Submission ID #: 61728
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18827088

Title: Atomic Force Microscopy Combined with Infrared Spectroscopy as a Tool to Probe Single Bacterium Chemistry

Authors and Affiliations: Kamila Kochan[1], Anton Y. Peleg[2,3], Philip Heraud[1,2], and Bayden R. Wood[1]

[1]Centre for Biospectroscopy and School of Chemistry, Monash University, Clayton Campus
[2]Infection and Immunity Program, Monash Biomedicine Discovery Institute and Department of Microbiology, Monash University, Clayton Campus
[3]Department of Infectious Diseases, The Alfred Hospital and Central Clinical School, Monash University

Corresponding Author:
Kamila Kochan
kamila.kochan@monash.edu

Co-Authors:
anton.peleg@monash.edu
phil.Heraud@monash.edu
bayden.Wood@monash.edu

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **38**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Kamila Kochan**: This method allows nanoscale studies of bacteria. It can provide detailed insight into chemical alterations in antimicrobial resistance and contribute to our understanding of AFM and drug development **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Kamila Kochan**: The nanoscale size of bacteria significantly limits the research tools capable of probing their chemistry. AFM-IR enables their study in a non-destructive and observer-independent manner, even at the subcellular level **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Kamila Kochan**: By providing insight into chemical changes related to antimicrobial resistance, this method can help to identify and test molecular targets for new antimicrobials **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.4. **Kamila Kochan**: In addition to bacteria, AFM-IR can easily be applied to a range of cells, tissues, and even viruses **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Atomic Force Microscopy-Infrared (AFM-IR) Spectroscopy Sample Preparation

2.1. To prepare a sample for AFM-IR imaging, after growing the bacteria of interest on the appropriate medium under the appropriate culture conditions **[1]**, use a sterile loop to carefully pick bacteria from the top of the colonies for transfer to a glass tube **[2-TXT]**[3].

2.1.1. WIDE: Talent taking plate(s) out of incubator

2.1.2. Bacteria being picked with loop, with tube visible in frame *Videographer: Important/difficult step* **TEXT: Collect bacteria from top of colonies**

2.1.3. Added shot: Bacteria being added to tube

2.2. Add 1 milliliter of ultrapure water to the tube **[1]** and vortex for 1-2 minutes until the collected bacterial pellet is no longer visible at the bottom of the tube **[2]**.

2.2.1. Talent adding water to tube(s)

2.2.2. Tube(s) being vortexed

2.3. Estimate the rough turbidity of the solution by visual comparison between the prepared solution and McFarland standards **[1]**.

2.3.1. Tube being compared to standards

2.4. If the turbidity of the bacterial suspension appears to be very low, continue to add more bacteria and to vortex **[1]** until the rough turbidity of the solution is comparable to the 0.5 and 1 McFarland standards **[2]**.

2.4.1. Talent adding bacteria to tube

2.4.2. Shot of tube with turbidity between 0.5 and 1

2.5. Pellet the bacteria by centrifugation **[1-TXT]** and carefully aspirate the supernatant with a pipette **[2]**.

2.5.1. Talent placing tube(s) into centrifuge **TEXT: 5 min, 3000 x g, RT**

2.5.2. Supernatant being aspirated

2.6. Resuspend bacterial cell pellet in 1 milliliter of ultrapure water with vortexing **[1]** and sediment the bacteria with another centrifugation **[2]**.

2.6.1. Use 2.2.2. Tube being vortexed

2.6.2. Use 2.5.1. Talent placing tube(s) into centrifuge

2.7. After washing the bacteria up to three more times as just demonstrated, aspirate the supernatant **[1]** and vortex the pellet for at least 2 minutes **[2]** before adding 5 microliters of cells onto the experimental substrate **[3]**.

2.7.1. Use 2.5.2. Supernatant being aspirated *Videographer: Important step*

2.7.2. Use 2.2.2. Tube being vortexed *Videographer: Important step*

2.7.3. Bacteria being added to substrate *Videographer: Important step*

2.8. If the desired thickness is a monolayer or individual bacteria, immediately after depositing, add between 20-100 microliters of ultrapure water to the substrate **[1]** and gently mix the solution with a pipette tip **[2]**. Note: 2.8.1. and 2.8.2. merged

2.8.1. Talent adding water to plate *Videographer: Important step*
2.8.2. Solution being mixed *Videographer: Important step*

2.9. When the sample has air dried, use double-sided adhesive tape to mount the substrate onto an AFM metal specimen disk **[1]**.

