

## Interactions of Intestinal Epithelial Cells With Bacteria and Immune Cells

*Methods to Characterize Microflora and Functional Consequences*

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### Summary

Epithelial cells at all mucosal surfaces are potentially apposed to bacteria, particularly in the intestine. It is established that intestinal epithelial cells (IECs) represent an important barrier between lamina propria cells and the potentially harmful luminal contents. In addition, IECs are important immunoeffector cells with the capacity to release cytokines, chemokines, and other molecules involved in antigen presentation and immune defense. The interaction of IECs with intestinal bacteria can result in a decrease in barrier function and the development of inflammation, which is known to be an important factor in the development of intestinal pathology. The potential role of such crosstalk between bacteria and other intestinal cell types in normal physiology and/or pathophysiology is therefore a topic of intense investigation. In this chapter, we provide protocols for the identification of bacteria that are associated with the epithelium and mucosa in addition to functional assays examining the interactions of neutrophils with epithelial cells and epithelial cell-mediated killing of bacteria.

**Key Words:** Structural organization of intestinal microbiota; fluorescence *in situ* hybridization (FISH); bacterial–epithelial interactions; neutrophil transepithelial migration; bacterial killing by epithelial cells.

### 1. Introduction

Intestinal epithelial cells (IECs) represent the first line of defense against potentially harmful bacteria present in the lumen. The human intestine is colonized by a huge number of bacteria, with approx  $10^{12}$  bacteria contained in a gram of colonic content, for example (**1**).

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In addition, IECs are important immunoeffector cells with the capacity to release cytokines, chemokines, and other molecules involved in antigen presentation and immune defense (2,3). Although bacteria are clearly necessary for the development of normal immune function in the intestine, they also are involved in the pathogenesis of inflammatory bowel disease (IBD). Under normal circumstances, bacterial–epithelial interactions may result in the generation of tolerogenic signals, and no obvious inflammation ensues (2). The underlying mechanisms involved in normal individuals that are abrogated in IBD remain largely unknown. However, a significant number of both clinical and laboratory findings have provided evidence for the contribution of luminal bacteria to the pathogenesis of IBD (4), with considerable data from practically all animal models implicating bacteria in this process (5). The potential role of such crosstalk between bacteria and other intestinal cell types in normal physiology and/or pathophysiology is therefore a topic of intense investigation. In this chapter, we provide protocols for the identification of bacteria that are associated with the epithelium and mucosa in addition to functional assays determining the interactions of neutrophils with epithelial cells and the killing of bacteria by enterocytes. A large influx of neutrophils into the mucosa from the underlying vasculature occurs during intestinal bacterial infections (6,7). A marked infiltration of activated lymphocytes, macrophages, and granulocytes into the gut mucosa is also a salient feature in the pathology of IBD (8,9). This chronic influx and activation of immunocytes result in the sustained overproduction of reactive metabolites of oxygen and nitrogen, and it is thought that some of the intestinal injury and dysfunction observed in IBD results from the elaboration of these reactive species (10). Neutrophils traversing the intestinal epithelium can lead to the formation of crypt abscesses. Moreover, large-scale transepithelial polymorphonuclear (PMN) leukocyte migration causes decreased barrier function (11). Bacteria can use secretion systems to insert proteins into enterocytes and thus activate inflammatory signaling pathways. For example, SipA, a protein secreted by *Salmonella typhimurium*, is necessary and sufficient to drive PMN transmigration across model intestinal epithelia (12). The transepithelial migration protocol described in this chapter is of great use in understanding the dynamics and functional consequences of neutrophil interaction with enterocytes, the mediators produced by both cell types, how this process contributes to pathology, and how it might be prevented therapeutically (13).

The gastrointestinal expression of antimicrobial peptides is evolutionarily conserved (14) but it is a relatively recent discovery that epithelial cells have the capacity to actively kill bacteria. Indeed, antimicrobial peptides and proteins are thought to constitute an important facet of innate immunity in the intestine. Most antimicrobial peptides and proteins expressed by mammalian epithelial cells are

members of protein families that mediate nonoxidative microbial cell killing by phagocytes (15). Indeed enterocytes express members of the defensin family (3) and other antimicrobial proteins, such as BPI (16), which are thought to be involved in the maintenance of homeostasis toward intestinal microflora and their products such as lipopolysaccharide (LPS). It is noteworthy that a deficiency in the production of certain defensins may play a role in the aetiopathogenesis of Crohn's disease (17). The killing assay provided here can be used to determine the contribution of IEC to mucosal defense and whether this is subject to regulation.

Formation of sessile communities and their inherent resistance to antibiotics and host immune attack are increasingly identified as a source of many recalcitrant bacterial infections. These include periodontal disease, endocarditis, chronic obstructive lung disease, and foreign body-related infections (18,19). With the exception of *Helicobacter pylori*, the impact of biofilms on the pathogenesis of intestinal diseases is unknown. We describe an easy practicable fluorescence *in situ* hybridization (FISH) methodology achieving high-quality pictures by accurate resolution of spatial structure and the composition of the microbiota associated with intestinal tissues. Probes are inexpensive and can be purchased from many oligonucleotide manufacturers (such as MWG Biotech, Ebersberg, Germany) using different fluorochromes. Cy3, Cy5, or fluorescein isothiocyanate (FITC) fluorochromes do not bleach quickly, have little auto fluorescence background, and allow high-quality micrographs (Fig. 1). Cy3 provides better resolution than Cy5, and Cy5 is better than FITC. The results with other fluorochromes are less encouraging. Presently, the sequences of more than 200 FISH probes targeting the bacterial rRNA at domain group and species levels are described in the literature. Some of the probes frequently used for evaluation of fecal communities are listed in Table 1 (20–23).

