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3D Bio-etching of a Complex Composite-like Embryonic Tissue†

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Abstract

Morphogenesis involves a complex series of cell signaling, migration and differentiation events that are coordinated as tissues self-assemble during embryonic development. Collective cell movements such as those that occur during morphogenesis have typically been studied in 2D with single layers of cultured cells adhering to rigid substrates such as glass or plastic. *In vivo*, the intricacies of the 3D microenvironment and complex 3D responses are pivotal in the formation of functional tissues. To study such processes as collective cell movements within 3D multilayered tissues, we developed a microfluidic technique capable of producing complex 3D laminar multicellular structures. We call this technique "3D tissue-etching" because it is analogous to techniques used in the microelectromechanics (MEMS) field where complex 3D structures are built by successively removing material from a monolithic solid through subtractive manufacturing. We use a custom-designed microfluidic control system to deliver a range of tissue etching reagents (detergents, chelators, proteases, etc.) to specific regions of multilayered tissues. These tissues were previously isolated by microsurgical excision from embryos of the African claw-toed frog, *Xenopus laevis*. The ability to shape the 3D form of multicellular tissues and to control 3D stimulation will have a high impact on tissue engineering and regeneration applications in bioengineering and medicine as well as provide significant improvements in the synthesis of highly complex 3D integrated multicellular biosystems.

Abstract

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To investigate the collective movements within 3D multilayered tissues our group has focused on developing a novel microfluidic technique capable of creating complex patterns in multicellular structures.

1 Introduction

The ability to control the spatial geometries of materials is extremely important in areas such as microfabrication¹. The ability to chemically etch away micro- and nanometer sized areas in materials as part of subtractive manufacturing has allowed the development of diverse technologies ranging from advanced computer chips to microelectromechanical systems. In biology, the ability to control the spatial stimulation and shaping of tissues is similarly important. For example, tissue engineers have recently turned to decellularization techniques to isolate signaling factors embedded in tissues and to use those factors for regenerative medicine. Intriguing applications of decellularization include decellularizing the 3D heart matrices to engineer bioartificial heart, decellularizing livers to recellularize liver grafts and decellularizing human veins for vascular tissue-engineering scaffolds^{2–4}. In contrast to these bulk, or whole tissue decellularization approaches where all the cells are removed from a tissue we present a microfluidic etching technique for localized removal of targeted cells. We use this approach with a composite tissue consisting of epithelial and mesenchymal cell layers to expose interactions between layers in a 3D tissue. Microfluidics have been used to control the environment of developmental biology systems such as mechanochemical sensing in *Xenopus*^{5,6} and developmental changes in *Drosophila melanogaster* embryos⁷. Microfluidics applied in developmental biology provides the opportunity to study the coordination of cell behaviors in a 3D environment.

Microfluidics have been used in numerous cell biology applications where there is a need to control temporal or spatial stimuli in a microenvironment. Microfluidics have become a commonplace tool with the development of soft-lithography methods, which allow the easy fabrication of channels in which laminar flows can be combined with minimal mixing^{8,9}. Microfluidic technology enables spatiotemporal control over living cells¹⁰ and allows for high-throughput manipulation of the microenvironment from subcellular to multi-cellular scales. Microfluidics offer high molecular selectivity and micron scale spatial accuracy to spatially control sub-cellular processes; a level of control that is extremely challenging without laminar flow, since small molecules diffuse across the distance of a cell diameter within seconds¹¹. Spatiotemporal control offered by microfluidics allows long-term studies

of localized responses of cells and tissues to chemical stimuli, which are critical in numerous areas including developmental biology and biological materials^{6,11,12}.

One of the most important properties of 3D tissues during development is the structure and interactions between cells in the tissue. Cells during development and regeneration must orchestrate numerous signaling events; cells coordinate inputs from 3D cell-cell interactions mediated by receptors and cell-matrix adhesion to regulate cell fate^{13,14}. To understand these processes requires tools to control 3D cell-cell interactions; however, conventional approaches are mainly limited to 2D monolayer systems and do not allow manipulation of complex multilayer tissues^{15–17}. For example in controlling tissue structure and 3D cell-cell interactions, studies have been conducted on the behavior of epithelial monolayers of cultured cells such as human umbilical vein endothelial cells (HUVEC) or Madin-Darby canine kidney (MDCK) cells¹⁸ in response to laser cuts or scratch assays but these methods are restricted to monolayers^{19,20}. One limitation of current cell-based approaches is that tissues and organs are 3D, and thus traditional 2D cell culture studies (e.g. Petri dishes or flasks) lack important features necessary to accurately replicate cell function and tissue formation²¹. Cells that are grown on 2D substrates may respond and differentiate differently than those in more physiologically relevant 3D environments^{22–25}.

Here, we demonstrate a novel etching approach using microfluidics to spatially alter multi-level multicellular structures while altering the 3D cell-cell interactions in tissues. We apply etching to the complex tissues in the *Xenopus laevis* animal cap (AC), which is composed of stratified layers of epithelial and mesenchymal cells. We first demonstrate that we can remove all the cells within a local region of the multi-layered AC. We then show that etching can be refined to remove only the top layer of cells of the tissue. Finally we show that we can remove different widths of tissues located within defined layers allowing us step-wise spatial precision in cell layer removal for examining in the future 3D cell interactions in tissues. We believe our results will provide an approach that will greatly enhance the understanding of biological materials including tissue remodeling events, embryonic morphogenesis and wound repair.

