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Video Article

Measurement of Extracellular Ion Fluxes Using the Ion-selective Self-referencing Microelectrode Technique

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Abstract

Cells from animals, plants and single cells are enclosed by a barrier called the cell membrane that separates the cytoplasm from the outside. Cell layers such as epithelia also form a barrier that separates the inside from the outside or different compartments of multicellular organisms. A key feature of these barriers is the differential distribution of ions across cell membranes or cell layers. Two properties allow this distribution: 1) membranes and epithelia display selective permeability to specific ions; 2) ions are transported through pumps across cell membranes and cell layers. These properties play crucial roles in maintaining tissue physiology and act as signaling cues after damage, during repair, or under pathological condition. The ion-selective self-referencing microelectrode allows measurements of specific fluxes of ions such as calcium, potassium or sodium at single cell and tissue levels. The microelectrode contains an ionophore cocktail which is selectively permeable to a specific ion. The internal filling solution contains a set concentration of the ion of interest. The electric potential of the microelectrode is determined by the outside concentration of the ion. As the ion concentration varies, the potential of the microelectrode changes as a function of the log of the ion activity. When moved back and forth near a source or sink of the ion (*i.e.* in a concentration gradient due to ion flux) the microelectrode potential fluctuates at an amplitude proportional to the ion flux/gradient. The amplifier amplifies the microelectrode signal and the output is recorded on computer. The ion flux can then be calculated by Fick's law of diffusion using the electrode potential fluctuation, the excursion of microelectrode, and other parameters such as the specific ion mobility. In this paper, we describe in detail the methodology to measure extracellular ion fluxes using the ion-selective self-referencing microelectrode and present some representative results.

Video Link

The video component of this article can be found at http://www.jove.com/video/52782/

Introduction

All animal cells are surrounded by a lipid bilayer membrane which separates the cytoplasm from the outside environment. The cell maintains an electrical membrane potential, negative inside, by active transport of ions[1]. The membrane potential is a stored energy source which the cell can utilize to operate various molecular devices in the membrane[2]. Neurons and other excitable cells have large membrane potentials. Rapid opening of sodium channels collapses the membrane potential (depolarization) and produces the action potential which is transported along the length of the neuron[2]. Aside from these rapid electrical changes, many tissues and organs generate and maintain significant long-term electrical potentials. For example, skin and corneal epithelia generate and maintain trans-epithelial potentials and extracellular electric currents by directional pumping of ions (mainly sodium and chloride)[3].

While measurements of endogenous extracellular electric current using the vibrating probe[4-6] and measurements of membrane or trans-epithelial potentials using the microelectrode system[7-10] allow measurement of the electric parameters of cell membranes and epithelial cell layers, they give no indication of the ion species involved.

Microelectrodes with selective ionophore can measure specific ion concentration in solution. Ion gradients or flux could be measured with two or more electrodes at different positions. However, the intrinsic voltage drift of each probe would be different, causing inaccurate measurements or even detection of a gradient that was not present. A single electrode used in "self-referencing" mode whereby it moves at low frequency between two points solves this problem. Now the ion flux can be seen against the background of a relatively slow and stable signal drift (see **Figure 3B**).

The ion-sensitive measuring system uses ion-selective self-referencing microelectrodes to detect small extracellular fluxes of ions close to tissues or single cells. The system consists of an amplifier which processes the signal from the microelectrode and a micro stepper motor and driver to control the motion of the microelectrode. The ion-selective microelectrode and the reference electrode that close the circuit are connected to the amplifier via a headstage pre-amplifier (**Figure 1A**). Computer software determines the parameters of the microelectrode movement (frequency, distance) and also records the output of the amplifier. The stepper motor controls the microelectrode movement via

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a three-dimensional micropositioner. A low frequency vibrating ion-selective microelectrode was first developed in 1990 to measure specific calcium flux[11]. As well as calcium, commercially accessible ionophore cocktails are now available to make microelectrodes sensitive to sodium, chloride, potassium, hydrogen, magnesium, nitrate, ammonium, fluoride, lithium or mercury.

Basically, the self-referencing ion-selective microelectrode technique converts the activity of a specific ion dissolved in a solution into an electric potential, which can be measured by a voltmeter. The ionophore cocktail is an immiscible liquid (organic, lipophilic) phase with ion-exchange properties. The ionophore selectively complexes (binds) specific ions reversibly and transfers them between the aqueous solution contained in the microelectrode (electrolyte) and the aqueous solution in which the microelectrode is immersed (**Figure 1D**). This ion transfer leads to an electrochemical equilibrium and a variation of the electric potential between the microelectrode and the reference electrode is measured by the voltmeter. The voltage is proportional to the logarithm of the specific ion activity according to the Nernst equation allowing the calculation of the ion concentration (**Figure 2A** and **B**).

