User Quick Guide

elements
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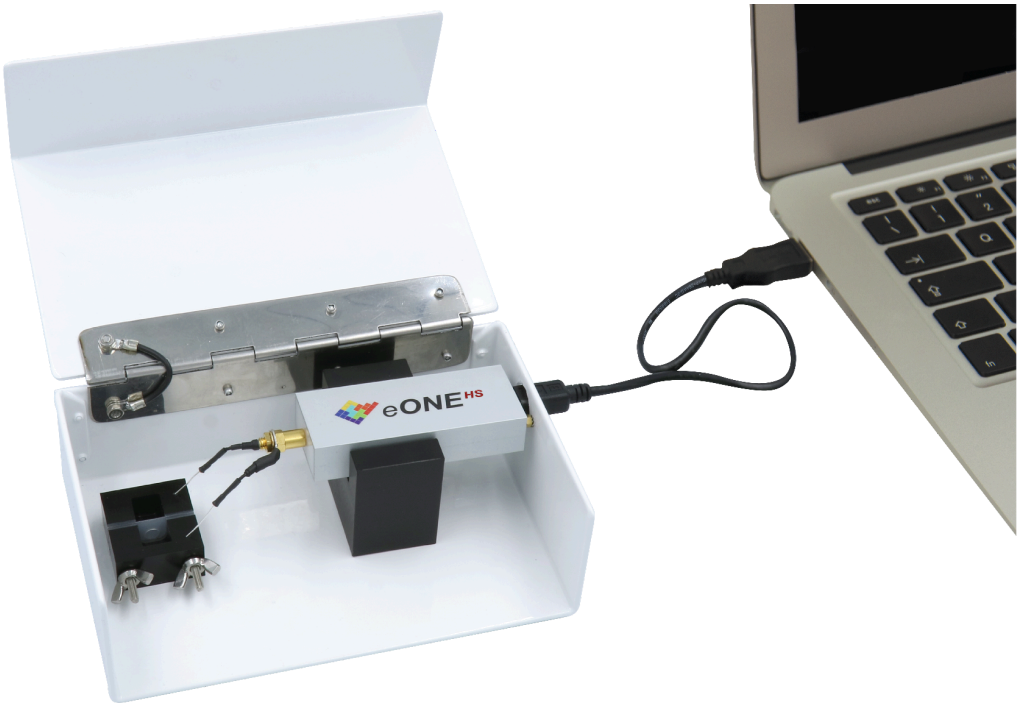
Montal-Müller technique in the Elements “BLM cuvette_MM”



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Revision History

Date	Version	Description
17/01/2024	1.1	Insert new entries on top
17/01/2024	1.0	First version of document

This document provides a detailed overview on how to paint lipid bilayers using either the Montal-Müller technique or the pseudo-air bubble method in the BLMkit recording chamber. Please note that the procedures described in this guide apply well to the new “BLM cuvette_MM” recording chamber, distributed from January 2024. It is particularly difficult to apply this procedure in the earlier “BLM cuvette_std” because of the low position of the septum aperture in the compartment. To learn more about the differences between the “std” and “MM” BLM cuvettes, please see the related guide ““Structural and functional details of the std and MM BLM cuvettes”.

The guide is divided in the following sections:

1) Instructions for software installation
2) BLM Kit content and setup
3) Instructions to properly setup the experiment and form a lipid bilayer membrane:
    1. Electrodes chlorination
    2. BLM formation and protein insertion
    3. BLM chamber’s cleaning

1) 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 support for EDR3 will end soon (except for major bug fixes). Please send an email to support@elements-ic.com and request a free firmware update to make your eONE device compatible with EDR4 (please attach a screenshot of the "device information" window accessible from the "?" menu to the email).

