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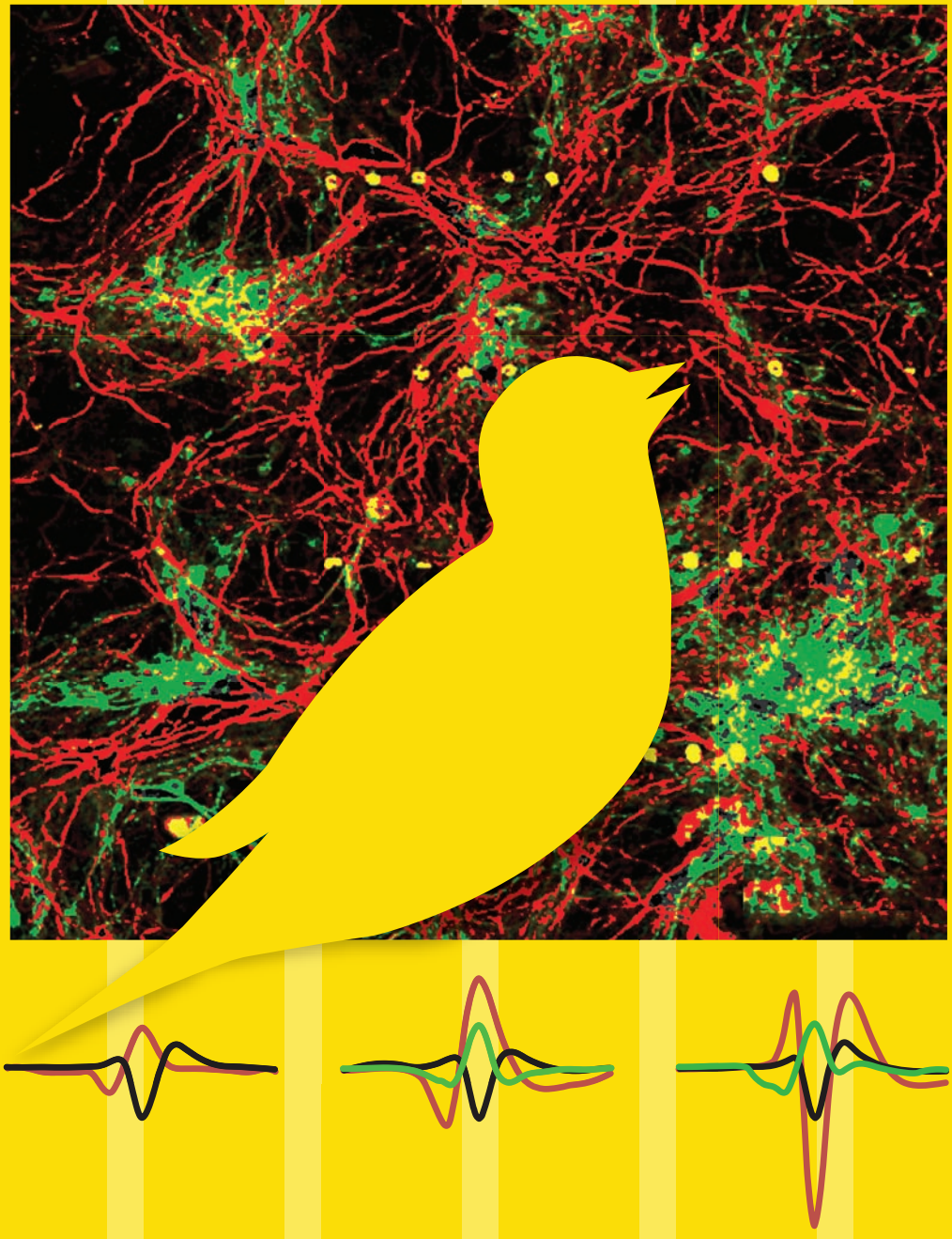

Thomas J. O'Shaughnessy • Naval Research Laboratory

Joseph J. Pancrazio • National Institutes of Health

In the sixth-century B.C. text *The Art of War*, Sun Tzu wrote, "The rising of birds in their flights is the sign of an ambush. Startled beasts indicate that a sudden attack is coming." Environmental toxicants—unseen molecular adversaries—are a major concern for deployed military forces and a growing issue for civilian communities. Sun Tzu's insight to make use of biological organisms for threat detection has been rediscovered throughout history. For example, it is well known that coal miners once brought canaries into the mines to detect potentially lethal levels of methane. During the Japanese police's raids of the Aum Shinrikyo cult in 1995, canaries were once again called into service as threat detectors (*1*). In 2003, Marine regiments in the Kuwaiti desert preparing for the Iraq conflict were issued pigeons to serve as detectors for gas threats (*2*).

