Chapter 5

Lithographically Defined Two- and Three-Dimensional Tissue Microarrays

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Abstract

Traditional methods to study normal and pathological development of tissues have been limited by difficulties in controlling experimental conditions and quantifying biological processes of interest. Here we describe methods to create microarrays of engineered tissues that enable controlled and quantitative investigations. Using soft lithography-based techniques, extracellular matrix proteins can be microcontact printed or micromolded to make two- and three-dimensional micropatterned scaffolds. The ultimate form and resulting properties of the tissue construct are dictated by the geometry of the patterned extracellular matrix components. This chapter describes elastomeric stamp fabrication, microcontact printing and micromolding of extracellular matrix proteins, cell culture in micropatterned substrata, and quantitative immunofluorescence analysis of micropatterned tissues.

Key words: Tissue engineering, Microfabrication, Organotypic culture, Epithelial

1. Introduction

Understanding the processes involved in both normal and pathological tissue development is crucial to the engineering of tissue constructs for therapeutic and diagnostic purposes (1). Studies in vivo are difficult to control, observe, and quantify. As a result, much effort has been directed toward culturing organs ex vivo. Despite progress made in this field, organ cultures tend to be difficult to maintain as they require fresh tissues and are often uncontrollable. Engineered tissues offer unique benefits over organ culture approaches by allowing for the investigation of developmental processes and cellular behaviors within tissues in a controlled and quantitative manner. By defining the properties of the extracellular matrix (ECM) environment and the specific

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biochemical factors that are presented, one can precisely control the spatial organization and behaviors of the cells that make up the engineered tissue. One can then study how properties of the tissue, such as geometry and form, contribute to the control of biological events including proliferation, apoptosis, gene expression, and differentiation. Geometry can be chosen to best recapitulate the system of interest with, for example, two-dimensional (2D) engineered tissues approximating epithelial sheets and three-dimensional (3D) engineered tissues approximating epithelial tubes. Additionally, engineered tissues are advantageous due to the fact that they can be multiplexed into microarray formats thereby enabling quantitative analysis and high-throughput assays.

Several techniques have been developed to pattern microarrays of ECM proteins in 2D onto rigid substrate. Early experiments used photolithographic methods to deposit adhesive islands of defined size onto nonadhesive substrate in order to study the effects of anchorage on cellular behavior (2). In recent years, soft lithographic techniques, which use elastomeric stamps to either print proteins using contact or adsorb proteins using microfluidics, have become popular (3–5). Soft lithography approaches have enabled the formation of complex patterns and gradients of ECM proteins on 2D substrate (6, 7). Similarly, elastomeric membranes containing holes can be used to mask regions of substrate thus enabling stenciling of proteins onto surfaces (8). More recently, microarrays of ECM proteins have been printed using both a standard DNA spotter and the atomic force microscopy technique of dip-pen nanolithography (9, 10). Sacrificial layers, such as aluminum thin films, have been used to pattern combinations of proteins and bioactive molecules onto silica (11). Of the techniques outlined, soft lithography offers the benefits of low cost and ease of use.

Recently, much effort has been directed toward tailoring the biochemical and mechanical properties of 3D ECMs to more closely mimic the natural cellular microenvironment. Photopolymerization has been used to form hydrogels through the activation of light-sensitive photoinitiator molecules to encapsulate cells and to create scaffolding materials (12). Through the use of multilayer photopatterning platforms, increased complexity can be built into engineered materials (13). Applications of 2D soft lithography have been extended to 3D by using elastomeric stamps as molds for macromolecular gels and hydrogel systems (14–19). Additionally, soft lithographic techniques have been used for layer-by-layer deposition of biopolymers, which exploits the use of alternating layers of cell-adhesive and cell-repellant polysaccharides and proteins, to pattern cellular co-cultures (20). Another strategy for patterning 3D ECMs relies on the use of a combination of microfluidics and sacrificial materials, such as

paraffin, matrigel, and gelatin, to create internal cavities, channels, and networks with gels (21, 22). Although progress has been made in patterning 3D ECMs, much work is still required in order to replicate the complex properties and architectures of ECMs found in vivo.

