

THE HUMAN INTESTINAL ORGANOID AS A MODEL SYSTEM FOR
ENTEROPATHOGENIC *ESCHERICHIA COLI*
PATHOGENESIS

by

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DEDICATION

To my dear friend, D. Gregory Smith who kept reminding me to, “Just Breathe.”

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ABSTRACT

Escherichia coli are Gram-negative, facultative anaerobic bacteria commonly found in the intestine of humans and warm-blooded animals. *E. coli* can be mutualistic or pathogenic that cause diarrheal disease (diarrheagenic *E. coli*, DEC). The colonization of *E. coli* begins with the successful adherence to intestinal epithelial cell (IECs); which is mediated by a variety of colonization factors on the bacterial cell surface. This is the first and most crucial step for *E. coli* colonization. Therefore, valuable model systems to study *E. coli* should recapitulate this adherence. Several model systems have been developed and successfully reproduce adherence and other important aspects of EPEC pathogenesis, but have significant limitations. However, human intestinal organoids (HIOs) are a 3-dimensional tissue culture composed of a single layer of mature, differentiated, columnar epithelial cells that surround a lumen. When compared to traditional cell cultures, animal models and ligated intestines, HIOs have the potential to be more representative human physiology.

Here, we begin to demonstrate the use of HIOs as an *in vitro* model to study *E. coli*. We hypothesized that HIOs could be used to study epithelial colonization dynamics of *E. coli*. In this study, we established working protocols for a novel experimental approach for investigating attachment factors involved in *E. coli* attachment.

HIOs are generated from embryonic or pluripotent stem cells into definitive endoderm that gives rise to 3-dimensional structures. These structures were routinely cultured to a diameter of approximately 3.0 mm and embedded in matrigel. An overnight culture of the prototypic EPEC strain, e2348/69 (O127:H6) was transfected with a plasmid carrying a green fluorescent protein that provided visualization by fluorescent microscopy. Approximately, 2.4×10^6 CFU was injected into individual HIOs using a microinjector and incubated for 12 hours at 35°C and 5% CO₂. HIOs were then fixed and either stained for fluorescence imaging or processed in 1% osmium tetroxide for examination by field emission scanning electron microscopy (FE SEM).

The observations that EPEC was intimately associated with the intestinal epithelium, supports the use HIO for *E. coli* colonization investigations.

BACKGROUND

Part I: The Intestine

The intestine is the lower portion of the alimentary canal and is lined with simple columnar intestinal epithelial cells (IECs). These IECs are comprised of 6 differentiated cell types that are organized into invaginations and protrusions, called crypts and villi. The crypts (also known as the crypts of Lieberkühn), located at the bottom of the villi, house a self-renewing stem cell that carries a leucine-rich repeat containing G protein-coupled receptor 5 (LGR5+). The LGR5+ cells are intercalated alongside of Paneth cells¹, and are responsible for auto-renewing and regenerating the various cell types that populate the intestine; Paneth, goblet, tuft, enteroendocrine, and enterocytes. The various cell types provide specific functions that maintain the health of the intestinal tract and host. The Paneth cells are responsible for the health of the self-renewing LGR5+ cell; goblet cells produce mucus that facilitates movement of luminal contents and promotes the IEC barrier; tuft cells have a putative function of chemosensory activity; enteroendocrine cells create the largest endocrine system of the human body and produce a variety of regulatory molecules that promote physiological and homeostatic functions; and absorptive enterocytes provide barrier function, absorption of nutrients, innate immune functions, such as antigen transport and chemokine/cytokine production²⁻⁴.

As these various cell types differentiate, they migrate from the crypts into the transient amplifying zone. Upon reaching maturity, the cells will migrate beyond the transient amplifying zone and provide their designated function. As the cells age, they are pushed further to the apical surface where they come in contact with the luminal

contents and will eventually slough off and be pushed into digestion ⁵. The cells, with the exception of the Paneth cells, have a turnover rate of approximately 3-5 days (Paneth cells turn over every 3-6 weeks) in murine models ⁵. This continual shedding of cells (approximately 10^{11} cells daily) provides innate defense against colonizing pathogens ¹.

On the apical surface of IECs, there are microvilli or hair-like projections that protrude into the lumen and increase the surface area of the intestine. The basal side secures the cells to fibrous collagens, which provides support and structure for the crypts, villi, and overall intestinal architecture. The intestine is segmented into 4 basic parts: duodenum, jejunum, ileum, and colon. This study focuses on the duodenum.

The Duodenum.

The duodenum is the first segment of the small intestine that begins after the pyloric sphincter, forming a “C” and eventually emptying into the jejunum. The duodenum, and the entire alimentary tract, forms a barrier between host circulation and luminal contents and also functions to continue the digestion of food. The duodenum receives partially digested food from the stomach known as chyme. The pancreas and gallbladder also aid at this point in digestion by releasing enzymes (bicarbonate, trypsin, amylase, lipase and bile) into the duodenum.

Part II: Diarrheagenic *Escherichia coli*

E. coli are Gram-negative, facultative anaerobic bacteria commonly found in the large intestine of humans and warm-blooded animals ^{6,7}. *E. coli* are among the first bacteria to colonize the gut upon birth, where they live mutualistically with their host ⁸. Some *E. coli* are pathogenic and cause diarrheal disease (diarrheagenic *E. coli*, DEC) ^{9,10}. DEC are commonly transmitted to susceptible individuals via contaminated food and/or water and can be differentiated into the following six groups based on the types of disease they cause ¹¹⁻¹³: enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC), and enterohaemorrhagic *E. coli* (EHEC) ^{6,14}. Each of these groups, or pathotypes, produces a unique set, or combination, of virulence factors (i.e. toxins, adherence mechanisms, and invasive machinery) that results in a variety of symptoms, from a transient watery diarrhea to a severe bloody diarrhea that can lead to other complications (Table 1.); ^{6,10}.

Enterotoxigenic *Escherichia coli*

Human ETEC infections typically occur among children in developing countries and also children and adults from developed countries that visit these areas ^{6,9,15,16}. Clinical features of ETEC infection are an abrupt onset of watery diarrhea with fever and vomiting, which is similar to a *Vibrio cholera* infection ⁶. The first and crucial step for ETEC, and all DEC pathotypes, is the ability to adhere to the IEC ⁶. ETEC infection begins with colonization of the human intestinal epithelium that is mediated by colonization factors, such as fimbriae and pili, located on the bacterial cell surface ^{17,18}.

Whether these factors require specific receptors on the IEC surface is unclear ⁵. After successful adherence, ETEC produces two hallmark enterotoxins; a heat-stable toxin (hST) and a heat-labile toxin (hLT) ^{9,16}. These toxins accumulate in the enterocyte, promote secretion of electrolytes and fluids, and eventually can lead to apoptosis ⁶.

There are two classes of the hST; STa and STb ⁶. The hST's are long-lived (i.e. stable), relatively small, and contain several cysteine residues. Disulfide bonds between cysteines likely provide toxin stability ⁶. STa is the smaller of the two classes and has a molecular weight of approximately 2-kDa ¹⁹. STa is secreted from the bacterial cell as an inactive pretoxin of 72 residues and cleaved by signal peptidase into the biologically active 53 residues ^{6,19}. During infection it binds the guanylyl cyclase receptor located on the IEC surface, causing activation of the guanylyl cyclase and the synthesis of cyclic guanosine monophosphate from guanosine triphosphate ²⁰. Cyclic guanosine monophosphate participates in ion channel maintenance (electrolyte homeostasis) and promotes a gut secretory response via a secondary messenger system ^{19,21,22}.

The second class of the hST, STb, is more than twice the size of STa (5.1-kDa) ⁶. The receptor for STb is a membrane trafficking molecule (an acidic glycosphingolipid, sulfatide) located on the apical surface of IEC ^{23,24}. STb is secreted by the bacterial cell as an inactive pretoxin of 71 residues, and is cleaved inside the IEC to a biologically active 48 residues ^{6,21}. Attachment of the STb to its receptor causes uptake of the toxin and activates an intracellular pertussis toxin-sensitive G protein ²³. This process is similar to the secondary messenger mechanism of the cyclic guanosine monophosphate by STa and causes an influx of the calcium ion through a receptor-dependent ligand-

gated channel and activation of the calmodulin-dependent kinase II, which helps maintain intracellular calcium homeostasis ^{23,25}. The increase of calcium causes arachidonic acid signaling, leading to the production of prostaglandin E₂ and serotonin, which leads to secretion of several important electrolytes (chloride, bicarbonate and sodium) ^{6,21,23}.

hLT of ETEC resemble toxins produced by *V. cholera* ^{6,26}. hLT's are separated into two classes, hLT-I and hLT-II, which present no antibody cross-reactivity ⁶. hLT-I is produced mainly by the class of ETEC that infects both humans and animals and hLT-II is produced more exclusively by ETEC that primarily infects animals ⁶. Despite their antigenic uniqueness, both classes of hLT function similarly to produce diarrhea. hLT is composed of a α subunit (approximately 28-kDa) and 5 β subunits (approximately 11.5-kDa each), which are organized into a hexameric (AB₅) complex ²⁷. The holo structure allows for precise binding to gangliosides ²⁷, found in the plasma membrane of IECs.

hLTs function similarly to STa, and act through the regulatory system that controls the adenylate cyclase system to produce cyclic adenosine monophosphate; ²⁸. The β subunit binds to GB₁ located on the plasma membrane and the enzymatically active tail of the α subunit transfers an adenosine diphosphate -ribosyl moiety to the G-stimulatory protein. This prevents the guanosine triphosphate from being hydrolyzed (normally used to inhibit the system), and so the adenylate cyclase system remains chronically active, producing cyclic adenosine monophosphate ²⁸. Accumulation of cyclic adenosine monophosphate increases secretion of chloride from crypt secretory cells into the intestinal lumen, resulting in watery diarrhea ^{6,27-29}.

Aggregative *Escherichia coli*

Aggregative *Escherichia coli* (AEC) infections typically result in watery diarrhea and a low-grade fever ⁶. These infections occur as sporadic cases and outbreaks among children in both developed and developing countries ^{6,30}. AEC has two distinct colonization phenotypes when cultured with HEp-2 cells; aggregative adherence (AA) and diffuse adherence (DA) ⁶. These phenotypes have been used to define the subclasses of AEC, EAEC and DAEC, respectively ³¹.

Enteragggregative Adherent *Escherichia coli*. Loosely adherent, aggregated bacteria on the mucosal surface that is located predominantly in the colon, characterize EAEC ²⁸. The AA phenotype of EAEC is the observation of “stacked-bricked” bacterial cells, formation of a thick mucous blanket, and mucosal pathology via cytotoxin production ^{28,30,32}. The AA phenotype and several toxins are encoded on a 65-MDa adherence-related plasmid, also known as the “pAA plasmid” [Johnson]^{28,33}. The pAA plasmid encodes for: 2 colonization factors (Pic and dispersin), aggregative adherence fimbriae I and II, and cytotoxins (enteroaggregative *E. coli* ST, EAST1; plasmid-encoded toxin, PET; and *Shigella* enterotoxin 1,shET1) ^{28,33-35}.

Approximately 1 mm of mucus covers the epithelial surface of the human colon to help protect the body from invading microbes ^{36,37}. Mucus is mainly composed of mucin, or glycosylated proteins, that is secreted by the goblet cells ³⁸. Two virulence colonization factors, Pic and dispersin, are pieces of the successful multi-factorial strategy employed by EAEC to colonize the intestine ^{35,36}. Pic belongs to the serine protease autotransporter of *Enterobacteriaceae*, or SPATE, and is secreted as a pretoxin

with a molecular weight of 146.5-kDa; which is cleaved upon entering the culture supernatant as a final 109.8-kDa protein ^{32,36}. The SPATE are assigned to a unique type V secretion system; which contain 3 functional domains: a signaling sequence, passenger domain, and a translocation unit ³⁹. Pic provides several putative roles for EAEC virulence including complement resistance, serum resistance, hemagglutination, and potential opposing roles involving the degradation and hypersecretion of mucin; however, unlike the plasmid-encoded toxin (Pet, another SPATE produced by EAEC), Pic does not have any cytotoxic activities or degrade host defenses found in the mucus ^{36,39}. One primary function of Pic is the degradation of mucin that allows EAEC access to the mucosal lining; this is accomplished by the dual functions of binding the monosaccharide and oligosaccharide side chains of mucin and the cleavage of serine bonds of the protein portion of mucin ³².

The second colonization factor protein, dispersin, allows for a slowing of adherence by EAEC to the mucosal border, and is thought to promote a greater EAEC distribution throughout the intestine ³⁵. Dispersin is a hydrophobic, 10-kDa protein that is secreted into the extracellular space and binds non-covalently to lipopolysaccharide located on the surface of EAEC (and other Gram-negative bacteria) ^{35,39}. The binding of dispersin lessens the attraction of the highly hydrophobic AAF fimbriae from other EAEC with attached AAF fimbriae, which slows the observed aggregation phenotype ³⁵.

