User Quick Guide

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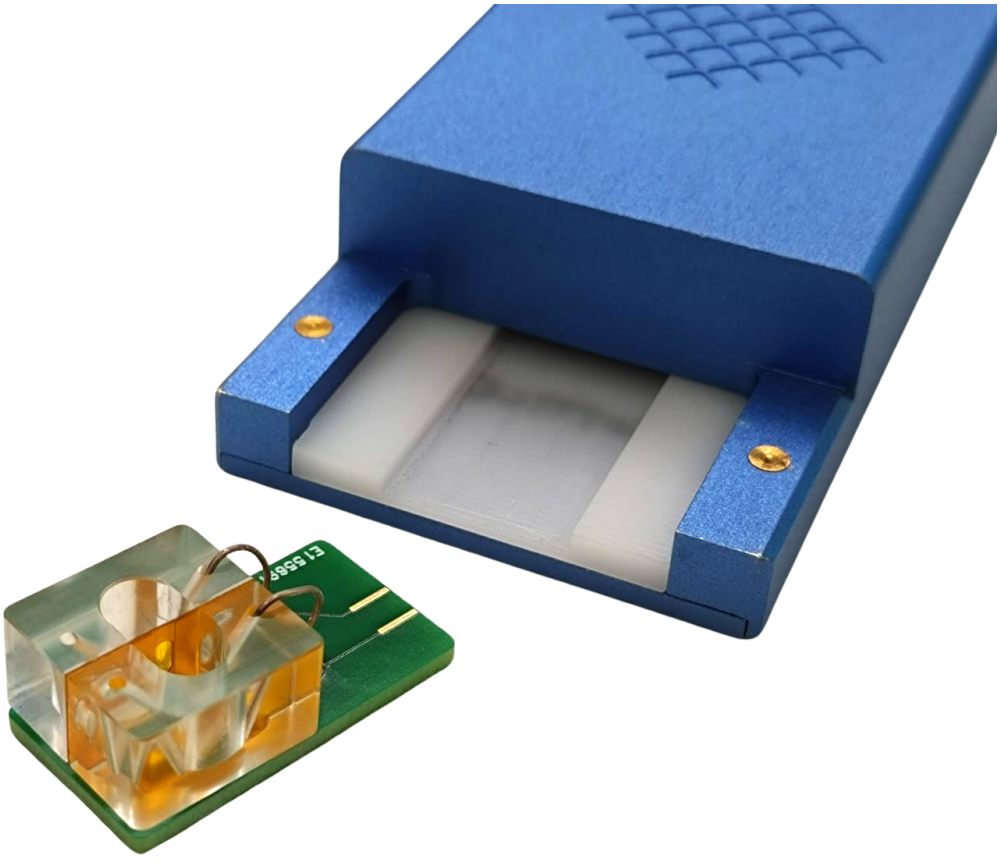
How to paint a lipid bilayer membrane in the BLM chip_MM



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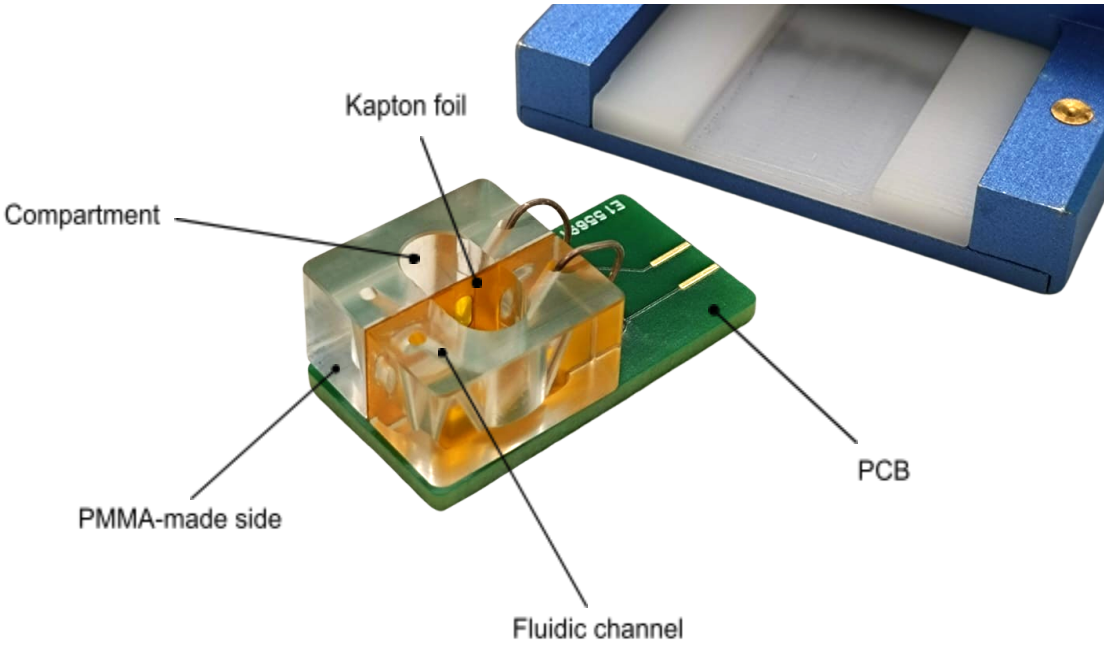
This document provides a detailed view showing how to paint lipid bilayer membranes using either the Montal Muller technique or the pseudo-air bubble method in the BLMchip_MM, the small fluidic cells designed for lipid bilayer painting with the Elements' eNPR 100kHz amplifier.

