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TITLE:

Visualization of Gut Microbiota-host Interactions via Fluorescence *In Situ* Hybridization, Lectin Staining, and Imaging

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SUMMARY:

This streamlined protocol details a workflow to detect and image bacteria in complex tissue samples, from fixing the tissue to staining microbes with fluorescent *in situ* hybridization.

ABSTRACT:

Measuring the localization of microbes within their *in vivo* context is an essential step in revealing the functional relationships between the microbiota and the vertebrate gut. The spatial landscape of the gut microbiota is tightly controlled by physical features—intestinal mucus, crypts, and folds—and is affected by host-controlled properties such as pH, oxygen availability, and immune factors. These properties limit the ability of commensal microbes and pathogens alike to colonize the gut stably. At the micron-scale, microbial organization determines the close-range interactions between different microbes as well as the interactions between microbes and their host. These interactions then affect large-scale organ function and host health.

This protocol enables the visualization of the gut microbiota spatial organization from distances between cells to organ-wide scales. The method is based on fixing gut tissues while preserving intestinal structure and mucus properties. The fixed samples are then embedded, sectioned, and stained to highlight specific bacterial species through fluorescence *in situ* hybridization (FISH). Host features, such as mucus and host cell components, are labeled with fluorescently labeled lectins. Finally, the stained sections are imaged using a confocal microscope utilizing tile-scan imaging at high magnification to bridge the micron to centimeter length scales. This type of imaging can be applied to intestinal sections from animal models and biopsies from human tissues to determine the biogeography of the microbiota in the gut in health and disease.

INTRODUCTION:

Microbial visualization techniques have origins that are as old as microbiology itself, when Antonie Van Leeuwenhoek used his microscope to observe bacteria (which he called “animalcules”) from tooth plaque and stool in the 17th century. Since then, numerous techniques have been developed to visualize the spatial organization of the consortium of bacteria, fungi, and viruses that live associated with a host—the microbiota¹. Elucidating the localization of these microbes is essential to determining their function within their animal host. The biogeography of bacteria is important in commensal and pathogenic taxa alike, as proximity to specific locations (e.g., the epithelium), nutritional substrates, and specific microbes may dictate bacterial production of metabolites underlying interspecies and inter-kingdom interactions.

A key structure separating bacteria and the host tissues in several disparate body sites, such as the oral cavity, intestine, or lung, is the mucus—a host-produced layer that both prevents microbial translocation onto the host epithelial cells and serves as a nutritional resource for the microbiota^{2–4}. Characterizing breaches and changes in this barrier is of key importance, leading to mechanistic insight into host-microbiota interactions that would not be obtained by sequencing alone^{5–7}. For example, imaging enabled the discovery that antibiotic exposure can disrupt the mucus layer and microbiota organization^{7,8}, and that laxatives may deplete the mucus, correlating with large changes in immune parameters⁵.

This protocol outlines a general framework for fixing, staining, and imaging the microbiota and the host tissue (**Figure 1**), built upon the work of Johansson and Hansson⁹. While this protocol is modeled in the context of intestinal sections, it can be easily adapted to other tissue types. This protocol enables the processing of either experimental animal or human clinical samples, and notes for processing both types of samples have been included. In the example presented here, the host epithelium and luminal bacteria have been simultaneously labeled with 4',6-diamidino-2-phenylindole (DAPI) staining, mucus with the fluorescein (FITC)-conjugated lectin, *Ulex europaeus* agglutinin I (UEA-1), and a specific bacterial taxon using FISH. FISH probes are usually designed against a taxon's 16S rRNA genes to ensure the high signal from binding a high-copy transcript.

In this example, the probe targets the 16s rRNA of the *Muribaculaceae* bacterial family (**Figure 2**). However, the stains are readily substituted with different FISH probes and/or lectins to accommodate the appropriate biological question. Previously validated FISH probes can be found on ProbeBase¹⁰, an online resource for rRNA-targeted oligonucleotide probes, or on Silva¹¹, a ribosomal RNA database. For the design of new probes, the reader may refer to new pipelines such as HiPR-FISH⁸ or Oligominer¹². Using this protocol, it is possible to observe the close packing of bacteria in the intestinal lumen and the different features of intestinal mucus throughout the digestive tract. The workflow described here enables the quantitative analysis of the microbiota in the spatial context of its host environment.

PROTOCOL:

All animal experiments and tissue collection described in this protocol were performed in compliance with the Canadian Council on Animal Care (CCAC) guidelines and were approved by

the Animal Care Committee at the University of British Columbia. Germ-free Swiss Webster mice were used. All animals were 8–10 weeks of age, both sexes were used, and all mice were co-housed with at least two mice per cage. Animals were euthanized using carbon dioxide with secondary cervical dislocation or cardiac puncture.

1. Designing an imaging experiment: considerations and sample collection

1.1. FISH probe design

1.1.1. If an appropriate FISH probe exists, select a pre-existing published one that shows a strong signal in labeled cells compared to the background.

1.1.2. Validate new probes *in vitro* to determine their efficiency of binding to fixed samples of the target bacteria and off-target binding to other bacteria.

1.1.3. Check all 16S FISH probes to be used against 16S sequences from the target organism or against 16S rRNA sequencing from the samples to be analyzed to ensure that the expected proportion of positive matches are present, and that probes will not bind non-specifically to other microbiota members.

1.1.3.1. Align the FISH probes against either full-length sequences or amplicon sequence variants using a tool, such as Clustal Omega¹³, to evaluate the potential binding of probes and their targets.

1.1.3.2. In addition to *in silico* testing, test the accuracy and level of FISH staining of unvalidated probes on pure cultures^{8,14}.

NOTE: When staining multiple community members, probes can be used combinatorially (e.g., the combination of a family-specific probe and a genus-specific probe) if the fluorophores used do not overlap in excitation and emission spectra (unless imaging on a system with the ability to perform linear unmixing). Additionally, specificity may be demonstrated by including specificity controls/scrambled probes or by competition with non-fluorescent probes.

1.2. Sample types and tissue collection

1.2.1. Using sharp and clean tools, cut intestinal segments from germ-free Swiss Webster mice for imaging. Minimize disturbing the sample as much as possible, and handle the sections by their edges to avoid affecting the imaging area. Fix the sample as soon as possible after dissection to prevent degradation.

NOTE: For animal studies, intact, unflushed intestinal sections are preferred; the membrane will help keep the luminal and mucosal organization intact.

1.2.2. Clean tools by wiping them with 70% ethanol between samples and sites. Obtain at least

three biological replicates per intestinal section/biopsy location, taking care to sample consistent intestinal locations, as the mucus and intestinal architecture as well as microbiota changes significantly in different locations.

2. Tissue sample fixation and infiltration

2.1. Fixation

2.1.1. Prepare fresh methacarn in a compatible container with the following ratios: 60% absolute methanol, 30% chloroform, and 10% glacial acetic acid. Dispense each of these chemicals in a fume hood. Choose a container that can be vented by cracking open the lid. As methacarn is not compatible with polystyrene, prepare it in a polyethylene container, using glass graduated cylinders/serological pipets for the chloroform and glacial acetic acid.