Just as canaries in a coal mine once provided a generalized warning of danger, living systems can offer a broadband means of detecting threats to troops and civilians.

The reliance on biological organisms rather than chemical agent detectors designed for specificity in these situations is not surprising. Although chemical agent detectors offer specificity and sensitivity, the trade-off is that one must assume a particular threat, or at least class of threats. In the absence of a priori knowledge of the nature of the threat, the use of broadly sensitive detectors that exploit functional biology offers a means of detecting unanticipated threats. The problem of assessing toxicity by using specific detectors, such as immunoassays or spectrophotometers, becomes even more daunting when the possibility of complex mixtures is considered. Combinations of toxicants, each at a level that would be considered safe independently, could result in cumulative or synergistic toxic effects. Instead of detecting very specific and anticipated analytes, living systems offer a broadband or generic means of detection.

In this article, we describe various approaches for biologically based detection of hazardous environmental toxicants. We emphasize the potential of mammalian-cell-based biosensors as broadband toxicity detectors. We also identify future directions that would improve the physiological relevance of such detectors and circumvent logistic hurdles for field-deployable devices.

Biological systems as toxicity detectors

There is a long history of the use of biological organisms as toxicity detectors, particularly for screening water supplies. A wide range of organisms have been exploited, including yeast, bacteria, algae, daphnia, and fish (*3*–*7*). Although it is tempting to assume that these living systems would automatically offer broadband sensitivity, this is not necessarily the case. The vast majority of bacterial systems have been engineered to exhibit some degree of specificity to a toxicant or class of toxicants. For example, recombinant *E. coli* coupled to fiber-optic sensors expresses the enzyme organophosphorus hydrolase, which allows detection of organophosphate pesticides (*8*). Likewise, heavy metals have been detected by bacteria with metal-induced promoter regions coupled with lux-based luminescence reporter systems (*9*). Lee et al. reported a bacterial array biosensor based on differential bioluminescent responses; this suggests the possibility of discriminating classes of toxicants (*10*). For many bacterial systems, the goal is, through a priori design, to express specific detectors that can identify and quantify toxicants. These systems perform well under controlled laboratory conditions in which only a single analyte rather than a mixture is presented to the detector.

Some efforts to achieve generic toxicity detection have been successful. One of the most widely used systems is Microtox, which is based on the use of the luminescent bacteria *Vibrio fischeri* (*11*). Luminescence is proportional to cellular respiration and metabolism; exposure to toxicants decreases respiration and therefore luminescence. In extensive testing of *Sinorhizobium meliloti*, toxicity is indicated by optical changes associated with the conversion of tetrazolium dye (*12*).

Two approaches make use of fish as aquatic sentinels for toxicity. McFadden's group developed an assay based on chromatophores derived from the scales of particular species of fish, such as the Nile tilapia and *Betta splendens* (*13*, *14*). The chromatophore approach relies on microscopic changes in the optical properties of single cells induced through pigment granule distribution, which can be altered in the presence of some toxicants (*15*). A library of responses to a variety of toxicants has been developed for the chromatophores; this raises the possibility that this approach can be used to enable some level of classification (*16*). The broadband detection methodology most similar to the use of the canary in the coal mine involves monitoring the physiological function of fish. The U.S. Army has developed a deployable biomonitoring system that performs continuous measurements of the muscle activity associated with ventilatory activity in fish to monitor surface water and wastewater effluents (*17*–*19*).

In spite of these successes, the central concern is that what is toxic to bacteria or fish is not necessarily toxic to mammals or humans. The aquatic biomonitor is extremely sensitive to certain insecticides at concentrations that have limited toxicity to mammals. Bacterial models that lack receptors to neurotoxins fail to respond to lethal levels of these agents. Conversely,

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antibacterial compounds are designed for medical purposes to exploit toxicity differences. This mismatch in toxicity response drives interest in detection approaches that harness tissue of mammalian origin.

