Tel: (509) 372-4524

Fax: (509)-372-6168

xiaoying.yu@pnnl.gov

March 17, 2017

Review Editor, Mala Mani, Ph.D.

JoVE

**Response to Reviewers Letter: Revision of 55944\_R0\_011717.docx**

Dear Dr. Mani:

Thank you very much for your insightful comments and suggestions! They are very useful for us to improve the manuscript. We find the review process extremely rewarding. We have made all suggested revisions to address your comments and suggestions.

Major revisions are summarized as following: First, several paragraphs covering discussion of the results were moved to the Representative Results section from the Discussion section. Second, the Discussion section was reconstructed in a 5-paragraph form following the Editorial comments: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. In addition, correction of typos, incorrect reference to protocol steps, revisions to figures and figure legends, some grammar/phrasing issues, and clarifications were also made as needed.

Here is a point-by-point summary of our response to your comments and suggestions. Your comments are listed first, and our responses follow each comment. The revised or added text is highlighted in blue font in the manuscript file.

We look forward to hearing from you about the decision of this paper in the near future.

Sincerely,



Xiao-Ying Yu, Ph.D.

Senior Scientist

Attachment

1. Response to reviewers’ comments;
2. Revised manuscript: a copy with text additions in blue and video sections highlighted in yellow, and a clean copy for transcript preparation;
3. Figures in tif format;
4. Revised materials/equipment list

cc: Yu, Rachel Komorek

# Editorial Comments:

*The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55944\_R0\_011717) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file.* ***Please download the .docx file and use this updated version for any future revisions.*** *The updated manuscript is also attached.*

**Reply:** Thank you! Revisions have been made to strengthen the paper based on your suggestions. Please see the detailed response below.

**Changes to be made by the Author(s):**

*1. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript (text and figures). Examples of commercial sounding language in your manuscript are: BD PlastiPak, Parafilm®, Origin Pro 2015, Scotch®, etc. All commercial products should be sufficiently referenced in the table of materials/reagents. Please replace all commercial sounding language in your manuscript with generic names that are not company-specific.*

**Reply:** “BD PlastiPak” was removed from step 1.2.4 and referred to as a syringe. “Parafilm” was removed from steps 1.3.3.1, 2.1.3, and 2.3.6 and was referred to as plastic paraffin film. “Scotch” tape was removed from step 2.3.6 and referred to as adhesive tape. “Origin Pro 2015” was removed from step 1.3.3.4 and 6.1 and referred to as a graphing software. In step 5.1 and in the Figure 2a description, “IonTof” software is mentioned but it was not removed because the protocol only teaches mass calibration using this specific software. The manuscript was checked for further use of commercial language but no other incidences were found.

*2. Please ensure that all items mentioned have been included in the Materials/Equipment list, and are accompanied by a catalog number. For e.g., Balch tube, etc.*

**Reply:** Some materials do not have a catalog number, in such cases N/A was specified as the catalog number, and we have tried to supply enough detail on where to purchase these items within the comments and company name, including the model. “Balch tube” in steps 1.1.2, 1.1.2.1, and 1.3.1.2 were changed to refer to “anaerobic culture tubes”, and these were added to the materials/equipment list.The manuscript was read for further use of supplies not in the Materials/Equipment list but no other incidences were found.

*3. Please define all abbreviations before use. For e.g., SALVI, ToF-SIMS, TSB, TSA, DI, etc.*

**Reply:** References to TSB and TSA were made clearer at the end of paragraph 3 of the introduction. Definition of TSB was removed within the note of 1.1.1 to avoid redundancy, as this was already defined at the end of paragraph 3 of the introduction. Acronyms for PTFE were fixed within step 1.2.3 and 1.2.4. Definition for UV/Vis was provided within step 1.3.3.3. DI water was defined as deionized within step 1.3.3.1. The manuscript was read for further incidences of lack of definition before acronym use but no other incidences were found.

*4. Please use h for hour(s), min for minute(s) and s for seconds throughout the manuscript (including figures and tables).*

**Reply:** Use of “hrs” within Figure 1b was changed to h. All use of “hours” in the document was changed to h. All use of “minutes” was changed to min. There were no uses of “seconds” found.

