

## Bio Currents Research Center Tutorial

### Self Referencing: The Basics

The material below has been adapted from several primary papers and reviews originating from the BRC. These should be referred to in publications and for the original source materials.

#### References:

1. *Smith, P.J.S., Sanger, R.S. and Messerli, M.A. (2007) Principles, Development and Applications of Self-Referencing Electrochemical Microelectrodes to the Determination of Fluxes at Cell Membranes. In: Methods and New Frontiers in Neuroscience. Ed. Adrian C. Michael. CRC Press. Ch. 18: 373-405.*
2. *Messerli, M.A., Robinson, K.R. and Smith, P.J.S. (2006) Electrochemical sensor applications to the study of molecular physiology and analyte flux in plants. In: Plant Electrophysiology - Theory and Methods. Ed. Alexander G. Volkov. Springer-Verlag. 73-107.*

### **Introduction**

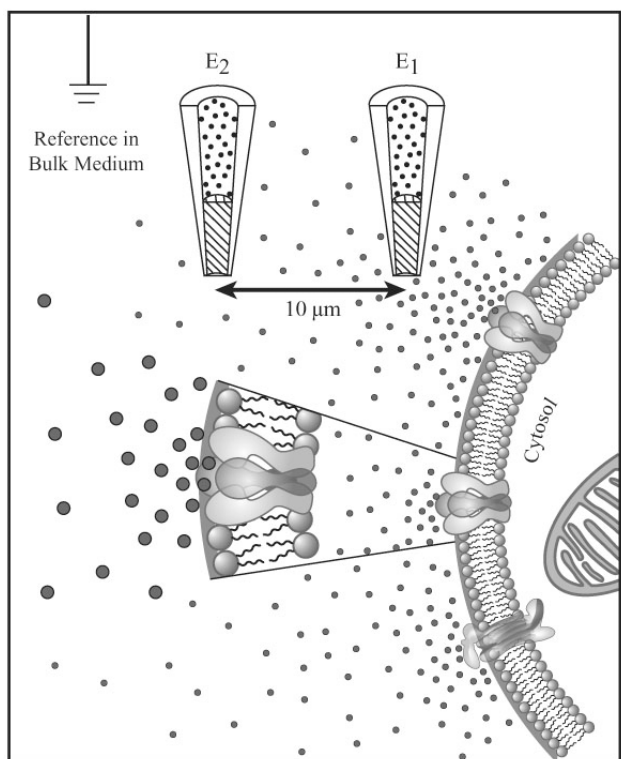
The complexity of the cellular microenvironment leads to two inevitable conclusions - *in vivo* studies are difficult and reduced preparations are simplistic. However, studying the brain environment and cellular function from both angles provides information that clearly advances our understanding of cellular function, signal processing and diseased states. Implanted sensors in whole brain, both electrical and electrochemical, have contributed to our knowledge of CNS organization and function. Recent moves to merge techniques of on-line capillary electrophoresis and detection of multiple chemicals simultaneously are exciting advances in understanding the CNS microenvironment.<sup>3</sup> Clearly, the chemical composition of this environment is important in not just sustaining the cells, but also in providing and controlling information transfer between cells. *In vivo*, however, studies are usually restricted to populations of cells and specific mechanisms are difficult to examine on a cell-by-cell basis. Reduced preparations and single cell studies offer insights into mechanisms, even if integration has been largely lost.