The adherent virulence factors of EAEC are mediated by one the two aggregative adherence fimbriae (the human pathogenic strain O42, which produces aggregative adherence 2 fimbriae, is discussed as an example) ³⁵. The aggregative adherence

fimbriae 2 is a member of the chaperone-usher family of adhesions ⁴⁰. The chaperone-usher family of adhesion is found throughout the family *Enterobacteriaceae* and contributes to the virulence of these pathogens ⁴⁰. The presence of the aggregative adherence fimbriae increases the hydrophobicity of bacterial cells, allowing for increased adherence to the IEC and to other planktonic bacterial cells in close proximity. However, as mentioned above, production of dispersin slows attraction to the IEC and other bacterial cells, allowing for a more dispersed infection ³⁴. Therefore, the actions of dispersin and aggregative adherence fimbriae appear to operate in counter balance with each other in order to maintain the appropriate level of adherence in the GI tract.

EAEC contains two cytotoxins, shET1 and EAST1, that have similar functions to the toxins produced by ETEC, which eliciting the cyclic guanosine monophosphate / cyclic adenosine monophosphate pathways ³⁹. The shET1 is composed of a 20-kDa protein that is associated with a pentamer of smaller 7-kDa proteins, arranged in the AB₅ conformation ^{39,41}. The receptor of shET1 is currently under investigation, but has been suggested to be the GB_x receptor of ETEC hLT ⁴¹. The shET1 toxin is thought to induce intestinal secretion through the cyclic adenosine monophosphate cycle and possibly the cystic fibrosis transmembrane conductance regulator, or CFTR ^{28,39,42}. The shET1 and Pic are encoded on the same chromosomal locus and are antisense of each other ³⁹.

EAST1 is a 41-kDa toxin that is structurally and functionally similar to guanylin, which is the ligand for the guanylyl cyclase C receptor on the IEC ^{39,43}. EAST1 binds to the guanylyl cyclase C and elicits similar cellular responses as hST-I produced by ETEC.

EAST1 increases cyclic guanosine monophosphate, which triggers CFTCR and the secretion of chloride, eventually leading to gut secretory responses ⁴⁴.

Pet is a 104-kDa cytotoxic SPATE produced by EAEC that causes a dilation of crypts and intercrypt regions, which promotes the rounding and extrusion of enterocytes by mediating cleavage of the cytoskeletal proteins. This eventually leads to the sloughing of the diseased enterocyte ^{39,45}. Pet uses clathrin-coated vesicles to pass through the IEC membrane, where it binds and cleaves fodrin ³⁹. Fodrin is a spectrin-like protein that crosslinks and assists in the stabilization of cytoskeletal actin filaments ⁴⁶. Fodrin cleavage causes the loss of structural support in the host cell, and promotes rounding of cultured IEC and eventually apoptosis ³⁹.

Diffusely Adherent *Escherichia coli*. DAEC can be found in both asymptomatic (children <12 months) and symptomatic children (>12 months) living in developing countries. It is sometimes associated with persistent watery diarrhea, but has been difficult to diagnose compared to other DEC ^{47,48 48,49}. DAEC are a sub-group of AEC characterized by a pattern of diffuse adherence to IECs, defined as a uniform bacterial covering of the majority of mucosal surface ⁵⁰.

DAEC has been separated into two classes based the expression of the afimbrial fimbrial adhesion (afa)/Cromer blood group antigen (Dr) adhesion factors ⁴⁷. Initial adherence of DAEC is provided by adhesion of (Dr to the decay-accelerating factor or DAF) receptor ⁴⁷. DAF is found on various cell types, including IECs. DAF is a 70-kDa membrane protein that directly interacts with complement cascade at the C3b/C4b step by impeding the uptake of C2 and factor B, which ultimately protects host cells from

complement damage (i.e. against autoimmune diseases and inflammation); ^{47,51,52}. The decay-accelerating factor (and two additional complement components, clusters of differentiation 59 (CD59) and C8-binding protein) is anchored to the cell membrane via a glycosphosphatidylinositol-anchor or GPI, which also functions in signal transduction ⁵¹. DAEC binds the decay acceleration factor to the glycosphosphatidylinositol-anchor and activates a calcium dependent-signaling pathway that ultimately causes re-structuring of IEC microvilli ⁵³.

Following DAF-GPI signaling cascade, a brush border lesions develops in association with activation of the phosphatidylinositol 3 kinase and mitogen-activated protein kinase systems. The sequence of events causes the development of long IEC extensions that envelope the adherent microbe ^{28,52}. These physical changes in the brush border alter tight junctions and promote intercellular permeability. In addition, IL-8 is produced, which induces neutrophil transmigration, which further up-regulates DAF and the MHC class I-related chain A or MICA ⁴⁷. This chain of events is the proposed mechanism for DAEC-induced host inflammation, which may play a role in the pathogenesis of inflammatory bowel disease ^{28,54}.

The DAEC produces an observable loss of microvilli that is localized to the immediate attachment site of the DAEC ⁴⁷. This is produced by the rearrangement and disappearance of core cytoskeletal proteins: f-actin, villin, fimbrin, and α -actinin ^{47,53}. These changes cause and elongation of the microvillus resulting in localized destruction of the microvilli and ultimately the successful production of the AE lesion ⁴⁷.

Once colonized, DAEC has effects on two major cellular junctions; the tight junctions and the adherence junctions ⁴⁷. Tight junctions provide cell-to-cell adhesion and a regulatory barrier that maintains cell polarity. DAEC excretes a toxin, Sat; which is an autotransporter toxin that produces a pronounced intraluminal fluid accumulation by effecting the tight junctions ⁴⁷. The accumulation of fluid increases the cyclic adenosine monophosphate intracellularly and eventually the permeability of the tight junctions. This progression of events leads to paracellular passage of mannitol (causing excess fluid secretion into the lumen) and transcellular blistering (accumulation of fluids on the basolateral membrane) ^{47,55}.

Enteroinvasive *Escherichia coli*

EIEC are closely related to *Shigella* spp. both genetically and in pathogenesis. Both pathogens promote a dysentery-like disease similar characterized by profuse diarrhea that sometimes containing blood and mucus and associated with high fever and abdominal cramping ^{6,9,56}. EIEC often cause sporadic diarrheagenic outbreaks and may be easily misidentified as *Shigella* spp. ⁶.

EIEC adherence to the IEC is mediated by adhesion located on the EIEC cell wall that causes hemagglutination of erythrocytes called the hemagglutinating factor ⁵⁷. There is a separate, but unidentified secondary mechanism that assists in adherence ⁵⁷. EIEC infections are typically localized and do not normally progress beyond the intestinal lamina propria ^{6,56}. EIEC and *Shigella* spp. share similar invasive strategies. Briefly, they invade the IEC layer by vacuole-mediated endocytosis, produce effector molecules, lyse

the endocytic vacuole, multiply intracellular, move through the cytoplasm to the epithelial membrane, and infect neighboring epithelial cells ^{6,28}.

The virulence genes necessary for infection are encoded on a large 120-kDa virulence plasmid that is shared by both *Shigella* spp. and EIEC ⁵⁶. This plasmid encodes a type-three secretion system (T3SS) that is responsible for the majority of toxic effects from EIEC ²⁸. The T3SS opens a pore into the IEC membrane to insert several key virulence proteins (effectors) that facilitate IEC cytoskeletal rearrangement, uptake of EIEC, and lysis of the endocytic vacuole ^{28,58}.

The virulence protein, IpaB has dual functions. It binds to CD44 on IEC surface and triggers cytoskeletal rearrangement for cell entry. It also has the ability to activate the macrophage caspase system, resulting in cellular apoptosis and release of interleukin 1(IL-1) ²⁸. IL-1 plays a central role in regulation of inflammatory response to infection. Three other virulence proteins, IpaC, IpaA and IpaD, play a critical role in bacterial cell uptake. These proteins work in a collaborative function where IpaC is involved in the actin polymerization that leads to lamellipodial extensions, while IpaA contours these extensions into a structure that promotes the uptake of the bacterial cell ²⁸. IpgD plays a critical role in reorganizing host-cell morphology by uncoupling the cellular plasma membrane and facilitates host cell blebbing ^{28,59}.

Enteropathogenic *Escherichia coli*

EPEC is an important pathogen of children in developing countries ⁶⁰, and is transmitted via the fecal-oral route usually in contaminated food and water ^{6,61,62}. The symptoms of EPEC infection presents are fever, malaise, vomiting, and diarrhea that is

dense with mucus and is typically non-bloody⁹. The symptoms result from an infection phenotype, called the attachment and effacement (AE) lesion. The pathogenesis of EPEC and formation of the AE lesion is unlike most other DEC that does not adhere as tightly to the GI epithelium. EPEC does not produce Shiga toxin or other verocytotoxins¹².

EPEC pathogenesis can be divided into 4 stages: initial adherence facilitated by the proteins *E. coli* secreted protein A (EspA) and the bundle-forming pili (BFP), formation of the T3SS and production of effector proteins, intimate and localized adherence to the epithelium, and formation of the AE lesion^{6,28,63-65}.

EPEC contains two main virulence-encoding loci; one is located on the chromosome (locus of enterocyte effacement, or LEE) and the other is on a plasmid (EPEC adhesion factor, or EAF)⁶. LEE is a pathogenicity island that encodes for effector molecules needed for AE lesion formation⁶⁶. LEE also encodes for the structural components of a T3SS, the translocated intimin receptor (Tir), and intimin^{12,67}. EAF has been shown to be absolutely required for infection and encodes for the BFP and pathogen bound intimin that is needed for the hallmark EPEC colonization phenotype, called localized adherence (LA)^{6,12,15,68}.

Once EPEC effector proteins have been delivered to the host epithelial cell, they produce significant alterations and membrane disruptions. These disruptions include tight junction permeability, loss of mitochondrial membrane potential, a releases cytochrome c, stalling of cell replication, the induction of apoptosis, and increased secretions of IL-8⁶⁶. Effector molecules also cause the rearrangement of the host cell

actin cytoskeleton to produce a pedestal that extends from the host cell surface and cradles the EPEC cell ⁶⁹.

EPEC has a two-step adherence process, initial adherence and secondary, or intimate, attachment. Initial adherence is facilitated by two proteins found on the surface of the EPEC cell, the BFP and EspA both encoded on the LEE. The receptors for these two ligands have not been fully identified and continue to be a topic of EPEC investigation ^{64 60}. EspA establishes a provisional adherence that permits the EPEC cell to deploy the T3SS and translocation of several effector proteins, including Tir. Tir translocates and insert into the host cell outer membrane, where it forms an extracellular hairpin loop ^{66,67}. Another LEE-encoded molecule, intimin, embedded in the EPEC outer membrane binds to the external Tir hairpin loop with strong affinity. The binding of intimin to Tir is referred to as intimate attachment ²⁸. This process and the activities of other effector molecules alter various host cell-signaling pathways and disrupt host cell membranes. These changes ultimately lead to the AE lesion ^{66,70}.

Enterohemorrhagic *Escherichia coli*

EHEC is a zoonotic, food-borne pathogen, and causes a wide range of symptoms; from mild watery diarrhea to fulminant, severe bloody diarrhea, and hemolytic uremic syndrome or HUS. HUS is a leading cause of renal failure in children ⁶⁶. EHEC infection is a disease of developed countries and is rarely observed in the same countries that have large outbreaks of EPEC and ETEC infections. EHEC is a significant health concern due to its ability to cause HUS and kidney damage in children ^{6,66,71}. Most infections are associated with hemorrhagic colitis, consisting of severe abdominal

cramping, watery and then bloody diarrhea, with minimal or no fever ⁶. HUS is an EHEC complications due to the action of Shiga toxin produced by the bacterial cells. Kidney toxemia by Shiga toxin precedes renal failure, thrombocytopenia, and microangiopathic hemolytic anemia ^{6,9,14}. EHEC infection often occurs in the lamina propria throughout the ascending and transverse colon that causes hemorrhage and edema throughout ^{6,72}.

EHEC pathogenesis is similar to EPEC in nearly every way, with the exception of Shiga toxin production. EHEC encode LEE and produce an AE lesion early during infection ^{6,12,66}. Shiga toxin contains a conserved A-B subunit structure. During infection, the A subunit is nicked to form A₁, which is a 28-kDa protein with enzymatic activity. A₂ is a 4-kDa protein that binds B subunit peptides and remains linked by a disulfide bond ⁷³. The B subunit is formed by 5 identical peptides that are 7.7-kDa ⁷³ and recognizes the glycolipid, globotriaosylceramide (CD77) on host cell surfaces ^{6,74}. Shiga toxin production occurs during the early to mid-phase of infection ⁶, and once produced, this toxin passes the intestinal epithelium, enters central circulation and targets the CD77 receptor located on both microvascular endothelial cells (located in the kidney) and intestinal epithelial cells, causing these targeted cell to enter apoptosis ⁷⁴.

Many EHEC strains carry an additional plasmid named pO157 ⁶. A portion of the pO157 encodes for the enterohemolysin toxin (Ehx) and a catalase-peroxidase enzyme. The catalase-peroxidase enzyme has heme-binding activity with a variety of functions that contribute to hydrogen peroxide resistance ^{6,75,76}. The toxins EAST1 and Ehx are two additional toxins are produced by EHEC. EAST1 is the same toxin produced in

EAEC with a putative function in EHEC that produce the symptom of non-bloody diarrhea ⁶. The Ehx toxin is located on a 90-kbp plasmid that is classified in the repeats-in-toxin (RTX) cytotoxin exoprotein family ⁷⁷. The RTX exoprotein family is found throughout many pathogenic Gram-negative bacteria, producing a vast assortment of biological functions ⁷⁸. The putative role for Ehx is facilitation of erythrocyte destruction, which releases iron and other nutrients that can help to support the growth of the pathogen ^{6,77,79}. The virulence factor, O157LPS can potentiate the cytotoxic effects produced by Stx on human endothelium and participate in a defensive role against host antimicrobial factors ^{6,80}.

