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Artificial Neural Network for Temporal Impedance Recognition of Neurotoxins

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***Abstract*— The design, development and *in-vitro* evaluation of an impedimetric neurotoxicity cell-based biosensor that is designed for real time monitoring of changes in electrophysiological behavior under the influence of neurotoxins is described. The electrical cell impedance sensing (ECIS) system [ECIS 8W1E element array of gold electrodes] is used as a substrate for the culture of rat pheochromocytoma (PC 12) cells. The neurotoxicity biosensor is a microfabricated solid state device that mimics the natural environment of PC 12 cells that are responsive to neurotoxins. The PC 12 neurotoxicity biosensors are complemented by artificial neural networks (ANNs) to recognize the impedance profiles of the cells under the influence of a neurotoxin. The neurotoxins were rotenone (Rot), okadaic acid (OA) and peroxynitrite (Per), which are all known to induce cell death in PC 12 cells. Three multilayer feedforward artificial neural network models were developed using a back-propagation algorithm for pattern recognition of neurotoxins. The neurotoxin network (NTN) and the neurotoxin concentration network (NTCN), were trained with data from all the neurotoxins and the cascade network (NTN_NTCN) was developed by combining both the NTN and NTCN. The cascade network was developed to screen against false positives. The neurotoxicity biosensor coupled with these networks allowed for the action of unknown agents (neurotoxins) to be deduced by the measured cellular response. Using back-propagation ANNs to distinguish neurotoxins under the cascade network, the highest success recognition rate for concentration identification were 96% for peroxynitrite, 88% for rotenone, and 96% for okadaic acid. The recognition rate for neurotoxin identification was 98%. The ANN models required less than ten minutes to train and demonstrated that back-propagation ANNs can be handled by commercially-available computers to train and assimilate neurotoxin impedance information, permitting high success rates in the neurotoxin recognition problems.**

***Index Terms*— Artificial neural networks, cell-based biosensors, neurotoxicity, pattern recognition**

I. Introduction

The principle of electric cell-substrate impedance sensing (ECIS), used for impedance stimulations, has become an integral part of neuron-to-electrode interface technology [1]-[8]. Electrical impedance techniques have been used to study the electrical properties of anchorage dependent cells in culture [9]. In this approach, cells were cultured on microelectrode arrays (MEAs) and the motility/viability/electrophysiology of the cells were electrically detected and monitored.

Traditionally, toxicity assessments involve animal studies, which are both time-intensive and costly [10]. Thus opportunities arise for the development of cell-based, high-throughput screening techniques that may be used for toxicity assessments and drug development. Cell-based biosensors are a key component in the development of practical methods for the screening of drugs for possible toxic side effects and for the monitoring of the effects of biochemical warfare agents; thus minimizing the use of experimental animals.

The work of Giaever *et al.* in 1984 [9] and Connolly *et al.* in 1990 [11] represent previous studies of the electrical impedance characteristics of anchorage dependent cultured cell lines. Many studies since Connolly *et al.* have investigated different designs pertaining to the combination of neuronal cells and electrodes based on contact adhesion [12]-[14]. Recently, other groups using different electrode structures have performed impedance studies of anchorage dependent cultured cells [15]. Slaughter *et al.* in 2004 focused on the development of a cellular measurement systems exemplified by the monitoring of the electrical impedance of rat pheochromocytoma (PC 12) cells [16] cultured on ECIS gold microelectrode arrays [7].

Currently, artificial neural networks (ANNs) have become the subject of study in many diverse research areas, such as neuroscience, medicine, engineering and economics, to solve problems that cannot be easily solved by other more established techniques. We have investigated the fundamental aspects of cell-neurotoxin interactions by developing techniques that couples the impedance responses generated from the PC 12 neurotoxicity biosensor with that of ANNs to exploit the properties of nonlinear modeling by artificial neural network models in order to solve the problems of pattern recognition of nonlinear dynamical systems. Useful insights into the toxin-induced cell death can be obtained through pattern recognition, thus increasing the speed of drug discovery/screening and minimizing the use of large-scale animal tests.

In this paper, we focused on the utilization of the laminin derivatized CA SAM [7] to monitor the impedance measurements of PC 12 in the presence of neurotoxins. A thorough toxicity evaluation is a vital step in the development of many products in the pharmaceutical industry and in the chemical industry. We report on the overall performance rates of three feedforward network models; (i) a neurotoxin network (NTN), (ii) a neurotoxin concentration network (NTCN), and

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(iii) a cascade network (NTN_NTCN). We address the neurotoxin network performance by increasing the training size, which was discovered to enhance the recognition rates. We also describe the design and development of the cascade network. We demonstrate the development of a PC 12 neurotoxicity biosensor that has the ability to detect and/or classify unanticipated threats (e.g. novel pathogens) with high success recognition rates and establish the principle that it is possible to recognize and differentiate between neurotoxins in real time utilizing temporal impedance signals and ANNs. Our ultimate goal is the development of techniques to allow the action of unknown agents (neurotoxins) to be deduced by the measured cellular response pattern.

