Journal of Visualized Experiments

A novel micro agar salt bridge electrode for analyzing proton turnover rate of recombinant membrane proteins

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58552R1
Full Title:	A novel micro agar salt bridge electrode for analyzing proton turnover rate of recombinant membrane proteins
Keywords:	Electrophysiology, Electrogenic transport, diffusion potential, Salt-bridge, protein turnover number, Nernst potential, uncoupling proteins, membrane transporter
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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Vienna, 18/07/18

Dear Editor,

We resubmit our manuscript with a modified title: "A novel micro agar salt bridge electrode for analysing proton turnover rate of recombinant membrane proteins'' by Elena E. Pohl and Jürgen Kreiter, which now addresses all of the points that were raised by the reviewers.
In the course of the manuscript revision we additionally changed the term "Liquid Junction Potential" to "Diffusion Potential", which is more precise for the described setup.
We very much appreciate all of the constructive feedback that our original submission received. After having addressed the issues that had been raised, we feel that the quality of the paper has improved and we hope you will also agree.

Sincerely yours,

TITLE:

A Micro-agar Salt Bridge Electrode for Analyzing the Proton Turnover Rate of Recombinant Membrane Proteins

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KEYWORDS:

Electrophysiology, electrogenic transport, diffusion potential, protein turnover number, Nernst potential, uncoupling proteins

SUMMARY:

In electrophysiological measurements, the presence of a diffusion potential disturbs the precise measurement of the reverse potential by altering the electrode potential. Using a micro-agar salt bridge, the impact of the diffusion potential is minimized, which allows a more precise measurement of substrate turnover numbers of reconstituted recombinant membrane proteins.

ABSTRACT:

To date, more than 50% of all pharmacological drugs target the transport kinetics of membrane proteins. The electrophysiological characterization of membrane carrier proteins reconstituted in lipid bilayer membranes is a powerful but delicate method for the assessment of their physicochemical and pharmacological properties. The substrate turnover number is a unique parameter that allows the comparison of the activity of different membrane proteins. In an electrogenic transport, the gradient of the translocated substrate creates a membrane potential that directly correlates to the substrate turnover rate of the protein. By using silver chloride electrodes, a diffusion potential, also called liquid junction potential, is induced, which alters electrode potential and significantly disturbs precise membrane potential measurements. Diffusion potential can be minimized by a salt bridge, which balances electrode potential. In this article, a micro-agar salt bridge is designed to improve the electrophysiological set-up, which uses micropipettes for the membrane formation. The salt solution is filled into a microcapillary pipette tip, stabilized by the addition of agarose, and can be easily mounted to a standard electrode. The electrode potential of a micro-salt bridge electrode is more stable compared to a standard electrode. The implementation of this system stabilizes electrode potential and allows more precise measurements of membrane potential generated by a pH gradient. Using this system, the proton turnover rates of the mitochondrial carriers UCP1 and UCP3 are reinvestigated and

compared to earlier measurements.

INTRODUCTION:

Membrane proteins are targeted by up to 60% of all known pharmaceutical drugs[1]. Electrophysiological measurements of membrane proteins are a powerful but delicate tool to analyze the electrogenic transport of substrates mediated by membrane carrier proteins. The modulation of the transmembrane current by the application of constant voltages or voltage ramps allows assessing the pharmacological and physical properties of the carriers, for instance, the activation and inhibition by substrates or the transport kinetics. Of special interest is the substrate turnover number, which displays the amount of substrate that is translocated by a membrane protein per time unit. It is a major parameter when comparing the kinetics of various membrane proteins. Establishing a concentration gradient of the charged substrate across the membrane generates an electromotive force from which the turnover number of the substrate is deduced.

Using an AgCl electrode, the presence of a chloride-free buffer creates a diffusion potential that alters electrode potential and leads to a shift in current-voltage measurements[2]. Although always present, it is negligible for standard conductance and capacity measurements since these parameters are either dependent on the slope of the current-voltage recording (conductance) or are the difference of a single recording (capacity), which cancels the potential. However, the recording of the reverse potential, which is created by the transport of substrate, can be significantly disturbed by the diffusion potential. Thus, for exact measurements of the reverse potential, the electrode potentials have to be kept constant.

The diffusion potential can be minimized by two methods: (i) in the presence of a bilayer membrane, a substrate concentration has to be increased on one side of the membrane[3,4], or (ii) a salt bridge balances the electrode potential[5]. The first method is highly dependent on the stability of the measurements. The membrane has to survive for several minutes, from the addition of substrate under stirring until the substrate is almost equally distributed in the solution. If the membrane ruptures in between, the substrate gradient is altered by the free exchange of charged molecules, and measurements turn inaccurate. The latter method balances the diffusion potential but is limited by the size of the set-up. Implementing a small but functioning salt bridge in a micro range to an electrophysiological set-up is challenging[6]. For the latter method, the salt solution is filled into a microcapillary tip and stabilized by the addition of agarose to prevent diffusion of the salt solution to the buffer solution.

