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Title: Super-Resolution Imaging of *Proteus mirabilis* Biofilm by Expansion Microscopy

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 19
Number of Shots: 32

Introduction

NOTE to SE: Please refer to JoVE_67932_shoot_list.PDF in the documents folder for shot numbers and video file details.

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Steffen Härtel:** The scope of this research is to provide the scientific community with an accessible super-resolution method for studying *Proteus mirabilis* biofilm architecture, assembly, and intracellular features beyond the diffraction limit **[1]**.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3B, 3C.*

What are the current experimental challenges?

1.2. **Dante Castagnini:** Quantitative microscopy of biofilms is challenging due to the limitations imposed by optical resolution. *Proteus mirabilis* Expansion Microscopy (or PmbExM) will contribute towards an improved morphological and topological description and analysis of these complex microbial communities **[1]**. NOTE: Statement modified

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3E, 3F.*

What advantage does your protocol offer compared to other techniques?

1.3. **Dante Castagnini:** Two main advantages of PmbExM is that, FIRST, it allows the super-resolution visualization of *Proteus mirabilis* biofilms using conventional, diffraction-limited microscopes and, SECOND, it does not rely on complex post-acquisition data processing routines **[1]**. NOTE: Statement slightly modified

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.1.*

How will your findings advance research in your field?

1.4. **Karina Palma:** Our method facilitates the nanoscale study of biofilm structure and organization to researchers lacking access to specialized super resolution equipment and without vast experience in digital image processing or analysis **[1]**.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.9.1, 2.9.2.*

What new scientific questions have your results paved the way for?

1.5. **Steffen Härtel:** The malleability of PmbExM supports its adaptation and modification for use with other biofilm species **[1]**.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 4*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. MA-NHS Anchoring and Acrylamide-Acrylate Polymerization for PmbExM Procedure

Demonstrator: Dante Castagnini

2.1. To begin, add 400 microliters of 1 millimolar methacrylic acid N-hydroxysuccinimidyl ester or MA-NHS *(M-A N-H-S)* in PBS to the stained biofilm samples and incubate them at room temperature for 1 hour with mild agitation **[1]**.

2.1.1. WIDE: Talent pipetting 400 microliters of 1 millimolar MA-NHS solution into the container with stained biofilm samples.

2.2. After one hour, gently wash the stained biofilm samples three times with 300 microliters of PBS at room temperature for 10 minutes each **[1]**.

2.2.1. Talent pipetting 300 microliters of PBS over the sample and washing the stained biofilm samples.

2.3. Remove the PBS **[1]** and add 300 microliters of monomer solution to the samples **[2]**. Incubate overnight at 4 degrees Celsius **[3]**.

2.3.1. Talent aspirating PBS.

2.3.2. Talent pipetting 300 microliters of monomer solution onto the sample.

2.3.3. Talent placing the sample container in a refrigerator.

2.4. To construct the gelation pre-chambers, use a glass slide as a base **[1].** Then cut, arrange, and attach two pieces of folded-over double-sided tape on the slide to act as 400-micrometer spacers **[2-TXT]**. NOTE: VO is modified for the modified shot.

2.4.1. Talent placing a glass slide on the work surface.

2.4.2. Talent cutting, arranging, attaching, and positioning folded double-sided tape on the slide with precise spacing. **TXT: Arrange the spacers 8 – 10 mm from each other** NOTE: Shot slightly modified.

2.5. Arrange wet chambers to protect samples from desiccation during polymerization **[1]**.

2.5.1. A shot of the wet chamber.

2.6. Now, prepare a fresh stock volume of gelling solution by mixing monomer solution, 10% tetramethylethylenediamine *(tetra-methyl-ethylene-di-amine)*, 0.5% 4-hydroxy-TEMPO *(T-E-M-P-O)*, and 10% ammonium persulfate in a 47:1:1:1 *(forty-seven to one to one to one)* ratio **[1]**.

2.6.1. Talent mixing the specified reagents in the specified ratio in a beaker or tube.

2.7. Immediately after preparing the gelling solution, remove the monomer solution from the samples **[1]** and replace it with 300 microliters of the gelling solution **[2]**. Incubate the samples at 4 degrees Celsius for 5 minutes **[3]**.

2.7.1. Talent aspirating the monomer solution from the samples.

2.7.2. Talent pipetting 300 microliters of gelling solution onto the samples.

2.7.3. Talent placing the samples in the refrigerator for incubation.

2.8. Meanwhile, remove the remaining protective cover from the double-sided tape strips arranged on the glass slides **[1]**. Pipette 40 microliters of gelling solution between the spacers **[2]**.

2.8.1. Talent peeling off the remaining protective cover from the tape strips on the glass slides.

2.8.2. Talent pipetting 40 microliters of gelling solution between the spacers.

2.9. Once the incubation of the sample is complete, use tweezers to lift each biofilm-bearing coverslip and place it on top of the 40-microliter drop of gelling solution on the slide **[1]**. Orient the coverslip so that the biofilm on its surface contacts the gelling solution **[2]**.