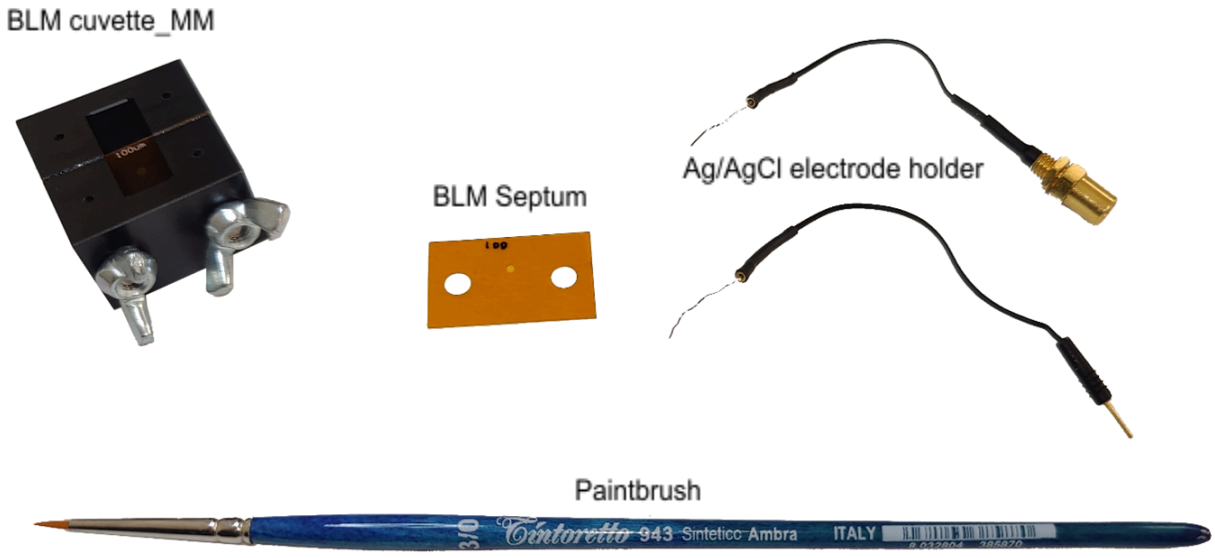
2) Unzip the archive and start the installation following the instructions.

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

2) BLM KIT content and setup

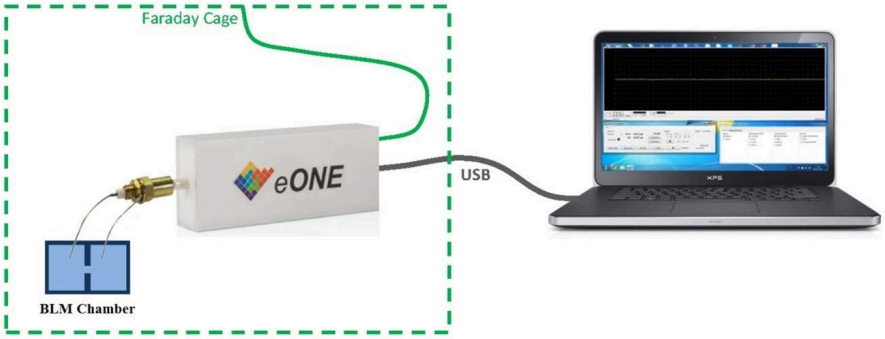
The kit is contained into a 16 x 10 x 5 cm (6.3 x 3.9 x 2 in) Faraday cage and includes:

- The amplifier belonging to the eONE family
- A recording chamber composed by two Delrin-made cuvettes embedding the holes for the Ag/Cl electrode. For painting membranes with the Montal-Mueller technique, we recommend using the “BLM cuvette_MM”.
- a set of Teflon-made septa embedding the micro-aperture (50, 75, 100 µm sized) and/or a set of Polyimide (Kapton) septa embedding the micro-aperture (100, 150, 200 µm sized).
- a paintbrush
- Ag electrodes and input connector to plug the eONE amplifier. Depending on the eONE amplifier you have purchased, the silver wires connectors might be different from the one shown in the picture below.



When everything is assembled, the kit looks as shown in the picture below. Please, watch the video shown in the BLMkit webpage to get a detailed view of the BLM kit assembly and connection.

BLM Kit experimental setup example

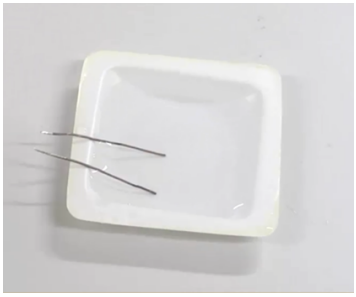


BLM Kit connections

Warning:
Do not short-circuit the electrodes with the Faraday cage!