In this protocol, a straightforward production of a micro-agar salt bridge and implementation into an electrophysiological set-up based on the pipette set-up[7] is described. A microcapillary tip is adjusted both to contain a 3 M KCl solution with 1 mol% (w/v) agarose and to bridge an AgCl electrode and buffer solution. The advantage of the micro-salt bridge is displayed by time recordings of the electrode potential shift and the more precise measurements of membrane potential at various pH gradients. In the model system of recombinant proteins reconstituted into liposomes, the turnover rates of mitochondrial carriers UCP1 and UCP3 produced under similar conditions are reinvestigated and compared to previous results[3,8].

PROTOCOL:

1. Production of Recombinant Uncoupling Proteins (UCPs) and the Formation of Planar Bilayer Membranes

1.1. Produce recombinant UCP1 and UCP3 as described by Rupprecht *et al.*[9] and Hilse *et al.*[10]

1.2. Form planar bilayer membranes on the tips of conventional dispensable plastic pipettes as described by Beck *et al.*[7]

2. Preparation of the Micro-agar Salt Bridge Electrode

2.1. Adjust a microcapillary pipette tip (see **Table of Materials**) to the appropriate length.

2.1.1. Mark the position on an empty tip at which the buffer-containing tip will be attached and use a sliding caliper to measure the length of the micro-agar salt bridge electrode.

CAUTION: The microcapillary tip must be long enough to enter the buffer solution for the measurements.

2.1.2. Cut the microcapillary tip with a sharp knife or blade and clean the cut surface with ethanol and water.

2.2. Prepare the AgCl-coated electrode.

2.2.1. Cut off an Ag wire of approximately 8 cm in length and clean it with ethanol and water.

2.2.2. Take a piece of sandpaper and smooth down the surface of 1-cm length at the end of the wire, which should be in contact with the salt solution.

2.2.3. Dip the smoothed end into a 3 M KCl solution and coat it electrochemically with chloride by using a DC supply at 1.5 V for 10 s.

2.2.4. Clean the electrode with water, dry it, and adjust the length of the AgCl electrode from the uncoated side so that it penetrates the microcapillary tip as deep as possible.

Note: The protocol can be paused here.

2.3. Prepare a 3 M KCl salt solution with 1% (v/v) agarose.

2.3.1. Weigh out 4.47 g of KCl and dissolve it in 20 mL of water by stirring it in a flask.

2.3.2. Remove the stirrer, weigh out 0.2 g of agarose, and add it to the flask.

2.3.3. Heat up the solution to 100 °C to melt the agarose and prevent it from clotting.

CAUTION: The flask will be very hot. Do not touch it with bare hands. Use gloves for handling.

2.4. Fill the microcapillary tip with the agarose salt solution.

CAUTION: The solution is hot. Protect hands and work carefully to avoid splashes.

2.4.1. If the agarose starts clotting, heat up the salt solution to fully melt the agarose again.

2.4.2. Soak 10 µL of the agar salt solution into the microcapillary tip. Soak it slowly and carefully to avoid air bubbles in the tip.

2.4.3. Remove the tip from the pipette and push in the AgCl electrode from the broader side of the tip. Ensure that the electrode penetrates the salt solution.

2.4.4. Cool down the electrode to room temperature and plug it into the amplifier.

2.5. Prepare the buffer for the measurements.

2.5.1. Weigh out 0.710 g of Na_2SO_4, 0.195 g of MES, 0.121 g of TRIS, and 0.023 g of EGTA, then add 100 mL of distilled water to a beaker and stir the solution.

2.5.2. Check for the pH value of the buffer by using a pH electrode and adjust the pH value to 7.32.

2.5.3. Check that the reference electrode and the agar salt bridge electrode are in electrical contact.

2.5.3.1. Add 1 mL of buffer to a plastic container.

2.5.3.2. Dip in the reference electrode and the agar salt bridge electrode and check for a signal response. If the signal response is correct, proceed to step 2.7.

2.6. If there is a false signal response or no electrical contact at all, perform the following troubleshooting.

2.6.1. Check if the electrode is in contact with the salt solution and push the electrode into the solution.

Note: If the solution is already too sticky, remove the micro-agar salt bridge and prepare a new microcapillary tip.

2.6.2. Check if there are air bubbles within the salt solution. If yes, prepare a new microcapillary tip.

2.6.3. Check if the salt solution is in contact with the buffer solution. If not, then cut off another 1 mm of the tube end of the tip.

CAUTION: Be sure that the tip is still long enough to penetrate the buffer-containing tip. If there is still no contact, prepare a new microcapillary tip.

2.6.4. If none of these steps help, prepare a new microcapillary tip.

2.7. For storage, dip the agar salt bridge electrode into a 3 M KCl solution.

Note: The protocol can be stopped here. For a pause overnight, store the electrode in 3 M KCl salt solution at 4 °C.

2.8. Prepare the buffer-containing plastic tip.

Note: If the electrode was stored overnight, take it out and let it heat up to room temperature for 30 min.

2.8.1. Take a microcapillary tip and bend the tube, 2 cm from the edge of the narrow part, approximately 90 degrees using a heating wire.

2.8.2. Use a very sharp knife or blade and cut off the tube around 5 mm from the bend.

2.8.3. Clean the surface at the end with ethanol and water and measure the diameter of the hole of the tip. From this, calculate the area of the surface.