1) BLMchip_MM overview
2) Software installation
3) Instructions to setup the experiment and form a lipid bilayer membrane:
    1. Electrodes chlorination
    2. BLM formation and protein insertion
    3. BLMchip cleaning

1) BLMchip_MM overview

The BLMchip_MM are designed to study the electrical activity of membrane proteins across a vertical lipid bilayer membrane. TheBLMchip_MM chips are made to fit into the Elements eNPR 100kHz amplifier via the provided small PCB. The fluidic cell consists of a Kapton-made foil sealed between two transparent PMMA-made parts that form two C-shaped compartments in contact with the two sides of the lipid membrane. Two tilted fluidic channels are drilled into each PMMA part to both host the silver wires and raise/lower the water level inside the compartment. At the center of the kapton foil, a micro sized aperture has been laser drilled to allow the lipid membrane formation. Four hole sizes are available: 50, 75, 100 and 140 um. Although you can choose your preferred painting method, we suggest forming the lipid bilayer membranes using the Montal-Mueller technique, as described in this guide.



2) Software installation

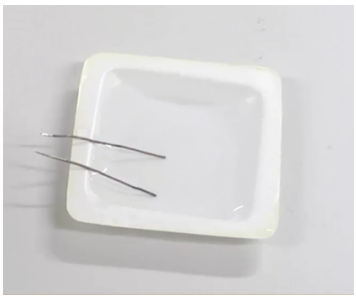
1) Download EDR4 software from http://elements-ic.com/downloads/. To date (January 2024), you may still control your eONE amplifier with EDR3 software (download from the same link) but soon EDR will be at the end of the support (except for major bug fixes). Please, write to support@elements-ic.com and ask for a free firmware update to make your eONE device compatible with EDR4 (please, attach to the email a screenshot of the "device information" window accessible from the "?" menu).

2) Unzip the archive and start the installation following the instructions.

3) Plug the eONE amplifier to any USB port of your computer and wait for hardware driver installation.

3) Set up an experiment

2.1) Instructions for chlorinating the Ag/AgCl electrodes



1) Before chlorinating, clean the Ag wires:
   - If it is the first usage, simply clean the wires with ethanol and rinse with double-distilled water.
   - If the wires were previously chlorinated, remove the old Ag/AgCl coating by abrading the surface with an extra fine grit sandpaper; then rinse with ethanol and double distilled water.
2) Chlorinate the silver wires:
   - The simplest method to chlorinate the silver wires is to immerse them into pure bleach until they become light gray coloured (typically 15 to 30 minutes are enough). The commercial bleach purchased in any supermarket is fine for this purpose. Important note: make sure not to chlorinate the portion of the wire that fits into the Ag/Cl electrode connector by keeping them outside the bleach, as shown in the figure above.
   - An alternative way that requires a little more effort but yields a deeper and more uniform chlorination is the electroplating method.

