



# Biochips and other microtechnologies for physiomics

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This paper presents a review of microtechnologies relevant to applications in cellular physiology, including biochips, electrochemical sensors and optrode sensing techniques. Microelectrodes have been the main tools for measuring cellular electrophysiology, oxygen, nitric oxide, neurotransmitters, pH and various ions. Optical fiber sensing methods, such as indicator-based optrodes, with fluorescence lifetime measurement, are now emerging as viable alternatives to electroanalytical chemistry. These new optrode techniques are possible because of recent advances in the optoelectronics industry and are comparably easier to miniaturize, have faster response times, do not consume the analyte and have lower operational costs. This review serves as a summary and predicts future trends for both electrochemical and optical luminescence lifetime sensing as components in lab-on-a-chip devices for physiological sensing.

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## Cellular physiology & challenges

Cell physiology includes the physiology of membrane transport, signaling, developmental polarity and metabolism, and is the key to curing many diseases including cancer. Overshadowed to some extent in the molecular biology era, researchers are now beginning to realize that the physiological integration of individual gene/protein activities is vital to the next era of advancement. For example, the cell plasma membrane comprises thousands of ionic pumps and channels necessary for the transfer of essential electrolytes and ions into and out of the cell, and various aspects of cellular signaling. Disorders in the function of these channels and pumps are the cause of many diseases, such as cystic fibrosis and central core storage disease. Similarly, malfunction in the glycolytic pathway or electron transport chain of a cell can lead to skeletal and muscle atrophy [1] or abnormal release of reactive oxygen species (ROS), disrupting control of apoptosis [2].

New approaches to studying cellular physiology would benefit basic biomedical research and pharmacological development. For example, better tools for monitoring the activity of the cell membrane, including the numerous Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup> and Ca<sup>2+</sup> channels, could be used

to study stress and drug responses, associated with many channelopathies. Also, a more in-depth observation of different signaling analytes and metabolites, such as oxygen, nitric oxide (NO), glucose, glutamate, ascorbate, dopamine and so on, both *in vitro* and *in vivo*, is necessary for the development of more efficient therapeutics. However, progress in this area has been hampered owing to a lack of technologies that can interface with a cell at the micro- and nanolevel. In this review, we will focus on some of the emerging electrochemical and optical microtechnologies that have the greatest potential of overcoming this barrier and bringing cellular physiology back to the forefront of biology.

## BioMEMS revolution

The recent decade has seen a major drive towards miniaturization of technology for performing sensing applications on a microscale. Research in the area of micro-electro-mechanical systems (MEMS), especially BioMEMS [3], which are targeted towards biological and biomedical applications, have enjoyed special attention. The advantages of miniaturization include reduced size, small sample volumes, multiple analyte detection,

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reduced analysis times and reduced reagents, used in devices that are highly uniform and composed of geometrically well-defined structures [4]. The integration of all these features on a single device, which can be as small as a penny, resulted in hundreds of lab-on-a-chip applications, also known as micro total analysis systems ( $\mu$ TAS).

The most popular method for fabricating these biosensors utilizes silicon as the building material. Various electrical, optical, microfluidic and structural features are then defined on silicon using manufacturing technology borrowed from the microelectronics industry. Recently, other cheaper and more disposable materials, such as polydimethylsiloxane (PDMS) and ceramics have been employed. Common sensing mechanisms employed by these 'biochip'-based devices include electrochemical or optical detection of analytes that are important in the context of cellular physiology. Along with biochip technology, other microtechnologies such as optical fibers that are in the order of a couple of microns in size, have also progressed. Some of the most significant advances in these technologies in the last 5 years are also discussed below.

#### Electrochemical BioMEMS for cellular physiology

##### *Patch clamp on a chip*

Since the invention of patch clamp technology by Neher and Sakmann, it has proved to be the most important breakthrough in ion channel research. Likewise, numerous attempts have been made to miniaturize, automate and parallelize this technology for high throughput. In the biochip method, instead of using a glass micropipette, micropores or pipettes are fabricated on a silicon, glass or polymer substrate. Micromachining technology enables the manufacture of multiple number of pores on a planar substrate enabling simultaneous multicellular recordings.

Matthews and Judy developed a microfabricated patch clamp device on a silicon substrate using deep reactive ion etching (DRIE) and anisotropic KOH etching, in which microfluidic channels were later formed on PDMS and integrated with the planar chip [5]. Patch site diameters ranging from 300 nm to 12  $\mu$ m were achieved and patch seals in excess of 1 G $\Omega$  were demonstrated on Chinese hamster ovary cells. The microfluidics were tested using human embryonic kidney cells and proved to be capable of driving them to patch clamp sites, subject to multiple media types. Pantoja *et al.* also fabricated a silicon-based planar patch clamp biochip with a pore size as small as 0.7  $\mu$ m [6]. PDMS was also used to form a holding chamber for the culture medium. Their device was able to achieve G $\Omega$  seals on Chinese hamster ovary (CHO)-K1 cells and RIN m5F cells. Two distinct potassium channel recordings were also demonstrated on HIT-T15 and RAW 264.7 cells. Pandey *et al.* also utilized the same fabricating processes, but applied analytical methods for electrical characterization of the biochip [7]. Their device also incorporates dielectrophoresis (DEP) electrodes, which adds the advantage of electrical manipulation and positioning of single cells over a patch site.