2.8.4. Coat the surface of the tip with 85:15 (v:v) hexane:hexadecane.

2.8.4.1. Pipette 3 µL of the solvent and remove it from the tip.

2.8.4.2. Wait 1 min so that all of the residual solvent in the tip has evaporated.

2.8.5. Fill the measurement tip with 3 µL of buffer and plug it into the salt bridge electrode.

Note: Check again if the salt bridge electrode and the reference electrode are in electrical contact by performing step 2.5.3.

2.8.6. If there is no electrical contact, check for the following:

2.8.6.1. Check whether the salt bridge electrode is in contact with the buffer solution. If not, then either increase the volume of the buffer in the tip or prepare a longer microcapillary tip.

2.8.6.2. If there is an air bubble, remove the tip from the micro-salt bridge electrode, refill the buffer in the tip, and plug it into the electrode again.

3. Measurement of the Electrical Parameters of the Membrane Reconstituted with Recombinant Protein

3.1. Apply a triangular alternating voltage signal with maximal voltage U_{max} = 50 mV and ΔT_{ramp} = 50 ms, which creates a rectangular alternating current response. From the mean values of the positive and negative currents (I_+ and I_-), calculate the capacity of the membrane according to the following formula:

$$C = \frac{(I_+ - I_-) \cdot \Delta T_{ramp}}{2 \cdot U_{max}}$$

3.2. Apply a voltage ramp ranging from -50 mV to + 50 mV and record the current. Fit a linear function to the data—the slope is the conductance—and calculate the x-axis intersection point of the fit.

3.3. Discharge the solution in the plastic tip and fill a new one with a buffer containing an increased substrate concentration.

3.3.1. If no membrane is formed within the first 20 - 30 s, discharge the volume and refill it. This guarantees that the concentration gradient across the membrane does not significantly change during the membrane formation.

3.3.2. After the formation of a membrane, perform steps 3.1 and 3.2 again to verify proper membrane formation and to get the x-axis intersection point.

4. Calculation of the Substrate Turnover Rate

Note: See previous work for details[3,7].

4.1. Estimate the amount of protein in the membrane from the molecular mass of lipid and protein (M_{Lipid} and $M_{Protein}$), the area of the membrane and of one lipid head group ($A_{Membrane}$ and A_{Lipid}), and the mass ratio of the protein per lipid (r).

$$N_{Protein} = \frac{M_{Lipid} \cdot A_{Membrane}}{M_{Protein} \cdot A_{Lipid}} \cdot r$$

4.2. Calculate the Nernst potential for the transported substrate. R is the gas constant, T the temperature, z the charge of the transported substrate, F Faraday's constant, and c_1 and c_2 the concentrations of the substrate of both sides of the membrane.

$$\Phi_{Nernst} = \frac{RT}{zF} \cdot \ln\left(\frac{c_1}{c_2}\right)$$

4.3. From the current-voltage recordings, take the reverse potential calculated with the difference of the x-axis intersection points from the linear fits in the presence and absence of the substrate gradient.

4.4. Calculate the proportion of substrate conductance, $G_{Substrate}$, to the total membrane conductance, G_{total}, by the ratio of reverse potential to Nernst potential[11].

$$G_{Substrate} = \frac{\Phi_{reverse}}{\Phi_{Nernst}} \cdot G_{total}$$

4.5. Calculate the substrate turnover number $\Delta N_{Substrate}$ per time unit ΔT from the substrate conductance ($G_{Substrate}$), the applied voltage U, and the charge of the substrate z:

$$\frac{\Delta N_{Substrate}}{\Delta T} = \frac{G_{Substrate} \cdot U}{z \cdot e}$$

4.6. From the ratio of transported substrate per time to the number of proteins, calculate the turnover rate κ.

$$\kappa = \frac{\Delta N_{Substrate}/\Delta T}{N_{Protein}}$$

REPRESENTATIVE RESULTS:

To verify the minimization of the diffusion potential, the stability of the current-voltage measurements of an intact membrane was measured. In **Figure 2A**, representative-current voltage recordings are depicted in the presence (white dots) and absence (black dots) of a pH gradient. According to the Nernst equation, the pH gradient induces a shift in voltage. From the x-axis intersection point of a linear fit to the data, the potential shift is calculated. In order to test both electrodes, the shift in x-axis intersection point was analyzed for a standard AgCl electrode (**Figure 2B**; white dots) and a micro-agar salt bridge (**Figure 2B**; black dots). A voltage ramp was recorded ten times in a row and the mean shift in the x-axis is depicted against time. Whereas the agar salt bridge electrode had a maximum shift of less than 5 mV even after 300 seconds of measurement, the standard electrode varied up to 30 mV in unpredictable, random, behavior.

Next, both electrodes were tested at different pH gradients (**Figure 3A**). For the standard electrode, a pH gradient of 0.35 and 1.0 (white dots) was generated; for the agar salt bridge electrode, pH gradients of 0.35, 0.7, and 1.0 (black dots). The shift in potential was analyzed in three independent measurements. In contrast to a gradient of 0.35, where the measured shift only varies slightly, the voltage shift significantly alters at the pH gradient of 1.0 in the absence of the micro-agar salt bridge. From a linear fit to the data, the slope of the function is 26.4 ± 2.3 mV/ΔpH for the standard electrode and 50.1 ± 4.6 mV/ΔpH for the micro-agar salt bridge electrode. According to the Nernst equation, the calculated potential shift is 60.7 mV/ΔpH at T =

32 °C.

