JoVE62035 "Assembly and Operation of an Acoustofluidic Device for Enhanced Delivery of Molecular Compounds to Cells"

Response to Comments

Thank you for the additional comments and details regarding the manuscript submission. We have made changes throughout the manuscript as requested. This revised version of the manuscript incorporates the requested changes. Our comments below are in blue text and all changes to the manuscript are indicated with red text to assist the Reviewers in identifying the revised text.

Reviewer comments:

1. Please reword the title to reflect the protocol being presented and not the device used for the protocol. Also please remove the "low cost" term from the title.
   e.g., Assembly and operation of an Acoustofluidic Device for Enhanced Delivery of Molecular Agents to Cells

We have revised the title as requested to focus on the protocol instead of the device:

"Assembly and Operation of an Acoustofluidic Device for Enhanced Delivery of Molecular Compounds to Cells"

2. We cannot have commercial terms in the manuscript. I have removed all the commercial terms from the protocol section. Please ensure these are present in the table of materials.
   We were not aware that commercial terms were still in the manuscript, so thank you for making modifications to the text to ensure that our manuscript matches the required criteria of JoVE.

3. Please include an ethics statement to show that the human blood collection protocol was performed following approval from the IRB ethics boards of your institute.

Thank you for pointing out this omission. An ethics statement has been added to specify our compliance with protocols approved by the institutional review board:

"Ethics Statement**:** Whole blood donations were collected from healthy donors following protocols approved by the institutional review board at the University of Louisville."

4. Please include the significane of preparing this as a note with citations if any.

The significance of using ultrasound contrast agents has been added to the manuscript:

"NOTE: Ultrasound contrast agents significantly enhance acoustofluidic delivery of molecular compounds by transiently increasing permeabilization of nearby cellular membranes.[19] Molecular delivery is very limited without ultrasound contrast agents in this system."

5. Which solution?

This statement was revised to clarify:

"Add 25 µL of ultrasound contrast agent solution per 1 mL of cell solution immediately before pumping the combined contrast agent/cell mixture through the acoustofluidic device. The cell solution can be modified as desired by the user, but in our studies the cell solution consisted of primary T cells in step 4.21, and A549 lung cancer cells in step 5.7, respectively."

6. Where was the ultrasound contrast agent used in this example. Please bring out clarity.

Additional steps were added (4.21 and 5.7) to clarify where the ultrasound contrast agent was added:

"4.21. Add 25 µL of ultrasound contrast agent solution as previously described in step 3.11."

"5.7. Add 25 µL of ultrasound contrast agent solution as previously described in step 3.11."

7. Citations if any to show how this is done.

We have added additional details and citations as requested:

"Density gradient separation containing a substrate is commonly utilized to separate PBMCs from whole blood.[24-26]"

8. Ethics statement for the use of human materials required.

We have added an ethics statement as noted above.

9. Cell number and volume per vial of PBMCs?

Thank you for pointing out these omissions. We have added these details as requested.

Step 4.3: "Dilute thawed PBMCs 1:10 with PBS in a 15-mL centrifuge tube. Each 1-mL vial contains approximately 10 million PMBCs."

10. To what cell number?

This is an excellent point. The text has been modified to specify the cell number:

Step 4.14: "Separate primary T cells with a commercially available benchtop magnetic sorting instrument using the "depletes separation" setting following manufacturer's protocol. This step should yield between 5-10 million T cells after cell sorting."

11. Cell number for experiment being presented?

We have added this detail as requested:

"Count T cells using an automated cell counter or hemocytometer and aliquot 1 million/mL for experiments."

12. So the acoustofluidic device is being used to incorporate fluorescein in this case?

That is correct. The acoustofluidic device was being used to deliver fluorescein intracellularly in primary T cells. The text has been revised to clarify this:

Step 4.22: "Process 1-mL aliquots of cells using the acoustofluidic system (see steps 2.10-2.11). This step enhances delivery of fluorescein into primary T cells."

13. Speed?

Thank you for pointing out this omission, we have added information on centrifugation speed and duration after treatment.