Contrary to expectations, none of the FISH probes that we have tested are absolutely specific. Depending on the microbial community investigated (e.g., human or animal intestine, pancreatic duct, gallstones, biliary stents) all FISH probes demonstrated some kind of cross-hybridization at the conditions of optimal stringency. Many probes delivering specific results in human intestine were widely cross hybridizing in murine material. Cross-hybridization of probes observed when characterizing biofilms within biliary stents and gallstones did not occur while investigating colonic microbiota even within the same patient. The choice of FISH probes must therefore be adjusted to the specific requirements of the investigation. FISH is an excellent tool for the assessment of spatial structure. However, care must be taken when interpreting the results, and the presence of specific bacterial groups must be confirmed using alternative methods such as culturing, polymerase chain reaction with subsequent cloning, and sequencing. Taken together, the methods

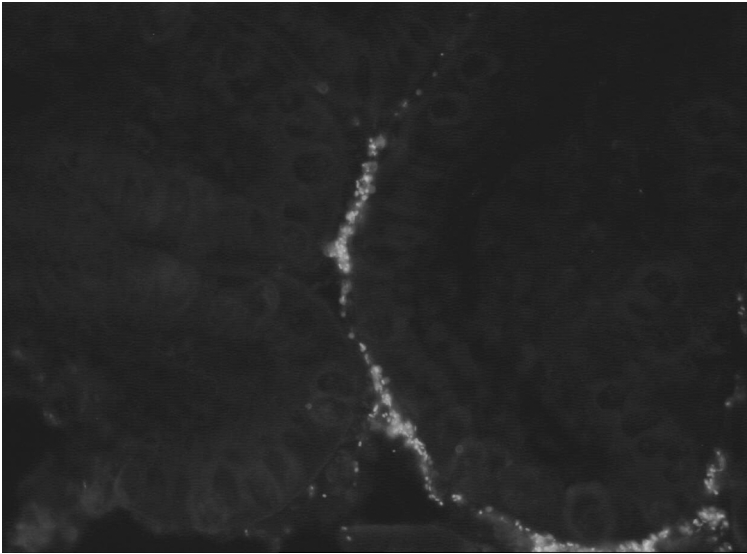


Fig. 1. Bacterial flora attached to the ileal mucosa in a patient with Crohn's disease (at a magnification of  $\times 1000$ ). The triangle of lumen is at the bottom right. Bacteria are tightly attached to the intact mucosal surface. *Bacteroides* is detected using an orange-labeled Cy3 probe, Erec (*Eubacterium rectale* *Clostridium coccoides* group), is stained red (Cy5). No bacteria are found in normal controls (data not shown). (Please see color insert following p. 50 for a color version of this figure.)

**Table 1**  
**FISH Probes**

Name	Target	Reference
Eub338	Virtually all bacteria, Kingdom ( <i>Eu</i> ) <i>Bacteria</i>	20
Ebac	<i>Enterobacteriaceae</i>	21
Erec482	<i>Clostridium coccoides</i> - <i>Eubacterium rectale</i> group	22
Chis150	<i>Clostridium histolyticum</i> group	22
Clit135	<i>Clostridium lituseburense</i> group (incl. <i>Clostridium difficile</i> )	22
Strc493	<i>Streptococcus</i> group	22
Ecy1	<i>Eubacterium cylindroides</i> and other	23
Phasco	<i>Phascolarctobacterium faecium</i> group	23
Veil	<i>Veillonella</i> group	23
Rbro, Rfla	<i>Ruminococcus bromii</i> , <i>Ruminococcus flavefaciens</i> and other	23

provided here are of considerable use in characterizing intestinal bacterial flora and examining their interactions with other cell types in this environment to elucidate functional implications in health and disease.

## 2. Materials

### 2.1. Fluorescence In Situ Hybridization

1. Carnoy-solution (ethanol 6:glacial acetic acid 3:chloroform 1 from Sigma, St. Louis, MO) stored in the refrigerator.
2. Clean, filtered paraffin wax held at 2–4°C above its melting point (Kendall, Mansfield, MA).
3. A cold plate to rapidly cool the wax.
4. A supply of molds in which to embed the tissues.
5. SuperFrost® glass slides (Fisher Scientific, Pittsburgh, PA).
6. PAP-PEN (Birmingham, UK).
7. Forceps.
8. Xylene (Sigma).
9. Absolute alcohol.
10. FISH Probes. For commonly used probes, *see Table 1*.
11. Hybridization buffer for (2 mL): 360 µL of 5 M NaCl; 40 µL of 1 M Tris-HCl, pH 7.4, x mL (*see Table 2A*) formamide, deionized, 10 µL of 10% sodium dodecyl sulfate (the amount will vary depending on the probe). With the exception of formamide, which must be deionized, all stock solutions can be stored at room temperature (RT) for 1 mo.
12. Posthybridization wash buffer (for 50 mL): 1 mL of 1 M Tris-HCl pH 7.4, y mL of 5 M NaCl (depending on the formamide concentration in the hybridization buffer, *see Table 2B*), 500 µL of 0.5 M EDTA (if formamide is used), 50 µL of 10% sodium dodecyl sulfate. This buffer should be made up fresh. Buffers can be made according to **Table 2B**.
13. DAPI (Sigma), store at –20°C. Dilute to a working solution of 0.5 or 1 µg/mL.
14. For mounting SlowFade (Molecular Probes), ProLong anti-fade (Molecular Probes), or CitiFluor (CitiFluor, Ltd.) can be used.

