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Using Artificial Neural Networks for Optical Imaging of Fluorescent Biomarkers

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***Abstract*—**The article presents the results of the application of artificial neural networks to separate the fluorescent contribution of nanodiamonds used as biomarkers, adsorbents and carriers of drugs in biomedicine, from a fluorescent background of own biological fluorophores. The principal possibility of solving this problem is shown. Use of neural network architecture let to detect fluorescence of nanodiamonds against the background autofluorescence of egg white with high accuracy - better than 3 ug/ml.

***Keywords*—**Artificial neural networks, fluorescence, data aggregation.

I. Introduction

DUE to the intensive development of biomedicine there is a need to develop ultra-sensitive and highly reproducible methods for the detection of proteins, single cells and genes, as well as imaging biomarkers. The most common way of imaging is the method using fluorescence (FL). The main difficulty of this method is to separate the desired signal of fluorescent biomarker from the background fluorescence of fluorophores of biological tissues. The most important autofluorophores are tryptophan, phenylalanine, tyrosine, collagen, flavoproteins and flavin, beta-carotene, porphyrins, nucleic acids, cofactors, vitamins, pigments and other. Tissue autofluorescence spectrum is the result of superposition of a large number of FL bands of these fluorophores and occupies range from 250 nm to 700 nm (Fig. 1).

This autofluorescence makes it difficult to observe the processes and movement of fluorescent nanoparticles. Therefore it is important to elaborate a method of separation of the fluorescent signal of the nanoparticles from the background of the fluorescence of biological tissues and the control of its change to ensure tracking biomarkers.

There are two major approaches to overcome the problem of background fluorescence: 1) synthesis of new biomarkers with minimum overlap of their emission spectra with the background fluorescence [2]-[21]; 2) development of advanced experimental techniques permitting one to decrease the background signal [22]-[30].

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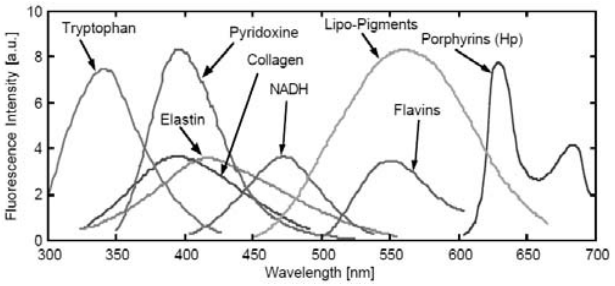

Fig. 1 Spectra of fluorescence of own fluorophores of biological tissues [1]

In this paper, a new approach to solve the inverse problem of separation of the fluorescent signal of the nanoparticles from the autofluorescence background using artificial neural networks (ANN) is suggested [31]. A row of methods and models that can be used for detection and recognition of objects by their images was proposed by authors [32]-[34]. An especial feature of using such methods to solve specific practical problems is that the solution of each of them requires a new specific additional research and development. Therefore, along with the use of well-known methods in their classical form, an active research towards further improvement of the accuracy and efficiency of neural network solution of different inverse problems is continued. [35]-[37]. It is very promising now to use ANN for solution of such problems as classification of proteins, selection of genome fragments, recognition of signal peptides and transmembrane helices etc. [38]. In [39], [40] the method of breast cancer diagnosis based on artificial neural network classification was proposed. The inverse problem of autofluorescence recognition of civilized cell culture and cancer cells was solved. Total synchronous fluorescence spectra of normal skin, nevus and melanoma samples were used as input for training of artificial neural networks. Two different types of artificial neural networks were trained, the self-organizing map and the feed-forward neural network. Histopathology results of investigated skin samples were used as the gold standard for network output. Based on the obtained classification success rate of neural networks, Dramircanin, Zekovirc et al. [39] concluded that both networks provided high sensitivity with classification errors between 2 and 4%. Despite the extremely wide application of pattern recognition methods in biomedicine, this paper is the first application of these methods for detection of fluorescence of nanoparticles in the presence of background autofluorescence to the authors' knowledge.