II. Pattern Recognition

A. Basic Biosensor Paradigm

To date, many toxin recognition strategies have been proposed, however many methodologies including signal processing, artificial neural networks, artificial intelligence, statistics, probability theory have not been extensively examined [17]. Toxin recognition and detection has been an area of significant interest, and an active research topic at many institutions. The aim is to predetermine the impact neurotoxins will have on humans before human exposure.

Currently, neurotoxicity cell-based biosensor test only contribute in a small way to this "risk assessment" process, where they are only used to reduce the amount of animal testing. However, the possibility that a neurotoxicity cell-based biosensor data processed using ANN will eventually make a significant contribution to, and perhaps improve, our determination of human risk. Ideally, this process must occur in a rapid, reliable, and cost-effective manner.

At present, there is no universal assay that is reliable and accurate for all agents or capable of functioning as an alternative to the animal model. By incorporating ANN, neurotoxin recognition will be based on a continuous decision-making process that will evaluate all relevant information about the agent impedance signature in the present of PC 12 cells. Since the PC 12 neurotoxicity biosensor is in its early stage of development, there must be a strategy that will allow us to learn from both our successes and our failures. This strategy is aim at evaluating the possibility of developing ANNs to satisfy the neurotoxin recognition and serve as a tool for refining our ability to predict neurotoxin impedance signatures.

The diagram in Fig. 1 shows the general structure of a typical biosensor paradigm. The digitized signal first transformed into a set of useful measurements or features at a fixed frequency and sine wave voltage of 4kHz and 50 mV p-t-p, respectively. The instrumentation used for monitoring the impedance profiles of cells has been programmed to select a suitable sampling rate for monitoring anchorage dependent cells. The impedance measurements were collected, typically once every 1-4 minute (15 points/hr). Moreover, several sampling rates (i.e., 60 points/hr and 120 points/hr) were examined and no significant changes were observed from one point to the next compared to using the sampling rate generated by the instrument (15 points/hr). The selected frequency (4kHz) is adequate for the detection of the activities of anchored cells, since the real impedance of the electrode and the faradaic resistance is several times larger than the constriction resistance. Thus, under these conditions, the activities of anchored cells are clearly revealed [4]. These measurements are used to identify the profile of the test agent (neurotoxin), making use of positive, negative, and blank controls. These samples are then processed to produce a representation of the profile as a sequence vector containing different parameters. The parameters are usually measured every 1-4 minute, resulting in a frame. Consecutive frames overlaps to insure "data" continuity, i.e. any such sharp change in the data are preserved. This data is then analyzed using statistical data analysis and ANN.

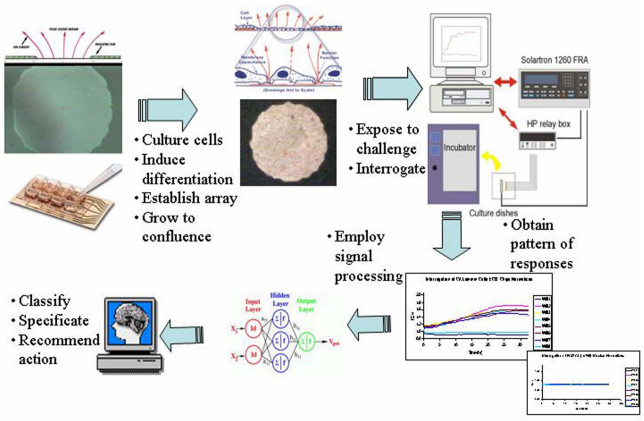

Fig. 1. Schematic illustration of the basic biosensor paradigm.

B. Data Analysis

There are two types of mathematical methods that can be used to analyze the impedance data obtained: one is the statistical data analysis to determine the robustness of the data and the other is the connectionist using ANN to identify the patterns generated from the measurement data. In the statistical approach, analysis of sample mean and variance (ANOVA) with multiple comparison tests is utilized for the recognition of neurotoxin patterns. This allows for the comparison of all possible pair-wise arrangement of the neurotoxin concentrations.

C. Artificial Neural Network

Recent research shows that an ANN has some advantages over the statistical data analysis, thus justifying its application for chemical recognition and toxin recognition [18], since it can be used for feature extraction and pattern recognition. These new, biologically motivated modeling approaches show promise for extending the traditional analysis of chemical sensor results into the realm of neurotoxicity cell based biosensors. Fig. 2 shows the general framework of a training process and a validation process for a neural network

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classifier.

A three layer fully interconnected feed-forward ANN with a back-propagation algorithm is used to update the weights during training to map the input patterns to the corresponding target output. It is a specific technique for implementing the gradient descent method to minimize the error for a multilayer feedforward network. There are three layers in this network. The first one is the input layer, whose number of neurons is the same as the number of the elements of the features set (sensors). The second layer is the hidden layer, where the number of neurons is adjusted accordingly to achieve better classification (fixed by trial). The third layer is the output layer, and the number of neurons equals the number of unit categories of neurotoxin requiring discrimination.

