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Using Fluorescence Spectroscopy to Diagnose Breast Cancer

Tatjana Dramićanin and Miroslav Dramićanin

Additional information is available at the end of the chapter

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Abstract

Optical spectroscopy methods have had considerable impact in the field of biomedical diagnostics, providing novel methods for the early or noninvasive diagnosis of various medical conditions. Among them, fluorescence spectroscopy has been the most widely explored mainly because fluorescence is highly sensitive to the biochemical makeup of tissues. It has been shown that tumors were easily detected on account of altered fluorescence properties with respect to fluorescence of ordinary tissue. Breast cancer is one of the most commonly diagnosed cancers among women in the world and also it is one of the leading causes of deaths from cancer for the female population. However, when detected in early stage, it is one of the most treatable forms of cancer. Therefore, fluorescence technologies could be highly beneficial in early detection and timely treatment of cancer. This chapter presents main results and conclusions that have been reported on the use of fluorescence spectroscopy for the investigation of breast cancer. It also gives an overview on the instruments and methodology of measurements, on the main endogenous fluorophores present in tissues, on the tissue fluorescence, and on the statistical methods that aid interpretations of fluorescence spectra. Finally, examples of using various fluorescence techniques, such as excitation, emission and synchronous spectroscopy, excitation-emission matrices, and lifetimes, for the breast cancer diagnosis are presented.

Keywords: fluorescence, breast cancer, fluorophores, tissue fluorescence, cancer diagnosis

1. Introduction

According to World Cancer Research Fund International (WCRFI), 1.7 million women have been diagnosed with breast cancer in 2012 [1]. With 25% share of all diagnosed cancers, breast cancer

is a leading malignant disease in female population throughout the world. It is also present in male population, but with about 100 times less incidence that is fairly stable over the last 30 years. Breast cancer incidence is highest in North America, West Europe, and Oceania (in highly developed countries); in 2012, record-high values of age-standardized rate per 100,000 were recorded in Belgium (111.9) and Denmark (105.0). Breast cancer is age- and hormone-status-related, being highest for woman 50–70 years old. Fortunately, prognosis is very good for early diagnosed disease. Five-year survival rate (see **Table 1**) is 100% for 0 and I stage conditions [2]. Therefore, the early diagnosis of breast cancer (and also all cancers) is a key for the successful treatment. On the other hand, in the absence of any early detection or screening and treatment intervention, patients are diagnosed at very late stages when curative treatment is no longer an option [3].

Stage	0	I	II	III	IV
5-year survival rate	100%	100%	93%	72%	22%

Table 1. Five-year survival rate of patients diagnosed with breast cancer of different stage.

Generally, tumor-observing methods can be categorized to screening, diagnostic, and monitoring tests. Clinical breast examination, mammography, and ultrasonography are the most common methods used for screening. Clinical examination by a specialist is usually the first step in breast cancer detection. However, this test is quite subjective and the results depend on the experience and skill of the examiner and most importantly, it does not detect small-size tumors. Mammography is the most cost-effective test, but with moderate sensitivity of 67.8% and specificity of 75%. Mammography combined with clinical breast examination slightly improves sensitivity (77.4%). For screening, ultrasonography is usually recommended to younger persons (premenopause), but it fails to detect microcalcifications and shows poor specificity (34%). Modern imaging techniques, such as positron emission tomography (PET), magnetic resonance imaging (MRI), and computed tomography (CT) are important tools for cancer detection and treatment. Some important features of the most common breast cancer tests are listed in **Table 2**.

Medical diagnostics of breast cancer based on optical spectroscopy and optical imaging are in the early stage of development in comparison with the traditional methods. But the need for sensitive and early cancer detection along with advances in technology played, and still plays, an essential role for the extensive research in this field. For example, lasers have provided a new technology for excitation, and microchips and miniaturized sensors have eased signal detection, while optical fibers have transformed the ways of access to the object of examination. The most attention among a variety of optical techniques is given to fluorescence, Raman spectroscopy, diffuse reflectance, elastic scattering spectroscopy, Fourier transform infrared microspectroscopy, near-infrared imaging, and optoacoustic tomography. These techniques offer several principal advantages over the traditional methods, including (a) noninvasiveness through the use of safe, nonionizing radiation, (b) display of contrast between soft tissues based on optical properties, and (c) a facility for continuous bedside monitoring. Detailed description

of optical methods in cancer research is beyond the scope of this chapter, which is devoted to fluorescence techniques. Those interested in more information on this subject can read some of topical books (e.g., Biomedical Photonics Handbook edited by T. Vo-Dinh [4]).

Method	Benefit	Deficiency
Clinical Breast Examination	no equipment needed	highly subjective, large FP and FN values
Mammography	suitable for screening	large FP and FN values, use of X-ray radiation
Ultrasonography	use of non-ionizing radiation, noninvasive, able to analyze lesions in dense breasts	large FP and FN results, not sensitive enough
Magnetic Resonance Imaging (MRI)	able to detect small DCIS, use of non-ionizing radiations	expensive, can not be used with patients with metal implants
Computed tomography (CT)	painless, noninvasive, accurate	expensive, use of X-radiation
Positron Emission Tomography (PET)	can indicate cancer from disordered metabolism, accurate	expensive, allergic reactions to RP, use of injection RP

Note: FP – false positive, FN – false negative, RP – radioactive pharmaceutical, DCIS – ductal carcinoma in situ.