At present, several systems allow measurement of ion flux using a similar concept or principle. For example, the Scanning Ion-selective Electrode Technique (SIET)[12,13] or the Microelectrode Ion Flux Estimation (MIFE) technique developed by Newman and Shabala[14-16] are commercially available and widely used by the research community in order to determine specific ion fluxes occurring at cell membrane and tissue across a variety of animal, plant and single living cell models. Ion-selective microelectrodes have been used to measure hydrogen, potassium and calcium flux across plant roots[17], chloride flux in rat cerebral arteries[18] and in pollen tubes[19], hydrogen flux in skate retinal cells[20], calcium flux in mouse bone[21], various ion fluxes in fungal hyphae[22] and in rat cornea[23], and finally calcium flux during single cell wound healing[12,24]. See also the following review for detailed information on ion-selective self-referencing microelectrodes[25].

The following article describes in detail how to prepare and perform measurement of endogenous extracellular ion fluxes using the ion-selective self-referencing microelectrode technique at the single cell level.

Protocol

1. Ion-selective Self-referencing Microelectrode Preparation

1. Preparation of ion-selective microelectrode
    1. Heat pull thin walled borosilicate capillaries without filament (1.5 mm outer diameter, 1.12 mm inner diameter) using a microelectrode puller.
       Note: This gives tips 3-4 µm in diameter. Smaller tips have higher resistance which makes microelectrodes more susceptible to electronic noise and is also associated with a slower response to a change in ion concentration. Useful information can be found in the paper published by Smith *et al.*[26].
    2. Silanize the electrodes to render the inner surface hydrophobic to aid retention of the lipophilic ionophore cocktail. Place the microelectrodes in a metal rack and heat O/N in an oven at >100 °C to dry them. The rack is a metal plate with 2 mm diameter holes drilled part of the way through. Place the electrodes in the holes tip upwards with a 250 ml glass beaker over them.
    3. In the morning, turn the oven off and while wearing insulated gloves, carefully remove the metal rack with the electrodes and beaker in place. Close the oven door to retain the heat.
    4. Wear latex or nitrile gloves, lab coat and eye protection. With a plastic Pasteur pipette, place a drop of silanization solution I at the base of each electrode (keep the beaker in place; use the pouring lip for pipette access). The silanization solution is vaporized by the hot plate and silanizes the inside of the electrodes. Use a chemical extractor fume hood for this stage. Place the rack/beaker/electrodes back in the hot oven for a few hours to allow any remaining silanization solution to evaporate.
       Note: For safety reasons, do not turn the oven back on. Place a label on the oven indicating it must not be switched on as it may contain harmful and flammable vapor.
    5. After cooling, store the microelectrodes in a microelectrode storage jar inside a glass desiccator with 400 g of desiccant. Microelectrodes can be stored thus for many weeks.
       Note: An alternative silanization method is described in Smith *et al.*[26]
    6. Back-fill the microelectrode with 50 to 100 µl (a length of about 1 cm) of a solution containing 100 mM of the ion to be measured (see **Table 1** and **Figure 1B**). Use a disposable plastic Pasteur pipette heat pulled in a Bunsen burner to a fine filament. Rinse the pipette in dH_2O afterward to prevent blockage.
       Note: Alternatively, adjust the ion concentration of the backfilling solution to match the concentration of ion in the external solution[27].
    7. Observe the microelectrode under a dissecting microscope to ensure the absence of air bubbles.
        1. If bubbles are present tap the microelectrode lightly with a finger nail while holding the electrode vertically (tip down) and/or push the bubbles out the tip by applying back pressure using a syringe modified with a silicone tube replacing the needle.
    8. Tip-fill the microelectrode with 15 to 20 nl (a length of 30-50 µm) of ion-specific ionophore cocktail (see **Table 1**). Place a small droplet of ionophore cocktail on the short edge of a microscope slide. Observe the microelectrode tip under a dissecting microscope and move it toward the microscope slide until the microelectrode tip touches the ionophore cocktail for only about half a sec. Draw the ionophore cocktail into the microelectrode by capillary pressure.
       Note: Avoid a long column of ionophore cocktail as this increases the probe's electrical resistance which can make it susceptible to electronic interference (noise) and also slows the response time.
    9. Mount the microelectrode in a straight microelectrode holder with a gold 1 mm male connector and $AgCl(Ag^+)$ wire (**Figure 1B**).
    10. Attach the microelectrode holder to the head stage mounted on a three-dimensional computer-controlled electronic micropositioner (**Figure 1A**).
    11. Place the microelectrode tip in measuring solution appropriate for the sample to be measured (physiological saline, culture medium, *etc.*) to allow the microelectrode to stabilize for an hr or two, or even overnight.
2. Preparation of the reference electrode

