

Journal of Visualized Experiments

Visualization of Candida Albicans in the Murine Gastrointestinal Tract Using Fluorescent In Situ Hybridization (FISH)

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60283R1
Full Title:	Visualization of Candida Albicans in the Murine Gastrointestinal Tract Using Fluorescent In Situ Hybridization (FISH)
Keywords:	Candida albicans; gut commensal; Fluorescent in situ hybridization (FISH); mucosa; localization; morphology
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	San Francisco, California

University of California
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May 17, 2019

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Dear Dr. Upponi:

Attached please find our invited manuscript, entitled "Visualization of *Candida albicans* in the murine gastrointestinal tract using fluorescent *in situ* hybridization (FISH)." We have attempted to make the protocol as clear as possible, and look forward to any suggestions for improvements proposed by you and the reviewers.

With best regards,

A handwritten signature in blue ink that reads "S Noble".

Suzanne Noble MD PhD
Associate Professor of Microbiology and Immunology
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TITLE:

Visualization of *Candida Albicans* in the Murine Gastrointestinal Tract Using Fluorescent In Situ Hybridization (FISH)

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KEYWORDS:

Candida albicans, gut commensal, fluorescent in situ hybridization (FISH), mucosa, localization, morphology

SUMMARY:

The purpose of this protocol is to visualize *Candida albicans* cell shape and localization in the mammalian gastrointestinal tract.

ABSTRACT:

Candida albicans is a fungal component of the gut microbiota in humans and many other mammals. Although *C. albicans* does not cause symptoms in most colonized hosts, the commensal reservoir does serve as a repository for infectious disease, and the presence of high fungal titers in the gut is associated with inflammatory bowel disease. Here, we describe a method to visualize *C. albicans* cell morphology and localization in a mouse model of stable gastrointestinal colonization. Colonization is established using a single dose of *C. albicans* in animals that have been treated with oral antibiotics. Segments of gut tissue are fixed in a manner that preserves the architecture of luminal contents (microorganisms and mucus) as well as the host mucosa. Finally, fluorescent in situ hybridization is performed using probes against fungal rRNA to stain for *C. albicans* and hyphae. A key advantage of this protocol is that it allows for simultaneous observation of *C. albicans* cell morphology and its spatial association with host structures during gastrointestinal colonization.

INTRODUCTION:

Candida albicans is a fungal commensal as well as an opportunistic human pathogen. This yeast lacks a defined environmental niche and instead propagates within the gastrointestinal (GI) tract,

skin, and genitourinary tract of humans and other mammals¹. Whereas early research on *C. albicans* focused primarily on its virulence potential, several recent reports suggest that commensally propagating organisms within the gut may play important roles in normal health, including the immune development of the host²⁻⁴. To facilitate investigations of *C. albicans* commensalism within the mammalian gut, we developed a mouse model of stable GI colonization and a fluorescence in situ hybridization (FISH)-based method to visualize fungal yeast cells and hyphae within the intestinal lumen.

With some exceptions⁵, laboratory-bred mice typically exhibit resistance to fungal colonization of the digestive tract. Colonization resistance is thought to be mediated by specific bacterial species; however, this can be overcome by treatment of the animals with antibiotics^{6,7} or use of a chemically-defined diet that presumably alters bacterial species composition^{8,9}. Similarly, in humans, the use of broad-spectrum antibiotics has been associated with *C. albicans* overgrowth and dissemination¹⁰. Our murine colonization model uses broad-spectrum antibiotics to establish *C. albicans* colonization of immunocompetent, conventionally reared mice. Penicillin and streptomycin are provided in the animals' drinking water for one week prior to gavage with 10⁸ colony forming units (CFUs) of *C. albicans*. As long as the antibiotic-infused water is continued, *C. albicans* will propagate through the GI tract, reaching fecal titers of 10⁶–10⁸ CFUs/g. Despite the high level of fungal colonization, animals remain healthy and gain weight at the same rate as uninfected controls. This model has been used successfully to screen for and characterize multiple *C. albicans* commensalism factors^{11,12}.

Like other members of the fungal kingdom, *C. albicans* is capable of enormous morphological plasticity¹³. Under in vitro conditions, it has been shown to transition among at least six unicellular yeast cell types, as well as multicellular hyphae and pseudohyphae. The yeast-to-hypha transition is one of its best-characterized virulence attributes, and hyphae and pseudohyphae predominate in most mammalian disease models, as well as in infected human tissues. To determine *C. albicans* localization and cell morphology within the murine digestive tract, we developed a FISH technique for staining yeasts and hyphae in fixed histological sections. The probes consist of fluorescently labeled DNA oligonucleotides that hybridize to fungal 23S ribosomal RNA (rRNA), which is distributed throughout the fungal cytoplasm. Because host tissues are fixed in a manner that preserves the three-dimensional architecture of the gut, including the host mucosa, digestive material, the bacterial microbiota, and mucus within the gut lumen, this technique permits the localization of fungal cells with respect to these landmarks when stained. The FISH technique compares favorably to traditional histological stains for fungi, such as Periodic Acid Schiff (PAS) or Gomori's Methenamine-Silver (GMS), as well as commercially available antifungal antibodies, because these reagents are not specific for *C. albicans*. Moreover, standard fixatives remove the mucus layer and disrupt other contents of the gut lumen^{14,15}.

In this article, we provide detailed instructions for establishing high-grade *C. albicans* colonization of the mouse GI tract, for dissection of the digestive tract from euthanized animals, for tissue fixation in a manner that preserves luminal architecture, and for detection of *C. albicans* within host tissues using FISH. In addition to wild type and mutant strains of *C. albicans*, the gavage technique can be used to deliver other microorganisms. The fixation technique would be useful

for any study in which preservation of gut contents is desired. The FISH procedure can be completed within a day and can be used to localize multiple fungal species by using multiple, differentially labeled probes.

PROTOCOL:

The steps described below have been approved by the UCSF Institutional Animal Care and Use Committee (IACUC).

1. Gastrointestinal colonization of mice with *C. albicans* using oral gavage

1.1. Provide housing for 18–21 g male or female mice (8–10 weeks old) as approved by the local IACUC. Use autoclaved food and water starting on the first day.

NOTE: The use of sterilized cages, bedding, food, and water reduces the risk of contamination with antibiotic-resistant environmental bacteria that can potentially displace *C. albicans* from the gut. Further, depending on the experimental question, individual housing of animals should be considered because mice are coprophagic and gut contents will be shared among cohoused animals.

1.2. The following day, replace the autoclaved drinking water with autoclaved water containing 5% glucose, 1,500 units/mL penicillin G, and 2 mg/mL streptomycin.

1.3. For convenience, prepare 200x antibiotic stock solutions (**Table 1**) in advance, filter sterilize, and freeze at –20 °C.

1.4. Maintain animals on antibiotics for one week.

1.4.1. On days 3 and 6 after antibiotics have been started, assess each animal's feces for contamination with antibiotic-resistant aerobic bacteria. Holding an animal with one hand, place an uncapped microfuge tube near the anus and collect at least one fecal pellet.

1.4.2. Resuspend each fecal pellet in 1 mL of sterile water and plate 100 µL on Luria broth (LB), yeast extract peptone dextrose (YEPD), or brain heart infusion (BHI) plus blood agar plates. Incubate the plates overnight in a standard incubator (not anaerobic) at 37 °C and assess for bacterial growth.

NOTE: Plates should exhibit minimal growth (0–20 colonies). If >20 colonies of bacteria are recovered from animals treated with penicillin and streptomycin, then it is unlikely that high-level colonization with *C. albicans* will be established and the experiment should be aborted.

1.5. Three days prior to gavage, streak *C. albicans* from frozen glycerol stocks to YEPD + 2% agar plates and incubate at 30 °C for 2 days.

1.6. One day before gavage, inoculate one colony of each *C. albicans* strain to be tested into 5 mL of liquid YEPD. Incubate at 30 °C overnight with shaking.

1.7. The morning of gavage, dilute the saturated culture of *C. albicans* to an optical density at 600 nm (OD₆₀₀) of 0.1 in 100 mL of YEPD prewarmed to 30 °C. Shake in an orbital shaker at 200 rpm at 30 °C for between 4 h and 5 h until OD₆₀₀ reaches 1 (mid-log growth).