Part III: Laboratory Models

EPEC is a major cause of acute pediatric gastroenteritis in developing countries and repeated bouts of EPEC infection can lead to growth and nutrition defects, such as failure to thrive, and even death ^{66,81,82}. EPEC was first identified in the mid-20th century as a diarrheal inducing pathogen, but the mechanisms underlying EPEC virulence were not elucidated until the 1980's. EPEC possess both chromosomal- (locus of enterocyte effacement, LEE) and plasmid- (EPEC adherence factor, EAF) encoded virulence factors that are required for pathogenesis. These factors produce an AE lesion on IEC surface and promote secretory diarrhea and other EPEC-specific symptoms ^{12,66,69,83}. The AE lesion results from a sequence of key interactions between the IEC and bacterial cell that involve initial adherence, deployment of a bacterial T3SS and effector molecules, intimate attachment, and finally host cell rearrangement ⁶⁴.

The histopathological characterization of the EPEC AE lesion was accomplished previously by infecting a human duodenum IVOC ⁶⁹. Scanning electron microscopy demonstrated the effacement of microvilli on the IEC surface and attachment of EPEC ⁶⁹. Cradled EPEC cells created a footprint on the IEC surface prior to replicating in localized adherence patterns and the eventual formation of localized micro-colonies. The EPEC AE lesion is a multifaceted, sequential set of bacteria-host interactions, which is an important and necessary criterion that must be reproduced in an effective model system ⁶⁹. *In vitro* and *In Vitro* models have both their advantages that have effectively recapitulated the various individual aspects of the EPEC infection process; contrasting to these advantages are significant limitations when trying to completely replicate the natural infection process ⁸⁴. It is critical to understand both advantages and disadvantages to the various model systems when investigating the use of a new potential model system.

Immortalized Cell Culture

Immortalized cell culture has advantages of being readily available, cost effective and is easily propagated *in vitro* ⁸⁴. The main disadvantage of using cell culture is that it may not adequately reflect the actual pathophysiological events that lead to diarrheal illness *In Vitro* ⁸⁴. Cell culture is a selective *ex vivo* model of cell membranes and/or internal cellular function upon infection and research is limited to bacteria-host-cell interaction ⁸⁴.

EPEC has specificity to host IEC; however, EPEC demonstrates a loss of this specificity when cultured on *in vitro* cell culture ⁸⁴. The loss of this IEC specificity allows the use of a wider variety of cell cultures that can be manipulated to evaluate the

roles of host genes and proteins during EPEC infection ⁸⁴. There are a variety of cell cultures currently used for EPEC pathogenesis, while the type of cells and reasons used are ultimately guided by the individual research design, the more widely used models will be reviewed. Research is not limited to the ones that will be discussed here.

There are two variations of immortalized cell culture that have been used to study EPEC, unpolarized and polarized models ⁸⁴. Polarization is defined as having a readily available apical membrane with an enclosed basement membrane. The absence of polarization has been an effective model to describe the effector protein interactions on the host cell; the polarized cells have provided key concepts for cell-cell interaction ^{30,84,85}. The two commonly used unpolarized cell culture lines to study EPEC are the Henrietta Lacks (HeLa) cell and human epithelioma cell-line #2 (HEp-2).

Henrietta Lacks Cells. HeLa cells were originally isolated from a cervical cancer cyst in 1951, from a patient named Henrietta Lacks. This cell line has the advantages of being well described and easily maintained over several generations ⁸⁶. The HeLa cell culture has been used to describe the internalized infrastructure reorganization that occurs during EPEC infection; such as the evaluation of the Tir interaction with α -actinin, describing the recruitment of cytoskeletal structure to form the pedestals that represent a piece of the classic attachment-effacement (AE) lesions produced by both EPEC and EHEC ⁸⁵. HeLa cells have been used to investigate and describe the actin pedestal formation by EHEC; a pathogen process that enhances host cell attachment and concomitant type III translocation ⁸⁷.

Human Epithelioma Cell-Line #2. HEp-2 cells were originally isolated in 1952 from a primary larynx tumor. This cell line has the advantages of rapid growth, and like HeLa cells, are readily available and easily maintained over several generations ⁸⁸. EPEC was first observed to adhere to HEp-2 cells that instigated further research to discover that specific serotypes of *E.coli* contain the 35-kbp plasmid (EAF), which encodes for the effector molecules needed to confer this adherence ^{31,89}. HEp-2 cells have provided basis of novel diagnostic test for EPEC and EHEC infections ⁹⁰.

The next two cell models move a step towards mimicking a more complex platform are the human colonic adenocarcinoma cells (T84) and heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2). These two cell cultures provide a polarized, but uniformed cell monolayer.

Heterogeneous Human Epithelial Colorectal Adenocarcinoma Cells. The Caco-2 cells are derived from colonic cells, grow in single layer confluent lawns and provide a polarized architecture, in contrast to the HeLa cell and the HEp-2 cell. These characteristics give Caco-2 cells an advantage over other cell lines. This line has been exploited for over four decades because of its ability to express these morphological and functional characteristics ⁹¹. This model system has been used to demonstrate the dynamics of brush border remodeling induced by EPEC ⁹².

Human Colonic Adenocarcinoma Cells. The T84 cells are from human colonic adenocarcinoma cells and contain goblet-like cells that are similar to the human IEC ⁹³. This system has been exploited heavily due to their ability to have a selective

permeability of electrolyte transport, various hormones and neurotransmitters⁹³. This cell line has been used to investigate the differences between healthy IEC that present with restrictive passive barrier function for luminal content transport and during EPEC infection. The contrast is when the tight junctions are modified this allows passive transport of luminal contents that potentially contribute to the diarrheal response⁹⁴.

These various cell cultures, both unpolarized and polarized, provide a readily accessible, cost effective human based culture system. They have successfully pieced together a vast amount of understanding into EPEC molecular pathogenesis; however, these cultures are uniformed (containing only a single cell type), and are limited to a molecular understanding that is solely provided by these individual cell types. The *In Vitro* intestine contains a population of cell types that have a variety of host functions that range from mucus and bactericidal peptide production to signaling events between various systems that effect the pathogen:host interactions. The investigations into these various host responses require a more complex system, such as *in vitro* organ cultures.

In Vitro Organ Culture

Cell culture findings can be further evaluated by a more advanced model called, *in vitro* organ culture (IVOC), which consists of tissue isolated from ligated human or selected animal bowels⁸⁴. IVOC represents the most complex of tissue culture system that is available to study EPEC infection^{69,84}. This system, as like cell culture, can be inoculated with pathogens and observed for pathological effects and is the closest to an *In Vitro* human model, *ex vivo*^{84,95}. Compared to traditional cell culture, IVOC is a human architecture needed to replicate host infection and supports the natural infection process

⁹⁶. Although, IVOC has superior physiology when compared to traditional cell culture; contrastingly they require advanced laboratory technique requiring a high oxygen pressure to delay ischemia, are not readily available requiring coordination with surgical teams and can be cost prohibitive ⁸⁴.

There are two types of IVOC's, similar to immortalized cell types, conventional or unpolarized IVOC (cIVOC) and polarized (pIVOC). The major difference is the ability for bacteria to access the apical surface, basolateral surface, or both membrane surfaces ⁹⁶. A drawback of the cIVOC model is minimal control of bacterial restriction to infection site, which may lead to an artificial infection progression ⁹⁶. The pIVOC (achieved by affixing a cIVOC to an acrylic glass disk to seal the mucosal side) permits a greater degree of control over membrane access ⁹⁶; however, both models fail to provide considerations for *In Vitro* factors such as gradient osmolality, low oxygen levels naturally found on the apical membrane, underlying host disease, surgical trauma, loss of interactions with deep tissues, and variations in gut flora ⁹⁵.

Various Animal *In Vitro* Organ Culture. The use of animal IVOC models to investigate bacteria-host interactions and extrapolate the roles of effector proteins during the formation of human AE lesions have guided further research into EPEC infection. However, animal studies do not accurately reflect the pathogenesis found in human infection possibly due to the lack specificity in virulence factors and their dependent responses ^{95,97}. For example, when a swine and newborn piglet ileo-loops were used to evaluate AE activity following infection with human EPEC, there was a comparatively mild infection due to less severe AE lesions ⁹⁸. A swine's large intestines develop with

age and infection severity could be partially dependent on the complex flora of the swine; the piglet model showed that absorption of most food occurs prior to reaching the proximal intestine⁹⁸. Rabbit ileo-loop models, when infected with human EPEC showed destruction of goblet cells located in the ileo-loop, which contrasted with what occurs in humans, the goblet cells are spared⁴⁹. These various models have a significant limitation due to the variation of the specie environment^{49,98}.

Animal Models

Complete animal models provide a multifaceted system for evaluation of the EPEC infection process. Maintenance of research animals can be expensive and like the animal IVOC system, the responses to human pathogens can vary and not accurately reflect the human response to infection.

The Murine Model. The murine is the least costly, most convenient and requires the least amount of space in comparison to other animal models⁹⁹. This model can be exploited to better understand the mechanisms involved the forming of the AE lesion, through genetic manipulation to emulate human characteristics, when infected with serotypes isolated from individuals with diarrheal illness^{100,101}. When the mouse is infected with human associated EPEC, there is a high frequency and sporadic absences of AE lesions within the mouse model^{14,99,101}. Because of these fluctuations, the use of this infection model does not directly translate into the clinical practice for EPEC infection¹². Alternatively, *Citrobacter rodentium* shares similar infectivity traits with human EPEC, the AE lesions formation and similar effector proteins are produced by *C. rodentium*¹⁰². An example is the phosphorylation events of the tyrosine residue 474 (Y₄₇₄) was not

needed for Tir in the murine host, but *C. rodentium* has an equivalent phosphorylation event at Y₄₇₁¹⁰³. This is contrary to the human model where the Y₄₇₄ phosphorylation is absolutely required for modification of which is essential for its actin-nucleating activities that ultimately leads to the AE lesion¹⁰⁴. *C. rodentium* has a high prediction value to assess the role of *E. coli* virulence factor in the human model, but it is not a human model¹⁴.

EPEC molecular and infection process' that lead to host pathogenesis has been effectively pieced together using various models. Each of the model systems has limits that potentially alter an *In Vitro* host response. The HeLa and Hep-2 cell lines, although human based are unpolarized and EPEC loses the boundaries of the apical and basement membranes; Caco-2 and T84 cell cultures, also derived from human, are uniformed cell cultures that do not reflect the population cell types found in the *in vivo* human intestines; the non-human models (various animals) elicit different molecular and physiological effects when challenged with a human pathogen; and the human IVOC system is often from a previously diseased tissue, has been challenged with variations of microbial ecology, and potentially have underlying genetic mutations. The newest model, the human intestinal organoid (HIO), overcomes many of these obstacles and variations previously discussed and it demonstrates a strong potential to be developed into the new standard for human based gut research.

Part IV: The Human Intestinal Organoid

A recent advance towards an *in vitro* model of the human gut, referred to as HIOs, was recently made by Spence et al.¹⁰⁵. HIOs are comprised of a single cell layer of

differentiated IECs that resembles a sterile, naive human duodenum. This tissue culture is compositionally similar to duodenal tissue with respect to the types of IECs and their organization (crypts and villi) ¹⁰⁶. HIOs have some distinct advantages over previously used *in vitro* tissue culture. First, HIOs are a naive tissue that has not been previously exposed to pathogens. HIOs are generated from embryonic or pluripotent stem cells and so human genetic variation can be accounted for. HIOs are human tissue with similar structure and function to that found in humans. Thus, this system has great potential to further understand bacterial pathogenesis in the GI tract. The advantages can guide a more effective therapeutic control of pathogen and treatment ⁸⁴. This research model could potentially become a valuable model system EPEC pathogenesis ^{12,84}.

Table 1. The Virulence Characteristics and Symptoms of DEC Pathotypes

Pathotype	Virulence gene loci	Colonization factors	Toxins	Symptoms
ETEC	Chromosomal	fimbriae, pili	hST, hLT	abrupt onset of watery diarrhea, fever, vomiting
EAEC	pAA Plasmid	Pic, Dispersin AAFI/II	EAST1, Pet shET1	watery diarrhea, low-grade fever
DAEC	Chromosomal	afa, Dr	Sat	watery diarrhea, low-grade Fever
EIEC	120-kDa Plasmid	HA, HAF	IpaC/A/D	profuse diarrhea, fever, abdominal cramping
EPEC	Chromosomal, pMar2 (EAF)	EspA, BFP, Tir, Intimin	none	watery-mucous diarrhea, malaise, vomiting
EHEC	Chromosomal, pO157, 60-MDa Plasmid	Tir, Intimin	Stx, Ehx, EAST1, O157LPS, CP	mild-watery diarrhea to HUS

THE HUMAN INTESTINAL ORGANOID AS A MODEL SYSTEM FOR ENTEROPATHOGENIC *ESCHERICHIA COLI* PATHOGENESIS

Introduction

A variety of experimental models have been used to study EPEC pathogenesis, including eukaryotic cell culture (unpolarized or polarized), *in vitro* organ culture (IVOC), and animal models. Each of these experimental models is selected based on the research design and investigational needs. There are advantages to these models (e.g. cost effectiveness, readily available, previously established protocols) and disadvantages (e.g. lack of differentiated cell populations, surgical trauma from incision sites, ethical considerations, non-human infection responses) that effect their ability to recapitulate certain *in vitro* infection characteristics that are important for relevant pathogen research⁸⁴. There is a need to further investigate and identify relevant human-based research models that can demonstrate the pathogen related disease progression from healthy to diseased tissue, and possibly beyond.