NOTE: During mixing, fumes are produced and may cause pressure to build up inside the container. It is advisable to keep the container relatively tightly closed once removed from the fume hood if samples are not being actively added. Chloroform is toxic; do not inhale, swallow, or absorb through the skin. Methanol is toxic and highly flammable; do not inhale, swallow, or absorb through the skin. Glacial acetic acid is flammable and highly corrosive to the skin and the eyes.

2.1.2. After dispensing, close the container, swirl to mix and then vent; repeat this until off-gassing has stopped (and pressure no longer builds up appreciably under the lid).

NOTE: Do not dispose of methacarn in drains; collect and dispose of as hazardous chemical waste as per institutional guidelines. Use a pencil for labeling histology cassettes, as many pen inks will come off in the methacarn solution.

2.1.3. Place the intestinal sections in histology cassettes by delicately holding an edge of the tissue with tweezers. Close the cassette and completely submerge it in fresh methacarn solution. Ensure that the solution is not older than a few hours upon immersion of the cassettes.

2.1.4. For clinical samples that will be collected in a clinical suite without access to a fume hood, use a polyethylene storage container with a flap cut into the lid that will permit the passage of the histology cassettes. Tape this flap shut when not passing samples to prevent the escape of toxic fumes.

NOTE: It is important to use masking tape to avoid the dissolution of glue onto the container—some types of tape (e.g., plastic packing tape) may not hold up well to the methacarn fumes.

2.1.5. Fix samples for a minimum of 3 h and a maximum of two weeks.

NOTE: Thicker samples may require fixation times longer than 3 h; however, fixation time may be chosen to enable the combination of cassettes for paraffin infiltration (e.g., to simultaneously perform paraffin infiltration on groups of samples collected and fixed over two weeks).

2.2. Paraffin infiltration and embedding

2.2.1. Place the paraffin in a heat-resistant container and melt it in an oven at 60 °C overnight. As paraffin pellets take up more space before melting, ensure that sufficient paraffin is melted to cover all the cassettes.

NOTE: The temperature should not be higher than 60 °C as it can give rise to artifacts.

2.2.2. Wash the tissue by pouring out the liquid in the appropriate waste receptacle and replacing it immediately with the following chemicals: first 30 min incubation in absolute methanol, second 30 min incubation in absolute methanol, first 20 min incubation in absolute ethanol, second 20 min incubation in absolute ethanol, first 15 min incubation in xylenes, second 15 min incubation in xylenes. Exchange the washes quickly, taking care to avoid letting the cassettes dry. Ensure that each wash fully covers the cassettes in the beaker or container.

NOTE: Xylenes are flammable, and if inhaled, the vapors may depress the central nervous system with potential long-term neurological consequences upon exposure to high concentrations (>200 ppm). Handle xylenes only within a fume hood. As xylenes are hazardous, take care to use the minimum amount necessary to cover the histological cassettes.

2.2.3. During embedding, orient the intestinal segments parallel to the length of the cassette (as opposed to upright) using tweezers to provide longitudinal, transverse sections instead of cross-sectional doughnuts to provide more potential sample area for quantitative imaging (**Figure 1B**).

2.2.4. Open the cassettes slightly (~1–2 cm or 0.5 inches) to allow the paraffin to enter without losing the tissue segments. Use double gloves for this step or tweezers to prevent overheating or mild burning of fingers. Submerge and close the cassettes in the container of melted paraffin, placing the container back into the 60 °C oven. Ensure that the cassettes are filled with paraffin, and no large air bubbles remain.

2.2.5. After incubation for 2 h at 60 °C, remove the container from the oven. Using forceps, carefully remove the cassettes and lay them individually on a piece of aluminum foil to cool. Store them at room temperature until ready for embedding and sectioning.

2.2.6. Section the paraffin blocks with a microtome, sectioning deep enough into the block that luminal contents are exposed, enabling a longitudinal section of villi and/or crypts in addition to luminal contents and mucus. Take care to replace the blades as fecal material dulls the blade rapidly compared to tissue. Cut sections to 4 µm of thickness for optimal sharpness of the images. Transfer the sections to regular uncoated slides.

2.2.7. For FISH staining, stain the intestinal sections as soon as possible (i.e., within a few weeks of sectioning) to achieve a strong FISH signal. If omitting FISH staining, lectin + DAPI staining can be performed on less fresh (i.e., months-old) samples without significant loss of signal.

3. Staining bacteria and host features

3.1. Preparation

3.1.1. Heat the oven to 60 °C. Pre-warm a Coplin jar and enough volume to cover the glass slides in the jar twice with xylenes in a glass bottle, taking care to parafilm around the lid to prevent evaporation of the xylenes. Allow the temperature of the xylenes to reach 60 °C.

NOTE: A standard Coplin jar fits 8 slides, and the slides can be covered with roughly 50 mL of liquid (may vary between 40 and 60 mL depending on the brand). Xylene substitutes are less toxic and have been successfully used by other groups for the de-waxing step (step 3.2)^{8,9}.

3.1.2. If a separate oven is available, pre-warm a hybridization oven to 50 °C. Otherwise, this step may be performed with the same oven used for baking the slides after step 3.2.3.

3.1.3. Prepare the FISH hybridization solution: 20 mM Tris-HCl, pH 7.4; 0.9 M NaCl; 0.01% (w/v) sodium dodecylsulfate (SDS) in nuclease-free water. If required, add 5–50% (v/v) formamide.

NOTE: Formamide is an amide that acts as a teratogen; handle formamide with gloves and goggles. In small doses and upon exposure to eyes, skin, or mucous membranes, it is irritating. In large amounts, formamide vapor can require medical intervention. Formamide can be stored at 100% in a -20 °C freezer.

3.2. Preparing the slides for staining: deparaffinization

3.2.1. Place the slides in the Coplin jar, ensuring that the sections do not come in contact with other slides or the jar, and bake the slides at 60 °C for 10 min.

3.2.2. In the fume hood, fill the Coplin jar with pre-warmed xylenes from the oven, taking care not to pour directly on top of the samples and potentially dislodge the tissues. Place the Coplin jar back in the 60 °C oven. Leave the bottle with the remaining xylenes in the fume hood.

3.2.3. Pour the used xylenes into a proper waste container for disposal, taking care not to disturb the tissue sections on the glass slides and using a pair of forceps to keep the slides from falling out of the Coplin jar. Replenish the Coplin jar with the remaining xylenes and incubate for 10 min at room temperature in the fume hood.

3.2.4. Incubate the sections in 99.5% ethanol for 5 min at room temperature. After this incubation step, remove the slides from the Coplin jar, wipe the back of the slides on a laboratory wipe or paper towel, and briefly air-dry until the ethanol droplets are gone.

NOTE: Wiping the back of the slides enables better visualization of the drying progress.