Here we describe soft lithography-based techniques to pattern 2D and 3D epithelial tissue microarrays using microcontact printing and micromolding approaches, respectively. In both the 2D and 3D patterning methods the geometry of the ECM is controlled, thus dictating the geometry and form of the tissue and the individual and collective behaviors of the cells that make up the tissue. Micropatterned tissues can be treated with biological molecules of interest, and the behaviors of the cells within the tissues tracked statistically by analyzing the spatial distributions of specific cellular markers within the tissue constructs. Here, we outline the procedures for (1) casting elastomeric stamps from patterned templates; (2) microcontact printing islands of ECM proteins onto slides to create 2D tissue microarrays; (3) micromolding collagen gels to create 3D tissue microarrays; and (4) immunofluorescence analysis of micropatterned tissues.

2. Materials

2.1. Stamp Preparation

1. Patterned silicon wafer.
2. Poly(dimethyl siloxane) (PDMS; Sylgard 184, Dow Corning).

2.2. Two-Dimensional Tissue Microarrays

1. Patterned PDMS stamp.
2. 22-mm glass coverslips (Fisher Scientific).
3. Spin-coater.
4. Extracellular matrix protein (fibronectin, BD Biosciences).
5. Phosphate-buffered saline (PBS).
6. Pluronic F108 Pastille, 1% (w/v) solution in PBS (BASF Corporation).

2.3. Three-Dimensional Tissue Microarrays

1. Patterned PDMS stamp.
2. 35-mm tissue culture dish.
3. Ethanol.
4. Bovine serum albumin (BSA, Calbiochem), 1% (w/v) solution in PBS.
5. 10× Dulbecco's Modified Eagle's Medium (DMEM/F12, Sigma).

6. 1:1 Dulbecco's Modified Eagle's Medium:Ham's F12 Nutrient Mixture (DMEM/F12 (1:1), Hyclone), supplemented with 2% fetal bovine serum (Atlanta Biologicals), 50 μg/mL gentamicin (Invitrogen), and 5 μg/mL insulin (Sigma).
7. 0.1 N NaOH.
8. Collagen (bovine dermal or rat tail, BD Biosciences).
9. Glass coverslips, 15-mm diameter.
10. Ice.

2.4. Immunofluorescence Staining and Image Analysis

1. PBS.
2. Paraformaldehyde, 4% (w/v) solution in PBS (Electron Microscopy Sciences).
3. IgePal CA 630, 0.5% (v/v) solution in PBS (Sigma).
4. Block buffer and antibody dilution buffer: 10% (v/v) goat serum/0.1% (v/v) Triton X-100/PBS.
5. Primary antibody: Phospho-p44/42 MAP Kinase (Thr202/Tyr204) Antibody (Cell Signaling Technology).
6. Secondary antibody: Alexa 594 goat anti-rabbit (Invitrogen).
7. Nuclear stain: Hoechst 33258 (Invitrogen).
8. Glass coverslips (Fisher).
9. Mounting medium: Fluormount G (Southern Biotech).
10. Photoshop, ImageJ, or another image analysis program.

3. Methods

Here, we describe soft lithography-based methods for creating 2D and 3D tissue microarrays (Fig. 1). First, PDMS stamps with defined patterns are prepared from a silicon wafer master. The patterned PDMS stamp is then used for microcontact printing or micromolding of ECM proteins for 2D and 3D ECM microarrays, respectively. Cells are then seeded on the ECM microarrays to form engineered tissues. The tissues can then be fixed, stained, and analyzed for spatial distributions of specific cellular markers.

The methods described outline the following:

1. Preparation of the stamp.
2. Fabrication of 2D tissue microarrays.
3. Fabrication of 3D tissue microarrays.
4. Staining and imaging the tissues.
5. Analysis of the spatial distribution of cellular behaviors.