2 Materials and Methods

2.1 Embryos and explant preparation

Eggs were collected from hormonally stimulated female *Xenopus laevis* frogs. Eggs were fertilized *in vitro*²⁶, dejellied in 2% Cysteine solution (pH 8)²⁷ and cultured in 1/3 X Modified Barth's Solution (MBS) until the early gastrula stage (stage 10²⁸). At stage 10, the embryos were transferred into Danilchik's For Amy solution (DFA) and forceps were used to remove viteline membranes. We used hair-loops and hair-knives to micro surgically isolate animal cap tissues from whole embryos. Microsurgically isolated explants were positioned in the channels using negative pressure and allowed to adhere for 4 hours prior to flow studies. All experiments were carried out at 21°C (room temperature) within the permissive range for developing *Xenopus* embryos.

All experiments in this paper were performed in compliance with the relevant laws and institutional guidelines. This project used embryos collected from the African claw-toed

frog, *Xenopus laevis*, under guidelines established by the University of Pittsburgh Division of Laboratory Animal Research (DLAR). All procedures adhered to our approved Institutional Animal Care and Use Committee (IACUC) protocol #15025409.

2.2 Microscopy and image analysis

To record the kinematics of bio-etching in microfluidic channels, a digital charge-coupled device (CCD) camera (The Imaging Source Europe GmbH) mounted on a stereomicroscope (SZX12, Olympus Corp., Waltham, MA) was used to acquire images at room temperature. A laser scanning confocal scanner (Leica TCS SP5; Leica Microsystems) mounted on an inverted compound microscope (DMI6000, Leica Microsystems) was used to collect high-resolution time-lapse confocal images. The image acquisition and subsequent analysis of time-lapses sequences used custom-written macros and plug-ins, and image analysis software (Fiji)²⁹. As the visualization of the streams was very important, two drops of food coloring (McCormick, green) were added to a 20 mL central reservoir as previously described³⁰.

2.3 Microfluidic device design

Modular microfluidic devices were fabricated using conventional soft-lithography techniques⁸ from polydimethylsiloxane (PDMS; SYLGARD 184, Dow Corning, MIDLAND, MI, USA). Modular resistances (R10-100×50), (R20-100×50) and (R60-100×50) were produced from microfluidic channels 10, 20 and 60 mm long each with each having a cross sections that were 100 μm wide and 50 μm high³⁰. The microfluidic channel for the AC explants included three inlet channels with rectangular cross sections with dimensions 500 μm wide, 300 μm high, and 5 mm long. These inlet channels converged to a single outlet channel (rectangular cross section 1500 μm wide, 300 μm high, and 10 mm long). The reservoirs and pressure sensors were connected to the microfluidic device using polyethylene tubing (Intramedic, inner diameter of 0.78 mm). Before using, microfluidic channels were coated with fibronectin at 4°C overnight.

3 Result/Discussion

3.1 Experimental technique to etch a composite tissue through 3D Bio-etching

To etch 3D composite tissues, we used our custom-designed microfluidic control system to deliver of a range of tissue etching reagents, adopted from decellularization protocols, as controlled streams to microsurgically isolated early stage embryonic tissue (Figure 1A). Controlling the flow rate at the microchannel inlets individually enables precise and dynamic chemical control over the 3D tissue³¹. Our system implemented a pressure-driven nitrogen gas approach and included two variable-volume fluid reservoirs with etchants and culture media (Danilchik's For Amy solution, DFA)³², three microfluidic resistances and a 3-inlet converging microfluidic channel (Figure 1B). The main microfluidic channels and fluidic resistance modules were fabricated with conventional soft lithography techniques from polydimethylsiloxane (PDMS). After fabrication, the main channels were coated with fibronectin. In the main T-shaped, 3-inlet microfluidic channel, the two side inlets had the identical high-flow rate flows, which were controlled by microfluidic resistances³⁰ with the center inlet having a lower flow rate (Figure 1D). These fluidic resistances altered the flow

rates that defined the width of the etching streams in the main microfluidic channel and thus the width of the areas exposed to the etchants over the apical surface of the tissue (Figure 1B). Inlet pressures can be dynamically changed to deliver etchant to any location over the apical surface and width of the tissue. The tissue used in these experiments was microscopically isolated from gastrula stage *Xenopus laevis* embryo and positioned inside of the main channel through the outlet. The tissue was first allowed to adhere to the fibronectin coated glass surface for 4 hours. Once adhered, we collected time-lapse sequences of the process of etching and the response of the remaining tissues.

Decellularization of organs through using etchants has been used to create extracellular matrix (ECM) for a variety of reconstructive surgical applications and regenerative medicine strategies for tissue and organ replacement³³. However, current decellularization approaches remove all cells to produce foundational scaffold ECM to regenerate an organ. In our approach, the goal is to remove only select cells in controlled areas to understand collective 3D cell reaction to etching. Our bio-etching technique allowed the removal of specific layers of tissue through exposure to a narrow etchant stream, which removed layers of cells to expose lower layers of cells or even to the supporting substrate such as glass (Figure 1C).

