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Supporting Information

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High-Density, Multiplexed Patterning of Cells at Single-Cell Resolution for Tissue Engineering and Other Applications**

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Supporting Information

EXPERIMENTAL METHODS

Mold and device fabrication. A 5-inch chrome mask drafted with the mold design (using AutoCAD software) and 4-inch silicon wafers were sent to a semiconductor processing foundry (Integrated Systems, Inc. ISSYS) for DRIE processing to create a silicon hard mold with 40 μm thick features. The design consisted of a series of twenty 10-μm wide serpentine channels (with 3 turns) spaced 30 μm apart. The hard mold was then placed in a chlorotrimethylsilane (TCMS) vapor chamber for 20 minutes. A PDMS mixture (10 parts Sylgard 184 A:1 part Sylgard 184 B curing agent) was poured onto the mold in a petri dish, degassed for 1 hour in a vacuum dessicator, and then baked at 80 ^{o}C until the samples were softly cured (~20 min). The PDMS mold replica was then cut out of the bulk PDMS and through-holes were punched at the locations of the channel inlets and outlets. The microfluidic mold replica was then rinsed with IPA and DI water, airgun dried, and dust was removed with scotch tape. The device was then centered on a clean polylysine-coated glass slide and bonded by baking for 4 hours at 80 ^{o}C.

Validation of oligonucleotide set. The oligonucleotide sequences were chosen based on a pre-validated set of 12 orthogonal 40-mer oligos, each containing a unique 20-mer sequence followed by a 20-mer poly-A tail as shown in **Supplementary Table 1**. These oligos had originally been screened for having very low calculated melting temperatures for hair-pin structures, self-hybridization, and cross-hybridization. Three of the sequences served as anchor sequences, with their complements serving as the “tail” portions of the bridging sequences. The other 9 sequences served as the “head” portions of the bridging sequences, with their complements serving as the conjugate strands for the cells. The 20-mer head and tail sequences

were appended to each other via a 20-mer polyA sequence, creating 60-bp bridging sequences as shown in **Supplementary Table 2**. For the anchor oligonucleotides, a length of 80 base pairs (two identical 40-mer sequences (appended end-to-end) was found to be optimal in ensuring sufficient contact area with the polylysine surface to anchor the cells.

First flow-patterning step. Approximately 100 μM solutions of oligos **A**, **B**, and **C** ("anchor" sequences) were flowed into channels 1, 2, and 3 respectively. This was simultaneously repeated for each additional set of three channels. The solutions were flowed in at around 3 psi via external 23-gauge pin adapters connected to Tygon lines, which were hooked up to an external pressure source and pressure gauge. The solutions were flowed until 1 μL droplets formed at the outlets, indicating the channels were completely filled. The device was then stored in a dessicator at room temperature for 2 days to allow for complete evaporation of water, leaving behind the DNA stripes on the substrate. The PDMS was then peeled away and the patterned glass slide was baked at 80 ^{o}C for 2 – 4 hours to facilitate DNA bonding to the polylysine-coated slide. In the meantime, a second PDMS mold replica was fabricated and rinsed thoroughly with IPA and DI H_2O, then airgun dried, and dust was again removed using scotch tape. Once removed from the oven, the slide was allowed to cool to room temperature and rinsed briefly with DI H_2O to remove excess salts and unbound DNA, then dried with an airgun. The second PDMS mold replica was then placed on the patterned slide oriented 90^{o}-clockwise with respect to the original position of the first PDMS mold replica in the first patterning step. The device was then baked at 80 ^{o}C for 4 hours (for stronger bonding).

Second flow-patterning step. A 3 % BSA/PBS blocking buffer solution was then flowed into all device channels for one hour at 3 psi. For 3 x 3 Arrays: Solutions **1**, **2**, **3** (bridging sequences) were flowed into channels 1, 2, & 3, respectively, for one hour at 3 psi. This was simultaneously repeated for each additional set of three channels in the device. **Solution 1**: 50 μM each of oligo strands **A′-i**, **B′-ii**, **C′-iii** in 3 % BSA/PBS; **solution 2**: 50 μM each of oligo strands **A′-iv**, **B′-v**, and **C′-vi** in 3 % BSA/PBS; **solution 3**: 50 μM each of oligo strands **A′-vii**, **B′-viii**, and **C′-ix** in 3 % BSA/PBS. For 3 x 1 Arrays: Solution **1** was flowed into multiple adjacent channels. A 3 % BSA/PBS buffer solution was then flowed into each channel for 1 hour to remove excess unbound DNA. The PDMS was then peeled off, and the slide was incubated in a 3 % BSA/PBS bath (to block areas previously in contact with the PDMS), followed by rinsing with PBS, and airgun drying.

