Supplementary information

Under-oil autonomously regulated oxygen microenvironments for cell culture

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table S1. Reported oxygen solubility and diffusivity of materials used or referred in this study.

Materials	**O_2 solubility (ml of gas/1 l of fluid @ 1 bar of air)**	**Diffusion coefficient of O_2 ($\times 10^{-5}$ cm^2/s @ 1 bar of air)**
DI water	5.4 (37 °C) (*58*)	1.9-2.3 (25 °C) (*59*)
Typical cell culture media + 10% FBS	5.3 (37 °C) (*31*)	2.69 (37 °C) (*31*)
Silicone oil (**fig. S3**)	51.9 (5 cSt, 38 °C) (*60*) Data not found (1000 cSt)	0.5 (500 cSt, 30 °C) (*61*) Data not found (5 cSt, 1000 cSt)
Mineral oil	49.5 (hexadecane, 22 °C) (*60*)	2.49 (hexadecane, 22 °C) (*60*)
Fluorinert FC-40	76.8 (25 °C) (*60*)	8.3 (22 °C) (*60*)
PDMS elastomer	310 (27 °C) (*62*)	16 (27 °C) (*62*)
Polystyrene (PS)/Polypropylene (PP)/Polycarbonate (PC)	Data not found	0.04-0.02 (25 °C) (*63*)
Polymethyl methacrylate (PMMA)	Data not found	0.0025 (25 °C) (*63*)

table S2. Compiled information of cell types and culture media.

Cell type (tissue origin)	Name	Culture media
Endothelium (blood vessel)	HUVEC (human umbilical vein endothelial cell)	Endothelial basal medium-2 (EBM-2) (Lonza, 0019086) + 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, 10437010) + 1% Penicillin-Streptomycin (Pen-Strep) (Thermo Fisher Scientific, 15070063)
Epithelium (colon cancer)	Caco-2 (cancer coli-2)	Eagle's minimal essential medium (EMEM) (Sigma Aldrich, M4655) + 20% FBS + 1% Pen-Strep
Epithelium (breast cancer)	MDA-MB-231	Dulbecco's Modified Eagle's medium (DMEM) (Thermo Fisher Scientific, 11960051) + 10% FBS + 1% Pen-Strep
Fibroblast (normal)	Colon fibroblasts	Fibroblast media (ScienCell, C2301), 384-well plates coated with gelatin solution (Thermo Fisher Scientific, S25335) at 37 °C for 15 min and then aspirated.
Fibroblast (tumor-associated)	CAF (cancer-associated fibroblasts) (breast)	DMEM + 10% FBS + 1% Pen-Strep
Blood cells (monocytes)	THP-1	Roswell Park Memorial Institute (RPMI) 1640 (Thermo Fisher Scientific, 11875085I) + 10% FBS + 1% Pen-Strep + 1% lactose
Blood cells (neutrophils)	Isolated from whole blood	EBM-2 + 5% FBS + 1% Pen-Strep
Primary epithelium (colon)	Colon organoids	Intestinal stem cell media [45% L-WRN conditioned media (*64*), 45% midgut media (*51*), 10% FBS, 50 ng/ml epidermal growth factor (EGF), 500 nM A-83-01, 10 μM SB202190, 10 nM [Leu^{15}]-Gastrin-1, 1 mM N-Acetylcysteine, 10 μM Y-27632, 2.5 μM CHIR99021, 2.5 μM Thiazovivin, and 100 μg/ml Primocin]
	Colon monolayer	Intestinal stem cell media (see above) Differentiation media (5% L-WRN conditioned media, 85% midgut media, 10% FBS, 50 ng/ml EGF, 500 nM A-83-01, 10 nM [Leu^{15}]-Gastrin-1, 1 mM N-Acetylcysteine)
Fungi	*Candida albicans* (*C. albicans*, CMM 16 PES1 mutant)	RPMI 1640
Bacteria	mCherry-labelled *Bacteroides uniformis* (*B. uniformis*, DMS 6597)	Anaerobe Basal Broth (Oxoid, CM0957) Brain Heart Infusion Broth (Sigma Aldrich, 53286) (for conjugation)

table S3. The panel of genes in RT-qPCR and related protein function.

