Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biosensors and Bioelectronics 20 (2005) 2218–2227
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

A low-cost, portable generic biotoxicity assay for environmental monitoring applications

Vivek Sharma[a,*], Arthi Narayanan[b], Thirumalai Rengachari[a], Gabor C. Temes[a], Frank Chaplen[b], Un-Ku Moon[a]

[a] Department of Electrical Engineering and Computer Science, 220A Owen Hall, Oregon State University, Corvallis, 97331 OR, USA
[b] Department of Bioengineering, Oregon State University, USA

Received 16 June 2004; received in revised form 18 July 2004; accepted 22 July 2004
Available online 21 August 2004

Abstract

Fish chromatophores have been shown to be promising biosensors for the detection of hostile agents in the environment. However, state-of-art methods for such applications are still based on extensive use of data/signal processing, in conjunction with need for a skilled human observer to carry out the detection. As a result, conventional methods are complex, costly and cumbersome rendering them useless for field applications requiring low-cost portable solutions capable of fast detection. A new technique is proposed based on the popular scheme of observing the aggregation response in chromatophores for detection of toxicity, and a solution using optical detection and electronic processing is outlined. This scheme has the advantage of being low in cost while providing simple, fast and reliable detection.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Biotoxicity assay; Biosensors; Chromatophores; Optical detection; Portable; Ecological canary

1. Introduction

There is considerable interest in developing a generic biosensor capable of serving as an ecological canary in various applications in order to provide an early warning regarding the presence of life-threatening agents in the environment. Potential applications include detection of toxic effluents in the water supply, detection of poisonous gases in industrial and military scenarios, detection of microbial poisoning in food, etc. Typical biosensors and bioassays use a biological sensing element to convert a change in the environment to a signal suitable for processing. Typically, the sensing element is a living organism, and the underlying sensing scheme relies on monitoring certain physiological changes in this organism under the effect of an environmental stimulus.

An ideal biosensor should detect the presence of any toxic agents rapidly, and at concentration levels much lower than those capable of affecting human beings adversely. Considering the various potential applications, it is desirable that such a sensor be small in size, low in cost and easy to operate. The ideal biosensor would be a battery-powered device no larger than a wristwatch.

Considerable research has been done in the field of biosensor design and several solutions have been proposed based on various techniques (Owicki and Parce, 1992; Paddle, 1996; Rogers, 1995; Rainina et al., 1996; Skladal and Mascini, 1992). Some of the popular schemes are based on monitoring the ventilation activity of fish (van der Schalie et al., 2001), immune cell response (Kumar et al., 1994; Narang et al., 1997; Pancrazio et al., 1998), cardiac myocyte activity (Gray et al., 2001), neuronal network response (Gross et al., 1995, 1997; Keefer et al., 2001), aggregation in fish chromatophores (Chaplen et al., 2002a) and changes in bacterial bioluminescence using the Microtox® test. Some of the existing schemes do not use the response of the cell itself, but are based on monitoring the reactions in nucleic acid samples (Pollard-Knight et al., 1990; Watts et al., 1995),

* Corresponding author. Tel.: +1 54 1737 9291; fax: +1 54 1737 1300.
E-mail address: sharmavi@ece.orst.edu (V. Sharma).

0956-5663/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2004.07.023

membrane ion channels (Stenger et al., 1991) and enzyme samples (Larsson et al., 1998; Niwa et al., 1998). A detailed review of various biosensing techniques is presented in Pancrazio et al. (1999).

It is evident that there are several different biosensing techniques to choose from. However, many of these methods are too selective to detect all possible agents of interest, and it is necessary to use an array of different schemes in order to improve the range of detection. Also, almost all these schemes rely on extensive data analysis and pattern recognition in order to perform reliable detection. This mandates use of a desktop PC running customized software which makes these schemes unattractive from the point of portability and cost. Additionally, most of the schemes require a trained human observer capable of interpreting the results. All these factors reduce the efficacy of these methods in real-life applications where such observers may not be available, or else the risk of hazard too great to allow anything but automated detection.

Among the commercially available schemes, the most attractive option is based on monitoring the metabolic activity of the bioluminescent bacteria *Vibrio fischeri* (AZUR environmental systems). This scheme utilises freeze-dried bacteria with a long shelf-life and the resulting sensor is relatively compact and portable. However, this approach alone cannot detect all possible agents of interest. Additionally, the operational life of each cell sample is limited to some extent as the bacteria are free to divide during the detection phase and the elevated metabolic activity during this time requires frequent changes in cell media and prevents this technique from being used for a round-the-clock standalone biological early warning system (BEWS). Thus, it would be of interest to explore other options.

In this work, we propose a novel biological assay which offers potential for a fully automated, portable, low-cost, generic biosensor which is much more robust and suitable for use in a standalone BEWS. This scheme can be used independently or in conjunction with other methods as part of an array of biological assays in order to reliably detect a wide variety of agents. Our scheme is based on monitoring the aggregation response of fish chromatophores. Previous research has shown that fish chromatophores offer a promising solution for detection and identification of a wide variety of environmental toxins and bacterial pathogens (Chaplen et al., 2002b; Danosky and McFadden, 1997). Also, they are easy to culture and handle. The fish chromatophores used in this scheme are terminally differentiated and have low energy needs. This increases the operational life of the assay and allows its use for several days without requiring change in cell media. The existing scheme based on fish chromatophores (Chaplen et al., 2002b) utilises pattern recognition and feature analysis in order to achieve extremely high levels of sensitivity and reliability, and requires a considerable amount of hardware and customized software in addition to a skilled observer. Thus, it is unsuitable for applications where a portable, automated, low-cost solution is required. We propose an alternative detection scheme that offers the advantages of simpler detection, lower cost and portability.