3.3. Bacterial staining with FISH

3.3.1. Use a liquid blocker or PAP pen to limit the area of liquid expansion by circling the area of each tissue section. Ensure that the ink does not come in contact with the section itself. Create a circle as close to each tissue section as possible without contacting the tissue to minimize the surface area that needs to be covered by the hybridization solution.

3.3.2. Prepare the hybridization solution: for every 50 μ L of pre-warmed hybridization solution, add 0.5 μ g probe (e.g., 0.5 μ L of 1 μ g/ μ L probe). Pipette the hybridization solution onto the sections on the slide (~20 μ L/section, depending on the section/circle size). Protect the hybridization solution and the slides from light from this point onward.

3.3.3. Ensure that the volume of liquid used covers the entire section upon overlaying with a flexible plastic coverslip. Incubate the slide in a humid chamber to reduce evaporation. Create a humid chamber with a pipette tip box with wipes or paper towels at the bottom that have been soaked with excess hybridization solution or phosphate-buffered saline (PBS) to provide humidity. Incubate the sections at 45–50 °C for >3 h depending on the probe set.

3.3.4. Warm up the FISH washing buffer: 20 mM Tris-HCl, pH 7.4; 0.9 M NaCl to 50 °C during the incubation period. Prepare enough washing buffer to cover the slides in the Coplin jar.

3.3.5. Remove the plastic coverslips; incubate the slides in FISH washing buffer pre-warmed to 50 °C, placing the Coplin jar back into the 50 °C oven for 10–20 min. For thick samples, remove the coverslips in the Coplin jar by adding the buffer and gently dislodging the coverslips to avoid smearing the slice.

3.4. Staining the mucus and host features

3.4.1. Prepare a general DNA/mucus counterstain in PBS: final 10 μ g/mL DAPI + 40 μ g/mL mucus stain UEA-1. Prepare enough counterstain to cover the spots in the absence of a coverslip to avoid damaging tissue with additional coverslips.

NOTE: Covering the spots in the absence of a coverslip will require larger volumes than the 20 μ L used in 3.3.2. For average-sized sections (~10 mm diameter), 200 μ L is enough to cover one spot; test this volume beforehand on a dummy slide.

3.4.2. Remove the FISH washing buffer and replace it with PBS in the Coplin jar. Immediately after refilling the Coplin jar with PBS, decant the PBS.

3.4.3. Remove the slides from the jar and pipette the counterstain on top of the section while ensuring to not touch the tissue with the pipette tip. Cover the entire section with a bubble. Incubate at 4 °C for 45 min in the dark.

3.4.4. Wash the stains 3 times quickly with fresh PBS: while holding the slides in place using tweezers, pour out the PBS in a sink and immediately refill with fresh PBS.

3.4.5. Wiping the back of the slides against a wipe or paper towel, let most of the PBS evaporate off the sections, aided by a vacuum line connected to a pipette tip. Once again, avoid touching the section with the pipette tip.

3.4.6. Mount the sections using a mounting medium (without DAPI) and let it set at room temperature covered by glass coverslips, ensuring that the coverslips are flat and there are no air bubbles in the mounting medium.

3.4.7. Affix the coverslips to the slide by painting along the edges of the coverslip with clear nail polish, taking care to stay away from the edge of the slide to ensure that the slide will sit level in the microscope slide holder during imaging.

3.4.8. Store the slides at 4 °C in the dark for a few weeks before the fluorescence is depleted.

4. Imaging and image analysis

4.1. Visualizing the stains

4.1.1. Select an area of the tissue that contains both tissue and lumen to image the microbiota-host interface. For quantitative imaging of mucus thickness and luminal biogeography, select fields of view where the slices of the epithelial villi and/or crypts are longitudinal and not cross-sectional. Avoid areas with large gaps between the mucus and the epithelium to avoid sectioning artifacts.

4.1.2. Locate tissue and correct the focal plane, utilizing the DAPI signal for locating and focusing to avoid photo-bleaching of the FISH fluorescence signal.

4.1.3. Adjust the imaging settings. To visualize the bacterial DAPI stain, increase the laser power and gain to the point where the DAPI signal from epithelial cells is oversaturated or “blown out.”

NOTE: Do not oversaturate excessively, as this will lead to photobleaching and visual artifacts in the nuclei that cannot be recovered.

4.1.4. For all other channels, use the minimum amount of laser power that will provide a clear signal above the background, and be careful not to oversaturate.

NOTE: This is especially important when performing tile-scans, as photobleaching will be problematic when the overlap between tiles is imaged.

4.1.5. Use 40x or higher magnification to image individual bacteria.

4.1.6. **Acquire the images.**

4.1.6.1. Acquire tile scans to obtain quantitative data on mucus thickness and spatial distribution of microbes within the lumen. Ensure 15% overlap between the tiles and check for vignetting/uneven illumination, which may be exacerbated by <1x zoom. When setting up a tile scan, pay close attention to whether the tissue is in focus in all the tiles, as blurry/out-of-focus images cannot be analyzed (Figure 3C).

4.1.6.2. If possible, minimize the number of tiles needed to image a given length of epithelium by rotating the scan area so that the epithelium and mucus layer are parallel to the tile and not at an angle. Otherwise, find a section of the epithelium that is parallel or perpendicular to the imaging area to achieve a similar effect.

REPRESENTATIVE RESULTS:

To investigate the localization of specific gut commensal bacteria in the mouse intestine, germ-free Swiss Webster mice that had been colonized with individual bacterial isolates were used in this study. For this experiment, the bacterial species labeled were i) a human isolate of the *Muribaculaceae* family⁵, *Muribaculum intestinale*, an abundant and prevalent family of bacteria in the murine microbiota¹⁵, as well as ii) *Bacteroides thetaiotaomicron*, a genetically tractable and common gut bacterium. Following two weeks of equilibration, the mice were euthanized, and a segment of the colon containing a fecal pellet was sectioned and fixed in freshly prepared methacarn. After two days of fixation, the sections were dehydrated by processing through methanol, ethanol, and xylenes, infiltrated with paraffin, embedded, and then sectioned to luminal 4 μ m slices. These samples were then stained with a FISH probe (3' Cy3-tagged) specific for the *Muribaculum* isolate, designed using Oligominer software¹², and checked for secondary and tertiary structure and minimal non-specific binding using NuPACK and mathFISH^{16,17}. The samples were also counterstained with the Rhodamine-bound lectin UEA-1 (which stains fucosylated glycans in mucus) and DAPI. The stained sections were imaged using a 40x oil objective and super-resolution module.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of visualization of the microbiota-host interface in the intestine. (A) An illustrated workflow for the pipeline. (B) The two sectioning planes will yield either transverse sections (preferred for this pipeline) or cross-sectional “doughnuts.” (C) Embedding tissues of very different thicknesses may result in slices that are only optimal for one tissue or another. Abbreviations: FISH = fluorescence *in situ* hybridization; DAPI = 4',6-diamidino-2-phenylindole.