Gene		Protein function	Source
Proliferation	MKI67	Cell proliferation marker	Thermo Fisher Scientific, Hs04260396_g1
Differentiation	Axis inhibition protein 2 (Axin2)	A surrogate marker of intestinal stem cell activity (targeting Wnt signaling pathway)	Thermo Fisher Scientific, Hs00610344_m1
	Trefoil factor 1 (TFF1)	An enterocyte marker (stabilization of mucus layer, healing of the epithelium)	Thermo Fisher Scientific, Hs00907239_m1
	Sucrase-isomaltase (SI)	An enterocyte marker (digestion of dietary carbohydrates)	Thermo Fisher Scientific, Hs00356112_m1
	Villin	Microvilli marker	Thermo Fisher Scientific, Hs01031739_m1
	Mucin 2 (MUC2)	Goblet cell marker (epithelial lining)	Thermo Fisher Scientific, Hs03005103_g1
Housekeeping (Reference genes)	GAPDH	N/A	Thermo Fisher Scientific, Hs01922876_m1
	HPRT	N/A	Thermo Fisher Scientific, Hs02800695_m1
	RPLP0	N/A	Thermo Fisher Scientific, Hs99999902_m1

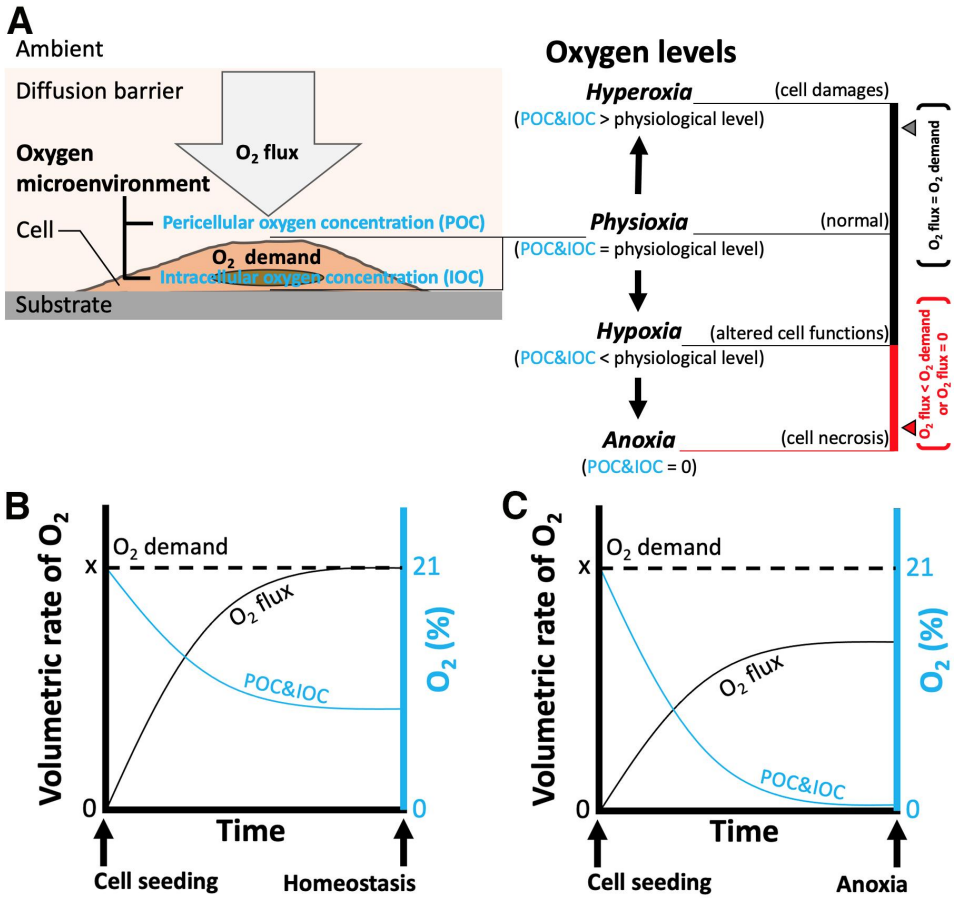

fig. S1. Oxygen levels and representative kinetics of supply-demand-defined oxygen microenvironments. (**A**) Schematic shows the possible oxygen levels in a supply-demand-defined oxygen microenvironment. (**B**), (**C**) Schematics show the theoretical kinetics of POC and IOC over time after cell seeding per a constant O_2 demand. In the condition of homeostasis with O_2 flux matching O_2 demand, POC and IOC can be stabilized anywhere between 0% O_2 and 21% O_2, which gives physioxia, hyperoxia, or hypoxia. In the condition with O_2 flux less than O_2 demand, POC and IOC drop to 0% O_2 in anoxia.

