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

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Abbreviations: ECM, extracellular matrix; PDMS, polydimethylsiloxane; 2D, two-dimensional; 3D, three-dimensional

Abstract

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 one to three days. 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 co-opted 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 thirty 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 identified surface treatments for PDMS that would allow the molded ECM gels to detach easily from the stamps without distorting the patterned features[14]. 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, 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 non-adhesive 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 (for example, luminal epithelial and myoepithelial) within the molded cavities. The heterotypic mixture of cells reorganizes to form a bi-layered 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

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

EQUIPMENT

- Vacuum chamber (e.g., vacuum desiccator without desiccant)
- Lithographically patterned silicon master
- Oven
- Equipment for culturing mammalian cells
- Fluorescent microscope for evaluation of stained samples

PROCEDURE

Preparation of stamps

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

PAUSE POINT: Polymerized PDMS can be stored for several months 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 bio-safety cabinet (cell culture hood), sterilize stamps by washing briefly in ethanol. Allow to dry completely.

4| Coat the feature-side surface of the stamp with 1% BSA in PBS for at least 30 min at room temperature, or overnight at 4℃. Ensure that all air bubbles are removed from the surface of the stamp prior to starting the incubation.

CRITICAL STEP: Sufficient BSA coating is essential to permit the stamp to be un-molded from the collagen gel. Do not allow the drop of BSA to roll off the surface, but cover completely for best results.

? TROUBLESHOOTING

Molding of gels

5| Prepare a neutralized collagen solution by mixing the stock collagen with 0.1N NaOH and 10X buffer on ice, according to the manufacturer's instructions. A final volume of 1 mL is needed for every 8 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 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!

? TROUBLESHOOTING

6| Prepare collagen 'lids' by pipetting 30 μL of neutralized collagen onto the surface of a sterile coverslip. Make one lid for each stamp.

7| Aspirate the BSA solution from the stamps. Wash the BSA-coated surface twice with neutralized collagen. Place a final drop (~30 μL) of neutralized

collagen on the surface of the stamp. Flip the coated stamp over into a 35-mm Petri dish. Place dishes and lids in 37℃ incubator fo r 30 min.

CRITICAL STEP: Work quickly to prevent premature gelling of collagen. To prevent the stamp from settling to the bottom of the surface, it helps to balance the inverted stamp on two flat slabs of PDMS in the bottom of the dish.

Patterning of cells

8| Prepare a concentrated suspension of cells (~10^6 – 10^7 cells/mL). 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.

? TROUBLESHOOTING

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 pipeting 400 μL of cold media across the surface to remove excess cells. Place sample in cell culture incubator for 5 min to allow patterned cells to adhere to the collagen.

? TROUBLESHOOTING

11| After cells have started to adhere to the gel, gently cover the gel with a collagen lid. Fill the 35-mm dish with 2.5 mL growth medium, and return patterned cells to the incubator. 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., EGF or 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.

Quantifying cell positions

13| Remove samples from incubator. Aspirate medium and replace with PBS. Gently remove glass coverslip from 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℃ in original dishes, or mounted on glass slid es.

15| Evaluate under a fluorescent microscope. To quantify position of cells, align 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 grey-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 grey-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.

TIMING

Steps 1-4: Stamp preparation: Casting stamps, ~30 min + 2 h polymerization; coating stamps, ~ 30 min + 30 min or overnight, depending on incubation temperature.

Steps 5-7: Gel molding: Washing stamps, ~15 min; gelling collagen, 30 min.

Steps 8-11: Cell patterning: Preparing cell suspension, ~15 min; loading cells into wells, ~30 min, depending on number of samples.

Step 12: Cell culture, 24-72 h, depending on application.

Steps 13-14: Fixation and staining, ~30 min for fixation.

Steps 15-16: Image acquisition and processing, ~30 min per sample.

? TROUBLESHOOTING

See **Table 1** for troubleshooting guidance.

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% (w/v) in PBS, and that the coating time is at least 30 min at room temperature.
	Stamp had air bubbles on surface during coating process.	Dislodge air bubbles prior to beginning BSA incubation, either

		mechanically with a pipette tip, or thermally by placing the stamps at 4℃ 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 10X 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℃. 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.
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”.

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 **Fig. 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[13]. 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. Aspects of morphogenesis and differentiation (cellular movements, gene expression changes, etc.) can be easily quantified by measuring differences in fluorescence intensity at different time points or via live imaging.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Figure 1 | Schematic of method to pattern multicellular tissues in micro-molded 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 refer to 200 μm in (a-f) and 50 μm in (g, h).