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In Vitro monitoring of extracellular pH in real-time

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TITLE:

In Vitro monitoring of extracellular pH in real-time

AUTHORS AND AFFILIATIONS:

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KEYWORDS:

Neutrophil, acidification, pH, transmigration

SUMMARY:

This article represents a useful *in vitro* assay to measure changes in extracellular pH during neutrophil (PMN) transepithelial migration (TEM)

ABSTRACT:

Early accumulation of neutrophils (PMN) is a hallmark of acute intestinal inflammation. This acute inflammation is either resolved or progresses to chronic inflammation. Without efficient PMN clearance at sites of infiltration, PMN can accumulate and contribute to chronic inflammatory conditions, including the intestinal diseases ulcerative colitis (UC) and Crohn's Disease (CD). The pH in the distal colon in individuals with active UC can range between a pH of 5 and 6, whereas healthy individuals maintain colonic pH in the range of 6.8-7.4. Extracellular pH has been shown to influence both intestinal epithelial cells and the infiltrating immune cells. More specifically, extracellular acidosis significantly impacts PMN. At pH below 6.5, there are increases in the production of H_2O_2, inhibition of apoptosis, and increases in the functional lifespan of PMN. Given the significant presence of PMN and extracellular acidification at sites of inflammation, we developed a novel model that allows for the monitoring of extracellular pH during PMN transepithelial migration in real time. Here, we describe this model and how it can be utilized to measure both the apical and basal pH during PMN trafficking. This model can be utilized to monitor extracellular pH under a wide range of conditions; including, hypoxia, PMN transepithelial migration, and for extended periods of time.

INTRODUCTION:

The extracellular microenvironment has been shown to play a significant role in modulating the inflammatory response. One aspect of the microenvironment which is often underappreciated is extracellular acidification. Extracellular acidification is often observed at sites of active

inflammation, including mucosal disorders such as inflammatory bowel diseases. The luminal pH in the distal colon from patients with UC can range between a pH of 5 and 6, whereas healthy individuals have colonic pH's in the range of 6.8-7.4[1,2]. This decrease in colonic pH is of particular interest because extracellular pH has been shown to broadly influence IEC and infiltrating immune cell function. Acidic microenvironments, for example, have been shown to extend the functional life spawns of infiltrating PMN, stimulate H_2O_2 production, and inhibit PMN apoptosis[3,4]. The exact mechanism by which the extracellular environmental becomes acidic remains unclear, highlighting the need to develop techniques to study acidification over time.

In a recent study, it was demonstrated that PMN TEM results in a significant acidification of the microenvironment and that IEC rapidly respond to this acidification through the adaptive upregulation of SLC26A3, the major chloride-HCO_3 transporter in mucosal tissue[5,6], thereby promoting pH homeostasis[7]. The exact mechanism(s) by which PMN TEM induces extracellular acidification remains unclear. It is currently believed that tissue acidification is caused by increased accumulation of lactic acid resulting from enhanced glycolysis and it was recently observed that PMN TEM stimulates the release of lactate from IEC[7,8]. However, the levels of secreted lactate do not fully account for the acidification observed during PMN TEM. A better understanding of the mechanisms involved in extracellular acidification will allow for the potential identification of novel therapeutic targets. To better understand the mechanism involved there needs to be a system which allows for monitoring of pH over an extended period of time, while allowing PMN to transmigration in the physiologically relevant apical to basal direction. Using a pH probe is time intensive and requires repeated manipulation of the culture system. Currently, there are several non-invasive techniques to monitor pH over time. One commonly used assay utilizes 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF), a dye which is internalized by the cell, however this assay is limited to the study of internal pH [9]. There are several pH plate assays, but most are only available in a 96-well format or require the addition of pH sensitive dyes. The protocol described below is based off a commercially available pH sensing plate which was designed for non-invasive monitoring of pH over an extended period of time[10]. Cells are grown as a monolayer in a 24-well plate that contains a pre-calibrated pH sensor. This sensor contains a luminescent dye which is excited by specialized plate reader. The luminescence lifetime, which is dependent on pH, is also measured by the plate reader. However, this model lacks an apical and basal compartment, preventing the study of transmigration or basal/apical differences in acidification.

