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Title: A Modular Workflow for Quantitative, Structural and Functional Analysis of *Leptospira* Biofilms

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

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

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

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **12/19/2025**

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Current Protocol Length

Number of Steps: 20
Number of Shots: 47

Introduction

INTRODUCTION:

1.1. **Roman Thibeaux:** We study biofilms as protective structures enabling environmental and host persistence, investigating their formation dynamics, molecular composition, adaptive features, and contribution to infection.

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

1.2. **Roman Thibeaux:** Combining crystal-violet assays, time-lapse imaging, confocal microscopy, SEM, and transcriptomics in our lab advances biofilm research through integrated multidimensional analysis.

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

CONCLUSION:

1.3. **Grégoire Davignon:** Our optimized protocol synchronizes complementary readouts from identical cultures, increasing robustness, reducing variability, and revealing dynamic and structural information unavailable with single-method approaches.

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

1.4. **Grégoire Davignon:** By integrating complementary analyses, our findings standardize biofilm assessment, clarify *Leptospira* dynamics and architecture, link structure to virulence, and strengthen reproducible comparative research.

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

1.5. **Grégoire Davignon:** Future work will examine mutants to link regulation with biofilm morphology and dynamics, clarify environmental persistence, and evaluate strategies disrupting formation or promoting dispersal.

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

Protocol

2. Preparation of the Biofilms

Demonstrator: Grégoire Davignon

2.1. To begin, grow *Leptospira* cells in EMJH medium in flat-bottomed, screw-cap glass tubes **[1-TXT]**. Incubate the cultures under aerobic conditions at 30 degrees Celsius without shaking until they reach mid-logarithmic phase **[2]**.

2.1.1. WIDE: Talent inoculating *Leptospira* cells into EMJH medium in screw-cap glass tubes. **TXT: EMJH: Ellinghausen-McCullough-Johnson-Harris**

2.1.2. Talent placing the inoculated tubes in an incubator set to 30 degrees Celsius.

2.2. Ensure the cultures reach an optical density between 0.2 and 0.4 at 405 nanometers, which corresponds to 2 to 5 x 10^8 cells per milliliter **[1]**. Using a dark-field microscope at 20x magnification, verify that the cells are motile and not clumped **[2]**.

2.2.1. Talent placing the sample in a spectrophotometer.

2.2.2. Talent examining the sample under dark-field microscope.

2.3. Dilute the verified mid-logarithmic phase culture at a 1 to 100 ratio in fresh EMJH medium to obtain approximately 1 x 10^6 cells per milliliter **[1]** and mix gently by inversion without vortexing **[2]**.

2.3.1. Talent preparing a 1 to 100 dilution of culture in fresh EMJH medium. **TXT: Use a biosafety level 2 hood**

2.3.2. Talent gently inverting the tube to mix without vortexing.

2.4. Now, use sterile forceps to place one 0.1-micrometer sterile hydrophilic polycarbonate membrane flat at the bottom of each well in a sterile 24-well plate with lid **[1-TXT]**. Add 1 milliliter of sterile EMJH medium to each well **[2]** and pre-soak the membrane for 2 hours at 30 degrees Celsius **[3]**.

2.4.1. Talent placing sterile membrane into the wells of a 24-well plate using sterile forceps inside the biosafety hood. **TXT: Alternately, use 12 mm sterile glass coverslip** **Authors**: I have mentioned membrane in this section and coverslips for the next sections so that our viewers can see both. Please let us know after

the shoot if the membrane or coverslip needs to be swapped in the narration for any of the shot/steps

2.4.2. Talent pipetting 1 milliliter of sterile EMJH into each well.

2.4.3. Talent placing the plate into a 30 degrees Celsius incubator.

2.5. Next, remove the soaking solution from each well without displacing the membrane **[1]** and add 1.5 milliliters of diluted bacterial suspension, ensuring the membrane remains firmly in place at the bottom **[2]**.