This paper shows fundamental possibility of optical

imaging fluorescent biomarkers in a biological environment using neural network algorithms. In addition, in this work the quantitative threshold of the sensitivity of the method was determined: the estimation of the minimal concentration of nanoparticles that can be confidently detected by ANN against the background of autofluorescence. The search of the ways to improve the sensitivity of the method, and a study of the practical stability of the solution of this inverse problem, were performed.

II. Experiment

A. Objects of Research

Nanodiamonds (NDs) G01 (PlasmaChem) were used as fluorescent biomarkers. Nanodiamond particles are very promising material for biomedicine, because of the stable and intense properties of fluorescence and absorption [12], [13], as well as NDs are biocompatible particles, unlike organic dyes and semiconductor quantum dots. Bidistilled deionized water was used for the preparation of aqueous suspensions of nanodiamonds. Aqueous suspensions were prepared with a concentration of G01 1 g/l. Suspensions were treated during 2 hours in ultrasonic bath (Bandelin Sonorex rk 31).

Egg white was chosen as a biological environment. This choice is justified by the fact that in this case nanoparticles are delivered directly into the cell, because the whole egg white is a single cell. Also the choice associated with representative natural fluorophores in the egg white. Later in the elaboration of neural network techniques of solution of stated problem in order to improve the stability of the trained ANN to changes in biological objects egg whites of different periods and different manufacturers were used.

B. Experimental Setup

Fluorescence and Raman spectra of aqueous suspensions of nanodiamonds and biological objects containing nanoparticles were excited by a diode laser (wavelength 405 nm, power incident on the sample ~50 mW). Spectra were recorded by PMT in the range 430-750 nm. Practical spectral resolution was 0.5 nm. Temperature of the samples was stabilized at 22.0±0.1ºC. Spectra were corrected for the laser power and data acquisition time. Further mathematical data processing consisted of the subtraction of the background caused by light scattering in the cuvette with sample and normalization of the obtained spectra to the area under the Raman valence band of water.

C. Analysis of the Fluorescence Spectra of Egg White with Introduced Nanodiamonds

Fig. 2 shows Raman and fluorescence spectra of water, egg white, aqueous suspensions of NDs and egg white with introduced nanoparticles. The spectral band near 470 nm is the Raman valence band of water (percentage of water in the egg white is 85%).

As it can be seen from Fig. 2 when fluorescence signal is excited by radiation with wavelength 405 nm, ND fluoresces in the region 430-680 nm with the maximum near 520-525 nm. Egg white fluoresces in the region 420-700 nm with maximum which varies for different eggs within 480-520 nm. The FL spectrum of egg white is a result of a superposition of different organic compounds: pyridoxine, NADH, flavin, lipo-pigments. The weak FL bands in the region 640 - 670 nm are due to porphyrins fluorescence. Thus, fluorescence spectra of nanoparticles and egg white are largely overlapping structureless bands. They differ in the position of maximum and boundaries of fluorescence bands. It is a precondition for successful extraction of contribution of nanoparticles.

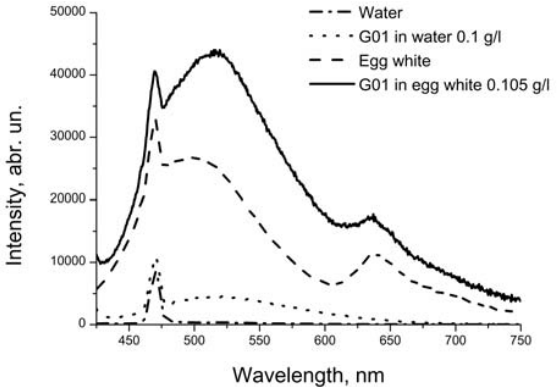

Fig. 2 Raman and fluorescence spectra of water, egg white, aqueous suspensions of NDs (G01) and egg white containing NDs

Obviously, if the concentration of nanoparticles in egg white changes, the band of total fluorescence varies significantly for several reasons. The main reasons are as follows 1) at change of concentration of nanoparticles the intensity of their own fluorescence changes; 2) due to the different interactions of NDs with components of white, both fluorescence of white and that of nanoparticles change. These interactions are very complex and still not interpreted. For these reasons, it is impossible to construct a mathematical model of change of total fluorescence of egg white and nanoparticles at change of their concentrations (for example, during movement of nanoparticles in biological tissue) by conventional methods. This means that the traditional mathematical methods cannot solve either direct or inverse problem of extracting the fluorescent contribution of varying amount of nanoparticles against the background of the fluorescence of egg white. This is one more reason to use the algorithms of artificial neural networks (ANN).

