FINAL SCRIPT: APPROVED FOR FILMING

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Title: Analysis of the Lipid Composition of Mycobacteria by Thin Layer Chromatography

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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. **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**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

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

Current Protocol Length

Number of Steps: 12
Number of Shots: 37

NOTE by videographer: 01 INTWS (5) *>> 5 files and 10,35 GB.*
02 PROTOCOL (46) *>> 46 files and 40,26 GB.*
03 PHOTOS (5) *>> 5 files*
04 NOTES (1) *>> 1 file*

Introduction

1. Introductory Interview Statements

NOTE to VO talent: Please record the introduction and conclusion statements.
REQUIRED:

1.1. Mycobacteria lipids are an important characteristic of the genus and are critical in host-mycobacteria interactions.

1.1.1. *2.1.2*

1.2. Speed and versatility are the main advantages of this procedure.

1.2.1. *3.1.1*

NOTE: Vo narration for shot 1.2 was changed.

OPTIONAL:

1.3. This method can be used to understand the role of lipids in the pathogenicity of mycobacteria and their immunostimulatory effect.

1.3.1. *2.2.1*

Protocol

2. Extraction of The Total Non-Covalent-Linked Lipids from Mycobacteria

2.1. For the non-covalent-linked lipids extraction, add 15 milliliters of 1:2 chloroform to methanol solution to the mycobacteria under a laminar flow hood **[1]**. Then, scratch 200 milligrams of mycobacteria from a solid medium **[2]** and add the mycobacteria to a glass tube with a PTFE *(P-T-F-E)* liner screw cap **[3]**.

2.1.1. WIDE: Talent adding solution to the tube. *Videographer: This step is important!*

NOTE: Shot 2.2.1 and VO narration were placed at the beginning before step 2.1.1.

2.1.2. Talent scratching a solid media.

2.1.3. Talent adding mycobacteria to tube.

2.2. Incubate the tube overnight with constant stirring **[1]**.

2.2.1. Shot of the tube placed on shaker.

2.3. On the next day, filter the organic solvents through a glass funnel lined with filter paper into a glass tube **[1]**. After using nitrogen gas flux to evaporate the liquid phase in the tube **[2]**, fill the tube with nitrogen gas **[3]** to store the tube at 4 degrees Celsius **[4]**.

2.3.1. Talent filtering the solvents.

2.3.2. Talent using a nitrogen flux for evaporation. *Videographer: This step is important!*

2.3.3. Talent filling the tube. *Videographer: This step is important!*

2.3.4. Talent storing tube in the fridge.

2.4. Then, add 15 milliliters of a 2:1 chloroform to methanol solution to the cellular debris **[1]** and incubate the tube overnight with constant stirring **[2]**.

2.4.1. Talent adding chloroform:methanol solution to the tube.

2.4.2. Shot of the tube kept for stirring.

2.5. On the next morning, allow the mixture to rest for 1 hour **[1]** before using a Pasteur pipette to transfer the organic solvents into a filter paper-lined, glass funnel into the same glass collection tube **[2]**. Then evaporate the liquid phase as demonstrated **[3]** before re-filling the tube with nitrogen gas for 4 degrees-Celsius storage **[4]**.

2.5.1. Talent turning off shaker.

2.5.2. Talent adding solvents to funnel over glass tube.

2.5.3. Talent evaporating organic phase.

2.5.4. Talent filling tube.

3. Mycolic Acid Extraction by Acid Methanolysis

3.1. For mycolic acid extraction, add 2 to 5 milliliters of esterifying solution **[1]** and 200 milligrams of mycobacteria biomass to a hermetic glass tube with a PTFE liner screw cap **[2]** and mix the contents by vortexing **[3]**. Incubate the mixture inside a dry bath at 80 degrees Celsius overnight **[4]**.

