December 17, 2021

Dr. Deepika Mittal
Review Editor
JoVE

Dear Dr. Mittal,

Attached please find a revised version of our manuscript, “Isolation and Characterization of Cyanobacterial Extracellular Vesicles”. We thank you and the reviewers for providing such detailed, helpful and constructive suggestions. We have addressed all of the comments (please see our detailed responses below), and agree that these changes have resulted in a much improved and clearer protocol and discussion.

We hope that this revised draft is now suitable for publication in JoVE. If you have any further questions or require additional changes, please do not hesitate to contact us.

Sincerely,

/Paulo Oliveira
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Editorial comments:

Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
AUTHORS: All authors have proofread and approved of this revised manuscript, and we hope that any spelling or grammatical errors have been corrected.

2. Please provide a summary between 10-50 words. The current summary is 52 words.
AUTHORS: The revised summary is within the word limit.

3. Please revise the Introduction to include all of the following with citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
AUTHORS: We have reformatted the end of the Introduction to more directly address the points raised here.

4. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Any text that discusses the protocol should be included in the Discussion section.
AUTHORS: We have changed our approach toward “Notes” as suggested and only include them in a few locations where we feel they are most relevant for helping readers. The other points we had previously included as ‘Notes’ have now been consolidated into the Discussion or protocol steps themselves as requested.

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
AUTHORS: As indicated in the response to point 4 above we have moved these as requested.

6. 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: Milli-Q, Nanosight, etc.
AUTHORS: Commercial language has been removed from the manuscript, and we now just refer to the Nanosight instrument as a 'nanoparticle tracking analysis device'.

7. 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 avoid going beyond the 3rd sub-step.
AUTHORS: We have reorganized our layout to only include up to 3 sub-parts of a protocol step.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Readers of all levels of experience and expertise should be able to follow your protocol.
AUTHORS: We have provided additional detail throughout the protocol as requested, including addressing the many helpful suggestions made below. We have tried to strike a balance between sufficient and unnecessary levels of detail, and also with overall protocol length in mind; we believe that the revised level of detail is consistent with what we see in other related JoVE manuscripts. However, if there remain portions of the protocol which remain unclear please let us know and we will be happy to correct them.

Line 98: Please provide literature citations to determine the optimum growth conditions. Also how is consistent steady-state growth assessed? Please specify.
AUTHORS: We have revised the wording of section 1 to avoid reference to 'optimum' growth as this is not a necessary requirement for these types of experiments.

Line 105: How were the gradual transfers carried out? Please provide the steps for doing this.
AUTHORS: Thank you for pointing this out; we now provide a suggested series of culture volumes to use when scaling up cultures.

Line 113: How much culture was used for inoculating the medium? What were the growth conditions? Please provide all steps for doing this.
AUTHORS: In 1.1.2 we now specify 1:20 dilutions as typical for culture inoculations, and in 1.1.3 detail a progression of culture volumes which results in reproducible growth of large culture volumes.

Line 163: How was the tubing and vessel cleaned?

AUTHORS: We have added the requested detail into 3.1.1: Tubing and vessel are washed with water and mild detergent, and thoroughly rinsed with double distilled and deionized water. Prior to utilization, material should be rinsed with type I ultrapure water.

Line 195-199: What was the steady rate maintained? How was the TFF run? Please provide all associated steps.
AUTHORS: We have increased the level of detail in our discussion of tangential flow filtration. Given differences between devices we must leave some specifics of setup (how to properly attach the filter module, tubing diameters, clamp setups, etc.) to recommendations from individual manufacturers, but hope that this provides a better guide to carrying out the process.

Line 244: What does too large vesicle volume mean? How much volume of vesicle and 45% iodixanol was mixed? Please clarify.
AUTHORS: We have clarified the wording in this section. Given that ultracentrifuge rotor capacities vary wildly and are quite expensive pieces of infrastructure, we do not think it is appropriate to specify precise volumes which are not consistent with the rotors available to a user. We hope that our language provides sufficiently clear guidance about relative volumes and the overall process to be broadly useful.

Line 275: How was glow discharge or charging of the formvar-coated TEM grid done? Please provide all associated steps.
AUTHORS: The process for this step can once again vary between whether a lab has a home-made or commercial discharge device, and we must leave it to the user to follow the manufacturer's instructions. This point has been clarified in the text.

Line 279: How was vesicle concentration determined.
AUTHORS: Vesicle concentration is determined by nanoparticle tracking analysis-based technology, as detailed in the manuscript. We have added a reference to the appropriate step where this is described.

Line 284, 285: How was the grid floated?
AUTHORS: The grid was placed on top of a 2% uranyl acetate drop sufficiently large (20-50 µL) for the grid to float. Text has been changed to better explain this part.

