Electronic Supplementary Material (ESI) for Lab on a Chip.
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Supplementary Information

1. Preparation of Wnt3A, R-spondin 2, and Noggin-conditioned media

HEK-293 cells producing Noggin protein were prepared by transfecting wild type HEK-293 cells with mouse noggin cDNA (Origene #MC209047) by DreamFect transfection reagent (OZ Biosciences #DF40500). L-cells and HEK-293 cells producing and secreting Wnt-3A protein and R-spondin-1 protein, respectively, were a kind gift from Dr. Jeffery Whitsett (Cincinnati Children's hospital). The cells were cultured in advanced DMEM/F12 medium supplemented with 10% FBS, GlutaMAX (1×), penicillin (100 unit/mL), streptomycin (100 μg/mL), and gentamicin (5 μg/mL). Selective antibiotic G418 sulfate (400 μg/mL) was added in the medium for the passage of the cells, but it was not added for the preparation of the conditioned media.

To prepare the Wnt-3A-conditioned medium, 5×10^5 L-cells suspended in 10 mL culture medium (*note: no addition of G418 sulfate*) were plated in a 10-cm, tissue-culture dish. The cells were allowed to grow for 3-4 days until they reached to 80-90% confluency. The medium was removed and centrifuged at 3,000×g for 10 min. The supernatant constituted the first batch of medium and was stored at 4 °C. 15 mL fresh medium was added to the dish and the cells were cultured for an additional 3 days. The medium was taken off and centrifuged at 3,000×g for 10 min. This supernatant was the second batch of medium. The first batch and second batch of media were mixed, filtered through a 0.22 μm membrane, and stored at -80 °C.

R-spondin-2 conditioned medium was prepared in the same manner as the Wnt-3A conditioned medium using the HEK-293 cell line that produced R-spondin-2 protein.

Noggin conditioned medium was prepared in the same manner as the Wnt-3A conditioned medium using the HEK-293 cell line that produced Noggin protein.

2. Preparation of the PDMS microwell array

PDMS microwells labeled numbers/alphabets were prepared in three microfabrication steps as illustrated in Fig. S1.

(1) Fabrication of the master mold on a glass substrate (Fig. S1A). The master mold contained an array of microwells firmly adhered to the glass substrate. The layer was a 150-μm thick epoxy film with array of wells of 150 μm in diameter. Letters and numbers were created on the borders to label the addresses of the wells. The popular SU-8 photoresist was not used to fabricate the mold due to the poor adhesion of SU-8 to the glass substrate. Instead, a less rigid, 1002F epoxy photoresist was used which has much improved adhesion to the glass substrate. [1]

A glass slide (75 mm × 50 mm × 1 mm, Corning) was cleaned using a plasma cleaner (Harrick Plasma) for 5 min. A 150-μm thick layer of 1002F photoresist was spin-coated onto the glass slide using 1002F photoresist (formulation 10) on a spin coater (Laurell Technologies) at 500 rpm, acceleration setting of 1 (108 rpm/second), 10 s followed by 1500 rpm, A = 4, 30 s. The photoresist was baked in a 95 °C oven for 60 min to evaporate the solvent. The photoresist was then exposed to UV light (800 mJ/cm^2) through a photomask to pattern the resist. The post-exposure baking was performed in a 95 °C oven for 10 min. The sample was then developed for 20 min, rinsed with propylene glycol monomethyl ether acetate (PGMEA) and isopropyl alcohol (IPA), and dried by purging with a stream of nitrogen. The sample was hard baked on a 120 °C hotplate for 60 min to harden the film. Finally, this 1002F master mold was treated with octyltrichlorosilane in a vapor-phase silanization process in a polycarbonate desiccator (Fisher Scientific). 50 μL octyltrichlorosilane was added in a small vial and placed in the desiccator. The dessicator was degassed using an oil-free pump for 2 min and then sealed for 16 h.