The HIO has several assumed advantages over previous models. These advantages are the ability to be derived from human embryonic or pluripotent stem cells, containing a population of cells that are found *in vitro*, a naive tissue culture, and cost effectiveness when compared to IVOC and animal models.

The goals of our study were to create laboratory protocols to maintain, inoculate, and evaluate the use of HIOs as an appropriate model for EPEC pathogenesis for our lab. The use of these protocols demonstrate the ability in which to observe the lumen of sterile HIOs and compare to HIOs that are colonized with EPEC with previously

established models (to determine whether an EPEC produced AE lesions or other hallmark phenotypes such as footprinting, localized adherence, and colony formation). The presence of these phenotypes supports the use of the HIO as a potential research tool for EPEC pathogenesis investigations.

Materials and Methods

Growth and Maintenance

Day Zero. Immature HIO's were obtained from the Michigan State University from the Spence Lab. The HIO's were shipped overnight in a sealed fifteen-milliliter conical tube that contained intestinal growth media complete (cIGM) and matrigel (Biosciences). HIO's were removed from the conical tube, placed in a sterile Petri dish that contains incomplete IGM (icIGM). Using a dissecting scope, the HIO's were carefully separated from previous matrigel. Several HIO's were grouped together in fresh matrigel, placed in a 24 Nunclon tissue culture plate (ThermoScientific Multidish 24 wells Nunclon Delta Surface) and allowed to rest in the 5% CO₂ 35°C incubator for thirty minutes. After incubation a 500ml of cIGM overlay was applied and plate placed back in incubator.

Daily Checks. The plates were checked daily for various visual signs of contamination (cloudiness and film inside the wells) and yellowing of the cIGM (demonstrates metabolic activity [IGM contains a buffering system that is comparable to the *In Vitro* system]). In wells are suspect of contamination, 70% ethanol was added to

the well and all contents were removed. The media was exchanged if yellow, and all wells were changed every 3 days, regardless of the presence or absence of yellowing.

Bi-Weekly Checks. The HIO's mature to an ideal size of approximately 2.5 to 3.0 mm. The HIO are maintained in a matrigel matrix. The manufacturer recommendation is that the matrix is sustainable for up to 7 days. It has been my observation that the matrix can readily support continual growth of the HIO for up to fourteen days. The matrix should be replaced (as described in Day 0) as needed, or every 14 days.

Preparation for Inoculation. The cIGM contains the antibiotic Penicillin-Streptomycin (or pen/strep). Prior to infection, the HIO must undergo a minimum of 3 days in antibiotic free cIGM to washout the antibiotic, with daily exchanges. The HIO should be prepped as referred on Day 0, with the only exception that all cIGM is made without pen/strep and individual HIOs placed in individual wells of the 24 Nunclon plate. Ampicillin can be added to the cIGM to a final concentration of 100ug/ml to assist in preserving the sterility during the 3 days of pen/strep washout. This is due to the pGen222 plasmid (discussed in next section) that encodes for an Ampicillin resistance.

ECOR2 Preparation. The ECOR2 was grown from freezer stock in 100ml Luria Broth (LB) overnight at 37°C. A sample of the broth was placed in a Luria Agar (LA) plate and streaked for isolations. An isolated colony was placed back in LB broth and incubated overnight to achieve a CFU/ ml of approximately 10^9 . To visualize the potential adherence of ECOR2, it was transfected with plasmid pGen222 that encodes for

a green fluorescent protein (Gfp) and ampicillin resistance protein (Amp). After transformation, ECOR2 was streaked for isolation on LA with Ampicillin at the concentration of 100ug/ml. GFP positive (GFP+) colonies fluoresce under ultraviolet light (wavelength 385-400 nm), which allowed for selection. The GFP+ ECOR2 colonies were then transferred into 100ml of LB containing 100ug/ml of Ampicillin and grown over night at 37°C.

Microinjection

HIO Preparation. HIOs that resemble a clear contact lens were selected and cultured to a diameter of 2.5 to 3.0 mm. Mature HIOs were carefully excised from the maintenance matrigel using a cut p200 micropipette tip and re-plated into individual wells of a 24-well Nunclon cell culture plate. Place the newly transferred HIOs in the incubator at approximately 35°C with 5% CO₂ for 15 minutes. Overlay with 0.5 mL of antibiotic free cIGM. Incubate HIOs at approximately 35°C with 5% CO₂ in cIGM. The HIO is incubated for ≥ 72 hours with exchange of cIGM and exchange overlay every 24 hours or upon turning yellow.

Syringe Preparation for Microinjection. Fill 1- 60mL BD syringe (BD 60 mL Syringe Luer-Lok top) with ice cold sterile water; 2- 10 mL BD syringe (BD 10 mL Syringe Luer-Lok top) with 70% ethanol (Pharmco-Aaper ethyl-alcohol, ACS/USP grade, 200 proof and TekNova Water; RNase, DNase Free); 1- 10 mL BD syringe with Phosphate buffered saline with calcium and potassium (Corning/Cellgro, Delbucco's Phosphate Buffered Saline 1x with calcium and magnesium) (PBS+). Place all syringes on ice.

Assembly of the Microinjector Line. The assembly of the microinjector should be placed and assembled in an area that provides for the ease of use by a single user. There is 1 meter of ethylene tetrafluoroethylene (ETFE) tubing included from the manufacturer that allows for a comfortable placement of the microinjector. The assembly set-up should be in the area where it will be used prior to priming. Movement after priming of the line may interfere with the prime. Assemble the Xenoworks® Analog Microinjector (Sutter Instruments) as per manufactures instructions. Attach a 50 uL gas-tight syringe (Sutter Instruments) to left side of a three-way stop-cock valve (first port) (Hamilton), leaving top valve open (second port), connect the ETFE tubing (Sutter Instruments tubing kit) to the port located 180° opposite of attached gas-tight syringe (third port) and fix the opposite end of the ETFE tubing to the micropipette holder (Sutter Instruments) that holds a thin-wall glass capillary needle (World Precision Instruments). Fix the micropipette holder to the manual micromanipulator (MMM) (World Precision Instruments) needle advancer. *After assembly, the user should practice alignment and advancement of the needle.

Cleaning and Flushing the Microinjector Line. *The following protocol describes how to clear, clean and flush the microinjection line with the use of a three-way stopcock valve and to prepare for HIO inoculation.* Turn the valve key to position 2, attach the previously prepared 10 mL BD syringe filled with room air to the second port of three-way stop-cock valve, flush line, remove air filled syringe. Attach the 10 mL BD 70% ethanol filled syringe and fill line with 70% ethanol, allow to sit for 3 minutes. Remove

the ethanol syringe. Attach the 60 mL BD sterile water syringe; slowly flush line with approximately 10 ml of sterile water. Remove the sterile water syringe. Attach the 10 mL air filled syringe; inject 0.5 mL of air. Remove air syringe. Attach the 10 mL PBS+ syringe; flush line with 10 ml of PBS+. Remove the PBS+ syringe. Attach the 10 mL air syringe; inject 0.5 mL of air. Turn three-way stopcock valve to position 3. All syringes, when not in use should be kept on ice.

*When the tubing is not in use for a period of time, it should be flushed with air.

*The tubing can be sterilized between uses by autoclaving for cycle 15 minutes at 121°C.

Priming the Line for Inoculation of Bacteria. Wipe all ports with 70% ethanol prior and after removing syringes. Place the syringes on ice when not in use. Prior to adding the inoculum, the line should be primed with PBS+ and a 0.5 ml air pocket should separate inoculum. Briefly vortex the inoculum to ensure that cells are suspended. Fill a 3.0 mL BD syringe (BD 3.0 mL syringe Luer-Lok Tip) with 1.0 mL of inoculum and 2.0 mL of sterile ice cold PBS+ using an 18 gauge hypodermic needle (BD PrecisionGlide™ Needle, 18G x 1½) and mix by inverting the syringe several times. Attach the 3.0 mL inoculum syringe to the top port of three-way stopcock valve and turn the valve to position 2. Slowly prime the line with inoculum until the air pocket that separates the inoculum is pushed through the thin-walled capillary tube. At this time, the line should be completely filled with inoculum; turn three-way stopcock to position 3 and remove the inoculum needle. Attach the 10mL air filled syringe and turn three-way stopcock to position 2 and slowly add 0.5 mL of air into the line. Do not remove air filled syringe and

turn three-way stopcock to position 3. Carefully clean thin-walled capillary needle with 70% ethanol, wipe with clean Kimwipe and allow to dry.

*If inoculum is not at completely at the edge of the capillary needle. Turn the three-way stopcock valve to position 2 and prime line with additional air to ensure inoculum is at the edge of glass capillary tube and re-clean glass needle.

Turn three-way stopcock valve to position 3, which will allow flow from the gas-tight syringe. Test the microinjector to ensure 1-3 ul of inoculum can be successfully pushed from the system by turning the dial on microinjector and gauging the amount of inoculum by the amount of air via the syringe. Turn three-way stopcock to position 2.

Aligning Needle and Performing Injection. Gently clean the external side of the thin-walled glass capillary needle. Place the 24 well Nunclon plate on scope base (Zeiss Stemi 2000-C Stereo Microscope) and grossly identify the position for injection of the HIO by aligning the HIO to center view and continue to grossly align the glass capillary needle while under magnification, using the multi-dimensional advancer. When aligned the HIO will be in center view and the tip of the needle will appear above the HIO. Turn the three-way stopcock to position 3 and slowly advance the Z-axis on the multi-dimensional advancer needle to the HIO, gently pierce the membrane and careful not to pierce posterior side of the HIO. Carefully inject 1 uL of inoculum into HIO by turning the fine tune dial on the microinjector. The gas-tight glass syringe will push positive pressure into the system (this may take a few seconds to reach through the system), evaluate for cloudiness of inoculum in the interior HIO. Repeat the injection up to 2 times or as needed for research design. Turn three-way stopcock to position 2 and slowly

retract thin-wall glass capillary needle. Clean needle as described previously. Immediately replace the overlay of cIGM- and place in 35°C with 5% CO₂ for experimental amount of time. cIGM- should be replaced every 1-2 hours to prevent overgrowth of bacteria. Flush and clean microinjector tubing.

Fixation and Staining

Primary Fixation for Microscopy. The primary fixation is typically 2.5% glutaraldehyde solution that is prepared at physiological pH in a sodium phosphate buffer. Glutaraldehyde is preferred for HIO imaging for electron microscopy because of the rigid stability of cytoskeletal structures, shortened fixation time, irreversibility of fixation, and the sensitivity of the microvilli to osmotic changes.

*All fixatives must remain in same pH buffer throughout staining process.

Remove the HIO from matrigel and place it in a solution of 2.5% glutaraldehyde that has been prepared in a 0.1M sodium phosphate buffer at pH 7.2-7.4 for a minimal of 3 hours to several days at 4°C.

For Secondary Fixation. The secondary fixation for EM imaging is by osmium tetroxide (OsO₄). OsO₄ is a strong oxidizing agent that embeds a heavy metal (Osmium) between the phospholipid cell membranes and allows for primary contrast that can be used in the absence of a sputter coating. However, in this study, to examine the brush border structure of the HIO, sputter coating with an Iridium target was used for definitive contrasting and for providing primary grounding of the multidimensional HIO.

Grounding the sample prevents charging by the electron beam when imaged by the FE SEM and provides a flow path for the electrons. 'Charging' is a phenomenon that is

caused by an accumulation of static charges within the sample that results from improper grounding. Charging of the sample creates an image that fluctuates in intensities and prevents a clear focus. Continued imaging of an ungrounded sample with 'charging' will cause irreversible damage to the sample (fig. 1).

*OsO₄ is **extremely** toxic- follow laboratory safety protocol while working with OsO₄. OsO₄ should ONLY be used in GLASS containers.

Remove the HIO from primary fixative with large bore p200 pipette tip and place in secondary fixative for approximately 4-24 hours at room temperature in chemical hood. The sample will turn black. Remove from the OsO₄ and wash 4x 10 minute washes in PBS (Corning/Cellgro, Delbucco's Phosphate Buffered Saline without calcium and magnesium). After final wash, begin the ethanol dehydration portion.

Dehydration Preparation of
Electron Microscopy Samples. In glass vials (Fisherbrand), make 1.0 mL of each concentration of ethanol (Pharmco-Aaper, ethyl alcohol, ACS/USP grade 200 proof)/water (TekNova water, RNase, DNase free) respectively: 25%, 50%, 75%, 95%, and 100%. Use a sterile large bore p200 to transfer HIO from PBS into 25% ethanol concentration, allow equilibrating for 15 minutes. Repeat transfer technique from 25% into 50% ethanol concentration and allow equilibrating for 15 minutes. Repeat transfer technique from 50% into 75% ethanol concentration. Allow the sample to equilibrate for no longer than 15 minutes. Repeat transfer technique from 75% into 95% ethanol concentration and allow to equilibrate for 30 minutes. Finally, place HIO into 100% ethanol.

*If HIO is not going to be used for immediate FE SEM imaging, the HIO can be maintained in 100% ethanol at 4°C for several days, with an ethanol exchange every 24 hours.