Table 2. Some important features of the most common breast cancer tests.

In the past several decades, fluorescence spectroscopy has been applied to many different types of samples, ranging from individual biochemical species to organs of living people. It has been applied so far for almost every type of cancer, both *in-* and *ex vivo*, and it has demonstrated advantages over other light-based methods in terms of sensitivity, speed, and safety. Since the early twentieth century, it is known that tissues fluoresce when exposed to light of a suitable wavelength and that infiltrating tumors can be detected on account of altered fluorescence signals. These alternations are the result of the exceptionally high sensitivity of fluorescence on the biochemical makeups of tissues, and are premise for the diagnoses of tissue pathologies by fluorescence. Tissue fluorescence comprises emissions of a number of natural fluorophores (endogenous fluorophores) that have unique spectral characteristics when excited with ultraviolet or visible light. Among them, regarding fluorescence, the most important are tryptophan, reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), elastin, collagen, flavins, and porphyrins. The changes in the concentrations and microenvironments of fluorophores alter tissue fluorescence to a sufficient extent to detect metabolic and pathological changes related to precancerous and cancerous growth, even though fluorescence measurements are not capable of detailing structural changes in tissues. The fluorescence of tissue and tissue fluorophores are discussed later in detail in a separate section.

It is impossible to present beauty, complexity, versatility, and usefulness of fluorescence in a single chapter. Many good treatises could be used for this purpose [5–7]. Thus, with this chapter

we intended to provide an overview of applications of fluorescence spectroscopy for the breast cancer diagnosis. This application is a small part of the extremely large and growing field of biomedical fluorescence. To do so, brief descriptions of basic principles of fluorescence, instruments, and techniques are given in Section 2. Tissue fluorescence, management, and interpretation of fluorescence data are explained in Sections 3 and 4. Fluorescence measurements ought to be subjected to mathematical quantification and interpretation for obtaining appropriate data for cancer diagnosis. Useful mathematical tools, mainly modern statistical tools, are described in Section 5. Examples of applications are given in Section 6, and they are selected to cover most of the fluorescence techniques listed in Section 2. These examples are mainly drawn from our own work, simply because we are most familiar with them.

2. Fluorescence spectroscopy methods and instrumentation

Photoluminescence is the physical process of light emission from any substance that has not been heated and takes place from the electronically excited states. Depending upon the nature of the excited state, luminescence can be strictly divided into two types, fluorescence and phosphorescence. Fluorescence is a rapid and spin-allowed emission of light from singlet excited states, while in phosphorescence emission of light comes due to spin-forbidden transitions from triplet excited states to the ground state. The fluorescence process, depicted in **Figure 1**, is ruled by three important events: (1) excitation of a molecule by an incoming photon from the ground state S_0 , (2) vibrational relaxation of S_1' excited state electrons to the lowest excited energy state S_1 , and, finally, (3) emission of a lower-energy photon and return of the molecule to the ground state.

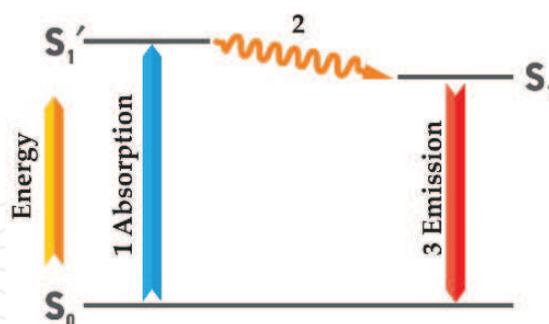


Figure 1. One form of a Jablonski diagram explaining the process of fluorescence.

Fluorescence measurements are the core of any fluorescence technology, and they are presently widely utilized by scientists from many disciplines. Depending on the temporal nature of excitation and detection, fluorescence measurements can be classified as steady-state (implemented with constant illumination and observation) and time-resolved (performed with pulsed excitation). Generally, steady-state measurements are simpler than time-resolved since they require less complex instrumentation and are easier for interpretation. Also, they are sufficient for many applications, so they are more common in practice. However, being simply

an average of time-resolved phenomena, steady-state measurements do not account all of the molecular information imparted to fluorescence. Characteristic examples are information on the distribution of emission decays or the nature of fluorescence quenching.

Fluorescence measurements can also be classified according to the type of observed data. Emission spectra are obtained by recording emission intensity over the spectral range of interest for the fixed energy of excitation. Excitation spectra are created by measuring the variation of emission intensity at the fixed energy (wavelength) while changing excitation energy in the desired spectral region. Generally, these spectra are symmetric (mirror image) because the same transitions are involved in both absorption and emission, and similar vibrational levels are present in both ground and excited state. Certainly, many exceptions may occur, but their explanation is beyond the scope of this chapter. Synchronous fluorescence method involves simultaneously scanning both emission and excitation wavelengths while maintaining the interval constant between emission and excitation (constant-wavelength mode) or maintaining the frequency gap constant (constant-energy mode). When selected interval corresponds to the difference between the excitation and emission maxima of a specific molecule, the emission of that molecule is maximally intensified in the measured spectrum. Consequently, synchronous luminescence spectra have sharper spectral characteristics than conventional, characteristics that are particularly valuable for discrimination between different biological tissues.

