

Bio Currents Research Center Protocol

Data Collection

Introduction

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.*

Theoretical considerations for signal detection and increased signal to noise

Self-referencing of electrochemical sensors involves the extraction of small electrical signals, μV or fA differences on top of relatively large offset signals, $\pm 100\text{s mV}$ or $\pm 10\text{-}100\text{s pA}$. The offset signals are usually large enough that only low-level gain can be used in order to keep the signal within the dynamic range of the amplifier. In order to supply sufficient gain to resolve the small electrical differences either 1) a nearly equal and opposite electrical offset must be supplied before amplification (sample hold mode) or 2) a running average of the low gain measurement can be subtracted from the real-time input before amplification (RC subtract mode). Sample hold mode either applies an offset selected by the user, or collects a voltage at a set point in time from the electrode output. It then applies the same magnitude offset of opposite sign to nullify the offset potential before applying 103 times gain. The primary disadvantage for this mode is that drift can take the system back out of the dynamic range of the amplifier so that a new potential must

be applied regularly. The advantage is that it does not need an additional correction factor to compensate for the signal lost due to the filtering that occurs in RC subtract mode. In RC subtract mode a high-pass filter is used to collect a running average potential that is subtracted from the potentials collected in the near and far pole. The signals are then amplified 103 times before digitizing. We use a high-pass filter that has a time constant of 10 s. Figure 1 shows a $200\ \mu\text{V}$ peak to peak square wave input, passed through the BioCurrents Research Center amplifier using Sample Hold and RC subtract mode acquisition.

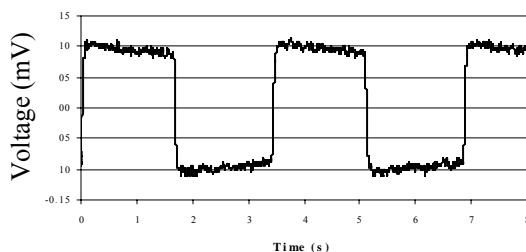
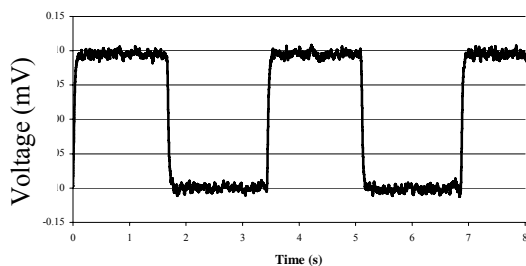


