# Journal of Visualized Experiments

## Selection of transporter-targeted inhibitory nanobodies by SSM-based electrophysiology

--Manuscript Draft--

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|---|---|
| Article Type: | Methods Article - JoVE Produced Video |
| Manuscript Number: | JoVE62578R2 |
| Full Title: | Selection of transporter-targeted inhibitory nanobodies by SSM-based electrophysiology |
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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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1    **TITLE:**
2    Selection of Transporter-Targeted Inhibitory Nanobodies by Solid-Supported-Membrane
3    (SSM)-Based Electrophysiology
4
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17    **KEYWORDS:**
18    membrane transporter, membrane proteins, solid-supported-membranes electrophysiology,
19    nanobodies, inhibitory nanobodies, nanobodies selection
20

21    **SUMMARY:**
22    Nanobodies are important tools in structural biology and pose a great potential for the devel-
23    opment of therapies. However, the selection of nanobodies with inhibitory properties can be
24    challenging. Here we demonstrate the use of solid-supported-membrane (SSM)-based elec-
25    trophysiology for the classification of inhibitory and non-inhibitory nanobodies targeting elec-
26    trogenic membrane transporters.
27

28    **ABSTRACT:**
29    Single domain antibodies (nanobodies) have been extensively used in mechanistic and struc-
30    tural studies of proteins and they pose an enormous potential as tools for developing clinical
31    therapies, many of which depend on the inhibition of membrane proteins such as transport-
32    ers. However, most of the methods used to determine the inhibition of transport activity are
33    difficult to perform in high-throughput routines and depend on labeled substrates availability
34    thereby complicating the screening of large nanobody libraries. Solid-supported membrane
35    (SSM) electrophysiology is a high-throughput method, used for characterizing electrogenic
36    transporters and measuring their transport kinetics and inhibition. Here we show the imple-
37    mentation of SSM-based electrophysiology to select inhibitory and non-inhibitory nanobodies
38    targeting an electrogenic secondary transporter and to calculate nanobodies inhibitory con-
39    stants. This technique may be especially useful for selecting inhibitory nanobodies targeting
40    transporters for which labeled substrates are not available.
41

42    **INTRODUCTION**
43    Antibodies are composed of two identical heavy chains and two light chains that are respon-
44    sible for the antigen binding. Camelids have heavy-chain only antibodies that exhibit similar
45    affinity for their cognate antigen compared to conventional antibodies[1,2]. The single variable
46    domain (VHH) of heavy-chain only antibodies retain the full antigen-binding potential and has
47    been shown to be very stable[1,2]. These isolated VHH molecules or "nanobodies" have been

48 implemented in studies related to membrane proteins biochemistry as tools for stabilizing
49 conformations[3,4], as inhibitors[5,6], as stabilization agents[7], and as gadgets for the structure de-
50 termination[8-10]. Nanobodies can be generated by the immunization of camelids for the pre-
51 enrichment of B-cells that encode target-specific nanobodies and subsequent isolation of B
52 cells, followed by cloning of the nanobody library and selection by phage display[11-13]. An al-
53 ternate way to generate nanobodies is based on *in vitro* selection methods that rely on the
54 construction of libraries and selection by phage display, ribosome display, or yeast display[14,15-
55 20]. These *in vitro* methods require large library sizes but benefit from avoiding animal immun-
56 ization and favor the selection of nanobodies targeting proteins with relatively low stability.
57
58 The small size of nanobodies, their high stability and solubility, strong antigen affinity, low
59 immunogenicity, and relatively easy production, make them strong candidates for the devel-
60 opment of therapeutics[21,22,23]. In particular, nanobodies inhibiting the activity of multiple
61 membrane proteins are potential assets for clinical applications[5,24-26]. In the case of mem-
62 brane transporters, to evaluate whether a nanobody has inhibitory activity, it is necessary to
63 develop an assay that allows the detection of transported substrates and/or co-substrates.
64 Such assays usually involve labeled molecules or the design of substrate-specific detection
65 methods, which may lack a universal application. Furthermore, the identification of inhibitory
66 nanobodies generally requires the screening of large numbers of binders. Thus, a method that
67 can be used in a high-throughput mode and that does not rely on labeled substrates is essen-
68 tial for this selection.
69
70 SSM-based electrophysiology is an extremely sensitive, highly time-resolved technique that
71 allows the detection of movement of charges across membranes (e.g., ion bind-
72 ing/transport)[27,28]. This technique has been applied to characterize electrogenic transporters,
73 which are difficult to study using other electrophysiology techniques due to the relative low
74 turnover of these proteins[29-35]. SSM electrophysiology does not require the use of labeled
75 substrates, it is suitable for high-throughput screening, and either proteoliposomes or mem-
76 brane vesicles containing the transporter of interest can be used. Here, we demonstrate that
77 SSM-based electrophysiology can be used to classify transporter-targeted nanobodies with
78 inhibitory and non-inhibitory properties. As a proof-of-principle, we describe the reconstitu-
79 tion of a bacterial choline transporter into liposomes, followed by detail steps for immobili-
80 zation of proteoliposomes on the SSM sensors. We next describe how to perform SSM-based
81 electrophysiology measurements of choline transport and how to determine the half-maxi-
82 mal effective concentration ($EC_{50}$). We then show how to use SSM-based electrophysiology
83 to screen multiple nanobodies and to identify inhibitors of choline transport. Finally, we de-
84 scribe how to determine the half maximal inhibitory concentrations ($IC_{50}$) of selected inhibi-
85 tory nanobodies.
86
87 **PROTOCOL:**
88
89 **1.     Membrane protein reconstitution**
90
91 1.1. Mix 3 mL of *E. coli* polar lipids with 1 mL of phosphatidylcholine in a round bottom flask
92 under a ventilated hood.
93
94 1.2. Dry the lipid mixture for 20 min under vacuum using a rotary evaporator and a water

