the bio molecules of these changes, such as collagen, tryptophan (15), porphyrin etc are highly fluorescent. The difference in the concentration and distribution of these fluorophores could be monitored either by spectral features and/or by their excited state decay times. Such investigation could be done on tissues or body fluids (16).

A number of workers, particularly Alfano *et al.* have done pioneering work in this line, in characterization of normal and malignant tissues in terms of emission, Stokes' shift (17) and time resolved spectroscopy (TRS) (18). In a set of recent papers, they have been able to show distinct difference in the decay time of the normal and malignant prostate tissue. The present paper is a logical extension to look for the differences among normal, benign and malignant prostate tissue, and also see differences, if any, for a similar set of breast tissues.

Materials and Methods

Small piece of excised tissue of malignant, benign and normal tissue samples kept in frozen condition in dry ice were obtained from Cooperative Human Tissue Network of Philadelphia (CHTN), USA under IRB approval, The samples were unpacked and allowed to thaw slowly to room temperature (20°C) and then loaded as such, without any further processing, into the experimental holder which consist of a metal plate with clips to hold the tissue intact. The laser beam of less than an mm in diameter is allowed to fall on the tissue at 45°. The fluorescence and scattered radiation collected for each sample were allowed to pass through a band pass filter of 450 nm or 500 nm and get focused on the entrance slit of the streak camera which captures only the fluorescence signal from the tissue.

Time resolved spectra were taken for 24 excised samples, out of which were 12 breast samples (normal 4, benign 2, and malignant 6) and 12 prostate samples (normal 8, benign 2, and malignant 2). In order to reduce the error arising out tissue handling and transportation, normal and benign or normal and malignant section of the same patients were selected. We were able to get only four such sets for breast tisuues (2 normal and 2 benign, 2 normal and 2 malignant) and another four such for prostates. For each tissue four data were collected for different position of tissue. Thus we had 32 sets of data for breast tissue and similar 32 set of data for prostate tissue. The mean and standard deviations were reported only for these data (see Tables).

The experimental set up used for this work was similar to one reported earlier. In brief, 780 nm and 100 fs laser pulses from mode locked Ti sapphire laser (Coherent Mira 900), with pulse repetition rate (PRT) of 78 MHz was allowed to get frequency doubled and split into two beams, one intense and other weak. The intense beam was allowed fall on the sample to excite fluorescence signal. The weaker one was used to reach a photodiode, the electrical signal of which was used to trigger and synchronize the streak camera. The streak camera has time resolution of 20 ps and the fluorescence decay was captured on charge-coupled device (CCD) of spectral resolution 20 μm/pixel. The unpolarised fluorescence emission signals from the tissue were collected through 500 nm or 450 nm band pass filter. The fluorescence decay curves were analyzed for single exponential and bi-exponential with the inbuilt software.

Results

The main objective of the paper is to distinguish spectral features of the malignant prostate/breast tissue from benign and normal ones based on their excited state decay time of some important fluorophores.

It is well known that tryptophan, collagen, Nicotinamide Adenine Dinucleotide (NADH), Flavin Mononucleotide (FAD) and porphyrin are the crucial bio molecules acting as fluorescent bio molecules of malignancy (12). Out of this, tryptophan needs to be excited at 300 nm at which femto second laser pulses are not easily available. Collagen, with excitation band around 320-360 nm, gets easily swamped by the strongly fluorescent NADH (with excitation ranging from 360 to 400 nm). Further, FAD is accepted to be well elevated in almost all cancer tissue and this has a strong absorption band ranging from 380 nm to 470 nm, with a peak at 450 nm. Hence we chose to excite the tissue at 390 nm so that we could excite both NADH and FAD. It is important to note that at this wavelength, porphyrin also could be excited, But the signal to noise ratio, as monitored at 620 nm, was too poor to consider any further.

