

Supplementary Information

Double-exclusive Liquid Repellency (Double-ELR): An Enabling Technology for Rare Phenotype Analysis

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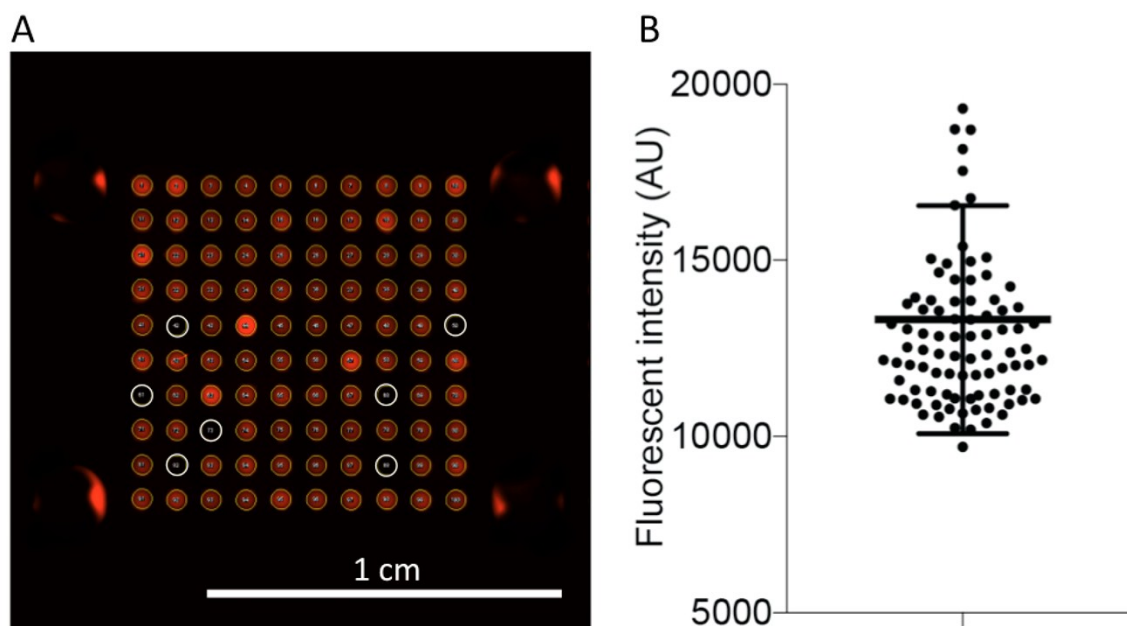


Fig. S1. Variation of volume distribution from underoil sweep patterning on a glass-PDMS patterned slide (containing 600 μm spots in a 10 x 10 array). **(A)** Regions of interest (ROIs) around each spot were denoted by yellow circles for measurement of mean fluorescent intensity using ImageJ. Empty spots (i.e., spots with no volume distributed) were labeled with white circles. **(B)** Scatter plot of the measured mean fluorescent intensity of the spots with the empty spots excluded. The coefficient of variation was calculated as 24% for this case. A significant part of the variance is due to inherent variance of milling used for the preparation of the silicone rubber mask. We anticipate that this variation can be improved by using a more precise surface patterning method (e.g., photo mask and UV-based surface patterning).

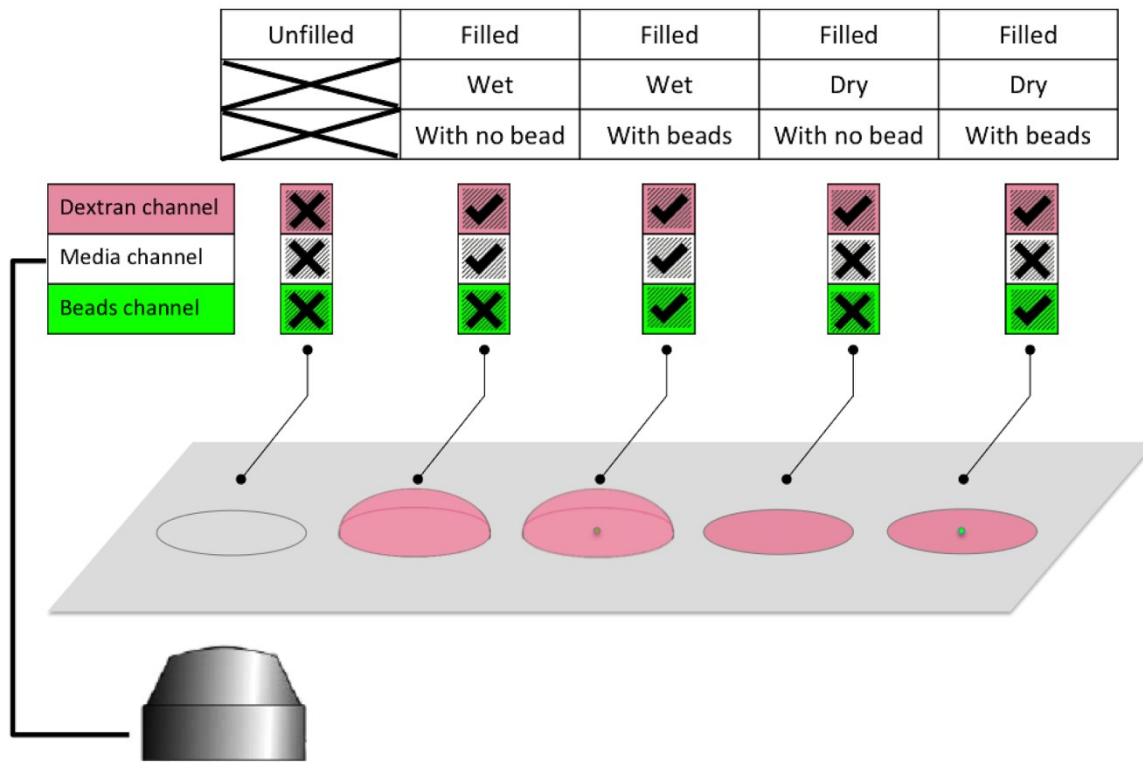


Fig. S2. Composite channels, i.e., dextran channel (560/607 nm), media channel (BF), and beads channel (485/525 nm), employed to characterize combinations of the possible outcomes of sample distribution.

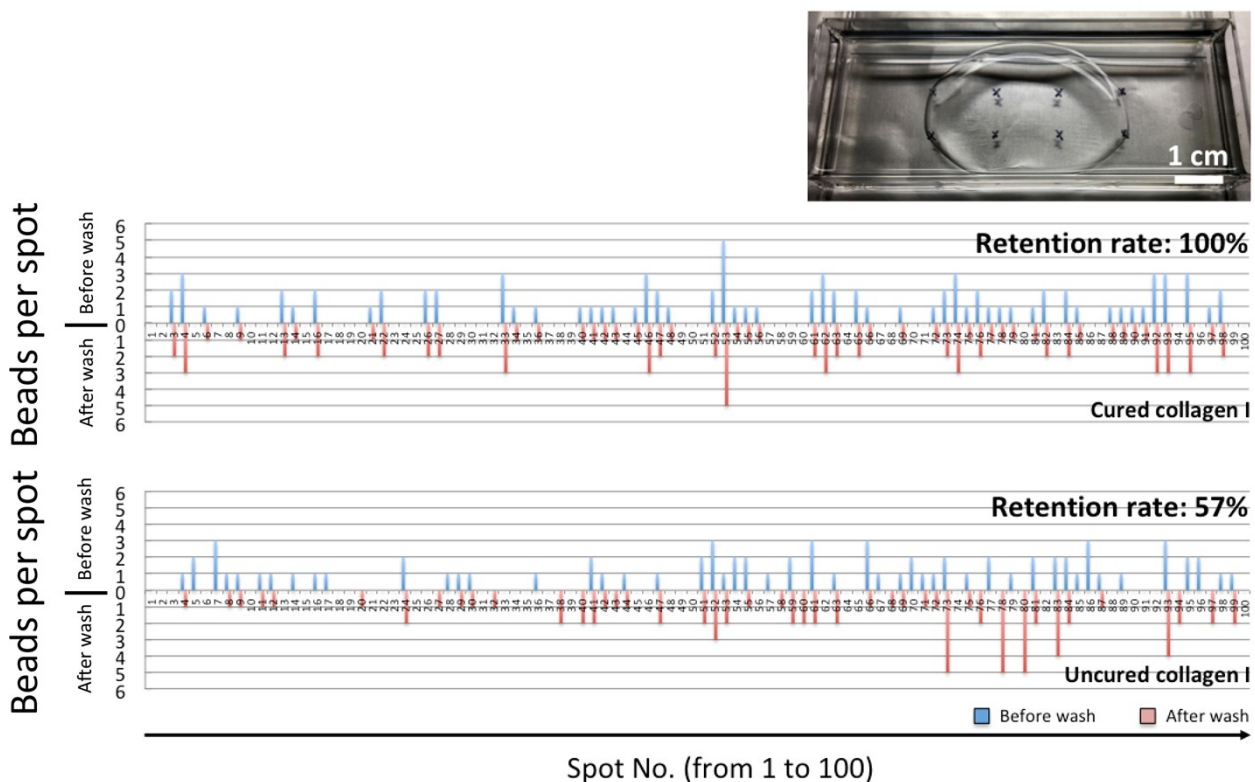


Fig. S3. Underoil washing of spots (400 μm in diameter in a 10 x 10 array) containing beads embedded in ECM (cured vs. uncured).

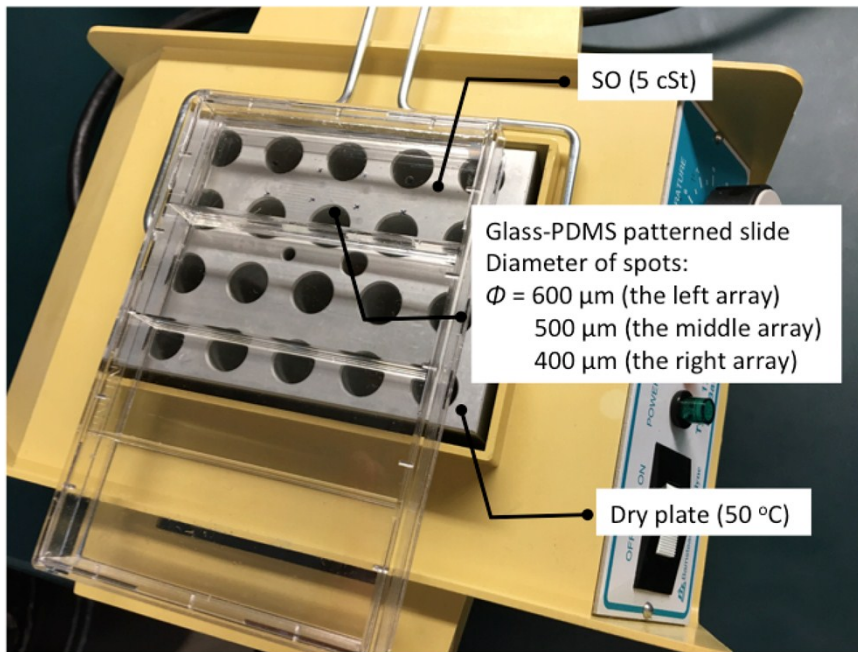


Fig. S4. Setup for preparation of highly aligned collagen fibers under oil. A glass-PDMS patterned slide was housed in a 4-well dish filled with SO on top of a dry block set to 50 °C. 20 μ L collagen I solution (rat tail, 2 mg/mL in PBS) was added onto the slide for each row of 10 spots via sweep.

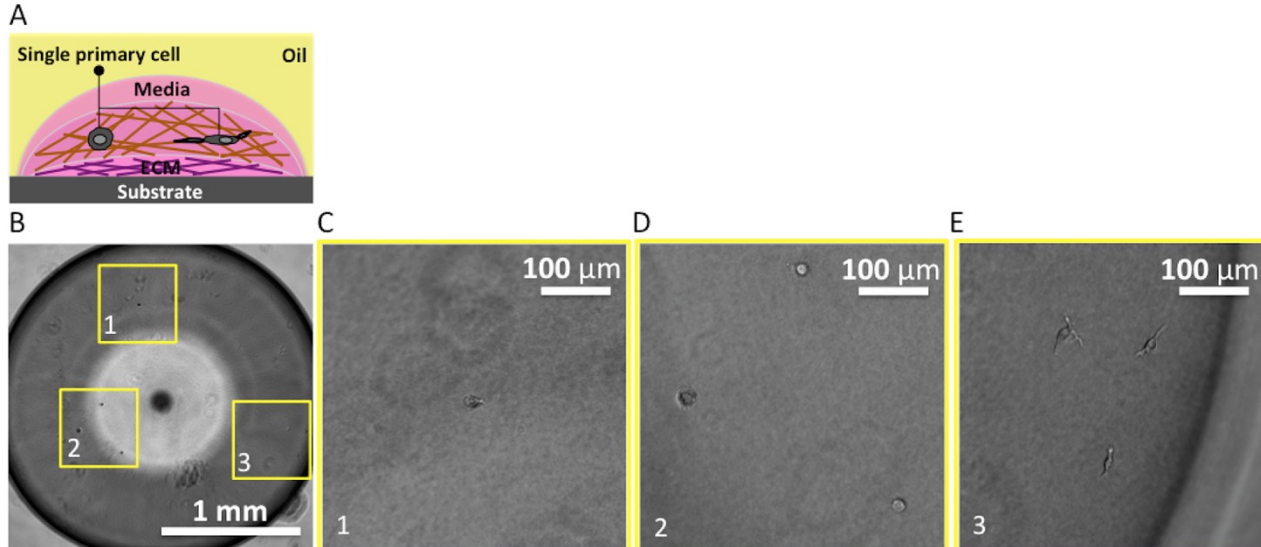


Fig. S5. Underoil 3D cell culture of primary cells from patient with prostate cancer disease. (A) Schematic of the layout. Matrigel (2 μ L, 5 mg/mL) was applied as the first layer of ECM. Cells (2 μ L, 5 cells/ μ L) were embedded in the second layer of ECM (collagen I, bovine, 2 mg/mL). Culture media (2 μ L) was added onto the spot at last. FC was used as the oil phase. (B) Spot image (BF) showing seven single cells in ECM. (C)-(E) High-magnification images of the single cells.