Using the micro-agar salt bridge, the proton turnover number, κ, of mitochondrial UCP1 and UCP3 was measured and compared to previous measurements (**Figure 3B**). Similar to **Figure 3A**, ΔpH = 1.0 was generated and the reverse potential was measured. The amount of protein in the membrane was estimated according to the formula provided in section 4 of the protocol, with a protein to lipid ratio of 4 µg/(mg of lipid), a molecular mass of 33,000, and 750 Da for the protein and lipid, a membrane surface area of 3.53×10^{-4} cm^2, and an area per lipid of 7.8×10^{-15} cm^2. The obtained κ was 5.56 ± 0.38 s^{-1} and 4.10 ± 0.71 s^{-1} for UCP1 and UCP3, respectively (**Figure 3B**).

FIGURE AND TABLE LEGENDS:

Figure 1: The electrophysiological set-up with the micro-agar salt bridge. (**A**) This panel shows a sketch of the set-up. The microcapillary tip containing the agar salt solution (depicted in orange) is placed between the electrode (black) and the buffer-containing tip (blue). The membrane is formed at the surface at the end of the buffer-containing tip (indicated by the arrow). (**B**) This panel shows an image of the electrophysiological set-up with the implementation of the micro-agar salt bridge. The arrows point to the electrode, the micro-agar salt bridge, the reference electrode, and the container with buffer solution.

Figure 2: The comparison of a micro-agar salt bridge and a standard AgCl electrode. (**A**) This panel shows a representative current-voltage measurement in the presence (grey dots) and absence (white dots) of a pH gradient of 1. The lines represent a linear fit to the data, from which conductance and x-axis intersection values are obtained. The voltage shift is evaluated by the difference of the intersection values of both recordings. (**B**) This panel shows the shift of membrane potential of a standard AgCl electrode (white dots) to a micro-agar salt bridge electrode (black dots) in time. Ten current-voltage measurements were recorded in a row and the mean voltage shift by diffusion potential is plotted against time. In all experiments, the membrane was made of 45:45:10 mol% DOPC:DOPE:CL reconstituted with 15 mol% arachidonic acid at a concentration of 1.5 mg/mL. The buffer contained 50 mM Na_2SO_4, 10 mM MES, 10 mM TRIS, and 0.6 mM EGTA at pH = 7.34 and T = 32 °C.

Figure 3: Proton turnover number of UCP1 and UCP3 calculated from the reverse potential in the presence of a pH gradient. (**A**) This panel shows the shift in potential of a UCP1-containing membrane of various pH gradients of a standard AgCl electrode (white dots) and a micro-agar salt bridge electrode (black dots). The lines represent a linear fit to the data. (**B**) This panel shows the proton turnover number of UCP1 (first data set) and UCP3 (second data set) as calculated from the ratio of voltage shift to Nernst potential according to the formulas in section 4 of the protocol. The first bar of each set represents turnover rates measured with the micro-agar salt bridge. The second bar of each data set represents previous measurements using a standard AgCl electrode. Values for UCP1 and UCP3 were taken from Urbankova *et al.*[3] and Macher *et al.*[8]. In all measurements, the membrane was made of 45:45:10 mol% DOPC:DOPE:CL reconstituted with 15 mol% AA and UCP1/UCP3. The concentration of lipid and protein was 1.5 mg/mL and 4 µg/mg of lipid, respectively. The buffer contained 50 mM Na_2SO_4, 10 mM MES, 10 mM TRIS, and 0.6 mM

EGTA at pH = 7.34 and T = 32 °C. The pH of the buffer for the gradient measurements was increased to 7.66, 8.00, or 8.33 by adding TRIS and was changed in the solution-containing pipette. The values are the mean ± standard deviation of three independent measurements.

DISCUSSION:

The implementation of the micro-agar salt bridge with the electrode minimizes its diffusion potential and allows more precise measurements of membrane potential generated by a pH gradient. In the presence of various transmembrane pH gradients, the potential shift of both electrodes was acceptable at ΔpH = 0.35 when comparing to the theoretical value of the Nernst equilibrium potential (Φ_{Nernst} = 23.8 mV for ΔpH = 0.4). However, at more physiological pH gradients, as for instance in mitochondria between the matrix and intermembrane space, the standard AgCl electrode failed to precisely measure the potential shift at ΔpH = 1 (**Figure 3A**). The electrode bridged with micro-agar salt delivered the values which were much more comparable to the theory.

Diffusion potential may also occur at the AgCl reference electrode if the buffer solution is changed during the experiment. Chloride-free buffer solution was used in the experiments since uncoupling proteins were suggested to transport chloride ions, and the pH was adjusted using Tris or MES. The electrode potential, in the absence of a substantial concentration of chloride, primarily depends on chloride impurities in the buffer solution. As its composition is unchanged during the experiments, it will simply result in a constant offset potential. However, for the measurement of an absolute potential difference between the two electrodes, a simple agar salt-bridge system (Ag/AgCl 3 M KCl) could also be used for the reference electrode.