2

95   bath at 37 ˚C to remove chloroform. If needed, dry further under nitrogen or argon gas.
96
97   1.3. Using TS buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl) containing 2 mM β-mercaptoeth-
98   anol, resuspend lipids to 25 mg/mL.
99
100  1.4. Resuspend lipids in 500 µL aliquots, flash freeze in liquid nitrogen, and store at -80 °C.
101
102  1.5. Thaw one 500 µL aliquot of lipids and dilute 1:1 using TS buffer containing 2 mM β-mer-
103  captoethanol.
104
105  1.6. Extrude the lipid suspension 15 times using a 400 nm membrane.
106
107  1.7. Dilute the lipid suspension to have a final lipid concentration of 4.4 mg/mL.
108
109  1.8. Add n-dodecyl-β-D-maltoside (DDM) to have a final concentration of 0.2% and leave it
110  rotating for 1 h at 200 rpm at room temperature (RT).
111
112  1.9. Add the purified protein to the lipids using a lipid-to-protein ratio between 1:10 and
113  1:100 (w:w).
114
115  NOTE: The ratio needs to be adjusted depending on the strength of the signal detected in
116  SSM-electrophysiology measurements of substrate transport (see below). To obtain larger
117  signals, use smaller lipid-to-protein ratios.
118
119  1.10. Incubate the mixture rotating at 200 rpm for 1 h at RT.
120
121  1.11. Add 30 mg/mL of polystyrene adsorbent beads, pre-washed in TS buffer.
122
123  NOTE: Add polystyrene beads stepwise.
124
125  1.12. Incubate the beads-lipids mixture for 30 min at RT under slow stirring.
126
127  1.13. To remove the beads, let the beads-lipids mixture stand so that the beads settle down.
128  Transfer the solution to a new tube and leave the beads behind. Add 30 mg/mL of fresh pol-
129  ystyrene adsorbent beads to the separated lipid mixture.
130
131  1.14. Incubate the mixture for 1 h at 4 °C under slow stirring.
132
133  1.15. Separate the beads from the mixture as described in step 1.13 and add 30 mg/mL of
134  fresh polystyrene adsorbent beads.
135
136  1.16. Incubate the mixture for 16 h at 4 °C.
137
138  1.17. Separate the beads from the mixture as described in step 13 and add 30 mg/mL of fresh
139  polystyrene adsorbent beads.
140
141  1.18. Incubate the mixture for 2 h at 4 °C for a fourth and final wash.

142

1.19. Centrifuge at 110,000 x *g* for 30 min at 4 °C.

143

144

1.20. Wash the pellet with 500 µL of TS buffer containing 2 mM β-mercaptoethanol.

145

146

1.21. Centrifuge again at 110,000 x *g* for 30 min at 4 °C.

147

148

1.22. Resuspend the pellet to a final lipid concentration of 25 mg/mL in TS buffer with 2 mM β-mercaptoethanol.

149

150

151

1.23. Estimate the protein concentration using an in-gel or amido black assay[36].

152

153

1.24. Aliquot the proteoliposomes, flash freeze in liquid nitrogen, and store at -80 °C.

154

155

156 **2. Chip preparation**

157

158 2.1. Fill a single sensor chip with 50-100 µL of 0.5 mM 1-octadecanethiol solution (resuspended in isopropanol).

159

160

161 2.2. Incubate the chip with the solution for 30 min at RT.

162

163 2.3. Remove the thiol solution by tapping the chip on a tissue.

164

165 2.4. Rinse the sensor 3 times with 5 mL of pure isopropanol.

166

167 2.5. Rinse the sensor 3 times with 5 mL of double distilled water.

168

169 2.6. Dry the sensor by tapping on a tissue paper.

170

171 2.7. Apply 1.5 µL of 7.5 µg/µL 1,2-diphytanoyl-sn-glycero-3-phosphocholine (lipids dried in a rotatory evaporator and resuspended in n-decane).

172

173

174 2.8. Immediately after, fill the sensor with 50 µL of non-activating SSM buffer, which does not contain the substrate. This will lead to a spontaneous formation of the SSM layer.

175

176

177 NOTE: The SSM buffer should be optimized beforehand to determine optimal conditions that have low background noise. A general buffer containing 30 mM HEPES pH 7.4, 5 mM MgCl$_2$, 140 mM NaCl, can be used as a starting point. The SSM buffer without the substrate is used for washing before and after the measurement (non-activating buffer). To avoid buffer mismatch, use the non-activating buffer to prepare the buffer containing the substrate (activating buffer). The substrate can be added either directly as a powder or in a small volume from a high concentration stock to avoid dilutions.

178

179

180

181

182

183

184

185 2.9. Thaw proteoliposomes from step 1.24 at RT.

186

187 2.10. Dilute proteoliposomes between 1:5 and 1:100 (proteoliposomes:buffer, (v:v)) in the non-activating SSM buffer (here 1:20).

188

189

190   2.11. Sonicate proteoliposomes for 20-30 s or 3 times for 10 s, placing on ice in between son-
191   ication, if necessary. Here a water bath sonicator at 45 kHz was used.

192

193   2.12. Apply 5-10 µL of the diluted sonicated proteoliposomes sample on the surface of the
194   sensor without touching it.

195

196   2.13. Centrifuge the chips with the solution at RT for 30 min using a speed between 2,000 and
197   3,000 x *g*.

198

199   NOTE: Use 50 mL tubes with a flat bottom. Carefully place the sensor chips upright using
200   tweezers. 6-well plates and a centrifuge with a plate holder can also be used.

201

202   2.14. Use the sensor chips on the same day.

203

204   **3. Measuring the solute transportation: determination of saturation conditions**

205

206   NOTE: As proof-of-principle, these experiments were performed using a bacterial choline
207   transporter reconstituted in liposomes following the protocol described above. The step-by-
208   step process of determining saturating conditions of the substrate choline prior to the meas-
209   urement of inhibition by nanobodies is shown here.

210

211   3.1. Prepare 1-2 L of the non-activating SSM buffer.

212

213   NOTE: Prepare and use the same SSM buffer stock for all activating and non-activating buffers
214   throughout all measurements.

215

216   3.2. Take 10 clean tubes and transfer 10 mL of the non-activating SSM buffer into each.

217

218   3.3. Add the substrate into the tubes from step 3.2 using a series of concentrations around
219   the expected half maximum concentration (here 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001
220   mM of choline) to prepare the activating SSM buffers. Use a high concentration stock to avoid
221   dilutions.

222

223   3.4. Switch on the SSM machine.

224

225   3.5. Start the SSM software and let the machine initialize automatically. Set the saving path
226   for data and confirm by hitting the **OK** button. Select the standard **Initial Cleaning Protocol**
227   in the **workflow** options and click **Run**.

228

229   3.6. Mount the proteoliposome coated chip on the socket, move the arm to lock the chip, and
230   close the mounted chip with the cap.

231

232   3.7. Select the program **CapCom** in the workflow and let it **Run** to determine the conductivity
233   and capacitance. Confirm that the conductivity is below 5 nS and the capacitance is between
234   15 and 35 nF before using it for the measurement.

235

NOTE: A capacitance value of 15-35 nF and conductance below 5 nS are recommended by the manufacturer when using a 3 mm chip.

3.8. Transfer the activating solutions into vials and position the buffers in the probe sampler.

3.9. Transfer the non-activating buffer into a reservoir and position it next to the chip holder at the reservoir position on the right.

3.10. Create a protocol for the workflow using a sequence of non-activating (B), activating (A), and non-activating (B) solutions (B-A-B sequence) and a loop that performs three measurements and moves to the next activating buffer for all 10 buffers prepared in step 3.3. Use the default flow rate at 200 µL/s using 1 s – 1 s – 1 s flow times for the B-A-B sequence. Click **Play** to start the measurement.