Preparation of HIO for Imaging on the FE SEM. The purpose of Critical Point Drying the sample for FE SEM imaging is to visualize and evaluate the HIO brush border architecture that has been fixed *ex vivo* (fig. 2).

*Care should be taken when preparing sample on the observation pedestal for imaging in order to prevent inaccurate orientation, particulate contamination and charging of the sample. Part of this protocol will require making an appointment with MSUiCAL for use of the Critical Point Dryer (CPD(ing)) and FE SEM.

The sample should be in 100% EM grade ethanol. Carefully transfer the HIO to a sterile Petri dish filled with enough ethanol to completely cover the HIO. Place under the stereomicroscope (do not allow the HIO to dry out, maintain the level of ethanol in the dish) and carefully cut the HIO (fig. 3). The HIO, after fixation should maintain the contour structure, allowing for identification of the luminal side. Replace HIO into the vial containing 100% ethanol. Transfer the HIO into a pre-labeled (with pencil) Kimwipe that has been saturated with ethanol. Fold the Kimwipe carefully (fig. 4) to fully encase the HIO and place in the CPD (Tousimis Samdri-795) and follow manufacturer's instructions for operation. When finished, carefully remove the Kimwipe from the CPD with forceps to avoid manipulation of the HIO.

*The HIO at this time is extremely fragile and any physical manipulation will destroy the cellular structures to be examined.

Place a small piece of two-sided carbon tape (Ted Pella) on a pin mount. Carefully, unfold the Kimwipe and remove the HIO under stereomicroscopy and identify the luminal side of the HIO. Lift the HIO with forceps and place it on the carbon lined pin mount luminal side up (once placed, the HIO cannot be removed). To help prevent charging, carbon paste should be used to contour the outer ridges of the HIO. Place pin mount in the sputter coater (Emitech K575X) with a target of Iridium and follow the manufacturer instructions and sputter coat for 90 seconds. When transferring HIO to and from devices, maintain a dust shield to prevent particulate contamination.

*HIO is now prepared for FE SEM imaging, follow manufacturer instructions for use of FE SEM (Zeiss Supra 55VP). If charging is present, repeat sputter coating.

Preparation of HIO for TEM Imaging. The HIO should be in 100% ethanol, as previously described. To prepare the HIO for embedding, carefully remove the HIO from 100% ethanol and transfer to a 1 ml aliquot of 100% propylene oxide (PO). Allow the HIO to equilibrate for 15 minutes and exchange out PO. Allow to equilibrate for an additional 15 minutes. In a new glass vial make a solution of PO/Spurr's resin in a 2:1 v/v ratio; place the HIO into this solution and allow to equilibrate and additional 60 minutes at 4°C. After 2:1 infiltration, place the HIO in 100% Spurr's resin for an additional 12-16 hours at 4°C.

*If unable to bake the resin after the 100% infiltration, replace Spurr's resin with new resin every 12-16 hours.

Transfer HIO into a BEEM capsule and fill with fresh Spurr's resin. Bake HIO in BEEM capsule for 12 hours at 70°C.

TEM sectioning. For thick sectioning, adjust the microtome to a degree of approximately $+4^{\circ}$. Place solid BEEM capsule in the chuck of the microtome. Fill the reservoir of the blade with sterile water level with the edge of the blade. Grossly advance the blade to the edge of the sample and fine tuning the blade with the second handle. Finally tune the blade with the third handle- moving the tertiary dial so that it cuts 2 nm each rotation of the cutting dial. Continue this step until smooth sections of the sample are released in the water. Continue for several cuts. Use a hair-loop scoop to carefully lift the sections from the blade reservoir into a drop of water on a glass slide. Place a drop of 1% toluidine blue stain at the edge of the water and examine sections on a light microscope. Once the sample image is identified, continue to the thin sections. For thin sectioning, replace the glass blade with the diamond blade. Follow gross adjustment of the blade, as described in thick sectioning. The cut of the sample is measured by the index of refraction (1.5) and should appear grey to silver when sliced (60-90 nm thick). Cut a ribbon consisting of several sections. Pick up an uncoated grid using jeweler's forceps by gentle bending the edge of the grid. Using the dull side down, slowly immerse the grid in the reservoir and lift out several sections onto the grid. Repeat this for several grids.

Staining Sections for TEM. The Primary stain is Uranyl acetate. Place two depression slides into a petri dish that contains a few milliliters of absolute menthol. Fill 1 of the depressions with Uranyl acetate and the other with absolute methanol. Immerse grids, sectioning side up, in the Uranyl acetate for 5 minutes and cover to avoid

evaporation. Rinse the grids by placing in absolute methanol for 30 seconds. Hold grid over a 600 ml beaker and gently rinse with the following gradients of methanol to remove the stain residue: 100%, 90%, 70%, 50%, 30%, 15%, and finally sterile distilled water.

Place grids on filter paper and allow drying in a covered petri dish.

Secondary staining is a Lead citrate stain.

*minimalize the lead citrate from contact with ambient air. The exposure to CO₂ will precipitate the lead citrate and cause contamination of the sample.

Place a drop of lead citrate stain in a petri dish, place a few pellets of sodium hydroxide (NaOH) pellets on the opposite side. Gently place the sample face down on the drop of lead citrate, cover petri dish and allow staining for 3 minutes. Rinse the slide with a gentle stream of 0.02N NaOH solution and rinse with sterile distilled water. Place on clean filter paper, specimen side up, to dry. Follow operation guide for imaging of the TEM.

Immunofluorescence Fixation. Fix the HIO with same steps except exchange the gluteraldehyde for a 4% paraformaldehyde solution. Remove from fixative and rinse sample in 1 ml of 1x PBS for 5 minutes, repeat 3x. Remove from PBS and place in plastic base mold. Remove excess PBS by using p200 to carefully aspirate PBS. Immediately overlay with a small dollop of Optimal Cutting Temperature compound (O.C.T.) (Tissue-Tek®). Then gently maneuver the HIO into the center of the dollop with a p200 tip and allow to equilibrate in O.C.T. for 60 minutes. Place in -80°C and allow to set for ≥ 20 minutes. Remove and immediately fill mold with O.C.T. and return to -80°C, for ≥ 2 hours (alternative to the -80 setting, use solid carbon dioxide mixed with

absolute ethanol (200 proof) to set initial fixation). Remove frozen O.C.T. block from plastic base mold and adhere to cryostat puck using O.C.T. Follow cryostat manufacturer protocol to section 8 micron thick slices, adhere to tissue culture slides (VWR) and place in dark box at 4°C for 12-24 hours.

Immunofluorescence Staining. Wash pre-set slides in 1x PBS for 15 minutes at room temperature. Then place slides in a blocking buffer for 30 minutes at room temperature. Remove from buffer and wash 2x 5 minutes in PBS. Place slides into a solution of 1:10⁻⁶ PBS/Phalloidin-Tetramethylrhodamine B isothiocyanate (Sigma-Aldrich) for 40 minutes in the dark at room temperature (tinfoil works well). Remove from primary stain in darkened area and wash 2x 5 minutes in PBS in dark box. Allow slides to dry in the dark and add 1:10⁻³ vector shield/DAPI (Vector Laboratories: Life Technologies,) stain to slides, fix cover slips using acrylic nail polish. Allow to rest in 4°C 12-24 hours. Image on fluorescent microscope.

See Table 2 for timeline.

Results

Characterization of Sterile HIO

HIOs are comprised of naïve and sterile tissue that has not been exposed to microorganisms. The IEC apical surface, when imaged, is expected to have microvilli similar to that seen in Caco-2, T84, and IVOC cultures. To test this expectation, protocols for field emission scanning electron microscopy (SEM) were designed and used to image the HIO IEC surface (fig. 5). The SEM demonstrated a uniform brush border

with microvilli covering the entire surface of individual cells. SEM also revealed cell junctions and the possibly different cell types based on observable differences in the microvilli shape and size (fig. 5).

To evaluate the immunofluorescence imaging capabilities of HIO's, sterile HIOs were examined cross-sectionally by staining the cell nuclei (4',6-diamidino-2-phenylindole, DAPI) and F-actin (TRITC-labeled Phalloidin) (fig. 6). Phalloidin-stained actin was observed in the expected columnar IEC confirmation with mesenchymal cells perpendicularly arranged at the bottom of the IEC layer. DAPI-stained nuclei were also clearly visible. In humans, IEC nuclei tend to lie toward the basement membrane of the cell, and the general location of nuclei helps to orient the direction of cells according to apical and basement membranes. The basement membrane is comprised of layers of undifferentiated mesenchymal cells. These cells are easily identified by their similarity to pseudo-stratified squamous cells.

Evaluation of Both GFP+ *E. coli* and Fluorescent Capabilities of the HIO

A mutualistic strain of *E. coli* from the *E. coli* reference (ECOR) collection, ECOR2, was selected because it was isolated from a human host and expected to adhere to the brush border of the HIO following inoculation and incubation. To image the ECOR2 strain, it was transfected with a plasmid pMAR22, which contains a gene encoding for green fluorescent protein (Gfp) to facilitate visualization with fluorescence microscopy. Gfp+ ECOR2 was inoculated directly into the lumen of the HIO using small-gauge needles (see materials and methods). Immunofluorescence following 12

hours revealed green bacterial cells associated with the brush border of the IEC surface in an aggregated arrangement (circle in figure 7). This aggregate suggests a small microcolony was formed (fig. 7). The observed confirmed the use of the fluorescence protocol and the localization of ECOR2 on the IEC surface suggested that the HIO can host microbial colonization.

HIO EPEC Infection

The prototypic EPEC strain, e2348/69, was obtained from the STEC Center at Michigan State University and transformed with the plasmid pMAR22, the same one used to transfect ECOR2. EPEC has been well characterized in adherence and colonization phenotypes. EPEC was expected to associate with the brush border, provide rearrangement of the brush border, produce localized adherence patterns, and micro-colony phenotypes. EPEC was inoculated into a HIO and incubated for 12 hours. The HIO was removed, fixed, stained and imaged using fluorescence and FE SEM. After 12 hours EPEC cells were found burrowed into the IEC brush border in a footprint that is consistent to what has been previously observed in a human IVOC of the small intestine (fig.8).

As expected, EPEC produced observable localized aggregates (or distinct cell aggregates) (fig. 9). EPEC micro-colony formation typically follows a localized adherence (LA) phenotype, characterized by small distinct aggregation of EPEC on the mucosal surface that occur as a result of intimate attachment. This was expected and was found to be consistent with LA that was observed with FE SEM (fig. 10). Although our images did not capture the initial steps of EPEC adherence, observation of the LA

phenotype and micro-colony formation indicates that these steps had taken place. A more detailed image of EPEC micro-colony was captured using SEM (fig. 11). The FE SEM images suggest that both fixation and imaging protocols for FE SEM are successful.

Discussion

The goal of this project was to determine whether HIOs were an appropriate model to study *E. coli* colonization and EPEC pathogenesis, in conjunction with verification of laboratory protocols. Our results suggest that *E. coli* can readily colonize the HIO lumen and that EPEC produce at least three hallmark virulence phenotypes (footprinting, LA, and micro-colony formation). Shaw et al, demonstrated the footprinting phenotype, where the brush border on the IEC surface was remodeled, forming a cradle for the EPEC cell⁹⁷. To show this, Shaw and colleagues used an IVOC system composed of human duodenal tissue and an EPEC mutant (Δtir) strain⁹⁷. Tir is produced by adherent EPEC cells and translocated into the IECs after via the TTSS. This molecule then migrates to the IEC surface and acts as the receptor for the EPEC-produced molecule, intimin. Tir-intimin binding promotes an intimate attachment and stabilizes the EPEC cell to the IEC⁹⁷. In the absence of Tir, the EPEC cell is expected to initially adhere and deploy effector molecules, but be deficient in intimate attachment. As a result, EPEC cells detach from the IEC surface, leaving an observable footprint. We observed EPEC cells attached to the IEC surface and SEM revealed that these cells were cradled (fig. 8 in circles). Had cells been removed, it is likely that a footprint would have been easily observed (fig. 8 identified by arrows), leading us to conclude that this phenotype was readily produced in HIOs.

The second phenotype, LA, that is produced by bacteria-bacteria interaction facilitated by the extracellular fimbriae, Bfp⁶. The Bfp provides two putative functions: loosely binding to the mucosal brush border (providing initial adherence) and interaction to other planktonic bacteria⁶⁴. Once Bfp binds to the IEC, effector proteins are deployed causing the EPEC cell to become anchored via Tir-Intimin association the Bfp interacts with surrounding planktonic EPEC cells⁶⁴. This interaction creates small aggregates, known as LA^{6,64}.

Nataro, et. al. demonstrated the LA phenotype on a Hep-2 cell using *wtEPEC*⁶. Similarly, the HIO was inoculated and incubated for 12 hours with *wtEPEC*, LA patterns in both immunofluorescence and FE SEM imaging were observed (fig. 9 and 10). The LA observed are similar to the observation by Nataro on his HEp-2 cell culture⁶.

The third phenotype, micro-colony formation was demonstrated by Saldaña et. al. on HeLa cells¹⁰⁷. The micro-colony formation demonstrated by Saldaña is similar in structure to the LA adherence patterns found by Nataro, except on a larger scale. These micro-colonies eventually form the AE lesion that is the hallmark of EPEC and cause the diarrheagenic symptoms. Similarly, when the HIO was infected and allowed to incubate for greater than 12 hours, these large micro-colonies formed (fig. 11).