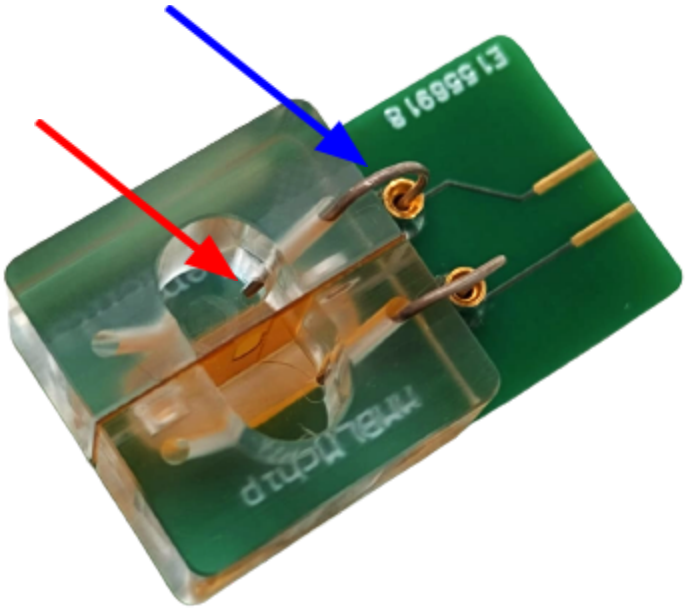
     Chlorinating a silver wire using the electroplating is achieved by making it positive relatively to a solution containing NaCl (0.9%) or KCl (1 M) and passing a current at a rate of approximately 1 mA/cm2 for about 1 minute (or until the wire is adequately plated and becomes gray coloured). For example, to chlorinate a 1 cm length of a 0.5 mm Ag wire (that is the diameter of the wires used in the kit) requires 0.15 mA of current. While plating, occasionally reverse the polarity for several seconds; this helps to deepen the chloride coating and yields a more stable electrode.

The chlorination must be performed every time the current baseline is not stable overtime. It is recommended to perform a chlorination every 5 hours of continuous experiments.

3.2) Lipid Bilayer Membrane formation and troubleshooting.

1) **Assemble the BLMchip** by firmly inserting the two silver wires into PCB sockets. Afterwards, insert them into the dedicated channels drilled in the plastic part, as shown in the figure below. Before installing new wires not provided by Elements, check the diameter is 0.5 mm to properly fit into the PCB sockets. The wires must be carefully pushed to the end of the fluidic channel. Check that the two final parts of the wires appear within the compartment, as indicated by the red arrow in the figure below. Check that the curvature indicated by the blue arrow adheres as closely as possible to the plastic part so as to reduce the total height of the fluidic device as much as possible. Important note: if the two silver wires are not kept low, they might get in contact with the blue lid of the eNPR amplifier, resulting in a short circuit between the two electrodes.

We suggest assembling the chip as shown in this video to reduce the mechanical stress of the silver wires and extend their life. The video also provides a detailed view of the next steps described below.

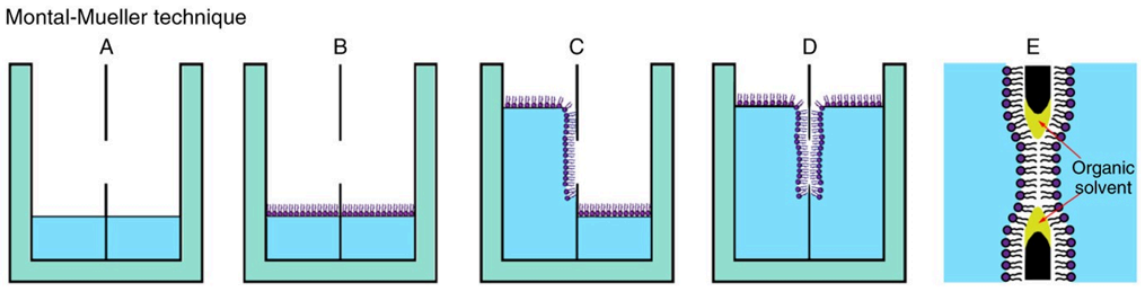


2) **Prepare the lipid solution**: in this example we use DPhPC lipids. For BLM chip_MM with a hole size of 100 or 150 um, we suggest to dissolve DPhPC at at 15 mg/ml in 100% n-hexane. For the smaller hole sizes, reduce the concentration to 10mg/ml in n-hexane.

3) **Pre-treat the aperture of the septum** with 1% hexadecane in n-hexane solution using the provided paintbrush to increase li-pids affinity to the septum in the area surrounding the hole. Wait 5 minutes for the solvent evaporation before filling the compartments (step 5).