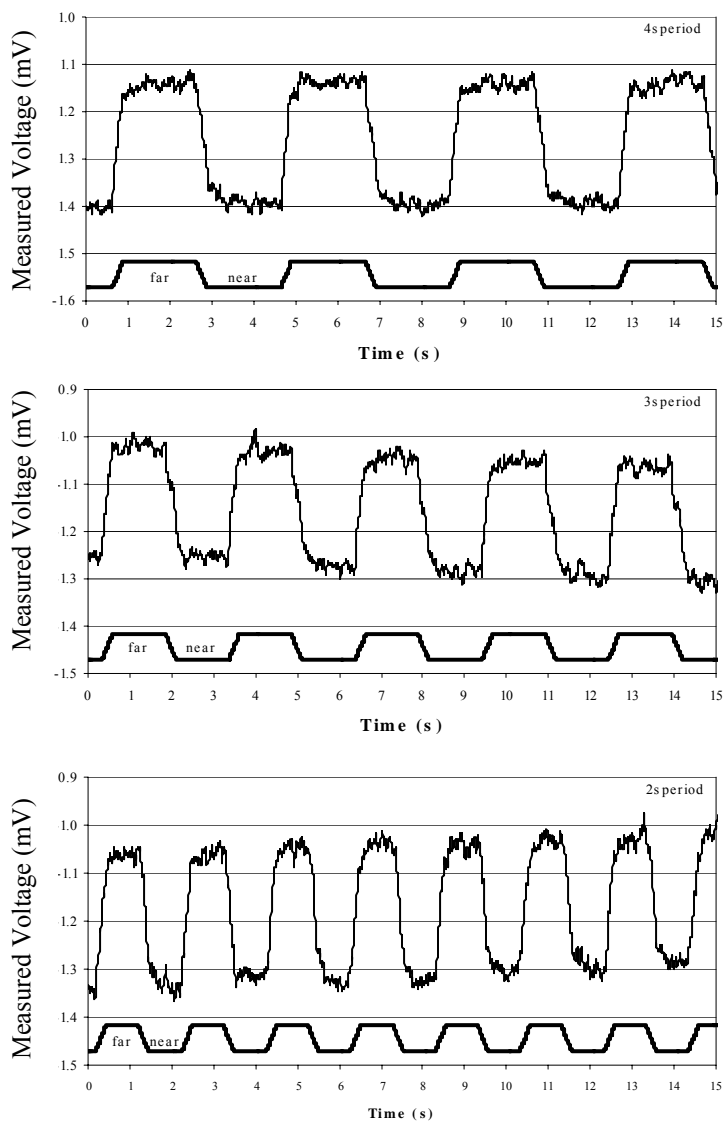
Figure 1: Comparison of the waveform distortion that occurs between 'sample hold' and 'RC subtract' mode. No distortion occurs for a square wave input during 'sample hold' acquisition mode. The highpass filter used during 'RC subtract' mode does cause waveform distortion that needs to be accounted for when determining the differential concentration.

With sample hold mode, the acquired signal is a square wave. The RC subtract mode however, has been distorted due to the filter. RC subtract allows amplification for systems with large drift but involves a correction factor to offset the high-pass filter. The correction factor will be dependent on the time constant of the high-pass filter and the period of data acquisition. For our normal conditions a period of 3.3s (0.3 Hz translation frequency), $40\ \mu\text{m/s}$ translation speed, 10 s time constant of filter along with data selection of the last 70% of the half cycle (more below) we calculate that the signal is 7% smaller than a square wave with similar rise time.

The primary reason for the added level of amplification is to make use of poor resolution digital systems. A dynamic range of ± 10 V provides only 4.9 mV/bit resolution for a 12-bit system, and 0.3 mV/bit for a 16-bit system. Therefore, amplification is necessary before digitization to provide adequate bit resolution at or below 1 μ V. Now that 24-bit A/D cards exist, signal resolution of 1.2 μ V can be attained over a dynamic range of ± 10 V.

Data collection

In order to accurately measure the analyte concentration at two points in a gradient with an electrochemical sensor in self-referencing mode, gradient disruption by the electrode must be minimal and the movement time and response time of the electrode must be significantly faster than the excursion period. Gradient disruption will be minimal if a sufficient amount of time is allowed to pass for the gradient to be reestablished after sensor movement, as movement of the sensor may disturb the gradient. The slow movement of the probe, usually 40 μ m/s, 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 μ m diameter, or even up the shaft where the diameter reaches 50 μ m, are in the range of 10^{-4} - 10^{-3} , respectively, indicating that only laminar flow occurs around the tip of the sensor. However, if some disturbance does occur, how much time must pass to allow the gradient to be rebuilt? Considering the reestablishment of a K^+ gradient over the excursion distance of the probe, 10 μ m, we find that it will only take about 8.3 ms, for K^+ to diffuse this short 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 very quickly.



Unfortunately there is no simple rule concerning the selection of the correct translational frequency and excursion distance. These are influenced by the specific conditions of cells and electrodes. Put simply, an investigator should ensure that both poles of translation are within the gradient - preferably over a 'linear' portion - and that the frequency is adjusted to allow a reasonable sampling period but one that avoids the impact of drift. The electrode must also have time to fully respond before data is collected. Messerli et al. deal with these matters in more depth.² Within the BRC a default starting position of 0.3 Hz over a 10 μ m translation is usually used.

