Hands-on learning membranes and ion channels biophysics through a small and powerful device: the eNPR

This practical course aims to teach students the fundamentals of the electrical properties of a cell membrane and the ion transport across it.

What should students know and be able to do as a result of this course?

- Describe the biophysical properties of a cell membrane
- Describe the basic features of an ion channel protein
- Use the bilayer method to build an artificial cell membrane and examine pore forming molecule by analysing the currents produced by their activity
- Learn the basics of electrophysiology and single channel data analysis

The combination of high performance and low cost of our eNPR device and the easy availability of the material make this course suitable also for low budget. Below is described a simple experimental protocol that was already performed by ungraduated biological sciences students at the University of Milan.

The laboratory session that we propose lasts only one working day. The session starts with a brief lecture focusing on the electrical properties of the cell membranes and the ion channels features. The students are reminded that a passive cell membrane is electrically equivalent to a capacitor in a RC circuit and the membrane capacitance is proportional to the membrane surface area. Each ion channel inserted in the membrane is a small resistor in parallel with the capacitor (i.e with the membrane). In addition, the Nerst equilibrium potential in both a single and multiple ion system is explained.

The pore forming protein that we use in this course is the Gramicidin, transmembrane antimicrobial peptides that bind and interacts with the bacterial membrane causing cell disruption by passing monovalent cations. Due to their small size, ready availability and low cost, gramicidins serve as excellent models for transmembrane channels. Gramicidin D is a heterogeneous mixture of gramicidin A, B, and gramicidin C, where these three antibiotic compounds combine as dimers to form a potential antimicrobial peptide called gramicidin D. The channel gating is known to occur by a well-defined conformational change (breaking of hydrogen bonds at the monomer interface) that is the formation and dissociation of a transmembrane dimer. Gramicidin channels share important structural features with real ion channels. An essential feature of ion channels is the ability to select for specific ions using a selectivity filter. Remarkably, both the gramicidin channel and the selectivity filter of the K^+ channels are lined by the polar carbonyls of the peptide backbone; ion selectivity in both cases arises due to backbone interactions with ions.

Finally, the operation of the voltage-clamp circuit is explained together with the main functions of the data acquisition software. After the introduction, the students are divided in small groups (2-3 students per group) ready to start with the practical session.

Materials:

1) eNPR amplifier (Elements srl) equipped with a BLMchip (100µM diameter hole size)
   https://elements-ic.com/nanopore-reader/

2) Personal computer with either EDR (Elements Data Reader) and EDA (Elements Data Analyzer) software installed (Elements srl). Alternatively, for the data analysis, Clampfit software (Molecular Devices) can be used.

3) 100 mM of KCl, HCl, NaCl and CsCl

4) DPhPC lipids (Avanti, CAS number 207131-40-6). Dissolved in n-Octane organic solvent (Sigma Aldrich) to make 10mg/ml.

5) Gramicidin D (Sigma Aldrich). Dissolve in methanol (Sigma Aldrich) to make 5 mg/ml.

The practical session is divided in three steps corresponding to a progressive approach of the different aspects of the course.

Step 1: Set up the recording apparatus and paint a stable bilayer membrane using the "bubble method"

Step 2: Functional study of the electrical properties of the bilayer membranes: apply an alternated triangular and square wave voltage stimulus and analyse off-line the current response to calculate the membrane capacitance (Cm), the membrane resistance (Rm), the membrane conductance (g) and the area of the formed membrane.

Step 3: Add the gramicidin peptide to the recording solutions and evaluate the current response at different voltages using a voltage step stimulus protocol. Repeat the experiment using different cation solutions and by varying its concentration in one side of the recording chamber. Analyse the acquired data, build the I/V relationship and calculate i) the single channel conductance using different cation-containing recording solutions; and ii) the E_{rev} value varying the cation concentration within one side of the chamber.

