Ion-selective self-referencing probes for measuring specific ion flux

Brian Reid1 and Min Zhao1,2,∗

1Department of Dermatology; 2Department of Ophthalmology; University of California; Davis, CA USA

Key words: ion, selective, electrode, probe, self-referencing, flux

Cells generate and maintain a significant electrical membrane potential by active transport of ions, mainly via the sodium-potassium exchange pump, which transports Na+ ions out of the cell and K+ ions in. This membrane potential allows the cell to function as a battery, powering a number of membrane processes including transport of sugars and other ions. The membrane potential of the mitochondria drives the production of ATP, the cell’s energy source. Excitable cells such as neurons have large membrane potentials. Rapid collapse of this potential by opening of sodium channels and in-rush of Na+ ions (depolarization) produces the action potential which is the basis of nerve transmission and brain function. Many tissues and organs also have long-lasting electrical potentials. Epithelia (skin, cornea, etc.) generate and maintain trans-epithelial potentials by directional pumping of ions (Na+ and Cl−). Many years ago it was discovered that wounds on human skin generate significant electrical potentials.1,2 The development of the vibrating probe allowed these small bio-electric currents generated by cells and tissues to be measured non-invasively.3,4 This ultra-sensitive probe can measure extracellular currents in the micro-Amp (μA) range, but gives no indication of the ion species involved.

To measure specific ion flux one could use two or more ion-selective electrodes in different positions to detect the concentration gradient. However; the inherent voltage drift of the probes would be different, causing a change in signal even if the ion flux was stable. The solution was to use self-referencing whereby a single ion-selective probe is moved at low frequency between two points close together. Now, even if the electrode potential drifts, the flux measurement is stable because the drift is relatively slow so that the probe moves before significant drift can occur (see Fig. 2B). A low frequency vibrating ion-selective electrode was first developed in 1990 to measure specific calcium flux.5 The tip of the glass micro-electrode contains an ionophore permeable to only Ca2+ ions, so the electrode is sensitive to changes in [Ca2+]. As the electrode moves back and forth in a gradient (flux) of Ca2+, pausing at each position, the electrochemical voltage of the electrode changes in proportion to the size of the ion flux. The electrode signal is amplified and recorded to computer. The ion flux can be calculated using Fick’s law of diffusion: $J = C \frac{\Delta c}{\Delta x}$ where $C$ is the ion concentration in the solution; $u$ is the ion mobility; and $\Delta c$ is the concentration difference over distance $\Delta x$.

The electrode potential in standard solutions containing different ion concentrations is used to construct a calibration curve to calculate the actual ion concentration. As well as Ca2+, commercially available ionophores are now available to make electrodes sensitive to Na+, Cl−, K+, H+, Mg2+, nitrate, ammonium, fluoride, lithium, mercury, etc. (see www.sigmaaldrich.com). Ion-selective probes have been used to measure H+, K+ and Ca2+ flux across plant roots,5,7 Cl− flux in rat cerebral arteries,8 Cl− flux in pollen tubes,7 H+ flux in skate retinal cells,10 Ca2+ flux in mouse bone,11 H+ flux in skate cornea,13 and various ion fluxes in fungal hyphae,11,12 and various ion fluxes in rat cornea.14 See also the following reviews for detailed information on self-referencing ion-selective electrodes.15-17 Interesting recent developments include amperometric self-referencing detectors of oxygen, nitric oxide and neurotransmitters dopamine and glutamate.18,19 Amperometric sensing is based on a chemical reaction at the sensor tip. New fiber-optic microprobes (optrodes) have been developed to measure non-invasively oxygen metabolic flux with high selectivity and sensitivity.20,21 There is now also an enzyme-based nanoparticle-coated probe sensitive to glucose.22

Ion-selective microelectrodes are best made with thin-walled glass capillaries such as World Precision Instruments (WPI)

The metal vibrating probe developed in the 1970s to measure electric current is sensitive down to the micro-Amp range, but detects only net current due to flow of multiple ions and is too large to measure from single cells. Electrophysiological techniques which use glass microelectrodes such as voltage clamping can be used on single cells but are also non-specific. Ion-selective probes are glass microelectrodes containing at their tip a small amount of ionophore permeable to a particular ion. The electrode is therefore sensitive to changes in concentration of this ion. If the probe tip is moved at low frequency between two points in a concentration gradient of this ion then the electrochemical potential of the solution inside the electrode fluctuates in proportion to the size of the ion gradient. This fluctuation is amplified and recorded and is used to calculate the actual ion flux using Fick’s law of diffusion. In this mini-review we describe the technique of ion-selective self-referencing microelectrodes to measure specific ion fluxes. We discuss the development of the technique and describe in detail the methodology and present some representative results.