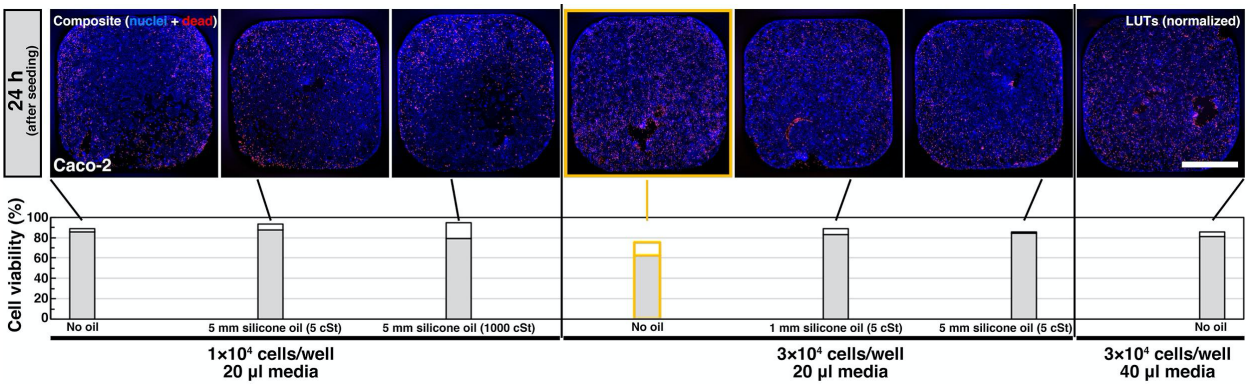

fig. S2. Cell viability of Caco-2 cultured with and without oil (silicone oil, 5 cSt) overlay (24 h after cell seeding with hypoxia dye). For a standard 384 well, 10 µl leads to about 1 mm in depth. The fluorescent images were all processed with normalized LUTs for visualization. Scale bar, 1 mm.

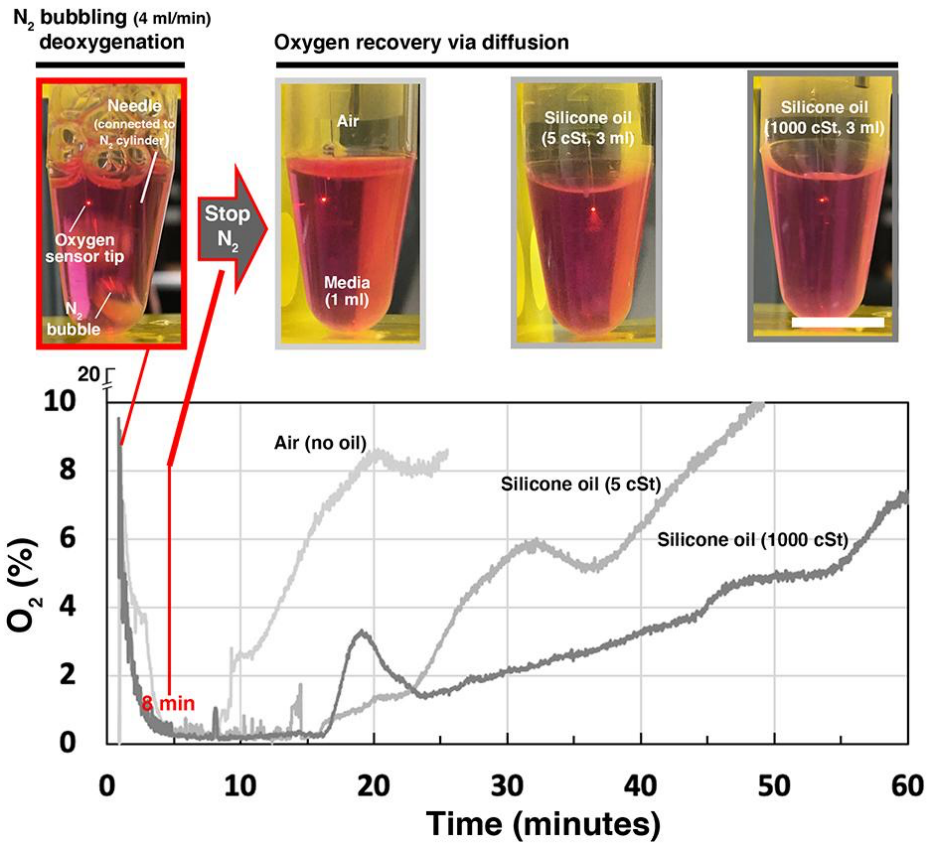

fig. S3. Oxygen diffusion test of silicone oil with different viscosities. Cell culture media (DMEM + 10% FBS) was deoxygenated to 0% O_2 by N_2 bubbling at a gas flow rate of about 4 ml/min. N_2 bubbling was stopped at around 8 min. The media was overlaid with no oil (purged with air) or silicone oil in 5 cSt and 1000 cSt, respectively. The oxygen recovery process was recorded with the oxygen sensor tip kept at about 1 mm below the air/media (or oil/media) interface until it reached about 10% O_2. The 3 ml of oil added on top the media in the 5 ml centrifuge tube led to about 18 mm in the oil depth. Note that the O_2 signal fluctuations recorded in the conditions with oil overlay were caused by the metastability and spontaneous adjustment of the oil/media meniscus during measurements. Scale bar, 10 mm.

