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Discrimination Between Normal and Malignant Breast Tissues by Synchronous Luminescence Spectroscopy

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Abstract

Studies of fluorescence from endogenous molecules in tissues are common for applications such as detection or characterization of early disease. Breast cancer is one of the most common malignant tumor among women and good screening methods are therefore of considerable interest. We have applied synchronous luminescence spectroscopy (SLS) to examine specimens of excised breast tissues. Measurements have been made in the 330 nm to 650 nm range of excitation wavelengths and constant wavelength interval varying from 30 nm to 120 nm on 21 normal and 21 malignant breast tissue samples. Significant differences between SLS patterns of normal and malignant tissues are detected and related to the discrepancy in concentrations of extracellular proteins and co-enzymes. Malignant tissue identification criteria are established on the basis of spectral peak areas of SLS spectra that correspond to constant wavelength intervals where the most pronounced differences are observed. These characteristic intervals are chosen from the difference of averaged three-dimensional SLS patterns of normal and malignant breast tissues. Using observed statistically significant spectral differences as classification criteria we tested classification success rate of presented SLS method.

Keywords: breast tumor, synchronous luminescence spectroscopy, cancer diagnosis.

1. Introduction

The breast cancer is one of the most common malignant tumors among women in the world[1] and if not diagnosed at proper time it delivers high mortality rates. On the other hand, if observed in early stages breast cancer is one of the most treatable forms of cancer. The important task of oncology is the development of methods for the early detection of tumors and tumor pre-stages, because a successful therapy essentially depends on the point in time at which the disease is detected, making possible to improve patient quality of life and survival rates. The most efficient screening methods for breast cancer detection are mammography and physical exam. The mammography has contributed significantly to reduce the mortality rates of this disease. Although these are most useful and most widely used they have some weaknesses. Detection of small tumors can be difficult by physical exam and mammography produces a considerable number of false positive results.[2]

Optical spectroscopy techniques are increasingly used in the field of cancer diagnosis.[3,4] These techniques have three major advantages over other investigation methods: they are fast, noninvasive and quantitative. Recently, photoacoustic (optoacoustic) imaging techniques showed very promising results[5-8] in breast cancer screening.

Luminescence spectroscopy is an optical method that can provide rapid differentiation between tumor and normal tissue in a variety of epithelial organ systems. The initial luminescence *in vitro* studies[9-11] were significant as the potential approach for *in situ*, noninvasive diagnostics. Luminescence spectroscopy and imaging techniques are able to characterize various metabolic and pathological changes in precancerous and cancer vs. normal tissues. These alternations that occur as tissue progresses from a normal to a diseased state are reflected in the spectral characteristics of the measured luminescence. The endogenous fluorophores such as NADH, FAD, collagen, elastin, amino acids, vitamins, lipids and porphyrins,[12] have a significant variation in the concentration in different tissue types. These differences, together with alternations in the local environment within the tissue, are the basis for the discrimination between tumor and normal tissue by luminescence spectroscopy.

The first luminescence measurements of malignant and normal breast tissues have been made by Alfano *et al.*[13] Later investigations were performed to evaluate feasibility of luminescence spectroscopy for breast cancer detection.[14-21] They used emission and/or excitation

spectroscopy, and more recently excitation-emission matrices (EEM).[19,21-24] The most recent represents a series of emission spectra, each of which is measured for different excitation wavelength, covering in that manner excitation region of interest.

The first report on application of synchronous luminescence spectroscopy (SLS) has been given by Lloyd[25] and Vo-Dinh[26,27] who provided the basic theory. The SLS method involves scanning simultaneously both emission and excitation wavelengths while keeping a constant wavelength interval (constant - wavelength mode) or frequency (constant-energy mode) between them. The synchronous spectrum is considered to be the characteristic "fingerprint", because it is unique for a given system. By the comparison of synchronous spectra of two similar systems, it is possible to reveal and identify differences or anomalies in their composition.[28] Compared with ordinary emission spectra, a synchronous spectrum often has more features and thus provides more information, in part because varying both excitation and emission alters the contribution of a larger number of components to the spectrum. The SLS measurement provides for decreased bandwidths and greater selectivity and can be used to detect subtle differences in the fluorescence emission of the biochemical components of cells and tissues.[29]

Fluorescence spectroscopy has emerged as a promising modality in the discrimination of normal from pathologically diseased cells and tissues. As tissues are highly heterogeneous with many native fluorophores, emission spectra at one or more excitation wavelengths or excitation spectra corresponding to one or more emission wavelengths are used for diagnostic purpose. This could be improved to a large extent by applying synchronous luminescence spectroscopy. The SLS method can be used to analyze tissue in vivo or to investigate spectral differences in normal and neoplastic cells in vitro.[30]

In our recent paper[31] we introduced ordinary and first derivative 3D-SLS measurements for the investigation of normal and malignant human breast tissues. The central idea of this work is to find spectral subsets of 3D-SLS that have enough distinguished spectral elements to be used for tissue discrimination based solely on comparison of measured spectral characteristics. These spectral subsets should be selected in a manner that they can be measured routinely in clinical applications.