The beginning of EPEC pathogenesis is strictly dependent on the bacteria's ability to adhere and successfully transfer effector molecules. These three EPEC phenotypes were chosen because each phenotype cannot be achieved without the one preceding it; and none of the steps can be achieved without initial adherence. This evidence concludes that the HIO can be a potential viable research model for various *E. coli* strains. The HIO

is a relatively new experimental model and the protocols for observing adherence patterns were limited due to its overall structure and maintenance required. The protocols for observing the internal structures (i.e. microvilli, cellular junctions, *E. coli*, etc...) were developed in parallel with and for the adherence studies.

Table 2. Maintenance and Inoculation Timeline

Day	Event
Zero	HIO Received from Michigan State University and placed in matrigel
5-7	HIO mature to 2.5 to 3.0 mm in diameter and placed in individual wells. Begin antibiotic washout
9	Prepare inoculum for injection
10	Inoculate HIO and incubate for 12 hours
11	Place HIO in fixative and prepare for imaging

Figure 1. Charging on the FE SEM sample. SEM image of the critical point dried (CPD) apical surface of an EPEC infected HIO. This image shows the various effects of electron beam charging interference when imaging under the SEM. The arrows identify a hazy lightened sample area where the static charge has not successfully been discharged and the large arrow identifies a sample area where the image is distorted due to incomplete grounding.

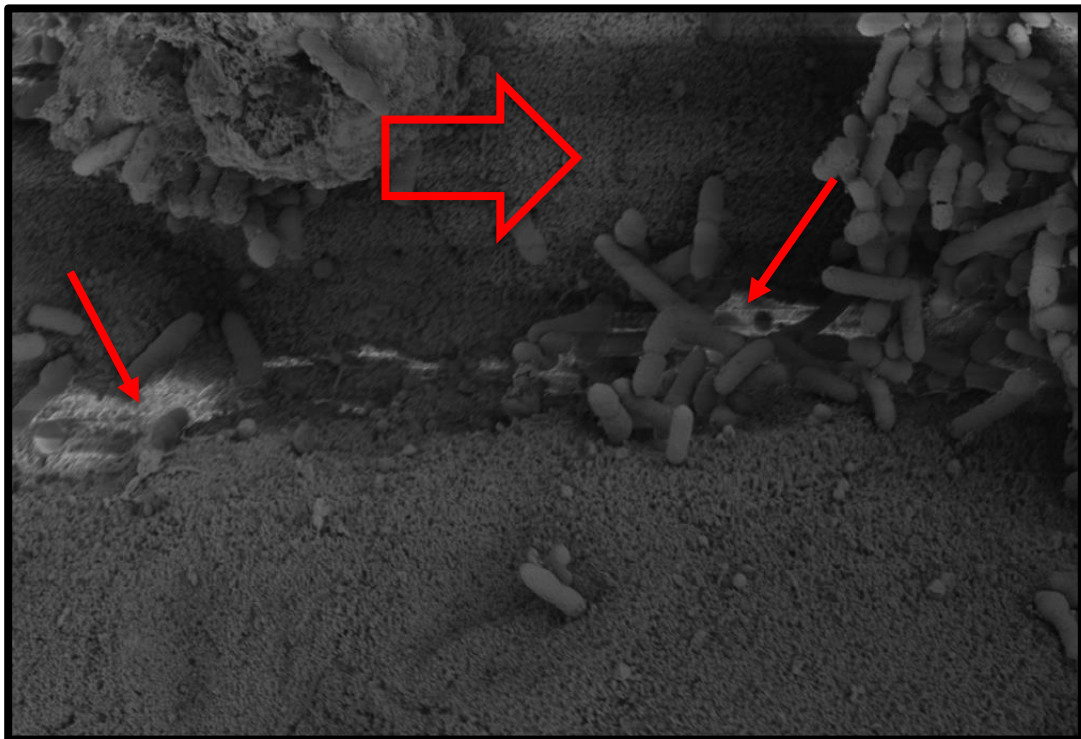


Figure 2. SEM image of the critical point dried (CPD) apical surface of an uninfected HIO viewed at approximately 13,000 X. This image shows the naive, uniform brush border contained within the HIO.

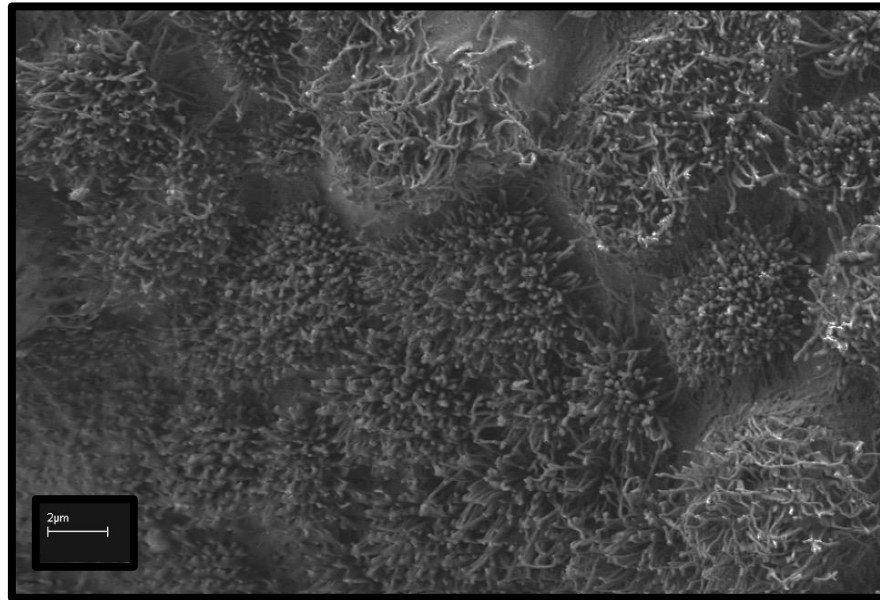


Figure 3. A. A clay representation of a HIO that has completed both primary and secondary fixation. The red band represents the sagittal plane. B. After dissection along the sagittal plane, the lumen is exposed (concave center). C and D. The red markers define the incomplete slices to provide maximum exposure that was used for FE SEM imaging. The HIO after fixation should maintain a concave shape similar to that of a soft contact lens.



Figure 4. A. Cut KimWipe in half and label in upper right corner with pencil. B. Place HIO face-up in center of KimWipe. C. Fold lower third up to carefully cover HIO (do not apply pressure to HIO). D. Fold upper third down to carefully enclose HIO. E. Fold right third over HIO. F. Fold left third over HIO. G. When flipped the HIO should be placed in the center of the folded KimWipe.

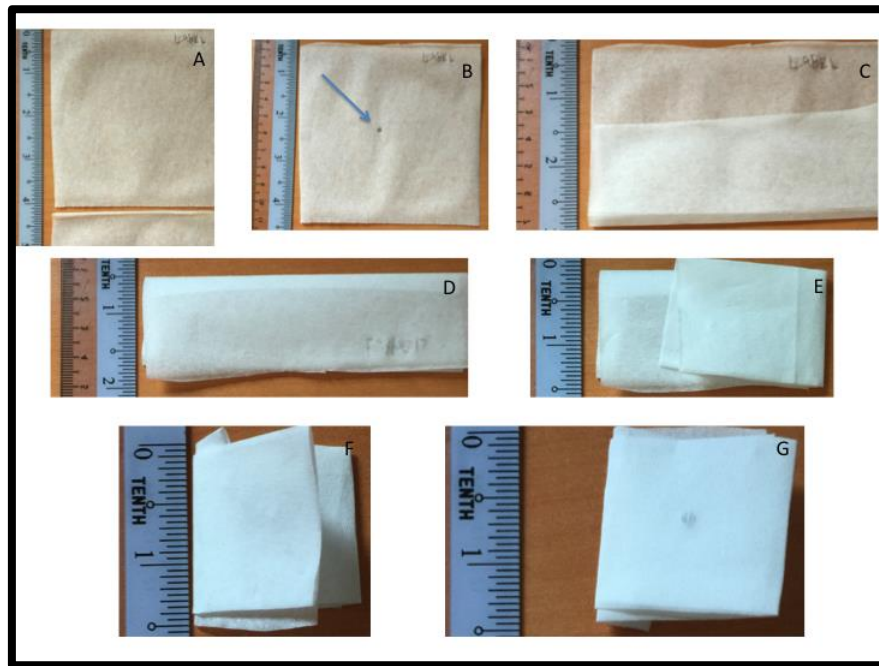


Figure 5. SEM image of the critical point dried (CPD) apical surface of an uninfected HIO viewed at approximately 10,000 X. This image shows the naive, uniform brush border contained within the HIO. This image shows the naive, uniform brush border contained within the HIO. There is variability of the brush border that suggests different cell types with clear cellular junctions found within the HIO.

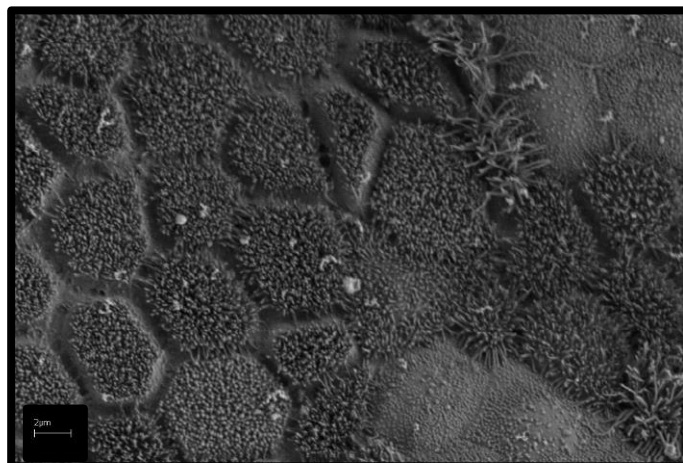


Figure 6. Immunofluorescence cross-section of a healthy and sterile HIO that has been fixed in paraformaldehyde, frozen in O.C.T. and section to 8 microns thick. The cellular F-actin is stained with a TRITC labeled Phalloidin and the nucleus is stained with DAPI. The right side of the image is the uniformed simple columnar epithelial cells with a round nucleus. The left side, contains the mesenchymal cells that appear to be a pseudo-stratification squamous cell. The mesenchymal cells cover the external portion of the HIO.

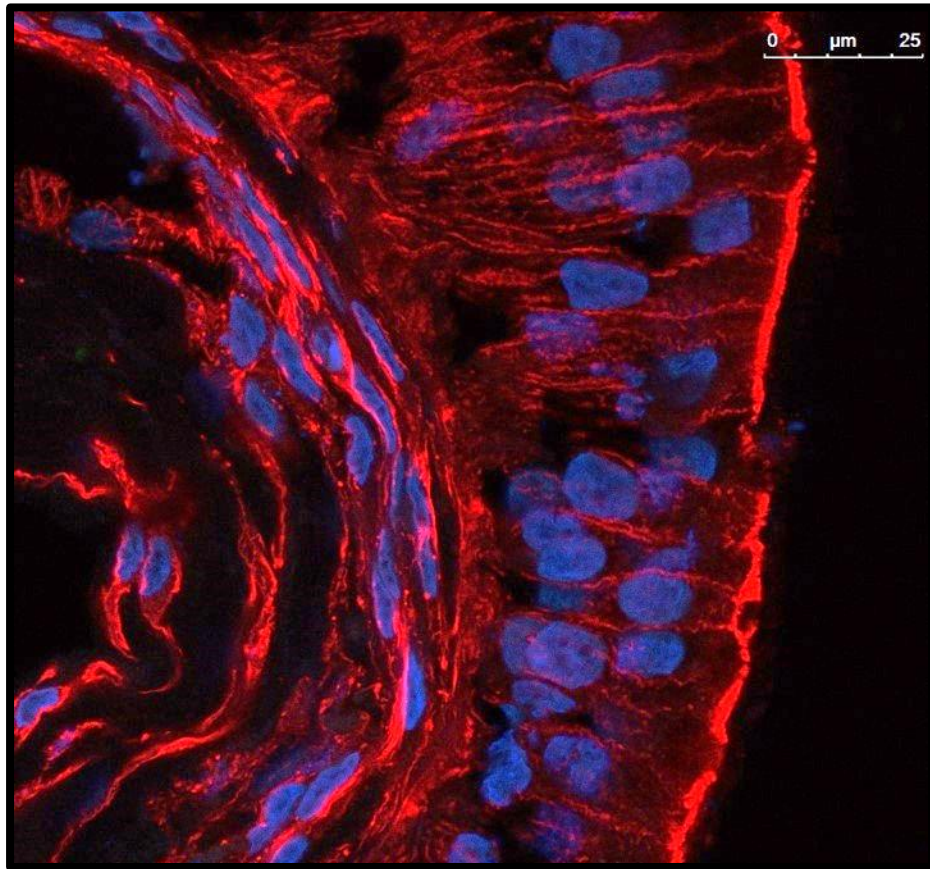


Figure 7. An 8-micron cross-section of the HIO viewed under differential interference contrast (DIC) with green fluorescent protein (Gfp) labeled ECOR2 and DAPI stained host cell nuclei. The ECOR2 is visible with the green fluorescent protein and appears to be in association with the HIO apical surface that can be visualized under DIC.