(2) Fabrication of a PDMS mold by replica molding (Fig. S1B). Two PDMS spacers (thickness, 200 μm) were placed at the ends of the master mold. PDMS pre-polymer was spread on the master mold, and trapped air bubbles were removed by degasing under house vacuum. A glass slide (75 mm × 50 mm × 1 mm) was treated with air plasma for 5 min and placed on the master mold. Two paper clips were applied at the ends to ensure the flatness of PDMS sandwiched between the master mold and the glass slide. The assembly was then baked in a 95 °C oven for 10 min. After separating the glass slide from the master mold, PDMS remained on the glass slide (since PDMS has very good adhesion to plasma cleaned glass, but it has very poor adhesion to an octyltrichlorosilane treated surface). The PDMS mold was baked on a 120 °C hotplate for 60 min to fully polymerize the elastomer. Finally, the PDMS mold was treated with plasma for 2 min, and reacted with octyltrichlorosilane in a vapor-phase silanization process for 16 h.

The PDMS mold was replicated from the master mold so that it contained an array of posts (150 μm in height, 150 μm in diameter).

(3) Fabrication of a PDMS microwell array by replica molding (Fig. S1C). PDMS pre-polymer was spread on the PDMS mold, and trapped air bubbles were removed by degasing under house vacuum. PDMS was cured in a 95 °C oven for 30 min. After cooling to room temperature, the PDMS microwell array was easily detached from the glass slide to form a freestanding film.

The array of microwells had a dimension of 7 mm × 7 mm. The array had 9 subregions. Each subregion was labeled by two capital letters (*e.g.* "AC" in Fig. S1D) and had 10×10 microwells. The address of wells was designated by lowercase letters (for rows) and numbers (for columns). The array was glued to a polycarbonate circular chamber by PDMS and cured at 95 °C for 30 min. The chamber had an inner diameter of 12 mm, an outer diameter of 20 mm, and a height of 12.7 mm (Fig. S1E).