Mammalian-cell-based toxicity detectors

Human alveolar epithelial cells were used by Riley and colleagues in a sensor platform based on a colorimetric assay. The conversion of tetrazolium dye via normal cellular metabolism is the basis for detecting micromolar to millimolar concentrations of Zn (*20*). This approach has been refined and implemented on optical fibers to capitalize on the modulation of the evanescent wave as a means of performing sensitive spectroscopic analyses of toxicant response profiles (*21*). One issue with the use of dyes to assess metabolism is that the cell-based sensor can be used only for single assays rather than for continuous monitoring. Another approach makes use of Raman spectroscopy to discriminate ricin and sulfur mustard toxicity in exposed cells (*22*). Spectroscopic analyses raise the possibility of generating fingerprints for cellular responses to at least identify pathways or processes that might be affected by toxicants. However, the spectral alterations may overlap, making such interpretations very difficult (*21*).

In addition to changes in metabolism, alterations in cell adhesion on substrates and monolayer confluence have been proposed as indicators of exposure. When forming a confluent monolayer, human umbilical vein endothelial cells cultured over ion-selective membranes exhibit no ion transport. In the presence of toxicants, the monolayer becomes permeable, and ion transport can be measured (*23*). Another interesting approach involves measurements of impedance at electrodes coated with cells. A change in endothelial cell monolayer integrity assessed through impedance is the basis for a commercially available system that responds in 5–20 minutes to a range of toxicants (*24*, *25*). More detailed measures of cell function—including spreading, mitosis, and proliferation on electrode contacts—have been proposed. Toxicant-induced changes in cell impedance on a fibroblast cell line and a hepatic cell line have been described (*26*, *27*); however, an exposure time of hours was required to detect significant differences.

Sensor responses and human toxicity

The key to the effectiveness of any whole-organism or mammalian-cell-based biosensor is the relationship between the responses of the system to a compound and the toxicity of that compound to human beings. Responding to a large number of nontoxic compounds, or toxic compounds well below the danger level, is just as problematic as failing to detect chemicals harmful to humans at appropriate levels. One of the goals in switching to mammalian cells has been to close the gap between the sensor element and the human organism. However, in switching to mammalian cells one loses the whole-organism aspects and introduces added variability because not all mammalian cell types respond the same to all compounds. For example, mammalian neurons will respond to a wide variety of neurotransmitter and synaptic inhibitors that are lethal to humans; however, a system based on mammalian epithelial cells would fail to respond to these compounds because they lack the required receptors.

Only a few studies compare the responses of a series of biologically based biosensors to the same challenges. Pancrazio and colleagues reported on the results of the EILATox-Oregon Biomonitoring Workshop, in which a variety of whole-organism and cell-based sensors were tested against a panel of blinded compounds (*28*). In this workshop, the *B. splendens* fish chromatophore system was the only one to detect all 14 of the chemicals tested. Systems based on *Thamnocephalus platyurus* or *Daphnia magna* mortality were also highly effective, followed by a system based on murine neurons. The bacterial-based systems had an ~50–60% response rate; the fish ventilatory response system also detected 50% of the compounds. The lowest response rate was from a system based on a rat hepatic cell line. Mammalian-cell-based systems performed at both the high and low ends of the spectrum; this suggests that each system will need to be evaluated individually. It is likely that no one of these generic detectors will be able to respond to the full range of threat compounds, and a combination of several systems will be needed. When van der Schalie et al. evaluated a series of cell-based sensors for drinking water against a panel of 12 threat compounds, they found that they needed three systems to detect 75% of the threats (*25*).

Although chemical agent detectors offer specificity and sensitivity, the trade-off is that one must assume a particular threat, or at least class of threats.

Practicality of biologically based sensors

Once a system has been determined to predict human toxicity at a sufficient level, practicality must be considered. Can the system be designed in such a manner as to be placed in the field with a minimum amount of intervention while providing easily interpreted results? The answer will depend on the biological system chosen, but in some cases, these systems can be designed to be robust enough for field use.

The aquatic biosentinel system mentioned previously can operate without intervention for up to 2 weeks and can remotely alert operators to detection events. The limiting factor in this system is the useful life span of the biological element,

in this case fish. The lifetime of the biological elements and the logistics of supplying them to the field are the biggest hurdles in most biologically based sensor systems. A significant hurdle for cell-based sensors is the reliance on cell culture media. Optimal neuronal culture requires maintenance of pH via the introduction of CO_2 and phosphate bicarbonate buffering. In addition, culture media contain serum, which has a limited lifetime and is inherently chemically ill-defined. Defined culture media and pH buffering strategies that reduce dependence on CO_2 have been developed, but their utility is not yet clear.