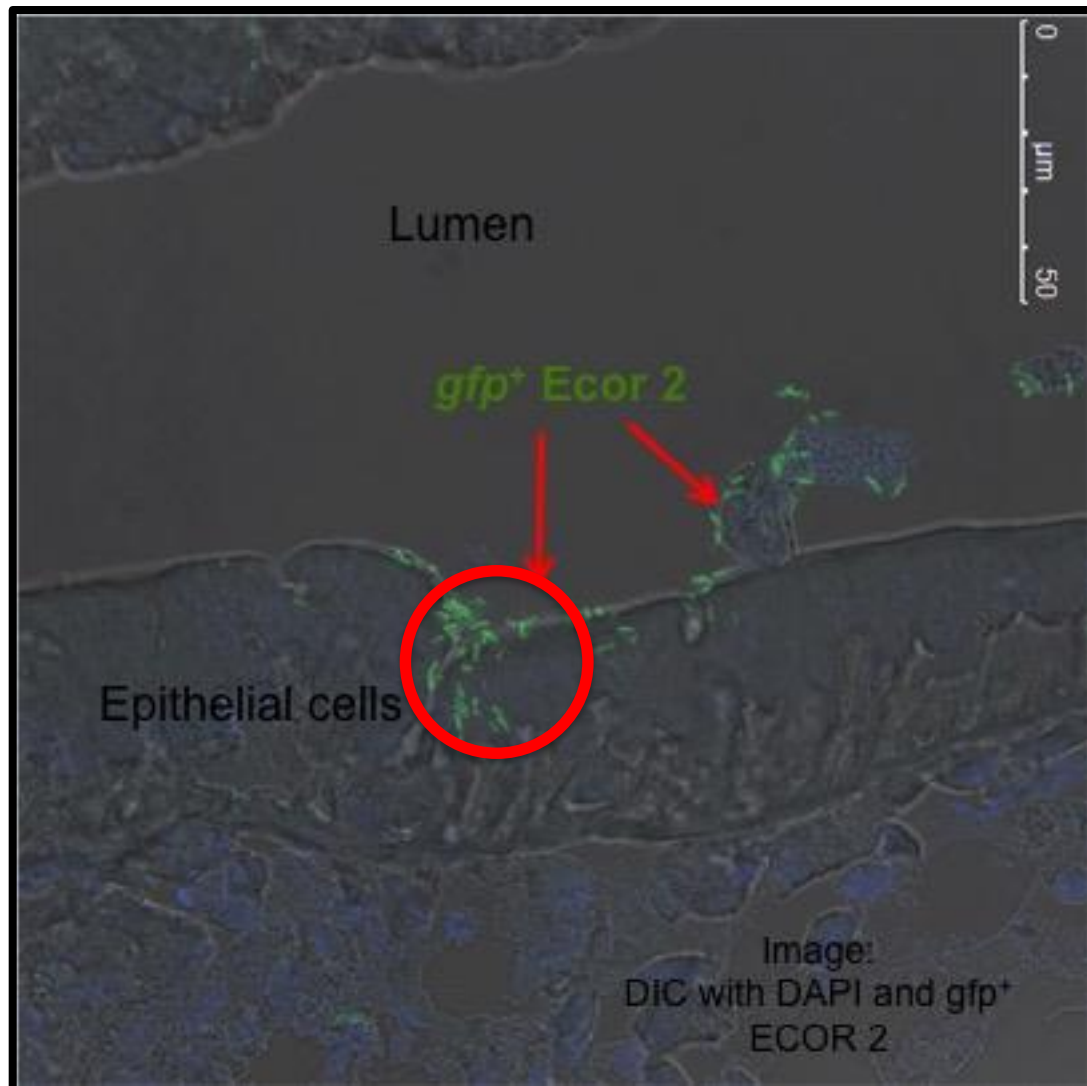


Figure 8. SEM image of the critical point dried (CPD) apical surface of an EPEC infected HIO viewed at 14,000 X with observed adherence. This image shows the rearrangement of the HIO brush border caused by the adherence of EPEC and cradling of the EPEC cells (circles), known as footprinting (arrows).

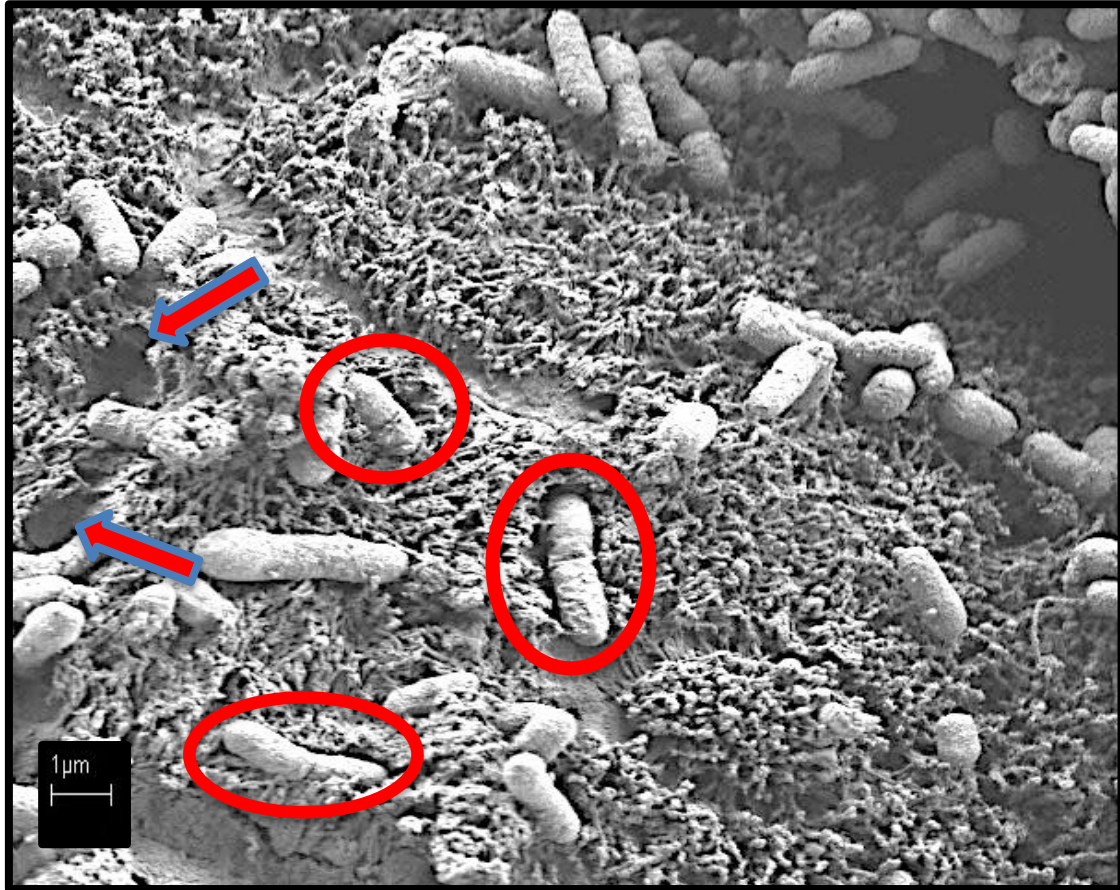


Figure 9. An 8-micron cross-section of the HIO viewed under differential interference contrast (DIC) with green fluorescent protein (Gfp) labeled EPEC and DAPI stained host cell nuclei with observed localized adherence. The EPEC is visible with the green fluorescent protein and appears to be in association within the HIO apical surface. The association of EPEC appears to be in a phenotypic adherence pattern known as localized adherence (LA) (round circles). The LA adherence phenotypes are facilitated by the BFP. This is secondary to intimate adherence that is secondary to the translocation of EPEC produced effector molecules and initial adherence.

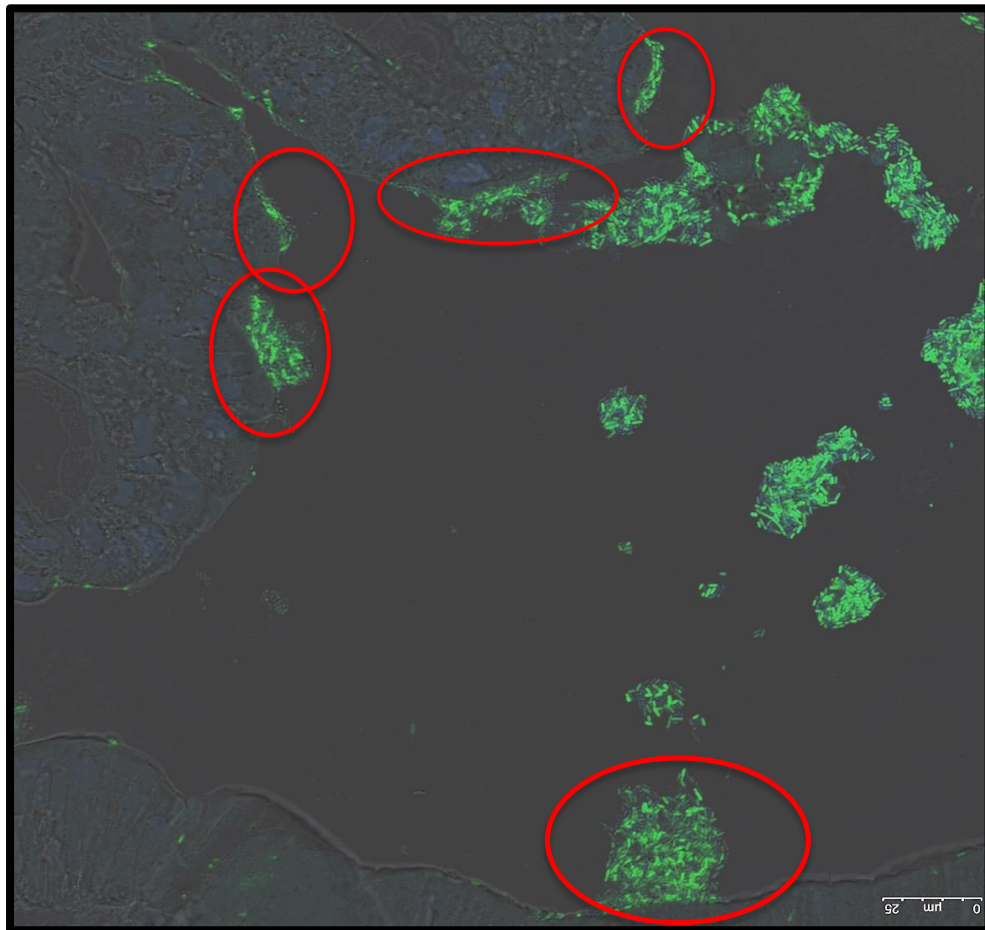


Figure 10. SEM image of the critical point dried (CPD) apical surface of an EPEC infected HIO viewed at approximately 7,000 X with observed localized adherence. This image shows the EPEC localized adherence phenotype on the HIO brush border (arrows). LA is a phenotype typical of EPEC colonization. The far right of the image shows a micro-colony formation that occurs from large aggregates of LA.