Biological materials are complex and comprise many different fluorophores, chromophores (molecules that absorb light but do not emit one), and light scatters. Because of that, the measurement of a single spectra, either emission or excitation or synchronous, is sometimes insufficient for the analyses and diagnostics. In this case, more comprehensive measurements are required to thoroughly combine excitation and emission features of tissue; commonly, this type of measurement is referred as multidimensional fluorescence measurement. Excitation-emission matrices (EEMs), also termed excitation-emission landscapes, are the most widely used type of multidimensional measurements. They combine in a three dimensional (3D) space the set of emission spectra excited by the light of different wavelengths. One can say the EEM of a sample is its characteristic fluorescent fingerprint, taking that the majority of the fluorescence characteristics of the sample are included in EEM. Alternatively, three-dimensional total synchronous fluorescence spectroscopy (3D-TSFS) can be used for the same purpose. During 3D-TSFS measurements, a series of SFS spectra are recorded for a range of synchronous intervals; therefore, 3D-TSFS is a multidimensional extension of SFS. The term "three-dimensional" refers to the space defined by the excitation or emission wavelength, synchronous interval, and the fluorescence emission intensity.

For medical diagnostics, measurements of quantum yield, polarization, and excited state lifetime may also be valuable. Quantum yield is the ratio of the number of photons emitted from fluorophore to the number of photons absorbed. Polarization gives information on the movement of fluorophore, if there is any, during the time between the absorption and emission of light, namely during the excited state lifetime.

To stimulate and measure fluorescence from a sample, one needs to use instrument with five basic components: (1) light source, (2) wavelength selector elements on the excitation and

emission paths to/from sample, (3) sample holder/positioner, (4) polarizers, and (5) detector (Figure 2).

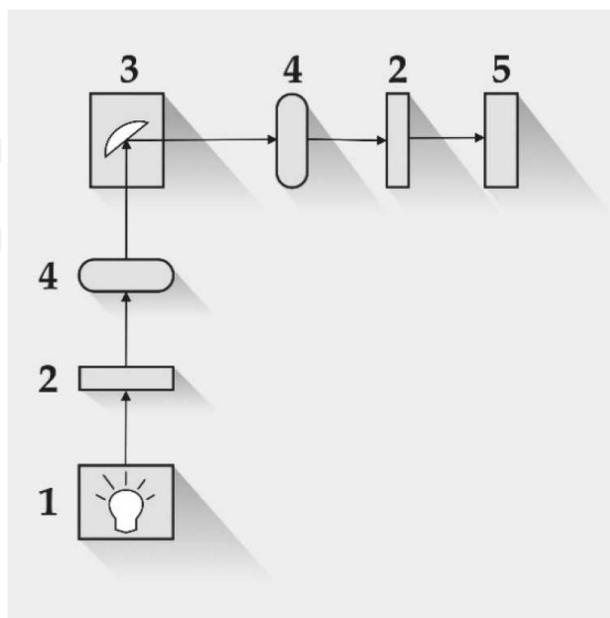


Figure 2. Five basic components of spectrofluorometer.

Polychromatic light from light source is dispersed on a dispersing element from which light beam of selected wavelength is directed on a sample to excite fluorescence. Most frequently, Xe arc lamps are used. These days, deuterium, tungsten, or halogen lamps are rarely utilized for excitation, since they are relatively weak sources for fluorescence. Lasers, laser diodes, and light-emitting diodes (LEDs) are also frequently used. They produce intense monochromatic light, so there is no need for the wavelength-selecting element on the excitation path. The drawback is that excitation spectra cannot be measured with these light sources. Tuneable and supercontinuum lasers are an exception, which can produce emissions over the given spectral interval, but these are rather expensive devices. Light dispersion can be accomplished with prisms and diffraction gratings; the latter is dominantly used in the modern spectrofluorometers. The detector can be either single-channeled or multi-channeled. The single-channeled detector, usually photomultiplier tube (PMT) or semiconductor, detects the intensity of one wavelength at a time. Multi-channeled detectors, such as charge-coupled device cameras (CCDs), record the intensity of emission over the range of wavelengths simultaneously. In addition, modern instruments include some other important components, such as polarizers, filters, and optical fiber connectors. Laboratory spectrometers are complex and robust devices capable of versatile and sensitive measurements. However, for many applications, and for the convenience and mobility of measurements fluorimeters can be designed as miniaturized devices. These devices are particularly suitable for clinical investigations of tissue fluorescence [8].

3. Breast tissue fluorescence

Native fluorescence (autofluorescence) of breast tissue comes from a number of fluorescent biochemical species (fluorophores) whose excitations and emissions are strongly influenced by the absorption and scattering of tissue components. Since the latter processes are both depth- and wavelength-dependent, the observed tissue fluorescence is subject not only to the concentration and microenvironment of fluorophores, absorbers (chromophores), and scatters but also it substantially depends on the geometry of excitation and observation.