A new approach in patch clamp on a biochip technology is to use horizontal patch sites rather than vertical sites. Ionescu-Zanetti *et al.* fabricated a PDMS-based patch clamp array that has 12 horizontal channels, each of them acting as a single patch site [8]. Each channel is 3.1- $\mu$ m high and they are arranged with a gap of 20  $\mu$ m between them. The channels are in a reservoir, where the cells are introduced. The cells are pulled into the patch sites by applying a negative pressure on the channels. Successful recording of voltage-activated potassium channel Kv2.1 were performed on the chip and compared with traditional recordings. Lao *et al.* fabricated a similar device but with raised horizontal channels [9]. This provides a more natural deformation to the cells, preventing undue stress on them. Another very interesting application utilizing horizontal patch sites was developed by Seo *et al.* [10]. The biochip was fabricated entirely on PDMS using silicon and SU-8 patterns as molds for forming the channels. A total of 14 patch pipette channels are located along the periphery of a circular reservoir, each of which traps a single cell via suction. Attachment of HeLa cells and G $\Omega$  seal formation was demonstrated on the biochip. Many of these research-based planar patch clamp biochips have now been commercialized. Nanion Technologies GmbH (Munich, Germany) markets its automated Nano-Patch-Clamp<sup>®</sup> technology [11], while Axon Instruments PatchXpress Device uses planar glass biochips developed by Aviva Biosciences [101].

##### *Potentiometric biochips*

High-throughput, biochip-based patch clamp technologies are unquestionably driving ion channel research in an entirely new and promising direction. However, the incorporation of patch clamp technology into the MEMS format is still plagued with the same problems as its parent micropipette technique. First, it is invasive in that it injures the cell being studied. This limits the potential for subsequent analysis of the cells using other techniques. Perhaps the most serious limitation associated with patch clamping is the lack of specificity of the measurements. Since the technique measures electrical signals based on membrane potential (voltage) or membrane transport (current), it is not possible to directly determine which ion species is driving the measured electrical event. Therefore, the technique requires multiple ion-replacement control experiments to determine which ion is responsible for the measured electrical signal. This means that large numbers of parallel control experiments must be performed and this ultimately limits the throughput of this approach.

The self-referencing ion-selective electrode technology is the alternative approach to studying ion channel physiology. Developed originally by Kuhnreiter and Jaffe [12], it is perhaps equally important to the patch clamp technology. Various ionic species, including Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, NH<sub>3</sub><sup>+</sup> and H<sup>+</sup>, can be detected using this technology with unsurpassed selectivity. The basic technology involves an ion-selective membrane immobilized at the tip of a glass micropipette, which is electrically in contact with an Ag/AgCl electrode through a

salt bridge. The potential developed across the ion-selective membrane, because of an ionic concentration, is referenced with another Ag/AgCl electrode. Thus, the potential difference thus measured corresponds to the ionic concentration, based on the Nernst equation. Since the actual quantity being measured is a voltage, these sensors are known as potentiometric sensors.

As with the patch clamp technology, there is also a drive towards miniaturizing ion-selective electrode technology and integration on a biochip platform. This has resulted in the development of some very interesting applications in recent years. Wygladacz *et al.* reported on the development of a Na<sup>+</sup> selective biosensor based on the field effect transistor (FET) sensing mechanism [13]. Doped silicon was used as the substrate with a Ag/AgCl electrode as the gate of the FET on which the Na<sup>+</sup> ion selective membrane is coated with poly(2-hydroxyethyl methacrylate) (pHEMA) as an intermediate layer. Photopolymerizable membranes isodecylacrylate (IDA) and acrylonitrile (ACN), crosslinked with hexanediol diacrylate (HDDA), were examined, rather than the commonly used polyvinylchloride (PVC). Excellent Nernstian response was observed and the sensor had a lifetime of more than 8 months.

Hisamoto *et al.* demonstrated the fabrication of a multiple analyte detection biochip by integrating square microcapillaries into channels formed on a PDMS substrate [14]. Each capillary is filled with a different ion-selective membrane for sensing Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup>. The sensor array included a pH indicator, K<sup>+</sup> and Ca<sup>2+</sup> ion-selective microelectrodes. A solid-state potentiometric biosensor for simultaneous detection of pH, Na<sup>+</sup> and K<sup>+</sup> was also developed by Liao *et al.* on a PDMS substrate [15]. The detection system was integrated with a micropneumatic pump that can continuously drive fluids into the microchannel through sensors at flow rates ranging from 52.4 to 7.67  $\mu\text{L min}^{-1}$ . The sensor array microfluidic device demonstrated near-Nernstian responses with slopes of  $62.62 \pm 2.5 \text{ mV pH}^{-1}$ ,  $53.76 \pm 3 \text{ mV } -\log[\text{K}^+]^{-1}$  and  $25.77 \pm 2 \text{ mV } -\log[\text{Ca}^{2+}]^{-1}$  at  $25 \pm 5^\circ\text{C}$ , and a linear response within the pH range of 2–10, with potassium and calcium concentrations between 0.1 and  $10^{-6}$  M.