The proposed scheme uses chromatophores from the Siamese fighting fish, *Betta splendens*. Chromatophores are pigment cells found in fish, amphibians and other lower vertebrates. They perform the functions of pigmentation and camouflage under the direction of the nervous and endocrine systems. Details of chromatophore types and their response can be found in the literature (Fujii, 1993, 2000).

The chromatophores show a marked change in appearance when exposed to most toxins, with the typical response involving a movement of the pigment granules to the center of the cell giving the cell a shrunken appearance. The cell itself does not shrink during this process. This response is known as aggregation and is shown in Fig. 1. The opposite response known as dispersion is also seen in reaction to some agents. However, aggregation is the dominant response and the discussion will concentrate only on this. The change caused by aggregation can be detected by observing magnified images of the cells as is the case in the current approach. Alternatively, aggregation can be detected by monitoring the change in the intensity of light transmitted through a chromatophore sample. Aggregation will cause an increase in this variable. This change can be measured using a sensitive silicon photodetector and analyzed using precision electronic circuitry, all of which are easy integrate into a single-chip electronic solution. This is the key to portability.

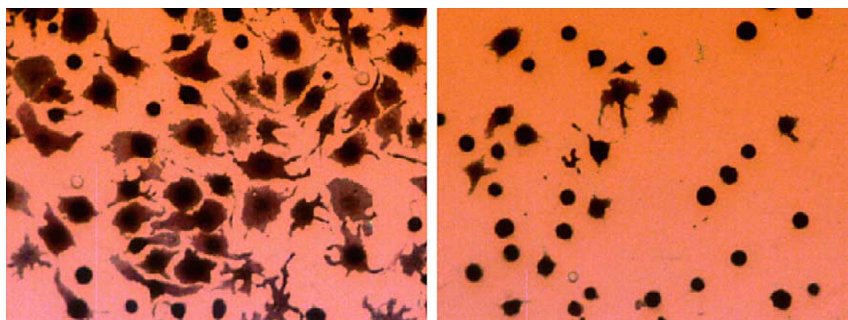

Fig. 1. (a) Chromatophores before exposure and (b) after exposure to norepenephrine (1 μg/ml).

2. Principle of operation

Fish chromatophores exhibit a marked change in appearance when exposed to most environmental toxins and bacterial pathogens. Most toxins elicit a very quick response resulting in an appreciable amount of aggregation within a minute or two. The *B. splendens* chromatophores have been demonstrated as effective biosensors for a wide variety of toxins including heavy metals, organophosphate pesticides, polynuclear aromatic hydrocarbons (PAHs), bacterial pathogens and microbial toxins. Control experiments have also demonstrated that the chromatophores do not show such a response when treated with nontoxic control agents and nonpathogenic bacteria such as *Lactococcus lactis* and *E. coli* (Chaplen et al., 2002b). Table 1 lists some of the agents that can be detected using this technique.

The change in apparent cell area of the chromatophores can be monitored by observing the intensity of light transmitted through the sample. A silicon photodetector is used to convert the transmitted light intensity to an electric current, which is then amplified and processed by sensitive electronic circuitry to complete the detection process. To facilitate processing, the signal is digitized and stored in an on-chip memory. Thus, the entire system can be made very small and operated with a 6 V battery.

Detection is accomplished if the change in signal level exceeds a predetermined threshold chosen experimentally according to the guidelines presented in Section 3.5. For ease of handling, the test experiments can be carried out using adrenergic agents such as norepenephrine and clonidine which simulate the effect of actual toxins by inducing aggregation commensurate with that seen during exposure to real toxins (Danosky, 1996; Teng, 2002).

Table 1
Classes of environmental toxins that cause changes in chromatophore pigment granule distribution

Class of agent	Specific agent
Heavy metals	Pb, Cu, Hg, others
Organophosphate pesticides	Paraoxon Mipafox EPN (*o*-ethyl *o*-*p*-nitrophenyl phenylphosphonothioate) Mevinphos Dichlorvos Trichlorfon
Live microbes, microbial toxins	*Bacillus cereus* *Salmonella enteriditis* Cholera toxin from *Vibrio cholera* α-Hemolysin from *S. aureus*
Polynuclear aromatic hydrocarbons (PAHs)	Pyrene 1,2-Benzanthracene Fluoranthene Acenaphthene Twelve others

Table taken from Chaplen et al. (2002a, 2002b).

3. Materials and methods

3.1. Chemicals and solutions

All chemicals were of reagent grade unless otherwise indicated. Antibiotic/antimycotic (penicillin, streptomycin and fungizone from Gibco-BRL 15240-062, diluted 1:100); phosphate buffered saline (PBS: 128 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.46 mM KH_2PO_4, antibiotic/antimycotic; pH 7.3), skinning solution (PBS with 1 mM NaEDTA; pH 7.3), digestion solution (30 mg collagenase type I: 220 U/mg (5 mg/ml) from Worthington and 3 mg hyaluronidase: 348 U/mg (0.5 mg/ml) from Worthington dissolved in 7 ml PBS and filter sterilized with a 0.2 μm acrodisc filter immediately before use), culture media (L-15 from GIBCO BRL with antibiotics/antimycotic), fetal bovine serum (FBS) (from HyClone Laboratory, Inc., Logan, UT, USA).