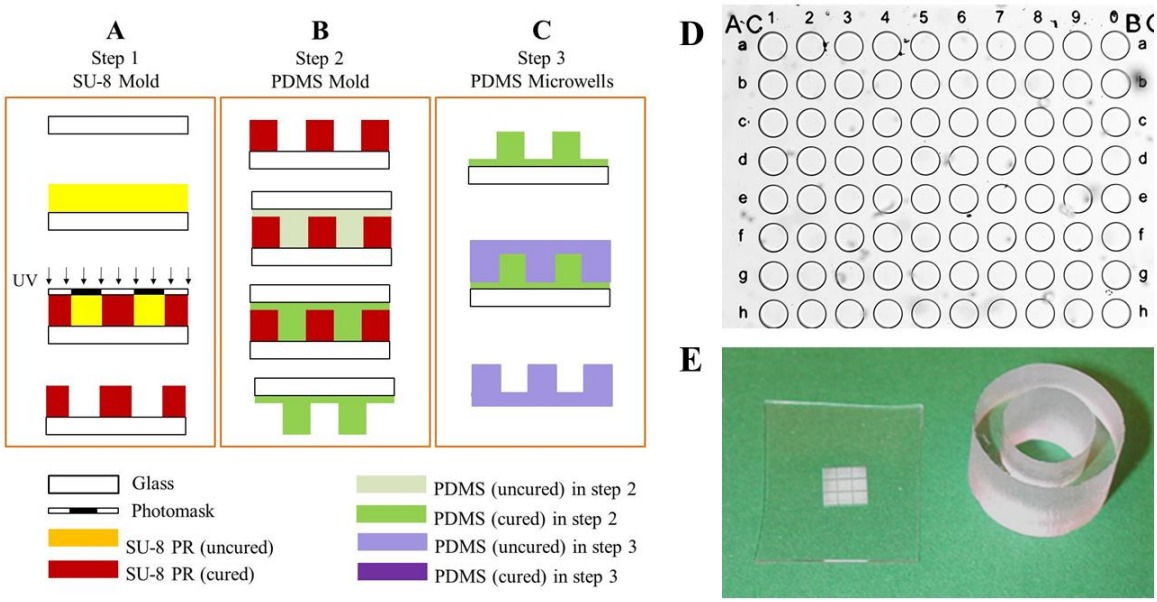

Fig. S1. *Three fabrication steps for PDMS microwell array. (A) Step 1: making an epoxy master mold. (B) Step 2: making a PDMS mold by replicate molding. (C) Step 3: making a PDMS strainer array by replica molding under pressure. (D) 4× microscopic image of the array. (E) Photo of the PDMS microwell array and a polycarbonate circular chamber.*

3. Comparison of 2D and 3D culture of crypts on a planar PDMS surface

The non-proliferative 2D culture of crypts on a PDMS surface was supported by two lines of evidence: (1) The average number of cells in 2D culture slowly declined over time, from 7,400 ±2,000 at day 1 to 6,500 ±1,500 at day 7 (Fig. 2C). (2) The complete loss of EGFP fluorescence starting at day 2. EGFP expression is driven by the Sox9 promoter and is thus a marker of cells with proliferative capacity (*i.e.* stem/progenitor cells).

The morphological difference of 2D and 3D culture was clearly shown in the culture of a high density of crypts (a total of 2,000 crypts per surface) at day 7, where 2D culture formed a continuous monolayer of cells while 3D culture generated isolated colonoids (Fig. S2).