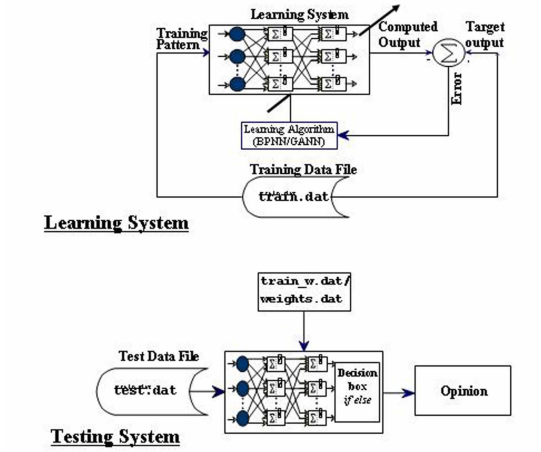

Fig. 2. A three layer feedforward network is used. There are 346 nodes in input layer, the number of nodes in output layer is equal to number of neurotoxins or neurotoxin concentrations requiring discrimination, and number of nodes in hidden layer is determined by optimization trials.

The impedance results were then extracted into Microsoft Excel and preprocessed by normalizing the data. The ANN development and simulation were carried out on a PC with a Pentium IV 1.8Ghz processor, 256MB of RAM, and the Windows 98 operating system. Impedance profiles were processed by the Neural Networks Toolbox in Matlab of Matlab v5.3. The impedance data were stored on hard disk as excel files in text format. The system is coded and integrated using Matlab 5.3, which is a powerful tool for scientific modeling and experiments. Three toxins were used, rotenone, okadaic acid, and peroxynitrite, which were conducted in 6-replicates at three concentration levels each. A total of 346 input features for each neurotoxin were recorded. For each toxin, two samples were used for training, and the other four samples were used for testing. So among 6 sample replicates, 2 sample replicates were used for training, and the other 4 sample replicates for testing. As mentioned, the training data required formatting to make it usable to the network. It was formatted into input vectors and output targets, using hand labeling of each sample in order to construct the target for the network. Thus, each individual neurotoxin was labeled with a unique category. The target file is used to record the labeling information and to be read by the program to create the output target for training.

For the input vectors, the training file is read as a vector, which contains 346 input elements. For the output targets, the corresponding target file is read in while the input training file is processed. The labeling information is also stored in a vector. While the input vector is processed, its location can be calculated and compared to the labeling information. Then, the input sample can be placed in a category to obtain the output target vector. A typical training set, input vector and output target, might appear as follows:

Input vector: [1 0.97512692 0.954564322 0.942463042 0.931830518 0.921197995 0.911204061…0.816756601 0.815255915 0.814329959 0.81528784 0.814393818 0.813882946 0.81618187 0.815287844 0.816628883 0.817012037 0.817107826 0.81618187 0.816756601 0.814745043….]

Output target: [0 0 0 1]

These two vectors are extracted from the training the file "Rot". This input has 346 elements, and the output target has four elements. The forth element of the output target is 1, which shows that this sample belongs to category Rotenone.

The transfer functions of different layers are the logarithmic sigmoid transfer function. Two recognition problems were attempted as objectives. The first objective required that the ANN classify and recognize neurotoxins as "Rot", "OA" or "Per". This ability is sufficient in areas that have one distinct neurotoxin or neurotoxins with similar impedance signatures. The second objective required that specific neurotoxin and its concentration levels be recognized and identified. An ANN with this ability is clearly applicable to a wider range of neurotoxins. The neurotoxin network is trained with data from all the neurotoxins.

D. Training of Network

After the setup, the network was initialized. The purpose of initialization is to randomly choose the value of weights, otherwise the weights equal zero and the initial error is too large, and thus, it will take a longer time to train the network.

The training algorithm used in this experiment is the back-propagation with Levenberg-Marquardt optimization, updates the network weights and biases in the direction in which the negative gradient of the performance function (steepest decent training):

$$\Delta W_{ij}(k+1) = -\eta \delta out_j + \alpha \Delta W_{ij}(k) \qquad (1)$$

where $W_{ij}(k)$ is the current weights and biases, α is a small positive constant selected by the user, δout_j is the current gradient and η is the learning rate. The learning rate multiplies the negative of the gradient, to determine the changes to the weights and biases. The larger the learning rate is, the bigger the step and if the learning rate is too large, the algorithm becomes unstable. However, if the learning rate is too small, the algorithm takes a long time to converge. Thus, a value of 0.01 was used. The training is done in a batch mode, where the weights and biases of the network are updated only after the entire training set has been applied to the network. The gradients calculated at each training example

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are added together to determine the change in the weights and biases.

The Levenberg-Marquardt algorithm is a pseudo-second order method, like the quasi-Newton methods, which estimates the Hessian matrix using the sum of the outer products of the gradient. Levenberg-Marquardt method therefore uses a search direction that is a cross between the Gauss-Newton direction and the steepest descent and significantly outperforms the gradient descent and conjugate gradient method. This training algorithm gave a reasonable convergent speed during the experiment.

The training data are based on normalized impedance data for three neurotoxins at three different concentrations, therefore input vector and output targets are generated neurotoxin by neurotoxin and concentration by concentration. Two types of neural networks were created, one to identify neurotoxins, and the other to identified concentrations of neurotoxin. For the neurotoxin networks, examples of each neurotoxin are used in the training sets. A maximum of 4000 epoch and a maximum error of 0.0000001 were used when training this network. For the neurotoxin concentration network, examples of each concentration were used in the training sets. The maximum epoch of 20000 and a maximum error of 0.0000001 were used when training this network. The Neurotoxin Network and the Neurotoxin Concentration Network were combined to form the Cascade Network.