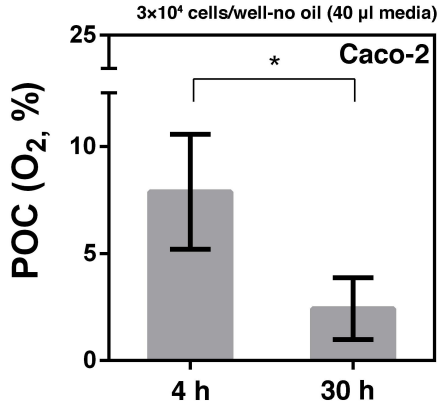

fig. S4. POC of Caco-2 from the condition of large media volume (40 µl/well on a 384 well plate for 4 mm in media depth) without oil overlay. Error bars, mean ± s.d. *$P \leq 0.05$.

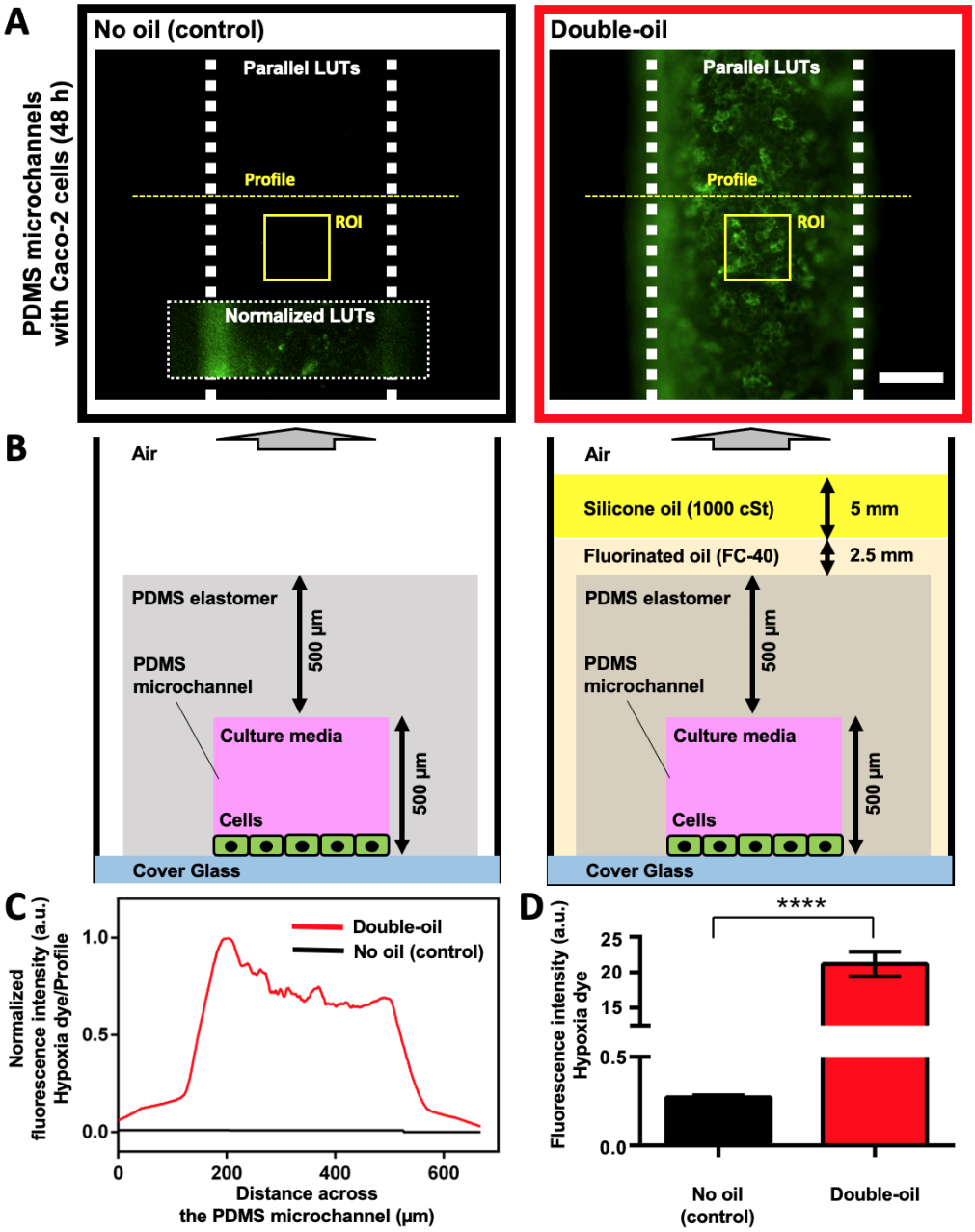

fig. S5. Comparison of hypoxia generation in PDMS microchannels with and without oil overlay. (**A**) The fluorescent images of hypoxia dye from each microchannel [no oil (control), left; double-oil, i.e. fluorinated oil (Fluorinert FC-40) + silicone oil (1000 cSt), right] with a confluent (Caco-2) cell monolayer (cultured for 48 h). Parallel LUTs (with exposure time of 500 ms), were applied for the comparison of fluorescence intensity. [Inset, no oil (control)] A fluorescent image with normalized LUTs to visualize the cells in the microchannel. The white dashed lines indicate the boundary of the microchannels. The channel dimensions are about 2500 µm in length (not fully shown in the images), 600 µm in width, and 500 µm in height. Scale bar, 200 µm. (**B**) Schematics show the cross section of the microchannels perpendicular to the length direction. (**C**) The profiles of fluorescence intensity (normalized) across the microchannels (the yellow dashed lines in **A**). (**D**) The bar graph of IOC (fluorescence intensity of hypoxia dye) of each microchannel. The ROIs are shown by the yellow boxes in **A**. Error bars, mean ± s.d. ****$P \leq 0.0001$.