Another interesting approach was used by Basu *et al.* [16]. Their design consisted of an electrolyte–insulator–semiconductor capacitor (EISCAP) fabricated on silicon, that shows a shift in the measured C–V with changes in the pH of the electrolyte. Thus, tributyrin and urea, which form acidic and basic solutions in the presence of the enzymes lipase and urease respectively, can be detected by observing the pH. Purvis *et al.* developed a potentiometric immunosensor biochip 1 mm<sup>2</sup> in size by screen printing gold electrode on a polyethylene terephthalate (PET) substrate [17]. This potentiometric biosensor detects enzyme-labeled immunocomplexes formed at the polypyrrole coating on the screen-printed gold electrode. Detection is mediated by a secondary reaction that produces charged products. A shift in potential is measured at the sensor surface, caused by local changes in redox state,

pH and/or ionic strength. The magnitude of the difference in potential is related to the concentration of the formed receptor–target complex. Hepatitis B surface antigen, troponin I, digoxin and TNF assays were successfully performed with this sensor.

Another novel design was developed by Errachid *et al.* that integrates H<sup>+</sup> and K<sup>+</sup> ion-selective FET-based sensors and a temperature sensor on a needle-like microprobe made from a silicon substrate [18]. Guenat *et al.* fabricated a two-part ion-selective biochip that contains silicon nitride micropipettes formed on a silicon substrate with another glass layer bonded below it [19,20]. This layer contains platinum electrodes and microchannels. The microchannels serve as inlets through which Ca<sup>2+</sup>, K<sup>+</sup> and NH<sub>4</sub><sup>+</sup> selective membranes are filled. The biochip demonstrated Nernstian response and demonstrates promise for *in vitro* physiology applications.

In our lab, we are focusing on investigating cellular physiology at the single-cell level, both in animal and plant model systems. We recently reported on the development of a cell electrophysiology lab-on-a-chip (CEL-C) device for monitoring real-time Ca<sup>2+</sup> currents across developmentally polarized single cells [21,22]. This biochip is a logical extension of the self-referencing ion-selective electrode technology originally developed by Kuhlreiber and Jaffe [12]. The device is manufactured using state-of-the-art silicon microfabrication technology. The CEL-C biochip consists of 16 pores on a silicon substrate each having four Ag/AgCl electrodes, at the polar positions. A SU-8 layer (a UV crosslinked epoxy) is used to form an insulating layer over the electrodes, as well as an encapsulating well around each pore. Finally, the electrodes are coated with a Ca<sup>2+</sup> selective membrane to impart selectivity for Ca<sup>2+</sup>. The final configuration creates 16 measurement chambers, each capable of measuring Ca<sup>2+</sup> ionic activity at four different points around a single cell. The footprint of this BioMEMS component of the device is only 9 × 11 mm. Another innovation is the incorporation of the biochip with a high-density data acquisition system [23]. This allows for real-time data acquisition, analysis and manipulation, at the same time providing a platform that can be used by minimally trained individuals. Transcellular Ca<sup>2+</sup> currents associated with the development and growth of the cells were recorded in normal environments, as well as when subjected to channel and pump blockers, such as nifedipine and eosin yellow. The two most important benefits of the CEL-C biochip are the use of ion-specific sensors and noninvasive monitoring of ion channel activity in living cultured cells. This ensures that all the signals measured are devoid of misleading artifacts associated with disruption of the cell membrane in patch clamped cells. To the best of our knowledge, this device is the only one to successfully demonstrate noninvasive, long-term (in excess of 24 h) physiological recording capabilities on a biochip platform. Currently, we are furnishing the CEL-C biochip with integrated microfluidics and on-board heating systems for sustainable growth and physiological analysis of mammalian cells.

### Amperometric biochips

The basic concept underlying amperometric sensors involves oxidation or reduction of an electroactive species on the surface of a platinum, gold or carbon electrode. The electrode is usually polarized at a fixed voltage corresponding to the redox peak of the analyte in question. The current generated on the electrode surface as a result of the redox reaction is measured and corresponds to the analyte concentration. Inherently, electroactive species, such as ascorbate, dopamine and NO, can be directly detected on the sensor surface. Other analytes, such as glucose, lactate, glutamate, choline, acetyl choline and ATP, are not electroactive, but can be measured using enzyme-based biosensors. The most common are oxidase-based biosensors where the enzyme oxidizes the analyte and in the process creates an electroactive species, hydrogen peroxide, which is in turn oxidized on the electrode. The measured current is proportional to the original analyte concentration.

