Video Article

Ex Vivo Intestinal Sacs to Assess Mucosal Permeability in Models of Gastrointestinal Disease

Sean W. Mateer[1], Jocelle Cardona[1], Ellen Marks[1], Bridie J. Goggin[1], Susan Hua[1], Simon Keely[1]

[1]School of Biomedical Science and Pharmacy, University of Newcastle

Correspondence to: Simon Keely at simon.keely@newcastle.edu.au

URL: https://www.jove.com/video/53250
DOI: doi:10.3791/53250

Keywords: Medicine, Issue 108, Intestinal permeability, drug absorption, epithelial barrier, inflammatory bowel disease, paracellular transport, mucosal permeability

Date Published: 2/9/2016

Citation: Mateer, S.W., Cardona, J., Marks, E., Goggin, B.J., Hua, S., Keely, S. *Ex Vivo* Intestinal Sacs to Assess Mucosal Permeability in Models of Gastrointestinal Disease. *J. Vis. Exp.* (108), e53250, doi:10.3791/53250 (2016).

Abstract

The epithelial barrier is the first innate defense of the gastrointestinal tract and selectively regulates transport from the lumen to the underlying tissue compartments, restricting the transport of smaller molecules across the epithelium and almost completely prohibiting epithelial macromolecular transport. This selectivity is determined by the mucous gel layer, which limits the transport of lipophilic molecules and both the apical receptors and tight junctional protein complexes of the epithelium. *In vitro* cell culture models of the epithelium are convenient, but as a model, they lack the complexity of interactions between the microbiota, mucous-gel, epithelium and immune system. On the other hand, *in vivo* assessment of intestinal absorption or permeability may be performed, but these assays measure overall gastrointestinal absorption, with no indication of site specificity. *Ex vivo* permeability assays using "intestinal sacs" are a rapid and sensitive method of measuring either overall intestinal integrity or comparative transport of a specific molecule, with the added advantage of intestinal site specificity. Here we describe the preparation of intestinal sacs for permeability studies and the calculation of the apparent permeability (P_{app}) of a molecule across the intestinal barrier. This technique may be used as a method of assessing drug absorption, or to examine regional epithelial barrier dysfunction in animal models of gastrointestinal disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/53250/

Introduction

The intestinal epithelial barrier of the gastrointestinal tract is a mucosal surface area estimated at 400 m^2 in the human adult. Consequently, it is constantly exposed to challenge from microbes, ingested drugs, nutrients and bacterial toxins. The host must not only distinguish between tolerable commensal bacteria and potential pathogens, but must prevent these species and their secreted molecules from crossing the epithelial barrier, while at the same time allowing absorption of nutrients. Thus, the role of the intestinal epithelium is to act as a selective barrier to the luminal contents [1]. This is achieved, in part, by the innate epithelial defense system at the mucosa, which acts through a responsive biological system consisting of constitutive and inducible mechanisms [2].

Loss of epithelial barrier function is a pathology that is characteristic of a number of gastrointestinal diseases. *In vivo* examination of epithelial barrier function may be assessed through oral gavage of a tracer molecule and subsequent serum analysis [3]. However, this technique offers no indication as to the site of barrier dysfunction. In *vitro* and *ex vivo* assessment of transepithelial resistance using Transwell systems [3] and Ussing chambers [4,5] respectively, are commonly employed as surrogate markers of epithelial barrier function, but lack the contributing disease physiology of animal models [6]. In this protocol we describe an *ex vivo* tissue preparation model that allows direct and localized assessment of intestinal integrity and which may be used to assess mucosal barrier function at a number of levels. Importantly, this technique may be applied to animal models of disease, or may be pharmacologically manipulated to allow in depth interrogation of mucosal barrier dysfunction.

Protocol

All animal work in this protocol is performed with strict adherence to University of Newcastle Animal Ethics Committee approved procedures.

