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Spectral Analysis to Detect Benign and Invasive Malignant Tumors in Breast Biopsy Specimens using Excitation Spectroscopy

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The UV excitation spectra from normal and malignant breast tissues revealed changes of proteins in the cellular and extracellular matrices. Based on the histogram analysis of the spectral intensity ratios at well define wavelengths, invasive carcinoma samples can be differentiated from normal and mixed carcinoma samples. This work indicates that changes in the excitation spectrum can be related to the degree of invasiveness of carcinoma.

Keywords: excitation spectroscopy, membrane proteins, malignant tumor, invasive carcinoma, breast tissue.

INTRODUCTION

Determining whether, and to what extent, a cancer is *in situ* or invasive is a major object of histological diagnosis, since invasive tumors may metastasize, and often require radiation and chemotherapy. To demonstrate that this distinction can be made without morphological analysis, we have studied the optical excitation spectra of benign and malignant breast tissue (Yang

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TABLE 1
Pathological classification of specimens

No.	Description	Total
Normal:		
1.	benign breast tissue	15
2.	fibrocystic change	44
3.	benign lymph node	1
4.	loctating breast	3
Malignant:		
1.	ductal carcinoma	80
2.	lobular carcinoma	6
3.	metastatic breast carcinoma	7
4.	mucinous carcinoma	3
5.	Poorly differentiated carcinoma	5
6.	pleomorphlic sarcoma	2

et al., 1997; Yang et al., 1997). Certain critical ratios of excitation spectra, based on the amounts of tissue matrix protein, appear to distinguish benign lesions, *in situ* and invasive tumors from each other, prior to biopsy. The results have been confirmed by pathological examination, and also appear valid for breast, colon, head and neck neoplasm (Yang et al., 1998). Over the past several years, there have been many studies using fluorescence spectroscopy on benign and malignant breast tissues to develop a non-invasive diagnostic method using lamp and lasers sources (Alfano et al., 1984: Alfano et al., 1991; Alfano et al., 1987; Gupta et al., 1997), with high sensitivity and specificity.

In this paper, we report on the correlation between excitation spectra of excised, unfixed breast biopsies and the presence of benign lesions, mixed *in situ* and invasive disease, and purely invasive tumor, in the specimens, as confirmed by subsequent pathological analysis. These spectral variations are presumed to be due to differences in the amounts of fibronectin, collagen IV and laminin in the interstitial spaces of benign, *in situ* and invasive lesions, which have been demonstrated by immunohistochemical studies of cancer (Travis 1997; Christensen 1990; Clavel et al., 1988; Uitto et al., 1991). The spectral differences between different types of breast lesions were assigned to the presence of fibronectin, collagen IV and laminin in the interstitial space. These assignments are supported by a theoretical fitting of the spectra to the emission properties of those proteins, which are altered in cancer development. The least squares fitting method was used to create models of

the spectra of malignant and benign breast tissue based on these proteins' spectra.

MATERIALS AND METHODS

Excised benign and malignant breast tissue samples were obtained from St. Vincent's Hospital, Memorial Sloan Kettering Cancer Center, and the National Disease Research Interchange (NDRI). 63 normal breast tissues and 103 malignant breast tissue samples have been measured. The pathological classification of these specimens is given in Table. 1. The size of each specimen is about 1cm×3cm×0.3cm. The specimens were refrigerated, but not frozen, immediately after the excision, and stored for less than 24 hours before measurement. Specimens were not chemically treated prior to spectroscopic measurements. Samples of random shapes were mounted in a 1cm×1cm×10cm standard quartz cuvette cell and attached to its inner wall to obtain its spectrum. The breast tissue specimens were classified into normal tissue, invasive carcinoma, and mixed *in situ* and invasive (part *in situ* and part invasive) using the pathology report. The proteins: fibronectin (from human plasma, 0.1% solution, 1mg/ml in 0.05M Tris buffered saline, pH=7.5); collagen IV (1mg/ml, in acetic acid 0.1mM); and laminin fragment 929-933(TYR-ILE-GLY-SER-ARG) purity 99%, peptide content 73%, 1mg/ml in 0.05M Tris buffered saline, pH=7.5 were commercially obtained from Sigma Co. All measurements were performed at room temperature.