DOI: 10.4161/cib.4.5.16182

Submitted: 04/26/11; Accepted: 04/27/11
*Correspondence to: Min Zhao; Email: minzhao@ucdavis.edu
borosilicate glass capillaries without filament (10 cm long, 1.5 mm outer diameter, 1.12 mm inner diameter, cat # TW150-4). Electrodes are heat-pulled using a Sutter P-97 electrode puller with the following settings: heat 470, pull 13, velocity 15, delay 1. This gives tips 3–4 μm in diameter. Smaller tips have higher resistance which makes them more susceptible to electronic noise. The electrodes are now dried and rendered hydrophobic by silanization. Electrodes are placed in a metal rack and heated overnight in an oven at 200°C. About 1–2 ml of silanization solution I (Sigma-Aldrich, cat # 85126) is placed in a glass Petri dish in the bottom of the oven. Electrodes are kept in the oven until all the silanization solution had vaporized; then the oven is turned off. After cooling, electrodes are stored in an electrode storage jar inside a glass desiccator. Electrodes can be stored thus for many weeks.

To prepare an ion-selective probe (Fig. 1A); the electrode is first back-filled to a length of about 1 cm with a solution containing 100 mM of the ion to be measured (see Table 1). This is done using a disposable plastic Pasteur pipette heat-pulled in a Bunsen burner to a fine filament. To eliminate the air bubble at the electrode tip, the electrode is attached to a 3 ml syringe via a silicon tube (3 mm o.d, 1.5 mm i.d). The electrode is secured with sticky-tack on the stage of a Nikon inverted microscope and observed at low power (x4 lens) while pressure is applied by the syringe to push the air bubble out the tip. The electrode is then tip-filled with a length of 30–50 μm of ion-specific ionophore (see Table 1). A small droplet of ionophore is placed on the short edge of a microscope slide. The electrode tip is observed with a x10 lens and the microscope slide moved towards it until the electrode tip touches the ionophore. The ionophore is drawn into the electrode by capillary pressure. A long column of ionophore should be avoided as this increases the probe’s electrical resistance which can make it susceptible to electronic interference (noise). The electrode is mounted in a straight microelectrode holder with a gold 1 mm male connector and Ag/AgCl wire (Fig. 1B) (Warner Instruments, cat # QSW-A15P). The electrode holder is mounted on a Newport 3-dimensional computer-controlled electronic micro-positioner (see below). The electrode tip is place in measuring solution appropriate for the sample to be measured (physiological saline, culture medium, etc.) to allow the electrode to stabilize for an hour or two, or even overnight.

Reference electrodes (Fig. 1A) are the same glass capillaries as above; cut into 5 cm lengths and fire-polished at each end for 1–2 sec in a Bunsen flame. These electrodes are filled with a 3 M solution of NaCl, CH₃CO₂K (potassium acetate) or KCl,
Communicative & Integrative Biology Volume 4 Issue 5

with 2% agar. The solution is chosen depending on the ion to be measured (the reference electrode cannot contain the ion being measured; see Table 1). Agar (0.2 g) is added to 10 ml of solution and brought to the boil on a hotplate. The reference electrode is attached to a plastic Pasteur pipette and the hot solution drawn into the capillary. The electrode is then dropped into cold 3 M NaCl, CH₃CO₂K or KCl solution and stored in 3 M solution in Petri dishes prior to use. Reference electrode with air bubbles are discarded. The reference electrode is mounted in a straight microelectrode holder (pre-filled with 3 M solution) with a Ag/AgCl pellet inside and a gold 2 mm male connector (Fig. 1B) (WPI, cat # MEH3S) and mounted on a Newport 3-dimensional micro-positioner.

Before experiments, consideration must be taken of the sample to be measured, and how the sample is to be immobilized and mounted for measurements. For example, for cornea measurements we glue two wire loops into a 9 cm or 5 cm plastic Petri dish (Fig. 1C). The loops hold the eye stable but allow it to be rotated or tilted to give the electrode access to different parts of the cornea. For tadpole measurements we glue a plastic ‘shelf’ and fine wire into a 5 cm Petri dish (Fig. 1C). The loop in the wire holds the tadpole stable without damage.