2. Results and Discussion

Three-dimensional synchronous luminescence spectral (3D-SLS) patterns consists of normalized luminescence emission intensity values measured for each point in the excitation wavelength (EW) – synchronous interval (Δλ) plane. Following the SLS measurements on the surgically removed 21 normal female breast tissue specimens and 21 malignant, 3D-SLS patterns are created for each case in the EW:330-650 nm and Δλ: 30-120 nm spectral domain. These are then averaged with respect to the pathological findings to produce patterns for the normal breast tissue and for the malignant. The difference between them ("normal" minus "malignant") is presented in a form of contour graph on Figure 1. Positive values on the graphic represent spectral regions where normal tissue specimens exhibit stronger luminescence emission while negative values stand for the stronger malignant tissue emission.

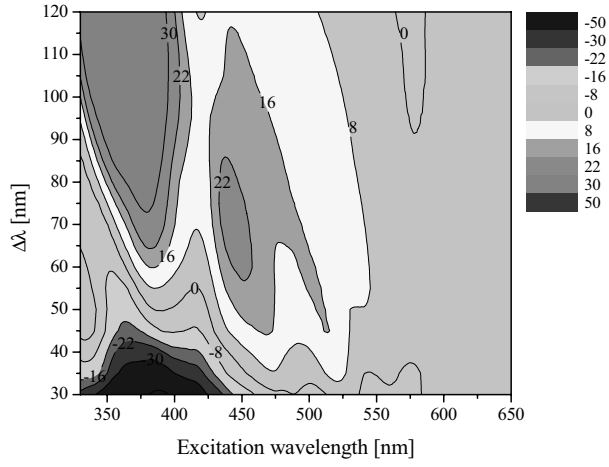

Figure 1. Difference between averaged 3D-SLS patterns of normal breast tissue and malignant tissue; positive values stand for the stronger luminescence of normal tissue.

There are three spectral regions where the contrast of a luminescence emission among tissues is very pronounced. The first one is located on the 330-425 nm EW and 30-45 nm Δλ showing significantly stronger malignant tissue emission, especially on the lowest used synchronous interval of 30 nm. The second region is again on the 330-425 EW range but on the higher Δλ, from 60 to 120 nm. In this case normal tissue has a stronger emission then malignant and also, but not to the same extent, in the third region defined by EW:425-550 nm and Δλ:40-115 nm. Although beyond the scope of this paper it is worth mentioning that 3D-TSLS patterns indicate discrepancy in concentrations of extracellular proteins and co-enzymes in normal and malignant breast tissues: collagen, elastin, NADH and FAD.

Three-dimensional SLS patterns have capability to thoroughly represent luminescent properties of the investigated specimens and they are, in some way, characteristic "fingerprint" of material.[28] It is

shown[32] that SLS provides several advantages over classical emission techniques when dealing with multi-fluorophoric systems, the one example of which is a tissue specimen. However, the experimental time required for the measurement of one 3D-SLS pattern is about one hour and it is hardly acceptable for regular clinical application. So, we need to choose data subsets from the 3D-SLS (Figure 1) in a way that they reflect observed luminescence differences between tissue samples and that can be measured in a considerably shorter time, appropriate for clinical investigations. Having in mind previous discussion on the most pronounced differences in 3D-SLS contour graph, we find that best candidates for data subsets are SLS spectra taken for $\Delta\lambda = 30$ and 80 nm. These are shown in the Figure 2. a) and b), respectively. The low synchronous interval spectra ($\Delta\lambda = 30$ nm) cuts previously discussed first spectral region of 3D-SLS, while the higher synchronous interval spectra ($\Delta\lambda = 80$ nm) cuts both the second and the third.