As in the case of potentiometric sensors, numerous attempts have also been made to create MEMS-based amperometric biosensors. These would enable researchers to measure concentrations of an analyte at the cellular level creating a better understanding of their role in cellular physiology. There have also been significant efforts to create MEMS-based implantable microelectrode arrays for *in vivo* cellular physiology, particularly in neurophysiology research. The most significant among them, in terms of potential for long-term, *in situ* monitoring and commercial viability, are also reviewed here.

### *In vitro* amperometric sensors

Various interesting applications on a biochip platform have been developed by different research groups for amperometric detection of physiologically relevant analytes. Chen *et al.* realized an array of 16 pyramidal wells on a silicon substrate each having a single gold electrode in it [24]. Each well was designed to hold a single chromaffin cell. PDMS was used as the insulating material and to form measurement chambers. Carbamylcholine-induced release of catecholamines was successfully detected on the electrodes. Cui *et al.* also developed a silicon-based chip with 25 gold disc electrodes each having a diameter of 30  $\mu\text{m}$  [25]. M9ND and PC12 cells were successfully cultured on chip and potassium-evoked dopamine release from the cells was detected amperometrically. Schoning *et al.* used a glass substrate to make an amperometric biochip with platinum electrodes [26]. Microfluidic channels for sample containment and capillary electrophoresis-based transport were formed in PDMS. The biochip was calibrated for dopamine and catecholamine and detection limits of 2 and 10  $\mu\text{M}$ , respectively, were achieved.

In another approach, Kovarik *et al.* designed a PDMS microfluidic device with 35- $\mu\text{m}$  wide and 12- $\mu\text{m}$  deep channels [27]. Carbon electrodes were micromolded into the channels and Nafion coated for increased selectivity. Catechol and dopamine detection was performed in a flow injection analysis setup. Roy *et al.* utilized the electrical properties of multiwalled carbon nanotubes (MWCNT) on a silicon-based biochip [28].

Vertical MWCNTs were grown on silicon on top of which cholesterol oxidase and horseradish peroxidase was immobilized for cholesterol detection. Two biochips that could have applications in studying mitochondrial respiration and associated diseases were developed by Krylov *et al.* [29] and Chang *et al.* [30]. The first group designed a ceramic-based biochip with screen-printed electrodes and integrated microfluidics for simultaneous detection of ROS and  $\text{H}_2\text{O}_2$ , using xanthine oxidase and superoxide dismutase. The second group employed a similar enzymatic configuration but used a transparent glass substrate. This allowed them to perform simultaneous intracellular and extracellular measurement of ROS in A172 glioblastoma cells using fluorescence and amperometry respectively on the same biochip.

Future amperometric biochips are geared towards the measurement of multiple physiological parameters on the same biochip. Popovtzer *et al.* fabricated a silicon-based biochip with platinum electrodes [31]. An eight-well array was formed from SU-8 (a UV crosslinked polymer), each well dedicated to a particular analyte measurement. Although actual multiple-analyte detection was not performed, physiological response of genetically engineered *Escherichia coli* to phenol was recorded on chip. Our research group is also working on the next generation of CEL-C devices, capable of performing multianalyte detection on single cells. Details will be reported in upcoming publications.

### *In vivo* amperometric sensors

The most popular type of amperometric sensors are the probe-type biosensors based on the Michigan probe design. These were first developed by KD Wise's group at Michigan, Ann Arbor, USA and have since advanced through numerous design modifications [32]. The basic technology consists of a silicon micromachined probe. Platinum is usually used as the electrode material. Use of microfabrication technology produces small probes used for studying neuronal physiology. The small size allows them to be minimally invasive so that actual physiological signals rather than injury currents are measured. Numerous advances have been made in this technology over the last 5 years. Johnson *et al.* developed a silicon probe with platinum-blackened electrodes, and Nafion coated for increased selectivity [33]. *In vivo*, neurophysiological measurements of dopamine were successfully performed on three rats. The sensor revealed 74% increased sensitivity of dopamine and 89% decreased sensitivity of other common interferents.

Greg Gerhardt's group at University of Kentucky, KY, USA, initially used Michigan probes for neurophysiological recordings, but faced problems related to electrode crosstalk and shunt capacitance. To overcome this problem, they used a ceramic substrate to develop what are now commonly known as Gerhardt probes. These represent the state of the art in neurophysiology sensors and have been extensively used for neurotransmitter recordings. Day *et al.* used these probes for amperometric measurements of  $\text{K}^+$ -stimulated glutamate release in rat hippocampus [34]. Similar configurations have also been used for measurement of choline [35], acetylcholine [36], L-lactate [37] and nitrous oxide [38] in rat brains *in vivo*. The sensors have

demonstrated sensitivity in the  $\mu\text{M}$  range and lifetimes in excess of 4 months *in vivo*. Significant research is being conducted to enhance the biocompatibility, lifetime and sensitivity of these sensors and they will definitely be at the forefront of *in vivo* cellular physiology in the near future.