The past two decades have seen tremendous advances in our approach to single cell studies. Electrophysiology has been dominated by the powerful derivatives of the original patch clamp technique, addressing the biophysics of single channels or whole cells.<sup>4</sup> Imaging has advanced our understanding of architectural dynamics and intracellular ion activities.<sup>5,6</sup> Indeed, the success of the latter displaced the use of intracellular, chemically-selective electrodes where voltage changes and competing ions make the technique difficult and the interpretation complex. Despite these limitations electrochemical sensors have continued to demonstrate strength and versatility when applied to the external boundary layers of single cells and tissues. For example, correlation between vesicle fusion and cell capacitance was demonstrated for dopamine release by Travis and Wightman<sup>7</sup>, and 5-HT with histamine electrochemistry has been used to study quantal corelease.<sup>8</sup> Both of these examples require the placement of a carbon fiber, amperometric microelectrode, in close apposition to the plasma membrane where strong electrochemical signals can be derived over low background. These cases are special in that a discrete cellular event carries a clear electrochemically detectable, phasic, signal into the boundary layer. All cells, however, modify the diffusive boundary layers as a result of physiology, for example transporter activities or respiration. These signals can be weak and often imposed on top of significant background levels of the same chemical - oxygen and calcium flux detection are examples. Problems in measuring changes of such chemical signals are further compounded by the instability of any electrode design. How can these limitations be overcome to make boundary layer measurements with high temporal and spatial fidelity?

Two approaches to making measurements in the boundary layer can be proposed. Firstly, the gradient can be constrained by a restrictive space, effectively 'amplifying' and defining the signal to be detected. Secondly, detection can be coupled directly to the chemical gradients radiating into or out of the cell. Two elegant examples of the first approach are available. Poitry et al. sought to measure the oxygen consumption and nucleotide levels, under closely matching conditions, for single cells.<sup>9</sup> To acquire the oxygen levels single rod photoreceptors were captured in glass micropipettes such that they lodged approximately 10  $\mu\text{m}$  from the tip. A Whalen style oxygen electrode<sup>10</sup> was then inserted through the pipette tip and measured oxygen consumption in a static configuration. Kang and Hilgemann<sup>11</sup> recently published results

from a similar approach but for the study of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger in a cardiac muscle macropatch. Here an ion-selective electrode was advanced into the body of a pipette supporting the macropatch. As the pipette geometry could be precisely modeled, Kang and Hilgemann were able to derive the stoichiometry of this electroneutral transporter.<sup>11</sup>

Coupling directly to the diffusive boundary layer, by alternating the placement of a chemically selective microelectrode in the proximity of active tissues or cells, was first reported by Gow et al.<sup>12</sup> These investigators monitored pH changes proximal to a growing fungal hypha. The process of microelectrode placement and modulation was automated by Jaffe and Levy and applied to biological preparations by Kühtreiber and Jaffe.<sup>13,14</sup> In a more recent study Kang et al. made measurements of the transmembrane potassium transport by 'self-referencing' through movement of the macropatch relative to a  $\text{K}^+$  ion-selective electrode.<sup>15</sup> The development of this 'self-referencing' technique (defined below) has occurred primarily at the BioCurrents Research Center, funded as a national resource by the NIH:NCRR since 1996, but also in other laboratories.<sup>2</sup> The term 'self-referencing', as it applies to this technique, was coined by Smith et al.<sup>16</sup>



In a static configuration, drift, sensitivity, and chemical selectivity, or the lack of it, can have complicated implications for the use of these sensors, whether potentiometric or amperometric. In a self-referencing, non-invasive, mode of operation, many of these conventional problems can have a minimal impact, although other aspects influence the quantification of data recovered.

**Figure 1:** Self-referencing electrodes operate in the diffusive boundary layers surrounding cells and tissues. Within these layer analytes form gradients depending on cellular activity, such as channel openings, as illustrated, or metabolism. This figure illustrates an ion selective microelectrode located at both the near pole and far pole within an ion gradient. The voltages recorded,  $E_1$  and  $E_2$ , will depend on the activity of the ion. Comparing these two values generates a differential value minimizing the impact of non-relevant voltages generated by background levels of the analyte, junction potentials and competing analyte levels. The same principle applies to amperometric microelectrodes.