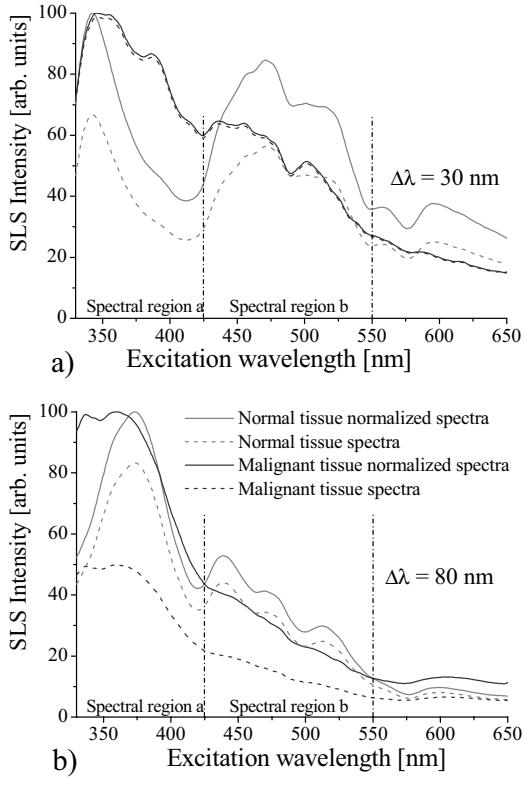

Figure 2. Synchronous luminescence spectra taken on a) $\Delta\lambda = 30$ nm synchronous interval and b) $\Delta\lambda = 80$ nm; red lines represent the average of spectra of 21 normal breast samples and blue lines the average of spectra of 21 malignant breast samples; full lines stand for additionally normalized spectra and dashed lines for raw spectra .

In the Figure 2. red lines represent the average of spectra of normal breast tissues and blue lines the average of spectra of malignant. Normalization of spectra is standard procedure in optical biopsy applications and the purpose is to account for differences in sample morphologies. The normalized spectra are shown in Figure 2. (full lines) together with the raw spectra (dashed lines). When Figure 2. is examined one can observe clear differences between normal tissue spectra and malignant. In order to quantify these differences and to investigate whether they are suitable for the tissue discrimination in optical biopsy applications we have defined two smaller excitation wavelength subregions. These are labeled "subregion a" and "subregion b" in Figure 2. and have ranges from 330 nm to 425 and 425 nm to 550 nm, respectively. For the spectral quantification we introduce areas under normalized spectral curves in subregions: Sa30, Sb30, Sa80 and Sb80, where "a" and "b" denotes "subregion a" and "subregion b," while "30" and "80" denotes SLS obtained for 30 nm and 80 nm synchronous intervals, respectively. These selected spectral areas together with six area ratios: Sa30/Sb30, Sa80/Sb80, Sa30/Sa80, Sb30/Sb80, Sa30/Sb80 and Sb30/Sa80, make a set of spectral properties whose significance for tissue discrimination need to be evaluated.

From the measured SLS data the above specified areas and the area ratios have been calculated for each tissue specimen. In this way we have obtained two data groups composed of 21 records for normal group and malignant group, for each spectral property on which we wanted to perform statistical analysis.

In the first step of analysis we have completed *Shapiro-Wilk* normality test on the 0.05 significance level[33] for all spectral property groups. The test results, listed in Table 1., confirms that data in all groups follow a normal distribution.

Table 1. Results of the *Shapiro-Wilk* normality test; the test confirms that distributions of all investigated spectral properties are normal at 0.05 significance level.

Spectral property	Normal tissue samples		Malignant tissue samples	
	W statistic	P value	W statistic	P value
S_{a30}	0.943	0.247	0.964	0.579
S_{b30}	0.981	0.927	0.928	0.124
S_{a80}	0.960	0.506	0.982	0.943
S_{b80}	0.978	0.877	0.949	0.323
S_{a30}/ S_{b30}	0.971	0.740	0.965	0.611
S_{a80}/ S_{b80}	0.968	0.671	0.924	0.101
S_{a30}/ S_{a80}	0.964	0.582	0.920	0.084
S_{b30}/ S_{b80}	0.956	0.435	0.975	0.815
S_{a30}/ S_{b80}	0.964	0.577	0.961	0.529
S_{b30}/ S_{a80}	0.944	0.251	0.967	0.659

In the second step we analyzed if there are statistically significant differences between spectral properties of normal tissue and malignant. To do so

we have performed hypothesis testing on all spectral properties using the *two-tailed t-test*.[34] We adopted standard threshold probability value of 0.05 and accepted the *null hypothesis* (the *null hypothesis* simply states that there is no difference between the groups). Then we computed *t* value (*t* value is defined as the ratio between the group means and the variability of groups) and the probability value, *p*, and based on this we made decisions about statistical significance of differences in the spectral properties observed with normal and malignant tissues. The results of hypothesis testing are given in the Table 2. In the cases where *p* value is less than the threshold we reject the null hypothesis and state that the difference is statistically significant. The levels of significance are determined on the basis of *p* values and statistical power analysis[34] and they are also given in Table 2. There NS, S, VS and ES denote "not significant," "significant," "very significant" and "extremely significant" difference.

Table 2. Results of the statistical hypothesis testing using *two-tailed t-test* implemented on spectral property groups obtained from SLS spectra of 21 normal tissue specimens and 21 malignant.

Spectral property	Difference of means	*t*	*p*	Power	Decision [a]
S_{a30}	2600.920	-6.854	$3 \cdot 10^{-8}$	0.999	ES
S_{b30}	656.287	1.209	0.234	0.212	NS
S_{a80}	-756.310	-2.251	0.030	0.590	S
S_{b80}	1162.171	2.430	0.020	0.657	S
S_{a30}/ S_{b30}	-0.647	-5.790	$3 \cdot 10^{-6}$	0.999	ES
S_{a80}/ S_{b80}	-0.336	-1.856	0.071	0.435	NS
S_{a30}/ S_{a80}	-0.409	-10.009	$< 10^{-10}$	1.000	ES
S_{b30}/ S_{b80}	-0.108	-0.720	0.475	0.105	NS
S_{a30}/ S_{b80}	-1.622	-6.891	$2.6 \cdot 10^{-8}$	0.999	ES
S_{b30}/ S_{a80}	0.146	2.012	0.051	0.497	NS

[a] Decision on statistical significance is made in a traditional way on the basis of probability value for null hypothesis, *p*: > 0.05 Not Significant (NS), 0.01 to 0.05 Significant (S), 0.01 to 0.001 Very Significant (VS), and < 0.001 Extremely Significant (ES).

The test shows 4 no significant, 2 significant and 4 extremely significant spectral differences between SLS of normal and malignant tissue measured on synchronous intervals of 30 nm and 80 nm. The number spectral properties that exhibit statistically extremely significant difference together with their almost maximal statistical power are excellent arguments to conclude that selected SLS spectra give enough elements for the tissue discrimination.

To test success rate in determination of tissue types we compared SLS results with findings achieved by histopathology for the test group of 67 biopsies. Analysis is carried out using for the criteria set of three areas under normalized spectral curves exhibiting the largest differences (S_{a30}, S_{a80} and S_{b80}), and also criteria based on the set of three ratios of areas under normalized spectral curves (S_{a30}/ S_{b30}, S_{a30}/ S_{a80} and S_{a30}/ S_{b80}). Table 3 depicts the diagnostic data and demonstrates a sensitivity of 77.4% and specificity of 86.1% for classification using area values, and sensitivity of 90.3% and specificity of 94.4% when using values of area ratios. In the first case seven malignant biopsies and five normal biopsies were misclassified, yielding a positive predictive value of 82.8% and a negative predictive value of 81.6%. In the second case three malignant biopsies and two normal biopsies we misclassified, yielding a positive predictive value of 93.3% and a negative predictive value of 91.9%.

Table 3. Comparison of classification accuracy[b] of SLS using areas under normalized spectral curves and ratios under areas under normalized spectral curves, respectively.

Criteria	Test result	Gold standard	
		Histology = Malignant	Histology = Normal
S_{a30}, S_{a80} and S_{b80}	Malignant	24	5
	Normal	7	31
S_{a30}/ S_{b30}, S_{a30}/ S_{a80} and S_{a30}/ S_{b80}	Malignant	28	2
	Normal	3	34

[b] Sensitivity is the proportion of those with disease according to the gold standard who are labeled positive by the test: 24/(24+7)= 0.774; 28/(28+3)= 0.903

Larger test group is necessary for more accurate determination of the classification accuracy of presented SLS method. We plan in the future, as our dataset enlarges, to reinvestigate classification based on observed significant differences in spectral properties and to explore alternate classification schemes which will include criteria established on differences in first derivative synchronous luminescence spectra described in our previous work.[31]

Finally, when discussing results, it is important to be aware that the tissues were frozen and thawed prior to measurement. In developing luminescence spectroscopy systems for the in vivo detection of pre-cancer and cancer, it is often necessary to perform testing on tissue biopsies. Time constraints in a clinical setting limit the number of synchronous intervals at which luminescence spectra can be measured from the tissue at a given time. Also, generally it can be difficult to obtain institutional approval to do in vivo studies before proof-of-principle in vitro studies have been performed. Therefore, in vitro studies are first used to fully characterize the spectroscopic properties of the tissue and establish optimal synchronous intervals for subsequent in vivo studies. Current standard protocols call for the tissue to be immediately frozen after biopsy and latter thawed for spectroscopic analysis.