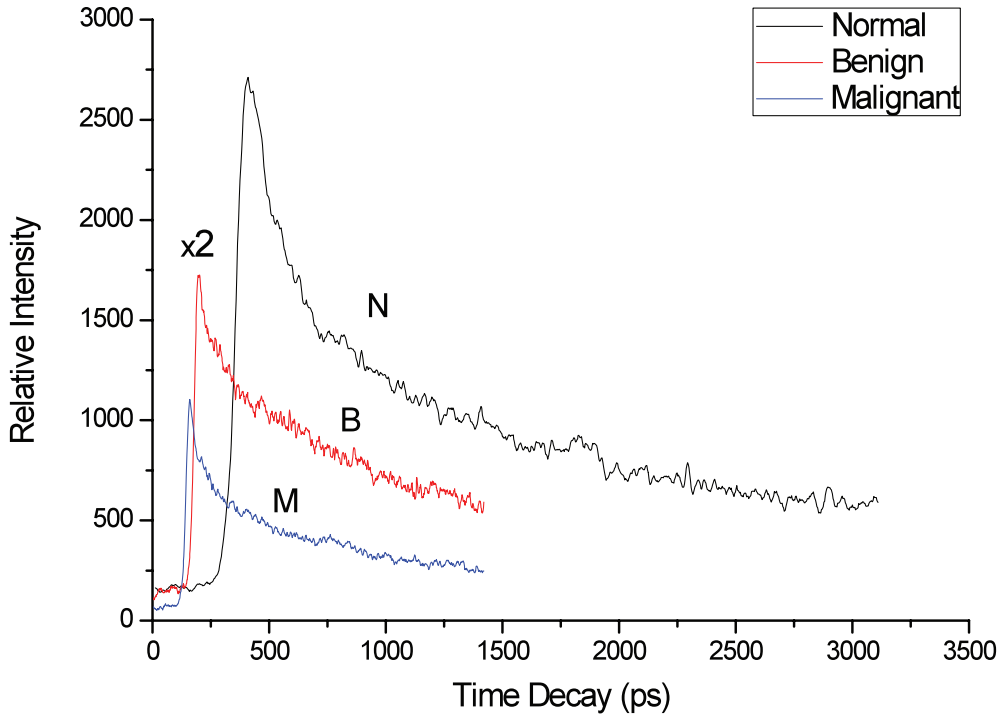

Figure 1: Time resolved fluorescence intensity profile for normal, benign and malignant breast tissue samples, as monitored with a 500 nm band pass filter. The profiles were quite similar when monitored with 450 nm band pass filter, but less in intensity.

For each tissue sample, TRS were obtained from four different sites and the average of the time decays were obtained. It is important to mention here that the amplitudes of signal varied many fold for different sites, but the decay constant, only marginally. Hence, as in the case of fluorescence emission spectroscopy, the actual intensity of one sample to another are NOT to be compared which depends upon many instrumental and sample artifacts; but the decay time constants, the intrinsic parameters of the sample, only are be considered Figure 1 shows the time resolved fluorescence intensity profile for normal, benign and malignant breast tissue samples, as monitored with a 500 nm band pass filter. The profiles were quite similar when monitored with 450 nm band pass filter, but less in intensity.

Table I shows the single exponential time decay constant for breast tissue for 500 nm and 450 nm intensities. It can be seen that all the three types of tissues fall into distinct category and that the decay constant measurement by 500 nm or 450 nm do not differ much. That is, the malignant tissue has 271ps for 500 nm and 265ps for 450 nm window respectively.

Table I

Single exponential time decay constant (in picoseconds) for breast tissue as monitored at 500 nm and 450 nm.

Window	Normal	Benign	Malignant
500 nm	551 ± 7.2	535 ± 13.6	271 ± 6.3
450 nm	563 ± 8.5	465 ± 9.3	264.5 ± 4.8

As our main interest in this paper is to distinguish the three type of tissues, a contrast ratio parameter is defined as $R_1 = (\tau_{bb}/\tau_{mb}) = 2.13$ for 500 nm window and 1.76 for 450 nm window. It is the ratio between the lifetime of the benign breast tissue and the malignant one. Note for all the above evaluation of time decay constants, R, the reliability factor of curve fitting, varied from 0.78 to 0.95.

In order to get better accuracy and contrast, we tried bi-exponential fit, which gave $R > 0.98$ for all. The results (as monitored at 500 nm) are presented in Table II. The bi-exponential fit showed that the fast component has a major contribution and the slow component has only a minor contribution. For example for breast tissue normal, the fast component showed 123 ± 18 ps with amplitude of 46793 and the minor with 1135 ± 168 ps with amplitude of 2506. That is, the major fast component is 18 times stronger in contribution than the minor slow component.

It is to be noted that the fast component is dominant in all (normal, benign and malignant tissues) and all the above fall into three distinct categories. We define a contrast parameters $R_2 = (\tau_{NB}/\tau_{MB}) = 2.67$ and $R_3 = (\tau_{NB}/\tau_{MB}) = 1.98$ and

Table II
Bi-exponential time decay constant (in picoseconds) for prostate and breast tissues as monitored at 500 nm.

	Prostate		Breast	
Tissues	τ_1 (Major fast component) (ps)	τ_2 (Minor slow component) (ps)	τ_1 (Major fast component) (ps)	τ_2 (Minor slow component) (ps)
Normal	124 ± 10 (A1 = 73726)	1177 ± 210 (A2 = 5251)	123 ± 18 (A1 = 46793)	1135 ± 168 (A2 = 2506)
Benign	60 ± 15 (A1 = 15281)	773 ± 111 (A2 = 1176)	62.5 ± 8.2 (A1 = 5392)	1103 ± 125 (A2 = 613)
Malignant	42 ± 8 (A1 = 1076)	610 ± 58 (A2 = 188)	46.1 ± 8.2 (A1 = 11122)	580.5 ± 68.3 (A2 = 463)

$R_4 = (\tau_{BB}/\tau_{MB}) = 1.34$. Here R_2 is the contrast parameters between the fast decay component of normal breast (NB) and that of the malignant breast tissues (MB), similarly R_3 is between normal and benign and that R_4 between benign and malignant.

