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KEYNOTE PAPER

STOCHASTIC FREQUENCY SIGNATURE FOR CHEMICAL SENSING VIA NONINVASIVE CELL-ELECTRONIC INTERFACE

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ABSTRACT

Cell based biosensors offer the capability for quickly detecting chemical and biological agents with high sensitivity in a wide spectrum. Membrane excitability in cells plays a key role in modulating the electrical activity due to chemical agents. However, the complexity of these signals makes the interpretation of the cellular response to a chemical agent rather difficult. It is possible to determine a frequency spectrum also known as the signature pattern vector (SPV) for a given chemical agent through analysis of the power spectrum of the cell signal. I will describe a system for the measurement of extracellular potentials from live cells isolated onto micromachined planar microelectrode arrays. Fast Fourier and wavelet transformation techniques are used to extract information related to the frequency of firing and response times from the extracellular potential. Quantitative dose response curves and response times are obtained using local time domain characterization techniques.

NOMENCLATURE

Cell based sensors, extracellular potential, microarrays, signature pattern vector, microfluidics.

Techniques to develop highly sensitive biosensors are largely dependant upon the properties of the material and its associated interactions[1-3]. Current biosensor technologies encompass antibody-antigen interactions[4], hormone-receptor interactions[5, 6], and nucleic acid based assays[7-9]. These sensors are useful in narrow band applications requiring high specificity for agent identification[10, 11]. Mammalian cells have excitable cell membranes that function as novel sensing platforms by producing a variation in the extracellular potential based on the chemical stimulus[12]. We have developed single cell based sensors by integrating the biological tool of dielectrophoresis[13] with the micro fabrication technology[14, 15]. Our technique is capable to detect a large number of chemical agents, reject false alarms, characterize the chemical agent functionality and determine the associated sensitivity limit, and the physiological response in terms of the calcium transients for each specific chemical agent that produces synergistic effects on humans. Our technique is capable for detecting chemical agents in cascaded[16,17,18,19] and mixed form[20,21,22] as well.

Briefly, the microelectrode array is fabricated on a silicon/silicon nitride substrate adopting conventional micro fabrication techniques of optical lithography, electron beam vapour deposition; plasma enhanced chemical vapour deposition and oxygen plasma etching to produce a 5x5 array of platinum electrodes with a nitride passivation layer, that are 80μm in diameter and with a 200μm centre-to-centre spacing with an intrinsic skew in the chip geometry to enable the establishment of dielectrophoretic traps. The optical probe station along with the chip assembly is enclosed by an acrylic chamber (Figure 1). The environment in the chamber is controlled so as to maintain a constant temperature of 37^{o}C. A heat gun inside the chamber heats the air in the chamber and this is linked to a temperature controller that stops the heat gun from functioning above the desired temperature. A 6" fan inside the chamber circulates the hot air to maintain temperature uniformity throughout the chamber and is monitored by a J-type thermocouple probe attached to the temperature controller. The carbon dioxide concentration inside the chamber is maintained at 5% and is humidified to prevent excessive evaporation of the medium. This chamber with all of its components will ensure cell viability over long periods of time and stable cell physiology in the absence of the chemical agents.

Cell cultures are prepared in a manner whereby the confluence of the cells allows individual cell positioning. As an example, primary rat osteoblast cells are cultured to a concentration of 10,000 cells in 1mL for sensing experiments. To achieve the patterning of a single cell over a single electrode, a 10 μL of cell culture solution was mixed with 500 μL Dulbeco modified eagle medium (DMEM; Gibco, Grand Island NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island NY), 100μg/mL penicillin, and 100 μg/mL streptomycin (P/S; Gibco, Grand Island NY). The cells were centrifuged and re-suspended in 1mL of separation buffer consisting of 1:9 dilutions of Phosphate Buffer Saline/250mM Sucrose (Sigma, St Louis) and de-ionized water (w/v). The conductivity of the separation buffer was 4.09mS/cm and with a pH of 7.4. The separation buffer is replaced with a test buffer ((DMEM)/ Fetal Bovine Serum (FBS)/Phosphate Buffer Saline (PBS)) with conductivity of 2.5 mS/cm and a pH of 7.4.