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1. Reference electrodes (**Figure 1C**) are the same capillaries as above. Cut the capillary with a diamond pencil into 5 cm lengths and fire-polished at each end for 1-2 sec in a Bunsen flame.
2. Fill these electrodes with ~200 µl of a 3 M solution of NaCl, CH_3CO_2K (potassium acetate) or KCl with 2% agarose. Choose the solution depending on the ion to be measured (the reference electrode should not contain the ion being measured; see **Table 1**). Mix the agarose and the solution and heat to almost boiling in a microwave. Stir to dissolve the agarose (the solution goes clear).
3. Attach the reference electrode to a plastic Pasteur pipette and draw the hot solution into the capillary.
4. Drop the electrode into cold 3 M NaCl, CH_3CO_2K or KCl solution and store in this 3 M solution in sealed tubes prior to use. Discard any reference electrodes with air bubbles.
5. Mount the reference electrode in a straight microelectrode holder (pre-filled with 3 M solution) with an AgCl(Ag^+) pellet inside and a gold 2 mm male connector (**Figure 1C**) and attach the electrode and holder onto a manual micro-positioner mounted on a magnetic stand.

2. Ion-selective Self-referencing Microelectrode Calibration

1. Preparing calibrating solutions containing the ion of interest as in the reference solution; see **Table 1**. Bracket the concentration that is in the solution the sample will be in (*e.g.* culture media, physiological saline). That is, one calibration solution must contain a lower concentration of ion than in the measuring solution, and one higher.
    1. For example, use saline that contains 1 mM of K^+. To bracket this concentration, dissolve KCl powder in deionized water to a concentration of 10, 1 and 0.1 mM in serial dilutions. Use these calibration solutions. Alternatively, use at least two of these solutions.
2. Immerse the ion-selective microelectrode and the reference electrode in each calibration solution and let the voltage value stabilize for 1 to 3 min before recording the corresponding voltage using the dedicated software (see **Table 1**).
3. As the software saves the data (amplifier output) as a txt file, copy the data into a spreadsheet file. Plot the microelectrode output (mV) against the logarithm of the molar ion concentration (**Figure 2A**).
4. Apply a linear regression and calculate the Nernst slope, intercept and R^2 value. Accept the microelectrode if the Nernst slope is 58 ± 11 mV/decade for monovalent ions and 29 ± 11 mV/decade for divalent ions (for cations, the Nernst slope is positive, for anions it is negative). Additionally, good microelectrodes should have a strong linear correlation (R^2 >0.9; **Figure 2B**).
Note: The mV output of the amplifier used here gives mV reading with a tenfold gain. Values have to be divided by a factor of ten.
5. Use the linear regression formula to convert the raw mV output of the microelectrode into actual ion concentration (**Figure 2B**).

3. Validation of the Ion-selective Microelectrode Technique

1. Preparing an artificial source
    1. Artificial source capillaries are the same capillaries as above. Heat pull the capillary using a microelectrode puller as in step 1.1.1.
    2. Backfill these capillaries with 200 µl of a 1 M solution of NaCl, KCl, $CaCl_2 2\ H_2O$ or pH 4 buffer. Choose the artificial source solution depending on the ion to be measured (see **Table 1**).
    Note: Alternatively, pull electrodes with bigger tip diameter (~20 µm) and tip-fill with the same solutions but containing 0.5-1% agarose (agarose will prevent any bulk flow of solution).
    3. Mount the artificial source capillary on a micromanipulator and immerse it in the solution used to measure the flux of the ion in the samples. Leave the artificial source in the solution for 30 min to 1 hr to allow stabilization of the gradient.
2. Validation of the ion-selective microelectrode
    1. Immerse the ion-selective microelectrode about one centimeter away from the artificial source capillary in the solution used to measure the flux of ion on the samples and close the circuit with the reference electrode as before. Let the voltage value stabilize for 1 to 3 min before recording the corresponding voltage using the dedicated software for 1 to 2 min. This value corresponds to the buffer value (in literature also referred as reference, background or blank value).
    2. Move the ion-selective microelectrode to about 5 µm from the artificial source and let the voltage value stabilize for 1 to 3 min before recording the corresponding voltage using the software for 1 to 2 min.
    3. Repeat the above procedure by placing the ion selective microelectrode at 10, 20, 40, 80, 160, 320, 640 and 1280 µm away from the artificial source capillary.
    4. Extract the data as a txt file and copy the values into a spreadsheet file.
3. Calculate the ion concentration corresponding to the mV values in the same way as for the calibration values. Plot the value.
Note: If an ion flux is present, the microelectrode detects a difference in ion concentration between the two positions **(Figure 3B**). If the artificial source contains more ions of the species measured than the solution, the concentration should be higher close to the source than far away, validating the ability of the ion-selective microelectrode to correctly detect the direction of an ion flux (in this case efflux; for an artificial sink, with lower specific ion concentration than measuring medium, it should be influx).
    1. Calculate the ion flux using Fick's law of diffusion: $J = c\ \mu\ (dc/dx)$ where *c* is the ion concentration in the solution (mol cm^{-3}), μ is the ion mobility (mol cm N^{-1} sec^{-1}), and *dc* is the concentration difference over distance *dx* (cm) (**Figure 2C**). Ion flux data are usually presented in $pmol\ cm^{-2}\ s^{-1}$ or $nmol\ cm^{-2}\ sec^{-1}$.
    Note: An alternative method of ion flux calculation described by Smith *et al.*[26]. can be used. Main differences include the use of the diffusion coefficient instead of the ion mobility and the subtraction of the background ion flux (also voltage drift or correction factor) calculated from measurement of ion flux in saline solution without sample.
    2. Plot the mean of the ion fluxes of each step against the distance from the source (**Figure 2D**). Moving away from the source, observe an exponential decrease of the flux value validating the ability of the ion-selective microelectrode to sense different magnitude of ion fluxes.
    3. Do the artificial source validation once for each specific ion intended to be recorded in order to validate its correct direction and magnitude measurements with a large signal-to-noise ratio.