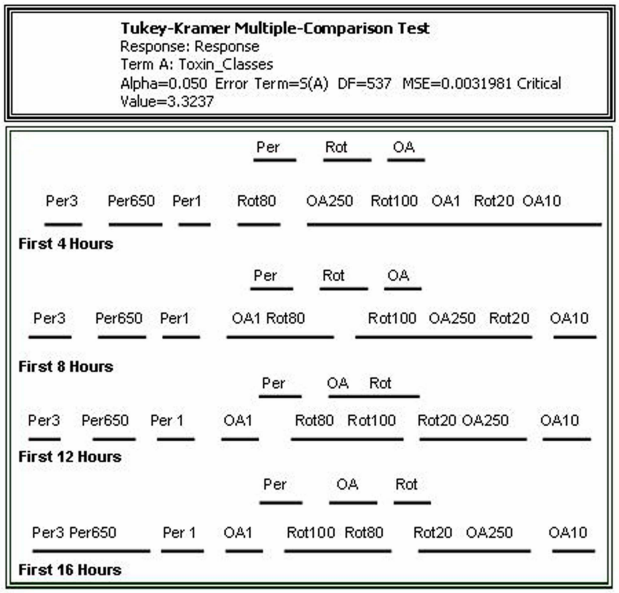
III. Experimental Results and Discussion

A. Statistical Data Analysis

The impedance obtained from all three neurotoxins at three different concentration levels on separate ECIS chips were used to determine the differences between the impedance signatures generated by these neurotoxins. Table I shows a comparison of all the measured neurotoxin-induced apoptosis impedances for the first four hours of data sampling. Using ANOVA at a significance of $P < 0.05$, with Tukey-Kramer Multiple-Comparison procedure [19], it is shows that rotenone (Rot), okadaic acid (OA) and peroxynitrite (Per), are significantly different from each other, thus resulting in three groups. And the assertion that all the neurotoxins generate the same impedance response was rejected decisively at level 0.05.

The differences in impedance responses among the neurotoxins at different concentration levels were then identified using Tukey-Kramer Multiple-Comparison procedure. For the first four hours, the neurotoxin concentrations seem to divide into five groups rather than nine as inferred from for the impedance profile generated (55.56% recognition); with no significant differences within each group, but with all "between-group" differences being significant. Thus, all the three peroxynitrite concentrations (3mM, 1mM & 650µM) are significantly different from each other and the other neurotoxins; 80µM rotenone is significantly different from 20µM rotenone, 100µM rotenone, and all the three okadaic acid concentrations (1µM, 250nM & 10nM). Thus, 20µM rotenone, 100µM rotenone, and all the three okadaic acid concentrations (1µM, 250nM & 10nM) are not significantly different from one another, but are significantly different from the other neurotoxin concentrations in their true content. A comparison of all the measured neurotoxin-induced apoptosis impedances for the first eight, twelve, and sixteen hours of data sampling, using ANOVA at a significance of $P < 0.05$, with Tukey-Kramer Multiple-Comparison procedure were also conducted and similar results as to the ones seen during the first four hours are shown in Table I. Thus, from neurotoxin to neurotoxin the pattern in impedance profile does not remains the same, where all peroxynitrite concentrations are significantly different than the other neurotoxin concentration in their impedance signature.

Table I
Multiple Comparison Engineering Plot
The Underlining of Homogeneous Subsets



B. Performance of Neurotoxin Identification Network

In the first approach used, an ANN was trained to distinguish neurotoxins from each other. The architecture of the ANN was (346, 6, 4). During training, the outputs, specified for each neurotoxin, were in binary format as follows: if the neurotoxin was of "Rot", the target output was set to [0 0 0 1], "OA", the target output was set to [0 0 1 1], for "Per" the target output was set to [0 1 1 1] and for the unknown (UNK) the target output was set to [0 0 0 0]. This coding mechanism was chosen to improve the training speed and accuracy of the network. Initial coding methods used just two outputs to encode the four choices; however this did not yield suitable results. Thus, the new coding method was derived utilizing the mild correlation between Rot [0 0 0 1] and OA [0 0 1 1] and OA [0 0 1 1] and Per [0 1 1 1] and as seen in the Tukey-Kramer multiple comparison engineering plots. Testing was performed by selecting and presenting the remainder four set of neurotoxins impedance data of all

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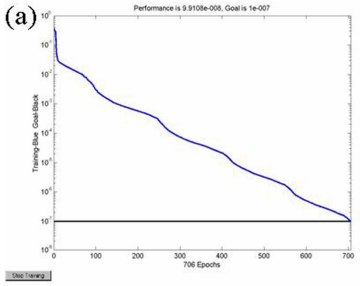
neurotoxins in question. The neurotoxins used for validation were different from the neurotoxins used for training. The testing outputs ranged from zero to one, and the classification criterion was based on the relative values of the four outputs.

