November 8th, 2021

Dear Vidhya Iyer, Ph.D.
Review Editor
The Journal of Visual Experimentation
625 Massachusetts Ave.,
2nd Floor, Cambridge, MA

Dear Vidhya,

Thank you very much for submitting your letter indicating that our protocol has passed the second round of review and is considered for publication in the JOVE contingent upon revising the reviewers' points.

Once more, we are very grateful to the reviewers and editors. Their comments have continued improving the overall quality of our work, which we feel will help others adopt this novel and quickly evolving methodology. Please, find attached the point-by-point letter addressing each of the referees' comments in the pages continuing below.

We have indicated the pages and lines of the most critical points raised by the reviewers and kept "track changes" to facilitate the revision of the manuscript. We hope the current version of the manuscript is acceptable for publication in the Journal of Visual Experimentation.

We look forward to hearing from you.

Yours sincerely,

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Point by point address to reviewers' comments:

Reviewer #2:
Manuscript summary:
The authors have sufficiently addressed my comments in the revised manuscript and I recommend acceptance.

Answer: Once more, we would like to thank the reviewer for all the suggestions to our work.

Reviewer #3:
Manuscript Summary:
The present JoVE manuscript by Cespedes-Donoso and Dustin illustrates different protocols for including relevant proteins regulating cell-cell interaction in bead supported lipid bilayers, for measuring the density of proteins on the surface of beads and cells and for estimating the proteins released by cells that are conjugated with these functionalized beads. These authors have previously used these methodologies in relevant articles describing the transfer from T cells to APCs of different effectors and mediators thanks to the T cell ability to release vesicles at this cell-cell contact. These are very useful and interesting protocols for researchers in the field and abroad. The manuscript is clearly organized; however, some points may be clarified by authors.

Major Concerns:
A major issue is that authors do not include the possibility of other kind of vesicles, apart from the ectosomes or the attack particles, which can be delivered by T cells or other cells to contacting cells. As an example, some cytokines are known to be secreted in a directional form from T cells to APCs, as well as intraluminal vesicles from multivesicular bodies or lytic granules. All these elements are transported by vesicles or organelles and delivered to the APCs, and could be potentially detected at the surface of the beads. This is an important concept that should be clearly included in the introduction and discussion sections.

Answer: *We thank the reviewer by pointing out to this very important concept in the biology of T cell communication. We have mentioned the biology of cytokine secretion at the immune synapse in the Introduction. In the discussion section, we have also mentioned that this is a non-validated application of the method, which we are currently developing. Please refer to page 2, lines 65 to 70; and page 22, lines 1076 to 1080 of the revised manuscript.*

Other Concerns:
Line 46: BSLBs instead of BSLB? Can authors revise the text to fix singular and plural forms?

Answer: *We thank the reviewer for pointing to this error. We have corrected the manuscript accordingly.*

Line 51: do these BSBLs allow the study of other kind of synaptic-delivered vesicles, apart from ectosomes? Are BSBLs useful to distinguish between these vesicle populations? Please, include this information.

Answer: *We thank the reviewer for suggesting this change. We have amended the introduction to include other types of trans synaptic particles including CD63+ exosomes, and supramolecular attack particles, which derive from intracellular stores. Please refer to page 2, lines 63 to 65 of the revised manuscript.*

Line 59: 1) the use of antibodies with known numbers of fluorochromes per antibody (F/P): Can authors secure the number of antigens (1-2) bound by each antibody, and is it included in their calculations for their estimations of physiological density of molecules? A comment on this may be useful to readers here, even if lines 554-557 state that: "Since we use the same antibodies to calibrate the number of recombinant proteins on the surface of cells and BSLBs, there is no need to correct the antibody binding valency as this remains constant…."