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Note: Ion flux measurement of the buffer without samples indicates the background level or noise. Typically, buffer measurement shows no clear fluctuation of the ion concentration leading to very small flux that displays variable directions.

4. Preparation of Measuring Chamber

Note: Before experiments, consider the sample to be measured and how the sample is to be mounted and immobilized for microelectrode measurements.

1. For *Xenopus laevis* oocyte measurements cut a 1 cm square of an 800 µm nylon mesh (nitex mesh) and glue it into a plastic Petri dish (**Figure 1E**).

5. Ion Flux Measurement

1. Measure the ion concentration present in the buffer used to perform the measurements on the sample in the same way as for the calibration solution. *X. laevis* oocytes require Mark's Modified Ringer (MMR). Dissolve NaCl, KCl, CaCl, MgCl and HEPES into deionized water to reach a final concentration of (mM): 100 NaCl, 2 KCl, 2 CaCl, 1 MgCl and 5 HEPES. Adjust the pH of the buffer to 7.5 using NaOH.
2. Place the sample into the measuring chamber and bring the ion-selective microelectrode close to the sample (about 10 µm away) using the micropositioner to define the close position of the microelectrode (**Figure 3A**).
3. Start the low frequency (0.3 Hz) excursion (100 µm) of the microelectrode between the close position and a position away from the sample (distant) using the dedicated software. Ensure that the movement of the microelectrode is perpendicular to the surface of the sample.
   Note: The excursion of the microelectrode can be set on the software. Large excursion increases the gradient read allowing an easier detection of small fluxes during the measurement while lengthens the sampling interval and decreases the temporal resolution. See **Figure 3A** for an example.
4. Start the recording using the software. The microelectrode pauses at each position and the electric potential in mV is recorded on the computer. Obtain measurements for at least 2 min, allowing signal stabilization. For short time-lapse experiments, record potential variations at the position of interest for the whole time course.
5. Extract the data as a txt file and copy the values into a spreadsheet file.
6. Calculate the ion concentration corresponding to the mV values in the same way as for the calibration values. Plot the value.
   Note: If an ion flux is present, the microelectrode detects a difference in ion concentration between the two positions (**Figure 3B**).
7. Calculate the ion flux using Fick's law of diffusion as before (step 3.3.1).
8. Repeat the buffer measurement before measuring a new sample and repeat the procedure of flux measurement and calculation for every new sample.

6. Statistical Analysis and Data Presentation

1. Test the independent effects of the position and/or of the time on ion fluxes under the control condition using an ANCOVA model with mixed effects[28].
   Note: Analysis of covariance (ANCOVA) is a general linear model that mixes regular ANOVA and regression by allowing both categorical and continuous measures to be used as independent variables. In addition, in the presence of correlated errors induced by repeated measures per individual and eventual nested effects, mixed effects models are used to model accurate estimates of both fixed and random effects.
2. Calculate pairwise comparisons using Student *t*-test between group levels with Bonferroni correction for multiple testing[28].
3. Generate boxplots to summarize ion flux measurements according to position and time. Include *p* values from the pairwise Student *t* described above (**Figure 3D**) and indicate significance levels of *p* values as follows: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$[29]

Representative Results

We have previously shown that calcium influx appears after single cell wounding[24]. We therefore asked whether other ion fluxes occur upon single cell wounding. We used the *X. laevis* oocyte, a well-established model for single cell wound healing[30-34] and electrophysiological recording[24,35-39]. Interestingly, potassium ions are more concentrated inside *X. laevis* oocytes (about 110 mM)[40] than in the extracellular solution used (in MMR 1x: 1 mM) suggesting an efflux of potassium upon wounding. In order to confirm that hypothesis, we measured the potassium flux during the course of *X. laevis* oocyte cell membrane healing using the ion-selective self-referencing microelectrode.