*5. Please include spaces between all numbers and units.*

**Reply:** A space was inserted between the number and unit in Figure 1c. A space was inserted between the number and unit in step 1.2.4. Additionally, a space was inserted in step 2.1.1 and 2.1.3.

*6. Your Short Abstract exceeds our 50 word limit. Please revise the Short Abstract so that it clearly states the goal of the protocol within 50 words*

**Reply:** The Short Abstract was simplified such that it now equals 50 words, such that it is more concise but still contains the same meaning. The word “natural” was removed, as it may be extraneous information for the short abstract. The phrase “mutated to express” was removed before green fluorescence protein, and “to illustrate the process” was removed, as these words are extraneous and do not add to the overall understanding of the short abstract.

“This article presents a method for growing a biofilm for in situ time-of-flight secondary ion mass spectrometry for chemical mapping in its hydrated state, enabled by a microfluidic reactor, System for Analysis at the liquid Vacuum Interface. The Shewanella oneidensis MR-1 with green fluorescence protein was used as a model.”

*7. Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.). Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*

**Reply:** Text including the phrase “should be” was moved to a note section within 1.3.3.1, 1.3.3.4, 2.3.6, and 3.1.6.Text including “should be” was revised to imperative tense within step 1.3.3.2, 3.1.4. Text including “would be” was revised to imperative tense within step 3.1.6. Some minor grammar changes were made throughout the procedure to be worded in imperative tense.

*8. 1.1.1: Please provide the composition of the “nanowires” medium in the Table of Materials.*

**Reply:** The composition of “nanowires” has been provided within one of the reference articles, additionally we have provided a reference to this paper at the first mention of “nanowires” medium within the third paragraph of the introduction (Hill, E. A. *Effects of Electron-Transport-System Impairment on Hydrogen Gas Production by the Bacterium Shewanella oneidensis MR-1* Master's thesis, Washington State University, (2007).), and have provided an additional sentence clarifying that the composition of this medium has been established within previous research. Also, all ingredients which are used for the “nanowires” are specified as such within the comments, and contain a reference to the paper to which its’ composition can be found.

*9. 1.2.5: Does the bubble trap include the syringe and rubber stopper?*

**Reply:** Parenthesis (the PTFE tubing, rubber stopper, and syringe) were inserted into the text to clarify the nature of the bubble trap. Additionally, a sentence “this is the bubble trap” was added at the end of 1.2.4 for clarity as to the nature and components of the bubble trap.

*10. 1.3.2.3: Incubate for how long?*

**Reply:** A sentence was added to specify how long incubation was necessary; “Incubate until the first OD600 data point is taken, within step 1.3.3.”, as the incubation duration will not be covered until this step, but rather step 1.3.2 is only meant to specify the preparation for this.

*11. 1.3.3.4 (first): This step should be made a NOTE.*

**Reply:** This step was changed to be a note.

*12. 2.1, 2.3, 2.4.6 NOTES: Please move the discussion of the protocol to the Discussion section.*

**Reply:** Notes discussing protocol was moved and integrated within the text of the Discussion section. The first note of 2.1 was removed, as it is already present within the Discussion section. Pertinent steps to the Discussion section were placed within the “critical steps within the protocol” paragraph.

*13. 2.2.2: Which prepared serum bottle?*

**Reply:** This step was revised to refer to the serum bottle prepared in step 1.1.1.

*14. 2.2.3: Select how? How much medium? Mix how?*

**Reply:** Additional text and a note were added to this step for clarification.The step now reads:

“2.2.3) Select an individual CFU from the agar plate and, using a sterile syringe with an attached 22 gauge needle, deposit enough growth media onto to dislodge the colony from the plate without touching any neighboring colonies and mix the colony with the medium with the tip of the needle.

Note: A singly colony should be selected that is far enough away from other bacteria on the plate, such that medium can be deposited onto it without touching any other colonies.”

*15. 2.3.1: Obtain how?*

**Reply:** The text on this step was revised from “obtain” to “use a new SALVI device”, a note was added to show that device fabrication was seen in our patents in references 5 and 6 and Li Yang’s 2011 Lab Chip paper (reference 8). Moreover, we added to the note to clarify that we prepare a SALVI device fresh for each experiment.