1. Preparation of Instruments, Culture Media and Dishes

1. Pre-warm Media 199 (TC199) or Dulbecco's Modified Eagle Medium (DMEM) media to 37 °C. Pre-oxygenate the medium by bubbling with 95% O_2/5% CO_2. Check that the medium has a final pH of 7.3.
2. Prepare suture by cutting two 5 cm sections for each sac. Loop the sutures into an unclosed knot.

2. Dissection and Preparation of the Gastrointestinal Tract

1. Withdraw solid food 12 hr before euthanasia. If desired, place animals on nutrient gel supplements during this time.
2. Euthanize mice by sodium pentobarbitone overdose ([200 mg/Kg], intraperitoneal injection) followed by cervical dislocation in accordance with institutional ethics protocols and spray 70% ethanol onto the abdomen and thorax.
3. Using a scissors, make a horizontal incision in the middle of the abdomen and expose the peritoneum.
4. Proceed to separate and the remove the gastrointestinal tract by cutting the upper small intestine from the stomach at the pyloric sphincter and cutting the large intestine at the anal verge. Use a forceps to gently remove the mesentery. Place the intestinal tract in pre-warmed, oxygenated medium.
5. Identify the section of intestine to be assessed for permeability (**Figure 1**) and cut this section free from the rest of the intestinal tract.
    1. In order to maintain consistency between animals, measure sections of duodenum and jejunum relative to the stomach, and measure sections of colon and ileum relative to the cecum.
    2. When selecting tissue segments, note the presence of mucosa-associated lymphoid tissues such as Peyer's patches. These can be identified as small nodules on the serosal side of the lumen.
    3. Using a 1 ml syringe, gently flush the luminal contents of the intestinal segment into a petri dish with pre-heated PBS (37 °C). These faecal contents may be discarded or stored at -80 °C for future analysis as desired.

3. Preparation of Intestinal Sacs

1. Prepare a 1 ml syringe with a 300 µl volume of the test compound or molecule. For mucosal integrity, a 1 mg/ml solution of FITC-Dextran M.Wt. 4,400 may be used. Probes ranging from 4,400-70,000 Da in size may be used for increased sensitivity. Securely fit a small animal vascular catheter onto the syringe.
2. Measure 5 cm from the opening of the intestinal segment and tie the segment securely closed with a suture-loop at this point. Gently place a pre-tied suture-loop around the opening of the intestine and insert the blunted catheter. Pull the noose closed so as to secure the intestinal segment and release the 300 µl volume from the syringe into the intestine, ensuring that all the solution is injected.
3. Gently remove the catheter while simultaneously pulling the suture noose to secure closure of the intestinal sac. Cut the intestinal sac loose from the intestine and place into a 50 ml conical tube filled with 20 ml of oxygenated medium, preheated to to 37 °C.

4. Measurement of Permeability

1. Place conical tubes containing the intestinal sacs in a heated water bath set to 37 °C. At 0, 30, 60, 90 and 120 min time points, take a 100 µl sample from the conical tube and transfer to a 96 well plate, replacing the volume with 100 µl of fresh media in each instance.
2. After the final sample is taken, cut open sacs at the point of suture and down the length of the segment, exposing the mucosal surface.
3. Measure the length and width of each intestinal segment. If desired, snap freeze the segments and store at -80 °C for protein or biochemical analysis, or alternatively, store in RNA stabilization solution for molecular assays.
4. Construct a standard curve of log dilutions for FITC-tagged molecules ranging from 1 to 1 x 10^{-6}.
5. Measure samples and standards for FITC on a fluorescent plate reader, FITC excitation/emission: 495 nm/519 nm.

