Laser Induced Fluorescence Spectroscopy from Native Cancerous and Normal Tissue*

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Abstract—**The visible fluorescence spectra have been measured from cancerous and normal rat kidney and prostate tissues, and from cancerous rat and mouse bladder tissues. The spectral profiles from the cancerous and normal tissues are substantially different; showing characteristic principal and secondary maxima. These peaks are assigned to fluorphors such as flavins and porphyrins in different environments in the two types of cell media.**

INTRODUCTION

FOR over 50 years, luminescence and absorption spectroscopy has served as a useful and important microscopic probe to characterize various physical and chemical processes in materials. Utilizing luminescence and absorption, chemists, biologists, and physicists have acquired a great deal of fundamental knowledge about the molecular structure and energy transferring mechanisms of materials [1], [2]. Chemists investigate emission and absorption properties of organic molecules such as dyes to obtain information about the radiative and nonradiative processes. Physicists investigate luminescence and absorption in semiconductors, and impurity doped dielectrics for possible laser sources and optoelectronic devices. Biologists, on the other hand, utilize luminescence and absorption to understand the dynamics and constituent make up of large complex organic molecules. The majority of the molecular systems investigated are made out of proteins, nucleic acids, and lipids, with fluorescing and nonfluorescing chromophores. Such systems are known to predominantly luminesce in the ultraviolet (UV) and visible spectral region [3], [4]. By measuring the luminescence and absorption spectra of a system one can characterize its physiological state in relation to a normal system.

Fluorescent dye stains for complex molecular systems have enabled researchers to obtain information about conformation changes in muscle and nerves, polarity of the surrounding environment, secondary structure of the DNA and RNA, and dynamical conformation of molecules in membranes [5]. Hematoporphyrin derivative (HPD) is currently employed as a fluorescent marker for cancer detection, as well as a photosensitizer of tumors for photoradiation therapy. The ability of tumors to preferentially absorb and retain HPD have made this an effective mode of therapy [6], [7]. However, such extrinsic fluorescent markers present themselves as foreign agents, and they are known to interact with the normal native cellular environment [7]. Hence, there arises a need to develop new microscopic techniques to detect pathological changes such as malignant tissues without interfering with the normal tissues.

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There are limited data reported on the native luminescence and absorption properties of different types of tissues. Fluorescence from the cellular macromolecules such as proteins, nucleic acids, and free nucleotide coenzymes had been studied in the late 1950's and early 1960's [8]. It was discovered that the fluorescence spectra of intact cells coincided with tryptophan containing proteins. The most intensely fluorescing component in the cell was found to be the mitochondrion. This is attributed to the proteins bound to respiratory coenzymes in mitochondria. Duysen [9], in the late 1950's, examined the fluorescence from reduced diphosphopyridine nucleotide to follow oxidation-reduction changes within cells. Chance and Baltscheffsky [10], using microfluorometric techniques, where able to follow changes in the oxidation state of pyridine nucleotides in mitochondria. Shudel, and, at the same time, Konev and Lyskova, reported that changes in the UV fluorescence spectra of mitochondria arose from the structural changes within the mitochondria which they had been artificially induced [8].

This leads us to believe that UV fluorescence spectroscopy may be a promising technique that could be used as a diagnostic as well as a monitoring tool for researching the physiological state of cells. One does not necessarily have to confine oneself to the UV region. There are natural fluorophors within the cells which fluoresce in the visible regions. Flavins, for instance, are known to fluoresce and exhibit spectral changes when transforming from the oxidized to the reduce state. Riboflavins are known to fluoresce in the visible spectrum and are part of the coenzyme flavin adenine dinucleotide which are responsible for oxidation-reduction in the mitochondria. Chance *et al.* [11] demonstrated flavin fluorescence in mitochondria of pigeon heart cells with the fluorescence peaked at 522 nm. It has been shown that the spectral maxima of flavins span from 520 to 535 nm depending on the environment [12]. Furthermore, porphyrins have been found in nature to be highly fluorescent. Naturally occurring porphyrins are found in hemoglobin, urine, stool, and in erythrocytes. The principal porphyrins fluorescence bands lie between 597-634 nm [3]. More recently, we have observed the fluorescence in the visible region from keratin and melanin. The emission maxima from these macromolecules are also found to be in the visible region located at 525-540 nm [13]-[15].