Single cells are trapped over the positive dielectrophoretic traps enabled by an interaction between the induced non-uniform dipole over the cell membrane and the gradient applied voltage (Figure 2). Due to a non uniform applied electric field the resulting force on each side of the cell is different. Thus depending on the relative polarizability of the cell with respect to the surrounding medium it will be induced to move towards high electric field region (positive dielectrophoresis) or towards the region of low electric field (negative dielectrophoresis). By controlling the parameters of the conductivity of the buffer to 1.2 mS/cm, the pH to 7.4, the applied AC voltage to 2 Volts peak-to-peak and the applied frequency to 1.2 MHz, an array of cells having identical biological conditions were isolated and localized over sensing/recording sites. The patterned cells adhered to the electrode site due to the pre-treatment of the chip for reliable data acquisition. The chip is coated with 100μL of collagen (type II, 1μg/mL) and incubated for three hours, at 37^{o}C to achieve better cell adhesion to enhance the signal detected. The chip is enclosed by a silicone chamber with microfluidic inlet and outlet connected to a syringe pump system that feeds the buffer solution and the sensing media in parts to the cell sensor at an inflow rate less than 40μL/min and an outflow rate less than 20μL/min.The low flow rates are required to prevent disruption of the adhered cells, which is required to maintain long term viability and enable reliable recordings.

Sensing performed over the array of these individually positioned cells produces comparable signals due to similarity in the cells' biological states and viabilities. This reduces false alarm generation which occurs in current cell based biosensors where there are variations in individual cells states in a population of cells. Next we modelled the electrical field distribution involved in the generation of positive dielectrophoretic traps over the chip surface. The cells were simulated based on the ion distribution along the cell surface and in the buffer solution (Figure 2). High electric field (Green colour) is seen around the edge of the microelectrode where the osteoblast will be attracted due to positive DEP. In order to demonstrate the patterning technique; we positioned single osteoblast cells over individual electrodes (Figure 2, inset).

For demonstration, we will present results from testing of primary rat osteoblast cells for one chemical agent. The cell membrane of an osteoblast cell comprises of gated ion channels with selective permeability. This generates an ionic gradient which in turn produces an extracellular potential that is modulated in the presence of a chemical agent. The modulated activity can be accurately and non-invasively measured due to the reliable contact between the cell membrane and the electrode surface. The cell membrane thus functions both as a sensing and a transduction element. Pertaining to a specific chemical agent, associated modulated electrical signals are generated.

The power spectrum of the signal is a better indicator of the signal modulation and its associated physiological state in comparison with the recorded peak to peak amplitude. Fast Fourier Transformation (FFT) analysis, identifies the shifts in the signals' power spectrum by forming the frequency spectrum that indicates the control and modified bursting rates due to the action of a specific chemical agent. This is termed as the "signature pattern" which is unique to each chemical agent. These frequencies in the signature pattern that correspond to the maximum relative amplitude and represent the cell's stable characteristic firing rate are called "Eigen Vectors".

The associated amplitudes are known as "Eigen Values". Signal processing using wavelet transformation (WT) enables the extraction of functional information pertaining to the response time and in determining the sensitivity limits.

The sensing of a single osteoblast cell due to a physiologically toxic agent, pyrethroid[23], is presented in Figure 3 in the form of a frequency spectrum. The chemical agent is pre-mixed with the sensing buffer and introduced into the sensing system. The action of each chemical agent at decrementing concentration ranges (step size in the higher concentration range of the spectrum: 500 ppm, lower concentration range (<1000 ppm) : 50 ppm) was monitored, at 30 sec intervals over a period of 180 sec which constitutes three phases in a particular sensing cycle. Each sensing cycle on an average comprised of three phases. The number of phases was determined to be inversely dependant on the concentration of the chemical agent. The instant at which the chemical is added to the chip system is denoted by t=0 sec. Osteoblasts have an unmodulated firing rate of 668 Hz. This corresponds to the frequency of firing of osteoblast in the absence of a chemical agent. Three Eigen Vectors associated with pyrethroid are generated simultaneously during the first 30 sec after the action of the chemical agent (129 Hz, 565 Hz and 873 Hz). During the next 30 sec, there is a shift in the low frequency subsidiary peak (257 Hz) but the high frequency subsidiary peak remains unchanged (873 Hz). The control frequency of 668 Hz is generated as in the previous cases before the action of the chemical agent.

