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Review

Measuring metabolism and biophysical flux in the tissue, cellular and sub-cellular domains: Recent developments in self-referencing amperometry for physiological sensing

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Abstract

Ultimately, advances in genomics, proteomics and metabolomics will be realized by combining these approaches with biophysical sensors for understanding the functional and structural (physiological) aspects of sub-cellular systems (cytomics). Therefore, the emergence of the new fields of cytomics and physiomics will require new technologies to probe the functional realm of living cells. While amperometric sensors have been used, their sensitivity and reliability are significantly improved through the development of new strategies and data acquisition systems for the operation of the sensors. This includes the application of the principles of the vibrating or self-referencing microsensor to the operation of amperometric sensors. The development of self-referencing amperometry (SRA) is significant because it effectively converts static concentration sensors into dynamic biophysical sensors that directly monitor physiological flux. SRA has been developed for analytes such as O_2, NO, H_2O_2 and ascorbate. These sensors have been validated against non-biological microscopic flux sources that were theoretically modeled, before being applied to biological research. This new sensor technology has been shown, through research in a wide variety of biological and biomedical research projects, to be an important new tool in the arsenal of the cell biologist. SRA technology has been adapted through SRA–H_2O_2 and SRA–NADH sensors, for electrochemically coupled enzyme based self-referencing biosensors (SRB) for glucose, glutamate and ethanol. These developments in self-referencing sensor technologies offer great promise in extending electroanalytical chemistry and biosensor technologies from the micro to the nanoscale where researchers can study physiology at the sub-cellular and organellar levels.

Keywords: Biosensors; Nitric oxide; Oxygen; Ascorbate; Ethanol; NADH; Hydrogen peroxide; Self-referencing; Vibrating probe

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1. Introduction

Electrochemistry has been readily adaptable in the development of sensors for agricultural, biological and medical research. Based on electroanalytical chemistry, it is possible to measure gaseous molecules that are directly relevant to basic metabolism and biochemistry (oxygen) as well as complex pathophysiology and signaling (nitric oxide). Our basic understanding of neurochemical signaling is based on sensors that were developed based on the distinct electroanalytical properties of individual neuromediators (catecholamines, epinephrine, etc.) for measurements in the intact brain, in tissues and in isolated single cells. Early success in developing and adapting electrochemically coupled enzyme based biosensors for basic research was also pursued by neurophysiologists for measuring in vivo glucose metabolism (Hu and Wilson, 1997) and neurogenic glutamate release (Hu et al., 1994) in the rat brain. Recently biosensor approaches have even been adapted for measurement of ATP (Llaudet et al., 2005).

Now as the scope of use and application of these technologies has advanced, scientists have become more attuned and aware of problems and artifacts associated with the use of these sensors, especially when they are used invasively. It is obvious to us now that the use of invasive microelectrodes can also introduce experimental error due to damage to the electrode and the biological system being studied. In general, the use of static concentration measurements, whether invasive or not, to characterize a component of the dynamic activity of biological systems is subject to limitations. This is especially true at the level of making cellular recordings, where the signal to noise characteristics of the recording instrumentation and electrodes hinders the ability to detect the intricate biophysical patterns that are present.

These limitations related to the mode of operation of the sensor can be avoided by using the self-referencing microsensor (SRM) technique. This technique allows non-invasive monitoring of biophysical transport activity (flux) from single cells or whole tissues in real time. This approach has been reinvented numerous times and with each new rediscovery a new name has been coined. The basic approach has been referred to as the vibrating probe, the self-referencing microelectrode, the microelectrode ion flux estimation and the microelectrode flux estimation techniques. What these sometimes-subtle variations all have in common is that a single microelectrode is used to sample two distinct positions within a biologically derived gradient. In this paper, we will describe the technique and detail recent advances in developing self-referencing amperometry (SRA) and self-referencing biosensors (SRB).

2. History of the self-referencing microsensor

As far as we know, this approach was first pioneered by Zisman (1932), who used a metal electrode as a probe to measure low magnitude currents. This involved measuring the voltage differences between two distinct points in an electric field by moving a single electrode. This has come to be referred to as the vibrating voltage probe and this has been applied where biologists seek to measure bulk ion fluxes. Despite the non-specificity of the technique, in terms of chemical analyte, this approach has provided a practical method to show total electrical currents in a number of biological systems. The first biological use of the technique was for plant research (Bluh and Scott, 1950), but was later used to measure net ion fluxes on skeletal muscle fibers (Davies, 1966). A notable rediscovery of the vibrating probe (Jaffe and Nucitelli, 1974) resulted in significant improvements of the technique based on the use of a commercial lock-in amplifier. The development of this new sensor modality was very significant as it led to numerous new studies and breakthroughs in our general understanding of ion currents and bioelectric processes in living systems (for examples, refer to Borgens, 1984; Kunkel, 1991; Nuccitelli and Jaffe, 1974; Robinson, 1979; Shi and Borgens, 1995; Shipley and Feijo', 1999; Smith and Shipley, 1990; Weisenseel et al., 1975; Woodruff and Telfer, 1980; Miller et al., 1988; McCaig and Robinson, 1982; Jaffe and Stem, 1979).

While the vibrating voltage probe technique did enable a whole new realm of biophysical sensing it is limited in that the sensor cannot directly describe the ionic composition of the biological currents it measures due to the non-specificity of the sensor. The functional ability to measure the active flux of specific ions came with the development of the self-referencing ion-selective (SRIS) microelectrode. This approach differs from the vibrating probe in that it is based on the use of ion-selective microelectrodes instead of the original metal electrodes and the frequency of probe movement is substantially slower (0.1–0.3 Hz versus 300–1000 Hz). This is a key difference, as the ion probe requires a slow movement to allow for the electrode to respond and to insure the chemical gradient is not disrupted by mechanical convection. Initially, investigators drove the probe movements manually to sample the biological gradients (Newman et al., 1987). Later Khütrieber and Jaffe (1990) used computer driven electrode movement and data acquisition to automate the measurement of calcium ion flux. The automated SRIS approach was subsequently diversified to measure other ions like H^+, K^+ (Kochian et al., 1992) and even heavy metals like Cd^{2+} (Pineros et al., 1998). The self-referencing technique has also recently been used to measure the biological flux of non-ionic compounds through the development of SRA (Porterfield et al., 1998; Land et al., 1999; Porterfield and Smith, 2000) and, more recently, the SRB.