#### 2.1.1. Cell Culture

1. Cell culture medium: T84 intestinal epithelial cells are grown as monolayers in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 Nutrient mixture (Invitrogen) supplemented with 14 mM NaHCO<sub>3</sub>; 15 mM HEPES buffer, pH 7.5, 40 mg/L penicillin, 8 mg/L ampicillin, 90 mg/L streptomycin (all Sigma); and 5% newborn calf serum (Sigma).
2. Solution of trypsin (0.25%) and EDTA (1 mM) from Invitrogen.
3. Hank's Balanced Salts Solution (HBSS) buffers. HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup> is termed HBSS+ (for 1 liter: 990 mL of deionized-distilled H<sub>2</sub>O, 10 mL of 1 M HEPES, 9.75 g of HBSS+ powder [Sigma], 0.05 mL 10N NaOH; pH 7.4). HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> is termed HBSS– (for 1 liter: 990 mL of deionized-distilled

**Table 2A**  
**Hybridization Buffer Composition**

Formamide (%)	Formamide ( $\mu\text{L}$ ) (=x)	Distilled $\text{H}_2\text{O}$ ( $\mu\text{L}$ )
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400
65	1300	300

**Table 2B**  
**Posthybridization Wash Buffer Composition**

Formamide (%)	NaCl mM ( $\mu\text{L}$ )	5 M NaCl ( $\mu\text{L}$ ) (=y)	0.5 M EDTA ( $\mu\text{L}$ )
0	900	9000	0
5	636	6300	0
10	450	4500	0
15	318	3180	0
20	225	2150	500
25	159	1490	500
30	112	1020	500
35	80	700	500
40	56	460	500
45	40	300	500
50	28	180	500
55	20	100	500

$\text{H}_2\text{O}$ , 10 mL of 1 M HEPES, 9.5 g of HBSS- powder [Sigma], 0.05 mL 10 N NaOH; pH 7.4). All chemicals are obtained from Sigma.

- Polystyrene T162 Tissue Culture Flasks with Phenolic Cap supplied by Fisher Scientific (Costar).

5. 0.33-cm<sup>2</sup> (5  $\mu$ m pore size) collagen-coated polycarbonate filters (Costar Corp., Cambridge, MA).
6. Triton X-100 (10% solution; Sigma).
7. *N*-formyl-Met-Leu-Phe (1  $\mu$ M stock solution; Sigma).

### *2.1.2. Solution of Rat Tail Collagen*

1. Rat tails.
2. 1% acetic acid (Sigma).
3. Spectropor no. 1 dialysis tubing (Fisher Scientific).
4. 70% ethanol.

### *2.1.3. Bacterial Growth Medium*

1. Luria-Bertani broth (LB), per liter: 10 g tryptone/5 g yeast extract/10 g NaCl (Difco Laboratories Inc.).
2. Luria agar is LB containing 12 g of Bacto agar (Difco Laboratories Inc.) per liter.
3. MacConkey agar (Difco Laboratories) is prepared according to package instructions (50 g powder per liter of deionized-distilled H<sub>2</sub>O).
4. Gentamicin (Sigma): 50  $\mu$ g/mL prepared in deionized-distilled H<sub>2</sub>O.

### *2.1.4. Neutrophil Isolation*

1. 2% Gelatin (Sigma) prepared in HBSS. Make up 2% gelatin fresh per each use by preparing 3 g gelatin in 150 mL of HBSS— as follows: Preheat HBSS— to no more than 45°C (gelatin has collagen in it that could degrade). Then, add gelatin while stirring continually. Visually confirm that all of the gelatin is dissolved and let the solution cool to 37°C before using (do not over cool). Prepare 50 mL of 2% gelatin per 60 mL of blood drawn.
2. 13% Acid-citrate-dextrose per liter: 13.7 g of citric acid, 25 g of sodium citrate, 20 g of dextrose (all chemicals from Sigma).
3. Lysis buffer (keep at 4°C), per liter: 8.29 g of NH<sub>4</sub>Cl, 1 g of NaHCO<sub>3</sub>, 0.038 g of EDTA (all chemicals Sigma).
4. Hemocytometer.
5. Siliconized Pasteur pipets (Sigmacote; Sigma).
6. 2% Acetic acid (Sigma).

### *2.1.5. Myeloperoxidase Assay*

1. 2,2'- azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution. ABTS is made fresh before every use: 28 mg ABTS, 45 mL H<sub>2</sub>O, 5 mL citrate buffer.
2. Citrate buffer (store at 4°C), for 500 mL: 400 mL of deionized-distilled H<sub>2</sub>O, 73.5 g of sodium citrate, 52.5 g of citric acid, pH 4.2.
3. 30% H<sub>2</sub>O<sub>2</sub>. All chemicals obtained from Sigma.
4. 96-well plates.