3.2. Cell culture

B. splendens (Siamese fighting fish) were anaesthetized in ice cold water for 10 min. Fins were removed by scalpel in PBS and washed with 10 ml skinning solution 8–10 times. Fins were then added to 7 ml digestion solution in a small beaker and rotated on an orbital shaker until the digestion solution appeared cloudy (5–15 min). The digestion solution was separated from the fins and centrifuged for 2 min at 400 $\times g$ to pellet cells. The fins were resuspended in the clarified digestion solution (7 ml) for the next harvest. The cell pellet was suspended in 7 ml L-15 medium following each harvest and then centrifuged for 2 min at 400 $\times g$ and 20 °C. The first harvest was discarded as it consisted mostly of epithelial cells. Cells were collected following subsequent harvests and plated in petridishes approximately 3 cm in diameter (Fisher Scientific, Inc.) with 1–2 ml of L-15 culture medium/10% FBS. Cells were plated in the center of each petridish as the optical density measurements were sensitive to initial cell densities. A small drop of cell solution was placed in the center of each well for 15 min. 1.5 ml of L-15 was added followed by 80 μl of L-FBS after 30 min for a final concentration of 5% (v/v) FBS. Medium was exchanged 12–24 h following cell plating and 12–24 h prior to experiments. Cultures were maintained at 23–24 °C on the lab bench for 3–10 days as needed.

3.3. System design

A simplified system diagram is shown in Fig. 2. A light source is needed to provide illumination. One can use white LEDs, or even the ambient if it is guaranteed to be stable over the course of the experiment. A simple photodiode (FIL-100C) is used for converting the transmitted light intensity into current. This photodiode is connected to the low-noise front-end amplifier, which provides a suitable bias to allow operation of the photodiode and measures its output photocurrent. This current is then amplified and converted to a

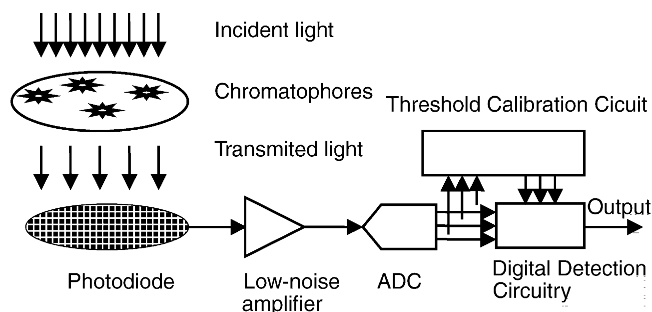

Fig. 2. Block diagram of the proposed bioassay.

voltage signal by the low-noise front-end amplifier. The output voltage from this amplifier is digitized using a low-power cyclic analog-digital converter, and the outputs are stored in a small on-chip memory and processed with simple digital circuitry. The threshold is evaluated for each run and stored on-chip. This evaluation is performed as part of a calibration phase, where the absolute threshold is computed based on the initial value of the signal and a pre-programmed relative threshold, computed according to the model presented in Section 3.5. The automatic calibration phase precedes the detection phase, and is performed each time the chromatophore sample is replaced. Once the setup is calibrated, the unit can work independently for several hours or even a couple of days until the cell media is due for a change. If the signal variation exceeds the threshold during the detection phase, an indicator or alarm is activated to warn the observer about the presence of a possible biohazard. Multiple assay units can be used to guarantee reliability of detection in field conditions. These units may be physically isolated and spread over the region of operation. Some of these may also be combined in form of an array of biological assays whose outputs are further processed using digital circuitry to decide whether the outputs represent a false alarm or the actual detection of an aggregation response. If needed, additional circuitry can be added to allow the individual units to communicate their warnings to a central monitoring station. All of this circuitry can be integrated on a printed circuit board. The current implementation uses an off-the-shelf photodiode. However, the entire electronic circuitry including this photodetector can be integrated onto a single-chip complementary metal oxide semiconductor (CMOS) integrated circuit. This offers the advantages of increased robustness along with lower cost and smaller size.

3.4. Theory—modeling of cell response using first-order kinetics

A simple model is presented here for the characterization of the expected cell response. This model has the advantage of offering physical insight and providing the basis for characterization of response and choosing the threshold.

For simplicity, it is assumed here that all the chromatophores are identical in terms of number of pigment granules and react similarly. Also, we assume that the agent of interest is present in uniform concentration throughout the cell sample, and that the concentration of the agent is not the limiting factor in the biological reaction. Thus, we can characterize the rate of reaction in terms of the cell density. This rate of reaction determines the time variation of the signal. With these assumptions, one can describe the reaction using a first-order kinetic model. The reaction and rate equation are shown below:

$$\mathrm{A} + \mathrm{B} \rightarrow \mathrm{C} \quad (1)$$

$$r = \frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{C}] = -\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{A}] = \lambda[\mathrm{A}] \quad (2)$$

$$\Rightarrow [\mathrm{A}] = [\mathrm{A}]_0\, \mathrm{e}^{-\lambda t} \quad (3)$$

$$\Rightarrow [\mathrm{C}] = [\mathrm{C}]_\infty (1 - \mathrm{e}^{-\lambda t}), \qquad \tau = \frac{1}{\lambda} \quad (4)$$

$$R = \frac{S_\infty}{S_0} \quad (5)$$

where A is the chromatophores in relaxed state; B the agent of interest; C the chromatophores after aggregation; λ the rate constant of reaction; τ the time constant of reaction; r the rate of reaction; [M] the concentration of reactant M in mol/l; R the signal range-index; S the signal value; 0 corresponds to the instant at which the reaction begins (system is in a relaxed state), while 5τ is assumed to be a suitable approximation for ∞ (completion of reaction). This model forms the basis for the threshold selection process outlined in the following section. The important point to note is that the system can be completely characterized by two parameters—R and τ, and we only need to estimate these.

3.5. Parameter estimation and threshold selection

The time constant τ is a random variable possibly dependent on the cell sample, the agent in question and environmental factors. The first task is to obtain an estimate for this based on measurements conducted with various agents and cell samples, and then to deduce a threshold taking this information into account, along with the distribution of the signal range-index.

