Three-dimensional lithographically defined organotypic tissue arrays for quantitative analysis of morphogenesis and neoplastic progression

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Here, we describe a simple micromolding method to construct three-dimensional arrays of organotypic epithelial tissue structures that approximate *in vivo* histology. An elastomeric stamp containing an array of posts of defined geometry and spacing is used to mold microscale cavities into the surface of type I collagen gels. Epithelial cells are seeded into the cavities and covered with a second layer of collagen. The cells reorganize into hollow tissues corresponding to the geometry of the cavities. Patterned tissue arrays can be produced in 3–4 h and will undergo morphogenesis over the following 1–3 d. The protocol can easily be adapted to study a variety of tissues and aspects of normal and neoplastic development.

INTRODUCTION

The ability to recapitulate normal and diseased tissue histology faithfully and reproducibly in culture would revolutionize science and medicine. Engineered tissues could be used by cell and developmental biologists to investigate the basic processes underlying normal morphogenesis, by cancer biologists to study how those control processes are coopted or circumvented during neoplastic progression and by clinicians as therapeutic replacements for diseased organs. Indeed, a few relatively simple models used extensively over the past 30 years have yielded insight into the normal and diseased development of mammary gland acini[1,2], renal cysts[3,4] and microvascular endothelial cords[5,6]. In these now-traditional assays, cells are embedded in gels of extracellular matrix (ECM), usually reconstituted type I collagen or an extract of basement membrane. Although they produce tissue structures with some similarity to their *in vivo* counterparts, the methods rely primarily on cell-driven self-assembly and are poorly controlled either spatially or temporally. The resulting tissues are therefore heterogeneous in size, geometry and composition and are difficult to analyze quantitatively.

Reproducing *in vivo* tissue structure requires building three-dimensional (3D) systems with micrometer-scale resolution and control. A plethora of techniques have been developed to create patterns of proteins and cells in two dimensions (2D)[7,8]. Most rely on variations of photolithography (light-based patterning) or soft lithography (contact-based patterning using elastomeric stamps to transfer pattern). Only recently have investigators succeeded in adapting these techniques for 3D systems. Several groups have focused on creating synthetic hydrogels containing specialized chemical moieties that can be polymerized into complex microscale topologies using patterns of light[9,10]. Combined with optical or electrophoretic methods to direct the location of cells, this approach can be used to define the geometry and position of microscale colonies of cells[11,12]. However, in studies published to date, although the cells achieve differentiated function, they fail to cohere into a tissue or to faithfully recapitulate *in vivo* structure, likely due, in part, to the artificial nature of the synthetic hydrogels and the resulting lack of appropriate biochemical signals.

To build microscale topologies using native ECM proteins, we and others have developed contact-based techniques using elastomeric stamps of polydimethylsiloxane (PDMS) to mold microscale features into ECM gels[13]. Tien and colleagues[14] identified surface treatments for PDMS that would allow the molded ECM gels to detach easily from the stamps without distorting the patterned features. Defined cavities can be created within monolithic gels by using sacrificial elements such as paraffin or gelatin[15–17], and stacking multiple gels can generate more complicated multilayered structures[14,18]. These techniques have been used successfully to construct simple endothelial tubes with correct histology and physiology that are capable of being perfused with blood or other solutions[19].

Here, we describe a technique that uses replica micromolding and layer-by-layer assembly to generate geometrically precise arrays of multicellular epithelial tissues in 3D ECM gels (**Fig. 1**). In brief,