In this paper, we report on the visible luminescence spectra from native animal tissues. We show that the spectral character of the emission from cancerous and normal tissues (kidney, bladder, and prostrate) are different. Furthermore, there appears to be well-defined spectral fingerprints characterizing both the cancerous and normal tissues. We hope that such a salient difference will provide a stimulus to develop a research program for further research in the visible spectral region for use as an *in vivo* cancer diagnostic tool.

Materials and Methods

The experimental arrangement used to measure the luminescence spectra from the various tissues is shown in Fig. 1. A 100 mw Argon ion laser operating at 488 nm was focused on the front surface of the tissue to a spot size of about 100 μm. The luminescence from the front surface was collected into a double Spex-1/2 m grating scanning spectrometer blazed at 500 nm. A photomultiplier tube (PMT) RCA 7265 (S-20) located at the exit slit of the spectrometer measured the intensity at different wavelengths. The spectral resolution was 1.8 nm. The output of the PMT was connected to a Princeton Applied Research lock-in recorder combination to display the spectrum. Both the laser and reference signal were chopped at 200 Hz. The spectra were not corrected for the spectral response of the system. Each sample emission spectrum was run three times for reproducibility. The measured spectra were stable in time and different regions yielded similar spectra.

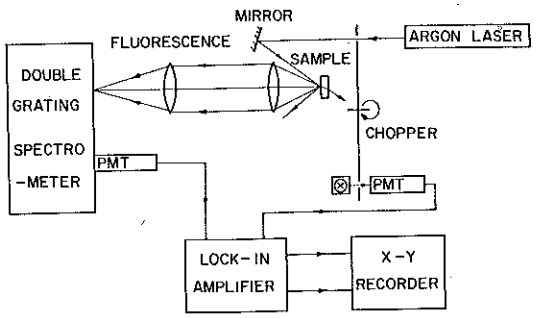

Fig. 1. Schematic diagram of the experimental setup.

The luminescence emitted from cancerous and normal tissues from rat prostate and kidney were investigated. The spectra from a rat female bladder tumor and a mouse bladder tumor were also measured. All tumors were subcutaneously implanted. Rat prostate tumors (R 3327 C.P.) were implanted in Fischer/Copenhagen male (F_1) rats and were five weeks old at the time of the testing. Rat kidney tumors were implanted in Wilson/Lewis rats and were four weeks old. Rat bladder tumor (TCT-4909) was implanted in a female Fischer rat and was four weeks old at the time of testing. Mouse bladder tumor (MBT-2) was implanted in a female C_3HHe mouse and was also four weeks old. All tissue samples were nonnecrotic, clean free and approximately 1 gm in weight. All tissue samples were solid chunks not cut to any particular specificity, and were few millimeters thick. Each tissue sample was placed in a clean pyrex test tube for these luminescence studies.

Results

The spectral curves for the cancerous and normal tissues are displayed in Figs. 2-4. One notices the differences in the spectra between the normal and cancerous tissues. The prominent maxima in the spectra from rat prostate tumor [Fig. 2(a)] and rat normal prostate [Fig. 2(b)] are located at 521 and 533.5 nm, respectively. The prostate tumor spectrum has two subsidiary maximas located at 552 and 593 nm while no additional maxima are recorded in the normal prostrate spectrum. In the prostate tumor spectrum there are four points of inflections located at 538.3, 571.7, 587.0, and 619.5 nm. On the decreasing side of the normal prostate curve there are two points of inflection located at 571.7 and 603.3 nm, as shown in Fig. 2(b).