2.5.1. Talent aspirating the soaking solution from the wells carefully.

2.5.2. Talent adding 1.5 milliliters of diluted bacterial suspension to each well and adjusting the membrane to ensure it lies flat.

2.6. Then, place a water-filled tray inside the incubator to maintain humidity **[1]**. Incubate the plate at 30 degrees Celsius under static conditions to allow biofilm formation, leaving the plate for up to 3 weeks for slow-growing strains **[2]**.

2.6.1. Talent placing a tray of water inside the incubator.

2.6.2. Talent placing the 24-well plate in the incubator and closing the lid.

2.7. At the desired timepoint, carefully aspirate as much culture medium as possible without disturbing the biofilm **[1]**. Rinse each well gently with 1 milliliter of sterile PBS while keeping the membrane flat against the bottom **[2]**.

2.7.1. Talent aspirating the culture medium slowly from each well.

2.7.2. Talent rinsing each well with PBS while holding the plate steady to avoid disturbing the biofilm.

3. Crystal Violet–Based Quantification of Biofilms

3.1. Add 1 milliliter of 4 percent paraformaldehyde in PBS to each well **[1]** and incubate at 37 degrees Celsius for 30 minutes to fix the biofilm samples **[2]**. After incubation, remove the fixative **[3]** and gently rinse twice with 1 milliliter PBS **[4-TXT].**

3.1.1. Talent adding paraformaldehyde to each well.

3.1.2. Talent placing the plate into a 37 degrees Celsius incubator.

3.1.3. Talent removing the fixative from the well.

3.1.4. Talent adding PBS to each well. **TXT: Ensure that PBS is completely removed from the sample after the washes Authors:** We say wash twice in the narration, but we can show it only once in the video

3.2. Add 1 milliliter of 0.1 percent weight by volume crystal violet solution to each well **[1]** and incubate at room temperature for 15 minutes, making sure the coverslip is fully submerged **[2]**.

3.2.1. Talent pipetting crystal violet solution into each well.

3.2.2. Talent setting a timer for incubation.

3.3. Then, discard the crystal violet dye from each well **[1]** and rinse twice with 1 milliliter PBS **[2]**.

3.3.1. Talent discarding the crystal violet dye from the wells.

3.3.2. Talent adding PBS to each well.

3.4. Tilt the plate and drain all remaining liquid **[1]**. Leave the plate at room temperature to air-dry until the substrate appears completely dry, preferably overnight **[2]**.

3.4.1. Talent tilting the plate slightly to facilitate pipetting and ensure complete removal of any remaining liquid.

3.4.2. Talent placing the plate on a bench top to dry completely.

3.5. Next, add 500 microliters of elution buffer, composed of 50 percent ethanol and 50 percent glacial acetic acid by volume, to each well. Pipette up and down to fully dissolve the stain bound to the biofilm **[1]**.

3.5.1. Talent pipetting elution buffer into each well and pipetting the contents up and down to dissolve the stain.

3.6. Now, transfer 200 microliters of each sample to an optically clear 96-well microplate **[1]**. Measure absorbance at 570 nanometers and subtract background values from uninoculated controls processed through all steps **[2]**. Record the mean and standard deviation for at least three technical replicates **[3-TXT]**.

3.6.1. Talent transferring 200 microliters of sample into wells of a 96-well plate.

3.6.2. Show the spectrophotometer interface measuring absorbance at 570

nanometers.

3.6.3. Talent making entries on a computer. **TXT: Dilute the samples with elution buffer if absorbance exceeds the spectrophotometer's linear range**

4. Biofilm Visualization Using Scanning Electron Microscopy (SEM)

Demonstrator: Roman Thibeaux

4.1. Obtain the biofilms in well-plates as demonstrated earlier **[1]**. Add a solution of 4 percent paraformaldehyde and 1 percent glutaraldehyde in 0.2 molar sodium cacodylate buffer at pH 7.4 into the wells **[2]**.

