Electronic Supplementary Material (ESI) for RSC Advances.

Supplemental Information

Optimizing Wnt-3a and R-Spondin1 Concentrations for Stem Cell Renewal and Differentiation in Intestinal Organoids Using a Gradient-Forming Microdevice

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Supplemental Methods

Fabrication of the 5-mm Gradient Device. The microfabricated polydimethylsiloxane (PDMS) gradient device possessed two reservoirs (each being of 16-mm length, 16-mm width, 5-mm height) connected by a central gradient-generating region (5-mm length, 5-mm width, 300-μm height). The device was constructed in three steps. In the first step, a master mold was fabricated using standard photolithography. A layer of 1002F polymer (250 μm) was spin-coated onto a glass slide and baked at 95 °C for 12 hours. The 1002F layer was irradiated with a UV light source (800 mJ) through a mask to crosslink the 1002F. The 1002F layer was then heated at 120 °C for 1 hour to complete polymerization.[33] Unhardened material was removed by rinsing with SU-8 developer. The resulting master mold was silanized prior to addition of PDMS to improve demolding. A 5-mm layer of PDMS (Dow Corning, Midland, MI) was poured onto the master and cured by baking at 65 °C for 12 hours. After removal of the cured PDMS from the master, the reservoirs were created by cutting square holes in the PDMS. The PDMS device was then attached to a glass slide coated with a 25 μm layer of PDMS after each had been plasma treated for 2 min. The two structures were aligned and brought into conformal contact to form a permanently bonded assembly. The assembled device was then baked at 95 °C overnight to enhance the bond strength.

Quantification of Colonoid Budding. Crypts were isolated from a CAGDsRed mouse and cultured within 12-well plates for 5 days under either the conventional or reduced growth factor conditions (Table S1). Colonoids were fixed and imaged using an Olympus MVX10 research macro zoom fluorescence microscope (0.25N.A. objective and 0.63× demagnification, depth-of-focus of 91 μm). Colonoids possessing DsRed and an area of $\geq$15,000 μm^2 in the 2-D image

slice were evaluated for the presence of budding, solidity, perimeter, and area were quantified from binary images derived from the DsRed fluorescence image. Solidity is a measure of the proportion of the pixels in the convex hull that are also within the colonoid (MATLAB). The perimeter specifies the distance around the colonoid boundary and the area is represented by the number of pixels within the perimeter. The relationship between solidity and its area/perimeter were graphed for a training set of colonoids manually judged to be budding or not budding (n = 30 colonoids for each type, Fig. S5). Support vector machine (SVM) learning was used to classify developed colonoids as either budding or non-budding. A SVM model was generated in MATLAB utilizing a test set of n = 30 colonoids that were accepted as either budding or non-budding, respectively. Budding colonoids in the training set possessed a solidity of 0.68 ± 0.09 and area/perimeter of 26.1 ± 10.2. Training-set colonoids that were non-budding displayed a solidity of 0.94 ± 0.03 and area/perimeter of 86.1 ± 10.9. The two groups formed statistically-significant clusters (Fig. S5). Subsequently, colonoids cultured under the conventional or factor reduced conditions were tested with the learned SVM model to determine the budding characteristics under both experimental conditions.

Passaging efficiency of cultured colonoids. Crypts were isolated from a CAGDsRed mouse and cultured under reduced growth factor or conventional culture conditions (Fig. 4A and Table S13). After 7 days of culture, both sets of colonoids were retrieved from the Matrigel using collagenase digestion (15 min, 37 °C) and then fragmented using trypsin/EDTA (3 min, 37 °C in 0.02 mM trypsin and 0.48 mM EDTA). The slurry was pipetted vigorously for 60 s to break the fragments into single cells. The cells were then rinsed, counted and re-embedded in Matrigel. Cells in Matrigel patties were placed into a 12-well plate (10,000 cells/well, Table S14) and then

cultured under either reduced growth factor or conventional culture conditions. After 5 days, the total number of colonoids formed were counted for each condition and the culture process was repeated. Three passages were conducted. Colonoids were identified as DsRed fluorescent objects with an area of $\geq$10,000 μm^2.

Supplemental Figures

Figure S1. Cross-sectional schematic of the gradient-generating region of the microdevice.