1.8. Transfer each 100 mL culture to two 50 mL conical tubes and centrifuge at 3,000 x *g* for 5 min at room temperature (RT).

NOTE: *C. albicans* propagates relatively slowly at RT. If adapting this protocol for another species, the inocula may need to be kept on ice to prevent continued growth.

1.9. Discard the supernatant, resuspend the pelleted cells in 20 mL of sterile saline per 50 mL of culture, and pool the duplicate resuspended pellets into single conical tubes. Centrifuge at 3,000 x *g* for 5 min.

1.10. Discard the supernatant and resuspend in 20 mL of sterile saline. Vortex briefly and remove 20 µL for OD₆₀₀ measurement. Dilute 1:50 in sterile normal saline. Centrifuge the remaining resuspended cells at 3,000 x *g* for 5 min.

1.11. Discard the supernatant. For an inoculum size of 1 x 10⁸ CFU, resuspend the pelleted cells to approximately 5 x 10⁸ CFUs/mL in sterile normal saline based on the estimated concentration determined from OD₆₀₀ measurement.

NOTE: Typically, an OD₆₀₀ of 1 corresponds to ~ 1 x 10⁷ yeast cells/mL. This value may vary by spectrophotometer and should be verified. If a change in concentration is desired for infections with a different microorganism, plan for an inoculum volume of ≤10 µL/g mouse to minimize discomfort to the animal.

1.12. Verify the concentration of the inoculum by counting a 1:1000 dilution with a hemocytometer. Adjust the volume of cells to be gavaged based on the concentration determined using the hemocytometer.

1.13. Using an animal feeding needle (**Figure 1A**), gavage each animal with the calculated volume of a given inoculum. See steps 1.13.1–1.13.8 for the gavage procedure.

NOTE: Training for gavage should be obtained from an experienced practitioner, and the procedure should be mastered in advance. A successful procedure for a single animal typically takes no longer than 1 min.

1.13.1. Attach an autoclaved animal-feeding needle to a 1 mL syringe and fill the syringe with one gavage volume, removing any air bubbles to avoid an inaccurate dose. Place on a sterile surface.

1.13.2. Secure the animal for gavage. Hold the animal at the base of the tail with the dominant hand and slide nondominant hand up the animal's back to the loose skin just behind the ears.

1.13.3. Using the forefinger and thumb of the nondominant hand, gather the loose skin together tightly; this is the "scruff". Pick up the animal by its scruff and loop small finger of the same (nondominant) hand around the tail to immobilize animal against the palm of the hand. Gently extend the animal's head so that its head and body (and therefore neck and esophagus) are in a straight line.

NOTE: Attempting a gavage while the animal's body is bent or twisted may result in complications such as esophageal perforation and death of the animal.

1.13.4. Pick up the syringe with the dominant hand and hold perpendicular to the animal, with the curved needle pointing down (**Figure 1B**). Using the ball of the needle, gently hook an inside corner of the mouth, gently advance the needle along the side of the mouth to the back of the throat, and finally move the needle to the center.

NOTE: Introducing the needle along a lateral path helps to avoid resistance from the animal's teeth and tongue.

1.13.5. Once the ball of the needle is at the back of throat, tilt the needle up so that it is parallel with the animal's body line (**Figure 1C**).

NOTE: At this point, the animal will exhibit a gag reflex. This is a good sign that indicates that the needle is positioned correctly, and the gagging motion helps to guide the needle down the esophagus. If there is no gag reflex, the needle may be in the trachea rather than the esophagus. Gently withdraw the needle and proceed back to step 1.13.1.

1.13.6. Allow the animal to swallow the inoculum until only a few millimeters remain visible (**Figure 1D**). Advance the needle smoothly.

NOTE: If the needle does not advance, avoid using force, which can damage the esophagus. Sometimes advancing the last half centimeter will require an extra-large gag from the animal. If the needle seems to be stuck, slowly withdraw it and try again from step 1.13.4.

1.13.7. Test that the ball of needle is in the correct location (stomach) by gently advancing the plunger. If there is no resistance, continue delivering the inoculum. Once the inoculum has been administered, slowly withdraw the needle.

1.13.8. Replace the animal in its cage and continue to monitor the animal for 5–10 min for signs of labored breathing or distress. Verify that the animal resumes normal activity soon after gavage.

1.13.9. If the same inoculum will be used with the next animal, clean the needle with an alcohol

wipe. Proceed back to step 1.13.1. If a different inoculum will be used, use a new needle and proceed back to step 1.13.1.

NOTE: It is important to inspect animals the day after gavage. Animals exhibiting signs of distress (e.g., hunched posture, labored breathing) should be humanely euthanized, as they have likely suffered esophageal rupture, damage to the lungs, or another internal injury from which recovery is unlikely.

1.14. Following gavage of the animals, plate dilutions of the inocula for dose verification.

1.14.1. Prepare 10-fold serial dilutions up to $1:10^5$. Plate one gavage volume of the $1:10^5$ dilution onto a 100 mm plate of Sabouraud dextrose agar.

1.14.2. Incubate the plate for 2 days at 30 °C. Count CFUs to confirm inoculum dose.

2. Preparation of gastrointestinal tissues for histology

NOTE: This section of the protocol is adapted from Johansson and Hansson¹⁶.

2.1. Prepare 500 mL of methacarn solution (60% methanol, 30% chloroform, 10% glacial acetic acid) in a large screw cap plastic jar for every 2 animals dissected.

CAUTION: Methanol and chloroform are harmful if inhaled and should be manipulated in a chemical hood. Glacial acetic acid can be corrosive and should be handled with the proper personal protective equipment. Methanol, chloroform, and glacial acetic acid should be discarded as hazardous waste.

2.2. At the experimental endpoint, euthanize animals humanely according to a protocol approved by the local IACUC.

NOTE: In antibiotic-treated animals, *C. albicans* colonization typically ranges from $\sim 10^6$ CFUs/g on day 5 up to $\sim 5 \times 10^7$ CFUs/g by day 25.

2.3. Sterilize dissection tools with 10% bleach and rinse with 70% ethanol.

2.4. Secure each animal to a dissection surface, with ventral side facing up. Use 30 G needles to secure the limbs to the dissection surface (**Figure 2A**). Spray the animal with 70% ethanol to clean the fur and minimize sticking to the tools.

2.5. Using blunt-ended forceps, pinch a section of abdominal skin with nondominant hand. Using scissors with dominant hand, incise the skin and underlying fascia near the base of the pelvis. Extend the incision in a U shape along each side of the peritoneal cavity and up to the rib cage. Lift the skin out of the way.

2.6. Using a pencil, prelabel histology cassettes for the GI segments to be assessed. For example, for each animal, label separate cassettes as “stomach,” “proximal small intestines,” “mid small intestines,” “distal small intestines,” “cecum,” and “large intestines.” Place a foam pad on the bottom of each cassette (**Figure 2A**).

NOTE: **Figure 2B** outlines suggested sections to place in cassettes.

2.7. Using blunt-ended forceps, gently extract the cecum from the peritoneal cavity. Use scissors to sever connections between the cecum and the small and large intestines.

NOTE: Cecal tissue is very delicate and easy to rupture. Take care when manipulating.

2.8. Place the entire cecum into a histology cassette so that it is untwisted and lays flat (**Figure 2C**). Place a second foam pad on top and close the cassette. Place the cassette into a screw cap jar containing methacarn.

2.9. Excise a section of the large intestines that contains 1–2 fecal pellets, with a length less than that of the cassette.

2.10. Place the tissue section into the cassette, keeping the tissue flat and untwisted. Overlay with a second foam pad and close the cassette. Place the cassette into methacarn.

2.11. For each section of the small intestines to be sampled, excise a 1–2 cm segment that contains some digestive material.

2.12. Place the segment into a histology cassette, keeping the tissue flat and untwisted. Overlay with a second foam pad and close the cassette. Place the cassette into methacarn.

2.13. For the stomach, sever the connections to the esophagus and small intestines. Place in a cassette, keeping the tissue flat and untwisted. Overlay with a second foam pad and close the cassette. Place the cassette into methacarn.