The estimated distributions for the time constant and the range-index are denoted by F_τ and F_R, respectively. Again, we make simplifying assumptions considering these to be normally distributed. This assumption is not necessary and can be altered appropriately. However, we expect the two parameters of interest to show a somewhat Gaussian distribution, and attempt to find a best-fit normal distribution for each case in order to simplify our task. In addition, we expect to see multiple peaks in the distribution of the range-index, corresponding to the control samples, and the samples treated with the agent. Based on these assumptions, if the estimated mean and variance of the time constant and range-index are

$\mu_\tau, \mu_R, \sigma_\tau^2, \sigma_R^2$, respectively, then we can write the estimated distributions as shown below:

$$\hat{F}_\tau \sim N(\mu_\tau, \sigma_\tau^2) \tag{6}$$

$$\hat{F}_R \sim N(\mu_R, \sigma_R^2) \tag{7}$$

The distribution of the time constant is used to find the input sampling rate of the analog-digital converter. As our method relies only on detecting the difference between the initial reading and future readings, it can perform reliable detection even when the system is sampled at an extremely low frequency. However, there is a possibility that the effect of a large and sudden spurt of harmful environmental stimulus may be detected too late. Thus, a minimum sampling rate is necessary. At the same time, sampling the system at a rate which is too high adds redundant samples, increases power consumption, and requires the use of more on-chip memory so as to ensure that significant change can be observed even with slowly varying signals. These conditions are translated into loose empirical bounds on the sampling frequency as shown below. Here, M can be interpreted as the size of the on-chip memory used to store sample values:

$$\frac{5}{\mu_\tau} < f_s < \frac{M}{\mu_\tau + 5\sigma_\tau}, \quad M \sim 10\text{–}20 \tag{8}$$

We can choose the threshold so as to provide an optimal trade-off between the probability of false alarm, and the probability of a miss. For a general case, where there is substantial overlap between the distributions corresponding to the control samples and the samples treated with the agent, one can use the crossover point of the two distributions as the threshold. Thus, the threshold Γ is chosen according to the criteria:

$$1 - \hat{F}_R(\Gamma) = \hat{F}_{R(\text{control})}(\Gamma) \tag{9}$$

This step and the approximation of the distributions by best-fit Gaussian distributions was performed using MATLAB®. However, it is noteworthy that we need to do this only to arrive at the value of a suitable threshold. None of these compute-intensive tasks need be performed during the actual on-chip detection as this is merely part of modeling the system and its behavior.

3.6. Methodology for parameter estimation

A batch of 21 samples was used for the experiments. Three samples were exposed to control agents (L-15), and the remaining 18 samples were divided into 2 groups of 9 samples. One set of nine samples was exposed to clonidine, while the other was treated with cirazoline (CRZ), both of which are adrenergic agonists. Each set was further sub-divided into subsets of three, with each subset exposed to low (10 nM clonidine; 1 μm cirazoline), moderate (100 nM clonidine; 10 μm cirazoline) and high concentration (500 nM clonidine; 100 μm cirazoline) levels of the respective agent. The value of 5τ was estimated as the time at which the sample–sample variation of the signal fell below 1% limit. The value of R was estimated as the ratio of the signal levels at $t = 0$ and 5τ.

For control samples, the value of R is estimated as the maximum change in signal level seen during the course of the entire observation interval (>10 min). τ cannot be estimated from these values because the control samples do not behave according to the model outlined earlier.

Since our model is not valid for low concentration levels of the agent, the results from these trials were not used for estimation of τ. However, the value of R is estimated from these samples in the same manner as for the control samples by computing the maximum change seen in the signal level during the course of the experiment. It should be noted that the rate of reaction is typically much slower in this case when compared to the case where the concentration level of the agent is appreciable. Therefore, we need to observe the system for much longer than the nominal interval of time, and this requirement along with finite on-chip memory determines the upper bound to the sampling frequency (8).

3.7. Testing and validation

As explained in the previous section, the first-order kinetic model was verified by testing the scheme with 21 different chromatophore samples, subjected to aggregation-inducing agents in varying concentrations. The relevant results are presented in later sections. Both the agents used for threshold estimation are adrenergic agonists which induce aggregation and thus simulate the effect of the various toxins listed in Table 1 Actual toxins were not used for the testing owing to safety concerns and difficulties involved in handling these, especially the microbial pathogens. However, the objective here was to determine the feasibility of using the proposed approach in order to detect aggregation response in chromatophores, and compare its sensitivity to the existing approach based on the use of fish chromatophores (Chaplen et al., 2002b; Danosky, 1996), which is extremely sensitive but more intensive in terms of resource requirements.

The threshold Γ was also determined using the measurements obtained from these 21 samples, according to the methodology outlined in the previous sections. This threshold was then programmed into the system. The system was then tested with 3 batches of chromatophores, each comprising 10 cell samples, 2 of which were used as control samples. Each chromatophore sample was treated with various aggregation-inducing agents including clonidine, norepenephrine (NE) and melanin concentrating hormone (MCH). Norepenephrine is a catecholamine neurotransmitter hormone synthesized in higher organisms and with a biological function similar to epenephrine. MCH is a cyclic neuropeptide responsible for regulating skin color. Both these compounds can be found in higher organisms. However, they are not freely available in the environment otherwise. Clonidine is an adrenergic agonist drug as described earlier and is not found naturally in living organisms or the environment. These agents have been used extensively for

purposes of testing and validation of other chromatophore-based biosensors and bioassays and have been demonstrated as suitable for such purposes (Danosky and McFadden, 1997; Narayanan, 2003; Rengachari, 2004; Teng, 2002). Therefore, these provide a sound basis for comparison of the proposed approach with the existing scheme.