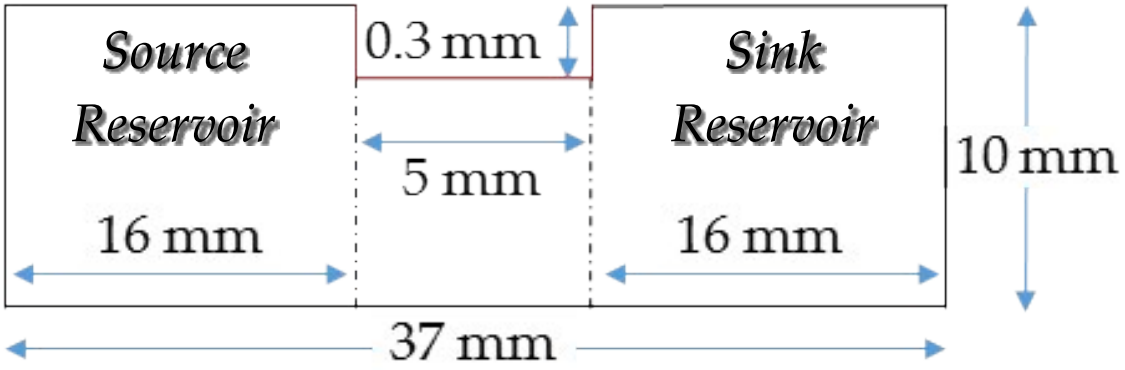
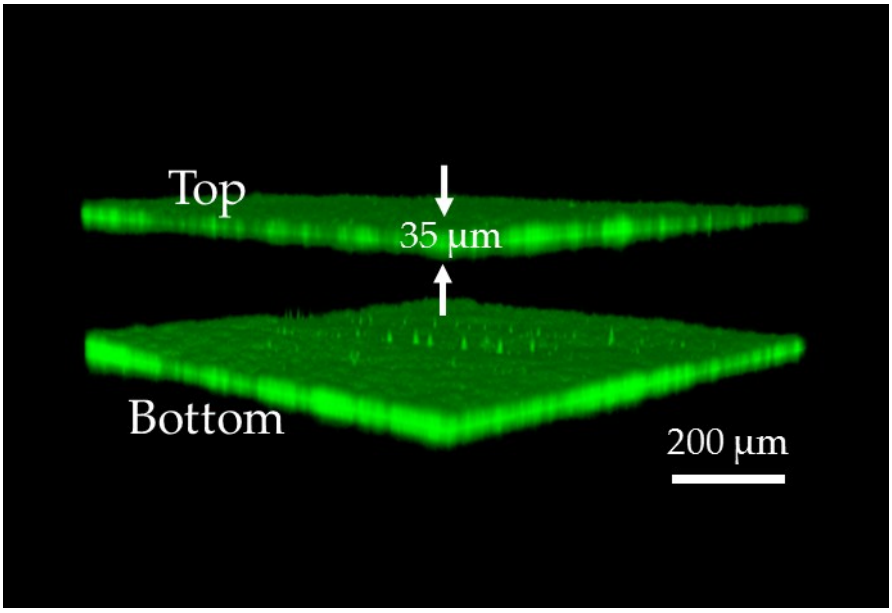


Figure S2. Matrigel pre-coat characterization. Shown is a reconstructed confocal image through a device pre-coated with Matrigel mixed with fluorescein-dextran. The coating on the top and bottom surfaces of the channel are visible as green sheets (top panel) or green lines (bottom panel), but the side walls are out of the field-of-view. The top panel is a tilted 3-D reconstruction while the lower panel is a single reconstructed Z-slice. The coatings were highly reproducible with the average coating thickness of 35 ± 5 μm, surveyed across 3 independent devices.



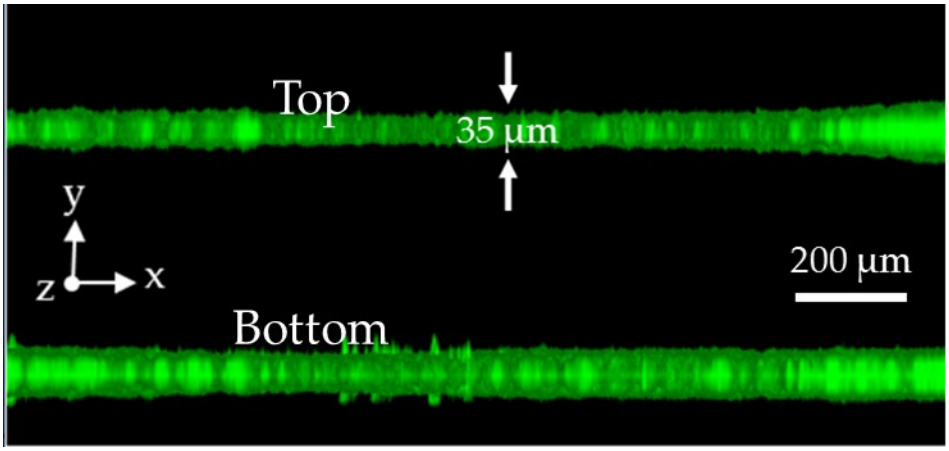


Figure S3. Culture of colonoids in the presence of a Wnt-3a gradient. Colonoid data is shown at days 1 (A, C, E) and 5 (B, D, F). Overlaid red-green images of the colonoids are shown (A,B). The scale bar represents 500 µm. Boxplots were used to represent the non-normal distribution of the area (C, D) or EGFP fluorescence (E, F) per colonoid. Colonoid area is represented as μm^2 ($\times 10^4$) and integrated EGFP fluorescent intensity is represented as RFUs ($\times 10^5$). The R-spondin1 concentration in the source and sink was 175 ng/mL while the Wnt-3a concentration was 0 ng/mL (sink) and 120 ng/mL (source). The threshold concentration (60 ng/mL) coincided at the interface between region 2 and region 3 and is marked by the arrow.