3.1.1. WIDE: Talent adding esterifying solution in a glass tube. *Videographer: This step is important!*

3.1.2. Talent adding mycobacteria to the glass tube. *Videographer: This step is important!*

3.1.3. Talent vortexing the tube.

3.1.4. Talent placing the tube in a dry bath.

3.2. The next day, when the tube has cooled room temperature, add 2 milliliters of n-hexane **[1]** and mix the contents for 30 seconds by vortexing **[2]**. Allow the tube to settle until two clear phases appear **[3]** and transfer the upper, n-hexane phase to a new tube **[4]**.

3.2.1. Talent adding hexane to the tube. *Videographer: This step is important!*

3.2.2. Talent vortexing the tube.

3.2.3. Talent placing tube into rack on bench.

NOTE: Shot number 3.2.3 was labeled as 3.2.2 by the videographer.

3.2.4. Shot of layers, upper layer being collected and transferred to a new tube. *Videographer: This step is important!*

3.3. Mix 2 additional milliliters of n-hexane with the tube contents **[1]** and collect the upper layer again **[2]**. Then evaporate the tube contents by nitrogen flow **[3]** and store the sample, covered in nitrogen, at 4 degrees Celsius as demonstrated **[4]**.

3.3.1. Talent adding hexane and vortexing the tube.

3.3.2. Talent collecting upper layer and transferred to same tube, previously used.

3.3.3. Talent evaporating the organic phase.

3.3.4. Talent placing tube at 4 °C.

4. Thin Layer Chromatography (TLC) Analysis

4.1. To analyze the samples by TLC, cover one wall of the TLC chamber with a piece of filter paper. Add Vaseline on the chamber corners to seal the chamber. Decant the solvent mixture over the filter paper **[1-TXT]** and add the remaining volume of the solvent to the bottom of the TLC chamber **[2]**. Close the TLC chamber for at least 20 minutes to saturate it **[3]**.

4.1.1. WIDE: Talent placing filter paper and adding vaseline on corners of the chamber. Talent decanting solvent mixture over the filter paper. **TEXT: See text for mobile phase preparation details**

NOTE: The shot description for step 4.1 was changed and actions were added in the shot 4.1.1.

4.1.2. Shot of the mobile phase in the chamber.

4.1.3. Talent closing the chamber.

NOTE: New shot 4.1.3 was added here. This is shot 4.2.1 that was replaced after 4.1.2.

4.2. Meanwhile, dissolve the lipids in the glass tube in 200-1000 microliters of chloroform **[1]** and use a capillary glass tube to apply 10 microliters of the lipid-chloroform suspension directly onto the TLC plate **[2]**. *Videographer: This step is important!*

4.2.1. Talent dissolving lipids in chloroform.

4.2.2. Talent applying sample on the TLC plate.

4.3. After allowing the sample to dry for 5 minutes, insert the plate into the saturated TLC chamber **[1]** and allow the mobile phase to run through the TLC **[2]**. When the solvent reaches 1 centimeter from the upper end of the plate **[3]**, place the plate under laminar flux until the silica is completely dry **[4]**.

4.3.1. Talent placing TLC plate in the chamber.

4.3.2. Mobile phase running through TLC.

4.3.3. Shot of solvent 1 cm from end of plate.

4.3.4. Talent placing plate under laminar flux.

4.4. Then spray the dried plate with 15 to 20 milliliters of 10% molybdatophosphoric acid hydrate in ethanol **[1]** until the plate is bright yellow **[2]** and heat the plate for 2 to 5 minutes at 120 degrees Celsius **[3]**. *Videographer: This step is important!*

4.4.1. Talent spraying the TLC plate with solution.

4.4.2. Shot of the plate with bright yellow stain.

4.4.3. Talent placing plate at 120 °C, waiting for 2-5 min until observing the appearance of lipid spots and removing the plate from the heater.

NOTE: Additional actions in shot 4.4.3

Results

5. Results: TLC Analysis of the Lipid Composition in Different Species of Mycobacteria

5.1. In this representative analysis **[1]**, the mycolic acid extracted from various mycobacteria species were analyzed through one dimensional TLC using two different elution systems **[2]** and two-dimensional TLC. Two types of methylation procedures were also performed to confirm the presence of type five mycolic acid **[3]**.