Since the threshold was chosen based on tests with clonidine and cirazoline, actual validation using NE and MCH offers a good indication of the reliability and portability of the model in the presence of unknown agents that may induce aggregation and is necessary to ensure that the scheme is truly generic in its operation.

4. Implementation details and issues

There are several issues that affect the reliability of operation. These include handling of the chromatophores, selection of an appropriate threshold, accounting for variations in cell samples, variations in environmental conditions and ensuring low-power consumption for the overall system to ensure battery-powered operation. Some of the important issues are addressed here.

4.1. Handling of chromatophore samples

It is very important to handle the chromatophores with proper care as sudden environmental shock may induce spurious aggregation or affect their sensitivity adversely. This work was based largely on erythrophores obtained from the *B. splendens* fish. These cells were found to be very robust and easy to handle, and capable of surviving for a few days without requiring a change of cell media. Nonetheless, it is important to ensure that the cells are completely submerged in the cell media at all times. Also, the cells should not be subjected to sudden variations in pH, salinity, temperature, etc. and should be maintained in the vicinity of room temperature (∼23–25 °C) at all times. The petridishes containing the chromatophore samples should be placed on a flat surface during normal operation and should be transported carefully to avoid causing excessive turbulence in the cell media. Most of these difficulties stem from the absence of a solid-state solution for growing the chromatophores.

The erythrophores used in this work are not affected adversely by ambient light and no special measures are required to shield them from such light. However, it is a good idea to enclose the entire setup in an opaque chamber as the sensing scheme utilises optical detection, and ambient light may act as an undesirable interference, especially if it shows significant variation during the detection phase. However, if the ambient light is well-controlled and strong enough, then this may be used as the source of illumination. If other chromatophore types such as melanophores are used, then it is absolutely necessary to shield these from any external light sources as these are very sensitive to light and may aggregate even without any other environmental stimulus.

If all these measures are taken, then each chromatophore sample can be preserved in a state of good health for at least a couple of weeks, and possibly up to 1 month.

4.2. Threshold selection

Perhaps, the most important issue is the choice of the threshold used for automated detection. This threshold must be chosen on the basis of tests conducted with a wide range of agents in varying concentrations. In addition, it is a good idea to choose this value as a predetermined fraction of the initial signal level instead of fixing it in the absolute sense. This is desirable because the initial signal level can vary widely depending on many factors, the most important one being the cell density of the sample in use. This can cause the initial reading to vary significantly. Thus, the choice of a relative threshold has the effect of normalizing the signal levels. This is the approach adopted in our scheme. The relative threshold Γ is determined using the analysis presented in Section 3.5 and the absolute threshold is computed on-chip during the calibration phase. The reliability of the scheme can be boosted further by testing it with several other agents in varying concentrations and fine-tuning the threshold value. In this study, the threshold was selected on the basis of the aggregation response observed in the presence of just two agents.

4.3. Variations in cell samples and environment

As described above, different chromatophore samples are likely to have a different number and density of chromatophores. Additionally, the chromatophores may differ in their sensitivity towards the agents of interest as different chromatophore samples may come from different fish. If the fish are not healthy, then the yield and sensitivity may be poor. The problem of variability can be addressed to some extent by appropriate choice of threshold as explained in Section 4.2. However, it is imperative that healthy fish are used for the cell culture. Also, it is desirable to ensure that the cell culture process is carried out according to a strict set of guidelines to ensure consistency in cell sample density. However, there will always be some amount of variation. Thus, the cell samples should be visually inspected after culture and samples showing unusually high or low cell density should be discarded. If further improvements are desired, then an additional calibration step can be added to the front-end low-noise preamplifier in order to adjust its gain level during the calibration phase. These measures were not included in the current implementation as the system displayed sufficient immunity to sample variations.

Environmental variations may also cause a change in chromatophore behavior. Specifically, it is recommended that the guidelines for proper handling of chromatophores be followed and sudden environmental shock avoided. This is a fundamental limitation arising from the fact that we are using living organisms as the sensing element. Development of solid-state or gel-based cultures of chromatophore cultures

can reduce the effect of some of these variables. However, such solutions are not yet available for the preparation of fish chromatophore samples.

4.4. Power consumption and circuit performance

From the perspective of circuit design, there are several ways of improving the system performance and battery life. First, the system ought to have a calibration mode where some form of automatic gain control is incorporated in order to ensure that the absolute signal level stays within the range of the processing circuitry, regardless of variation in the cell sample density, illumination level, photodetector sensitivity, etc. This calibration needs to be performed each time a new cell sample is used, and can be easily built into the system. Currently, such calibration is not built into the system. However, there is provision for manual calibration although our setup showed sufficient immunity to variations so as to obviate the need for any such adjustments.

Another important task is to ensure low-power circuit operation and small die area for the system to be truly portable. In order to meet this constraint, we use an algorithmic analog-digital converter (Rengachari, 2004), which is optimally suited for this application. Also, we use an automatic control that places the system in a standby mode when no data is being processed. Most of the electronic circuitry is powered down during this period. As the time-scale of the signal response in biological systems is of the order of a few seconds, while it is merely a few microseconds in electronic systems, significant improvement in battery life can be achieved by doing this. This feature has been added to the design of our analog-digital converter, and can be used to turn off the light source as well, thus saving considerable amounts of power.

5. Results and discussion

5.1. Model verification and threshold estimation

Key results include an example of the data used for estimation of time constant and range-index (Fig. 3), density functions for the estimated time constant (Fig. 4), the range-index (Fig. 5) and the decision criteria. In Fig. 3 the time origin represents the system in a relaxed state just before introduction of the agent. One can clearly see that the time-domain response closely matches the exponential response predicted by our simple first-order kinetic model. We also see that the distribution of the time constant can be approximated by a Gaussian density function, although it shows a wide spread. (In all the plots, the number of samples is expressed as a percentage of the total samples used for the experiment.)