Spectroscopic measurements were performed by using an automated lamp spectrophotometer (Mediscience Technology Corp CD Scan.). The instrument is based on the Perkin-Elmer LS50 spectrophotometer with additional key software. There are two monochromators, one in the excitation beam path and the other in the emission beam path. This instrument can be used for obtaining both emission and excitation spectra by fixing the excitation wavelength or the emission wavelength and scanning the other wavelengths. The excitation spectrum was obtained by fixing the emission wavelength at 340nm and scanning the excitation wavelength from 250nm to 320nm. The light source is a Xenon flash lamp. The average power of the excitation light incident on the tissue is less than 0.5 μW. The beam size on the sample is 1mm×8mm. The fluence on the specimen is approximately 30 $\mu J/cm^2$. This value is 70 times lower than the minimal erythemal dose of 20 mJ/cm^2 for UVB exposure (13,14). The process of spectroscopic measurements on most of the tissue was taken in a blind study. (We did not know the

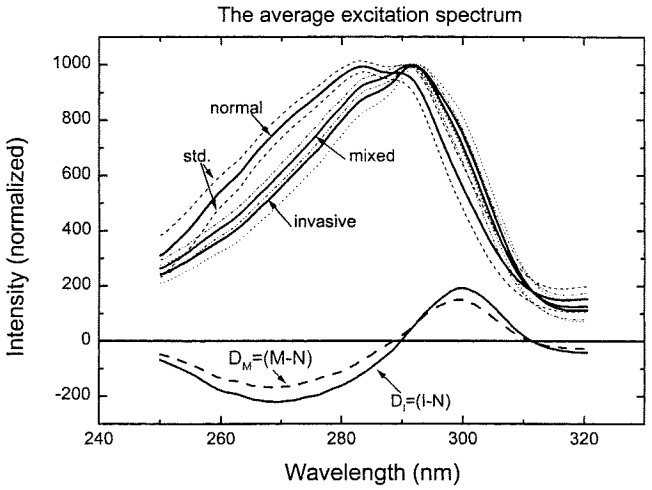

FIGURE 1
Averaged excitation spectra of normal, invasive and mixed *in situ* and invasive breast tissue for emission at 340 nm. $\mathcal{D}_I$=(I-N) and $\mathcal{D}_M$=(M-N) are the difference spectrum, where N is normal, 'std' is standard deviation, I is invasive carcinoma, and M is mixed *in situ* and invasive.

pathological result during optical measurements.) After spectroscopic measurements, most of the samples were fixed by formalin and returned to the hospital for pathological determination. The results of these two methods were compared and the pathology was used as the "gold standard" At least three sites on each specimen were studied. The measured site was selected step by step from one side to another side and from top to bottom with no preference.

RESULTS

The average normalized excitation spectra of normal, invasive carcinoma and mixed *in situ* and invasive (part *in situ* and part in invasive) breast tissues are shown in Fig. 1 for emission at 340nm. Each curve was normalized to its peak then averaged by computer using developed software made for the averaging process. The difference spectra $\mathcal{D}_I$=(N-I) and $\mathcal{D}_M$=(N-M), are also displayed in Fig. 1, where N denotes normal, I denotes invasive carcinoma, and M denotes mixed *in situ* and invasive. The average spectra clearly show not only several salient spectral differences between malignant and

