University of Basel
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Molecular Life Sciences

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.

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

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.