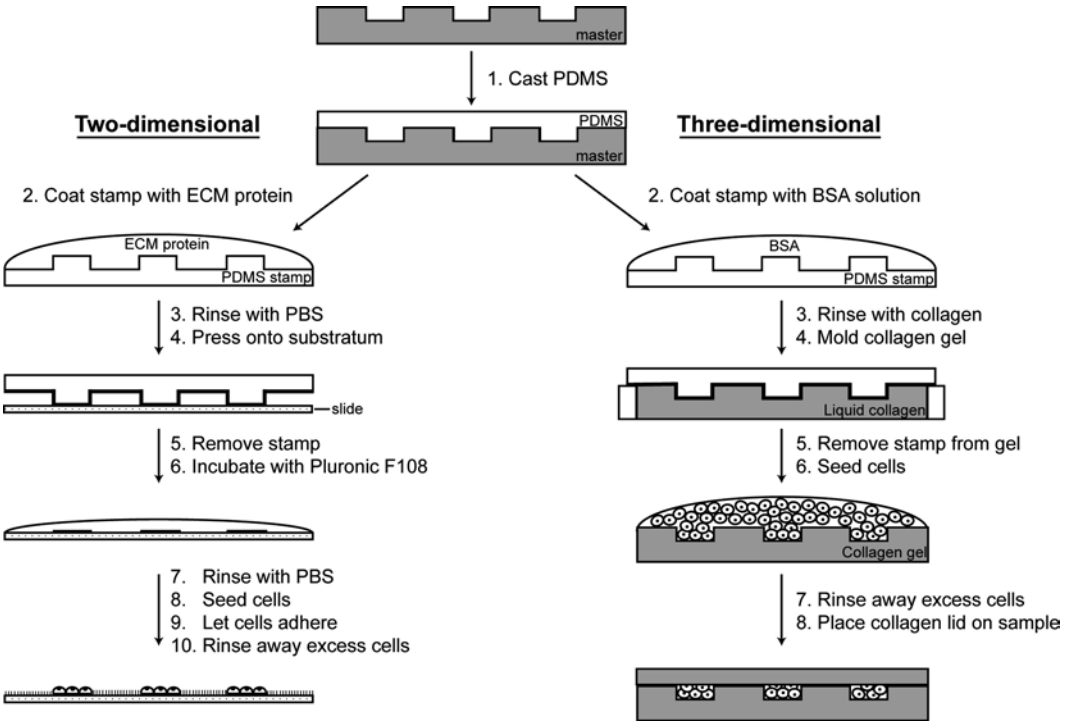

Fig. 1. Schematic of patterning two- and three-dimensional tissues.

3.1. Stamp Preparation

1. Prepare 60 g of 10:1 (w/w) PDMS polymer:curing agent solution. Mix thoroughly and place the mixture in a vacuum dessicator to remove air bubbles.
2. Pour degassed PDMS mixture onto patterned silicon wafer master (see Note 1).
3. Bake at 60°C for 2 h to cure the PDMS.
4. Carefully peel the PDMS from the surface of the master.
5. Cut PDMS patterned by master into stamps of the desired size.

3.2. Two-Dimensional Tissue Microarrays

1. Spin coat a thin layer of PDMS onto the surfaces of glass coverslips.
2. Bake at 60°C for 2 h to cure PDMS.
3. Treat PDMS-coated coverslips for 7 min in UV/ozone cleaner before use in 2D microarray patterning. This oxidizing treatment increases the wettability of the PDMS substration thus allowing for both microcontact printing of protein and adsorption of Pluronics F108 (see steps below) (23).
4. Sterilize PDMS stamps with ethanol. Dry the stamps thoroughly with a vacuum aspirator.

5. Coat the PDMS stamps with a solution of 25 μg/mL of fibronectin in PBS. Incubate for 2 h at room temperature.
6. Rinse once with PBS.
7. Dry the stamps with a steady stream of compressed nitrogen.
8. Stamp fibronectin onto the surface of a UV/ozone-treated PDMS-coated glass coverslip. Press lightly and then lift directly upward to remove stamp.
9. Flood the dish with a solution of 1% (w/v) Pluronics F108 in PBS. Incubate for 15 min.
10. Rinse twice with PBS. Leave patterned coverslips in PBS until ready to plate cells.
11. Plate cells on fibronectin patterned coverslips in cell culture media. Place in incubator and allow cells to adhere to fibronectin islands. Rinse to remove excess cells that have not adhered (see Note 2).