5.1.1. LAB MEDIA: Figure 3 A, B.

5.1.2. LAB MEDIA: Figure 3 A, B. *Video Editor: Emphasize elution system text in upper left corner of each plate.*

5.1.3. LAB MEDIA: Figure 3 A, B. 7 *Video Editor: Emphasize lanes 1 to 4 together, then lanes 1' to 4' together.*

5.2. In both elution systems, the mycolic acids were located approximately in the middle of the plate **[1]**, although the spot corresponding to type five mycolic acid was observed only after saponification **[2]**.

5.2.1. LAB MEDIA: Figure 3 A, B. *Video Editor: Emphasize spots around I, II, and IV in both plates*

5.2.2. LAB MEDIA: Figure 3 A, B. *Video Editor: Emphasize V spot in lane 2' of both plates.*

5.3. Two-dimensional TLC also allows the identification of individual mycolic acid types **[1]**.

5.3.1. LAB MEDIA: Figure 3 C.

5.4. For example, *Mycobacterium brumae* expresses only type one mycolic acids **[1]** *Mycobacterium bovis* BCG expresses type one and four **[2]**, *Mycobacterium fortuitum* expresses type one and five **[3]**, and *Mycobacterium abscessus* expresses type one and two mycolic acid profiles **[4]**.

5.4.1. LAB MEDIA: Figure 3 C. *Video Editor: Emphasize type I spot in M. brumae plate.*

5.4.2. LAB MEDIA: Figure 3 C. *Video Editor: Emphasize type I and IV spot in Mycobacterium bovis plate.*

5.4.3. LAB MEDIA: Figure 3 C. *Video Editor: Emphasize type I and V spot in Mycobacterium fortuitum plate.*

5.4.4. LAB MEDIA: Figure 3 C. *Video Editor: Emphasize type II spot in Mycobacterium abscessus plate.*

5.5. The total lipid extracts from various mycobacteria species were analyzed in function of their polarity and size **[1].** It reveals that most apolar lipids, such as PDIMs *(P-D-E-M's)* are present in *Mycobacterium bovis BCG* (B-C-G) , but not in *Mycobacterim fortuitum, Mycobacterium brumae* **[2-TXT]** and the smooth morphotype of *M. abscessus* **[3]**.

5.5.1. LAB MEDIA: Figure 4 A, B

5.5.2. LAB MEDIA: Figure 4 A, B. *Video Editor: Emphasize the presence (in* M. bovis *BCG) and the absence (in* M. fortuitum*) of PDIM in 4B plates lanes 1, 2, and 4 in all plates.* **TEXT: PDIM: phthiocerol dimycocerosate**

5.5.3. LAB MEDIA: Figure 4 C, D, E. *Video Editor: Emphasize lane 3 in all plates.*

5.6. Phenolic glycolipids are present only in *M. bovis BCG* **[1]** while glycopeptidolipids are observed only in samples from *Mycobacterium abscessus* **[2]**.

5.6.1. LAB MEDIA: Figure 4 C. *Video Editor: Emphasize PGL spots in lane 1 in all plates.*

5.6.2. LAB MEDIA: Figure 4 D, E. *Video Editor: Emphasize GPL spots in lane 3 in 4D and lane 3 in 4E plates.*

5.7. Similar to mycolic acids, acyl glycerols, and PDIMs, PIMs (pems) can also be easily visualized through one dimensional TLC or 2D-TLC analyses **[1]**. Two types of stains were used to reveal PIMs in one dimensional TLC **[2-TXT]**.

5.7.1. LAB MEDIA: Figure 5 A, B.

5.7.2. LAB MEDIA: Figure 5 C. **TEXT: PIM: phosphatidil-inositol mannoside**

Conclusion

6. Conclusion Interview Statements

NOTE to VO talent: Please record the introduction and conclusion statements.

6.1. The most important thing to remember when attempting this protocol is to not use any plastic instruments throughout the procedure.

6.1.1. *2.5.2*