Figure 2: Voltage response of a H^+ -selective electrode collected continuously during translation between two position near a H^+ sink. The measurements were acquired in low Ca^{2+} Dickinson's medium. The three graphs correspond to the period of translation listed in the upper right of each trace. The voltage response of the H^+ -selective electrode is shown at the top of each graph. The electrode equilibrated quickly with the new position, similar to the response time of the sensor collected in the flow streams listed in Table 1. The waveform at the bottom of each graph corresponds to the position of the electrode in the gradient as it changes with time. Translation of the electrode between the two poles is not instantaneous and must be taken into consideration when collecting a differential concentration measurement.

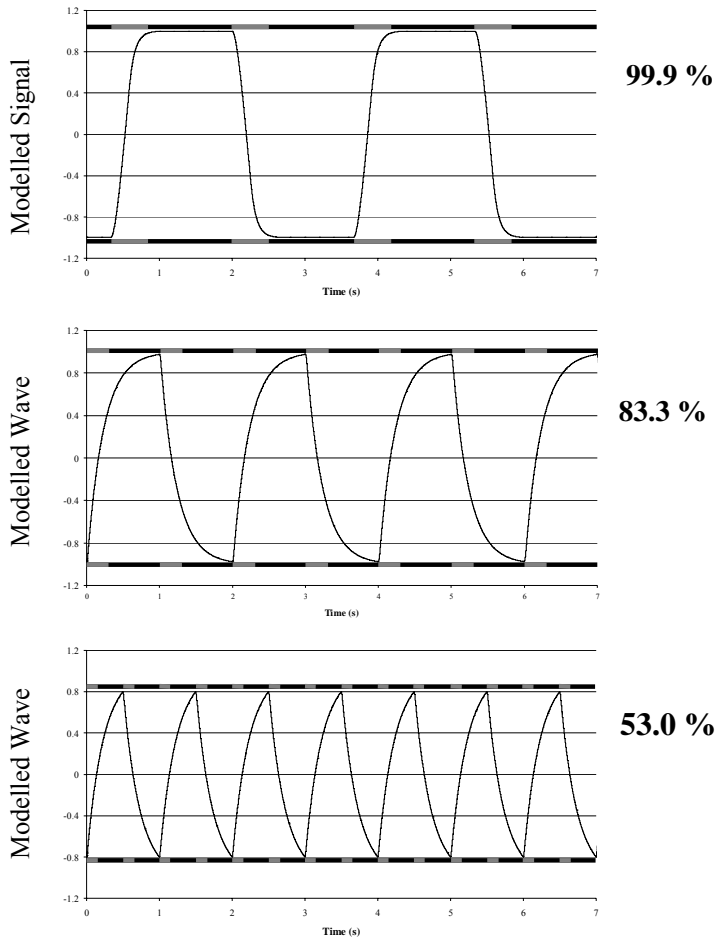


Figure 3: Models of the voltage response for H^+ -selective electrodes with different response times and collection periods. The bars at the top and bottom of each graph correspond to the 30% of the half cycle that is ignored as it accounts for translation and equilibration (gray) while the average of the remaining 70% (black) is used to determine a concentration measurement. The top graph was generated based on a sensor response time of 223 ms with an excursion time of 250 ms (translation speed 40 $\mu\text{m/s}$), similar to conditions used by Messerli et al. (1999). The response time is sufficiently fast and these collection parameters allow nearly 100% collection efficiency of concentration. The middle and lower graph correspond to the average response time of the H^+ -selective electrode determined in the culture medium used by Feijó et al. (1999) (675 ms) along with the shorter collection periods that they used 2s (middle graph), 1s (bottom graph). The medium causes the electrode to respond more slowly such that it does not reach equilibrium before data collection occurs. This leads to only 83% and 53% efficiency, using the same 30%:70% collection scheme.