Step 1:

Connect the eNPR device to a computer, launch the EDR software and connect the device by pressing the "connect" button. Place a BLM chip (the recording chamber designed by Elements srl) previously chlorinated in the specific slot of the eNPR device. Fill the bottom and top chambers of the BLMchip with 30 µl and 60 µl of recording solution, respectively. For a detailed description of the BLMchip and its use please visit the section "how to" of the eNPR webpage.

Apply an alternate triangular and square wave voltage protocol (Fig. 1A) in order to check the correct conductive-path formation by using the dedicated software command located in top-right of the GUI (Fig 1B).
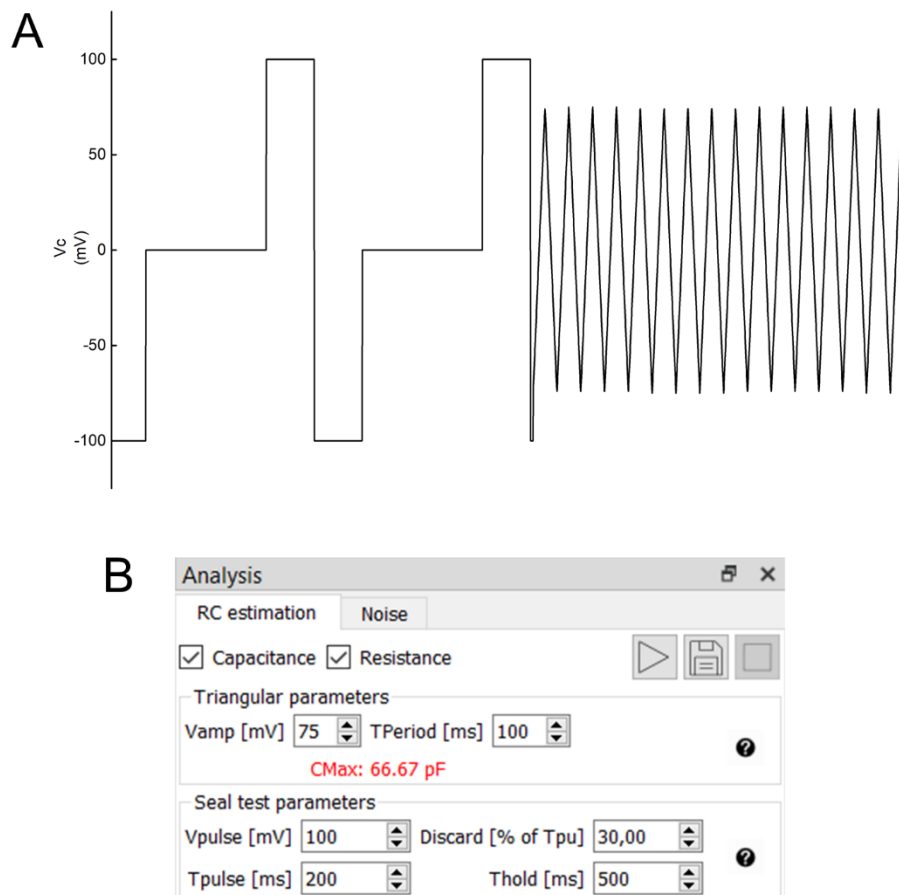
Figure 1: snapshot of the alternate squared wave and triangular wave voltage stimulus applied to check the membrane formation (A). Parameters of the two waves are set as reported in panel (B).

If the cavity is properly wetted, the resulting current signal should alternate between the positive and negative maximum of the selected current range. If not, use a p200 pipette to stir the solution in proximity of the hole until you see the correct formation of the path.