3) Setting up an experiment

3.1) 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 (it typically takes 15 to 30 minutes). 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 to 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% w/v) or KCl (1 M) and passing a current at a rate of approximately 1 mA/cm^2 for about 1 minute (or until the wire is adequately plated and becomes gray coloured). For example, chlorination of a 1 cm-long 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 to a more stable electrode.

Chlorination must be performed every time the observed 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 (with the Montal-Mueller technique) and troubleshooting.

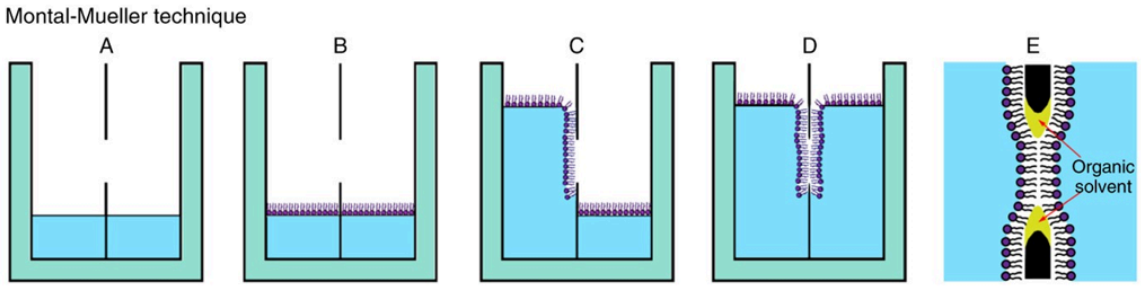
1) **Assemble the BLMkit** and the recording chamber as shown in the video uploaded in the BLMkit webpage. The video also provides a detailed view of the next steps described below.

2) **Prepare the lipid solution**: in this example we use DPhPC lipids at 15 mg/ml in 100% n-pentane (for this painting method, DPhPC lipids can be dissolved in n-hexane too).

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

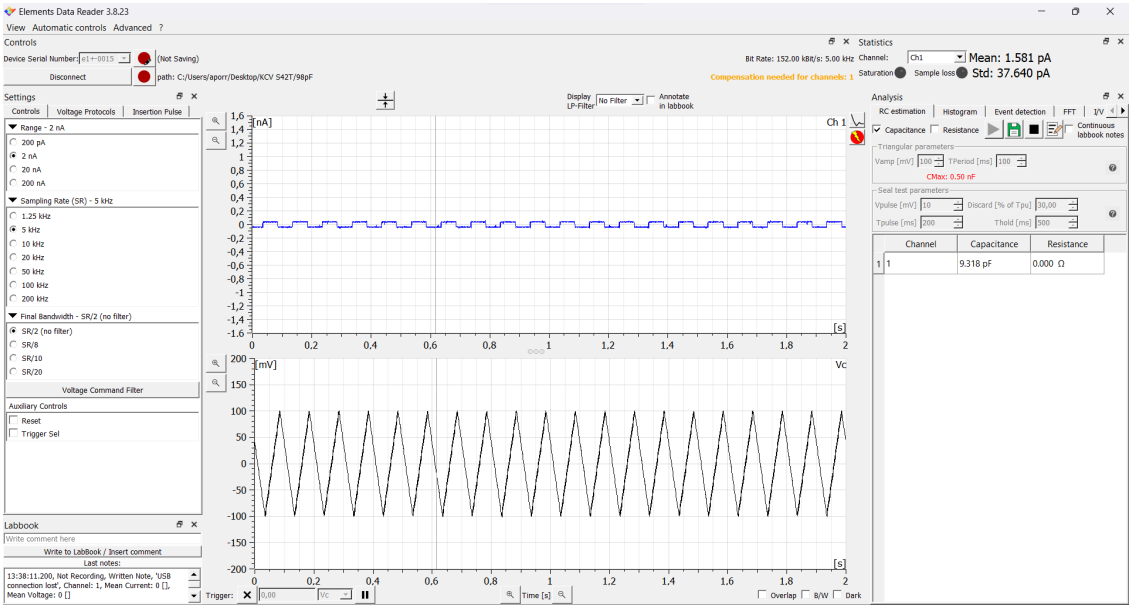
4) **Setup the software**: connect the amplifier and open EDR software. Set the 2 nA range and disable the voltage command filter tool (for more details, please see the EDR related "how to" guides or watch the video). Use the RC compensation tool to apply a triangular wave stimulus and estimate in real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