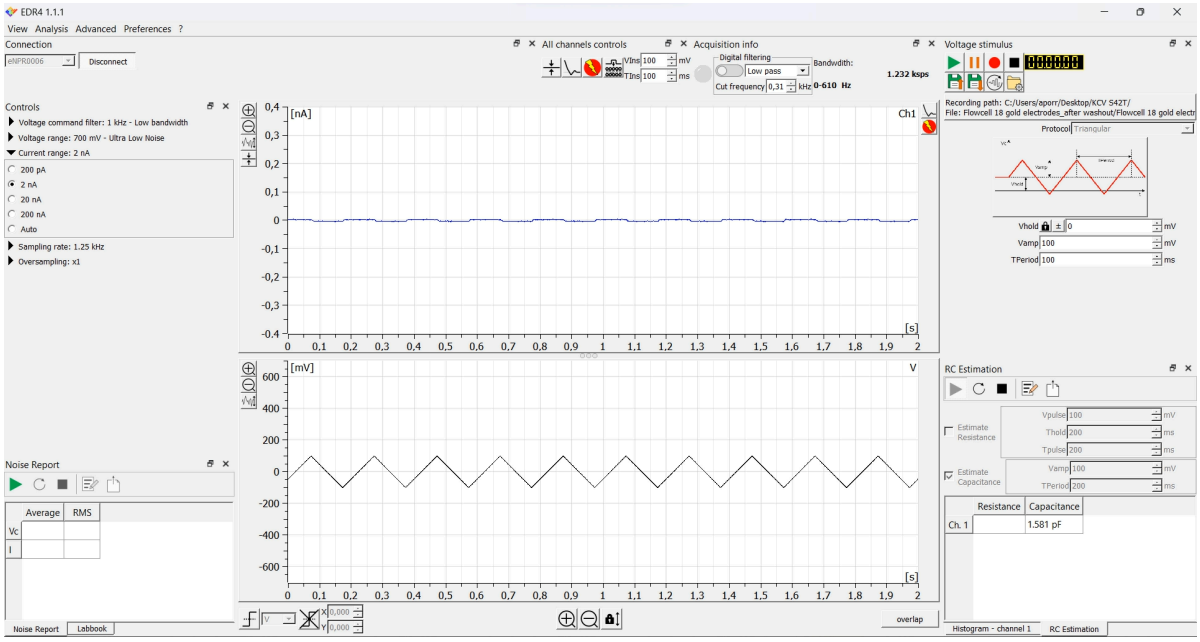
4) **Setup the software**: connect the amplifier and open EDR software. Set the 2nA range and disable the volt-age command filter tool (for more details, please see the EDR related "how to" guides or watch the video). Use the RC compensation tool to apply a triangular wave stimulus and estimate in real time the capacitance of the circuit.

5) **Fill each BLM chip_MM compartment** with 30 ul of electrolyte solution. We suggest starting with 0.1 M KCl. Before using, electrolyte solutions should be filtered with a 0.2 µm syringe filter. The top level of the solution must be below the hole of the septum, as shown in the scheme below, step A. Be careful not to wet the region surrounding the hole with the electrolyte solution.



*Montal-Mueller technique; modified from Gutsmann, T., Heimburg, T., Keyser, U. et al. Protein reconstitution into free-standing planar lipid membranes for electrophysiological characterization. Nat Protoc **10**, 188–198 (2015). https://doi.org/10.1038/nprot.2015.003*