NOTE: A typical experiment consists of the sequential flow of non-activating (B), activating (A), and non-activating (B) solutions (written as B-A-B; see **Figure 1A**). The immobilized proteoliposomes on the sensor will be washed with the solutions. Therefore, the B-A solution exchange generates a substrate concentration gradient, which drives the electrogenic transport reaction.

3.11. Save the protocol and let the workflow run by clicking on the **Play** button. Perform the same type of experiment but using protein-free liposomes. This is highly important as it would show the intensity of background currents. This should be considered when analyzing data of electrogenic transport measured with proteoliposomes (**Figure 1C**).

3.12. Use any preferred software for data analysis to plot the measured current versus time. Read out the peak current manually, or if using the software, use the function for peak height estimation in the range of the addition of the activating buffer.

3.13. Plot the peak current against the substrate concentration to determine the $EC_{50}$ of the substrate via the nonlinear regression (**Figure 1B,C**). Read out the lowest concentration at which the peak current reaches a maximum value, this concentration corresponds to saturating conditions.

NOTE: It is important to consider that the number of proteoliposomes that remain immobilized vary from chip-to-chip. This variation is evident as the peak currents at identical measurement conditions will show different heights. Therefore, it is necessary to normalize the current amplitudes of measurements performed on each chip separately before comparing measurements among different chip preparations.

**4. Serial classification of inhibitory and non-inhibitory nanobodies**

NOTE: This section shows how to measure choline transport in the presence of nanobodies that bind specifically to the bacterial choline transporter. Smaller peak currents in the presence of nanobodies indicate transport inhibition. Non-inhibitory nanobodies will not impact substrate transport, i.e., no decrease of the peak current signal.

283    4.1. Prepare 1-2 L of non-activating SSM buffer.

284

285    4.2. Transfer 50 mL of the non-activating SSM buffer into a clean tube. Add the substrate
286    choline to a final concentration of 5 mM (saturating conditions). Use this for a positive control
287    measurement.

288

289    4.3. Transfer 10 mL of the non-activating SSM buffer into a clean tube. Add the substrate
290    choline to a final concentration of 5 mM (saturating conditions) and add nanobody to a final
291    concentration of 500 nM.

292

293    4.4. Repeat step 4.3 for each nanobody to prepare the activating solutions.

294

295    NOTE: If the purified nanobodies are resuspended in a different buffer than the SSM buffer,
296    their addition to the activating and non-activating buffers will lead to a buffer mismatch. A
297    buffer mismatch should be avoided since it can lead to high noise. Exchanging the buffer of
298    the purified nanobodies with the SSM buffer can help to avoid this issue. Furthermore, using
299    nanobody concentrations that allow to reach saturating conditions is recommended. Consid-
300    ering that the binding constants of nanobodies are generally below 100 nM, a nanobody con-
301    centration of 500 nM is recommended for this experiment. However, it is important to pre-
302    screen for optimal concentrations.

303

304    4.5. Start the SSM machine and measure the capacitance and conductivity of the proteolipo-
305    some coated chip as described in steps 3.4-3.7.

306

307    4.6. Transfer the activating solution without a nanobody into a vial and place the buffer in the
308    probe sampler. Transfer the non-activating buffer without a nanobody into a reservoir and
309    position it in the probe sampler.

310

311    4.7. Transfer the activating solutions containing nanobodies into vials and position the buffers
312    in the probe sampler. Transfer the non-activating buffers containing nanobodies into vials
313    and position the buffers in the probe sampler.

314

315    4.8. Create a protocol for the workflow using a sequence of non-activating (B), activating (A),
316    and non-activating (B) solutions (B-A-B sequence).

317

318    4.9. Create a loop that performs the following: three measurements of the B-A-B sequence
319    using buffers without nanobody, two measurements of the B-A-B sequence with buffers con-
320    taining a nanobody, 120 s delay time for incubation with the nanobody, then 3 measurements
321    of the B-A-B sequence with buffers containing the nanobody.

322

323    4.10. Save the workflow and let it run by clicking the **Play** button.

324

325    NOTE: This workflow will measure the initial conditions of the transport without inhibition by
326    running the B-A-B protocol 3 times using non-activating (B) and activating buffers (A) without
327    the nanobody (**Figure 1B,C**), followed by the measurement of the nanobody effect on the
328    transport by running the B-A-B protocol 5 times with the non-activating and activating buffers
329    containing nanobodies (**Figure 2A**). The second order binding kinetics dictate the interaction

of nanobodies and their target proteins. Therefore, it is important to use a time delay in the B-A step in order to give enough time for nanobodies to bind to transporters in proteoliposomes on the chip. Optimal times depend on the nanobody concentration i.e., at lower concentrations longer times are required. The first two measurements are required to adapt the system to the new conditions and the second run should be performed after a delay time of 120 s. Only the following three measurements should be used for data analysis.

4.11. Create a new protocol for the workflow using a sequence of non-activating (B), activating (A), and non-activating (B) solutions (B-A-B sequence) and a loop of 5 measurements to wash out the reversibly bound nanobody.

NOTE: Optionally, include an incubation step to allow dissociation of nanobodies with slow kinetics.

4.12. Save the workflow and let it run by clicking the **Play** button.

4.13. Compare the last peak current of the measurements with the initial substrate-only measurement in step 4.10. The nanobody has been successfully washed out and the initial conditions have been reestablished if the peak current reaches the initial value, otherwise repeat steps 4.12-4.13 or change to a new chip.

4.14. Repeat steps 4.6-4.13 and use individual chips for each nanobody screen (**Figure 2C**) or repeat with multiple nanobodies using the same chip (**Figure 2D**).

4.15. Use any preferred software for data analysis to plot the measured current versus time. Read out the peak current manually, or if available, in the used software, automatically select the function for peak height estimation in the range of the addition of the activation buffer.

4.16. Normalize the peak current in presence of the nanobody, based on the preceding substrate-only measurement. Plot the peak currents in a histogram and compare the peak currents of the substrate only measurements to the peak currents measured in the presence of nanobodies (**Figure 2C,D**) to identify inhibitory nanobodies.

NOTE: Normalization of the determined peak currents of each individual run with a nanobody should be performed considering the peak current in the absence of the nanobody from the preceding measurement. Also, since the number of proteoliposomes that remain immobilized vary from chip-to-chip, it is important to normalize the current amplitudes of measurements performed on each chip separately before comparing measurements among different chip preparations.

## 5. IC$_{50}$ measurement with inhibitory nanobodies

NOTE: After identifying inhibitory nanobodies, it is possible to determine their half maximal inhibitory concentration (IC$_{50}$). This is done by measuring the transport of choline at constant concentration, while varying concentrations of the inhibitory nanobody.

5.1. Prepare 1-2 L of the non-activating SSM buffer.

377

5.2. Transfer 50 mL of the non-activating SSM buffer into a clean tube. Add the substrate choline to a final concentration of 5 mM (saturating conditions). Used this as the activating solution for positive control.