5) **Fill the BLM cuvette** with 300 µl of electrolyte/recording solution. The top level of the solution must be below the aperture of the septum, as shown in the scheme below, step A. Be careful not to wet the region surrounding the hole with the recording 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*

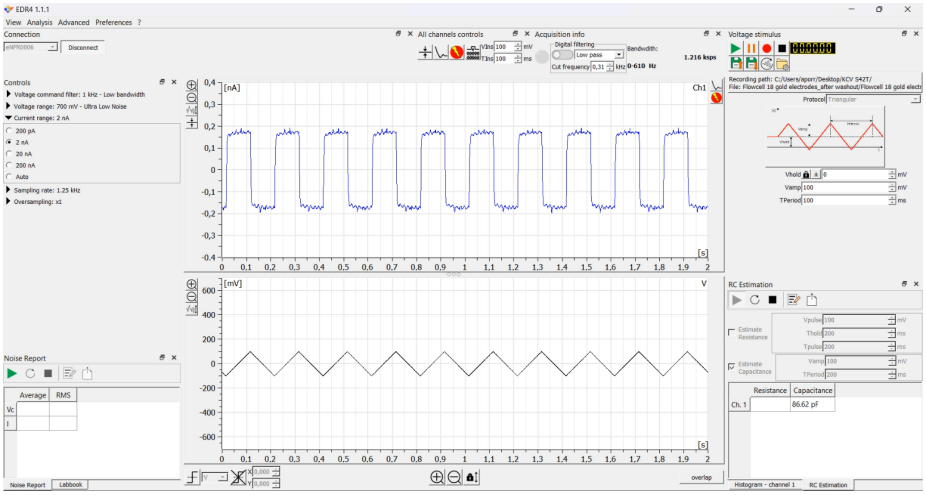
Close the Faraday cage and check the current signal appears as a squared wave fluctuating around zero, as shown in the screenshot below.



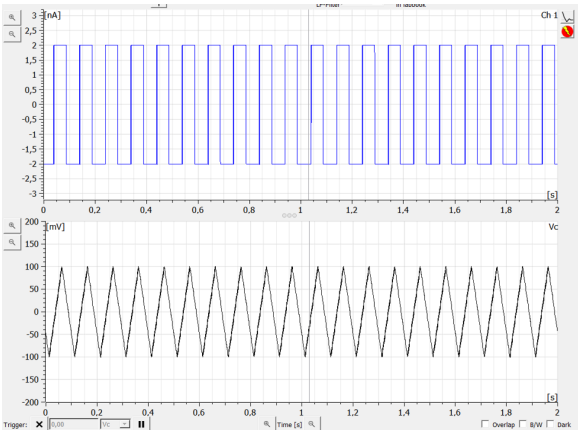
If a much larger current signal is observed, or the waveform appears as mixed squared-triangular, there is a leak in the recording chamber, possibly due to an incorrect assembly. In this case, disassemble the recording chamber, clean all the components as described at the end of this guide and restart from scratch. Important note: if the recording solution contains a membrane protein (e.g. channel, porine etc..) the waveform may appear as mixed squared-triangular even when a perfect lipid membrane is formed. This is due to the resistive component that the membrane protein adds to the RC circuit.

6) **Add the lipids:** slowly add 15µl of the lipid containing solution to each compartment of the recording chamber and wait 5 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**: after 5 min, slowly add 500 µl of the electrolyte solution in each compartment of the recording chamber (step C and D in the scheme shown above). While raising the volume, check that the electrolyte solution covers completely both sides of the hole (the surface of the solution should be a few millimeters above the hole level). 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 estimation panel.