## 2.2. Epithelial Cell-Killing Assay

1. Cell culture: Caco2 cells are cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL Streptomycin sulfate. All reagents were obtained from Sigma. Other necessary cell culture materials are listed in **Subheading 2.1.1**.
2. Sterile HBSS– (Sigma). Store at 4°C. Killing assays are conducted in the presence of HBSS– as calcium and magnesium inhibit BPI-mediated activity (**24**); see **Note 1**.
3. Sterile water.
4. Sterilized Eppendorf tubes (Fisher Scientific).
5. Cell scrapers (Fisher Scientific).
6. Luria Bertani broth and agar plates, prepared as detailed in **Subheading 2.1.3**.

## 3. Methods

### 3.1. Fluorescence In Situ Hybridization

For Probe fixation, see **Note 2**.

1. Place the biopsy in an Eppendorf tube with 1.5 mL of cold Carnoy solution. Leave large probes longer in Carnoy (on average 1 h for each millimeter of tissue thickness).
2. Transfer the biopsy from Carnoy to the chilled absolute ethanol twice and from ethanol to xylene twice. Incubate the biopsy for 15 min in each. Embed the biopsy in liquid paraffin.
3. Paraffin embedding (see **Note 3**). Select the mold; there should be sufficient room for the tissue allowing for at least a 2-mm surrounding margin of wax.
4. Fill the mold with paraffin wax.
5. Using warm forceps, select the tissue, taking care that it does not cool.
6. Chill the mold on the cold plate, orienting the tissue and pressing it into the wax with warmed forceps. This step ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.
7. Insert the identifying label or place the labeled embedding ring or cassette base onto the mold.
8. Cool the block on the cold plate, or carefully submerge it under water when a thin skin has formed over the wax surface.
9. Remove the block from the mold.
10. Cross check block, label, and worksheet.
11. Cut the paraffin blocks to 2- to 10-µm thick slices and put them on super frost glass slides (two slices for each appointed FISH probe). It is important to have a properly fixed and embedded block and sharp knife or many biases can be introduced in the sectioning. Common biases include tearing, ripping, "venetian blinds," holes, and folding.
12. Once sections are cut, they are floated on a warm water bath, which helps remove wrinkles. They are then picked up on a glass microscopic slide.



13. Place the glass slides in a warm oven at 50°C for approx 60 min to help the section adhere to the slide.
14. Deparaffinize the slides by running them through xylene to alcohol shortly before hybridization: 4X 3-min incubations in xylene and 4X 3-min incubations in absolute ethanol; all steps can be carried out at RT. Change the ethanol and xylene solutions every 40 slices containing intestinal contents to prevent fecal bacteria floating in the fluid and being found on the slices.
15. Heat the slices once more in a warm oven at 50°C for approx 25 min.
16. Demark the area of the hybridization with a PAP-PEN and air-dry.
17. Prepare the hybridization chamber using a Tupperware container that seals well. Darken the chamber to avoid bleaching of the fluorescence. A platform can be created for the slides, that must be absolutely horizontal over the hybridization time. Soak several paper towels with hybridization buffer to keep the chamber humidified so the hybridizations do not dry out. Individual slides can also be hybridized in humidified, 50-mL plastic screw cap tubes.
18. Mix 16  $\mu\text{L}$  of hybridization buffer with 2  $\mu\text{L}$  of the FISH probes (~10 ng of each probe) and distribute it over the biopsy surface. Be careful not to scratch the probe while distributing it. Do not use cover slips; they can mechanically disturb the mucus layer and spatial structures.
19. Place slides in box and incubate for 60 to 90 min in a moisture chamber at probe-specific temperature (*see Note 4*).
20. Posthybridization washes; remove the slices from the hybridization chamber. They should not be dry! Rinse the slides with MQ  $\text{H}_2\text{O}$  and transfer them immediately into a 50-mL tube with washing buffer prewarmed to the required hybridization temperature (two slides in each tube). Wash for 5 min in a water bath at the required hybridization temperature.
21. After 5 min of washing, rinse the probes with MQ  $\text{H}_2\text{O}$ , air-dry.
22. DAPI counter staining; apply 20  $\mu\text{L}$  of DAPI to each section, stain for 5 min at RT, wash with MQ  $\text{H}_2\text{O}$ , and air-dry.
23. Mounting; apply anti-fading mountant to the section. Add cover slip and view under an epifluorescent microscope and take pictures as desired (*see Note 5*). Because the slides fade quickly, they should be viewed within 2 d and stored in the dark.

### **3.2. Enteric Pathogen Interactions With Model IEC-Polarized Monolayers**

#### **3.2.1. Preparation of Rat Tail Collagen**

The isolation of rat tail collagen is performed by the method of Cereijido et al. (25).

1. Strip the skin from the rat tail; the tendons are almost pure collagen.
2. Soak 0.75 g of collagen in 70% ethanol for 20 min to dehydrate. Next, solubilize the tendons in 100 mL of 1 % acetic acid (v/v) precooled to 4°C. Stir the mixture overnight at 4°C.

At this point, the solution should be viscous and some undissolved material will remain.

3. Spin the collagen solution at 25,000g for 30 min at 4°C. Discard only the pellet and put the collagen preparation into dialysis tubing (Spectropor no. 1), which has been presoaked in distilled-deionized H<sub>2</sub>O for 1 h.
4. Place the tubing in a 2 L beaker, add cold distilled-deionized H<sub>2</sub>O, and dialyze for 24 h at 4°C against two to three changes of H<sub>2</sub>O.
5. Remove the collagen from the dialysis tubing and dilute the solution if necessary. The collagen solution should be a little thinner than the consistency of honey.
6. Store at 4°C for 2 mo.