Figure 2: Imaging the colon shows the localization of *Muribaculum intestinale* relative to mucus in a gnotobiotic mouse model. Sections were stained with DAPI (blue), *Muribaculaceae* FISH probe (red), and UEA-1 (green). (A) Distal colon of mouse mono-colonized with *Muribaculum intestinale* and (B) distal colon of mouse bi-colonized with *Muribaculum intestinale* and *Bacteroides thetaiotaomicron*. Scale bar = 20 μ m. (C and D) Cy3-FISH (left), DAPI (middle), and combined Cy3-FISH + DAPI channels for luminal inset portions of (A) and (B), respectively. Scale bar = 5 μ m. In (A) and (C), all bacteria-shaped and bacteria-sized DAPI signals are labeled with Cy3 (single arrowheads), and in (B) and (D), in addition to these Cy3- and DAPI-double-positive cells (single arrowheads), there are DAPI-stained bacterial cells that are Cy3-negative (double arrowheads), as expected for mono- and bi-colonization states. With the exception of longer filamentous bacteria, larger (>4 μ m in length) DAPI-positive structures (blunt arrows) are plant material or nuclei from host cells. Abbreviations: FISH = fluorescence *in situ* hybridization; DAPI = 4',6-diamidino-2-phenylindole.

Figure 3: Common problems. (A) Shallow sectioning will not provide luminal slices of the intestine. (Left) An intestinal segment that has been sectioned at a depth that provides a view of the lumen as well as longitudinal views of the epithelium. (Right) An intestinal segment that has been sectioned too shallowly, revealing only cross-sections of crypts and no constant mucus layer or bacteria. (B) Uneven tissue/coverglass coverage may result in blurry and unevenly illuminated tile-scans. A tile-scan was set up with positions that were out of focus (top right corner). (C) Example of normal background (left) and high background (right). In this example, for the DAPI signal—note the signal coming from the epithelium, outside the nuclei. All scale bars = 20 μ m. Abbreviation: DAPI = 4',6-diamidino-2-phenylindole.

DISCUSSION:

The protocol described above provides a reproducible method to visualize the host-microbiota interface. These assays have considerably benefited from protocol development, starting from the optimization of FISH labeling¹⁸ to mucus preservation and imaging⁹. Fixation and embedding also provide useful storage of samples; furthermore, paraffin-infiltrated cassettes can be mailed without any restrictions, as the samples are completely fixed and inert.

Significance and alternative methods

The combination of FISH and mucus staining enables the analysis of microbiota composition at specific tissue locations and the interaction between individual bacteria and the host. Alternative techniques that involve the analysis of specific sites within the microbiota are usually unable to explore the single-cell nature of these interactions, such as in the case of laser capture microdissection coupled with sequencing¹⁹. However, sequencing-based techniques have the advantage of being able to capture the genetic make-up of the microbiota at a finer level and more broadly than 16s rRNA probes.

A pitfall of the protocol presented is that it provides a single-time snapshot of the microbiota in relation to the host. For real-time imaging in live animals, special imaging techniques are required to facilitate the acquisition of a fluorescence signal in deep tissue (e.g., intravital two-photon

microscopy and light-sheet fluorescence microscopy^{20,21}). In these methods, the model organism is either colonized with bacteria that are stained with molecules that bind to their envelope²⁰ or genetically modified bacteria that harbor fluorescent proteins²¹. In the latter case, the maturation of fluorescence proteins is a key issue as most standard fluorescent proteins require oxygen to emit light. Therefore, model organisms that have at least nanaerobic environments (nanomolar concentration of oxygen) are required, such as the aerobic zebrafish gut²¹.

In this protocol, methanol-Carnoy is used as a fixative instead of paraformaldehyde or formalin because it does not contain water. This prevents hydration, dehydration, and collapse of the mucus layer during processing and facilitates accurate measurements of mucus thickness. While methacarn fixation and paraffin embedding have become a standard in the field, different fixatives and embedding techniques have been investigated to optimize mucus preservation^{9,22}, with some studies indicating that resins may be superior to paraffin embedding²². Another important limitation to paraffin embedding and methacarn fixation is the loss of fluorescent protein fluorescence, which can be avoided by using alternative fixatives (e.g., formalin or paraformaldehyde) or modified embedding techniques²³. Each fixative has benefits and drawbacks, and the choice of the fixative depends on the imaging priorities. Imaging modalities can be combined if biological replicates are fixed in either PFA and methacarn and images are obtained from both samples.

FISH has been combined with immunofluorescence in isolated instances with specific anti-Muc2C3 rabbit polyclonal antibodies^{9,24}. These results have not been widely reproduced with other Muc2 antibodies, indicating that the choice of antibodies may be critical in the success of the FISH-immunofluorescence combination. The conditions for successful antibody staining for a given antibody may not permit retention of FISH staining.

Troubleshooting

In this protocol, sample collection, sectioning, and staining represent critical steps to prevent the creation of imaging artifacts. For example, pressing on the tissue upon collection and before embedding can lead to mislocalization of the microbiota and affect mucus quality. Another important consideration is to avoid combining segments of very different thicknesses in a single cassette, such as a relatively empty piece of the small intestine and a pellet within the distal colon. The different thicknesses lead to difficulties in imaging: after embedding, transverse sectioning at a specific depth for one segment may be at the wrong depth for imaging for the other one (e.g., the lumen will be at different depths for each tissue) (**Figure 1B,C** and **Figure 3A**). Furthermore, for imaging animal model stool pellets, they may be wrapped in peritoneal membrane²⁴. In this technique, a small section of freshly isolated peritoneum is gently folded around the stool and preserves the structure of the pellet during the wash steps outlined in the protocol.

Unlike processing animal tissue with content, imaging human intestinal samples presents some challenges. Specifically, luminal contents and mucosal lining will likely be disrupted by the colonoscopy bowel preparation usually performed before intestinal biopsies or resection procedures. Additionally, when biopsies are collected, it is helpful to note the tissue orientation

to aid in the identification of specific features (e.g., luminal vs. submucosa) in the absence of intestinal contents. Pre-embedding with 3% agar and/or agar:gelatin mixtures may be useful in maintaining tissue polarity²⁵; however, there are issues with the dehydration and sectioning of agar, as reported in the literature, and processing times may need to be altered.

A key aspect of achieving good FISH staining comes from adjusting the formamide concentration for specific FISH probes. Formamide will control the hybridization stringency of FISH probes to their targets. It is important to ensure that a) the proper formamide concentration is chosen for staining a given community, and b) that a suitable formamide concentration is even possible for the probe combination that is being utilized—this may affect the optimal probe choice when utilizing multiple probes. Websites such as mathFISH (<http://mathfish.cee.wisc.edu/>) may help with calculating the proper formamide concentrations for a given probe. In the absence of information about appropriate formamide concentrations, this protocol can be tested with 0% and 10% formamide.

