Coupling of neurons with biosensor devices for detection of the properties of neuronal populations

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The *in vitro* detection of the neural biophysical chemistry of populations of neurons is an important emerging area of research. This *critical review* describes the current methodologies, challenges and future prospects for this exciting field of research. There are different classes of techniques for the study of neuron-based systems. These include devices to measure inter-neuron contact and connectivity, microelectrodes for the determination of extracellular metabolic products, and sensors employed for the evaluation of complex neuron–small molecule interactions, toxicity, and mutagenicity of anti-tumor drugs.

Since the neuron is an electrogenic cell and a complex biological entity capable of effecting recognition, the main emphasis of this article will be placed on devices based on nerve-cell networks that are able to electrically detect neuron-active compounds and specific pharmacological activity. Such neuron-based devices can be used to measure numerous neurological events with a high degree of sensitivity. Examples include the influence of different neuro-active compounds on neuronal function, the effects of neurotransmitters and neuro-modulators, changes in membrane potential, transmission effects that influence the propagation of the action potential, and the manner through which neuro-chemicals can influence ion channels. Moreover, these devices posses promising potential for the testing and development of novel neuron-active drugs and fundamental neurological research to further the understanding of brain activity. The inner workings of the human mind remain largely unknown and the key to comprehending it may rely on how molecules can initiate and influence synchronous neural oscillations, and the phenomenon of resonance in neural cells. The knowledge acquired in such detailed investigations can lead to the future development of regenerative medicines, neurochips and biocomputers, intelligent prosthetic devices and new applications that integrate neurobiology with molecular electronics (69 references).

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Introduction

A biosensor is a device that consists of a biological component possessing a bio-recognition mechanism coupled to a physical transducer that is capable of the quantitative detection of a

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Pasquale Benvenuto graduated recently from the University of Toronto. In undergraduate school he worked on the label-free detection of biochemical events on surfaces by scanning Kelvin nanoprobe. Pasquale is now in graduate school at the same University. His research interests are in the behavior of various types of biological cell on surfaces, with a special focus on implantable devices.

Professor Thompson's research is centred on the fundamental aspects of molecular recognition at the solid–liquid interface, with particular emphasis on biosensor technology in diagnostics and drug discovery. He has published over 200 papers, 15 patents and 2 books, and has been invited to present more than 200 lectures. Thompson has received several awards for his work and in 1998 he was made a Fellow of the Royal Society of Canada. He is currently Scientific Editor of the Analyst.

specific analyte. The latter is usually a biological species such as a biomarker, although the technology is often considered to include non-biological moieties, particularly compounds of pharmaceutical significance. By far the vast majority of biosensing systems involve the use of biochemical components such as enzymes, peptides, antibodies, nucleic acids (both DNA and RNA) and membrane receptors as molecular recognition elements. However, recent times have seen the introduction of systems which incorporate whole living cells such as endothelial, cancer and bacterial cells.[1–4] The use of the entire living entity, either as a single cell or population of cells, offers unique possibilities for bio-detection, drug discovery and research in biology and medicine.

The neuron as a sensor component is of particular value because it already possesses receptors displaying specificity for exterior stimuli and signal transduction cascades that amplify signaling instigated by receptor–ligand binding events. Measurable cellular responses, such as the release of a wave of Ca^{2+} in response to a stimulus, and any resultant alteration in the membrane potential thereof can be detected. The various signaling pathways can act as specific and sensitive detection configurations with respect to the interaction of the cell with neurotransmitters, drugs, neurotoxins or viruses. The main advantage of neurons is that they, like all living cells, are self-sustaining machines. When provided with the appropriate nutrients and environmental conditions, they are capable of the continuous maintenance of their components. Moreover, neurons can perform as useful genetic tools for genetic bioassays due to their expression of reporter genes. They can also detect and convey information on the toxicity of unknown or unanticipated threats. In complex networks, neurons present emerging properties that are reflective of novel aspects of physics and biology at the micro- and nanoscales.

Cellular variability constitutes a substantial problem in neural biosensor research since even genotypically identical organisms posses functional differences due to the phenotypic variability associated with cellular microenvironments, protein expression and number of receptors. Even if the biological components of neuronal biosensors are relatively unpredictable, the calibration and engineering of a stable neuron–substrate interface will be essential for the development of neuronal biosensors that display adequate reproducibility. Lastly, past studies of neuronal cells were difficult to perform because they could not be produced in continuous cell lines. This problem has now been overcome through the use of immortalized embryonic cells modified with a cancer gene. This modification allows the embryonic cells to self replicate because the introduced gene disrupts the mitosis cycle that normally prevents the self-replication of neurons.[5] Despite the advantages gained by this modification, the altered physiological properties of cells resulting from genetic changes must be considered. The trade-off between normal function and cell variability must be carefully balanced for each particular application.

In this paper, we discuss existing analytical detection methods such as transistor and microelectrode arrays and ion-selective electrodes, and present new methods based on acoustic and Kelvin fields that are capable of monitoring complex biochemical information.

The neuron–substrate interface

An important issue with respect to the isolation and study of neuron populations is the nature of their interaction with the substrate employed to fabricate hybrid detection systems. It is mandatory to attach cells to a surface in a manner in which they can remain viable, sterile, functional and stable. The surface physical chemistry requirements for this purpose are presently the subject of intense research. Integration of neurons and neuron tissue with non-biological substrates, whether inorganic or organic in nature, requires special strategies to preserve the biological activity and complete function of the living moiety. Neurons are fastidious when it comes to the substrate upon which growth is successful. Attaching them to inorganic substrates requires modification of the surface. Furthermore, subsequent characterization of the surface chemistry and surface topography at sub-cellular dimensions is necessary.

While the importance of cell–surface interactions has been long recognized as an area of scientific and practical importance, only recently have efforts been directed at high-resolution surface patterning. In earlier work, various forms of surface roughening and coatings have been used with respect to dental and orthopedic implants. Recent years have seen increasing use of micro-fabrication and nano-fabrication technology in the study and practical applications of cell patterning.[6–8] While patterned substrates are primarily used for cell culturing, they are also being applied to experiments conducted *in vivo*, especially for the study of tissue rejection phenomenon associated with medical implants and the overall effectiveness of such structures. Conventional photolithography, contact printing and lift-off approaches, once used only for producing microelectronic devices, are now being employed for the complex chemical patterning of surfaces for a number of applications connected to cell attachment. Once again, we witness the merging of ideas from the microelectronics and biological systems areas.

Production of neuron populations on an underlying material can be achieved by trapping the neurons in porous materials such as those generated by polymeric matrices, and by both chemisorption and physisorption onto planar surfaces, although the use of the former may create barriers to diffusion that would hinder the transport of stimulants and analytes. With respect to inorganic materials, considerable attention has been given to surface modification in order to construct a supposedly more compatible interface. This has primarily involved the use of proteinaceous material coatings. For example, polylysine, bovine serum albumin, protein A, fibronectin, laminin and various antibodies have been patterned by microcontact printing for the construction of artificial neuron cell networks.[5,9–15] In a similar manner, cell-electrode coupling can be enhanced by placing proteins immediately adjacent to electrode sites in order to improve the recording of extracellular field potentials.[10] Among proteins, polylysine is a popular choice for surface modification and has been previously printed onto silicon oxide, silicon nitride, indium tin oxide and glass surfaces to control the stability and longevity of long-term cultures.[16–21] Neuron growth guidance necessitates a controlled surface chemistry consisting of hydrophobic and

hydrophilic regions achieved through the printing of octadecyltrichlorosilane (OTS) molecules onto silicon wafers and the treatment of the substrate with a solution of dirthylenetriamine (DETA), respectively. Such patterned surfaces have been employed successfully to culture astroglial cells, yielding guided attachment and sufficient spreading.[22–24]

Epoxysilane, which is known to form a dense, cross-linked multilayer film on surfaces, can be used for the covalent linking of protein molecules onto substrates to promote long-term cell growth and superior compliance.[25] Interestingly, the electrical impedance of the cell-coated electrodes prepared by the aforementioned chemistry remains low over long periods of time and thus does not affect the quality of the recorded electric signals, even in long term implantation.

The viability of neuron cells and cellular growth have also been studied on porous silicon, scratched glass, ultra-fine quartz gratings, quartz microgrooves[26] and on nanostructures such as multi-walled carbon nanotubes.[27] The unique properties of carbon nanotubes, such as their strength, electrical conductivity and chemical functionality can be used for the fabrication of nanometer-scale neural probes. Another form of carbon, C_{60}, which displays remarkable electrical, mechanical and optical properties, promotes significant adhesion and cellular activation on surfaces. With regard to silicon, neurons have shown a surprising preference for topographical structures, such as silicon pillars (pillar-patterned surfaces), as opposed to flat silicon surfaces. However, a discernable preference for the height of the pillars is not evident.[28–30] Parylene cages, developed as an alternative to neuron-wells, allow neuron cell bodies to be dissociated by their insertion into a cage.[31] Once incarcerated, the neurons are cultured and this results in the growth of neurites through channels and the formation of living networks. The fabrication process on either silicon or glass substrates incorporates electrodes into cages for the purposes of stimulating and recording action potentials. Integrated SU-8 clustering structures,[32,33] ion-selective electrodes for extracellular measurements of essential ions, micropipette-based ion-selective electrodes for intracellular measurements and microfluidics have all been added to substrates on micro-fabricated platforms.

There has been significant interest in placing populations of neuronal cells on substrates for various applications. However, much of the previous work has been primarily empirical in nature, done at the micro-scale level and reliant upon the assumption that cells can preferentially grow and function on a "like" material such as proteins. It is expected that future nano-scale research will yield a better understanding of the influence that physical and chemical properties of materials have on the interaction between living neurons and the types of substrates to which they are attached. This will not only enable optimized growth of such cells, but also allow studies of other important features such as the role played by surface coverage, density of cells, or alternatively, neuron dissociation.

Detection technologies

The transduction of neuronal output to solid-state signals at the molecular level remains to be well defined. The most significant problem lies in the interface between ionic conduction (in living cells) and electronic conduction (in solid-state sensing devices) since there is a large difference between the mobilities of the two different charge carriers. Detection strategies require the specific engineering of the interface in order to convert manifestations of neural responses such as action potential, membrane depolarization and Ca^{2+} flux into quantifiable and measurable signals.

There are several detection strategies that can be employed to interrogate cell function and response to stimuli. For instance, cells can be genetically modified to produce fluorescent reporter proteins detectable with optical methods, they can be grown on arrays of transistors and microelectrodes that respond to changes in the action potential, or cells can be cultured on acoustic wave devices that can detect changes in membrane and cytoplasm properties. Additional techniques for electrical detection are related to the possible culturing of neurons on gold, indium tin oxide, or chemically modified silicon substrates. Here, detection is based on alterations in dielectric permittivity and through the use of impedance spectroscopy. Finally, detection using a non-invasive Kelvin vibrational field that examines whole cells in an integrated manner will be also discussed in this review.

Detection using field-effect transistors

One representative type of neural biosensor developed by the Fromherz group is based on a silicon microstructure, an example of which is shown in Fig. 1. Miniaturized field-effect transistors (FET) monitor changes in the electrical potential of cells. Extensive work in this field has also been performed by other research groups.[34–37] Interfacing of individual nerve cells and silicon microstructures has been achieved through capacitive coupling of the cell membrane to field effect transistors operated with an open gate (Fig. 2). The silicon dioxide layer that covers the silicon is an electrochemical barrier, but at the same time induces a low signal-to-noise ratio. Through this barrier the coupling of ionic signals from a neuron to the electronic signal in the semiconductor takes place through electrical polarization.[38] The electric field across the membrane is created by neuron activity and this polarizes the silicon dioxide so that the electronic band structure of silicon and the source–drain current of the integrated transistor are affected. In a reciprocal fashion, the electric field that is generated across the silicon dioxide by a voltage applied to the transistor polarizes the membrane in such a way that the conformation of field-sensitive membrane proteins and voltage-gated ion-channels become affected. As a result, the applied field becomes an exterior stimulus for the cell.

The electrochemical mechanism responsible for signals originating from silicon-based devices is complex in terms of the characteristics of the neuron-substrate interface. Protein molecules that protrude from the cell membrane (integrins, glycocalix) are the first to be deposited on the silicon dioxide surface and mediate cell adhesion. These proteins keep the membrane at a certain distance from the substrate, forming a gap filled with the electrolyte. This conductive gap shields the electrical field and suppresses a direct mutual polarization of the silicon dioxide and the membrane. Despite this condition, currents along the gap induce a displacement current in the

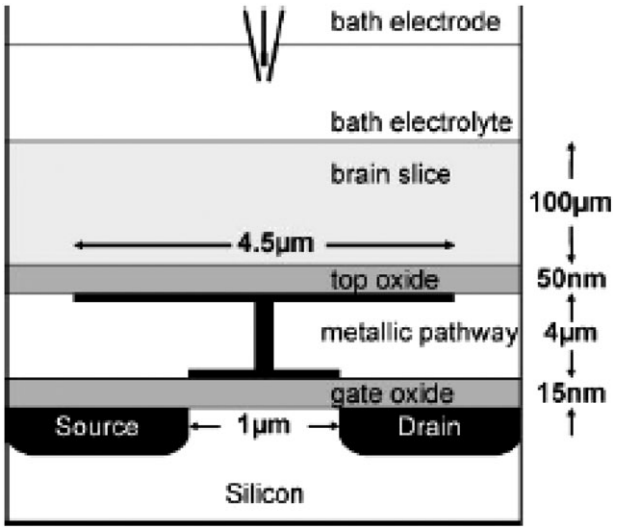

Fig. 1 Schematic of a measuring chamber in which neural tissue is exposed to the open gate of an integrated field transistor. (*Reprinted with kind permission of Elsevier*).

silicon dioxide dielectric that, in turn, influences the transductive extracellular potential arising from the activity of an excited neuron. The action potential drives an electrical current through the cell membrane and along the gap between the membrane and surface. The superposition of all the above capacitive and ionic currents induces the extracellular potential that modulates the band structure in the silicon. Detection or stimulation takes place at this very complex ionic-electronic interface.

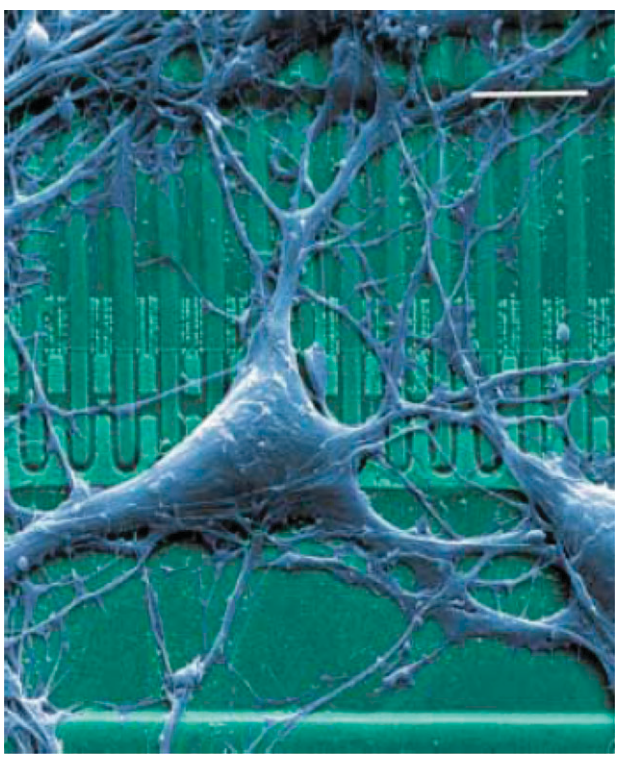

Fig. 2 Image of a neuron grown on an integrated field effect transistor illustrating the dimensional match between neurons and microelectronics. (*Reprinted with kind permission of Elsevier*).

Related to the model outlined above, it is remarkable to note that Hodgkin and Huxley[39] were the first researchers to empirically establish the ionic currents that flow through the channels of excitable membranes. They predicted the total membrane current through the following expression:

$$I_M = C_M \frac{dV_M}{dt} + I_{ionic} \quad (1)$$

where V_M is the transmembrane potential, C_M is the membrane capacitance per unit area, and I_{ionic} is the total ionic current through the cellular membrane. When neurons are cultured on an oxidized silicon surface, eqn (1) becomes:

$$\frac{V_J}{R_J} = C_M \frac{d(V_M - V_J)}{dt} + I_{ionic} \quad (2)$$

where V_J is the transductive extracellular potential, V_M is the transmembrane potential, and R_J is the electrical resistance in the gap. It is necessary to couple this treatment with theory connected to the operation of the silicon-based device in order to explain signals obtained from neurons. This is a complex exercise since the real behaviour of the ionic channels in the membrane cannot be simplistically reduced to that of plain electrical circuit components or sub-circuits. For example, an appropriate model should include sodium and potassium currents to account for complementary transmembrane processes. The current through each channel can be expressed as:

$$i_i = \alpha \cdot \bar{g}_i (V_M - E_i) \quad (3)$$

where g_i is the conductance of the ion i, E_i is its equilibrium potential and α is a dimensionless parameter that depends on the type of channel. In the Hodgkin and Huxley model, the potassium and sodium currents are connected to time-varying linear conductances with associated activation and inactivation parameters, while the so-called leakage current is referred to as a time-invariant conductance. Such assumptions lead to contradictions and "anomalous impedances".[40] To solve the impasse, the potassium activation parameter and the sodium activation and inactivation parameters are considered as proportional to the state voltage-variable of a linear capacitor[41] while the time-varying linear conductances for the sodium and potassium currents can be replaced and modeled by more complex sub-circuits.[42]

Detection using CMOS structures for nerve regeneration

A particularly interesting application for nerve regeneration involves the use of complementary metal-oxide-semiconductor (CMOS) structures that are the integrated circuits currently used in digital logic circuits and microprocessors. The complementary *p*-type and *n*-type metal-oxide-semiconductor field effect transistors (MOSFETs) have particularly high noise immunity, which is very attractive for measuring neuronal activity where noise is a major concern. This type of device has been used with some success in exciting studies of neuroplasticity.

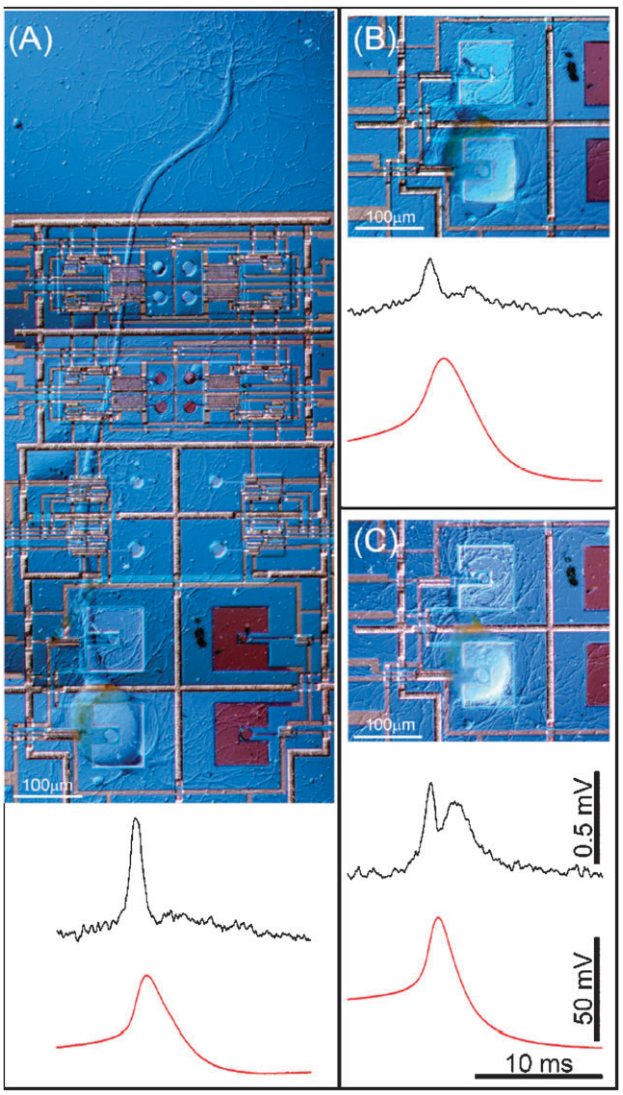

Fig. 3 Recorded potentials from a neuron cultured on a CMOS chip (A). After axotomy (B). 24 h later neurites are re-grown from the cut tip (C). (*Reprinted with kind permission of Elsevier*).

Scientists have long believed that humans lack the capacity to repair or replace damaged neurons in the brain or spinal cord. Recent research[43–45] shows that the human brain is capable of lifelong neuroplasticity and neurogenesis. Although neurogenesis is mainly restricted to the hippocampus, prefrontal cortex and olfactory bulb, neuroplasticity is a general property of the entire brain. The brain is flexible and plastic, continuously reorganizing and changing in structure and function by rewiring itself. New synapses are formed while others disconnect. Some dendrites grow longer and sprout new branches. Others are pruned away and thus making the brain a wonderfully adaptive system. Fig. 3 shows the recorded potential for an intact *Aplysia* neuron grown on a CMOS chip (A), after cutting away the axon (B) and following the re-growth of neurites from the cut tip (C).[34] This experiment demonstrated that the shape and amplitude of field potentials are not only proportional to the first derivative of the intracellular voltage as previously stated in the Fromherz model,[46] but also on the electro-anatomy of a particular neuron, which generates a particular field potential.

Such experiments could be easily adapted to the investigation of neuron regeneration and neuron plasticity in order to shed light on preconceived beliefs that brain cells are a fixed entity. Perhaps one of the most interesting recent findings is neurogenesis, the production of nerve cells from stem cells. The generation of neural stem cells happens deep within the brain where they subsequently migrate to become part of the brain's circuitry. Neuro-electronic devices have tremendous research potential in evaluating the best course of action for the use of stem cells in the repopulation or replacement of brain cells devastated by injury. There also exists the possibility to help patients suffering from strokes, Alzheimer's, Parkinson's, epilepsy, stress, leukemia or depression.