A micro-agar salt bridge balances the diffusion potential by an equilibration of the electrode potential. In order to stabilize the salt solution, 1% (w/v) agarose was added to prevent the mixing of the salt solution with the buffer solution. The salt ions K^+ and Cl^- have similar mobilities in liquid and balance the electrode potential. To properly install the salt bridge, the agar salt solution has to be sufficiently heated up to fill the microcapillary tip without any air bubbles and to cover the AgCl electrode. Before further usage, electrical contact between the salt bridge electrode and the reference electrode has to be checked. Depending on the time the salt bridge is used, the salt solution has to be sufficiently gelled to prevent any mixing of the salt solution with the buffer. This is especially critical if K^+ or Cl^- transporters are investigated. The salt bridge was used for a very short time and the elution of agarose is negligible in this time range. A higher concentration of agarose of up to 5%, or of agar (3% - 5%), allows using the salt bridge for a longer period of time[6,12].

This method allows determining the transport kinetics of a membrane transporter (i) with low turnover rates and (ii) of mitochondrial proteins of the inner membrane, which can hardly be investigated in standard patch clamp set-ups[13]. Its precision is mainly dependent on the reverse potential measurement, which accuracy is decreased at a low total membrane conductance and small concentration gradients which induce a membrane potential below the noise of recording.

Using this set-up, the turnover rates of UCP1 and UCP3 as produced under the same conditions

were measured. Due to the higher pH gradient, the obtained rates seem to be more precise and unperturbed by artefacts resulting from the minor electrode potential shift. It can be used to further analyze and compare mitochondrial membrane transporters produced under similar conditions.

ACKNOWLEDGMENTS:

This work was supported by the Austrian Research Fund (P31559-B20 to E.E.P.). The authors thank Sarah Bardakji for the excellent technical assistance in the production and reconstitution of mouse UCP1 and UCP3 into proteoliposomes.

DISCLOSURES:

The authors have nothing to disclose.

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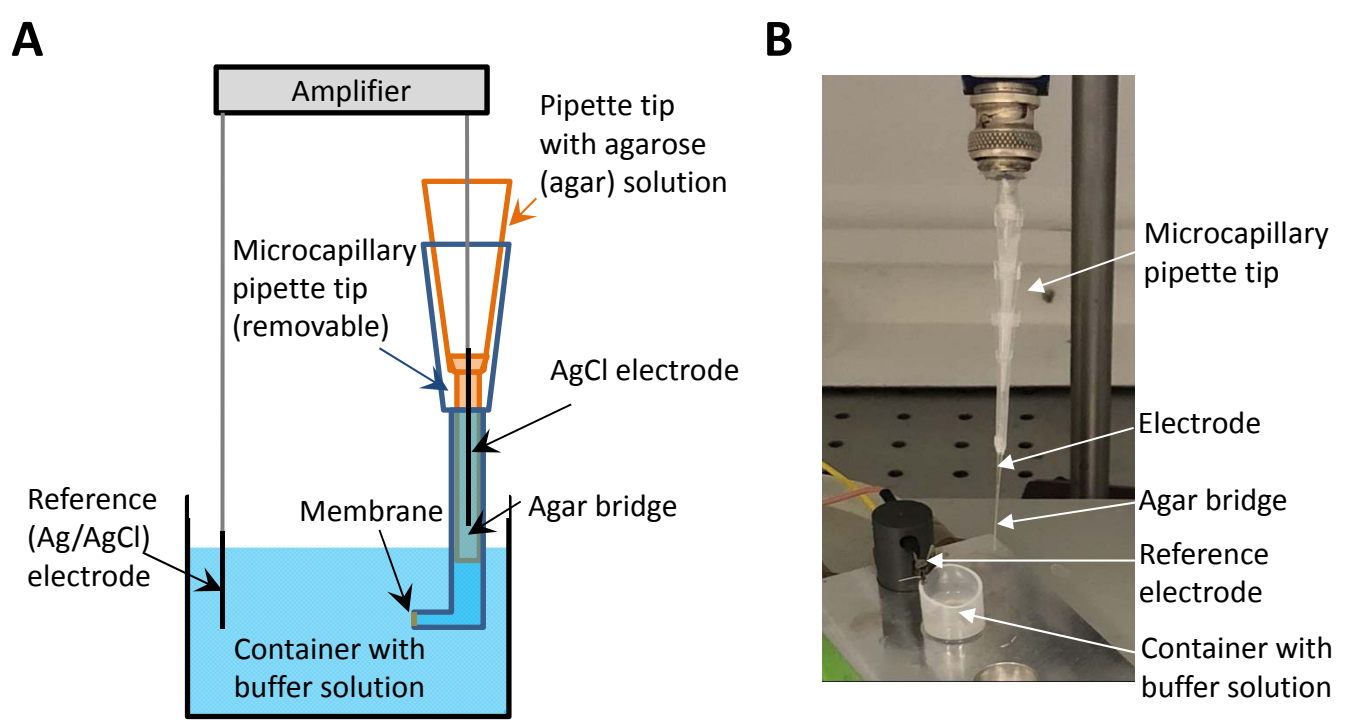
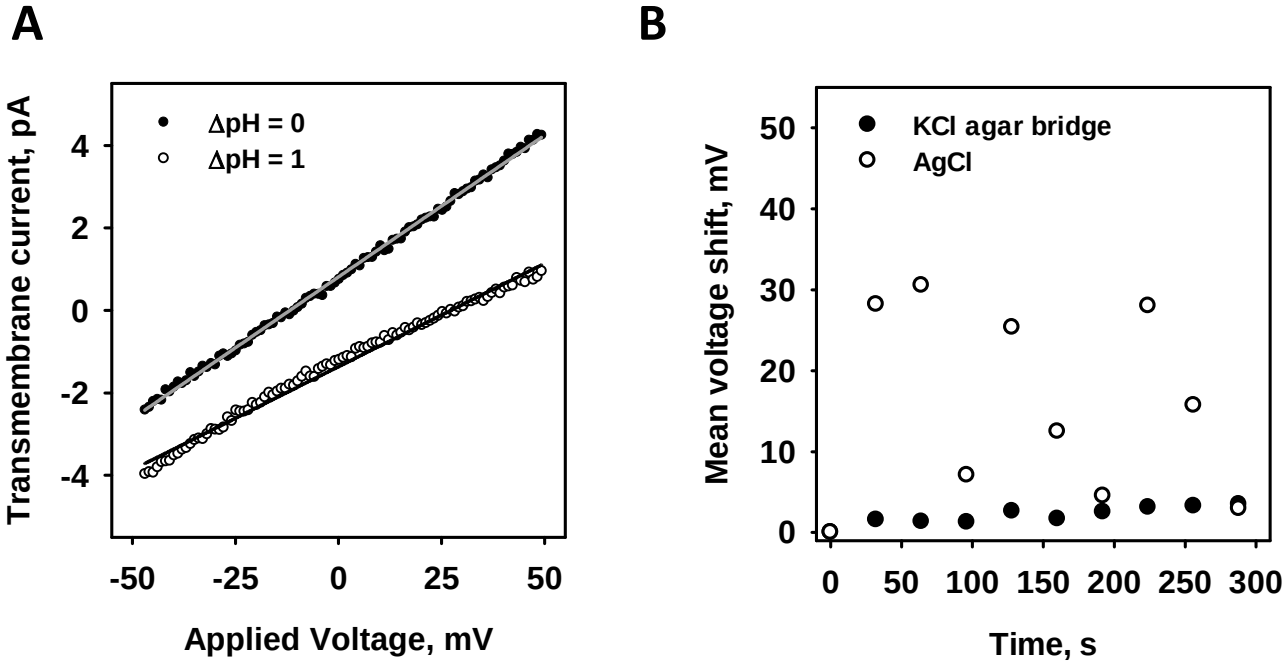

