

## Bio Currents Research Center Tutorial

### Problems and Pitfalls

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.*

#### **Potentiometric: Selectivity**

It cannot be overemphasized that all the electrochemical techniques used in a self-referencing mode to date are selective not specific. There is always a finite chance that an interferent can be detected under experimental conditions designed for the primary analyte. As discussed above, the differential approach, inherent to self-referencing a microelectrode, will greatly reduce the probability of simple background interferents being detected. However, unexpected problems can happen. Chloride detection using an ISM built with the Fluka Cl<sup>-</sup> selective liquid ion exchanger cocktail A, is a good example. This ISM should more accurately be described as an anion sensor, even detecting a H<sup>+</sup> flux via sensitivity to the unprotonated anionic form of a Good buffer.<sup>3,4</sup> This ISM is also sensitive to a pharmacological agent used to block chloride transport, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS).<sup>5</sup> Measurements of Cl<sup>-</sup> with the more commonly used Cl<sup>-</sup> ionophore I, cocktail A (Fluka Chemical Co) can sense or be poisoned by chloride channel blockers such as 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid (NPPB), indanyloxyacetic acid<sup>6</sup> (IAA-94), and 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS).<sup>7,3</sup>

An additional area of interference is caused by exposing an ISM to excitation light during imaging. Hydrogen flux detection, while imaging the cytosolic pH sensor BCECF, is not possible with global illumination. In this case the microelectrode couples to the excitation wavelength altering the Nernstian values and sensitivity (unpublished observation - Anthony Molina). Caged calcium has been released while using the amperometric oxygen microelectrode without adverse effect.<sup>8</sup>

#### **Amperometric: Selectivity**

Amperometric electrodes are also susceptible to the detection of multiple molecules. For example, nitric oxide (NO) oxidation at a carbon fiber begins to plateau between +0.8 - +0.9.<sup>9</sup> Neurotransmitters, ascorbic acid, nitrite and H<sub>2</sub>O<sub>2</sub> can also be oxidized at this high voltage. A charged coating on the carbon fiber surface can eliminate passage of charged compounds.<sup>9,10</sup> However, H<sub>2</sub>O<sub>2</sub>, an uncharged molecule, will be oxidized as it can pass through the coatings. Addition of a layer of catalase to the charged coating layers, similar to the enzyme layers used by Csöregi et al.<sup>11</sup>, can eliminate H<sub>2</sub>O<sub>2</sub> before it reaches the electrolytic surface.

#### **Mixing**

As self-referencing involves movement there is an inevitable risk of disrupting the gradient. This disruption can be minimal if a sufficient amount of time is allowed to pass for the gradient to be reestablished after sensor movement. The slow movement of the self-referencing microelectrodes, usually 40 μm/sec, does not cause turbulence that can be detected visually. Also, the Reynolds numbers calculated for the speed of movement of either the small tip, 1-4 μm diameter, or even up the shaft where the diameter reaches 50 μm, are in the range of 10<sup>-4</sup> - 10<sup>-3</sup>, respectively, indicating that only laminar flow occurs around the tip of the electrode. However, in the worst case scenario, if gradient disruption does occur how long must the microelectrode remain stationary for the gradient to be reestablished? If we consider the case of reestablishing the potassium gradient, after a 10μm move between the near and far pole, we find that it will take about 8.3 msec for K<sup>+</sup> to diffuse this distance. This indicates that an ionic gradient would be rebuilt in a few tens of milliseconds. As long as turbulent flow does not occur, most small analyte gradients will be rebuilt quickly.

### **Positional Artifacts**

Positional artifacts can be broken into two types. The first concerns access or egress of analytes to or from the microelectrode tip. Specific cases are discussed below. The second potential artifact comes from a change in the geometrical relationship between the electrode and the target cell or tissue. By definition the amplitude of a differential signal will depend on the position from the source and the recording position within the gradient. If this is not controlled then apparent flux changes will occur that actually represent a changing electrode to target distance. Although the motion controller design developed by the BRC shows exceptional stability<sup>12</sup>, cells can move. This has constrained most studies to recordings made perpendicular to the visible edge of a cell or tissue, thus stabilizing distance by visual observation and software based manual adjustment. Recently a new approach has been developed for the amperometric microelectrode that allows positional control through coupled electrochemical techniques.<sup>13</sup> In this case an AC signal of 10mV at 100kHz is superimposed around the -0.6V working potential of a self-referencing oxygen electrode. The monitored amplitude of this signal is dependent on the access resistance and, therefore, sensitive to the distance from an object. There is no interference between the two voltages and oxygen flux changes from a single INS-1 cell can be recorded while correcting for nanometer changes in electrode to target distance.<sup>13</sup>

### **Positional Artifacts: Potentiometric**

The positioning of ISMs near surfaces can generate artifacts. Of particular relevance to the self-referencing technique is the possibility of ions emanating from the tip of the sensor and accumulating between the electrode tip and the target cell or tissue. The source of this potential artifact is a combination of current leakage from the electronics and zero-current (electroneutral) ion exchange of the primary ion for an interfering ion. This phenomenon has been shown for  $K^+$  and  $Ca^{2+}$ -selective solvents.<sup>14,15</sup> The artifacts are most apparent in solutions of a low background primary ion. Reducing the concentration of the primary ion in the backfilling solution has been one compensation method.<sup>14</sup> Additionally, an applied current can control the flux across the membrane.<sup>15</sup> Pergel et al. used a current to compensate for the zero current phenomenon with  $Pb^{2+}$  detection.<sup>16</sup> None of these solutions have as yet been applied to self-referencing microelectrodes.

### **Positional Artifacts: Amperometric**

In amperometric applications the analyte is actually being consumed on the electrode surface. Therefore, an analyte depletion zone can form. An ideal electrode is built such that the analyte-depleted region is located primarily within the electrode itself. However, it can quickly be envisioned that even a small depletion of the analyte in the bulk can give rise to a measurement artifact. In the extreme case, placing the electrode up against an insulator will prevent any analyte from reaching the surface of the electrode resulting in no change in current for a change in analyte. Backing the electrode away from the insulator, such that analyte can now diffuse, albeit in a restricted manner, will still give rise to a value below the real level. Moving back further, such that diffusion of analyte is not impeded, will result in maximal current for a change in analyte concentration. This phenomenon has been observed with the  $H_2O_2$  product of the glucose electrode reaction building up between the electrode and a cluster of Hamster Insulinoma Tumor cells.<sup>17</sup> In that specific case the artifact was removed by the addition of catalase to the medium.

### **Voltage Fields**

In low conductivity medium, cellular currents can generate substantial voltage gradients next to the cells, coexisting with the chemical gradients of the analytes being measured. The potentiometric electrodes may sense these voltage gradients. Therefore, the voltage difference measured by the potentiometric electrode will be the sum of the voltage differences, due to the analyte concentration difference and the voltage difference, based on the current density and medium conductivity. In an animal system, however, the effect of voltage fields will be minimal due to the conductivity of the medium. Earlier studies using the vibrating voltage electrode confirm that these voltages are in the  $nV$  range and will not impact significantly on the ISMs.<sup>18</sup>

Static surface charges exist within the glycocalyx of cells as a result of molecular and ionic charge distribution. These charges will project their electrostatic effects into the bulk medium in accordance with the Debye length,  $1/K$ . In the case of a mammalian Ringer this is  $7.8\text{\AA}$  (0.78nm).<sup>19</sup> All self-referencing microelectrode studies have been conducted at significantly greater distances.

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