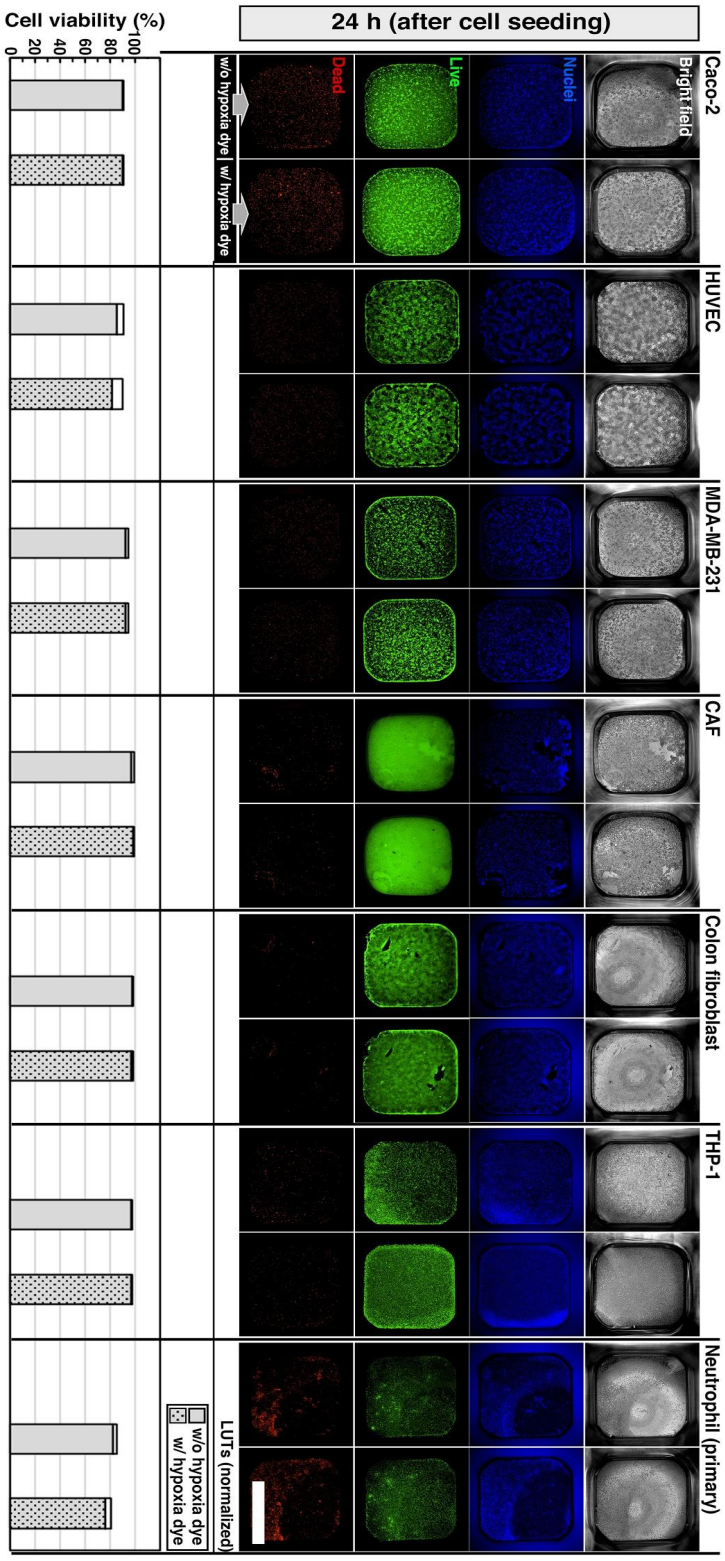

fig. S6. Cell viability of different cell types cultured under oil (24 h after cell seeding with and without hypoxia dye). Each cell type was seeded at 3×10^4 cells/well on a 384-well plate with 20 µl/well of media (for 2 mm in media depth), overlaid with 50 µl of silicone oil (5 cSt) (for 5 mm in oil depth), and cultured up to 24 h for a parallel comparison. The fluorescent images were