**“**Note: SALVI devices are prepared fresh for each experiment following the device fabrication detailed in previous research and patents.5,6,8”

*16. 2.3.2: Draw how? Is this done manually or using a software?*

**Reply:** Instances of “draw” were changed to “take” within the entire protocol section, to avoid any confusion that this refers to the intake of liquid to a syringe rather than an incorrect use of the term.

*17. 2.4.6: Refill how?*

**Reply:** Additional text was added to this step for clarification.

“Refill fresh medium as it runs out within the BSC by filling a new sterile syringe with 10 mL of growth medium and attaching to the SALVI after the previous growth medium has run out.”

*18. 3.1.2: Clamp how?*

**Reply:** Additional text was added to this text for clarification.

“Clamp the slide to the stage of the microscope by placing the glass slide between the stage clamps on the platform.”

*19. 3.1.6: Please add details on how to attach the medium flow again or refer to the relevant steps.*

**Reply:** This step was clarified by adding text to refer to the setup of medium flow through the tubing system within step 2.3.6. “To attach to medium flow once again, refer to step 2.3.6.”

*20. After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.*

**Reply:** The protocol begins on page 4 and ends on page 13, thus it is below the 10-page limit for protocol text. Including notes, filmable content now comprises less than 2.75 pages of text; to do this, one section of highlighted content was removed, this being step 3, optical imaging of the biofilm within the SALVI microchannel, as SALVI users should typically know how to view the window with simple microscopy, and additional coverage of this content is not necessary in comparison to all other highlighted areas.

*21. Results: Please move the discussion of the results from the Discussion section to the Representative results.*

**Reply:** Three paragraphs including discussion of results were moved from Discussion to Representative Results.

*22. Figures: Please define the error bars (SD, SEM, etc.) in the legend. For all microscope images in the figures, please include scale bars and define their sizes in the associated legends.*

**Reply:** Information that the error bars are based on SD were added to the legend of Figure 1b. A scale bar was included in the microscope image in Figure 1c.

*23. Figure 1 legend: Please provide a title for Figure 1.*

**Reply:** A title has been added to the legend of Figure 1, “Experimental Schematic”.

*24. Please expand your discussion to cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

**Reply:** Detailed edits have been made to the discussion section following the outline provided above, the Discussion section was rearranged following this outline, and new content was written to cover limitations of the technique.

*25. References: Please abbreviate all journal titles.*

**Reply:** All journal titles within the reference section have been abbreviated.

*26. JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.*

**Reply:** DOIs have been added for all papers which have a DOI available.

*27. Please revise the protocol text to avoid the use of any pronouns (i.e. "we", "you", "your", “our” etc.).*

**Reply:** Uses of the word “you” were removed from the text and the text was reworded to accommodate this change in step 1.3.1.3.The manuscript was read for further use of pronouns but no other incidences were found.

*28. Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors.*

**Reply:** We have thoroughly proofread the manuscript, and revisions have been made as needed.

# Reviewers' comments:

# Reviewer #1:

**Manuscript Summary:**

*This article that explains how to use grow a biofilm in a SALVI device, and then to analyze the hydrated film while it is bathed in aqueous solution with time-of-flight secondary ion mass spectrometry (TOF-SIMS). The manuscript not only describes how to culture the bacteria in the SALVI device, but also how to experiments, namely cell growth curves, that are used to identify the duration of the culture period, and microscopy experiments that are used to assess whether a satisfactory biofilm has been produced. I expect that this technique will be of interest to other scientists, and this article will allow them to use it.*

**Major Concerns:**

*I have no major concerns, but I have some minor issues that need to be addressed (see below).*

**Minor Concerns:**

*These procedures were clearly, though I believe that I may have found a few mistakes involving referring the reader to the wrong step to find a procedure (described below). There were also a few places in the manuscript where it seemed redundant. These minor issues and a few other minor questions I have are described below. I expect that it will be easy for the authors to address them.*

**Reply:** Thank you for your insightful comments! Revisions have been made within the paper to match your suggestions.

*1. Step 1.2.4 says, "…. and fit the rubber stopper from step 1.2.3 into the open end of the syringe. Fit the rubber stopper into the syringe." Is the second sentence simply repeating the previous sentence, or are multiple rubber stoppers being fit into multiple syringes? If the second sentence is only repeating the previous sentence, please delete it. If the second step refers to a different task than the first one, please explain the second task more clearly.*

**Reply:** Thank you for finding this mistake! The sentence referring to the rubber stopper was indeed redundant, and the second sentence was deleted to avoid this mistake within step 1.2.4. This step now reads:

“Remove the plunger of a 5 mL syringe and fit the rubber stopper from step 1.2.3 into the open end of the syringe. The 2 cm end of PTFE tubing is within the barrel of the syringe. This is the bubble trap.”