Step 2:

Manually paint a lipid bilayer by spreading the lipids over 100 µM hole via a lipid covered air bubble. This easy technique helps to form a stable membrane slightly avoiding the formation of undesired lipid plugs. Procedure: take a p10 pipette. Immerse the p10 tip of the pipette in the solution containing the lipids without aspirating any of it. Due to capillary action, a small amount of lipid solution remains in the tip. Squeeze out any of the residues from the pipette tip. Place the tip in proximity to the hole, dispense a small bubble of 3 µl over it and aspirate it back. In doing so the lipids that are coated over the cavity rearrange to form a bilayer. Follow the formation of the membrane by checking the current signal as well as the capacitance and resistance values automatically calculated by the software. If nothing happens, just dispense a new bubble until good and constant capacitance and resistance values are obtained (do not dip the tip a second time; this could cause the formation of lipid plugs). Use the ZAP tool to judge the status of the bilayer and to exclude the formation of a lipid plug. A lipid plug cannot be destroyed by the ~1V voltage pulse generated by the ZAP tool. On the contrary, a thin lipid bilayer would break.

When both the capacitance and resistance values are stable over time (Fig. 2), press the "quick save" button to save data to disk. At the end of the session, open the saved file using EDA software and analyse the current signal in order to manually calculate the resistance, the capacitance and surface occupied by the lipid bilayer. Alternatively, data can be analysed using Clampfit software (pClamp software package, Axon Instruments). Depending on the analysis software that the user intends to use, the right saving data format (.dat or .abf) must be selected before the data acquisition. For further details, please visit the "user guide" section within the [EDR software website page](#).

Below are reported the formulae needed to calculate the Resistance (R), the Capacitance (C) and the membrane surface (A).

$$I = C \cdot \frac{dV}{dt} \qquad C = I \cdot \frac{\frac{T}{2}}{V_{pp}} = \varepsilon \frac{A}{d} \qquad C = C_s \cdot A \qquad A = \frac{C}{c_s} \qquad R = \frac{V}{I}$$

Where ε (permittivity) and d (thickness of the membrane) strictly depend from the lipids. They are the so-called C_s, the specific capacitance of the lipids. The DPhPC used in this experiment, have a $C_s \sim 0.5\ \mu F \cdot cm^{-2}$. T and V_{pp} are the period and the peak to peak voltage of the triangular waveform, respectively.
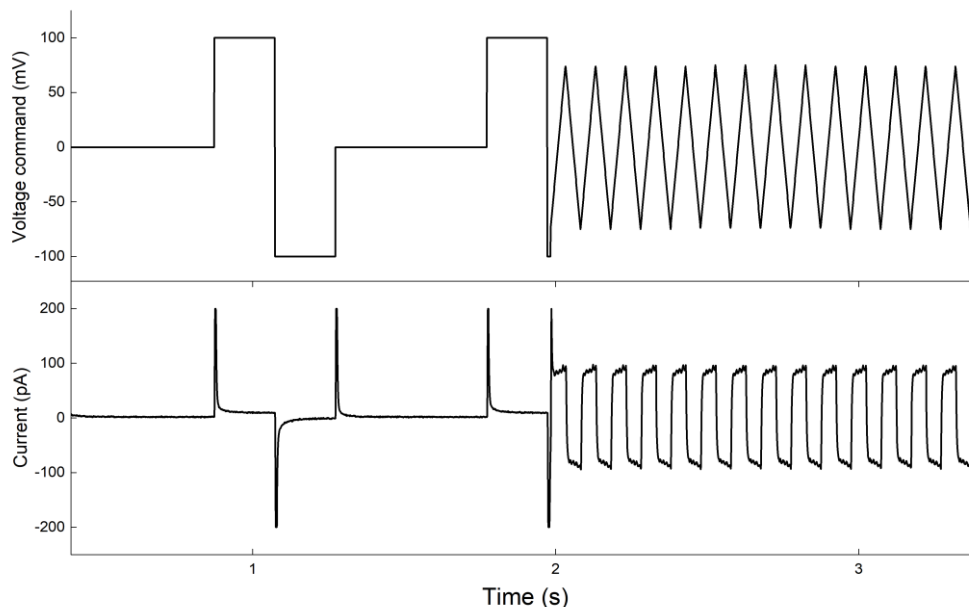

