Journal of Visualized Experiments

In Situ Characterization of Shewanella oneidensis MR1 Biofilms by SALVI and ToF-SIMS

--Manuscript Draft--

Manuscript Number:	JoVE55944R1
Full Title:	In Situ Characterization of Shewanella oneidensis MR1 Biofilms by SALVI and ToF-SIMS
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	SALVI, ToF-SIMS, biofilm, in situ, molecular imaging, microfluidics
Manuscript Classifications:	92.23.1: chemical analysis techniques; 95.51.26: microbiology
Corresponding Author:	Xiao-Ying Yu, Ph.D. Pacific Northwest National Laboratory Richland, WA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	xiaoying.yu@pnnl.gov
Corresponding Author's Institution:	Pacific Northwest National Laboratory
Corresponding Author's Secondary Institution:	
First Author:	Rachel Komorek
First Author Secondary Information:	
Other Authors:	Rachel Komorek
	Wenchao Wei
	Xiaofei Yu
	Eric Hill
	Juan Yao
	Zihua Zhu
Order of Authors Secondary Information:	
Abstract:	Bacterial biofilms are surface-associated communities that are vastly studied to understand their self-produced extracellular polymeric substances (EPS) and their roles in environmental microbiology. This study outlines a method to cultivate biofilm attachment to the System for Analysis at the Liquid Vacuum Interface (SALVI) and achieve in situ chemical mapping of a living biofilm by time-of-flight secondary ion mass spectrometry (ToF-SIMS). This is done through the culturing of bacteria both outside and within the SALVI channel with our specialized setup, as well as through optical imaging techniques to detect the biofilm presence and thickness before ToF-SIMS analysis. Our results show the characteristic peaks of the Shewanella biofilm in its natural hydrated state, highlighting upon its localized water cluster environment, as well as EPS fragments, which are drastically different from the same biofilm's dehydrated state. These results demonstrate the breakthrough capability of SALVI that allows for in situ biofilm imaging with a vacuum-based chemical imaging instrument.
Author Comments:	N/A.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a	07-21-2017

certain date, please indicate the date below and explain in your cover letter.	

TITLE:
***In Situ* Characterization of *Shewanella oneidensis* MR1 Biofilms by SALVI and ToF-SIMS**

AUTHORS:
Rachel Komorek
Earth and Biological Sciences Directorate
Pacific Northwest National Laboratory
Richland, Washington
rachel.komorek@pnnl.gov

Wenchao Wei
Earth and Biological Sciences Directorate
Pacific Northwest National Laboratory
Richland, Washington
wenchao.wei@pnnl.gov

Xiaofei Yu
Wiley Environmental Molecular Sciences Laboratory
Pacific Northwest National Laboratory
Richland, Washington
xiaofei.yu@pnnl.gov

Eric Hill
Earth and Biological Sciences Directorate
Pacific Northwest National Laboratory
Richland, Washington
eric.hill@pnnl.gov

Juan Yao
Earth and Biological Sciences Directorate
Pacific Northwest National Laboratory
Richland, Washington
juan.yao@pnnl.gov

Zihua Zhu
Wiley Environmental Molecular Sciences Laboratory
Pacific Northwest National Laboratory
Richland, Washington
zihua.zhu@pnnl.gov

Xiao-Ying Yu
Earth and Biological Sciences Directorate
Pacific Northwest National Laboratory
Richland, Washington

xiaoying.yu@pnnl.gov

CORRESPONDING AUTHOR:
Xiao-Ying Yu
xiaoying.yu@pnnl.gov

KEYWORDS:
SALVI, ToF-SIMS, biofilm, in situ, molecular imaging, microfluidics

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.

LONG ABSTRACT:
Bacterial biofilms are surface-associated communities that are vastly studied to understand their self-produced extracellular polymeric substances (EPS) and their roles in environmental microbiology. This study outlines a method to cultivate biofilm attachment to the System for Analysis at the Liquid Vacuum Interface (SALVI) and achieve *in situ* chemical mapping of a living biofilm by time-of-flight secondary ion mass spectrometry (ToF-SIMS). This is done through the culturing of bacteria both outside and within the SALVI channel with our specialized setup, as well as through optical imaging techniques to detect the biofilm presence and thickness before ToF-SIMS analysis. Our results show the characteristic peaks of the *Shewanella* biofilm in its natural hydrated state, highlighting upon its localized water cluster environment, as well as EPS fragments, which are drastically different from the same biofilm's dehydrated state. These results demonstrate the breakthrough capability of SALVI that allows for *in situ* biofilm imaging with a vacuum-based chemical imaging instrument.

INTRODUCTION:
Bacterial biofilms are surface-associated communities which have evolved over time as a defense for bacteria to survive varying adverse physical and mechanical stimuli, wherein cells are able to attach and survive in many possible environments.[1,2] Biofilms are vastly investigated and have applications in many fields such as biomedicine, biomedical engineering, agriculture, and industrial research and development.[1,2] Understanding the chemical mapping of these complex microbial communities, including their self-produced extracellular polymeric substances (EPS) and their local water-cluster environment, is essential to gaining an accurate and detailed depiction of their biological activities.[2]

Biofilms exist and grow within a highly hydrated state. This presents a great challenge in using vacuum-based surface analysis techniques such as time-of-flight secondary ion mass spectrometry (ToF-SIMS) due to the difficulty in studying volatile liquids in vacuum. As a result, vacuum-based surface analysis techniques have been limited almost exclusively to studying biofilm samples at only their dried state. However, studying a biofilm in its dried state inhibits

the accurate investigation of its true biological microenvironment. It often causes drastic changes to the EPS integrity and biofilm morphology, which has been demonstrated after comparing dry biofilm mass spectral results to *in situ* liquid studies.[3,4] This article presents a solution for studying biofilms within their natural hydrated state by employing the use of our System for Analysis at the Liquid Vacuum Interface (SALVI)[5,6], a microfluidic reactor that contains liquid under its thin silicon nitride (SiN) membrane in a microchannel made of polydimethylsiloxane (PMDS), thus providing direct access to the secondary ion probe beam while still maintaining the structural integrity of the liquid matrix within a vacuum chamber.[7,8]

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 micrometers.[4,12] While this paper will only discuss the confirmation of biofilm's presence through fluorescence microscopy, the SALVI is compatible with other optical imaging methods such as super-resolution fluorescence imaging (i.e., structured illumination microscopy (SIM)[9]) and confocal laser scanning microscopy (CLSM) imaging[4]). Optical imaging can serve to measure the biofilm thickness, and obtain a 3D image of, the shape of the biofilm as it appears, confirming its thickness and its attachment to the SiN window.[9] While GFP was used in the SIMS analysis, *S. oneidensis* without GFP was used for the growth curve, as this only required measurement of optical density and did not require any fluorescent imaging. Generally, the difference between the GFP tagged and untagged species in the growth curve is insignificant. Additionally, while this protocol uses *S. oneidensis* MR-1 GFP as a model organism to describe the procedure, this procedure is designed for any bacterial strain that may be needed for cultivation within SALVI. Although, given knowledge of the bacterial strain needed, some growth conditions such as time, temperature, and oxygen environment may need to be modified to accommodate the strain of bacteria to be used. For growth medium, this procedure uses "nanowires" medium, tryptic soy broth (TSB) without dextrose, and tryptic soy agar (TSA) without dextrose for culturing. 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]

Our previous protocol on *in situ* liquid ToF-SIMS has illustrated the benefit that SALVI has to offer for protein immobilization and attachment to the SiN, as well as a detailed protocol on ToF-SIMS analysis and data reduction.[12] Rather than reiterate data reduction steps, this paper will serve to instead focus on the unique approach of setting up and cultivating biofilms within our SALVI microchannel, as well as the imaging steps to detect biofilm presence and thickness prior to ToF-SIMS analysis. While biofilms have been previously limited to only dried samples within the chamber of vacuum-based surface analytical techniques, detailed EPS and biofilm chemical mapping of live biofilms can now be obtained *in situ* because of this new capability.

PROTOCOL:

1. Preparation of Materials

1.1) Preparation of Medium Tubing

1.1.1) Serum Bottles (one needed per biofilm culture and three needed per growth curve)

Note: As mentioned in the introduction, any growth medium suitable to provide the nutrients needed for the strain of bacteria of interest can be utilized for this procedure; in this case, "nanowires" media and TSB without dextrose medium was used for the growth of *S. oneidensis* MR-1 GFP.[13]

1.1.1.1) Deposit 20 mL of growth medium into one 70 mL serum bottle, cap the stopper and crimp the bottle. Cover the top with a piece of clean sterile aluminum foil.

