

Supporting Information

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Under-Oil Autonomously Regulated Oxygen Microenvironments: A Goldilocks Principle-Based Approach for Microscale Cell Culture

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Supplementary Information for

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This PDF file includes:

Figures S1 to S14 Tables S1 and S2 Supplementary References 1 and 2 SI Section - COMSOL Tutorial



Figure S1. Camera pictures showing the setup of UOMS in air with a 384-well plate and the optical oxygen sensor system for the measurement of POC. The well contains 20 μ l of media (with blue food color for visualization), overlaid with 50 μ l of silicone oil (5 cSt). For a standard 384 well, 10 μ l in volume leads to about 1 mm in depth. In UOMS cell culture, cells are seeded on the bottom of a well. The oxygen sensor is mounted on a linear translation stage for accurate position control in z-direction, resting on the cell layer during the measurement. The temperature sensor is submerged in a spare well filled with deionized water (50 μ l) for real-time temperature compensation. Scale bar, 1 cm.



Figure S2. 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 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 . 3 ml of oil added on top of the media in the 5 ml centrifuge tube leads 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.



Figure S3. Oxygen diffusion in PDMS microchannels with and without oil overlay. a) The kinetics of IOC (monitored by the hypoxia dye, green) in 48 h from a PDMS microchannel with a confluent (Caco-2) cell monolayer and double-oil overlay. Scale bar, 500 μ m. b) The fluorescent images of hypoxia dye from the microchannels with no oil (control, left) and double-oil [i.e., fluorinated oil (Fluorinert FC-40) + silicone oil (1000 cSt), right] cultured for 48 h. Parallel LUTs (with exposure time of 500 ms), were applied for the comparison of fluorescence intensity. [Inset of 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 μ m in length (not fully shown in the images), 600 μ m in width, and 250 μ m in height. Scale bar, 200 μ m. The schematic under each microchannel shows 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 (b)]. d) The bar graph of IOC (fluorescence intensity of hypoxia dye) of each microchannel. The ROIs are indicated by the yellow boxes in (b). Error bars, mean \pm s.d. *****P* \leq 0.0001.



Figure S4. Raw UPLC-MS media analysis results. a) EICs (raw data, unsmoothed) of the five identified lipophilic compounds with their HLB values. Intensity of the signal at a chosen mass-to-charge (m/z) value is plotted against the retention time (RT). "Treatment: Control" is for the internal standard condition. See Methods for details of the other conditions. b) ms/ms fragments spectra of each compound.



Figure S5. The proposed mechanisms for high retention of lipophilic molecules in media-fluorinated oil and media-silicone oil conditions. Studies showed that the hydrocarbon surfactants and biopolymers can get accumulated at the media-oil interfaces in a very fast rate (less than a second), which acts as a barrier that mitigates or prevents the extraction of low or nonvolatile molecules by the oil phase. In addition, the chemical inertness of fluorinated oil leads to insolubility of most of the hydrocarbon/organic molecules.



Figure S6. 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 in volume leads to about 1 mm in depth. The fluorescent images were all processed with normalized LUTs for visualization. Scale bar, 1 mm.



Caco-2, 1×10⁴ cells/well, no oil, 24 h (bright field)



Caco-2, 1×10⁴ cells/well, no oil, 24 h (hypoxia dye)



Caco-2, 1×10⁴ cells/well, media-2 mm, silicone oil-5 mm-1000 cSt, 24 h (bright field)



Caco-2, 1×10⁴ cells/well, media-2 mm, silicone oil-5 mm-1000 cSt, 24 h (hypoxia dye)



Caco-2, 3×10⁴ cells/well, no oil, 24 h (bright field)



Caco-2, 3×10⁴ cells/well, no oil, 24 h (hypoxia dye)



Caco-2, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



Caco-2, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)

Figure S7. Large images showing the typical cell morphologies in Fig. 4a. Scale bar, 100 μ m.



Figure S8. POC of Caco-2 from the condition of large media volume (40 μ l/well on a 384-well plate for 4 mm in media depth) without oil overlay. Error bars, mean ± s.d. **P* ≤ 0.05.



Figure S9. 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 an additional 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.



C. albicans, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



C. albicans, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)



Caco-2, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



Caco-2, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)



HUVEC, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



HUVEC, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)



MDA-MB-231, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



MDA-MB-231, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)



CAF, 3x10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



CAF, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)



Colon fibroblast, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



Colon fibroblast, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)



THP-1, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



THP-1, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)



Neutrophil, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (bright field)



Neutrophil, 3×10⁴ cells/well, media-2 mm, silicone oil-5 mm-5 cSt, 24 h (hypoxia dye)

Figure S10. Large images showing the typical cell morphologies in Fig. 5b. Scale bar, 500 μ m.