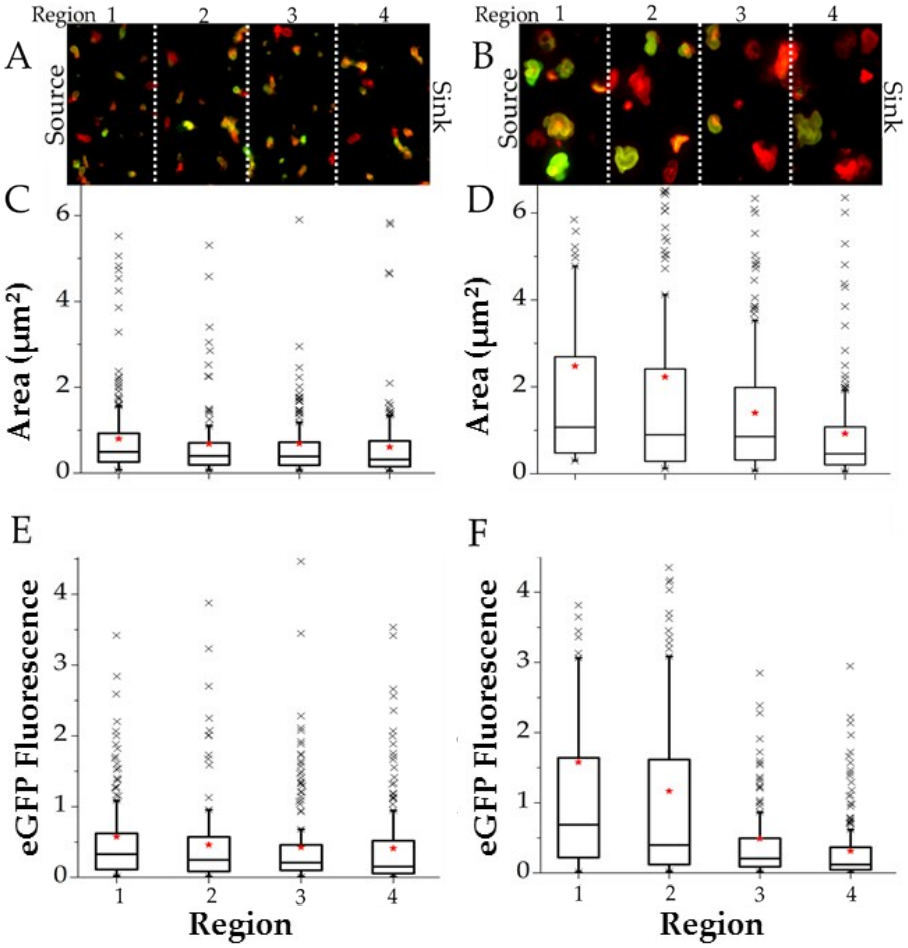


Figure S4. Culture of colonoids in the presence of an R-spondin1 gradient. Colonoid data is shown at days 1 (A, C, E) and 5 (B, D, F). Overlaid red-green images of the colonoids are shown (A, B). The scale bar represents 500 μm. Boxplots as described in the legend of Fig. 2 were used to represent the non-normal distribution of the area (C,D) or EGFP fluorescence (E, F) per colonoid. Area is represented as μm^2 ($\times 10^4$) and integrated EGFP fluorescence intensity is represented as RFUs ($\times 10^5$).The Wnt-3a concentration in the source and sink was 120 ng/mL while the R-spondin1 concentration was 0 ng/mL (sink) and 175 ng/mL (source). The threshold concentration (90 ng/mL) coincided at the interface between region 2 and region 3 and is marked by the arrow.