2.14. Leave the cassettes in methacarn solution at RT for at least 3 h and less than 2 weeks.

NOTE: There are several points during which the fixed GI segments can be transferred to a commercial histology service. These tissues can be difficult to section without disruption of luminal contents, and optimal results will be obtained by an experienced technician. If the commercial service is willing to perform washes prior to embedding the tissues in paraffin, the cassettes can be sent at this stage along with instructions for step 2.16.

2.15. Begin melting enough paraffin wax to cover all tissue cassettes in a large beaker in a preheated 70 °C hybridization oven.

2.16. Following fixation, remove the foam pads and return each intact tissue section back into its

cassette. Wash the tissues with the following solutions at RT with shaking: twice for 35 min in 100% methanol, twice for 25 min in 100% ethanol, twice for 20 min in xylene.

CAUTION: Xylene is flammable, toxic, and can cause damage if inhaled. Use in a chemical hood with proper protection. Dispose of xylene via hazardous waste procedures.

2.17. Pat the cassettes dry on a paper towel and place in premelted paraffin wax for 2 h at 70 °C in a hybridization oven. Ensure that the cassettes are filled with paraffin and no bubbles remain.

NOTE: This step allows the wax to infiltrate the GI segment and to stabilize the tissue and contents.

2.18. Remove the cassettes from wax, allow the excess wax to drain, and store at RT until they are embedded in wax blocks. Discard the wax containing trace amounts of xylenes as hazardous waste.

NOTE: The Noble Lab uses a commercial histology service for embedding and sectioning of tissues. For standard visualization of *C. albicans* yeasts and hyphae, 4 µm sections are used. To evaluate connectivity of hyphal segments, which typically extend through multiple planes, 8 µm sections can be used. Depending on the ability of FISH probes to penetrate into the specimen, it may be possible to use even thicker sections.

3. Validation of newly designed probes

NOTE: The following protocol for validating *C. albicans* probes was adapted from Swidsinski et al.¹⁷.

3.1. Streak *C. albicans* SC5314 and closely related comparator species (e.g., *Candida dubliniensis*, *Candida tropicalis*) to YEPD + 2% agar plates. Incubate for 1–2 days at 30 °C.

3.2. Inoculate 5 mL of liquid YEPD medium with a single colony.

3.3. Pipette 1 mL of the suspended cells to a microcentrifuge tube and centrifuge at 3,000 x *g* for 5 min. Discard supernatant.

3.4. Resuspend the pellet in 100 µL of phosphate-buffered saline (PBS).

3.5. Pipette 5–10 µL of resuspended cells and spot onto a glass slide. Spread into a larger circle and allow to dry.

NOTE: If cells are not adherent, use poly-L-lysine coated slides.

3.6. Circle the cell spot with a PAP pen and cover with 50 µL of 2.5% paraformaldehyde (PFA) in PBS. Incubate at RT for 10 min.

CAUTION: PFA is flammable and can cause health problems if inhaled or in contact with skin. Items contaminated with PFA should be discarded as hazardous waste.

3.7. Wash 3x with PBS. Tap liquid off onto paper towel and cover with 100 μ L of PBS. Repeat twice. Discard final wash.

3.8. Prepare slides for multiday storage. If performing FISH on the same day, proceed to step 3.9 without the steps in this section.

NOTE: It is preferable to perform the hybridization immediately but if short on time follow step 3.8 before storing. Slides can be stored for several days at 4 $^{\circ}$ C.

3.8.1. Cover the spot with 60% ethanol. Incubate at RT for 3 min. Discard the solution.

3.8.2. Cover the spot with 80% ethanol. Incubate at RT for 3 min. Discard the solution.

3.8.3. Cover the spot with 100% ethanol. Incubate at RT for 3 min. Discard the solution. Proceed to step 3.9.

3.9. Place the slides uncovered in a hybridization oven at 50 $^{\circ}$ C for 1 h. If step 3.8 was followed, store the slides at 4 $^{\circ}$ C. If not, proceed to step 4.2 to perform the hybridization protocol.

4. FISH staining in GI tract tissues

4.1. Dewax paraffin-embedded histological sections.

4.1.1. In a hybridization oven, prewarm a Coplin jar filled with xylene to 60 $^{\circ}$ C.

4.1.2. Insert the slides into the xylene-filled jar. Place at 60 $^{\circ}$ C for 10 min. Discard the xylene wash. Keep the slides in the jar and pour off the xylene into a waste container.

4.1.3. Pour fresh RT xylene into the jar and incubate at 60 $^{\circ}$ C for 10 min. Discard the second xylene wash.

4.1.4. Fill the jar with 100% ethanol and incubate at RT for 5 min. Remove the slides from the jar and air dry.

4.1.5. Set the hybridization oven to 50 $^{\circ}$ C for steps in section 4.2.

4.2. Stain *C. albicans* with the FISH probe.

4.2.1. Prepare 1 mL of fresh hybridization solution (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% formamide in RNase-free water). See **Table 1** for sample

calculations.

4.2.2. Mix together 50 μ L of hybridization solution with 1 μ L of *C. albicans* probe (5' Cy3-ACAGCAGAAGCCGTGCC 3'; 50 μ g/mL in RNase-free water) for a final probe amount of 0.5 μ g per slide.

NOTE: Many laboratory-bred mice do not contain any fungi among their microbiota. In this case, it is possible to substitute a pan-fungal probe (5' Cy3-CTCTGGCTTCACCCTATTC 3') that recognizes 23S rRNA of most fungal species, not just *C. albicans*. In the authors' experience, the panfungal probe yields brighter staining than the *C. albicans*-specific probe.

4.2.3. Protect the solution from light and prewarm to 50 °C.

4.2.4. Using a PAP pen, draw a circle around tissue section from section 4.1.

4.2.5. Pipette 50 μ L of probe-containing hybridization solution onto the fixed tissue and gently spread over the tissue section with a pipette tip. Overlay the liquid with a hybridization coverslip making sure to prevent bubbles.

4.2.6. Seal slides in a watertight hybridization chamber and incubate the sections for 3 h at 50 °C in the dark in a hybridization oven.

4.2.7. Meanwhile, prepare 50 mL of FISH washing solution (20 mM Tris-HCl, pH 7.4, 0.9 M NaCl in RNase-free water). See **Table 1** for sample calculations.

4.2.8. Move the washing solution to 50 °C to prewarm.

4.2.9. After 3 h, add the washing solution to a Coplin jar. Then, carefully remove the coverslip from the slide with forceps and place the slide in the washing solution.

4.2.10. Cover the jar with aluminum foil and incubate at 50 °C for 20 min.

4.2.11. During this incubation, prepare mucin-nuclei staining solution (20 ng/mL 4',6-diamidino-2-phenylindole [DAPI], 1.6 μ g/mL fluorescein isothiocyanate [FITC]-lectin in PBS). See **Table 1** for sample calculations. For stomach and large intestines, use FITC-Ulex europaeus agglutinin 1 (UEA-1). For small intestines and cecum, use a combination of FITC-UEA-1 and FITC-wheat germ agglutinin (WGA).

NOTE: Lectins from different species recognize different sugar modifications of glycoproteins such as mucin. The lectin combinations recommended here were determined empirically for optimal staining of mucus in different GI compartments.

4.2.12. Wash the slides by pouring off the FISH washing solution and refilling the jar with RT PBS. Pour off the PBS immediately and refill with PBS. Pour off the PBS for a total of 2 washes.

4.2.13. Wick away the PBS with a delicate task wiper tissue and circle the intestinal tissue section with a PAP pen. Place the slides in an opaque plastic container and add 50 μ L of mucin-nuclei staining solution to the tissue section. Cover the container with aluminum foil to protect from light. Incubate at 4 °C for 45 min.

4.2.14. Place the slides in a Coplin jar and wash quickly twice in PBS at RT.

4.2.15. Tap away the excess PBS on a paper towel, and then wick away the final drops of PBS with a tissue.

4.2.16. Place one drop of mounting medium on each section and overlay with a glass coverslip, preventing bubbles. Let the mounting medium spread under the entire coverslip. For immediate imaging, anchor the coverslip in place with nail polish at the corners. For long term storage, seal around the coverslip with nail polish.