Described is a protocol that allows for *in vitro* evaluation of extracellular acidification during PMN TEM, using human T84 intestinal epithelial cells grown on transwell inserts, human PMN, and a non-invasive fluorescent based pH sensor. Provided is a successful example where the extracellular pH is examined over the course of 10 hr and demonstrate active PMN TMN results in extracellular acidification. Although a specific example is presented, this protocol can be utilized to evaluate the effect of any number of factors; including, metabolites, cell types, and potential therapeutics, on extracellular pH.

PROTOCOL:

1. Cell Preparation

Day 1

1.1. Warm cell culture media (DMEM/F12 with 5% FBS, 2 mM L-alanyl-L-glutamine dipeptide, and Pen Strep) in a 37 °C water bath for 20 min.

1.2. Prepare the tissue culture hood by spraying it with 70% ethanol and wiping down the surfaces with paper towels.

1.3. Spray and wipe tissue culture flasks and media, PBS, and trypsin bottles with 70% ethanol and bring them into the tissue culture hood.

1.4. Aspirate the media from the cell culture flask and wash the cells with 12 mL of phosphate buffered saline (PBS).

1.5. Aspirate the PBS and add 1 mL of trypsin to the cells. Incubate at RT for 10-20 min, until the cells detach.

1.6. Add 11 mL of cell culture media to the trypsin and mix by pipetting up and down 3-5 times.

1.7. Invert a 24-well cell culture dish containing 5 μm pore inserts and gently pipette 100 μL of the cell suspension to the underside of the insert (**Figure 1**).

1.8. Place the inverted plate in a 37 °C incubator with 5% CO_2.

Day 2

1.9. Warm cell culture media in a 37 °C water bath for 20 min.

1.10. Prepare the tissue culture hood by spraying it with 70% ethanol and wiping down the surfaces with paper towels.

1.11. Spray and wipe tissue culture flasks and media, PBS, and trypsin bottles with 70% ethanol and bring them into the tissue culture hood.

1.12. Right the tissue culture plate and gently add 1 mL of media to the basal compartment and 180 μL to the apical compartment.

1.13. Place the plate in a 37 °C incubator with 5% CO_2.

Day 3-7:

1.14. Monitor the transepithelial resistance (TER) daily, using a epithelial volt/Ohm meter, until the TER have stabilized and the cells have formed a confluent monolayer.

2. PMN Isolation

2.1. Room temperature gradient solutions, 50 mM K_2EDTA, and Hanks' Balanced Salt Solution (HBSS) without calcium/magnesium (HBSS-) to room temperature.

2.2. Prepare double density gradients in 50 mL conical tubes.

2.3. Coat a 60 mL syringe with 6 mL of 50 mM K_2EDTA and collect 54 mL of blood from donor.

2.4. Overlay 15 mL of blood to the double density gradients prepared above.

2.5. Centrifuge at 700 x *g* for 30 min at room temperature, with the brake off.

2.6. Aspirate the serum, peripheral blood monocyte, interphases and transfer the layer containing PMN to a new 50 mL conical tube and resuspend to 50 mL in HBSS-.

2.7. Centrifuge at 700 x *g* for 10 min at room temperature.

2.8. Aspirate the supernatant and resuspend the pellet in 40 mL of cold red blood cell lysis buffer.

2.9. Centrifuge at 700 x *g* for 10 minutes at 4 °C.

2.10. Aspirate the supernatant and combine the pellets from all tubes into 3 mL of HBSS-.

2.11. Determine the concentration of PMN and dilute to $5x10^4$ using the appropriate about of HBSS-.

3. *In Vitro* pH Monitoring Assay

3.1. Warm HBSS with calcium/magnesium (HBSS+) in a 37 °C water bath for 20 min.

3.2. Prepare the tissue culture hood by spraying it with 70% ethanol and wiping down the surfaces with paper towels.

3.3. Spray and wipe HBSS+ with 70% ethanol and bring them into the tissue culture hood.

3.4. 2-3 h before the assay is performed, equilibrate the pH sensing plates by adding 1 mL of HBSS+ to each well and placing in a 37 °C incubator.