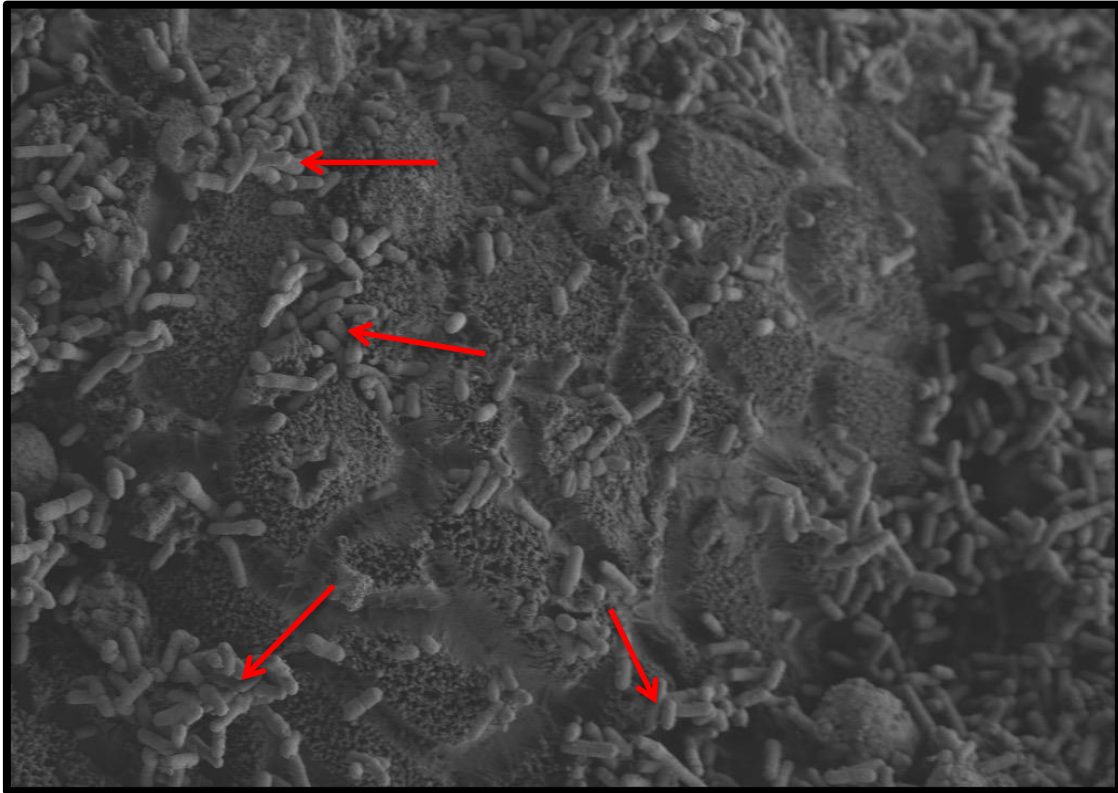
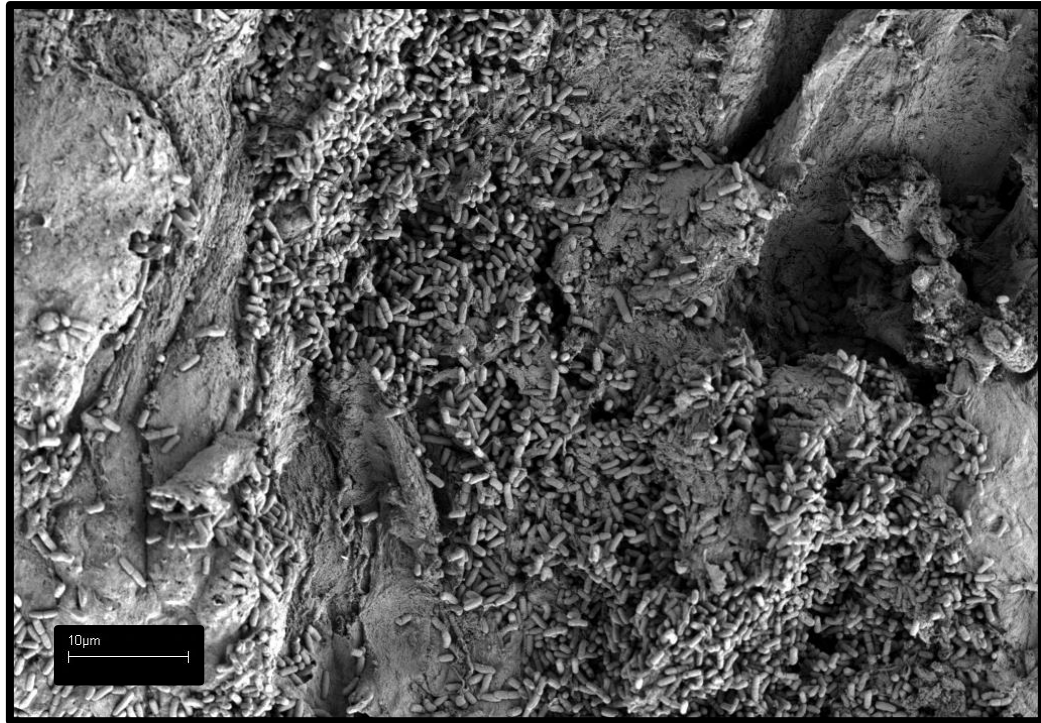


Figure 11. SEM image of the critical point dried (CPD) apical surface of an EPEC infected HIO viewed at approximately 4,000 X with observed micro-colony. This image shows the EPEC micro-colony phenotype on the HIO brush border. The micro-colony phenotype is typical of EPEC colonization that occurs from the formation of large aggregates of LA.



FUTURE DIRECTIONS

The HIO has several advantages over other model systems that include i) replication of the population of cells found within the *In Vitro* human model, ii) derived from human embryonic or pluripotent stem cells, and iii) has a naïve and intact brush border. The research contained in this thesis has demonstrated that the HIO is a suitable model for *E. coli* adherence with the potential to recapitulate an *In Vitro* disease progression, *in vitro*. However, this system has structural limitations that need to be addressed before the later can materialize.

HIOs currently suffer from two limitations: i) they are closed structures (fig. 12) and ii) waste products (necrotic tissues and mucus) build up within the structure (fig. 13). By-products and necrotic tissues may form an internal positive pressure that in turn could build up within the HIO, causing the overall HIO to stop growing. HIOs are therefore unlike the *In Vitro* duodenum, where waste products are excreted during normal digestive function. It could be that pressure affects the ability of *E. coli* to adhere robustly, thus our observed attachment may be conservative with respect to pathogenesis. It is also possible that waste products inhibit adhesion by preventing microbial contact with the mucosal brush border and/or by mucus build-up that forms a formidable and electrostatic barrier against invading pathogens.

Working with HIOs requires a significant investment in tissue culture and trial-and-error. When the inoculation needle punctures the basement membrane, an artificial trauma similar to a surgical incision occurs. This trauma, is a wound that spans the

membranes and provides a potential access of bacteria to otherwise inaccessible tissues.

This injury could modify HIO tight junctions and influence *E. coli* infection.

Inoculation of the HIO is currently variable with respect to the amount of inoculum successfully delivered (bacterial CFU/ μ L). Other influences, such as changes in oxygen gradients and access of growth media and matrigel to the brush border may also influence this model system.

The research outlined in this thesis will be taken forward by investigating how the closed HIO structure can be manipulated. Two potential ways that this can be done are:

i) creating a flow-through chamber with a mature HIO, or ii) growing the HIO from original cells on a scaffolding material. For the flow-through chamber, the working design is to create a limiting space for growth, provide external structural support, artificially produce entrance and exit ports simulating the *In Vitro* intestine, and provide a constant flow of nutrients to the HIO. The flow-through design has been attempted using several materials including glass, various tubing, and polydimethylsiloxane (PDMS).

There are functional characteristics that the material for the flow-through chamber must demonstrate. These characteristics are gas permeability, rapid access to the HIO, and ability to be sealed to ensure sterility. The best material to date has been PDMS. This polymer can be formed and molded to support the HIO, is gas permeable, and can be designed to provide rapid access. However, the exact design is still a work-in-progress.

A second option that should be explored is growing the HIO from seeded stem cells in a scaffold material. Dr. Jason Spence demonstrates that the HIO stem cells form a confluent layer on a sterile culture plate prior to them “blebbling” into small closed

spheroids¹⁰⁵. Such growth would allow the HIO to develop and adhere to a material where the structure could be controlled for further manipulation. Once in a more open system, internal contents can be more easily manipulated, allowing further qualitative and quantitative investigation of EPEC pathogenesis.

Further investigations should be taken with the new designs mentioned above to better identify host receptors for EPEC initial adherence factors, EspA and BFP. The flow-through design combined with the ability of the HIO to be derived from human stem cells gives investigators a unique opportunity to manipulate the genetic constructs of the tissue. This uniqueness of the HIO has great potential to explore important the differences between healthy and unhealthy states (e.g. patients with known GI issues) as well as various pathogen infections. The HIO could be expanded to host a complex microbiome and further research into this relatively new and emerging field.

In conclusion, the HIO model system is in development stages and demonstrates great potential to further provide understanding to the insight of the human gut. The possibilities of this human-based model are limitless.

Figure 12. An immunofluorescence 8-micron cross-section of a healthy and sterile HIO that has been fixed in paraformaldehyde, frozen in O.C.T.. The cellular F-actin is stained with a TRITC labeled Phalloidin and the nucleus is stained with DAPI. The image presents the uniformed simple columnar epithelial cells with a round nucleus and empty lumen. This HIO presented as a clear membrane in culture. The top contains the mesenchymal cells that appear to be a pseudo-stratification squamous cell. The mesenchymal cells cover the external portion of the HIO.

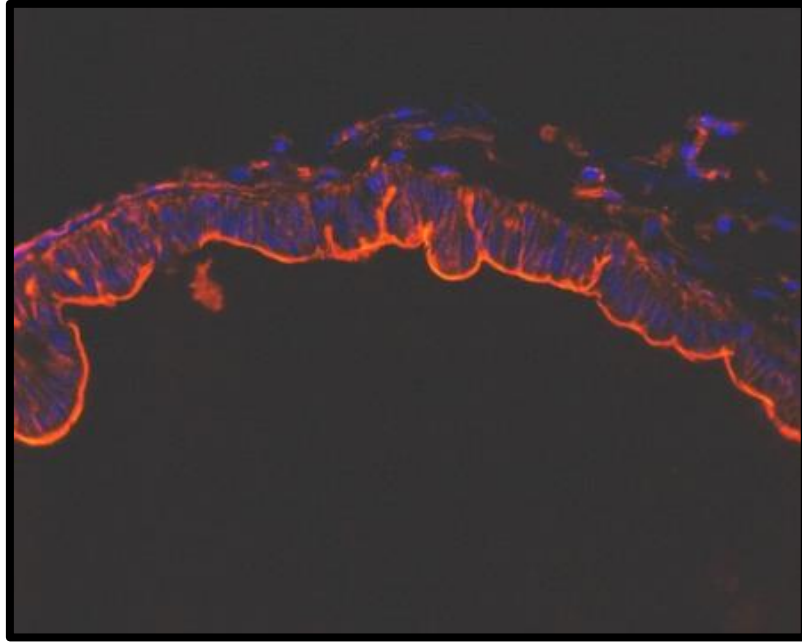


Figure 13. An immunofluorescence 8-micron cross-section of an aged and sterile HIO that has been fixed in paraformaldehyde, frozen in O.C.T.. The cellular F-actin is stained with a TRITC labeled Phalloidin and the nucleus is stained with DAPI. The image presents the uniformed simple columnar epithelial cells with a round nucleus and full lumen. This HIO presented as opaque and dense in culture. The internal lumen is filled with necrotic tissues, cellular by-products, and mucus build-up. The top contains the mesenchymal cells that appear to be a pseudo-stratification squamous cell. The mesenchymal cells cover the external portion of the HIO.

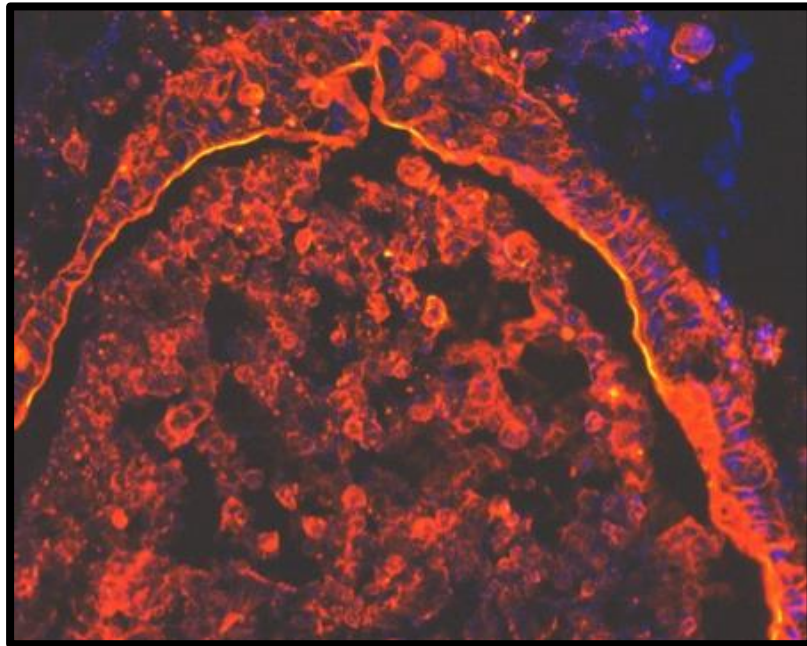
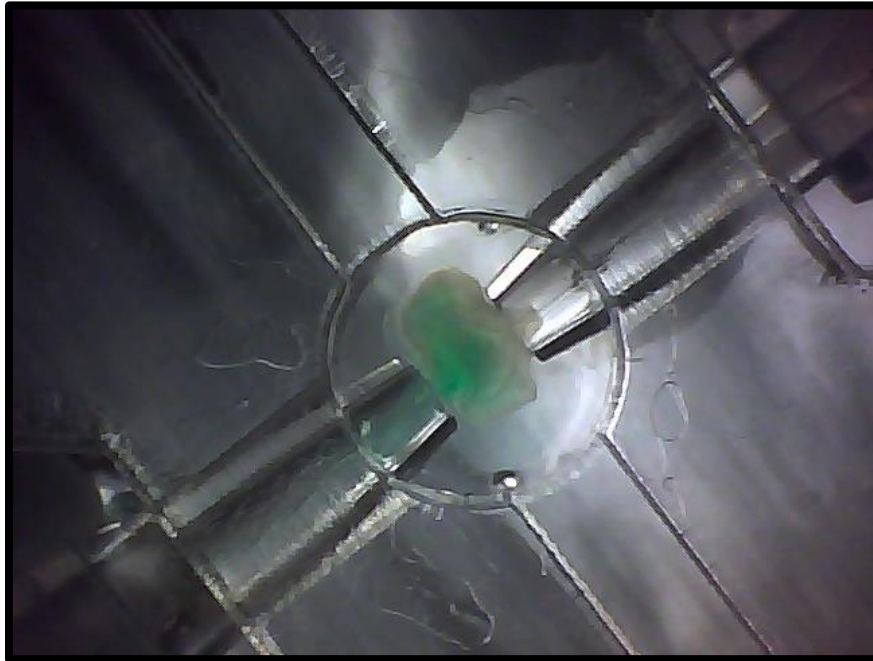


Figure 14. A mature HIO secured in a PDMS chamber with cradle pipettes allowing needle ports to be inserted in opposing directions. The affluent needle has injected green food coloring. Image was taken with an optical camera attached to a stereomicroscope seen at approximately 10X.



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