Sensor Technologies for Monitoring Metabolic Activity in Single Cells—Part II: Nonoptical Methods and Applications

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***Abstract*—A review of solid-state chemical and electrochemical sensors to detect metabolic activity at the extracellular, single-cell level is presented in the context of the development of lab-on-a-chip research instrumentation. Metabolic processes in cells are briefly reviewed with the goal of quantifying the role of metabolites within the cell. Sensors reviewed include both research and commercial devices that can noninvasively detect extracellular metabolites, including oxygen, carbon dioxide, and glucose. Metabolic activity can also be sensed nonselectively by measuring pH gradients. Performance metrics, such as sensitivity, sensor size, drift, time response, and sensing range, are included when available. Highly suitable sensor technologies for monitoring cellular metabolic activity include electrochemical sensors, scanning electrochemical microscopy, ion-sensitive field effect transistor sensors, and solid-state light-addressable potentiometric sensors. Other less-suitable, but still potentially viable, solid-state sensing technologies are also reviewed briefly, including resonant chemical sensors (surface acoustic wave and quartz crystal microbalance), conductivity or impedance sensors, and sensors with multiple transduction stages. Specific biological applications which benefit from detection of extracellular metabolic events at the single-cell level are discussed to provide context to the practical use of these sensor technologies; these applications include case studies of various diseases (cancer, diabetes, mitochondrial disorders. etc.), cell and tissue differentiation; cell and tissue storage; cell life cycle and basic cellular processes; and developmental biology.**

***Index Terms*—Carbon dioxide sensors, cell metabolic activity, chemical sensors, electrochemical sensors, glucose sensors, ion-selective membranes, ion-sensitive field effect transistor (ISFET), light-addressable potentiometric sensors (LAPS), microelectrodes, oxygen sensors, pH sensors, quartz crystal microbalance, scanning electrochemical microscopy (SECM), single-cell detection, surface acoustic wave.**

I. Introduction

SOLID-STATE and electrochemical sensors designed for resolving events at the single-cell level are restricted by a different set of requirements than those designed for other sensing applications. For example, the design of highly selective chemical membranes is often of paramount importance and plays a determining role in the final size, complexity, and sensitivity of the sensor. Other design factors include the tradeoff between signal-to-noise ratio (SNR) and overall size, long-term stability, repeatability, and lifetime of the sensor. Classes of sensors that are compatible with detecting metabolic events at a single-cell level include electrochemical sensors, scanning electrochemical microscopy (SECM), field effect transistor (FET)-based chemical sensors, and light-addressable potentiometric sensors (LAPS). Electrochemical sensors have been relatively successful in resolving single-cell events through the development of microelectrodes, which are usually wire electrodes encapsulated within pulled glass capillaries. If these microelectrodes are physically translated, or rasterized, through space, a concentration image can be obtained with resolutions as low as 2 μm, and this type of microscopy is called scanning electrochemical microscopy (SECM). Solid-state sensors of interest include the FET-based chemical sensors, where chemical reactions change underlying transistor characteristics, and light-addressable potentiometric sensors (LAPS), where photodiode structures are used to monitor changes in surface charge on a chemically sensitive surface. By using a scanning laser, the resolution of the LAPS device has been shown to be as low as 5 μm. Other electrochemical and solid-state sensor technologies include resonant sensors, conductivity or impedance sensors, and sensors with multiple transduction steps. Due to size, detection range, or sensitivity incompatibilities, these sensors have not yet become compatible with resolving events at the single-cell level but are discussed here because of their future potential to become competitive with electrochemical, SECM, LAPS, and FET-based sensors.

The analytes emphasized in this review of sensing technology are related to the metabolic activity in single cells (Table I). Solid-state and electrochemical-sensing technologies are particularly suited to the monitoring of extracellular metabolites including oxygen, carbon dioxide, and glucose. Fluorescent means of detection dominate the monitoring of intracellular events because fluorescent probes can be easily injected inside cells for sensing purposes. For long-duration experiments, such as those required to obtain meaningful metabolic activity information, however, fluorescent detection methods are often limited by photobleaching and cytotoxicity, two limitations which are not present in solid-state and electrochemical approaches. The detection of pH is also reviewed, since it is a general indicator of metabolic activity in the extracellular space. The first half of this review addresses the use of nonoptical methods for sensing extracellular metabolic analytes and the second half focuses on efforts directed at addressing

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TABLE I
COMMON CELL METABOLITES AND RELEVANT SENSING METHODS

Metabolite or Metabolic By-Product	Detection Range of Interest (for single cells)	Large-Scale Detection Methods	Spatially-Resolved (Small-Scale) Detection Methods
Oxygen	0-280μM at 0.1μM resolution [1][2]	Colorimetric, *ISFET, electrochemical*	PEBBLEs, *SECM*
Carbon Dioxide	0.1-10mM at 0.1μM resolution [3]	*ISFET, electrochemical*	Colorimetric
pH	5.2-8.5 at 0.1 resolution [4]-[7]	*ISFET, electrochemical*	Luminescent, Colorimetric, *LAPs*
Glucose	3-23mM [8]	*LAPS, ISFET, electrochemical*	Luminescent, Colorimetric, *SECM*
ATP	0-5mM [9][10]	Colorimetric	Luminescent
NADH, $FADH_2$	0-125μM [11]	N/A	Luminescent

- Detection methods in *italics* are non-optical sensing methods discussed in this paper.
- ISFET: ion selective field effect transistor
- PEBBLEs: probes encapsulated by biologically localized embedding
- SECM: scanning electrochemical microscopy
- LAPS: light addressable potentiometric sensors

system-level biological applications including monitoring of various diseases (cancer, diabetes, mitochondrial disorders, etc.), cell and tissue differentiation, cell and tissue storage, cell life cycle and basic cellular processes, and developmental biology.

II. SINGLE-CELL SENSING AND DETECTION

In order to develop a more complete understanding of inherently heterogeneous cell populations, measurement and analysis of a wide variety of parameters from individual living cells is necessary. At the present time, most studies tend to be focused on multiple cells, where averages from populations are correlated to behaviors, expressions, and processes of interest. Multiple-cell measurements are more compatible with the limitations of existing instrumentation technology; however, the need for a wide variety of sensing and measurement techniques to analyze cells at a single-cell level is more and more in demand in the biological research community. Single-cell characteristics play a key role in not only determining population characteristics but also in determining the transient dynamics that lead to future cell expression and behavior at a system (population) level.

In order to accomplish an understanding of single cells, instrumentation and sensing technology must meet stringent detection limit, sensitivity, and signal-to-noise constraints. These technologies must also be constructed in a way that enables multiple-sensing mechanisms to be employed simultaneously, because the understanding of single cells is linked to a broad range of activities and analytes of interest. Such analysis includes not only measuring the intent of the cell (genetic sequence and protein expression), but also resulting behavior of the cell. One of the most important tasks a cell accomplishes is metabolism, keeping itself alive and supporting reproduction. The quality and rate of metabolism not only determine the cell's current health and level of activity but also plays a significant role in determining the future health of the cell and the population to which it belongs. Unusual metabolic changes are often linked to early onset of disease and other potential detriments to cell viability.

A number of research groups and research centers are currently emphasizing the analysis of single-cell behavior and characteristics. For example, the Microscale Life Sciences Center at the University of Washington is developing microsystems to measure multiple parameters in individual living cells in real time to correlate cellular events with genomic information. Specific biological applications are being studied to understand response at the cellular level [12]. The Center for Genome Research (The Broad Center) at MIT has a number of research interests in single-cell activities related to genomics and biomedical research. Broad Center projects include a collaboration with the Department of Energy's "Genomes to Life" program, which emphasizes understanding life functions of single-cell organisms at the microbial level and developing means to address national concerns in energy and the environment. The Institute for Genomic Research (TIGR) in Rockville, MD, and partners at George Washington University have research interests in structural, functional, and comparative analysis of genomes from viruses to single-cell eukaryotes. The Nanobiotechnology Center, led by Cornell University, emphasizes the merging of nanostructure fabrication and biotechnology methods (biomolecular devices and analysis, biomolecular dynamics, cellular microdynamics, cell-surface interactions, nanoscale materials, and nanoscale cell biology) and includes collaboration with Princeton University, Wadsworth Center, Oregon Health Sciences University, Clark Atlanta University, and Howard University. Through interdisciplinary research interests, this center seeks to merge nanostructure fabrication and biotechnology methods. The Beckman Institute at California Institute of Technology has been developing new biological imaging techniques, and have recently developed methods to image multiple fluorescent proteins within a single cell [13]. Research at the Cell Biology and Metabolism Branch (CBMB) of the National Institute of Child Health and Human Development (NICHD) focuses on studying metabolism and cell cycle at the single-cell level [14]. Researchers at Johnson and Johnson Pharmaceutical Research

and Development have developed microarrays to profile gene expression at the single-cell level [15].