Microelectrode arrays

More than a decade of persistent work with field-effect transistors has shown that the response to action potentials can be detected as complex patterns of burst signals. However, the noise level is still too high and the amplitude of the extracellular recordings is small due to the high conductance of the junction. The small magnitude of this signal is further exacerbated in mammalian neurons due to their small size. Leech and snail neurons, and brain slices are much easier to investigate from this point of view, although there are different problems related to the recording of "natural populations" of neurons as they appear already organized in a brain slice. Since the preparation of brain slices causes an unavoidable area of damaged/dead cells on either side of the slice ($\sim$50 μm), even greater resistance is encountered relative to dissociated neurons randomly dispersed over and in direct contact with the electrodes. As shown in Fig. 4, a parallel strategy is based on interfacing neurons with microelectrode arrays (MEAs). Usually the array of photo-etched electrodes is placed in a special measurement chamber with ion-selective electrodes (pH, oxygen sensors *etc.*) that are connected to the back of the neural chip. The system includes reference and counter-electrodes and an instrument to conduct measurements. Spontaneous activity and induced action potentials can be detected extracellularly *via* the ion flux associated with

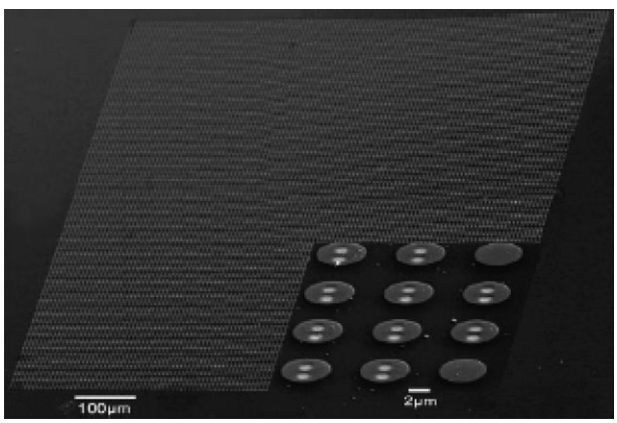

Fig. 4 Area of a manufactured array of microelectrodes. (*Reprinted with kind permission of Elsevier*).

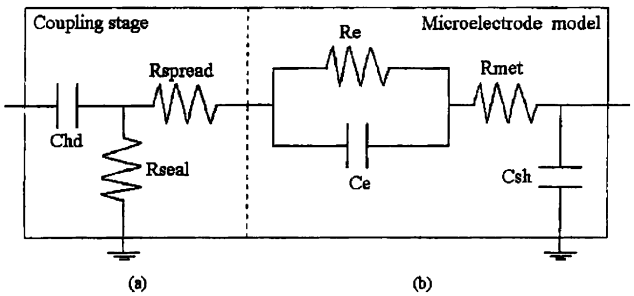

Fig. 5 The electrical model of the neuron (a)–microelectrode (b) interface. (*Reprinted by kind permission of the IEEE*).

membrane channel receptors. The principle of measurement is based on detecting the electrical capacity measured between a chemically-modified microelectrode and the neuron axon using AC-coupled amplifiers with high input impedance. The whole configuration is connected to multiple recording channels, operational amplifiers, filters and long-term data acquisition instrumentation with high resolution. The configuration also contains electrical stimulation systems and real-time signal visualization. Parameters such as the time of burst occurrence, duration between the first and last spikes, the interval between bursts and the burst amplitude are all recorded and subjected to subsequent analysis. A TV camera can be connected to an optical microscope positioned over the array. This method has already been developed and applied to the detection of neurotoxins and chemical agents.[33,47–53]

The interpretation of signals measured by arrays of microelectrodes is based on the complex neuron-microelectrode junction model presented in Fig. 5. The metal microelectrode has been first modeled by Robinson[54] and contains passive circuit elements. The metallic resistance, R_{met}, represents the resistance of the connecting paths, the C_e term models the capacitance of the microelectrode–electrolyte interface, R_l is the leakage resistance that takes into account the charge carriers crossing the double layer, and C_{sh} is the shunt capacitance which takes into account all the shunt capacitances to the ground. For the neuron component, R_{seal} is the sealing resistance between the cell and microelectrode and is related to the strength of attachment to the metal. This represents the cleft of electrolyte between the microelectrode surface and the neuronal membrane. R_{seal} is directly proportional to the resistivity of the electrolyte solution and surface overlapping, and inversely proportional to the distance between the microelectrode and the membrane. R_{spread} is the spreading resistance related to the signal loss due to the distance between the microelectrode and the neuron and acts perpendicular to the membrane. C_{hd} is the cell membrane–electrolyte capacitance corresponding to the polarization layers of the electrolyte solution near the cell and the microelectrode. C_{hd} is composed of the capacitance of the Helmholtz layer C_h and the Gouy–Chapman diffuse layer capacitance C_d of the classical double layer theory. Recent advancement in planar microelectrode arrays (PEA) in the form of active CMOS-integrated electrode arrays[32] opens new perspectives for the development of high-performance microelectrode arrays. The technology is enhancing the functional characteristics of MEAs using a larger number of electrodes and the integration of the amplification circuit, thus attaining a higher spatial resolution and a better signal-to-noise ratio. High-resolution active-pixel-sensor based microelectrode arrays (APS-MEA) exploit the APS technology, which is used in CMOS cameras for fluorescence and light-sensitive measurements. Such interfacing platforms offer new tools for studying the dynamic features of large assemblies of neurons and can thus lead to promising new perspectives for both fundamental and applied neurophysiology.

Extra-cellular signals recorded from nerve cell cultures are very sensitive to neuroactive compounds.[55] The latter are water-soluble molecules that can have direct metabolic effects which may increase, decrease or stop activity (cAMP, cyanide) and cause specific synaptic effects (neurotransmitters and neuromodulators, *e.g.* strychnine). Additionally, they can result in effects that stop the propagation of action potentials. For example, tetrodoxin blocks the Na^+ channel and oubain causes a similar effect on the sodium pump preventing the maintenance of the membrane potential. There are also generic membrane effects mediated through non-synaptic Ca^{2+}, or K^+ channels. Both neural biosensor systems presented above (cells cultured on field effect transistors or on microelectrodes) are able to detect the effects of such neurochemicals through the changes in membrane potential during an action potential that directly affects the gate of the field effect transistor, or the electrical capacity of a microelectrode.

There still remain challenges to overcome for the detection strategies presented so far. For instance, the low signal-to-noise ratio due to the resistance between the neurons and the field-effect transistor or microelectrode requires improvement in the acquisition and processing of multiple noisy signals. This can be possibly remedied through the use of low-noise transistors and CMOS technology.[56] With regard to optimizing neuron–electrode coupling, patterning with aminosilanes or adhesion proteins such as laminin or fibronectin can be employed.

Another major problem to consider is the fact that the number of total active microelectrodes is usually low. Fabrication of smaller electrodes will only worsen the signal-to-noise ratio. Alternative technologies such as CMOS and APS-MEAs that increase the number of measuring sites are currently under development. The data obtained from all the electrodes is usually overwhelmingly large and difficult to analyze. However, computational approaches for pattern-recognition in neural response and data mining strategies can be utilized to enhance the high specificity of neural receptor interaction without reducing sensitivity.[57] New bioinformatics and system biology methods to interpret, classify and link the measured data to neurophysiologic responses are also explored. Since single neurons possess the intrinsic ability to resonate and oscillate at particular frequencies, probing their activity at cellular levels using transistor and microelectrode arrays might reveal the mechanisms of generating patterns of significant neural signaling.