The breast, or mammary gland, is an organ composed of four major structures: skin, subcutaneous tissue, breast tissue, and the nipple centered on the round pigmented skin area (areola). In the breast tissue, distinct optical characteristics show glandular, adipose (fat), and fibrous tissue. The differences in autofluorescence of these tissues come from the structural and compositional differences of which the concentration of fluorophores and their distribution has the largest influence. Most fluorophores are associated with the structural matrix of tissues, such as collagen and elastin, or are involved in cellular metabolic processes such as reduced nicotinamide adenine dinucleotide (NADH) and flavins. Other fluorophores include the aromatic amino acids (e.g., tryptophan, tyrosine, phenylalanine), various porphyrins, and lipopigments (e.g., ceroids, lipofuscin) that are the end products of lipid metabolism [9]. Absorption and fluorescence emission spectra of the most important tissue fluorophores are shown in **Figure 3**. The excitation (λ_{ex}), emission (λ_{em}), and absorption (λ_{ab}) maxima for some important tissue fluorophores are given in **Table 3**.

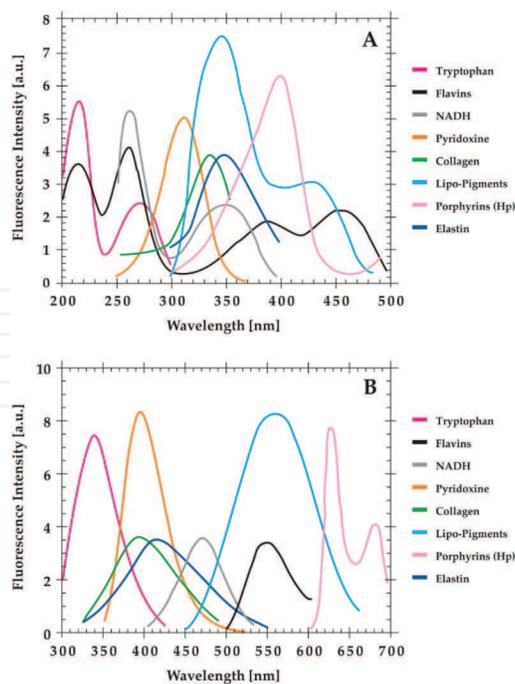


Figure 3. Absorption (A) and fluorescence emission (B) of the most important fluorophores present in tissue. (Adapted from Wagnières et al. [9] with permission of John Wiley and Sons).

Endogenous fluorophore	λ_{ex} (nm)	λ_{em} (nm)	λ_{ab} (nm)
<i>Amino acids</i>			
Tryptophan	280	350	280
Tyrosine	275	300	275
Phenylalanine	260	280	257
<i>Structure proteins</i>			
Collagen	325	400,405	325
Elastin	290,325	340,400	325
<i>Coenzymes</i>			
FAD, flavin	450	535	450
NADH	290,351	440,460	260
NADPH	336	464	340
<i>Vitamins</i>			
Vitamin A	327	510	300 - 350
Vitamin K	335	480	249
Vitamin D	390	480	280
<i>Vitamin B6 complex</i>			
Pyridoxine	332,340	400	291
Pyridoxamine	335	400	-
Pyridoxal	330	385	-
Pyridoxal 5'-phosphate	330	400	-
4-Pyridoxic acid	315	425	307
Vitamin B12	275	305	361
<i>Lipids</i>			
Phospholipid	436	540,560	234
Lipofuscin	340-395	540,430-460	335, 435
Ceroid	340-395	430-460,540	-
<i>Porphyrin</i>	400-450	630,690	408

Table 3. Tissue fluorophores and approximate values of their excitation (λ_{ex}), emission (λ_{em}), and absorption (λ_{ab}) maxima.

Regarding breast cancer, it has been shown that distinct fluorescence response of tumors compared to one ordinary tissue is a result of notable differences in concentrations of collagen, elastin, NADH, and flavin adenine dinucleotide (FAD) [10]. Generally, fluorescence measurements have indicated lower concentrations of collagen and FAD and increased concentrations of NAD(P)H in malignant tissues compared to normal breast tissue [11]. Transformation from

normal to malignant tissue leads to degradation and changes in the cross-links of collagen; breaking the cross-links in collagen is a consequence of the increased presence of collagenase in the tumor cells [12]. Modulation of the extracellular matrix is a common characteristic of invading tumor cells and usually involves increased production of collagenases by the tumor cells or stromal fibroblasts [13]. It has also been shown that the changes in the metabolic status of tissue have influence on the variation in concentrations of both NADPH and NADH. A shift from aerobic to anaerobic metabolism accompanied with damaged mitochondrial metabolism caused by malignant alterations leads to an increased concentrations of electron carriers such as NADH. Increased levels of these coenzymes have been observed in a high-grade malignant tissue [12, 14].

4. Interpretation of tissue fluorescence data

As in all fluorescence measurements, acquiring experimental data on tissue fluorescence is associated with procedural and instrumental/technical difficulties. One should note that unlike some other types of spectroscopy measurements, for example, absorption measurements, the intensity of recorded fluorescence signal and the shape of spectra strongly depend on the characteristics and settings of the instrument. One can see later in the text that establishing diagnosis from fluorescence measurement can be successfully done only if data were acquired from a statistically significant number of samples. With this in mind, each study related to tissue fluorescence should be done on a single instrument and with exactly the same instrument settings (such as PMT voltage and monochromator slit widths) for each sample. Even so, if measurements are done over the longer times some of instrument characteristics may change, such as the strength of the excitation lamp. Samples most frequently have different surface morphologies and can be differently or partly illuminated when they are small sized. All of this may produce unwanted artificial differences in fluorescence spectra, commonly in the intensity of observed signal. Normalization of measured spectra may resolve this problem; however, there is no universal method or procedure for this task. For *ex vivo* measurements, it is important to note that all excised tissues change over the time. The fluorescence measurements on different tissue samples should be done, in principle, at the similar time interval after excision.

It is also important to keep in mind distinction between technical (uncorrected for instrument characteristics) and molecular (corrected) spectrum. Modern instruments commonly produce both types of spectra. In most cases, technical emission spectra are sufficient for the analysis and presentation. However, when it is important to compare emission spectra obtained on different instruments, corrected spectra must be used. Excitation spectra, and conversely SFS, EEMs, and 3D-TSFS, are more instrument-dependent, being largely influenced with spectral characteristic of excitation sources. These spectra are, therefore, analyzed and presented after corrections. In addition, spectra may contain features that originate from physical processes apart from fluorescence, such as Rayleigh and Raman scatters, which should be excluded from subsequent analyses.

Fluorescence spectra of tissues are generally poorly resolved since they are composed from a broad emission of many fluorophores and affected by strong re-absorption processes. When needed, better-resolved spectra can be achieved with synchronous scanning.

5. Mathematical tools for fluorescence-based diagnosis of breast cancer

To draw conclusions from experimental data of tissue fluorescence measurements, these data ought to be interpreted using adequate mathematical methods, most frequently statistical methods. Even if one analyze fluorescence from a single type of tissue, one must take into account that tissue characteristics considerably differ between individuals, statistically speaking they exhibit “a large in-group variances”. For this reason, proper analysis is achieved only if it is done on a statistically significant number of samples and measurements. The differences in fluorescence, for example, differences in wavelengths of emission maxima, integrated emission intensities over some spectral range, excited state lifetimes, etc., between groups of different-type tissues can be asserted by methods of exploratory data analysis, one of which is hypothesis testing. One-tailed and two-tailed *t*-tests are commonly used to find if there is a statistically significant difference between the mean values of analyzed parameters in two data groups. Analysis of variance (ANOVA) and Tukey’s test are used when more than two groups of data should be compared and studied. ANOVA is considered the most used and most useful statistical technique in biomedical research, even though this method is not easy to learn and should be implemented with caution.

Frequently, data from measurements of tissue fluorescence are vast, especially in cases of EEM and 3D-TSFS measurements. The size of data can be significantly reduced without loss of variance using some of statistical tools available for reducing the dimensionality. Principal component analysis (PCA) is a common tool for this purpose, and it is also capable of revealing hidden structures in a data set. Generally, PCA is used to recognize the main variations in a data set in an unsupervised way. It is also able to study these variations and to aid in visualizing them. PCA transforms and compresses input variables, which may be correlated, into uncorrelated variables of fewer numbers in such a way that they preserve the majority of variance of the input data. These new variables are called principal components (PCs) and they are obtained by a linear orthogonal transformation of input variables, so that each PC is, in fact, the linear combination of inputs. The largest portion of the input data variance is accumulated in the first PC, then less variance in the second PC, and so on. Then, for further analyses, one can use just few PCs, which help to alleviate numerical problems associated with large data sets and enable efficient visualization and interpretation of data.

Many features of fluorescence differ between healthy and cancer tissues; the selected examples are shown and discussed in the following section. Even though fluorescence measurements contain information needed for the cancer diagnosis, the observed data are subtly related in ways that are often difficult to express in the form of diagnostic rules just by observing spectra and must be processed for tissue classification purposes. To do so, mathematical algorithms ought to be developed and optimized to classify tissues into their respective histological categories. Several methods have been successfully used for this purpose.

Linear discriminant analysis (LDA) can be used for the discrimination of two or more groups from one or more linear functions (latent variables) of input data. As a consequence of singularity problems (caused by fewer samples in group than variables or highly correlated variables), LDA is incapable of solving high-dimensional data problems. In such cases, reduction of data dimensionality is required for the successful application of LDA.

Partial least squares (PLS) discriminant analysis (DA) is a method based on the PLS regression, which constructs linear discriminant models with no restriction for processing high-dimensional data. Input data are transformed by the PLS-DA into a set of linear components (latent variables) further used to predict the dependent variable. The dependent variable is in fact a dummy variable that only serves to show whether a particular sample belongs to the specific class. With the LDA model, one is able to perform classification of data, that is, to predict the class to which new, unknown samples belong.

Artificial neural network (ANN) is a model used to approximate unspecified relations between a large number of input data in a robust manner. ANN learns from data, so it can be regarded as a machine-learning method and is able to handle a variety of problems including complex ones such as those involved in medical diagnostics. For example, ANNs can perform data classifications in both a supervised and unsupervised way, that is, both with prior knowledge of the data class and without. Unlike human decisions, those made by ANNs are invariably consistent since they are not liable to suffer from fatigue or bias. Kohonen's self-organizing map (SOM) and feed-forward neural network (FFNN) are among the most popular neural network architectures. SOM converts high-dimensional nonlinear statistical relationships into simple geometric relationships in an unsupervised way. On the other hand, FFNN uses a supervised training method which requires data on sample's group membership entered along with other data at the network inputs.

Support vector machine (SVM) is a statistical technology developed by the machine-learning community that can be used for both classification and regression. Compared with other machine-learning methods, SVM has such advantages as it does not require a large number of training samples for developing model and is not affected by the presence of outliers. Having high generalization procedure and feasibility to extract higher order statistics, the SVM has become extremely popular in terms of classification and prediction. In the context of classification, SVM is transforming original data space into a much higher dimension space in which classification groups would be linearly separable. For the data that belong to one of two classes (binary classification), SVM aims to derive the hyperplane in the transformed space so that the data of one class are on one side of the plane, and the data of other class are lying on the other side of the plane. The position of the hyperplane should be such that the greatest possible fraction of data is correctly placed and that the distance of both classes from the hyperplane is maximal. Such conditions minimize the risk of misclassifying data.

The performance of binary classification, the most probable classification case in breast cancer diagnoses, can be evaluated from a receiver operating characteristic (ROC) curves. The ROC curve is in fact graphic that shows the performance of a classification at different discrimination threshold values. It helps in finding how good classification model discriminates samples belonging to the particular group from all other samples. To construct the ROC, sensitivity and

specificity of the model are calculated for different threshold values and plotted. Generally, an excellent model has an area under the ROC curve between 0.9 and 1.

Some other mathematical tools may also be useful for the study of breast cancer fluorescence. We believe that it is important to mention parallel factor analysis (PARAFAC) since this method is capable of modeling fluorescence response of complex fluorescence systems, the category in which all biological systems fall. Nowadays, PARAFAC has commonly used with EEMs in the analysis of fluorescence in many fields since it is a multi-way decomposition method capable of analyzing complex high-dimensional data and perform second-order calibration. Its main advantages are the uniqueness and simplicity of its solutions. When the correct number of components and multilinear data are used to build the PARAFAC model, the true underlying phenomenon is revealed. An additional advantage of this form of analysis is that it can predict the concentrations of different chemical compounds in complex systems, a phenomenon known as second-order advantage. For these reasons, PARAFAC is used to detect fluorophores in multi-component systems and precisely calculate their concentrations.

6. Examples of applications of fluorescence spectroscopy in breast cancer research

So far, different types of fluorescence measurements have been employed for the breast cancer diagnosis and they are listed in **Table 4** along with comments on the used instrumentation and obtained sensitivity and specificity of detection.

Measurement type	Instrumentation specifics	Measurement setup (nm)	SE, SP (%)	Comment	Ref.
Emission spectra	Argon laser	Ex 457.9, 488 Em 460-700	-	<i>Ex vivo</i>	[15]
Emission spectra	N ₂ laser	Ex 337 Em 360-560	99.6, 99.6	<i>Ex vivo</i>	[16]
Excitation spectra	lamp-based spectrophotometer	Ex 250-320 Em 340	> 90, -	<i>Ex vivo</i>	[17]
Emission spectra	N ₂ laser	Ex 458 Ex 480-700	100, 100	<i>Ex vivo</i>	[18]
EEM	lamp-based spectrophotometer with fiber optics probe	Ex 300-460 Em 310-600	70, 91.7	<i>Ex vivo</i>	[19]
EEM	lamp-based spectrophotometer	Ex 260-540 Em 275-700	-	MDA231 MCF10 T47D cell line	[20]

Measurement type	Instrumentation specifics	Measurement setup (nm)	SE, SP (%)	Comment	Ref.
EEM	lamp-based spectrophotometer	Ex 335-470 Em 430-640	83.9, 88.9	<i>Ex vivo</i>	[11, 21]
3D-TSFS	lamp-based spectrophotometer	330-650, $\Delta\lambda$ 30-120	100, 100	<i>Ex vivo</i>	[10, 26] [27]
Synchronous spectra	lamp-based spectrophotometer	Ex 340-650 $\Delta\lambda$ 30, 80	93.3, 91.9	<i>Ex vivo</i>	[22]
Polarized fluorescence spectra	Argon laser	Ex 488 Em 540-700	92.6, 91.2	<i>Ex vivo</i>	[23]
Emission spectra	laser diode Argon laser Ex	Ex 405, 458 Em 428-700, Em 470-750	-	<i>In vivo</i>	[8]
Synchronous spectra	lamp-based spectrophotometer	Ex 250-650 $\Delta\lambda$ 40	90, 79	<i>Ex vivo</i>	[24]
Lifetime	Supercontinuum pulsed laser	Ex 447 Em 532, 562, 632, 684	89.8, 93.8	<i>Ex vivo</i>	[25]

Note: Ex – excitation, Em – emission, $\Delta\lambda$ – synchronous interval, SE – sensitivity, SP – specificity.

Table 4. Fluorescence methods used for breast cancer diagnosis.