Individual groups working on the analysis of single cells include the Single-Molecule Research Group at the Old Dominion University [16], Dr. Virta's group at the University of Turku [17], the Weijer Laboratory at University of Dundee [18], and Molin's Molecular Microbial Ecology group at the Technical University of Denmark [19]. The Saykally Group at the University of California, Berkeley, is studying biochemical properties of individual organelles in living cells, which required single-cell measurements of live human lung cells. Dr. E. Yeung at Iowa State University develops analytical and fluorescent methods for use with micro-scale samples, which include analyzing the chemical content of the fluid in a single red blood cell. The Nie Group at Indiana University seeks to improve measurement capabilities in the study of macromolecules and other parts of a living cell. Research in single-molecule dynamics, single ligand-receptor interactions (which include the effects of radio frequency radiation and electric fields on binding properties and electrochemiluminescence interactions), and novel bio/nanotechnologies are being developed by Dr. X. N. Xu, at Eastern Virginia Medical School, to study living systems at the single-molecular level in real time. D. Discher's laboratory at the University of Pennsylvania is researching "functional materials aspects of polymers, cells, and biomolecules to develop single-molecule and single-cell manipulation methods, microscopies, molecular and cell biological systems and reagents," and other projects. These groups, both large (center-based) and small, are a mention of only a few groups in the vastly expanding area of interdisciplinary research and development of single-cell analysis.

At the single-cell level, when analyzing metabolism, the analytes of most interest are inorganic molecules such as dissolved oxygen and carbon dioxide; organic molecules such as glucose, adenosine triphosphate (ATP), NADH, and reduced flavin adenine dinucleotide ($FADH_2$), and pH, a general indicator of cellular metabolic activity. Possible measurement techniques that can be used to analyze these metabolites, at a microscale resolution include luminescent spectroscopy, colorimetry, electrochemical techniques (including scanning electrochemical microscopy, or SECM), solid-state potentiometric sensors (such as the LAPS microscope), and combinations of these sensor technologies. Following a discussion of nonoptical-sensing techniques appropriate for single-cell analysis, this paper will address representative biological applications that can make substantial use of metabolic information from single cells.

III. Overview of Metabolic Activity in Cells

The objective of this section is to provide a brief overview of basic metabolic activity in most eukaryotic (contains a nucleus) and prokaryotic (no nucleus) cells. An excellent overview of cellular metabolic processes can be found in introductory biology texts such as [20]. Significant differences occur among cells, plants, and animals; however, the focus of this section is to identify common and primary analytes and units of interest from a sensing perspective in terms of extracting and interpreting information from living cells in a dynamic and changing environment.

Most organisms aim to stay alive, grow, and reproduce. In order to do so, they must generate chemical energy for their daily activities and they must produce complex molecules to maintain life. Chemical energy is used to produce fuel for daily activities and is derived almost exclusively from a molecule called ATP. ATP easily undergoes hydrolysis to form adenosine diphosphate (ADP), an inorganic phosphate molecule, and energy. Any metabolic function associated with making ATP (and, therefore, fuel) is called a *catabolic pathway*. Molecules that contain carbon are used to make DNA, RNA, proteins, fatty acids and other molecules necessary for short and long term maintenance and regulation of the cell life cycle (including reproduction). Any metabolic function associated with assembling these more complex molecules from carbon-containing molecules is called an *anabolic pathway*.

The catabolic pathway, or production of ATP, as a means of storing energy for easy release when required by the demands of cellular activity, is dominated by the breakdown of glucose into a form that releases sufficient energy to make ATP. Thus, the catabolic pathway is simply the transfer of energy stored in glucose bonds to phosphate bonds. Donor electrons are loosely bound in glucose but tightly bound in oxygen; since loosely bound electrons have far more potential energy than tightly bound electrons, transfer of electrons from glucose to oxygen results in a release of energy that is sufficient to convert (phosphorylate) ADP molecules to ATP molecules. If glucose were to transfer electrons directly to oxygen, however, the release of energy would be so sudden that a large amount of heat would be produced, which would cause cell damage and would be extremely inefficient. By releasing this energy through a series of intermediate reactions, a similar amount of energy is released to produce ATP without causing cell damage.

The first intermediate reaction that glucose undergoes is glycolysis, where glucose sugar is "loosened" into a compound called pyruvate. In addition to pyruvate being produced during glycolysis, some energy is directly released (to convert ADP to ATP) and a compound called nictoniamide adenine dinucleotide (NAD^+) is reduced to become NADH. As a product of glycolysis, pyruvate is used to initiate a series of chemical reactions known as the Krebs cycle. During the Krebs cycle, more NADH, and a related compound $FADH_2$, are produced. The Krebs cycle converts some ADP molecules to ATP and also completes the conversion of glucose to carbon dioxide (CO_2). The energy stored in NADH and $FADH_2$ is used to create a proton gradient across the mitochondrial inner membrane. Energy is released via the electron transport chain, which consists of a series of molecules with slightly different oxidative energies. An electron is released through oxidation of NADH or $FADH_2$, and the potential energy of the electron is slowly released as it bonds to different molecules in the electron transport chain. A protein called ATP synthase uses the energy in the proton gradient to ADP to ATP.

In an aerobic metabolic cycle, oxygen is the final electron acceptor. When oxygen is not readily available for accepting electrons at the end of the transport chain, cellular respiration does

not occur, and instead, electrons are transferred from glucose to some other organic, electron-accepting molecule. A transfer of electrons to any other molecule than oxygen, however, is not as efficient of a process and does not produce fuel as efficiently as when oxygen is present. The process by which ATP is produced via electron transport from glucose to a nonoxygen molecule is called fermentation and is essentially a secondary process for the cell to produce fuel in the absence of oxygen. The use of other electron acceptors (nonoxygen molecules) in the transport chain produces different byproducts than regular cellular respiration, including lactate (in muscle cells of the human body) and ethanol (in yeast). Respiration (an aerobic process) is always preferred by eukaryotic cells over fermentation (an anaerobic process), because it is a more efficient means to produce fuel. Many bacteria, however, use anaerobic processes (of which fermentation is one) as a primary means for producing fuel. These types of cells may use compounds other than glucose to initiate the production of fuel (e.g., H_2, H_2S, and CH_4) or may use electron acceptors other than oxygen in the electron transport chain (Nitrate NO_3^- and Sulphate SO_4^{2-}).

Other compounds, including other carbohydrates, fuels, and fat, can be used to feed the catabolic pathways of a cell (and subsequent production of ATP). All of these molecules are first broken down into pyruvate, which is then used in the Krebs cycle. Carbohydrates of interest include glycogen (animals) and starch (plants) which can be converted to glucose through enzymatic reactions. Fats are broken down by enzymes into pyruvate, and proteins can be broken down into constituent amino acids which are then broken down into amino groups (NH_3 which is excreted from the cell) and carbon compounds which are eventually converted to pyruvate.

The anabolic pathways are the other class of metabolic pathways in living cells. Typically, in order to convert carbon-containing molecules to more complex molecules, the anabolic pathway uses both energy and products produced from the catabolic pathway. If enough ATP is already available in the cell, pyruvate (from respiration) and lactate (from fermentation) can be used to make more glucose which is then converted to glycogen and stored for future production of fuel. Many of the amino acids needed by cells to assemble proteins can be made from molecules taken from Krebs cycle reactions. Acetyl CoA, which is the starting point for the Krebs cycle (produced from pyruvate), is also a starting point for many anabolic processes that produce fatty acids. Finally, byproducts of glycolysis can be used to manufacture DNA and RNA, critical components to continued cell reproduction and regulation.

In summary, the combination of catabolic and anabolic processes (or pathways) make up cellular metabolic activity (chemical reactions in cells). This section has provided an overview of how these pathways proceed and interact. In the context of sensing metabolic activity, the list below summarizes analytes of primary interest in understanding how well a cell is supporting proper metabolism through both catabolic and anabolic means.

- Extracellular Oxygen (O_2): indicates how well the cell is able to carry out respiration.
- Extracellular Carbon Dioxide (CO_2): reflects how well the Krebs cycle is being completed in the cell; in conjunction with glucose concentration, CO_2 concentrations reflect the overall viability of the cell.
- Extracellular Glucose: reflects the capacity for the cell to produce fuel and to carry out anabolic functions.
- Intracellular ATP: indicates how much fuel is immediately available to the cell.
- Intracellular NADH and $FADH_2$: reflects the successful completion of glycolysis in the cell.
- pH: intracellular pH differences (or a proton gradient) across the mitochondrial membrane drive the electron transport chain, which converts electron energy to phosphate bonds. Extracellular pH is an indication of cellular respiration, especially the production of CO_2.
- Extracellular H_2, H_2S, and CH_4: alternative electron donors to glucose in some cells.
- Extracellular Nitrate NO_3^- and Sulphate SO_4^{2-}: alternative electron acceptors to oxygen in some cells.