Electrode movement and data recording are controlled by IonView32 software (BioCurrents Research Center; Marine Biological Laboratory, Woods Hole, MA). The ion-selective electrode moves at low frequency (0.3 Hz) between two points 30 μm apart (Fig. 2A). The electrode pauses at each position and the electrode potential in mV is recorded on the computer (Fig. 2B). If an ion flux is present, the electrode detects a difference in ion concentration between the two positions. The actual ion flux is calculated using Fick’s law of diffusion: \[ J = \frac{Cu}{dx} \] where \( C \) is the ion concentration in the solution, \( u \) is the ion mobility, and \( dc/dx \) is the concentration difference over distance \( dx \). Ion flux data are presented in pmol/cm²/sec or nmol/cm²/sec.

Before and after experiments, electrodes are calibrated in three standard solutions. These solutions should contain ion concentrations above and below the ion concentration in the measuring solution. For example, for Na⁺ cornea measurements in artificial tear solution (BSS + Intraocular Irrigating Solution, Alcon Laboratories, Inc.), which contains ~150 mM Na⁺, calibration solutions contain 10, 100 and 200 mM NaCl. The Ca²⁺ concentration is much lower so a Ca²⁺ electrode is calibrated in 0.1, 1 and 10 mM CaCl₂.2H₂O. Plotting the electrode output (mV) against the logarithm of the molar ion concentration usually gives a linear correlation with an \( R² \) value close to 1 (Fig. 2C). The formula describing the line is used to convert the raw output of the electrode in mV into actual ion concentrations, and in turn the ion flux is calculated using the formula above.

We measured Ca²⁺ and K⁺ fluxes at a cornea wound over time (Fig. 3, data adapted from Vieira et al. 2011). The data are normalized because the K⁺ flux is much larger than the Ca²⁺ flux. Before wounding (time zero) cornea has small outward flux of both ions (efflux). After wounding, K⁺ flux increases dramatically but then drops back down after 20 min. This suggests that the K⁺ flux is leakage from damaged cells, which have a high intracellular [K⁺]. We confirmed this using a high external K⁺ concentration. In high [K⁺] the initial peak of K⁺ flux was

| ION  | Ionophore I cocktail A (cat # 21048) | Test Solution (100 mM) | Ref. Solution (3 M) |
|------|-------------------------------------|------------------------|--------------------|
| Ca²⁺ | calcium ionophore I cocktail A      | CaCl₂,2H₂O             | KCl                |
| Na⁺  | sodium ionophore II cocktail A      | NaCl                   | KCl                |
| Cl⁻  | chloride ionophore I cocktail A     | NaCl                   | CH₃CO₂K (potassium acetate) |
| K⁺   | potassium ionophore I cocktail A    | KCl                    | NaCl               |

Also shown are appropriate solutions to place in the test (ion-selective) electrode and reference electrode (see Methods). Catalog numbers are Sigma-Aldrich.

Figure 3. Cornea wound measurements. Cornea wounding induces different K⁺ and Ca²⁺ fluxes. After wounding (at time zero), K⁺ flux rises and falls rapidly, suggesting this is passive leakage from damaged cells, which contain high [K⁺]. In contrast, Ca²⁺ flux rises slowly and is maintained at a high level, suggesting that Ca²⁺ efflux is an active response to corneal injury. Data adapted from Vieira et al., 2011.
absent. In contrast to K⁺ flux, Ca²⁺ flux increased slowly and was maintained at a significantly higher level. This suggests that Ca²⁺ efflux is an active response to cornea wounding. Chemical fixation of the cornea eliminated the Ca²⁺ flux.

In conclusion, ion-selective self-referencing probes are extremely useful tools where small fluxes of specific ions need to be measured from tissues or even single cells. They have proved useful in a wide variety of biological applications. New adaptations of self-referencing include the use of amperometric, optical and nanoparticle-coated sensors.

Acknowledgments
This work was supported by the National Institutes of Health National Eye Institute grant 1R01EY019101 (to M.Z. and B.R.). The authors thank the Wellcome Trust for continuous support (068012). This work was also supported in part by Research to Prevent Blindness, Inc., an NSFC grant (30628026), and UC Davis Dermatology Department developmental fund. M.Z. is also supported by grants from the California Institute of Regenerative Medicine RB1-01417, NSF MCB-0951199.