Sectioning the embedded samples requires cutting the block to a sufficient depth to obtain luminal slices, as sectioning too shallowly into a block will result in a cross-sectional view of the villi/crypts with no luminal contents (**Figure 3A**). Stain the samples soon after sectioning for optimal FISH signal, and use fresh DAPI (or DAPI stored at -20 °C) to resolve background issues (**Figure 3B**). FISH staining of sections that are more than a month-old may result in poorer/inconsistent staining. If the tissue or coverslip is not perfectly flat with respect to the slide, the sample may not be in focus at every position within a tile scan (**Figure 3C**), leading to wasted effort and potential photobleaching. This can be solved by taking smaller tile scans in which all positions have been verified to be in focus. Sectioning with dull blades can also lead to the detachment of the mucus and luminal contents, which appear as large dark areas while imaging. Finally, the evaporation of the solution or bubbles during the hybridization step can lead to uneven staining of the FISH probes in the tissue.

Another critical parameter that affects the efficiency of the FISH probe binding is the competition between different probes. A useful check to verify that the FISH probe is labeling bacteria is to examine the colocalization of the signal from the FISH probe with DAPI (**Figure 2A,B**, inset). In samples that require the use of multiple rRNA probes, adjusting parameters such as hybridization temperature, probe concentration, and formamide becomes critical to ensure that the probes are binding to the correct species efficiently¹⁸.

Notes about imaging

FISH-stained bacteria are best visualized using a confocal microscope and an objective of at least 40x magnification. While 63x magnification is preferred to image bacteria at the single-cell level, 40x magnification can also be used by maximizing the digital zoom or employing a super-resolution system. Systems equipped with super-resolution capabilities may also provide sub-cellular resolution of individual bacterial cells. Lower magnification objectives may be utilized to get a general sense of bacterial localization and mucus thickness.

Acquiring 16-bit images instead of 8-bit images is also highly recommended, as the higher dynamic range will help to capture the wide range of the DAPI signal from the extremely bright nuclei of the epithelium and the relatively weak signal of luminal bacteria. Aside from using the imaging setup described above, an important imaging consideration is the choice of fluorophores. For best separation between bacterial types, ensure that the fluorophores used do not overlap in excitation and emission spectra (unless imaging on a system with the ability to perform linear unmixing). Additionally, probes can be used in combination (e.g., the combination of a family-specific probe and a genus-specific probe) to identify additional community members. If using linear unmixing or unvalidated probes, it is essential to test the accuracy and level of FISH staining on pure cultures^{8,14}.

Once the images are collected, multiple tools are available for the quantitative analysis of the host-microbiota interface, such as Bacspace²⁶, HiPR-FISH⁸, and other segmentation tools²⁷. BacSpace is a MATLAB software that allows the segmentation of the tissue and luminal components and the removal of plant material from images²⁶. The program also provides analysis of mucus thickness in 2D and quantification of spatial distribution based on pixel distances in different channels²⁶. Conversely, the HiPR-FISH Image Analysis software allows the segmentation of FISH-stained cells⁸. Finally, other bacterial segmentation tools that have been optimized for *in vitro* experiments²⁷ could also be adapted to this application.

Conclusion

In situ imaging enables the quantitative analysis of individual microbial taxa in the context of other community members and the various microenvironments created by diet, mucus, and host epithelial crypts. The interaction between organisms dictates the biology of host-associated microbial systems and provides an essential analysis of change to these structures during perturbation that has implications for health. Notably, the ability to image the microbiota *in situ* through the protocol described above provides an incredible window into the biogeography of the microbiota in relation to the animal host.

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DISCLOSURES:

The authors have no conflicts of interest.

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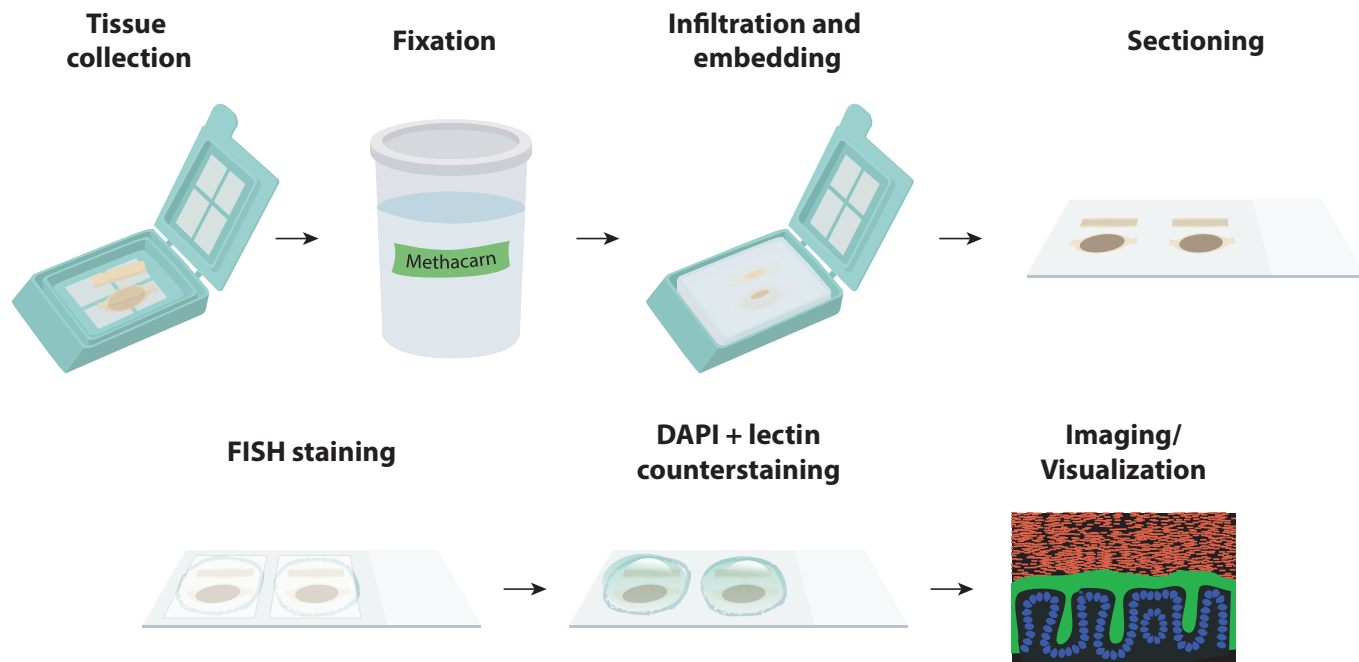
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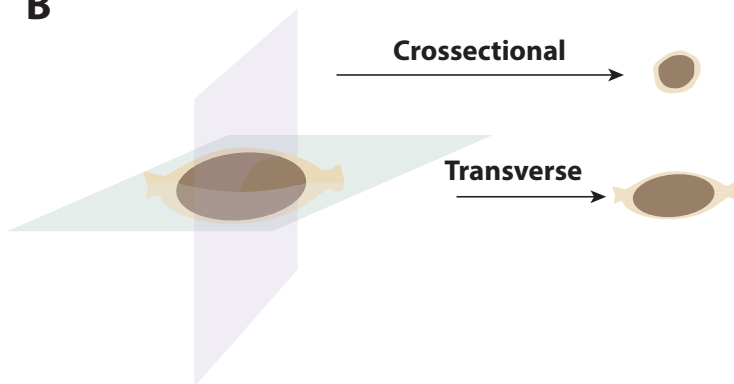
Figure 1