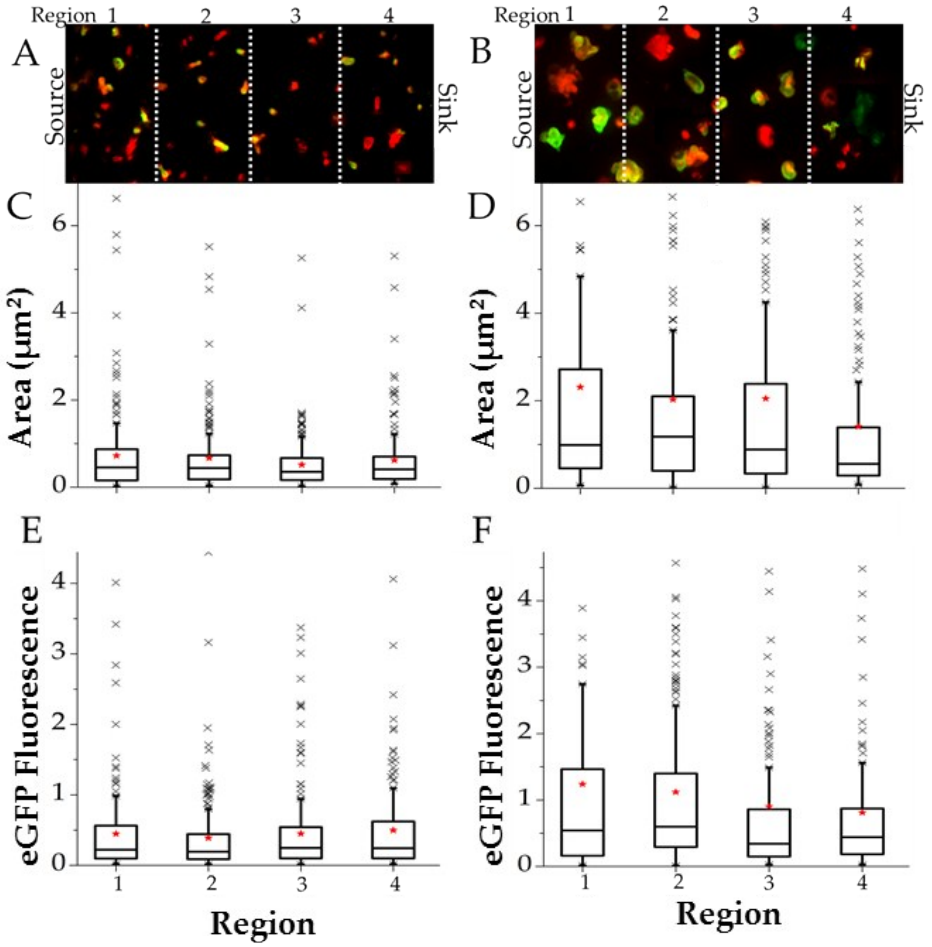


Figure S5. Training colonoid set to used to classify budding *vs* nonbudding colonoids based on area/perimeter and solidity with a learned Support Vector Machine model. Colonoid solidity was plotted against colonoid area divided by colonoid perimeter for colonoids manually identified as budding (n = 30, solid circles) or nonbudding (n = 30, open squares).

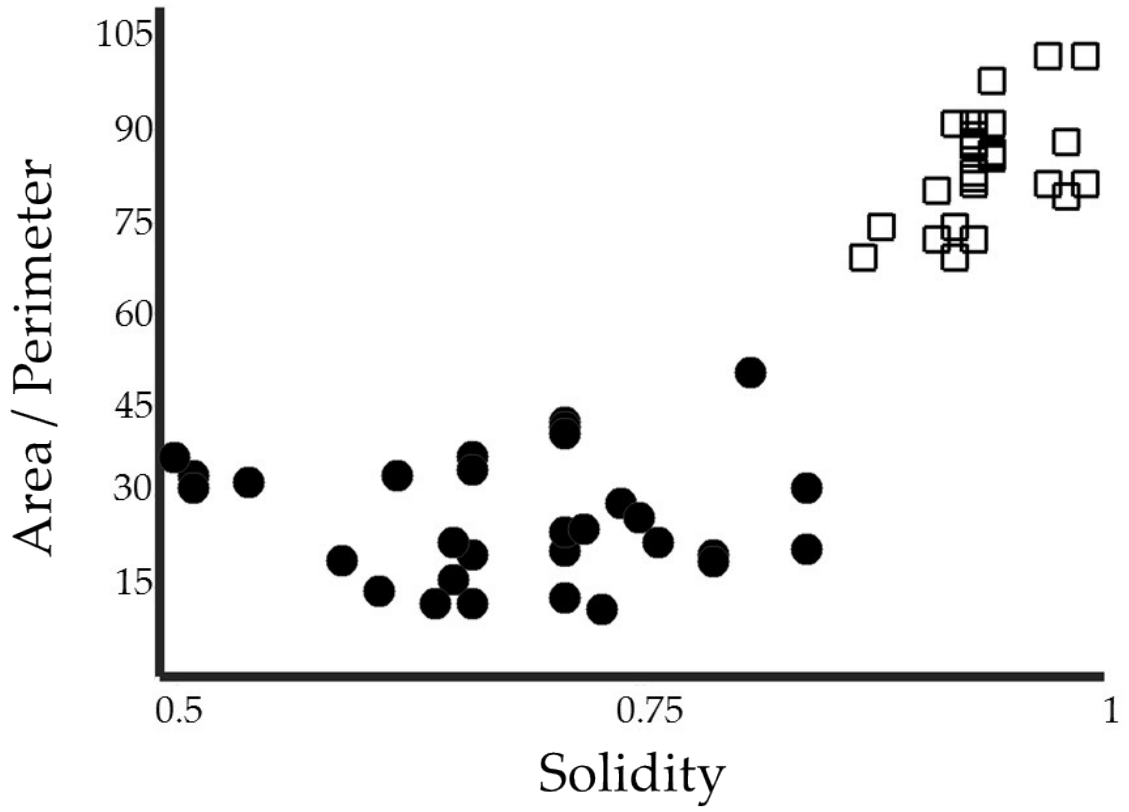


Figure S6. Fluorescence images of colonoids on day 5. Shown are immunofluorescence images for mucin 2 (Muc-2, top row) and chromogranin A (Chg-A, 2nd row). The 3rd row shows a fluorescent EdU-based stain while the final row is EGFP expression (under a Sox9 promoter). The columns are colonoids grown under conventional (left) or reduced factor (right) culture conditions. The scale bars are 150 µm.