Light-addressable potentiometric sensors (LAPS)

These devices hybridize living cell-semiconductor substrate-coupled systems with optical interrogation. The LAP sensor

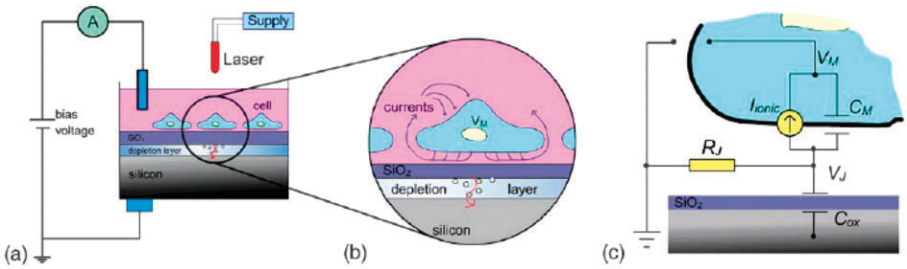

Fig. 6 Principle of a LAPS neuronal biosensor. (a) Schematic of the cell-based biosensor; (b) simplified cell–semiconductor interface; (c) circuit model. (*Reprinted with kind permission of Elsevier*).

detects the surface potential based on the following principle: a light pointer illuminates the semiconductor substrate, inducing a transition in the energy bands of the semiconductor due to the absorbed energy, producing electron-hole pairs. When polarized, the width of the depletion layer is a function of the local value of the surface potential. Neurons cultured on the surface of the device cause changes in potential through ionic currents generated by ions such as Na^+ and K^+. This induces fluctuations in the photocurrent which detects the changes in the extracellular potential (Fig. 6).[50] In a similar manner to the cell–transistor junction equation presented previously (eqn (2)), ionic and capacitive currents flow through the membrane when changes take place in the cell. The concomitant currents along the gap give rise to the potential between the cell and the chip, V_J, and this is equal to the change in the bias voltage of the LAPS. The transductive extracellular potential, V_J, represents the general extracellular potential detected by LAPS.

One application of the technique is the biomimetic "electronic nose" for odor detection where cultures of olfactory receptor neurons present on the surface of the LAPS sensor are monitored for patterns of extracellular potential as a fingerprint for a specific combination of odorants. The use of LAPS for continuous monitoring of cell metabolism through pH changes, cytotoxicity of chemotherapeutic drugs, and of activation of acetylcholine receptors has also been described in the literature.

The vibrational field and acoustic wave detection

This technique has its origins in the piezoelectric effect. In the thickness-shear mode acoustic wave device (TSM) a wafer of quartz, or other piezoelectric material is caused to vibrate at MHz frequencies by an oscillating electrical field imposed by gold electrodes.[59] The bulk acoustic wave travels through the quartz with very little dissipation and is reflected at the solid interface to maintain a standing wave. When operated as a chemical sensor or biosensor in the gas or liquid phase, receptor species can be attached to the electrode in order to ideally bind analyte molecules in a selective fashion. With respect to the gas phase environment, the thickness of the substrate and attached layer can be monitored as changes in the resonant frequency (f_s) of the standing wave where in the purely adsorptive limit a decrease in resonant frequency corresponds to an increase in the thickness of the attached layer. Until now this theory has led to the use of the term "quartz crystal microbalance" (QCM). However when this device is operated in the liquid phase, the pure "thickness" model becomes invalid under these conditions because many factors influence both the resonant frequency of the device and its level of energy dissipation. Important considerations for liquid matrices are acoustic coupling between the device and the medium, the electric double layer and the viscoelastic properties of the adsorbed layer. In addition to monitoring changes in the resonant frequency, it is also important to detect changes in energy dissipation. This is achieved through measurement of the motional resistance (R_m) of the device, which arises as a viscous damping force.

For experiments where neurons are attached to the surface of a TSM device, the acoustic wave will penetrate the interface with the cell by approximately 500 nm. Accordingly, instigated changes in the neuron result in an alteration of coupling at the membrane–sensor interface, and of signals associated with cytoplasm properties. These effects will be reflected in changes in both f_s and R_m. Fig. 7 illustrates the basic principle of the acoustic wave experiment for neurons (a N-38 neural cell line) cultivated on the surface of the Au electrodes of a TSM device operated at 9 MHz. The variations in acoustic signals (f_s and R_m) over time for a neuron population exposed to the drug betaferon are depicted in Fig. 8.[60] These plots of acoustic parameters illustrate the oscillation in signals that are then followed by a damping effect instigated by the drug (betaferon is known to have strong effects on neural tissue). The former result is interesting and likely connected to the synchronous behavior of neurons. The reasons behind this behavior currently remain obscure, although it is clear that an acoustic manifestation of changes in cellular physical properties is being observed. Whatever the nature of the oscillatory characteristics of the initial signal, perturbations in the signal are in fact evident after the introduction of the drug; the amplitude is drastically reduced while the period of the oscillation remains unchanged. For such sensitive cells, we have surprisingly observed in our experiments that the neurons are exceptionally adaptive in that they are tolerant to relatively deleterious conditions and will return to normal function when the right parameters are re-established. Normal metabolic activity is partially restored when neurons are re-exposed to a fresh medium following recovery from the influence of certain drugs and toxins.

The Kelvin field

The long history of this phenomenon dates back to the pioneering work of Lord Kelvin. He discovered that current

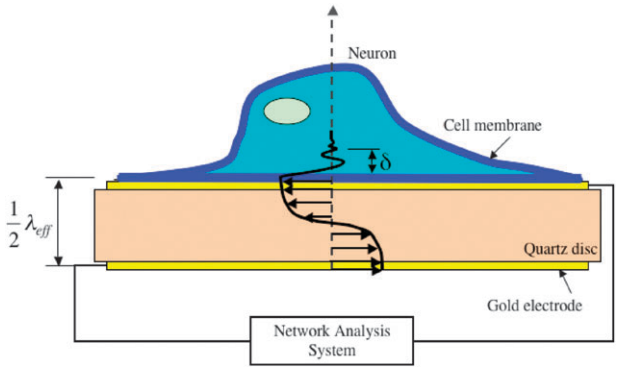

Fig. 7 Schematic representation of a single neuron on a TSM surface. (*Reprinted with kind permission of the Royal Society of Chemistry*).

could be caused to flow in an external circuit when two different metals are brought into close proximity and electrically connected. It is now known that this effect is associated with an equilibration between the two Fermi levels of the respective materials. This difference can be realized as a measurement of the difference in work function of the two substrates. Recently, the Kelvin technique has been placed in a scanning configuration by employing a tip as one of the Kelvin substrates.[61] This instrument has the capability to obtain both the work function and a topographical map of a surface with high spatial resolution (nanometric). Any sample subject to tip scanning can be imposed on a substrate consisting of various materials such as metals or semiconductors.

There is no definition of work function for complex biological molecules, or for living neurons. However, we have been able to detect extremely subtle events when applying the scanning Kelvin nanoprobe (SKN) to biomolecules. For instance, detection of mismatches in a an oligonucleotide strand at the level of single-base pairs has been achieved.[62] An entirely new physics based on the electronic states of biomolecules and their large-scale electrical and dielectric properties is currently emerging, which is a direct consequence of the high sensitivity exhibited by the SKN.[63] Relating the measured work function to biological bio-potentials through electron band theory may provide a detailed understanding of the transformation of chemical energy into biological energy.

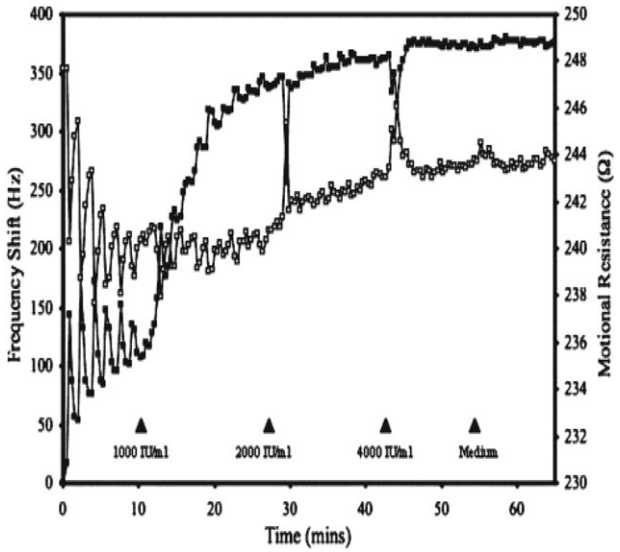

Fig. 8 Effect of neurotrophic factors (betaferon) on a neuron population immobilized on a TSM surface. Oscillations are detected both on the resonant frequency (dark squares) and motional resistance (open squares). (*Reprinted with kind permission of the Royal Society of Chemistry*).