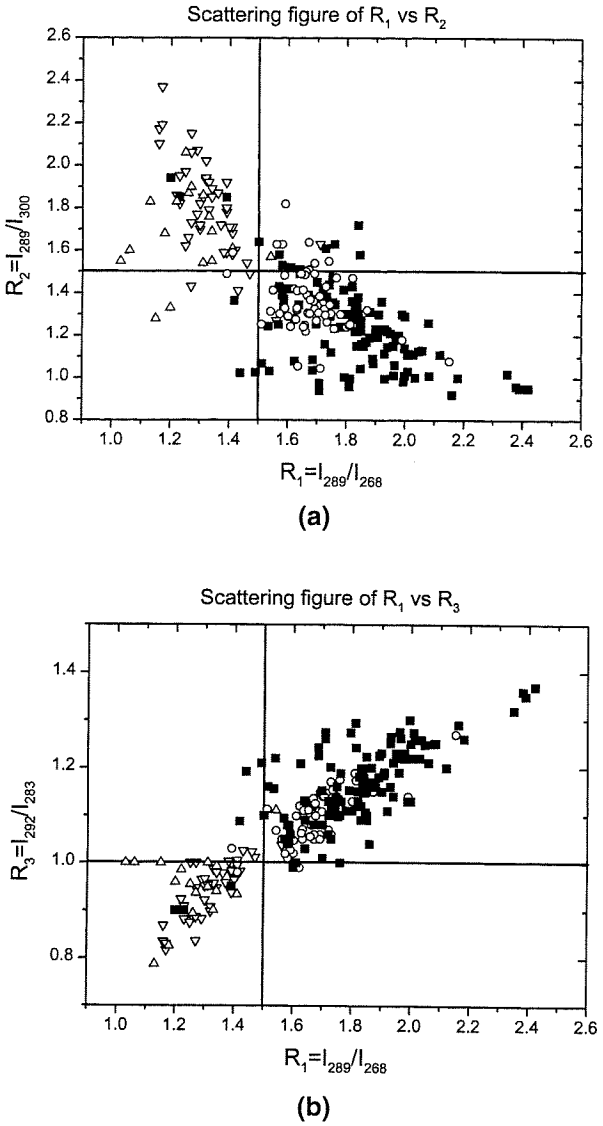

FIGURE 2
Scatter ratio figures for different specimens in terms of parameters R_1 vs R_2 coordinates. (2a), and R_1 vs R_3 coordinates (2b). The specimens were obtained from St. Vincent's Medical Center on Staten Island, Memorial Sloan-Kettering Cancer Center and The National Disease Research Interchange. (In figure: Δ up triangle is benign breast tissue, ∇ down open triangle is fibrocyctic change, ■ solid square is infiltrating carcinoma. ○ open circle is mixed *in situ* and invasive carcinoma.

TABLE 2
Statistical results of normal and malignant breast tissue by using spectroscopic method.

	$R_1=I_{289}/I_{268}$		$R_2=I_{289}/I_{300}$		$R_3=I_{292}/I_{283}$	
	Correct	False	Correct	False	Correct	False
Normal						
Benign breast tissue (15)	14	1	13	2	14	1
	93%	7%	87%	13%	93%	17%
Fibrocystic change (44)	43	1	41	3	40	4
	98%	2%	93%	7%	91%	9%
Benign lymph node (1)	1	0	0	1	1	0
Loctating breast (3)	2	1	2	1	2	1
Total (63)	60	3	56	7	57	6
	95%	5%	89%	11%	90%	10%
Malignant						
Ductal carcinoma (80)	78	2	78	12	75	4
	97%	3%	85%	15%	95%	5%
Lobular carcinoma (6)	1	5	0	6	3	3
Metastatic breast carcinoma (7)	7	0	7	0	7	0
Pleomorphic sarcoma (2)	2	0	2	0	2	0
Mucinous carcinoma (3)	3	0	1	2	2	1
Poorly differentiated carcinoma (5)	5	0	5	0	5	0
Total (103)	96	7	83	20	94	8
	93%	7%	80%	20%	92%	8%

normal tissues but also between invasive and mixed carcinoma. To quantify these spectral differences and determine their diagnostic potential, three diagnostic ratio intensity parameters: $R_1 = I_{289}/I_{268}$, $R_2 = I_{289}/I_{300}$, and $R_3 = I_{292}/I_{283}$ are introduced. I_{292} and I_{283} are the normalized peak intensities. I_{268} and I_{300} are the relative intensities of the peak positions of the difference spectra. I_{289} is the normalized intensity at the isosbestic point. The parameters R_1 and R_3 are related to the change of excitation spectrum from 250nm to 290nm. The parameter R_2 is related to the change of excitation spectrum

from 290nm to 320nm. Each specimen or each measured point has three parameters to describe the changes in the excitation spectrum.

DISCUSSION

Scatter plots R_2 and R_3 as functions of R_1 are displayed in Figs 2(a) and 2(b), respectively. From this figure one can observe that most normal specimens were located in zones $R_1<1.5$, $R_2>1.5$ and $R_3<1.0$. The malignant specimens were located in zones $R_1>1.5$, $R_2<1.5$ and $R_3>1.0$. The statistical results using these parameters as compared to the pathological results are shown in Table 2. The specificity (% of true negative) and sensitivity (% of true positive) is above 93% for R_1. Furthermore, it was noted that the ratio values R_1 and R_3 of invasive carcinoma have larger values than those tissues consisting of mixed *in situ* and invasive. To quantify these observations, the histograms of R_1, R_2 and R_3 have been plotted. From the histogram of R_1 one finds that two kinds of benign specimens (normal breast tissue and abnormal tissue) have nearly the same distribution and peak position of the Gaussian curve. For two groups of malignant specimens (invasive carcinoma and mixed *in situ* and invasive), the peak position of the Gaussian distribution curve of invasive carcinoma has a larger value than those consisting of mixed invasive and *in situ* carcinoma (See Fig.3a). For histograms of R_3 and R_2, similar differences of the Gaussian peak positions of the two malignant groups were found (See Fig.3b & 3c). The averaged values and standard deviations of R_1, R_2 and R_3 are given in Table 3.