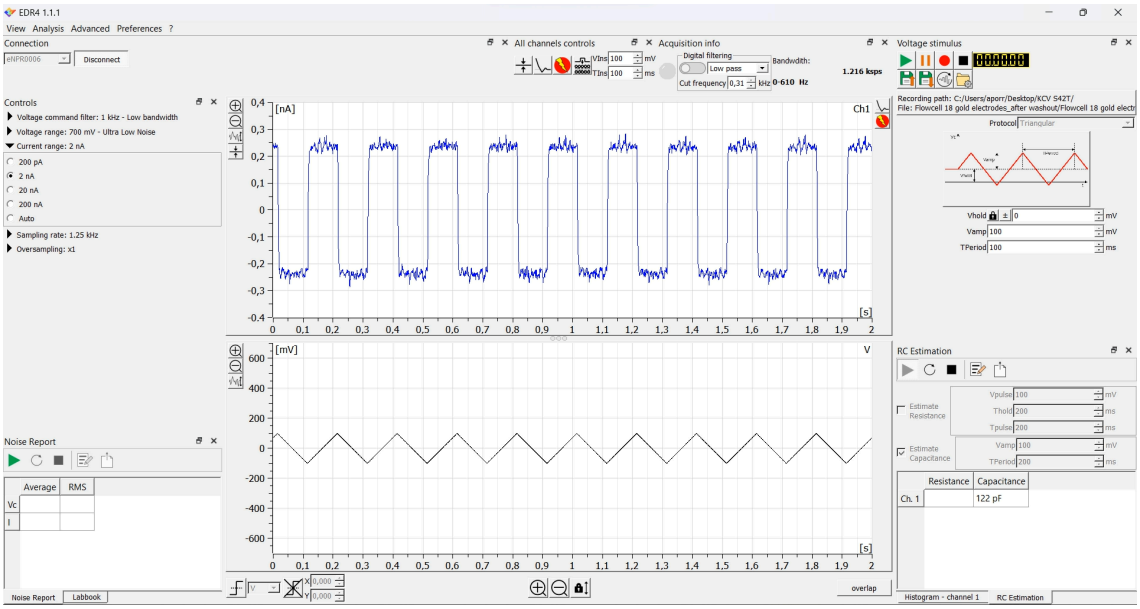
Close the Faraday cage and check the current signal appears as a squared wave fluctuating around zero, as shown in the screenshot below.



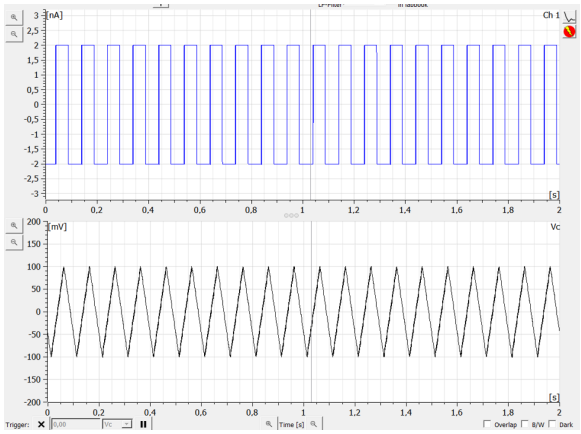
If a much larger current signal is observed, or the waveform appears as mixed squared-triangular, there is a leak in the BLMchip. If the unit is new (never used before), please contact us at support@elements-ic.com. On the contrary, if the chip has been used for some time, try with another BLm chip_MM and make sure you have properly washed it according to the instructions at the end of this guide. The chip can be washed and re-used up to 20 times without affecting the sealing agent.

6) **Add the lipids:** slowly add 2.5ul of the lipid containing solution to each compartment of the recording chamber and wait 1-2 minutes for the solvent evaporation (step B in the scheme shown above). In this time, check again that the current response fluctuates around zero, as before. Small changes in the current due to the addition of the solvent in the electrolyte solution might be observed.

7) **Create the membrane**: slowly add 80 µl of the electrolyte solution in each compartment (step C and D in the scheme shown above). While raising the volume, check that the electrolyte solution covers completely both sides of the aperture. The formation of the membrane can be seen as a change in the current response: a squared wave current should be observed, as shown in the figure below. Its amplitude is proportional to the membrane capacitance, which value is displayed in the RC esti-mation panel.

