

Stereolithographic Printing of Ionically-Crosslinked Alginate Hydrogels for Degradable Biomaterials and Microfluidics

Thomas M. Valentin, Susan E. Leggett, Po-Yen Chen, Jaskiranjee K. Sodhi, Lauren H. Stephens, Hayley D. McClintock, Jea Yun Sim, and Ian Y. Wong

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Pattern Fidelity Image Matching Matlab Algorithm:

The following image matching algorithm uses light microscope images that have been processed and binarized using Matlab as described in the Methods. It takes the original spiral CAD file and compares it to the binarized image, calculating overcure and undercure. Overcure is quantified as printed area that is not part of the original CAD template, and undercure is quantified as part of the original CAD template that was not printed.

Step	Input	Matlab Function	Parameters/Options	Output
Binarize brightfield image	Brightfield image	im2bw	Threshold: 0.7	Binarized image (binImage)
Compare to CAD template	CAD file, binarized image	normxcorr2	binImage, origTemplate	Binarized image matched to CAD (matchedTemplate)
Calculate total area		bwarea	binImage	Total area (totalArea)
Calculate overcure + undercure		~bsxfun	@and, binImage, matchedTemplate	Difference in area (diffArea)
Calculate undercure		bsxfun	@and, matchedTemplate, diffArea	Undercure
Calculate overcure		bsxfun	@and, binImage, diffArea	Overcure

Alginate Recoating Method:

Layer thickness was controlled by manually mimicking the SLA recoating method without the use of a recoater blade by adding 1000 μL of alginate, allowing the alginate to flow over the printed part, and then removing 825 μL . This ideally would leave 175 μL of alginate precursor which is roughly equivalent to 175 μm of layer thickness.

Quantification of Evans Blue Diffusion:

Evans Blue diffusion is quantified by taking video of diffusion through agarose over 2hrs. These video files are loaded into Matlab, greyscaled, cropped, and the intensity values quantified.

Step	Input	Matlab Function	Parameters/Options	Output
Load video frame	Video file	VideoReader		Video frame
Greyscale image	Video frame	rgb2gray		Greyscaled image

COMSOL Multiphysics Simulation:

COMSOL Multiphysics v5.1 simulations (COMSOL, Inc.) were calculated in 2D based on Time-Dependent Transport of a Diluted Species in Porous Media (*tds*), with no convection, no adsorption in porous media, and no dispersion in porous media. For Even channel simulations, both channels had dimensions of 1.5 mm in width and 100 μm in height. For uneven channel simulations, both channels had dimensions of 1.5 mm in width. The source channel had a height of 500 μm and the sink channel had a height of 100 μm . No Flux boundary conditions were established along the outer perimeter of the simulation. Evans Blue (EB) concentrations were held constant for both source channel (2 mg/mL for initial and boundary concentrations) and sink channel (0 mg/ml for boundary concentration). Diffusion was governed by Fick's Law:

$$J_i = -D_i \nabla c_i$$

where J_i is the diffusion flux, D_i is the diffusion coefficient and c_i is the concentration. There is assumed to be no convection due to the source and sink channels being at equal pressure.

The diffusion coefficient of EB was determined by Levick & Michel, 1973¹ to be $1.64 \times 10^{-6} \text{ cm}^2/\text{s}$. To determine the permeability (κ) of the 1.5% agarose hydrogel, first the volume fraction (Φ) of agarose was calculated to be 0.0146 from Pluen et al., 1999² according to the following formula:

$$\Phi = C/\rho\omega$$

where C is the concentration of the agarose (in mg/mL), ρ is the dry agarose density (1640 mg/mL), and ω is the mass fraction of agarose in a fiber (0.625). The permeability was then calculated to be 762 nm^2 from Johnson & Deen, 1996³ according to the following formula:

$$\kappa(\Phi) = (0.0244)\Phi^{-2.45} (\text{nm}^2)$$

Concentration profiles were taken across the width of the agarose hydrogel every 100 μm at a height of 100 μm for the even and uneven channels.

10% Gelatin and 2% Silk Fibroin hydrogel preparation:

Microfluidic channels were also encapsulated using 10% (w/v) gelatin (Sigma, G1890) and 2% (w/v) silk fibroin (SF) hydrogels. Gelatin and SF microfluidic channels were fabricated using the same strategy as for agarose. Gelatin was prepared by dissolving gelatin powder in hot water. Molten gelatin was pipetted on top of the alginate parts and allowed to cool. SF solution was prepared as previously described by Rockwood et al., 2011. Prior to encapsulating the alginate, β -sheet crosslinking of the 2% SF was initiated using ultrasonication. The sonicated SF solution was pipetted on top of the alginate parts and allowed to crosslink overnight at 37°C. Finally, reservoirs were created in the gelatin and SF hydrogels with a 3mm-diameter biopsy punch, the alginate degraded using 100 mM EDTA, and the channels filled with 0.2% EB. Images were acquired using a Celestron microscope.

Cell Viability Assay:**Methods:**

Cell viability was assessed over 24 h to control for potential toxicity from (1) confluence of the cell sheet, (2) printed alginate parts, and (3) EDTA treatment. This was done using 3mm x 3mm printed alginate squares. Cells were seeded around these parts and grown to confluence. The following conditions were used: Cells Only (no alginate or EDTA), Alginate (cells + alginate, no EDTA), Alginate + EDTA (cells + alginate + 20 min 10 mM EDTA), EDTA (cells + 20 min 10 mM EDTA, no alginate). For conditions exposed to EDTA, growth media was aspirated and the wells were washed with 1X PBS. The PBS was then aspirated, and 10mM EDTA was added to degrade the alginate parts for 20min. Following degradation, the EDTA was aspirated and the wells were again washed with 1X PBS and fresh growth media was added. For conditions without EDTA, growth media was aspirated, the wells were washed with 1X PBS, and the growth media replaced. DRAQ7 (0.3 mM stock) was diluted in growth media (1:150 dilution) and 1mL was added to each well.

Cells were imaged immediately after EDTA treatment (0 h), 1 h after treatment, and 24 h after treatment. To account for the 3D nature of the confluent cell sheet, a 10 μm z-stack was taken by imaging 2.5 μm below the focal plane, and 2.5 μm and 5 μm above the focal plane. For wells that contained an alginate square, the cells immediately to the left of the printed square were imaged. For wells that did not contain an alginate square, cells in the middle of the well were imaged.

NIS Elements .nd2 files were converted to Imaris .ims files using Imaris File Converter 8.2.0. Imaris files were then opened in Imaris 8.2.0 and live / dead cells were detected using Spot Detection.

Cell viability was calculated as $(n_{\text{total}} - n_{\text{dead}}) / n_{\text{total}}$

Detection Parameters:

Parameter	
Enable Region Growing	Off
Enable Tracking	Off
Source Channel	RFP / TRITC for Total count Cy5 for Dead count
Estimated Diameter	9 μm
Background Subtraction	On
Quality	Above 9.50
Exported Statistics	Number of Spots per Time Point

Collective Cell Migration Segmentation Parameters:

The following parameters are for Bitplane Imaris 8.1 Spot Detection. Imaris spot detection statistics were laser processed using Matlab to detect the wound front.

Methods:

NIS Elements .nd2 files were converted to Imaris .ims files using Imaris File Converter 8.2.0. Imaris files were then opened in Imaris 8.2.0 and cells were detected using Spot Detection.

Detection Parameters:

Parameter	
Enable Region Growing	Off
Enable Tracking	Off
Source Channel	RFP / TRITC
Estimated Diameter	14 μm
Background Subtraction	On
Quality	Above 9.50
Exported Tracking Statistics	Position Time

Exclusion criteria: Fluorescence Images

Fluorescence images of samples were excluded in the case of a discontinuous cell sheet (e.g. gaps in the cell front) at $t = 0$ h, 36 h and 48 h time points were excluded as the cell migration front had filled the entire field of view and could not be reliably detected in its entirety.

Exclusion criteria: Spot Detection

All detected spots were verified against the phase and GFP channels to confirm they correspond to a cell. If a spot was erroneously detected (does not correspond to a cell), the spot was excluded. Furthermore, if a cell was not physically attached to the cell sheet, it was also excluded. In limited cases where cells began to invade from the top or the side of the image, spot detection was cropped using Region of Interest to include only the initial cell front.

Migration Front Detection:

Cell coordinates were exported from Imaris as a csv file and imported into MATLAB. Cell coordinates were offset in order to center the origin at the middle of the monolayer. A single conforming boundary around the cell positions was calculated using the BOUNDARY function with shrink factor $s = 1$ to envelope the points as closely as possible. The monolayer migration front was then determined by excluding the portions of the boundary on the sides ($-550 \mu\text{m} < x < 550 \mu\text{m}$), as well as the portions on the bottom ($y < 0$). For both straight and convex initial geometries, the mean position of the migration front was calculated as the average y value of all the cells comprising the front.

Leader Cell Detection:

Leader cells were detected and counted manually by 3 individuals (TMV, SEL, JYS) using a pathological scoring system based on published literature. To be counted as a leader cell, potential candidate cells needed to satisfy at least four of the following criteria:

1. One cell at the apex, dragging followers in a finger-like projection
2. Leaders feature ruffled lamellipodia at the front
3. Leaders are ~2X larger than followers
4. Leaders are elongated in direction of travel
5. Followers maintain cuboidal, epithelial phenotype.

Candidate cells that appear to be breaking away from the migration front and are not dragging follower cells behind them were not included in the leader cell count.

References:

1. J. R. Levick and C. C. Michel, *Q J Exp Physiol Cogn Med Sci*, 58, 67-85 (1973).
2. A. Pluen, P. A. Netti, R. K. Jain and D. A. Berk, *Biophys J*, 77, 542-552 (1999).
3. E. M. Johnson and W. M. Deen, *AIChE J*, 42, 1220-1224 (1996).
4. D. N. Rockwood, R. C. Preda, T. Yücel, X. Wang, M. L. Lovett and D. L. Kaplan, *Nat Protoc*, 6, 1612-1631 (2011).
5. X. Wang, J. A. Kluge, G. G. Leisk and D. L. Kaplan, *Biomaterials*, 29, 1054-1064 (2008).

Sample	Mass (mg/mL)				Molar Ratio (Cation / Alginate)			
	BaCO ₃	CaCO ₃	MgCO ₃	PAG	0.09 (0.5X)	0.18 (1X)	0.36 (2X)	0.72 (4X)
1	1.51			5.26	[Bar chart showing 0.18 molar ratio]			
2	3.03			10.53	[Bar chart showing 0.36 molar ratio]			
3	6.05			21.06	[Bar chart showing 0.72 molar ratio]			
4	12.11			42.11	[Bar chart showing 1.44 molar ratio]			
5		0.77		5.26	[Bar chart showing 0.18 molar ratio]			
6		1.54		10.53	[Bar chart showing 0.36 molar ratio]			
7		3.07		21.06	[Bar chart showing 0.72 molar ratio]			
8		6.14		42.11	[Bar chart showing 1.44 molar ratio]			
9			0.65	5.26	[Bar chart showing 0.18 molar ratio]			
10			1.29	10.53	[Bar chart showing 0.36 molar ratio]			
11			2.59	21.06	[Bar chart showing 0.72 molar ratio]			
12			5.17	42.11	[Bar chart showing 1.44 molar ratio]			
13	0.30	1.38		10.53	[Stacked bar chart showing 1:9 ratio]			
14	0.76	1.15		10.53	[Stacked bar chart showing 1:3 ratio]			
15	2.27	0.38		10.53	[Stacked bar chart showing 3:1 ratio]			
16	2.72	0.15		10.53	[Stacked bar chart showing 9:1 ratio]			
17	0.30		1.16	10.53	[Stacked bar chart showing 1:9 ratio]			
18	0.76		0.97	10.53	[Stacked bar chart showing 1:3 ratio]			
19	2.27		0.32	10.53	[Stacked bar chart showing 3:1 ratio]			
20	2.72		0.13	10.53	[Stacked bar chart showing 9:1 ratio]			

Figure S1 – Formulations Table: Alginate precursor formulations were prepared per the following table. 1X is denoted as a molar ratio of cation to alginate of 0.18. All masses of cation and photoacid generator were mixed directly into 3% (w/v) alginate.

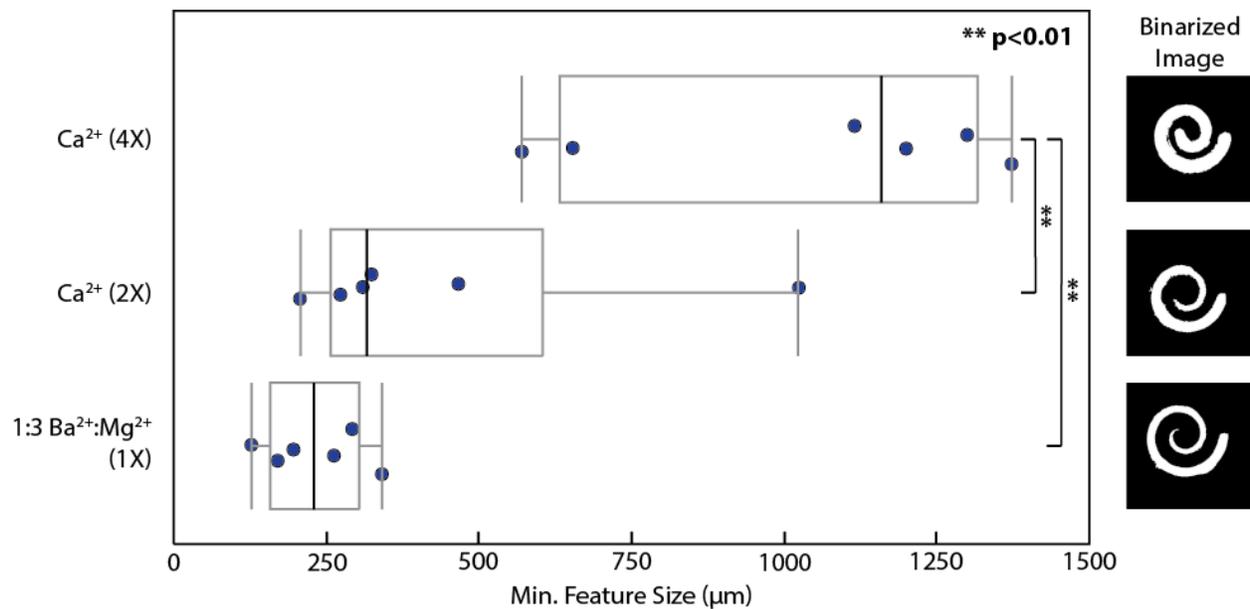


Figure S3 – Minimum Feature Size: Minimum feature size for varying formulations of alginate precursor solution. The formulation with the best pattern fidelity (1:3 Ba²⁺: Mg²⁺) also exhibited the smallest feature size. $n = 6$ samples were processed using ImageJ. Means compared for significance using Student's t-test, and ** indicates $p < 0.01$.

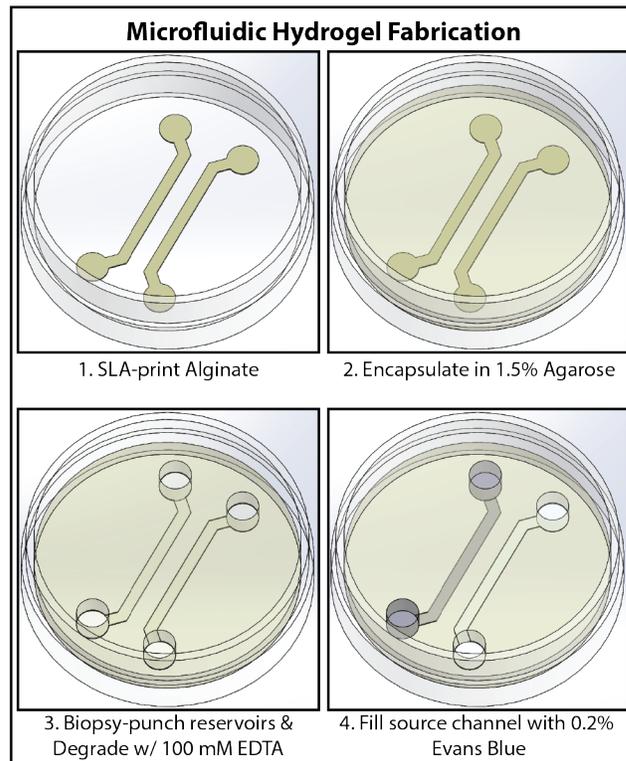


Figure S5. Fabrication steps for agarose microfluidic channels. 3% alginate with 1:3 Ba^{2+} : Mg^{2+} was SLA-printed in a 35 mm diameter Petri dish, and washed with DI water. Alginate template structures were encapsulated in molten 1.5% agarose, and allowed to cool to form a hydrogel. Reservoirs were biopsy-punched and the alginate degraded with 100 mM EDTA. The source channel was filled with 0.2% Evans Blue and the sink channel with DI water.

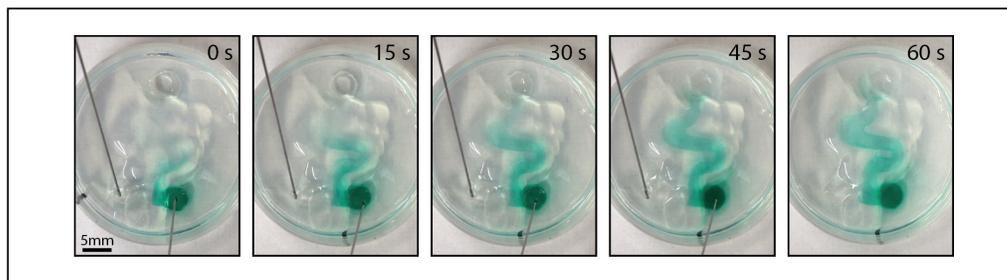


Figure S6. Gravity-driven microfluidic mixing of green dye mixing with water in an agarose T-junction device over 60 s

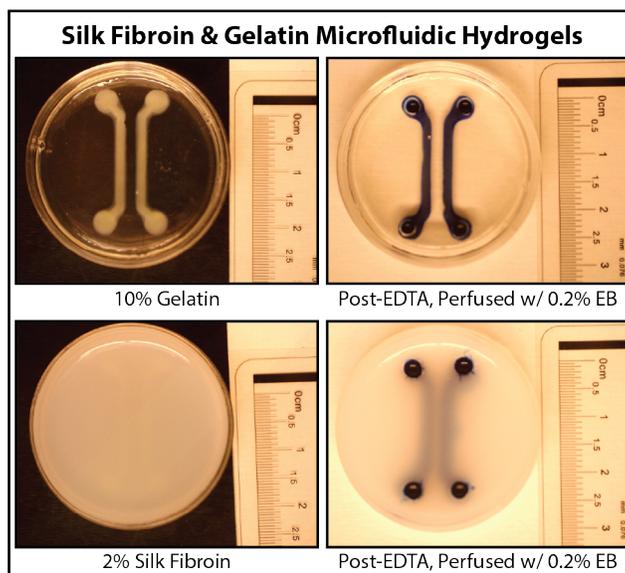


Figure S7. Representative images of 3% Alginate with 1:3 Ba^{2+} : Mg^{2+} parts encapsulated in 10% gelatin or 2% silk fibroin hydrogels, and with both channels filled with 0.2% Evans Blue.

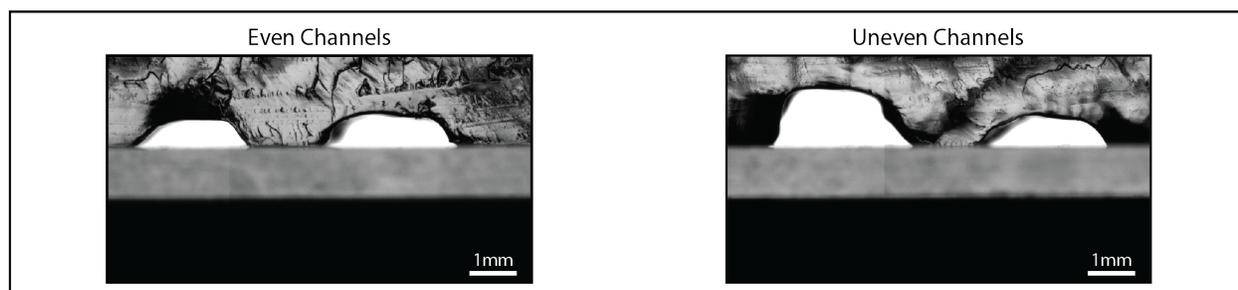


Figure S8. Cross-sectional images of the agarose hydrogel after EDTA degradation of the alginate templates for both even and uneven height channels.

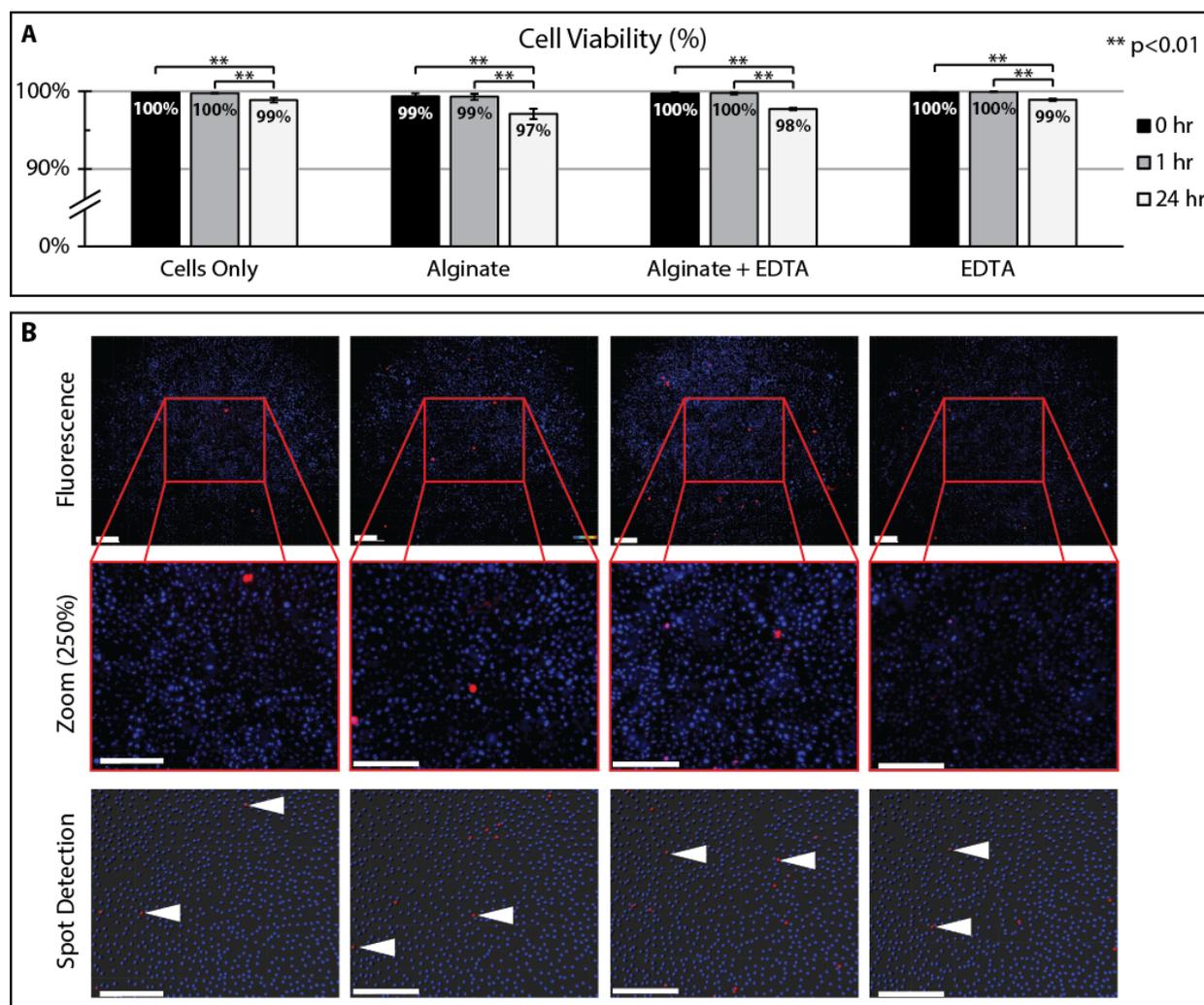


Figure S9: (A) Live / dead counts using mammary epithelial cells (MCF-10A) on a 12-well plate were taken at 0hr, 1hr, and 24hr time points following EDTA degradation. The “cells only” condition was a negative control with cells cultured without alginate or EDTA. The “alginate condition” consisted of cells seeded around a printed 3 mm x 3 mm alginate square. The “alginate + EDTA” condition consisted of cells seeded around a printed 3 mm x 3 mm alginate square, which was subsequently degraded using 10 mM EDTA for 20 min. The “EDTA” condition consisted of cells treated with 10 mM EDTA for 20 min. Cells were grown to confluency prior to EDTA degradation. All conditions exhibited > 97% cell viability over 24hrs. Data sets were compared using a Student’s t-test and statistically significant differences between conditions are indicated by * ($p < 0.05$) and ** ($p < 0.01$). Error bars represent standard deviations. (B) Fluorescence images, and snapshots of spot detection of live and dead cells in confluent cell sheets at 24hr timepoint. White arrows indicate dead cells. Conditions from L to R: Cells Only, Alginate, Alginate + EDTA, EDTA. Live cells are false colored blue (actually RFP nuclei), dead cells are false colored red (actual Cy5 stain) For conditions containing alginate, cells were imaged immediately to the left of the printed square (edge of printed square visible on the right edge of both fluorescence and spot detection images). Scale bar = 150 μ m.

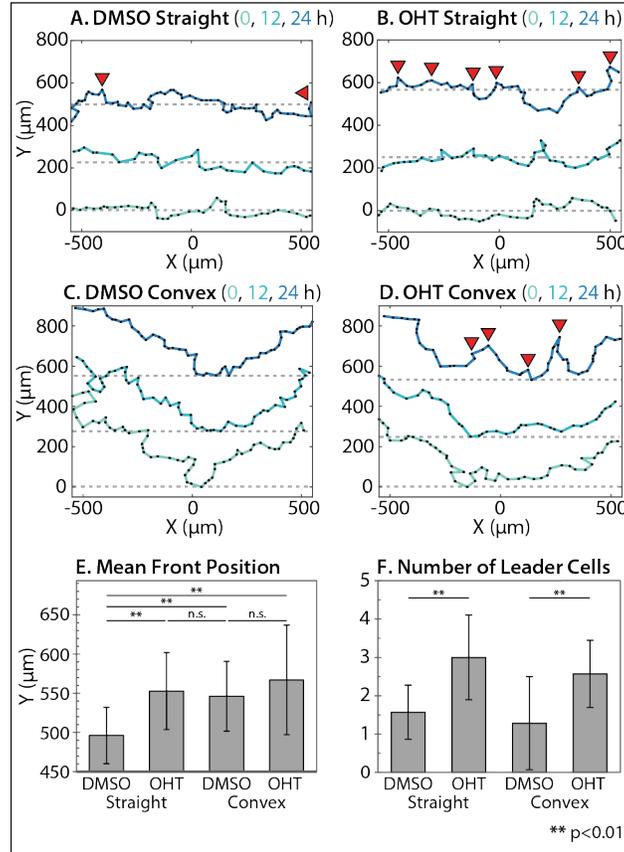
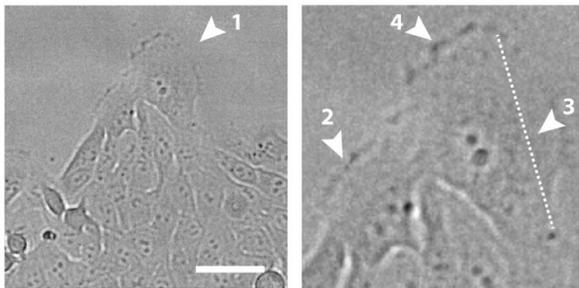


Figure S10. Collective migration front profiles at 0 h, 12 h, and 24 h, respectively. **(A)** DMSO control from initially straight geometries, **(B)** OHT treatment from initially straight geometries. **(C)** DMSO control from initially convex geometries, **(D)** OHT treatment from initially convex geometries. **(E)** Comparison of the mean or minimum y-positions of the migration front. Bar height denotes the mean. Error bars indicate standard deviation. Data sets were compared using a Kolmogorov-Smirnov test and statistically significant differences between conditions are indicated by ** ($p < 0.01$). **(F)** Comparison of the number of leader cells per condition (mean), where error bars are the standard deviation. Data sets were compared using a Student's t-test and statistically significant differences between conditions are indicated by ** ($p < 0.01$). $n > 10$ for all conditions.

**Leader Cell Characteristics:**

1. At the front of a finger-like projection
2. Large cell area (~2x) compared to followers
3. Elongated morphology in the direction of travel
4. Presence of lamellipodia at the leading edge, "ruffles"

Figure S11. Phase contrast image depicting a representative example of an identified leader cell at the migration front (white arrow, left) and zoomed in image (middle) with arrows to highlight leader cell attributes (right). Scale = 50 μm .