The possibility of developing an ANN that could correctly classify the concentration levels of the three specific neurotoxins was also explored. There were three outputs in this ANN instead of four, with definitions analogous to those in the previous section: i.e., the expected outputs in the training file were [1 0 0] for 100μM rotenone, [1 1 0] for 80μM rotenone, [1 1 1] for 20μM rotenone and [0 0 0] for UNK. The same four set of impedance data for each neurotoxin used in the previous model validation were used to test the ANN. Because this problem was several times more complex due to the lack of data diversification, the number of processing elements (PEs) in the hidden layer was 16. When the neurotoxins were used to validate the ANNs, each neurotoxin was recognized by its concentration level. Validation is generally used to ensure full and thorough training of the ANN model; therefore it was attempted for all the case where the neurotoxins were to be recognized by the ANN model. The validation was done and the success recognition rates and the corresponding 95% confidence intervals were computed.

The criterion of the performance is the identification rate defined below. This criterion is also applied to the NTCN and NTN_NTCN networks.

$$Identification\ rate = \frac{number\ \ of\ correc\ tly\ identified\ neurotoxins}{total\ number\ of\ neurotoxins} \quad (2)$$

The testing results for Neurotoxin Network 1 (NTN1) neurotoxin identification are shown in Fig. 3 along with the completed training module.



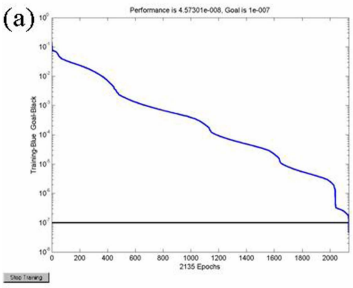
(c)

	Training Set	Testing Set					Overall
	1W	2W	3W	4W	5W	6W	
Identification rate	10/10	10/10	10/10	8/10	8/10	9/10	55/60
The overall performance							55/60= 91.667%
95% confidence interval							[0.847, 0.987]

Fig. 3. The testing results for (NTN1) neurotoxin identification: (a) 3-Layer structure (346:18:4) training simulation result (trained on one data set), (b) training module output for NTN1, and (c) testing NTN1on five data sets.

The overall performance accuracy is 91.667% (P<0.05). Several misclassifications where observed when training with one data set and testing on five. The firing of the neurons was strong for these misclassifications. The misclassifications were observed in the testing of data set 4W [OA was misclassified as Rot, and Per was misclassified as an unknown (UNK)], 5W [OA was misclassified as Rot, and UNK was misclassified as Per], and 6W [UNK was misclassified as Per]. Moreover, we are 95% confident that the true proportion of correct classifications is between 0.847 and 0.987.

The accuracy of the NTN1 system was increased appreciably upon incrementing the training data from using one training set to two training sets and testing on four (NTN2). In addition, fewer misclassifications were observed for NTN2. The trained network was applied to the four sets of neurotoxin test patterns. The results are shown in Fig. 4.



(c)

	Training Set	Testing Set				Overall
	1 & 2W	3W	4W	5W	6W	
Identification rate	20/20	10/10	9/10	10/10	10/10	59/60
The overall performance	Misclassify UNK as Rotenone in 4w					59/60= 98.333%
95% confidence interval						[0.951, 1.016]

Fig. 4. The testing results for (NTN2) neurotoxin identification: (a) 3-Layer structure (346:6:4) training simulation result (trained on two data set), (b) training module output for NTN2, and (c) testing NTN2on four data sets.

From Fig. 4, it is apparent that differentiation between neurotoxins and the unknown was the most successful in this test with an overall performance accuracy of 98.33% (P<0.05), identifying 59 of 60 test vectors correctly. The misclassification rate was low enough to be insignificant from the point of view of decision-making. The misclassifications were observed in the testing of data set 4W [UNK was misclassified as Rot]. Moreover, there was a strong firing of the neuron to classify UNK as rotenone. Thus, this shows that increasing training sample size results in better performance accuracy of the network. The high success recognition rates for the three neurotoxins may have been the result of sufficient training of the ANNs. We are highly confident that at least 95% of the true proportions of correct classifications are between 0.95 and 1.02. Compared to the NTN1, whose interval was wider, NTN2 narrow interval indicates substantial certainty about correct classifications than NTN1. The difference in performance accuracy between the two networks as measured was statistically significant (P<0.05).

The initial results using ANN for the performance of neurotoxin identification network were sufficiently successful with an overall performance accuracy of 98.33% (P<0.05). Thus, ANN was used to determine the performance of neurotoxin concentration recognition networks and the cascade network. The combination of neurotoxin recognition and concentration identification implements the function of the general trained network (NTN2) with an additional class

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identified as "unknown" for unknown agents. These unknown agents can then be trained by the network into a know class, thus creating an updated database of neurotoxins. When the testing data comes in, the neurotoxin network recognizes the neurotoxin, and selects the corresponding neurotoxin for concentration identification.

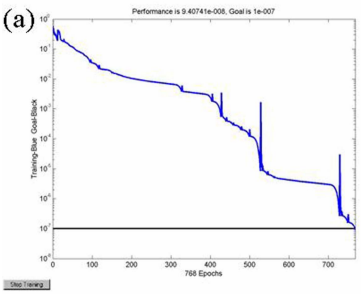
C. *Performance of Neurotoxin Concentration Network*

For NTCN, the results are shown in Fig. 5-7, which depict the trained network that was applied to the 4 sets of neurotoxin test patterns for rotenone, okadaic acids and peroxynitrite respectively. The overall recognition rate for these three networks is 93.06% (identifying 67 of 72 test vectors correctly) and it is shown in Fig. 8. It is apparent that the overall performance of concentration dependent networks has a lower recognition rate than the neurotoxin trained networks. This is evident because it is difficult for the network to discriminate between the narrow concentration range given by the impedance data generated due to lack of diversification in the concentration data set and visual plots indicates that there are similarities between the concentrations.