The main maxima in the spectra from male rat kidney tumor [Fig. 3(a)] and normal male rat kidney [Fig. 3(b)] are also located at 522.0 and 530.6 nm, respectively. After the first prominent peak, the spectrum from the rat kidney tumor decreases monotonically and there are three small peaks located at 592, 612, and 638 nm. Along this declining side of the

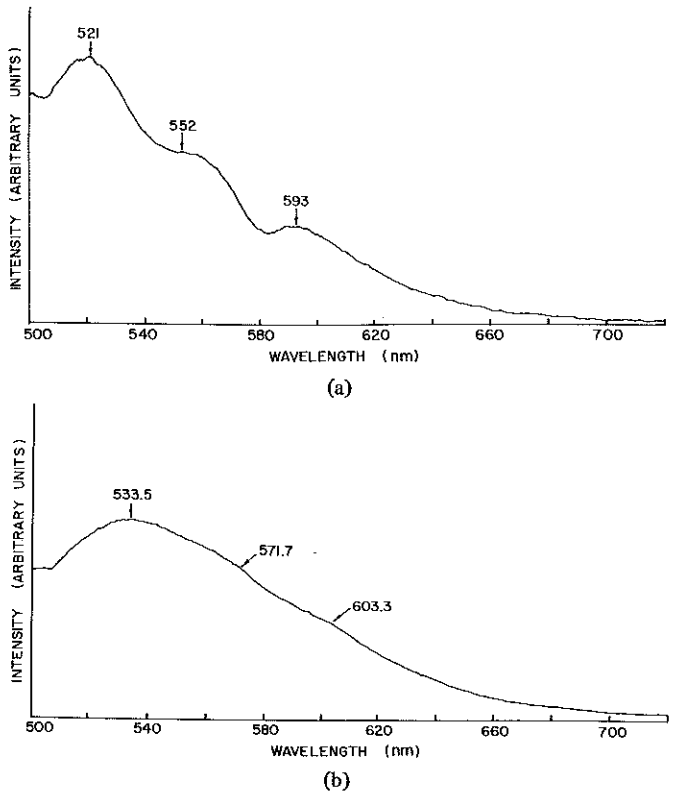

Fig. 2. (a) Fluorescence spectrum of rat prostate tumor. (b) Fluorescence spectrum of normal rat prostate.

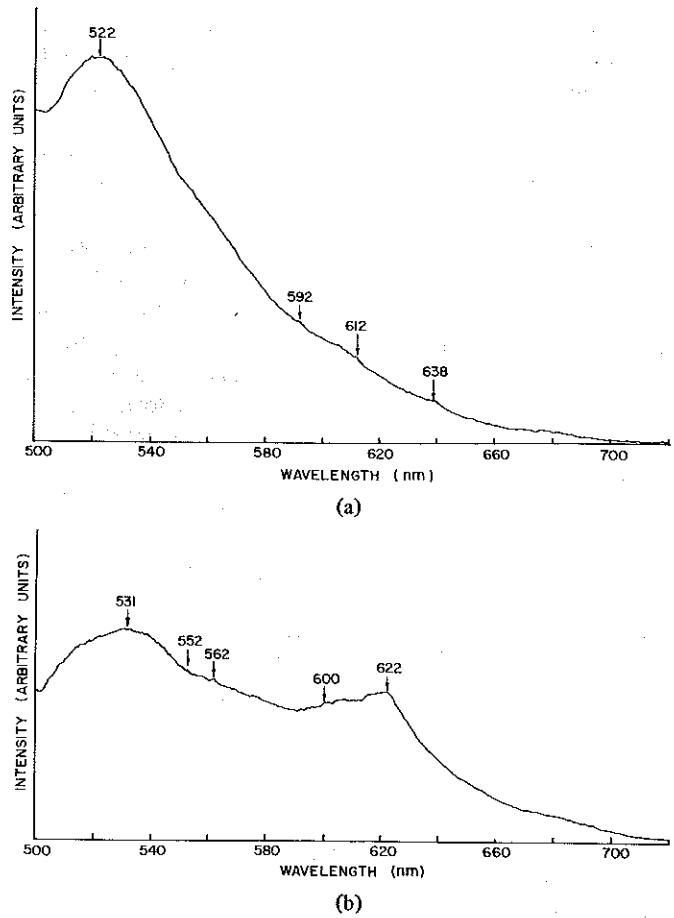

Fig. 3. (a) Fluorescence spectrum of rat kidney tumor. (b) Fluorescence spectrum of normal rat kidney.