The use of a Gaussian approximation is not very good for the probability density function of the range-index. However, for simplicity, we use this approximation here. The distribution shows multiple peaks in the density function. The first

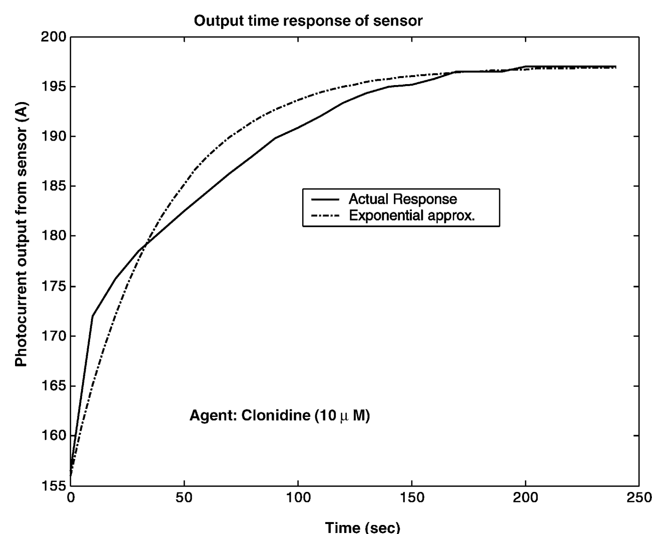

Fig. 3. Time response for a cell sample exposed to clonidine.

of these corresponds to the samples tested with the control agent, while the other peaks correspond to samples exposed to agents in low (10 nM clonidine; 1 μM cirazoline), moderate (100 nM clonidine; 10 μM cirazoline) and high levels (500 nM clonidine; 100 μM cirazoline) of concentration, respectively. As there is a clear demarcation between the control region and the rest, we can easily choose the threshold without using (9). In this case, we choose $\Gamma = 10\%$. The plot for range-index depicts the results from all 21 samples. It is also of interest to observe the effect of each individual agent. Such a plot is shown in Fig. 6 where only the nine samples treated with clonidine are shown. One can see clear demarcations between the regions exposed to low, moderate and high levels of clonidine. This is consistent with our expectations of a stronger stimulus eliciting a stronger response. However, this trend is sustained only till a certain range, beyond which

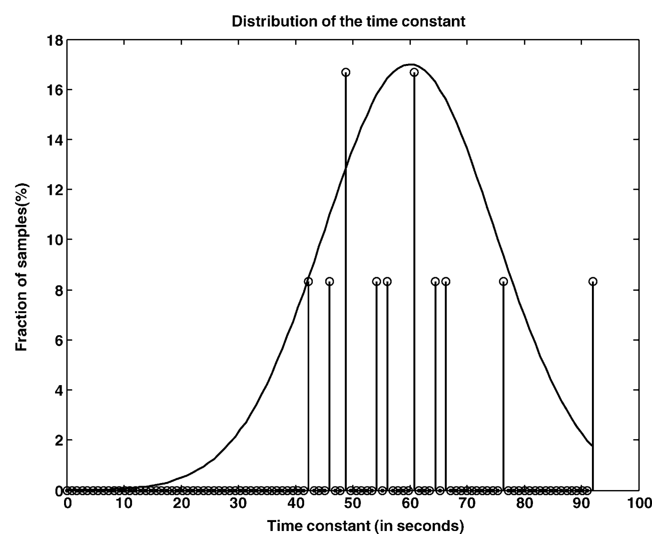

Fig. 4. Distribution of the time constant.

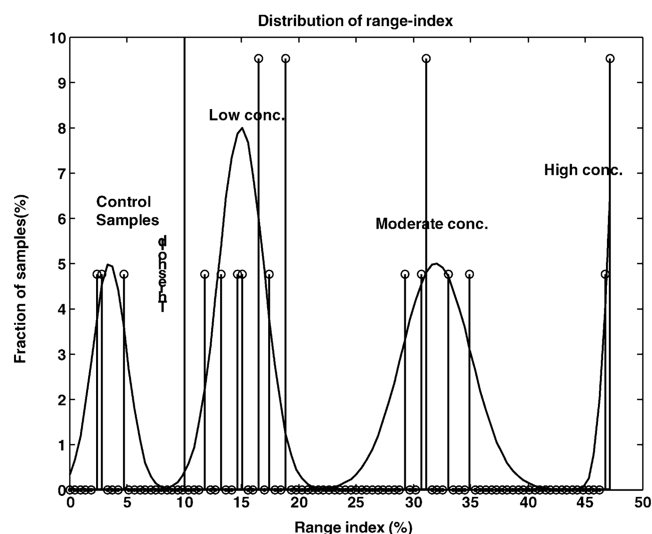

Fig. 5. Distribution of the range-index (all samples).

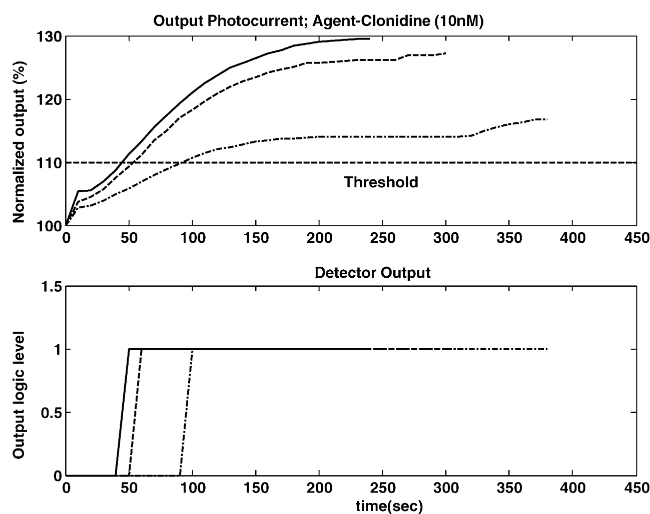

Fig. 7. Typical detection results. The output signal in the upper graph is normalized with respect to the initial reading.

the intensity of response flattens out as the chromatophores exhibit a saturation of response.