A second issue to consider is that the sensor must be allowed to remain at each pole of excursion for a period long enough for it to acquire a steady state signal. This will inevitably depend on the response times of the sensors. As discussed above, most sensors can reach 95% of their final value within 40-250 ms in physiological solutions. However, contaminants of ISMs can slow the electrode response and cause underestimation of concentration differences. Figure 2 shows the voltage response of an H^+ -selective electrode, self-referencing near a H^+ sink, in low Ca^{2+} Dickinson's Medium, a culture medium used for growing pollen tubes (Messerli and Robinson, 1999). A batch of these electrodes was characterized with the perfusion system and shown to possess an average response time of 223 ± 37 ms with an average Nernstian response of 27.8 ± 0.5 mV between pH 5.5 and 6.0 for a tip size of 1-2 μm listed in Table 1. The positional information has been added to the bottom of each graph to show the position and movement of the probe during translation. Figure 2 shows that the sensor reaches equilibrium at each pole with translation periods of 4, 3 and even 2 seconds because it can reach steady state very quickly. For our typical operating period, 3.3 s, we allow 30% of each half cycle, 0.5 s, for translation and electrode equilibration and then collect the average of the remaining 70%, 1.16 s, to use to determine the concentration. Figure 3 shows a model used to calculate the collection efficiency of an acquisition scheme using 250 ms movement time, 223 ms electrode response time and an excursion period of 3.3 s (0.3 Hz) similar to our normal operating parameters. For these parameters the system possesses 99.9% efficiency in collecting the concentration at either pole because the electrode has nearly reached equilibrium before the data has been collected. However, if an electrode was used with a slower response time and/or a shorter oscillation period, with a similar data collection scheme, a significant underestimation of the concentration difference will result.

Table 1

Electrode	Column length	Tip Size	Medium	Response times ($t_{95\%}$ msec) for concentration ranges			
				10-1 mM	1-0.1 mM	0.1-1 mM	1-10 mM
K ⁺	100 μ m	1 μ m	1	195 \pm 59	376 \pm 86	165 \pm 41	114 \pm 26
				369 \pm 91	516 \pm 86	247 \pm 54	191 \pm 46
	1000 μ m	1 μ m	1	41 \pm 3	77 \pm 4	53 \pm 4	44 \pm 3
w/ 450 mM Na ⁺	100 μ m	2-3 μ m	2	64 \pm 9	225 \pm 6	91 \pm 8	69 \pm 15
H ⁺	30 μ m	1 μ m	3	pH 6-7	pH 7-8	pH 8-7	pH 7-6
				209 \pm 12	220 \pm 15	214 \pm 27	202 \pm 13
	300 μ m	1 μ m	3	251 \pm 21	269 \pm 14	245 \pm 11	244 \pm 17
	30 μ m	2-3 μ m	3	135 \pm 23	130 \pm 32	126 \pm 30	124 \pm 35
Ca ²⁺	30 μ m		4	10-1 mM	1-0.1 mM	0.1-1 mM	1-10 mM
				58 \pm 9	81 \pm 10	48 \pm 7	53 \pm 10
Electrode		Surface area	Medium	Response times ($t_{90\%}$ msec) to different partial pressures.			
O ₂	n.a.	2 μ m ²	4	Air – N ₂ saturated		N ₂ – Air saturated	
				17.95 \pm 5.26		17.03 \pm 5.75	
NO	n.a.	30 – 40 μ m ²	4	Saline – NO bubbled		NO bubbled – Saline	
				49.63 \pm 13.21		61.00 \pm 14.33	

Table 1. Characteristics and response times determined for a variety of electrochemical microsensors under different conditions. All tip diameters and reactive areas are approximate.

Medium 1. 100 mM HEPES (pH 7.0) with 0.1, 1.0 or 10 mM KCl.

Medium 2. Medium 1 with 450 mM NaCl.

Medium 3. 100 mM MES (pH 6), 100 mM HEPES (pH 7,8) set with KOH

Medium 4. in mM, 120 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂ 10 HEPES (pH 7.4)