To wound the oocyte, first pull a capillary electrode with a large tip size (~50 µm). Attach the electrode to a straight electrode holder and mount on a manual micro-positioner. Wound the oocyte by touching the membrane with the electrode tip[24]. Soon after wounding we detected a large efflux of potassium (up to 250 nmol cm^{-2} sec^{-1}; **Figure 3B-D**). As the membrane wound healed, this flux diminished, returning to unwounded flux values seen in the intact membrane (~5 nmol cm^{-2} sec^{-1}) when the wound healed (up to 16 min after wounding; **Figure 3B-D**). ANCOVA revealed a significant effect of time after wounding on potassium flux measurements ($p < 0.001$). *Post hoc* analyses revealed significantly increased potassium efflux at 1-2 min ($p < 0.001$) and at 5-6 min ($p < 0.05$), but not at 15-16 min after wounding when compared to intact cell membrane condition (**Figure 3D**). We concluded that upon single cell wounding, an efflux of potassium appears at the level of the wound that decrease during the course of healing.

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Ion	Ionophore cocktail	Electrolyte solution (100 mM)	Reference solution (3 M)	Artificial source solution (1 M)
Ca^{2+}	Calcium Ionophore I Cocktail A (cat# 21048)	$CaCl_2\ 2H_2O$	KCl	$CaCl_2\ 2H_2O$
Na^{+}	Sodium Ionophore II Cocktail A (cat# 71178)	NaCl	KCl	NaCl
Cl^{-}	Chloride Ionophore I Cocktail A (cat# 24902)	NaCl	CH_2CO_2K (Potassium Acetate)	NaCl
K^{+}	Potassium Ionophore I Cocktail A (cat# 60031)	KCl	NaCl	KCl
H^{+}	Hydrogen Ionophore I Cocktail A (cat#95291)	pH 7.0	KCl	pH 4.0

Table 1: Examples of commonly used ionophore cocktails. Also shown are appropriate solutions to place in the microelectrodes, for the artificial source and to perform calibration. Catalog numbers are from Sigma-Aldrich.

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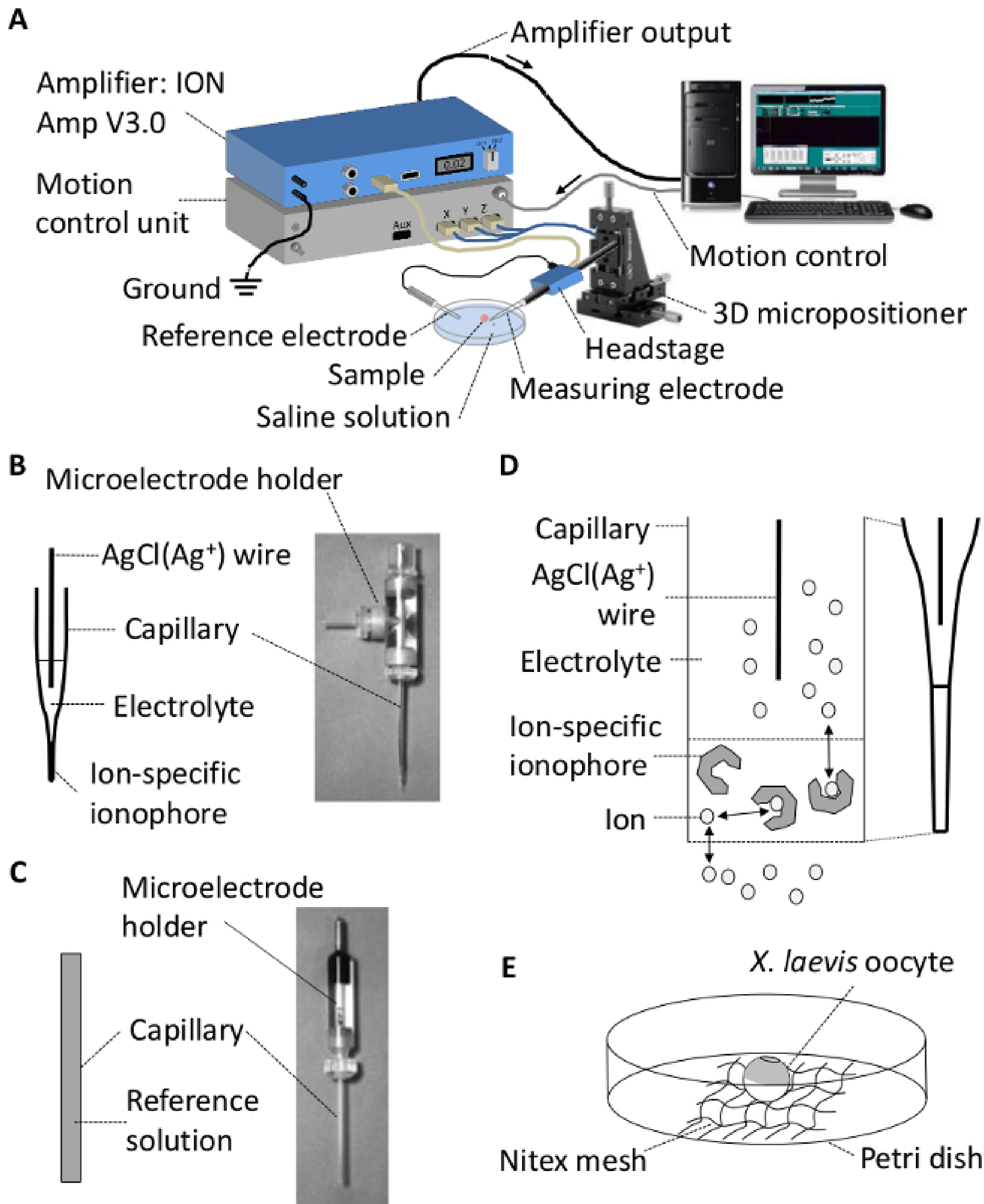

Figure 1: Ion-selective microelectrodes. (**A**) Schematic representation of the ion-selective self-referencing microelectrode system. (**B**) Ion-selective microelectrode. (**C**) Reference electrode. (**D**) Ion exchange between the external solution and the microelectrode via the ionophore. (**E**) Scheme of the measuring chamber used for *X. laevis* oocyte.

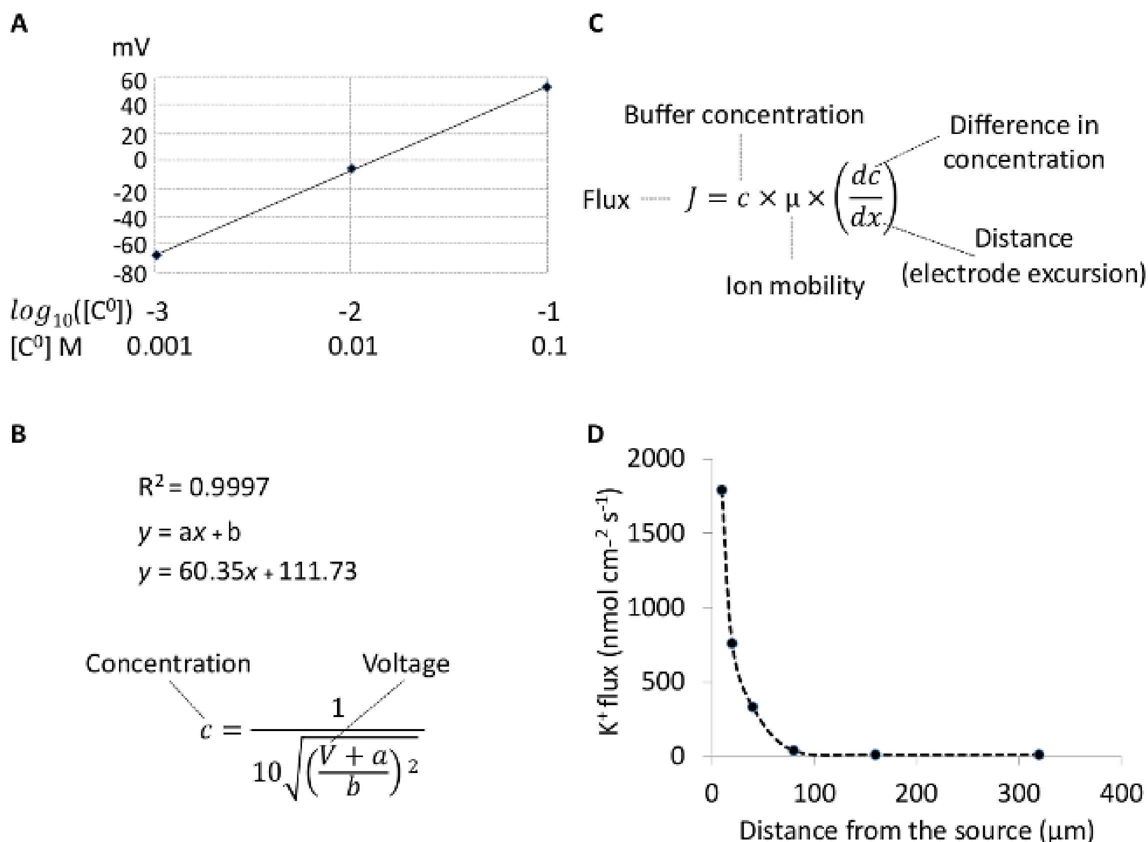

Figure 2: Ion-selective microelectrodes calibration, artificial source and flux calculation. (**A**) Calibration curve. (**B**) Equation of the calibration curve and calculation of the ion concentration. (**C**) Calculation of the ion flux. (**D**) Ion flux measured at specific distances from the artificial source (1 M KCl).