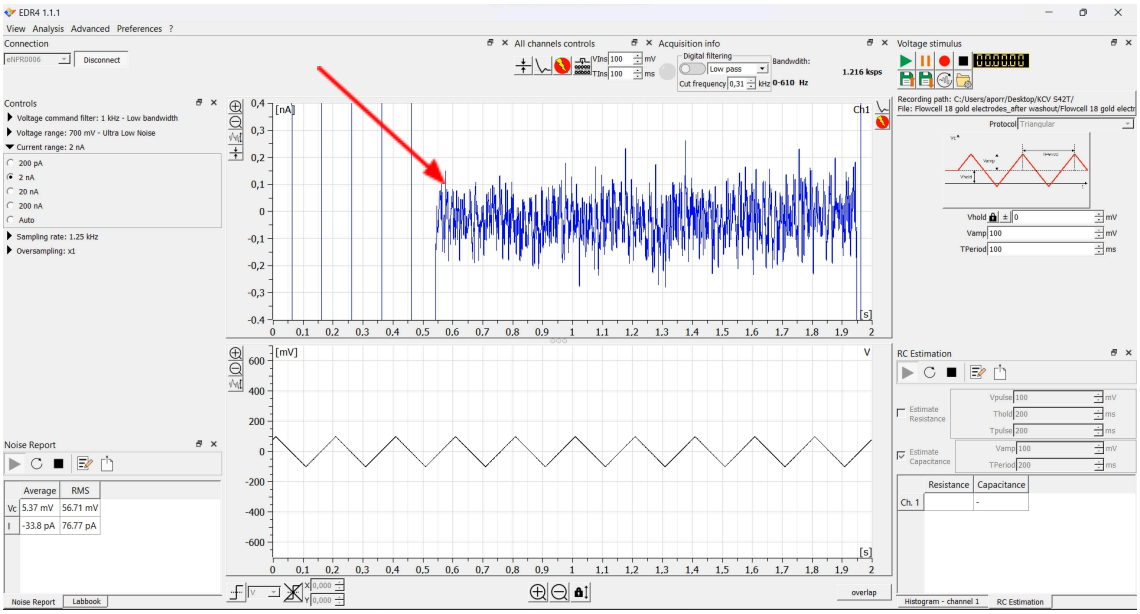


If no membrane forms, the current signal appears as a squared wave, stepping between the max and min values of the selected current range, as shown in the figure below.



In this case, lower the water level below the aperture in one of the two compartments and raise it up again to allow a new membrane formation. If no membrane forms, do the same in the other compartment.

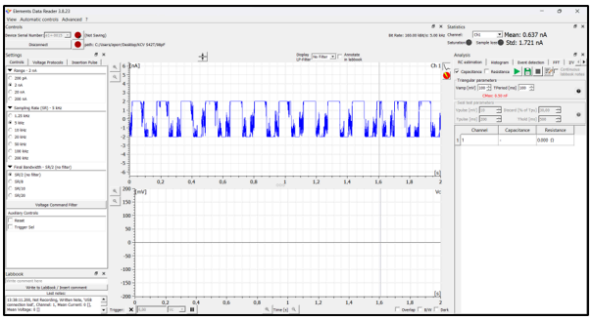
Important note: while lowering the water level, when it is below the aperture, check that the current signal fluctuates around zero and the current does not saturate the amplifier as shown in the figure below. The the arrow indicates the moment when the water level dropped below the hole.



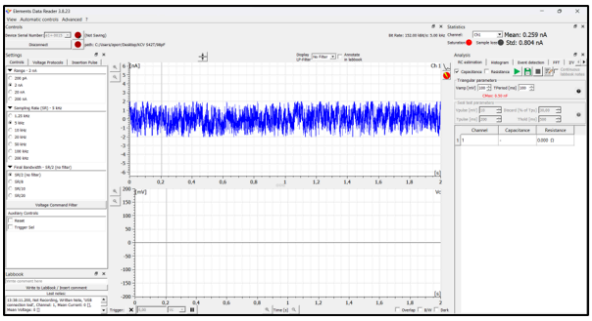
If this does not happen in either compartment, the pretreatment with 1% hexadecane in n-hexane was not effective.

In this case, try to paint the membrane with the pseudo-air bubble method (see step 11) or clean the BLMchip as described at the end of this guide and make the pre-treatment by adding more hexadecane + n-hexane solution. .

8) **Voltage offset compensation**: this procedure is required to set the current at 0 pA when a conductive path is present between the two electrodes. The operation must be performed only when there is no membrane formed in the aperture. Therefore, if the membrane forms at the first trial of step 7, break it using the ZAP tool (for more details on the ZAP tool, please see the EDR related "how to" guides or watch the video). On the contrary, if no membrane forms (i.e. the current signal saturates the selected current range), you can directly proceed with the compensation. Before proceeding to the compensation, it is convenient to apply a 0 mV constant voltage value to better follow the zeroing of the current signal. Compensate the electrode offsets by clicking on the "Voltage offset compensation" button. Depending on the EDR version this button can be located in different positions. Please refer to the EDR related "how to" guides to find it. The full compensation is achieved when the current fluctuates close to 0, as shown in the figure below. Afterwards, disable the digital compensation tool before proceeding to any other step. Important note: if not disabled, everything measured afterwards is meaningless.