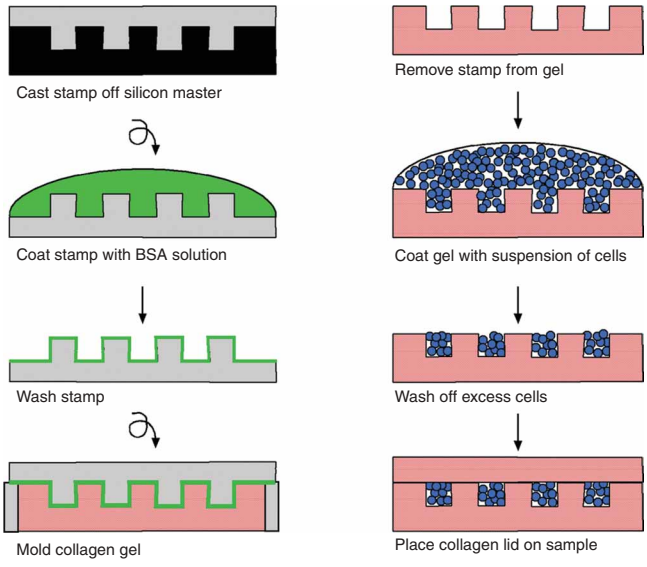

Figure 1 | Schematic of method to pattern multicellular tissues in micromolded gels.

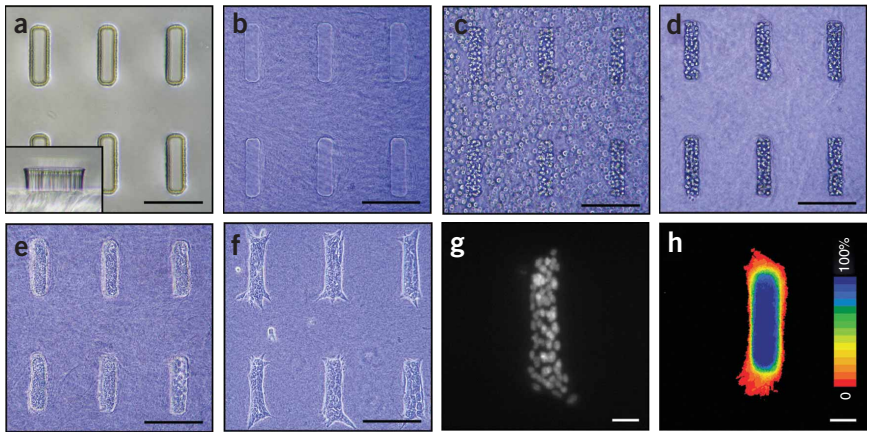

Figure 2 | Images of different stages of the patterning process. (**a**) PDMS stamp (inset: vertical section through one post); (**b**) molded collagen gel; (**c**) molded gel during addition of cells (note that cells are both in the wells and on top of the gel); (**d**) molded gel after washing away excess cells; (**e**) tubules; (**f**) branched tissues 24 h after addition of EGF to the sample; (**g**) one branched tissue stained for nuclei with Hoechst 33258; (**h**) frequency map depicting quantification of 50 branched tissues. Scale bars, 200 μm (**a**–**f**) and 50 μm (**g**,**h**).

an elastomeric PDMS stamp containing a relief of the desired tissue architecture is used as a releasable mold. The stamp is treated with a solution of inert protein to render the surface nonadhesive to the ECM gel. Modified stamps are placed on a drop of liquid-neutralized collagen or Matrigel under conditions that favor gelling of the ECM polymers. Removal of the stamp reveals microscale indentations within the gel that correspond to the bas-relief pattern on the stamp. A concentrated suspension of cells or primary organoids is allowed to settle within the micromolded gel cavities. Excess cells are removed by gentle washing, and the cavities containing cells are sealed by placing a slab of unpatterned gel on the surface.

The 3D patterning technique is flexible—it can be used to mold microscale features into a wide range of natural and synthetic polymers. Sharply defined features down to <1-μm resolution can be introduced into the gels[14]. We have successfully used this procedure to pattern multicellular tubules of human and murine mammary epithelial cells[20], kidney epithelial cells as well as microvascular endothelial cells. The geometry of the tissue is dictated by the geometry of the molded cavities, which is determined by the features on the surface of the stamp, which are determined *a priori* by the investigator (**Fig. 2**). The assay is readily quantifiable with a high level of statistical confidence because each sample consists of an array of hundreds of multicellular tissues, each having the same initial geometry. We have used this principle to analyze quantitatively the spatial and temporal dynamics of gene expression changes and alterations in cell positions during branching morphogenesis of mammary epithelial tubules[20].