all processed with normalized LUTs for visualization. Scale bar, 2 mm.

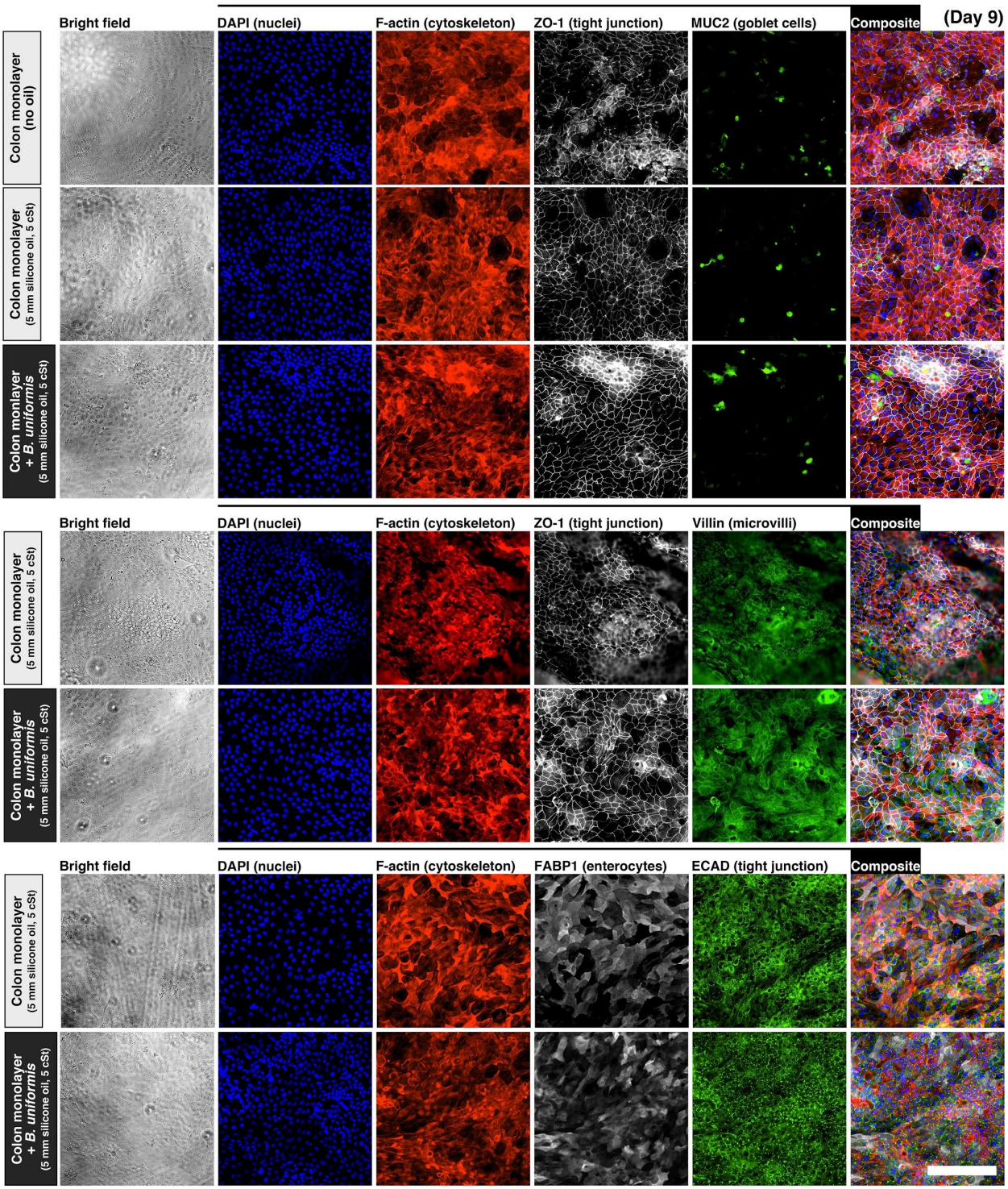

fig. S7. Immunofluorescence staining (IFS) images of primary colon epithelium from mono-culture (no-bacteria control) and co-culture with *B. uniformis* under oil on Day 9 (i.e. 24 h after inoculation of the bacteria). The fluorescent images were all processed with normalized LUTs for visualization. Scale bar, 200 μm.