### 3.2.2. Preparation of T84 Cells Grown on Collagen-Coated Polycarbonate Filters

1. Conventional inserts are prepared for the use in bacterial invasion assays (26). A 50-μL aliquot of the rat tail collagen mixture is placed in each well. Care should be taken to make sure collagen is evenly distributed across the filter and that the collagen stock solution is maintained at 4°C.
2. Allow plates to completely dry (3–24 h).
3. Next, the T84 cells, which are grown on 162-cm<sup>2</sup> flasks are trypsinized, and  $7.5 \times 10^5$  cells are then suspended in 75 μL of media and are placed in each transwell™ (Costar). A total 900 μL of cell culture media is placed in the outside chamber. Polarized monolayers prepared in this fashion can be used 6 to 14 d after plating. A steady-state resistance is reached 4 to 6 d after plating, with variability largely related to the cell passage number.
4. Inverted monolayers are prepared for use in neutrophil transmigration assays (26). The transwells (Costar) are removed from their respective wells and are placed upside down in a large gridded Petri dish; one invert to each petri dish grid square (each Petri dish holds 24 inverts).
5. A 50-μL aliquot of rat tail collagen is placed on the filter. When adding the collagen to the filter it is important to keep the solution at 4°C. Let the collagen dry on the filter overnight in the covered Petri dish.
6. Carefully add 75 μL of a  $7.5 \times 10^5$  T84 cell suspension to each invert and incubate at 37°C overnight. The next day, use forceps to aseptically flip the inverts into a 24-well plate. Add 1 mL of cell culture medium to the outside well and 200 μL to the inside of the invert. Polarized monolayers prepared in this fashion can be used 6 to 14 d after plating. A steady-state resistance is reached 4 to 6 d after plating, with variability largely related to the cell passage number.

### 3.2.3. Growth of Bacteria

Nonagitated microaerophilic cultures of *S. typhimurium* are prepared by inoculating 10 mL of LB broth with 0.01 mL of a stationary-phase culture, followed by overnight incubation (18 h) at 37°C. Bacteria from such cultures

are in late logarithmic phase of growth and represent approx  $5-7 \times 10^8$  colony-forming units (CFUs)/mL. CFUs are determined by serially diluting the bacteria and plating onto MacConkey agar medium.

### 3.2.4. Neutrophil Isolation

The neutrophil isolation procedure is based on the method of Henson and Oades (27).

1. From a healthy human volunteer, draw 60 mL of blood directly into a 50-mL syringe that contains 6 mL of 13% acid-citrate-dextrose (pH 4.0).
2. Spin for the buffy coat (400g, 20 min at RT).
3. Using prepared, siliconized pasteur pipets, remove top yellow serum layer down through the white interface by gentle aspiration. The interface contains lymphocytes, platelets, and monocytes, while the neutrophils are in the red layer, and just below the white interface.
4. Once the buffy-coat is removed, dispense 20 mL of the blood per 50 mL Falcon™ centrifuge tube and then add the 2% gelatin bringing the final volume to 50 mL (i.e., should have three tubes/60-mL blood draw). Let stand for 25 min at 37°C, during which time the red blood cells will settle to the bottom of the tube.
5. Using a 10-mL pipet, combine as much of the clear supernatant as possible in new tubes and do not contaminate with the bottom red blood cell layer.
6. Spin the clear supernatant at RT in a tabletop centrifuge for 10 min at 400g.
7. Remove the supernatant by aspiration and gently resuspend the white pellet in 3 mL cold (4°C) lysis buffer per 50-mL tube.
8. Next, bring the volume of cold lysis buffer to 50 mL, and centrifuge at 4°C for 10 min at 1200g. Aspirate off the supernatant and discard.
9. Gently resuspend the pellet in 5 mL cold HBSS– and wash with 50 mL of cold HBSS–. Resuspend the pellet in 3 mL cold HBSS– and carefully break up the pellet by gentle pipetting.
10. To count the neutrophils, remove 5  $\mu$ L of the suspension and place this in 500  $\mu$ L of 2% acetic acid. Acetic acid prevents swelling of the neutrophils and lyses the red blood cells, permitting one to view the morphology of cells. Add 12  $\mu$ L of the neutrophil suspension to each side of the hemocytometer and count the number of cells in each of the chambers and average the counts.
11. Resuspend the neutrophils at a final concentration of  $5 \times 10^7$ /mL in HBSS–. PMN are kept on ice in HBSS– and are used within 1 hr of being isolated. It is also important to keep the PMN in HBSS– buffer since the addition of divalent cations will cause them to become activated.

### 3.2.5. Invasion of *S. typhimurium* Into T84 Intestinal Epithelial Monolayers

Infection of T84 monolayers is performed by the method of Lee and Falkow (28) with slight modification (26).