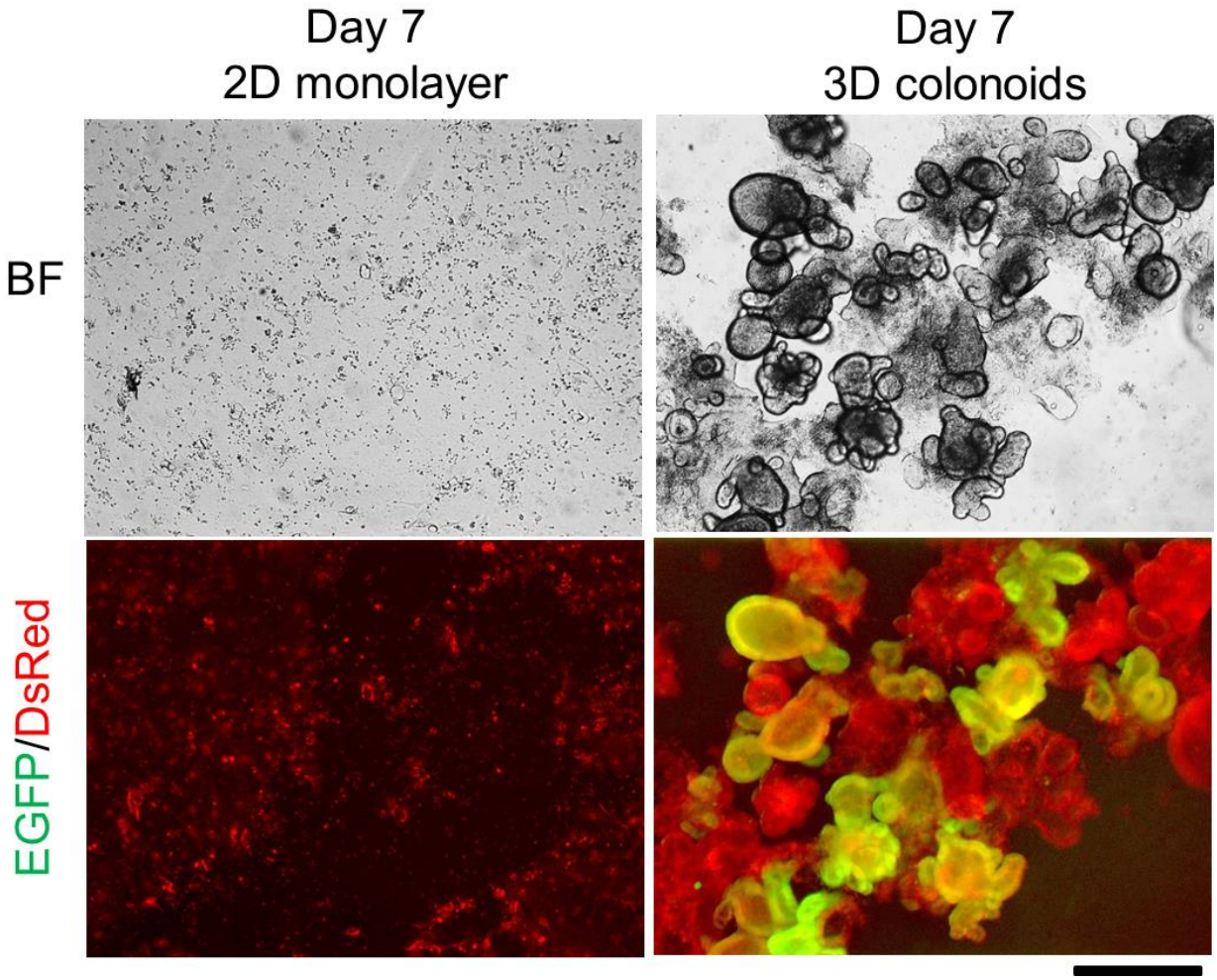

Fig. S2. *2D and 3D culture of crypts on or over a planar PDMS surface. 4× microscopic image of a cell layer generated by 2D (left) and 3D (right) on a PDMS surface at day 7. 2,000 crypts were loaded on each surface. Both brightfield (top panel) and overlaid EGFP and DsRed fluorescence (bottom panel) images are shown. Scale bar = 500 μm.*

4. Time lapse imaging of 2D/3D hybrid culture of crypts in microwells

Video 1. 3D to 2D transition of a crypt when it was cultured in a microwell from 48-96 h. It first expanded in the well as a 3D spheroid. When the colonoid reached the top of the well where Matrigel was absent, the cells began growing as a 2D monolayer.

Video 2. A continuous 2D monolayer connected adjacent crypts located in 5 separated wells. The culture time was 48-96 h. At 96 h, five colonoids in separate wells formed a single tissue.

5. Wide-field-of-view of 2D/3D growth of crypts on the microwell array

Fig. S3A shows a wide-field-of-view of 80 microwells. 49 of 80 wells were filled with crypts at day 0. The crypts first underwent 3D expansion inside the wells. At day 2, only 7.1 ± 5.3% colonoids transited to 2D/3D hybrid structure (n = 3 arrays). At day 6, 96.5 ± 4.3% colonoids formed a 2D/3D hybrid structure. The 2D monolayer on the top of the wells started to merge at around day 4. The tissue area coverage was quantified by Image J, which clearly show the progressive generation of a continuous colonic epithelial tissue in 2D/3D culture model (Fig. S3B). Most of the 3D-2D transition occurred around day 2-5, and the surface coverage rapidly increased from 36 ± 4% (day 2) to 77 ± 7% (day 5). A continuous tissue was formed around day 6 when 3D colonoids in adjacent microwells were connected by a 2D monolayer.