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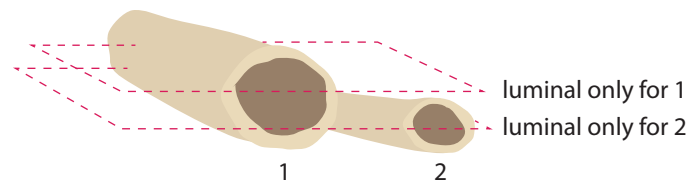
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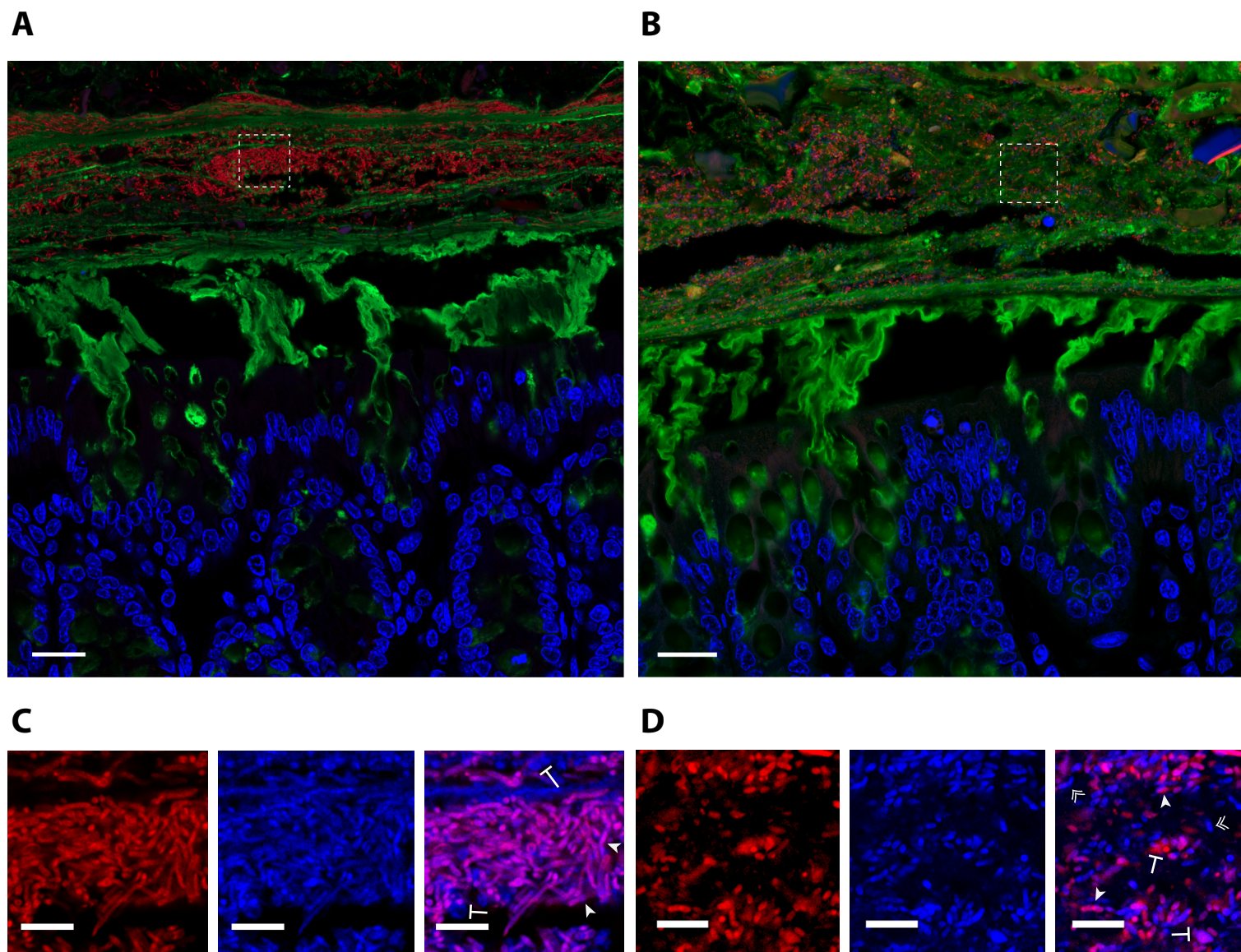


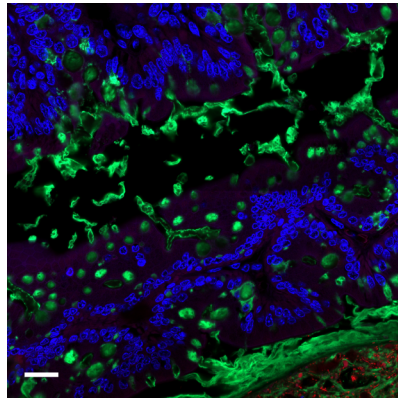
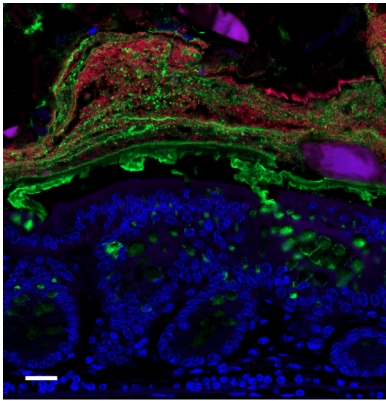
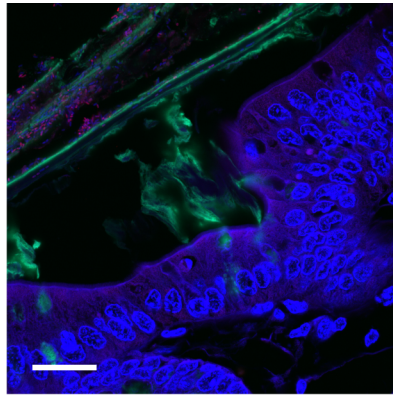
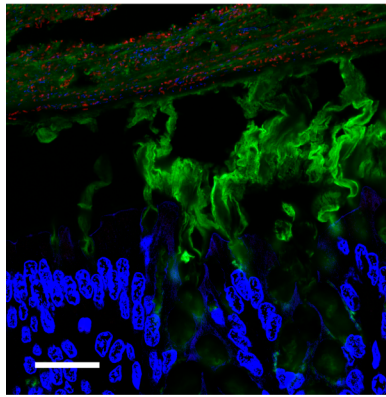
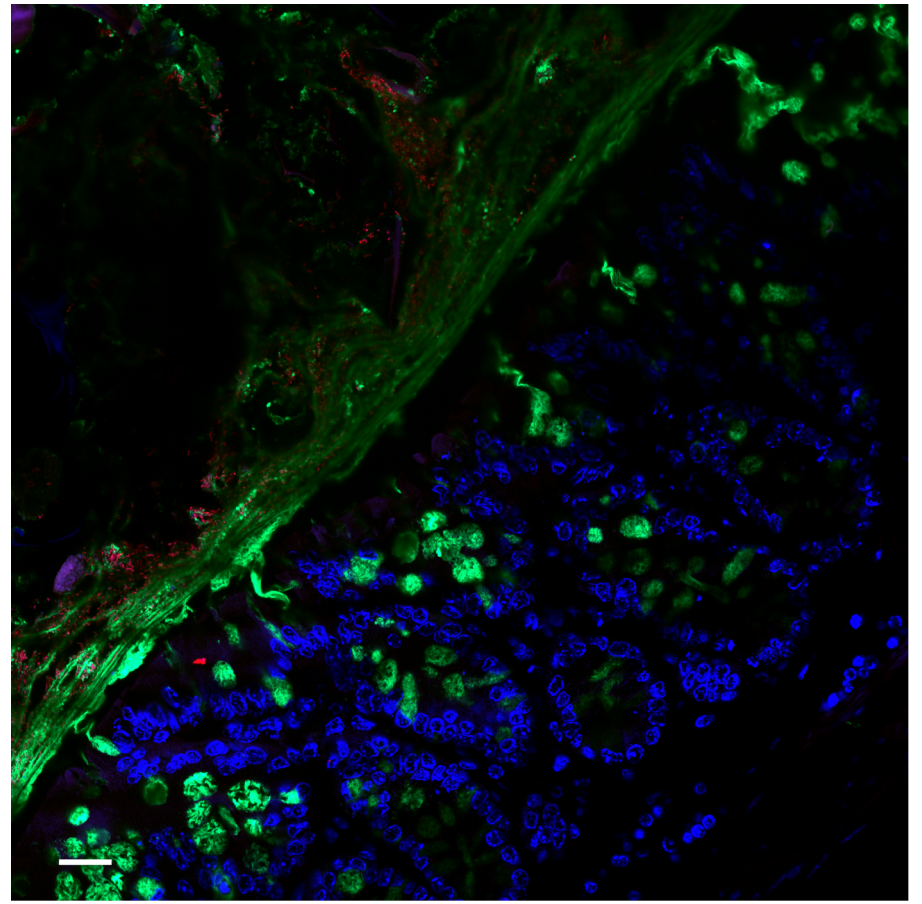
B



