Sensor Technologies for Monitoring Metabolic Activity in Single Cells—Part I: Optical Methods

Rachel A. Yotter, *Member, IEEE*, Linda A. Lee, and Denise Michelle Wilson, *Member, IEEE*

***Abstract*—A review of optical, chemical, and biological sensors to detect metabolic activity at the single-cell level is presented in the context of the development of lab-on-a-chip research instrumentation. The sensors reviewed include optical sensors, at both research and commercial levels, that can optically detect intracellular metabolites including adenosine triphosphate, nicotinamide-adenine dinucleotide, reduced flavin adenine dinucleotide, and other metabolites, including oxygen, carbon dioxide, and glucose. Methods to optically detect pH changes which are a general indicator of activity in extracellular space are also briefly reviewed. Performance metrics such as sensitivity, sensor size, drift, time response, and sensing range are included when available. Highly suitable optical sensor technologies for monitoring cellular metabolic activity include luminescent (fluorescent, phosphorescent, and chemiluminescent) and colorimetric optical probes. Different approaches to extracting luminescent and colorimetric information are reviewed, including benchtop techniques, fiber-optic approaches, and the use of probes encapsulated by biologically localized embedding. A brief discussion of alternate optical sensor technologies, such as surface plasmon resonance and infrared absorption spectroscopy, is also presented.**

***Index Terms*—Absorption spectroscopy, carbon dioxide sensors, cell metabolic activity, chemiluminescence, colorimetric sensors, dissolved oxygen sensors, evanescent wave sensors, fiber-optic chemical sensors, fluorescence, luminescence, optical sensors, probes encapsulated by biologically localized embedding (PEBBLEs), pH sensors, phosphorescence, single-cell detection, surface plasmon resonance.**

I. Introduction

OPTICAL chemical sensors used to detect single-cell events must meet performance requirements that are much more stringent than for other sensors used in more common applications. Foremost is the need for an optical sensor that is both highly sensitive and selective to the analyte being detected. If the optical sensor responds to other analytes, the signal from the analyte of interest can be lost due to interference from other false chemical signals. Other factors involved in optical-sensing system design include minimizing the interference of optical tags with biological parameters and events, matching the analyte absorption or emission to the sensing range of the optical sensor, deconvoluting the optical signal, and transducing the optical output to an electronic output. Furthermore, single-cell detection has the added requirement that events separated by subcellular dimensions must be spatially resolved within the sensing instrument.

For single-cell detection, two popular classes of optical chemical sensors are used to study events in living cells. These optical chemical sensors include luminescent (fluorescent, phosphorescent, and chemiluminescent) sensors and colorimetric sensors. The chemical behavior of the two types of optical sensors differ in that luminescent sensors absorb and then emit photons, while colorimetric sensors only absorb photons (thereby changing color). Luminescent sensors have the advantage that they can be placed directly within the cell to detect intracellular conditions. They can also be made selective to a virtually unlimited array of macromolecules (proteins) and some small analytes. However, luminescent sensors have limitations that influence their performance and capability both outside (extracellular) and inside the cell (intracellular). Photobleaching both inside and outside a cell results in signal attenuation proportional to the time and intensity of exposure to an excitation light source. This is an issue that can be resolved through such engineering solutions as pulsed sample/light excitation, improved optical coupling (to reduce the required amount of excitation), and alternative measurement approaches such as luminescence lifetime detection. A primary obstacle involved in placing luminescent tags inside a cell for intracellular measurements is the interaction of the luminescent agent with cellular components and processes. In the worst case, these interactions are cytotoxic, resulting in a decrease of cell viability; other nonideal interactions between luminescent agents and intracellular components include the disruption of metabolic and other cellular processes, partial degradation or denaturation of luminescent tags induced by cellular dynamics, and influences of cellular components that attenuate luminescence (e.g., temperature, pH, and heavy metal ion concentrations). In many cases, overcoming these obstacles to intracellular introduction involve the reengineering of the luminescent agent or the use of another luminescent agent altogether. Some potential solutions include creative modification of the biological experiment to mark an alternative (and, perhaps, secondary) cellular component using a different fluorescent marker (such as lipids, nucleic acids, organelles, etc.), using a chemical amplification scheme to increase the optical output signal, and using caged luminescent probes which are activated (or released) only within a localized region that is initially optically excited. If the luminescent probe leaks from the cell under study, the effect is an increase in the noise of the system. The leaked probe increases the background light level so that it is more difficult to resolve low-signal events inside of the cell. This effect can be eliminated by introducing a

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The authors are with the Department of Electrical Engineering, University of Washington, Seattle, WA 98195-2500 USA (e-mail: yotter@ee.washington.edu; leeli@u.washington.edu; denisew@u.washington.edu).

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TABLE I
COMMON CELL METABOLITES AND RELEVANT SENSING METHODS

Metabolite or Metabolic By-product	Detection Range of Interest	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	0.1-10μM 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 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

second luminescent probe with a different nonoverlapping excitation profile, photobleaching the original dye and re-introducing a probe, or restarting the experiment to restore leakage to its original minimum.

Unlike their luminescent counterparts, colorimetric sensors involve only the absorption of photons of light rather than both absorption and emission of photons. For this reason, they do not suffer as substantially from photobleaching as luminescent tags, nor do they typically leak or significantly affect cell viability, in and of themselves. Colorimetric absorption characteristics change as function of changing concentration of an analyte of interest. They are most often bound to the tip of a fiber-optic cable through either chemical or physical means. These fiber-optic probes are then used to measure both extracellular and intracellular analyte concentrations through the use of tapered fiber-optic tips (less than 20-nm diameter). The control and placement of the probe at this size is the most significant barrier to intracellular monitoring. In general, the colorimetric sensors do not impact cellular processes and conditions as significantly as luminescent probes. For this reason, even though colorimetric sensors are not as popular or as varied as luminescent means, barriers to their introduction to intracellular monitoring are not as significant.

Other optical-sensing methodologies appropriate to monitoring cellular metabolic activity are the evanescent wave sensors which include surface plasmon resonance sensors and infrared absorption spectroscopy. Although these optical methods have several advantages not available with luminescent or colorimetric sensors, the technology has not developed with these sensor types to the point that they can resolve events within a single cell. Nevertheless, these popular optical-sensing technologies are also discussed briefly in this review, with an emphasis on the barriers to their use in single-cell detection.

The analytes emphasized in this review of sensing technology are related to the metabolic activity in single cells (Table I). Fluorescent means of detection dominate the monitoring of intracellular events because fluorescent probes can be easily injected inside cells for sensing purposes. The detection of pH is also reviewed, since it is a general indicator of metabolic activity in the extracellular space.

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.

To understand the behavior 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. Some 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.

The recent development of microelectromechanical systems (MEMS) technology has improved detection technology of events at the single-cell level. The size scale of MEMS is

compatible with the size of cells, which enables the precise manipulation of biological material down to the single-cell or single-molecule level. Advances in microfluidics, a subset of MEMS technology, is also capable of maintaining cell cultures in an automated way. Microfluidic pumps can often help deliver a controlled and steady amount of oxygen and nutrients to cells, maintaining cell viability over a longer period of time. Microfluidics also reduces the amount of waste products for chemical interactions, an advantage often exploited by micro-total-analysis systems (microTAS) or lab-on-a-chip devices, which are small systems which aim to integrate an entire chemical experiment onto a single chip. Through soft lithography and other polymer microfabrication methods, a wide variety of new devices have emerged. The polymers are often transparent, increasing compatibility with optical detection methods. These surfaces are either intrinsically biocompatible, or they can often be modified using simple procedures to create stable biocompatible substrates.

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. 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. 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 & 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, Dr. Virta's group at the University of Turku, the Weijer Lab at University of Dundee, and Molin's Molecular Microbial Ecology group at the Technical University of Denmark. The Saykally Group at UC Berkeley is studying biochemical properties of individual organelles in living cells, which required single-cell measurements of live human lung cells. Dr. Edward Yeung at Iowa State University develops analytical and fluorescent methods for use with microscale 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. Nancy Xu, at Eastern Virginia Medical School, to study living systems at the single-molecular level in real time. D. Discher's lab 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), nicotinamide-adenine dinucleotide (NADH), and reduced flavin adenine dinucleotide ($FADH_2$), and pH, a general indicator of cellular metabolic activity. Possible optical measurement techniques that can be used to analyze these metabolites, at a microscale resolution are reviewed here and include luminescent spectroscopy, colorimetry, surface plasmon resonance and infrared spectroscopy.