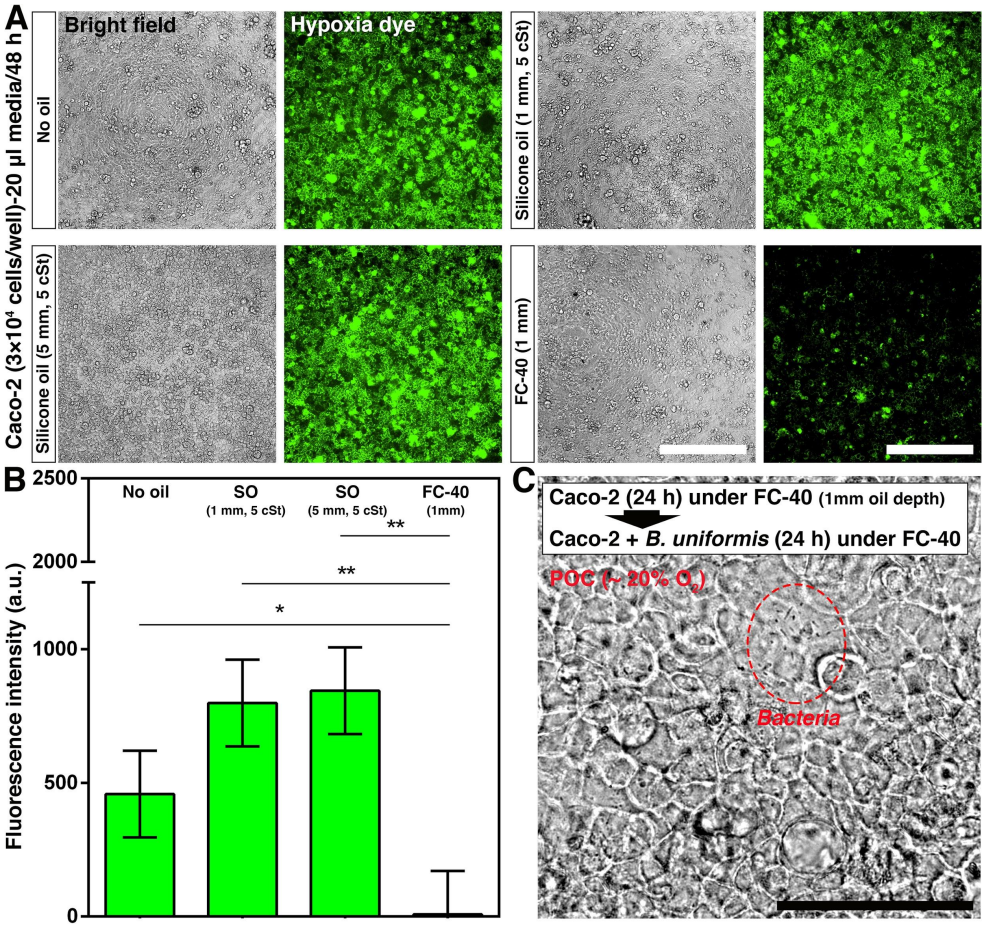

fig. S8. Comparison of hypoxia generation between silicone oil (5 cSt) and fluorinated oil (Fluorinert FC-40). (**A**) Microscopic images (bright field, left; fluorescent, right) of Caco-2 monolayers [3×10^4 cells/well, 20 µl/well of media (for 2 mm media depth), 10 or 50 µl/well silicone oil (5 cSt) (for 1 or 5 mm oil depth, respectively) overlay, 10 µl/well fluorinated oil (FC-40) (for 1 mm in oil depth)] cultured on a 384-well plate for 48 h. The fluorescent images of hypoxia dye were processed with parallel LUTs. Scale bars, 500 µm. (**B**) IOC (fluorescence intensity of hypoxia dye) of each condition. Error bars, mean ± s.d. $^*P \leq 0.05$, and $^{**}P \leq 0.01$. (**C**) Co-culture of Caco-2 monolayer (from **A**) with *B. uniformis* [inoculum density, OD_{600} = 0.1, 1:20 v/v ratio (1 µl bacteria:20 µl media)] under fluorinated oil (FC-40, 1 mm oil depth). POC was measured for about 20% O_2 with the fluorinated oil overlay. The bacteria (the red dashed line circle) showed little growth after 24 h co-culture under FC-40. Scale bar, 200 µm.