2.9.1. Substrate being taped/mounted onto disk Use take 3

3. Instrument preparation

3.1. To prepare the AFM-IR spectroscopy instrument for an analysis **[0]**, press **Initialize** after turning on the software and laser **[1]** and confirm that the laser shutter is in the **Open** position **[2]**.

Added shot 3.1.0. WIDE: Shot of setup
3.1.1. WIDE: Talent pressing Initialize

3.1.2. Talent checking laser shutter position

3.2. During this process, the **Stage Initialization** window will pop-up. When the initialization process is finished, click **Initialize** and **OK [1]**.

3.2.1. SCREEN: 3.2 *Video Editor: please speed up*

3.3. If possible, turn on the nitrogen flow to purge the instrument with nitrogen **[1]** and adjust the nitrogen purge to achieve a stable humidity level **[2]**. Note: 3.3.1. and 3.3.2. merged

3.3.1. Talent turning on nitrogen flow
3.3.2. Talent adjusting purge

3.4. Carefully place the sample in the sample chamber without damaging the tip and click **Load** in the software **[1]**[1.1.]. Follow the wizard to load the sample, focusing on the tip and on the sample in each step. Then click **Approach** to approach the sample without engaging **[2]**.

3.4.1. Talent placing the sample in the chamber and clicking load, with monitor visible in frame *Videographer: Important/difficult step*
3.4.1.1. Added shot: Close up of sample loading
3.4.2. SCREEN: 3.4 and 3.5: 00:15-02:22 *Video Editor: please speed up*

4. Single Spectrum Data Collection

4.1. Before engaging the sample, select **Tools**, **IR background calibration**, and **New [1]**. In the pop-up window, set the resolution and spectral range according to the aim of the analysis and set the **Co-averages** and the **Backgrounds To Average** values **[2]**.

4.1.1. WIDE: Talent selecting Tools, IR background calibration, and New, with monitor visible in frame
4.1.2. SCREEN: 4.2: 00:15-00:43 *Video Editor: please speed up*

4.2. Select **Enable**. In the pop-up window, set the **Start** and **End** parameters and click **Accept** and **Acquire [2]**.

4.2.1. SCREEN: 4.2: 00:47-01:18 *Video Editor: please speed up*

4.3. When the background data has been acquired, click **Save [1]**. To engage the tip to the sample, click **Engage**. The system will approach the sample surface until direct contact is detected **[2]**.

4.3.1. SCREEN: 4.3: 00:00-00:25 *Video Editor: please speed up*
4.3.2. SCREEN: 4.4: 00:04-00:14

4.4. Click **Scan** to collect an initial larger area, low spatial resolution AFM (A-F-M) image to visualize the surface ~~**[1]**~~ before moving the tip to a measurement spot of interest **[1]** ~~**[2]**~~.

4.4.1. SCREEN: 4.5: 00:47-02:10 *Video Editor: please speed up*
~~4.4.2. Tip being moved to spot~~

4.5. To align the infrared laser to a wavenumber at which the sample will absorb, enter the wavenumber at which the sample will absorb in the **Wavenumber** field and click **Start IR**. At least one clear peak should be observed in the amplitude versus frequency graph. The deflection versus time graph should show a periodic waveform **[1]**.

4.5.1. SCREEN: 4.6: 00:28-00:44 *Video Editor: please speed up and please emphasize red peak with amplitude vs frequency and blue data line with deflection vs time*

4.6. Click **Optimize** to select a conventional infrared bacteria spectrum to identify the positions of the bands and use the spectrum to optimize the hot spots at various wavenumber values from various spectral regions **[1]**.

4.6.1. SCREEN: 4.7: 00:04-42 *Video Editor: please speed up*

4.7. When the infrared spots for selected wavenumber values have been optimized, define the spectral resolution and range and number of co-averages and click **Acquire** to collect the AFM-IR spectrum **[1-TXT]**.

4.7.1. SCREEN: 4.8 and 4.9: 00:06-00:34 *Video Editor: please speed up* **TEXT: Caution: Higher laser powers may cause sample damage**

5. Imaging Approach Data Collection

5.1. To collect an intensity distribution image for a selected wavenumber value, after recording a single AFM-IR spectrum, record an AFM image of the selected sample area **[1]** and select the wavenumber values for AFM-IR imaging **[2]**.