A
Amplifier
Pipette tip
with agarose
(agar) solution
Microcapillary
pipette tip
(removable)
AgCl electrode
Reference
(Ag/AgCl)
electrode
Membrane
Agar bridge
Container with
buffer solution
B
Microcapillary
pipette tip
Electrode
Agar bridge
Reference
electrode
Container with
buffer solution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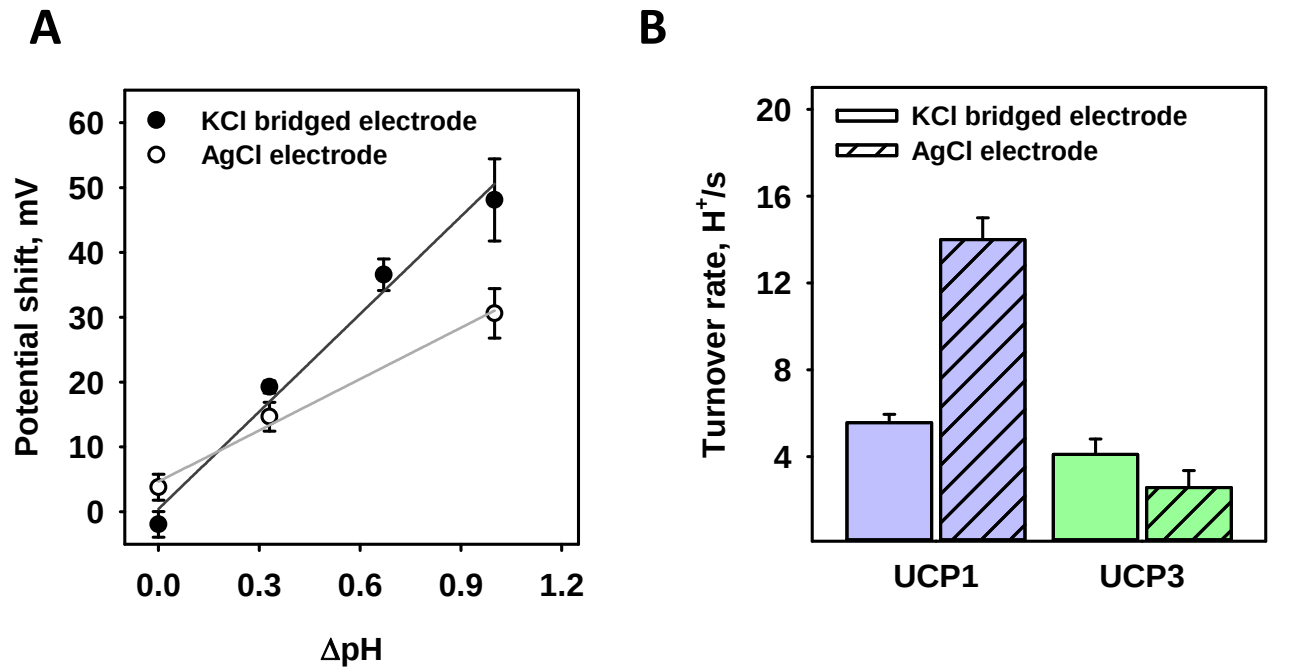
ΔpH = 0
ΔpH = 1
Transmembrane current, pA
4
2
0
-2
-4
-50
-25
0
25
50
Applied Voltage, mV
B
KCl agar bridge
AgCl
Mean voltage shift, mV
50
40
30
20
10
0
0
50
100
150
200
250
300
Time, s