4.2.17. Image the slides using a fluorescent microscope.

REPRESENTATIVE RESULTS:

Following the instructions provided, the outcome of this technique will be fluorescent labelling of nuclei in the host epithelium, mucus, and *C. albicans* in sectioned GI tissues. Wild type *Candida albicans* cells should appear as either round, unicellular yeast cells or highly elongated, sometimes branching, multicellular hyphae (cell-cell divisions will not be apparent in the hyphae). Mutants of *C. albicans* may additionally appear as smaller, slightly elongated “gray” yeasts or as larger, slightly elongated “GUT” (gastrointestinal-induced transition) or “opaque” yeasts.

Figure 1 depicts the feeding needle used for oral gavage, as well as key positions of the needle during the gavage procedure. **Figure 2** provides a typical setup used for organ dissection and the appearance of GI segments of the mouse.

Figure 3 displays FISH staining of different *C. albicans* cell types following propagation in vitro and within the mouse model. **Figure 3A** shows fluorescence and phase images of fixed, permeabilized, in vitro-propagated hyphae. Hypha formation was induced with liquid Lee’s medium¹⁸ with 2% N-acetylglucosamine, pH 6.8 at 37 °C. **Figure 3B** shows fluorescence and phase images of fixed, permeabilized, in vitro-propagated yeast cells. **Figure 3C,D** shows fixed, permeabilized, in vitro-propagated GUT cells and opaque cells, respectively. GUT and opaque cell types are morphologically indistinguishable except when visualized by scanning electron microscopy. Please note that FISH staining of in vitro-propagated cells will be suboptimal if the cells are not well permeabilized. An example of weak and diffuse staining is shown in **Figure 3C**. For best results, cell fixation and permeabilization conditions should be determined empirically for each cell type and species to be investigated. Fortunately, the recommended protocol for visualizing *C. albicans* in sectioned tissues produces more consistent permeabilization.

Figures 3E-G depict *C. albicans* in mouse large intestines, stained with the *C. albicans*-specific probe (**Figure 3E**) or the panfungal probe (**Figure 3F,G**). *C. albicans* appears red in these images, host cell nuclei are blue, and the mucus layer is green. Both round yeasts and highly elongated hyphae (white arrowheads) occur in the large intestines. Please note that staining is brighter with the panfungal probe. **Figure 3G** depicts a *ume6* mutant in the same compartment, stained with the panfungal probe. *Ume6* encodes a transcription factor that is required for hypha formation under in vitro conditions¹⁹. Interestingly, the yeast-locked phenotype displayed by this mutant under in vitro conditions is not recapitulated within the gut, suggesting that a redundant factor must be activated within the host¹². **Figure 4** depicts the appearance of wild type *C. albicans* in different segments of the murine GI tract, including the regions immediately adjacent to the host mucosa and more central regions of the gut lumen.

FIGURE AND TABLE LEGENDS:

Figure 1: Key feeding needle positions during gavage. (A) Feeding needle. (B) Feeding needle ball inserted to the side of the tongue. (C) Feeding needle in the upright position with insertion in the esophagus. (D) Feeding needle inserted into the stomach with a few millimeters visible above the nose.

Figure 2: Example dissection layout. (A) Dissection pad and tools. (B) Excised GI tract. Proximal (esophagus) to distal (anus) sections: I. Stomach. II. Proximal small intestines. III. Medial small intestines. IV. Distal small intestines. V. Cecum. VI. Large intestine. Pink dashed border boxes indicate the tissues to be fixed. (C) Tissues positioned in cassettes. Roman numerals are the same as in panel B.

Figure 3: Representative images of FISH-stained *C. albicans*. (A) FISH of *C. albicans* hyphae grown in liquid Lee's media¹⁸ with 2% N-acetylglucosamine, pH 6.8. (B) FISH of *C. albicans* round yeast cells grown on YEPD + 2% agar plates. (C) FISH of *C. albicans* GUT cells (ySN1045) grown on YEPD + 2% agar plates. (D) FISH of *C. albicans* opaque cells grown on YEPD + 2% agar plates. (E) Wild type *C. albicans* (ySN250) in large intestines, stained with the *C. albicans*-specific probe. (F) Wild type *C. albicans* in large intestines, stained with the panfungal probe. (G) *Ume6* mutant (ySN1479) in large intestines, stained with the panfungal probe: red is *C. albicans*, blue is host epithelial nuclei, and green is mucin. Arrowheads indicate hyphae. Arrows indicate yeast. Scale bars are 20 μ m. Images F and G are adapted from Witchley et al.¹².

Figure 4: Appearance of FISH-stained *C. albicans* in different gut compartments. Wild type *C. albicans* is shown in the indicated segments of the mouse GI tract. Within each compartment, images depict the area adjacent to the host mucosa and the central region of the gut lumen. Staining was performed with the panfungal probe, and the scale bar corresponds to 20 μ m. Images are adapted from Witchley et al.¹².

Table 1: Typical volumes and concentrations of solutions used in this protocol.

DISCUSSION:

The method described here allows for visualization of *C. albicans* yeasts and hyphae in the GI tracts of commensally colonized mice of any sex or strain. The FISH probe hybridizes to 23S rRNA, which is distributed throughout the fungal cytoplasm. Our method was adapted from a previously reported protocol for visualizing gut bacteria²⁰. Because *C. albicans* changes its morphology within the host, the method is useful to monitor fungal cell shape as well as localization. For example, we have used this method to disprove the hypothesis that yeasts predominate throughout the digestive tract, and to expose discrepancies between the in vivo and in vitro phenotypes of certain “filamentation-defective” mutants¹².

Several mouse models exist for *C. albicans* commensal colonization. The microbiota of laboratory mice and humans are distinct and, in mice, the use of antibiotics or a specialized diet is required to establish stable colonization. Treatment with antibiotics also enhances *C. albicans* colonization of humans and is a major risk factor for disseminated disease¹⁰. The antibiotics used in this study are relatively inexpensive and yield reliable decreases in the bacterial microbiota. Note that antibiotics are used to decrease the burden of antagonistic bacterial species, not to eliminate all bacteria from the animals. If researchers wish to study *C. albicans*-host interactions in the absence of bacteria or to avoid the use of antibiotics or a special diet, germ-free animals can be substituted for conventionally reared animals; however, gnotobiotic mice exhibit certain immune and anatomic abnormalities and therefore may not be suitable for all purposes.

Several steps are important for successful FISH staining: The recommended fixation method is critical to preserve the structural integrity of GI tissues, particularly the fragile contents of the gut lumen, such as the mucus layer and the three-dimensional organization of fungi and bacteria¹⁶. Please note that many commonly used fixation solutions that contain water are highly damaging to the luminal architecture. The fixatives and solutions for post-fixation washes recommended in this protocol do not contain water, and it is important to avoid contamination with water. Another critical step is the hybridization step, where it is important to avoid evaporation of the hybridization solution during incubation of slides in the hybridization oven. We suggest placing the slides in a watertight container to avoid this problem. Alternatively, one can use watertight hybridization chambers such as those originally used for hybridization of microarrays.

A *C. albicans*-specific FISH probe is described in this protocol. However, because many laboratory-reared mice do not contain *C. albicans* or other fungi as part of their natural gut microbiota, a panfungal probe may specifically stain *C. albicans* in experimentally colonized animals. Substitution of a panfungal probe may be desirable because of its superior hybridization characteristics and higher signal-to-noise ratio. If the panfungal probe is used as a pseudospecific probe for *C. albicans*, it is important to document a lack of staining in uninfected animals (i.e., treated with antibiotics but not *C. albicans*). Staining of a noncolonized control animal is also useful to assess background staining that may result from adherence of FISH probes to food particulates. Overall, the use of FISH probes offers enhanced specificity over most other methods of staining fungi, such as Calcofluor white (which stains chitin, a cell wall component that may vary between cell types), GMS, or commercially available antifungal antibodies. Moreover, FISH

staining allows for costaining of multiple organisms using differentially labeled FISH probes to species-specific rRNAs.

One caveat of this technique is that certain *C. albicans* yeast cell types appear very similar by FISH (for example, the opaque and GUT cell types). To distinguish among these cell types, it would be useful to develop hybridization probes to cell type-specific fungal mRNAs. Nevertheless, in its current form, the FISH technique has already revealed surprising discrepancies between the in vivo and in vitro behaviors of *C. albicans* mutants evaluated under both conditions¹², indicating complex interactions in the natural commensal environment that are not adequately mimicked by existing in vitro assays. Further studies of different *C. albicans* stains in additional wild type and mutant hosts are likely to yield additional insights into fungal-host interactions. In particular, it will be instructive to profile *C. albicans* in models of inflammatory bowel disease, which is associated with high titers of *C. albicans* in humans²¹, and other models of *C. albicans* overgrowth and disease. The FISH technique may also be combined with immunohistochemistry to stain specific host cells. Overall, the method presented here provides a reasonably quick and reliable means to determine *C. albicans* localization and morphology within the mammalian GI tract.