Extracellular analytes associated with primary aerobic processes are emphasized in this paper. Nonoptical-sensing technologies do not yet have widespread applicability to monitoring analytes within live cells. For this reason, this review focussed on extracellular monitoring of the primary aerobic metabolites: oxygen, carbon dioxide, and glucose (and a related parameter, extracellular pH).

A. *Chemical Approaches: Electrochemical Sensors*

The basic operating principle behind an electrochemical sensor relies on the transfer of electrons between an electrode and a chemical redox reaction. A thorough handling of the principles underlying electrochemical sensors can be found in [21]. One of the most successful electrochemical sensors for detecting metabolic products is the Clark-type oxygen sensor, which uses an electrode (usually made of platinum or palladium) to catalyze the reduction (or gain of electrons) of oxygen and water

$$O_2 + 2H_2O + 2e^- -> H_2O_2 + 2OH^-. \quad (1)$$

Two aspects of this chemical reaction are important. First, the analyte of interest (oxygen) is consumed in this process, so it is no longer available for the cell to use for metabolic processes. Second, the reaction requires two electrons, which are usually taken from an electrode. The movement of electrons from the electrode to the chemical reaction generates a current, which can then be measured amperometrically. The electrode that drives this reaction is called either the cathode, the reducing electrode, the working electrode, or the sensing electrode (since the current generated by this electrode is ultimately used to measure the oxygen concentration). To avoid confusion, the term "sensing electrode" will be used exclusively. The sensing electrode can be implemented with a variety of fabrication methods to measure dissolved oxygen; Table II summarizes the performance characteristics of commercial and research Clark-type electrochemical oxygen sensors. Both detection range and resolution can be correlated to the information provided in Table I to establish the usefulness of each sensor approach to single-cell operation limits.

TABLE II
SUMMARY OF ELECTROCHEMICAL OXYGEN SENSOR PERFORMANCE

Ref	Sensitivity	Drift	Sensing Area Size (μm^2)	Sensing Range	Response Time	Features
[23]	~4pA/μM	low	~7500	0-60μMO_2	-	Commercial, Clark-style microelectrode, integrated reference electrode
[24]	0.78μA/μM	-	~7.5x10^5	0-310μMO_2	2.6min (90%)	Micromachined, integrated reference electrode in anisotropic etched wells
[25]	<83pA/μM	-	5026.5	0-601μM	0.7s	Micromachined, 4x4 array, metrics cited for single 80μm electrode
[26]	5.48μA/μM	-	2500	>2μM	35-45s	Micromachined, integrated reference electrode and membrane, metrics cited for 50μmx50μm electrode
[27]	10.78nA/μM	-	<7.1	-	<1s	Ultramicroelectrode, cellulose acetate membrane
[28]	12.8μA/μM	<5%/h	7854	0-3890μM	30s	Linear, array of 8 electrodes, transparent substrate, metrics cited for a single electrode

In the reaction described in (1), peroxide is formed from oxygen and water. The peroxide either escapes from the proximity of the electrode or it can undergo one of two subsequent reactions

$$H_2O_2 + 2e^- -> 2OH^- \quad (2)$$

$$2H_2O_2 -> 2H_2O + O_2. \quad (3)$$

Higher electrode potentials increases the likelihood that the first reaction will occur rather than the second reaction [22]. The first reaction is important because it results in the production of OH^- ions, thereby changing the pH which can detrimentally impact cell viability. The second reaction generates oxygen which in turn supports metabolism. Because the sensing electrode is optimally small, though, it is more likely that the peroxide will diffuse away from the electrode before undergoing the second reaction, and an oxidizing electrode is often used instead to minimize oxygen consumption.

Diffusion can have a significant effect on the response of the sensor to oxygen. When the sensor is initially turned on, the reaction of oxygen proceeds rapidly. If oxygen is catalyzed too quickly, though, oxygen is depleted in the regions closest to the electrodes. The overall rate of the reaction then slows as the reaction starts to depend on the rate of diffusion of oxygen to the electrode from the surrounding bulk fluid. The sensor response then becomes diffusion limited rather than reaction limited; response linearity changes and response time becomes slower.

For detecting single cells, one of the main disadvantages of electrochemical cells is that analyte is consumed. One method to reduce this disadvantage for oxygen sensors is to integrate an oxidizing electrode with the sensor. Other terms commonly used for the oxidizing electrode are counter electrode and anode. The oxidizing electrode is commonly made of either copper [29] or gold [25], [30]. The chemical reaction at the oxidizing electrode is

$$4OH^- -> O_2 + 2H_2O + 4e^-. \quad (4)$$

This reaction offsets the consumption of oxygen in the sample being measured. Usually a reference electrode is still required to maintain the potential difference between the sensing and oxidizing electrodes. To detect single cells and cell populations, the size of the sensing electrode must be at most a few micrometers in size. Miniature oxygen-sensing Clark electrodes are commercially available, and currently available tip diameters range from 5 to 250 μm (Micro Clark Style Oxygen Electrode, Diamond General, Ann Arbor, MI). Intracellular microelectrodes for sensing oxygen are also available, and the tips range in size from 2 to 3 μm (Intracellular Oxygen Microelectrode, Diamond General, Ann Arbor, MI). One drawback to a smaller active area for electrochemical sensors is that the SNR decreases [31].

Recent research has focused on new methods to integrate the sensing electrode with the reference electrode for simpler fabrication, development of solid-state electrolytes to simplify the fabrication process further, or development of new materials to make the sensor easier to use and maintain. For example, some research has focused on increasing the surface area of the reference electrode while keeping the overall sensor size small by depositing Ag/AgCl inside anisotropically etched grooves, and fabricating the catalytic metal of the sensing electrode on planar surfaces on the etched structure [24]. The grooves increase the surface area of the reference electrode without increasing the overall sensor size. Another research group built a microfabricated solid-state oxygen sensor that incorporated a proton conductive matrix (PCM) to eliminate the need for rehydration, and used microfabrication techniques to eliminate manual fabrication steps [25]. In this sensor, the electrodes are arranged in a 4×4 array, with electrode sizes ranging from 10 to 80 μm. Electrochemical sensors are also capable of detecting CO_2, pH, and glucose, usually through the addition of semipermeable membranes, electrolytes, and/or enzymes. Table III summarizes the performance of these types of sensors. Both detection range and resolution can be correlated to the information provided in Table I to establish the usefulness of each sensor approach to single-cell operation limits. A review of transduction princi-

TABLE III
SUMMARY OF ELECTROCHEMICAL SENSOR PERFORMANCE FOR CO_2, pH, AND GLUCOSE

Analyte	Ref	Sensitivity	Drift	Sensing Area Size (μm^2)	Sensing Range	Response Time	Features
CO_2	[33]	5.65nA/ sqrt(mMCO_2)	~42pA/h	$5.7x10^8$	0.04-0.1%	~0.5min	PTFE membrane, PtO electrode, 0.1M KCl electrolyte, nonlinear response, vulnerable to several interferents
CO_2	[37]	0.4mV/mMCO_2	~17μV/h	$7x10^6$	0.1-10mM	1min	Commercial, gas-permeable membrane with electrolyte
CO_2	[34]	0.51μA/ sqrt(mMCO_2)	3.96mV/h	$3.5x10^8$	0-56.8mM	~0.5min	PTFE membrane, RuO_2 electrode, 0.1 KCl electrolyte, nonlinear response, improved selectivity
CO_2	[35]	10.23nC/mM	<1%/h	$8.41x10^6$	0-41.5mM	2min	Silicone membrane, solid PNVP hydrogel electrolyte, RuO_2 electrode
CO_2	[38]	43mV/decade	-	-	2.15-8.2μM	1min	Solid-state, sintered ceramic electrolyte
CO_2	[39]	4.033μA/mM	0.2%/h	$3x10^6$	0-41.5mM	2min	Quasi-linear for <8.2mM, nonlinear for >8.2mM, sensitivity given for <8.2mM, interleaved comb structure Pt electrode, pH change due to copper dissociation
pH	[40]	59mV/pH	-	~44	6-11pH	<5s	Commercial, beveled tip, 6.5pH NaCl electrolyte, #823
pH	[41]	60mV/pH	-	$3.85x10^5$	4-9pH	-	Bilayer lipid membrane with TCNQ, Au electrode
O_2	[42]	78.9mV/decade	0.14mV/h	$9.5x10^5$	1.9-8.3mM	60s	Combined blood sensor, logarithmic response to O_2, 1.1mm total tip diameter
CO_2		57.5mV/decade	0.46mV/h		0.8-3.9mM	78s	
pH		59.3mV/decade	0.004pH/ h		7.0-7.8pH	9s	
glucose	[43]	6.5pA/mM	-	$2x10^5$	0-100mM	120s	Coated with biocompatible copolymer, uncharged membrane, resists protein adhesion
glucose	[44]	0.13μV/mM	-	$>9.1x10^6$	1-10mM	<2min	Glucose oxidized by *E. coli* cells, sensitive to pH and temperature changes
glucose	[45]	0.94 log pA/ log mM	0.6%	$7x10^6$	0.04-21.9mM	<10sec	Al_2O_3 sol-gel enzyme matrix, platinized glassy carbon electrode, 2.05% repeatability, some interference
glucose	[46]	34.8 nA/mM	-	1.8×10^6	0.1-10mM	48 sec	PbO_2/CA/PEG-type oxidant-removing membrane
glucose	[47]	20nA/mM	0.04%/ hour	1.4×10^7	0-35 mM	10 sec	Low-interference, Nafion and acetylcellulose membranes
glucose	[48]	160nA/mM		3.1×10^6	0-13 mM	90-120 sec	Glucose-oxidized by *A. niger* cells in carbon paste, linear range up to 6mM

ples for electrochemical sensors targeted for biochemical applications can be found in [32].