Another major issue is the availability of cell sources. Systems based on bacteria have a distinct advantage because many types of bacteria can be freeze-dried and quickly reconstituted. However, the use of mammalian cell lines represents a potentially renewable source of cells if the process of cell passage (splitting a population of cells to keep them alive under cell-culture conditions) can be automated. For terminally differentiated cell types such as neurons, other options must be sought. Our group is exploring using stem cells as a renewable source of neurons for a cell-based sensor. Whether the logistic issues can be overcome remains to be seen, but they will likely be dealt with on a case-by-case basis.

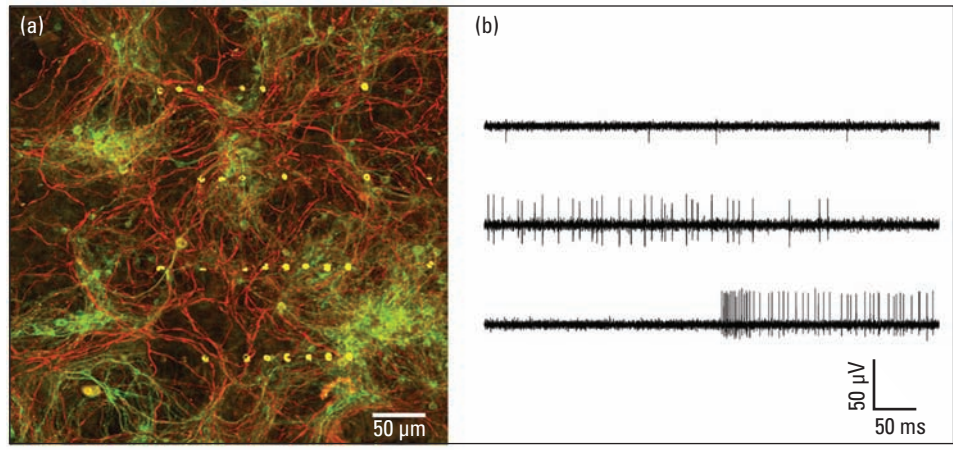

FIGURE 1. Neurons as a biosensor.

(a) Network of spinal cord neurons growing on an MEA. Neurons have been stained red for neurofilament (axonal marker) and green for MAP2 (cell body and dendrite marker). The horizontal rows of yellow fluorescing circles are a portion of the 16 × 4 matrix of electrode recording sites. Electrode diameter: 10 µm. (b) Extracellular recordings from three channels taken simultaneously with the neuron-based biosensor at a sampling rate of 40 kHz per channel.

Electrically active cells as canaries

Electrically active cells offer the ability to readily tap into physiologically relevant cellular signaling as a basis for detection. Cardiac myocytes and neurons follow the excitation–contraction and stimulus–secretion paradigms, respectively, in which biopotentials are central to function. Unlike cell types in which gene transcription and protein translation govern function with temporal profiles of minutes to hours, electrically active cells exhibit action potentials that occur over millisecond to second time spans.

The classic approach for the measurement of action potentials is the use of intracellular recording techniques, which involve one or more glass microelectrodes and a vibration isolation table. Clearly, this approach is not amenable to field use; however, the capacity to perform whole-cell recordings on planar chips has emerged (*29*). Nevertheless, invasive recordings are not practical for field devices because the integrity of the recording is lost over a time frame of minutes to hours. Instead, noninvasive recordings can be achieved with the use of microelectrode arrays (MEAs) in which activity is monitored as extracellular potentials (*30*, *31*).

Even with noninvasive recordings, the development of a cell-based sensor system entails several other challenges. One is to collapse a rack full of laboratory equipment into a portable instrument capable of making microvolt-level recordings outside the constrained environment of a laboratory. The second challenge rests in characterization and testing of the biosensor. This phase of testing is concerned not with the validation of the electrical interface between cells and electrodes but with the validation of the biosensor's sensitivity to toxicants. In fact, no amount of engineering will circumvent the need to build a library of response profiles for characterization of a cell-based sensor system.

The feasibility of using cardiac myocytes cultured over MEAs in portable cell-based biosensor systems was initially demonstrated when we collaborated with Kovacs (*31*, *32*). More refined field-portable systems relying on cultured myocytes were developed, including a handheld system (*33–35*). In one application, the multielectrode recordings of cardiac cell monolayers offer the ability to probe the effects of candidate antiarrhythmic therapeutics and perhaps of other drugs on classes of cardiac ion channels (*36–39*). With respect to toxicant detection, it was recently reported that extracellular cardiac action potentials are sensitive to pyrethroid pesticides at concentrations ≥10 µM (*40*). Overall, however, relatively few published papers have explored the utility of the cardiac cell model for environmental toxicity detection.