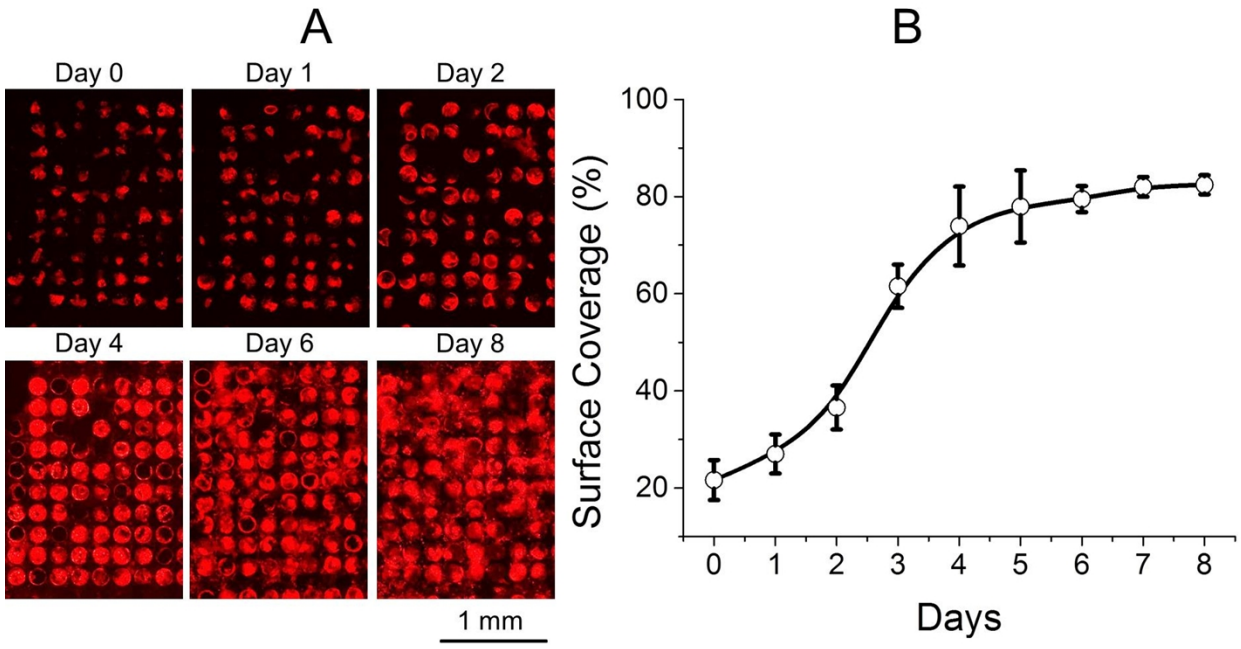

Fig. S3. *Progressive generation of a continuous colonic epithelial tissue in a 2D/3D hybrid culture model. (A) 4x DsRed fluorescence images of the array at the same location, imaged at day 0, 1, 2, 4, 6 and 8. (B) Surface coverage vs. days.*

6. Comparison of 2D, 3D, and 2D/3D hybrid culture of crypts in microwells

Control experiments were performed by culturing crypts in microwells in 2D culture conditions only, 3D culture conditions only, and 2D/3D hybrid culture conditions (Fig. S4).

In 2D culture, crypts were loaded into microwells without filling the wells with Matrigel. As expected, crypts formed monolayers in and out of the microwells, and all cells lost EGFP fluorescence. Typical images are shown in Fig. S4A, top panel.

In 3D culture, crypts were loaded into microwells and overlaid with a thick layer of Matrigel that filled the wells and the space above the surface of the array. For this experiment, the Matrigel was not aspirated from the array such that a conformal layer of Matrigel was formed over the entire array. Crypts underwent 3D expansion first inside the wells; however, the colonoids continued to expand when the cells reached the top surface of the microwells into the overlying Matrigel layer. Typical images are shown in Fig. S4B, middle panel. EGFP fluorescence was retained in all parts of the colonoid. Thus, the microwell alone cannot guide the growth of crypts into a crypt-like architecture.

In 2D/3D hybrid culture, crypts were loaded in microwells and overlaid with 200 µL liquid Matrigel (4 °C). Matrigel was then aspirated form the array. The microwells retained Matrigel to generate isolated Matrigel pockets embedding the crypts. The crypts expanded into colonoids, then the cells spread across the upper surface in a monolayer. Typical images are shown in Fig. S4C, bottom panel. EGFP fluorescence was retained only inside the wells. Both the PDMS microwells and Matrigel pockets were required to guide the growth of crypts in this unique 2D/3D hybrid culture to form an architecture that mimicked the crypt.