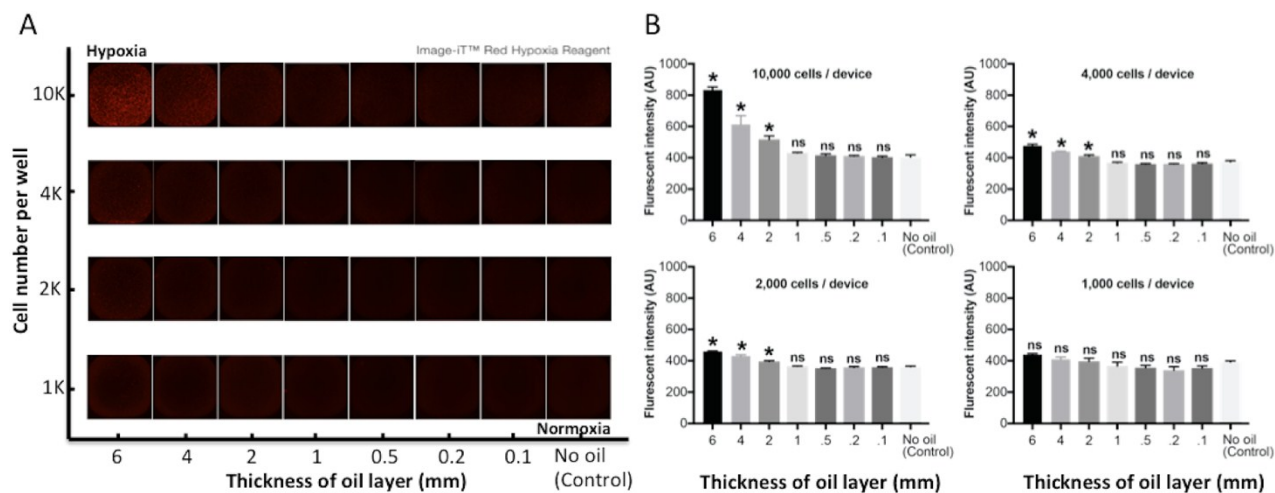


Fig. S6. Results of underoil hypoxia test (from 2-day culture of LNCaP cells) with varying cell densities and thicknesses of oil layer. **(A)** An array of fluorescent images with each corresponding to a single 384 well (3.45 x 3.45 mm). **(B)** Bar chart of fluorescent intensity for each condition in **(A)**. Conditions with asterisk represent hypoxia compared with no oil control.

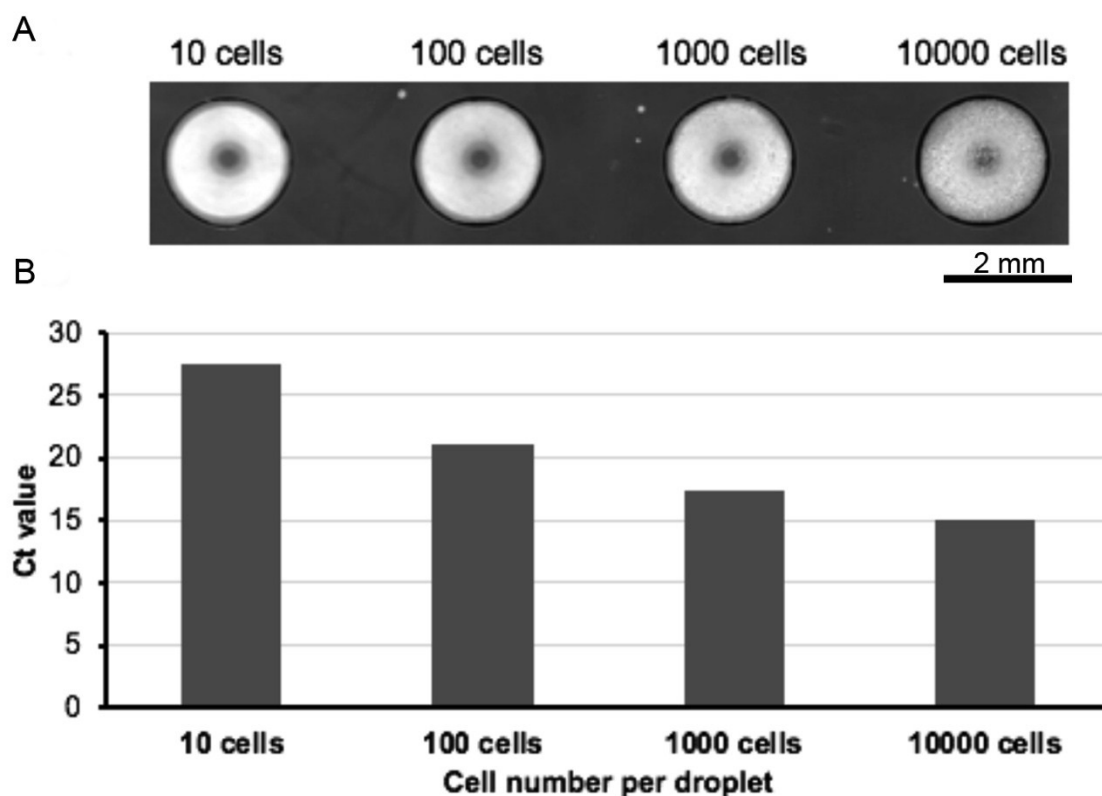


Fig. S7. mRNA extraction with oligo-dT magnetic beads from underoil cultured THP-1 cells. **(A)** Spots (from left to right) with a seeding density of 0, 10, 100, 1k, and 10k cells, respectively. **(B)** RT-qPCR result of the extracted mRNA quantified using for housekeeping gene RPLP0. Cycle 35 is defined as the cut-off cycle.



Fig. S8. Electrostatic perturbation applied via anti-static gun to accelerate underoil sweep patterning. The gun was held about 5 cm above the oil-air interface while positive and negative charges were alternatively applied to the oil layer. The oil layer vibrated during this process, which adds momentum to the aqueous drop under oil and thus help it to penetrate the thin layer of oil underneath. Typically, the momentum from one positive and negative cycle is enough to trigger a complete replacement of oil by aqueous media.

Table S1. Undermedia CAs.

		Glass	PS	PDMS
W-in-O	FC	6.2°	102.7°	139.7°
	SO	60.4°	146.5°	180°
	MO	17.0°	133.0°	155.0°
O-in-W	FC	180°	88.5°	51.9°
	SO	180°	67.3°	71.3°
	MO	158.0°	84.7°	56.5°

Measurement error: $\pm 2^\circ$

Table S2. Model S/W/O systems with different types of liquid repellency.

Surface chemistry of solid	Liquid repellency	Model S/W/O systems
Heterogeneous (e.g., Glass-PDMS)	Double-ELR	# * * # Glass-PDMS/W/SO
	ELR-FLR	# # Glass-PDMS/W/FC
	Double-FLR	Glass-PDMS/W/MO
Homogeneous	ELR-FLR	# # Glass/W/FC # # Glass/W/SO * * PDMS/W/SO
	Double-FLR	Glass/W/MO PS/W/SO PS/W/MO PDMS/W/FC PDMS/W/MO PS/W/FC

*ELR of water on the paired surface under oil. #ELR of oil on the paired surface under water. On a homogeneous substrate, water and oil can be only loaded on one surface. On a heterogeneous substrate, the default loading pattern of the liquids follows each preferred surface. For example, on a glass-PDMS patterned slide, water is loaded on glass spots and oil is loaded on the PDMS background.

Movie S1. Dextran drop (100 μ L) on glass-PDMS patterned slide for underoil sweep patterning enabled by double-ELR.

Movie S2. Dextran drop (100 μ L) on O₂ plasma patterned PS dish that gets pinned on the substrate due to FLR.

Movie S3. Formation of a liquid “Cloud Gate” by bridging two spots (2 mm in diameter with 7 μ L dyed water on each) and adding extra volume (20 μ L DI water) in between.

Movie S4. Debridge two spots by removing the extra volume in the liquid bridge.

All movies were recorded in real time.

Materials and Methods

Preparation of substrates: (I) Glass: Glass slides (Premium microscope slides, Fisher Scientific, 12-544-1) were treated with O₂ plasma (Diener Electronic Femto, Plasma Surface Technology) at 60 W for 3 minutes. (II) PS: 4-well dishes (Nunc 4-well dishes, PS, non-treated sterile, Fisher Scientific, 267061) were used without treatment. (III) PDMS: PDMS precursors (Sylgard 184 elastomer clear Kit, Dow Corning) were mixed with curing agent in 1 : 10 mass ratio. The mixture was thoroughly degassed and then poured into a 4-well dish for a thorough curing in an oven (VWR INCU-Line) at 60 °C for 48 hours. (IV) PDMS-grafted glass: Glass slides were treated first with O₂ plasma at 60 W for 3 minutes then transferred into a vacuum desiccator (Bel-Art F420220000, Fisher Scientific, 08-594-16B). 10 µL PDMS-silane (1,3-dichlorotetramethylsiloxane, Gelest, SID3372.0) was added to vaporize under pumping for 3 minutes and then condensed onto a glass surface under vacuum at room temperature for 10 minutes. The PDMS-grafted glass slides were thoroughly rinsed with toluene (ACS reagent, > 99.5%, Fisher Scientific), ethanol (anhydrous, 99.5%, Fisher Scientific) and DI water then dried with compressed air for use. (V) Glass-PDMS patterned slide: Custom silicone rubber mask (press-to-seal, 0.5 mm thick, varying spot sizes, Grace Bio-Labs) was added onto PDMS-grafted glass and pressed tight to remove air pockets. The masked slide was treated with O₂ plasma at 60 W for 1 minute. After patterning the mask was removed by tweezers. The glass-PDMS patterned slide was taped down in a 4-well dish filled with oil (6 mL/well to achieve an approximately 5 mm thick layer of oil) for use. (VI) O₂ plasma patterned PS dish: A 4-well dish was treated using the same protocol as described above in (V).

Cell line origin and authentication: The human prostate cancer cell lines (C4-2, DU145 and LNCaP), and the human monocyte cell line (THP-1) were all acquired from the American Type Culture Collection (ATCC) and used in low passages (less than P14 for C4-2, DU145 and LNCaP, less than P7 for THP-1). Cells were cultured and passaged in RPMI 1640 with 10% fetal bovine serum (FBS), 2% Glutamax, and 1% PenStrep. All cells were maintained in an incubator at 37 °C (humidified, 5% CO₂). All cell lines were authenticated by the University of Wisconsin Department of Pathology and Laboratory Medicine TRIP Laboratory.

Prostate cancer patient tissue acquisition and processing: A punch biopsy core (4 mm in diameter and 3 mm in length) was obtained from prostatectomy specimen with lesion of prostate cancer at University of Wisconsin Hospital. All patients were informed and consented after surgery under the auspices of a protocol approved by institutional review board (IRB). The tumorous tissues were confirmed by frozen section diagnosis, which is the standard practice. Patient samples were kept in Clin-Ess buffer (Bio-Ess) in a 4 °C refrigerator until the digestion was initiated. The digestion media was made out of 37 °C RPMI 1640 with 25 mM HEPES, 10% glutamax, 1% PenStrep, 1% DNase and 0.28% collagenase I (all from Gibco). This digestion media was aliquoted into a 6-well plate with 2 mL per well. The samples were removed from the ClinEss buffer and each sample was placed in a separate well. Surgical blades were used to pull apart the tissue and break it into smaller pieces. The digestion plate was placed in an incubator at 37 °C (humidified, 5% CO₂). The mixtures were pipetted hourly for the first two hours to further disassociate the tissue pieces. After the first two hours the mixtures were checked every 30 minutes for complete digestion, with no collagen connective tissue pieces, and each time lightly pipetted. The majority of the tissues took around three hours to fully digest. The digestion mixtures were spun down for 4 minutes at 1000 rpm. The supernatant was discarded, and 37 °C RPMI 1640 with 10% FBS, 10% glutamax, and 1% PenStrep was used to resuspend and culture. Primary cells were used at the passage numbers of P2-7.

Characterization of undermedia CAs: All CAs were measured on a standard goniometer (Ramé-Hart 200-00). For W-in-O, DI water drops (3 µL) were added under oil by a pipette. For O-in-W,

varying volumes of oil drops [FC (51142-49-5), SO (317667), and MO (M8410), Sigma-Aldrich] were dispensed under water through a stainless steel needle. Due to the lower density and the ultra-high repellency on glass under water, SO was attached to the end of a stainless steel needle during measurement and MO was added onto substrate first then overlaid with water. At least three measurements from different spots on a substrate were performed for each condition. Measurement error was within $\pm 2^\circ$.

Fouling test on homogeneous substrates in ELR and FLR: Dextran (Texas Red, 10000 MW, Fisher Scientific, D1863) was dispersed in phosphate-buffered saline (PBS) for 1.0 mM solution. A 4-well dish was used with two wells directly as PS surfaces and the other two coated with PDMS as PDMS surfaces. SO and MO were added to each well to give four combinations, i.e., PS/MO, PS/SO and PDMS/MO for FLR, and PDMS/SO for ELR. 1 mL dextran solution was added and then removed by a pipette in less than 10 seconds. Fluorescent images of the contact areas were taken on Nikon Eclipse Ti (4x objective, 560/607 nm, no LUTs applied) to monitor fouling. Mean fluorescent intensity was measured for each ROI using ImageJ default function (Analyze - Measure). Auto-fluorescence levels were subtracted from each measurement. Graphing and statistical analysis was performed using GraphPad Prism 7 software.

Underoil sweep patterning: (I) A glass-PDMS patterned slide and an O₂ plasma treated PS dish (containing spots 600 μm in diameter in a 10 x 10 array) were housed in a 4-well dish filled with SO. 100 μL dextran solution (labeled with Texas Red, 10000 MW, 1.0 mM in PBS) was added to the glass-PDMS patterned slide under oil via sweep then removed by a pipette. On O₂ plasma patterned PS dishes, sweep patterning became impractical due to FLR to water on PS under oil. 1 mL dextran solution was added instead to cover all of the spots then removed by a pipette. The substrates were then imaged on Nikon Eclipse Ti [4x objective (with 6 x 6 stitching), BF, and 560/607 nm]. The image was then imported into ImageJ software (<https://imagej.nih.gov/ij/>) for image analysis. An ROI array outlining each of the 100 underoil sweep-distributed dextran droplets was generated using the ROI manager of ImageJ and then the mean fluorescence intensity of each ROI region was measured and exported into Microsoft Excel. Data analysis for variation of volume distribution was performed using GraphPad Prism 7 software. (II) Fluorescent beads (melamine resin-based, carboxylate modified, FITC-marked, 12 μm in average diameter, Sigma-Aldrich, 90287-5ML-F) were used as model particles. The beads were dispersed in PBS containing 1.0 mM dextran (Texas Red labeled, 10000 MW) and 2 mg/mL collagen I (bovine, PureCol, Advanced BioMatrix) to give a stable suspension (i.e., showing no settling of the dispersed particles over time). Two sweep concentrations of the beads suspension (100 beads/ μL and 10 beads/ μL) were prepared. 100 μL beads suspension was added to glass-PDMS patterned slides (containing spots 100, 200, 300, 400, 500, and 600 μm in diameter and each in a 10 x 10 array) under oil via sweep. The beads suspension was removed from under oil after sweep by a pipette. Three microscopic channels on the Nikon Eclipse Ti, including the “dextran channel” (560/607 nm), “media channel” (BF), and “beads channel” (485/525 nm), were employed to check different combinations of the possible outcomes of sample distribution. The dextran channel indicates whether or not any volume successfully dispersed onto a spot. The media channel indicates whether or not the spot is still wet (or not fully dried) with media. The beads channel indicates whether or not any beads were successfully distributed onto a spot. The frequency (or the probability, P) of a given bead count per spot was calculated using the equation of: $P = \text{No. of spots with a given bead count} / \text{No. of total spots (100 in this case)} \times 100\%$. Four outcomes of beads distribution (unfilled, no volume dispersed; no-bead, volumes dispersed with zero beads; single-bead, volume dispersed with one bead; and multi-bead, volumes dispersed with more than one bead) were quantified.

Underoil reconfigurable co-culture with liquid bridge: Day 0: Media deposition (1st sweep) was done first by adding RPMI 1640 and PMA for THP-1 and RPMI 1640 only for DU145 onto a glass-PDMS patterned slide (containing spots 2 mm in diameter and 2 mm in edge-to-edge

spacing in a 5 x 15 array) in adjacent rows under oil via sweep. Then 2 μL cell stocks (THP-1 and DU145, 500 cells/ μL in RPMI 1640 with no ECM) were added to each corresponding row under oil by a pipette. Cells were cultured in an incubator at 37 °C (humidified, 5% CO_2) for 24 hours. Day 1: The slide was imaged on Nikon Eclipse Ti [4x objective (with 7 x 21 stitching) and 20x objective (with 1.5x magnification lens), BF]. Replacement of old media (2nd sweep) was done by sweeping each row of the cells with 50 μL RPMI 1640 under oil. The THP-1/DU145 spots ready for co-culture were bridged by adding 20 μL RPMI 1640 each pair in between. 2 μL RPMI 1640 was added to each spot for monoculture. Cells were further cultured for 48 hours. Day 3: The co-culture spots were debridged (3rd sweep) by sweeping each row of cells with 50 μL PBS under oil. The slide was re-imaged for analysis.

Underoil washing: Two glass-PDMS patterned slides (containing spots 400 μm in diameter in a 10 x 10 array) were housed in a 4-well dish filled with SO. 100 μL fluorescent bead suspension [100 beads/ μL in PBS with 2 mg/mL collagen I (bovine)] was added to the slides under oil via sweep. The slides were imaged on Nikon Eclipse Ti [4x objective (with 6 x 6 stitching), BF and 485/525 nm] before washing. One slide was washed by adding 5 mL PBS and then removed after 15 minutes of rest without having the ECM further cured, and then re-imaged. The second slide was kept at 37 °C for 30 minutes to thoroughly cure the ECM, subjected to the same washing procedure, and then re-imaged. The number and location of the beads for each spot were analyzed using the images from before and after washing.

Underoil layer-by-layer cell stacking (Tri-color stacking with THP-1 cells): Three separate THP-1 cell stocks were loaded with different fluorescent tracers (CellTrace-Oregon green, 488/520 nm, Fisher Scientific, C34555; CellTracker-red, 577/602 nm, Fisher Scientific, C34552; MitoTracker-deep red FM, 644/665 nm, Fisher Scientific, M22426) according to the manufacturer's protocol. Cells were brought to a final concentration of 100 cells/ μL in RPMI 1640 with 5 mg/mL matrigel (Corning 356234, Fisher Scientific, CB-40234). Cell stocks were added one at a time to a glass-PDMS patterned slide (containing spots 500 μm in diameter in a 5 x 5 array) under oil via sweep. The slide was kept in an incubator at 37 °C (humidified, 5% CO_2) for 30 minutes, and then imaged on Nikon Eclipse Ti [4x objective (with 3 x 3 stitching), BF, 485/525 nm, 560/607 nm, and 648/684 nm] following each sweep.

Underoil 3D growth of single-cell derived tumor spheroids: A layer of matrigel [5 mg/mL in a mixture of conditioned media (from C4-2 flask and 0.2 μm filtered) and fresh media (RPMI 1640) in 1 : 1 volume ratio] was first applied to a glass-PDMS patterned slide (containing spots 2 mm in diameter and 2 mm in edge-to-edge spacing in a 5 x 15 array) under oil via sweep. The slide was kept at 37 °C for 30 minutes to fully cure the ECM. C4-2 cells were brought to a final concentration of 10 cells/ μL in the same media with 2 mg/mL collagen I (bovine) and then added via a second sweep. The slide was kept at 37 °C for another 30 minutes to fully cure the ECM. 5 μL media (with no ECM) was added to each spot under oil at last by a pipette. The slide was kept in an incubator at 37 °C (humidified, 5% CO_2) for 16 days, and imaged on Nikon Eclipse Ti [4x objective and 20x objective (with 1.5x magnification lens), BF].