3. Self-referencing microsensor theory

As previously stated, the SRM approach allows a researcher to actively measure biophysical transport from single cells or whole tissues based on the diffusionary movement of molecules within the gradient (Newman et al., 1987; Khütrieber and Jaffe, 1990; Porterfield et al., 1998; Land et al., 1999; Porterfield and Smith, 2000). This is accomplished by controlling the translational movement of a selective microelectrode in the gradient. The basic components of an SR recording system include a microscope with a head-stage sensor amplifier driven by a translational motion control system. The microscope allows the user to visualize the position of the sensor relative to the cell being studied. Typically, the cell is growing on a cover slip that is mounted onto the bottom of a Petri dish. In other cases, the cell simply is allowed to sink to the bottom of the dish. By adjusting the range of magnification, while manually operating the motorized micromanipulator motion controls system, it is possible to bring the probe to a position in the field of vision of the microscope. Final positioning and fine adjustments of the sensor relative to the living cell is done by controlling the probe position using the computer driven motion control system. Gross adjustment and alignment is done by hand by visualizing the sensor and cell in the microscope oculars, while fine adjustments are visualized via a camera and monitor on the microscope.

The microscope, camera and motion control system is mounted on an anti-vibration table and housed within a Faraday cage. The motion control system allows the electrode tip to be moved through the gradient at a known frequency and between known points (commonly 10–50 μm apart). The automated electrode movement induces a phase dependant waveform on the electrode output signal, where the amplitude of said waveform is proportional to the differential analyte concentration within the gradient (Fig. 1). Operating the sensor in this way offers many advantages that initially may not be obvious. The technique basically converts a static concentration sensor into a dynamic biophysical flux sensor. This is a huge advantage in the realm of biology and medicine where the interesting and important aspects of transport physiology and exchange typically are not easily detectable as concentration changes, in or out of the cell or tissue. The reason that the approach extends the functional sensitivity of the sensor is because phase coupled detection acts as a dynamic filter that effectively minimizes the impact of random noise and drift on the differential sensor output (Fig. 2).

Whereas the original vibrating probe is an ac signal domain technique, there are two main approaches to acquiring and analyzing amplitude of the waveform or the electrode differential signal for SRIS and SRA. Some of the first automated systems adapted the original ac coupling approach for slower signals. In this approach, the electrode signal coming from the preamplifier is capacitively coupled through a lock-in amplifier to non-discretely subtract off the baseline output signal so that the differential waveform signal can be amplified enough to digitally analyze these minute signals. The problem with this approach is, in 0.1–.5 Hz time domain, capacitive decay in the lock-in leads to signal collapse just when the differential signal is being analyzed. This invariably results in degradation of the differential signal (Khütrieber and Jaffe, 1990) and in an underestimation of the true flux values (Smith et al., 1999). The problem can be overcome by discrete subtraction of the baseline signals before amplification. This is referred to as dc-coupling and it allows for baseline signal subtraction without the associated problems of differential signal degradation due to capacitive discharge (Shipley and Feijo', 1999). The dc-coupling approach is preferred as it does a better job of preserving the signal integrity.

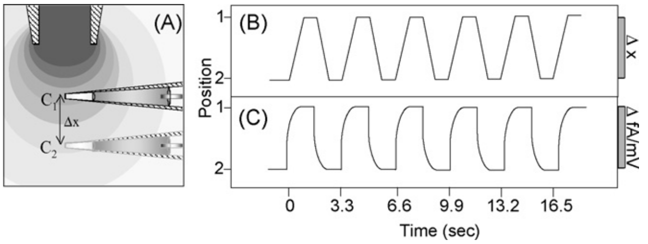

Fig. 1. Diagrams showing the basic procedure for conducting a self-referencing microelectrode flux measurement. In panel (A) a schematic of an experiment is depicted where a generated diffusional gradient is established outside of a micropipette. The relationship between probe positions (A) and electrode output (B) during the probe movement cycle are plotted to illustrate how a waveform is induced on top of the electrode output signal. For automated systems, a PC typically controls the translational frequency, allowing signal analysis software to extract voltage changes in phase with the probe moving through the gradient. The relationship between the concentration differential (C1 − C2) is directly proportional to the amplitude of the waveform of the differential electrode output value (ΔfA for amperometric electrochemical sensors and ΔmV for potentiometric ion selective sensors).

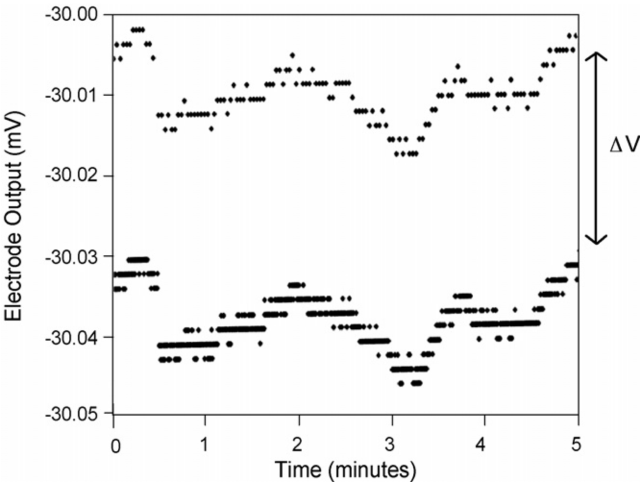

Fig. 2. Data showing how the self-referencing technique effectively filters out noise based on the basic principles of phase-sensitive detection. The data were collected only during the time when the probe was stopped at the two positions and the data that were collected are plotted here as a scatter plot. This clearly shows that the data segregate into two distinct groups representing the two measuring positions. Note how electrode drift and noise patterns are common to both groups of data while the differential (ΔV) between the two populations stays relatively constant.

Once the measurements of the differential electrode signals within the gradient have been acquired, they must be converted into a measure of differential concentration before flux can be calculated. The formulas for doing the conversion from differential electrode output to differential concentration are another area where the ac-coupling and dc-coupling approaches diverge. Because of the signal degradation and the way that the ac-coupled method logs data, it is impossible to directly calculate these values (Smith et al., 1999). Because the dc-coupling discretely subtracts off the baseline signal, this allows for logging of the actual electrode output values at both measuring positions. Thereby it allows calculation of these concentration differentials without having to make any types of assumptions regarding background electrode signals and measurement efficiency.

Instrumentation and software platforms for building a system to make these measurements are now commercially available (Science Wares, Mass; Applicable Electronics) and are being advanced through partnerships with academia (UMASS Vibrating probe facility; Purdue University, Physiological Sensing

Facility). The NIH also funds the BioCurrents Research Center, formally known as the National Vibrating Probe Facility, which was founded by Lionel Jaffe at the Marine Biological Laboratory at Woods Hole. Another place associated with innovation in this area is the University of Tasmania, which primarily has focused on ion sensing and applications in plant systems.

For sensing diffusionary flux using the self-referencing approach, calculations are all based on the Fick equation: $J = -D(\Delta C/\Delta X)$, where J = flux, D is the diffusion coefficient, ΔC is the differential concentration and ΔX is the distance between the two electrode measuring positions. Note that this assumes flat planar geometry of the surface being analyzed. Some researchers, using a variation of the Fick equation, have gone to great lengths to calculate flux accounting for non-planar geometry, but this approach was compared to the planar flux model using real data and quantitatively shown to be unnecessary (Kochian et al., 1992). The planar model yielded almost identical results to the cylindrical model, as would be expected given the small electrode tip size and excursion distance. When possible, the less complex planar approach is preferred as estimations of some of the values required for the cylindrical formula may actually introduce analytical error.