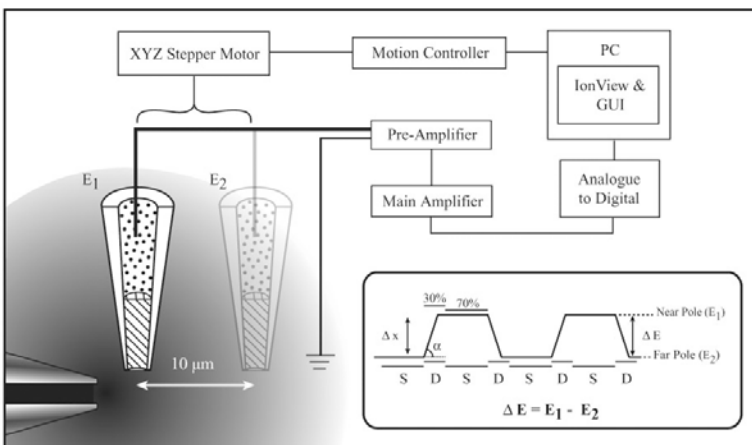
In the context of the electrochemical technique reviewed here, self-referencing refers to a modulation approach. The method belongs to a family of techniques employing position modulation of microprobes to enhance signal detection. Variants can be seen in scanning electrochemical microscopy, atomic force microscopy, and scanning reference electrode techniques used in both biological and materials science.<sup>17-20</sup> In the application described here the term implies that a single sensor is modulated such that signal values obtained at the near pole (closest to the tissue or cell) can be compared to the values at the far pole (a known distance away). As will be discussed below this delivers significant advantages in signal analysis with background, drift, and noise reduction - the electrode is effectively referring to itself at two locations. A second and independent reference electrode is also included to complete the circuit. When positioned in a chemical gradient surrounding a single cell, or tissue surface, this type of electrochemistry generates a differential output that can be converted to a flux value through calibration and Fick's 1st Law. As the strength of voltage fields in conductive animal media will be negligible, there is no need to use the more complex solution embedded in the Nernst-Einstein equation. Using this approach with potentiometric electrodes differential signals of low  $\mu\text{volts}$  can be observed and with amperometric electrodes values of femtoamps are measured. The technique can be coupled to electro-optical and positional methods.<sup>21,22</sup> Overall, self-referencing of an electrochemical sensor offers the advantage of low signal detection, observation of non-electrogenic transport, use of ion-selective and redox based reactions, and enzyme-assisted flux detection, all at the single cell level.

## Self Referencing Principles

Cells modify the immediate chemical composition of the medium surrounding them. This is an inevitable consequence of physiologically driven molecular movements, both active and passive. For example, as a cell respire, oxygen will be taken up from the medium and a shallow depletion gradient will exist around the plasma membrane, particularly in locations where mitochondria are localized.<sup>23</sup> Similarly the opening of a channel, or the activity of a pump, will locally modify the chemical concentrations/activities in a regional manner (Fig 1). These gradients reveal aspects of cell physiology in both normal and diseased states, making it of interest to couple to the gradients and thus gain insight into cell function. To measure a gradient one needs two points of reference, a known distance apart, recording an identifiable analyte, with a known diffusion coefficient.