#### Optical microtechnologies for physiological sensing

Developments in optical sensing methods provide alternative means for cellular physiologists to obtain information about bioactive molecules or ions inside cells. This area is based on the use of fluorophores as sensors, which have in the past been used and applied directly to cells for imaging-based detection. Potential pitfalls of direct fluorescence indicators include:

- Limited kinetics of the dye
- pH effects on the indicator  $K_d$
- Cytotoxicity of the dye and direct illumination
- The significant problem of analyte buffering by the dye

While there has been some work to account for and understand these artifacts based on analytical modeling [39], these issues will remain as limitations in the application of fluorophores for sensing. However, these limitations can be overcome by immobilizing the indicator dye into a solid-state sensor format where the immobilized dye is not free in the cell, the immobilization materials can be engineered to buffer the pH microenvironment for the indicator and indicator dyes with limited solubility in aqueous systems can be used.

#### Fiber optic sensors & opt(ro)des

An optical fiber sensor is called an optrode (optode), derived from the words optical and electrode. The combination of optical fiber techniques and fluorescence spectroscopy, together with new materials for indicators and sensor immobilization, has greatly contributed to the progress of optical chemical sensors in recent years [40–44].

Fiber optic sensors can be divided into direct sensors and indicator-based sensors [44]. Most of the applications of such sensors in biological sensing are indicator-based, where the luminescence of an immobilized indicator is monitored [45]. Fiber optic chemical sensors can be created for specific analytes by placing a chemically sensitive layer at the distal end of the fiber. The optrodes can be made from multimode silica glass optical fibers with a tip size of 10–20  $\mu\text{m}$  or a single-mode fiber with a submicron (0.1–1  $\mu\text{m}$ ) tip size. In a fiber optic nanobiosensor, after the photons have traveled as far down the fiber as possible, evanescent fields continue to travel through the remainder of the tip, and only species in extremely close proximity to the fiber tip can be excited, thus preventing excitation of interfering fluorophore species in the neighborhood [46].

In biomedical applications, the detection of chemical parameters by means of optical fibers has some advantages. Their high degree of miniaturization, considerable geometrical versatility and robustness, make it possible to continuously monitor numerous parameters (such as pH, oxygen and carbon dioxide

partial pressure, calcium, potassium and glucose). Compared with injecting fluorescent ion-sensitive dyes directly into a cell and using microscopy for detection, optrodes entrap the dyes within a solid-state matrix, thereby minimizing undesired interactions between the fluorescent dyes and the cells. The optrode immobilization matrix allows ions or neutral analyte species to diffuse through and interact with the indicator, but prevents mobility of the indicator dye. In addition, optrodes are capable of localized measurements, because only the area where the biosensor is located will contribute to the signal.

Micro-optrodes do not need a reference electrode as do electroanalytical sensors. In addition, fiber optic sensors have the advantages of intrinsic immunity against electromagnetic interference, increased sensitivity, fast response times, capacity of remote and *in situ* sensing, and relatively low cost [47–49]. Optrode-based biosensors have fast response times, for example, a few tenths of a second for oxygen micro-optrodes, compared with electrochemical methods with seconds to minutes of response time. Optrodes were implemented from a cellular physiology standpoint to measure important analytes, such as pH, oxygen, carbon dioxide, potassium, sodium, calcium, chloride, ammonia, urea and glucose [50–52]. Recently, optrodes have been tailored for investigation of more complex physiological events even at the single cell level.

One application is the use of an optrode to measure cellular toxicity response to heavy metals such as mercury [53]. Single *E. coli* cells were immobilized on the distal end of an optical fiber bundle. These *E. coli* cells were genetically modified containing the *lacZ* reporter gene fused to the heavy metal-responsive gene promoter *zntA*. To identify the location of cells, a plasmid carrying enhanced cyan fluorescent protein (ECFP) was also introduced. Fluorescent data were acquired by using a charge-coupled device (CCD) camera and a fluorescent microscope. Cells were located on the images using ECFP fluorescent signals and cellular response was measured by  $\beta$ -galactosidase substrate fluorescein di- $\beta$ -D-galactopyranoside (FDG). A unique characteristic of this biosensor is the use of a single cell response as an analytical signal. The biosensor integrates physiological measurements with molecular biology and this can open new doors to investigating the effects of molecular biology-induced changes at the genomic level.

Kasili *et al.* reported the application of optrodes to monitor the onset of the mitochondrial pathway of apoptosis in a single living cell by detecting the enzymatic activities of caspase-9 [54]. The tip diameter of the optrode is approximately 50 nm and smaller than the wavelength of light used for excitation. This leads to a diffraction-limited condition that does not allow photons from the laser beam to be transmitted through the tip of the optrode but rather allows energy to be transmitted in the form of an interfacial leaky surface mode, which is propagated as the evanescent field exciting molecules only at the periphery of the tip. This allows molecular interaction in the proximity of the optrode tip. Evanescent fields reduce exponentially with distance from the surface and approach zero at an approximate distance of 200 nm. The optrode tip is immobilized with