III. Methods and Approaches

In this study, to solve the pointed inverse problem of optical visualization the following methods and techniques were elaborated:

1) Method of Detection of ND Fluorescence against the Background of Autofluorescence of Biotissue by Total FL Spectrum of the Sample

The considered problem is the simplest variant of classification problem - determination of the belonging of a pattern to one of two non-crossing classes (nanoparticles present - no nanoparticles). In solution of this classification

problem, it is necessary to take into account specifics of input data, where there are no individual features allowing one to ascertain confidently belonging of the pattern, i.e. it is not a problem of classical spectral analysis. On the contrary, spectra of fluorescence of nanoparticles and biofluorophores almost completely overlap, although they have different shape. This peculiarity causes expediency of application of neural network methods for solution of the stated problem and requires elaboration of correct methodology of its solution. This methodology includes elaboration of procedure of data preparation, determination of optimal neural network architectures, algorithms and parameters of their training.

2) Method of Determination of ND Minimal Concentration, When the Presence of Nanoparticles is Confidently Detected against the Background of Proper Fluorescence of Biotissue

This means determination of the threshold of sensitivity of the method on the whole. It is clear that the numeric value of this threshold depends both on methodology of experiment and on further data handling.

3) Use and Comparison of Algorithms of Input Data Compression

One of the traditional problems arising in work with spectroscopic data is very high dimensionality of the input data, as a spectrum is usually registered in several hundred or even thousand channels. At the same time, the number of patterns for ANN training when using the “experiment-based” approach (ANN training using only experimental data) corresponds to the number of measured spectra, and therefore it is substantially limited [41]. Both of these factors reduce the ratio of the number of patterns and the number of input features. This is unfavorable for ANN training. Therefore, it is necessary to elaborate a method to reduce the dimensionality of the input data space.

Use of ANN for solution of inverse problems of optical spectroscopy is possible in the context of three approaches: “model-based”, “experiment-based”, “quasi-model” [41]. In the “model-based” approach, to obtain the data for ANN training, an existing analytical or computational model of solution of the direct problem is used. In the considered problem of recognition of fluorescent contribution of ND against the background of fluorescence of egg white, the “model-based” approach cannot be used because of lack of correct analytical description of fluorescence spectra of ND and egg white. In the “quasi-model” approach, model spectra are used to obtain representative data sets. A parametrical “quasi-model” is constructed for description of spectra on the base of a moderate set of experimental data, and then it is used to calculate the complete data array. Because of the object of study is a living biological material, and its state can vary significantly with time, it is especially important to train the ANN on real signals of objects containing noise. This is the reason why the inverse problem was solved by ANN in the frameworks of "experimental-based" approach.

In the "experimental-based" approach experimental data are used to train the neural network. The disadvantage of this approach is the low representativity of sets since obtaining of immense experimental material is a reasonably tedious work. The main advantages of this approach are the following: when ANN trains directly on experimental data, all molecular interactions are taken into account; the network is trained with real instrumental noise which raises accuracy of solution of inverse problems.

The considered problem in its original formulation is characterized by extremely unfavorable ratio of the number of examples in the training set and the number of input features. Thus, an important area of research is the use of algorithms for reducing the input dimension of the problem, which means reducing the number of input features. The previous studies made by the authors of this work [42] showed that one of the most efficient ways to reduce the dimensionality of the spectroscopic data is the aggregation of channels. New composed features constitute sums of intensities in several adjacent spectral channels. Apart from possible improvement of the quality of solution of the problem, this approach in case of success can allow using considerably less expensive equipment with significantly lower spectral resolution.