Electrochemical CO_2 sensors detect a pH change using a "Severinghaus-type" sensor. In this sensing scheme, an electrode is immersed in a fluid that is encapsulated in a membrane selectively permeable to CO_2. At the electrode, CO_2 is reduced to form bicarbonate. This reaction changes the pH of the solution surrounding the electrode, and the pH change can then be measured potentiometrically. CO_2 is reduced more efficiently with platinum oxide [33] or rubidium oxide [34] electrodes than with other electrode materials. The rubidium oxide electrode is approximately 100 times more sensitive to carbon dioxide than platinum oxide, and it is also much more selective. By replacing the electrolyte with a solid-state electrolytic membrane, the fabrication process can be further simplified, but it may be difficult to fabricate a sensor that performs as well as the liquid-electrolyte versions [35]. To increase the selectivity of these sensors, a gas-permeable PTFE membrane is usually integrated with the

sensor. A commercial CO_2 electrode is available with a 3-mm tip diameter (Mini Carbon Dioxide Electrode, Diamond General, Ann Arbor, MI). Commercial pH miniature electrodes are also available with beveled tips ranging from 2–10 μm diameters (pH Microelectrode, Diamond General, Ann Arbor, MI). Commercial sensors usually rely on a liquid electrolyte and a simple gas-permeable membrane to reduce the cost and complexity of the sensor.

Glucose is usually sensed amperometrically, but glucose needs to be converted to a form that can be reduced or oxidized by the electrode. To accomplish this, the enzyme glucose oxygenase first converts glucose plus one oxygen molecule into peroxide and a glucose byproduct. At an Ag/AgCl electrode, the peroxide is converted to oxygen, two hydronium ions, and two electrons. The electrons then generate a current that can be detected amperometrically [36]. The performance of the glucose sensor is determined primarily by how the enzyme is immobilized onto the surface of the electrode. Ideally, the enzyme would be covalently bonded so that it does not diffuse away into the surrounding bulk fluid, but it would also have a large surface area to enhance diffusion of glucose to the enzyme. The enzyme also needs to be close enough to the electrode so that the peroxide byproduct tends to interact with the electrode rather than escaping into the bulk solution. Ideally, the electrode would not oxidize other species and would be resistant to interference.

In another approach for sensing glucose, glucose oxygenase has been immobilized by chemically bonding the enzyme onto the electrode surface or inside of a membrane, such as crosslinking with glutaraldehyde [49], or physically trapping the enzyme in sol-gel or other hydrogels [45], in carbon paste [48], in semipermeable membranes, in electropolymerized films, or in thermo-shrinking polymers [50]. Two drawbacks to this type of glucose sensor include interference from other easily oxidized species and a dependency upon the partial oxygen pressure of the fluid. The magnitude of both of these effects can be reduced by either adding an oxidant membrane to the sensor, such as PbO_2 embedded in a polymer [46] or acetylcellulose and Nafion [47], or by using an electron transfer mediator embedded in a polymer, such a ferrocene-modified polysiloxane [51], polyacrylamide [52], ferrocene-modified poly(ethylene oxide) [53], ferrocene-modified polypyrrole [54] polyquinone [55], ferrocene-modified Nafion [56], polymetallocyanines [57], or osmium-modified polyvinylpyridine [58]. An excellent review of electron transfer mediators can be found in [59]. A novel approach to detecting glucose amperometrically is to use microorganisms to convert the glucose into analytes that can be detected by the electrode. *Escherichia coli (E. coli)* cells have been successfully incorporated into an amperometric sensor to detect glucose. The *E. coli* cells contain apo-glucose dehydrogenase, which oxidize glucose while reducing interference from other redox-active substances [44]. Another option is the fungus *Apsergillus niger*, which contains glucose oxidase, and these microorganisms can be directly mixed into a carbon paste that can be applied to the surface of the electrode [48].

One of the reasons that electrochemical sensors must be large is that a perm-selective membrane must be integrated with the sensor. An attractive option for increasing the selectivity of electrochemical sensors to metabolites while minimizing the size is to use a bilipid membrane layer. The bilipid membrane layer is similar in structure to the membrane of a cell and is only a few nanometers thick. It can be made permeable to specific ions by using proteins naturally found in cells. Recently, Tvarozek *et al.* constructed a pH sensor that is selective only to H^+ ions. To fabricate a bilipid membrane selective to hydrogen, mediating substances were used to create channel-like structures in the membrane. The resulting pH sensor was linear with a sensitivity of 60 mV/pH, but the stability of the bilayer lipid membrane limits the lifetime of the sensor [60]. Innovative approaches to improve the stability of the membrane layer would result in the ability to construct much smaller ion-selective electrochemical sensors.

Another option to reduce the size of an electrochemical sensor is to combine sensors into one structure. Meruva and Meyerhoff successfully combined three sensors to detect pH, pCO_2, and pO_2 into one sensing element [61]. The catheter-type sensor uses three electrodes and two chambers surrounded by a silicone membrane. One segment of the silicone membrane was impregnated with tridodecylamine to impart selectivity to H+ ions. One chamber contained an unbuffered bicarbonate solution, an Ag/AgCl electrode, and a cobalt electrode. The cobalt electrode detected pO_2, and the Ag/AgCl electrode detected a pH change. The other chamber was filled with a strong buffer and an Ag/AgCl reference electrode. The pCO_2 was detected via the membrane potential across this reference electrode and the Ag/AgCl electrode in the bicarbonate solution. Total tip diameter for the combined sensor is 1.1 mm, and the performance of this sensor is comparable to separate sensors.

Single-cell detection is ideally done by detecting the spatial distribution of metabolites. Spatial resolution using electrochemical sensors is accomplished by either using an array or by "rasterizing" the sensor. An example of an electrochemical array is the work done by Hermes *et al.* in which a 32×32 array of amperometric sensors was integrated onto one substrate [62]. Each sensor in the array was individually addressable and was 40×80 μm in size, and the total sensor size was 30×30 mm. Another example of an electrode array is the work by Sargent and Gough [28]. Their design had eight 100-μm diameter platinum electrodes with integrated counter-electrodes and reference electrodes. By fabricating these electrodes onto a glass wafer, the substrate was transparent and the device could be easily combined with fluorescent or other optical-sensing techniques. With advances in ultramicroelectrodes and microfabrication, it is theoretically possible to reduce the size of the array, but crosstalk between electrodes and SNR become issues as the electrodes become smaller and more closely spaced.

The other method to obtain spatial information using electrochemical sensors is "rasterizing" the sensor. In a "rasterizing" scheme, an electrode with a very small tip is physically moved through the fluid using a micromanipulator or some other mechanical motion control [63]. One common method to fabricate these electrodes is to encapsulate a Pt, Pd, Ir, C, or Au wire in a pulled glass microelectrode. A review of recent developments in microelectrodes and fabrication methods can be

found in [64]. Scanning electrochemical microscopes use these microelectrodes to measure or modify surface properties, and there are several commercially available microscopes of this type (SCM270, Uniscan Instruments, Buxton, U.K.).

Microelectrodes have also been used to monitor spatial distributions of oxygen concentrations in single cells. Jung and associates fabricated a microsensor having a tip diameter of less than 3 μm to monitor the spatial oxygen consumption of mouse pancreatic islets and single cells within the islet [65]. The electrode consisted of an etched Pt wire encapsulated in a pulled glass micropipette, and the tip was filled with cellulose acetate, which functioned as a gas-permeable membrane. Land *et al.* also measured the oxygen concentration across the membrane of a cell using two commercial polarographic oxygen sensors with 2–3-μm diameter tips (Diamond General Corp., model 723, Ann Arbor, MI). These electrodes were separated by a known distance, then rasterized near the cell to noninvasively obtain spatial information and oxygen flux [66]. In a comparative study between fluorescence methods and SECM methods of measuring cell viability and metabolic activity, it was found that SECM responded 15–20 min faster than the fluorescence method [67]. In this study, the SECM detected the spatial oxygen flux surrounding cultured HeLa cells with a resolution of 5 μm, and the changes in metabolic activity under three environmental conditions was compared to the fluorescent response of Calcein-AM, a fluorescent probe used to measure deformation of cell membrane and cell viability. SECM can also detect glucose by direct detection of peroxide, a byproduct of the oxidation of glucose, by using a microelectrode containing a carbon wire [68]. The resolution was approximately 20 μm, and the glucose oxidase enzyme was immobilized onto the substrate rather than the microprobe. With further advances in the construction of glucose microelectrodes and enzyme-embedded membranes, glucose SECM should improve in both resolution and fabrication simplicity.

It has been demonstrated that electrochemical sensors are capable of noninvasively detecting extracellular metabolites, including oxygen, carbon dioxide, and glucose. Through recent advances in microelectrodes, it is also possible to resolve spatial concentration gradients through the use of SECM. The compatibility between the size of the microelectrodes and cells has lead to the detection of chemical gradients generated by single cells and islets. Development in electrode and membrane materials, enzyme entrapment, membrane materials, and fabrication technology will continue to improve the sensitivity and resolution capabilities of these types of sensors. Overall, electrochemical approaches are a very promising means for monitoring single-cell activity.

B. Solid-State Approaches: FET-Based Sensing

The class of chemical sensors that use the FET as an underlying amplification method are called ChemFETs. A variety of ChemFET structures have been proposed and demonstrated; however, the most common class of ChemFETs in both commercial and research use is the ion-sensitive FET (ISFET). The ISFET is characterized by the absence of an explicit gate material; the insulating layer in the FET structure is used as the chemically sensitive layer instead. When the insulator layer is sensitive to enzymes and the gate layer is missing from the FET structure, the resulting ISFET (ChemFET) structure is typically called an enzyme selective FET (ENFET). All ChemFET-based structures work on the basic principle of measuring surface charge changes at the interface between the insulator layer and the overlying layer (whether it be the sensing solution as in an ISFET or the gate layer as in a more generic ChemFET). Changes in surface charges result in changes in the work function that are in turn, measured as a change in transistor threshold voltage.

The first ISFET ever reported was developed by Bergveld in 1970 [69]. In the following 30 years, improvements in Bergveld's basic ISFET structure have emphasized increasing SNR, repeatability, and temperature stability while reducing size, response time, and cost. The SNR has been improved through recent emphasis on the careful selection, combination, and design of new organic materials as the chemically selective layers [70]. Recent research has also explored the use of diamond-type carbon gate materials to improve repeatability [71]. More traditional approaches for improving repeatability, however, have involved the introduction of the traditional Ag/AgCl reference electrode used in larger sensing systems to the smaller ISFET structure; however, adding the Ag/AgCl reference electrode often significantly increases the size of the overall sensing system, since the reference electrode must be significantly larger than the sensing electrode to provide a stable reference [25]. As an alternative to the Ag/AgCl reference system, another solid-state reference electrode is often used to maintain repeatability while continuing to minimize size [72]. To be a valid reference, the reference ISFET (or REFET) should be insensitive to the analyte of interest and have no effect on the potential of the fluid [73]. In practice, however, the REFET usually has some sensitivity to the analyte and interacts with the surrounding fluid [74]. To reduce this undesired sensitivity, the REFET can be coated with a membrane such as polyACE, Vernis pour l'electronique (VE), polybutadiene (PBD), polybisacryl, poly(lauryl methacrylate)-ethylene glycol dimethacrylate (95:5), and others [75]. A two-layer cationic and anionic conducting membrane structure can also be used to cancel out e.m.f. contributions of the REFET to the surrounding fluid [73]. A solid-state REFET can also improve temperature stability when it is used in conjunction with the active ISFET in a differential sensing mode. In one design, two ISFETs were fabricated on the same substrate but with different gate materials. One ISFET used a Si_3N_4 gate while the other ISFET used an SiO_2 gate, causing the sensitivity of the ISFETs to the analyte to be unequal. Combined with integrated drain-source follower amplification circuits, this ISFET pair showed effective operation of a built-in reference electrode (REFET) as well as reduced temperature dependency generated by a differential measurement and appropriate circuit calibration. Overall sensitivity of this system was demonstrated at 34 mV/pH [80]. Another differential pH sensor design used three sensors: an ISFET with an SnO_2/ITO glass gate, a polypyrrole/SnO_2/ITO coated wire electrode, and an SnO_2/ITO glass reference electrode. The pH change is measured differentially between the ISFET and the coated wire electrode, and the overall

TABLE IV
ISFET PERFORMANCE FOR MEASURING pH

Ref	Sensitivity (mV/pH)	Drift (mV/hour)	Sensing Area (μmXμm)	Sensing Range	Features
[78]	58	3.96	600X20	2-10	0.5μm DPDM CMOS, ion-selective membrane; no additional mask layer
[80]	34	-	400X20	-	2.5μm CMOS differential source-drain follower
[81]	56	-	400x20	3-12	MOSFET reference electrode; CMOS
[77]	47	-	250x25	3-11	1.0μm CMOS, interdigitated
[82]	30.14	-	-	-	Tin oxide/indium tin oxide gate, differential sensing mode
[71]	57.8	0.124	-	1-13	Diamond-like carbon insulating layer
[70]	62	very low	10^3 X10^3	2-10	Organic poly(e-hexylthiophene) insulating layer
[83]	53	.083	10^4 X 10^4	1-13	Al_2O_3 gate, total size given for combined oxygen, carbon dioxide, and pH sensor, response time demonstrated in seconds.

sensitivity was demonstrated at 30.14 mV with considerable reduced temperature dependency as compared to single ISFET structures [82].

Reducing the size of the ISFET has been a challenge, because SNR typically degrades with decreasing device size. However, careful membrane design that includes reducing the size and thickness of the chemically sensitive layer in an ISFET also decreases the response time, which creates a complex tradeoff between size and response time. The tradeoff between device size and response time is often dependent on the sensitivity and detection constraints of the application to which the ISFET is targeted.

Finally, the cost of an ISFET-based system can be reduced substantially through the use of standard fabrication processes, such as the CMOS family of processes used for the majority of integrated circuit manufacturing lines. The most common method to create an ISFET in CMOS technology is to use the silicon nitride passivation layer common to most CMOS processes for chemical sensitivity. Compared to the other available insulator material in CMOS processes (SiO_2), ISFETs manufactured with an Si_3N_4 insulator are more sensitive due to the type of surface sites of the gate [76]. Using silicon nitride in a standard CMOS process to make ISFETs, Bausells *et al.* achieved a sensitivity of 47 mV/pH [77]. In order to circumvent the nonideality of the thickness of the chemically sensitive membrane inherent in a CMOS process, a contact window is often used to post-process (deposit) a membrane of desired thickness while eliminating additional photolithography steps and still maintaining the remaining devices (electronics) and structure in a standard CMOS fabrication scheme [78]. An example of such a device has demonstrated a pH sensitivity of 58 mV/pH. Unfortunately, both of these devices require an external, large Ag/AgCl reference electrode which limits the benefits of CMOS miniaturization and integration. Attempts to reduce the size and integrate the Ag/AgCl reference electrode on chip by patterning silver during fabrication and converting them to Ag/AgCl in a post-process dip in a $FeCl_3$ solution have limited the size and subsequent effectiveness of the reference electrode [79].

The ISFET, with an oxide material used for chemical sensitivity, is typically used to measure pH. Extensive research effort in the use of ISFETs to measure pH is summarized in Table IV. Insulator layers for these ISFETs typically consist of SiO_2 or Si_3N_4, but can also be constructed using Al_2O_3, Tn_3O_5, SnO_2, and other oxide layers. Typical sensitivities of ISFET-based pH sensors are 50–60 mV/pH for Si_3N_4 surfaces and 20–40 mV/pH for SiO_2 surfaces; response times are in the order of minutes. Although the range and resolution of ISFET performance in determining pH is well within the limits of those provided in Table I as a guideline for single cells, the large size of the ISFET limits the spatial resolution to a level that is too low to detect changes in pH among neighboring cells in extracellular activity.

To sense other metabolic products of extracellular interest (glucose, oxygen, and carbon dioxide), two primary approaches are used in the FET-based sensing technologies. One approach involves a two stage transduction process whereby the analyte of interest is first forced to react with an electrochemical electrode; the reaction causes a subsequent change in pH which is then measured by an ISFET structure similar to those discussed previously. The second approach uses a single-stage transduction process by depositing selective semipermeable membrane on top of the ISFET structure to generate a more generic ChemFET structure. These two approaches, the two-stage ISFET and the ChemFET, are summarized in Table V and also discussed in the text in terms of their ability to sense the more specific metabolic products of oxygen, carbon dioxide, and glucose.