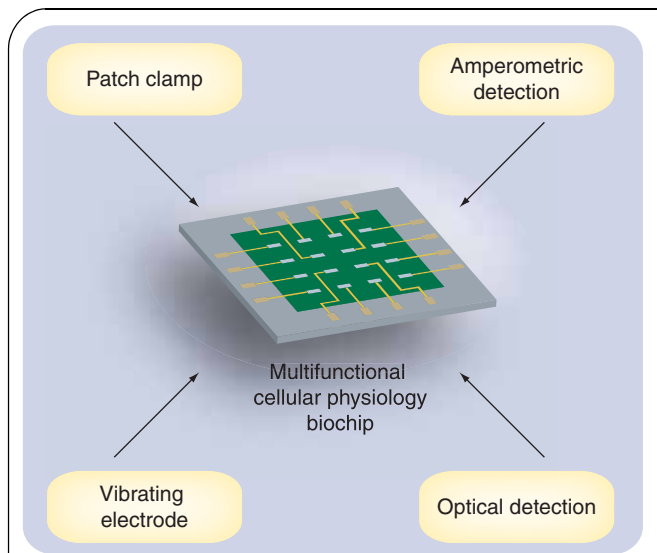


Figure 1. Future cellular physiology biochips will integrate various sensing methodologies on a single platform. Combined with microfluidics and electronic capabilities, insight into the role of every possible parameter in the physiology of a single cell will be possible.

leucine–glutamic acid–histidine–aspartic acid–7-amino-4-methylcoumarin (LEHD-AMC), which consists of a tetrapeptide, LEHD, coupled to a fluorescent molecule, AMC. LEHD-AMC exists as a nonfluorescent substrate prior to cleavage by caspase-9, and after cleavage, free AMC fluoresces when excited at 325 nm. The mitochondrial pathway for apoptosis was investigated under various conditions for single MCF-7 cells. By comparing the fluorescence signals from apoptotic cells induced by photodynamic treatment and nonapoptotic cells, caspase-9 activity was detected, which indicates the onset of apoptosis in the cells.

An investigation of the apoptotic pathway utilized optrodes for the measurement of intracellular cytochrome *c* in MCF-7 cells [55]. Apoptosis was induced in MCF-7 cells using  $\delta$ -aminolevulinic acid (ALA). Mouse anticcytochrome *c* was immobilized on the tip of the optrode. The optrode was moved into the cell cytoplasm and incubated for the antibody–antigen reaction to take place. After 5 min, the optrode was taken out of the cell and then enzyme-linked immunosorbent assay (ELISA) was performed on the optrode. Cytochrome *c* concentration was determined using fluorescence of cleaved enzymatic product *N*-dimethyl-dodecylamine (DDAO). Combination of ELISA and optrode measurements led to the detection of small quantities of cytochrome *c*.

Byars *et al.* also used optrode technology for multi-site optical recordings of cardiac membrane potentials [56]. The optrode consisted of a bundle of seven fibers, each with a diameter of 225  $\mu\text{m}$  arranged in a hexagonal pattern. The end of each fiber was immobilized with voltage-sensitive dye RH-237. Multichannel recordings were performed on rabbit hearts and step-back measurements were performed. The fluorescence signal reduced exponentially as the optrode was moved away from the experiment site.

Oxygen concentration measurement is one of the most important domains of cellular physiology. The biophysical oxygen demand of cells carries direct, relevant information regarding cellular life. In our laboratory at Purdue University, we developed the self-referencing optrode technology for oxygen concentration measurement [57]. Although self-referencing is a known technique for microelectrode-based measurements, this is the first time that it has been implemented for optrodes. Self-referencing optrode technology not only behaves as a dynamic oxygen flux sensor, but also reduces drift and noise commonly present in optrode measurements. The raw signals contain noise, drift and other artifacts, but stable measurements are obtained because they are subtracted in the self-referencing mode. We demonstrated the application of self-referencing optrode technology for measuring the effects of various electron transport chain inhibitors on rat tumor spheroids and plant cells. The optrode is immobilized with platinum porphyrin tetrakis (PtTFPP), which is an oxygen-sensitive dye. Fluorescence lifetime measurements are performed instead of fluorescent intensity. This also offers significant advantages; the performance of the optrode is not limited by the chemical fouling of the fluorophore, because fluorescence lifetime is a quantum mechanical property of the fluorophore. Fluorophores photobleach very rapidly making intensity-based measurements difficult for quantitative measurements. Lifetime-based sensing has several advantages over steady-state or intensity-based methods. Since the lifetime is independent of the total probe intensity, its measurement can provide quantitative sensing of many analytes without the requirement for wavelength-ratiometric probes [58]. It is possible to eliminate effects due to photodecomposition and small loss of fluorophores, since the lifetime is independent of the concentration of the fluorophore [59]. The measurement inaccuracy due to unsteadiness of the light source intensity, fluctuations in the light field, inhomogeneities of the dye concentration and photobleaching effects are strongly reduced [60]. Fluorescence lifetime-based sensing is not a new technique and has been applied in a variety of configurations to measure physiological analytes mentioned earlier [50–52,61,62]. In these configurations, however, the optrodes are static sensors. Incorporation of the self-referencing technique converts the optrode to a dynamic flux biosensor and at the same time reduces noise artifacts and drift present in the fluorescence lifetime static biosensors.