1. Inserts with attached monolayers are lifted from the wells, drained of media by inverting, and gently washed by immersion in a beaker containing HBSS+, warmed to 37°C. Cells are incubated in HBSS+ buffer because the absence of divalent cations will cause the opening of intestinal epithelial tight junctions.
2. Next, the monolayers are placed in a new 24-well tissue culture plate bathed with 1.0 mL HBSS+ in the lower (outer) well and 0.05 mL HBSS+ added to the upper (inner well) and are then equilibrated for 30 min at 37°C.
3. 7  $\mu$ L of each HBSS+ washed, bacterial sample (representing an inoculation ratio of 10:20 bacteria:epithelial cell) is added per monolayer for 1 h. If necessary, the bacterial invasion can be followed throughout a time-course of 1 h. Two populations of bacteria generally are assessed: cell associated and internalized. Cell-associated bacteria represent the population of bacteria adherent to and/or internalized into the T84 monolayers and are released by incubation with 0.1 mL of 1% Triton X-100 for 20 min at 4°C. Internalized bacteria are those obtained from the lysis of epithelial cells with 1% Triton X-100, 90 min after the addition of gentamicin (50  $\mu$ g/mL). Gentamicin, an aminoglycoside antibiotic, does not permeate eukaryotic plasma membranes and is therefore cytolytic only to extracellular populations of bacteria while intracellular bacterial populations remain viable (29).
4. For both cell associated and internalized bacteria, 0.9 mL of LB is then added and each sample is vigorously mixed and quantified by plating for CFUs on MacConkey agar. To determine the number of adherent organisms the internalized population is subtracted from the cell-associated population (i.e., cell associated – internalized = adherent).

### 3.2.6. PMN Transepithelial Migration

Evaluation of neutrophil migration across monolayers of polarized intestinal epithelia stimulated by *S. typhimurium* is performed by the method of McCormick et al. (26) and is illustrated in **Fig. 2**.

1. T84 inverted monolayers are extensively rinsed in HBSS+ to remove residual serum components.
2. *S. typhimurium* is prepared by washing twice in HBSS+ and then are suspended to a final concentration of  $5 \times 10^9$ /mL. Then, 100- $\mu$ L aliquots of the bacterial suspension are microcentrifuged at 13,000g for 3 min and resuspended in HBSS+ a final volume of 25  $\mu$ L.
3. Inverted cell monolayers are removed from each well and placed in a moist chamber such that the epithelial apical membrane surface is oriented upward.
4. The bacterial suspension is gently distributed onto the apical surface and incubated for 45 min at 37°C.
5. Nonadherent bacteria are then removed by washing three times in HBSS+ buffer. The monolayers are then transferred, by inverting, back into the 24-well tissue culture tray containing 1.0 mL of HBSS+ buffer, in the lower (apical membrane now colonized with *Salmonella*) reservoir and 160  $\mu$ L in the upper (basolateral interface) reservoir.

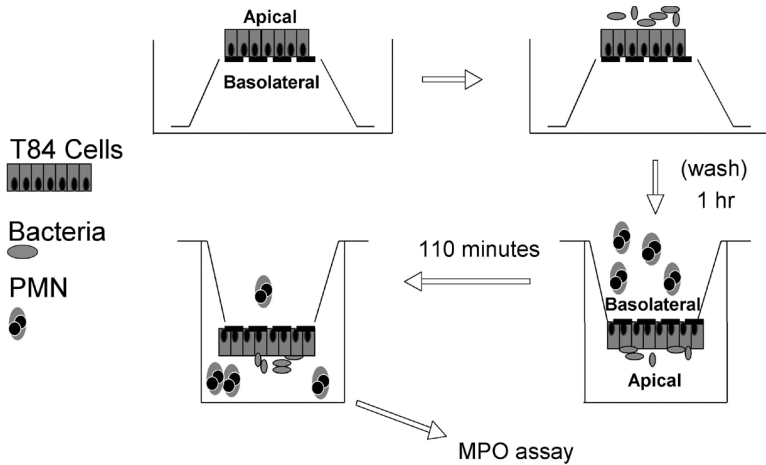


Fig. 2. Schematic for the protocol of *S. typhimurium*-induced neutrophil transepithelial migration. Inverted polarized, T84 cells are grown on the under-surface of, Transwell permeable supports. In this orientation, the apical surface of T84 cells was first colonized by *S. typhimurium* for 1 h. After the establishment of *S. typhimurium* infection, purified human peripheral blood neutrophils are then placed in the upper reservoir, interfacing with the epithelial basolateral membrane for 2 h. Such a maneuver is performed to achieve appropriate physiological orientation (i.e., neutrophils interacting basolaterally/*Salmonella* interacting apically).

6. To the basolateral bath, 40 mL ( $10^6$ ) of isolated neutrophils are added to each monolayer and incubated for 110 min at 37°C. Positive control transmigration assays are performed by the addition of chemoattractant (1  $\mu$ M fMLP) to the opposing apical reservoir. All experiments are performed at 37°C.
7. Transmigration is quantified by a standardized assay for the neutrophil azurophilic granule marker myeloperoxidase. After each transmigration assay, nonadherent PMN are washed from the surface of the monolayer and neutrophil cell equivalents, estimated from a standard curve, are assessed as the number of neutrophils that had completely transversed the monolayer (i.e., into the apical reservoir).

### 3.2.7. Myeloperoxidase Assay

This assay is used to determine neutrophil transmigration across epithelial monolayers (26).

1. To assess the transmigrated neutrophil population, 50  $\mu$ L of 10% Triton X-100 stock solution is added to the bottom reservoir. Rotate the plate carefully (setting ~150 rpm) at 4°C for at least 20 min.
2. During this time period, make up ABTS substrate solution and just before use add 1  $\mu$ L of 30%  $\text{H}_2\text{O}_2$  per mL of ABTS solution.