4.1.1. Talent placing the plate with biofilms on the bench.

4.1.2. Talent pipetting the fixative solution into each well.

4.2. Incubate the fixed samples for 30 minutes at 37 degrees Celsius **[1]**. Remove the fixative **[2]** and rinse the sample twice with PBS to preserve the surface-attached biofilm while minimizing detachment **[3]**.

4.2.1. Talent placing the plate into a 37 degrees Celsius incubator.

4.2.2. Talent aspirating the fixative.

4.2.3. Talent rinsing the wells with PBS.

4.3. Now, immerse the coverslip in 1 percent osmium tetroxide diluted in PBS **[1]** and incubate for 1 hour to enhance scanning electron microscopy contrast **[2-TXT]**. Then, rinse the substrate twice with PBS **[3]**.

4.3.1. Talent adding 500 µL of 1% osmium tetroxide solution directly into the well to fully cover the coverslip.

4.3.2. Talent placing the sample in an incubator. **TXT: RT; Use a BSL2 hood**

4.3.3. Talent rinsing the substrate twice with PBS.

4.4. Dehydrate the samples by immersing them sequentially for 10 minutes each in graded ethanol series **[1-TXT]**.

4.4.1. Talent sequentially adding increasing concentrations of ethanol directly into the wells for dehydration, replacing the solution with the next. **TXT: Ethanol concentrations: 25, 50, 70, 90, and 100% (v/v)** **Authors:** We can show only 1

replacement as showing the incubation with all solutions will make the video clip longer and we do not have that much of narration to cover. We need only a small 30-second clip for each shot to match the narration.

4.5. Next, add 500 microliters of hexamethyldisilazane and incubate for 5 minutes **[1]**. Then, replace with fresh hexamethyldisilazane and incubate for an additional 5 minutes **[2]**. Afterward, remove the excess solution and let the sample air-dry completely under a fume hood **[3]**.

4.5.1. Talent pipetting hexamethyldisilazane onto the sample and setting a timer.

4.5.2. Talent replacing the liquid with fresh hexamethyldisilazane and incubating again.

4.5.3. Talent draining the excess and leaving the sample uncovered in the fume hood to dry.

4.6. Now, mount the dried samples onto scanning electron microscopy stubs using double-sided conductive carbon tape **[1]**. Sputter-coat the samples with a thin layer, approximately 10 nanometers, of gold or platinum to enhance electron contrast for imaging **[2]**.

4.6.1. Talent affixing the dried samples to SEM stubs with carbon tape.

4.6.2. Talent sputter-coating the samples in the coating chamber.

4.7. Finally, load the prepared stubs into the scanning electron microscope using the appropriate sample holder **[1]**. Evacuate the chamber overnight if possible to improve imaging quality **[2]**, then acquire secondary electron images at 5 to 15 kilovolts using suitable magnifications to visualize the ultrastructure of the biofilm **[3]**.

4.7.1. Talent loading the stubs into the SEM and securing the sample holder.

4.7.2. Talent working at the computer attached to SEM.

4.7.3. SEM software interface showing secondary electron imaging at selected voltages and magnifications.

Results

5. Results

5.1. After 21 days of incubation, crystal violet staining revealed visible biofilm patterns on both polycarbonate filters and glass coverslips for *Leptospira interrogans* and *Leptospira biflexa*, with each species displaying distinct architectural footprints such as dot-like, branching, or reticulated forms **[1]**.

5.1.1. LAB MEDIA: Revised Figure 2A. *Video editor: Sequentially highlight the images*

5.2. Absorbance measurements at 570 nanometers confirmed greater crystal violet retention in *Leptospira biflexa* biofilms compared to *Leptospira interrogans* across all time points, indicating higher biomass accumulation in the saprophytic strain **[1]**.

5.2.1. LAB MEDIA: Revised Figure 2B. *Video editor: Highlight the green line representing L. biflexa*

5.3. Scanning electron microscopy of *Leptospira interrogans* biofilms captured early extracellular matrix deposits in 3-day-old biofilms **[1]**, a mature and channeled basal face with three-dimensional structure at 14 days **[2]**, and fully developed matrix consolidation with dense architecture by 21 days **[3]**.