Because neurobehavioral changes can have profound impacts on the readiness of deployed forces, the military is interested in broadly sensitive biosensors for neurotoxicant detection (*41–43*). Although tumor-derived lines, such as neuroblastoma and pheochromocytoma, have been popular in toxicity assays, it is important to recognize that many of these cell lines often fail to form functional, robust synapses. Clearly, a useful cell-based biosensor for neuroactive compounds should be based on physiologically relevant activity, including synaptic transmission.

Several groups have explored the potential of hippocampal brain slices as screens for neurotoxicity. The slice model, which

Table 1. Effect of a broad range of compounds on neuronal network activity.[a]

Compound	IP LD_{50} (mg/kg)	Oral LD_{50} (mg/kg)	Neuronal network EC_{50} (mg/L) (Ref.)
Aldicarb	0.28	0.3	0.0074 (*25*)
Brevetoxins	0.2	6.6	0.036 (*68*)
$CdCl_2$	9.3	60	1.28 (*63*)
Chloroquine	68	500	4.79 (*88*)
Domoic acid[b]	3.6	—	0.31 (—)
Ethyl alcohol	528	3450	1840 (*68*)
Fluoxetine	100	248	1.87 (*89*)
$HgCl_2$	3.9	6	5.43 (*70*)
Methamidophos	5.3	14	1.08 (*25*)
Phenol	180	270	5.94 (*25*)
Quinine	115	—	9.31 (*89*)
Saxitoxin	0.01[c]	0.263[c]	5.98×10^{-4} (*68*)
NaCN	4.9	6.44[d]	4.90 (*89*)
Strychnine	0.98	2	0.20 (*63*)
Tetrodotoxin	0.008	0.334	1.28×10^{-3} (*69*)
Trimethylolpropane phosphate	0.7[d,e]	—	6.00 (*90*)

[a] IP and oral LD_{50} values obtained from Sigma-Aldrich MSDSs or mouse unless otherwise indicated. The neuronal network EC_{50} data are for 50% inhibition of mean spike rate for murine-cultured neuronal networks.
[b] Unpublished Naval Research Laboratory data.
[c] www.cbwinfo.com.
[d] Data for rat.
[e] Ref. 62.

shows many properties of the in situ hippocampus in terms of organization, morphology, and function, provides a neural substrate that captures the physiologically relevant architecture and circuits. A hippocampal structure can be cut into five to seven slices of ~400 µm thickness; this allows access to a stereotypical trisynaptic circuit (*44*, *45*). The slice technique is a step beyond conventional glass and metal microelectrodes inserted at specific locations with micromanipulators; it has been applied to MEAs capable of stimulation and recording to enable neurotoxicant evaluation (*46*, *47*). Recognizing the nonlinearities inherent in hippocampal circuitry, Baudry's group has developed a method to generate parameters for stimulus–response dynamics based on the Volterra model (*48*, *49*). One of the major problems with the use of slices is that they last <1 day after preparation. Organotypic culturing methods relying on rotating vessels do allow slices to be maintained for more than a month (*48*), and the longest reported culture period is 3–4 weeks in a specialized tilting incubator system (*50*).

During the past three decades, techniques have emerged to realize extracellular recording through substrate-integrated microelectrodes from networks of cultured mammalian neurons (*31*, *51–53*). Gross and colleagues have developed and refined the culture methodology such that neuronal networks can be prepared routinely and can survive for months, thus allowing long-term monitoring of inherent spike activity (*54–58*). The methodology depends on the dissociation and seeding of embryonic neural tissue on appropriately prepared surfaces of MEAs, resulting in cell adhesion and maturation of networks (Figure 1a). After plating on substrates, the networks grow over a period of 1–2 weeks to form stable culture models. Simultaneous recordings from multiple electrode sites in the cultures are readily taken (Figure 1b); distinct signal-generating neurons or neuronal processes defined by characteristic waveforms will often appear at an individual electrode site (Figure 2).

Data from the networks, including spike and burst rates, burst durations, interspike intervals, and correlation or synchronization among units, can be quantified in several ways (*55*, *59–61*). A change in the mean spike rate across a network is usually the most sensitive and useful parameter for detection. However, if other parameters, such as bursting and synchronization, are examined, toxicant effects can be separated into inhibitory, excitatory, and disinhibitory classes. For example, disinhibitory neurotoxicants that block inhibitory synapses are associated with seizure induction. Disinhibitory compounds such as trimethylolpropane phosphate and strychnine increase bursting and synchronization of network activity (*62–64*).