3. Prepare neutrophil standards by serially diluting  $1 \times 10^6$  neutrophils down to  $5 \times 10^3$ .
4. Add 50  $\mu\text{L}$  of citrate buffer to each well.
5. Remove 100  $\mu\text{L}$  of sample from each well and add it into a 96-well plate in duplicate.
6. Add 100  $\mu\text{L}$  of the ABTS solution (supplemented with  $\text{H}_2\text{O}_2$ ) to the prepared 96-well plates.
7. Place at  $37^\circ\text{C}$  and let develop for 15 to 20 min.
8. Read the plate in an ELISA plate reader at a wavelength of 405 nm.

### 3.3. Epithelial Cell-Killing Assay

1. Culture *S. typhimurium* in LB broth at  $37^\circ\text{C}$  until the culture reaches an absorbance of 0.8 when spaced at 600 nm. This corresponds to approx  $1 \times 10^9$  CFU/mL. If the bacteria have grown further, dilute the culture to this absorbance in HBSS-. These bacteria are in log phase.
2. Centrifuge the bacterial culture at 3000g for 10 min. Aspirate off the broth and resuspend the pellet in 5 mL of prewarmed HBSS-. Repeat this procedure once to wash the bacteria. Resuspend the bacteria in 5 mL HBSS-.
3. Fill and label Eppendorfs with 1 mL of HBSS-, these are used to dilute the samples. For purposes of accuracy it is important to pipet exact volumes.
4. Fill a 15-mL sterilin for each well of cells with 5 mL ice-cold sterile water and keep the sterilins on ice.
5. Take the plate of epithelial cells, aspirate off the medium and wash gently with 1 mL of warm HBSS-.
6. Reaspirate and add 1 mL of HBSS- to the cells.
7. Then incubate the cells with 100  $\mu\text{L}$  of the bacterial suspension. We generally use 6 well plates of 7- to 10-d-old Caco2 cells but this method can be modified for cells cultured on inserts by adding HBSS- apically and basolaterally and incubating the cells with apically applied bacteria, thereby mimicking the physiological scenario. Incubate HBSS- and bacteria in an additional plate to serve as a bacterial control. This bacterial standard allows for control of bacterial death and growth independent of epithelial cells and will be used to calculate percentage killing.
8. Incubate the cell culture plates at  $37^\circ\text{C}$  on a rotating platform set to 60 rpm.
9. At desired time-points, scrape the cells using a cell scraper along with the supernatants into 5 mL ice-cold water to hypotonically lyse the epithelial cells and prevent further killing or growth of bacteria.
10. Take samples from epithelial lysates and the bacterial standard and serially dilute them by transferring 10  $\mu\text{L}$  of lysate into 1 mL HBSS- and 100  $\mu\text{L}$  of this dilution into 1 mL HBSS-. To reduce variability this volume should be added without touching the liquid in the Eppendorf. Plate triplicate 50- $\mu\text{L}$  drops of 1/1000 and 1/10,000 dilutions on agar (this allows for replicates to be plated) and culture

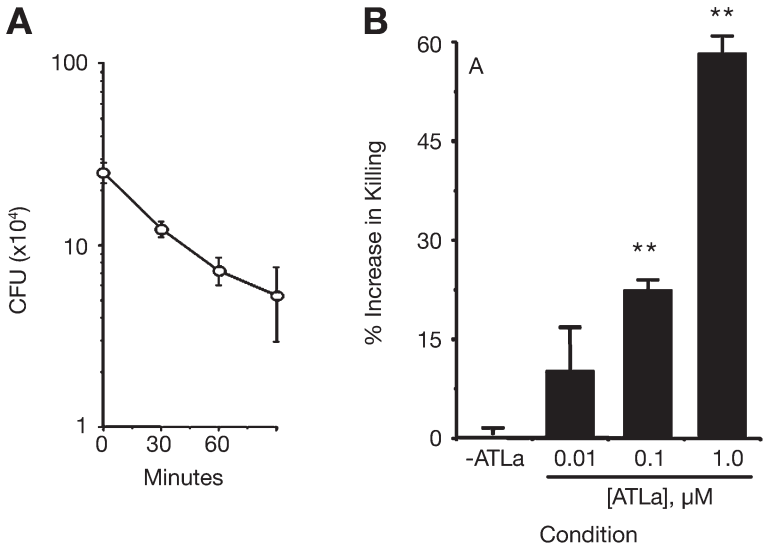


Fig. 3. (A) A time course depicting the killing of a smooth strain of *Salmonella typhimurium* by Caco2 cells. (B) Epithelial pre-exposure to the stable Lipoxin A<sub>4</sub> analog ATLa enhances this killing in a concentration-dependant fashion. Results are expressed as mean  $\pm$  SEM.

the plates at 37°C overnight. Determine the percentage bacterial killing using the following equation:

$$\frac{\text{Mean standard CFU} - \text{mean sample CFU}}{\text{Mean standard CFU}} \times 100 = \% \text{ killing}$$

where mean sample CFU was the mean of triplet drops for each dilution of the cellular lysate and standard was that of the bacteria alone. Results can also be expressed as CFU or CFU/mL. Alternatively, the plating may be performed using the “pour plate method” if desired. For Caco2 cells, significant killing has occurred by 15 min and, in our hands, it frequently is maximal by approx 60 min. For results of a typical experiment using a smooth strain of *Salmonella*, see Fig. 3. Care must be taken to ensure that the bacteria are not dying throughout the course of the experiment, thus negating the results. In this regard, *Salmonella* are viable for several hours in HBSS.