5.3. Take 8 clean tubes and add 5 mL of the non-activating solution into each. Add the substrate choline to a final concentration of 5 mM (saturating conditions). Add the inhibitory nanobody to the tubes at concentrations in the expected $IC_{50}$ range (here 500 nM – 1 nM).

5.4. Take 8 clean tubes and add 10 mL of the non-activating solution into each. Add inhibitory nanobody to each tube individually at the same concentration as in step 5.3. This corresponds to the non-activating buffer.

NOTE: This will generate a series of activating and non-activating buffer pairs at different concentrations of the same inhibitory nanobody.

5.5. Start the SSM setup and measure the capacitance and conductivity of the proteoliposome coated chip as described in steps 3.4-3.7.

5.6. Transfer the activating solution without nanobody into a vial and place it in the probe sampler. Transfer the non-activating buffer without nanobody into a reservoir and position it at the reservoir position next to the chip holder on the right.

5.7. Transfer the activating solutions containing nanobodies into vials and position the buffers in the probe sampler. Transfer the non-activating buffers containing nanobodies into vials and position the buffers in the probe sampler.

5.8. Create a protocol for the workflow using a sequence of non-activating (B), activating (A), and non-activating (B) solutions (B-A-B sequence). Include a loop to measure each concentration 2 times, incubate for 120 s and measure 3 more times. The workflow will start with the positive control of substrate-only followed by the lowest concentration of the nanobody. Each nanobody measurement will be followed by a subsequent measurement of the positive control with substrate-only to restore the initial peak amplitude, before moving to the next higher nanobody concentration.

NOTE: The second order kinetics dictate the binding of nanobodies. Therefore, it is important to use a time delay in the B-A step. Optimal times depend on the nanobody concentration i.e., at lower concentrations longer times are required; here 120 s was used with satisfactory results.

5.9. Use any preferred software for data analysis to plot the measured current versus time. Read out the peak current manually, or if using software, select the function for the peak height estimation in the range of the addition of the activation buffer (**Figure 3A**). Plot the peak currents against the nanobody concentration to determine the $IC_{50}$ via non-linear regression (**Figure 3B**).

NOTE: Normalize the current amplitudes of measurements performed for each individual chip

9

424 before comparing measurements among different chip preparations.

## 6. Cleaning of sensors

6.1. Rinse the single sensor chips after use with 10 mL of distilled water.

6.2. Dry the chip by tapping it on a tissue paper.

6.3. Fill the sensor cavity of the chip with 100 µL of pure isopropanol and incubate for 10 min at RT.

6.4. Place a cotton swab in pure isopropanol and incubate for 1-3 min.

6.5. Use the presoaked cotton swabs and gently rotate on the sensor's surface without pressure to remove residues.

6.6. Rinse the sensor with 5 mL of pure isopropanol.

6.7. Rinse the sensor with 10 mL of distilled water.

6.8. Dry the sensor by tapping the chip on a tissue.

6.9. Let the sensor dry overnight at RT, and store at RT under dry conditions.

NOTE: Sensors can be re-used up to 4-5 times when cleaned and stored properly.

**REPRESENTATIVE RESULTS**
SSM-based electrophysiology has been extensively used for the characterization of electrogenic transporters. In the protocol presented here, we show how to use SSM-based electrophysiology to classify nanobodies targeting a secondary transporter (here a bacterial choline symporter) based on their inhibitory and non-inhibitory properties. One of the most useful features of this technique is that it allows for the high-throughput screening of multiple buffer conditions. This particular characteristic is beneficial for the analysis of nanobody libraries, which after the selection of binders can be constituted from a few to dozens of nanobodies. In a standard experiment, a stable lipid monolayer is assembled on a sensor chip. After applying the proteoliposomes preparation containing the choline transporter, a check for the good conductivity and capacitance is performed as this is essential for the success of the experiment. In case that the integrity of the membrane is compromised during an experiment, which is easily observed due to the high noise background currents, changing to a new chip is recommended as recovering low noise conditions is rather difficult. In general, we have observed very good reproducibility among measurements of transport and inhibition by nanobodies when using different chips.

To decide about the substrate concentration to be used during a screening of nanobodies, electrogenic transport was first measured under different substrate concentrations to determine $EC_{50}$ (**Figure 1B,C**). A substrate concentration that corresponds to saturating conditions

470 was selected (**Figure 1C**). This substrate concentration was then kept constant in all activating
471 buffers. For this particular example, we selected 5 mM choline.
472
473 For the screening of nanobodies, the nanobody must be added to both non-activating and
474 activating buffers. When nanobodies were added to only the activating buffer, it was not pos-
475 sible to observe the inhibition of the electrogenic transport. We speculate that this is due to
476 an incomplete occupation of all nanobody binding sites in the transporter population on the
477 chip, thereby revealing the importance of pre-incubation with nanobodies in non-activating
478 conditions. To ensure that all sites are likely to be occupied, a time delay step was included
479 during the application of the first non-activating buffer step to allow the saturation of nano-
480 body binding sites on the transporter population. Incubation times ranging from 2-60 min
481 have been tested with reproducible results. Keep in mind that optimal times of incubation
482 depend on the nature of the nanobody binder and its concentration during the experiment
483 (as well as the concentration of transporter in proteoliposomes on the chip). Therefore, it is
484 recommended to try different incubation times. In any case, as a rule of thumb, the lower the
485 nanobody concentration, the longer the incubation time required. We tested incubation
486 times of 2 min, 20 min, 30 min, and 60 min for different nanobodies but did not detect further
487 transport inhibition.
488
489 The effect of inhibitory nanobodies on electrogenic transport is visualized from the decrease
490 of peak currents amplitudes (**Figure 2A,C,D**). Non-inhibitory nanobodies, on the other hand,
491 do not affect peak currents. After running the washing protocol to allow nanobodies unbind-
492 ing, a recovery of 80 to 95% of the initial peak current amplitude was observed (**Figure
493 2A,C,D**). We have performed a similar experiment but in the presence of liposomes without
494 the transporter protein. When changing from non-activating to activating conditions, no sig-
495 nificant artifact currents was introduced by nanobodies present in these buffers (**Figure 2B**).
496 Running this control experiment is recommended as it is important to know whether changes
497 in peak currents arise from artifacts or not.
498
499 After the selection of nanobodies with inhibitory properties, we determined $IC_{50}$ values for
500 individual nanobodies (**Figure 3A,B**). For this particular experiment, it is recommended to
501 start with a low concentration of nanobodies and then move towards the high concentration
502 during the assay. The calculation of the inhibition for each concentration was then performed
503 by comparing peak currents measured before and after the application of nanobody. To avoid
504 unspecific binding of nanobodies to surfaces, which can be particularly problematic when us-
505 ing low nanobody concentrations, it is advised to follow a similar protocol to that described
506 by Kermani et al.[37], where 50 µg/mL of bovine serum albumin was added to the buffers, pre-
507 venting this deleterious effect. Adding detergents such as Tween or Triton for this purpose
508 should be avoided as these would dissolve lipid membranes.
509
510 **FIGURES AND TABLES LEGENDS**
511
512 **Figure 1: SSM-based electrophysiology. (A)** Protocol for transient currents measurement. A
513 non-activating solution is replaced by an activating solution followed by the flow of non-acti-
514 vating solution to restore initial conditions. During the first step, nanobodies bind to the trans-
515 porter. When switching to the activating solution, the substrate gradient drives the electro-
516 genic transport (orange curve). In the presence of an inhibitory nanobody, the peak current