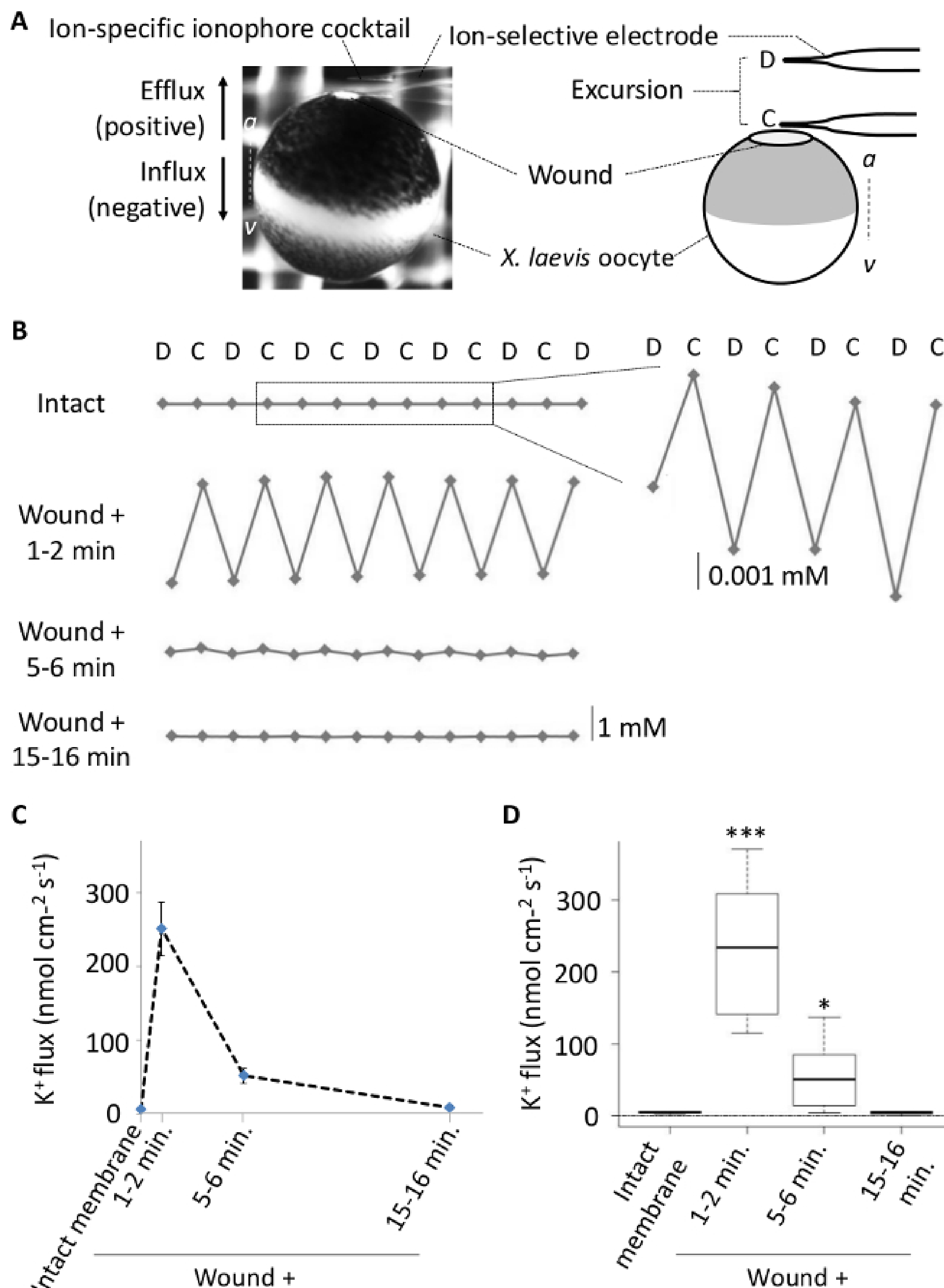

Figure 3: Evolution of the potassium flux at *X. laevis* oocyte wound during healing. (**A**) Photograph and illustration of the excursion of the ion-selective microelectrode measuring ion concentration at *X. laevis* oocyte wound; the dashed line between "a" and "v" represents the animal–vegetal axis. (**B**) Illustration of the variation of potassium ion concentration at *X. laevis* oocyte wound during healing. (**C**) Scatter (xy) plot showing the mean and the standard error of potassium flux measurement at the level of the wound at different time during *X. laevis* oocyte wound healing. (**D**) Boxplot showing potassium flux measurement at the level of the wound at different time during *X. laevis* oocyte wound healing (n = 16; p values indicated as follow: *: p <0.05; ***: p <0.001).

Discussion

The most critical steps for successful measurement of extracellular ion fluxes *in vivo* are: the reduction of the noise, the correct fabrication of the ion-selective microelectrodes and reference electrode, and the positioning of the sample and both electrodes.