4. Self-referencing amperometric (SRA) microsensors

Amperometric sensors mediate analyte detection by reducing or oxidizing an analyte. The redox changes in the analyte are driven by a polarized potential on the electrodes' sensing surface which facilitates electron transfers between the surface and the molecule being detected. The flow of electrons is therefore dependent upon the concentration of the analyte and these sensors typically show a linear relationship between analyte concentration and the resulting current. Specificity of analyte detection by electroanalytical sensors is mediated by the redox properties of the analyte, the polarization potential of the electrode and membrane coatings on the electrode surface that may facilitate phase or size exclusion of potential interferants.

4.1. SRA–O_2 sensor

Because of the importance of oxygen in the environment, biology and medicine there have been considerable efforts in development of sensing strategies for sensing this molecule. The central role of oxygen in cellular respiration is based on the electro-negativity of molecular oxygen, which, in turn, facilitates amperometric sensing. In biology and medicine, the primary approach for the use of these sensors has been in invasive probing of plant and animal tissues. While in vivo oxygen measurements have provided basic insight into many components of metabolism and physiology, this approach is problematic because the electrode is more often than not, obstructed or damaged. Even worse tissue invasion will evoke a damage response in the system that will affect the results. These limitations can be overcome by operating the electrode as a non-invasive, self-referencing microsensor.

In the initial development of the technique, commercially available (Diamond General, Ann Arbor, MI) Whalen style, polarographic oxygen microelectrodes (tip diameter = 2–3 μm) were used (Porterfield et al., 1998; Land et al., 1999). These electrodes involve filling a micropipette with woods metal, etching the woods metal from the electrode tip and then plating a gold surface onto the woods metal within the tip recess, before dip coating with an oxygen permeable membrane. Later researchers have turned to using various approaches for constructing these SRA oxygen sensors based on etching an immobilized Pt wire, which is subsequently insulated in glass (Osbourn et al., 2005) or by paint electro-deposition (Mancuso et al., 2000). Regardless of the mode of fabrication, the performance of any polarographic oxygen electrode can be assessed by observing current:voltage curves in air saturated medium. Typically, optimally functioning electrodes have a plateau region between −700 and −800 mV that corresponds with transport limiting sensor output. The electrodes output is correlated with oxygen based on calibration against known oxygen concentrations created by bubbling the appropriate medium with gasses of known composition.

Operational characteristics of the SRA–O_2 sensors are "calibrated" as flux sensors through tests that involve using gradients created from non-biological sources (Fig. 3). This arrangement is used to validate any new sensor and measurement instrumentation in terms of dynamic physical flux sensing. An obvious aspect of this process is the determination of the impact of moving the sensor within the gradient. Obviously if the gradient is physically stirred by the movement of the sensor, this will have a negative impact on our ability to measure physical flux in the gradient. In numerous experiments, it has been shown that movement of the sensor between 0.1 and 0.3 Hz does not disrupt the O_2 gradient (Land et al., 1999; Porterfield and Smith, 2000; Mancuso et al., 2000). This was ultimately

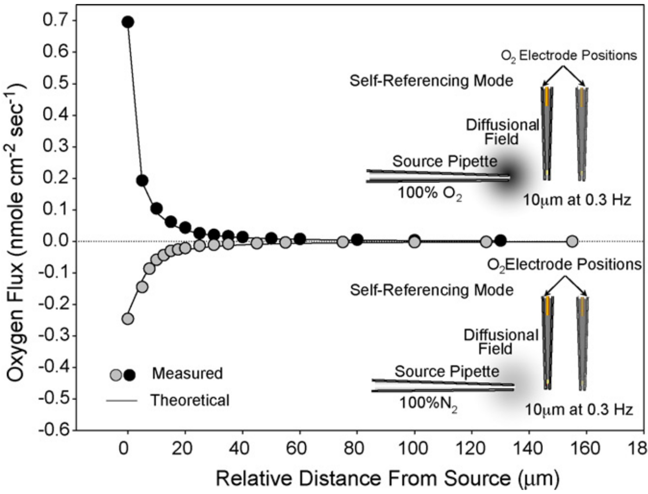

Fig. 3. Comparison of theoretical and measured oxygen flux as a function of distance from a source pipette (2 μm diameter) containing either 100% O_2 or 100% N_2. Excursion = 10 μm, frequency = 0.3 Hz. Theoretic values were calculated to model the relationship between flux and distance outside the micropipette sources (Land et al., 1999). The variability in the individual measured data points was very small. In the non-linear portion of the plot the coefficient of variance was less than 0.01, with individual standard deviations being smaller than the plotted data points for the entire plot.

proved by comparing measured oxygen flux values to those which had been mathematically modeled. With an optimized set-up the standard deviations of the individual data points are typically smaller than the plotting symbols. In the non-linear phase of the curve (1–40 μm in Fig. 1), the coefficient of variance is typically less than 0.01 and is typical for the technology.

Since development of the technique the SRA–O_2 sensor has been used in numerous biological and biomedical research studies on important aspects of physiology and pathology. This includes studies of respiratory oxygen consumption during egg fertilization (Dumollard et al., 2003) and mouse embryo development (Porterfield et al., 1998; Trimarchi et al., 2000). Lopes et al. (2005) also used a variation on the technique to study various aspects of bovine embryo development with in vitro produced embryos. Previously unpublished work on starfish egg fertilization (Fig. 4) demonstrates the utility of the approach in measuring changes in oxygen consumption associated with early events in fertilization of the egg by a sperm cell.

The technique is also now proving to be a valuable tool in the study of disease related physiology, including diabetes. Porterfield et al. (2000) demonstrated the occurrence of discrete temporal oscillations of respiration in insulin secreting beta cells responding to glucose stimulation. Work by Li et al. (2005) later used the technique to better understand the effect of low oxygen stress during diabetic pregnancy and MacLellan et al. (2005) studied the role of fatty acid metabolism and respiration in muscle cells. The technique has also proven to provide important insight in studies related to neurodegenerative diseases (Sandhu et al., 2005).