5.1.1. WIDE: Talent recording image, with monitor visible in frame
5.1.2. SCREEN: 5.2: 00:04-00:08

5.2. Confirm that the IR spot of the laser has been optimized for the selected wavenumber values and set the number of data points in the X and Y directions of the image area **[1]**.

5.2.1. SCREEN: 5.2: 00:09-00:44 *Video Editor: please speed up*

5.3. In the **General** window, set the laser power. In the **AFM Scan** window, define the **Scan Rate [1]**.

5.3.1. SCREEN: 5.3: 00:08-00:24 *Video Editor: please speed up*

5.4. Then check the **IR Imaging Enable** box and click **Scan** to begin imaging. The AFM-IR of the intensity of the signal at the selected wavenumber will be collected simultaneously with the AFM data from that area **[1]**.

5.4.1. SCREEN: 5.4: 00:04-00:37 *Video Editor: please speed up*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.1., 2.7., 2.8., 3.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.1. Collection of as clean as possible samples.
3.4. Loading the sample, as it needs to be conducted with care. This is the step upon which the tip can easily be broken. Slow approach towards the sample is recommended.

Results

6. Results: Representative AFM-IR Analyses of Single Bacterium Chemistry

6.1. This protocol enables the acquisition of a range of types of bacteria cell distributions on a substrate from single cells to monolayers **[1]**.

6.1.1. LAB MEDIA: Figures 1A-1H *Video Editor: please sequentially add/emphasize Figures 1B, 1D, 1F, and 1H*

6.2. The protocol can be used to monitor dynamic changes in living bacteria **[1]**, for example, in the formation of a septum during *S. aureus* cell division **[1]**.

6.2.1. LAB MEDIA: Figures 2A-2D
6.2.2. LAB MEDIA: Figures 2A-2D *Video Editor: please sequentially add/emphasize Figures 2A-2D*

6.3. The AFM-IR spectrum of the septum was characterized by a higher relative intensity of bands at 1240 and 1090 centimeters **[1]** compared to AFM-IR spectra collected from the cell area **[2]**, suggesting that the septum is made up of carbohydrate and phosphodiester groups of cell wall components **[3]**.

6.3.1. LAB MEDIA: Figures 2E-2G *Video Editor: please emphasize red line peaks in Figure 2G*
6.3.2. LAB MEDIA: Figures 2E-2G *Video Editor: please emphasize black line peaks in Figure 2G*
6.3.3. LAB MEDIA: Figures 2E-2G *Video Editor: please emphasize red square in Figure 2F*

6.4. The protocol can also be used to study differences in the chemical composition arising from the development of resistance **[1]**. As observed in this representative analysis, no morphological differences **[2]** were measured between vancomycin intermittent resistance and vancomycin susceptible *S. aureus* cells **[3]**.

6.4.1. LAB MEDIA: Figures 4A and 4C
6.4.2. LAB MEDIA: Figures 4A and 4C *Video Editor: please emphasize Figure 4A bacteria*
6.4.3. LAB MEDIA: Figures 4A and 4C *Video Editor: please emphasize Figure 4B bacteria*

6.5. Evaluation of the AFM-IR spectra and their second derivatives **[1]**, however, revealed a clear increase in the relative intensity of the bands associated with the carbohydrate and phosphodiester groups from the cell wall components in the resistant strain **[2]** compared to the susceptible counterpart **[3]**.

6.5.1. LAB MEDIA: Figures 4D-4G

6.5.2. LAB MEDIA: Figures 4D-4G *Video Editor: please emphasize 1240 and 1086 peaks in Figure 4F*

6.5.3. LAB MEDIA: Figures 4D-4G *Video Editor: please emphasize 1238 and 1082 peaks in Figure 4G*

Conclusion

7. Conclusion Interview Statements

7.1. **Kamila Kochan**: It's very important to prepare a clean sample. Take care to reduce the medium contribution from the beginning and to remove the medium completely through multiple washes **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.1., 2.5.-2.7.)

7.2. **Kamila Kochan**: Since AFM-IR is non-destructive, the sample can later be stained or studied with other techniques, such as Raman spectroscopy, to provide complementary data about the chemical composition of the sample **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera