Chapter 3

High-Throughput Assembly of Compositionally Controlled 3D Cell Communities for Developmental Engineering

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Abstract

Cell patterning for 3D culture has increased our understanding of how cells interact among themselves and with their environment during tissue morphogenesis. Building cell communities from the bottom up with size and compositional control is invaluable for studies of morphological transitions. Here, we detail Photolithographic DNA-programmed Assembly of Cells (pDPAC). pDPAC uses a photoactive polyacrylamide gel substrate to capture single-stranded DNA on a 2D surface in large-scale, highly resolved patterns using the photomask technology. Cells are then functionalized with a complementary DNA strand, enabling cells to be temporarily adhered to distinct locations only where their complementary strand is patterned. These temporary 2D patterns can be transferred to extracellular matrix hydrogels for 3D culture of cells in biomimetic microenvironments. Use of a polyacrylamide substrate has advantages, including a simpler photolithography workflow, lower non-specific cell adhesion, and lower stiction to ECM hydrogels during release of patterned hydrogels. The protocol is equally applicable to large (cm)-scale patterns and repetitive arrays of smaller-scale cell interaction or migration experiments.

Key words Cell micropatterning, 3D culture, Developmental engineering, Collective cell behavior

1 Introduction

1.1 Opportunities for Cell Patterning in Developmental Engineering

Many cell–cell and cell–matrix interactions couple with intracellular biochemical and mechanical signaling in developing tissues. These interactions build mature tissues by modulating cell biophysical properties, including proliferation, sorting, differentiation and collective migration (Fig. 1). Given the complexity of determining the contributions of multiple cell–cell and cell–matrix interactions to morphogenesis in vivo, an increased mechanistic understanding can often be achieved by augmenting traditional research with reductionist model systems built from the bottom up [1–4]. Reductionist model systems that pattern cells in precise microenvironments have

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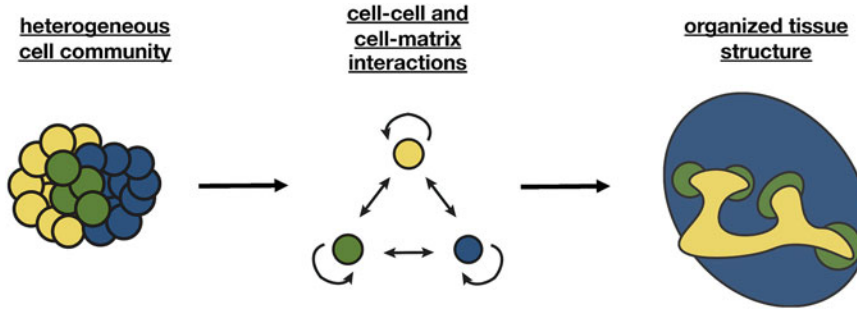

Fig. 1 Cell communities such as spheroids and organoids are increasingly powerful for studying tissue morphogenesis. For example, Taguchi et al. studied the in vitro development of higher-order kidney organoids by combining cultures of embryonic kidney epithelial, mesenchymal, and stromal cells [15]. Reductionist systems designed through precise cell patterning can supplement these technologies by isolating the contributions of specific cell–cell and cell–matrix interactions to processes in morphogenesis

two distinct advantages. Firstly, they allow individual parameters such as shape and stiffness to be quantitatively varied while maintaining other variables constant [5, 6]. Secondly, they enable real-time imaging, especially for models of organ systems that are not superficially located, which limits optical accessibility.

1.2 Defining Cell Communities by DNA-Programmed Assembly of Cells

A key requirement for reductionist tissue models is to build cell communities of precise size and composition in biomimetic, optically-accessible microenvironments suited to live culture. We and others have recently leveraged the binding specificity of ssDNA oligonucleotides to improve engineering control over the size and composition of cell communities. DNA-programmed Assembly of Cells (DPAC) allows for precise cell placement using lipid-DNA "tags" and substrate DNA patterns to temporarily attach cells to specific locations on a 2D substrate [7–9]. The lipid-ssDNAs incorporate passively into cell membranes, while substrate ssDNAs are covalently bound to glass substrates using amine-aldehyde reductive amination chemistry. Using ssDNA as a patterning strategy has a clear advantage over generic adhesive molecules such as fibronectin or RGD, since it enables multiplexed patterning of different cell types simply by changing the ssDNA sequence used for each one. In DPAC, the DNA pattern is created with a relatively slow droplet printer that motivates higher-throughput approaches such as photolithography [10–12]. These photolithographic methods allow for cell-patterning using more common laboratory equipment as well as allowing for the rapid generation of large ssDNA patterns. Here we present one such approach called Photolithographic DNA-programmed Assembly of Cells (pDPAC).

2 Materials

2.1 Fabrication of Hydrophobic Silicon Wafer with Shims for Gel Spacing

1. Hot plate.
2. Mechanical grade 100 mm diameter silicon wafer (University Wafer).
3. Microchem SU-8 2025 photoresist (Fisher Scientific).
4. Vacuum spin coater (SCK-300P, Instras Scientific).
5. 365 nm ultraviolet light source (M365LP1, Thorlabs).
6. Microchem SU-8 developer (Fisher Scientific).
7. 100% acetone.
8. 100% isopropanol.
9. Dichlorodimethylsilane (Sigma).
10. 10,000 dpi photomask with "shim design" (CAD/Art Services, Inc, outputcity.com).

2.2 Functionalization of Polyacrylamide Substrate Slides with Self-assembled Methacrylate Silane Monolayers

1. 1″ × 3″ glass microscope slides (Fisher Scientific).
2. 0.1% Triton-X-100.
3. 1 M sodium hydroxide.
4. Plate rocker.
5. Diamond scribe (McMaster-Carr).
6. 100% acetic acid (EMD Millipore).
7. 100% methanol.
8. 3-(Trimethoxysilyl)propyl methacrylate, 98% (Sigma).

2.3 Fabrication of Photoactive Polyacrylamide Gels

1. 10× Phosphate-buffered saline calcium magnesium free (PBSCMF).
2. Acrylamide/Bis-Acrylamide, 30% solution, 37.5:1 (Sigma).
3. Ultrasonic bath sonicator.
4. 5% Triton-X-100 in ultrapure water.
5. 5% sodium dodecyl sulfate (SDS) in ultrapure water.
6. 100 mM benzophenone methacrylamide (BPMAC) (PharmAgra) in dimethyl sulfoxide (*see* **Note 4**).
7. 10% w/v ammonium persulfate (Sigma) in ultrapure water prepared fresh.
8. 10% tetramethylethylenediamine (TEMED) (Sigma) in ultrapure water prepared fresh.
9. 1× PBSCMF.
10. Razor blade.
11. Delicate task wipes.

2.4 Photopatterning ssDNA

1. Glove box for nitrogenous environment (Bel-Art).
2. TAE buffer: 1 mM (Ethylenedinitrilo)tetraacetic acid (EDTA) disodium salt, 40 mM Tris base, 20 mM acetic acid, in ultrapure water.
3. Photoactive polyacrylamide gel (fabricated in previous steps).
4. 200 μL of 200 μM first patterning ssDNA in ultrapure water (*see* **Note 9**).
5. Quartz-chrome photomask of first ssDNA design.
6. Delicate task wipes.
7. 254 nM ultraviolet crosslinker (Stratagene) (*see* **Note 10**).
8. Razor blade.
9. TAE + 0.1% SDS.
10. Quartz-chrome photomask of second ssDNA design.
11. 75 μL of 1 μM FAM-ssDNA probe in TAE (IDT) (*see* **Note 11**).
12. Widefield fluorescence microscope.
13. 400 μL of 200 μM second patterning ssDNA in ultrapure water.

2.5 Cell Patterning on ssDNA-Functionalized Polyacrylamide Gel and Transfer to 3D Culture

1. 1″ × 3″ glass microscope slide.
2. 0.1% Triton-X-100.
3. Laser etcher (Universal Laser Systems).
4. Approximately 5×10^6 cells are necessary for cell patterning 8 flow cells (This is approximately the cells contained in one 90% confluent T175 flask).
5. Trypsin or other preferred cell detachment reagent.
6. 1× sterile PBSCMF.
7. 100 μM universal anchor lipid-DNA in deionized water (OligoFactory) (*see* **Note 17**).
8. 100 μM universal co-anchor lipid-DNA in deionized water (OligoFactory) (*see* **Note 17**).
9. 100 μM corresponding adhesion ssDNA to ssDNA patterned photoactive polyacrylamide gel (*see* **Note 18**).
10. Sigmacote (Sigma-Aldrich).
11. 2.5% w/v bovine serum albumin in ultrapure water.
12. Machined jig (*see* **Note 19**).
13. 0.01″ PDMS craft-cut gasket for flow cells (*see* **Note 20**).
14. Microarray hybridization cassette gasket (GAHC1X16, Arrayit).

15. #6–32 × ¾″ machine screws and #6–32 wingnuts (McMaster-Carr).
16. 1 mg/mL NHS-AlexaFluor555 in dimethyl sulfoxide (DMSO) (Thermo Fisher).
17. Rat tail collagen 3.9 mg/mL (Corning).
18. Growth factor-reduced Matrigel (Corning).
19. 10 M sodium hydroxide.
20. Sterile 10× PBSCMF.
21. "Deep" 15 cm Petri dish (150 mm × 25 mm) (Corning).
22. Desired cell media.
23. Dissection microscope.
24. Probe tool and forceps (fine science tools).
25. Razor blade.
26. 24-well coverslip-bottom tissue culture plate (Greiner).

3 Methods

In pDPAC, ssDNAs are captured on the surface of photoactive polyacrylamide gels in millions of locations simultaneously. This photoactivity is created by co-polymerizing benzophenone-methacrylamide into the polyacrylamide structure [12]. ssDNAs are then diffused throughout the gel, and a pattern of ultraviolet light created with a photomask locally activates the benzophenone and promotes covalent binding to the ssDNA molecules. The gel is then washed to reveal a spatial pattern of ssDNA on the surface. This process can be repeated with different ssDNAs and photomasks for multiplexed patterns on the same substrate. Next, cells tagged with lipid-DNA are introduced to the ssDNA-patterned gel surface in a 2 × 8 array of custom flow cells. ECM hydrogel precursor is then introduced to each flow cell and set to generate 3D matrix sheets bearing the desired cell pattern, which can then be cultured floating in media or adhered to a glass surface (Fig. 2).

We have previously used pDPAC to build large folding biological structures that guide epithelial network formation [12]. Here, we adapt pDPAC for high-throughput arrays of individual 3D collective cell migration experiments that can be analyzed at high-resolution in real-time (Fig. 3a, b). These cultures can then be processed for downstream assays including fixing and immunostaining for proteins of interest (Fig. 3c). This protocol thus details pDPAC for parallelized cell interaction experiments that model developmental processes.

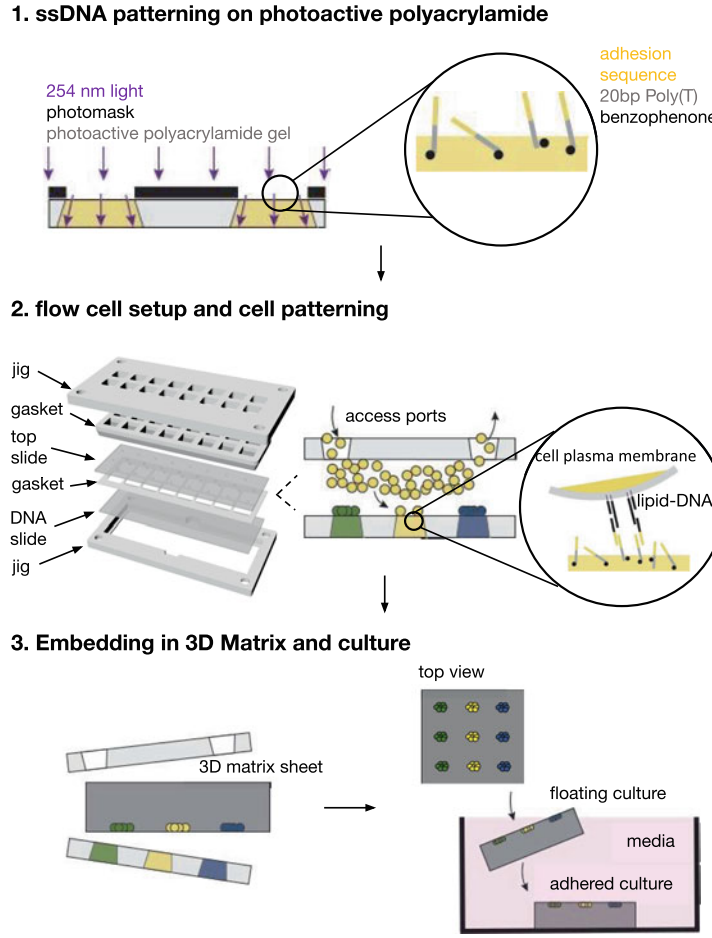

Fig. 2 Schematic outline of pDPAC. (1) Photomask-derived patterns of ultraviolet light spatially activate benzophenone in a photoactive polyacrylamide gel. Activated benzophenone binds thymine bases of ssDNA revealing a surface pattern of ssDNA adhesion sequences for cell attachment. (2) The ssDNA patterned slide is placed in a machined jig and flow cells are created using a gasket and top glass slide with flow access ports. Lipid-DNA tagged cells are introduced to the flow cell for attachment to the gel. (3) Hydrogel precursor is introduced to the flow cell to generate thin sheets containing cell patterns that can then be released from the flow cell and cultured floating in media or adhered to a glass surface

3.1 Fabrication of Hydrophobic Silicon Wafer with Shims for Gel Spacing

1. Design "shim" photomask. Two shims will run lengthwise to hold up a standard 1″ × 3″ microscope slide at a 30 μm offset from the wafer. CAD file with dimensions is included in the supplementary material.
2. Bake silicon wafer on a hot plate at 200 °C for 10 min.

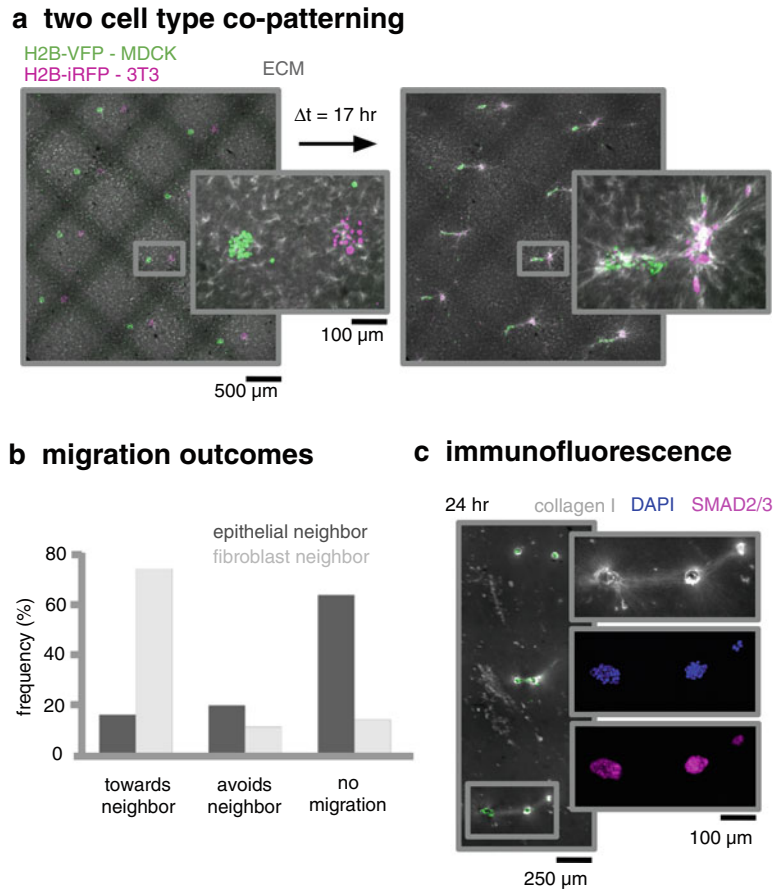

Fig. 3 Here, epithelial cell collectives efficiently elongate and migrate along tensile ECM "straps" generated in the matrix by nearby fibroblast clusters, similar to guidance of the developing mammary gland epithelium by aligned ECM fibers [16]. In this system, several parameters can be independently and quantitatively modulated, including the cell types, the initial cell cluster shapes and sizes, the cell-derived force, and 3D culture medium. (**a**) Co-patterned 100 μm diameter Madine-Darby Canine Kidney (MDCK) cell and 3T3 cell clusters at 250 μm spacing in Matrigel + 1 mg/mL Collagen—NHS—AlexaFluor555. (**b**) Qualitative assignment of MDCK migration modes at $t = 17$ h after patterning ($n = 35$ for 3T3 fibroblast neighbor, $n = 80$ for MDCK epithelial neighbor). (**c**) MDCK-MDCK migration pairs fixed and stained after 24 h. SMAD2/3 nuclear translocation occurs during activation of TGF-beta signaling, which is implicated in epithelial-mesenchymal transitions

3. Center the silicon wafer on a vacuum spin coater. Pipette 4 mL of SU-8 2025 photoresist onto the wafer without generating bubbles (*see* **Note 1**).
4. Spin the wafer at 500 rpm for 5–10 s followed by 2500 rpm for 30 s to generate an ~30 μm layer of photoresist on the wafer.
5. Bake the wafer on a hot plate at 95 °C for 5 min.

6. Place the wafer on a flat surface. Place the transparency flat on the wafer with the printed side of the transparency against the wafer. Expose the wafer to 365 nm ultraviolet light for a total of 150 mJ/cm^2 exposure energy (*see* **Note 2**).
7. Bake the wafer on the hot plate at 95 ° C for 5 min.
8. Develop the wafer by submerging in SU-8 developer and agitating gently for 10 min.
9. Clean the wafer with acetone and then isopropanol using spray bottles.
10. Dry the wafer using a stream of compressed air. If light diffracts off of the wafer in a rainbow pattern, the wafer is not fully developed and **steps 8–10** should be repeated.
11. Place the "shim" wafer in a vacuum chamber in a fume hood next to the bottom of a 10 cm Petri dish or other similar open plastic container.
12. Pipette 1–2 mL of dichlorodimethylsilane into the open container.
13. Secure the vacuum tank and apply vacuum for 30 min.
14. Remove the wafer. Rinse with deionized water, and dry with a stream of compressed air. The silicon wafer shim surface should now be very hydrophobic (*see* **Note 3**).
15. Set aside the wafer in a covered Petri dish until use. The wafer can be re-used for about 3–4 weeks. **Steps 11–14** can be repeated if the hydrophobic surface coating is lost after use.

3.2 Functionalization of Polyacrylamide Substrate Slides with Self-assembled Methacrylate Silane Monolayers

1. Submerge microscope slides in a Petri dish containing 0.1% Triton-X-100.
2. Remove slides one at a time and scrub with gloved fingers to remove grease on the slide surface. Rinse with DI water and submerge in a Petri dish containing 1 M sodium hydroxide.
3. Incubate for at least 15 min at room temperature on a plate rocker.
4. Remove slides one by one and rinse with deionized water to remove 1 M sodium hydroxide. Be careful not to touch the flat surfaces of the microscope slide to keep the surface clean. Dry slides with a stream of compressed air.
5. Clean sides can be stored in a Petri dish for future use.
6. Before continuing, mark the 'bottom' surface of the slide with a diamond scribe with a distinguishing mark to maintain the orientation of the slide for the rest of the procedure. The "bottom" surface is the surface that will not be used for gel attachment.

7. In a fume hood, make methacrylate solution by combining 3-(trimethoxysilyl)propyl methacrylate, acetic acid, and ultrapure water in a 2:3:5 ratio. Vortex until the solution becomes clear. About 100 μL of solution is needed per slide.
8. Lay the cleaned microscope slides flat in a fume hood with the 'top' surface facing up. Pipette about 150 μL of methacrylate solution onto the top surface of half of the slides in a large droplet.
9. Place a separate cleaned slide on top of the slides that have a methacrylate solution so that both 'top' surfaces are facing each other. Drop the slide down slowly at an angle so that a film of methacrylate solution is generated in between the two slides.
10. Incubate the 'slide sandwiches' for 30 min in a fume hood.
11. Remove slides from the slide sandwich and rinse in 100% methanol (*see* **Note 5**).
12. Rinse slides in deionized water and dry with a stream of compressed air. Make sure to keep the functionalized surface clean from this point onwards.
13. The functionalized slides can be stored in a Petri dish for 2–3 weeks for future use.

3.3 Fabrication of Photoactive Polyacrylamide Gels

1. Place the shim wafer flat on a surface and rest the methacrylate-functionalized glass slide on the shims with the functionalized glass surface facing the wafer.
2. Make gel precursor by diluting 30% Acrylamide/Bis-Acrylamide to 4% in 1× PBSCMF in a 1.7 mL eppendorf tube. Approximately 250 μL of gel precursor will be needed for each gel.
3. Degas gel precursor by poking holes in the top of the 1.7 mL tube using a 21G needle. Apply a vacuum line to the top of the tube while placing the bottom of the tube in an ulstrasonic bath sonicator for ~2 min to remove dissolved air.
4. Working quickly, add 20 μL of 5% SDS and 20 μL of 5% Triton-X-100 per 1 mL of gel precursor solution to promote BPMAC dissolution in the gel precursor. Do not vortex.
5. Add 20 μL of BPMAC stock solution per 1 mL of gel precursor solution to generate a 2 mM BPMAC gel.
6. Vortex gently and briefly, so as to reintroduce as little air as possible to the solution, until BPMAC fully dissolves in the gel precursor solution.
7. Add 6 μL of 10% TEMED and 6 μL of 10% APS per 1 mL of gel precursor solution. Vortex briefly and gently.
8. Pipet 150 μL of gel precursor at the short side of the glass slide in between the wafer and the slide. Allow the solution to wick

through to cover the slide surface without generating bubbles. Use a dry delicate task wipe to wick away excess gel precursor (*see* **Note 6**).

9. Cover the wafer and gel as it polymerizes with the top of a 15 cm Petri dish. Place a delicate task wipe dampened with deionized water under the Petri dish next to the wafer to maintain environmental humidity as the gel sets.
10. Incubate the gel for 30 min to allow for the gel to set.
11. Remove the Petri dish cover and cover the slide with 10 ml of 1× PBSCMF.
12. Insert a razor blade corner between the slide and silicon wafer, then slowly twist to gradually detach the slide bearing the gel from the wafer. Make sure to do this while the slide is submerged in 1× PBSCMF to prevent the gel from tearing.
13. The gel can be stored submerged in 1× PBSCMF in the dark at 4 °C for about 2 weeks before photopatterning.

3.4 Quartz-Chrome Photomask Design (See *Note 7*)

1. The gasket for generating flow cells for cell patterning in upcoming steps divides a 1″ × 3″ slide into 16 regions. The desired patterns must be contained within these subdivided regions of the gel. The dimensions and locations of the flow cells on a 1″ × 3″ slide are provided in a CAD file in supplementary material.
2. If patterning two distinct DNA strands in succession for the patterning of multiple cell types, two separate photomasks must be made with registration marks for manual pattern alignment. We have had success using a 1″ × 3″ outline of a microscope slide on each photomask for rough alignment and also using a "crosshairs" design in all four corners of the gel for more precise alignment (*see* **Note 8**). CAD files with dimensions are included in the supplementary material.

3.5 Photopatterning ssDNA

1. Dry the photoactive polyacrylamide gel using a gentle stream of compressed air and place the dry gel in a closed 10 cm Petri dish with a dampened delicate task wipe.
2. Place the 10 cm Petri dish containing the gel and the quartz-chrome photomask in the glovebox and purge it with nitrogen.
3. Prepare a 15 mL conical tube containing 10 mL of TAE. Degas the TAE similarly to the gel precursor in Subheading 3.3.
4. Place the de-gassed TAE in the nitrogen-purged glove box and bubble gaseous nitrogen into the TAE.
5. Hydrate the photoactive polyacrylamide gel by pouring the nitrogenated TAE into the Petri dish containing the gel.

6. Dry the gel within the glovebox with a stream of compressed nitrogen and set aside.
7. Introduce pipettor and first ssDNA solution to the glove box.
8. Pipette 175 μL of first ssDNA solution onto the patterned side of the quartz-chrome photomask. Pipette in 5–10 large droplets equally spread on the photomask pattern.
9. Place the dry photoactive polyacrylamide gel onto the photomask slowly at a decreasing angle so that a film of patterning DNA solution is generated between the gel and the photomask. Slide the gel back and forth along the photomask to release any bubbles that may have been generated in the DNA solution.
10. Use a delicate task wipe to wick away any excess patterning solution (*see* **Note 12**).
11. Remove the photomask and gel from the glove box and place in the UV crosslinker with the photomask facing the light source. Expose for 2 min at a power of ~7 mW/cm^2 (*see* **Note 13**).
12. Remove the photomask and rest it on a flat surface slide side up.
13. Pipette 5–10 mL of TAE to submerge the entire slide. Slide a razor blade corner in between the slide and photomask and twist slowly to release the gel from the mask. Make sure the slide is submerged during this process to prevent the gel from tearing.
14. Place the gel slide in a 10 cm Petri dish and incubate for 10 min at room temperature in TAE + 0.1% SDS on a plate rocker.
15. Place the gel in a new Petri dish containing TAE Buffer × 2 exchanges for 10 min on a plate rocker to remove SDS.
16. The gel can be stored at 4 °C in TAE for 2–3 weeks until ready for second ssDNA strand patterning or cell patterning.
17. The next steps are only necessary for second ssDNA patterning.
18. Gently dry the first ssDNA patterned photoactive polyacrylamide gel with a stream of compressed air and place it on a flat surface.
19. Pipette 15 μL of FAM-ssDNA probe solution in a droplet onto each of the four corners of the gel in the general location of the registration marks (*see* **Note 14**).
20. Incubate the gel at room temperature for 15 min. Place a dampened delicate task wipe next to the gel and cover with a Petri dish cover to prevent the gel from drying out.

21. Use a dry delicate task wipe to wick away the FAM-ssDNA probe solution droplets at each corner.
22. Rinse the four corners of the slide with a TAE spray bottle. Angle the gel so that the TAE buffer runoff does not flow through the middle of the gel (*see* **Note 14**).
23. Incubate the gel at room temperature for 10 min submerged in 15–20 mL of TAE in a 15 cm Petri dish to remove any remaining unbound FAM-ssDNA probe.
24. Use a widefield fluorescence microscope to confirm FAM signal in the registration "crosshair" design in all four corners.
25. Store at 4 °C in TAE. Perform second ssDNA patterning shortly after marking the registration patterns for alignment. Can store up to 1–2 days if necessary.
26. Repeat **steps 1–7** for the second ssDNA pattern.
27. Pipette 350 μL of second ssDNA solution onto the patterned side of the second strand photomask in 5–10 large droplets across the pattern (*see* **Note 15**).
28. Place the dry gel slide onto the photomask similarly to the first ssDNA round, except do not use a dry delicate task wipe to wick away excess fluid (*see* **Note 15**).
29. Remove the photomask and gel from the glove box and place the photomask on the stage of a widefield fluorescence microscope. Adjust the gel position by sliding it on the photomask while viewing the registration marks through the microscope (*see* **Note 16**).
30. When the gel is aligned, use a delicate task wipe to wick away excess fluid generating a capillary suction between the gel and the photomask holding the gel in place.
31. Repeat **steps 11–16**.

3.6 Cell Patterning on ssDNA-Functionalized Polyacrylamide Gel and Transfer to 3D Culture

1. Prepare slides that will create the 'top' surface of flow cells by washing 1″ × 3″ slides with 0.1% Triton-X-100.
2. CAD file for the location of through-holes for flow cell access ports on a 1″ × 3″ slide is included in the supplementary material.
3. Use a laser etcher to generate through-holes for flow cell access ports. Use 20–30 passes of a 50 W etching laser at 100% power, 15% speed, 350 pulses per inch. Keep track of the slide's top and bottom surfaces from now on (*see* **Note 21**).
4. Wash the slide thoroughly in 0.1% Triton-X-100 and deionized water to remove any glass debris.
5. Pipette 150 μL of Sigmacote onto an untreated glass microscope slide and place the 'top slide' onto this slide slowly and at

an angle to generate a thin film of Sigmacote against the bottom of the top slide. Incubate at room temperature for 10 min.

6. Wash the 'top slide' in 100% ethanol then deionized water and incubate at room temperature in a Petri dish submerged in 2.5% w/v bovine serum albumin for 1 h. This generates a protein coating on the side of the 'top-slide' that will face the cells and 3D culture medium. This prevents cell and 3D culture medium attachment to the top slide.
7. Detach cells from the tissue culture flask or dish using trypsin or other cell dissociation agent.
8. Centrifuge cells to generate a pellet. Remove and resuspend in 10 mL 1× Sterile PBSCMF. Repeat.
9. Pellet cells again and resuspend in 100 μL of 1× Sterile PBSCMF per 5×10^6 cells used and transfer to a 1.7 mL tube. Cells can be apportioned 100 μL at a time into separate tubes here for multiple lipid-DNA labeling reactions (*see* **Note 22**).
10. Add 3 μL of anchor DNA stock for a final concentration of 3 μM. Incubate the tube at room temperature on a gentle vortex (approximately 5 Hz) for 5 min (*see* **Note 23**).
11. Add 3 μL of co-anchor DNA stock for a final concentration of 3 μM. Incubate the tube as in **step 9**.
12. Add 3 μL of adhesion ssDNA stock for a final concentration of 3 μM. Incubate the tube as in **step 9**.
13. Resuspend in 1 mL of Sterile 1× PBSCMF and pellet cells. Repeat 3 times to remove any unincorporated lipid-DNA from solution.
14. Resuspend the cell pellet in 150 μL of 1× sterile PBSCMF per 5×10^6 cells to generate a cell-dense solution of lipid-DNA labeled cells.
15. Maintain the cell solution on ice until ready for patterning.
16. Dry the DNA-patterned gel slide using a gentle air stream. Store in a Petri dish containing a dampened delicate task wipe to maintain a humid environment.
17. In a biosafety cabinet, place the dry DNA-patterned slide in the jig bottom.
18. Using forceps, place the gasket on top of the polyacrylamide gel so that it is flush against and is roughly in the center of the gel (*see* **Note 24**).
19. Dry the top slide with a stream of compressed air.
20. Pipet 100 μL droplets of ice-cold 1× PBSCMF onto each of the 16 gel areas to hydrate and prime the gel (*see* **Note 25**).

21. Gently place the top slide down at an angle onto the gel and gasket so that the entry and exit ports are located within each flow cell. Drop the top slide down slowly to help prevent bubbles from forming within the flow cells (*see* **Note 26**).
22. Place the hybridization cassette gasket on top of the slide sandwich to demarcate the flow cells in separate wells.
23. Place the top of the jig setup on the top slide and screw into place with screws and wingnuts to assemble the jig. Screw in just tightly enough so that the jig setup is secured.
24. Maintain the jig setup on ice from this point on.
25. Flow 100 μL of ice-cold PBSCMF through each flow cell to further prime them (*see* **Note 27**).
26. Pipette 18 μL of the labeled cell solution into each flow cell for patterning. Observe periodically under a simple light microscope to confirm there is a confluent "blanket" of cells throughout the flow cell.
27. Incubate the flow cells on ice for 10 min.
28. Pipette another 18 μL of cell solution into each flow cell. Can use the ejected cells from the previous flow cell to inject into the next flow cell.
29. Incubate the flow cells on ice for 10 min.
30. Remove un-patterned cells by pipetting 100 μL of ice-cold PBSCMF into each flow cell in the entry port. Rotate the jig and repeat except pipetting the PBSCMF through the exit port. Observe pattern fidelity through a widefield microscope. Repeat washes until there is a low background of un-patterned cells.
31. Prepare desired 3D culture medium. The following instructions are for generating 1 mg/mL collagen-NHS-AlexaFluor555 in growth factor reduced Matrigel.
32. Prepare 0.5 M sodium hydroxide in 10× PBSCMF for collagen neutralization by combining 1 μL of 10 M sodium hydroxide and 199 μL 10× PBSCMF. Mix by vortex. Maintain on ice.
33. Combine 175 μL of collagen 3.9 mg/mL, 20 μL of 0.5 M sodium hydroxide in 10× PBSCMF, and 5 μL of 1 mg/mL NHS-AlexaFluor555 in DMSO. Mix by gentle pipetting. Incubate on ice for 15 min (*see* **Note 28**).
34. Combine 180 μL of growth factor-reduced Matrigel and 70 μL of neutralized collagen on ice. Mix by gentle pipetting without introducing bubbles.
35. Inject 40 μL of Matrigel/collagen mixture into each cell-pattern containing flow cell. Remove excess fluid from the flow cell exit ports.

36. Observe the flow cells for a pink hue to confirm that 3D culture medium is in the flow cell.
37. Place the jig in a "deep" 15 cm Petri dish and incubate at 37 °C for 30 min to allow for the 3D culture media to set within the flow cells.
38. Fill a "deep" 15 cm Petri dish with cell culture medium enough to submerge the flow cells in the jig. Remove any bubbles that form. Submerge the jig setup in the culture medium.
39. Disassemble and remove the jig while keeping the top and bottom slides submerged and sandwiched together.
40. Hold the top and bottom slides together to prevent them sliding across each other. Slide a razor blade corner between the top and bottom slides. Slowly twist the razor blade to release the top slide (*see* **Note 29**).
41. Place the Petri dish under a dissection microscope for observation while manipulating.
42. Use a probe to gently release bubbles from the sheets and then to release sheets that are adhered to the polyacrylamide gel or the top slide into the media (*see* **Note 30**).
43. Trim the edges of the sheet with a razor blade.
44. Make a "wide-mouth" pipette tip by using a razor blade to remove the tip of a P1000 pipette tip. Use a pipette with the wide-mouth pipette tip to aspirate and transfer each sheet to a well of a 24-well glass coverslip bottom plate.
45. Repeat **steps 40–42** for all sheets.
46. View the sheets in the 24-well plate under a widefield microscope while "flipping" the sheet with the probe tool to discern the cell-patterned side of the sheet. Orient the sheet so that the cell-patterned side is facing up (*see* **Note 31**).
47. To adhere the sheet to the cover-slip bottom, hold the sheet down against the glass with the probe tool while aspirating the cell culture medium in the well. Make sure the sheet is flush against the plate bottom. Aspirate as much media as possible. Remove the media slowly to avoid tearing the sheet.
48. Repeat **steps 44** and **45** for all wells.
49. Incubate the 24-well plate at 37 °C for 30 min to adhere sheets to the glass.
50. Gently add 500 μL of desired cell culture medium to each well. Pipet gently against the side of the well to maintain sheet attachment to the glass.
51. The 24-well plate containing the sheets can now be cultured and imaged using confocal microscopy (*see* **Note 32**).

52. ECM sheets can be fixed for immunofluorescence microscopy at the termination of the experiment. Again, reagents should be pipetted gently against the side of the well during fixation and staining to maintain adherence.

4 Notes

1. We use scissors to cut a transfer pipette to generate a "wide mouth" opening to prevent bubbles forming in the photoresist when transferring.
2. It is acceptable to overexpose the wafer rather than underexpose in this case.
3. Large droplets of water with high surface contact angles should form on the wafer while rinsing due to the hydrophobic surface. Repeat **steps 11–14** if you do not see this.
4. Benzophenone methacrylamide can be synthesized as previously reported (Hughes, PNAS 2012). However, we purchased a custom synthesized lot (PharmAgra).
5. We rinsed in methanol using a glass container in a fume hood. Use forceps to submerge the functionalized slides in methanol and gently agitate for 1 min.
6. Generating bubbles in the gel is a common failure point. Shortly after pipetting and before the gel solidifies, you can slide the top slide along the shims to expose the bubbles to air. This will remove the bubbles from the gel. However, work quickly because oxygen can prevent acrylamide polymerization.
7. It is important to use a quartz-chrome photomask rather than the more widely used soda lime glass-chrome photomasks. Quartz will allow 254 nm ultraviolet light necessary for BPMAC photoactivation to pass through transparent sections of the photomask, while soda lime will not. Quartz-chrome photomasks can be ordered from various vendors, we procured quartz-chrome photomasks through the Singh Center for Nanotechnology at the University of Pennsylvania.
8. The registration mark for the first photomask is a crosshair design. For the second photomask we included a window in the crosshair design so that the first DNA pattern can be easily viewed while manually aligning the second photomask. These registration marks are in the same exact location on the first and second photomasks. Note that the registration marks are located 2.5 mm from the corners of the glass slide. This is to account for the photoactive polyacrylamide gel not forming all the way to the edge of the slide surface. After the first ssDNA is patterned, the left-right orientation of the slide must be maintained so that asymmetrical patterns can be aligned.

9. The patterning ssDNA sequence is 5′-T20-X20-3′. We have found that the 20 bp Poly(T) sequence is important for the ssDNA to efficiently bind to the photoactive polyacrylamide gel. The X20 sequence is used for cell attachment. We utilized X20 "adhesion" sequences "F" and "G" in 2-pattern experiments as previously described. X20 for strand "F" is 5′-AGAAGAAGAACGAAGAAGAA-3′, and X20 for strand "G" is 5′- AGCCAGAGAGAGAGAGAGAG-3′. These sequences in addition to other adhesion sequences can also be found in previous works [7, 9, 12]. New "adhesion" sequences can also be designed. Newly designed "adhesion" sequences should be predicted to have a little non-specific binding to other sequences used in the same experiments.
10. We measured this 254 nm ultraviolet crosslinker at a power of ~7 mW/cm^2. The 2 min exposure time can be adjusted for the specific power of the light source used. The major absorption peak of benzophenone is 240–280 nm [13, 14]. Choose a light source that will emit in this range. We did not achieve sufficient photoactivation of BPMAC utilizing 365 nm LED light sources.
11. The ssDNA probe sequence is the reverse-complement of the "adhesion" sequence of the first patterning ssDNA.
12. Placing a flat edge of a dry delicate task wipe along the length of the slide against the photomask will wick away any excess fluid. This will also generate a kind of "seal" between the gel and the photomask. This is helpful to keep the gel in place against the photomask while transporting the gel and photomask for ultraviolet light exposure.
13. If using a 5″ × 5″ quartz-chrome photomask it may be helpful to use glass slides placed around the edges of the photomask to help balance the photomask on top of the photoactive polyacrylamide gel as the photomask faces the light source.
14. Make sure the gel is sufficiently dry so that there is sufficient surface tension to keep a droplet of FAM-ssDNA probe solution from spreading. If the FAM-ssDNA probe diffuses and binds to the first photopatterned ssDNA, cell patterning may be blocked in those locations. For this same reason, it is also important not to let the FAM-ssDNA probe solution flow through the middle of the gel when washing the solution off of the corners of the gel.
15. The second pattern requires more ssDNA solution during patterning so that the gel can be more easily readjusted on the photomask for alignment without tearing the gel. It is normal to have some excess fluid around the slide at this point.

16. Work as quickly as possible during alignment to prevent oxygenation of the gel by room air. We have found that aligning for 5–10 min before ultraviolet exposure still allows for second ssDNA patterning.
17. "universal Anchor" DNA (5′- TGGAATTCTCGGGTGC CAAGGGTAACGATCCAGCTGTCACT-C24 lipid (lignoceric acid)-3′) and "universal co-anchor" (5′-C16 lipid (Palmitic acid)- AGTGACAGCTGGATCGTTAC -3′) were custom synthesized from OligoFactory. The lipid-DNAs were resuspended and aliquoted as 100 μm stocks in deionized water in 1.7 mL tubes. The stocks were stored at −80 °C until use. Working aliquots were stored at −20 °C for 1–2 months.
18. The "adhesion" ssDNA sequence is 5′-CCTTGGCACCCGA GAATTCCA-T_{20}-Y_{20}-3′, where Y_{20} is the reverse complement of the corresponding ssDNA that was patterned on the photoactive polyacrylamide gel.
19. Jig parts were designed in Rhino 3D modeling software (Robert McNeel & Associates) and custom CNC-milled in 6061-T651 aluminum (ProtoLabs). Cad file is in the supplementary material.
20. 0.01″ thick PDMS (SSP Inc.) membrane was cut using a craftcutter (Silhouette Cameo 3) to demarcate a 2 × 8 array of flow cells. CAD file for the gasket design is in the supplementary material.
21. The surface of the glass slide that is nearest the laser source will have a wider opening for the through hole than the farther surface. The wider opening should be the "top" of the glass slide and face away from the gel when assembling the flow cells. The wider openings allow for fluid entry when injecting fluid into the access ports.
22. We found that carrying out the reaction in 100 μL volumes with approximately 5×10^6 cells worked well. We worked in this volume and cell density even when patterning larger numbers of cells.
23. Cells may resettle to the bottom of the tube during the incubations. If this happens, resuspend the cells gently using a pipette.
24. It is important that the gasket is flush against the gel to generate a seal between the separate flow cells. We gently pressed the gasket against the gel with forceps and gently rolled out any wrinkles in the gasket. The flow cells contain "sharp" and "rounded" corners. Make sure the gasket is oriented so that the "sharp" corners are near the flow cell entry and exit ports. Rounded corners in the flow cell prevent dead areas within the flow cell that limit cell wash-out. It is also important to make sure the gasket is relatively centered at this stage. This is so that

the flow cell entry and exit ports in the top slide are located within each flow cell. The entry and exit ports can become blocked by the gasket if it is off-center. You can check this by holding the top slide with flow access ports above the gel and gasket and observing the entry and exit port locations. You may have to lift off and reposition the gasket on the gel multiple times. Be careful and work slowly when peeling the gasket off of the gel so as not to tear the gel.

25. If the gel is sufficiently dry, the droplets should be prevented from spreading by surface tension. Be careful to prevent PBSCMF from flowing under the gasket. If this happens, the gel will need to be re-dried and the gasket positioned again.
26. If bubbles are formed within some flow cells, it may be possible to remove them by forcefully flowing ice-cold PBSCMF through the flow cells after the jig has been set up. The bubbles can be expelled through the entry and exit flow ports.
27. When injecting fluid into the flow cells only, go to the first stop on the pipettor. This prevents air bubbles from being introduced into the flow cells. Air bubbles will block fluid flow and prevent cell patterning.
28. Collagen concentration varies by batch. The proportions can be adjusted for the desired final collagen concentration.
29. This is a very delicate step. Be careful not to allow the slides to slide along each other. This can generate tears in the thin 3D culture medium sheets. Be careful to keep both slides submerged at this stage since the sheets may stick to the top slide, the bottom slide, or they may float away into the Petri dish. It may be difficult to keep track which flow cell each sheet came from. It is useful to have a method for discerning separate patterns later on.
30. Air bubbles are commonly formed under the sheets in this step. These bubbles can be released by poking them using the probe. It is important to work gently in these steps to prevent the gel from tearing. Additionally, the sides of the sheets are commonly attached to the sides of the gasket. Remove these attachments by gently sliding the probe along the sheet and gasket interface.
31. The cells will be patterned in 3D just slightly under the matrix surface on one side of the sheet. It is important that the cells face away from the glass coverslip bottom while adhering the sheet to the plate.
32. We found that using an objective with a working distance of at least 4 mm was necessary to view the top of adhered sheets when using a coverslip-bottom plate.

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