Underoil 3D culture of primary cells from patient with prostate cancer: 2 μL matrigel [5 mg/mL in a prostate cell growth media (PrEGM BulletKit, CC-3165 & CC-4177, Lonza, CC-3166)] was added first to a glass-PDMS patterned slide (containing spots 2 mm in diameter and 2 mm in edge-to-edge spacing in a 5 x 15 array) on each spot under oil (FC40) by a pipette. The slide was kept at 37 °C for 30 minutes to fully cure the ECM. Primary prostate cancer cells were brought to a final concentration of 5 cells/ μL in the same media with 2 mg/mL collagen I (bovine). 2 μL cell stock was added to each spot under oil by a pipette. The slide was kept at 37 °C for another 30 minutes to fully cure the ECM. 5 μL media (with no ECM) was added to each spot under oil at last by a pipette. The slide was kept in an incubator at 37 °C (humidified, 5% CO_2) for 24 hours, and then imaged on Nikon Eclipse Ti [4x objective and 20x objective (with 1.5x magnification lens), BF].

Underoil formation of self-organized highly aligned collagen fibers: A glass-PDMS patterned slide (containing spots 600 μm in diameter and 400 μm in edge-to-edge spacing in a 10 x 10 array) was housed in a 4-well dish filled with SO and pre-heated on a dry plate set to 50 $^{\circ}\text{C}$. 20 μL collagen I solution (2 mg/mL in PBS) (rat tail, Fisher Scientific, 354249) was added onto the slide for each row of 10 spots via sweep. After sweep, the fibers were kept on the dry plate for another 30 minutes to complete curing, and then imaged on Nikon Eclipse Ti [4x objective (with 6 x 6 stitching) and 20x objective (with 1.5x magnification lens), BF].

RNA extraction and RT-qPCR: mRNA was extracted from underoil cultured THP-1 cells using a protocol modified from manufacturer's instructions for the Dynabeads mRNA DIRECT Kit (Fisher Scientific, 61012). In brief, after a one day underoil cell culture with various seeding densities from 0 to 10k cells per sessile droplet on a glass-PDMS patterned slide (containing spots 2 mm in diameter and 2 mm in edge-to-edge spacing in a 5 x 15 array), the culture media was removed/replaced by sweeping a large 1 mL droplet of PBS over the entire patterned surface. Subsequently, a 2 μL solution containing oligo-dT functionalized paramagnetic beads and Lysis/binding buffer [100 mM Tris-HCl at pH 7.5, 500 mM LiCl, 10 mM EDTA at pH 8, 1% LiDS, 5 mM dithiothreitol (DTT)] was added to each spot by pipette and allowed to incubate at room temperature for 5 minutes to lyse cells and hybridize the released mRNA onto the oligo-dT beads. The beads were subsequently collected by a pipette into an eppendorf tube and washed with washing buffer (10 mM Tris-HCl at pH 7.5, 0.15 M LiCl, 1.0 mM EDTA, 0.1% LiDS). The mRNA was eluted from the beads with nuclease free water, then reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA Kit (Fisher Scientific, 4387406). A qPCR reaction was performed for quantifying the cDNA of the ribosomal protein lateral stalk subunit P0 (RPLP0) housekeeping gene using TaqMan real-time PCR master mix and primers (Fisher Scientific) in accordance to the manufacturer's protocols.

Underoil hypoxia test: 20 μL LNCaP cell stock was added in a 384 well plate (PS, Flat Bottom, Tissue Culture Treated, Corning, 3701) in four final concentrations (in RPMI 1640 with no ECM) of 50 (1k cells per well), 100 (2k cells per well), 200 (4k cells per well), and 500 cells/ μL (10k cells per well), respectively. Each concentration was brought into an 8 x 4 array on the plate for eight different thicknesses of the SO layer [0 (no oil), 0.1, 0.2, 0.5, 1, 2, 4, and 6 mm, respectively] and four replicates in each condition. In a standard 384 well (3.45 x 3.45 mm), 10 μL liquid results in a liquid layer that is approximately 1 mm thick. A real-time oxygen detector (Invitrogen, Image IT Red Hypoxia Reagent, Fisher Scientific, H10498) was pre-mixed in the cell stocks following the manufacturer's instructions to monitor the oxygen stress. Briefly, it is non-fluorescent when live cells are in an environment with normal oxygen concentrations and becomes fluorescent when oxygen levels are decreased. Cells were cultured under oil in an incubator at 37 $^{\circ}\text{C}$ (humidified, 5% CO_2) for 2 days, and then imaged on Nikon Eclipse Ti (4x objective, 560/607 nm, no LUTs applied). Mean fluorescent intensity was measured for each condition using ImageJ default function (Analyze - Measure). Auto-fluorescent level was subtracted from measurement. Graphing and statistical analysis was performed using GraphPad Prism 7 software.