5.2. Assay response to aggregating agents

The threshold chosen in Section 5.1 was used in several experimental trials, each comprising a batch of 10 cell samples (2 control samples per batch). The results from one such batch are shown in Fig. 7. The control samples are not shown here as they exhibit no change in terms of the detector output. The sensing scheme was found to be successful in detecting the presence of aggregating agents in a significant number of cases, and failed detection was seen only in only 1 of the 30 samples in response to extremely low concentration levels of agents (<10 nM clonidine). No false alarms were observed in any of the control samples. This represents a reasonably high level of reliability. This reliability can be boosted further by fine-tuning the threshold, and using other measures to ensure consistency in cell sample preparation. Thus, the automated approach is nearly as sensitive as some much more sophisticated techniques based on monitoring chromatophore response.

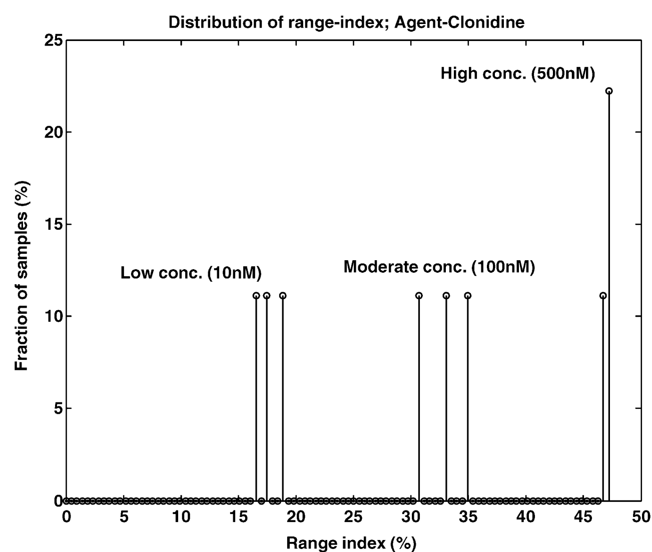

Fig. 6. Distribution of the range-index (samples exposed to clonidine).

Currently, the final detection in most other methods is performed by human observers looking at magnified images of cell samples captured by cameras, or by measuring other variables of interest, which are processed further using expensive signal-processing and pattern-recognition software. The proposed approach eliminates the need for expensive and bulky hardware and software and requires no observer.

There is a small possibility of false alarm in our scheme owing to certain benign changes in the environment that might trigger an aggregation response. This could be owing to agents that induce aggregation but are not toxic or drastic changes in pH or temperature. In fact, most of our feasibility analysis was conducted using agents such as norepenephrine and clonidine which are non-toxic, but capable of inducing aggregation. However, these agents are not found in the environment. Thus, the risk of false alarm can be ignored as long as the cells are handled carefully.

6. Further directions

Although the proposed scheme offers several advantages, it also inherits some of the limitations of the conventional approach based on chromatophores. First, the proposed scheme can only detect agents which induce response in chromatophores, and despite the wide range of agents falling in this category, there are still some that cannot be detected by this method. However, the reliability and range of detection can be improved by using different types of chromatophores, and by using suitable modifications of other techniques that

rely on some form of optical detection such as schemes that measure change in bio-luminescence. The current scheme suffers from variations in the quality of cell samples, which is inherent in every technique. Also, the portability of any scheme based on the proposed approach is hampered to some extent owing to the use of liquid media. Although the chromatophore samples may be transported, they must be placed on a level surface during the detection phase to prevent turbulence in the liquid media. Thus, true portability cannot be achieved at the moment despite the significant reductions in size and power consumption. Further research is required in order to devise techniques to reduce this variability and to culture the cells on solid or semi-solid gel substrates to allow for enhanced portability.

Another point of interest would be to find ways of prolonging the active life of chromatophore samples. Since the chromatophores cannot divide, fresh samples must be harvested from new fish, and each sample can be typically used just once. It would be of interest to find ways to recycle chromatophore samples so as to avoid use of too many new fish.

Another important aspect concerns the choice of the threshold. There is still need for extensive testing with a wide range of agents before a universally optimal threshold is found. Higher-order models can also be used to account for cases where the current model breaks down, e.g. extremely low agent concentrations, non-Gaussian statistics, etc. Since the modeling computation is carried out off-line, use of a more intricate and compute-intensive modeling process does not add to the complexity of the electronic system.

7. Conclusion

A novel approach was proposed for simpler and faster automated detection of toxic agents in the environment. The method relies on using the aggregation response of fish chromatophores. The approach is shown to be capable of realizing a low-cost, generic and portable biotoxicity assay, and is much simpler than the existing state-of-art. It is expected that improvements in methods for the preparation of cell samples and extensive field testing will further improve the reliability of this approach.

Acknowledgements

The authors would like to thank the Catalyst Foundation, New York, USA for funding the research on electronic cytosensors. In addition, we would like to thank Dr. Rosalyn Upson and Cheng-Yan Peng from the Department of Bioresource Engineering, Oregon State University for providing the erythrophore cell samples used for testing.