5.3.1. LAB MEDIA: Revised Figure 2C.

5.3.2. LAB MEDIA: Revised Figure 2D. .

5.3.3. LAB MEDIA: Revised Figure 2E and F

1. Leptospira
   Pronunciation link: https://www.merriam-webster.com/dictionary/Leptospira
   IPA: /ˌlɛp.təˈspaɪ.rə/
   Phonetic Spelling: lep·tuh·spy·ruh
2. interrogans
   Pronunciation link: https://www.merriam-webster.com/dictionary/interrogans
   IPA: /ˌɪn.təˈrɑː.gænz/
   Phonetic Spelling: in·tuh·rah·ganz
3. biflexa
   Pronunciation link: No confirmed link found

IPA: /baɪˈflɛk.sə/
Phonetic Spelling: bye·flek·suh

4. logarithmic
Pronunciation link: https://www.merriam-webster.com/dictionary/logarithmic
IPA: /ˌlɔː.gəˈrɪð.mɪk/
Phonetic Spelling: law·guh·rith·mik
5. spectrophotometer
Pronunciation link: https://www.merriam-webster.com/dictionary/spectrophotometer
IPA: /ˌspɛk.troʊ.foʊˈtɑː.mə.tər/
Phonetic Spelling: spek·troh·foh·tah·muh·ter
6. polycarbonate
Pronunciation link: https://www.merriam-webster.com/dictionary/polycarbonate
IPA: /ˌpɑː.liˈkɑːr.bə.neɪt/
Phonetic Spelling: pah·lee·kar·buh·nayt
7. hydrophilic
Pronunciation link: https://www.merriam-webster.com/dictionary/hydrophilic
IPA: /ˌhaɪ.droʊˈfɪl.ɪk/
Phonetic Spelling: hy·droh·fil·ik
8. paraformaldehyde
Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
IPA: /ˌper.ə.fɔːrˈmæl.də.haɪd/
Phonetic Spelling: pair·uh·for·mal·duh·hyde
9. glutaraldehyde
Pronunciation link: https://www.merriam-webster.com/dictionary/glutaraldehyde
IPA: /ˌgluː.təˈræl.də.haɪd/
Phonetic Spelling: gloo·tuh·ral·duh·hyde
10. cacodylate
Pronunciation link: https://www.merriam-webster.com/dictionary/cacodylate
IPA: /kəˈkɑː.də.leɪt/
Phonetic Spelling: kuh·kah·duh·layt
11. osmium tetroxide
Pronunciation link: https://www.merriam-webster.com/dictionary/osmium%20tetroxide
IPA: /ˈɑːz.mi.əm tɛˈtrɑː.k.saɪd/
Phonetic Spelling: oz·mee·um teh·trok·side
12. hexamethyldisilazane
Pronunciation link: https://www.merriam-webster.com/dictionary/hexamethyldisilazane
IPA: /ˌhɛk.səˌmɛθ.əl.daɪˈsɪl.ə.zeɪn/
Phonetic Spelling: hek·suh·meth·ul·dye·sil·uh·zayn
13. sputter-coat
Pronunciation link: https://www.merriam-webster.com/dictionary/sputter
IPA: /ˈspʌt.ər koʊt/
Phonetic Spelling: sput·er koht
14. kilovolt
Pronunciation link: https://www.merriam-webster.com/dictionary/kilovolt

IPA: /ˈkɪl.oʊ.voʊlt/
Phonetic Spelling: kil·oh·vohlt

15. ultrastructure
Pronunciation link: https://www.merriam-webster.com/dictionary/ultrastructure
IPA: /ˈʌl.trəˌstrʌk.tʃər/
Phonetic Spelling: ul·truh·struk·cher
16. saprophytic
Pronunciation link: https://www.merriam-webster.com/dictionary/saprophytic
IPA: /ˌsæp.rəˈfiθ.ɪk/
Phonetic Spelling: sap·ruh·fith·ik