A
KCl bridged electrode
AgCl electrode
Potential shift, mV
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50
40
30
20
10
0
0.0
0.3
0.6
0.9
1.2
ΔpH
B
KCl bridged electrode
AgCl electrode
Turnover rate, H+/s
20
16
12
8
4
UCP1
UCP3

Table of Materials

Click here to download Table of Materials JoVE_Materials_073118.xlsx

Name of Material/ Equipment	Company
Microloader tips	Eppendorf
Ethanol 99%	AustrAlco Österr. Agrar-Alkohol Handelsges.m.b.H
Kaliumchlorid	Carl Roth GmbH + Co. Kg
DC supply	Voltcraft
Agarose Standard	Carl Roth GmbH + Co. Kg
Patch Clamp Amplifier	Heka
Sample tube	Carl Roth GmbH + Co. Kg
Na2SO4	Carl Roth GmbH + Co. Kg
MES	Carl Roth GmbH + Co. Kg
TRIS	Carl Roth GmbH + Co. Kg
EGTA	Carl Roth GmbH + Co. Kg
Hexane	Sigma-Aldrich
Hexadecane	Sigma-Aldrich
Heating wire	Voltcraft

Catalog Number	Comments/Description
5242956.003	Microcapillary pipette tip
AAAH-5020-07025-230317	
6781.3	
V10/CPG 1940 -01	
3810.2	
5863.1	
8560.3	
4256.2	
AE15.2	
3054.1	
296090-100ML	
296317-100ML	
USPS-2250	

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Title of Article: A novel micro agar salt bridge electrode for analyzing proton turnover rate of recombinant membrane proteins.

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Response to Reviewers

We thank the reviewers for their helpful and constructive comments. Below we have addressed point by point all issues raised by the reviewers. The newly introduced/modified text is underlined in the resubmitted manuscript.

Reviewer #1:

The manuscript by Kreiter et al. deals with the elaboration of the electrophysiological technique for the reconstitution and functional characterization of the reconstituted carrier proteins. The results are sound, the manuscript is well written and quite interesting.

We thank the reviewer for the positive feedback.

1) I have however, several comments which can potentially improve the impact of the work. 1). I did not find the description of the reference electrode preparation in the manuscript. It seems that the authors did not use it. I think that it is necessary to test the effect of the agar bridge presence on the reference electrode on the measurements of reverse potentials. Since the micro agar salt bridge improved substantially the results of the measurements, the presence of the agar bridge on the reference electrode may also be helpful.

We thank the reviewer for addressing this point. Indeed, if substrates would be added directly to the buffer reservoir containing the reference electrode, the usage of the salt bridge would balance substrate-related diffusion potential. However, we used buffer solution which is chloride-free to exclude the putative possibility that uncoupling proteins transport chloride ions. For the adjusting of pH we used Tris or MES. The electrode potential in the absence of substantial concentration of chloride is primarily dependent on chloride impurities in the buffer solution. As its composition is unchanged during the experiments it will simply result in a constant offset potential. However, for a different situation where it is required to obtain an absolute potential difference between the two electrodes, a simple agar salt-bridge system (Ag/AgCl 3 M KCl) could also be used for the reference electrode.

We made a corresponding comment in the revised manuscript (underlined).

2). The manuscript would benefit if the authors describe in more details the background and the meaning of the equations which transform the measurable parameter, a reverse potential, into another parameter, i.e. proton turnover rate. For example, it is hard to understand the

relationship between the proton turnover rate and delta_T which is a time parameter of the triangular alternating voltage applied.

We thank the reviewer to mention this point. The more detailed deviation of the conversion of the measurable parameter, reverse potential, to the turnover number is described in Urbankova et al (2003) JBC and Beck et al (2007) FASEB. We adjusted the symbols to ΔT_{ramp} to indicate the time constant of the voltage ramp in step 3 in order to distinguish it from the time unit of the substrate turnover rate.

3). I suggest to run control measurements with a protonophore (i.e. CCCP), K+-carrier (i.e. nonactin), and H+/K+-channel (gramicidin) which can validate the approach.
We appreciate the suggestion of the reviewer. Our main goal in this study was to develop a salt bridge for the rapid and stable determination of the substrate turnover number and we therefore did not focus on reverse potential measurements and conversions to substrate turnover rates per se, as it was already established in Urbankova et al. 2003. Proposed experiments will substantially confirm the method, but are out of the scope for this work.