These results from training the ANN with 16 PEs in the hidden layer to classify each neurotoxin by its concentration levels are clearly satisfactory. The best recognition rate for rotenone concentration identification is 87.50%, the best recognition rate for okadaic acid is 95.83% and the best recognition rate for peroxynitrite is approximately 96.83%. In other words, the system would recognize approximately 88% of rotenone concentrations, 96% of both okadaic acid concentrations and peroxynitrite concentrations.

The relatively low performance rate observed for rotenone in regards to the other performance rates indicates the high difficulty for the ANNs to recognize this neurotoxin and its concentration levels when compared to okadaic acid. This problem may be caused by high similarity of impedance profiles between these two neurotoxins, thereby impeding the ANNs ability to distinguish between these two neurotoxin concentration levels and resulting in false positives. However, these false positives were eliminated with the use of the cascading network, thus implying that a properly trained ANN model is ideally suited for real time neurotoxin applications in toxin detection which requires very fast execution times.

(a) (b)



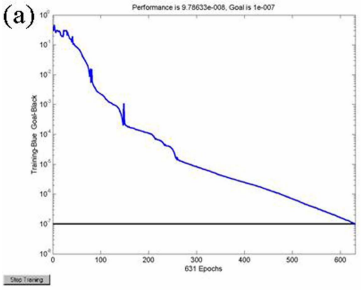
epochs=20000
goal=.0000001
Performance goal met at epoch 768/20000
MSE 9.40741e-008/1e-007
Time: Approximately 10 minutes

(c)

	Training Set	Testing Set				Overall
	5 & 6W	1W	2W	3W	4W	
Identification rate	7/8	4/4	3/4	4/4	3/4	21/24
The overall performance	Misclassify 20mM Rot as 100mM Rot in 2w & 4w					21/24= 87.5%
95% confidence interval						[0.743, 1.007]

Fig. 5. Neurotoxin concentration network (NTCN_ROT). The testing results for (NTCN_ROT) neurotoxin concentration level identification: (a) 3-Layer structure (346:16:3) training simulation result (trained on two data set), (b) training module output for NTCN_ROT, and (c) testing NTCN_ROT on four data sets.

(a) (b)



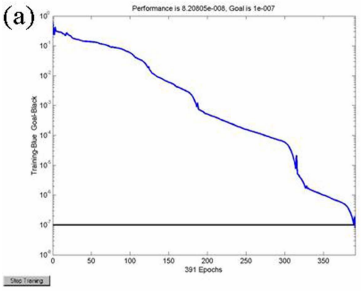
epochs=20000
goal=.0000001
Performance goal met at epoch 631/20000
MSE 9.78633e-008/1e-007
Time: Approximately 10 minutes

(c)

	Training Set	Testing Set				Overall
	5 & 6W	1W	2W	3W	4W	
Identification rate	7/8	4/4	4/4	4/4	4/4	23/24
The overall performance	No Misclassification					23/24= 95.83%
95% confidence interval						[0.878, 1.038]

Fig. 6. Neurotoxin concentration network (NTCN_OA). The testing results for (NTCN_OA) neurotoxin concentration level identification: (a) 3-Layer structure (346:16:3) training simulation result (trained on two data set), (b) training module output for NTCN_OA, and (c) testing NTCN_OA on four data sets.

(a) (b)



epochs=20000
goal=.0000001
Performance goal met at epoch 391/20000
MSE 8.20805e-008/1e-007
Time: Approximately 10 minutes

(c)

	Training Set	Testing Set				Overall
	5 & 6W	1W	2W	3W	4W	
Identification rate	8/8	4/4	4/4	3/4	4/4	23/24
The overall performance	Misclassify 3mM Per as 1mM Per in 3w					23/24= 95.83%
95% confidence interval						[0.878, 1.038]

Fig. 7. Neurotoxin concentration network (NTCN_PER). The testing results for (NTCN_PER) neurotoxin concentration level identification: (a) 3-Layer

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structure (346:16:3) training simulation result (trained on two data set), (b) training module output for NTCN_PER, and (c) testing NTCN_PER on four data sets.

	Training Set	Testing Set	Overall
Network for Rotenone	7/8	14/16	21/24
Network for Okadaic Acid	7/8	16/16	23/24
Network for Peroxynitrite	8/8	15/16	23/24
Overall	22/24	45/48	67/72
Overall Recognition rate for these three networks is 69/72 = 93.06%			
95% confidence interval			[0.872, 0.989]

Fig. 8. Schematic representation of the overall performance of NTCN.

D. Performance of Cascade Neurotoxin Network

As mentioned, the combination of NTN and NTCN implements the function of a general trained network NTN2. When testing data comes in, the neurotoxin network recognizes the neurotoxin, and then selects the corresponding neurotoxin concentration network for concentration recognition. The testing results are given in Fig. 9.