Figure 2: snapshot of the current response when an alternate squared and triangular wave voltage stimulus is applied to the painted bilayer membrane.
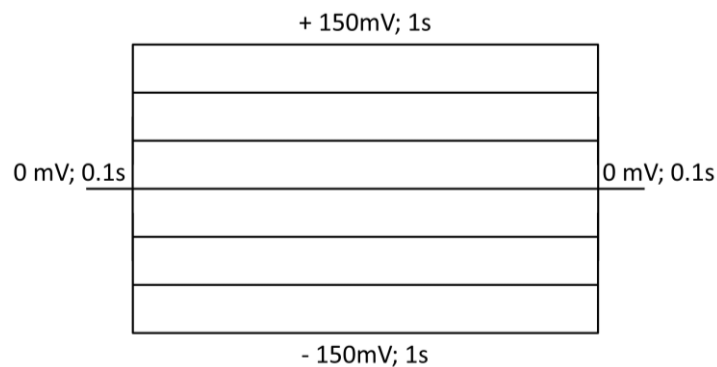
Students will learn that the cell membrane act as a leaky capacitor. The total current flowing in the equivalent RC circuit is produced by the capacitive current when a high frequency voltage stimulus (i.e. the triangular waveform) is applied to the circuit. On the other hand, when a constant voltage is applied (i.e the constant portions of the squared wave pulse) the current is purely resistive. The

combination of these two voltage stimuli allow the students to calculate the resistance and the capacitance of the membrane.

Step 3:

Add 1 µl and 0.5 µl of Gramicidin D (previously dissolved in methanol) in the top chamber and in the bottom chamber respectively. Apply a constant voltage stimulus (e.g -50 mV) and check the current signal in time, until you see the raise of single current steps (you might have to wait for several tens of seconds to see something. This time is required for the formation of Gramicidin dimers that mediate the flow of cations across the membrane).

Once a single channel activity is detected, apply a voltage step protocol previously designed as depicted below (customize the protocol n°4 within the protocols list of the software) in order to evaluate the current response at different potentials.

Afterwards, vary the cation concentration of the recording solution to shift the E_{rev} potential: gently add 30 µl of Milli-Q water into the top chamber of the BLM chip and apply again the voltage step stimulus protocol. By doing so, the cation concentration within the top chamber will be half-concentrated, thus changing the Nerst potential.

At the end of the session, analyse the data measuring the amplitude of the current steps at the acquired voltages. Plot the dataset into a I/V graph and fit data to a linear function y= mx + b in order to obtain the slope of the line (i.e. the single channel conductance) and the intercept with the X axis (i.e the reversal potential). Compare the experimentally obtained E_{ion} value to the theoretical one calculated using the Nerst equation

$$E_{ion} = \frac{RT}{zF} \, ln \, \frac{[ion]_{out}}{[ion]_{in}}$$

Repeat the above described experiment using different recording solutions (e.g. CsCl, HCl, KCl and NaCl) to analyze the change of the single channel conductance.