Line 297: What was the volume of glutaraldehyde added for sample fixation? Were the samples fixed at a specific temperature? If yes, please specify.
AUTHORS: We have clarified these details as suggested in 5.2

Line 301, 304: Volume of osmium tetroxide, ethanol added?
AUTHORS: We have clarified these volumes as suggested in 5.2.

Line 310: How were the samples stained and visualized? Please provide all the associated steps. If this step needs to be filmed, please make sure to provide all the details such as “click this”, “select that”, “observe this”, etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.
AUTHORS: We have included all the steps carried out for staining the grids. However, the process to visualize the grids by the TEM can vary between different microscopes. Therefore, for the visualization we leave it to the user to follow the manufacturer's instructions.

Line 317: Please provide all the steps for visualizing and setting up the particle visualizer? What are the instrument settings and parameters?
Line 325, 337: How much sample was added? What is the linear range for measurement? What are the predefined limits?
Line 328: What are the video settings for the instrument?
AUTHORS: Concerning the above comments, we have provided more explicit instructions as to the use of the nanoparticle tracking instrument in section 5.3.

Line 359: Please provide references which show how the densitometry analysis is done?
AUTHORS: Two references have been included to show how the analysis is performed (section 5.5).

Line 366: How was the culture acclimated? What are the desired media conditions?
AUTHORS: We have provided more details and rationale for culture acclimation. We have changed the phrase “desired media conditions” to better reflect our intent which was more about the user’s experimental conditions. Given the nature of this protocol as one that can apply to many different cyanobacterial strains, we do not think it is necessary nor appropriate to detail the exact experimental conditions one might want to use.

Line 368: At what time points were the sample collected?
AUTHORS: We have provided more detail specifying guidelines for the number and relative distribution of sampling timepoints required.

9. Please include a single line space between each step, substep, and note in the protocol section. Please 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.
AUTHORS: Lines are now included in the protocol section. Moreover, relevant sections of the protocol have been highlighted in yellow to indicate the essential steps that should be filmed.

10. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures.
AUTHORS: A paragraph of text is now included to explain the representative results, referring specifically to what is presented in Figures 1 and 2.

11. Please define all abbreviations used in the figures in the figure legends.
AUTHORS: Abbreviations used in the figures are now defined in the respective captions.

12. As we are a methods journal, please also include in the Discussion the following in detail along with citations:
a) Any limitations of the technique
b) The significance with respect to existing methods
c) Any future applications of the technique
AUTHORS: While these topics were indirectly discussed, we hope that our revised discussion makes these points more clearly. Relevant points are now discussed in a separate “Conclusions” section at the end of the Discussion.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes protocols for isolation of extracellular vesicles (EV) from Cyanobacteria. In general, the techniques described are modifications of standard protocols for isolating and characterizing EVs across a broad range of bacterial species.

Major Concerns:
* In the discussion, the authors state that Cyanobacteria present some unique challenges with regard to EV sampling and analysis: low maximum cell density in culture, poor growth in pure culture. I suggest moving this text on the challenges to the Introduction instead, as they provide the rationale for using large scale cultures and capsule filtration and subsequent tangential flow filtration. Moving the challenges up would enhance the novel aspects of these protocol modifications.
AUTHORS: Thank you for the helpful comment. As suggested, we now introduce these concepts at the end of the Introduction. We have also made other wording changes in order to avoid making unnecessary and/or excessive claims about the novelty of these protocol modifications.

* It‘s not clear which aspects of the protocols for isolating EVs are unique to Cyanobacteria. Most modificiations relate to the low cell density. Perhaps providing additional specific information related to growing conditions for large-scale exponential phase cultures would potentially be more useful to researchers (see below for specific suggestions).
AUTHORS: Thank you for pointing this out. As also noted above, we have changed the wording in the abstract and introduction to avoid giving the impression that any of these steps for working with vesicles, other than the culture cultivation conditions, are necessarily unique to Cyanobacteria. Instead, these protocols represent a combination of smaller adjustments and detailed considerations which are critical for handling samples like these with low cell densities and which can grow at seawater salinities. We also now include citations to other publications (refs 20-22) detailing vesicle purification from microbial systems which work with much smaller volumes of samples and further do not include methods such as density gradient purifications which we (and others) argue are critical for experimental rigor.

Minor Concerns:

* Line 25: delete "some"
AUTHORS: Changed as suggested.

* Line 35: delete "of most, if not all,"
AUTHORS: Changed as suggested.

* Line 36: delete "help
AUTHORS: Changed as suggested.

* Line 59: replace" for questions surrounding" to "because of"
AUTHORS: Changed as suggested.