C





A**C****B**



Name of Material/ Equipment	Company	Catalog Number
Aluminum foil		
Chloroform	Fisher	C298-500
Coplin jar	Fisher	08-813E
Cover glass, 22 × 22 mm, no #1	VWR	CA48366-227-1
DAPI	Sigma-Aldrich	D9542
Empty pipette tip box(es)		
Ethanol, anhydrous, molecular grade	--	--
FISH probes		
FISH hybridization solution	--	--
FISH washing buffer	--	--
		FL-1061 (Fluorescein-labeled) or RL-1062-2 (Rhodamine-labeled)
Fluorescently-labeled Ulex Europaeus Agglutinin (UEA-1) lectin	Vector Laboratories	
Forceps		
Formamide	Sigma-Aldrich	F9037
Fumehood		
Glacial Acetic Acid	Fisher	A38-212
HybriSlip flexible plastic cover slips, 22 x 22 mm × 0.25 mm	Sigma Aldrich	GBL722222
ImmEdge Hydrophobic Barrier PAP Pen	Vector Laboratories	H-4000
Kimwipes		
Methanol	Fisher	A412
Microtome		
Multipurpose Specimen Storage Containers, 473 mL	Fisher	14-955-117A
Nail Polish	Electron Microscopy	72180
Oven		
Paraffin: Leica Paraplast	Leica	39601006
PBS, Phosphate-buffered Saline (10x solution)	Fisher	BP3991
Sodium chloride	Fisher	S271
Sodium Dodecyl Sulfate (SDS), 20% Solution, RNase-free	Invitrogen	AM9820
Tissue-Loc HistoScreen Cassettes	Thermo Scientific	CA83009-999
Trizma hydrochloride solution, 1M Tris-HCl, pH 7.4	Sigma-Aldrich	T2663-1L
Vectashield	Vector Laboratories	H-1000
Water, nuclease-free	Fisher	BP2484100
Xylenes	Fisher	X3P-1GAL

Comments/Description

Toxic

Resuspended to 5 mg/mL
To melt paraffin

20 mM Tris-HCl pH 7.4, 0.9 M NaCl, 0.01% (w/v) SDS in nuclease-free water.
Optional addition of 5–50% (v/v) formamide
20 mM Tris-HCl pH 7.4, 0.9 M NaCl

UEA-1 labels fucosylated glycans, so it is not appropriate for germ-free mice or for samples from fucosyltransferase 2 (FUT2)-deficient hosts.

Corrosive and flammable
for FISH hybridization steps, can substitute glass coverslips

Toxic and flammable
for sectioning

Can substitute with a glass or polyethylene container of your choice. Methacarn is not compatible with polystyrene or metal.
Any nail polish should work
Necessary range: 45-60 °C

Dilute to 1x for protocol

Toxic and flammable

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for these comments. We have performed a thorough proofreading of the manuscript.

2. Please provide an institutional email address for each author.

We have added both of our email addresses.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text to avoid any personal pronouns.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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.

For example: Kimwipes, Vectashield, Carl Zeiss Microscopy's Airyscan detector, Leica Microsystems, Zeiss 900, Zen Blue, etc.

We have removed commercial language from the protocol to the Table of Materials.

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

We have added an ethics statement. "All animal experiments and tissue collections described in this protocol were performed in compliance with the Canadian Council on Animal Care (CCAC) guidelines and were approved by the Animal Care Committee at the University of British Columbia."

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. Please include all safety procedures and use of hoods, etc.

We have changed the text to use imperative tense and condensed the notes.

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.

We have removed the extra text that is not directing the reader to do something specific to the discussion and intro.

We have kept in the protocol notes that are deemed to be essential to be read within the protocol.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have added details to the protocol steps to answer the "how" question.

9. Lines 116-133: Please move the cautions pertaining to a specific solvent/chemical to the specific steps mentioning its usage.

We have moved the cautions to each specific step as a NOTE.

10. Line 134-139: Please specify the steps of infiltration. How much xylene is used?

The steps are specified in 2.3.2. We have added a note to describe the amount of each wash needed: "The volume of each wash needed should be the minimum amount required to fully cover the cassettes in the beaker or container."

11. Line 165-170/338-344: Please refrain from using bullets or dashes.

Thank you, we have changed the bullets and dashes to numbered list.

12. After incorporating the revisions/ changes, please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have included a one-line space between each step and have highlighted steps of the protocol that identify what should be filmed.

13. Lines 351-357: Please move the lines describing the colonization of germ-free Swiss Webster mice with individual bacterial isolates to the protocol section.