1.1.1.2) Autoclave the bottle with a liquid cycle for 30 min at 121 °C. Afterward, store the sterilized serum bottle at room temperature.

1.1.2) Anaerobic Culture Tubes

1.1.2.1) Deposit 5 mL of growth medium into an anaerobic culture tube with air headspace, put on the stopper and crimp the bottle. Cover the top with sterile foil.

1.2) Preparing Bubble Trap Tubing

1.2.1) Using a 22 gauge needle, carefully punch a hole through a serum bottle rubber stopper.

1.2.2) Cut 20 in of 1/32" polytetrafluoroethylene (PTFE) tubing with a razor such that one end of the segment of tubing is pointed.

1.2.3) Using the pointed end, thread the PTFE tubing through the hole in the top portion of the rubber stopper. Push the tube through until roughly 2 cm is through the stopper and cut the pointed end of the PTFE tube with a razor to have a flat end.

1.2.4) 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.

1.2.5) Wrap the bubble trap (the PTFE tubing, rubber stopper, and syringe) made in step 1.2.4 with aluminum foil and secure with autoclave tape. Autoclave the foil package to sterilize the tubing with a gravity cycle at 121 °C for 30 min. Store the sealed foil package at room temperature until ready for use in step 2.4.

1.3) Bacterial Growth Curve

Note: This protocol uses *S. oneidensis* MR-1 GFP as a model organism for growing the biofilm in SALVI. However, this procedure can be adapted to accommodate other bacteria growing aerobically or anaerobically. Depending on which strain is used, growth time and environment may need to be adjusted accordingly.

Note: -80 °C bacterial freezer stocks consisted of a 1:1 mixture of TSB without dextrose and glycerol. The growth curve example shown in **Figure 1B** was prepared using a starter culture of TSB with no dextrose, transferred in respective 0.2 mL quantities three times to 70 mL serum bottles with 20 mL "nanowires" medium to remove traces of TSB. However, this protocol assumes that only one growth medium will be used, and that subsequent transfers will not be conducted, as this was done with these particular medium solutions for *S. oneidensis*. Although not outlined in this protocol, extra transfers like this are recommended as initially depositing the bacteria to the rich TSB helps the frozen cells recover; and three subsequent transfers to "nanowires" ensures that all TSB is gone and that typical growth dynamics are occurring.

1.3.1) Inoculating the Starter Culture

1.3.1.1) Remove the bacterial glycerol stock from the -80 °C freezer and warm the stock with a gloved hand to thaw as quickly as possible. Move this freezer stock to the biological safety cabinet (BSC).

1.3.1.2) Within the BSC, use a 1 mL syringe with a 22G needle attached to transfer 0.1 mL of freezer stock to a capped and crimped anaerobic culture tube containing 5 mL of growth medium with no dextrose, prepared in step 1.1.2. Dispose of the remaining freezer stock in the appropriate biological waste container, as freezing again could shock the cells.

1.3.1.3) Incubate the starter culture in a shaker/incubator at 30 °C at 150 rotations per minute (rpm) and be sure to take an optical density at 600 nm (OD_{600}) reading when ready for use. Record the time of growth and OD_{600} such that it can be done at that same time for every subsequent use, such that results are reproducible. As an example, this starter culture was allowed to grow for 15 h and used at an OD_{600} of approximately 1.0.

1.3.2) Inoculating Growth Medium

1.3.2.1) Take the three serum bottles prepared in step 1.1.1.2, and the starter culture prepared in 1.3.1.3, and bring both to the BSC.

1.3.2.2) Within the BSC, using a sterile 1 mL syringe with a 22G needle attached, transfer 0.1 mL of solution from the starter culture to each of the serum bottles, respectively.

1.3.2.3) Sterilize the top of the serum bottles with 70% ethanol and cover with sterilized

aluminum foil, label appropriately, and transfer to an orbital shaker within an incubator. Set the orbital shaker to 150 rpm and set the incubator to 30 °C. Incubate until the first OD_{600} data point is taken, within step 1.3.3.

1.3.3) Obtaining OD_{600} Data Points

1.3.3.1) Prepare a blank by depositing 100 µL of filter-sterilized distilled deionized (DI) water into a sterile cuvette. Wrap plastic paraffin film around the top of the cuvette so that the water will not be contaminated. Store at room temperature. Use this blank with the UV/Vis Spectrophotometer before taking any data points for the growth curve by inserting the cuvette and pressing "blank".

Note: Every 48 h a new blank should be prepared to avoid incorrect reading, as regularly replacing a blank is good practice.

1.3.3.2) For each OD_{600} data point, remove the serum bottles prepared in step 1.3.2.3 from the incubator and transfer to a sterilized BSC. Take 0.1 mL of the inoculated medium from each serum bottle and deposit into three separately labeled sterile cuvettes.

1.3.3.3) After blanking, insert cuvette containing the culture into the ultraviolet visible (UV/Vis) Spectrophotometer and read the OD_{600} one at a time, and record all three readings for that time point.

Note: For *S. oneidensis* MR-1, data points were taken at 1, 14, 28, 32, 41, 57, 81, and 105 h after inoculation. The growth is complete when the bacteria has completed the death phase. These points should be adjusted accordingly if there is lack of knowledge on the particular growth time and tendency of bacteria used in this protocol. If there is uncertainty about the growth tendency, data points should be collected more frequently, for example, every three h for the first 12 h, every six h for subsequent 36 h, at 12 h intervals for the following 100 h, and at 24 h intervals for the final 124 h.

1.3.3.4) Using the OD_{600} data points as the y-axis and the time as the x-axis, graph a curve of the average of the three points taken with standard deviation error bars for each time point using a graphing software. The *S. oneidensis* MR-1 growth curve is displayed in **Figure 1B** in the representative results section.

1.3.3.5) Using the graph prepared in step 1.3.3.4, identify the timeframe of the log-phase section of the growth curve. For *Shewanella*, this is between 12 and 33 h of growth; therefore it can be inferred that at 24 h of growth, *Shewanella* will be within its log-phase of growth, assuming that the bacteria will be cultured using the same media and treatment before use that was used for the growth curve.

2. Culturing the Bacteria

2.1) Day One: Inoculating the Agar Plate

Note: This section of the procedure was used with an agarose plate to take one colony-forming unit (CFU) at log-phase, instead of the liquid starter culture used for the growth curve. This could be assumed to reproduce the same growth conditions, due to the fact that TSA without dextrose was used and subsequently transferred to “nanowires” medium in the growth curve procedure, and TSA has the same ingredients that comprise TSB.

2.1.1) Remove *S. oneidensis* MR-1 GFP bacteria stock from -80 °C freezer and place into an ice bucket, place this bucket inside of sterilized BSC.

2.1.2) Within the BSC, use a sterile 1 µL inoculation loop to scrape the surface of the frozen bacteria stock and use the loop to T-streak the surface of an agarose plate.

2.1.3) After sealing the sides of the plate with plastic paraffin film, invert the plate and store in a 30 °C incubator for 24 h, until individual colonies appear.

2.2) Day Two: Inoculating the Serum Bottle

2.2.1) Remove the plate from the incubator and open within the BSC.

2.2.2.) Within the BSC, clean the surface of the rubber stopper on a prepared serum bottle from step 1.1.1 with 70% ethanol in DI water.

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 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.

2.2.4) Using the same syringe, extract the liquid from the surface of the plate.

2.2.5) After tapping out bubbles within the syringe, inject the liquid into the serum bottle, and place onto an orbital shaker within 30 °C set at 150 rpm for 24 h.

Note: Tapping the bubbles out of the syringe is important in order to avoid introducing more oxygen to the serum bottle, as introducing more oxygen to the bottle can change experimental conditions and reduce consistency between growth times for bacteria.