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 [16]. Significant differences occur among cells, species, and plant and animal; 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 by- products 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, 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, by-products 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, $FADH_2$: 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.

The last two elements are associated with cells that do not produce fuel predominantly by respiration (transferring electrons from glucose to oxygen) and are outside the scope of this paper. All other elements are taken into consideration in the following review of optical microsensing technology applied to monitoring the metabolic activity of cells.

IV. Optical-Sensing Technologies

This section provides an introduction to the most viable optical sensor technologies for cell analysis (*in vitro*). Basic transduction mechanisms are discussed here, along with advantages and disadvantages as applied to intracellular and extracellular detection of analytes at concentrations relevant to single-cell analyses (refer to Table I). Many types of optical sensors are available to detect essential analytes that are present in cellular metabolic processes. While recognizing that sensor technology continues to diversify at a rapid rate, a selection of sensors is discussed here to describe the design, method, and application of recent optical technologies that are most viable to the life-on-a-chip problem. Sensors reviewed in this section include both research and commercial devices that can optically detect intracellular metabolites (ATP, NADH, and $FADH_2$), extracellular metabolites (oxygen, carbon dioxide, and glucose), and changes in pH.

A. Luminescence

Luminescence is the process by which a molecule or compound emits light in response to an input stimulus. In some cases, such as the formation of the burning flame or the characteristic glow of the firefly, a chemical reaction causes the emission of light, a process called *chemiluminescence*. The reactions of many molecules that cause them to emit (output) light in response to the absorption (input) of light are processes categorized as *fluorescence* (instantaneous emission of light) or *phosphorescence* (longer lifetime emission). By far, fluorescence is the most common means by which luminescence is used to analyze biological analytes because it is most readily manipulated to behave in a way that is associated with an analyte or event of interest and to emit light in a way that is sufficiently measurable.

The dividing line between fluorescence and phosphorescence is not well defined because some molecules, like transition metal-ligand complexes, emit light via both phosphorescent and fluorescent means, leading to "moderate" emission lifetimes. Typical fluorescence lifetimes are on the order of tens of nanoseconds (10^{-9}), while lifetimes for phosphorescence are much slower, on the order of milliseconds to seconds (10^{-3} to 10^{0}). The determining factor between fluorescence and phosphorescence is the nature by which electrons, excited by impinging light, return to their ground states. In fluorescent events, the electron in the excited state (or orbital) is paired to a second electron in the ground state orbital that has opposite spin to the excited electron. This situation is called an excited single state, and return of the excited electron to its ground state orbital is spin-allowed (because the paired electrons are of opposite spin) and occurs rapidly, accompanied by the emission of a photon. During a phosphorescence event, however, light is emitted through a situation called a triplet excited state; the electron is excited by impinging light into an excited state of the same spin as its paired electron in the ground orbital. Transition of the excited electron back to the ground state is forbidden, causing emission of light via this mechanism to be very slow. Fluorescence, since it is an "allowed" process at the electron transition level, is inherently more sensitive than phosphorescence, and it is by far the more common means for identifying parameters associated with cell expression and activity. Molecules which exhibit fluorescence are called fluorophores.

Fluorophores can be divided into three categories: *extrinsic, intrinsic, and indicator*. *Intrinsic* fluorophores occur naturally; in other words, the molecule of interest fluoresces of its own accord. Intrinsic fluorophores include such molecules as NADH (a coenzyme active in many cellular reactions), flavins (related to vitamin B2), and elastin and collagen. Although fluorescence in the molecule of interest is useful, it is rarely as sensitive as an extrinsic fluorescence unit. A fluorophore, when it is attached to a molecule of interest rather than being of interest in and of itself, is called *extrinsic*. A very common extrinsic fluorophore is green fluorescent protein (GFP) which is derived from the jellyfish *Aequorea victoria*, and is available in several colors (green, blue, yellow, and cyan) at varying levels of stability and sensitivity to environment interferents (e.g., pH and temperature). Extrinsic fluorophores (reporters and probes) tend to be more sensitive than intrinsic fluorophores but are not as reliable, since their properties can sometimes change independent of the molecule to which they are attached. Fluorophores, whose fluorescent properties (emitted light intensity) change as a function of what the fluorophore is doing (e.g., binding to another molecule) rather than to concentration alone, are called *indicator* fluorophores. Of the three fluorophore types, the *extrinsic* fluorophores which are attached to analytes of interest, in a highly specific manner, are the most popular choice for monitoring biologically processes [17].

The first step in designing a viable, useful fluorescence analysis system for the analysis of living cells is selection of an appropriate fluorophore, which can attach to or detect the molecule of interest. At its most basic level, a fluorophore must be designed so that it both targets the molecule of interest appropriately and also generates a response proportional to the appropriate molecular properties, such as concentration. *Targeting* involves not only attaching the fluorophore to the molecule of interest, but doing so in such a way that the attachment does not interfere with the normal operation of the molecule in the cellular system. While the fluorophore should not interfere with the molecule of interest, it should generate a *response* proportional to the molecule to which it is attached or to some behavior of the molecule. Once the fluorophore is targeted to a molecule of interest and has an appropriately designed response, additional complications are introduced by the intent to extract a fluorescent response while the fluorophore is part of a functioning cellular system. *Delivery* of the probe to the targeted molecule must occur in a way that no major damage or mutation of the physiological and structural properties of the cell occurs. The fluorophore, especially when attached to molecules that undergo metabolic changes, must be appropriately designed to indicate *state specificity* as well as *type specificity*. For example, a molecule that has been metabolized by a cell may no longer be of interest, yet its fluorophore may still be present, generating a false

TABLE II
OPTICAL-SENSING INSTRUMENTS APPROPRIATE FOR SINGLE CELL ANALYSIS

Instrument/Device	SNR	λ Range	Light Source	Single Cell Application
Fluorometers				
Hitachi F-2500 [21]	450*	220-730 nm	150W Xenon	-
Oceanoptics SF 2000 [22]	250*	360-1000 nm	Pulsed Blue LED	-
PTI QM-1[23]	1000*	200-2000 nm	Pulsed Xenon	Intracellular NADH [24] Intracellular O_2 [25]
Shimadzu RF 5301PC [26]	150*	220-900 nm	150W Xenon	pH [27][28]
Shimadzu RF 1501PC [29]	300*	220-750 nm	150W Xenon	-
Varian Cary Eclipse [30]	750*	190-1100 nm	Xenon Pulse (80 Hz)	pH [31]
Spectrophotometers				
Hitachi U-2810** [32]	Unspec SPD	190-1100 nm	Not provided	pH [33]
Perkin Elmer Lambda 650** [34]	See R-955	190-900 nm	Not provided	CO_2 [35]; pH [31]
Shimadzu UV-1601 PC [36]	Unspec SPD	190-1100 nm	50W Halogen	pH [37]
Shimadzu UV-2401 [38]	See R-928	190-900 nm	50W Halogen	-

- *SNR given with reference to the Raman scattering of water
- **Specifications for instrument in referenced literature not available; latest generation provided.

reading and increasing the noise floor of the system. In addition, as the fluorophore becomes part of a sensing system, *matching* to spectroscopic capability of the instrumentation must be considered; detection capability must be matched to the spectral emission of the fluorophores. Finally, *deconvoluting capacity* in the signal processing of fluorescence signals has to be taken into account when monitoring multiple-complex cellular interactions, so that the emissions from multiple fluorophores can be discriminated at maximum resolution and sensitivity [18].

