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Title: An Ex vivo Assay to Study Candida albicans Hyphal Morphogenesis in the Gastrointestinal Tract

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **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? **Yes**

   If **Yes**, how far apart are the locations? **Walking distance, approximately a 5-minute walk.**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Shankar Thangamani:** The protocol demonstrated in this video allows us to understand the role of gut metabolites on fungal hyphal morphogenesis.

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

1.2. **Shankar Thangamani:** This *ex vivo* technique models the closest approximation of the gut for studying fungal hyphal morphogenesis, in a way that no *in vitro* study can replicate.

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

OPTIONAL:

1.3. **Ross Monasky:** This method can be applied to study the role of gut metabolites on other enteric pathogens.

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

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Midwestern University.

Protocol

2. Mouse Dissection

2.1. Dissect mice using autoclave-sterilized sharp ended scissors and forceps **[1]**. After euthanasia, secure the animal to a dissection surface by pinning all limbs to expose the abdomen **[2]**. Spray the abdominal region with 70% ethanol to prevent fur from sticking to forceps, scissors, or gut sections **[3]**.

2.1.1. WIDE: Establishing shot of talent at the workstation, preparing to dissect the mouse.

2.1.2. Talent pinning the mouse to the dissection surface.

2.1.3. Talent spraying the mouse with ethanol.

2.2. Use forceps to pinch and lift a section of skin at the base of the abdomen and create a small incision through the skin and underlying fascia, taking care to avoid puncturing the cecum or intestinal wall **[1]**.

2.2.1. Talent pinching the skin and making the incision.

2.3. Extend this cut to the rib cage, partially exposing the peritoneal cavity **[1]**. Then, make a cut starting at the point of the initial incision on either side and extending upward and laterally **[2]**. Pull these flaps laterally and pin them to the dissecting surface to fully expose the peritoneal cavity **[3]**.

2.3.1. Talent extending the cut.

2.3.2. Talent making the side cuts.

2.3.3. Talent pinning the flaps to the dissection surface.

2.4. Extract the GI tract with forceps while using scissors to make cuts superior to the stomach and at the distal region of the large intestine to ensure collection of the greatest amount of gut content **[1]**. *Videographer: This step is important!*

2.4.1. Talent extracting the GI tract while making the cuts.

2.5. When removing the GI tract, take care to avoid rupturing the individual components. Separate the stomach, small intestine, cecum, and large intestine at their proximal and distal ends **[1]**.

2.5.1. Talent carefully removing the GI tract and separating the components.

2.6. For collection of gut contents from each section, make a single incision at the distal end **[1]**, then manually expel the gut content into a 1.5-milliliter microcentrifuge tube with forceps **[2]**. Store the samples at -80 degrees Celsius for ex vivo assays **[3]**. *Videographer: This step is important!*

2.6.1. Talent making an incision to separate a section.

2.6.2. Talent expelling the gut contents into a tube.

2.6.3. Talent putting the sample tubes in the freezer and closing the door.

3. Ex Vivo Prep and Exogenous Addition of Metabolites to the Gut Homogenate Extracts for Hyphal Morphogenesis Assay

3.1. Streak a fresh culture of *C. albicans* SC5314 onto a YPD agar plate **[1]** and incubate it overnight at 30 degrees Celsius **[2]**. On the next day, pick two to three medium-sized individual colonies **[3]** and re-suspend them in 1 milliliter of PBS **[4]**. *Videographer: This step is important!*

3.1.1. Talent streaking the fungal cells on the plate.

3.1.2. Talent putting the plate in the incubator and closing the door.

3.1.3. Talent picking colonies from the plate.

3.1.4. Talent resuspending the cells in the PBS.

3.2. Retrieve frozen gut contents from the freezer and thaw them at 25 degrees Celsius **[1]**. Transfer approximately 150 milligrams of gut contents into a new 1.5-milliliter tube **[2]** and resuspend them with 150 microliters of PBS **[3]**.

3.2.1. Talent taking the gut content samples out of the freezer.

3.2.2. Talent transferring the gut contents to a new tube.

3.2.3. Talent resuspending the gut contents.

3.3. Vortex the sample at high speed for 30 seconds to homogenize the gut contents and allow it to sit at room temperature for about a minute **[1]**. Centrifuge the homogenates at 1000 x *g* for 3 minutes **[2]**, then transfer the supernatant to a new 1.5-milliliter tube, taking care to not transfer any debris **[3]**. *Videographer: This step is difficult!*

3.3.1. Talent vortexing the sample.

3.3.2. Talent putting the sample in the centrifuge and closing the lid. *Videographer: Obtain multiple usable takes, this will be reused in 3.5.2.*

3.3.3. Talent transferring the supernatant to a new tube. *Videographer: Obtain multiple usable takes, this will be reused in 3.5.3.*

3.4. After repeating the centrifugation, add 10 microliters of the *C. albicans* inoculum to the supernatant, mix well **[1]**, and incubate the sample at 37 degrees Celsius for 4 to 5 hours **[2]**.