The potential for the use of cultured neuronal networks as biosensors has been widely recognized (*30*, *31*, *41*, *65–68*). Working with Gross's group, we developed a portable biosensor specifically to accommodate their neuronal network format in a manner conducive to cell-culture handling. The biosensor provided robust control of temperature and flow conditions as well as low-noise recording of microvolt-level extracellular potentials (Figure 3; *69*). Recording levels are virtually identical to those achieved with laboratory-based systems. Because of the network's robustness, the biological components can be shipped to end users and treated as modules for the portable system (*70*, *71*). Moreover, because of the standardization of these networks, results between laboratory and field settings can be compared.

Broadband sensitivity

Unlike the case with cardiac cell models, a substantial body of published work demonstrates the sensitivity of cultured neuronal networks to environmental toxicants (Table 1). It is important to note that the networks are not overly sensitive to any and all compounds. For example, neuroactive agents that are considered to be highly potent, such as botulinum toxin and marine algal toxins, exert profound effects on cultured neuronal networks at picomolar and nanomolar concentrations, whereas compounds with much lower toxicity, such as ethanol and chemical agent hydrolysis products, are effective at millimolar concentrations (*42*, *68*, *72*).

An important question to address is the relationship between the sensitivity of the networks to a toxicant and the expected toxicity of that compound in vivo. We examined the concentration required to reach 50% of the maximal effect (EC_{50}) on mean spike rate in the neuronal networks for a series of compounds and compared these values to the known oral and/or intraperitoneal (IP) dose that produces lethality in 50%

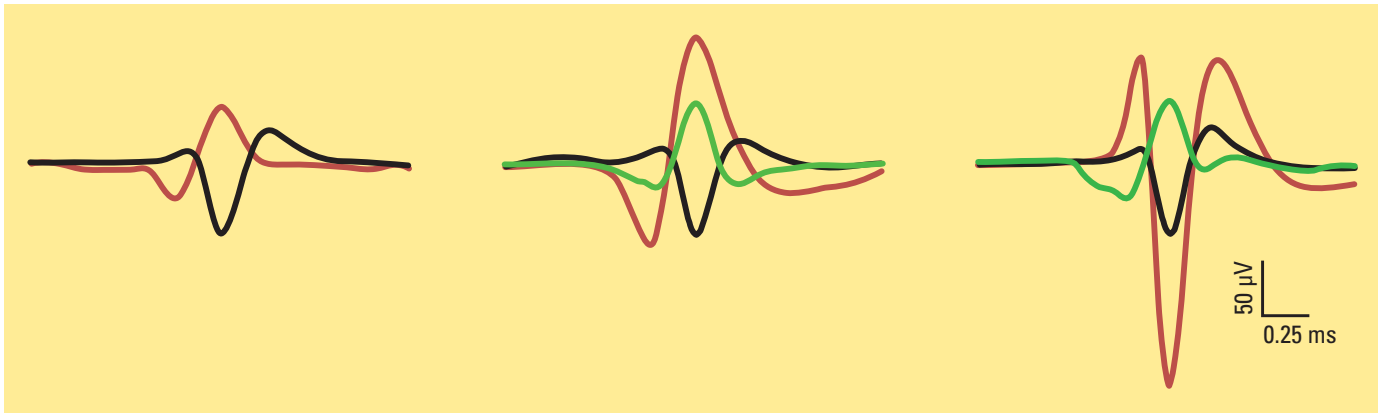

FIGURE 2. Multiple neurons (units) can be detected on a single MEA channel.

Data for three channels are shown. The first channel had two units, and the other two channels had three units each. The waveforms are the signal averages of 1000 action potential spikes for each channel, discriminated by a hierarchical clustering algorithm. (Left) 87% of spikes fitted the black waveform, and 13% matched the red. (Middle) The distribution was 45% black, 41% red, and 14% green. (Right) The distribution was 54% black, 41% red, and 5% green. The percent distributions are a function of the differing firing rates of the given units.

of cases (LD_{50}; Table 1). A strong positive correlation exists between the sensitivity of the neuronal networks as determined by log EC_{50} and the toxicity of the compound, represented by log LD_{50} (Figure 4). These data suggest that the prediction capacity for the log LD_{50} values from neuronal network results is ~±2 log units.