Identification rate for Cascade Network	Testing Set						Overall
	1W	2W	3W	4W	5W	6W	
Rotenone_CL	4/4	3/4	4/4	3/4	3/4	4/4	21/24
95% confidence interval							[0.743, 1.007]
Okadaic_Acid_CL	4/4	4/4	4/4	4/4	3/4	4/4	23/24
95% confidence interval							[0.878, 1.038]
Peroxynitrite_CL	4/4	4/4	3/4	4/4	4/4	4/4	23/24
95% confidence interval							[0.878, 1.038]
The overall performance							67/72= 93.06%

Fig. 9. Identification rate of the cascade network (NTN_NTCN).

The overall performance accuracy of the cascade network is 93.06%, $P<0.05$ (identifying 67 of 72 test vectors correctly). Fig. 10-11 are schematic depiction of the comparison of testing results as well as the misclassification of the cascade combination network, respectively. The 95% confidence intervals are also given in the table enabling classification to be made with a known level of confidence. Therefore, if the confidence bounds are tight, there is little uncertainty in the prediction and vice versa.

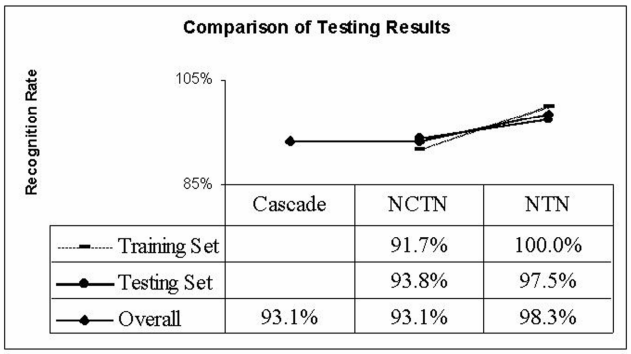

Fig. 10. Comparison of recognition rates of all networks, training set (–), testing set (•), and overall performance (♦).

	1W	2W	3W	4W	5W	6W
Rotenone_CL	100% Classification for all ROT concentrations & UNKs	20μM Rotenone was misclassified as 100μM ROT	100% Classification for all ROT concentrations & UNKs	20μM Rotenone was classified as 100μM ROT **UNK was misclassified as ROT in NTN2 & was then classified as UNK at the Rotenone CL**	100mM Rotenone was misclassified as unknown to network	100% Classification for all ROT concentrations & UNKs
Okadaic_Acid_CL	100% Classification for all 3 OA concentrations & UNKs				1mM Okadaic Acid misclassified as UNK	100% Classification for all 3 OA concentrations & UNKs
Peroxynitrite_CL	100% Classification for all 3 PER concentrations & UNKs		3mM PER was misclassified as 1mM PER	100% Classification for all 3 PER concentrations	100% Classification for all 3 PER concentrations & UNKs	

Fig. 11. Classification and misclassification in cascade network.

The cascade network correctly classified neurotoxins and selected the appropriate neurotoxin concentration network for concentration recognition. For the concentration level of rotenone, the network correctly classified 88% of the concentration levels presented, for okadaic acid concentration levels, they are correctly classified at a rate of 96%, and similarly, peroxynitrite concentration levels are also correctly classified at a rate of 96%. As mentioned previously, NTN2 misclassified unknown as rotenone, however when this information was analyzed using the cascade network, the misclassified unknown was classified as an UNK at the rotenone concentration level. This is perhaps the most encouraging case to prove ANN's ability for neurotoxin and concentration level recognition, because it is equally important to recognize a neurotoxin and its concentration level as such.

In Fig. 3-9, the 95% confidence intervals are provided for comparison. These statistics are all based on the network's output. Generally, it can be said that there is a 95 percent probability of the error values falling within two standard deviations of the mean. Therefore, the larger the standard deviation, a greater range of error will be observed. The NTN2 is the most accurate classifier of the neurotoxins, therefore supporting the theory that identifying a neurotoxin is more accurate than identifying a concentration level of a given neurotoxin, due to diversification in the NTN data sets.

Visually examining Fig. 3-9, all network statistic charts, clearly shows the NTCN for rotenone has the greatest standard deviation. Differentiation between the three individual NTCNs is easily attainable by looking at the overall performance of the networks, suggesting that both the statistics and the identification rates provide the true ranking.

An attempt was made to see whether the introduction of a new class of impedance data (forskolin) to the trained general network and the neurotoxin concentration network will result in false positives. As shown in Fig. 12, the ANNs correctly classified forskolin as UNKs and unknown to the network for all networks developed in this study.

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Networks	Forskolin
Neurotoxin_ID	UNK
Rotenone_CL	UNK
Okadaic_Acid_CL	UNK
Peroxynitrite_CL	UNK to Network

Fig. 12. Classification and misclassification: false positives.

IV. Conclusion

We describe the design and development of a PC 12 neurotoxicity biosensor coupled with ANNs that can successfully discriminate between different neurotoxin impedance patterns. This neurotoxicity biosensor serves as a reliable method for rapid screening of unknown agents (neurotoxins), which significantly increase the efficiency of the drug development process and minimize the use of experimental animals. This work demonstrates the feasibility and practicality of using neural networks as a classification tool for neurotoxins and validates the development of a neurotoxin network that achieved a 98.33 percent performance rate for identifying a neurotoxin, and a 93.06 percent performance rate for identifying a neurotoxin concentration. It is concluded that neural networks do have the capability to classify neurotoxins if properly trained.