*2. Step 1.2.5 says "Wrap the bubble trap made in step 1.2.2…". I believe that is the wrong step because step 1.2.2 refers to cutting tubing. Please change step 1.2.5 so it refers to the step where the bubble trap was made. I'm not really sure which item is the bubble trap. Please consider adding a sentence that says something like, "This is the bubbler trap," at the end of the step in which the bubble trap is made.*

**Reply:** This was revised to show that the bubble trap was finished in step 1.2.4 within step 1.2.5, and a sentence was added to the end of 1.2.4 to show that the bubble trap is completed and identified stating “this is the bubble trap” at the end of step 1.2.4, and clarification in parenthesis (the PTFE tubing, rubber stopper, and syringe) within step 1.2.5.

*3. Step 1.3.1.2 instructs the reader to transfer some of the freezer stock to a Balch tube that contains dextrose-free medium, prepared in step 2.1.2. When I read this, I assume that step 2.1.2 would describe how either the medium, freezer stock, or Balch tube was prepared. Instead, step 2.1.2 describes streaking agarose plate with frozen bacterial stock; is that the step they meant to refer to? If it isn't, please revise accordingly.*

**Reply:** Thank you for finding this typo!Step 1.3.1.2 was changed to refer to step 1.1.2 instead of step 2.1.2.

*4. I believe that the last sentence of the 2nd paragraph section on culturing the bacteria (line 281) contains a small typo. "…and TSA has the same ingredients the comprise TSB" is probably supposed to say "…and TSA has the same ingredients that comprise TSB".*

**Reply:** This typo has been corrected, and the sentence now contains the word “that” instead of “the”.

*5. The first sentence of the second paragraph of section 2.3 (line 318) says "To increase sterility". Sterility means that something is free of viable microorganisms, so technically, one cannot "increase" sterility. Did the authors mean that this procedure increases the likelihood that the device would remain sterile, or perhaps to ensure that the tubing is not contaminated? Alternatively, authors may wish to use "maintain" or "promote" in place of "increase".*

**Reply:** Thank you for your insight on this misuse of vernacular regarding sterility! The word increase in the note of section 2.3 was changed from “increase” to “promote”, as per your suggestion.

*6. In step 4.2.3, the authors describe what to do after punching through the SiN window. Can the authors add a sentence or two that explains how one knows whether they have punched through the window? If this cannot be described in 1 or 2 sentences, perhaps they can reference a prior publication that explains how one knows that punch-through occurred.*

**Reply:** A sentence was added to the end of step 4.2.3 to describe how a ToF-SIMS operator can know when the window has been punched through.

**“**After punch-through, the counts will increase significantly within the depth profiling region. After stabilizing, this can be referred to as the high-intensity region.”

*7. The first 4 paragraphs of the REPRESENTATIVE RESULTS section describe the results that are shown in figures 1 and 2 (lines 557 - 597). I do not understand why paragraphs 6 & 7 of the same section (lines 599 - 619) re-summarize the results shown in figures 1 and 2. Are paragraphs 6 & 7 of the REPRESENTATIVE RESULTS section actually figure captions, and not text that will appear in the REPRESENTATIVE RESULTS section?*

**Reply:** Sorry for the confusion. Paragraphs 6 & 7 are actually figure captions, and not text which will appear in the representative results section.