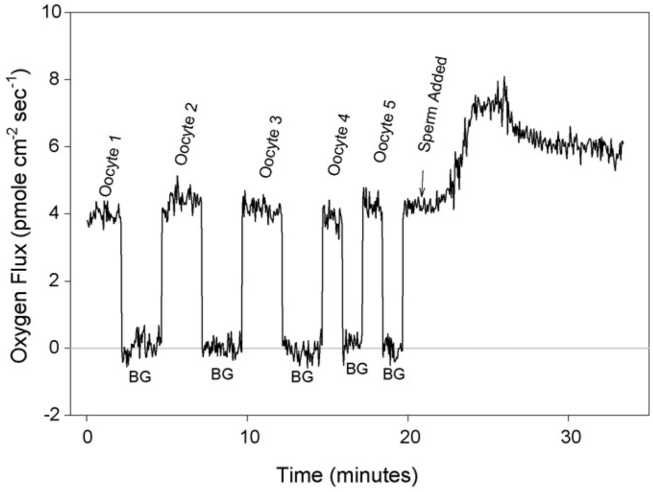

Fig. 4. Real time measurement of oxygen flux from a starfish egg during fertilization in aftificial seawater media. The starfish (*Asterias forbesi*) gametes were obtained by dissecting the gonads from adult starfish. Sperm were collected from the testes in concentrated form with a needle. The ovaries were treated with 10 μM 1-methyl adenine to induce egg release. Once collected, the eggs were put into a dish containing artificial seawater for the experiments. The probe was maintained at the surface of each individual egg long enough to obtain a stable signal, then alternatively moved to a background position 200 μm away. Each individual data point is plotted here at a frequency of collection of 0.3 Hz. Oocyte number 5 was monitored during the intervening time after addition of the sperm to the dish. The electrode was operated at 0.3 Hz over a distance of 10 μm starting from a position that was within 1 μm of the cell.

The first plant cellular experiments involved studies of photosynthesis and cellular respiration in the filamentous green algae *Spirogyra* (Land et al., 1999; Porterfield and Smith, 2000). This work was the first to demonstrate the true sub-cellular resolution of the technique by mapping and scanning oxygen flux patterns that were correlated with the position of the chloroplast within the cell. Serikawa et al. (2000, 2001) used the technology to study the marine algae *Acetabularia* and correlated oxygen and proton fluxes with sub-cellular patterns of calcification and cellular mRNA. Experiments to demonstrate the technique in studying plant root physiology were first shown by Mancuso et al. (2000) in studies that mapped patterns in olive roots. The technique was also applied to study the physiology of grape (Mancuso and Boselli, 2002) and corn roots (Porterfield, 2002). In addition to work with plants and animals Lew and Levina (2004) demonstrated subcellular hetererogeneity in the tips of growing fungal hyphae. While the SRA–O_2 technique has already proven to be an important technology; perhaps the greatest innovation is in the extension of the SRA platform to detect other target analytes.

4.2. SRA–NO sensor

Nitric oxide is a gas produced in biological systems by specific enzyme systems. These enzymes called NO sythatases, come in various forms that involve specific physiology in both plants and animals. Understanding this molecule is now considered key because of the astonishing array of physiological and pathological processes the molecule mediates. In human physiology, it mediates immune responses through bactericidal or cytotoxic reactions. It also regulates smooth muscle relaxation, neurotransmission and modulation of inflammation in a number of organ systems.

There are literally hundreds of papers published on NO sensing but for development of the SRA–NO sensor technique a basic carbon fiber electrode is modified with *o*-phenylenediamine (OPD) and Nafion. The basic electrode was constructed by modifying and combining two previously described protocols (Cahill and Wightman, 1995; Freidman et al., 1996). Briefly this involved pulling a 5 μm carbon fiber (Amoco, Greenville, SC) in a glass microcapillary. When the glass was heated and pulled it was drawn down to make contact with the fiber. The fiber was stabilized and sealed in the pulled glass pipette using epoxylite (Epoxylite Corp., Westerville, OH). After the epoxylite was allowed to cure in an oven at 110 °C for 5–10 h the electrode was backfilled with a graphite/epoxy paste (PX-grade Graphpoxy, Dylon Industries, Cleveland, OH). A copper wire was then inserted to make electrical contact with the carbon fiber through the graphite epoxy paste. After the graphite epoxy past had cured (110 °C for 5–10 h) the excess carbon fiber was chopped with a scalpel and beveled to approximately 30°. The use of OPD and Nafion to impart selectivity for the oxidation of NO was first described by (Freidman et al., 1996). Nafion is a polysulfonated Teflon that caries an intrinsic negative charge repelling electrochemically active anions (nitrate and nitrite, ascorbate). For our electrodes we first coated the

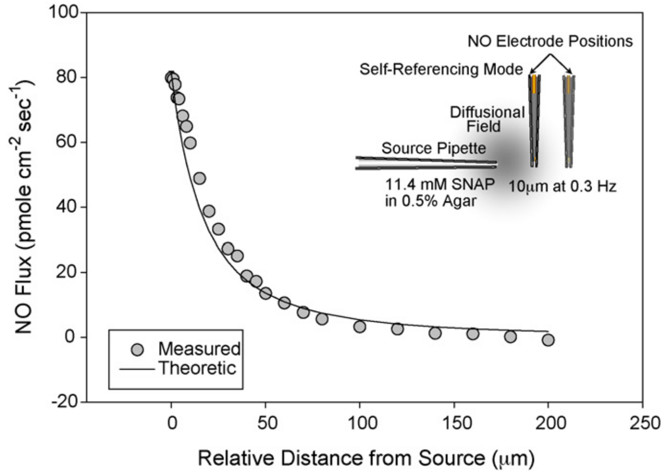

Fig. 5. Comparison of theoretical and measured nitric oxide flux as a function of distance from a source pipette containing a spontaneous nitric oxide donor (SNAP) immobilized in an agar matrix. Excursion distance = 10 µm, frequency = 0.3 Hz. Theoretic values were calculated using the differential of the modeled relationship between concentration and distance outside the artificial source (Land et al., 1999). In the non-linear portion of the plot the coefficient of variance was less than 0.01, with individual standard deviations being smaller than the plotted data points for the entire plot.

electrodes with Nafion (5% in aliphatic alcohols, Aldrich) using three coats that were dried in-between at 110 °C for 5–10 min. The OPD coating is an electrochemically active material, but is thought to impart selectivity to NO oxidation by size exclusion of non-charged interferents such as electrochemically active catecholamines. An OPD plating solution containing 5 mM *o*-phenylenediamine dihydrochloride (Sigma) with 100 mM ascorbic acid in 100 mM PBS (pH 7.4) was freshly made for each plating session. The OPD was plated at a constant +0.9 V potential until a stable current with desirable noise characteristics is obtained. The modified carbon fiber electrodes had a final tip diameter of 7–8 µm and were operated with an Ag/AgCl reference electrode which completed the circuit in solution via a 3 M KCl/5% agar bridge. Polarization voltage during operation was 0.90 V and the electrode was operated in self-referencing mode as previously described for the self-referencing oxygen microelectrode.

Before and after use, the electrode was calibrated against known concentrations of NO (Freidman et al., 1996) and tested for specificity against ascorbic acid. The SRA–NO technique was validated using artificial gradients (Fig. 5) created using the NO donor *s*-nitroso-*n*-acetylpenicillamine (SNAP). A source micropipette (diameter 10 µm) was filled with a 0.5% agar solution containing 11.4 mM SNAP in phosphate buffer (pH 7.4). After this was allowed to gel the source was maintained in a dish that contained phosphate buffer. First the gradient was characterized and modeled so that measured NO flux values could be compared to theoretically derived values as done during the validation of the oxygen system (Porterfield et al., 2001). Since development the technique has been used in several studies investigating the role of NO in biological systems (Billack et al., 2001; Kumar et al., 2001; Porterfield et al., 2001) including the induction of NO release from depolarized neurons (Fig. 6).