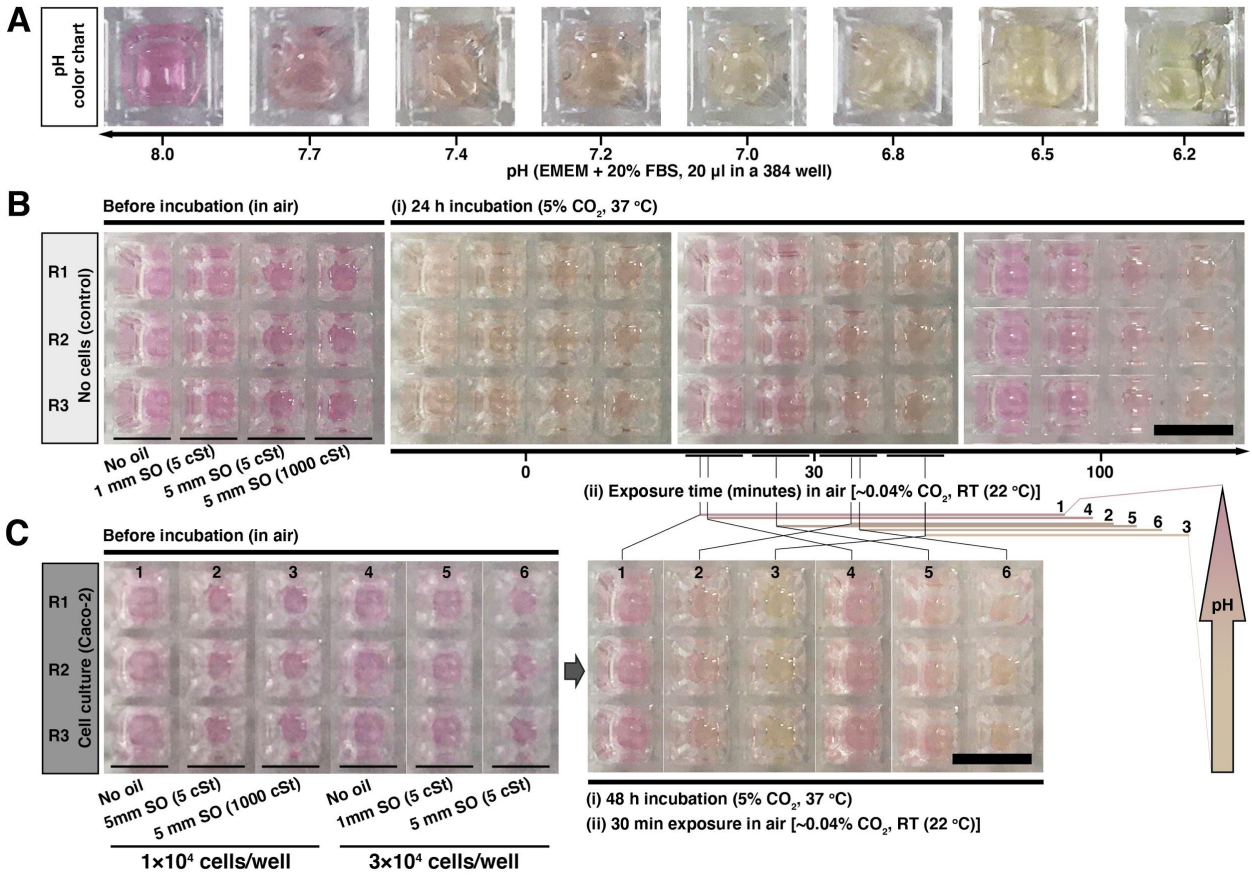

fig. S9. Colorimetric analysis of pH of the culture media before, after incubation and exposure in air. (**A**) A pH color chart of phenol red (30 µM in EMEM + 20% FBS, 20 µl/well on a 384-well plate). (**B**) The control of a no-cell plate with different oil [silicone oil (SO)] overlays. (**C**) The Caco-2 plate [1×10^4 or 3×10^4 cells/well, 20 µl/well of media (for 2 mm in media depth)] with different oil overlays. CO_2 dissolved in the culture media diffused out through the oil overlay over time, which led to the different recovery rates of pH to basic across the tested conditions. The oil overlay stabilized the pH in culture media during device transfer or operation in an atmospheric ambient environment. Scale bars, 4 mm.