2.3) Day Two: Sterilization of the SALVI Microchannel

Note: To promote sterility of the tubing system, steps that require detaching the syringe from the tubing and replacement with a new syringe should be done within the BSC. To do this,

simply detach the syringe from the syringe pump by unscrewing the metal syringe holder, and bring the syringe with tubing attached to a sterile BSC. When tubing system is mentioned in the procedures, this refers to the syringe containing the liquid reservoir, attached with a polyetheretherketon (PEEK) injector to the PTFE tubing, attached to the drip chamber, which has a PEEK injector fitting attached to the SALVI inlet tubing, as well as the outlet container that the SALVI outlet tubing is sealed to.

2.3.1) Use a new SALVI device and attach a PEEK fitting and injector to one end of a PTFE tubing.

Note: SALVI devices are prepared fresh for each experiment following the device fabrication detailed in previous research and patents.[5,6,8,15]

2.3.2) Take 2 mL of 70% ethanol into a syringe and connect to the PEEK fitting on the SALVI. After connecting the syringe to a syringe pump and attaching the outlet of the SALVI to a waste bottle, allow the ethanol DI water solution to run through the SALVI at 20 µL/min for 1.5 h.

2.3.3) Take 4 mL of sterilized DI water into a syringe and connect to the inlet of the same SALVI. After connecting the syringe to a syringe pump, allow the water to run through the SALVI at 20 µL/min for at least 3 h.

2.3.4) Within a BSC, open the foil packet prepared in step 1.2.5 and connect a sterile PEEK injection fitting to the end of the 5 mL syringe and sterile PEEK fittings to the end of the TFE tubing. Take ~ 3 mL of sterile medium into a syringe and attach to the TFE tube. Invert the 5 mL drip chamber and, using a sterile syringe, inject the growth medium into the drip chamber until it reaches a total volume of 1 mL.

2.3.5) Connect the end of the 5 mL syringe drip chamber to the inlet of the SALVI.

2.3.6) Take 10 mL of growth medium into a sterile syringe and connect to the inlet of the drip tubing. After connecting the syringe to a syringe pump, allow the medium to run through the SALVI at 20 µL/min for 12 h (or overnight). Use adhesive tape to secure these parts. Cover the outlet bottle with foil or plastic paraffin film to minimize the probability of dust particles and organisms contained therein to contaminate the medium. A detailed depiction of how this setup should look can be found in **Figure 1A**.

2.3.6.1) Allow the drip tubing to be vertical, meaning that the syringe pump should be placed on an elevated surface with drip tubing secured with tape, and with the SALVI secured on a flat surface below.

2.3.6.2) Run medium through the SALVI for 12 h to ensure that all traces of ethanol have been removed from the microchannel and tubing of the SALVI before inoculating with bacteria.

2.3.6.3) For added protection, keep the SALVI microchannel chamber within a sterilized Petri

dish, with the sides cut to fit the inlet and outlet tubing. Additionally, keep tape always on the window to protect the SiN membrane and channel prior to imaging analysis.

2.4) Day Three: Inoculation of the SALVI Microchannel

2.4.1) Remove the whole tubing system (syringe connected to drip chamber connected to SALVI connected to the waste bottle) from the syringe pump and bring it to a sterilized BSC. Additionally, remove the serum vial from step 2.2.5 from the incubator and bring it to the BSC.

2.4.2) Clean the surface of the serum bottle stopper with 70% ethanol to prevent any contamination, and then use a sterile syringe with an attached sterile 22G needle to extract 4 mL of bacteria from the bottle.

2.4.3) Detach the SALVI from the 5 mL chamber of the drip tubing, and connect the syringe with inoculated medium directly to the inlet of the SALVI. Leave the drip tubing within a sterile aluminum foil packet or within the BSC.

2.4.4) Attach the syringe with SALVI and outlet bottle to the syringe pump to run at 20 µL/min for 3 h to inoculate the channel of the SALVI, in order to allow for multiple volume changes of the liquid contained within SALVI.

2.4.5) After inoculation, disconnect the syringe with SALVI from the syringe pump and bring to the BSC. After attaching the inlet of the SALVI back to the 5 mL drip chamber from step 2.4.3, take 20 mL of growth medium into a sterile syringe and attach to the inlet of the drip tubing.

2.4.6) Bring the tubing attached to the SALVI from the BSC to the syringe pump and allow the medium to pass through the tubing at a rate of 2 µL/min for six to ten days, or until fluorescence imaging (discussed in step 3) shows favorable visible biofilm growth for ToF-SIMS analysis. Refill fresh medium as it runs out within the BSC by filling a new sterile syringe with 10mL of growth medium and attaching to the SALVI after the previous growth medium has run out.

Note: After beginning inoculation at 2 µL/min, the flow rate should never be increased or decreased, as changing the flow rate would create shear-stress within the channel and detach the biofilm. It is critical that this flow rate should never be changed; to prepare for this, ~24 h before SIMS, observe the tubing closely to make sure there are no bubbles so that there is no need to push more medium at a faster rate throughout the tubing.

2.4.6.1) Avoid bubbles in the microchannel, as they can push the biofilm out of the channel. It is important to be cautious of bubbles within the bottom of the 5 mL drip tubing where it connects to the SALVI tubing. Fresh medium can be injected into the end of the PEEK injector to ensure that no air will be forced into the SALVI.

3. Optical Imaging of the Biofilm within the SALVI Microchannel

3.1) Fluorescence Microscopy Imaging

Note: The *Shewanella* used as a model organism within this protocol is mutated to express GFP; as such, it does not require staining. If the bacteria require staining, this should always be done at the same flowrate (e.g., 2 µL/min) to avoid biofilm detachment. Additionally, when cells go without nutrient availability, they will detach. Therefore, if any additional staining is required, the stain should be injected to the growth medium rather than water before supplying to stain the cells.

3.1.1) Detach the SALVI from the drip tubing inside the BSC and close the SALVI by screwing the PEEK fittings to finger-tighten the PEEK union.

3.1.2) Tape the tubing of the SALVI microchannel chamber securely onto a glass slide, such that the window is completely flat and facing upward. Remove the protective tape from the window. Clamp the slide to the stage of the microscope by placing the glass slide between the stage clamps on the platform.

3.1.3) Lower the stage of the microscope such that the top of the SALVI is positioned close enough to the end of the 10x objective, where adjustment of the focus will not cause the lens to touch the window.

3.1.4) Switch on the backlight and find the channel using the 10x microscope objective by adjusting the focus. At this time, make sure the presence of bacterial attachment to the SiN window of the SALVI is apparent in comparison to the window of a control channel with no inoculated bacteria. For closer imaging of cells, switch to the 20x objective. When compared to an empty SALVI channel, the presence of bacteria attached to the window should have a strong green fluorescence.

3.1.5) Turn off the backlight and turn on the microscope mercury source. Wait 2-3 min and switch to the FITC filter set on the microscope to view and capture images of the biofilm attached to the window. As an example of what the matured biofilm will look like when ready, refer to **Figure 1C**.

3.1.6) Turn off the mercury source and detach the SALVI from the slide. If needed, attach to 2 µL/min medium flow once again to allow for more growth before imaging again, or transfer the SALVI to secondary containment use for ToF-SIMS analysis. This is needed if biofilm thickness is concluded to not be thick enough, and more time for growth is required before ToF-SIMS analysis. To attach to medium flow once again, refer to step 2.3.6.

Note: If put back under medium flow, fluorescence imaging should be done again before ToF-SIMS analysis.

3.1.6.1) Give the biofilm growth medium to feed it until ToF-SIMS analysis can be conducted.

While not recommended, if needed, the closed SALVI can be stored within 4 °C overnight, but should be allowed to warm to room temperature before inserting to the vacuum chamber of the ToF-SIMS. However, analyze the biofilm immediately after detached from medium flow to reduce biofilm detachment.

4. ToF-SIMS Data Acquisition

Note: This section serves as an overview, and is discussed in greater detail within our earlier protocol describing adsorbed protein molecule liquid SIMS analysis.[12]

4.1) Install SALVI into the ToF-SIMS Loadlock Chamber

Note: Gloves should be worn at all times when handling the SALVI device and installing it onto the ToF-SIMS stage to avoid potential contaminations during surface analysis.

4.1.1) Mount SALVI onto the stage and fix it with several screws. Make sure that the SiN window is flat by adjusting screw tightness and inserting silicon wafer pieces at the bottom of the PDMS microchannel chamber. Remove the protective tape from the window, then load the stage into the ToF-SIMS loadlock chamber.