Correlations alone do not necessarily indicate that cultured neuronal networks make for good environmental biosensors. To be useful, a broadband biosensor should not only exhibit the above correlation but also be sufficiently sensitive to detect toxic levels before people are exposed to lethal levels. To assess the ability of cultured neuronal networks to detect toxicants in the aqueous phase, the concentration-dependent effect of various threats can be compared with lethal levels. For example, Cd induces significant alterations in neuronal network activity at ~1 µM. If we apply the rat oral LD_{50} for $CdCl_2$ of 88 mg/kg for a 70 kg human, a molecular weight of 183 g/mol, and a conservative estimate of 10 L of water consumption, the LD_{50} for Cd corresponds to 4.8 mM. The cultured neuronal network shows a significant response well below these levels, which suggests potential for environmental threat detection. Similar relationships can be observed for a wide range of toxicants.

Future directions

For the full potential of neuronal network biosensors to be realized, substantial advances in engineering and biology are needed. The technology itself can become smaller; certainly, MEAs can be made from readily manufacturable yet disposable materials, incorporate a much larger number of recording sites, or have electronics embedded directly in the substrate (*73–76*). A difficult engineering challenge for any detection device making a transition from the laboratory bench to the field is the user interface. Automated sample introduction and processing coupled with a statistically validated but simply represented "red light–green light" interface are essential for minimizing the training and sophistication required of the user.

The challenges in biology pose significant logistic barriers that are more difficult to surmount. For neuronal network biosensors, cell source and shelf life are the major hurdles. With regard to cell source, biosensors require robust synapses and therefore have relied on animal dissection and veterinary support. An exciting alternative to primary cultures would be stem cells, which proliferate while maintaining the ability to differentiate into any of a large class of cell types.

Stem cells offer the potential advantages of primary cells and tumor-derived cell lines without the inherent drawbacks of either. Embryonic stem cells could be used to generate cardiac and neuronal cells for drug screening and toxicological investigations and also open a window for access to human tissue (*77*, *78*). Embryonic murine stem cells that are capable of

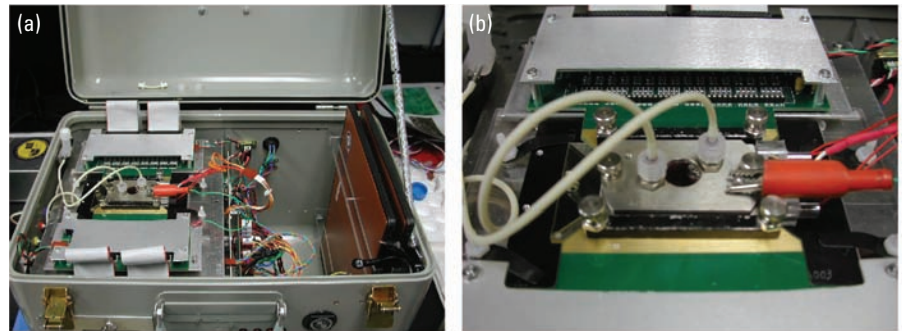

FIGURE 3. Biosensor in a box.

(a) Interior of the neuron-based biosensor. The left half of the device houses the amplifier circuits, pumps, heaters, and cell chamber (visible on top); the right half contains the batteries and power distribution system. (b) Close-up of the cell chamber. The stainless steel chamber containing the MEA can be inserted and removed from the system without harming the neuronal networks. The chamber doubles as a shipping container; cells can be shipped overnight by commercial carrier and still be viable.