In our experiments on neurons, the scanning Kelvin nanoprobe vibrates over a living neuron cell without contact and discerns any changes in the membrane and cytoplasm of the cell. This enables an integrative view of the whole neuron. The technique operates in an electrodeless manner, with detection being obtained non-invasively. Unlike the patch-clamp technique where intracellular recording is obtained by "impaling" the cell with a thin glass capillary, the Kelvin measurement does not destroy the neuron and is therefore able to continuously monitor signals before and after the addition of a drug or other chemical stimulant.

The SKN can perform both scanning and localized real-time measurements that reveal charge and dipolar redistribution at the surface of a neuron, or structural changes in its cell membrane. Fig. 9 illustrates the application of the SKN to surface-attached neurons. The probe vibrates with a high frequency at a distance d_0 from the neurons and with small

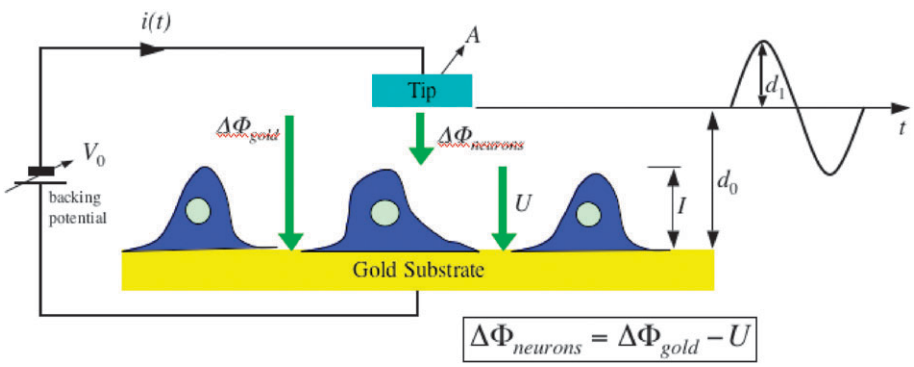

Fig. 9 Schematic representation of the SKN detection principle for a neuron cell culture. The tip vibrates at a distance d_0, with an amplitude d_1 over the cells which are grown on a gold substrate. The presence of the neurons alters the gold work function by a potential difference reflecting the dielectric characteristics of the cell membrane and cytoplasm. The detected Kelvin current, $i(t)$, is very sensitive to any change in the metabolic state of the neurons. (*Reprinted with kind permission of the Royal Society of Chemistry*).

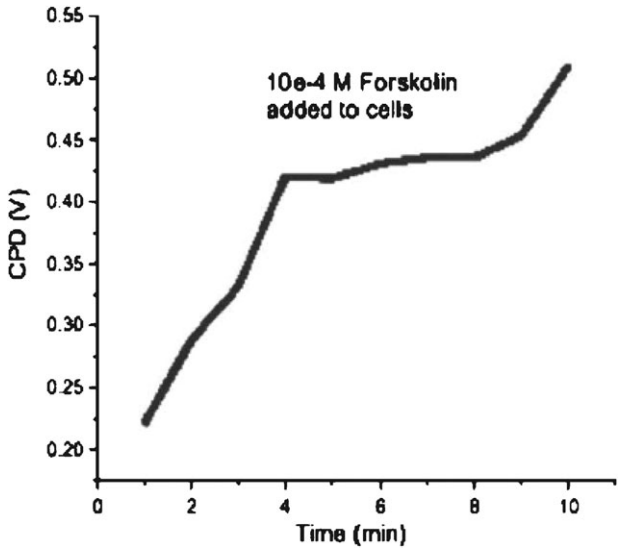

Fig. 10 Change in the work function through time over the surface of a neuron during the addition of 10e−4 M of forskolin. (*Reprinted with kind permission of the Royal Society of Chemistry*).

amplitude, d_1. The neurons, which are grown on a gold surface, alter the work function of the metal by their presence *via* dielectric properties. The Kelvin current is extremely sensitive to any variation in the properties of neurons. Biochemical cascades arising from intracellular mechanisms and ionic influx can be directly observed under the SKN probe. For example, Fig. 10 illustrates the effect of forskolin (a neuron stimulant acting at the cell receptor level) on the work function values measured on a specific area of a neuron.[60]

When forskolin was added at the critical concentration of 10^{-4} M, a significant change of 300 mV in electrical contact potential was detected by the SKN. This result interestingly correlates with observations from a classical immunochemical ELISA assay for cAMP accumulation.[60] For the same neuron line, a dramatic 850% variation in cAMP levels was detected. This change only occurs at the 10^{-4} M threshold concentration of forskolin. It is worthy to note that reproducible results were obtained with different cultures. This is in contrast to the results from the behaviour of random populations cultured on microelectrode arrays. The likely reason for this lies in the fact that the signal is not emanating from a large number of electrodes that suffer from high noise, or from electrodes, which may or may not be in contact with the surface of a neuron.

Such experiments illustrate the potential application of the SKN detection strategy for the evaluation of the efficacy, and/or toxicity of pharmaceuticals. However, the realization of such an application relies on developing an understanding of the physiological and biological relevance behind the measurements of neuron cultures.

Oscillations and neural resonance

In order to understand the origins of neuron activity sensed by different types of neural biosensors, a discussion of the important underlying physiological mechanisms is required. Since Berger recorded the first alpha brain rhythm (of 8–12 Hz) in 1929,[64,65] electroencephalographic patterns have been studied intensively during wake and sleep intervals. When oscillatory activity was also later recorded in anesthetic or epileptic states, where loss of consciousness is expected, the interest in associating brain oscillations to complex cognitive operations diminished. However, recent observations that single neurons can oscillate and resonate at specific frequencies within neural networks have renewed interest in oscillatory explanations of cognition. Moreover, oscillatory patterns during sleep can be related to those occurring during a previous wake period, albeit at smaller amplitudes.[58] This introduces the possibility that perception, temporal representation and short and long-term consolidation of information might be the result of synchronized network activity that spans over five orders of magnitude in frequency. Interestingly, the different classes of measured brain rhythms and their related patterns of behaviour are being phylogenetically preserved throughout mammalian evolution. This may support the proposition that there is a universal mechanism inherent to the brains of separate species.

Oscillations and oscillatory synchronicity are the most energy efficient mechanisms through which information is conveyed. The non-dissipating solitary wave is an example of this. How the brain stores patterns and generates creativity using little energy may be explained by such an energy-effective mechanism.