4.1.2) Open the ToF-SIMS loadlock, hold and set the stage horizontally onto the loading platform, and close the loadlock door. Initiate the vacuum pump and wait to ensure that suitable vacuum is reached.

4.1.3) Move the SALVI into the main chamber once the vacuum is stabilized to $1x10^{-7}$ mbar.

4.2) Depth Profiling and Collecting Data Points

4.2.1) Find the microfluidic channel using the optical microscope equipped in ToF-SIMS.

4.2.2) Select positive or negative mode before data acquisition. Use the 25 keV Bi_3^+ beam as the primary ion beam in all measurements, and use the electron flood gun to neutralize the surface charging during all measurements.

4.2.3) Scan the Bi_3^+ beam with 150 ns pulse width on a round area with a diameter of ~2 μm with 64 pixels by 64 pixels resolution. After punching through the 100 nm SiN window, continue to scan for another 150 s to collect high intensity data for imaging. 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.

4.2.4) Reduce the pulse width to 50 ns for data collection to acquire spectra with better mass resolution. Continue this acquisition for about another 200 s.

4.2.5) Repeat these steps for collecting at least three positive and three negative data points.

Note: Be sure to space the punch-through areas such that the SiN window will not break and leak into the chamber of the vacuum from compromised window integrity.

5. ToF-SIMS Data Analysis
5.1.) Mass Calibration using IonToF Software

5.1.1) Open the analysis software of ToF-SIMS (Measurement Explorer) and then click the “profiles", "spectra" and "image" buttons, respectively, to process depth profile, m/z spectrum, and image data.

5.1.2) Open the data files obtained throughout ToF-SIMS data acquisition.

5.1.3) Select the depth profiling data of interest and reconstruct the spectra according to the depth profile temporal series.

5.1.2) Press “F3” to open the “mass calibration” window. Choose peaks for calibration based on chemical compounds which are expected to exist in the specific sample.

5.2) Peaks of Interest Selection

5.2.1) As peak selection is necessary for sample analysis, determine characteristic peaks of the sample according to literature or other previous findings, if any. Compare the intensity of peaks of interest in the m/z spectra. If necessary, add new peaks or delete interference peaks in the peak list.

5.2.2) Click on a peak, find the red lines at the left bottom window. Move the red lines to enclose the whole peak.

5.2.3) Select a peak matching the chemical formula in the main spectrum window, and then to click the “add peak” button above the window.

5.3) Export Mass Calibrated Data

5.3.1) To export a peak list, in the “Peak List” menu, select “Save…” the peak list in the “itmil” format.

5.3.2) To export a depth profile, in the “Profile” window, click on the “File” menu, then select “Export”, and then select “Save as” a “.txt” file.

5.3.3) To export an m/z spectrum file, in the “Spectra” window, click on the “File” menu, then select “Export”, and then select “Save as” a “.txt” file.

5.3.3) To export an image file, in the “Image” window, print screen and save as the image file.

6. ToF-SIMS Data Plotting and Presentation

6.1) Use a graphic tool to import the data.

6.2) Make a plot using the m/z as the x-axis and peak intensity as the y-axis to show the reconstructed spectrum. An example is given in **Figure 2A**.

6.3) Combine reconstructed two-dimensional (2D) images of different m/z and form a matrix to show either positive or negative ion mapping. An example is given in **Figure 2B**.

REPRESENTATIVE RESULTS:

These representative results serve to show how the chemical profile of the attached biofilm can be identified and interpreted, as obtained through ToF-SIMS. After plotting mass spectra from ToF-SIMS data acquisition, highlighted briefly in the procedures section, peak identification should be conducted in order to assign identities to each respective m/z value. This can be done through extensive literature review on mass spectrometry on bacteria and specific chemical fragments that are expected to be present within the bacteria studied, such as various water clusters, fatty acids, and protein fragments.[16-20] These representative results only show the negative mass spectra as obtained by the *S. oneidensis* MR-1 biofilm and a DI water sample. Interpretation of positive spectra follow a similar procedure.

Figure 1A shows the schematic setup according to this protocol when cultivating a biofilm in the SALVI microchannel. On the top left, the syringe is attached to a syringe pump, which keeps medium flowing at a constant rate throughout the PFTE tubing system and inside the microchannel. The syringe pump is placed above the drip chamber, which prevents backwards contamination to the medium reservoir to prevent motile organisms from swimming up-stream. The medium flows from the drip chamber reservoir directly into the PFTE tubing of the SALVI, which passes through the microchannel and through the outlet tubing to a sealed outlet bottle. The dotted lines point from the SiN window to an image of a matured *S. oneidensis* MR-1 GFP cells captured by fluorescence microscopy, detailed in step 3.1. **Figure 1B** shows an example of a growth curve established using the model organism for this protocol, *S. oneidensis* MR-1. From the growth phases in this graph, it can be concluded that the log-phase of growth occurs between 15-32 h in these specific cultivation conditions. Additional information from this graph shows that 0-15 h is the lag phase of growth, and 33-105 h represents the stationary phase of growth. **Figure 1C** is an image acquired with fluorescence microscopy showing an example of a matured biofilm.

Figure 2A shows the negative mass spectra of a hydrated *S. oneidensis* MR-1 biofilm within the microfluidic channel, as obtained by *in situ* liquid ToF-SIMS. This graph shows an example of the selected mass range (m/z 100-350) comparison between the biofilm of *S. oneidensis* MR-1 and DI water, the latter was obtained as a system control. **Figure 2B** displays a comparison of 2D images of peaks of interest in the MR-1 biofilm and DI water obtained by ToF-SIMS. Once interesting m/z values can be identified from studying the mass spectra, potential peak identification of m/z values are properly attributed to chemical fragments, as supported by the IonToF SIMS software library and literature survey. Peaks of interest in **Figure 2A** include water

clusters (i.e., m/z 107 $(H_2O)_5OH^-$, 125 $(H_2O)_6OH^-$, 143 $(H_2O)_7OH^-$, 161 $(H_2O)_8OH^-$, 179 $(H_2O)_9OH^-$, 197 $(H_2O)_{10}OH^-$, 215 $(H_2O)_{11}OH^-$, 233 $(H_2O)_{12}OH^-$, 251 $(H_2O)_{13}OH^-$, 269 $(H_2O)_{14}OH^-$, 287 $(H_2O)_{15}OH^-$, 323 $(H_2O)_{17}OH^-$, 341 $(H_2O)_{19}OH^-$))[9], as denoted by red lines above respective peaks, quorum sensing related compounds biomarkers (i.e., m/z 175 $C_{10}H_9NO_2^-$), EPS byproducts (m/z 123 $C_2H_4PO_4^-$, 159 $P_2O_8H^-$, 216 $Cr_2O_7^-$, 285 octadecanoethiols, 325 polar compounds, C_{12} Polar compounds)[15,16,18], and fatty acid chain fragments (i.e. m/z 127 $[C_2H_3(CH_2)_3COO]^-$, 255 C16:0 fatty acid, 279 linoleic acid, $C_{18}H_{31}O_2^-$, 325 $C_{21}H_{40}O_2^-$, 341 surface lipids, 18-MEA bound through thioester linkage, stearic acid $C_{18}H_{35}O_2^-$, ions of monoacylglyceryls of palmitic acid $C_{19}H_{17}O_6^-$).[16,19]

Since the biofilm is hydrated, it is not surprising that some peaks seen in the biofilm sample are similar to those found in DI water. These water cluster peaks are marked with a red line above each peak in **Figure 2A**, including m/z 107, 125, 143, 161, 179, 197, 215, 233, 251, 269, 287, 323, and 341 and corresponding to $(H_2O)_5OH^-$ $(H_2O)_6OH^-$ $(H_2O)_7OH^-$ $(H_2O)_8OH^-$ $(H_2O)_9OH^-$ $(H_2O)_{10}OH^-$ $(H_2O)_{11}OH^-$ $(H_2O)_{12}OH^-$ $(H_2O)_{13}OH^-$ $(H_2O)_{14}OH^-$ $(H_2O)_{15}OH^-$ $(H_2O)_{17}OH^-$ $(H_2O)_{19}OH^-$, respectively.[9] As there is prevalence of many characteristic water clusters in this mass spectrum, this results provides a strong evidence of the chemical water cluster environment present in a hydrated biofilm. Additionally, non-water cluster related peaks observed in both spectra can commonly be attributed as interference peaks, typically from the PDMS, a component of the microfluidic channel. For example, one common known interference peak from PDMS is the m/z 137 in the negative ion mode.