ACKNOWLEDGMENTS:

The authors would like to thank Carolina Tropini, Katharine Ng, Justin Sonnenburg, and KC Huang for guidance in developing the FISH technique. Teresa O'Meara provided helpful comments on the manuscript, and Miriam Levy assisted with photography. This work was supported by NIH grants R01AI108992, R01DK113788, and a Burroughs Wellcome Award in the Pathogenesis of Infectious Disease.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Noble, S. M., Gianetti, B. A., Witchley, J. N. Candida Albicans Cell-Type Switching and Functional Plasticity in the Mammalian Host. *Nature Reviews in Microbiology*. **15** (2), 96-108 (2017).
2. Bacher, P. et al. Human Anti-fungal Th17 Immunity and Pathology Rely on Cross-Reactivity against Candida albicans. *Cell*. **176** (6), 1340-1355 (2019).
3. Shao, T. Y. et al. Commensal Candida albicans Positively Calibrates Systemic Th17 Immunological Responses. *Cell Host and Microbe*. **25** (3), 404-417 (2019).
4. Jiang, T. T. et al. Commensal Fungi Recapitulate the Protective Benefits of Intestinal Bacteria. *Cell Host and Microbe*. **22** (6), 809-816 (2017).
5. Iliev, I. D. et al. Interactions Between Commensal Fungi and the C-Type Lectin Receptor Dectin-1 Influence Colitis. *Science*. **336** (6086), 1314-1317 (2012).
6. Fan, D. et al. Activation of HIF-1 alpha and LL-37 by Commensal Bacteria Inhibits Candida Albicans Colonization. *Nature Medicine*. **21** (7), 808-814 (2015).
7. Koh, A. Y., Kohler, J. R., Coggs, K. T., Van Rooijen, N., Pier, G. B. Mucosal Damage and Neutropenia Are Required for Candida Albicans Dissemination. *PLoS Pathogens*. **4** (2), e35 (2008).
8. Yamaguchi, N. et al. Gastric Colonization of Candida Albicans Differs in Mice Fed Commercial and Purified Diets. *Journal of Nutrition*. **135** (1), 109-115 (2005).

615 9. Kadosh, D. et al. Effect of Antifungal Treatment in a Diet-Based Murine Model of Disseminated
616 Candidiasis Acquired via the Gastrointestinal Tract. *Antimicrobial Agents and Chemotherapy*. **60**
617 (11), 6703-6708 (2016).

618 10. Perlroth, J., Choi, B., Spellberg, B. Nosocomial Fungal Infections: Epidemiology, Diagnosis, and
619 Treatment. *Medical Mycology*. **45** (4), 321-346 (2007).

620 11. Pande, K., Chen, C., Noble, S. M. Passage Through the Mammalian Gut Triggers a Phenotypic
621 Switch That Promotes *Candida Albicans* Commensalism. *Nature Genetics*. **45** (9), 1088-1091
622 (2013).

623 12. Witchley, J. N. et al. *Candida Albicans* Morphogenesis Programs Control the Balance between
624 Gut Commensalism and Invasive Infection. *Cell Host and Microbe*. **25** (3), 432-443 (2019).

625 13. Noble, S. M., Gianetti, B. A., Witchley, J. N. *Candida Albicans* Cell-Type Switching and
626 Functional Plasticity in the Mammalian Host. *Nature Reviews in Microbiology*. **15** (2), 96-108
627 (2017).

628 14. Balish, E., Filutowicz, H., Oberley, T. D. Correlates of Cell-Mediated Immunity in *Candida*
629 *Albicans*-Colonized Gnotobiotic Mice. *Infection and Immunity*. **58** (1), 107-113 (1990).

630 15. Guarner, J., Brandt, M. E. Histopathologic Diagnosis of Fungal Infections in the 21st Century.
631 *Clinical Microbiology Reviews*. **24** (2) (2011).

632 16. Johansson, M. E., Hansson, G. C. Preservation of Mucus in Histological Sections,
633 Immunostaining of Mucins in Fixed Tissue, and Localization of Bacteria with FISH. *Methods in*
634 *Molecular Biology*. **842** 229-235 (2012).

635 17. Swidsinski, A., Weber, J., Loening-Baucke, V., Hale, L. P. & Lochs, H. Spatial Organization and
636 Composition of the Mucosal Flora in Patients with Inflammatory Bowel Disease. *Journal of Clinical*
637 *Microbiology*. **43** (7), 3380-3389 (2005).

638 18. Lee, K. L., Buckley, H. R., Campbell, C. C. An Amino Acid Liquid Synthetic Medium for the
639 Development of Mycelial and Yeast Forms of *Candida Albicans*. *Sabouraudia*. **13** (2), 148-153
640 (1975).

641 19. Banerjee, M. et al. UME6, a Novel Filament-Specific Regulator of *Candida Albicans* Hyphal
642 Extension and Virulence. *Molecular Biology of the Cell*. **19** (4), 1354-1365 (2008).

643 20. Earle, K. A. et al. Quantitative Imaging of Gut Microbiota Spatial Organization. *Cell Host and*
644 *Microbe*. **18** (4), 478-488 (2015).

645 21. Sokol, H. et al. Fungal Microbiota Dysbiosis in IBD. *Gut*. **66** (6), 1039-1048 (2017).

Figure 1

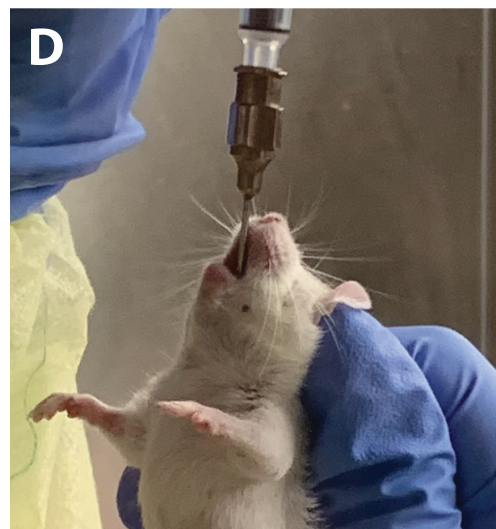
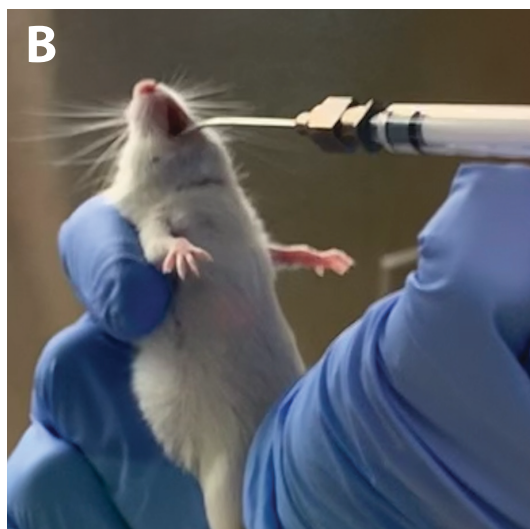