Other means for using fluorescence as a sensing mechanism have been explored including the use of fluorescence resonance energy transfer (FRET), inorganic nanoparticles, two-photon fluorophores, and probes encapsulated by biologically localized embedding (PEBBLEs). FRET is a fluorescent means by which interactions between different cellular units, such as protein-protein interactions, can be measured. It involves the use of a Forster excitation energy transfer from a donor (a higher energy molecule) to an acceptor (a lower energy molecule). The distance between the acceptor and donor affect the emitted fluorescence, thus enabling the detection of interactions between acceptors and donors (e.g., certain proteins form FRET acceptor/donor pairs) [19]. Certain inorganic nanoparticles are also of interest in fluorescence spectroscopy because of the ease in which their emission spectra can be manipulated. For example, the size of semiconductor quantum dots consisting of nanoparticles of CdS and CdSe can be increased to increase the corresponding emission wavelength during fluorescence. Similarly, the addition of light from a CW laser (exciting at 974 nm) in addition to a standard excitation source can increase the emission wavelength of rare-earth doped nanocrystals. Certain fluorophores, both organic and inorganic, are capable of emitting at two primary (peak) wavelengths, thereby enabling less filtering in the emission path in order to remove the excitation signal from the emission signal. The corresponding reduction in optical losses and increase in signal-to-noise performance allows these two-photon fluorophores to be used at lower intensities of excitation and lower concentrations of fluorophore which increases cell viability [17].

PEBBLEs address a particular concern in the systematic monitoring of cell activity where the shear number of fluorophores required for measuring intracellular activity can impact cell viability. Fluorophores, when inserted into the cell as a dye, often leak out of the cell, inadvertently bind with proteins (causing performance degradation), or contribute some toxicity to cell viability. An optrode, or matrix of fluorophore, and a protective coating is an ideal solution to this problem, since the optrodes allow the intracellular analyte of interest to penetrate the matrix while preventing the fluorophore dye from leaking out into the cell. Until recently, however, optrodes were too large and bulky to be effectively inserted into cells for systematic monitoring of multiple analytes. The PEBBLE has addressed this limitation by making optrodes that are 20–200 nm in diameter, a size compatible with multiple-optrode insertion into living cells without detrimentally impacting cell viability. Fluorophores are integrated and entrapped into a polyacrylamide matrix and then injected into cells where they can be analyzed using standard fluorescent spectroscopy instrumentation [20].

Whether by conventional or by modified means discussed above, fluorometry is a very common and attractive technique for biological analysis. Fluorescence is most commonly measured with specialized instruments called spectrofluorometers, with more generic laboratory instruments for measuring properties of light called spectrophotometers, or with custom testbeds including fluorescent microscopes, specially designed optics, and highly sensitive photodetectors. A sampling of benchtop instruments and associated photodetectors appropriate for detecting cellular metabolite concentrations at single-cell levels are summarized in Table II and Table III. Because single-cell analyte detection limits are not explicitly correlated to instru-

TABLE III
PHOTODETECTORS APPROPRIATE FOR SINGLE-CELL ANALYSIS

Product	ENI	Gain	Sensitivity	Application/Instrument
Hamamatsu R-928 PMT [39]	1.3 x 10^{-16}W	1.0 x 10^7	7.4 x 10^5A/W	Shimadzu 2401 PC
Hamamatsu R-955 PMT [39]]	1.3 x 10^{-16}W	1.0 x 10^7	7.4 x 10^5A/W	Perkin Elmer Lambda 650
Hamamatsu R-647-04 PMT [39]	Not provided	2.2 x 10^6	80 mA/W	Intracellular ATP[9]
Roper Scientific 256B CCD [40]	7e- rms @100 kHz	0.5, 1x, 2x	Not provided	Intracellular pH [41]

ment specifications, each type of instrument is correlated to one or more efforts in the research literature that have successfully used that instrument in an application relevant to single-cell metabolite detection. Other similar instruments that demonstrate comparable performance [especially signal-to-noise ratio (SNR)] are also summarized to provide the reader with a guideline for appropriate instrumentation for measuring fluorescence at single-cell levels. The SNR compares the clarity of the desired signal to the interference from certain standard signals, classified as undesirable (noise). Unwanted interference in a sample medium of cells may include detection from other fluorescing molecular compounds, residual products, chemical reactions, autofluorescence, or other cellular components. Signal-to-noise level in spectrofluorometers is calculated by dividing the lowest measurable signal by the (noise) spectrum generated from Raman scattering of water. Signal-to-noise levels in spectrophotometers are characterized by the detection limits of their photodetectors; photodetector detection limit is expressed as noise equivalent power (NEP) or equivalent noise input (ENI), which is the amount of input (optical) power required to produce an output signal level comparable to the noise intrinsic to the photodetector itself.

Other instruments have been demonstrated for applications in single-cell detection and analysis; however, specifications were not available. These instruments are listed below for reader reference.

- Unspec SPD: Unspecified silicon photodiode used as photodetector (SNR not available).
- Spectrophotometers: Comspec 2H [31], Genesys 5 [42], Perkin Elmer Lambda 3 [35] and Lambda 16 [31], Phillips PU 862 [43] and PU 8750 [37], [43], Shimadzu RF 5001 PC [27], and Unicam 8625 [35].
- Photodetectors: Hamamatsu H5701-02 [44], Hamamatsu H5702-50 [45] and R-268 [8], Olympus LX-70 Fluorescence Microscope [25], PTI R1527P [24], and Zeiss Axiovert 100 A [9].
- Other: Monochromator Monospec18 ThermoJarrel and Oriel n1925 Ash Photodiode [46].

The effective signal to noise performance of an optical analysis (spectroscopic) instrument is not a simple expression of the limiting (highest) detection limit of any of the system components; rather, it is a more complex function of the matching of system components, across wavelength, noise performance, and other parameters. The following discussion addresses briefly factors which influence the amount of light delivered to the photodetector in the system; efficient delivery of signal (light) to the photodetector is essential for single-cell applications.

An analysis system using modified or natural fluorescence, consists of an excitation (light) source, an excitation optical path, a sample micro environment, an emission optical path, and a photodetector. The light source can be a polychromatic source such as a xenon lamp, a laser, or a narrow-band device such as an LED (light emitting diode). Except in the case of a laser, the light emitted from the source is typically filtered to extract desired spectral bands. Light is collected, focused, transferred (using appropriate lenses), and filtered along the input (excitation) and the output (emission) optical path to reduced the influence of unwanted light. At a particular wavelength λ_{i}, the maximum possible light that is transmitted through the sample, rather than absorbed by fluorophore in the sample can be expressed, according to Beer's Law, in terms of the extinction coefficient $\varepsilon(\lambda_{\mathrm{i}})$

$$I_{L\_\max}(\lambda_i) = I_{IN}(\lambda_i) \times \left((10)^{-\varepsilon(\lambda_i)cL}\right) \times F_L(\lambda_i) \times A_L \quad (1)$$

where $I_{\mathrm{in}}(\lambda_{\mathrm{i}})$ is the total excitation light (Watts) impinging on the sample at wavelength λ_{i}; $\varepsilon(\lambda_{\mathrm{i}})$ is the extinction coefficient (liters moles^{-1} cm^{-1}) at wavelength λ_{i} and is given by the excitation spectrum for the fluorophore (it is essentially the efficiency with which light is absorbed by a fluorophore and emitted as fluorescence); c is the concentration of fluorophore in the sample (moles per liter); L is the sample path length (cm); $F_L(\lambda_{\mathrm{i}})$ accounts for filters in the excitation optical path; and A_L is an attenuation factor which accounts for losses in the optical path as well as pH, temperature, heavy metal ion concentration, and other components of the sample that absorb/interfere with emitted fluorescent light before it reaches the output signal path. Optical losses are typically 8% per lens used in the system and can be substantially reduced by the use of fiber optics rather than conventional optics. Light lost through filtering $[F_L(\lambda_{\mathrm{i}})]$ can be restored through the use of alternative waveguides (e.g., liquid) that collect light orthogonal to the sample rather than axially, providing a means for emission light to reach the detection stage independent of excitation light. This approach drastically reduces the need for optical filtering but typically exposes a smaller amount of the sample volume to excitation, hence reducing the overall intensity of fluorescence available for collection at the back end of the system.