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Answer: *We thank the reviewer for suggesting this change. We have included a statement in the abovementioned paragraph to clarify that correcting for the antibody avidity is possible by using fluorescent recombinant proteins with known F/P ratios. Please refer to page 2, lines 89 to 94.*

Line 158: "Acquire relevant samples and a minimum of 104 target cells for quantification". Is this minimum number of cells enough to assess differences in the density of molecules? Please, include an estimation/comment on this issue.

Answer: *We thank the reviewer for pointing to this key point. The number of target cells to analyse will depend on the abundance of the cell subset and the abundance of starting tissue material. In some cases, recording 10,000 events might require half an hour of acquisition per sample, or ultimately be limited by the total number of cells collected from a given human tissue. We have added a note to indicate that a more robust analysis requires the collection of data from multiple donors and across independent experiments. Please refer to page 5 and lines 276-279 of the revised manuscript.*

Line 159: do authors find relevant to wash the cytometer between samples to facilitate acquisition? If the answer is yes, please, include a note on this.

Answer: *We thank the reviewer for suggesting this note. We have added a note recommending activating either the sit flush option or the washing option in high throughput samplers to reduce the noise from sample to sample carry over. Please refer to lines 285-291 in pages 5 and 6 of the revised manuscript.*

Line 257: point 5.2 should not be there.

Answer: *We thank the reviewer for pointing to this error. We have amended the manuscript accordingly.*

Line 258: How do authors avoid sedimentation of silica beads usually?

Answer: *We have included a comment in the manuscript to clarify this point. Please refer to new page 8, lines 428 to 429, and page 10, lines 488-489.*

Line 287: Can authors explain how they add the argon/nitrogen gas to the tube containing the BSLBs during mixing?
Answer: *We have included a comment in the manuscript to clarify this point. Please refer to current page 10, lines 481 to 485.*

Line 427: include the medium to resuspend the cells (some information is missing).

Answer: *We have included the step in which the synaptic transfer assay medium was prepared and clarified its composition. Please refer to current page 14, lines 673 to 676.*

Line 459: Does "in absence of CO2" mean "in an non-CO2 incubator"? I recommend using this later term.

Answer: *We thank the reviewer for suggesting this correction. We have amended the manuscript accordingly.*

Line 503 and others: please use a unique term when referring to the BD HTS cytometer.

Answer: *We thank the reviewer for suggesting this correction. Since Hight Throughput Samplers are sell by several vendors and for a variety of commercially available instruments, we have used a more general term instead.*

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Reviewer #4:

Manuscript Summary:

This protocol provides both a wealth of details into the practical, in-the-lab aspects of this assay and a high-level overview of the underlying foundations. The overall concept of replacing antigen-presenting cells with bead-supported lipid bilayers is powerful, and does deserve a discussion as accomplished in this manuscript. The revisions are responsive to comments in the original review, and improve the manuscript. I recommend accepting this manuscript for the next stage of production with minor revisions.

Minor Concerns:

There are organizational issues. While additional copy-editing will be done, I would like to illustrate specific problems:

1) Line 256, there appears to be an extra line return, which breaks the numbering system. Fixing this could have a cascading effect on sections, so should be carefully addressed.

Answer: *We thank the reviewer for pointing to this error. We (and the reviewing editor) have modified the text accordingly to correct for any structural and spelling errors while keeping the unique format of JOVE.*

2) Line 427, something is missing at the end of the highlighted text.

Answer: *We thank the reviewer for pointing to this error. We have modified the manuscript to improve its readability.*

3) Line 42, "BSLB" is used in the introduction before definition. There is a definition in the Abstract, but if the Abstract is meant to stand alone, the acronym should be defined again in the Introduction.

Answer: *We thank the reviewer for pointing to this error. We have modified the revised manuscript accordingly.*

4) The Comments/Description section (starting page 47 of the review document) is missing indicators from the main text.

Answer: *We thank the reviewer for pointing the unclarity of this section. We (and the reviewing editor) have modified the text and associated tables accordingly to correct for any structural and spelling errors while keeping the unique format of JOVE.*

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