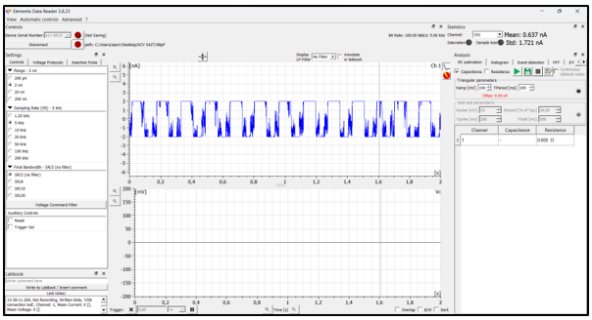
If no membrane forms, the current signal appears as a squared wave, stepping between the max and min values of the selected current range, as shown in the figure below.



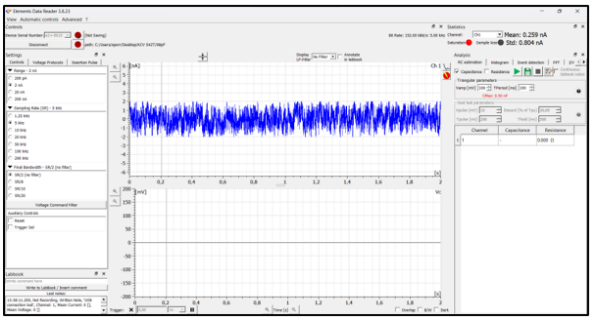
In this case, lower the water level below the aperture in one of the two compartments and raise it up again to allow a new membrane formation. If no membrane forms, do the same in the other compartment.

Important note: when the water level is below the aperture, check that the current signal fluctuates around zero, as shown in the video (i.e. the current signal does not saturate the amplifier). 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 disassemble the recording cham-ber, clean all the components as described at the end of this guide and restart from scratch.

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 is measured afterwards is meaningless.



Current signal during the voltage offset compensation

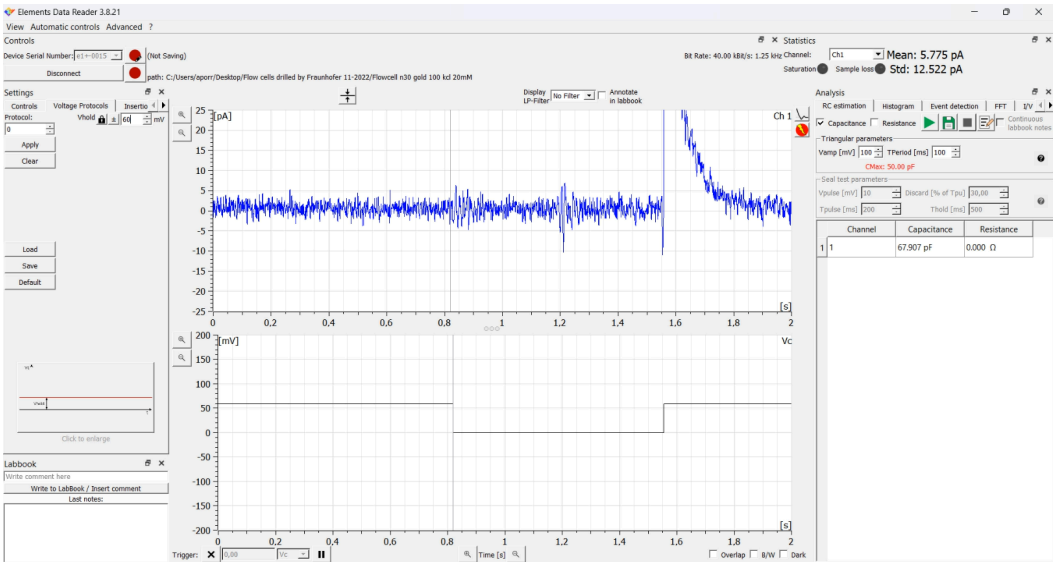


Current signal after disabling the automatic voltage offset compensation tool

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.