3.5. 30 min before the assay remove the pH sensing plates from the incubator and add 1 μM fMLP to each well.

3.6. Place the pH sensing plate on the SDS reader at 37 °C and record the pH to establish a baseline for the experiment.

3.7. Once the pH reading has stabilized remove media from inserts and add 180 µL HBSS+ to the top well.

3.8. Add 20 µL HBSS+ to control wells or 20 µL PMN.

3.9. Remove the HBSS+ and transfer the inserts to the pH sensing plate.

3.10. To the bottom well, add 1 mL of HBSS+ with and without 1 µM fMLP.

3.11. To the top well, add 180 µL ofHBSS+.

3.12. To the top well, add 20 µl of HBSS- containing 0 or 1 x 10^6 PMN.

3.13. Place the pH sensing plate on the SDS reader at 37 °C (**Figure 2**).

3.14. On the attached computer, open the pH monitoring software and set up the software to record the pH at designated time intervals (every 1-10 min).

REPRESENTATIVE RESULTS:

The results are usually via line graph to show change in pH over time (example shown in F**igure 3A**) or as a scatter plot showing extracellular pH at a single point in time (example shown in **Figure 3B**). Depending on experimental need, additional controls and treatments can be included. Additionally, this assay can be modified to monitor extracellular under a wide range of conditions. For example, the SDR reader can be placed in a hypoxic chamber and the extracellular pH can be monitored.

FIGURE AND TABLE LEGENDS:

Figure 1: Inverted plating of T84 IEC. 100 µl of T84 IEC cell suspension was plated on the underside of 5 µm pore permeable inserts.

Figure 2: Image of plate reader and 24-well pH sensing plate

Figure 3: Change in extracellular pH over time. (A) Changes in extracellular pH over time. Extracellular pH in T84 cultures was recorded every minute for 600 min in the presence or absence of 1 x 10^6 PMN ± fMLP. (B) At 600 min the difference in pH between 0 min and 600 min were calculated. Data expressed as the mean ± SEM.

DISCUSSION:

In this protocol, there are several key steps. Monolayers should be confluent, but not overconfluent. For T84 IEC, they should be used 7-10 days after plating. Human and murine

enteroids grow at different rates than T84 IEC and the researchers should determine how long it takes each line to reach confluency. It is important that the researchers need to use minimally buffered media to ensure shifts in extracellular pH are observed. HBSS+ contains glucose and is suitable for experiments shorter than 12 h. T84 IEC incubated in HBSS+ for periods longer than ~16 h begin to loss barrier function. If researchers are interested in extended experiments complete media, containing FBS, should be used and the plates incubated in a 37 °C incubator with 5% CO_2.

Researchers can modify this assay for different applications. For example, both IEC and PMN can be pre-treated with various compounds targeting metabolic or signaling pathways. Additionally, this assay can be expanded to include the use of other immune cells or the impact of various stimuli on IEC alone. Likewise, a similar model using an oxygen-sensitive fluorescent probe has been used to monitor oxygen consumption in real-time during PMN TEM[11,12]. Finally, researchers can use cell lines other than T84 human IEC. Human/murine enteroids can be grown as 2D monolayers and assayed as described here[13].

All assays have their limitations. For this assay, the experiment is being carried out in a closed system. Extended incubations might be influenced by the depletion of a crucial nutrient or the accumulation of metabolites. Additionally, this assay lacks potential signaling from other parenchymal cells within the tissue environment. Therefore, further confirmation of the *in vitro* findings using other *in vitro* and *in vivo* methods would be beneficial.