DNA microarray validation. Once these DNAs were patterned into densely packed 3 x 3 grids or 3 x 1 grids, the patterned slide underwent a second cross-talk validation assay (now using just the 9 dye-conjugated complements **i′** through **ix′**) to ensure that no new crosstalk had been introduced by having two orthogonal sequences tethered into a single bridging sequence. This assay also served to assess whether there was leakage between microfluidic patterning channels, which would manifest as smearing of the fluorescent signal between oligo spots in the grid.

Conjugating cells with DNA. Prior to conjugation, media was decanted from the cell culture dishes and the dishes were rinsed 3 times with 1x PBS. Each dish was then incubated at room temperature for 10 minutes with 3 mL of a 40 μM solution of NHS-biotin in PBS (Solulink protocol). The culture dishes were then rinsed 3 times with media to remove excess unreacted

biotin. Next, 3 μL of 1 mg/mL SaC-oligo in 1 mL of 3 % BSA/PBS was added to each dish, followed by incubation at 37 ^{o}C for 5 minutes. The biotin-streptavidin binding reaction is complete in under 1 minute, but the reaction can proceed longer without causing harm to the cells. **iii′**-SaC was used to address α cells and neurons; **ii′**-SaC was used to address β cells and astrocytes. The culture dishes were then rinsed again with 3 % BSA/PBS 3 times to remove excess unbound SaC-oligo. Approximately 3 mL of warm (37 ^{o}C) trypsin was added to each culture dish and allowed to incubate for 3 minutes at 37 ^{o}C (to detach cells), followed by 5 mL trypsin-neutralizing solution and 5 mL of media. Each cell suspension was then centrifuged at ~150 RCF for 5 min in a 15 mL falcon tube. The supernatant was aspirated, and oligo-conjugated cells were resuspended at a concentration of 10 million cells / mL in 2 μM EDTA in 3 % BSA/PBS.

Cell culture. All cell cultures were incubated in a 37 ^{o}C humidity-controlled incubator with 5 % CO_2/95 % air. Mouse cell-line derived pancreatic α cells (alpha TC1 clone 6, ATCC, Manassas, VA) were cultured in DMEM (Invitrogen Cat. No. 31600-034) containing: 10 % FBS (heat-inactivated), 15mM HEPES, 0.1 mM non-essential amino acids, 0.02 % BSA, 1.5 g/L sodium bicarbonate, 3.0 g/L glucose. Mouse cell-line derived pancreatic β cells (Beta-TC-6, ATCC) were cultured in 15 % FBS/DMEM. Experiments were conducted a week after plating. Human Primary Neurons (ScienCell Research Labs, Carlsbad, CA) were cultured in Neuronal Medium (ScienCell), and used for experiments 2 days after plating. Human Primary Astrocytes (ScienCell) were cultured and continued to divide in Astrocyte Medium (ScienCell) and were used 4 days after plating.

Cell patterning. Two distinct experiments were carried out, one with neurons and astrocytes and one with α cells and β cells. In both cases, 200 μL of a single oligo-conjugated cell type (2 million cells total) was pipetted onto a pre-blocked ultra-dense oligo-patterned slide, followed by incubation at 37 ^{o}C for 30 min. The cell-patterned slide was then swirled lightly in 3 % BSA/PBS to remove non-specifically bound cells. This procedure was then repeated on the same slide (either immediately or within a couple days) using the second oligo-conjugated cell type. A dye-conjugated DNA reference marker (50 nM **i′**-Cy3) was added prior to patterning of the cells to assess whether the cells were binding to the correct spots (relative to the reference marker).

Hydrogel encapsulation of cells[5]**.** A 10 % solution of polyethylene glycol diacrylate (PEG-DA) (MW 3400) in DMEM was filtered through a filter membrane with 0.2 μm pores. Separately, 30 mg of 2,2-Dimethoxy-2-phenylacetophenone (DMPA) photoinitiator was dissolved in 100 μL N-vinylpyrrolidone. Then, 10 μL of DMPA solution was added to 1 mL of PEG-DA solution and vortexed. Approximately 150 μm-thick polished glass coverslips were then placed on the cell-patterned polylysine slide on either side of the cell-patterned area. A third coverslip was positioned such that it bridged the first two, creating a channel in which the cell-patterned glass substrate served as the floor, and the 3 coverslips served as the walls and ceiling. The PEG pre-polymer solution was then pipetted into this channel and cured for 5 minutes under a UV lamp at 4 mW/cm^2 to form a thin (150 μm) hydrogel layer encasing the cells. The hydrogel layer was then peeled off keeping the cell pattern within intact. By stacking and aligning multiple such layers on top of each other, a three-dimensional tissue-like structure was created. The tissue construct was then placed in a culture dish with media at 37 ^{o}C.

Islet cell function and viability assessment. Approximately 200 μL of a 5 μg/mL **i′**-anti-insulin Ab (R&D Systems, Minneapolis, MN) DNA-antibody conjugate in 15 % FBS/DMEM media solution was added to an islet cell patterned slide and allowed to hybridize to the spots of **i** DNA, followed by incubation at 37 ^{o}C for 2 days to allow insulin from nearby β cells to bind the conjugate. The slide was then gently rinsed three times with media followed by addition of a 200 μL solution of 2 μg/mL biotinylated insulin detection antibody (GeneTex, Irvine, CA) in media, which was allowed to incubate at 37 ^{o}C for 1 hour. Again, the slide was gently rinsed three times with media, followed by a 1 hour incubation at 37 ^{o}C of a 200 μL solution of 5 μg/mL streptavidin-Cy5 conjugate (eBioscience, San Diego, CA) for fluorescence detection. A final triple rinse with media was performed. Cell viability was examined by labeling with calcein AM (5 μM) and ethidium homodimer (2.5 μM) (live/dead) fluorescent stains (Molecular Probes).

Oligo orthogonality validation method. To validate that the designed oligo set was truly orthogonal, cross-talk assays were performed on the twelve oligos. First, the twelve primary strands were array-spotted on a polylysine-coated glass slide, baked at 80 ^{o}C for 2 – 4 hours, rinsed briefly with DI water, and airgun dried. A thin slab of PDMS with 12 pre-cut square holes was then bonded to the spotted glass slide, forming 12 assay wells. The wells were then blocked with 3% BSA/PBS for an hour. Twelve solutions, each containing 50 nM of a distinct dye-conjugated complementary strand in 3 % BSA/PBS, were prepared and pipetted into the wells, such that each well was incubated with a different complementary strand for an hour. An additional complementary oligo (also at 50 nM) with a distinct dye color was added at this step as a reference marker. The same reference marker was used in all twelve wells. The wells were

then rinsed with 3 % BSA/PBS, the PDMS was peeled off, and the slide was rinsed with 1X PBS followed by DI water, then airgun dried. The slide was then scanned using a fluorescent microarray scanner to determine if more than one of the twelve spots fluoresced, suggesting cross-talk. If this occurred, the sequence assayed would be considered insufficiently orthogonal to the rest of the panel and would be replaced by a different sequence.

Our lab has previously validated a set of 12 orthogonal oligos, whose sequences (not including the 20-mer polyA tail) are shown in **Supplementary Table 1**. The first 3 sequences (A, B, C) can serve as the anchor sequences, as follows:

2X A-3'-polyA

ATCCTGGAGCTAAGTCCGTAAAAAAAAAAAAAAAAAAAAATCCTGGAGCTAAGTCCGTAAAAAAAAAAAAAAAAAAAA

2X B-3'-polyA

GCCTCATTGAATCATGCCTAAAAAAAAAAAAAAAAAAAAGCCTCATTGAATCATGCCTAAAAAAAAAAAAAAAAAAAA

2X C-3'-polyA

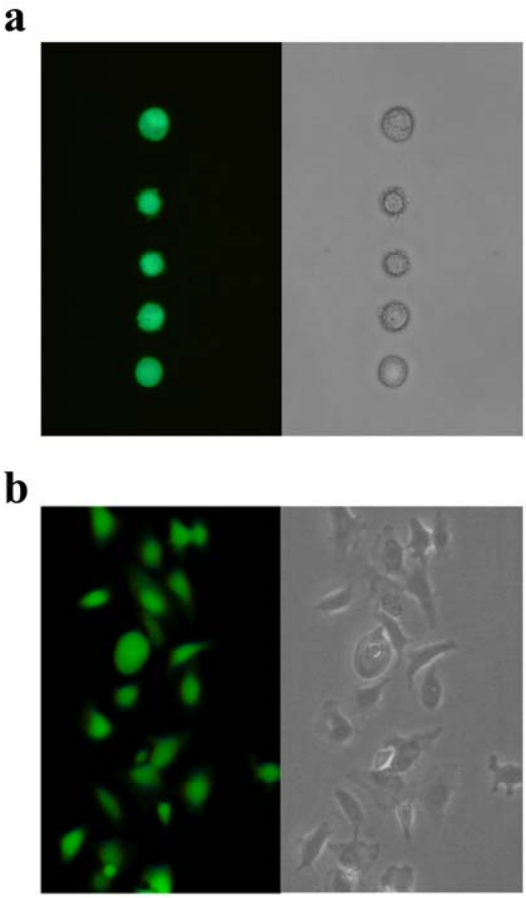
GCACTCGTCTACTATCGCTAAAAAAAAAAAAAAAAAAAAGCACTCGTCTACTATCGCTAAAAAAAAAAAAAAAAAAAA

Supplementary Table 1. List of 12 orthogonal DNA oligomers and their complementary sequences.

A	ATCCTGGAGCTAAGTCCGTA	A'	TACGGACTTAGCTCCAGGAT
B	GCCTCATTGAATCATGCCTA	B'	TAGGCATGATTCAATGAGGC
C	GCACTCGTCTACTATCGCTA	C'	TAGCGATAGTAGACGAGTGC
i	ATGGTCGAGATGTCAGAGTA	i'	TACTCTGACATCTCGACCAT
ii	ATGTGAAGTGGCAGTATCTA	ii'	TAGATACTGCCACTTCACAT
iii	ATCAGGTAAGGTTCACGGTA	iii'	TACCGTGAACCTTACCTGAT
iv	GAGTAGCCTTCCCGAGCATT	iv'	AATGCTCGGGAAGGCTACTC
v	ATTGACCAAACTGCGGTGCG	v'	CGCACCGCAGTTTGGTCAAT
vi	TGCCCTATTGTTGCGTCGGA	vi'	TCCGACGCAACAATAGGGCA
vii	TCTTCTAGTTGTCGAGCAGG	vii'	CCTGCTCGACAACTAGAAGA
viii	TAATCTAATTCTGGTCGCGG	viii'	CCGCGACCAGAATTAGATTA
ix	GTGATTAAGTCTGCTTCGGC	ix'	GCCGAAGCAGACTTAATCAC

Supplementary Table 2. The complements to the anchors and the 9 remaining sequences which are coupled together to form bridging DNA sequences.

A'-i	TACGGACTTAGCTCCAGGATAAAAAAAAAAAAAAAAAAAATGGTCGAGATGTCAGAGTA
B'-ii	TAGGCATGATTCAATGAGGCAAAAAAAAAAAAAAAAAAAATGTGAAGTGGCAGTATCTA
C'-iii	TAGCGATAGTAGACGAGTGCAAAAAAAAAAAAAAAAAAAATCAGGTAAGGTTCACGGTA
A'-iv	TACGGACTTAGCTCCAGGATAAAAAAAAAAAAAAAAAAAAGAGTAGCCTTCCCGAGCATT
B'-v	TAGGCATGATTCAATGAGGCAAAAAAAAAAAAAAAAAAAAATTGACCAAACTGCGGTGCG
C'-vi	TAGCGATAGTAGACGAGTGCAAAAAAAAAAAAAAAAAAAATGCCCTATTGTTGCGTCGGA
A'-vii	TACGGACTTAGCTCCAGGATAAAAAAAAAAAAAAAAAAAATCTTCTAGTTGTCGAGCAGG
B'-viii	TAGGCATGATTCAATGAGGCAAAAAAAAAAAAAAAAAAAATAATCTAATTCTGGTCGCGG
C'-ix	TAGCGATAGTAGACGAGTGCAAAAAAAAAAAAAAAAAAAAGTGATTAAGTCTGCTTCGGC



Supplementary Figure 1. Viability assay for astrocytes: a) upon assembly and b) after 2 days of incubation. These cells continued to divide and propagate throughout the slide area over this time period as their pattern was not fixed by a hydrogel film.* For the freshly assembled astrocytes, only 1 cell out of 100 was found to be non-viable. After two days of incubation, all cells appeared viable, likely because the few dead cells had detached from the slide surface. Cell viability was examined by labeling with calcein AM (5 μM) and ethidium homodimer (2.5 μM) (live/dead) fluorescent stains (Molecular Probes). Live cells exhibit green fluorescence, while dead cells fluoresce red.

*In our studies of encasing patterned cells in hydrogel films, cell division and mobility within the hydrogel matrix was not observed, likely due to the strong mechanical barrier provided by the rigid PEG membrane. The use of lower PEG concentrations or reduced UV light power would likely decrease this mechanical barrier, but at the expense of the rigidity needed to maintain the pattern integrity.