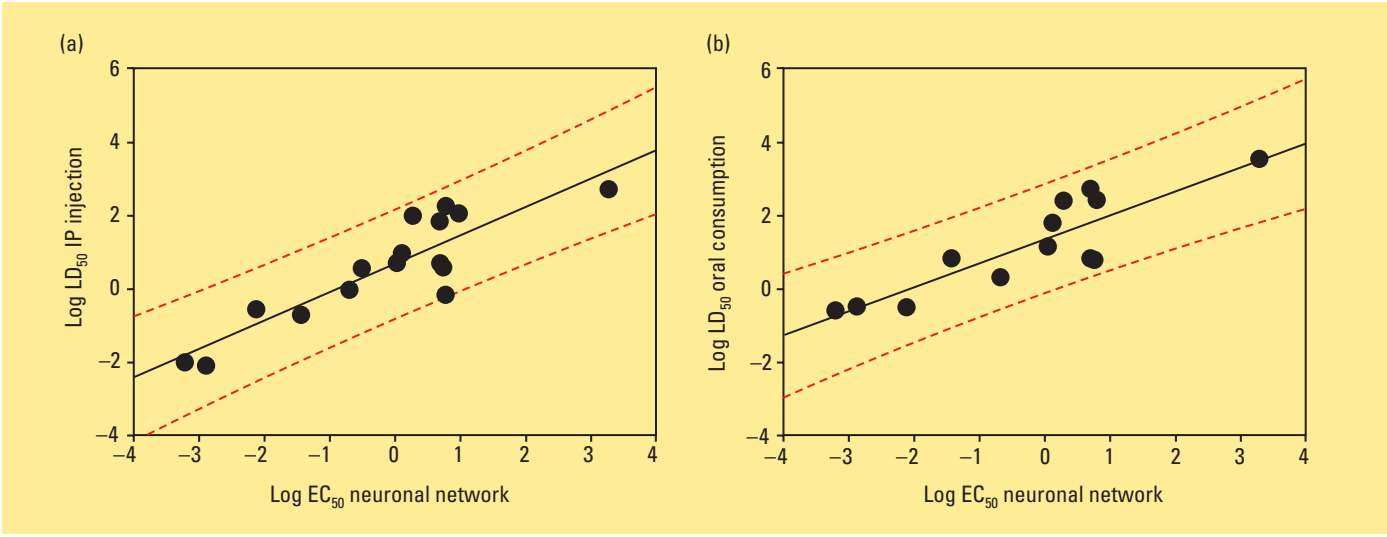

FIGURE 4. Strong positive correlations between the sensitivity of the neuronal networks (log EC_{50}) and the (a) IP and (b) oral (log LD_{50}) values.

Data are for the compounds listed in Table 1. The black lines are a linear fit of the data, and the red curves indicate the 95% prediction intervals.

producing functional neurons and cardiomyocytes that exhibit action potentials with utility for biosensors have been reported (*79*). In addition, neural progenitor cells that have the capacity to proliferate but are committed to a neural phenotype are also of interest (*80*). Mistry and colleagues demonstrated spontaneously active neuronal networks derived from hippocampal progenitors (*81*). Our group has conducted preliminary experiments demonstrating that rat neural precursor cells can differentiate into networks of neurons and glial cells with morphological and electrical properties similar to the primary-culture neuronal networks.

Although stem cells represent a renewable source of cells for biosensors, they do not overcome the issue of shelf life. Shipping live networks overnight by commercial carrier is viable for scientific laboratories but impractical for military field operations. Cryopreservation may provide increased shelf life for neuronal networks. Cryopreserved embryonic neurons in suspension have been reported to form intact neuronal networks once thawed (*82*). However, this approach requires 1–2 weeks for the thawed cells to re-form surface-adherent neuronal networks. To overcome this limitation, our group has explored the possibility of cryopreserving intact, attached neuronal networks in the hope that the directly thawed networks would retain their functional synaptic activity. We have demonstrated that a slow-freezing protocol that uses DMSO and trehalose as cryoprotectants and a collagen gel layer to stabilize the neuronal network has promise (*83*). When attached neuronal networks were frozen by this protocol and later thawed, cell viability was such that active recycling of synaptic vesicles was seen via the fluorescent dye FM-143. This initial work suggests that cryopreservation of intact neuronal networks may be an option for supplying cells to the field.

We envision more complex in vitro models for toxicity detection. Leveraging advances in microfluidics and cellular patterning (*84*), these biosensors would extend detection beyond neurotoxicants by representing multiple physiological systems. Shuler's group has developed a cell-culture analog device for toxicity and drug-testing applications (*85*). Consisting of multiple chambers to represent various organ systems with integrated fluidics, this microscale device allows transport, metabolism, and bioaccumulation to be simulated (*86*, *87*). A multiorgan approach may be essential for detecting toxicants for which the metabolized form is physiologically active.

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Thomas J. O'Shaughnessy is a research biologist at the Naval Research Laboratory. His research interests focus on cell-based biosensor applications and the use of neuronal networks as model systems to study toxicity and cellular interactions. Joseph J. Pancrazio is a program director at the National Institutes of Health. His research interests include neural engineering and neural prosthesis. Address correspondence about this article to O'Shaughnessy at Naval Research Laboratory, Code 6900, 4555 Overlook Ave., SW, Washington, DC 20740 (Thomas.oshaughnessy @nrl.navy.mil).

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