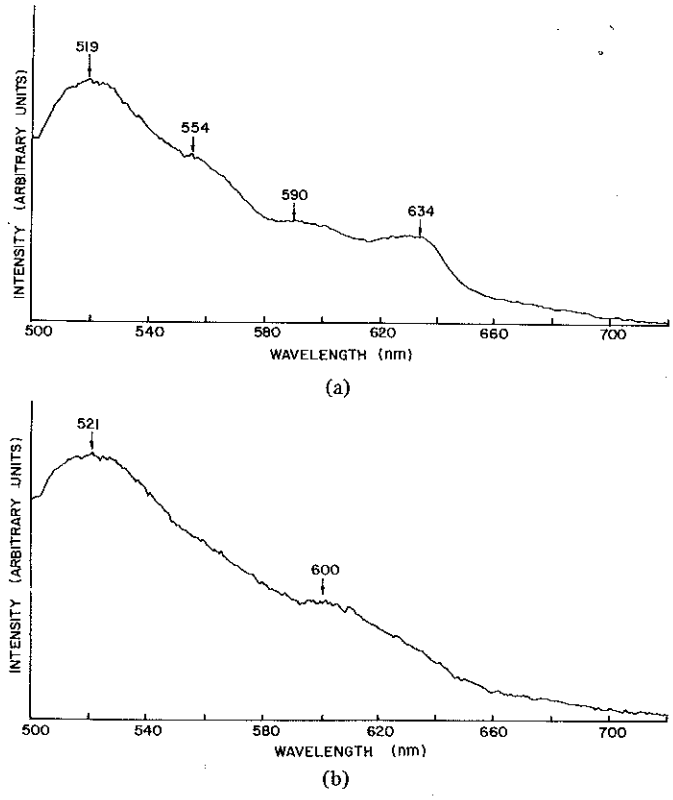

Fig. 4. (a) Fluorescence spectrum of rat bladder tumor. (b) Fluorescence spectrum of mouse bladder tumor.

curve there are four inflectionary points located at 548.7, 559.3, 581.3, and 604.2 nm. However, after the first prominent peak for the normal male kidney, the spectrum declines monotonically until it reaches a wavelength at 590.8 nm where it starts to increase. Along the declined portion of the curve there are three smaller peaks located at 562, 600, and 622 nm. The spectrum also contains three inflectionary points located at 552 and 595 nm.

The salient features of the rat bladder tumor spectrum are its four peaks [Fig. 4(a)]. The first prominent peak is located at 519.1 nm: other smaller peaks are located at 554, 590, and 634 nm. The spectrum also contains two inflectionary points located at 567.0 and 605.2 nm. After the minima at 614.7 nm the curve starts rising to the last peak at 634.0 nm, after which there is a fall off to zero intensity.

The salient features of the mouse bladder tumor spectrum are its two wide peaks [see Fig. 4(b)]. The first prominent peak is located at 521.0 nm, and the other at 600.0 nm. The spectrum starts declining from 610 to 648 nm after which its slope changes and decays slowly to zero. There are two points of inflection in the spectrum, one located at 559.3 nm and the other at 648.2 nm.

The summary of the results from the fluorescence measurements are displayed in Table I. The salient features that are found in common among the tumor spectra are as follows.

1) Locations of the prominent maxima of the tumor spectra all occur at about 521.0 nm.
2) The width of the prominent maxima are virtually the same, approximately spanning 15 nm.
3) Secondary peaks which are in common to all tumors occur between 590–600 nm.