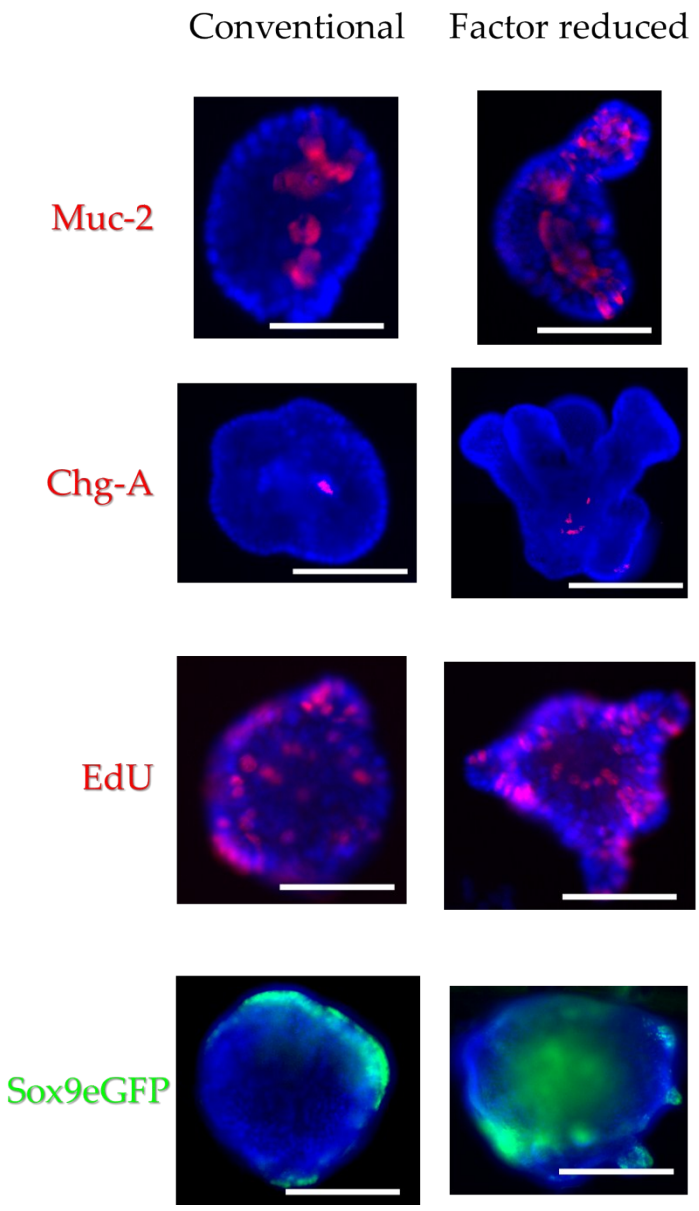


Figure S7. Quantile-quantile plots for the log transformed eGFP fluorescence intensity for the Wnt-3a + R-spondin gradient (Fig. 3E,F) on day 5. The vertical axis of each plot represents the log-transformed experimental data (eGFP fluorescence intensity) while the horizontal axis displays the normal theoretical quantiles. The theoretical quantiles plotted on the x-axis are the predicted values if the data followed a purely Gaussian distribution. The dashed line is the best-fit straight line through the data. R^2 is the adjusted coefficient of determination for the fit to the straight line.