The potential applications of the 3D patterning protocol are diverse. Basic studies of cell–cell interactions can be performed by simultaneously patterning two cell types (e.g., luminal epithelial and myoepithelial) within the molded cavities. The heterotypic mixture of cells reorganizes to form a bilayered structure that approximates *in vivo* histology[20]. Epithelial/mesenchymal interactions can be studied by patterning epithelial cells in the cavities and interspersing fibroblasts or other mesenchymal cells in the bulk ECM gel. Patterning cancer-derived cells or cells with oncogenic mutations can be used to analyze aspects of neoplastic progression such as loss of polarity, luminal filling and uncontrolled cellular invasion. The tissue arrays could also conceivably be used to screen libraries to identify potential drug targets or therapeutic agents. The final tissues produced are only limited by the properties of the ECM gel (softer gels, in general, fail to retain pattern), the supply of cells and the resolution of the lithographic techniques used to create the initial mold. Highly compliant ECM gels that lose their structure when patterned with the technique described here may still be successfully employed as scaffolds using laser-guided approaches[21].

MATERIALS

REAGENTS

- PDMS (Sylgard 184, Dow Corning)
- Ethanol
- PBS, Ca^{2+}- and Mg^{2+}-free
- Bovine serum albumin (BSA; Calbiochem)
- Collagen (e.g., bovine dermal or rat tail collagen; BD Biosciences)
- 0.1 N NaOH
- 10× PBS or 10× DMEM (hereafter referred to as 10× buffer)
- Glass coverslips, 15-mm diameter, #1 thickness
- Paraformaldehyde

EQUIPMENT

- Vacuum chamber (e.g., vacuum desiccator without desiccant)
- Lithographically patterned silicon master (with 1:1 height/width aspect ratio)
- Oven
- Equipment for culturing mammalian cells
- Fluorescent microscope for evaluation of stained samples

PROCEDURE

Preparation of stamps

1| Mix the PDMS prepolymer and curing agent at a 10:1 (wt/wt) ratio. Remove entrapped air bubbles by degassing in a vacuum chamber (~15 min). Pour the bubble-free mixture onto the silicon master (~35 g in a 100-mm diameter Petri dish yields the correct height of stamp). Cure the PDMS in an oven at 60 °C for at least 2 h.

■ **PAUSE POINT** Polymerized PDMS can be stored for several months under ambient conditions before cutting into stamps.

2| Carefully peel the PDMS from the silicon wafer. Using a clean razor blade, cut the polymerized PDMS into stamps (~5-mm cubes), making one stamp for each sample. Place the stamps feature side up in a clean Petri dish.
■ **PAUSE POINT** Stamps can be stored for several months in a dust-free atmosphere.

3| In a biosafety cabinet (cell culture hood), sterilize stamps by washing briefly in ethanol. Allow ethanol to evaporate completely (~2 min).

4| Coat the feature-side surface of the stamp with 30 µl of 1% (wt/vol) BSA in PBS for at least 30 min at room temperature (22 °C) or overnight at 4 °C. Ensure that all air bubbles are removed from the surface of the stamp before starting the incubation.
▲ **CRITICAL STEP** Sufficient BSA coating is essential to permit the stamp to be unmolded from the collagen gel. Do not allow the drop of BSA to roll off the surface, but cover completely for best results.