In order to minimize the noise, the recording system should be in an earthed (grounded) Faraday cage preferably with a metal-topped (vibration isolation) table which is also earthed. In addition, the microscope chassis should also be earthed. Sources of electric noise include the light source. A fiber-optic 'fan-less' light source causes minimal electric noise. Finally, keeping the silver wire and pellet in the microelectrode holders chlorided also minimizes noise (dip in sodium hypochlorite bleach and rinse in dH_2O).The presence of air bubbles in the ion-selective microelectrode or in the reference electrode will result in measurement failure as the conductivity of the microelectrode will be nil or compromised. Thus, it is crucial to verify the electrodes under the microscope before mounting them on the holders. See the protocol for detailed procedure to remove air bubbles. The correct positioning of both the sample and the microelectrodes are required in order to ensure reliable and reproducible results. The measurement of ion flux is dependent on the excursion of the microelectrode and its position relative to the sample. It is important to identify precisely the area of interest that will be measured on the sample and position the microelectrode to have a perpendicular movement from the sample. Any excursion of the microelectrodes in a way that is not perpendicular to the sample will result in altered ion fluxes measurements and increased variability among samples.

Ionophore cocktails dedicated to measure specific ions, for example potassium, may also sense the presence of other ions, such as sodium. In the case that the measuring solution contains a high amount of a competing ion for the ionophore cocktail, it is important to determine the selectivity of the ionophore cocktail by using the artificial source experiment. Here, the solution used to culture *X. laevis* oocytes (MMR) contains a high concentration of sodium. Thus, it is important to assess whether the potassium ionophore cocktail used also senses sodium. By using the potassium ionophore cocktail filled microelectrode, we can attempt to measure a sodium flux using an artificial source that contains high sodium concentration (1 M NaCl; see **Table 1**) keeping the same measuring solution. The chemical gradient favors the efflux of sodium, but ideally no sodium flux should be detected by the potassium-specific ionophore cocktail. If a significant flux is measured, the experimental condition should be optimized. For example, the concentration of the competing ion could be lowered down to the point the microelectrode does not sense it anymore, while this may affect the sodium flux across the plasma membrane leading to potential interference during potassium flux measurements. Ideally, a correcting factor calculated from the artificial source experiment can be applied to the data, or another ionophore cocktail can be tested. Ion flux measurements using the ion-selective self-referencing microelectrode allow the measurement of ion fluxes occurring at cells and tissue in aqueous solution. Measurements of ion fluxes in cells or tissues that are normally in contact with an air environment requires the presence of a solution that is not naturally present in their environment and that can alter the ion flux and exchange that occurs under normal conditions. Specific attention has to be made to define the content of such solution and minimize the deviation from the original, physiological environment. The spectrum of ions that can be measured by the ion-selective self-referencing microelectrode technique depends on the availability and existence of specific ionophore cocktails selective to the ion of interest.

Ion flux measurements performed with the ion-selective self-referencing microelectrode are done in solution, usually near the surface of cells or tissues, allowing the non-invasive measurement of extracellular ion fluxes. This method does not allow the measurement of ion fluxes inside tissues, between cell and intercellular space. The ion-selective self-referencing microelectrode is not the only method that allows measurement of ion fluxes *in vivo*. An alternative new method uses fluorescent bioelectricity reporters[41] which enables the measurement of ion fluxes that are not possible using microelectrodes. These dyes permit measurements of ions fluxes inside tissues and cells and can achieve subcellular localization. This technique can acquire spatial information of the ion flux inside tissues and cells but not ion exchange between the tissue and the extracellular space. Furthermore, the fluorescent bioelectricity reporters usually generate semi-quantitative data. The use of microelectrode-based technology to measure ion fluxes is still valid and necessary and brings additional information to the use of fluorescent bioelectricity reporters, making them complementary rather than competing techniques. In addition, interesting recent developments include amperometric self-referencing detectors of oxygen, nitric oxide and neurotransmitters dopamine and glutamate[42,43]. Amperometric sensing is based on a chemical reaction at the sensor tip. New fiber-optic microelectrodes ("optrodes") have been developed to measure non-invasively metabolic oxygen flux[34,35] and pH[44] with high selectivity and sensitivity[45,46]. There is now also an enzyme-based nanoparticle-coated probe sensitive to glucose[47].

We have seen that the ion-selective self-referencing microelectrode enables measurements of extracellular ion fluxes *in vivo*. Ions are not only exchanged between the cells/tissues and extracellular space but also between cells and tissues within living organisms. It is important to combine this technique with others such as fluorescent bioelectricity reporters in order to appreciate the spatial resolution of the ion fluxes inside tissues in addition to the actual measurements of the ion fluxes near its surface. In addition, ion fluxes represent an important part of the bioelectric state that defines cells and tissues together with cell membrane potential, trans-epithelia potential or extracellular electric currents. It is important, in addition to the measurement of ion fluxes, to measure, in combination, cell membrane and trans-epithelia potential as well as extracellular electric currents[24].

Disclosures

The authors declare that they have no competing financial interests.

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