Emitted light from the fluorophore is attenuated further once it reaches the emission path by additional optics losses, as well as the scattering of light and imperfect collection efficiency. The emission optical filters along the output path, designed to eliminate excitation light that leaks through to the output path, cause

even further losses. When it finally reaches the photodetector in the system, the total light emitted by the fluorophore, I_{E} is the light absorbed by the fluorophore attenuated by a factor A_{Q}

$$I_E = (I_{IN} - I_{L\_\max}) \times A_Q \tag{2}$$

where the factor A_{Q} includes the effects of attenuation in the sample and imperfect optical collection efficiency along the output optical path. Optical losses included in this attenuation factor can be reduced in ways similar to the excitation path. The total emitted light is then distributed across wavelength λ_{i} according to the emission spectrum of the fluorophore (the area under the emission curve is equal to I_{E}) to arrive at the emission intensity at each wavelength $I_{\mathrm{E}}(\lambda_{\mathrm{i}})$. Finally, the photodetector has its own spectral properties $[F_{\mathrm{P}}(\lambda_{\mathrm{i}})]$ and inherent attenuation that impact the fluorescence signal before it is converted to its final electrical form for analysis. In its final form, the output signal of a fluorescence analysis system can be generally expressed as

$$\begin{aligned} J(\lambda_i) &= J_E(\lambda_i) + J_L(\lambda_i) \\ &= F_E(\lambda_i) F_P(\lambda_i) \times A_P \times (I_E(\lambda_i) + I_L(\lambda_i)). \end{aligned} \tag{3}$$

The variable J is used to express current density in order to avoid confusion with the intensity variable I and also to express current independent of photodetector area. J_L is the current density in the photodetector due to leakage light (from the excitation process) and J_E is the current density in the photodetector due to emitted light from the fluorophore under analysis. The photodetector area can be broadened to increase the gain factor A_{P} which represents the loss of light at the photodetector due to reflection, limitations in quantum efficiency, and other attenuating factors. The total current J extracted from the photodetector is the integral of all components $J(\lambda_{\mathrm{i}})$ across wavelength. The spectral properties of the photodetector must be matched to both the detection limit and the spectral properties of fluorophore emission. Simple selection of a photodetector according to detection limit is ineffective since the specifications provided by a manufacturer are not necessarily extracted at the wavelengths of interest to the analysis of particular fluorophores.

Fluorescence is highly sensitive, as inherently 50% to 80% of absorbed light is converted to emitted light; however, as can be seen from the above analysis, the fluorescence instrumentation, optical path, and filter losses as well as photodetector limitations can drastically reduce the efficiency of output emission detection. Despite such losses, the high power excitation sources (50–450 W) of benchtop systems still provide a level of sensitivity at least two orders of magnitude above comparable optical techniques such as absorption spectroscopy (refer to Table II).

In conjunction with the engineering of a wide variety of extrinsic fluorophores, the availability of commercial instruments has contributed to the explosion in the use of fluorescence to analyze biological systems. Fluorescence analysis (fluorometry), however, does have some fundamental limitations. Fluorometry, by definition, is not compatible with compounds that are not naturally fluorescing or are incompatible with fluorescent reporters. In addition, biological units that are tagged with fluorescent reporters are dynamic systems; changes in the biological unit are often not communicated or imperfectly communicated to the attached fluorescent reporter, making it difficult to extract states of interest from those that are no longer useful to the analysis at hand. When fluorescence is used in interpreting and analyzing complex biological systems such as dynamic cell behavior and composition, a variety of influencing factors can substantially influence the effective signal-to-noise ratio of the analysis system. For example, naturally fluorescing compounds can interfere with the emission signal of a fluorescent reporter to varying degrees, as the dynamics and concentration of these natural interferents change over the lifetime and dynamics of cell behavior. Likewise, the mutation of recently tagged biological units is not just a possibility but a certainty in cell-based analysis, making the understanding of what happens to the corresponding fluorescent tag very necessary. Finally, although fluorescence remains a highly sensitive, specific, and attractive means for monitoring cell activity, the number of fluorescent reporters that can be analyzed simultaneously, while accounting for naturally fluorescing interferents, photobleaching, biological processes, and other factors, is limited by the engineering of the spectral excitation (at the system input) and the signal processing (deconvolution) capability at the system output. Therefore, fluorescence analysis, at the level of analyzing whole cells, should be limited to a trade-off between 1) the biological units of most interest and 2) the efficiency (high signal-to-noise, sensitivity, low interference) by which certain biological units can be effectively tagged with fluorescent reporters.

Fluorescence is well suited to the analysis of intracellular conditions. Since few sensing technologies are compatible with insertion into living cells, the use of fluorescent probes has seen an explosion in recent years for monitoring DNA, RNA, proteins, and other intracellular units of interest. For example, between 1975 and 1994, only 42 articles appeared on the Web of Science database on green fluorescent protein, one of the most common fluorescent probes, extracted from the jellyfish *Aequorea victoria*. In contrast, in the years from 1995 to 2003, over ten thousand articles were published on this same subject. The explosion in the use of fluorescent probes and indicators to monitor biological systems, units, and analytes has been dependent on the ability to clone and express fluorescent protein genes in a wide variety of cells and organisms. Advances in fluorophore engineering, including improvements in stability, toxicity, brightness, and targeting, have also contributed to this explosion.

Fluorescent probes can be naturally occurring (intrinsic) or engineered to bind to the analyte of interest. The primary intracellular analytes of metabolic interest emphasized in this paper are NADH, forms of flavin adenine dinucleotide (FAD and $FADH_2$), and ATP. Both NADH and FAD are natural (intrinsic) fluorophores. Although fluorescence is a convenient characteristic of NADH and FAD that can be seen in viable living cells, the study thus far of these metabolic units has largely emphasized the interfering effects they have on measurements using green fluorescent protein and other extrinsic fluorophores that emit in the 400–600-nm range. Often, it is the task of distinguishing the much larger signal of the extrinsic fluorophore from the interfering effect of auto-fluorophores (intrinsic) that is the focus of research studies [47].

FAD has an emission maximum at 530 nm (when excited by blue light), but has low (dim) fluorescence efficiency, especially compared with its relative flavin mononucleotide (FMN), which is ten times brighter than FAD. The autofluorescing properties of flavins have been exploited since the 1940s for studying vitamin content in biological organisms, including the American roach [48], and observed in scientific studies for a variety of cell types [49]. However, FAD detection in single cells has not received extensive research attention in the past decade, even with the explosion of fluorophore development and usage, because of its prohibitively dim (intrinsic) fluorescence.

On the other hand, NADH has been detected in a variety of applications including resolving concentrations in large numbers of chinese hamster ovary cells (0.1 million cells/ml) [24], single mammalian cells [50], single rat cells [51], and single pancreatic β cells [8]. Its autofluorescence is 1–2 orders of magnitude brighter than that of the flavins, but the emission maximum occurs at a wavelength of approximately 460 nm when excited at 360 nm (which is in the ultraviolet range). Since it is an intrinsic fluorophore, NADH requires a highly sensitive photodetector for single-cell measurement; NADH must also be excited with ultraviolet (UV) light which can damage cell viability. The most common detection method for measuring NADH uses a fluorescence microscope and photomultiplier tube (PMT) or charge coupled device (CCD) camera for detection of emitted light. This method works well only with monolayers for analyzing single cells; flow cytometry can resolve cells to single monolayers, but there are limits in the amount of time that a single cell can be tracked and monitored. Confocal microscopy is a practical alternative to resolve multiple layers of cells; however, the UV signal needed for NADH excitation is degraded by the optics in confocal systems. To address many of these limitations, two photon microscopy, in combination with a confocal or flow cytometry approach, allows light at twice the requisite UV wavelength to excite NADH, thereby reducing cell photodamage as well as optical losses in the analysis system [52]. Since the prevailing approach to autofluorescence of NADH and flavins in cells has been to remove the autofluorescing "noise" from these systems, it remains unclear whether deconvolution algorithms can be developed to distinguish autofluorescing components in a system that contains both intrinsic and extrinsic fluorophores.