Kwok *et al.* also demonstrated the investigations of microbial oxygen demand in waste water samples using optrode technology [63]. Sample vials were coated with oxygen-sensitive dye tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) (Ru(dpp)) on the bottom. Microbial samples were added and then the vials were excited with light. The fluorescence intensity signal was recorded using optrodes. The optical signals were converted to electrical signals and quantified. Effects of temperature and pH were also investigated. Toxicity effects of heavy metals were studied. This work demonstrated the potential of optrode technology to be a high-throughput tool for waste water management.

### Probe encapsulated by biologically localized embedding

The basic probe encapsulated by biologically localized embedding (PEBBLE) nanosensors are composed of a spherical polymer matrix that encapsulates an analyte-sensitive fluorophore along with a reference dye. PEBBLEs can vary in size from 20 to 200 nm and can be synthesized using several different matrices, including polyacrylamide, sol gel and polydecyl methacrylate. The small size allows for measurements in domains where larger, micrometer-sized tips induce an unacceptable degree of physical perturbation. On the other hand, the volume that the PEBBLE itself takes up in any given cell is negligible (1 ppm or less) with respect to the volume of the cell itself; therefore, any physical perturbations to the cellular conditions of interest are minimized. The inert matrix serves to protect the fluorophore from the cellular environment and vice versa, also minimizing chemical perturbations to the cell. This dual protection allows for the use of potentially toxic fluorophores and concurrently eliminates any nonspecific binding to proteins, organelles and other intracellular species. In contrast, the use of toxic dyes is undesirable and incompatible with the primary aim of ionic measurements in viable cells. In addition, biological fluorescence intensities are often affected by nonspecific protein binding, thereby distorting the measurements being made. These problems are avoided by the use of PEBBLEs. Another advantage of the PEBBLE is the biocompatibility of the matrices used [64]. Kopelman's group at the University of Michigan has implemented PEBBLE-based imaging techniques for intracellular physiological measurements of  $Mg^{2+}$ , glucose,  $O_2$ ,  $Na^+$  and  $Fe^{2+}$  [65–68].

### Optical biochips

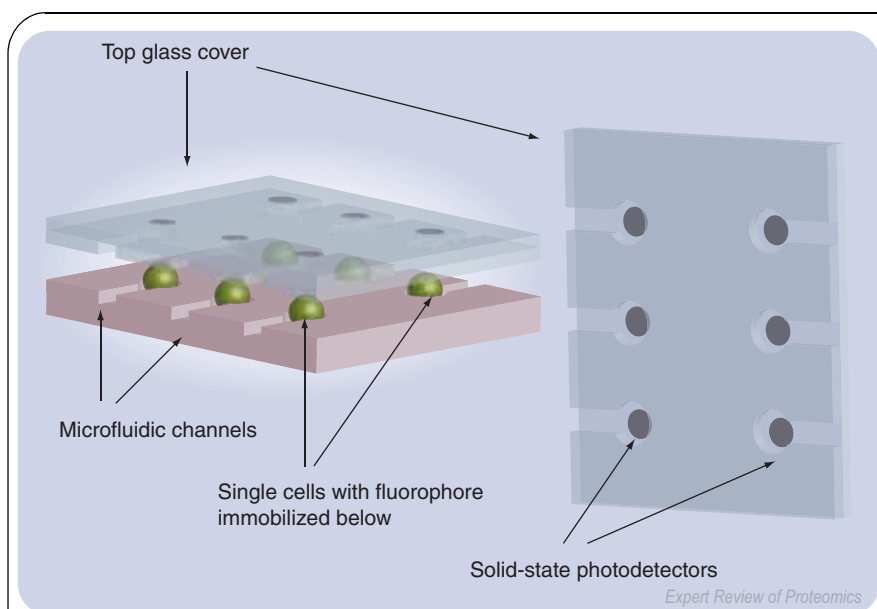
The field of fully developed and integrated biochips is still in its infancy. With the pregression and application of microfabrication processes to MEMS development, there have been various efforts to incorporate optical components on microchips. Microchips containing optical fibers or spin-on-glass waveguides have been manufactured [69–75]. Although silicon and PDMS have been the most common substrates, recently soda lime glass has been used to fabricate waveguides and couplers [76]. One aspect is common amongst all these developments; they still have to interface with macroscale optical components such as photomultiplier tubes (PMT), lock-in amplifiers and microscopes. In the future, miniaturization and high-level integration will lead to stand-alone microscale optical biochips.