Reviewer #2:
This manuscript clearly demonstrates that using such a 'novel micro agar salt bridge electrode configuration' gives vastly superior results compared to the large inaccuracies that resulting from the use of a simple Ag/AgCl electrode as the test electrode for analysing proton turnover rate of membrane proteins reconstituted in the lipid bilayer membrane. The problem with the use of a simple Ag/AgCl electrode results from the fact that its electrical potential in a salt solution is highly dependent on the Cl- activity in the solution (e.g., Eqs. 1 & 2 in Raynauld & Laviolette, 1987 paper) and that in the absence of a significant Cl- concentration, the electrode potentials are poorly defined and unstable. This creates a major problem at the internal membrane interfacial solution in the absence of a significant Cl-, and where the solution composition is being altered. The problem is essentially overcome with the use of this novel 3M KCl micro-agar electrode, as described in this JoVE manuscript.
Thank you for the positive comment.

There are some relatively minor suggestions, comments and questions, which the authors should take note of:

1. With respect to the Ag/AgCl electrode in the large external Cl- -free buffer solution, there should also be some small comment indicating that while its electrode potential is poorly defined and primarily dependent on chloride impurities in that solution, as its composition is unchanged during the experiments, it will simply result in a constant offset potential, during an experiment. However, for a different situation where it was required to obtain an absolute potential difference between the two electrodes, a simpler Ag/AgCl 3 M KCl agar salt-bridge system could also be used for the reference electrode.

We appreciate the advice of the reviewer and introduced the following comment to the text of the article.

Since uncoupling proteins were suggested to transport chloride ions, chlorid- free buffer solution was used and the pH was adjusted using Tris or MES. The electrode potential in the absence of substantial concentration of chloride is primarily dependent on chloride impurities in the buffer solution. As its composition is unchanged during the experiments it will simply result in a constant offset potential. However, for a different situation where it is required to obtain an absolute potential difference between the two electrodes, a simple agar salt-bridge system (Ag/AgCl 3 M KCl) could also be used for the reference electrode.

2. With respect to the micro agar salt bridge on Line 77, I was surprised that the agarose concentration used was only 1%, as the more standard concentration in most salt-bridges 3-4% (w/v) agarose is used (Barry et al., 2013, Eur Biophys J 42-631-646; cp Kleene, 1993, who even used 5% agarose for his salt bridges) and I wondered if the salt-bridge in this JoVE manuscript is sufficiently gelled to retain the agar in the salt bridge for a reasonably long time.

We thank the reviewer for addressing this point. Indeed, the concentration of agarose in the salt bridge is lower than compared to the before mentioned references. Since our measurements are relatively short (less than 5 minutes) and we prepare new salt bridges for each new measurement, the usage of 1 % (w/v) agarose is enough to gel the salt bridge in this short period. Generally we suggest using agar in a concentration range of 1 – 5% (w/v), depending on the time scale of the

measurements and how often the salt bridge is reused. We also introduced the recommended citations.

3. I found the statement that the 'salt ions K+ and Cl- have equal mobility in liquid' (Lines 316f) was strictly inaccurate and misleading with respect to the role of 3 M KCl at such junctions. The relative mobilities of the Cl- and K+ ions uCl/uK = 1.04, and 3 M KCl microelectrodes or salt-bridges are useful for minimizing liquid junction potentials (LJPs), because of the high concentration of the 3 M KCl and because the mobilities of the Cl- and K+ ions are similar [u(Cl)/u(K) = 1.04] BUT NOT EQUAL , so that the 3 M KCl LJP contribution swamps the effects of the diffusion of the solution ions into the 3 M KCl agar salt bridge and will be relatively solution independent. If the Cl- and K+ mobilities had been equal, there would be no contribution from the 3 M KCl salt -bridge and the solution ions alone diffusing into the salt-bridge would by themselves contribute to the LJP at that junction, so that the LJP would be solution-dependent and could even be large.

We apologize for the inaccuracy regarding the mobilities of the ion species. The values are of course not equal but similar. We have corrected the phrases in the manuscript (underlined) to avoid incorrect statements.

4. Fig. 1a. With respect to the precise construction of the Microload pipette, it is difficult to see from Fig. 1A, the precise relationship between the chlorided part of the Ag wire and the part of it in contact with the 3 M KCl agar-Ag/AgCl region (compare Fig. 1 in Shao & Feldman). It could also help to replace 'Agar - Salt' by 'Agar - 3 M KCl' in Fig. 1A).

We have made changes to Fig. 1 A so that the contact side of the chloride part of the Ag wire and the 3 M KCl agarose solution is outlined more clearly.

5. Section 3.1 (Lines 191-195). It would be more accurate to define ΔT as "...and the duration of each ramp as ΔT, calculate…" especially, as 'time constant' tends to have a well-defined usage in electrophysiology and electricity. I also take it that Umax = 100 mV?

We have defined the parameters of the voltage ramp - the maximum voltage U_{max} (50 mV) and the time of the ramp ΔT_{ramp} - more clearly.