In general, brain oscillations appear to be the result of an optimized interplay between intense intracellular activity and the dynamic properties of neural networks and circuits. Neuronal networks and circuits behave surprisingly like miniature electrical oscillating circuits in which the membrane capacity and the conductance of ionic channels decide the resonant behavior. Rhythmic oscillations are a basic feature of membrane potentials found in spontaneously active neuronal cells or neuron networks. These cells or networks generate the patterns responsible for walking, breathing, chewing and other rhythmic movements. There are several mechanisms through which oscillatory activity can be produced. These include interactions among ion channels, inhibitory interactions among neurons in cyclic networks, cascades of metabolic reactions, and/or cyclic transcriptions of genes. It should be noted that the aforementioned mechanisms each operate with different time periods. For example, one such mechanism is established by the action of the hyperpolarisation-activated current, a cationic current critical for the neuronal pacemaker activity. It is a slowly developing inward current (depolarizing) activated by hyperpolarisation of the membrane beyond the resting potential and produced by a mixed Na^+/K^+ conductance. The time constant of current activation varies from 1–2 s at close to rest potential, to 100–400 ms at maximum hyperpolarisation. An increase in intracellular Ca^{2+} regulates the current such that it operates at more depolarized membrane potentials.[66]

Another example of the molecular underpinnings of oscillatory behaviour is related to the action of a specific glutamatergic receptor. In pacemaker neurons the NMDA (*N*-methyl-D-aspartic acid) glutamate receptor generates the pacemaker rhythms and provides a mechanism for synaptic plasticity. NMDA is an amino acid derivative that acts as a specific

agonist to the NMDA receptor, and therefore mimics the action of glutamate on that receptor. In contrast to glutamate, NMDA binds to and regulates the above receptor only and does not bind to any other glutamate receptors. NMDA is a water-soluble synthetic substance that is not normally found in biological tissue. First synthesized in the 1960's, NMDA is an excitocin which has applications in behavioral neuroscience research. Researchers apply NMDA to specific regions of an animal brain or spinal cord and subsequently test for aberrant behavior. If behavior is compromised, it suggests that the destroyed tissue was part of a brain region that made an important contribution to the normal expression of that particular behavior.[67]

The cation pore of the NMDA receptor allows the diffusion of both Ca^{2+} and Na^{+}, however these processes are blocked by the presence of Mg^{2+}. When the membrane is strongly depolarized, Mg^{2+} is cleared away and Ca^{2+} and Na^{+} are allowed to diffuse through the membrane. As a result of the Ca^{2+} influx, intracellular Ca^{2+} or Mg^{2+} activates a Ca^{2+} dependent K^{+} current that eventually hyperpolarizes the membrane when there is a sufficient build up of the K^{+} efflux. Membrane repolarisation closes the Ca^{+} channel. The K^{+} current gradually decays as Ca^{2+} is pumped out of the cytoplasm. Provided that the neuron is receiving tonic excitatory input from some glutamatergic source, the NMDA receptor will reopen after the hyperpolarisation has dissipated. The NMDA receptor transforms a steady excitatory drive into an alternating rhythm of bursts of spikes separated by silent periods of hyperpolarisation, a very useful feature of pattern generators. The rhythm of NMDA oscillators is in the range of 0.1–3 Hz.[68]

Simple pacemaker neurons cannot produce complex rhythmic patterns of activity. Synchronized oscillations can be created either by neurons sharing, or distributing the timing function among themselves through mutually exciting or inhibiting one another *via* synaptic connections. When large numbers of neurons are involved and phase relationship requisites are met, then the cyclic behaviour is best produced by an interactive network of neurons, the network oscillators. Breathing, locomotion and other rhythms are generated by reticular networks that allow for changes in relative timing, and in phase magnitude with respect to the other constituent neurons of that particular network. The basic design of an oscillatory network involves a diffuse excitatory input from an extraneous source so that the discharge is automatic when neurons are not being inhibited. Such inhibitory connections among the network of neurons determines the order and timing of the activity patterns. Oscillating networks generally follow a ring structure with directed excitatory and inhibitory interconnections. Each node or group of neurons functions as a burst generator that drives a particular phase of the movement cycle, and tends to inhibit its immediate predecessors. It is the retrograde inhibitions that are the most important and constant feature of the ring. A ring cycles freely in the direction counter to the inhibitory connections. Because the entire network is subjected to diffuse excitation, firing in any node is mainly due to the spontaneous depolarisation following the removal of the inhibition. The rate of cycling is therefore dependent on the strength and duration of the inhibitory effects. The stronger and longer-lasting the inhibition, then the slower the cycle frequency.

The coupling of individual oscillators can occur in several ways. Phase-coupling entrains the start of a cycle in one oscillator to a specific phase of another so that a traveling wave of activity will pass rhythmically. Relaxation coupling is the manner in which a group of oscillators become abruptly synchronized. Their individual cycles are concurrently reset by the simultaneous inhibition of all the individual oscillators. When the inhibition subsides, all of the oscillators resume their synchronous discharge. This is the mechanism responsible for synchronizing populations of thalamic neurons that project to the cerebral cortex. Synaptic connections between excitatory and inhibitory thalamic neurons force each individual neuron to conform to the rhythm of the group. These coordinated rhythms are then passed to the cortex by the thalamocortical axons which excite cortical neurons. This is how a small group of centralized thalamic cells can impose synchronicity to a much larger group of cortical cells.

Membrane potential oscillations are always entrained throughout populations that are joined by gap junctions. Such oscillations occur in many neurons at sub-threshold levels, meaning that action potentials need not be triggered to maintain the oscillation. The origin of this background oscillation implies a fast persistent Na^{+} current and a slow non-inactivating K^{+} current. The kinetics of the latter establishes the frequency of the oscillation. The Na^{+} channel is a different one than that involved in action potentials. It opens at sub-threshold potentials (-60 to -65 mV) and closes only when the membrane hyperpolarizes as a result of the ensuing depolarisation-activated K^{+} current. The background level of membrane depolarisation determines the strength of the Na^{+} current and the frequency of the oscillation. The increasing depolarisation increases the Na^{+} current which, in turn, increases the frequency up to a maximum of approximately 20 Hz.

Synchronized neuronal assemblies in the cerebral cortex generate an oscillating membrane potential that may be part of the basis of EEG rhythms (such as the 6–12 Hz alpha rhythm). Ion channel oscillations, membrane capacitance and resistance determine resonance behavior at a preferred frequency. The resonance of cortical neurons ensures that they respond to inputs arriving at the preferred frequency and without any time delays. They are effectively tuned to respond to specific dynamic rhythms. Such models as the ones described above might explain the oscillatory and resonant behavior of neurons, and possibly the effects of melotherapy and an individual's musical preference. However, all the molecular dynamics that produce resonance phenomena are still unknown. At another scale, oscillations detected from neurons cultivated outside the brain on biosensing surfaces which have been observed to form dendrites, connect with one another, and initiate synchronous oscillation as shown in Fig. 8. This raises additional interesting questions concerning the possible relation of certain brain rhythm frequencies to particular behaviors. For example, magnetic transcranial stimulation experiments reveal that frequencies less than 1 Hz induce brain inhibition, whereas over 1 Hz they induce excitation. Brain activity at 1 Hz frequency generally corresponds to deep meditation and hypnotic phases, both of which

are the only non-destructive methods for mind relaxation known today. Relaxation takes place by coordination and deep synchronization of different brain areas. A possible hypothesis is that these synchronous oscillations are somehow hardwired in the structure of cells and can consequently be manifested in brain rhythms and thus determine human behaviour.

Applications of neural biosensors

There are a number of significant potential applications of the neuron-based devices discussed in this review. Combining the pattern-based computation of artificial neural networks with the capabilities of *natural* neural networks (networks using biological neurons and their natural processing of input signals) in an environment that exploits the high-speed signal processing and memory capabilities of microelectronic and MEMS technologies can lead to a new generation of hybrid devices. Numerous possibilities can emerge from this partnership. These range from the fundamental study of the behavior of brain neurons in two- and even three-dimensional network structures, to medical diagnostics, nanomedicine and drug discovery.