When comparing the biofilm SIMS mass spectra to that of DI water, as shown in **Figure 2A**, some peaks are not present in the DI water, yet they appear in the biofilm. For example, the peak at m/z 255 ($[CH_3(CH_2)_{14}COO]^-$, palmitic acid) is present at high intensity in the biofilm sample, but not at all in the DI water sample. This would suggest that the signals come from in the biofilm, most likely the biofilm and/or its self-generated EPS. Many interesting peaks were identified in **Figure 2A**. For example, EPS related peaks include m/z 123$^-$, found to be $C_2H_4PO_4^-$, 159 ($P_2O_8H^-$), 216 (Cr_2O7^-), 285 (octadecanoethiols), and 325 (polar compounds, C_{12} Polar compounds).[17,18,20] Peaks associated with fatty acids were found to be 127 ($[C_2H_3(CH_2)_3COO]^-$), 255 ($[CH_3(CH_2)_{14}COO]^-$, palmitic acid), 279 ($[CH_3(CH_2)_{15}COO]^-$, linoleic acid), 317 ($C_{21}H_{33}O_2^-$), 325 ($C_{21}H_{40}O_2^-$), and 341 (Surface lipids, 18-MEA bound through thioester linkage, stearic acid $C_{18}H_{35}O_2^-$, ions of monoacylglyceryls of palmitic acid $C_{19}H_{17}O_6^-$).[16,19] Lastly, a quinolone signal quorum sensing signal related peak of $C_{10}H_9NO_2^-$ was observed at m/z 175.

Figure 2B displays 2D images of the distribution of four different interesting ions between the *S. oneidensis* MR-1 biofilm (top row) and DI water (bottom row). The m/z value 107 ($(H_2O)_5OH^-$) shows a water cluster of significant intensity within the samples, the m/z value 175 ($C_{10}H_9NO_2^-$) depicts a quorum sensing related signal, m/z 255 is a fatty acid ($[CH_3(CH_2)_{14}COO]^-$, palmitic acid), and m/z 341 ($C_{21}H_{41}O_2^-$) may represent both lipid fragments and water clusters due to the 1 amu mass accuracy.[9] Brighter regions of the 2D pictures indicate a higher count of the molecular ion present in the image. As expected, signals for the QS compound, and lipids were far greater in quantity within the biofilm sample. While the signal for the water cluster (m/z 107) was stronger throughout the biofilm sample, it was also present in the DI water sample, as

expected. Lastly, the signal at m/z 341 was found to be homogeneous within the biofilm sample, but very weakly within the DI water sample. While m/z 341 was a water cluster peak, it was also representative of several different fatty acid and surface lipids.[16] Its stronger presence within the biofilm sample after normalization of data suggests that the presence of lipid fragments far outweighs the presence of water clusters.

Figure 1: Experimental Schematic.
(A) Displays the experimental schematic of the biofilm setup as it appears within the laboratory. Starting at the top left, medium flows from the syringe (1), which is attached to the syringe pump (2), controlling the rate at which liquid moves through. Arrows indicate the flow direction throughout the system. The syringe (1) is connected via a PEEK injector fitting (3) to the TFE tubing (4). Medium flows through a drip chamber (5) to avoid contamination, and eventually moves through the SALVI (6) to the sealed outlet container (7). The syringe pump is positioned on an elevated surface (9) such that the drip chamber (5) can be perpendicular to the flat surface (8) that the SALVI (6) is placed upon. **(B)** Growth curve for Shewanella oneidensis MR-1 in "nanowires" medium. Time 0-15 h represents lag phase, time 15-33 h represents log phase, time 33-105 h represents stationary phase, and time 105 h or longer represents death phase. Error bars represent standard deviation. **(C)** A picture of a matured biofilm acquired with fluorescence microscopy.

Figure 2: Comparisons of ToF-SIMS negative spectra of the hydrated *Shewanella oneidensis* biofilm and MR-1 medium in the SALVI microchannel.
(A) The m/z SIMS mass spectra of the *Shewanella oneidensis* biofilm in MR-1 medium and DI water. The latter was used as a control. Red lines indicate locations of characteristic water cluster peaks. **(B)** 2D image comparison of peaks of interest between the biofilm and DI water. From left to right, images display a water cluster (m/z 107 $(H_2O)_5OH^-$), a quorum sensing/hormone signal (m/z 175, $C_{10}H_9NO_2^-$), a fatty acid (m/z 255, $[CH_3(CH_2)_{14}COO]^-$, palmitic acid), and surface lipids/EPS fragments (m/z 341, 18-MEA, $C_{21}H_{41}O_2^-$).

DISCUSSION:
After inoculating at log-phase, it is important to test the number of days and temperature at which the biofilm should grow before it is healthy and thick enough for imaging, as described in step 3.1. This procedure specifically covers culturing a S. oneidensis MR1 biofilm at room temperature; however different room temperatures can influence the rate of growth. Therefore, it is critical to use optical imaging to understand whether the biofilm is ready before proceeding to ToF-SIMS analysis. Similarly, different strains of bacteria require different growth conditions and length to achieve a desirable thickness for analysis. While the growth curve in Figure 1b depicts log phase 200of the bacteria to be 12-32 h, and this was used at 24 h throughout the procedure, it is important to note that this time cannot be used as log-phase for other strains of bacteria without establishing the growth curve independently. While the log phase was between 12 and 33 h of growth for S. oneidensis, 24 h was chosen due to the fact that it was in the portion of growth where oxygen was starting to limit the growth-rate of the organism, towards the end of log-phase. Experiments showed that this was the time cells began to produce nanowires, thus primed to form successful biofilms that could survive in low oxygen

environments.[3,13] Consequently, the time chosen to use the bacteria during log phase should be influenced by research experience and knowledge gained from literature on experimental study of the bacteria being studied. Additionally, a separate growth curve must be established to understand the log-phase for all different strains of bacteria that will be used for biofilm growth using this approach. The growth rate of 2 µL/min was calculated so as not to exceed the maximum growth-rate of S. oneidensis MR-1, and the total time was chosen to get a mature biofilm that was not overgrown and prone to detaching. These rates can be adjusted accordingly to knowledge of different bacteria to be used with this setup.

Some limitations to using SALVI for biofilm growth include biofouling within the channel as well as the probability of bacteria to attach to the flat walls of the channel. Despite growth at a constant rate, recommended to be 2 µL/min within this protocol, biofouling can still occur if the biofilm is allowed to grow for too long. In such a case and without warning, the biofilm can detach from the window and exit the SALVI to the outlet containment. Biofouling can also occur simply by adding mechanical force (i.e., moving) the SALVI, which cannot be avoided. However, this can be kept in check by viewing the attachment of the biofilm to the SiN window before ToF-SIMS analysis under a light microscope. One other limitation includes the smoothness of the PDMS microchannel which can make it difficult for some bacteria to attach. However, this can be changed in the design of the SALVI to accommodate by creating ribbed edges to the channel, if attachment of the bacteria is an issue. Lastly, cell counts can be done in lieu of OD_{600} readings for growth curve determination. For example, studies have shown direct and consistent correlation of cell counts to OD_{600} readings.[21] Therefore, OD_{600} is deemed sufficient in evaluating biofilm growth.

Limitations to this technique are few, as the SALVI essentially acts as a culture dish which has much versatility for use in many applications, such as anaerobically within a glove box, or within any temperature-controlled environmental chamber. Some minor limitations include uncontrollable room conditions such as relative humidity, temperature, placement near sunlight, which are still possible to be accommodated for, for each specific bacteria's optimal growth conditions. In addition, some of these technical challenges can be overcome with an incubator with temperature or RH mediation features in the biofilm setup.