IV. Results and Discussion

A. Obtaining Experimental Data

Two series of Raman and fluorescence spectra were obtained for two different egg whites with injected NDs. The concentration of NDs varied in the range from 0 to 30 ug/ml with increments 1.5 ug/ml. Fig. 3 shows some of experimental Raman + FL spectra of NDs with egg white with different concentrations. The obtained datasets for ND were used for work with ANN.

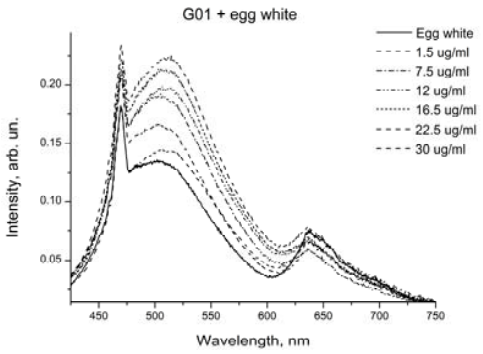

Fig. 3 Some of the spectra from one of the series of Raman and fluorescence spectra of egg whites with different concentrations of introduced ND

B. Use of ANN

For realization of "experimental-based" approach to ANN training, the experimental data was divided into three sets: training, test and examination. Training set is used for actual ANN training (weights adjustment), test - for periodic testing of the learning process in order to determine the moment of termination of the training and to prevent network overtraining, examination - to check the quality of network on independent data. In this paper, this partitioning was carried

out randomly in the ratio of 70:20:10 (training: test: examination). As a result, for the spectra of egg white with NDs the sets 45:12:6 (total 63) were obtained. For an objective evaluation of the quality of networks, averaging over ten random partitions for each type of dimension reduction of input data was conducted.

The following adaptive algorithms were used to work with this problem:

1) A perceptron with a single hidden layer (with 10 neurons in a single hidden layer), trained by back propagation algorithm [31], [43] with the following parameters: the transfer function in the hidden and output layers - hyperbolic tangent, learning rate 0.01, moment 0.5, the initial dispersion of weights 0.3, random order of presentation of examples, stop after 100,000 events after the minimum of the error on the test dataset.
2) A General Regression Neural Network (GRNN) [28]. For GRNN, two variants were considered: with unified smoothing factor (USF) selected by step by step method, and with individual corrections to the smoothing factor (ICSF) for every input feature. In the second case, the values of the corrections, as well as the values of the main smoothing factor, were selected by genetic algorithm [31]. In both cases, selection was performed by minimal squared error on test set.

Software package NeuroShell 2 [44] was used for all calculations.

Table I shows the results obtained using the perceptron with a single hidden layer and two variants of GRNN on three data sets (training, test, exam). The results obtained on the examination set are the most informative. As statistics values, Table I shows the coefficient of multiple determination R square [41] and the mean absolute error (MAE) of measuring the concentration of ND.

TABLE I
VALUES OF THE COEFFICIENT OF MULTIPLE DETERMINATION R SQUARED ON DIFFERENT DATA SETS FOR DIFFERENT ALGORITHMS OF DATA PROCESSING

Algorithm \ Data set	Training	Test	Exam	MAE of exam set, ug/ml
Perceptron	0.995	0.897	0.879	2.41
GRNN, USF	0.987	0.878	0.793	3.04
GRNN, ICSF	0.991	0.890	0.868	2.78

As it can be seen from Table I, the best results on the examination data set are obtained using the perceptron with a single hidden layer. Further calculations were carried out only using this architecture of ANN, which provided the error in determination of the concentration of ND in the egg white 2.41 ug/ml for a given data partition.

C. Use and Comparison of Algorithms of Input Data Compression

As already mentioned, the problem is characterized by extremely unfavorable ratio of the number of examples in the training set (45) and the number of input features (651). Therefore, the compression of the input data was carried out in two ways: aggregation of the channels of the spectrum and selection of the most essential characteristics using standard deviation of the channel values, proportional to the amount of information contained in that channel.

Tables II and III show the best results obtained with a perceptron with a single hidden layer on exam sets, for one partitioning into training, test and examination sets, and then averaged over 10 random partitionings - for the original dataset and after compression of input features. Aggregation is carried over 2, 4, 6 and 8 channels. The best results were demonstrated by aggregation over 4 channels, and they are shown in Tables II and III.

TABLE II
RESULTS FOR THE BEST PARTITIONING BY ERROR ON EXAMINATION DATASET

	Type of preprocessing	Number of channels	R^2 on the training set	Average error on the examination set, ug/ml
ND, 63 examples; partition 45:12:6	Without preprocessing	651	0.9948	2.41
	Aggregation	165	0.9954	1.04
	Selection	330	0.9954	0.91

TABLE III
RESULTS OF AVERAGING OVER TEN DIFFERENT PARTITIONINGS: MEAN ± STANDARD DEVIATION

	Type of preprocessing	Number of channels	R^2 on the training set	Average error on the examination set, ug/ml
ND, 63 examples; partition 45:12:6	Without preprocessing	651	0.837±0.106	4±2
	Aggregation	165	0.832±0.112	3.5±1.6
	Selection	330	0.910±0.084	2.5±1.0

As it can be seen in Table III, the error on examination set for the initial array of data for ND is on the average 4 ug/ml. The high value of the coefficient of multiple determination R^2 should be noted. It indicates effective learning of ANN.

It can be seen from these results, that both methods of reduce of dimension of input features allow to decrease the error. In the case of aggregation, significant features are concentrated in a smaller number of channels without loss of representativity. In the case of neglecting unimportant features the same principle operates: the network is trained better when more information is concentrated in fewer input features without loss of representativity. Table I shows the best results obtained by the aggregation over 4 channels. However, the removal of uninformative channels turned out to be more effective. As a result, the average error on the examination set decreased by one and a half.

Besides the possible improvement of the quality of the problem solutions, compression of input features, if successful, can afford to use much less expensive equipment with a significantly lower spectral resolution. Thus, the aggregation over 4 channels corresponds to "desensitization" of spectral resolution of registration devices by 4 times (from 0.5 nm to 2 nm). Spectral range of registration is usually chosen in such a way to include the entire bands of objects. Discarding unimportant input features allows to select the most informative region of the spectra and to use the device with registration in the narrow spectral range.

Thus, the results of the use of ANN showed the principal

possibility of solving the problem of separation of the fluorescent contribution of nanodiamonds from the background autofluorescence. After reducing the dimension of the input data features, the attained accuracy of determination of the concentration (on examination set) in the studied volume is up to 3 ug/ml for ND, which is also equal to the minimum detectable concentration of nanoparticles.

V. Conclusions

In this paper the principal possibility of solving the inverse problem of optical imaging – extraction of fluorescence of nanoparticles against the background of the autofluorescence of the biological environment using neural network algorithms was demonstrated. It is shown that used methods allow to detect ND fluorescence against the background of the autofluorescence of egg white with a sufficiently low threshold of detecting concentration. It was also shown that the use of the input data compression by aggregation or selection of initial spectral channels can further improve the accuracy of solving the inverse problem in 1.5 times.

Some advantages of the proposed method of imaging fluorescent nanoparticles in biological tissues should be noted.

1. In this study the successful application of ANN for detection of nanoparticles in biological objects using the fluorescent signal (i.e. for the case of fluorescence spectroscopy, when only simple and cheap equipment is required) was demonstrated. Obviously, in this case it is possible to operate with the blood, skin or subsurface vessels. But so far it is impossible to obtain a fluorescence signal from the deeper layers of the biological system. Nevertheless, the elaborated method can be used for detection of signal from deeper layers of bioobject (for example, when X-ray sources of excitation are used).
2. An important advantage of using ANN is that the training of neural networks occurs already considering all possible interactions of nanoparticles with biomacromolecules. Of course, here it is intended to work with specified object. For another bioobject and other nanoparticles new training ANN is required using appropriate experimental sets.

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ANN computations in this study were performed with NeuroShell 2 software package by Ward Systems Group, Inc. (http://www.wardsystems.com/neuroshell2.asp).

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