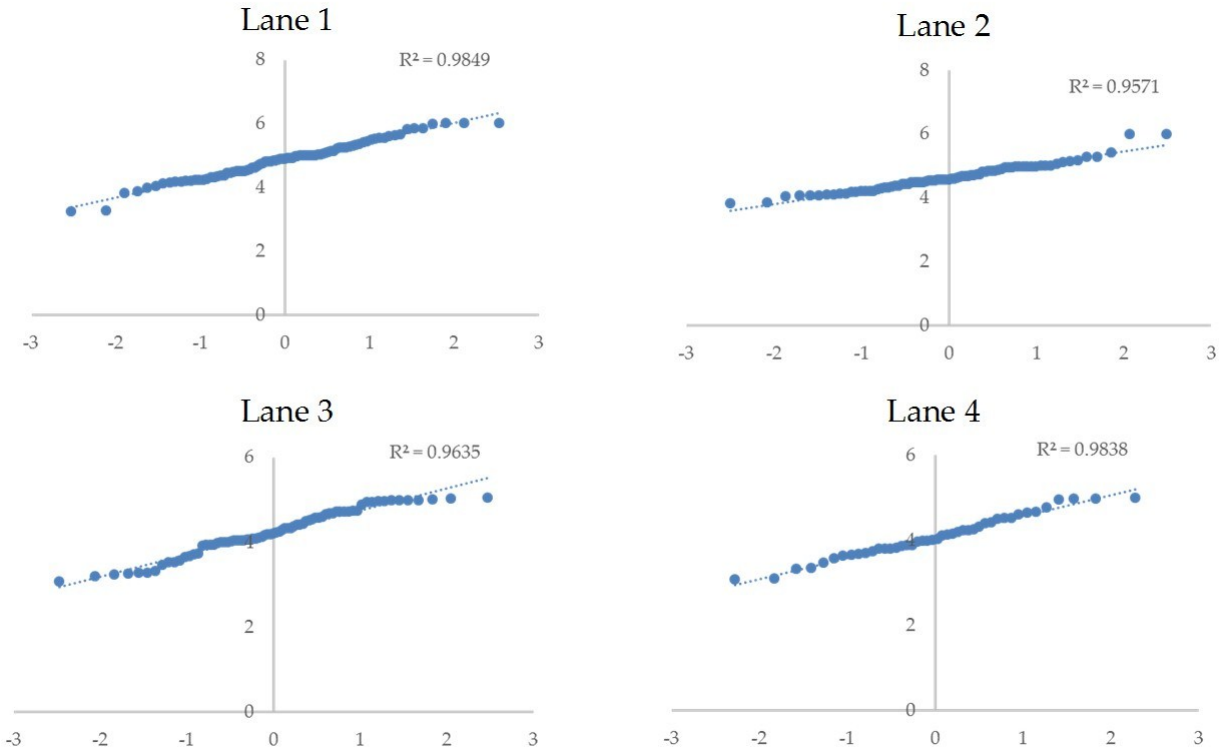


Figure S8. Brightfield (A,C) and fluorescence (B,D) images of colonoids after 15 days in culture with conventional growth factor (A,B) or reduced growth factor (C,D) conditions. The fluorescence images are overlays of DsRed/eGFP fluorescence. The scale bars represent 300 µm for all of the images.

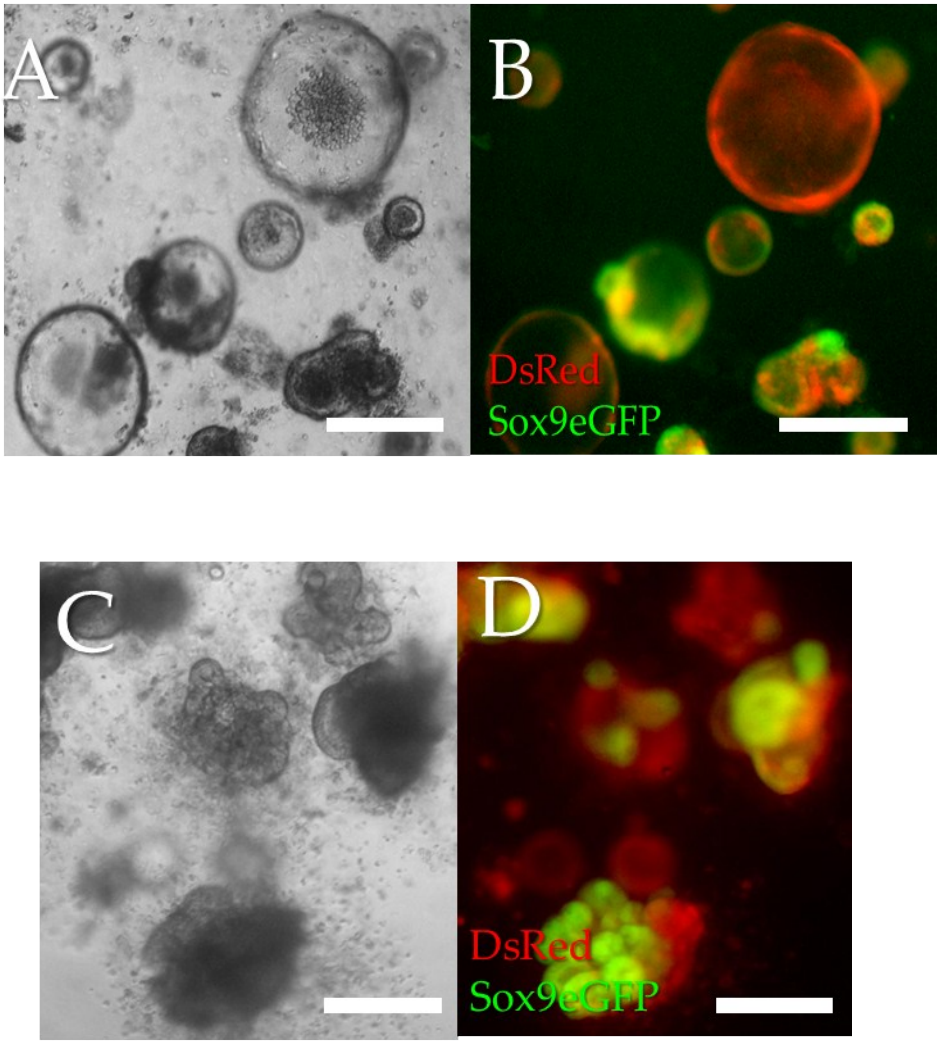


Table S1. Growth factor concentrations in the 4 regions of the microdevice

Region	Wnt-3a ng/mL range (Average)	R-spondin1 ng/mL range (Average)
1	91-120 (105)	131-175 (153)
2	61-90 (75)	89-130 (109)
3	31-60 (45)	44-88 (66)
4	0-30 (15)	0-43 (22)

Table S2. Area occupied by each colonoid in a 2-D image slice in the absence of a gradient after 1 and 5 days of culture on the microdevice.

Conditions	Day	Number of Crypts/Colonoids	Quartile 1	Median	Quartile 3
Microdevice	1	30	3,655	4,326	5,748
Standard	1	30	3,912	4,760	6950
Microdevice	5	30	11,550	15,010	28,434
Standard	5	30	10,044	16,425	25,140

Table S3. Area occupied by each colonoid cultured in a 2-D image slice in the absence of a gradient after 1 and 5 days of culture on the microdevice.