It is also important to know how fast live cells would respond to different chemical agents. Actually, the initial signal is composed of so many frequency subsets that it is difficult to extract the useful information. The WT analysis is performed on the acquired data to yield the local time domain characteristics in order to extract the first modulated maxima corresponding to the first Eigen Vector of the response. We first pick up the first modulated maxima corresponding to the first Eigen Vector of the response from the frequency domain. Then we decompose the initial signal to different frequency subsets using Wavelet Transformation (WT). Finally we reconstruct the first modulated maxima frequency to time domain to extract the response time. The response time is defined as the time taken for the functional sensing element- osteoblast, to respond to the specific input- chemical agent, and reach its first extreme value. We determined that there was an inverse relationship between the response time and the concentration of the chemical agent. The average response time due to the action of pyrethroid was obtained to be 0.23 sec for n = 15 (Figure 4). The detection limit which is a measure of the sensitivity of the technique was determined for each chemical agent. The sensitivity limit of a single osteoblast for ethanol[18] was 19 ppm, for hydrogen peroxide[18] was obtained to be 25 ppm and for pyrethroid[18] the value was 890 ppb.

Cell based sensing technology could change the paradigm of chemical and biological warfare detection from "detect-to-treat" to "detect-to-warn", since it has the capability to directly access the physiological changes and the resulting human performance decrements.

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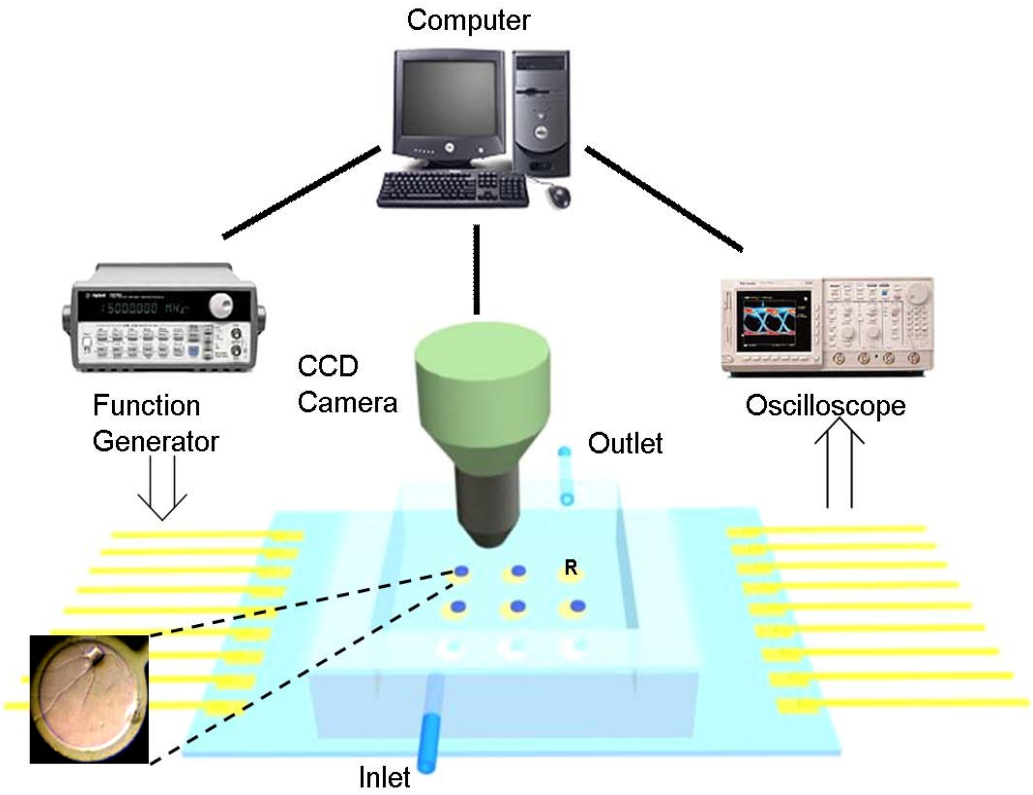

Figure 1. Schematic of the microelectrode array based single neuron chemical-sensor. Electrical and optical monitoring of the neurons is performed using the oscilloscope and the charge coupled (CCD) camera. Extracellular electrical activity measurements are performed non-invasively by probing the electrode pads connected to the electrodes. All the extracellular electrical measurements from the single neuron cell membrane are performed with respect to the reference electrode denoted by "R" which has no neuron on the top. All the measurable data is quantified and analyzed to generate the "Signature Pattern Vector".