This protocol is agnostic to the sample type, and both animal and human sample processing is described. It is assumed that colonization (If necessary) happens prior to this protocol, we have therefore not added this in. To clarify the sample type, we have included this sentence: "This protocol enables processing of either experimental animal or human clinical samples, and notes for processing both types of samples have been included."

14. Figure 2/3: Please include scale bars in all the images of the panel. Please provide the details of the magnification in the figure legends.

We have added scale bars for all images and provided details for the magnification used.

Reviewers' comments:

Reviewer #1:

A contributive protocole suite and report, warrants publication as so.

We thank Reviewer #1 for their appreciation of our protocol and report!

Reviewer #2:

In the manuscript by Ng and Tropini, the authors provide a protocol for the visualization of the microbiota by fluorescence in situ hybridization (FISH) while maintaining the spatial organization in the intestine. The protocol will be of great benefit for research questions relevant to microbiota-host interaction. The protocol is detailed and well described.

I have 2 points of somewhat major concern:

1) In the abstract, the authors mention that "host features [...] and host cell components are labeled with fluorescently labeled antibodies and lectins". I could not find any methodology referring to antibody staining in the manuscript. I think this is an important issue as it is often difficult to combine with FISH. It would be very helpful for the scientific community to include dos and don'ts of immunofluorescent staining in this context.

We thank the reviewer for the comment and we removed references to antibodies, as we have had issues combining antibody staining with FISH as well.

2) An important control for FISH are specificity controls, such as scrambled or mutated nucleotide probes or blocking with non-fluorescent probes. This should be included in the protocol.

This is an important addition and we have added a note in 1.1.3 to incorporate this comment: "Additionally, demonstrating specificity may be performed by including specificity controls/scrambled probes or by competition with non-fluorescent probes."

Minor points:

1) For better overview, it could be helpful to list the required buffers/solutions that need to be prepared also in the material list.

We have added a list of the required buffers. Specifically, we have added the FISH hybridization solution and FISH washing buffer.

2) Lines 163-170: it may be clearer to state that the tissue is washed/incubated twice for 30 min in methanol, twice for 20 min in EtOH, etc.

Thank you for this suggestion, we have edited this to reflect this:

"

1. First 30 minute incubation in absolute methanol
2. Second 30 minute incubation in absolute methanol
3. First 20 minute incubation in absolute ethanol
4. Second 20 minutes incubation in absolute ethanol
5. First 15 minute incubation in xylenes
6. Second 15 minute incubation in xylenes"

3) Line 266: what does "quick wash" mean exactly?

We have edited for clarity, thank you for your comment. Specifically, the text now reads as "immediately after re-filling the Coplin jar with PBS, decant the PBS."

4) Line 280: should it state "to prevent the slide not NOT sitting level"?

Thank you for this comment, we have edited this to read "to ensure the slide will sit level in the microscope slide holder during imaging."

5) Line 308: It seems that there is something missing or the "either" should be deleted.

Thank you for this comment, the sentence now reads as "Ensure 15% overlap between tiles and check for vignetting/uneven illumination, which may be exacerbated by <1X zoom."

Reviewer #3:

Manuscript Summary:

In this manuscript, the visualization of specific bacteria using FISH technique in combination with host cells

and mucus staining is described.

Major Concerns:

The authors presented a protocol as well as pitfalls for staining intestinal tissue. Although it is a very nice written protocol, some questions arise.

Section 1.1.3.: The authors mentioned that FISH probes should be checked for specificity but they do not describe how to do that; for example using BLAST. As this is a very important step it would be helpful to find more information in the manuscript.

We thank the reviewer for their thorough reading of the protocol. Regarding checking for specificity, we have added a note which explains:

"To check, align the FISH probes against either full length sequences or ASVs using a tool such as Clustal Omega to evaluate potential binding of probes and their targets. In addition to in silico testing, test the accuracy and level of FISH staining of unvalidated probes on pure cultures."

Here it would also be helpful to point out difficulties using more than one probe as the authors did in section 4.1.3.

This is a very useful suggestion, and we have added a new note that states: "When staining multiple community members, probes can be used combinatorially (e.g. the combination of a family-specific probe and a genus-specific probe), as long as fluorophores used do not overlap in excitation and emission spectra (unless imaging on a system with the ability to perform linear unmixing)."

Section 2.2.: Here it would be helpful if the authors can write something how to store the Carnoy and how to discard it. Could the authors please give any suggestions about the fixation time of tissue? Is it dependent on tissue size or on the tissue itself?

We thank the reviewer for this comment. In section 2.1, storage in polyethylene is already described, but a note has been added about disposal: "Methacarn is not compatible with polystyrene, so prepare in a polyethylene container, using glass graduated cylinders/serological pipets for the chloroform and glacial acetic acid. Do not dispose of methacarn in drains; collect and dispose as hazardous chemical waste as per institutional guidelines."

Fixation time is largely dependent on logistical considerations that enable paraffin processing of multiple samples. A note has been added to address this, specifically: "fixation time may be chosen to enable combination of cassettes for paraffin infiltration (e.g. to simultaneously perform paraffin infiltration on groups of samples collected and fixed over the course of two weeks)."

Section 2.3.: The authors did not deparaffinize the samples or use any antigen retrieval is that right? What kind of slide did they used? Did they test how long the sections can be used for FISH staining? Is there any possibility to use it longer than a few weeks?

Thank you for bringing up this important clarification - we do perform deparaffinization in section 3.3—the title has been changed to reflect that. No antigen retrieval was performed.

Normal glass slides are used and we have added this clarification in the text: "Transfer sections to regular uncoated slides."

Regarding the length of time sections can be used for, we have not systematically tested this and loss of signal is anecdotal. We have added this clarification in the discussion: "FISH staining of sections that are more than a month old may result in poorer/inconsistent staining."

Section 3.2.: The concentration of formamide is dependent on what? Could the authors give any recommendations? Unfortunately, the website Mathfish is not working.

We are sorry the website does not work for the reviewer, on our end we are able to load it correctly. In the protocol we recommend 0% and 10% as starting concentrations in the Troubleshooting section of the discussion.

Section 3.4.2.: Do the author would suggest diluting the probe to detect the correct concentration of the probe? In this section they use 20µl/section in section 3.5.1. they use 200µl is it a typo? From this section on it is important to protect the section from light. Please include this pitfall.

Thank you for noticing the difference, this is not a typo, the difference comes from the fact that larger volumes are required when a coverslip is not used. We have included the clarification "this will require larger volumes than the 20 µl used in 3.3.2".

Figure 2 and 3 are without any bars. Arrows to show the different areas in the section will be helpful. Are the smaller images magnifications of the bigger picture? If yes from which area?

We thank the reviewer for these comments. We have added scale bars to Figure.

Figure 2b: Did the authors use the same fluorophore for staining *M. intestinale* and *B. thetaiotaomocron*?

Thank you for this question: *M. intestinale* was doubly stained with FISH probe and DAPI, *B. thetaiotaomicron* was stained only with DAPI and this is reflected in the legend.

At the end, it would be helpful to include the used technique to the title of the manuscript.

This has been incorporated into the title, thank you.