**Additional Comments to Authors:**

N/A

# Reviewer #2:

**Manuscript Summary:**

*The manuscript describes a method for growing a biofilm for in situ time-of-flight secondary ion mass spectrometry for chemical mapping in its natural hydrated state, enabled by a microfluidic reactor, System for Analysis at the liquid Vacuum Interface. Also, Shewanella oneidensis MR-1 mutated to express green fluorescence protein was used as a model to illustrate the process.*

*This manuscript presents good results, however, in my opinion this study needs more clarifications of S. oneidensis MR-1.*

**Reply:** Thanks for your comments!

The third paragraph of the introduction introduces *S. oneidensis* MR-1 as our model bacteria for the experiment:

“*S. oneidensis* MR-1 mutated to express green fluorescence protein (GFP) was chosen as a model organism for this biofilm procedure illustration due to its metabolic versatility and common use in environmental and applied microbiology, which was based heavily on its unique capability for metal reduction and extracellular electron transfer.9-11 Additionally, the presence of GFP allowed for easy continuous biofilm-thickness monitoring through fluorescence microscopy, using a fluorescein isothiocyanate (FITC) filter. Our previous studies have shown evidence of this bacteria favoring attachment to the SiN window using *in operando* fluorescence imaging for biofilm growth to a thickness of up to one hundred micronmeters.4,12”

Some words were added to the first sentence to provide further clarification for our use of S. oneidensis as our model bacteria for the experiment, as per your suggestion, these are highlighted in orange. We simply chose this bacteria to demonstrate the attachment and biofilm growth within the SALVI in this protocol, and those following the protocol are likely to use other bacteria for growth within the SALVI.

*Furthermore, more limitations and alternative approaches need to be discussed.*

**Reply:** Thank you for your suggestion! More limitations to the experiment have been added as a second paragraph within the Discussion section.

*Line 121,189, 665: For instance, "nanowires" media needs to be clarified in detail because it is not widely known as the medium of Geobacter. Recent work has shown S. oneidensis MR-1 nanowires are not pili (Lovley DR. Energy Environ Sci 2011, 4: 4896-4906), but extensions of the outer membrane and periplasm that appear to form from chains of outer membrane vesicles (McCormick et al., Energy Environ. Sci., 2015, 8, 1092-1109)*

**Reply:** *(McCormick et al., Energy Environ. Sci., 2015, 8, 1092-1109)* was cited and further clarification of both composition and use was added for “nanowires” media within the third paragraph of the introduction, where the media is first mentioned.

*“*The composition of “nanowires” medium has been specially formulated for the growth and for monitoring of extensions of the membrane and periplasm of *S. oneidensis* that appear to take the shape of small wires, and the medium composition has been established within previous research.13,14”

*Line 238-240: Additionally, OD600 is not accurate enough for quantification, direct cell counting is suggested.*

**Reply:** We have developed a growth curve which associates the growth of *Shewanella* directly from OD600 to the cell count, and have proven the result is the same with this growth curve regardless whether OD600 or cell count is used in this particular experiment. Further, successful experiments have been published with the use of OD600 for growth curves correlating to exact counts of CFU, e.g. Peñuelas-Urquides, Katia et al. “Measuring of Mycobacterium Tuberculosis Growth. A Correlation of the Optical Measurements with Colony Forming Units.” Brazilian Journal of Microbiology 44.1 (2013): 287–289. PMC. Web.

A discussion point has been added to the limitations (2nd) paragraph within the discussion:

“Lastly, cell counts can be done in lieu of OD600 readings for growth curve determination. For example, studies have shown direct and consistent correlation of cell counts to OD600 readings.[21](#_ENREF_21) Therefore, OD600 is deemed sufficient in evaluating biofilm growth.”

**Major Concerns:**

N/A

**Minor Concerns:**

N/A

**Additional Comments to Authors:**

N/A

# Reviewer #3:

**Manuscript Summary:**

*This manuscript entitled "In Situ Characterization of Shewanella oneidensis MR1 Biofilms by SALVI and ToF-SIMS" described a protocol of analyzing a hydrated biofilm via the "SALVI" tool. The method could be expanded and potentially suitable for other many in situ analyses within the hydrated state. The detailed description of each of these steps clearly rebuilt the whole experiment. In sum, the manuscript seems to be suitable for publication.*

**Reply:** Thanks for your comments!

**Major Concerns:**

N/A

**Minor Concerns:**

*The layout in the last two pages was confusing.*

**Reply:** The discussion section, which comprises the last two pages, was revised and reconstructed following the Editor’s suggestion, and is now comprised of paragraphs which follow these points:1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. Hopefully, this reconstruction of the discussion section is easier to follow than its previous organization.

**Additional Comments to Authors:**

N/A