3.3. Three-Dimensional Tissue Microarrays

1. Cut PDMS into stamps that are ~5 mm cubes. Cut two small rectangles per sample from a thin sheet of polymerized PDMS to use as supports. Place stamps into Petri dish feature-side up.
2. Sterilize the PDMS stamps, PDMS supports, and 15-mm diameter coverslips with ethanol. Dry thoroughly with a vacuum aspirator.
3. Coat the feature side of the PDMS stamps with a solution of 1% BSA in PBS. Using a pipette tip, gently scrape the surface of the PDMS to remove air bubbles from the PDMS surface. Incubate for at least 30 min at room temperature (see Note 3).
4. Prepare a neutralized solution of collagen by mixing stock collagen with 0.1 N NaOH and 10× DMEM on ice. Mix thoroughly without introducing air bubbles (see Note 4). Adjust to the desired collagen concentration by adding 1× DMEM/F12.
5. Aspirate the BSA from PDMS stamps with a vacuum pipette. Rinse the BSA-coated surface twice with neutralized collagen (~30 μL).
6. Pipette a drop of neutralized collagen to the top of the PDMS stamp (~30 μL).
7. Flip over the collagen-coated stamp and place on top of two supports.
8. Pipette ~30 μL of collagen to the center of 15-mm round coverslips to make lids.
9. Place the dishes and lids in a 37°C incubator for 30 min.
10. Prepare a concentrated suspension of cells and keep them on ice.
11. Remove PDMS stamp from collagen gels by lifting directly upward with sterilized tweezers.

12. Add ~30 μL of resuspended cells to the collagen gel. Monitor the sample with a phase-contrast microscope. When cells have settled into the wells, wash the sample by holding the dish at a 45° angle and gently pipetting 400 μL of cold media across the surface to remove excess cells. Repeat up to three times. Place the sample in a 37°C incubator for 5 min to allow the cells to adhere to the collagen.
13. Remove samples from the incubator and gently place a collagen lid on each sample. Add 2.5 mL of culture media to each sample and return the sample to the incubator. Observe the sample after ~24 h for tubule formation.

3.4. Immunofluorescence Staining and Analysis

After seeding cells onto the 2D and 3D micropatterned ECM arrays, cellular and tissue properties, such as projected cell area and tissue form, can be observed by phase-contrast microscopy (see Fig. 2a, d). Likewise, samples can be fixed and stained for markers of interest. Here, as an example we describe the spatial distribution of phosphorylated extracellular signal-regulated kinase (ERK1 and ERK2) in 2D and 3D epithelial tissues. After treatment with epidermal growth factor (EGF), ERK1 and ERK2 are phosphorylated and are then translocated to the nucleus where they promote transcription of target genes of the mitogen-activated protein kinase (MAPK) signaling pathway.