SALVI is a vacuum-compatible microfluidic interface. In this work, a 200x300 µL microfluidic channel was used to culture biofilms followed with optical and liquid SIMS imaging. Liquids present a technical challenge to study using vacuum based techniques, because they are volatile and difficult to retain in the liquid phase in vacuum. Thus vacuum techniques such as ToF-SIMS have been traditionally restricted to only dry and cryogenic samples.[22] Additionally, SALVI can be used for diverse imaging and spectroscopy using a variety of microscopy and spectroscopy techniques.[4,9] Our group has worked to continually expand various SALVI applications in *in situ* analysis of liquids and solid-liquid interfaces.[15,23,24] Our most recent effort has effectively shown bacteria attachment to the SiN membrane.[9] After initial attachment, continual flow of medium over time has been shown to successfully cultivate a biofilm directly on the SIN window of the SALVI. During ToF-SIMS, the primary ion beam is used to bombard holes of 2 µm in diameter. This dimension is similar to the cell length of the biofilm attached to

the SiN window, thus providing a chemical profile of the biofilm in its naturally hydrated microenvironment. This is highly important due to the difference of a biofilm's chemical identity, presence of EPS, and water cluster environment when comparing the biofilms in its hydrated state with dried samples.[9] Without the use of SALVI, ToF-SIMS could only be conducted on dry and cryogenic samples, providing chemical mapping which fails to capture the chemical profile of a sample in its natural hydrated state.

As shown in **Figure 1A**, it is important to keep the syringe pump above the drip tubing in order to allow for the drip tubing to be perpendicular to the SALVI microchannel. Additionally, bubbles will be less likely to become trapped within the tubing of the device, if the outlet PFTE tubing (within the outlet bottle) is lower than the inlet tubing (attached to the drip chamber). Another critical step of cultivating bacteria for SALVI growth is to first obtain a growth curve of the particular bacteria strain that will be used, as described within step 1.3. This can be done by obtaining growth curves with optical density measurements. A growth curve, as shown in **Figure 1B**, is important for understanding the timeframe at which bacteria will be within the log-phase of growth, when it can be assumed that the bacteria are healthiest. Utilizing bacteria within this phase of growth facilitates the creation of a healthy biofilm microenvironment and ensures that the biofilm will grow at its expected rate. The SALVI microchannel is sterilized in room temperature with 70% ethanol and DI water, as it cannot be autoclaved for sterilization prior to use with ToF-SIMS. This is due to the fact that autoclaving can both damage the window of the SALVI and introduce water vapor to the channel. After several days of growth, the microchannel should be checked with fluorescence microscopy to validate bacterial attachment. An example can be found in **Figure 1C**, this image was taken to show a matured biofilm. Due to the flow path of the medium, the bacteria more favorably attaches and grows along the edges of the microchannel, as this location is where flow and shear-forces are lowest.

In summary, SALVI is an excellent method for which to culture and study biofilms using *in situ* chemical mapping, as it allows for providing the living biofilm in its natural hydrated state to the vacuum-based imaging mass spectrometer. This unique approach can provide more information on a biofilm's water cluster microenvironment, as well as to gain a deeper understanding of its EPS byproducts. This information could be used to understand a biofilm's biological activities, and can be utilized in various applications such as in biomedicine, biomedical engineering, agriculture, and industrial research and development.

ACKNOWLEDGMENTS:
We are grateful to the Pacific Northwest National Laboratory (PNNL) Earth and Biological Sciences (EBD) mission seed Laboratory Directed Research and Development (LDRD) fund for support. Instrumental access was provided through a W. R. Wiley Environmental Molecular Sciences Laboratory (EMSL) General User Proposal. EMSL is a national scientific user facility sponsored by the Office of Biological and Environmental Research (BER) at PNNL. The authors thank Dr. Yuanzhao Ding for proof reading the manuscript and providing useful feedback. PNNL is operated by Battelle for the DOE under Contract DE-AC05-76RL01830.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Renner, L. D. & Weibel, D. B. Physicochemical regulation of biofilm formation. *MRS Bull.* **36** (5), 347-355, doi:10.1557/mrs.2011.65 (2011).

2 Flemming, H. C. & Wingender, J. The biofilm matrix. *Nat Rev Microbiol.* **8** (9), 623-633, doi:10.1038/nrmicro2415 (2010).

3 Aldeek, F. *et al.* Patterned hydrophobic domains in the exopolymer matrix of Shewanella oneidensis MR-1 biofilms. *Appl Environ Microbiol.* **79** (4), 1400-1402, doi:10.1128/AEM.03054-12 (2013).

4 Hua, X. *et al.* In situ molecular imaging of a hydrated biofilm in a microfluidic reactor by ToF-SIMS. *Analyst.* **139** (7), 1609-1613, doi:10.1039/c3an02262e (2014).

5 Yu, X. Y., Yang, L., Cowin, J. P., Iedema, M. & Zhu, Z. Systems and methods for analyzing liquids under vacuum. USA patent (2013).

6 Yu, X. Y., Liu, B., Yang, L., Zhu, Z. & Marshall, M. J. Microfluidic electrochemical device and process for chemical imaging and electrochemical analysis at the electrode-liquid interface in situ. USA patent (2014).

7 Yang, L., Yu, X. Y., Zhu, Z. H., Thevuthasan, T. & Cowin, J. P. Making a hybrid microfluidic platform compatible for in situ imaging by vacuum-based techniques. *J Vac Sci Technol A.* **29** (6), doi:Artn 061101.10.1116/1.3654147 (2011).

8 Yang, L., Yu, X. Y., Zhu, Z. H., Iedema, M. J. & Cowin, J. P. Probing liquid surfaces under vacuum using SEM and ToF-SIMS. *Lab Chip.* **11** (15), 2481-2484, doi:10.1039/c0lc00676a (2011).

9 Ding, Y. *et al.* In Situ Molecular Imaging of the Biofilm and Its Matrix. *Anal Chem.* doi:10.1021/acs.analchem.6b03909 (2016).

10 Yang, J., Ghobadian, S., Montazami, R. & Hashemi, N. in *Proceedings of the Asme 11th Fuel Cell Science, Engineering, and Technology Conference, 2013* (2013).

11 Yu, F., Wang, C. X. & Ma, J. Applications of Graphene-Modified Electrodes in Microbial Fuel Cells. *Materials.* **9** (10), doi:10.3390/Ma9100807 (2016).

12 Yu, J., Zhou, Y., Hua, X., Zhu, Z. & Yu, X. Y. In Situ Characterization of Hydrated Proteins in Water by SALVI and ToF-SIMS. *J Vis Exp.* (108), 53708, doi:10.3791/53708 (2016).

13 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).

14 McCormick, A. J. *et al.* Biophotovoltaics: oxygenic photosynthetic organisms in the world of bioelectrochemical systems. *Energy Environ Sci.* **8** (4), 1092-1109, doi:10.1039/C4EE03875D (2015).

15 Liu, B. *et al.* In situ chemical probing of the electrode-electrolyte interface by ToF-SIMS. *Lab Chip.* **14** 855-859, doi:10.1039/C3LC50971K (2014).