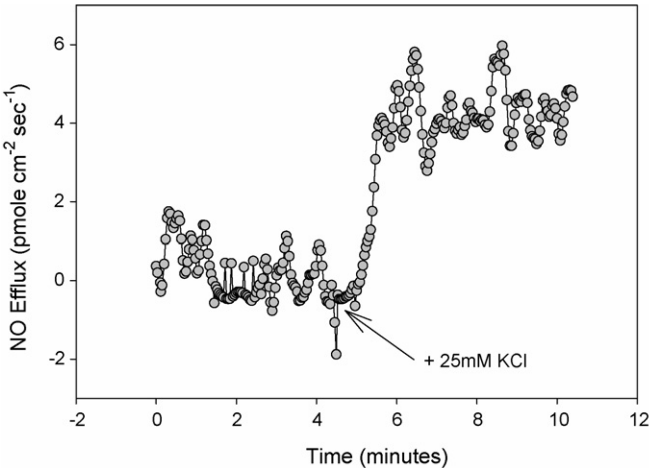

Fig. 6. Direct measurement of nitric oxide flux from a cluster of cultured neurons (GH3 cells). The cells were stimulated to release NO by depolarization caused by the application of KCl. When the cells were pretreated with an inhibitor of the enzyme that produces NO, the response is not observed. The electrode excursion distance was 10 µm and the frequency was 0.3 Hz.

Currently, there are three NIH funded studies which utilize this approach.

4.3. *SRA–ascorbate sensor*

Ascorbic acid (AA) is considered to be a prominent interferent by researchers using electrochemical probes to measure catacholamine release/uptake in the brain. That is why many strategies have been developed to exclude AA from electrochemical detection. The result is that a tremendous amount of information has been obtained about the electrochemistry of AA. Given the importance of this compound and its role as a biological antioxidant, it is not surprising to find that researchers are turning their attention to measuring this compound as opposed to excluding it. For the development of a self-referencing AA system we utilized an electrochemically modified carbon fiber electrode. These electrodes were built by modifying the basic carbon fiber electrode described for use in the NO system by electrochemical treatment (Gonon et al., 1981). A 70 Hz triangle wave that cycled between +3.0 and +1.5 V (7 s) was followed by a constant potential of +1.5 V (7 s), then held at 0.0 V (30 min). The finished electrodes with beveled tips were operated as described above for the oxygen and NO electrodes. The electrochemical treatment process decreases the polarization voltage required for operation down to the 0.05–0.1 V range. Before and after use the electrode was calibrated against known concentrations of ascorbic acid. This technique has been tested and validated against theoretic values in a gradient created with an ascorbic acid source (Fig. 7). For ascorbic acid a source pipette was prepared with 0.66 M ascorbate in a 0.33% agar matrix. As with the other methods the gradient was characterized and modeled so that measured flux values could be compared to the theoretically derived values. For research, this technique has been used to measure ascorbate fluxes from individual hamster zygotes (Fig. 8), pre-implantation embryos (Pepperell et al., 1999a,b) and isolated rat luteal cells (Pepperell et al., 2003).

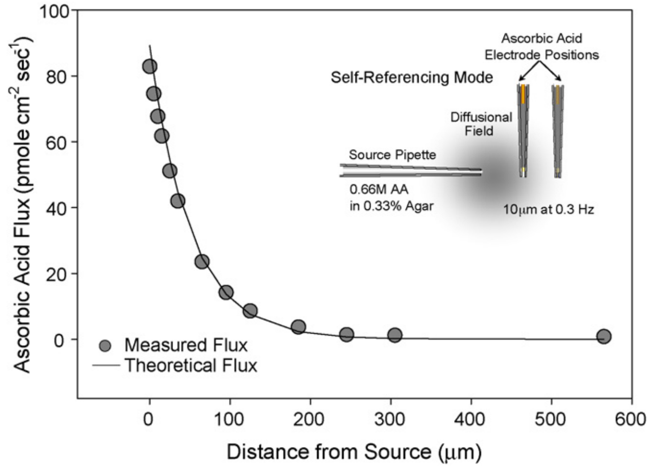

Fig. 7. Comparison of theoretical and measured ascorbic acid flux as a function of distance from an artificial source pipette containing ascorbic acid immobilized in an agar matrix. Excursion distance = 10 μm, frequency = 0.3 Hz. Theoretic values were calculated using the differential of the modeled relationship between concentration and distance outside the flux (Land et al., 1999). In the non-linear portion of the plot the coefficient of variance was less than 0.01, with individual standard deviations being smaller than the plotted data points for the entire plot.

4.4. SRA–H_2O_2 sensor

One of the by-products of many cellular reactions is hydrogen peroxide (H_2O_2), which is extremely toxic to living cells. All aerobic organisms use oxygen for respiration, but an often unwanted byproduct of this process involves reduction of molecular oxygen to hydrogen peroxide instead of water. There are also many oxidase enzymes that create H_2O_2 including those involved with conversions of amino acids during catabolism and conversion of lipids to carbohydrates. Some cells in the immune system also are known to produce hydrogen peroxide as a weapon to attack viruses and bacteria. Hydrogen peroxide can damage DNA, protein and lipid membranes and may even be a causative factor in cancer and general aging. Therefore, there is considerable interest in measuring hydrogen peroxide production from single cells.

Hydrogen peroxide is oxidizable on a variety of metal electrode surfaces including Pt and Au. The oxidation of hydrogen peroxide is the fundamental basis for electrochemically coupled enzyme biosensors that utilize oxidase enzymes. Hydrogen peroxide sensors were built by dip coating a 2 μm tip diameter Pt-Ir parylene insulated microelectrode (Microprobes Inc. Gaithersburg, MD) in a 10% cellulose acetate membrane followed by two coats of nafion. The sensor was tested in an artificial gradient (Fig. 9) but we found that the gradient tended to be unstable due to formation of gas bubbles in the micro-source pipette. The sensor was used to directly measure peroxide formation from a 250 μm diameter cultured cancer tumor spheroid model system (Fig. 10). The spheroids were cultured from of rat osteogenic sarcoma UMR-106 in RPMI 1640 medium with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% L-glutamine. The cells are transferred to a rotating bioreactor (Synthecon, Houston, TX) and they form spheroids that become visible in 7–10 days.