5. Calculation of Apparent Permeability for Each Individual Intestinal Sac

1. Convert time units to sec.
2. For each time point, calculate the cumulative concentration, *Q*
   $Q_t = (C_t * V_r) + (Q_{t\ sum} * V_s)$, Where:
   Q_t = Cumulative concentration at time t
   C_t = Concentration at time t
   V_r = Volume at receiver side
   $Q_{t\ sum}$ = Sum of all previous Q_t
   V_s = Volume sampled

3. Plot Q versus time (T) and calculate the slope: δQ/δt
4. Calculate the apparent permeability (P_{app})
   $P_{app} = (\delta Q/\delta t)/(A*Co)$, Where:
   A = Area of tissue
   C_0 = Initial concentration

Representative Results

This protocol may be used to examine regional changes in intestinal barrier function in animal models of gastrointestinal disease. By measuring the flux of a paracellular probe across the mucosal surface at varying areas of the gastrointestinal tract[7], the integrity of the epithelial tight junctions can be assessed. In addition, by varying the nature of the paracellular probe by size (**Figure 2**) or hydrophobicity (**Figure 3**), the degree of epithelial perturbation, or the integrity of the mucous gel layer, can also be measured. Employing larger molecular weight markers allows for a more sensitive interrogation of paracellular permeability of the mucosa, detecting discreet changes, that may not be apparent by electrophysiological measurements such as transepithelial electrical resistance (TEER), but which would be sufficient to allow paracellular transport (**Figure 2**). Mucosal inflammation may lead to a loss of goblet cells and reduction in the protective mucous gel that normally overlies and protects the epithelial interface. Using hydrophobic probes, the integrity of the intestinal mucous gel layer can also be examined (**Figure 3**). In addition, regional barrier integrity can be examined through the specific preparation of intestinal sacs from different intestinal areas. Regional changes in barrier function vary within different animal models of disease and thus, the use of intestinal sacs allows for a localised assessment of intestinal barrier function (**Figure 4**), when compared to oral gavage of probes and subsequent serum assay.

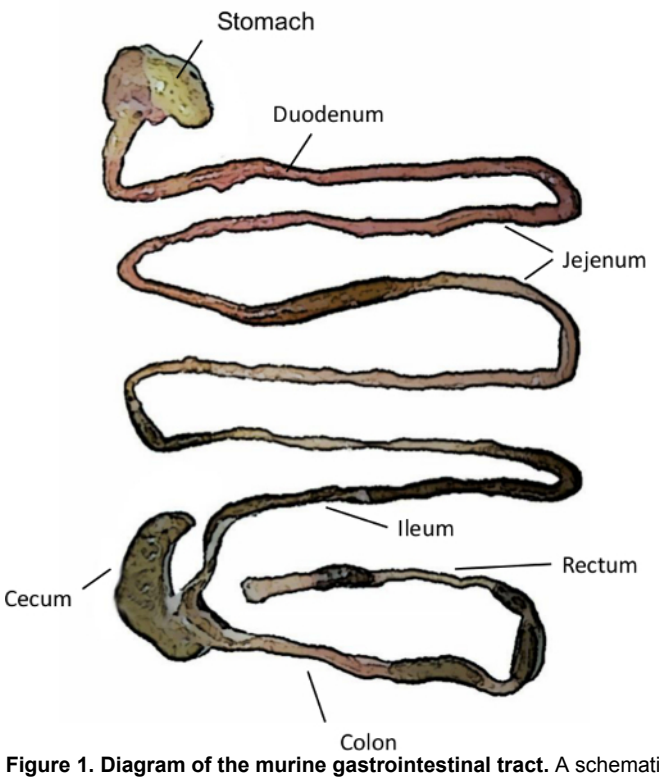

Figure 1. Diagram of the murine gastrointestinal tract. A schematic of the gastrointestinal tract of a C57Bl/6 mouse, from the stomach to the anus. The small intestinal junctions cannot be readily differentiated macroscopically and consistent sampling helps minimize mouse to mouse variation. For the purposes of creating intestinal sacs, a 5 cm segment distal to the pyloric sphincter will encompass the duodenum. A 10 cm section extending proximally from the cecum will encompass the ileum. The remaining small intestinal tissue represents jejunum. The rectum is located 2 cm proximal to the anus, with the remaining large intestine, extending to the cecum representing the colon [8]. Please click here to view a larger version of this figure.