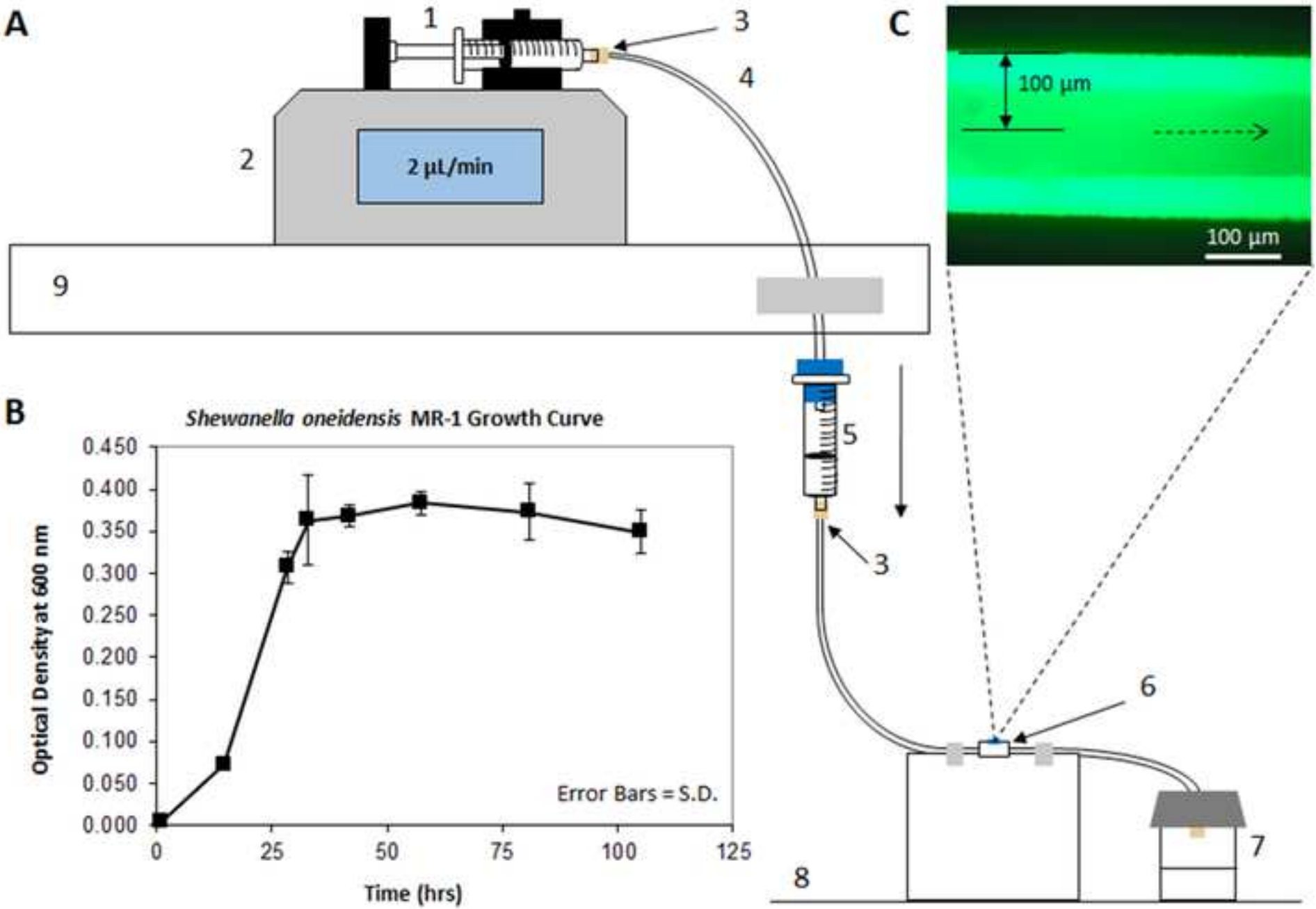
16 Keune, K., Hoogland, F., Boon, J. J., Peggie, D. & Higgitt, C. Evaluation of the "added value" of SIMS: A mass spectrometric and spectroscopic study of an unusual Naples yellow oil paint reconstruction. *Int J mass Spectrom.* **284** (1-3), 22-34, doi:10.1016/j.ijms.2008.10.016 (2009).

17 Lee, M. *Mass Spectrometry Handbook*. 988 (John Wiley & Sons Inc., 2012).

18 Petrovic, M. & Barcelo, D. Determination of anionic and nonionic surfactants, their degradation products, and endocrine-disrupting compounds in sewage sludge by liquid chromatography/mass spectrometry. *Anal Chem.* **72** (19), 4560-4567, doi:Doi 10.1021/Ac000306o (2000).
19 Vickerman, J. C. Molecular Imaging and Depth Profiling by Mass Spectrometry--Sims, MALDI or DESI? *Analyst.* **136** (11), doi:10.1039/c1an00008j (2011).
20 Weng, L. T., Bertrand, P., Stonemasui, J. H. & Stone, W. E. E. Tof Sims Study of the Desorption of Emulsifiers from Polystyrene Latexes. *Surf Interface Anal.* **21** (6-7), 387-394, doi:DOI 10.1002/sia.740210611 (1994).
21 Peñuelas-Urquides, K. *et al.* Measuring of Mycobacterium tuberculosis crowth. A correlation of the optical measurements with colony forming units. *Braz J Microbiol.* **44** (1), 287-289, doi:10.1590/S1517-83822013000100042 (2013).
22 Dohnalkova, A. C. *et al.* Imaging hydrated microbial extracellular polymers: comparative analysis by electron microscopy. *Appl Environ Microbiol.* **77** (4), 1254-1262, doi:10.1128/AEM.02001-10 (2011).
23 Yang, L. *et al.* In situ SEM and ToF-SIMS analysis of IgG conjugated gold nanoparticles at aqueous surfaces. *Surf Interface Anal* **46** 224-228 (2013).
24 Yu, J. C. *et al.* Capturing the transient species at the electrode-electrolyte interface by in situ dynamic molecular imaging. *Chem Commun.* **52** (73), 10952-10955, doi:10.1039/c6cc02893d (2016).

Figure

Click here to download Figure Fig1.tif



Figure

Click here to download Figure Fig2.tif

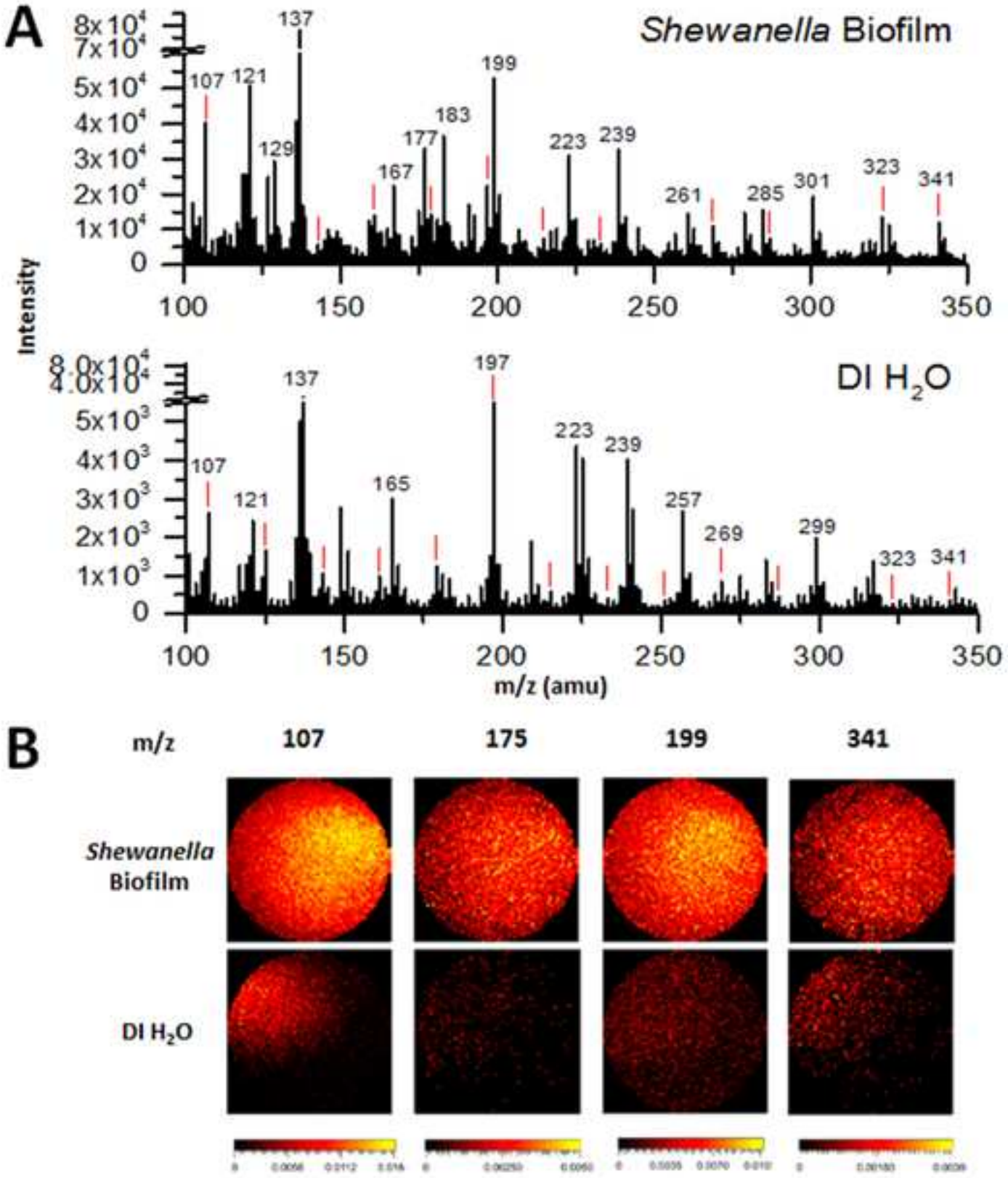


Table of Materials/Equipment

Click here to download Table of Materials/Equipment
JoVE_Materials_Komorek_03172017.xls