References

Chaplen, F.W.R., Upson, R., McFadden, P.N., Kolodziej, W.J., 2002a. Fish chromatophores as cytosensors in a microscale device: detection of environmental toxins and microbial pathogens. Pigm. Cell Res. 15, 19–26.

Chaplen, F.W.R., Upson, R., McFadden, P.N., Kolodziej, W.J., 2002. Method and system for classifying a scenario. International Patent Application No. PCT/US02/29085, filed September 12, 2002. Oregon State University, USA.

Danosky, T.R., 1996. Biological inspired biosensors from fish chromatophores. M.S. Thesis. Oregon State University, Corvallis, USA.

Danosky, T.R., McFadden, P.N., 1997. Biosens. Bioelectron. 9–10 (12), 925–936.

Fujii, R., 1993. Cytophysiology of fish chromatophores. Int. Rev. Cytol. 143, 191–255.

Fujii, R., 2000. The regulation of motile activity in fish chromatophores. Pigm. Cell Res. 13, 300–319.

Gray, S.A., Kusel, J.K., Shaffer, K.M., Shubin, Y.S., Stenger, D.A., Pancrazio, J.J., 2001. Design and demonstration of an automated cell-based biosensor. Biosens. Bioelectron. 16, 535–542.

Gross, G.W., Harsch, A., Rhoades, B.K., Göpel, W., 1997. Odor, drug and toxin analysis with neuronal networks in vitro: extracellular array recording of network responses. Biosens. Bioelectron. 12, 373–393.

Gross, G.W., Rhoades, B.K., Azzazy, H.M.E., Wu, M.C., 1995. The use of neuronal networks on multielectrode arrays as biosensors. Biosens. Bioelectron. 10, 553–567.

Keefer, E.W., Gramowski, A., Stenger, D.A., Pancrazio, J.J., Gross, G.W., 2001. Characterization of acute neurotoxic effects of trimethylolpropane phosphate via neuronal network biosensors. Biosens. Bioelectron. 16, 513–525.

Kumar, P., Colston, J.T., Chambers, J.P., Rael, E.D., Valdes, J.J., 1994. Detection of botulinum toxin using an evanescent wave immunosensor. Biosens. Bioelectron. 9, 57–63.

Larsson, N., Ruzgas, T., Gorton, L., Kokaia, M., Kissinger, P., Csöregi, E., 1998. Design and development of an amperometric biosensor for acetylcholine determination in brain microdialysates. Electrochim. Acta 43, 3541–3554.

Narang, U., Anderson, G.P., Ligler, F.S., Burans, J., 1997. Fiber optic-based biosensor for ricin. Biosens. Bioelectron. 12, 937–945.

Narayanan, A., 2003. Use of elicitor sets to characterize cellular singal transduction. M.S. Thesis. Oregon State University, USA.

Niwa, O., Horiuchi, T., Kurita, R., Torimitsu, K., 1998. On-line electrochemical sensor for selective continuous measurement of acetylcholine in cultured brain tissue. Anal. Chem. 70, 1126–1132.

Owicki, J.C., Parce, J.W., 1992. Biosensors based on energy metabolism of living cells: the physical chemistry and cell biology of extracellular acidification. Biosens. Bioelectron. 7, 255–272.

Paddle, B.M., 1996. Biosensors for chemical and biological agents of defense interest. Biosens. Bioelectron. 11, 1079–1113.

Pollard-Knight, D., Hawkins, E., Yeung, D., Pashby, D.P., Simpson, M., McDougall, A., Buckle, P., Charles, S.A., 1990. Immunoassays and nucleic acid determination with a biosensor based on surface plasmon resonance. Ann. Biol. Clin. 48, 642–646 (Paris).

Pancrazio, J.J., Bey, P.P., Cuttino, D.S., Kusel, J.K., Borkholder, D.A., Shaffer, K.M., Kovacs, G.T.A., Stenger, D.A., 1998. Portable cell-based biosensor system for toxin detection. Sens. Actuators B 53, 179–185.

Pancrazio, J.J., Whelan, J.P., Borkholder, D.A., Ma, W., Stenger, D.A., 1999. Development and application of cell-based biosensors. Ann. Biomed. Eng. 27, 697–711.

Rainina, E.I., Efremenco, E.N., Varfolomeyev, S.D., Simonian, A.L., Wild, J.R., 1996. The development of a new biosensor based on recombinant *E. coli* for the direct detection of organophosphorous neurotoxins. Biosens. Bioelectron. 11, 991–1000.

Rengachari, T., 2004. A 10-bit algorithmic A/D converter for a biosensor. M.S. Thesis. Oregon State University, USA.

Rogers, K.R., 1995. Biosensors for environmental applications. Biosens. Bioelectron. 10, 533–541.

Skladal, P., Mascini, M., 1992. Sensitive detection of pesticides using amperometric sensors based on cobalt phthalocyanine-modified composite electrodes and immobilized cholinesterases. Biosens. Bioelectron. 7, 335–343.

Stenger, D.A., Cribbs, D.H., Fare, T.L., 1991. Modulation of a gated ion channel admittance in lipid bilayer membranes. Biosens. Bioelectron. 6, 425–430.

Teng, A., 2002. Cytosensor project—process segmentation and multivariate time series modelling. Technical Report—Electrical and Computer Engg. Oregon State University.

van der Schalie, W.H., Shedd, T.R., Knechtges, P.L., Widder, M.W., 2001. Using higher organisms in biological early warning systems for real-time toxicity detection. Biosens. Bioelectron. 16, 457–465.

Watts, H.J., Yeung, D., Parkes, H., 1995. Real-time detection and quantification of DNA hybridization by an optical biosensor. Anal. Chem. 67, 4283–4289.