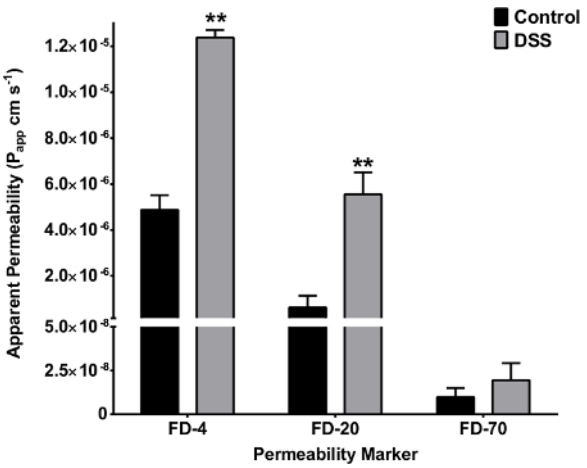

Figure 2. Size-dependent paracellular transport of FITC-Dextran molecules though the epithelial mucosa in control and DSS colitis animals. Intestinal sacs were prepared from the colons of DSS mice 10 days into the course of disease. Sacs were loaded with 1 mg/ml solution of FD-4 (MW 4,400 Da), FD-20 (MW 20,000 Da) or FD-70 (70,000 Da) and flux of the FITC-Dextran permeability marker was measured over 120 min. Age matched healthy animals were used as controls. N = 5, 2 technical replicates per N. **p <0.01, Student's t-test. Please click here to view a larger version of this figure.

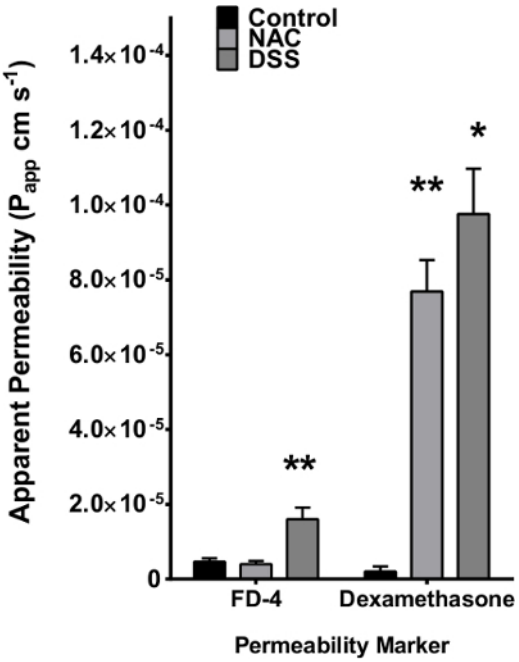

Figure 3:. The influence of mucous-gel layer on barrier integrity to hydrophobic compounds. Intestinal sacs were prepared from the colons of DSS mice 10 days into the course of disease. Age matched healthy animals were used as controls. For negative mucous-gel control, the intestines were loaded with 10 mM N-acetyl cysteine (NAC) (300 µl volume per 5 cm of intestine) and incubated at 37 °C for 15 min and flushed with fresh medium before sacs were prepared. Sacs were loaded with 1 mg/ml solution of FD-4 (MW 4,400 Da) and flux measured over 120 min. N = 3, 2 technical replicates per N. *p <0.05, **p <0.01, Student's t-test. Please click here to view a larger version of this figure.