ATP is another intracellular analyte of interest to monitor metabolic activity in cells. It is not a natural (intrinsic) fluorophore and therefore requires some engineering to construct biosensors to measure its presence using luminescent phenomena. For example, fluorescent reporters for ATP have been constructed using anthryl-functionalized open-chain polyaza-alkanes [53] and 7-amino-4-trifluormethylcoumarin [54], to measure ATP at 10 mM concentration levels using conventional fluorescence spectroscopy instrumentation. Using these alkane reporters, fluorescence is either quenched (diminished) or enhanced with increasing concentrations of ATP depending on whether the surrounding pH is acidic or neutral, respectively. Using the 7-amino-4-trifluoromethylcoumarin reporter, both ATP and ADP give strong fluorescent signals, which may limit the usefulness of this detection mechanism in determining the relative concentrations of ATP and ADP in cells; both of these reporters have not been tested inside cells. Fluorescence resonance energy transfer (FRET) measurements have alternatively been used in combination with biocyclomycin fluorescent probes to measure conformational changes induced by ATP (at single-cell concentration levels of 0–120 μM) when binding with a primary interaction site while the commercially available antibiotic biocyclomycin attacks *Escherichia coli (E. coli)* [55]. This work represents a demonstration that fluorescent reporting can not only be used to measure ATP concentrations but can also detect the activity of ATP in bonding with other elements of the cell. Fluorescence-based detection of ATP at the single-cell level has not yet been demonstrated in the published literature. The lack of research interest and successful demonstration of intracellular detection of ATP can be attributed to the combined difficulty in measuring ATP intracellularly, due to fluorophore limitations, and the increased importance of other intracellular metabolites (such as oxygen) over ATP itself.

Oxygen is of vital importance in intracellular and extracellular analysis, because it plays an important role in a variety of metabolic processes. In addition, sensing of oxygen is also used as a correlating factor to other metabolites of interest inside and outside of cells, such as glucose and ATP. For this reason, oxygen has gained a great deal of interest in the research community for cell-level detection. Single-cell resolution of oxygen concentration levels has been demonstrated using ruthenium chloride complex in J774 murine macrophages (0–280-μM concentration range) [25], a more stable ruthenium diimine complex [56], a ruthenium complex integrated onto a fiber-optic probe at 20–250 μM with 25-μM resolution [45], and a ruthenium diimine complex on the end of a submicron fiber-optic probe (15.6–625 μM) [57]. A combination of ruthenium complex and platinum porphyrins has been used to measure both oxygen and pH using fluorescence lifetime rather than intensity, in order to circumvent the instability effects often associated with ruthenium [58]. Ruthenium-based complexes also tend to leak easily outside the cell and are often sensitive to other factors including pH and temperature. Ruthenium can be delivered to the cell interior in a manner that overcomes some of these limitations through the use of lipobeads which combine the mechanical stability of beads with the biocompatibility of liposomes [56]. Beads allow oxygen to be sensed inside the cell without leakage, and liposomes allow the "sensor" to readily merge with the cell membrane to inject the sensing mechanism (the oxygen sensitive bead) into the cell without disrupting cellular processes. Another approach to resolving instability in a single ruthenium-based probe is to use multiple sensors on a single probe to resolve interference and instability effects. Walt *et al.* have fabricated a variety of multianalyte fluorescent probes including ones that can simultaneously resolve oxygen (20–100%), glucose (0.6–20 mm), pH (6.2–7.5), and penicillin in a single measurement [59]. In general, both lipobeads and multianalyte probes effectively determine concentration levels compatible with single-cell detection even though demonstrations in realistic single-cell environments are sparse in the published literature.

A variety of fluorescent means have also been used to monitor intracellular and extracellular pH in cells. Many

fluorophores are inherently sensitive to pH, a characteristic that causes instability and drift when sensing nonpH analytes but is useful for pH sensing. Often, the response of the fluorophore to pH is secondary and less sensitive than the response of the fluorophore to a primary analyte. In spite of this decreased sensitivity, some pH-sensitive fluorophores have been developed to detect pH using conventional static fluorescence measurement and instrumentation schemes. For example, 1,4-dihydroxyphthalonitrile (at two wavelengths) has been demonstrated for monitoring fermentation-based manufacturing processes [60]. GFPmut1 fluorescence, mutated from *Aequorea victoria* (GFP), was used in rabbit proximal tubule cells and BS-C-1 African Green Monkey kidney cells to detect intracellular pH in the range of 6.2–8.0, both *in vitro* and *in situ* [6]. However, conventional fluorescent analysis is not well suited to determining pH. One of the issues associated with monitoring pH using fluorescent means is the high excitation intensity typically required to generate a measurable pH-based fluorescence emission. High intensities typically lead to high photobleaching rates, limiting the useful life and accuracy of the fluorescent agent. To address this problem, research has been directed toward looking at fluorescent decay times of green-excited, Ca^{2+} competitive assays for glucose (5.3 μM to 125 mM concentration levels) which indicate pH while remaining immune to photobleaching effects and other changes in optical light loss [61]. Other time-based fluorescent analysis techniques applied to the detection of pH include static quenching, intermolecular proton transfer, intramolecular proton transfer, and excited state reversal, which are reviewed in [62] and [63]. These techniques involve selecting and manipulating a particular indicator in the acidic or basic form to interact with a measurand that responds to different fluorescent decay times. For example, Lippitsch *et al.* designed a pH sensor using diethylaminomethyl attached to a pyrene fluorophore, and used fluorescence quenching to detect changes in pH between 6 and 9 [64]. This method resulted in the transfer of an electron from the amino group to the pyrene (fluorescence quenching). Fluorescent lifetime-based pH sensing is useful in that the measurand responds only to the decay time, which is independent of fluorescence intensity and considers only the optics and electronics involved [62], [63], [65]. Time-based resonance energy transfer (FRET) techniques have also been successfully demonstrated in the detection of pH in solution, with similar resilience to changes in light intensity and signal level [66]. Yet, another nonconventional approach to measuring pH (using 6-carobxy fluorescein) at levels between 6–8 with a 1-s response time, as well as dissolved oxygen and glucose, has been to evaluate steady-state changes using FRET in light polarization between an analyte-sensitive fluorophore and a reference fluorophore (of known polarization) [67], [68]. Notably absent from these pH-sensing efforts, however, are successful demonstrations of measuring pH inside cells. Slavik *et al.* used fluorescent probes to detect cytoplasmic pH in three types of cells: *Saccharomyces cerevisiae* (at pH range of 4.7–5.8), *Tritrichomonas foetus* (at pH range of 4.6–5.6), and leukocytes (at pH range of 7.0–7.4). The cells were mainly stained with two pH sensitive dyes [2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluroescein (BCECF) and carboxy-SNARF-1], but the fluorescent probing technique did not involve penetration into the cell [69]. However, by using more complex sensing mechanisms that protect the cell from toxic side effects of the fluorophore, pH has been successfully detected inside cells (murine macrophages) using the oxygen-sensitive lipobeads discussed previously for 5.5–7.0 ranges, a resolution of 0.1, and 6 h of stability [41].

In addition to lipobeads, probes encapsulated by biologically localized embedding (PEBBLE) sensors have proven to be useful in fluorescence-based methods for analyzing single-cell activity, particularly pH. PEBBLE sensors based on matrices of polyacrylamide, cross-linked decyl methacrylate, and silica-based sol-gel are characterized in aqueous solutions and have been used to detect several types of intracellular analyte concentrations. Detection of analyte concentrations using PEBBLE sensors have been extensively researched in particular by Kopelman *et al.* PEBBLE sensors have been used to detect changes in glucose and oxygen concentrations [70], [71] and to sense pH (6.0 to 7.0), calcium (1–300 mM concentration levels), potassium (120–700 mM concentration levels), and sodium (5–100 mM concentration levels) ion concentrations intracellularly [72], [73]. The matrix characteristic of PEBBLE sensors permits the simultaneous detection and monitoring of multiple analytes at multiple locations in a single living cell [74]. Multiple dyes can also be combined in a single PEBBLE sensor to set a reference standard for calibration purposes in acquiring consistent responses by the sensor and to minimize error. This approach is also useful in detecting and distinguishing areas of high ion concentration from areas of lower concentrations within the intracellular environment. The protective polymer coating prevents the dyes from interacting with the intracellular environment, which prevents toxic fluorescent dye leakage and minimizes error resulting from protein binding and membrane or organelle sequestration. To effectively evaluate pH inside cells as part of a larger, systematic sensing scheme of many analytes, recent efforts have shown that PEBBLEs can effectively monitor intracellular pH with a 1-ms response time, with leaching of fluorophore from the probe at a rate less than half of conventional fluorescent methods. Ten different fluorescent dyes were inserted into a nanoscale acrylamide polymer matrix and evaluated for their stability, resolution, and range for measuring pH, and the ability to measure pH in the range from 5.8 to 8.0 was demonstrated [75]. Each PEBBLE sensor contained sulforhodamine 101 as the internal standard for calibration and consistent comparison, when injected into human C6 glioma and SY5Y neuroblastoma cells. At a similar size scale to PEBBLEs, submicron and nanoscale fiber-optic probes are capable of single-cell analysis. The probes are made sufficiently small to penetrate cells without detrimentally impacting cell viability. Submicron scale probes have been demonstrated for single-cell oxygen concentration levels [57] as well as for insertion into single cells [76]–[78]. Both PEBBLEs and ultrasmall fiber-optic probes are promising candidates for effective intracellular analysis of metabolites.