Current signal during the voltage offset compensation

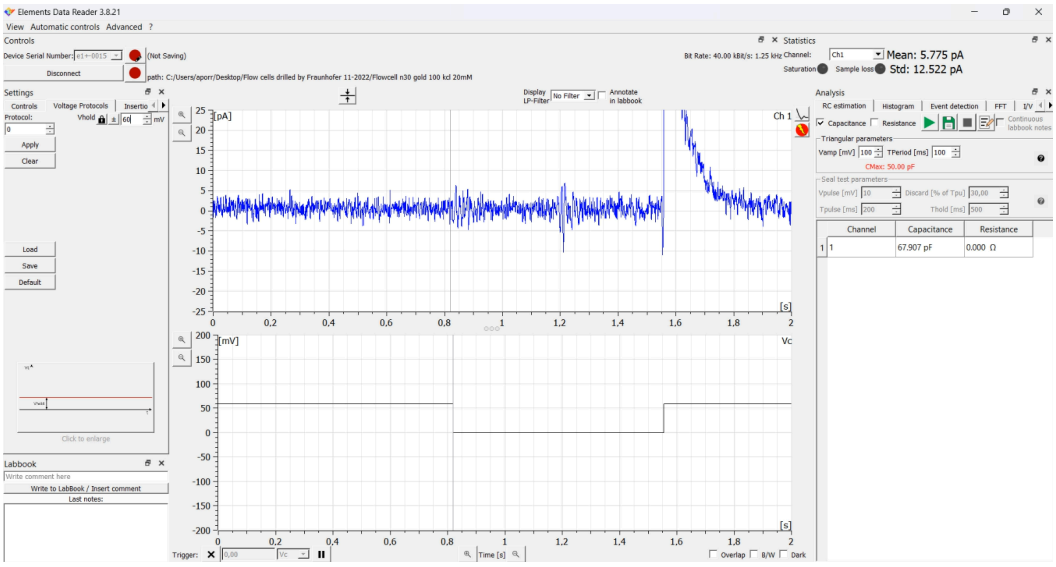


Current signal after disabling the automatic voltage offset compensation tool

9) **Create the membrane with an alternative method:** after the voltage offset compen-sation has been performed, restart the RC estimation tool to apply a triangular voltage stimulus and reform the membrane by lowering and raising the water level.

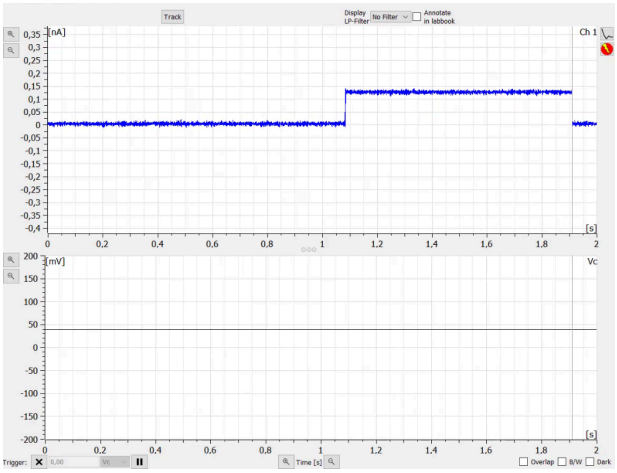
Alternatively, the membrane can be formed by forming one or more bubbles that must spread on the septum, just below the aperture of the septum, using a lab pipette. By moving up, the bubble will transfer some lipids from the reservoir (which was previously already formed in the region surrounding the hole at step 11) forming a new bilayer. This is the "blind" version of a method that was described for the first time by Braun and collaborators in this paper. Please watch the video in the BLMkit webpage to see how to apply it to our BLM chip.

10) **Test the membrane robustness**: once the membrane is formed, enable the voltage command filter option to increase the signal to noise ratio and test its stability overtime by applying a series of constant voltage values. If the membrane is well formed, after a short current peak due to the membrane capacitance, the current must be flat at 0 pA (see figure below).



11) **Add the pore forming protein**: add the membrane protein/molecule and wait for the reconstitution into the lipid membrane. Depending on the expected current amplitude, select the lowest current range available in order to increase the signal to noise ratio.