3.4.1. Talent adding the inoculum to the sample.

3.4.2. Talent putting the sample in the incubator and closing the door.

3.5. To test the effect of exogenous metabolites on *C. albicans*, add the desired concentration of metabolites to the gut content and PBS mixture **[1]**, then vortex the sample at high speed for 30 seconds, let it sit at room temperature for 10 minutes **[2]**, and perform centrifugation and inoculation as previously described **[3]**.

3.5.1. Talent adding the metabolite to the gut contents.

3.5.2. *Use 3.3.1.*

3.5.3. *Use 3.3.2.*

4. *C. albicans* Morphogenesis Assay

4.1. Centrifuge the fungal cell culture at 1000 x *g* for 2 minutes and discard the supernatant **[1]**. Fix the samples in 100 microliters of 2% PFA for 15 minutes **[2]**, then repeat the centrifugation and discard the supernatant **[3]**.

4.1.1. Talent putting the sample in the centrifuge and closing the lid. *Videographer: Obtain multiple usable takes, this will be reused in 4.1.3.*

4.1.2. Talent adding PFA to the cells.

4.1.3. *Use 4.1.1.*

4.2. Wash the samples twice with 1 milliliter of PBS according to manuscript directions **[1]**, then incubate the samples at room temperature in 100 microliters of PBS with polyclonal *C. albicans* antibody for 30 minutes **[2]**. After the incubation, wash the sample 3 more times with PBS, working in dim light to avoid photo bleaching **[3]**. *Videographer: This step is difficult and important!*

4.2.1. Talent adding PBS to the sample, with the PBS container in the shot. *Videographer: Obtain multiple usable takes, this will be reused in 4.2.3.*

4.2.2. Sample incubating at room temperature.

4.2.3. *Use 4.2.1.* NOTE: Because this step was described as occurring in dim light, it was filmed to distinguish it from 4.2.1. which is not in dim light.

4.3. In a dark room, incubate the samples at room temperature for 15 minutes in 100 microliters of PBS containing anti-Rabbit IgG Alexafluor 488 antibody at 1 to 500 dilution **[1]**. After washing the sample 3 times with 1 milliliter of PBS, resuspend it in 100 microliters of PBS **[2]** and transfer it to a 96-well plate for imaging **[3]**. *Videographer: This step is important!*

4.3.1. Talent placing the sample in a dark room to incubate.

4.3.2. Talent resuspending the sample in PBS.

4.3.3. Talent transferring the sample to a well in the plate.

4.4. Image the fungal cells with a fluorescence imaging microscope using 20x and 40x objective lenses and a green fluorescent protein filter **[1]**. *Videographer: This step is important!*

4.4.1. Talent using the microscope.

Results

5. Results: Effects of Antibiotic and Gut Metabolites on *C. albicans* Hyphal Morphogenesis

5.1. When *C. albicans* is grown ex vivo in gut homogenate extracts taken from the stomach, small intestines, and large intestines of untreated control and antibiotic-treated mice, it generally develops with a yeast morphology **[1]**.

5.1.1. LAB MEDIA: Figure 1 B. *Video Editor: Emphasize the stomach, small, and large intestine images in both rows.*

5.2. However, when grown in the cecal extract from antibiotic-treated mice, *C. albicans* readily undergoes morphogenesis, resulting in yeast and hyphae forms **[1]**. This indicates that that antibiotic treatment causes changes in the cecal environment, which induce hyphal morphogenesis of *C. albicans* **[2]**.

5.2.1. LAB MEDIA: Figure 1 B. *Video Editor: Emphasize the cecum antibiotic treated image.*

5.2.2. LAB MEDIA: Figure 1 B.

5.3. Exogenous addition of glucose to the cecal homogenate of antibiotic-treated mice showed a massive hyphal development ex vivo. These results suggest that addition of gut metabolites back to the cecal homogenate of the antibiotic-treated mice differentially regulates the morphogenesis of *C. albicans* **[1]**.

5.3.1. LAB MEDIA: Figure 2 B. *Video Editor: Emphasize the antibiotic + glucose image.*

Conclusion

6. Conclusion Interview Statements

6.1. **Ross Monasky:** When attempting this protocol, keep in mind that optimization of dilution of the gut content ensures sufficient collection of gut metabolites without collection of debris. Do not exceed a 2:1 media:gut content dilution, and aim for a lower dilution if possible.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.1 and 3.3.2.*

6.2. **Ross Monasky:** This technique is being used now to explore how specific metabolites in the gut impact hyphal development, allowing researchers to better understand the role of gut metabolites on fungal pathogenesis.

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