Figure 3

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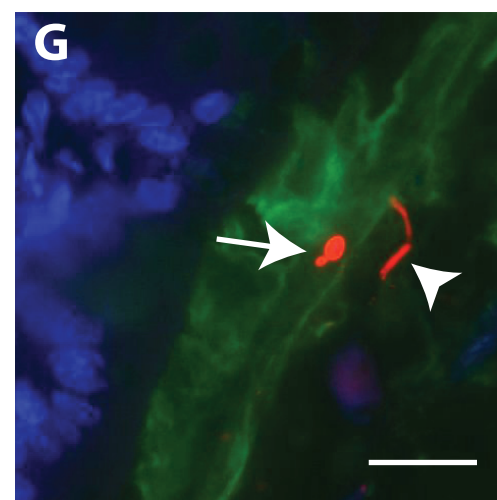
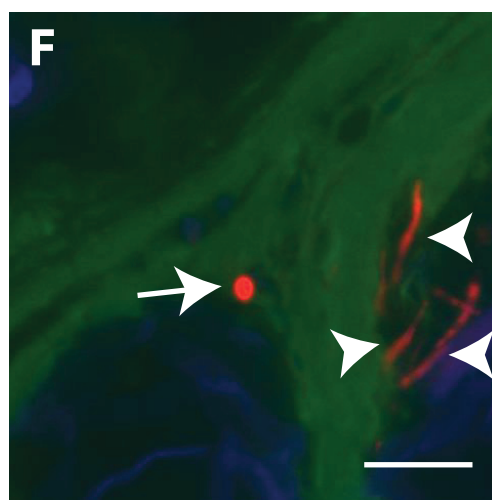
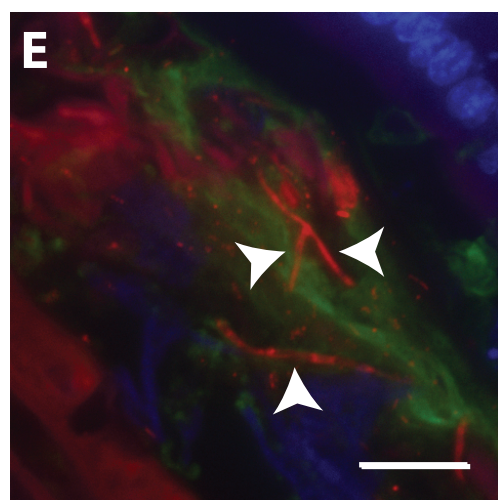
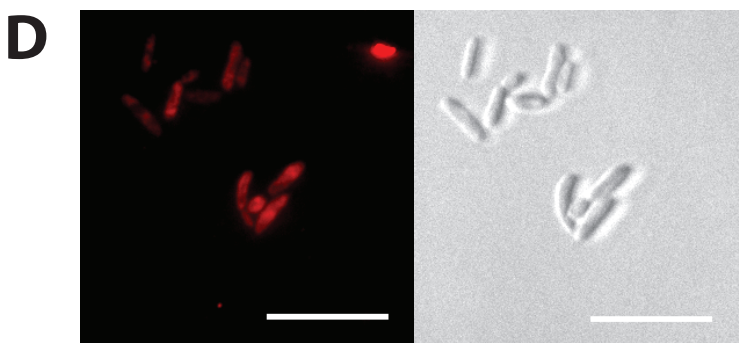
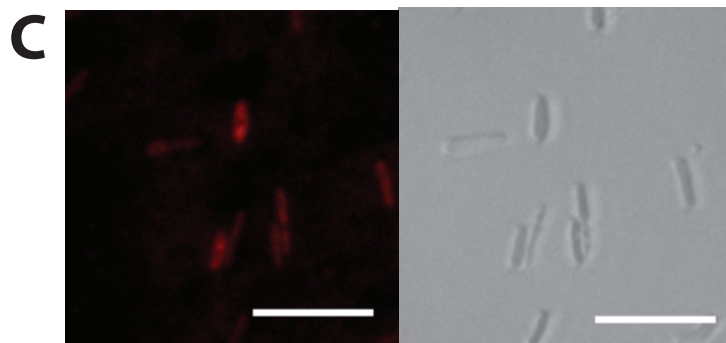
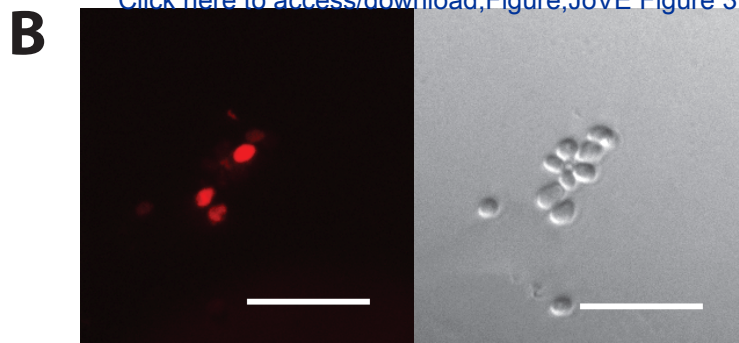
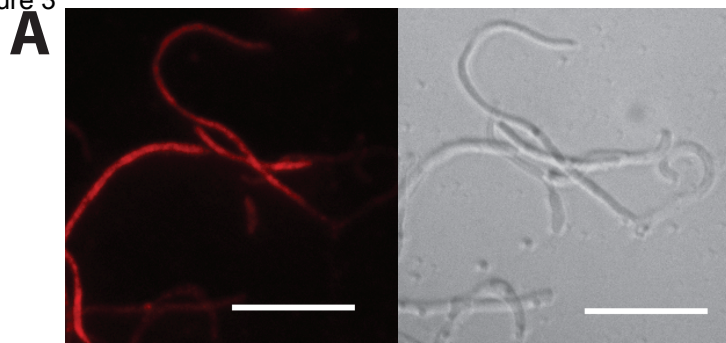


Figure 4

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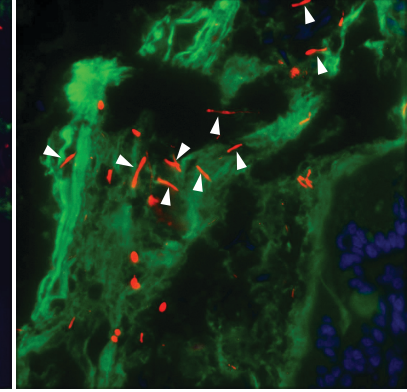
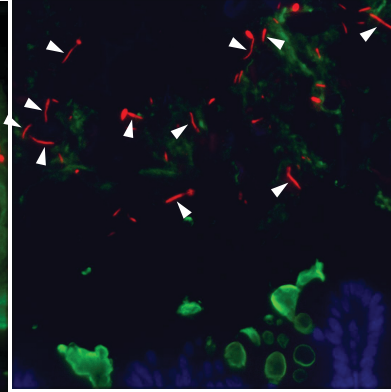
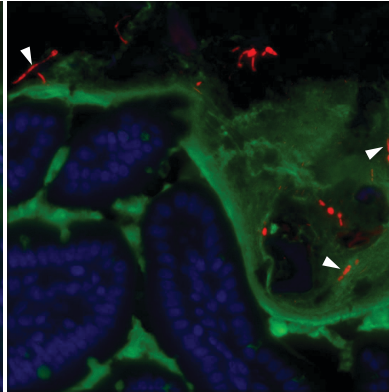
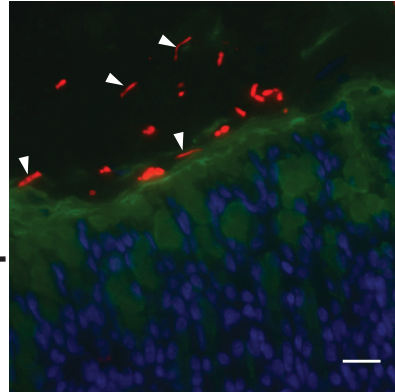
Stomach

**Small
intestines**

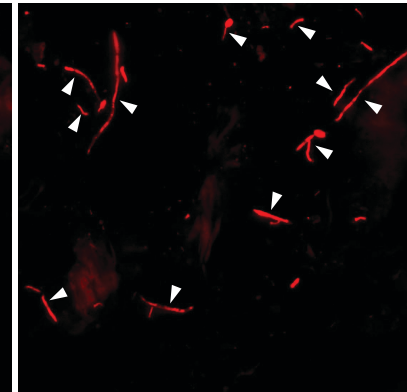
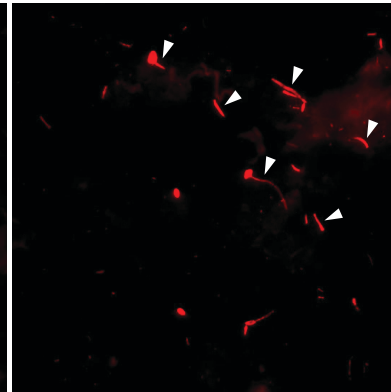
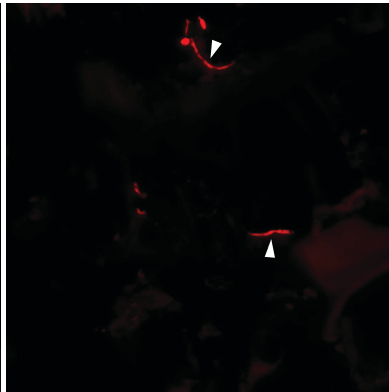
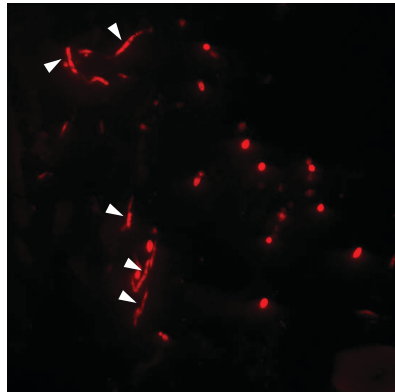
Cecum