In one example of the two-stage approach, an ISFET has been placed next to a Clark-type oxygen electrode; the Clark electrode produces OH^- in the presence of oxygen and the ISFET detects the resulting change in pH, proportional to the original oxygen concentration [84]. This approach tends to increase response time and to consume oxygen in the sensing process, which can limit their application for single-cell monitoring. This two-stage design was slightly improved by microfabricating a palladium electrode around the active area of the ISFET, and detection of both cell acidification and respiration by the same sensor was demonstrated [22]. Another alternative

TABLE V
ISFET AND CHEMFET PERFORAMANCE FOR MEASURING OXYGEN, CARBON DIOXIDE, AND GLUCOSE

Analyte	Ref	Sensitivity	Drift (mV/hour)	Sensing Area (μmXμm)	Sensing Range	Features
O_2	[84]	61-344μV/mM	-	-	0.06-0.94 mM	Pt electrode placed close to ISFET, logarithmic response, consumption of O_2, interference possible
O_2	[85]	0.66V/log mM	-	-	0.16-0.31 mM	Pt/LaF_3 carbon-paste membrane 60-70 second response time
O_2	[86]	300mV/decade	-	-	3-50 mM	Iridium oxide (III and IV) gate, ratio of Ir III to Ir IV depends on O_2 concentration, Nernstian pH dependency
O_2 and pH	[22]	45mV/pH	-	$2000\mu^2$ (O_2)	-	Pd electrode surrounds pH-sensitive area, O_2 consumption inherent to sensing process; interference from NO or other molecule
O_2	[83]	110nA/mM	very low	10^3 X10^3	0-10.9mM	10min 95% response time, amperometric
CO_2	[83]	49mV/decade	0.083	10^4 X 10^4	0-8.2mMr	2 minute response time, Severinghaus-type, Al_2O_3 gate, insensitive to external pH changes
glucose	[88]	3.25 mV/mM	-	-	0-3 mM	1μm-thick membrane, gluconolactonase and glucose oxidase ratio 1:3.14, linear response
glucose	[89]	0.01mV/mM	low	400 X 50	6-28 mM	enzyme membrane containing immobilized glucose oxidase, cross-linked with albumin-glutaraldehyde, 4.7% variance
glucose	[90]	50mV/log mM	-	-	0.2-20mM	LaF_3 solid electrolyte, 1-2 minute response time
glucose	[91]	8mV/decade	-	500 x 20	0.2-2μM	SOS substrate, PVA matrix; 30 minute response time

is to use a generic ChemFET structure other than an ISFET to sense oxygen. In one design, a thin-film carbon paste membrane containing platinum and LaF_3 changes the threshold voltage of the conductive gate in proportion to oxygen concentration [85]. An iridium oxide gate has also been used for oxygen sensing, since the ratio of IrIII to IrIV depends on the oxygen concentration and also changes the work function [86].

To fabricate two-stage FET-based sensors selective to carbon dioxide, the most common method employed is to use a Severinghaus-type sensor. In this sensor, the formation of carbonic acid results in a pH change, which can be detected by the active area of the underlying ISFET structure. These sensors must be integrated with a perm-selective membrane, which can be made out of polysiloxane [87] or PTFE. The fabrication process for these sensors remains complex, and, at this time, in combination with slow response times (minutes), this sensor design does not demonstrate a clear advantage over other sensing methods.

The ChemFET structure has been combined with a two-step transduction process to sense glucose by depositing a glucose oxidase gate on top of the insulating layer and measuring peroxide-induced work function changes between the gate and insulator generated from the conversion of glucose to peroxide by glucose oxidase. Adding only glucose oxidase, however, generates nonlinearities in the sensor response to glucose and undesired interference with other components in the sensing solution. In order to increase the range of linearity up to 3-mM glucose, the membrane can be composed of a mixture of glucose oxidase and gluconolactonase, an enzyme which converts the glucose product from oxidase to B gluconic acid [88]; a 30-mM linear range is desirable for physiological applications and has been approximately achieved by limiting the diffusion of glucose into the sensor using albumin-glutaraldehyde [89].

The size of both FET-based chemical sensors is significantly larger than the size of most single cells. These sensors also require several minutes to stabilize, which may limit their use in some applications; subdevice resolution is helpful in achieving higher spatial resolution than the size of the device itself, but novel approaches to FET signal readout for chemical sensing have been limited. The potential to detect single cells with FET-based sensors has just begun to be explored. To demonstrate detection of cell metabolism with ISFETs, at least two different groups of researchers have cultivated monolayers of fibroblasts (3T6 mouse embryo and LS 174T human colon ardenocarcinoma) onto ISFET sensors and measured the regional acidification increase due to cell metabolism [92], [93]. They were able to detect changes in local pH (calculated to be approximately 0.1 pH/min), but this required using an unbuffered or lightly buffered medium and encapsulating the cells in a microchamber. For this setup to be feasible, the cells must tolerate an environment with a fluctuating pH, must withstand the shear forces of fluid flow through a small volume, and may also need to survive through oxygen or nutrient deprivation since these resources are very limited in such a small volume. This limits the application of ISFET sensors to sensing metabolism of robust cells. As individual sensors, these technologies are not yet competitive with electrochemical and optical sensor technologies.

However, ChemFETs have an advantage through the ease with which they can be integrated into sensor arrays to detect multiple analytes. Several research groups have demonstrated ChemFET arrays that can detect multiple metabolic products on a larger scale. For example, Arquint *et al.* demonstrated a combined blood-gas sensor to detect pO_2, pCO_2, and pH on a 10×10 mm chip [83] based on FET technology. Oxygen was sensed using a Clark-type amperometric electrode with a polyacrylamide hydrogel membrane to serve as the electrolyte. The carbon dioxide sensor was a Severinghaus-type sensor with an Al_2O_3 FET integrated with a polyacrylamide hydrogel layer containing bicarbonate solution. These two sensors are protected from the surrounding solution by a gas-permeable polysiloxane membrane. The pH level is detected with an Al_2O_3 ISFET in direct contact with the solution. A later example of an integrated gas sensor is the work by Lauwers *et al.* who integrated sensors using a standard 1.2-μm CMOS process to measure pO_2, pCO_2, pH, conductivity, and potassium on a single chip. The total size of the sensor was 4.11×6.25 mm. This sensor combined a Clark-type electrode to measure oxygen, a Severinghaus-type pH-FET to measure carbon dioxide, an ISFET integrated with a potassium-selective membrane to measure potassium, and three electrodes to measure conductivity [94].

C. Solid-State Approaches: LAPS

In the ISFET, the underlying sensing principle relies on the change in the surface charge of the gate, which changes the gate voltage and modifies the operating characteristics of the FET device. Surface charge changes can also be detected using light. In a light-addressable potentiometric sensor (LAPS), the change in surface potential is detected by measuring a photo-induced current, and the surface charge depends on the pH of the fluid containing cells. A thorough review of the principles underlying LAPS can be found in [95].

A commercial version of LAPS is available from Molecular Devices (Cytosensor Microphysiometer, Molecular Devices Corporation, Sunnyvale, CA). This device does not provide spatial resolution of cellular events, and recent research has been focusing on methods to modify this sensor to resolve spatial events. Two methods to provide spatial resolution are to scan a laser beam across the device or to create apertures or windows which limit the passing of light through the sensor. By using a scanning laser beam to form a conductivity image, only one light source is required and the spatial resolution of the image is high. Using this system, Iwasaki's group measured the pH gradient surrounding *E. Coli* colonies after 8 h [96]. The resolution that the LAPS microscope was experimentally determined to be 50 μm. One of the difficulties with any conductivity or pH measurement is that it takes several hours for cells to produce enough metabolites to significantly change the pH. In this experiment, 8 h were required before the colonies changed the conductivity above the sensing threshold. One disadvantage of using a scanning laser beam is that it requires a very long time to collect data from 128×128 pixels if a conventional rasterizing algorithm is used, since each pixel must reach equilibrium before a measurement can be taken. Two methods were used to speed up the data acquisition process. In one method, a dc bias was applied across the device, hence eliminating the need to wait for equilibrium during each measurement cycle; the measured ac photocurrent was proportional to pH. A 1% change in the voltage amplitude corresponded to a pH change of 0.025, and the noise was less than 0.01 pH. Using this system, each pixel could be measured within 10–20 ms [97]. In the other method, the silicon substrate was thinned to 20 μm, and the laser beam emitted infrared light. This new system had a spatial resolution of 5–10 μm, and collecting an image of 128×128 pixels required only 15 min. This system was used to measure *E. Coli* colonies, and it was again verified that 8 h were required before the pH changed above the sensing threshold. The sensitivity was determined to be 0.02 pH, but the sensitivity would have to be 10^{-3} pH units or less to detect single cells within a reasonable time window [98]. Also, cells would ideally be directly cultured onto the silicon surface of the LAPS device, and cell adhesion was shown to be further improved by roughening the silicon surface without loss of sensitivity [99].