The last major category of fluorescent sensors relevant to monitoring the metabolic activity of cells are those that measure the concentration of glucose both inside and outside of cells.

Two broad categories of glucose fluorescent sensors are found in the literature. The first category, the most common, consists of the glucose–oxidase-based sensors, and the second consists of the affinity binding sensors. Both types of sensors are reviewed in [79]. The first category of sensors uses electroenzymatic oxidation of glucose by a glucose-oxidase (GOX) to produce a glucose-dependent signal. This sensing method takes advantage of the oxygen consumption in the reaction, which is dependent on the local concentration of glucose. By detecting the rate at which oxygen is consumed and by using a fluorophore sensitive to oxygen, the local concentration of glucose can be extrapolated and quantified. This approach reduces the sensing of glucose to a matter of sensing oxygen; methods to detect oxygen inside and outside of cells have been summarized previously. Creative means to enhance glucose sensitivity using oxygen-based sensing have included using a photopolymerization process to decrease the diameter of the optical fiber probe that detects the luminescence of tris(1,10-phenanthroline)-ruthenium(II) cation which is proportional to oxygen present in the GOX reactions [80]; this method achieved detection limits to glucose of 1×10^{-15} M. Another approach uses tetrathiafulvalence (TTF) as a mediator in the glucose-sensing process. TTF oxidizes directly into TTF^{+}, which then reacts with the reduced form of GOX; the concentration of TTF^{+}, a fluorophore, is then proportional to glucose concentration through this mediated process [79].

The second category of glucose sensors based on fluorescence are the affinity-binding sensors that utilize the competitive binding properties between glucose and a fluorescent compound to a common receptor site. Greater concentrations of glucose enable greater displacement of the fluorescent compound at the receptor site; glucose concentration is then inversely related to fluorescence. For example, a well-known affinity sensor method by Shultz *et al.* used fluorescein isothiocyanate (FITC) labeled dextran as the competing agent and concanavalin A (ConA) as the receptor site [81], [82]. These methods may be appropriate for extracellular monitoring of glucose. However, a significant and potentially prohibitive drawback of the affinity-based sensors in intracellular monitoring is that the introduction of the competing agent can have a significant impact on cell viability.

Much like pH sensor research, novel approaches to glucose sensing, such as lifetime-based and polarization methods, show promising alternative techniques that are independent of fluorescence intensity, or at least less dependent upon it. A lifetime-based fluorescence-sensing method using a ruthenium metal complex $[Ru(bpy)_2(dphen)]$ is described in [83]. The equilibrium established between cyclic-esters and glucose in the presence of 2-bromophenylboronic acid (BBA) makes the detection of cyclic-esters a valuable means to determine glucose concentrations. In another detection method that uses polarization measurements, the polarized emission from a stretch-oriented reference film was compared to that of a fluorophore tagged protein from *E. coli*, whose intensity changed proportional to the concentration of glucose (0–8 μM concentrations) [84].

As with pH sensors, many of the glucose sensors are designed for extracellular detection of glucose. Successful methods to measure glucose within the cell have yet to be achieved. However, promising technologies have been developed to miniaturize fiber-optic glucose sensors to submicrometer sizes with an improvement in detection of 2–3 orders of magnitude as compared to conventional sensors (1–10 mm glucose sensitivity) [57]. Loschenov *et al.* have developed an experimental method for monitoring glucose concentration levels in interstitial fluids within the range of 1–10 mM [85]. Fiber-optic penetration of cells has the potential to damage cell viability and interfere with cellular processes; however, submicron scale probes minimize the risk of such effects in intracellular measurements. In addition to miniaturized fiber-optic probes, the advancement, and miniaturization of microbead technology are likely to make significant contributions to all aspects of metabolic activity monitoring, both intracellular and extracellular, including glucose sensing [86].

Fluorescence has been the dominant means of using luminescence spectroscopy to monitor analytes related to metabolic activity. However, the use of chemiluminescence in monitoring analytes related to cellular metabolic activity is also present in recent literature. Coulet *et al.* report a fiber-optic probe using luciferase (a chemiluminescent agent) from fireflies and marine bacteria that provide emission correlated to concentrations of ATP (2.8×10^{-4} to 1.6 μM) and NADH (0.001 to 3 μM), respectively [87], [88]. These chemiluminescent probes have been demonstrated in biochemical manufacturing processes. For biological analysis applications, ATP concentrations at the low-end of single-cell limits (up to 1 μM) have been demonstrated using firefly luciferase on a fiber-optic sensing platform in noncellular experiments [88]. A very limited number of efforts have successfully demonstrated the detection of ATP in single cells using the chemiluminescence of the luciferin/luciferase couple in mouse myoblasts (0.01–1 mM concentration range) [10] and myoballs from Wistar rats (0.01–1 mM concentration range) [9]. Similarly, chemiluminescence has been used to measure bacteria in water using a luciferine/luciferase combination; however, this process requires lysing (breaking up) the bacteria prior to successful introduction of the chemiluminescent agent [89]. The fundamental limitation of chemiluminescence is that a chemical reaction is required to emit light. Artificially induced chemical reactions for the purposes of inducing luminescence have the unfortunate potential to disrupt cell activity in an often unpredictable and unmeasurable manner. Introducing chemiluminescent agents inside cells to targeted analytes is also substantially more difficult than introducing artificially engineered, extrinsic fluorophores for these same analytes.

Phosphorescence, which is different from fluorescence in its longer lifetime, is of note in monitoring the metabolic activity of living cells, because of its potential for monitoring dissolved oxygen (glucose and carbon dioxide) concentrations in biological applications. Phosphorescent palladium and platinum coatings on porphyrin in fiber-optic probe configurations have been demonstrated as a biosensing mechanism for both oxygen (1.8–8.8 mM) and glucose (1–300 μM) [90], [91]. In these luminescent mechanisms, phosphorescence (emitted light) is quenched (attenuated) with increasing oxygen concentration. Similarly, performance comparable (181–250 μM O_2 sensitivity) to the popular Clark-type amperometric electrode has been demonstrated using an oxygen-sensitive dye absorbed

TABLE IV
COLORIMETRIC SENSOR DYES AND SENSING PROPERTIES

Dye	Analyte	Sensing Method	Sensing Range	Ref
dipicrylamine	pH	colorimetric	1-4pH	[43]
bromine phenol blue	pH	colorimetric	3.0-5.0pH	[94]
bromothymol blue	pH	colorimetric	6.0-10.0pH	[94] [27]
phenol red	pH	colorimetric	7.0-8.5pH	[94]
thiazole yellow	pH	colorimetric	12.0-13.5pH	[37]
methylene blue	pH	evanescent	3-9pH	[98]
polyaniline (IR)	pH	colorimetric	5.4-10.5pH	[33] [31] [110]
polypyrrole (IR)	pH	colorimetric	6-12pH	[42]
viologen	oxygen	colorimetric/recovery	1.8-47μM	[105]
glucose oxidase	glucose	colorimetric	0-20mM	[46]
Prussian blue	H_2O_2/glucose	colorimetric	0-2.5mM glucose	[107]

onto silica gel particles in an optical biosensor probe [92]. Neither of these sensors have been demonstrated inside living cells; however, both distributed sensing mechanisms, such as beads, and point sensing mechanisms, such as fiber-optic probes, are viable technologies for extra cellular monitoring of oxygen and other analytes of metabolic importance, including glucose and carbon dioxide.