Conditions	Day	Number of Crypts/Colonoids	Quartile 1	Median	Quartile 3
Microdevice	1	30	9,458	23,250	61,280
Standard	1	30	10,760	26,810	55,434
Microdevice	5	30	35,966	60,830	147,415
Standard	5	30	37,840	66,610	163,320

Table S4. Area occupied by each colonoid in a 2-D image slice in the absence of a gradient after 1 and 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1 (μm^2)	Median (μm^2)	Quartile 3 (μm^2)
1	1	338	2,054	3,936	7,616
2	1	356	3,018	5,337	7,631
3	1	335	2,856	4,998	8,000
4	1	327	2,750	4,988	7,929
1	5	253	6,587	15,731	29,290
2	5	277	7,878	12,767	29,994
3	5	266	6,981	13,930	26,953
4	5	254	6,175	13,320	30,475

*Wnt-3a was at 120 ng/mL in the sink and source while R-spondin1 was at 175 ng/mL in the sink and source.

** Number of colonoids decreased from day 1 to day 5 as a result of merging colonoids and growth attrition.

Table S5. eGFP intensity per colonoid in the absence of a gradient after 1 and 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1	Median	Quartile 3
1	1	338	7,858	19,360	47,520
2	1	356	11,210	24,688	49,017
3	1	335	11,602	28,408	61,585
4	1	327	12,519	24,277	69,591
1	5	253	17,769	56,157	166,056
2	5	277	31,138	66,039	142,704
3	5	266	25,782	58,758	143,274
4	5	254	21,561	58,766	156,359

*Wnt-3a was at 120 ng/mL in the sink and source while R-spondin1 was at 175 ng/mL in the sink and source.

Table S6. Area occupied by each colonoid in a 2-D image slice in the presence of a Wnt-3a gradient after 1 and 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1 (μm^2)	Median (μm^2)	Quartile 3 (μm^2)
1	1	257	2,615	4,939	9,264
2	1	244	1,925	4,000	7,058
3	1	268	1,869	3,881	7,204
4	1	250	1,501	3,178	7,496
1	5	181	4,790	10,721	26,912
2	5	173	2,879	8,960	24,129
3	5	194	3,182	8,566	19,859
4	5	179	2,095	4,610	10,797

*Wnt-3a was at 0 and 120 ng/mL in the sink and source, respectively, while R-spondin1 was at 175 ng/mL in the sink and source.

Table S7. eGFP intensity per colonoid in the presence of a Wnt-3a gradient after 1 and 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1	Median	Quartile 3
1	1	257	11,013	32,826	62,141
2	1	244	8,386	24,665	57,161
3	1	268	9,977	20,941	45,992
4	1	250	5,547	15,183	51,707
1	5	181	21,910	68,773	164,134
2	5	173	12,109	39,738	161,655
3	5	194	8,912	20,605	48,852
4	5	179	4,711	12,082	36,403

*Wnt-3a was at 0 and 120 ng/mL in the sink and source, respectively, while R-spondin1 was at 175 ng/mL in the sink and source.

Table S8. Area occupied by each colonoid in a 2-D image slice in the presence of an R-spondin1 gradient after 1 and 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1 (μm^2)	Median (μm^2)	Quartile 3 (μm^2)
1	1	234	1,576	4,538	8,713
2	1	237	1,805	4,388	7,339
3	1	258	1,634	3,547	6,700
4	1	229	1,876	4,112	7,038
1	5	162	4,564	9,870	27,165
2	5	152	4,022	11,798	21,048
3	5	181	3,357	8,857	23,865
4	5	164	2,916	5,569	13,916

*R-spondin1 was at 0 and 175 ng/mL in the sink and source, respectively, while Wnt-3a was at 120 ng/mL in the sink and source.

Table S9. eGFP intensity per colonoid in the presence of an R-spondin1 gradient after 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1	Median	Quartile 3
1	1	234	9,761	22,194	56,434
2	1	237	8,694	19,452	44,429
3	1	258	9,833	24,665	54,164
4	1	229	9,890	24,401	62,144
1	5	162	15,966	54,298	146,460
2	5	152	19,220	59,967	140,036
3	5	181	14,931	34,149	85,976
4	5	164	18,332	43,982	87,193

*R-spondin1 was at 0 and 175 ng/mL in the sink and source, respectively, while Wnt-3a was at 120 ng/mL in the sink and source.

Table S10. Area occupied by each colonoid in a 2-D image slice in the presence of a Wnt-3a and an R-spondin1 gradient after 1 and 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1 (μm^2)	Median (μm^2)	Quartile 3 (μm^2)
1	1	221	2,737	5,448	8,364
2	1	233	2,515	5,314	8,057
3	1	220	2,397	5,374	8,696
4	1	225	2,799	5,297	9,114
1	5	147	4,973	13,923	32,335
2	5	163	3,775	9,343	19,165
3	5	152	3,762	7,975	17,616
4	5	148	3,751	5,963	14,630

*R-spondin1 was at 0 and 175 ng/mL in the sink and source, respectively, while Wnt-3a was at 0 and 120 ng/mL in the sink and source, respectively.