6.1. Breast cancer diagnosis using emission and excitation spectra

The first report on the characterization of human breast tissues by fluorescence emission spectroscopy has been published by Alfano et al. [15] They have obtained normal and cancerous breast tissues from two different individuals and have measured emission spectra (460–700 nm) after excitation with an argon ion laser (at 488 and 457.9 nm). However, the assessment of method's capability for diagnosis has been impossible because of small number of investigated samples. Gupta et al. [16] have measured N₂ laser-excited (337 nm) emission spectra (360–560 nm) from different sites on benign (fibroadenomas, 35 patients), cancerous (ductal carcinomas, 28 patients), and normal specimens (the uninvolved areas of the resected cancerous specimens). Intensities of emission were much higher from cancerous sites compared to those of benign tumor and normal breast tissue sites (**Figure 4**). In this *ex vivo* study, cancer tissues have been discriminated from benign and normal ones with a sensitivity and specificity of up to 99.6% using absolute intensity of emission (with aid of stepwise multivariate linear regression statistical method).

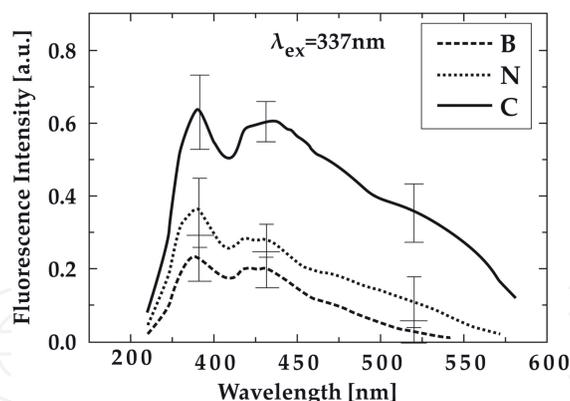


Figure 4. Mean emission spectra from 245 cancerous (C), 230 normal (N), and 436 benign (B) breast tissue sites. (Adapted from Gupta et al. [16] with permission of John Wiley and Sons.)

Yang et al. [17] have reported the use of excitation spectra for breast cancer detection. They have measured excitation spectra in the 250–320-nm spectral region recording emission at 340 nm from 103 malignant and 63 benign breast tissues. The averaged spectra and their difference (**Figure 5**) distinct spectral features can be clearly evidenced. Authors have estimated above 90% sensitivity and specificity of cancer diagnosis for this method.

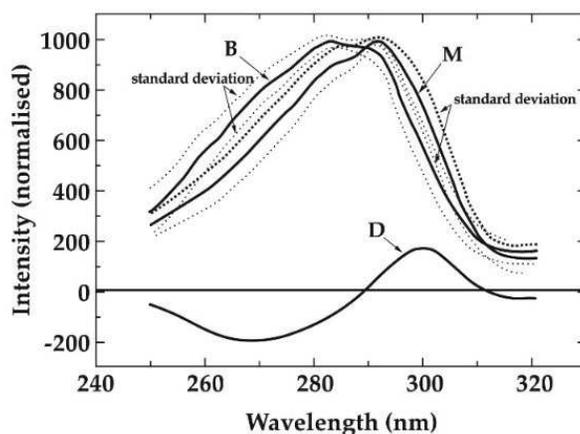


Figure 5. Averaged excitation spectra for emission at 340 nm from 103 malignant and 63 breast tissues. D is the difference spectrum (M – B). B is benign; M is malignant. (Adapted from Yang et al. [17] with permission of John Wiley and Sons.)

6.2. Breast cancer diagnosis using EEMs

Considering that EEMs incorporate more information pertinent for the breast cancer diagnosis than the single emission or excitation spectra, several studies have been conducted using this method (see **Table 4**). Palmer et al. [19] have measured EEMs (excitation range: 300–460 nm; emission range: 310–600 nm) of 20 malignant, 15 normal/benign fibrous, and 21 adipose tissue samples. They have found four peaks in the spectra of malignant and normal/benign fibrous tissues which occurs in similar locations (excitation/emission pairs of (300, 340), (340, 390), (360,

460), and (440, 520) nm). The spectra of adipose tissue were distinctively different; particularly, the peak at (340, 390) nm was weakly present and the peak at (360, 460) nm has been shifted to approximately (360, 520) nm. Using PCA as a data reduction technique and SVM for classification, authors have showed that EEM was successful in discriminating malignant and nonmalignant tissues with a sensitivity and specificity of 70% and 92%, respectively.