TABLE I
CHARACTERISTICS OF THE FLUORESCENCE SPECTRA FROM TISSUES

Fig.	Spectrum of	Number of Peaks	Prominent Maxima	Secondary Maxima Locations in nm			Points of Inflection
				α	β	γ	
2(a)	Rat Prostate Tumor	3	521.0 nm	552	593	–	538.3 nm, 571.7 nm 587.0 nm, 619.5 nm
2(b)	Rat Normal Prostate	1	533.5 nm	–	–	–	571.7 nm, 603.3 nm
3(a)	Rat Kidney Tumor	4	522.0 nm	592	612	638	548.7 nm, 559.3 nm 581.3 nm, 604.2 nm
3(b)	Rat Kidney Normal	4	530.6 nm	562, 600, 622			552 nm, 595 nm
4(a)	Rat Bladder Tumor	4	519.1 nm	554	590	634	567.0 nm, 605.2 nm
4(b)	Mouse Bladder Tumor	2	521.0 nm	600	–	–	559.3 nm, 648.2 nm

4) The secondary peak which is also in common with the rat prostate tumor and the rat bladder tumor is in the range of 552–554 nm.
5) The secondary peak which is also in common with the rat kidney tumor and the rat bladder tumor fall in the range of 634–638 nm.

Upon analysis of the data between the two normal spectra, one recognizes the prominent maxima are located at 530–533 nm and the width of the prominent maxima are broad, each spanning 38 nm.

The most salient differences between the cancerous and the normal tissues are that the spectral profiles are very different and that the cancerous prominent maxima are blue shifted and located around 521 nm, whereas the prominent maxima of the normal tissues spectra are located at about 531 nm.

DISCUSSION

Visible luminescence spectra emitted by natural fluorphors, within the intact cells, are most intriguing and clearly display a unique set of spectral features which characterize the state of cells making up the tissues. In view of our observations, one could designate the 521 nm wavelength of prominent peaks as the wavelength which is indicative of cancerous tissues. Similarly, one can designate the prominent peaks of the 531 nm wavelength as the characteristic spectral fingerprint indicative of normal healthy tissues. Such salient differences in the spectral profiles of malignant and normal tissues are most likely attributed either to changes in the environment of the fluorphors, or perhaps production of new fluorphors induced by the changes within the cellular environment. It is well documented that the internal environment of cancer cells are physiologically and biochemically different from their normal counterparts. Depending on the environmental conditions flavins are known to fluoresce between 520–535 nm. If no new fluorphors are produced (that fluoresce between 520–535 nm) due to the environmental transformations, then one may conjecture that the most likely fluorphors giving rise to our spectral signatures must be flavins in the mitochondria. Recently, keratin was found in thymona tissues. The fluorescence spectra from keratin are known to be broad with maxima located at 525 nm [15]. Hence, it is probable that keratin emission may have contributed in part to our observed spectra.

When protein containing fluorphors either gain positive charge ions or lose negative charge ions the fluorescence from the fluorphors have been noted to be blue shifted. The prominent maxima of all cancerous spectra exhibit in our results a 10 nm blue shift, suggesting an accumulation of positive ions, or a depletion of negative ions in the mitochondria of cancerous cells, thus causing the flavins to emit at 521 nm instead of 531 nm. Various mechanical as well as biochemical factors may be responsible in narrowing the width of the prominent peaks which are observed in all tumor spectra. The possibility that the visible emission arises from phosphorescence via a two step excitation process is ruled out here due to a measured linear dependence of the luminescence intensity on laser pump intensity on all spectra.

The secondary peaks which are in common to all tumor spectra between 590–600 nm may be attributed to the porphyins. Heme proteins containing metalloporphyrins are commonly found within hemoglobin and cytochrome in the mitochondria. When iron or copper are removed out of the heme prophyrins, the porphyrins are known to fluoresce in distinctive principal and secondary bands. Depending on the environment the principal band of various porphyrins lie between 597–634 nm. In an acidic environment coroporphyrin and protoporphyrin have their bands at 597 and 603 nm respectively. In organic solvents their respective principal bands are at 625 and 634 nm. In view of these results, all tumor spectra display signatures that may arise from free porphyrins. This would suggest that the environment within the cancerous cells may be appreciably transformed to such a degree that the

metal components dissociate from the porphyrin part yielding a high characteristic fluorescence of various porphyrins indicative of their regional environment. However, it is fair to point out that while visible necrotic tissues had been removed from the tumor samples, small residual necrotic region(s) could have contained small pools of enzymatically reduced heme proteins which could have contributed in part to our observation. The peak assignments require additional studies in cells and tissues in order to make firm conclusions about the origin of the luminescence.