Molding of gels

5| Prepare a neutralized collagen solution by mixing the stock collagen with 0.1 N NaOH and 10× buffer on ice, according to the manufacturer's instructions. A final volume of 1 ml is needed for every eight samples to be patterned, although the volume needed will scale with the surface area of the stamps. Mix thoroughly but gently so as to avoid introducing air bubbles into the collagen solution. The final pH should be ~7.2. Final concentrations of 2–4 mg ml^{-1} collagen have been used successfully.
▲ **CRITICAL STEP** The neutralized collagen will gel at room temperature. Read the collagen supplier's instructions carefully. Keep on ice!

6| Prepare collagen 'lids' by pipetting 30 µl of neutralized collagen onto the surface of a sterile coverslip. The collagen should form a drop in the center of the coverslip. Make one lid for each stamp.

7| Aspirate the BSA solution from the stamps. Wash the BSA-coated surface twice with neutralized collagen (~30 µl per stamp). Place a final drop (~30 µl) of neutralized collagen on the surface of the stamp. Flip over the collagen-coated stamp into a 35-mm Petri dish. Place dishes and lids in a 37 °C incubator for 30 min.
▲ **CRITICAL STEP** Work quickly to prevent premature gelling of collagen. Avoid using too large a volume of collagen to coat the surface, as this increases the likelihood of the collagen dripping off the stamp while the stamp is being flipped into the dish. It helps to balance the inverted stamp on two flat slabs of PDMS (~1-mm thick) in the bottom of the dish, preventing the stamp from settling at the bottom of the surface.

Patterning of cells

8| Prepare a concentrated suspension of cells (~10^6–10^7 cells ml^{-1}). Keep the cells on ice.

9| Carefully remove the stamp from the collagen gel by pulling straight up with sterilized forceps. Avoid shearing the gel to prevent distortion of the molded wells.

10| Immediately add a drop (~30 µl) of concentrated cells to the molded surface of the collagen gel. Under a phase-contrast tissue-culture microscope, monitor the sample. As soon as the wells are filled with cells (~1–2 min), wash the sample by tipping the dish at a 45° angle and gently pipetting 400 µl of cold media across the surface to remove excess cells. Place the sample in cell culture incubator for 5 min to allow patterned cells to adhere to the collagen.

11| After the cells have started to adhere to the gel, gently cover the gel with a collagen lid. Allow the collagen lid to settle for 2 min. At this point, the glass coverslip can be removed if desired. Fill the 35-mm dish with 2.5 ml of growth medium and return patterned cells to the incubator. The final thickness of the patterned collagen and lid is ~1–2 mm. Cells should reorganize into tubules or cysts (depending on the aspect ratio of the wells) within 24 h. A lumen may fail to form if using cancer cells.

Inducing morphogenesis

12| To induce branching morphogenesis, prepare a solution of growth medium containing growth factors (e.g., epidermal growth factor (EGF) or hepatocyte growth factor (HGF) at the desired concentration. Aspirate media from samples and replace with the growth factor-containing medium. Branches can be observed at ~20 h after addition of growth factors and will continue to grow over several days. Medium should be changed every 48 h.

Quantifying cell positions

13| Remove samples from incubator. Aspirate medium and replace with PBS. If it is still present, gently remove glass coverslip from the surface of sample. Aspirate PBS and replace with fixative solution (4% paraformaldehyde in PBS). Incubate at room temperature for 20 min.

14| Wash fixed samples twice in PBS. To analyze cell positions, incubate samples in a solution of DAPI or Hoechst 33258 to mark cell nuclei. Samples can also be stained for other markers using individual protocols.
■ **PAUSE POINT** Once fixed and stained, samples can be stored for several weeks at 4 °C in original dishes or can be mounted on glass slides.

15| Evaluate under a fluorescent microscope. To quantify position of cells, align the tissue in the center of the field of view using an eyepiece or stage micrometer. Take images of at least 50 aligned tissues.