When the tumor was challenged with cyanide *m*-chlorophenylhydrazone (CCCP), a metabolic uncoupler which causes the electron transport system to run unchecked, peroxide generation increased and could be measured from the tumor. The sensor was not detecting NO because the working potential of the sensor (0.65 V) was lower than that required for NO. Ascorbate was excluded by the nafion membrane. The peroxide flux was modulated by addition of catalase to the dish. These results are similar to those reported by

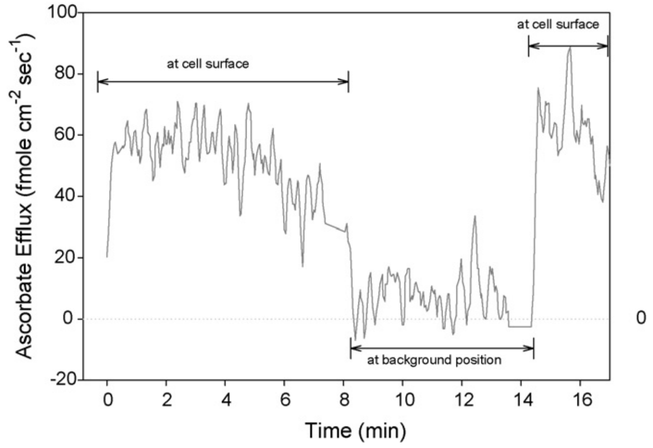

Fig. 8. Direct measurement of ascorbic acid efflux from an isolated hamster zygote. The electrode was operated with an excursion distance of 10 μm and at a frequency of 0.3 Hz. Ascorbate release and flux was detectable from the single-celled embryo. The electrode alternately moved to a background position 0.5 mm away to demonstrate that the differential flux dependent signal was spatially associated with the cell.

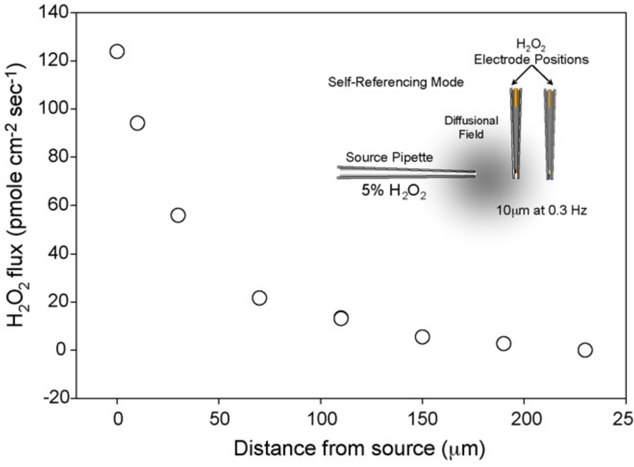

Fig. 9. Measured fluxes of H_2O_2 as a function of distance from an artificially generated gradient. The gradient was produced around a micropipette with a tip diameter between 1 and 2 μm. The source pipette contained 5% H_2O_2 but was not immobilized in an agar matrix due to the reactivity of the peroxide. The electrode was operated over a 10 μm excursion distance at a frequency of 0.3 Hz. Theoretic values were not calculated for this experiment due to the unstable nature of the artificial gradient. While the gradient was unstable the individual data point measurements was fairly constant In the non-linear portion of the plot the coefficient of variance was less than 0.02, with individual standard deviations being smaller than the plotted data points for the entire plot.

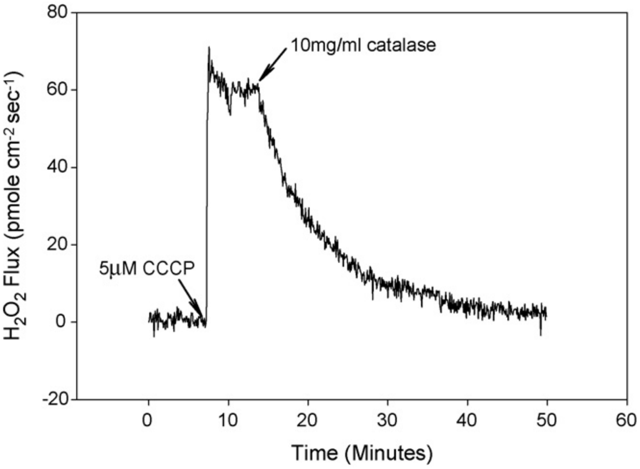

Fig. 10. Peroxide flux measured from 250 µm diameter cultured cancer tumor spheroid. The spheroids were cultured from rat osteogenic sarcoma UMR-106 in RPMI 1640 medium with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% L-glutamine. The cells are transferred to a rotating bioreactor (Synthecon, Houston, TX) and they form spheroids that become visible in 7–10 days. When the tumor was challenged with CCCP, a metabolic uncoupler which causes the electron transport system to run unchecked, peroxide generation increased and could be measured from the tumor. The peroxide flux was modulated by addition of catalase to the dish.

Twig et al. (2001) who measure peroxide flux from microglial cells.

4.5. SRA–dopamine sensor

The use of electrochemistry as an important tool for studying and understanding neurochemistry and neurophysiology is well established. Detection of catecholamines by cyclic voltammetry is a common tool in many research labs. We recently did some tests to determine if the self-referencing approach could be extended to measure flux of these neurotransmitters. For these sensors ascorbic acid (AA) is an interferent; therefore, we utilized a nafion coated carbon fiber microsensor. These electrodes were built exactly as described above, including tip beveling and dip coating of nafion. The electrodes were operated at 0.65 V and were calibrated against known concentrations of dopamine. The SR-Dopamine technique was validated for flux measurements in artificial gradient experiments (Fig. 11) where the source pipette contained 50 mM dopamine in 0.5% agar. These experiments showed that the sensor reliably measured flux as compared to theoretical values ($R^2 = 0.98$). We tested the sensor in experiments on a rat brain slice prep (Fig. 12). The sensor was positioned near the surface of the preparation, which was subsequently stimulated by KCl depolarization. Pulses of dopamine flux were measured only when nomifensin, a dopamine uptake inhibitor was added to the bath. Without nomifensin the dopamine is recycled back into the neuron from the synapse and therefore does not diffuse from the cell and tissue. Each subsequent stimulatory action on the system lowered the response until the prep was depleted. These results illustrate that other well developed schemes for sensing neurotransmitters using amperometry can be adapted for use in self-referencing mode.

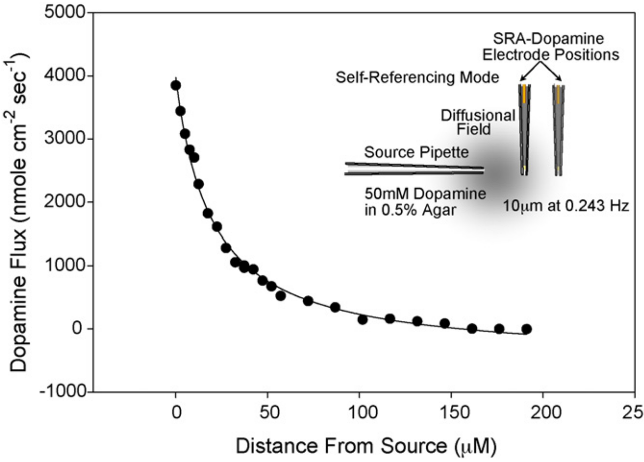

Fig. 11. A modified 5 µm carbon fiber (disk) microelectrode was first calibrated then tested as a self-referencing microsensor. A 20 µm glass micropipette was filled with 50 mM dopamine, which was allowed to sit for 30 min to allow the gradient to stabilize. The sensor was operated in self-referencing mode and stepped back from the source pipette. The measured flux values from each position are shown plotted against the theoretical flux (Land et al., 1999). The data show that the sensor reliably detected the dopamine flux within the artificially generated gradient. In the non-linear portion of the plot the coefficient of variance was less than 0.01, with individual standard deviations being smaller than the plotted data points for the entire plot.