6.3. Breast cancer diagnosis using SFS and 3D-TSFS

Synchronous spectrum, in majority of cases, has more features and provides more information than the conventional emission or excitation spectra. This comes as a result of simultaneous acquisition of both excitation and emission spectral characteristics of the sample, which brings more information to a single spectrum. It has been shown that SFS measurements have better selectivity and decreased bandwidths than emission or excitation spectra. For these reasons, they can be used to detect fine differences in the fluorescence of complex systems. Dramićanin et al. [22] have measured SFS with different synchronous intervals on 21 normal and 21 malignant breast tissue samples. The largest differences between spectra have been found for synchronous intervals of 30 and 80 nm, **Figure 6**. Diagnostic criteria have been established from the areas below different spectral peaks and from the ratios of these areas. The first

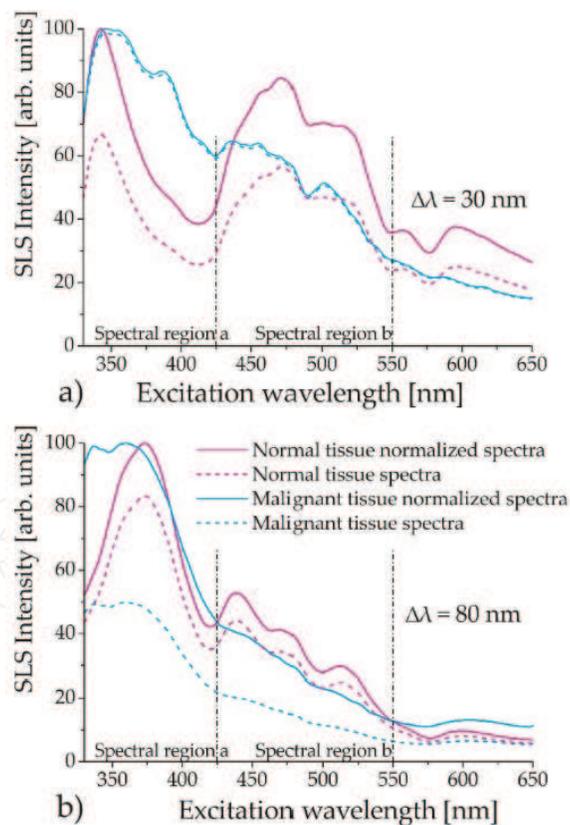


Figure 6. Synchronous fluorescence spectra taken with (a) 30 nm and (b) 80 nm synchronous intervals; the average spectra of 21 normal breast samples and 21 malignant are represented with the red and blue lines, respectively; dashed lines stand for the raw spectra and full lines for those additionally normalized. (Adapted from Dramićanin et al. [22].)

criterion yielded a sensitivity of detection of 77.4% and a specificity of 86.1%, while the second one presented a sensitivity of 90.3% and a specificity of 94.4%.

3D-TSFS and first derivate 3D-TSFS spectra have been measured on the same samples [10], and showed significant differences between normal and malignant tissues. Based on these differences, an artificial neural network (SOM) diagnosis method has been established [26], which provided a sensitivity of 87.1% and a specificity of 91.7% when using 3D-TSFS data, and 100% sensitivity and 94.4% specificity when using first derivate 3D-TSFS data. Diagnosis based on the use of support vector machine algorithm provided 100% specificity and sensitivity for both 3D-TSFS and first derivate 3D-TSFS [27].

6.4. Breast cancer diagnosis using lifetime and polarization measurements

In contrast to emission or excitation measurements, excited state lifetime measurements are not affected by the morphology of specimen and geometry of the measurements. Therefore, no normalization procedures are needed to compare results obtained with different instruments, which is quite important for the applicability of derived diagnostic methods. Sharma et al. [25] have measured fluorescence lifetimes at multiple emission wavelengths (532, 562, 632, and 644 nm) under excitation at 447 nm on 93 sites from 6 specimens (34 infiltrated ductal carcinoma, 31 benign fibrous, and 28 adipose sites). They have found that lifetime values measured at 532 and 562 nm significantly differ between normal and malignant sites, and achieved 92.3% accuracy for classifying infiltrated ductal carcinoma.

Choi et al. [23] have performed analysis of parallel and perpendicularly polarized fluorescence from 36 normal and 36 cancerous tissue samples. First, they have performed random forest algorithm to the four data sets: the fluorescence intensity and the curvature features for parallel and perpendicular components. Then, SVM classifiers were designed using the significant features from the random forest algorithm, which provided >90% specificity and sensitivity of breast cancer detection.

7. Conclusion

To conclude, fluorescence is sensitive to the biochemical makeup of tissue. There are several natural fluorophores that exist in tissues and cells that, when excited with ultraviolet and visible light, fluoresce over well-defined spectral regions. Among them, the fluorescence of collagen, elastin, NADH, and FAD contribute the most to the different fluorescence of cancerous tissue in respect to the normal because of altered concentrations and local environment of fluorophores. Distinct differences in the fluorescence of cancerous and normal tissues can be observed with almost all conventional fluorescence techniques. For this purpose, up to now, emission spectroscopy and EEMs have been used most extensively. However, even though fluorescence differences between tissues are obvious, the development of a sensitive diagnostic method based on these differences is not an easy task. First, a large number of specimens must be measured under identical conditions. The specimens should be taken from different individuals and some gold diagnostic standard must be provided (e.g., histopathol-

ogy). Fluorescence data ought to be processed with statistical tools that include analysis of variance, data reduction, and regression to obtain diagnostic criteria, and further with statistical tools to validate diagnostic results. On the other hand, once established, fluorescence methods are a great aid for early cancer detection, since methods can be noninvasive and do not require expensive and sophisticated equipment.

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Author details

Tatjana Dramićanin and Miroslav Dramićanin*

*Address all correspondence to: dramican@vinca.rs

Vinča Institute of Nuclear Sciences, University of Belgrade, Belgrade, Serbia

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