ACKNOWLEDGMENTS:
NA

DISCLOSURES:
No disclosures

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Figure 1

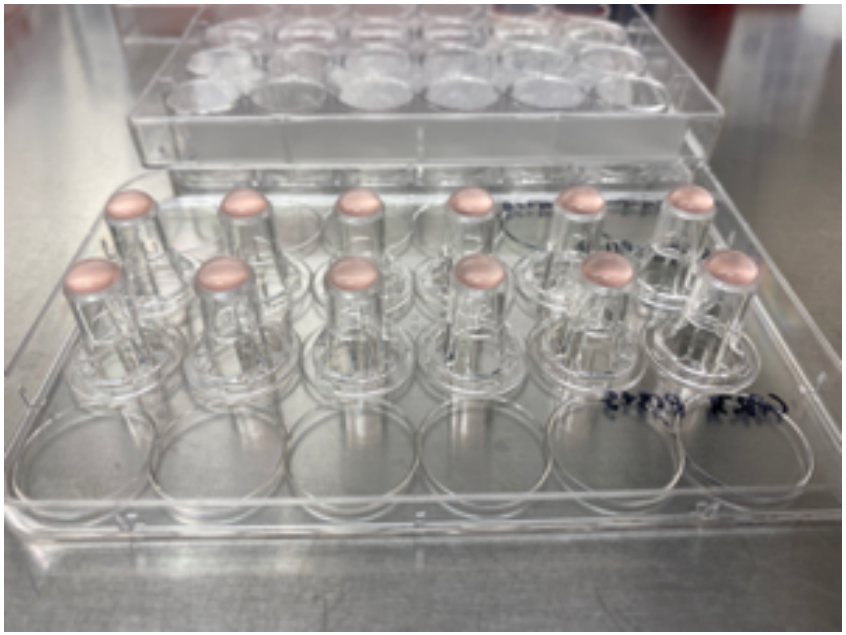


Figure 2

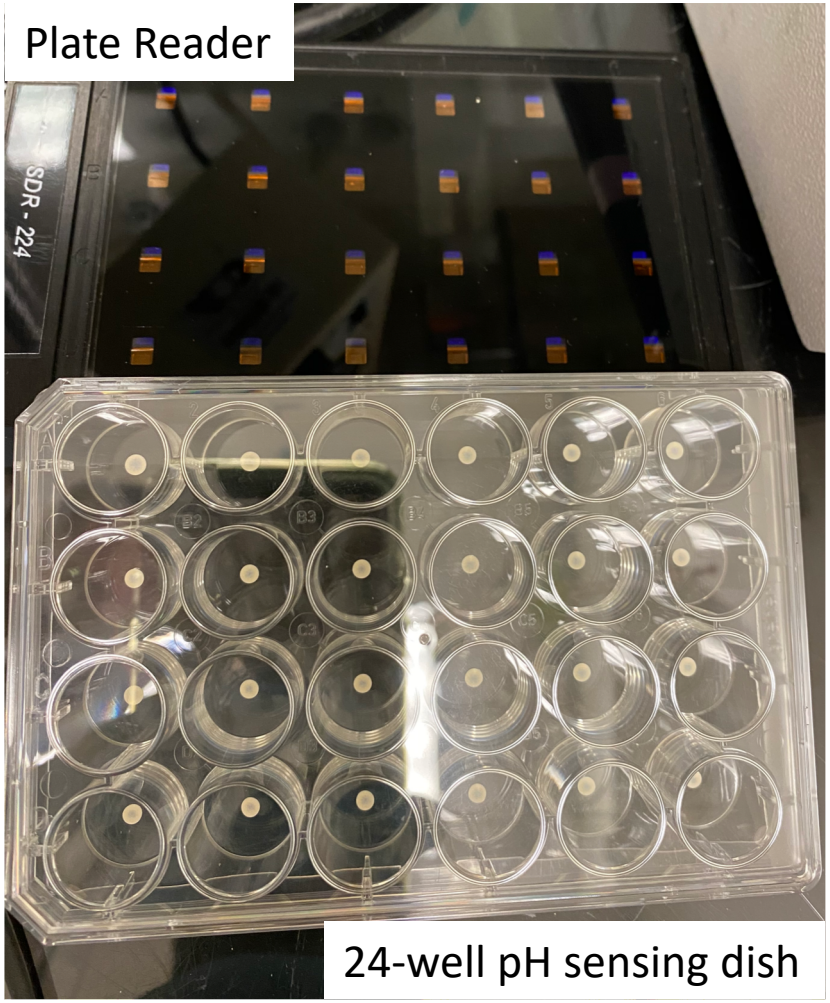
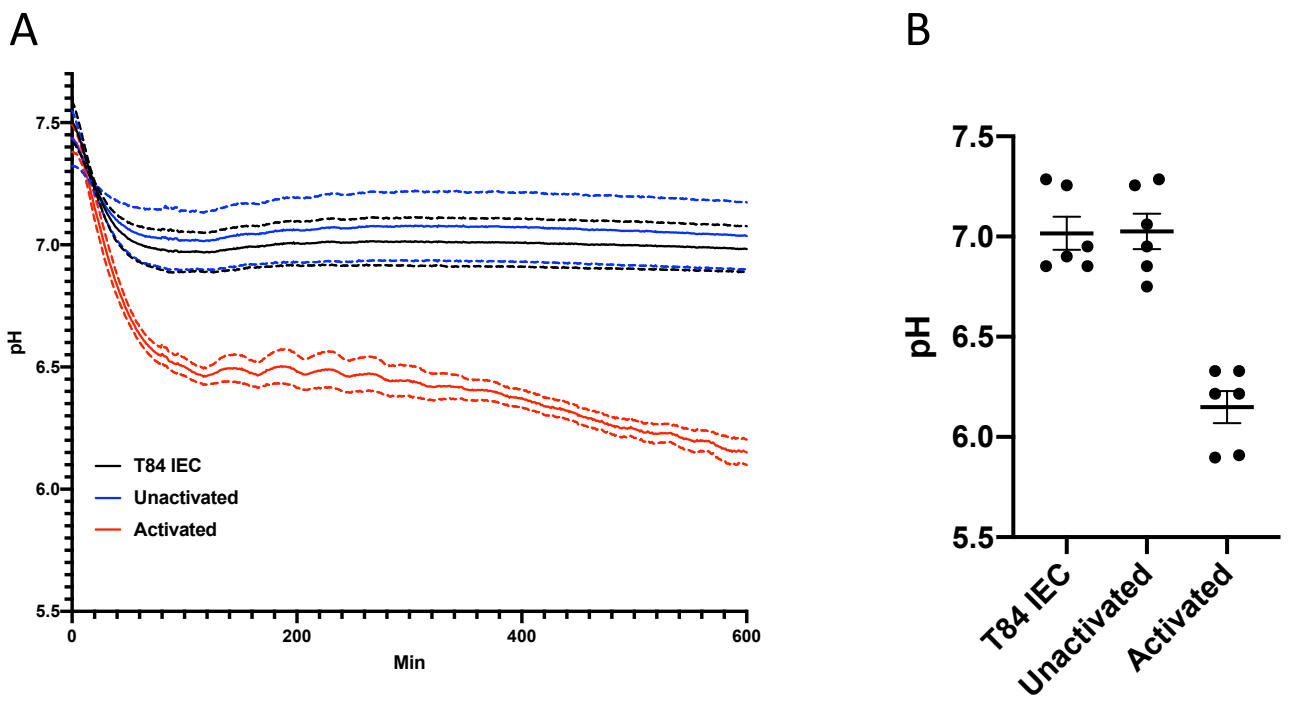


Figure 3



Name of Material/Equipment	Company	Catalog Number	Comments/Description
10 mL serological pipettes	Corning	4101	
24-well plate	Corning	CLS3527-100EA	
5 µm pore inserts	Corning	3421	
50 ml sterile conical tube	Corning	0553855A	
75 cm^2 flask	Corning	430641U	
DMEM/F12	Gibco	10565-018	
FBS	Gibco	26140	
GlutaMax	ThermoFisher	35050061	
HBSS-	Sigma-Aldrich	H4891-10X1L	
HBSS+	Sigma-Aldrich	H1387-10L	
Histopaque T1077	Sigma-Aldrich	10771-6X100ML	
Histopaque T1119	Sigma-Aldrich	11191-6X100ML	
HydroDish HD24	PreSens	NA	https://www.presens.de/products/detail/hydrodish-hd24
PBS	Gibco	14190-144	
Pen Strep	Gibco	15140-122	
RBC lysis buffer	ThermoFisher	00-4333-57	
SDR Reader	PreSens	NA	https://www.presens.de/products/detail/sdr-sensordish-reade
Trypsin	Fisher Scientific	25200114	