Name of Material/ Equipment	Company	Catalog Number
ToF-SIMS	IONTOF	TOF.SIMS 5
System for Analysis at the Liquid Vacuum Interface (SALVI)	Pacific Northwest National Laboratory	N/A
-80°C Freezer	New Brunswick Scientific	N/A
4°C Refrigerator	BioCold Scientific	N/A
Orbital Shaker	New Brunswick Scientific	N/A
Syringe Pump	Cole-Parmer	EW-74905-02
Incubator	Barnstead International	LT1465X3
Autoclave	Getinge	533LS
Spectrophotometer	Thermo Fisher Scientific	4001-000
Biological Safety Cabinet	Thermo Fisher Scientific	1385
Fluorescence Microscope	Nikon	N/A
pH Meter	Mettler Toledo	51302803
PEEK Union	Valco	ZU1TPK
5 Axes Sample Stage	IONTOF	N/A
Barnstead Nanopure Water Purification System	Thermo Fisher Scientific	D11921
Pipette	Thermo Fisher Scientific	21-377-821
Pipette Tip	Neptune	2112.96.BS
Razor Blade Handle	Stanley	N/A
Syringe	BD	309659
Syringe	BD	309657
Syringe	BD	309646
Syringe	BD	309604
Syringe	BD	302830
Disposable Pipette	Thermo Fisher Scientific	13-678-11
Electric Pipette Filler	Pipet-aid	P-57260
Serum Bottle	Sigma	33109-U
Anaerobic Culture Tube	VWR	89167-178
Rubber Stopper	Sigma	27235-U
Aluminum Crimp Seal (without septum)	Sigma	27227-U
Serum Bottle Aluminum Seal Crimper	Wheaton	224307
PTFE Tubing	Supelco	58697-U
Disposable Cuvettes	GMBH	759085D

Needle	BD	303015
Needle	BD	305120
Shewanella oneidensis MR-1 with GFP	N/A	N/A
Ethanol	Thermo Fisher Scientific	S25310A
TSA	BD	212305
PIPES Buffer	Sigma	P-1851
Sodium Hydroxide	Sigma	S-5881
Ammonium Chloride	Sigma	A-5666
Potassium Chloride	Sigma	P-4504
Sodium Phosphate Monobasic	Sigma	S-9638
Sodium Chloride	Thermo Fisher Scientific	S271-3
Sodium lactate	Sigma	L-1375
Sodium Bicarbonate	Sigma	S-5761
Nitrilotriacetic Acid Trisodium Salt	Sigma	N-0253
Iron (III) Chloride	Sigma	451649
Magnesium Sulfate	Sigma	208094
Manganese (II) Sulfate Monohydrate	Sigma	M-7634
Iron(II) Sulfate Heptahydrate	Sigma	215422
Calcium Chloride Dihydrate	Sigma	223506
Cobalt(II) Chloride	Sigma	60818
Zinc Chloride	Sigma	229997
Copper(II) Sulfate Pentahydrate	Sigma	C-8027
Aluminum Potassium Sulfate Dodecahydrate	Sigma	237086
Boric Acid	Sigma	B-6768
Sodium Molybdate Dihydrate	Sigma	331058
Nickel(II) Chloride	Sigma	339350
Sodium Tungstate Dihydrate	Sigma	14304
D-Biotin	Sigma	47868
Folic Acid	Sigma	F-7876
Pyridoxine Hydrochloride	Sigma	P-9755
Riboflavin (B2)	Sigma	47861
Thiamine Hydrochloride	Sigma	T-4625
Nicotinic Acid	Sigma	N4126

D-Pantothenic Acid Hemicalcium Salt	Sigma	21210
Vitamin B12	Sigma	V-2876
4-Aminobenzoic Acid	Sigma	A-9878
Thioctic Acid	Sigma	T-1395

Comments/Description

Resolution:>10,000 m/Δm for mass resolution;>4,000 m/Δm for high spatial resolution

SALVI is a unique, self-contained, portable analytical tool that, for the first time, enables vacuum based scientific instruments such as time-of-

U410 Premium Energy Efficient Ultra-Low Temperature Freezer

COLDBOX1

Innova 4900 Multi-Tier Environmental Shaker, set at 30 degrees Celsius for serum bottle and flask culturing, set at 150rpm.

Cole-Parmer Syringe Pump, Infusion Only, Touchscreen Control 74905-02, used for injecting liquid into the tubing system and SALVI at a cons

Lab-Line incubator, set at 30 degrees Celsius for plate culturing.

Used to sterilize PEEK fittings, tubing systems, serum vials, and medium. Model 533LS Vacuum Steam Sterilizer

GENESYS 20 spectrophotometer for OD600 readings of cuvettes for growth curves.

1300 Series AZ Biological Safety Cabinet

Nikon OPTIPHOT-2 fluorescence microscope with camera and super high pressure mercury lamp power supply.

Used to test the pH of the “nanowires” medium after finished and before autoclaving.

For connecting the inlet and outlet of SALVI, the syringe to the tubing system, and the inlet of the SALVI to the drip chamber of the tubing sys

Stage is self-made for mounting SALVI in ToF-SIMS.

ROpure LP Reverse Osmosis filtration module (D2716)

Range: 100 to 1,000 µL.

1,000 µL pipette tips

Stanley Bostitch Razor Blade Scraper with 5 Single-Edge Blades, used for cutting PTFE tubing

1 mL

3 mL

5 mL; Used for making the drip chamber

10 mL

20 mL

25 mL Fisherbrand™ Sterile Polystyrene Disposable Serological Pipets with Magnifier Stripe, for filling serum bottles.

Vacuum pressure electric serological pipette filler

Holds approximately 69 mL of liquid for culture growth, optimum for use of 20mL culture per bottle.

Anaerobic Tubes, 18 x 150 mm, Supplied with 20 mm Blue Butyl Rubber Stopper and Aluminum Seal.

Silicone stopper, used for sealing serum bottles and for creating the tubing system/drip chamber.

Aluminum seal for top of serum bottle for use with serum bottle crimper.

30 mm crimper with standard seal.

1.58 mm OD x 0.5 mm ID 50 ft. PTFE Teflon tubing, used for creating the tubing system.

1.5 Ml for use with spectrophotometer.

22G; used for serum bottle injection.
23G; used for punching-through rubber stopper to create drip tubing system.
Matthysse AG, Stretton S, Dandie C, McClure NC, & Goodman AE (1996) Construction of GFP vectors for use in Gram-negative bacteria other than E
95% Denatured
Tryptic soy agar for culturing the model organism (*S. oneidensis*) used in this protocol
Used for “nanowires” medium {Hill, E.A. 2007}
Used for “nanowires” medium {Hill, E.A. 2007}
Used for “nanowires” medium {Hill, E.A. 2007}
Used for “nanowires” medium {Hill, E.A. 2007}
Used for “nanowires” medium {Hill, E.A. 2007}
Used for “nanowires” medium, and used to make mineral solution used for “nanowires” medium {Hill, E.A. 2007}
60%(w/w) syrup @ 98% pure, d=1.3 g/mL, 7M, used for “nanowires” medium {Hill, E.A. 2007}
Used to make ferric NTA solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make ferric NTA solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make ferric NTA solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make minerals solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}

Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}
Used to make vitamin solution, used for “nanowires” medium {Hill, E.A. 2007}

-flight secondary ion mass spectrometry (ToF-SIMS) to analyze liquid surfaces in their natural state at the molecular level.

tant flowrate.

;tem.

scherichia coli. *FEMS Microbiol Lett* 145(1):87-94.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: In Situ characterization of Shewanella oneidensis Biofilms by SALVI and ToF-SIMS

Author(s): Rachel Konwrek, Wenchao Wei, Xiaofei Yu, Eric Hill, Juan Yao, Zihua Zhu, and Xiao-Ying Yu

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Xiao-Ying Yu

Department: Eearth and Biological Sciences

Institution: Pacific Northwest National Laboratory

Article Title: In Situ Characterization of Shewanella oneidensis MR1 Biofilms by SALVI and ToF-SIMS

Signature: [signature] Date: Jan. 5, 2017

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Pacific Northwest
NATIONAL LABORATORY

Proudly Operated by Battelle *Since 1965*

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] 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