Overall, luminescent-based sensing technologies have substantial potential for cohesive monitoring of metabolic activity in living cells. Of all methods and analytes addressed in this review, fluorescence-based luminescence, because of its high sensitivity and nonintrusive impact on cells, is the most popular and the most promising of these technologies; it has substantial purpose for the monitoring of intracellular components, which makes it stand apart from other, nonluminescent sensing means.

B. Colorimetry

While luminescent sensors rely on the absorption and reemission of photons from a luminescent probe, colorimetry relies on a change in the absorption of photons in response to an analyte. In colorimetric sensing, an optical dye interacts with the analyte, which causes the dye to shift its absorption band, thereby changing color or opacity. The color change can be detected by measuring the absorption or reflectivity of the matrix containing the dye. Other methods to detect the change in the dye properties emphasize the use of evanescent waves or the measurement of a phase difference using interferometry. These dyes are usually covalently bonded or physically trapped to the tip of a fiber-optic cable, to ensure that the dye remains at the tip of the fiber and does not dissolve into the solution. By "pulling" the fiber-optic cable, it is possible to reduce the tip of the sensor below diameters of 20 nm, and sensors within this size range can be used for single-cell detection [93]. An excellent review of electron transfer mechanisms for colorimetric dyes can be found in [17].

There are two general classes of colorimetric sensors. The first class relies on the direct detection of an analyte via changes in the colorimetric dye alone, and the second class combines luminescent and colorimetric dyes in which the luminescent dye is the photon donor and the colorimetric dye is the photon acceptor. An advantage of colorimetric dyes is a greatly improved response time for detecting certain types of analytes over other sensors, partially because of their small sensing area (nanometers to micrometers). Metabolites that can be detected using these types of sensors include pH, carbon dioxide, oxygen, glucose, and ATP. Because NADH and flavins are intrinsically luminescent, colorimetric sensors have not yet provided any performance advantage for sensing these two metabolites. Most of the research in colorimetric sensors has been in the development of pH sensors, due to the wide availability of pH-sensitive dyes, and CO_2 sensors, because colorimetric CO_2 sensor performance is highly competitive with other sensor classes. Typical dyes used in colorimetric and colorimetric/luminescent sensors are listed in Table IV, including polymer pH-indicative dyes that absorb infrared light.

One of the important design parameters is the method by which the dye is immobilized onto the tip of the fiber-optic cable. If the dye is directly placed onto the fiber-optic cable, diffusion of the analyte to the dye is restricted, and the surface area is greatly reduced. These two effects reduce the overall sensitivity of the sensor. Alternate approaches to place the dye in a matrix with greater surface area include physically containing the dye using a semipermeable membrane or physically trapping the dye. For colorimetric sensors, membranes are usually composed of PTFE, a silicone rubber, or some other semipermeable material. The dye can also be immobilized onto an intermediate substrate, such as polystyrene [94] or glass microspheres, in order to increase the sensing surface area. These microspheres are then contained with a semipermeable membrane or by physical trapping. Physical entrapment involves mixing the dye into a matrix. Ideally, the matrix material would trap the dye, reduce or eliminate dye leaching, and resist biofouling and interferents. Matrix materials include polyacrylamide, polyvinyl alcohol, polyvinyl chloride, epoxy, ethylcellulose [95], sol-gels, or Langmuir–Blodgett films. A review of entrapment methods can be found in [96].

For pH sensors, the available pH-sensing range depends on the properties of the dye. In order to broaden the sensing range, two or more sensors can be immobilized onto the same optical fiber and the absorption of both dyes can be measured. Using this method, a fiber-optic pH sensor sensitive to pH values between 1 and 13 has been demonstrated [97]. To remove nonlinearities in the signal, an artificial neural network was used to predict the pH based on the outputs of this sensor.

A novel approach to detecting pH colorimetrically is to detect a change in dye concentration using an evanescent field. To construct this type of sensor, a fiber-optic cable is stripped of its cladding and exposed to a solution containing a chemical such as methylene blue. The adsorption of methylene blue onto the surface of the unclad fiber changes linearly with pH, and the amount of methylene blue adsorbed onto the fiber can be measured evanescently [98]. Further improvements to this sensor technology include containing the methylene blue close to the fiber using a perm-selective membrane, and reducing the size of the sensing region of the fiber.

Carbon dioxide is sensed colorimetrically either with a Severinghaus-type sensing scheme, using a phase transfer agent, or using a combination of fluorescent and colorimetric dyes. For the typical Severinghaus-type colorimetric sensor, a bicarbonate solution is physically trapped to a region close to the dye with a gas-permeable membrane [99], [100]. One disadvantage of incorporating a gas-permeable membrane is that the membrane is also permeable to water, causing an osmotic pressure change which destabilizes the sensor and causes a long-term drift. Stability can be improved by using a phase transfer agent, such as a quanternary ammonium hydroxide [101], [102]. The dye/quanternary ammonium ion pair immobilized in a matrix eliminates the need for an electrolyte solution, and these sensors have been shown to have a high stability and long lifetime [95]. The response time is also greatly improved, and typical response times are on the order of 200 ms [35]. Another electron mediator used in colorimetric CO_2 sensors is ethylcellulose [103], [104], which combines with the indicator dye to form a hydrophobic polymer membrane. The membrane naturally repels water and ions due to its hydrophobic nature, reducing the effects of interferents on sensor behavior. Combination colorimetric/fluorescent CO_2 sensors are based on measuring the absorption band shift of pH-sensitive dyes due to the CO_2 concentration. Such pH dyes, such as bromothymol blue, cresol red, and phenol red, have an absorption band around 600 nm which shifts downward with increasing CO_2 concentration. This shift changes the amount of light absorbed from a luminescent reference dye which is insensitive to CO_2 concentration. The amount of light emitted from the luminescent reference dye is directly related to the CO_2 concentration. Some reference dyes used in these sensors include a ruthenium complex [44] and a europium(III) complex [27], [28]. The sensors can detect changes in CO_2 concentration up to 20% CO_2, and have response times of a few seconds and recovery times of approximately half a minute.

Colorimetric sensors have also been developed to detect oxygen, glucose, and ATP. However, oxygen-sensitive colorimetric sensors have not been extensively developed, mostly because of successful alternative technologies such as oxygen-quenching luminescence analysis and electrochemical-sensing means. A colorimetric oxygen sensor has been developed which measures the change in the recovery time of viologen after being exposed to UV light, which, in turn, is dependent on the partial oxygen pressure [105]. This sensor had a response time of less than 15 s. The detection of glucose relies on the conversion of glucose to peroxide and a glucose by-product, catalyzed by the enzyme glucose oxidase. One method to detect glucose is to use Prussian blue, an indicator dye that is sensitive to hydrogen peroxide. As glucose is converted to peroxide and another by-product, the optical properties of Prussian blue also changes due to its interaction with peroxide [107]. Another novel approach to detect glucose is to use the absorbance band shift of glucose oxidase itself. The conversion of glucose also reduces a FAD group on glucose oxidase, which shifts the absorption band of the enzyme [46]. This sensor would require further miniaturization in order to detect single cells. Some of these compounds are detected by developing a chromoionophore to detect cations or anions. ATP was sensed colorimetrically by developing an anion-sensitive chromoionophore derived from 1,3,5-triarylpent-2-en-1,5-dione, which turns from yellow to red in the presence of ATP [108]. The detection range of this colorimetric dye is between 0.05- and 1-μM ATP.

Colorimetric sensors have been extensively developed to detect pH changes and CO_2. It has been shown that colorimetric sensors can also detect other metabolites, including oxygen, glucose, and ATP, but it is not yet clear that colorimetric sensors provide an advantage over other sensor classes for these other metabolites. However, for detecting carbon dioxide, colorimetric sensors have been shown to perform better than other sensors due to their small size and fast response times. Further developments in indicator dyes and entrapment techniques will further increase the sensing capabilities and performance of this type of sensor.