Figure S11. IFS images of primary colon epithelium from monoculture (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.



Figure S12. RT-qPCR (Δ Ct) results for comparison of HIF-1 α and HIF-2 α gene expression (Table S2) of the colon epithelium. Lower Δ Ct values indicate higher gene expression. The POC of the no-oil controls is 17-19% O₂, and 2-8% from the under-oil culture (Figure 6c). Data were pooled and averaged with 3 replicates of each condition. Error bars, mean ± s.d. The *P* values are shown on the plot.



Figure S13. 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. * $P \le 0.05$, and ** $P \le 0.01$. c) Co-culture of Caco-2 monolayer from (a) with *B. uniformis* [inoculum density, OD₆₀₀ = 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% O₂ 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.



Figure S14. Colorimetric analysis of pH of the culture media before/after incubation, and after exposure in air. a) A pH color chart of phenol red (30μ M in EMEM + 20% FBS, 20μ I/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, 20μ I/well of media (for 2 mm in media depth)] with different oil overlays. CO₂ dissolved in the culture media diffused out through the oil layer over time, which led to different recovery rates of pH from acidic 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.

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, ^[1] 45% midgut media, ^[2] 10% FBS, 50 ng/ml epidermal growth factor (EGF), 500 nM A-83-01, 10 μ M SB202190, 10 nM [Leu ¹⁵]-Gastrin-1, 1 mM N-Acetylcysteine, 10 μ M Y-27632, 2.5 μ M CHIR99021, 2.5 μ M Thiazovivin, and 100 μ g/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	<i>Candida albicans</i> (<i>C. albicans</i> , 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 S1. Compiled information of cell types and culture media.

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
Hypoxia response	Hypoxia-inducible factor (HIF- 1α/HIF-2α)	Transcription factors (developmental response to hypoxia)	Thermo Fisher Scientific, Hs00153153_m1 (HIF-1α), Hs01026149_m1 (HIF-2α)
Housekeeping (Reference	GAPDH	N/A	Thermo Fisher Scientific, Hs01922876_m1
genes)	HPRT	N/A	Thermo Fisher Scientific, Hs02800695_m1
	RPLP0	N/A	Thermo Fisher Scientific, Hs99999902_m1

Table S2: The panel of genes in RT-qPCR and related protein function.

Supplementary References

- [1] K. L. VanDussen, N. M. Sonnek, T. S. Stappenbeck, Stem Cell Res. 2019, 37, 101430.
- [2] M. M. Mahe, N. Sundaram, C. L. Watson, N. F. Shroyer, M. A. Helmrath, J. Vis. Exp. 2015, DOI 10.3791/52483.

SI Section – COMSOL Tutorial

1. Install COMSOL Multiphysics 5.6 on a PC. Double-click the icon on desktop.



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7. Materials

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8. Physics (Transport of Diluted Species (*tds*) in the demo)

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You can switch to other physics modules using "Add Physics" (as highlighted by the blue box) from the database.

9. Mesh



You will see Mesh in the Settings (as highlighted by the red box):

The default element size is Normal. You can use coarser (faster) or finer (slower) mesh as needed.

10. Study 1 → Step 1: Time Dependent

You will see Time Dependent in the Settings (as highlighted by the red box):

	Multi-phase O2 Diffusion (3D)_Oil (SO5-1m	n)+Media (2mm)_30K.mph - COMSOL Multiphysics	- L X
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You can select a time unit and the range of output times. The tolerance can be defined as well.

11. Results \rightarrow 1D Plot \rightarrow Point Graph 1

You will see Point Graph in the Settings (as highlighted by the red box):

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Go to Selection and pick the point of interest for the output (as highlighted by the yellow circles). After everything is set, click "Compute" in Step 10 (as highlighted by the yellow circle).

12. Results \rightarrow 1D Plot or 3D Plot

Click tabs (1D Plot or 3D Plot) and you will see the 1D and 3D graphs.

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Right click on the tabs (Point Graph 1 or Volume 1) and you can "Add Plot Data to Export". Or Right click on the tabs (1D Plot or 3D Plot) and you can "Add Image to Export".

13. Export → Plot 1 or Image 1

Go to Export. You can save the plots in .txt (i.e., spreadsheet) and the images in a selected format (e.g., TIFF, JPEG, or PNG) (as highlighted by the red boxes).

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