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Mucosal Inflammation Program

Dear Dr. Bajaj,

Thank you for you review of our manuscript entitled "*In Vitro* monitoring of extracellular pH in real-time". The manuscript has been revised according to the recommendations of the reviewers and the editorial comments. Please find enclosed the revised manuscript and a detailed description of the changes made in a point-by-point fashion. All changes to the manuscript are marked in red font.

Editorial and production comments:

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

2. Please provide an institutional email address for each author.
   - We have added emails for all authors

3. Please revise the following lines to avoid previously published work: 25-26, 72-73, 94-95, 142-143, 178-179.
   - Manuscript has been revised to avoid previously published work.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
   - We have thoroughly edited the manuscript to remove personal pronouns

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For example: Glutamax, Histopaque, HydroDish, PreSens Measurement Studios software, etc.
   - References to trademarks have been removed

6. Please revise the Introduction to include all of the following with citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

- Introduction has been thoroughly revised to address the points above.

7. Line 70/89/92/103: Please insert the degree symbol between the number and the unit (revise “37 C“ to “37 °C“).

- Corrected throughout manuscript

8. Line 87/101/114/118/120/127: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.

- All SI units have been reformatted and now use standard abbreviations

9. Line 122/129/133/137: Please maintain a single space between the numeral and (abbreviated) unit (“700xg” becomes “700 x g”, “5x104” becomes “5 x 104”).

- Revised throughout

10. Line 147: Please specify the volume of HBSS used for washing.

- Clarified volume of all wash steps

11. Please title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

- References formatted using the Endnote file located on the JoVE website. https://www.jove.com/files/JoVE.ens

12. Figure 2: Please consider removing the figure as JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. All commercial products should be sufficiently referenced in the Table of Materials.

- The figure has been revised to remove commercial language

13. Please sort the Table of Materials in alphabetical order.

- The revised table of materials is now in alphabetical order

Video:

Please note that we are awaiting your JoVE style video so that it can be reviewed. If the video is ready, please upload a high-resolution version here: https://www.dropbox.com/request/bX194HcV4mlLjmA6fpNo?oref=e

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors describe a new model how HydroDishes can be utilized to measure changes in extracellular pH upon neutrophil trafficking.

Major Concerns:
(line 149-150) it is not self explaining what a HydroDish is, this dish should be introduced earlier

- We have introduced the hydrodish in the introduction, line 73, where we refer to it as a pH sensing plate.

(line 157-158) the same is true for the SDS reader and the physical principle of the test, the SDS reader should be introduced earlier

- The SDS reader, now referred to as a plate reader, is introduced and discussed in the introduction (line 70-76).

Minor Concerns:
(line 44-45) It should be mentioned that a pH of 5 and 6 refers to luminal pH (in contrast to tissue)

- Thank you for this comment, we have clarified that the pH refers to luminal pH.

(line 75-77) Name the cells here

- Corrected this to include human T84 intestinal epithelial cells.

(line 105-107) How is TER measured

- Use of a epithelial volt/ohm meter was included in line 130.

Reviewer #2:
Manuscript Summary:
The manuscript describes a novel procedure to measure the pH of micro-environments in real time. this correlates to the pH in the intestinal epithelium as polymorphonuclear leukocytes migrate across polarized monolayers of T84 intestinal epithelial cells. Such cells mimic the intestinal cells in the large bowel and the pH of the environment may correlate with what occurs in vivo.

Major Concerns:
no real major concerns

Minor Concerns:
to test the system, I would like to see if beginning at a low pH or a higher pH has any effect on the cells and if they might modify that pH

- The reviewer raises an excellent point here. We have added a reference to a paper which addressed this point in the introduction, line 73.

Sincerely,

Ian M. Cartwright, Ph.D.