C. Other Optical Sensor Technologies

Two other optical sensor technologies of particular note because of their potential to become competitive with luminescence- and colorimetric-based techniques are evanescent wave-based sensors [particularly, surface plasmon resonance (SPR)] and infrared absorption spectroscopy. Recent developments in reducing the optical train of SPR sensors combined with future developments in competitive assays can make SPR viable for detecting metabolites and metabolic events in single cells. Infrared spectroscopy, although less sensitive than luminescence spectroscopy for most analytes, has application in complex monitoring and sensing schemes where more analytes are to be measured than fluorophores are practically available, due to signal processing or delivery constraints. Because these two schemes can be used in concert with the dominant optical-sensing means for holistic interpretation of metabolic activity in cells, they are reviewed in this section for completeness.

SPR Sensors: Evanescent wave biosensors rely on the interaction of evanescent waves with analytes of interest in order to detect analyte concentrations. Evanescent waves are fields that extend beyond the structure of a waveguide that rapidly

decay with distance from the waveguide; as a result, only changes in the composition of the immediate region outside of the waveguide, such as analyte binding behavior or other surface properties, can be detected. Examples of waveguides used for chemical sensors include unclad or partially-clad optical fibers, planar waveguides, and channel waveguides (including capillaries). Sensors that are based on evanescent wave detection can be used to detect metabolites such as NADH [24], oxygen [111], carbon dioxide [112], and glucose [113]. Unfortunately, the size of the sensing region is usually on the order of several millimeters or larger. Since sensitivity is directly related to sensing area, construction of evanescent field sensors of suitable small size for single-cell analysis is nearly impossible. One possible exception to this limitation is the surface plasmon resonance (SPR) transduction scheme.

Surface plasmon resonance (SPR) sensors are a subset of evanescent wave sensors. SPR relies on plasmons that can be excited at the boundary between a dielectric and a metal. By measuring the amount of reflected light at different wavelengths from an SPR probe, changes in refractive index along the surface of an SPR probe due to chemical binding by an analyte of interest can be detected. Selectivity can be accomplished by chemically modifying the surface with a receptor for the analyte of interest. Typical sensing schemes using SPR transduction involve: 1) affinity assays in which a receptor for a large macromolecule is chemically bonded to the gold surface, 2) competitive assays in which the small analyte of interest competes with a large macromolecule for binding spots on the surface of the metal, and 3) multistep assays, in which the (very small) analyte must catalyze the bonding of two large macromolecules, one of which is covalently bonded to the surface of the metal, and the addition of the second macromolecule generates a signal large enough to be detected. Unfortunately, metabolites such as glucose, oxygen, carbon dioxide, ATP, NADH, and $FADH_2$ are too small to be detected directly, and the availability of competitive or multistep assays that involve these analytes to be detected indirectly is limited.

Another obstacle to the use of SPR in monitoring the metabolic activity of single cells is the size of the instrument, which is typically dominated by the size of the optical train. The typical SPR sensor uses an attenuated total reflection (ATR) scheme which incorporates a prism to guide the optical pathway to the dielectric-metal surface. Although amplitude information is typically the only parameter that is measured, additional information about the chemical can be obtained by measuring the phase as well, in an interferometric sensing scheme [114]. For single-cell detection, the main disadvantage of SPR sensors using the ATR scheme is that the prism is usually large and bulky. Recently, significant advances have been made in decreasing the size of SPR sensors. One promising development is an alternative sensing scheme which relies on the anomalous reflection (AR) of gold. This scheme uses a blue or violet light source which propagates down a fiber-optic cable. The light then enters a cell containing the fluid being sensed and a substrate consisting of gold and another dielectric layer. Since gold behaves as a dielectric for blue or violet light, it is transparent at these wavelengths, and the reflectivity strongly changes based on the amount of analyte binding [115].

In summary, SPR is a promising technique for the application of monitoring metabolic activity in single cells because the transduction mechanism (extraction of refractive index changes) itself is extremely sensitive. However, bulky (optical train) instrumentation limits its use in small areas (e.g., single cells); in addition, the current lack of competitive binding assays and other indirect binding techniques make the detection of small molecules, such as the metabolites emphasized in this review, very difficult. Further advances in chemically selective assays and new detection techniques for more compact sensors are needed before SPR sensors will be capable of resolving chemical events at the single-cell level.

Infrared Absorption Spectroscopy: Another optical-sensing technology is infrared-absorption spectroscopy, which can provide information about the structure of molecules, including the type of bonds, functional groups, and physical conformations. This technique measures the vibrational levels of the material. When molecules are illuminated with an infrared light source, some of the absorbed energy changes the vibrational levels of the bonds within a molecule, and the energy associated with this change is different depending on the structure of the molecule. A derivative of IR-absorption spectroscopy is Fourier transform infrared (FT-IR) spectroscopy, which includes a Michelson interferometer to obtain phase shift information and convert transmittance to frequency coordinates. Unfortunately, vibrational transitions are much weaker than electronic transitions such as luminescence and colorimetry. A review of current efforts to reduce the sample size for IR-absorption spectroscopy for biological applications can be found in [106]. Overall, infrared absorption spectroscopy, though less sensitive than luminescent methods, is a useful technique to complement fluorescence analysis; after the fluorescence capacity of a multiple-analyte sensing system is consumed, infrared absorption spectroscopy, like SPR, is a useful additional method for detecting remaining analytes of interest.

V. Summary

Within the MSLC group at the University of Washington, one of the focuses of the group is the development of new sensing technology to resolve metabolic activity at the single-cell level. This paper has emphasized the review of the sensing characteristics of optical-sensing technologies, such as luminescent spectroscopy, which is capable of detecting intracellular events in real-time with a high degree of selectivity, and colorimetry, which is capable of detecting extracellular analytes with high spatial resolution, and provides advantages for detecting carbon dioxide. Each sensor technology has been 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 $FADH_2$. Luminescent optical sensors are, perhaps, the most widely used chemical sensors for biological applications, but challenges faced when integrating luminescent optical sensors with lab-on-a-chip instrumentation include the propensity of fluorophores to photobleach, the cytotoxicity of fluorophores, and automated processes to inject the

fluorophore into the cell. Colorimetric sensors circumvent some of these difficulties since these sensors measure extracellular concentrations and do not suffer from photobleaching, but the number of analytes that can be sensed with these sensors is limited. SPR and infrared spectroscopy sensors are promising techniques to address analyte monitoring where colorimetric and luminescent means fall short. For all optical sensors, long-term stability is of primary importance, and applications in integrated technology systems require the development of better methods to extend the lifetime of these sensors.

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Rachel A. Yotter (M'01) received the B.S. degree in electrical engineering from the University of California, Berkeley, in 1998 and the M.S. degree in electrical engineering from the University of Washington, Seattle, in 2003. She is currently pursuing the Ph.D. degree in electrical engineering at the University of Washington.

Her research interests include neurophysiology and computational neuroscience. Other interests include MEMS, sensors, and biological instrumentation. Her current research topic is electrophysiological recordings from rat hippocampus.

Linda A. Lee received the degree from Whitman College, Walla Walla, WA.

She is currently with the Distributed Microsystems Laboratory, Department of Electrical Engineering, University of Washington, Seattle. The laboratory's research focus is on the integration of sensors into viable microsystems. Her research projects include developing miniaturized fluorescence analysis technology and pH sensors for biomolecular research.

Denise Michelle Wilson (M'89) was born in Chicago, IL, in 1966. She received the B.S. degree in mechanical engineering from Stanford University, Stanford, CA, in 1988 and the M.S. and Ph.D. degrees in electrical engineering from the Georgia Institute of Technology, Atlanta, in 1989 and 1995, respectively.

She is currently an Associate Professor with the Electrical Engineering Department, University of Washington, Seattle, and she was previously with the University of Kentucky, Lexington, in a similar position from 1996 to 1999. She was also with Applied Materials, a semiconductor capital equipment supplier, from 1990 to 1992. Her research interests focus on the development of signal processing architectures, array platforms, and other infrastructures for visual, auditory, and chemical-sensing microsystems.