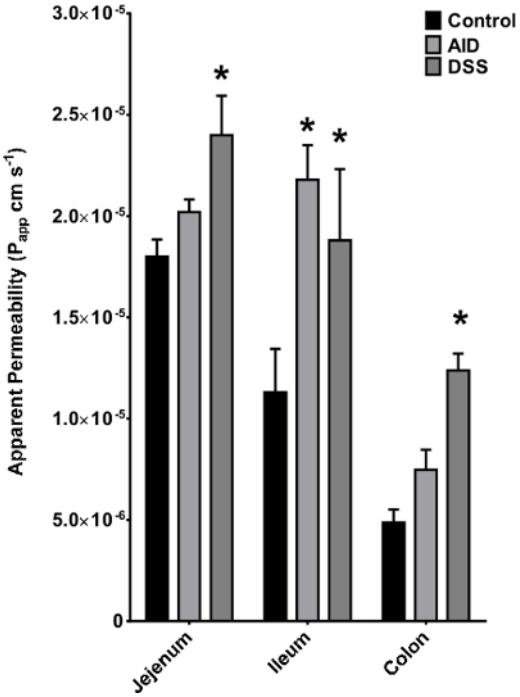

Figure 4. The regional permeability of the intestine to FD-4 in murine models of intestinal inflammation. Intestinal sacs were prepared from the jejunum, ileum or colon of healthy animals, DSS animals or antibiotic-induced dysbiosis (AID) animals. Sacs were loaded with 1 mg/ml solution of FD-4 (MW 4,400 Da) and flux measured over 120 min. N = 3, 2 technical replicates per N. *p <0.05, **p <0.01, Student's t-test. Please click here to view a larger version of this figure.

Discussion

Here, we have detailed the isolation and preparation of intestinal sacs to assess mucosal barrier function *ex vivo*. Intestinal sac preparations have primarily been utilized in pharmaceutical research, examining the absorption of candidate drugs across the intestine. However, this assay is equally well suited for the study of intestinal disease. Intestinal permeability may vary greatly by region and site specific assessment of permeability allows a better understanding of the regional importance of mucosal integrity in digestive diseases. The assay is robust and under the correct physiological conditions, isolated tissues remain viable for up to 6 hr post mortem. Following euthanasia, the speed of the tissue preparation is important and the intestine should be flushed of its contents and transferred to oxygenated medium as rapidly as possible. In order to ensure consistency between animals, it is essential that the correct intestinal regions are identified (**Figure 1**). It is advised that colonic and ileal segments are measured from the cecum, while segments of duodenum and jejunum are measured from the stomach. Because different strains, models or transgenic animals may be larger, and have longer GITs, it is worth characterizing the average length or each intestinal segment in your model before engaging in permeability assays.

In addition to regional consistency, it is important to ensure that each intestinal sac is cut to equal length and the sac is filled with the correct volume of fluid. Inconsistency in these factors will result in unequal distension of the mucosa between assays. Under-distension of the intestinal segment not only reduces exposure of the luminal contents to the mucosal surface, but also increases the thickness of the tissue through which the marker must travel. Over-distension of the segment may damage the tissue or activate stress-responses in the tissue, potentially confounding results. It should be noted, that for the calculation of P_{app} does not account for surface area of villous structure. While this leads to error in calculating the actual apparent permeability of the tissue, it does not affect comparative results between models, as the surface area is calculated as a constant. Any changes in surface area due to disease are offset by loss of epithelial integrity and the protocol is sufficiently robust to identify changes in permeability with disease progression [6].

The use of tissue culture medium is recommended. In studies performed by Barthe *et al.* the use of TC199 was found to significantly increase the longevity of epithelial viability and tissue histological architecture, when compared to simple salt buffers [9]. In our studies both DMEM and TC199 medium maintained at 37 °C provided optimal conditions for tissue survival [5,6,10]. The correct media supplies the epithelium with nutrients required to sustain tissue integrity, while maintaining temperature during the assay ensures optimal tissue metabolism which is essential tight junctional integrity and assays involving active transport via transcellular pathways. Temperatures below 37 °C cause loss of tissue viability, increasing paracellular transport and decreasing transcellular transport [11]. Thus, prolonging tissue viability with optimal conditions is essential and in doing so, the assay may be used not only to examine regional barrier integrity and but also to examine intervention studies and physiological and transcriptional responses.

While primarily used for drug absorption studies, techniques to examine apparent permeability (P_{app}) of a marker molecule across an epithelial barrier are a highly sensitive measurement of intestinal integrity [12-15]. In contrast to surrogate *ex vivo* measurements of barrier function, such as TEER, P_{app} is a direct and highly sensitive measurement of the intestinal barrier [4]. It is worth noting that TEER measurements and permeability as measured by the P_{app} of marker molecules do not always correlate. TEER measurements encompass the resistance of both the tight junctions and transcellular parallel resistors [16]. Therefore TEER is a measurement of the combined resistance offered by tight junctions and the cells themselves. If the cell-cell associations are weak (that is, the tight junctions are leaky) then this contribution to the overall resistance of a

monolayer will be low. Thus small changes in tight junctional resistances for leaky epithelium become negligible changes to the resistances of the epithelium as a whole. In contrast, P_{app} assays measure the ability of a molecule to cross the mucosal barrier [17] and indeed, the sensitivity of P_{app} measurements may be altered through selecting different sized marker molecules (**Figure 1**). The consideration of marker size is important in relation to the model of intestinal disease being examined. Models of allergy or functional disease [18,19], where loss of integrity is mild to moderate, may be more suited to lower molecular weight markers, which will allow identification of subtle changes to intestinal barrier function. In contrast, with models such as DSS colitis, which involves denuding of the intestinal epithelium, larger markers may be more appropriate to assess mucosal healing, as relatively small increases in barrier integrity will be highlighted.

While intestinal sacs offer a physiologically relevant model of GI barrier function, there are some limitations with respect to the variability of animal models which need to be considered. For instance, the secretory state or the epithelium can influence both paracellular transport across the intestine [20] and the integrity of the mucous gel layer [5]. While *ex vivo* assays incorporating electrophysiological measurements, such as Ussing chamber preparations, may account for this, intestinal sacs do not. Secondly, in preparing the intestinal sacs researchers should account for lymphoid structures, such as Peyer's patches, within sac preparations, as these may influence the permeability of the tissue [21]. Despite these considerations, unlike many cell culture models, used to assess epithelial integrity, the intestinal sacs offer the contribution of both a mucous gel layer and an underlying lamina propria. The contribution of the mucous gel layer, in particular, may be assessed through the use of mucolytic agents, such as N-acetyl cysteine, or hydrophobic tracer molecules, such as dexamethasone [5,17]. This may be particularly important in assessing intestinal permeability in models of cancer therapy or Inflammatory Bowel Disease, where loss of the mucous barrier can be an early pathology in the mucosal inflammation [22,23]. Similarly, in models of diarrheal disease, functional GI disease or dysbiosis, intestinal mucus production and overall mucous gel integrity may be altered at regional sites [5,19,24]. Oral gavage of permeability markers and subsequent serum sampling is another option for assessing intestinal permeability *in vivo*. While this method has minimal manipulation of the tissue, it is a composite measure of intestinal barrier function and it does not assess the regional contributions to the overall barrier. Different models of GI disease are likely to have different sites of relative importance which will not be accounted for by oral gavage approaches. The use of intestinal sacs, is therefore a rapid, sensitive and physiologically relevant assay which may be used to examine regional intestinal mucosal integrity in small animal models of disease.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This work was funded by National Health and Medical Research Project Grant APP1021582 and a Hunter Medical Research Institute grant sponsored by Sparke Helmore/NBN Triathlon and the Estate of the late Leslie Kenneth McFarlane.

References

1. Goggins, B. J., Chaney, C., Radford-Smith, G. L., Horvat, J. C., & Keely, S. Hypoxia and Integrin-Mediated Epithelial Restitution during Mucosal Inflammation. *Frontiers in immunology.* **4**, 272 (2013).
2. Otte, J. M., Kiehne, K., & Herzig, K. H. Antimicrobial peptides in innate immunity of the human intestine. *Journal of gastroenterology.* **38**, 717-726 (2003).
3. Robinson, A. *et al.* Mucosal protection by hypoxia-inducible factor prolyl hydroxylase inhibition. *Gastroenterology.* **134**, 145-155 (2008).
4. Feighery, L. *et al.* Increased intestinal permeability in rats subjected to traumatic frontal lobe percussion brain injury. *The Journal of trauma.* **64**, 131-137; discussion 137-138 (2008).
5. Keely, S. *et al.* Chloride-led disruption of the intestinal mucous layer impedes Salmonella invasion: evidence for an 'enteric tear' mechanism. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology.* **28**, 743-752 (2011).
6. Keely, S. *et al.* Contribution of epithelial innate immunity to systemic protection afforded by prolyl hydroxylase inhibition in murine colitis. *Mucosal immunology.* **7**, 114-123 (2014).
7. Sourisseau, T. *et al.* Regulation of PCNA and cyclin D1 expression and epithelial morphogenesis by the ZO-1-regulated transcription factor ZONAB/DbpA. *Mol Cell Biol.* **26**, 2387-2398 (2006).
8. Ruehl-Fehlert, C. *et al.* Revised guides for organ sampling and trimming in rats and mice--part 1. *Exp Toxicol Pathol.* **55**, 91-106 (2003).
9. Barthe, L., Woodley, J. F., Kenworthy, S., & Houin, G. An improved everted gut sac as a simple and accurate technique to measure paracellular transport across the small intestine. *European journal of drug metabolism and pharmacokinetics.* **23**, 313-323 (1998).
10. Marks, E. *et al.* Oral Delivery of Prolyl Hydroxylase Inhibitor: AKB-4924 Promotes Localized Mucosal Healing in a Mouse Model of Colitis. *Inflammatory bowel diseases.* **21**, 267-275 (2015).
11. Keely, S. *et al.* Hypoxia-inducible factor-dependent regulation of platelet-activating factor receptor as a route for gram-positive bacterial translocation across epithelia. *Mol Biol Cell.* **21**, 538-546 (2010).
12. Brayden, D. J., Bzik, V. A., Lewis, A. L., & Illum, L. CriticalSorb promotes permeation of flux markers across isolated rat intestinal mucosae and Caco-2 monolayers. *Pharmaceutical research.* **29**, 2543-2554 (2012).
13. Hubbard, D., Ghandehari, H., & Brayden, D. J. Transepithelial transport of PAMAM dendrimers across isolated rat jejunal mucosae in ussing chambers. *Biomacromolecules.* **15**, 2889-2895 (2014).
14. Keely, S. *et al. In vitro* and *ex vivo* intestinal tissue models to measure mucoadhesion of poly (methacrylate) and N-trimethylated chitosan polymers. *Pharmaceutical research.* **22**, 38-49 (2005).
15. Maher, S. *et al.* Evaluation of intestinal absorption enhancement and local mucosal toxicity of two promoters. I. Studies in isolated rat and human colonic mucosae. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences.* **38**, 291-300 (2009).

16. Balda, M. S. *et al.* Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *The Journal of cell biology.* **134**, 1031-1049 (1996).
17. Behrens, I., Stenberg, P., Artursson, P., & Kissel, T. Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. *Pharmaceutical research.* **18**, 1138-1145 (2001).
18. Stefka, A. T. *et al.* Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci U S A.* **111**, 13145-13150 (2014).
19. Keely, S. *et al.* Activated fluid transport regulates bacterial-epithelial interactions and significantly shifts the murine colonic microbiome. *Gut microbes.* **3**, 250-260 (2012).
20. Barrett, K. E., & Keely, S. J. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annual review of physiology.* **62**, 535-572 (2000).
21. Soni, J. *et al.* Rat, ovine and bovine Peyer's patches mounted in horizontal diffusion chambers display sampling function. *Journal of controlled release : official journal of the Controlled Release Society.* **115**, 68-77 (2006).
22. Justino, P. F. *et al.* Regulatory role of Lactobacillus acidophilus on inflammation and gastric dysmotility in intestinal mucositis induced by 5-fluorouracil in mice. *Cancer chemotherapy and pharmacology.* (2015).
23. Tran, C. D., Sundar, S., & Howarth, G. S. Dietary zinc supplementation and methotrexate-induced small intestinal mucositis in metallothionein-knockout and wild-type mice. *Cancer biology & therapy.* **8**, 1662-1667 (2009).
24. Musch, M. W., Wang, Y., Claud, E. C., & Chang, E. B. Lubiprostone decreases mouse colonic inner mucus layer thickness and alters intestinal microbiota. *Digestive diseases and sciences.* **58**, 668-677 (2013).