2.9.1. Talent removing a coverslip from the well using tweezers and placing it on top of the 40-microliter drop of gelling solution on the slide.

2.9.2. ECU: Talent orienting the coverslip and the biofilm contacts the gelling solution.

2.10. Finish constructing the gelation chamber by gently pressing the biofilm-bearing coverslip with tweezers to ensure adhesion to the tape spacers **[1]**.

2.10.1. Talent pressing the coverslip onto the tape using tweezers.

2.11. Place the assembled gelation chamber inside a wet chamber to allow polymerization

[1] and incubate at 37 degrees Celsius without agitation for 2 hours **[2]**.

2.11.1. Talent placing the assembled gelation chamber inside a wet chamber.

2.11.2. Talent placing the setup in an incubator.

2.12. After 2 hours, disassemble the gelation chambers **[1]** and use a surgical blade to trim the excess gel around the biofilm sample's region of interest **[2]**.

2.12.1. Talent disassembling the gelation chambers.

2.12.2. Talent trimming the excess gel around the biofilm sample's region of interest using a surgical blade.

2.13. Place the coverslips carrying the trimmed gels into a new 24-well plate with the gel side facing upwards **[1]**.

2.13.1. Talent placing the coverslips carrying the trimmed gels into a new 24-well plate with the gel side facing upwards.

2.14. After enzymatic digestion of the gelled samples with proteinase K solution **[1]**, remove the proteinase solution **[2]**, and transfer each gel to a separate 60-millimeter Petri dish for the expansion of the samples **[3]**.

2.14.1. A shot of the gelled samples with proteinase K solution after enzymatic digestion.

2.14.2. Talent aspirating the solution from the sample.

2.14.3. Talent transferring one gel into each individual Petri dish.

2.15. Fill each Petri dish with excess deionized water so the gel is fully submerged **[1]** and incubate under gentle agitation at room temperature for 20 minutes **[2-TXT]**.

2.15.1. Talent adding deionized water into the Petri dish until the gel is fully submerged.

2.15.2. Talent placing the Petri dish on a shaker. **TXT: Exchange the water 4 – 5x**

3. Procedure for Mounting the Expanded Samples

3.1. After completing the fifth water exchange, remove the deionized water covering the gel **[1]**. Use a small flat brush to gently push the sample onto a 24 by 50 millimeter glass coverslip **[2]**.

3.1.1. Talent aspirating the water covering the gel.

3.1.2. Talent pushing the sample onto a glass coverslip with a small flat brush.

3.2. Remove the excess water from the gel using tissue paper **[1]**.

3.2.1. Talent carefully blotting the gel with tissue paper to absorb excess water.

3.3. Carefully place the gel onto the glass surface of the imaging chamber, ensuring no air bubbles are trapped between the gel and the glass **[1]**.

3.3.1. Talent placing the gel onto the glass surface of the imaging chamber, ensuring no air bubbles.

3.4. Finally, add deionized water to the imaging chamber until the gel is fully submerged **[1]**.

3.4.1. Talent adding deionized water into the imaging chamber until the gel is completely covered.

Results

4. Results

4.1. Quantitative and spatial analyses were used to evaluate the expansion fidelity and morphological preservation of *Proteus mirabilis* biofilms following Proteus mirabilis Biofilm Expansion Microscopy or PmbExM *(P-M-B-E-X-M)* treatment **[1]**. The PmbExM technique expanded the biofilm structures by approximately 4.3-fold **[2]**, while maintaining both cellular morphology and topological organization **[3]**.

4.1.1. LAB MEDIA: Figure 3E, 3F.

4.1.2. LAB MEDIA: Figure 3E, 3F. *Video Editor: Highlight 3E.*

4.1.3. LAB MEDIA: Figure 3E, 3F. *Video Editor: Highlight 3F.*

4.2. This expansion significantly increased the visual resolution of bacterial cells **[1]**, making individual cell structures sharper and more defined **[2]**.

4.2.1. LAB MEDIA: Figure 3B, 3C.

4.2.2. LAB MEDIA: Figure 3B, 3C. *Video Editor: Highlight 3C.*

4.3. With the enhanced resolution, PmbExM also enabled the clear identification of multilayered bacterial arrangements within the biofilm that were previously unresolved **[1]**.

4.3.1. LAB MEDIA: Figure 3G, 3H.

4.4. PmbExM also facilitated the visualization of previously unresolvable subcellular structures, including distinct patterns of DNA organization **[1]**.

4.4.1. LAB MEDIA: Figure 4A, 4C, 4E.

4.5. These organizational patterns were confirmed by line intensity profiles **[1]**, which revealed multiple distinct fluorescence peaks in expanded samples **[2]** compared to the single peak observed in non-expanded cells **[3]**.

4.5.1. LAB MEDIA: Figure 4. *Video Editor: Highlight 4B, 4D, 4F.*

4.5.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize 4C-4F.*

4.5.3. LAB MEDIA: Figure 4. *Video Editor: Emphasize 4A, 4B.*