The cell of a carcinoma *in situ* is composed of an abundance of special proteins in the cytoplasm and irregular nuclei, commonly with prominent nucleoli. In invasive carcinoma, malignant cells invade the stroma, and changes in the extracellular matrix occur. There are qualitative and quantitative changes in the proteins of the basement membrane between normal and invasive carcinoma states (Travis, 1997, Christensen, 1990, 1992, Clavel, 1998, Uitto, 1991). The ratio values R_1 and R_3 are found to be more sensitive to pathologic changes than R_2. This data suggests that the increase of the ratio values R_1 and R_3 be connected to extracellular membrane protein changes when the tissue changes to invasive carcinoma. The ratio value R_2 may reflect the changes in proteins within the malignant cells (Chen, 1995, Niki, 1994, Easton, 1993).

To support our hypothesis, a linear regression model was used to fit the spectral profiles of malignant and normal tissues using the spectra of the

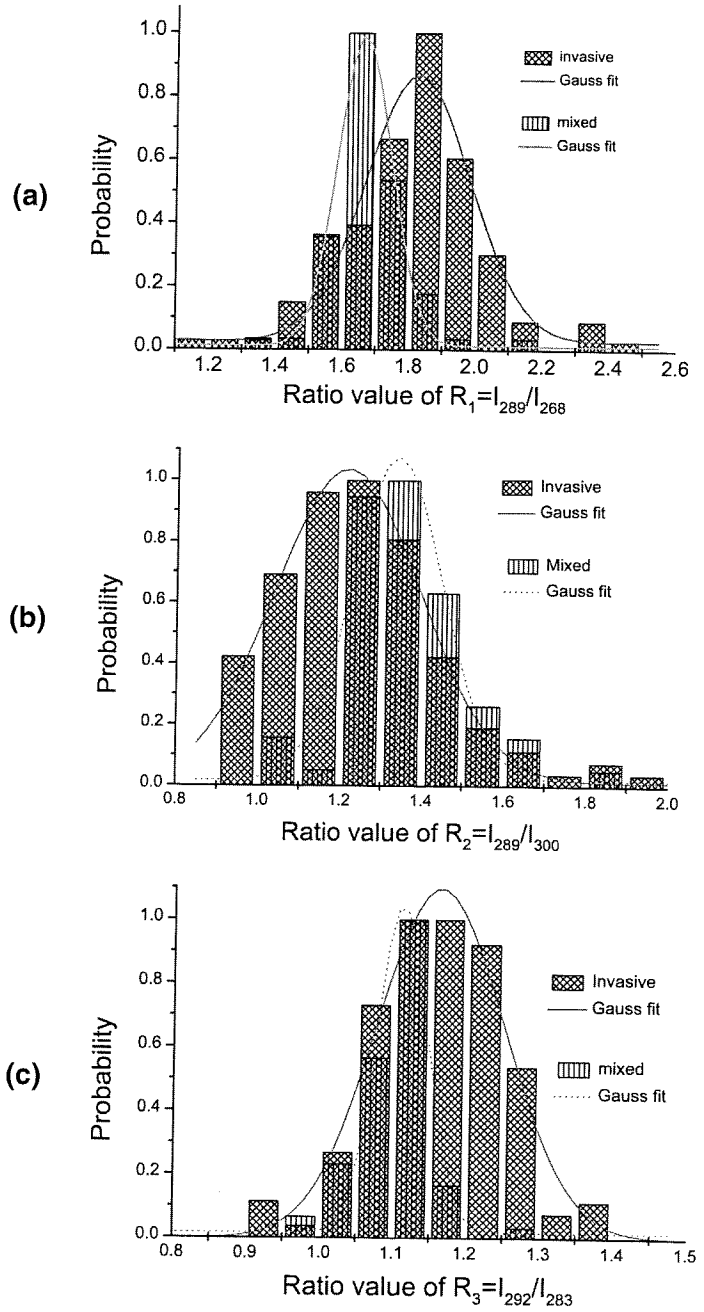

FIGURE 3
The histograms of the parameters R_1 (3a), R_2 (3b), and R_3 (3c) for invasive and mixed carcinoma tissue. See Color Plate I at back of issue.

TABLE 3
Critical ratio values R_1, R_2 and R_3 of different tissue types. R_M, R_I, and R_N are the ratio value of a mixture of *in situ* and invasive, invasive carcinoma, and normal glandular respectively.

Type	$\mathbf{R_1=I_{289}/I_{268}}$	$\mathbf{R_2=I_{289}/I_{300}}$	$\mathbf{R_3=I_{292}/I_{283}}$
Normal glandular	1.19±0.04	1.83±0.05	0.96±0.02
Benign breast tissue	1.25±0.122	1.68±0.20	0.95±0.07
Fibrocystic change	1.32±0.09	1.81±0.22	0.95±0.06
Average	1.29±0.12	1.74±0.22	0.95±0.07
Critical value	**1.5**	**1.5**	**1.0**
Mixed *in situ* and invasive	1.64±0.36	1.36±0.31	1.04±0.21
Change rate $\Delta=(R_M-R_N)/R_N$	0.38	-0.26	0.08
invasive carcinoma	1.85±0.23	1.30±0.21	1.14±0.10
Change rate$\Delta=(R_I-R_N)/R_N$	0.55	-0.29	0.19

TABLE 4
Coefficients of the matrix equation (1) of proteins for excitation spectra with emission at 340 nm. The function Φ (i) was normalized to its area:

	A(normal)	**B**(fibronectin)	**C**(collagen IV)	**D**(laminin)	**E**(constant)
Coefficient	1.05505	0.97983	-0.74060	-0.28554	-0.00035
Std. Err. of coeff.	0.14436	0.11505	0.09817	0.06193	0.00019

main component proteins of the extracellular matrix such as fibronectin, collagen IV and laminin fragments in various solutions and concentrations as mentioned above. The average spectrum of malignant tissue can be described by,

$$\Phi_{\text{av. malig}}\,(i) = \mathbf{A}\,\Phi_{\text{av. nor.}}\,(i)+\mathbf{B}\,\Phi_{\text{fib}}\,(i)+\mathbf{C}\,\Phi_{\text{coll4}}\,(i)+\mathbf{D}\,\Phi_{\text{lam}}+\mathbf{E}, \qquad (1)$$

where $\Phi_{\text{av. nor}}\,(i)$, Φ_{fib} (i), $\Phi_{\text{coll4}}\,(i)$, $\Phi_{\text{lam}}\,(i)$ and $\Phi_{\text{av. malig}}\,(i)$ are the excitation spectra of fibronectin, collagen IV, laminin fragments, malignant and benign tissue, respectively. (i refers to 0.5nm steps taken from 250nm to 320nm.). The functions Φ (i), are normalized by their area to avoid the effect of concentration. The coefficients **A**, **B**, **C**, **D** and the constant **E** are fitting parameters obtained by the least square method. The results are displayed in Table 4.