To prove that the killing is mediated by a specific peptide or protein, an inhibitory antibody or antisera may be used. In this case, prepare the antibody or antisera at the appropriate dilution and after washing the epithelial monolayer add 1 mL of this solution to the cells. Incubate for 30 min at 4°C. This is done to allow attachment of the antibody and prevent non-specific binding.

Thereafter, allow the plate to warm to 37°C, add the bacterial suspension and continue with the procedure above. Additional controls should be included to rule out the possibility of the antibody affecting bacterial growth.

#### 4. Notes

1. Initial growth curves should be conducted to relate absorbance to bacterial number. We have used this protocol to assess the contribution of BPI to bacterial killing mediated by intestinal epithelial cells but the assay can be adapted for other mucosal epithelial cells using a relevant strain of bacteria. Anionic sites in the Lipid A region of LPS are normally occupied by divalent cations ( $\text{Ca}^{2+}$  and/or  $\text{Mg}^{2+}$ ) which result in tight LPS packing. Bacterial/permeability increasing protein at antibacterial concentrations effectively competes for these sites in the LPS molecule and conversely, bound bacterial/permeability increasing protein can be displaced by these cations (24).
2. Before the spatial organization of intestinal microbiota can be investigated, it must be preserved. Shock frosting of the probes allows the preparation of native tissue slices with bacteria attached to the mucosa and intestinal contents. However, the fluorescent *in situ* hybridization takes place in an aqueous environment at temperatures of 46°C and higher. Hybridization occurs during a period of at least 90 min, which is necessary to achieve sufficient signals, but during this time bacteria are being progressively detached from the tissue and the spatial structure is lost. Formalin disintegrates spatial structures even before hybridization and tends to swell tissues rendering loose bacterial conglomerates within mucus more vulnerable to disintegration. The use of nonaqueous fixatives to preserve the mucus layer is crucial. We achieved excellent results preserving the material of human biopsies, surgically removed tissues and whole animal intestines with Carnoy solution. Once the structure of microbial communities is stabilized FISH proves to be a simple and reliable method.
3. The mechanical treatment of tissues while obtaining specimens leads to biases caused by dragging, crushing, and disrupting of normal anatomy. Fecal bacteria may be integrated into the distorted tissues and found at atypical anatomic sites and even within single cells. The optical integrity of the epithelial layer or of the tissue at the site of investigation is extremely important. Normally the autofluorescence background of human tissues allows good visualization and orientation within histological structures and the mucus layer. However, at least two additional stains (Alcian blue, hematoxylin and eosin, or other) for each biopsy or tissue specimens should be performed and are extremely helpful especially during optimization. No evaluation should be attempted when signs of tissue damage are obvious.
4. When optimizing probes for specificity, it is necessary to vary the hybridization temperature or formamide concentrations (increased temperature or formamide concentration means increased specificity). If possible do not use temperatures higher than 50°C or formamide concentrations higher than 55%. High temperatures result in mucus detachment and formamide introduces biases in eucaryotic tissues leading to bizarre fluorescent signals. Whenever possible, short hybridiza-



tion times of 90 min are preferable, and the hybridization should not exceed 6 h. In most cases, 90 min provides excellent resolution when Cy3, Cy5, or FITC fluorochromes are used.

5. The quantification of bacteria within spatial structures cannot be directly verified by stepwise dilution of bacteria. We use the approximation based on the following model. A 10- $\mu$ L suspension of bacteria of  $10^7$  cells per mL applied to a glass surface in a circle of 1 cm results in 40 cells per average microscopic field of 200  $\mu$ m diameter at a magnification of  $\times 1000$ . One bacterium thus occupies an area of 785  $\mu$ m<sup>2</sup>. One bacterium averages 0.8  $\mu$ m in size. Under these assumptions, a surface of 100  $\mu$ m<sup>2</sup> containing one (or 100) bacterial cells will correspond to a concentration of  $1 \times 10^9$  (or  $1 \times 10^{11}$ ) cells per microliter in tissue slides that are 10- $\mu$ m thick. 250 bacterial cells per 100  $\mu$ m<sup>2</sup> does not allow the visual distinction of single bacteria but spaces between bacteria can still be seen; cases such as this were therefore assigned a concentration of  $10^{11}$  cells per milliliter. A homogeneous carpet without any empty spaces between them occurs as bacteria increase to 2500 cells/100  $\mu$ m<sup>2</sup>; these cases were assigned a concentration of  $10^{12}$  cells per milliliter. In cases of extremely high bacterial concentrations sections of 4 or even 2  $\mu$ m in size can be performed. The spatial organization and the composition of the biofilm can be evaluated using a cumulative multi-step extension analysis which includes detection of bacteria using single FISH probes (Cy3) and DAPI DNA counter-stain. For each sample, probes that positively hybridize with more than 1% of the bacteria that are visualized by DAPI are further combined with each other in pairs and triplets and applied simultaneously using different fluorochromes in a single hybridization (Cy3/FITC/Cy5/ DAPI corresponds to orange/green/dark red/blue). This permits a three- or four-color analysis of the population structure within the same microscopic field. The hybridizations with different probe combinations are cumulatively extended until the position of all relevant bacterial groups to each other and their relative concentrations are clarified. When probes for unrelated bacterial groups hybridize with the same bacteria, it is necessary to adjust the hybridization stringency (*see Note 4*) until a clear differentiation of the bacterial groups is possible. Probes cross-hybridizing even under high stringency conditions should be excluded from the evaluation.

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