### Integrating new biochip devices

To realize the full potential of physiological sensing, we envision three stages of development in the future: miniaturization,

integration and automation. Miniaturization is an important step because it enables the compact arrangement of experimental equipment leading to high-throughput systems for the complex realm of cellular physiology, drug discovery and proteomics. In some ways, miniaturization of bulky laboratory apparatus is more important than continued miniaturization of the sensors, because this is the area that limits the development of microsensor technologies to the true lab-on-a-chip or systems-on-a-chip level. Currently, we envision miniaturization proceeding in two different domains; mesoscale and the micro–nano scale. Some examples of mesoscale miniaturization are evident. Traditional bench-top lock-in amplifiers are being replaced by preprogrammed digital signal processors (DSP). Combinations of specialized analog circuit components are also being used to perform dedicated functions of lock-in amplifiers. Light-emitting diodes (LEDs) are replacing the expensive and sensitive arc lamps and lasers. CCD are also being used instead of PMT, providing better spatial resolution and high throughput. This not only reduces costs, but also aids in mass production; a step toward high-throughput systems.

Developments in microfabrication have led to the birth of micro- and nanofluidics. Microchannels and structures are fabricated on various substrates, such as silicon and PDMS, in array formats. Biomolecules are then immobilized onto the channel surfaces and biological reactions are characterized and studied via optical or electrical methods. However, miniaturization is stalled beyond this point because these microchannels



**Figure 2.** Design of a future optical cellular physiology lab-on-a-chip (OCPL-C) being developed in our lab. The biochip will consist of measurement chambers on a silicon substrate, in which a single cell can be placed. Fluorophores for sensing various physiological analytes will be immobilized on the base of the chambers. A top glass cover containing a solid-state photodetector array will serve as the top cover. Both the substrate and top cover will have microfluidic channels for transporting cell nutrient media providing on-chip lab culture capabilities. The chip will be illuminated with light-emitting diode (LED) light sources, tuned for individual fluorophores through the transparent glass cover. The fluorescent lifetime optical signal will then be detected by the photodetectors and will relate to the physiological analyte concentration of the cells.

have to be interfaced with conventional sources and detectors leading to only partial miniaturization. In the future, we envision optical and electroanalytical microsensor devices with multiple levels of integration (FIGURE 1). All the essential components of physiological sensing will be organized in a multilayer multimodule fashion.

The next generation optical microsensor device will consist of an optical source, sample chambers, optical detection, signal processing and conditioning, data acquisition and wireless communication modules (FIGURE 2). All the modules except the sample chambers will be stationary. The sample chambers will be designed in an array format to provide high-throughput capabilities. The sample chambers can be modified with fluorophores on the bottom surface using an automated microinjection system. The back side of the sample array will include a microfabricated LED array and emission signals will be detected using the microfabricated photodiode array. The excitation and emission signals will then be guided to the signal-processing and -conditioning module where artifacts will be removed. The data acquisition module will acquire the conditioned signals and send them to a wireless module. The signals will then be wirelessly transmitted to a central network, handheld devices and computers for real-time viewing and archiving.

All the modules will be controlled with a central control software infrastructure with the capability of controlling the progress on each sample chamber in the array. The software will also contain error checking mechanisms. If there is any problem, the software will send an email or a text alert to the system administrator, or the person in charge, and also shut the whole system. Such multiple systems would function in tandem to conduct large sets of experiments. The software control will also enable data to be viewed from any sample chamber on any of the systems. This high level of throughput and control will fulfill the promise of high-throughput cell physiology technologies and usher in the physiomics era.

#### Expert commentary & five-year view

The authors expect that development in micromanufacturing and MEMS fabrication technologies will drive the development of the next generation of cell electrophysiology and optical cellular physiology biochips. High-throughput single cell electrophysiology will become a reality with the capability of detecting multiple analytes on the same platform. Preliminary demonstration of this has already been demonstrated by the CEL-C biochip. Improvements in stability, adhesion and biocompatibility of ion-selective membranes and enzyme immobilization layers will have a positive impact on the sensitivity, selectivity and lifetime of cell physiology biochips. The most significant breakthroughs are expected to arise from integration of optical technologies on a biochip platform. The inherent advantages of optical-sensing methodologies over electrochemical sensing make them an ideal candidate for producing miniaturized, reusable, long-lifetime and low-noise biosensors. The key, however, is the integration of all the aforementioned sensing modalities on one common integrated platform, as envisioned by the authors in FIGURE 1. Such a 'multifunctional cellular physiology' biochip will provide cell physiologists with a single tool for performing a wide array of sensing applications. The application of this biochip in high-throughput drug screening, cell signaling studies, cancer research and medical diagnostics will be immense. Ultimately, such an effort will only be possible through highly interdisciplinary collaborations between research groups. This has been realized by the research community and joint efforts have begun that will surely have a deep impact over technology development for cellular physiology in the next half decade.

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#### Key issues

- The cell electrophysiology lab-on-a-chip approach has tremendous potential, especially when used in a noninvasive, high-throughput approach for investigating protein function and cell physiology.
- Microsensor technologies including optical methods, such as optrodes, have not been fully developed as research tools or as tools for clinical applications.
- Success for the next generation of microsensor technology, both electrical and optical, will depend on miniaturization, integration and automation. Development in micro-electro-mechanical-systems fabrication technology and nanotechnology will be the enabling factors for realizing this.
- Due to the interdisciplinary scope, collaborations between scientists and engineers hold the key to success in the future.

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