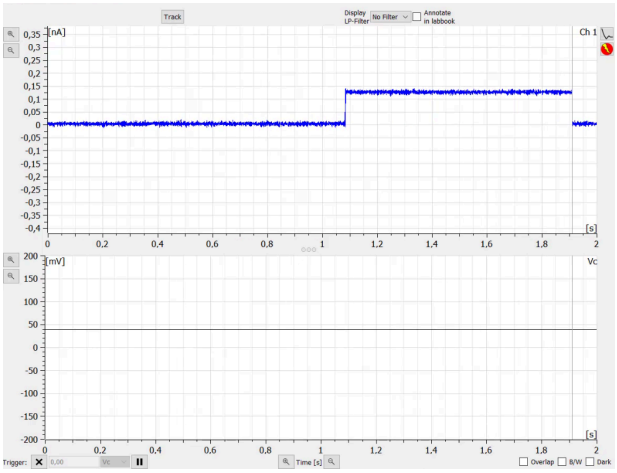
Alternatively, the membrane can be formed by forming one or more bubbles that must spread on the septum, just below the aperture of the septum, using a lab pipette. By moving up, the bubble will transfer some lipids from the reservoir (which was previously already formed in the region surrounding the 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 cuvette_MM.

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 α-hemolysin (αHL, 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 µl) 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).



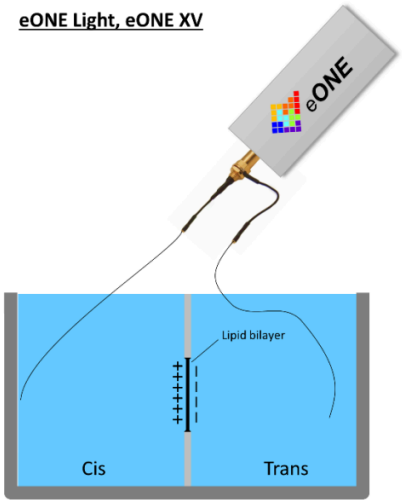
If you can't see any insertion, try to add some more protein in solution or improve the quality of the lipid bilayer. The latter can be easily evaluated by monitoring the membrane capacitance of the membrane. As a general rule, the larger the capacitance value the higher the quality. As a reference, 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 value.

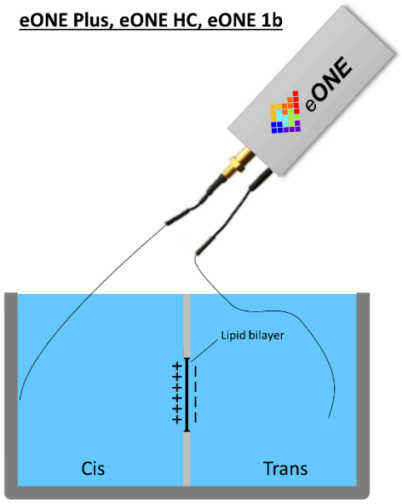
Material	Polyimide	Polyimide	Polyimide	Polyimide
Thickness	25 µm	25 µm	25 µm	25 µm
Hole size Ø	50 µm	75 µm	100 µm	150 µm
Typical. BLM capacitance (pF)	30 – 40	50 – 60	70 – 90	120 – 150

Membrane capacitances were measured using DPhPC lipids dissolved in n-hexane at 15mg/ml

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 the toxin in the cis chamber (negative electrode) when a constant positive voltage is applied.

Depending on the eONE amplifier version used, negative charged protein should be added to the cis chamber, according to the scheme shown below:





3.3) Instructions for septa and BLM chamber's cleaning

Warning:

Do not use Acetone to clean Teflon septum

The safest way is to clean the Teflon (or Polyimide) septum by using ethanol to remove lipids; then simply rinse the septum with double-distilled water. Acetone is compatible with the Delrin material and the Polyimide (Kapton) septa. Do not use acetone to clean Teflon septa.

Another procedure is to use trisodium phosphate (TSP, Na_3PO_4) solution (40-50 mM), which is a stronger cleaner than ethanol.

In this case you can use the following procedure to clean the supports:

1) Rinse the chambers with double-distilled water to remove the water-soluble components
2) Remove the Teflon (or Polyimide) support from the BLM chamber
3) Clean the hole in the septum using the trisodium phosphate (TSP) solution;
4) Rinse with dilute HCl solution (0.1% by volume) to remove any residual TPS;
5) Rinse with double-distilled water to remove any remaining HCl;
6) Gently dry the septum using a wipe or a low air jet.

Warning:

Since the Teflon septum is very fragile, be careful to not apply a high pressure on them while rinsing or drying.