The data in Table 4 shows that the difference in the excitation spectrum

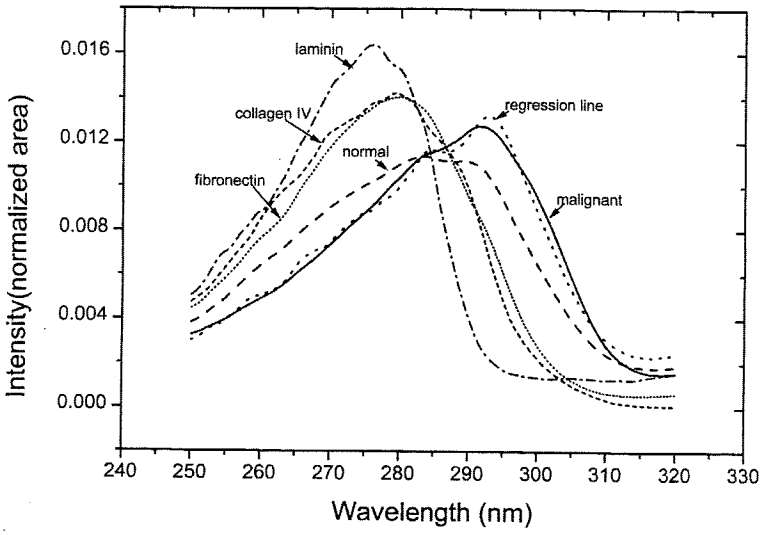

FIGURE 4
The excitation spectra for normal and malignant breast tissues together with the proteins; fibronectin, laminin fragment, collagen IV, and the fitted spectral profile (dotted line) for emission at 340 nm. To avoid the effect of concentration, the intensity was normalized by total intensity. The model malignant spectral profile (dotted line) is found from the benign and protein compounds using a least square method.

between normal and malignant tissues can be connected to the increase of the protein, fibronectin, (0.97983) and decrease of the protein collagen IV (-0.74060) and laminin fragments (-0.28554). The fitted curve (dotted line) of equation (1) and other related curves are displayed in Fig. 4.

Furthermore, a linear regression method was also used to fit the difference spectra $\mathcal{D}_I$=(I-B) and $\mathcal{D}_M$=(M-B), (as shown in Fig. 1) from the proteins in the extracellular matrix. Similarly the difference spectra $\mathcal{D}_I$=(I-N) and $\mathcal{D}_M$=(M-N) can also be described by,

$$\Phi_{\text{ben-mixture}}(i) = \mathbf{F}\,\Phi_{\text{fib}}(i) + \mathbf{G}\,\Phi_{\text{coll4}}(i) + \mathbf{I}\,\Phi_{\text{lam}} + \mathbf{J}, \qquad (2)$$

These coefficients **F**, **G**, **I** and the constant **J** are obtained. The results are displayed in Table 5. These coefficients give relative changes in these proteins for the mixed and invasive carcinoma compared to normal tissue. The

TABLE 5
Coefficients of the matrix equation (2) of proteins for the spectral differences $\mathcal{D}_M$ and $\mathcal{D}_I$, where $\mathcal{D}_M$ is the mixed carcinoma (M) minus normal (N) and $\mathcal{D}_I$ is the invasive carcinoma (I) minus normal (N).

	F(fibronectin)	**G**(collagen IV)	**I**(laminin)	**J**(constant)
$\mathcal{D}_M$=(M–N)	0.9995	–0.7734	–0.2222	0.0003
Std. Err. of coeff.	0.0469	0.0017	0.0286	0.0001
$\mathcal{D}_I$=(I–N)	1.1424	–0.9018	–0.2512	0.0002
Std. Err. of coeff.	0.0533	0.0702	0.0234	0.0001
$\Delta=(\mathcal{D}_I-\mathcal{D}_M)/\mathcal{D}_M$	14.29%	16.60%	13.05%	

percent changes in the proteins show an increase of 14.29% for fibronectin, a decrease of 16.60% for collagen IV and a decrease of 13.05% for laminin, when the group of invasive carcinoma is compared with the group of a mixed carcinoma.

These changes associated with fibronectin, collagen, and laminin are consistent with the results observed by immunohistochemistry (Travis, 1997, Christensen, 1990, 1992, Clavel, 1998, Uitto, 1991) where fibronectin was recognized as a marker in the differentiation between benign proliferate breast lesions and invasive breast carcinoma (Christensen, 1992). Laminin was found to be a discriminating marker between non-invasive breast carcinomas and *in situ*/invasive breast carcinoma (Christensen, 1992). Fibronectin was found to increase in all tumors (Christensen, 1990). In mammary carcinomas, proteins, particularly, type IV collagen and laminin were associated with tumor invasion (Clavel, 1988).

Optical excitation spectroscopy appears to offer a new and novel way to probe changes of key proteins in the development of cancer. Furthermore, it is anticipated that spectroscopy with fine optical fibers can be an additional aid to the current procedures of ultrasonic and stereotactic breast biopsies to achieve a better diagnostic outcome. Co-regulation of optical fluorescence with these approaches offers increased accuracy.

CONCLUSION

The excitation spectra of malignant and normal breast specimens for emission at 340nm are found to be different and are used to establish a criterion to distinguish malignant from normal breast tissue. A set of diagnos-

tic parameters has been determined which relate to the invasiveness of the carcinoma. It also appears that fluorescence excitation spectroscopy offers a novel tool to probe the mechanism of cancer.

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