The other option to obtain spatial information with a LAPS device is to fabricate apertures through which light can pass. Two approaches have been taken for this type of sensor. Bousse *et al.* fabricated multiple flow channels which could be measured independently using LAPS. A few thousand cells contained within a 0.3-μL chamber were successfully detected using this device [100]. Another approach is to have multiple light sources and matching apertures within the substrate. The prototype of this system had a resolution of approximately 3 mm, the distance between aperture windows [101]. One advantage of this configuration is that the light source can potentially be a light-emitting diode (LED) rather than a laser. LEDs are both more cost-effective and are more compatible with microfabrication processes. Furthermore, the speed of acquisition can be improved by modulating the light sources at different frequencies, and using a fourier transform to deconvolute the signals [102]. In both of these devices, the spatial resolution is limited by the size of the apertures or microfluidic channels, and it would be challenging to reduce the resolution of these devices to the single-cell level. The advantage that these devices have is the ability to detect chemical concentration gradients in the context of a simple and inexpensive fabrication process.

For laser-scanning LAPS systems, further developments in the LAPS conductivity microscope should improve the resolution of the sensor to below 5 μm. The outlook for single-cell detection with this type of sensor is promising, and the resolution of pH is well within the limits of those provided in Table I. The sensor also has a high potential to detect other analytes, since the surface can be either chemically modified or integrated with an ion-selective membrane to detect a particular metabolic analyte [103]. For instance, by immobilizing glucose oxidase onto the charge-sensitive surface, LAPS can be used to detect glucose concentrations [104]. It is also possible to integrate an electrode on the sensing surface to detect gases such as oxygen through the subsequent pH change [105]. By using clear conductive and semiconductive materials rather than a silicon substrate, a LAPS sensor could become optically transparent, making it possible to combine this type of sensor with optical (fluorescent) methods.

D. Other Solid-State Sensing Technologies

Other solid-state sensor technologies are available for detecting metabolites, but these sensor schemes are currently not suitable for single-cell detection due to size, detection range, or sensitivity incompatibilities. Recent research has focused on decreasing the size of these sensors, though, and some of these sensor classes may become compatible with single-cell detection in the future. Classes of sensors used to detect metabolites include resonant sensors such as the surface acoustic wave (SAW) and quartz crystal microbalance (QCM) sensors, conductivity or impedance sensors, and sensors with multiple transduction steps.

In resonant devices, the sensing principle relies on the chemisorption or physisorption of the analyte of interest onto the resonant structure. The amount of analyte sorbed onto the surface then changes the mass of the sensor, the permittivity of the material within the region through which acoustic waves pass, the viscosity, or the thickness of the sensor. Ultimately, these changes translate to a change in the resonant frequency or damping behavior of acoustic waves across the sensor. A review of the operating principles underlying the most common acoustic wave sensors can be found in [106]. In biological media, the usefulness of acoustic wave devices is limited by the selectivity of the coating on top of the resonant structure, as it is exposed to a wide variety of molecules that can interact with the surface. To date, surface acoustic wave (SAW) liquid sensors have been demonstrated to measure only the conductivity, density, and viscosity of an ionic fluid [107]. Surface acoustic waves are ill-suited for liquid solution-based problems because the acoustic wave is often damped or attenuated as it passes across the chemically sensitive coating, which decreases the sensitivity of the sensor. Bulk acoustic waves implemented in quartz crystal microbalance (QCM) sensors have been used to characterize the adsorption properties of biological molecules onto nonbiological substrates [108]; however, these sensors are also often unsuitable for sensing real-time changes of metabolites in liquids because of this same loss in amplitude and quality factor as SAW-based devices in liquids [109]. Alternatives to surface and bulk acoustic waves, such as shear horizontal surface acoustic waves (SH-SAW) that travel parallel to the sensing surface, rather than perpendicular, do not suffer as much attenuation and make better alternatives for liquid phase sensing. A review of these alternative acoustic wave sensors, including the applicability of the sensing technology to a liquid environment, can be found in [110]. In conclusion, in resonant transduction schemes, a tradeoff remains between sensor size and sensitivity which currently prohibits their use for single-cell detection.

Another approach to detect the metabolic activity of single cells is to measure the impedance change due to the generation of metabolic byproducts by the cell. By using microfabrication processes, it is possible to build arrays of electrodes that are small enough to be compatible with the size of single cells, but experimental evidence suggests that the time it takes to detect live cells depends logarithmically on the density of cells within a volume. For example, for single-cell detection in a 1-nl volume (equivalent to 106 cfu/ml), it requires approximately 2 h before the impedance change is large enough to be detectable [111]. Amplification of the signal or some other signal processing methodology may increase the sensitivity of impedance sensors so that the sensors are able to detect metabolic activity of single cells with a much shorter response time.

A third general class of solid-state chemical sensors for detecting metabolites are sensors that rely on multiple transduction schemes. Most of these sensors have the disadvantages that the signal is acquired over a large spatial area, and each transduction step is accompanied by some loss in signal strength, lowering the sensitivity. One novel example of this type of sensor uses hydrogel spheres that swell or shrink in response to carbon dioxide. These hydrogel spheres are embedded in a pressure sensor membrane, and a change in the CO_2 concentration results in a change in pressure [112]. Another example is the calorimetric detection of the oxidation of glucose by glucose oxidase. Although small changes in temperature can be thermoelectrically detected with a thermopile, the output from this sensor is still the summation of many chemical reactions occurring over a wide area [113].

IV. Applications: Single-Cell Detection and Analysis

There are a wide range of applications for sensors requiring the detection of concentration gradients of metabolites such as oxygen, carbon dioxide, and glucose. Although it is possible to use fluorescence methods to detect intracellular analytes, fluorescent probes are unsuitable for long-term use due to photobleaching and cytotoxicity. Injecting a cell with a fluorescent probe can also lead to experimental error, since biochemical mechanisms inside of the cell may interact with the probe. An intracellular probe also has disadvantages, since it can introduce experimental error due to the damage caused by the injection process [114]. Instead of detecting the intracellular concentration, information about metabolic processes can be obtained noninvasively by detecting the extracellular metabolite gradient. This gradient is related to the processes occurring inside of the cell, and advantages of sensing the external gradient include the ability to monitor metabolic activity for long periods of time and reduction of experimental error. Some of the biological applications for sensing the extracellular gradient of metabolic products include the study of disease, such as diabetes, cancer, and mitochondrial diseases, such as age-dependent infertility in women and Alzheimer's disease; cell and tissue differentiation; cell and tissue storage; cell life cycle and processes; and developmental biology.

A. Biological Applications: Understanding Disease

Approximately seventeen million Americans, or 6.2% of the population, suffer from diabetes [115]. To cure diabetes, it is necessary to understand the functioning of the β cells inside of the pancreas. Pancreatic β cells are responsible for releasing insulin in response to glucose influx. In these cells, glucose enters through a glucose transporter channel and is consumed by glycolysis. Through this process, NADH, ATP, and $FADH_2$ are produced, and the production of NADH increases the mitochondrial respiration, which in turn increases the intracellular ATP concentration. The increase in intracellular ATP results in the depolarization of the cell membrane via deactivation of potassium

channels, and the depolarization then opens voltage-gated calcium channels. The influx of calcium causes two competing effects: positive feedback effects such as the exocytosis of vesicles containing insulin (or insulin release), and negative feedback effects such as reduced glucose consumption and a change in mitochondria membrane potential. The result is that the metabolic cycle of β cells oscillates with a period of 4–13 min [116]. These oscillations are critical for insulin release, and questions to be answered include how this mechanism of insulin release breaks down in people with diabetes, how β cells synchronize their oscillations, and how to "repair" the oscillations or release of insulin through the addition of drugs. Islets of pancreatic cells mimic the behavior of the entire pancreas, and studying the islets *in vitro* can provide keys to understanding diabetes for the entire pancreas. These oscillations can be measured in real time using an oxygen-sensitive microelectrode, since microelectrodes respond fast enough to detect the oscillations. Jung *et al.* have developed a microelectrode system that can detect both the gradient from the entire islet as well as metabolic activity of single cells using a microelectrode [117]. Using this system, they have taken the first steps to correlating extracellular signals with insulin release by measuring the change in oscillation frequency due to the external glucose concentration. A combination of sensor technologies, for instance studying metabolic changes along with fluorescence from calcium, promises to give clues to the mechanisms behind cell synchronization and mechanisms of breakdown.