Table S11. eGFP intensity per colonoid in the presence of a Wnt-3a and an R-spondin1 gradient after 1 and 5 days of culture on the microdevice.

Region*	Day	Number of Crypts/Colonoids	Quartile 1	Median	Quartile 3
1	1	221	9,458	19,460	38,596
2	1	233	11,514	23,572	48,315
3	1	220	10,366	22,168	52,063
4	1	225	12,469	30,102	65,651
1	5	147	27,395	77,744	175,614
2	5	163	20,739	37,311	81,546
3	5	152	9,314	16,232	47,912
4	5	148	4,823	9,660	23,978

*R-spondin1 was at 0 and 175 ng/mL in the sink and source, respectively, while Wnt-3a was at 0 and 120 ng/mL in the sink and source, respectively.

Table S12. The cost-savings benefit of the reduced factor conditions identified in this research *vs.* conventional culture concentrations.

	Conventional		**Factor Reduced**	
Growth-Factor	Wnt-3a*	R-spondin 1*	Wnt-3a	R-spondin 1
Price/Amount	$230/2 µg	$315/25 µg	$230/2 µg	$315/25 µg
Stock Concentration	40 µg/mL	250 µg/mL	40 µg/mL	250 µg/mL
Working Concentration	100 ng/mL (400×)	1,000 ng/mL (250×)	60 ng/mL (667×)	88 ng/mL (2,841×)
Volume per Well**	10 µL / week	16 µL / week	6 µL / week	1.4 µL / week
Price per 12-Well Plate	$552.00 / week	$604.80 / week	$324.00 / week	$53.28 / week
Price per Year***	$16,560	$18,144	$9,720	$1,599
	$34,704 per year		**$11,319** per year	

* Wnt-3a and R-spondin1purchased from the vendors listed in table S13.

**Calculations were made for cultures consisting of a total of 1 mL of media. Cultures were maintained for one week with media and growth factor exchanges occurring every other day. Each well therefore each required 3 mL of total media for the entirety of the week.

***Calculations were made for a total of 30-independent cultures that were maintained throughout the duration of a year.

Table S13. Media preparation for the off-chip colonoid characterization experiments.

Reagent	Vendor	Catalog Number	Stock Concentration	Working Concentration	Resuspended in:
Matrigel (GFR)	BD Bioscience	354230	100%	50%	Media
Murine Noggin	eBioscience	34-8004	100 µg/mL	100 ng/mL	PBS + 0.1% BSA
Murine Wnt-3a	R&D	1324-WN-002	40 µg/mL	60-100 ng/mL	PBS + 0.1% BSA
Human R-spondin1	R&D	4645-RS	250 µg/mL	88-1,000 ng/mL	PBS + 0.1% BSA
Murine EGFP	Life Technologies	PMG8-041	1 mg/mL	50 ng/mL	PBS + 0.1% BSA
Y27632 Inhibitor	Sigma-Aldrich	Y0503	10 mM	10 µM	PBS
Adv DMEMD/F12	Life Technologies	12634-010	-	-	-
NAC	Sigma-Aldrich	A9165	500 mM	1 mM	H_2O
N2	Life Technologies	17502-048	100×	1×	-
B27	Life Technologies	12587-010	50×	1×	-
GlutaMAX	Life Technologies	35050-061	100×	1×	-
Pen/Strep	Life Technologies	15070-063	5,000 µg/mL	1 µg/mL	H_2O
HEPES	Life Technologies	15630-080	1 M	10 mM	H_2O

There were two main components of this culture technique: Matrigel preparation and media preparation. The components of the Matrigel preparation can be seen in the light orange shading. All of the above growth factors and small molecules were added to the Matrigel (100 µL of Matrigel was used to plate ~10,000 crypts). The components of the media preparation can be seen with the light blue shade regions. Additives were added to the base media to create the culture media. After Matrigel polymerization, the culture media was overlaid (1 mL per 12-well plate). Before plating of the crypts, the Matrigel (containing all of the growth factors) was diluted in media at a 1:1 ratio.

Table S14. Number of cells reseeded at each passage step.

	P1	P2	P3
Conventional	10.0 ± 1.7	10.0 ± 1.9	10.0 ± 2.4
Factor Reduced	10.0 ± 0.9	10.0 ± 2.5	10.0 ± 2.1

Number of Cells (× 10^3)

Table S15. Adjusted R-squared values indicating the log-transformed data approaches normality.

DsRed Fluorescent Areas (Column 1 lists the gradients)

	Lane 4	**Lane 3**	**Lane 2**	**Lane 1**
W + R	.953	.982	.987	.977
Wnt	.932	.963	.982	.986
Rspo	.952	.971	.980	.988

Integrated eGFP Fluorescent Intensities (Column 1 lists the gradients)

	Lane 4	**Lane 3**	**Lane 2**	**Lane 1**
W + R	.985	.957	.964	.984
Wnt	.984	.968	.983	.970
Rspo	.982	.971	.975	.988

References

1. A. A. Ahmad, Y. Wang, A. D. Gracz, C. E. Sims, S. T. Magness and N. L. Allbritton, *J Biol Eng*, 2014, **8**, 1754-1611.