Step 4.23: "Immediately after treatment, wash cells three times via centrifugation at 580 x g for 10 min with 500 µL of PBS to remove extracellular fluorescein. Cells should be washed within 10 min after adding fluorescein solution."

14. Again please bring out clarity for the use of acoustofluidic system here. Is it used for incorporating trehalose into the cells-please clearly mention this. Also where did you incorporate the contrast reagent in this case.

Thank you for this feedback. We have added additional details describing when the ultrasound contrast agents are added and what biomolecule is being delivered with this protocol. Please see below:

Step 5.7: "Add 25 µL of ultrasound contrast agent solution as previously described in step 3.11."

Step 5.8: "Process 1-mL aliquots of cells using the acoustofluidic system (see steps 2.10-2.11). This step enhances delivery of trehalose into A549 lung cancer cells."

15. Please include more details on figure 2 and 3. How the experiment was performed, how did you normalize the values, at what point the measurement was taken, number of cells to begin with, number of experiments as replicate, number of wells per experiment, etc.

Thank you for the feedback. The text was revised to provide these additional details as requested:

"Figure 2 demonstrates enhanced intracellular delivery of a fluorescent compound, fluorescein, to primary human T cells with acoustofluidic treatment compared to an untreated control group (p<0.05, n=3/group). T cells were suspended at a concentration of 1 million/mL in PBS with 100 µg/mL fluorescein solution and 25 µL/mL ultrasound contrast agent solution, and the mixture was passed through the acoustofluidic device for ultrasound treatment. Intracellular fluorescein delivery and cell viability were measured with flow cytometry after washing cells via centrifugation to remove extracellular fluorescein. T cells in the untreated control group were also suspended at 1 million/mL in PBS with 100 µg/mL fluorescein solution, but ultrasound contrast agent solution was not added and cells were not passed through the acoustofluidic device. The fluorescence intensity of T cells increased by 5-fold after acoustofluidic treatment relative to the fluorescence intensity of T cells in the untreated control group, indicating enhanced delivery of fluorescein. Cell viability decreased slightly after acoustofluidic treatment but remained above 80% (p<0.05, n=3/group)."

"Figure 3 demonstrates enhanced intracellular delivery of a preservative compound, trehalose, to human A549 lung carcinoma cells with acoustofluidic treatment compared to flow alone (no ultrasound contrast agents or ultrasound exposure) and compared to cells in the untreated control group (ANOVA $p<0.05$, n=3/group). A549 cells were suspended at a concentration of 100,000/mL in PBS with 200 mM trehalose solution and 25 µL/mL ultrasound contrast agent solution, and the mixture was passed through the acoustofluidic device for ultrasound treatment. A549 cells in the control groups ("Flow Only" and "No Treatment") were also suspended at 100,000/mL in PBS with 200 mM trehalose, but ultrasound contrast agent solution was not added and cells were not exposed to ultrasound treatment. Intracellular trehalose was quantified using a trehalose assay kit and normalized to the untreated control group. Cell viability was measured with trypan blue assay. There was no statistical difference in cell viability between groups (n=3-7/group)."

16. If any of the figures are reprinted from previously published figures, please include a reprint permission as well.

We confirm that none of these figures were previously published.

17. Please remove the word Aurdino from the figure as it is commercial.

Thank you for this helpful comment. We have removed the word "Arduino" from Figure 1 as requested.

18. Please expand on the limitation of the acoustic device.

Thank you for this feedback. We have added additional text to expand on the limitations of this system:

Line 428-432: "A limitation of this system is that the small acoustofluidic channels can easily become blocked by debris or cell aggregates. Thoroughly rinsing the channels between each sample will help prevent problems with channel blockage. In addition, multiple PDMS devices can be fabricated in each batch so that devices can be quickly replaced if necessary."

Line 474-476: "Acoustofluidic delivery of other biomolecules, such as proteins or DNA plasmids, is also possible, although a limitation of this system is that the efficiency of molecular delivery may be lower for larger compounds.[18,31]"