Diagnosis of diabetes may also be improved using sensors that can detect a concentration gradient. One of the symptoms of diabetes is the increase in acetone in the patient's breath. In an attempt to develop a noninvasive method to detect blood glucose levels, Zhang *et al.* developed a LAPS sensor that detected the concentration gradient of acetone from a patient's breath [118]. With improvements in repeatability and selectivity (or detecting another analyte that is also tied to diabetes), this type of sensor could provide a method to diagnose diabetes or measure blood glucose levels noninvasively.

Mitochondrial-based diseases such as age-dependent infertility in women and Alzheimer's disease can also benefit from sensors that monitor the extracellular gradient of metabolites [119]. There is some evidence that tissues within the brain degenerate in Alzheimer's patients due to impaired energy metabolism or oxidative stress. These types of studies have been conducted mostly *in vivo* or in brain autopsies, but there has been a recent push to study mechanisms of neurodegeneration *in vitro* [120]. Experiments would require long-term monitoring of metabolic processes in order to detect eventual neuronal tissue damage and loss of function and real-time monitoring of metabolic activity enables the monitoring of the effect of drugs that could potentially be used to counteract the effects of mitochondrial diseases.

For the study of cancer, the application of these sensors is in finding better chemotherapy treatments. SECM has been shown to be capable of quantifying the effectiveness of anti-cancer drugs on specific types of cancer cells by monitoring the effect of the drug in real-time, and this type of system can be used to evaluate new drugs for treatment of specific forms of cancer [121], [122]. Quantification of the effect of anti-cancer drugs can lead to more effective chemotherapy that is less damaging on the patient, leading to a faster recovery and shorter hospital time.

B. Biological Applications: Cell and Tissue Differentiation

The structure of cells varies widely depending on the type of organism from which it comes, and even cells within the same organism can differ widely. The most simplistic example of this is a comparison between plant, mammalian, and bacterial cells. While mammalian cells only have one membrane layer separating the cell from the external environment, plant and bacterial cells have two membranes (one of the membranes being the cell wall for plant cells). One question is how these differences affect the chemical gradients surrounding the cell. To date, the approach taken by researchers is to assume that charge transfer across the membrane is an indication of redox reactions occurring inside of the cell [123]. In a study by Mirkin's group, human breast cell redox reactions were studied using a SECM, which obtained topographic images and maps of chemical reactivity on the surface of the cell as well as measured the oxygen flux at the cell membrane. Redox reactions were detected by adding redox mediators and detecting the subsequent change in microelectrode current. SECM has also been successfully applied to the problem of differentiating between cells. Although this is a new field, one research group has already demonstrated the ability to differentiate between normal human breast epithelial cells and metastatic breast cancer cells using SECM [124]. Normal and cancerous cells can be differentiated even if the cells are closely packed in a monolayer culture [125]. Efforts were made to detect the membrane permeability differences between bacteria and mammalian cells in the presence of redox mediators, but more work needs to be done before there is a conclusive result [126].

Understanding tissue structure and differentiating between tissues is another application that would be enhanced by information obtained from instrumentation that resolves spatial differences. Although there are a wide range of potential applications, there is one application that has been studied extensively by these new technologies. Localized porosity of skin can be determined by scanning a tissue sample with SECM [127]. Transport across skin usually occurs at localized regions such as hair follicles and sweat glands, and the size of these openings are usually on the order of tens of microns, which is slightly larger than the resolution capabilities of SECM. Transport across skin also depends on any applied electric field and the concentration of ions, and these effects can be quantified using SECM [128]. Understanding the mechanism of transport across skin has applications in improving the efficiency of transdermal drug delivery, especially for iontophoresis, in which transport of the drug is assisted by an applied electric field.

C. Biological Applications: Cell and Tissue Storage

Medical implants, experimental biology, and the food industry often rely on the ability to store tissues and cells for a long period of time without losing tissue function. Some of the parameters that should be experimentally determined include the ideal temperature at which to store tissues, the optimum

time to freeze the sample after it has been extracted, and the best storage medium. The applications for cell and tissue storage is broad, ranging from organs [129] to stem cells [130] to yeast in bread [131]. Many of the applications in the food industry are focused on protecting food from being poisoned by salmonella, *E. coli*, and other bacterial pathogens during storage [132]. For all of these applications, viability of the cells after revival is determined in part by measuring the metabolic activity of the cells, usually by measuring the oxygen consumption [133]. Instruments such as LAPS and SECM provide a noninvasive way to quantify the metabolic activity of tissues and cells, and these devices are especially well-suited for these applications in which the sample will be used in further experimentation.

D. Biological Applications: Cell Cycle

As single-celled organisms grow and replicate, they progress through a series of steps in their development called the cell cycle. The cell cycle can be separated into a dividing phase called the M or mitotic phase and an undividing phase called interphase. The interphase can be further separated into three different phases: the first gap phase during which the cell grows, the S phase during which DNA is synthesized to make a copy, and a second gap phase right before cell division. Gap phases are necessary for the cell to generate excess cytoplasm, replicate mitochondria and other organelles, and prepare for cell division. During each phase, the energy requirements of the cell differ, and progression through these stages has been shown to be linked directly with the amount of nutrients available to the cell [20].

For any given population of cells, information that depends on the cell cycle is averaged out across the population, since the cells will be at different stages of the cycle. Elaborate schemes to synchronize the cell cycle in a population of cells have been developed. One of the most popular schemes is the "baby machine." In this cell cycle synchronization scheme, bacterial cells are immobilized in a single layer on a substrate. As these cells replicate, they release the new cell into the fluid above the substrate. By flowing fluid over the substrate to wash away the new cells, the cells at any given location are then approximately within the same stage of cell development [134]. Although this scheme is reasonably successful for synchronizing cells (as long as they are not stressed in any way), a simpler and more precise approach relies on the ability to detect activity at the single-cell level. By detecting metabolic activity at the single-cell level, it can be determined how the cell cycle changes in response to environmental conditions [135]. By detecting single cells, questions can be answered to a high level of precision, such as how long cells will continue to grow if the nutrients are removed suddenly or how different cells respond to the same external stimuli.

Another basic cell mechanism that can be studied using imaging instrumentation is the effect of the boundary layer at the membrane of the cell. The boundary layer is a gradual change in the concentration of a given analyte that occurs close to the surface of the cell. For example, a cell may actively or passively transport glucose into the cell, depleting the region directly outside of the cell of glucose. In this case, the glucose influx depends on diffusion of glucose from the surrounding bulk fluid into the boundary layer. Bulk concentration measurements of the cell environment do not give any information about the boundary layer or the true chemical gradients across the cell membrane. By using electrochemical microprobes, it is possible to directly measure the boundary layer at the membrane wall and to extract information that could determine the precise transport mechanisms across the cell membrane [136].

E. Biological Applications: Developmental Biology

A simplified development pattern for animals consists of the following steps: 1) egg fertilization, 2) cleavage, in which the single-nucleus embryo divides without growing into a multinucleated embryo called a blastula, 3) gastrulation, in which furrows form on the blastula and determine the anterior/posterior orientation, and 4) differentiation, in which cells become different from each other to form functional tissues. Depending on the organism, the energy to develop may come from a yolk that was initially deposited inside of the egg, or the embryo may rely solely on another organism for all of its nutritional requirements. Some questions to be answered include how much energy is required for an egg to be fertilized and for an embryo to develop into an organism, when cells begin to differentiate, and how the energy requirements of each type of tissue changes.

One of the applications of studying the developmental metabolics is to increase the chances of conceiving for females who are suffering from reproductive complications, or for livestock or other animals. This type of experiment requires that the sensing technology is noninvasive to ensure that no damage is done to the embryo. SECM is a promising sensing technology for such an application, and Shiku *et al.* have recently applied SECM to measure the oxygen consumption of single bovine embryos [137].

V. Summary

There is an ongoing effort to develop new sensing technology to resolve metabolic activity at the single-cell level. In this article, solid-state sensing technologies, such as ISFET and ChemFET-based chemical sensors and LAPS, have been reviewed. Each sensor technology is reviewed in the context of providing spatially-resolved information at a resolution that is comparable to the dimensions of a single cell. The analytes of interest included small molecules that are integral to cellular metabolic activity, including glucose, oxygen, carbon dioxide, ATP, NADH, and $FADH_2$. Current biological applications of these sensor technologies have also been summarized. Because fluorescent probes are unsuitable for long-term real-time experiments due to photobleaching and cytotoxicity, the focus of the biological applications is on extracellular methods to detect metabolites using solid-state, electrochemical, and colorimetric sensors. Further improvements in sensor materials, fabrication processes, and detection methods will broaden the availability of small sensors, reduce the response time, and lower the cost, increasing the compatibility of these sensors with single-chip analysis systems.

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