shows a smaller amplitude (blue curve). After finishing the protocol and running solutions without nanobody (wash), unbinding of nanobodies occurs. In the schematic, proteoliposomes with reconstituted protein (blue) are immobilized on the SSM sensor. Triangles and red circles represent nanobodies and substrate, respectively. (**B**) Electrogenic choline transport in the absence of nanobodies. Peak currents measured during activating conditions are shown for different substrate concentrations. (**C**) Representative measurement of currents during activating conditions in the absence of transporter protein at different substrate concentrations. (**D**) Plot of substrate concentration versus peak currents amplitude. The $EC_{50}$ determined was 95 ± 11 µM choline. Error bars indicate standard deviation (n=3 biological replicates, n=3 technical replicates).

**Figure 2: Screening and classification of inhibitory and non-inhibitory nanobodies.** (**A**) Electrogenic choline transport in the presence of a nanobody. Peak currents measured during activating conditions are shown in the absence of nanobody (blue), in the presence of an inhibitory nanobody (red), and after nanobody unbinding (green). (**B**) Measurement of currents during activating conditions in the absence of transporter protein. Traces show recordings in the absence of nanobody (blue), in the presence of an inhibitory nanobody (green), and in the presence of a non-inhibitory nanobody (red). (**C,D**). Histograms showing peak currents measured during activating conditions in the presence of nanobodies and after nanobody unbinding (recovery). Panel **C** shows the results of measurements using individual chips per nanobody. Panel **D** shows the results from a serial measurement using one chip. Nanobodies are indicated as Nb. Error bars indicate the standard deviation (n=3 biological replicates, n=2 technical replicates).

**Figure 3: Determination of $IC_{50}$ of an inhibitory nanobody.** (**A**) Electrogenic choline transport and inhibition by a nanobody. Peak currents measured during activating conditions are shown for different nanobody concentrations. (**B**) Plot of peak currents amplitude vs nanobody concentration from a serial measurement with an inhibitory nanobody. The $IC_{50}$ determined was 18 ± 2 nM. Error bars indicate the standard deviation (n=3 biological replicates, n=3 technical replicates).

**DISCUSSION:**
The technique presented here classifies nanobodies with inhibitory and non-inhibitory properties targeting electrogenic transporters. Assessing the substrate transport is possible due to the detection of the movement of charges through the transporter embedded in the membrane of proteoliposomes. Some of the critical steps during the setup of an experiment are reconstitution of active protein in liposomes, preparation of stable monolayers on SSM chips, and recovering of initial conditions after the application of the wash protocol to remove bound nanobody molecules. Once the membrane protein is reconstituted at an appropriate lipid-to-protein ratio, a general SSM protocol can be established using the native substrate. It is crucial to perform control experiments using protein-free liposomes to reveal noise currents that would need to be subtracted from the currents measured using proteoliposomes. However, if noise currents are too large, we recommend trying protein reconstitution using different lipids, or screen for buffer conditions that minimize these deleterious signals. After successfully establishing conditions for an SSM assay, screening of nanobodies can be performed. A very useful option that may help in faster screening of nanobodies is to perform high-throughput assays using a single SSM sensor chip. This reduces the time of manipulation

564  of chips and buffers and reduces the costs. However, because during this type of assay mul-
565  tiple nanobodies are applied sequentially, it is important to ensure that the applied nanobody
566  can be washed away after the measurement. A stringent washing cycle may need to be im-
567  plemented to unbind some nanobodies in case that reduced peak current amplitudes are de-
568  tected in the absence of nanobody. We recommend using, as the starting point, the washing
569  conditions described here. If increasing the washing volume or the number of cycles does not
570  help, individual chips would need to be used to screen each nanobody separately. In all the
571  cases examined here, the binding of nanobodies was reversible and a high-throughput proto-
572  col could be applied. In our experimental setup, we could not recover the full amplitude of
573  the initial peak current after measurements with nanobodies (**Figure 1A; Figure 2C,D**). How-
574  ever, in most cases, the magnitude of the peak currents recovered ranged between 80 and
575  95% of the amplitude measured before applying nanobodies (**Fig. 2C,D**). We speculate that
576  this could be a consequence of washing away a fraction of the proteoliposomes adhered to
577  the chip, or due to slow kinetics of unbinding of some inhibitory nanobodies, or a combination
578  of both. In either case, it was still possible to continue assaying further nanobodies as elec-
579  trogenic transport was measurable. This is shown in **Figure 2D**, where we screened six nano-
580  bodies using a single chip preparation.

582  The high-throughput characteristic is one of the most significant advances of the method pre-
583  sented. In addition, in contrast to other approaches, this method allows for the selection of
584  inhibitory nanobodies targeting electrogenic transporters for which labeled substrates are
585  not available. A fast identification of inhibitory nanobodies can help to speed up the research
586  aiming to identify novel applications of nanobodies as drugs. Their apparent advantages com-
587  pared to similar therapies such as antibody treatments are numerous, starting with a smaller
588  size which helps them propagate further into tissues or cells, to their low production costs
589  and high stability.

591  SSM-based electrophysiology has been used in the past for the characterization of electro-
592  genic transporters in membrane vesicles[29,38]. These types of experiments are advantageous
593  as they do not depend on the protein purification and reconstitution protocols. We speculate
594  that performing the selection of inhibitory nanobodies using membrane vesicles is feasible.
595  This would help to reduce costs and avoid manipulations of purified proteins.

597  SSM-based electrophysiology is a strong technique to screen for nanobody inhibitors of mul-
598  tiple membrane proteins that exhibit electrogenic transport. We envision that SSM-based
599  electrophysiology will become an important tool for the selection of inhibitory nanobodies
600  and other antibodies with potential clinical applications.

610  **DISCLOSURES**

The authors declare no competing financial interests.