This work also shows that the properties of nonlinear modeling and adaptability exhibited by artificial neural network models can offer effective solutions to problems that may be very difficult or intractable by other approaches. The use of such ANNs as an intermediate step between biochemical analysis and animal experiments holds the promise of great research efficiency through a rapid recognition and quantification of physiological tissue response. Several applications of this research involve implantable functional electrodes (retinal and cochlear implants), environmental monitoring, chemical and biological warfare detection, toxin detection and drug discovery, neural cell regeneration, and chemotherapy.

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References

[1] C. M. Lo, C. R. Keese, and I. Giaever, "Cell-substrate contact: another factor may influence transepithelial electrical resistance of cell layers cultured on permeable filters," *Exp. Cell. Res.*, vol. 250, pp. 576-580, Aug 1999.

[2] I. Giaever, and C. R. Keese, "Use of electric fields to monitor the dynamical aspect of cell behavior in tissue culture," *IEEE Transactions on Biomedical Engineerin*g, vol. 33, pp. 242-247, Feb 1986.

[3] I. Giaever, and C. R. Keese, "Fractal motion of mammalian cells," *Physica* D, vol. 38, pp. 128-133, Sep 1989.

[4] I. Giaever, and C. R. Keese, "Micromotion of mammalian cells measured electrically," *Proc. Natl. Acad. Sci*., vol. 88, pp. 7896-7900, Sep 1991.

[5] I. Giaever, and C. R. Keese, "A morphological biosensor for mammalian cells," *Nature*, vol. 366, pp. 591-592, Dec 1993.

[6] K. V. Sharma, C. Koenigsberger, S. Brimijoin, and J. W. Bigbee, "Direct evidence for an adhesive function of the noncholinergic role of acetylcholinesterase in neurite outgrowth," *J. Neurosci. Res.,* vol. 63, pp.165-175, Jan 2001.

[7] G. E. Slaughter, E. Bieberich, G. E. Wnek, K. J. Wynne, A. Guiseppi-Elie, "Improving neuron-to-electrode surface attachment via alkanethiol self- assembly: an alternating current impedance study" *Langmuir,* vol. 20, no. 17, pp.7189-7200**,** Aug 2004.

[8] J. Wegener, C. R. Keese, and I. Giaever, "Electric cell-substrate impedance sensing (ECIS) as a non-invasive means to monitor the kinetics of cell spreading to artificial surfaces," *Exp. Cell. Res.*, vol. 259, pp. 158-166, Aug 2000.

[9] I. Giaever, and C. R. Keese, "Monitoring fibroblast behavior in tissue culture with and applied electric field," *Proc. Natl. Acad. Sci*., vol. 81, pp. 3761-3764, Jun 1984.

[10] J. Zurlo, D. Rudacille, and A. M. Goldberg, Animal and Alternatives in Testing: History, Science, and Ethics, Mary Ann Liebert, Inc., 1994.

[11] P. Connolly, P. Clark, A. S. G. Curtis, J. A. T. Dow, and C. D. W. Wilkinson, "An extracellular microelectrode array for monitoring electrogenic cells in culture," *Biosensors and Bioelectronic*s, vol. 5, pp. 223-234, 1990.

[12] G. W. Gross, B. K. Rhoades, H. M. E. Azzazy, and M. C. Wu, "The use of neuronal networks on multielectrode arrays as biosensors," *Biosensors and Bioelectronic*s, vol.10, pp. 553-567, summer 1995.

[13] M. P. Maher, J. Pine, J. Wright, Y. C. Tai, "The neurochip: a new multielectrode device for stimulating and recording from cultured neurons," *J. Neurosci. Meth*. vol. 87, pp. 45-56, Feb 1999.

[14] C. R. Keese, and I. Giaever, "A whole cell biosensor based on cell-substrate interactions," *Annual International Conference of the IEEE Engineering in Medicine and Biology Societ*y, vol. 12, pp. 500-501, Nov 1990.

[15] R. Ehret, W. Baumann, M. Brischwein, A. Schwinde, K. Stegbauer, and B. Wolf, "Monitoring of cellular behaviour by impedance measurements on interdigitated electrode structures," *Biosensors and Bioelectronics*, vol 12, no. 1, pp. 29-41, 1997.

[16] L. A. Greene, and A. S. Tischler, "Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor," *Proc. Natl. Acad. Sci. U.S.A.,* vol. 73, pp. 2424-2428, Jul 1976.

[17] P. T. Moseley, J. Norris, and D. E. Williams, *Techniques and mechanisms in gas sensing*. New York: Adam Milger, 1991.

[18] M. L. Padgett, and J. L. Johnson, "Pulse coupled neural networks: biosensors applications," in *Proc. Intl. Conf. NN*, ICNN97, Houston TX, June, 1997, pp. 2507-2512.

[19] J. L. Devore, *Probability and Statistics for Engineering and the Sciences*. 6th ed., New York: Duxbury Press, 2004, pp.402-480.

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