A number of experimental drugs are thought to be effective in the treatment of neurodegenerative conditions such as dementia, Alzheimer's and Parkinson's. However, the action of these drugs remains largely unknown and finding the correct drug and dosage often relies on trial and error. These diseases progress slowly and are therefore difficult to detect, and the efficacy of the particular treatment depends on the individual patient. Moreover, many of the pharmaceutical treatments prescribed for these neurodegenerative conditions cannot traverse the blood–brain barrier and thus suffer in terms of uptake by the brain. There is no therapy available that reverses or cures these diseases. Treating the symptoms and curbing further degeneration is often all that can be currently achieved. It is expected that the devices outlined here can be employed to assess the efficacy and toxicity of candidate pharmaceuticals for these neurodegenerative diseases. Regenerative medicine holds the remarkable promise to not only treat symptoms, but to also restore neural functionality. The challenge is to convert this from conception to reality and neural biosensors may serve as a tool to assist in this goal. Fig. 11 depicts a possible pharmacological application of such a single neuron based device. As shown, an analyte interacts with a receptor located in the neuron wall. Subsequent to the interaction, an ion influx results from a signal cascade involving cyclo-adenosine monophosphate (*cAMP*). Localized release of chemical messengers (*e.g.* dopamine) can be also detected by specialized sensors. A silicon-based device that is located inside the substrate measures changes in the electric potential of the cell. Alternatively, the cell can be immobilized over a microelectrode or nanoelectrode array with integrated semiconductor structures. In the latter configuration, the cell may be stimulated by microelectrodes and the response can be detected by a second set of microelectrodes (the gate length in microelectronic devices has presently reached 1–2 nm). The immobilized cell may contain a fluorescence indicator that can be activated by the ion influx, or the activation can be initiated directly by a signaling cascade. A laser can excite the indicator and the emitted fluorescence can be detected and quantified. Calcium, potassium and sodium-selective electrodes and micropipettes can concomitantly measure the extracellular and intracellular concentration of significant compounds. The whole platform can be superposed over a microfluidic device that delivers minute amounts of the drug being investigated. A non-invasive Kelvin nanoprobe can vibrate over the whole structure and record the changes taking place in a localized area of the membrane.

Such devices may be also useful for applications in cell biology and stem cell research. Recent findings have shown that stem cells can now be controlled *in vivo* to de-differentiate, or differentiate into many different types of cells such as neuronal, precursor and sensory cells. Furthermore, the associated protocols no longer rely on embryonic sources and are thus free from moral and ethical scrutiny. Some major disorders of the central nervous system could benefit from safe and affordable therapeutic strategies to regenerate tissue,

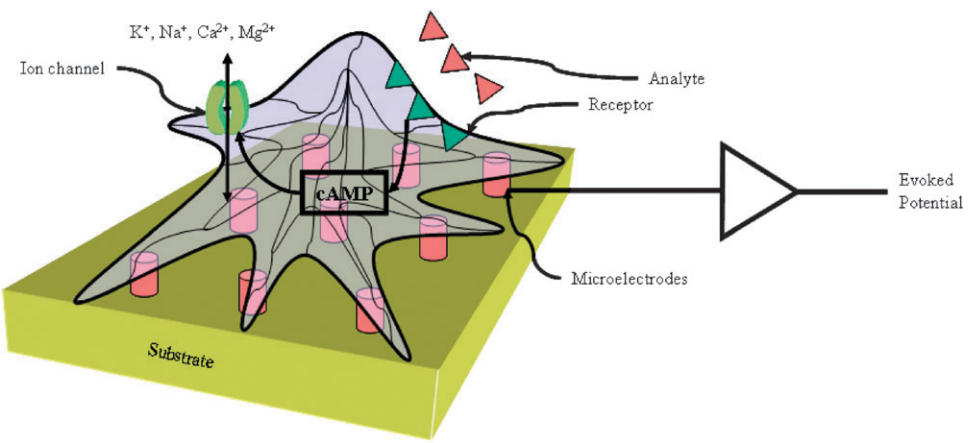

Fig. 11 Possible applications of complex neural biosensors in pharmacology. An analyte (triangles) interacts with a receptor located in the neuron wall. After interaction, an ion influx is caused by a signal cascade involving cyclo-adenosine monophosphate (*cAMP*). Changes in the electric potential of the cell are measured by a silicon-based device that is located inside the substrate. The cell may be stimulated by micro-electrodes and the response can be detected by another set of microelectrodes. Ion-selective electrodes detect the ion flux and ion channel activity is thus measured.

especially if the conditions are related to the malfunctioning of specific types of cells or the reduction of secreted neurotransmitters. A strategy for the treatment of advanced stages of these diseases would consist of regenerating the cells that secrete the proteins, metabolites and protective factors that keep the surrounding tissue functional. All this early research will surely require at least partial development of *in vitro* complex neural biosensing platforms.

Parallel to the applications in neurobiology and medicine, the possible employment of biological entities in the fabrication of devices capable of computer-like processing has long captured the imagination of scientists and the public at large. Integrating living neurons with microelectronics could lead to the opening of a new era in neuro-processing and information technology. The first step in this endeavor is to overcome the great challenges presented by the interface between the ionics of neurons and the electronics of solid state devices. The second step is to understand the mechanisms that govern the behavior of such complex biological systems so that their efficiency, plasticity and adaptability can be mimicked and used in well-designed and stable applications. Integrated systems are obtained by the growth of natural neuronal networks on the surface of the silicon chip by implementing electrical circuitry and two-way interfacing of the neuronal and electronic components. The ultimate goal would be a direct connection between the human brain and computers, which ideally will directly use the visual and motor cortex and thus removing the necessity for displays and keyboards. Until neuron-based processors become feasible, prosthetic structures (chips integrated in brains as opposed to brain tissue integrated in a computer) are a much more attainable goal. The cochlear implant is an example of a successful neuroprosthetic device that is currently being used in medical therapy. These implants electrically stimulate acoustic sensory neurons to restore lost hearing function. Retinal implants reportedly offer a partial restoration of vision in formerly blind patients who lost their vision due to the destruction of the photoreceptors in their retinas. In the study reported by Zrenner,[69] the still intact nerve terminations are stimulated with an array of 1500 microelectrodes and corresponding transistors. Implantable deep-brain stimulating electrodes are already used to alleviate symptoms of severe depression in patients who were otherwise resistant to standard types of treatment. Brain implantable devices which release a specific drug over an extended period of time, or that electrically stimulate regions of the brain are thus conceivable. Miniaturization and biocompatibility represent challenges for implantable neuro-devices. However, microtechnology may be able to effectively address these challenges in the future.

Lastly, another related and unusual potential application is the use of neural devices in virtual reality. In this setting, muscular and nervous inputs from an individual in the "real" world can be translated to motor manifestations in the virtual environment. Beyond the promising commercial prospective, such advances could result in improved interfaces in a severed nerve or in the retinal implants discussed above. Transistor-like bio-controllers connected to each nerve could provide the missing inputs for walking or visual orientation, providing a direct link between the nervous system and an implantable information processing device.

Final remarks

Integration of biology and microtechnology offers tremendous opportunities and represents an enormous potential for the future. Before living neuronal sensors can become commercially viable, critical issues concerning the understanding of complex neurobiological responses and the difficulties associated with the bioelectronic interface and transduction schemes must be unraveled. Until then, learning to communicate with single neurons or with intricate neuron networks will be the first step in understanding complex biological systems.

Neuron-based sensing requires detection of subtle phenomena that occur at the micro- and nanometre scales. Innovations in micro and nanotechnology fabrication, materials, microfluidics, microcontrollers and signal processing circuitry, as well as the development of a new microscale physics are all necessary for the development of new generation devices such as neurochips, invasive nanoscale probes, lab-on-a-chip or micro-analysis systems for biomarker detection.

Bioelectronics and biosensors represent multidisciplinary fields of study. As in any highly interdisciplinary scientific domain, new developments and future progress in integrating microtechnology and biology strongly depends on the collaboration between researchers from different disciplines. A key feature of such collaborative work is the ability of individuals to communicate and cooperate in endeavors that further the progression of this area. We are presently in the doorway of a new and spectacular field of research.

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