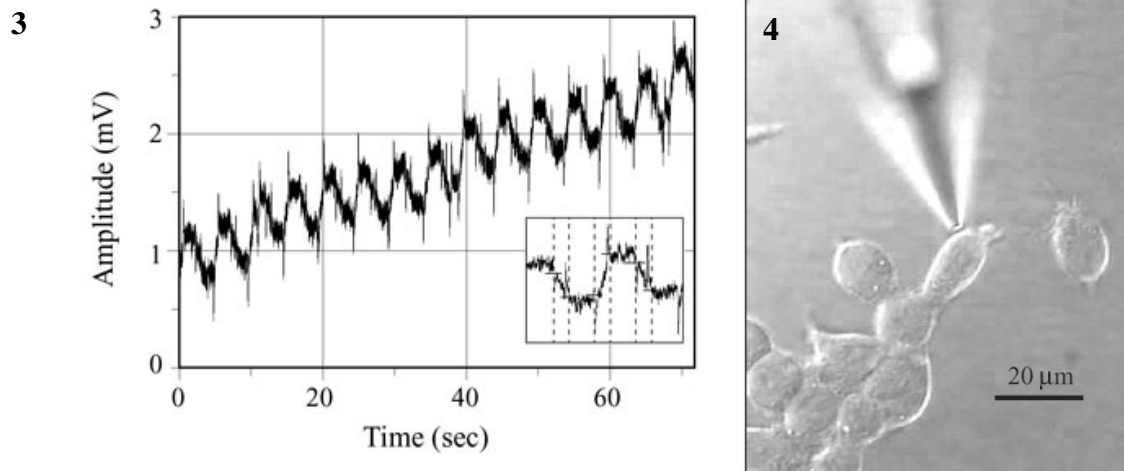
The basic operational configuration of a self-referencing electrode is shown in Figure 2, where an ion-selective microelectrode (ISM) is illustrated near a pipette source, forming a defined gradient in the bulk solution (for details of this procedure and comparisons to modeled gradients see Smith et al.<sup>16</sup>). The ISM is moved in a square wave between two points in the gradient, usually 10  $\mu\text{m}$  apart. This distance can be varied. The frequency of movement is usually 0.3 Hz. At that frequency the electrode comes to rest for approximately 1 second at each pole after translation. As with distance, frequency can be independently controlled. The point nearest the source is referred to as the near-pole and the other as the far-pole. The convention is followed that if the electrode detects a higher concentration at the near-pole the flux is given a positive value indicating efflux. Signals are sampled 1000 times a second with the first 30% collected during and after translation being discarded. A final differential value is stored along with the correlated DC value, which can be compared to an original calibration slope.

The key to the sensitivity of this approach is that drift and background are relatively common to both positions of translation. Thus, if we observe the signal seen by the amplifiers when an ion-selective electrode is placed in a steep ion gradient, from an artificial source, we get the result illustrated in Figure 3. No signal processing has taken place. A clear drift is seen in the signal from both poles of translation. Extracting a differential voltage or current, by subtracting the far pole signal from the near pole, minimizes the impact of drift and subtracts out background signals as well as contaminating voltages - such as junction potentials.



**Figure 2** A schematic illustration of the self-referencing set-up. In this case an electrode is shown near a source of the analyte, moving in a 'square' wave step between two positions 10  $\mu\text{m}$  apart. Data is collected at 1000 points per second with the first 30% collected during and after translation being discarded (D). Movement is frequently set for a 10  $\mu\text{m}$  displacement ( $\Delta x$ ) at a frequency of 0.3 Hz, thus data is collected at each pole for approximately 1 second or 70% of the cycle time (S). Comparing, in this case, voltages collected between the two extremes of movement, gives rise to a differential value ( $\Delta E$ ). See equipment and software protocol for description of components.

Although originally conceived for calcium flux detection<sup>14,16,24</sup> uses of ISMs for the detection of potassium and hydrogen<sup>25</sup> as well as chloride ion flux<sup>26,27</sup> have been popular extensions. Amperometric-based detection of nitric oxide, oxygen, glucose and ascorbate, have proved powerful additional applications. No other approach permits repeated non-invasive observations on the same intact cell over hours or days, recording flux over periods of seconds, with a spatial resolution of a few microns. Figure 4 illustrates a  $\text{Ca}^{2+}$  ISM in the near pole of measurement next to an INS-1 cell. In this sense the technique is unique and complements other methods available to observe the movements of analytes across membranes or within cells.



**Figure 3 & 4:** Raw data collected from a Calcium ISM moving in a step gradient generated by a 100 mM source. A clear stepping of the data can be seen as the ISM moves between the two poles of translation. Significant drift in the base line is also evident. The insert illustrates the time scale for two cycles. The broken lines divide the signal into the discard and save sections – see figure 2. Biological signals will normally be in the microvolt range and therefore buried in the noise.

**4** A real microelectrode near a real cell. This figure shows a  $\text{Ca}^{2+}$  ISM located at the near pole position for measurement from a cultured INS-1 cell. As the electrode is silanized, and the cocktail lipophilic, stability of electrode placement and movement is critical. The electrode must not touch the cell.

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