Conclusions

The visible luminescence spectra from native cancerous and normal tissues have been measured. The emission spectral characteristics of the cancerous and normal tissues are found to be substantially different, each displaying their own characteristic prominent maxima and other spectral marks. Currently, research in this area is in its embryonic stage and further research is warranted to check the consistency and reproducibility of these characteristic spectral fingerprints so that they may hopefully be utilized as a viable diagnostic tool in cancer research. One could then envision an optical fiber spectroscopy instrument [16] for cancer diagnostic which offers both high speed and spatial resolution for *in vivo* and surgical use.

References

[1] P. Pringsheim, *Fluorescence and Phosphorescence.* New York: Interscience, 1949.

[2] J. B. Birks, *Photophysics of Aromatic Molecules.* New York: Wiley, 1970.

[3] S. Udenfriend, *Fluorescence Assay in Biology and Medicine*, vol. 1. New York: Academic, 1962.

[4] —, *Fluorescence Assay in Biology and Medicine*, vol. 2. New York: Academic, 1969.

[5] J. Yguerabide, "Nanosecond fluorescence spectroscopy of biological macromolecules and membranes," in *Fluorescence Techniques in Cell Biology*, A. A. Thaer and M. Sernetz Eds. Berlin, Germany: Springer, 1973, pp. 311–331.

[6] T. J. Dougherty, J. E. Kaufman, A. Goldfarb, K. R. Weishaupt, D. Boyle, and A. Mittelman, "Photoradiation therapy for the treatment of malignant tumors," *Cancer Res.*, vol. 38, pp. 2628–2635, 1978.

[7] D. Kessel, "Hematoprophyrin and HPD: Photophysics, photochemistry and phototherapy," *Photochem., Photobiol., Yearly Rev.*, vol. 39, no. 6, pp. 851–859, June 1984.

[8] G. M. Barenboim, A. N. Domanskii, and K. K. Turoverov, *Luminescence of Biopolymers and Cells.* New York: Plenum, 1969, pp. 67–71.

[9] L. N. M. Duysens, and G. Sweep, "Fluorescence spectrophotometry of pyridine nucleotide in photosynthesizing cells," *Biochim. Biophysics. Acta*, vol. 25, no. 13, 1957.

[10] B. Chance and H. Baltscheffsky, "Respiratory enzymes in oxidative phosphorylation," *J. Biol. Chem.*, vol. 233, p. 736, 1958.

[11] B. Chance and B. Schoener, "Fluorometric studies of flavin compounds of the respiratory chain," in *Flavins and Flavoprotein*, E. C. Slater, Ed. Amsterdam, The Netherlands: Elsevier, 1966.

[12] G. D. Fasman, Ed., *Handbook of Biochemistry and Molecular Biology*, 3 ed. Cleveland, OH: CRC Press, 1975, pp. 205–210.

[13] J. M. Gallas, "Fluorescence of melanin," Ph.D. dissertation, Univ. Houston, Houston, TX, 1981.

[14] S. Kozikowski, H. Wolfram, and R. R. Alfano, "Fluorescence spectroscopy of eumelanins," *IEEE J. Quantum Electron.*, vol. QE-20, pp. 1379–1382, Dec. 1984.

[15] M. A. Alfano, "Fluorescence from nails–(keratin)," private communication.

[16] R. R. Alfano, patent pending.

Robert R. Alfano, for a photograph and biography, see this issue, p. 1342.

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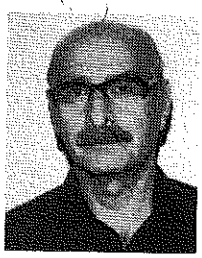

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