**Large
intestines**

epithelium



lumen



Antibiotic stocks (200x)	
Penicillin G	181 mg/mL
Streptomycin	400 mg/μL

Methacarn			
Stock	Final concentration	Volume	Units
Methanol	60%	300	mL
Chloroform	30%	150	mL
Glacial acetic acid	10%	50	mL

Hybridization solution			
Stock	Final concentration	Volume	Units
1 M Tris-HCl, pH 7.4	20 mM	20	μL
5 M NaCl	0.9 M	180	μL
10% SDS	0.10%	10	μL
100% formamide	1.00%	10	μL (kept in small aliquots at 20 °C between uses)
RNase-free water		780	μL

FISH washing solution			
1 M Tris-HCl, pH 7.4	20 mM	1	mL
5 M NaCl	0.9 M	9	mL
RNase-free water		40	mL

Mucin-nuclei staining solution			
DAPI, 10 μg/mL	20 ng/mL	2	μL
lectin, 40 μg/mL	1.6 μg/mL	40	μL
PBS, pH 7.4		958	μL

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 mL syringe	BD	309659	can be substituted from any vendor
BHI blood agar			can be substituted from any vendor
<i>C. albicans</i> FISH probe	IDT DNA Technologies	custom order	
chamber for hybridization			watertight chamber meant to reduce evaporation
incubation			
chloroform	Sigma	C2432	>=99.5%
DAPI	Roche	10236276001	can be substituted from any vendor
delicate task wiper tissues	Kimberley-Clark	34256CT	Kimwipes
D-glucose	Sigma	G7021-5KG	can be substituted from any vendor
ethanol	Sigma	E7023	molecular biology grade
feeding needles	Cadence, Inc.	7910	metal; 20 X1-1/2" W/2-1/4; can be autoclaved to sterilize
FITC-UEA-1	Sigma	L9006-1MG	other fluorophores available
FITC-WGA	Sigma	L4895-2MG	other fluorophores available
Foam pads	Fisher	22038221	Order foam pads that will fit within cassettes
formamide	Sigma	47671	molecular biology grade
glacial acetic acid	Macron Fine Chemicals	MK881746	ACS reagent, >=99.5%
glass Coplin jar	Fisher	08-815	hold up to 10 slides back to back
Histology cassettes	Simport	M512	Deep cassettes so cecum is not squished
hybridization coverslips	Sigma	GBL712222	RNase-free
hybridization oven			can be substituted from any vendor
LB			can be substituted from any vendor
Lee's media			prepared as described in Lee <i>et al.</i> 1975
methanol	Sigma	179337	ACS reagent, >=99.8%
	Charles River		
mice	Laboratories	028	adult BALB/c; 18-21 grams (8-10 weeks)
paraformaldehyde	Fisher	50-980-487	16% solution
paraffin wax	Sigma	P3558-1KG	Paraplast for tissue embedding

	UCSF Cell Culture		
	Facility Media		
PBS, pH 7.4	Production Unit	CCFAL003	calcium, magnesium-free; can be substituted from any ve
penicillin G	Sigma	PENNA-100MU	
Sabouraud dextrose agar			can be substituted from any vendor
saline	Baxter	2F7123	sterile, can be substituted from any vendor
sodium chloride (NaCl)	Sigma	S3014	can be substituted from any vendor; maintain RNase-free
sodium dodecyl sulfate (SDS)	Sigma	L3771	can be substituted from any vendor; maintain RNase-free
streptomycin	Sigma	S9137-100G	
super PAP pen	Life Technologies	8899	can be substituted from any vendor
Tris-HCl	Sigma	T3253	can be substituted from any vendor; maintain RNase-free
Vectashield	Vector Laboratories	H-1000	does not contain DAPI
xylenes	Sigma	214736	reagent grade
YEPD			can be substituted from any vendor

endor

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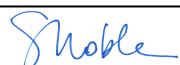
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Signature:	 <small>Digitally signed by Suzanne Noble DN: cn=Suzanne Noble, o, ou, email=suznoble@gmail.com, c=US Date: 2019.05.17 17:02:23 -07'00'</small>	Date:	May 17, 2019

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Responses to Editorial Comments

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been proofread.

2. Please define all abbreviations during the first-time use.

All abbreviations have been defined.

3. Please ensure that the Abstract is between 150-300 words.

Abstract is now at the recommended length.

4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Kimwipe (Kimberly-Clark 34256CT) , Vectashield, etc

Generic terms have been substituted.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

An ethics statement has been introduced.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

The imperative tense is now used throughout.

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

All discussion has been moved to the Discussion.

8. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Notes are now more sparing.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

The protocol has been simplified.

10. For all the protocol steps, please ensure you answer the “how” question, i.e., how is the step performed?

Steps now answer the “how” question.

11. 1.1. Any age, sex, strain specific bias with respect to the mouse?

Bias has been addressed in the discussion.

12. 1.2: Two notes cannot follow each other. Please consider making substeps.

Serial notes have been eliminated.

13. 4.2.1, 4.2.6, 4.2.10: For the volume and concentration, of the reagents use please make a .xlsx table and upload it separately to your editorial manager account. This can be referred wherever applicable.

An Excel table has been generated (Table 1)

14. Please expand the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Data from both successful and sub-optimal experiments can be included.

The discussion has been expanded per these instructions.

15. Figure 3 legend is mislabeled.

The legend has been relabeled.

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Editorial policy is now attached.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

Discussion has been revised to include these points

18. Please expand the journal title in the reference section.

Journal titles have been expanded.

Responses to Reviewer comments

Reviewer #1:

Manuscript Summary:

This article describes a detailed protocol for visualizing *C. albicans*, a major human fungal pathogen, during colonization in the mouse GI tract. Protocols are presented for *C. albicans* gut colonization, preparation of GI tissues for histology, validation of probes for fluorescent in situ hybridization (FISH) as well as FISH staining of GI tissues.

The article is well-written and the protocols are clearly described and supported by figures that demonstrate the efficacy of the procedures. A major advantage of this protocol is that it allows for direct visualization of *C. albicans* morphologies in the context of the natural architecture of the gut environment. Given that the GI tract is a predominant portal for the establishment of systemic *C. albicans* infections, the applications of this protocol are far-reaching.

Major Concerns:

There are no major concerns with the manuscript.

Minor Concerns:

1. As discussed in the Introduction, laboratory mice are not typically colonized in the mouse GI tract and treatment with antibiotics is thus required. Some additional discussion on how accurately this model may reflect *C. albicans* colonization in the human GI tract is warranted.

This point is now discussed in the text.

2. One concern is that the pan-fungal FISH probe for 23S RNA would detect a background level of commensal fungal species in the GI tract. Although this is an unlikely possibility for the murine model, given that fungal species do not typically colonize the mouse gut, it could be a concern for other hosts. In either case, it would be useful to show representative images using the 23S FISH probe in Figure 3.

A comparison of staining with the two probes is now included in the Figures.

3. From the protocol, it was not clear whether the entire contents of the GI tract are washed out in step 2.12 since complete segments of the gut, including digestive material, are excised in step 2.9. Would the presence of residual GI tract contents interfere with subsequent microscopy?

As described as a “critical step” in the revised discussion, the fixation method was developed specifically to avoid disturbing the contents of the GI lumen.

4. The manuscript could be strengthened by an expanded discussion of the potential future applications for this protocol. In the current manuscript, this discussion is limited to only two sentences (lines 517-519).