Similarly contrast parameters could be obtained comparing the slow components too. However, these would not be discussed further since the fast decay components gave better contrasts.

The experiments and analyses were repeated for a set of normal, benign and malignant prostate cancer tissues and the results are presented in Table II. It can be seen that the decay constant are very similar. That is, the normal breast tissues and prostate tissues both have similar decay time constant (123 ps and 124 ps respectively). Similarly the benign tissues of both fall into another category (62 and 60 ps; and the malignant tissues of both fall into yet another category (46 ps for breast malignancy and 42 ps for prostate malignancy).

Discussion

Cancer biomarkers are perennial quest for scientist and medical professionals, because they could give valuable information about the cause of cancer and also in progression or regression. Most of the investigations in this line are in terms of genomics, proteomics and metabolomics Out of a set of bio molecules indicative of cancer, only a few of them are fluorescent. The essence of OB is based on the set of fluorescent bio molecules going out of proportion due to abnormal biochemical and biophysical features initiated by cancer. It is important to note that in most cases of malignancy no new fluorescent biomarker is observed: the biomarkers already present in normal conditions go awry in tissue transformations.

In this context, the spectral features of bio molecules such as tryptophan, FAD *etc*. are measured through Fluorescence Emission or Stokes shift spectra (14) or monitored by time decay constants. The bio molecules, identified and quantified by spectral domain or time domain contribute two complementary windows to look at the tissue alterations.

The paper presented here is one of the series of reports to characterize different tissues. In this short report presented here we have been able to show that bi-exponential fit of collected data exhibiting better contrast between benign and malignant tissues. Also it could be seen that both normal (breast, prostate) have almost same decay time of about 125 ps and the malignant ones around 45 ps the benign ones around 62 ps. That is, from the TRS point of view one cannot discriminate malignant breast tissue form malignant prostate tissue. Similar arguments hold good for normal as much as benign tissues. On the other hand, the malignant breast tissue can be very well distinguished from normal breast tissue, since the contrast is about 300%, but, the contrast between the benign and malignant tissue is 50%. Similarly arguments are true for prostate tissues too.

The causes for such differences are rather difficult to postulate at this juncture; however, it looks like the inhomogenities and random orientation of the malignant tissues act as major scattering centers and this could reduce the excited state life time of the fluorophores. This is borne out by the fact that whether we monitor the emission at 500 nm or 450 nm for a particular tissue (say normal of breast), the decay times are about the same. In an earlier report by the same group , with excitation at 800 nm, with 100 ps pulse on prostate tissue and observation at 830 nm single exponential decay time was 311 ps as for cancer tissue and 517 ps for normal tissue. That is, the cancer tissue has 1.7 times shorter decay time than normal. This is to be compared with our results presented on breast tissue, with excitation by 390 nm laser and fluorescence being monitored at 500 nm. In our case the normal breast tumor has decay time of 551 ps and malignant breast tissue has 271 ps In an important paper based on Barrett's esophagus surveillance [???] the researchers have observed decay times in nanoseconds employing nitrogen and dye laser for excitation. For them also carcinomatous tissue has 45% decreased lifetime than the low risk, normal tissue (1 ns versus 1.5 ns). Another paper with a similar approach (337 nm, 700 ps pulses) on neck carcinoma has reported 1.7 ns for normal and 1.3 for malignant tissues.

One possible reason for such differences could be that our studies were based on excised, *in vitro* tissues and the last cited two were done *in vivo* clinical setting. May be this could be the reason that our samples have lifetimes in picoseconds time ranges and theirs in nanosecond ranges.

That is, the distinct difference between normal and malignant come not due to the intrinsic fluorescence decay of the fluorophores but mostly from the environment in which they are placed. It is the heterogeneous scattering centers which play more prominent role. This ties up well with the decay times of benign tissues which have values intermediate between normal and malignant. A benign tissue is overgrowth of the normal tissue, but it is still organized like the normal and not disorganized like the malignant and hence has intermediate values.

Conclusion

In this report on picoseconds TRS we have been able to show very good contrast between the normal and benign, normal and malignant, and benign and malignant tissues of breast and prostate. In all cases, malignant tissues have life time about 45 ps, benign about 60 ps and normal about 120 ps. All these have been done *in vitro*; only after an extension in the *in vivo* condition, as few others have done, the clinical advantage of this technique could be appreciated. Such study is to be done soon and this it could prove to be a non surgical OB of tissues.

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Conflict of Interest Statement

Nil.

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