The process freezing and thawing cause changes in the luminescence properties of tissue. These changes arise from a number of reasons of which the most important are tissue degradation, the oxidation of electron carriers (*e.g.* reduced nicotinamide adenine dinucleotide, reduced flavin adenine dinucleotide, etc)[35] and lack of blood flow to the specimen.[36] The loss of perfusion alters the contribution of hemoglobin absorption to luminescence spectra. Scattering properties of the tissue are changed too. However, these changes affect both the normal and the malignant tissue (in the same manner but not to the same extent) and in principle do not present a serious obstacle for making conclusions after in vitro investigations about the capacity of certain luminescent technique to discriminate normal and malignant breast tissues. Systematic comparison and analysis of tissue luminescence emission spectra in vivo, ex vivo (immediately after biopsy), and after the freeze and thawed process is presented by Palmer et al.[22]

3. Conclusions

Three-dimensional synchronous luminescence spectroscopy patterns clearly expose differences in the luminescence properties between the normal breast tissue and the corresponding malignant tissue. Their subsets, in the form of ordinary synchronous luminescence spectra measured on two synchronous intervals picked from the regions that show the largest differences, posses enough statistically significant elements required for the applications in optical biopsy. Measurement time needed for two SLS spectra is in order of 3 min which is quite adequate for routine clinical investigations.

In the view of the given results it is our opinion that presented method of synchronous luminescence spectroscopy application for the breast cancer identification has a good prospective in a breast cancer optical screening.

4. Experimental

The study involved tissue samples from female patients with breast cancer. Forty two samples are used for 3D-TSLS measurements, while sixty seven are used for testing classification accuracy. The breast tissue specimens were obtained from the Institute of Oncology and Radiology of Serbia. For the 3D-TSLS measurements both the tumor tissue and the normal tissue have been collected from patients. The samples were taken soon after the surgical resection, identified and stored at -80 ^{0}C until luminescence characterization. Their sizes varied from 0.2 × 0.5 × 0.5 cm to 0.3 × 1.0 × 1.5 cm. According to the histopathological exam, all malignant breast tissue samples included in the present study were infiltrating ductal carcinoma. Tissue specimens were collected after the signed Informed Consent was obtained from patients. The Consent was acquired according to the International Ethical Guidelines for Biomedical Research involving Human Subjects (CIOMS), Geneva 1993 and the Guidelines for Good Clinical Practice (CPMP/ICH/135/95), September 1997.

Synchronous luminescence spectra were measured at room temperature using Perkin Elmer Fluorescence Spectrophotometer LS45 in a synchronous luminescence constant wavelength mode. The spectra were automatically normalized to excitation power by the instrument. The spectra were collected at 200 nm/min scan rate in the excitation range from 330 to 650 nm. 3D synchronous luminescence spectra were obtained by repeatedly measuring spectra while varying constant wavelength interval (wavelength difference between positions of excitation and emission monochromators) from 30 to 120 nm. All 3D spectra were normalized in a manner that the point of maximal luminescence emission has a value of 100 in order to account for differences in specimen morphologies.

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Povzetek

Fluorescenco endogenih molekul v tkivih pogosto uporabljamo za ugotavljanje in karakterizacijo bolezni v zgodnji fazi. Rak dojke je eden najpogostejših malignih tumorjev pri ženskah, zato obstaja velika potreba po dobrih diagnostičnih metodah za njegovo odkrivanje. Za preiskave odstranjenega prsnega tkiva smo uporabili sinhrono luminiscenčno spektroskopijo (SLS). Meritve smo izvedli na 21 vzorcih normalnega in 21 vzorcih malignega tkiva z vzbujevalnimi valovnimi dolžinami v območju 330 do 650 nm in s stalnim intervalom med 30 in 120 nm. Zaznali smo pomembne razlike med spektri SLS zdravega in malignega tkiva, ki so povezane z razlikami v koncentracijah izvenceličnih proteinov in koencimov. Kriterije za identifikacijo malignega tkiva smo opredelili na podlagi razlik v povprečenih tridimenzionalnih spektrih SLS s stalnim intervalom valovnih dolžin, pri katerih smo opazili največje razlike med zdravim in malignim tkivom.