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Figure_1

Figure 2

Figure_3

**A**

**B**
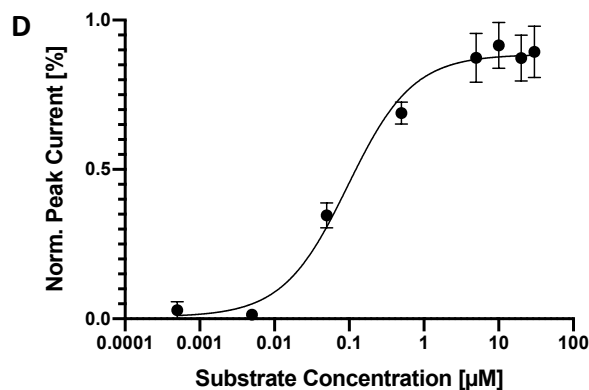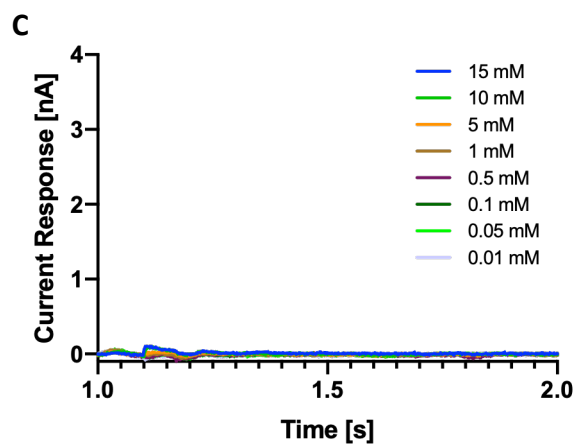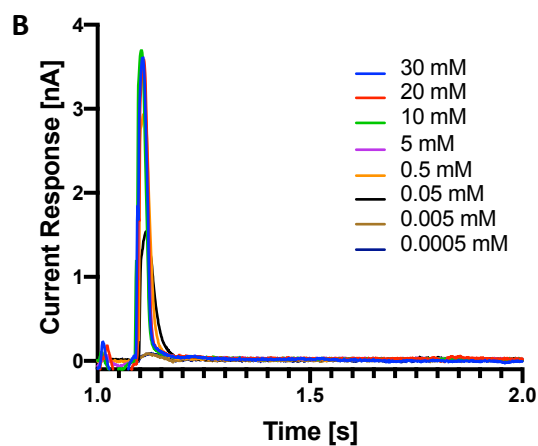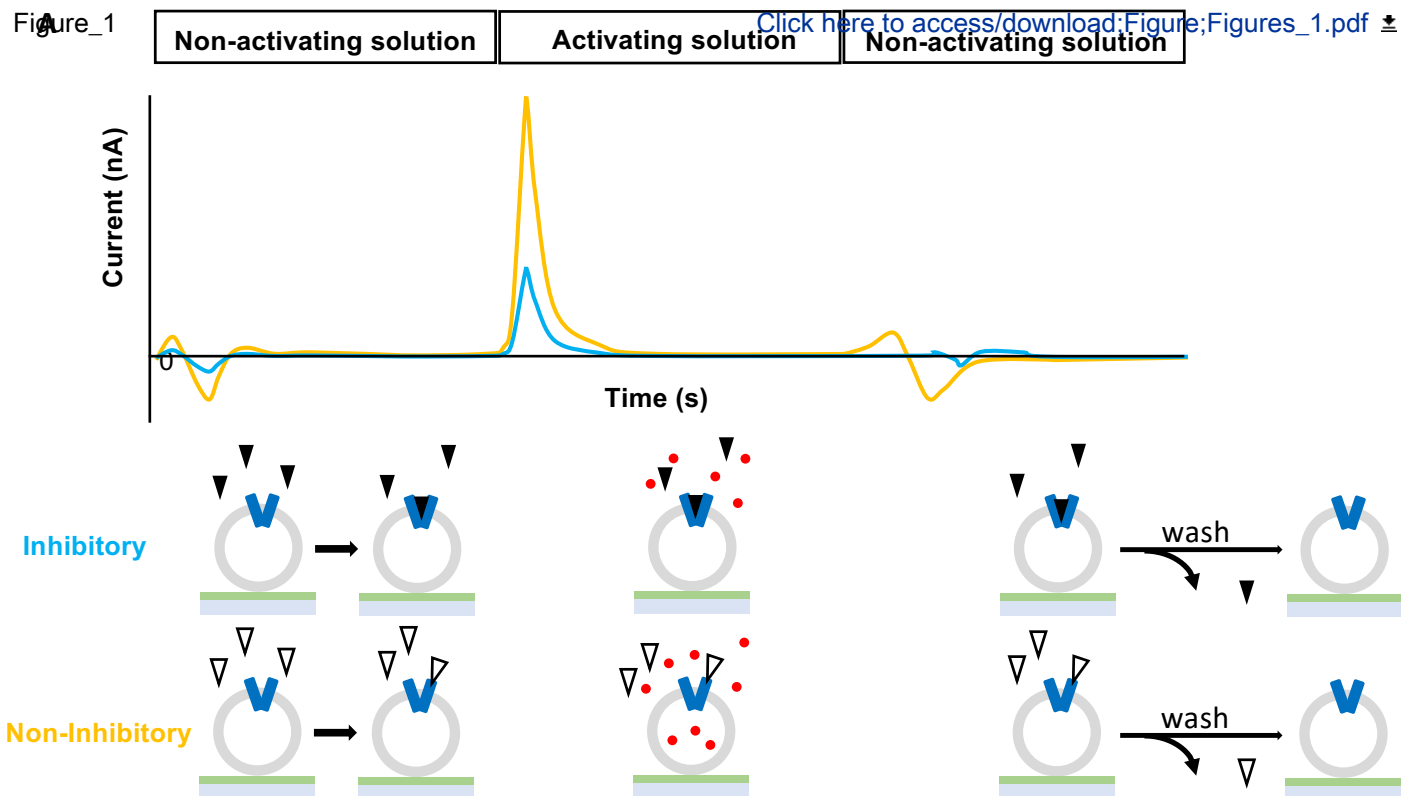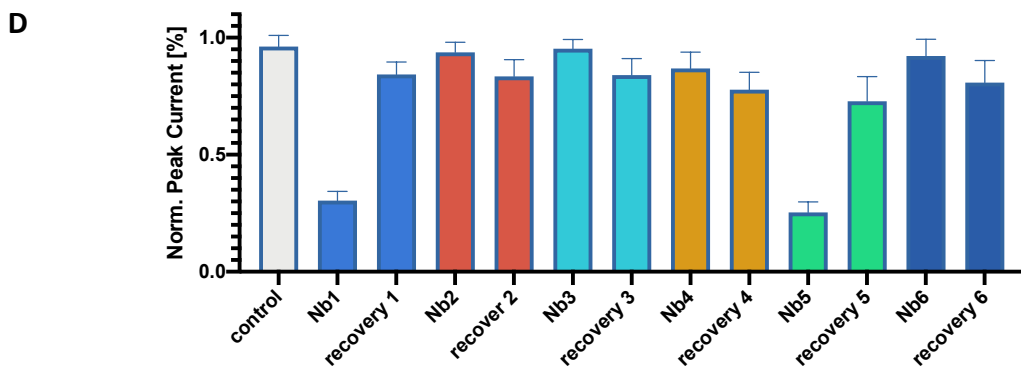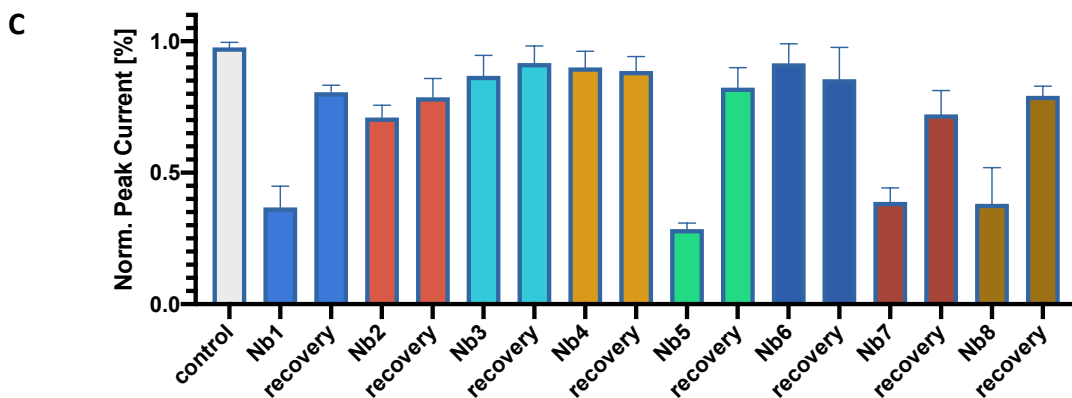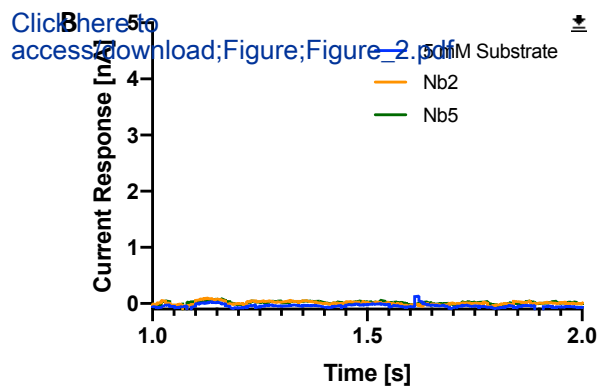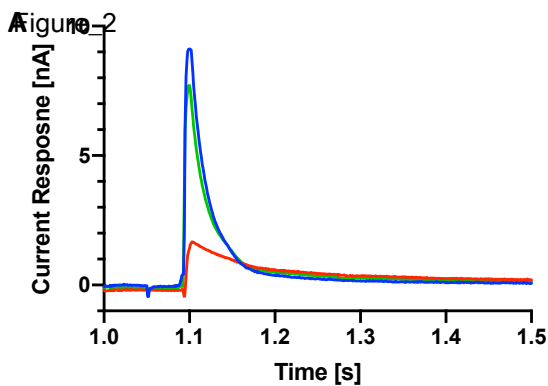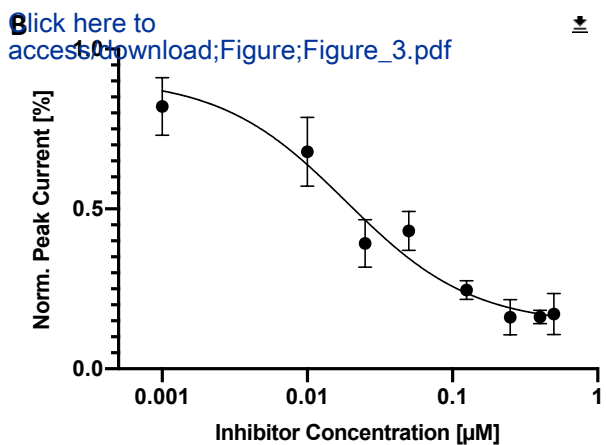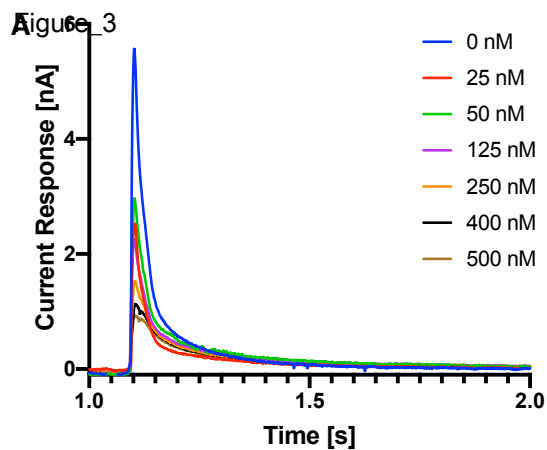
| Name of Material/Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 1-octadecanethiol solution | Sigma Aldrich | O1858-25ML | |
| 1,2-diphytanoyl-sn-glycero-3-phosphocholine | Avanti Polar Lipids | 850356C-25mg | |
| Bio-Beads SM-2 Adsorbent (Polystyrene adsorbent beads) | BioRad | #152-3920 | |
| PD 10 Desalting Columns | GE Healthcare | GE17-0851-01 | |
| Filter 200 nm membrane | Whatman Nucleopore | WHA800282 | |
| 2-Propanol | Merck | 33539-1L-R | |
| n-Decane | Sigma Aldrich | 8034051000 | |
| n-dodecyl-ß-D-maltoside (DDM) | Avanti Polar Lipids | 850520P-25g | |
| Sodium Chloride | AppliChem | 131659.1211 | |
| (SSM setup) SURFE2R N1 | Nanion | ----- | |
| SURFE2R N1 Single Sensor Chips | Nanion | # 161001 | |
| Trizma Base | Sigma Aldrich | T1503 | |
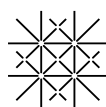
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**Manuscript Title:**
Selection of transporter-targeted inhibitory nanobodies by SSM-based electrophysiology
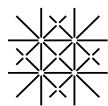
JoVE62578