16| To generate a frequency map of the position of cells, convert the 50 gray-scale images into 50 black-and-white images using the binarize function of any image analysis software (e.g., Scion Image). Stack the binary images by adding the images together. This will generate a new gray-scale image, where the intensity at every pixel denotes the percentage of samples that had a cell located at that position. The stacked image can be converted into a color-coded frequency map using the Indexed Color mode in Photoshop.
? TROUBLESHOOTING

● **TIMING**
Steps 1–4, preparation of stamps: casting stamps, ~30 min + 2 h polymerization; coating stamps, ~30 min + 30 min or overnight, depending on incubation temperature
Steps 5–7, molding of gels: washing stamps, ~15 min; gelling collagen, 30 min
Steps 8–11, patterning of cells: preparing cell suspension, ~15 min; loading cells into wells, ~30 min, depending on the number of samples
Step 12, cell culture, 24-72 h, depending on application
Steps 13 and 14, fixation and staining: ~30 min for fixation
Steps 15 and 16, image acquisition and processing: ~30 min per sample

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Collagen gel sticks to the stamp	Stamp insufficiently coated with BSA	Verify that the concentration of BSA is at least 1% (wt/vol) in PBS and that the coating time is at least 30 min at room temperature
	Stamp had air bubbles on the surface during coating process	Dislodge air bubbles before beginning BSA incubation, either mechanically with a pipette tip or thermally by placing the stamps at 4 °C for several hours
	Collagen did not gel	Verify that the neutralized collagen solution is at the proper pH and temperature for gelation. Refer to collagen supplier's instructions for optimum conditions
	Collagen formed a weak gel	Increase the final concentration of the neutralized collagen solution
Collagen wells are distorted or blurred	The gel was sheared during stamp removal	Remove stamp as gently as possible, pulling straight up with forceps
	Air bubbles were present in the collagen solution	Prepare neutralized collagen as gently as possible to prevent introduction of bubbles. Allow bubbles to float to the surface and pop before rinsing stamps
	Collagen gelled unevenly	Mix the neutralized collagen thoroughly on ice. The phenol red in 10× media can serve as a visual indicator that the solution is well mixed
		Work quickly to ensure that collagen does not start gelling before samples are placed at 37 °C
		Remove excess BSA by washing at least twice using liquid neutralized collagen. Many solutions of BSA are slightly acidic and can therefore affect the gelling of collagen

TABLE 1 | Troubleshooting table (continued).

Problem	Possible reason	Solution
Cells fail to form pattern	Cells did not settle into the wells	Gently shake the dish side to side while monitoring on the tissue culture microscope
	Cells were washed out of wells	Take care to wash with a very gentle stream of media. Avoid bubbles
Cells stick outside of collagen wells	Samples were not adequately rinsed	Rinse off excess cells at least three times before placing sample in the incubator
	Cells are too adhesive	Incubate cell suspension on ice for several minutes to half an hour before patterning
		Wash with ice-cold media
		Limit the amount of time cells are in trypsin. We have found that, for mammary epithelial cells, longer times lead to greater 'stickiness'
		Prepare neutralized collagen using 10× calcium- and magnesium-free PBS and plate cells in the same

ANTICIPATED RESULTS

The patterned tissues should precisely match the size, geometry and spacing of the pattern etched into the silicon master. Tissues down to one-cell diameter (~10 μm) can be reproducibly constructed. Stained tissues can be photographed under the microscope and quantified as represented in **Figure 2g,h**. We have found that for tissues constructed of phenotypically normal cells (mammary, kidney and endothelial), the invasion of the cells into the surrounding ECM is controlled by the initial geometry of the tissue[20]. We expect qualitatively similar results for epithelial cells derived from other branched organs, although the governing relationship is likely to vary from organ to organ. Over the time frame of mammary epithelial morphogenesis, we have observed no changes in cell viability as determined by tracking cell number per tubule in real time, although this can be determined explicitly by staining for apoptosis or necrosis *in situ*. Other aspects of morphogenesis and differentiation (cellular movements, gene expression changes, etc.) can also be easily quantified by measuring differences in fluorescence intensity at different time points or via live imaging.

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