We thank the editor and the reviewers for the preliminary acceptance as well as the insightful comments and critiques. The protocol has been modified to address the reviewers’ concerns and suggestions. We have included a point by point response to each question and/or remark.

**Editorial comments:**

1. All authors proofread the manuscript for spelling and grammar.
2. A total of 6 keywords/phrases are now included.
3. We have expanded the introduction to include relevant alternative techniques and previous studies. (Lines 58-68)
4. Generic names have been substituted for commercial product names in the text as requested.
5. Protocol steps were revised to imperative tense and suggested notes were moved to the discussion.
6. Approximate volume (5 mL) is now indicated in the text. (Line 91)
7. A short overview of enteroid/colonoid formation and maintenance in 3D culture has been added to the text (Lines 106-110). Many of the notes in the protocol that were better suited to the Discussion section were relocated accordingly (Lines 360-383 and 390-397).
8. Organoid Harvesting Solution and all other similar commercial product compositions are proprietary, so we are unable to list the specific components in the Table of Materials.
9. The discussion has been expanded to include limitations and future applications. (Lines 439-453)
10. The panels in Figure 1 have been resized to matching dimensions.
11. Table 1 has been uploaded as an .xlsx file.
12. Table of Materials has been alphabetized.

**Reviewer #1:**

1. Antibody and shRNA sources with catalog numbers were added to the Table of Materials.
2. Step 1.3 was amended as suggested.
3. Coated inserts can be stored at 4 °C for up to 1 week; this information has been added to the text. (Line 101)
4. A brief description of crypt isolation, 3D culture in basement membrane matrix (BMM/Matrigel), and apical and basolateral orientation has been added. (Lines 106-110)
5. The word ‘material’ is preferable in this case as we are referring to both BMM (Matrigel) and cells.
6. A brightfield image of colonoids post-trituration has been added to Figure 1, panel A.
7. We have added a sentence in the Discussion to explain that the optional incubation with trypsin and wash step is recommended if the user is having difficulties obtaining uniform fragments with trituration. (Lines 371-374)
8. We use transparent PET membrane Transwells, so no special handling is necessary to view cells on a standard light microscope.
9. The difference between 1 and 2 days is negligible and offers some flexibility, for example, when plating on a Thursday and then feeding on a Friday to avoid weekend maintenance. A standard 2 day interval between feedings is otherwise appropriate.
10. It does not appear to be standard practice in JoVE articles to refer to Figures in the Protocol section, so we are hesitant to breach this precedence. The author instructions designate Figures to be referenced in the Representative Results section.
11. We have included our recommendation for *E. coli* HS or EHECinfection and the suggestion that users measure the OD and infection time for their pathogen of interest. (Lines 385-388)
12. We have added a section on harvesting colonoid monolayers for immunoblotting. (Lines 240-257)
13. Author instructions requested a Representative Results section with reference to each of the figures (please also see our response to point #10). We have added asterisks to Figure 1 to designate the cell-free areas (black regions with no nuclei or F-actin present) and arrowheads to indicate F-actin perijunctional rings and brush border. Figure legends were adjusted to describe the days post-seeding for each image.
14. We and others have previously shown images of goblet cells (immunostained with MUC2 and/or WGA) and enteroendocrine cells (immunostained with CHGA) in 3D cultures and monolayers. Specific reference to these examples have been added (Lines 273-274). Clarke’s fixative preserves the external mucus layer (Fig 4) but does not allow for intracellular staining of the goblet or enteroendocrine cells. Due to the novelty of studying the secreted mucus layer, we focused on that particular protocol.
15. We have clarified when the transductions are performed. Lentiviral transductions are performed in 3D cultures in order to generate a KD culture over indefinite passages. The KD culture is plated as monolayers for experimental purposes. (Lines 288-291)
16. We have added a section on harvesting monolayers for immunoblotting.
17. We have moved these lines, per reviewer’s suggestion.
18. We and others have commented on the observation that EHEC appears to be colocalizing on or near the goblet cells. Our unpublished results and others’ data suggest that EHEC is attaching to a component of the mucus layer via flagella or fimbria and utilizing the mucus as a nutrient source. We are actively working on this question.

**Reviewer #2:**

*Introduction concerns*

* The term ‘serosal’ has been changed to ‘basolateral’.
* The microinjection citations have been added.
* The introduction now mentions specific withdrawal of inhibitors A-83-01 and SB 202190 during differentiation.

*Methods concerns*

* Criteria for including the optional trypsin digest before plating monolayers is now outlined in the Discussion. (Lines 371-374)
* Information about antibodies has been added to the Table of Materials.

*Results concerns*

* F-actin was chosen as a general marker that allows visualization of the entire cell morphology, including brush border, whereas TJ markers don’t give that information. Additionally, many TJ proteins are not uniformly expressed throughout the sub-confluent monolayers, which makes it difficult to follow the process of confluency formation.
* The images in Figure 2A are jejunal monolayers and has been updated in the legend; similar results are obtained from all segments. Our intent was to demonstrate that the general methodology works for any intestinal segment.
* Figure 3A shows the secreted mucus layer that covers all cells on the apical surface. This staining can only be performed when cells are subjected to an anhydrous acidic fixative such as Clarke’s solution. We were not staining for intracellular MUC2, which is better visualized by buffered paraformaldehyde fixation and detergent permeabilization in our hands. Additionally, mucus location relative to the cell membranes would be ideal, but Clarke’s fixation renders many epitopes unreactive to antibodies or phalloidin (for F-actin).
* Cell height/thickness, general growth rate to confluency, TER, and other measurable criteria are not significantly different between enteroids (small intestine) and colonoids (colon).

*Discussion concerns*

* To develop the technique for growing confluent enteroid/colonoid monolayers, we tested laminin, fibronectin, collagen IV and BMM (Matrigel). We were able to obtain small patches of monolayer-like growth on all the substrates, but only collagen IV supported reproducible formation of confluent monolayers. Therefore, we obtained negative data for the other ECMs. (Lines 407-408)
* We believe that human collagen IV as the insert coating is indispensible for longer-term confluent cultures than the previously published 6 d. If monolayers are kept in expansion medium, they remain stable and confluent for approximately 3 weeks, after which they were transitioned to differentiation medium for 1 additional week. We have not examined cultures beyond this period.