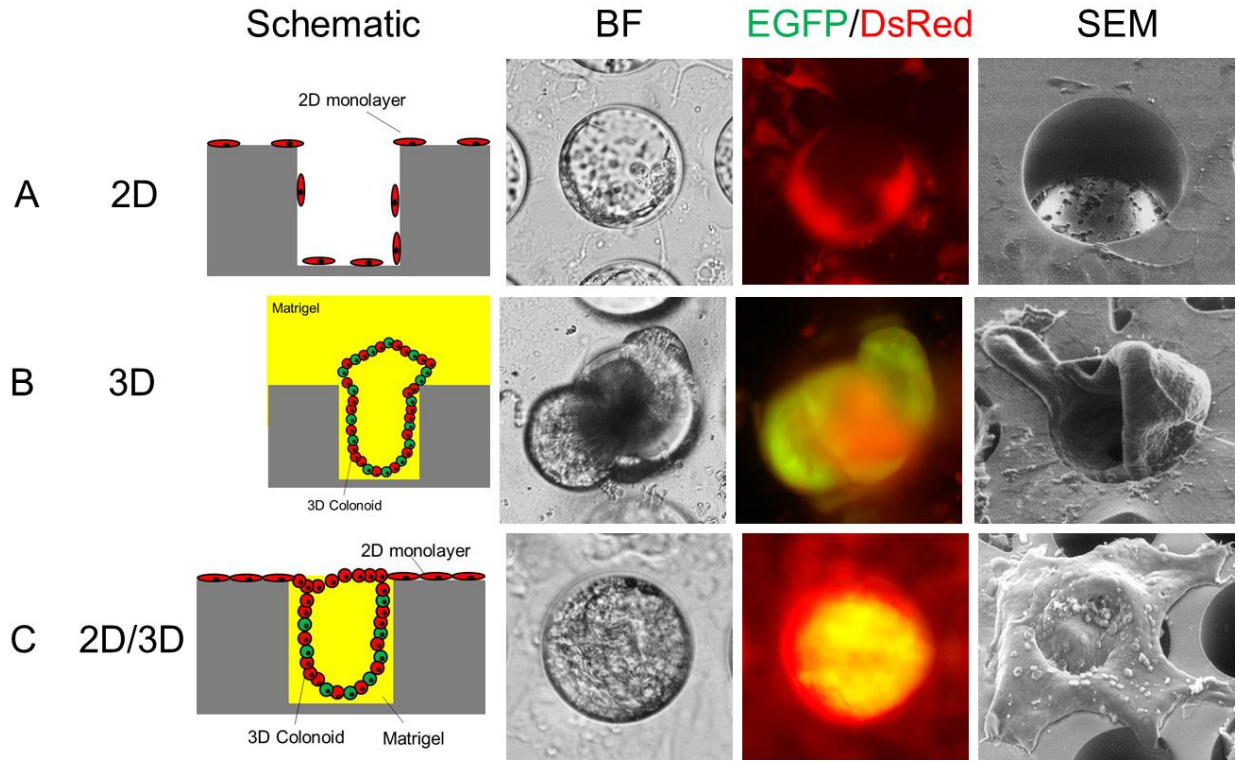

Fig. S4. *Comparison of (A) 2D, (B) 3D, and (C) 2D/3D hybrid culture of crypts in microwells. The diameter of wells was 150 µm. All images are at day 4 of culture.*

References

1. J. H. Pai, Y. Wang, G. T. Salazar, C. E. Sims, M. Bachman, G. P. Li and N. L. Allbritton, *Analytical Chemistry*, 2007, **79**, 8774-8780.