* Line 60: replace "and as important models" with "and are important models"
o Replace "Cyanobacteria can be" with "Cyanobacteria are"
AUTHORS: Changed as suggested.

* Line 67: replace: "found in" with "isolated from"
AUTHORS: Changed as suggested.

* Lines 108 - 109: authors note that larger culture may require addition of pH buffers and supplementary sodium bicarbonate or aeration. It could be useful for those trying to troubleshoot growth conditions to know what pH ranges are optimal during large batch culturing.
AUTHORS: We have clarified the suggested pH ranges and buffers.

* Line 110 - references to aeration >> does this mean shaking or bubbling in of air. Specified
AUTHORS: We meant ‘bubbling’ and have clarified this in 1.1.3.

* Lines 123: replace "soaps" with "soap"
AUTHORS: Changed as suggested.

* Line 138: I think the authors mean to say "sterilized receptacle" rather than "sterilized recipient"
AUTHORS: Thanks for catching this. Changed as suggested!

* Line 170: authors suggestion that if flow rate becomes too slow to use gentle force with a peristaltic pump. More specific conditions would help individuals who have not attempted these protocols before. For instance, how slow is too slow? What setting should the peristaltic pump be set at in order to be gentle? Authors

recommend backflushing the accumulated biomass. How much volume should be backflushed? 500 mL? 1L? or are there visible criteria that could be applied?
AUTHORS: We agree that more detail on these points should be included and have modified point 2.2.4. As the specific flow rate expected can vary markedly among different model filters, the revised manuscript now clarifies an approximate relative decrease in measurable flow rate (< 1/10th the starting rate, or if material is moving through dropwise as opposed to in a continuously flowing stream). We also now suggest backflushing the filter until the material coming off the filter is no longer visibly turbid.

* Line 183: I think authors mean "filtrate" instead of "run through"
AUTHORS: You are correct, we have changed "run through" to "filtrate".

* Line 463: replace "can not" with "cannot"
AUTHORS: Changed as suggested.

Reviewer #2:

Manuscript Summary:

The manuscript describes a robust isolation and characterization method of cyanobacterial EVs from 3 different cultures. All the methods fit to MISEV guidelines, moreover is replenished by several filtration and concentration processes necessary for bacterial EV isolation.

Major Concerns:

The characterization of cyanobacterial EVs should be amended. The presented methods and results deals mainly with morphology, only LPS analysis was suggested and performed. A protein and/or lipid concentration or determination of protein-to-lipid ratio should be also infromative. Moreover, despite that NTA method is included into the protocol, no indication on EV yield or data on vesicle production rate is presented. I think these value are indispensable for end-user researchers.

AUTHORS: We thank the reviewer for the helpful comments and for pointing out our inadvertent omission of these data. We agree that more information on typical yields is important and now include such values for *Synechocystis* PCC6803 in Representative Results (Fig 2).

Our protocol is designed around initial purification and basic physical characterization of vesicles. We have consciously avoided including downstream biochemical characterizations of vesicles, as the particular values that might be useful and the methods used for characterizing different types of cargo are constantly changing. We did include some basic LPS analysis for the stated reason of helping to validate that Gram-negative vesicles are present in a given purified sample. While proteins are frequently examined as a typical cargo in a vesicle population, they are not necessarily inherently part of all vesicles, and other groups have shown that protein content can change depending on growth phase (now discussed in the text - references 39 and 40). Thus, the protein to lipid ratio is perhaps of interest, but represents more of a biological variable than an expected characteristic of EV preps. In addition, there appear to be some discrepancies between different protein quantification approaches when applied to EVs, and we are not ready to recommend one protocol over another at this time.

Minor Concerns:

Indeed, NTA is the gold standard for EV size and concentration measurements, however, its potential is limited at small particle size. Microfluidic resistive pulse sensing (MRPS) represents a non-optical alternative for NTA, and it is getting to be recognized in EV research recently (PLoS ONE 16(4): e0249603. https://doi.org/10.1371/journal.pone.0249603; J Extracell Vesicles 2021

Apr;10(6):e12079. doi: 10.1002/jev2.12079. Epub 2021 Apr 6.). The detection limit of MRPS is independent from the size, hence it provides a more realistic size distribution of EVs in the size range below 100 nm.

AUTHORS: Thanks for raising the idea of MRPS as an exciting alternative to the described NTA analyses for characterizing cyanobacterial vesicle size distribution. In the Discussion, we have now expanded the list of possible techniques that can be used for EVs characterization to include MRPS as well as other approaches like Nano Flow Cytometry, and include references to the indicated papers. As we do not have access or experience with MRPS or some of these other devices, we cannot directly speak to their utility for cyanobacterial samples.