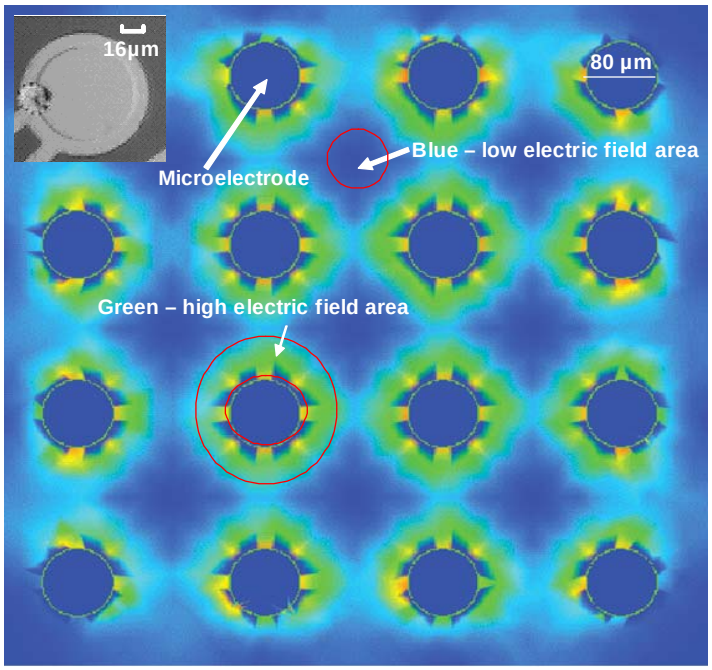

Figure 2. Simulation of the electric field distribution across the sensing platform due to the action of gradient electric field.

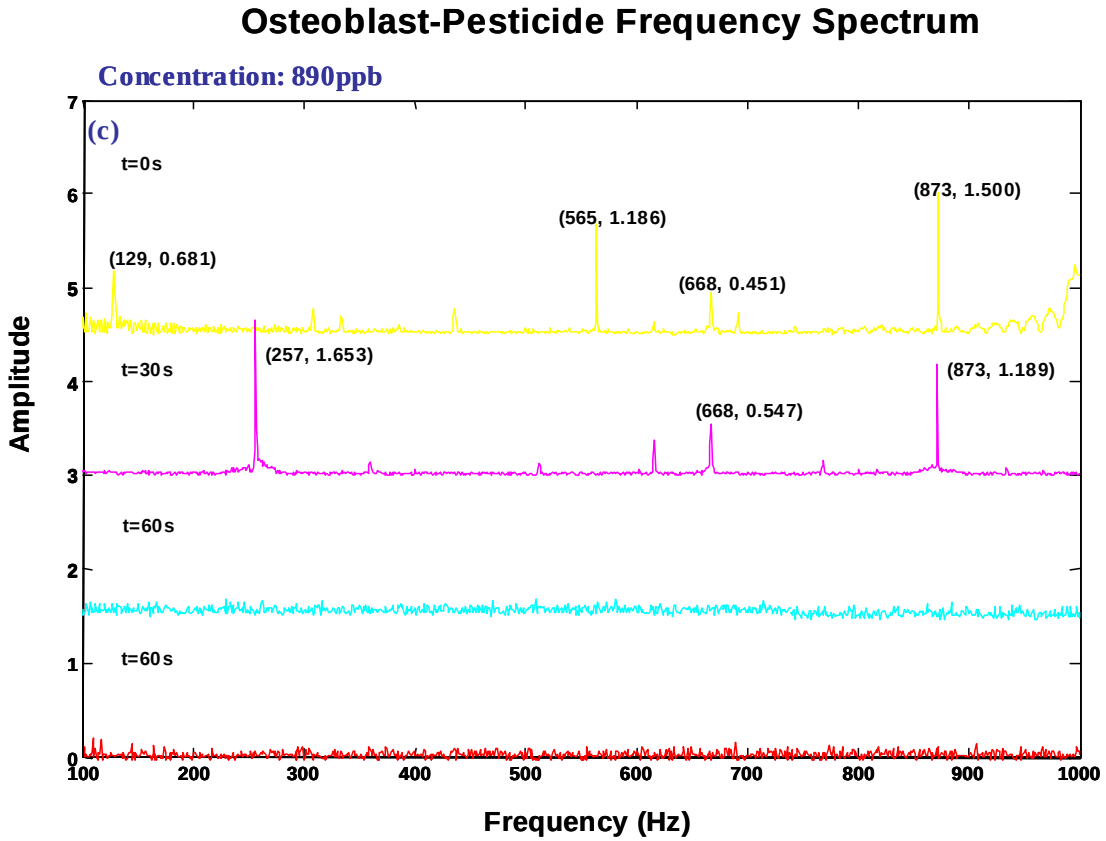

Figure 3. Signature pattern generated by a single osteoblast cell due to the action of pyrethroid at the detection limit. The detection limit for ethanol was determined to be 19 ppm; for hydrogen peroxide it was 25 ppm and that for pyrethroid it was 890 ppb. The signature patterns obtained were identical for varying concentrations of a specific chemical agent.