5. Self-referencing biosensors

Low molecular weight organic compounds are ubiquitous and important in a diverse array of metabolic and biochemical pathways, as well as numerous and physiological processes. These compounds are typically measured using GC-MS techniques, which limit the types of measurements that can be made. The biosensor concept utilizes a specific enzyme to convert an undetectable substance into a detectable signal via the production of

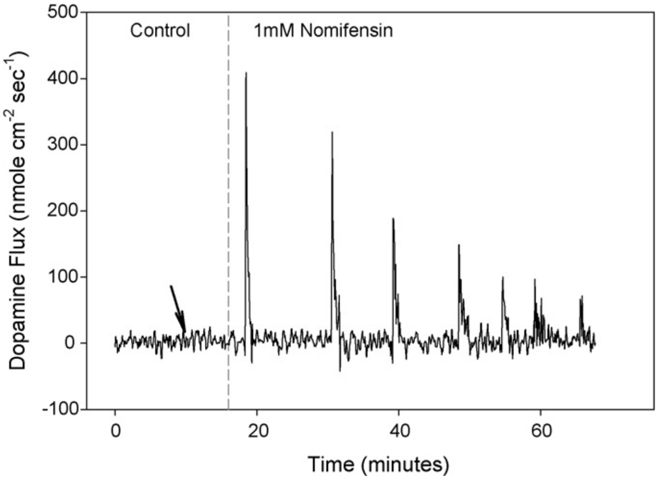

Fig. 12. Dopamine flux from a rat brain slice prep from the dorsolateral region of the striatum. The recordings were made in aCSF (artificial cerebrospinal fluid) and the tissue was a standard brain slice prep (Hoffman et al., 1998). In the absence of nomifensin KCl stimulation (arrow) did not produce a measurable flux of dopamine as the dopamine is rapidly taken back up from the synaptic cleft and recycled by the neurons. However, with nomifensin, a potent dopamine reuptake inhibitor, there is a signal that is attenuated by repeated stimulation (7 total).

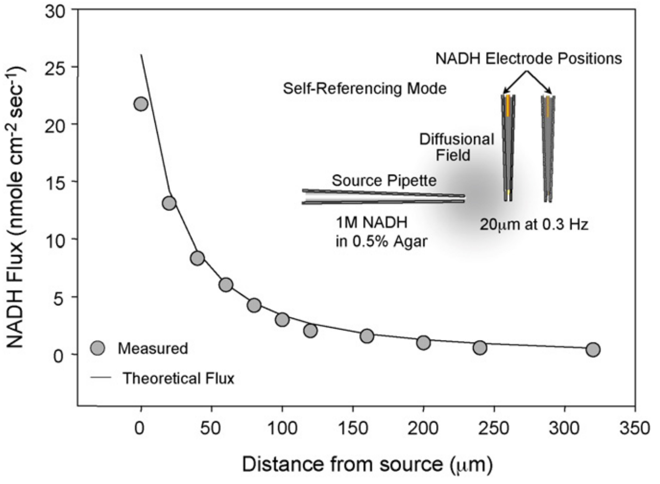

Fig. 13. Comparison of theoretical and measured NADH flux as a function of distance from a source pipette containing 1M NADH immobilized in an agar matrix. For this experiment a 5 μm carbon fiber electrode was modified for the detection of NADH (Pariente et al., 1994) and operated over a 20 μm excursion distance at a frequency of 0.3 Hz. Theoretic values were calculated using the differential of the modeled relationship between concentration and distance outside the artificial source (Land et al., 1999). In the non-linear portion of the plot the coefficient of variance was less than 0.01, with individual standard deviations being smaller than the plotted data points for the entire plot.

a measurable reporter (H_2O_2 or NADH). In general, there are two classes of electrochemical enzyme electrodes and these are based on the uses of either oxidase or dehydrogenase enzymes. Oxidase activity can be coupled to the electrochemical detection of hydrogen peroxide, whereas dehydrogenase activity is coupled to the measurement of NADH.

The first stage in development of the self-referencing biosensor (SRB) technique was to establish SRA sensors for the detection of the reporter compounds. For the oxidase-based SRB sensors the SRA–H_2O_2 sensor has been developed (Fig. 7). The SRA–H_2O_2 sensor is based on a composite cellulose acetate/Nafion membrane modified platinum-iridium microelectrode. The Nafion membrane layers are applied to the electrode as described above (NO electrode). We have focused initial development of the SRB technique to oxidase based sensors but have also validated a SRA–NADH sensor (Fig. 13) as a platform for development of dehydrogenase base SRBs.

The second stage of development of the oxidase based SRB technology was to impart selectivity for the sensor by immobilizing the appropriate enzyme on the electroanalytical surface of the microsensor. The focus in the initial development of SRB technology was miniaturization and adaptation of existing biosensors. For this we developed the self-referencing glucose biosensor (SRB–glucose) by adaptation of existing protocols (Hu and Wilson, 1997; Hu et al., 1994). This resulted in the development of the SRB–glucose sensor, which was subsequently validated and tested in artificially generated gradient experiments. Jung et al. (2001) also developed and utilized a SRB–glucose biosensor to measure glucose consumption in pancreatic beta cells. This was later adapted and used to validate a SRB–glutamate sensor that was tested in an artificial gradient experiment (Bogorff et al., 2003).

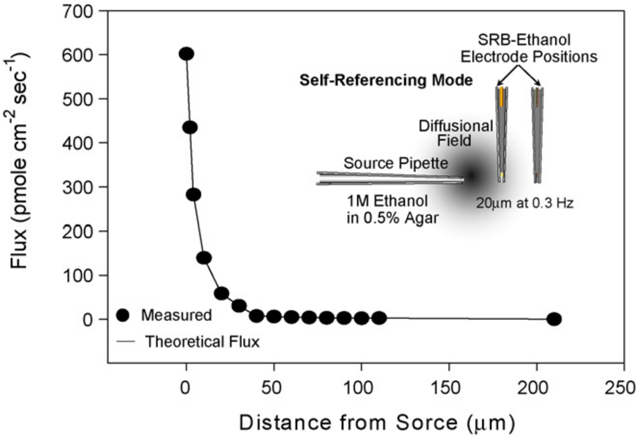

Fig. 14. Comparison of theoretical and measured ethanol flux as a function of distance from a source pipette containing 1M ethanol immobilized in an agar matrix. For this experiment a 5 μm platinum iridium electrode was modified by immobilization of the alcohol oxidase enzyme on a 5 μm Pt microelectrode based on 2,6-dihydroxynaphthalene (2,6-DHN) copolymerised with 2-(4-aminophenyl)-ethylamine (AP-EA) onto the Pt electrodes according to previously described protocols (Badea et al., 2002) The resulting sensor was calibrated and operated as self-referencing sensor (20 μm excursion distance at a frequency of 0.1 Hz). Theoretic values were calculated using the differential of the modeled relationship between concentration and distance outside the artificial source. In the non-linear portion of the plot the coefficient of variance was less than 0.01, with individual standard deviations being smaller than the plotted data points for the entire plot.