Dear Dr. Amit Krishnan,
Review Editor
JoVE

Thank you for your Email of March 9th, 2021, in which you invited us to revise our manuscript. We appreciate the insightful comments of the reviewers. We have responded to all the comments made by the editor and reviewers. The manuscript file contains track changes of the edits and a point by point response is found here below. We hope you find our manuscript acceptable after these modifications. Please do not hesitate to contact me in case of further comments or questions.

I look forward to hearing from you and I thank you for your efforts and consideration.


Yours sincerely,


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## Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
3. Please define all abbreviations before use (TS, DDM, etc.)
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Bio-Beads, SURFE2R N1, Nanion, etc.
5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
6. Line 70: Please elaborate the process of drying lipids using Rotary evaporator. Please include specific requirements to perform this drying technique.
7. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Line 78, 80, 86,118, 201, etc.)
8. For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2 (Line 81, 83, 85, 90, etc.)
9. Line 91: Please mention the conditions for centrifugation. Are they the same as in line 89?
10. Line 103/104: Please mention if there is any specific volume required for the solution used.
11. Line 114: Please mention if there is any specific frequency used for sonication.
12. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
13. As we are a methods journal, please revise the Discussion to include modifications/ troubleshooting steps of the technique and limitations of the protocol.
14. Line 278-307: Please move the Figure Legends section to the end of the Representative Results section.
15. Please do not use the &-sign or the word "and" when listing authors. Please title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Thanks for pointing this out. All these comments were addressed

## Reviewer #1:

*Major Concerns:*

Introduction is well written. I just note that there 2 impactful papers that have revised an old
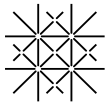
transport system using SSME and lead to ground-breaking results. These papers are cited in the Nanion website, and in my opinion should be cited in the introduction.

1 - Williamson G, Tamburrino G, Bizior A, Boeckstaens M, Dias Mirandela G, Bage MG, Pisliakov A, Ives CM, Terras E, Hoskisson PA, Marini AM, Zachariae U, Javelle A. A two-lane mechanism for selective biological ammonium transport. Elife. 2020 9:e57183. doi: 10.7554/eLife.57183

This paper should be cited after "This technique has been applied (…) turnover of these proteins".

2 - Mirandela GD, Tamburrino G, Hoskisson PA, Zachariae U, Javelle A. The lipid environment determines the activity of the Escherichia coli ammonium transporter AmtB. FASEB J. 2019 33:1989-1999. doi: 10.1096/fj.201800782R.

This paper should be cited as it presents a method to measure the effect of lipids on transport system, which have never been done before. It goes shows how to use SSME to measure external parameters on transport systems.

*Both references have been added to the manuscript.*

I am not sure what is the policy of the journal, but I find difficult to really assess and criticised the result as the transport system is not describe. It seems that the correct controls have been done, but again without knowing what transport system is use, it is difficult to evaluate the results.

*Thanks for pointing this out. Initially, we didn't want to distract the readership from the main aim of the manuscript. We now mention that this is a "bacterial choline symporter" and specify that the substrate used in these experiments is "choline".*

***Minor Concerns:***

Fig1 A. Should add "time (s)" on X axis.

*This has been added*

## **Reviewer #2:**
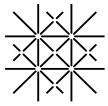
*We thank the reviewer for the very insightful comments.*

***Major Comments:***

None. The overall structure and aim of this protocol is good. Please provide additional clarification/elaboration on points raised in the minor comments below.

***Minor Comments:***

General - is there a reason not to say what the transported substrate and transporter are? Even though the protocol is generally applicable, it seems unnecessarily evasive not to tell the reader what you're measuring in the example data shown.

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Thanks for pointing this out. Initially, we didn't want to distract the readership from the main aim of the manuscript. We now mention that this is a "bacterial choline symporter" and specify that the substrate used in these experiments is "choline".

Section 3.
1) The end of this section would be a good place to mention the negative controls that are essential for any SSM experiment (doing the experiment with empty liposomes). You might want to make a big deal out of it, for example "It is essential to measure a negative control when designing these assays…"

Thanks for pointing this out. A note on this important point was added.

Section 4.
1) Step 1 - How much buffer is enough? For example, how much to go through the series of experiments with one antibody?

The required volume is now specified

2) Step 3 - why did you choose 500 nM for the antibody concentration? You may want to indicate a general range of concentrations typically seen for antibody binders.

This is now clarified

3) Step 9 -- This issue of the time delay before assessing binding is so important. I am wondering if you might want to elaborate on it a bit more. It would be more precise to describe the association kinetics (rather than "to interact better with proteoliposomes…") And mention that since nanobody association is a second order rate constant (rate is M-1s-1), at very low (nM) concentrations of nanobody this delay might need to be extended.
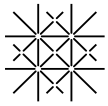
This is now clarified in section 4.3 "…Note: It is recommended to use nanobody concentrations that allow to reach saturating conditions. Considering that the binding constants of nanobodies are generally below 100 nM, we suggest to use 500nM for this experiment. However, it is recommended to pre-screen for optimal concentrations."

4) Step 10 -- After the wash step, in some cases you might need to wait again before measuring the recovery currents, especially for tight binders with slow dissociation kinetics.

A note mentioning this was added

5) Step 13 -- Although you do elude to this, it would be helpful for people that are not familiar with the technique if you were explicit about the fact that you can't compare absolute current amplitudes from chip-to-chip since the vesicle fusions are not well-controlled.

Notes mentioning this were added

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6) Frequently, when measuring protein-protein interactions, the proteins are "sticky" and adhere to surfaces, and this becomes especially problematic at lower antibody concentrations. How would an experimenter deal with non-specific binding? If we're assuming non-specialists, you may want to warn away from detergent additives people commonly use to prevent non-specific binding, like Tween/Triton. In my lab, we do similar SSM/antibody binding assays with addition of 50 ug/mL bovine serum albumin to combat this, and it works ok. (ie Kermani...Stockbridge Nat Comm, 2020. Please forgive the obnoxious insertion of my own work, and don't feel compelled to include the reference).

Thanks for this very helpful comment. Indeed, this is a very important point to take into account. We now mention this in lines 334-338 in the representative results section.

Section 5.
Step 5. Again, I think it's important to be precise that the binding kinetics are what dictate the length of the time delay. At low nM concentrations, longer time delays may be required since binding is a second order process (for example, at 1 nM, an antibody with a reasonable "on" rate of $1x10^6$ M-1s-1 is going to have a time constant for association of >10 minutes).

A note mentioning this was added

Section 6. Cleaning of sensors
1) Can you re-use sensors after this type of experiment? How many times, typically?

A note clarifying this was added at the end of this section

Discussion.
Page 6/Line 217 - What do you mean by "nanobody libraries are generally large"? Do you mean after selection of binders to a target? Is large on the order of 10 or 100 candidates?

This has been now clarified in line 283

Page 6/Line 222 - Reproducibility of what, specifically?

This has been now clarified in line 289-290

Page 6/Line 229 - I would make a stronger statement than "highly recommend" - I would say the antibodies must be added to both buffers.

This chance was done

Page 6/Line 232 - Instead of "inefficient" occupation, maybe use "incomplete" or mention the kinetics again.

The changes were made and a note clarifying the importance of taking into account the kinetics was introduced in lines 315-321
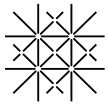
Page 6/Line 237 - What do you mean by satisfactory results? Stable transporter fusions? (Also you might want to mention that you tried varying the length of the delay in the actual protocol, in section 4/step 3.)

This is now clarified in this part and in the notes introduced in the protocol

Page 7/Line 264 - Change "Inefficient unbinding" to "slow kinetics of unbinding"

This change was incorporated

Figures:
You show the negative control (empty liposomes) for the transported substrate in Figure 2, but you might want to put it in figure 1 since it's really important to do this at the early stages of the protocol.

Thanks for noticing this. Indeed, this is a very important point. A new figure 1C was added.