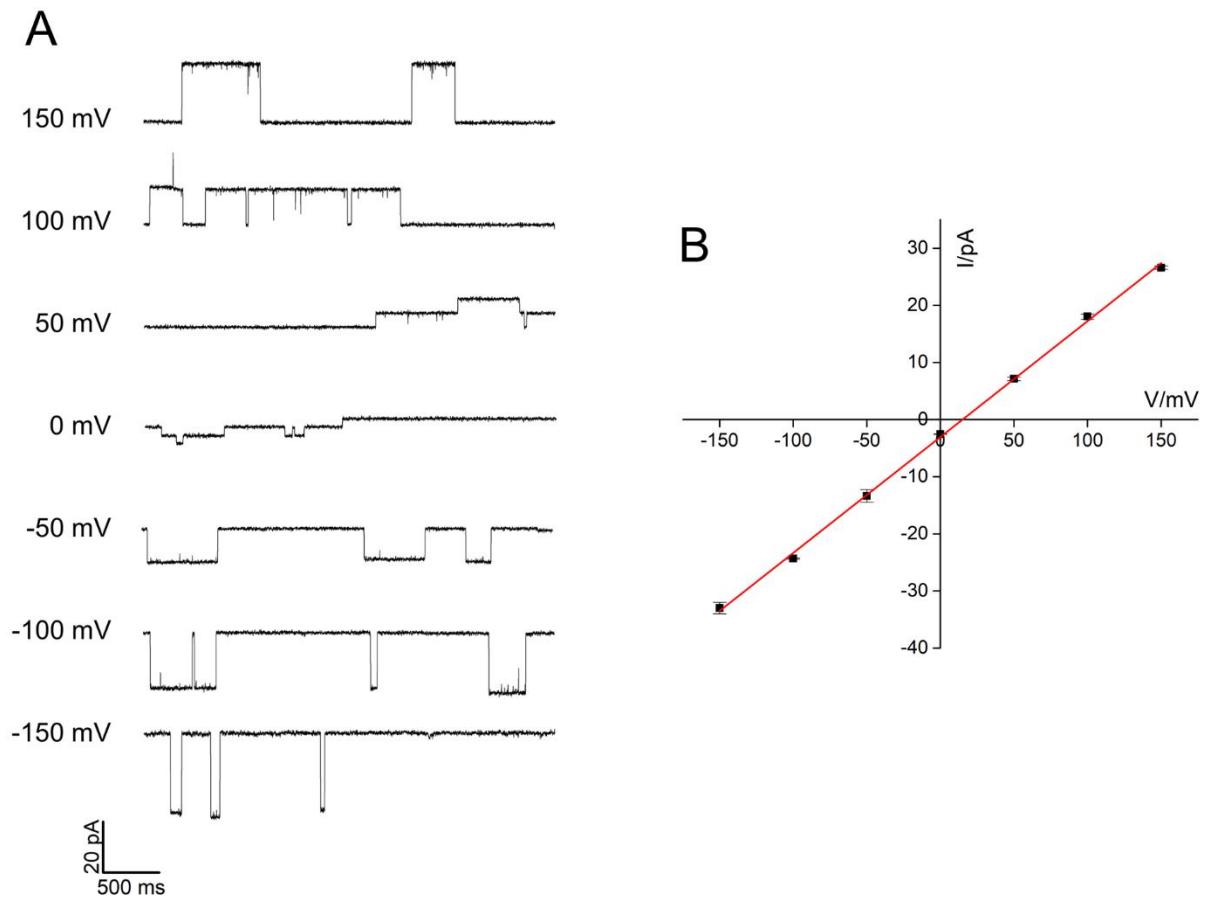Below is reported a representative result obtained in HCl solution.

Figure 3: (A) example single channel activity of Gramicidin D recorded at the indicated voltages in DPhPC membrane using asymmetric HCl recording solutions (100 mM and 200 mM). (B) current-voltage relationship measured from the current traces in panel A. Data fit to a linear equation yield the slope of the curve (i.e. the single channel conductance) and the X axis intercept (i.e. the E_{rev} potential).

Students will learn that ion channels form pores in the lipid bilayer allowing, transiently, the selective passage of ions acting therefore as electrical molecular switches. In addition, they will learn that the relation between the single channel current and membrane voltage is very close to a straight line that crosses the horizontal axis at E_{ion}, meaning that the channel, when is open, behaves as an ohmic resistor. Moreover, the students will go through the notion of ion channel selectivity for different ion species: since they have worked with a single-ion system, they will see that E_{ion} predicts E_{rev} and that such value strictly correlates with the ion concentration on both sides of the membrane.

The proposed experimental protocol is a simple example that can be further improved depending on both the time and the pore forming protein available for the lab sessions. For example, the experiments can be performed in presence of drugs with a predicted bilayer-modifying effect in order to study their impact on Gramicidin single channel activity. Alternatively, the possibility to

translate in vitro an ion channel protein (e.g KcsA) reconstituted into nanodiscs would open the possibility to introduce single point mutations within the amino acids sequence aimed to study their effects on the biophysical properties of the current.

For any question or suggestion please do not hesitate to contact us (info@elements-ic.com).