References
1. Du Bois-Reymond E. Vorläufiger abriss einer unter-suchung über den sogenannten froschstrom und die electromotorischen fische. Ann Phy U Chem 1843; 58:1-30.
2. Piccolino M. Animal electricity and the birth of electrophysiology: the legacy of Luigi Galvani. Brain Res Bull 1998; 46:381-407.
3. Jaffe LF, Nuccitelli R. An ultrasensitive vibrating probe for measuring steady extracellular currents. J Cell Biol 1974; 63:614-28.
4. Reid B, Nuccitelli R, Zhao M. Non-inverse measurement of bioelectric currents with a vibrating probe. Nat Protoc 2007; 2:661-9.
5. Kuhlreuber WM, Jaffe LF. Detection of extracellular calcium gradients with a calcium-specific vibrating electrode. J Cell Biol 1990; 110:1565-73.
6. Ryan PR, Newman IA, Shields B. Ion fluxes in corn roots measured by microelectrodes with ion-specific liquid membranes. J Memb Sci 1990; 53:59-69.
7. Kochian LV, Shafi JE, Kuhlreuber WM, Jaffe LF, Lucas WJ. Use of an extracellular, ion-selective, vibrating microelectrode system for the quantification of K⁺, H⁺ and Ca²⁺ fluxes in maize roots and maize suspension cells. Planta 1992; 188:601-10.
8. Doughty JM, Langton PD. Measurement of chloride flux associated with the myogenic response in rat cerebra l arteries. J Physiol 2001; 534:3:753-61.
9. Messori MA, Smith PJS, Lewis RC, Robinson KR. Chloride fluxes in lily pollen tubes: a critical reevaluation. Plant J 2004; 40:799-812.
10. Molina AJ, Verzi MP, Birthaun AD, Yanoah EN, Hammar K, Smith PJ, Malchow RP. Neurotransmitter modulation of extracellular H⁺ fluxes from isolated retinal horizontal cells of the skate. J Physiol 2004; 560:639-57.
11. Marenzana M, Shipley AM, Squirezob P, Kunkell JG, Rubinacci A. Bone as an ion exchange organ: Evidence for instantaneous cell-dependent calcium efflux from bone not due to resorption. Bone 2005; 37:545-54.
12. Ramos AC, Façanha AR, Feijó JA. Proton (H⁺) flux signals the transition of the arbuscular mycorrhizal fungus. New Physiol 2007; 178:177-88.
13. Lew RR. Ionic currents and ion fluxes in Neurospora crassa hyphae. J Exp Bot 2007; 58:3475-81.
14. Vieira AC, Reid B, Cao L, Mannis MJ, Schwab IR, Zhao M. Ionic components of electric current at rat corneal wounds. PLoS One 2011; 6:17411.
15. Smith PJS. Non-invasive ion probes—tools for measuring transmembrane ion flux. Nature 1995; 378:645-6.
16. Smith PJS, Hammar K, Porterfield DM, Sanger RH, Trimarchi JR, Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. Microse Res Technique 1999; 46:398-417.
17. Smith PJS, Sanger RH, Messori MA. Principles, development and applications of self-referencing electrochemical microelectrodes to the determination of fluxes at cell membranes. In: Michael AC, Borland LM, eds. Electrochemical Methods for Neuroscience. Boca Raton: CRC Press, 2007: 18.
18. Porterfield DM. Measuring metabolism and biophysical flux in the tissue, cellular and sub-cellular domains: recent developments in self-referencing amperometry for physiological sensing. Biosens Bioelectron 2007; 22:1186-96.
19. McAlmorie ES, Mohanty S, Shi J, Clausen J, Jedlicka SS, Rickus JL. Porterfield DM. A self-referencing glutamate biosensor for measuring real time neuronal glutamate flux. J Neurosci Methods 2010; 189:14-22.
20. Chami MR, Porterfield DM. Self-referencing electrode technology for non-invasive real-time measurement of biophysical flux and physiological sensing. Analyst 2009; 134:2224-32.
21. McAlmorie ES, Jaroch D, Chami MR, Porterfield DM. Self-referencing optodes for measuring spatially resolved, real-time metabolic oxygen flux in plant systems. Planta 2010; 232:1087-99.
22. McAlmorie ES, Shi J, Jaroch D, Clausen JC, Uchida A, Jiang Y, et al. A self-referencing platinum nanoparticle decorated enzyme-based biosensor for real-time measurement of physiological glucose transport. Biosens Bioelectron 2011; 26:2237-45.