Future applications are now expanded in the discussion.

5. Line 484: Figure 3 is labeled as Figure 2.

The figure has been relabeled.

Reviewer #2:

Manuscript Summary:

a method to visualize *C. albicans* cell morphology and localization in a mouse model of gastrointestinal colonization

Major Concerns:
none

Minor Concerns:

Line 437: Please explain why a two lectin combination is needed in these sites: For example in saliva UEA binds to low molecular weight and WGA binds to high MW mucins (Zhang XS, J Histochemisrty and Cytochemistry, 1994), is this the same reason? what is the mucin composition in the gut?

We now describe that lectin combinations were tested empirically to identify those that yielded the brightest staining in each compartment.

Figure 2 letter labels are wrong and also the wrong figure numbers are given in several places in the text.

Figure 2 has been relabeled, and figure numbers have been checked

Reviewer #3:

Manuscript Summary:

In this text portion of the JoVE manuscript, Noble and co-workers describe methodology for visualization of *Candida albicans* in murine GI tract tissue using FISH. Overall the methodology is nicely described and is suitable as a visualization manuscript. The methodology, while not novel, is new for *C. albicans* and will enable excellent ex vivo visualization of various morphological forms of *C. albicans* with distinct localization within the GI tract. While nicely done, I have several suggestions to strengthen this portion of the JoVE manuscript.

Major Concerns:

Representative Results: Suggestion to focus this section on the FISH staining for a more descriptive narrative. Fig. 3 is not really described other than the figure legend. Eliminate the references to Fig. 1 and 2. Show a few more representative images of *C. albicans* in other GI sections for comparison. This piece is the sole purpose of the method and it does not get adequate attention in the manuscript narrative to fully appreciate the applications of the methodology. The resolution of the images could be better as well.

The narrative has been expanded, and additional images have been included in the revised Figure 3 and new Figure 4.

Minor Concerns:

1. 1.3: Some parts of the GI tract are anaerobic, but only aerobic clearance is monitored in antibiotic-treated animals. It may be necessary to monitor the presence/clearance of anaerobes. Some discussion of this would be appropriate.

In the discussion, we now clarify that antibiotic treatment is required to lower the burden of antagonistic (to *C. albicans*) bacteria, not to eliminate all bacteria.

2. 1.6: the term 'inoculation' is more appropriate than 'infection'.

Infection has been replaced with inoculation or gavage.

3. 1.12.3 reference to Fig. 1: Suggestion to show an image of the entire gavage needle for reference of length and size of the gavage ball

Whole needle is now depicted.

4. 2.2: Can the authors provide a general range of fecal titers and fungal burden at select time points associated with the gut as CFU/g from their experience. This would enable some indication of how extensive/formidable the colonization is.

The range has been added.

5. 2.5 reference to Fig. 2: Suggestion to also show an image of the intestinal contents initially removed from the peritoneal cavity (still attached) as an orientation for eventual dissection.

We do not have such an image (but images from mouse dissection atlases are readily available on the internet).

6. 2.7 reference to Fig. 2C: Suggestion to label the actual GI sections referenced by the roman numerals.

Sections are now labeled.

7. 3.1: dubliniensis and tropicalis are misspelled

Spelling has been corrected.

8. 4.2.10: Suggestion to provide the reasons/advantages of using two lectins for mucin staining in different GI sections? Is there a difference in specificity?

Note has been added.

9. Figure 2 legend: Fig. 2A alone shows both dissection pad and instruments (not 2B). In Fig. 2B the term 'excised GI tract' instead of 'peritoneal cavity' is more appropriate.

These problems have been remedied.

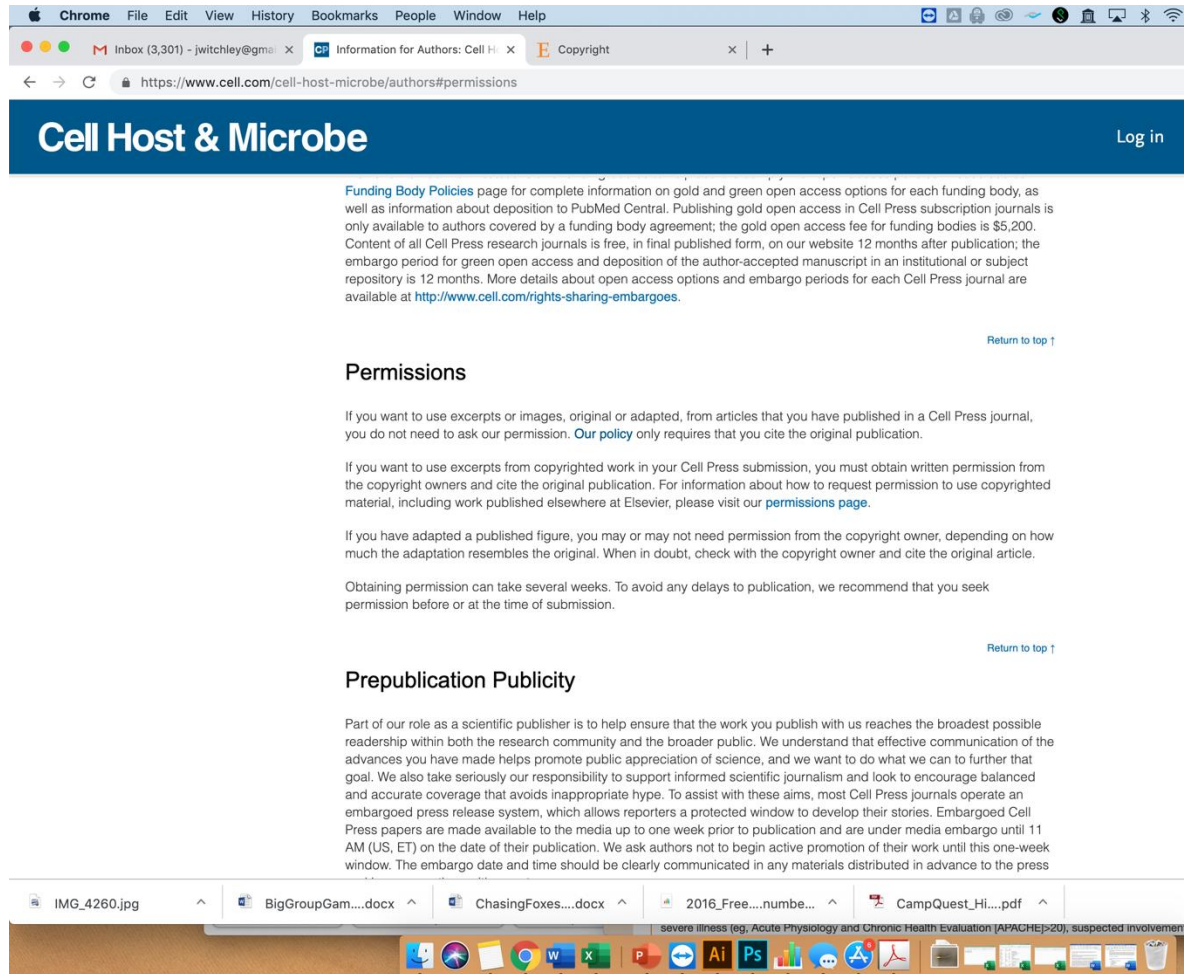
10. Figure 3 legend: The legend is mislabeled as Fig. 2. The frames in the figure (the two bottom panels) are mislabeled. In Fig. 3A, the culture medium is not mentioned in the protocol and not listed in the table. In Fig. 3B, GUT cells should be spelled out and defined. In Fig. 3E-F, *C. albicans* mutants used in the image should be described in the legend. Line 489....eliminate 'Red is *C. albicans*' since the white arrows have already defined *C. albicans*. Consider adding arrows for the mucin and epithelial nuclei

These problems have been remedied.

11. Discussion: Line 509-515, suggestion to address the Pros/Cons of using the FISH technique for *C. albicans* visualization over other methods. If staining with the pan-fungal probe is sufficient in this model (or superior in terms of signal intensity), how is it comparable to other conventional fungal staining methods (e.g. immunohistochemistry, Calcofluor white)? Is it simply the ability to visually localize the organisms within the tissue?

The discussion of pros and cons has been expanded as suggested.

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