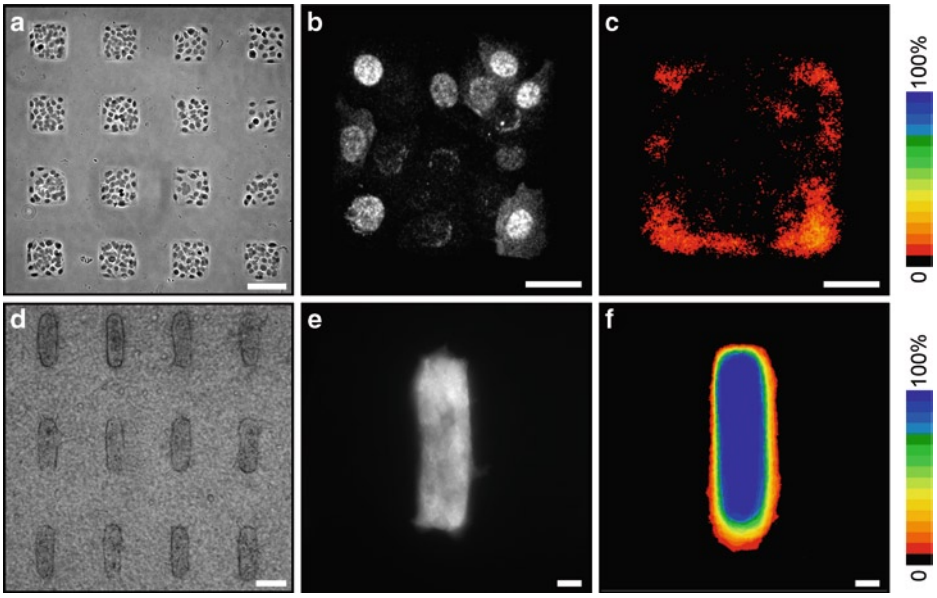

Fig. 2. Two- and three-dimensional mammary epithelial tissue microarrays. (**a**) Phase-contrast image of 2D mammary epithelial tissue microarray. (**b**) Gray-scale fluorescence microscopy image of 2D tissue stained for phosphorylated ERK1/2. (**c**) Color-coded frequency map of nuclear localized phosphorylated ERK1/2 in 2D tissue. (**d**) Phase-contrast image of 3D mammary epithelial tissue microarray. (**e**) Gray-scale fluorescence microscopy image of 3D mammary tissue stained for phosphorylated ERK1/2. (**f**) Frequency map of total phosphorylated ERK1/2 in 3D mammary epithelial tubule. Scalebars: (**a**, **d**) 100 μm; (**b**, **c**, **e**, **f**) 25 μm.

1. Treat sample with 25 ng/mL EGF and place in an incubator for 15 min.
2. Remove sample from the incubator and aspirate media. Rinse the sample once with PBS. Aspirate the PBS and replace with fixative solution (4% paraformaldehyde in PBS). Incubate at room temperature for 15 min. Wash fixed samples three times with PBS.
3. Incubate in 0.5% IgePal CA 630 in PBS twice for 10 min each time.
4. Incubate in 0.1% TritonX-100 in PBS for 15 min at room temperature.
5. Block sample with blocking buffer for 2 h at room temperature. Rinse sample once with PBS.
6. Apply diluted primary antibody (1:500) and incubate overnight at 4°C. For 2D samples, rinse sample with PBS three times for 5 min. For 3D, rinse with PBS for 5 h at room temperature.
7. Apply diluted secondary antibody (1:1,000). Incubate for 1–2 h at room temperature in the dark or overnight at 4°C for 2D and 3D tissues, respectively. For 2D samples, rinse sample with PBS three times for 5 min. For 3D, rinse with PBS for 5 h at room temperature.
8. Apply diluted nuclear stain (1:10,000) and incubate for 20 min at room temperature. Rinse sample with PBS. Mount samples on cover slides.
9. Observe and image using a fluorescence microscope. Take 50 images of tissues that have been aligned using the eyepiece or a stage micrometer on a fluorescence microscope.
10. Using an image analysis software convert gray-scale images into black-and-white images using binarize function.
11. Add the black-and-white images together.
12. Convert the gray-scale image into a color-coded frequency map using the Indexed Color mode in Photoshop (see Fig. 2c, f).

4. Notes

1. Silicon masters can be silanized to aid in removal of the PDMS. Place the master in a vacuum dessicator with a glass slide containing a drop of (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-tricholorosilane(Sigma-Aldrich). Evacuate the chamber. After 1–2 min isolate the chamber and allow for vapor silanization reaction to proceed for 2 h. Alternatively, a PDMS master can be made from the silicon master. First create a PDMS master with posts from the silicon wafer.

Silanize the PDMS master as described above. Use PDMS master with posts to make a PDMS master with holes.

2. Observe samples every half hour to determine when cells begin to adhere to ECM islands. The concentration of plated cells and the plating time can be modified to best achieve desired number of cells per island.
3. Stamps can be incubated overnight with BSA at 4°C.
4. The neutralized collagen solution can be quickly spun down in a centrifuge to remove air bubbles, if needed.

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