Building on this development, we have recently developed a SRB–ethanol sensor by substitution of the enzyme alcohol oxidase for glucose oxidase. Here, we tested a new protocol for immobilizing the enzyme (Badea et al., 2002). In this procedure, the alcohol oxidase enzyme was co-entrapped on a 5 μm Pt microelectrode based on 2,6-dihydroxynaphthalene (2,6-DHN) copolymerized with 2-(4-aminophenyl)-ethylamine (AP-EA). We tested the sensor in an artificial gradient (Fig. 14) and have been using it to study hypoxic stress in plant roots during soil

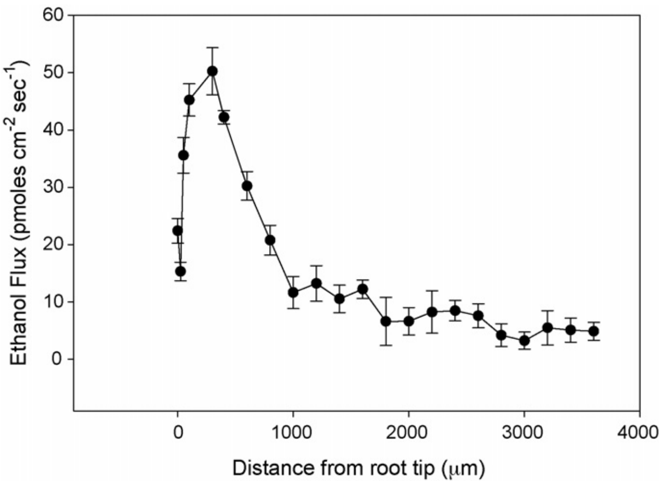

Fig. 15. Ethanol flux patterns along an *Arabidopsis thaliana* (Columbia) root tip as measured using an SRB–ethanol sensor. Here, the plant roots were submerged for 12 h before being scanned for ethanol produced by fermentative metabolism. The peak for ethanol production correlates with a position that is approximately 250 μm from the root tip and this is where we see peak oxygen consumption in normoxic roots.

flooding conditions (Fig. 15). Now that the initial development in SRB sensors has occurred it is not hard to imagine that the near-term development will include rapid diversification of these systems based on application of new enzymes for the sensors.

6. Conclusions

While the self-referencing sensor approach has been widely adapted and used for physiological sensing, the importance of the technique in terms of efficacy and sensitivity is not widely understood within the greater community of engineers, physicists and chemists who work in the fields of sensor science and engineering. The technique was originally developed and applied to study ion transport and has been broadly applied to study and understand some very important questions in agricultural, biological and medical sciences. Here, we have introduced and reviewed the major advances in the development of self-referencing amperometry, both for direct bio-analytical sensing and as a platform for development of self-referencing biosensors.

Biosensing has emerged over the last 10–20 years as being an important area of research and work in this interdisciplinary field is very extensive. From electrical engineering and micro-fabrication to materials science and biochemistry there is tremendous diversity in the field both in the breadth of the work and the backgrounds of the investigators. While most of this work is focused on expanding the scope and sensitivity of the sensors themselves, here we seek to improve and advance the modality of sensing in terms of biophysical transport and physiology. Essentially it is not what you sense, but instead how you sense it that is important. For a biologist or biomedical research scientist, being able to directly measure and map transport activity is a tremendous advantage and it represents a transition from concentration sensing to physiological sensing in the truest sense.

7. Future perspectives

While the ability to directly measure biophysical transport cannot be understated, it is important to understand the fundamental basis for how the technique improves the functional sensitivity of the sensor itself. By moving the sensor and modulating the signal we are using phase sensitive detection, which allows us to significantly improve the signal-to-noise ratio. This extends the range and sensitivity of the sensor such that it provides dynamic, multidimensional information about the biological process being studied. Understanding and adapting this concept to other sensing systems is going to be especially important as we advance sensing into the nanoscale range of sub-cellular domains. Develop of new sensors with expanded ranges in the types of analytes detected and the actual size of the sensor will undoubtedly continue and these sensors will be functionally enabled by the application of self-referencing technique.

Amperometry may see some advances but chemically most of the detectable analytes, accessible using this approach, have already been explored. Instead using amperometric coupling for biosensor development and application seems to be the most promising area here. We have recently adopted a novel approach to developing this technology. Previously biosensor development has depended upon the availability of enzymes for sensors from commercial vendors. Now we are using a genomics approach; to identify/clone the gene and produce the biosensor enzyme in a transgenic system. This starts with physiological identification of the specific enzymes in biological systems.

Another area that holds tremendous opportunity is in adaptation of sub-micrometer chemical sensing pioneered by Kopelman and co-workers over the last decade for self-referencing optrodes. This work developed optrodes for extremely small-scale (micro-nanoscale) sensing of intracellular pH (Tan et al., 1992a,b). By coating an optical fiber with aluminum but leaving a 100 nm tip exposed they created a sub-wavelength light source. Covalent immobilization of selective luminophores by photopolymerization on the tip converts the fiber into a chemical sensor. Because this method uses near field optics, the typical $\lambda/2$ diffraction limit does not apply to limit the degree of miniaturization. The resulting probe is capable of extremely fast response times (ms) in femptoliter volumes with excellent detection limits.

As the omics revolution advances and bionanotechnology emerges, we undoubtedly will witness a convergence based on a need to understand nanoscale sub-cellular mechanisms including biophysical transport. This convergence in the form of cytomics and physiomics will require that we advance sensor science and technology not only in terms of bio-analyte sensitivity and selectivity, but also in terms of biophysical transport activity and physiological sensing.

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The pioneering work of Lionel Jaffe in the areas of electrophysiology and physiological sensor development was the foundation for this work. I would like to thank Lionel for his advice and input over the years I spent at the Marine biological Laboratory. Others who deserve thanks include Kasia Hammar, Rick Sanger and Peter Smith of the Biocurrents Research Center, as well as Al Shipley and Eric Karplus who were formally members of the National Vibrating Probe facility. Much of the work presented was supported in one way or another by NASA, NIH, NSF, Purdue University-Discovery Park and the USDA.

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