As a test bench, we suggest adding a commercially available pore-forming cytotoxin, the a-hemolysin (Sigma Aldrich, SKU H9395-.5MG), which is easy to handle and, if properly stored, it is stable for several months. Briefly, dissolve the lyophilized toxin in MilliQ water to make a stock solution at 0.10 mg/ml that can be stored at 5 °C. The day of the experiment, prepare a 1:500 dilution in the electrolyte recording solution (3M KCl, 20mM TRIS, PH8). Add small amounts (1 ul) of this solution in the CIS side of the recording chamber and wait for the protein reconstitution into the lipid bilayer. The latter is visible as a current step from 0 to about -130 pA at+ 40 mV bias voltage (see screenshot below). If multiple steps of the same amplitude are visible, multiple toxins have been inserted into the lipid bilayer. In this case, it is convenient to further dilute the working solution (e.g. try making 1:1000).

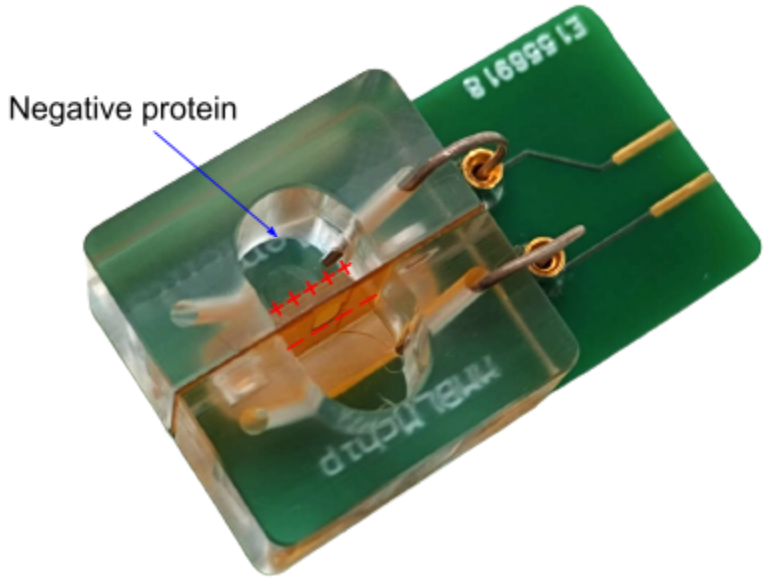


If you can't see any insertion, try to add some more protein in solution or improve the quality of the lipid bilayer. The latter can be easily evaluated by monitoring the membrane capacitance of the membrane. As a general rule, the larger the capacitance value the higher the quality. As a reference, the typical values obtained using the above-described procedure are shown in the table below (experimental conditions are reported in the cap-tion). Important note: changing the experimental conditions (e.g. different lipids or different electrolyte solution) might result in observing different capacitance values.

Hole size Ø	50 µm	75 µm	100 µm	140 µm
Hole Thickness	25 µm	25 µm	25 µm	25 µm
Typical BLM capacitance (pF)	20 - 40	40 - 60	60 - 90	90 - 140

Membrane capacitances were measured using DPhPC lipids with the protocol described above

Adding the protein to the right side of the recording chamber (cis or trans side of the figure below) may be crucial for the protein reconstitution in the lipid bilayer. For example, since the αHL protein is slightly negatively charged in buffer solution at pH 7, it could be useful to add it into the cis chamber when a constant positive voltage is applied, according to the figure below (the plus and minus sign show the polarity of the membrane when a positive voltage is applied).


Negative protein

3.3) Instructions for BLMchip_MM cleaning

Warning:

Do not use Acetone or absolute EtOH to clean the chip

Before proceeding to the cleaning, remove the plastic part from the PCB. We recommend cleaning the BLM chip_MM with isopropyl alcohol. You might use a lab pipette to add 100% isopropyl alcohol into the compartments and the fluidic channels. Do not leave the IPA inside the chip for more than 1 minute. Afterwards, rinse the chip with double distilled water and dry with a low pressure jet of air compressed. If you do not have the compressed air available, remove as much as possible the water from the fluidic channels using a lab pipette and let it dry completely. Do not proceed to a new pre-treatment until the chip is completely dry.

The plastic parts separating the Kapton foil are made of PMMA. Before using other solvents in your experiments and/or cleaning sessions, check their compatibility with the PMMA material. For this purpose, you might refer to this chart. The Kapton foil is sealed between the PMMA parts with a strong sealing agent. We do not ensure that other solvents outside of IPA will not damage the sealing compound.