3.2 Width and depth of the tissue etching regulated by microfluidic positioning and duration of detergent stream

After microsurgery, the freshly-isolated tissue was positioned and adhered in the microfluidic channel, the center inlet was connected to a reservoir containing the etchant sodium dodecyl sulfate (SDS; 0.3% sodium dodecyl sulfate in DFA). Using fluidic resistance modules to control the flow of the center inlet relative to the outer inlets, a narrow center stream delivered SDS to the epithelial surface of the multi layer animal cap tissue. With exposures greater than 3 minutes, we etch the tissue through its full thickness (Figure 1C, Movie S1). By reducing the exposure time we could reliably remove a single layer of epithelial cells (Figure 2, Movie S2). By controlling the width of the etchant and the exposure time a broader swath of the surface epithelial layer was removed. After the removal of a broad swath the resistance modules were changed and a second narrow stream was used to remove a single narrow band within the swath-exposed cells (Figure 3, Movie S3). With this novel 3D bio-etching, depending on the exposure time and position of the etchant, single or multiple cell layers can be removed.

As a first step we removed all of the layers in a spatially controlled region of the 3D *Xenopus* AC. To accomplish this, after positioning and adhering the microscopically excised in the microfluidic channel, the center inlet was connected to the SDS reservoir. We started the pressure driven flow and opened the valves to the side inlets. After the DFA was in the entire main channel and the flow equilibrated, the center inlet's valve was opened to start the etching process. Using modular fluidic resistance modules, a narrow center stream delivered the etchant to the apical layer of the multi layer animal cap tissue. By controlling the width of the etchant and the exposure time a broader swath of cells were removed. We also used this methodology to remove multiple layers of cells through the entire AC so that the interacting substrate was exposed, but only in the specified regions where cells were previously removed; we call this a "deep etching" approach (Figure 1C).

To create more complex structures we limited the amount of time the tissue was exposed to SDS. By reducing the exposure time a single layer of epithelial cells was removed (Figure 2A), which we called this 'single layer etching'. For single layer etching, after the first layer was removed, the middle inlet of the microfluidic system containing the etchant was closed to stop the flow of the etchant. This approach created a discontinuous region of epithelial cells (EL) over an intact, continuous layer of mesenchymal cells (ML) (Figure 2B, Movie S2). Where the EL is discontinuous it remains in contact with the deeper continuous ML. The width of the etched gap in the EL was controlled by modulating the central inlet flow rate by changing the microfluidic resistance modules (Figure 2C). With this approach we manipulated the etching depth and width of the epithelial layer in a spatiotemporally controlled manner. Single layer etching created a free margin in the epithelial cell layer that remained in contact with deep mesenchymal cells. The viability of the remaining cells in the tissue were confirmed with a Live-Dead Cell Assay (Figure S1). We know that the functional behavior of the cells exposed to etchant reagents does not change since the cells continued to move after culture media replaced the etchant. Cells transiently exposed to the etchant, but not washed away, appeared unaffected and remain motile for more than 10 hours.

3.3 Multi-step etching can create novel 3D tissues

By repositioning the microfluidic stream after removing a single epithelial layer we created a 3D 'tiered' tissue. After the removal of a broad swath of epithelial cells we switched resistance modules to produce a more focused stream and removed cells from the ML (Figure 3A). To etch tiered structures in the tissue required sequential steps (Figure 3B). As before the DFA reservoir was connected to a lower resistance module and then connected to two side inlets. The width of etchant was based on the ratio of resistance modules in the side inlets compared to the middle inlet. In the first step, the etchant reservoir was connected to two of the same resistance modules with a T connector. Two higher fluidic resistance modules, namely the R-60 -length of the channel, were connected in parallel to each other and the DFA reservoir was connected with the R-10 module. This configuration with the etchant inlet:side inlet resistance modules in the ratio of 1:3 removed a defined region of the EL with a relatively wide stream in the main channel. In step two, one of the resistance modules leading to the etchant channel was closed off to create an etchant:side ratio of 1:6. The increase in the resistance to the etchant flow caused the middle stream to become narrower in width (Figure 3D). The narrow stream of etchant then contacted only the newly exposed ML without contacting the EL. The resistances can be manually changed in a few seconds so that a narrow stream of etchant could be positioned within the wider region used to remove the EL (Figure 3C, Movie S3). With spatiotemporal control of the etchant our microfluidic technique enabled multiple rounds of etching, which we have used to create tiered margins within 3D animal cap tissue. The removal of the etchant was also analyzed when the inlet channels was closed (Figure S2).

3.4 Alternative etchants

In addition to the anionic detergent SDS, we tested other detergents, enzymes, and an alkaline base for their ability to remove cells from tissues. Anionic and nonionic detergents have been extensively used to decellularize tissues and organs by solubilizing cell

membranes and dissociating DNA from proteins³³. SDS, which is anionic detergent, is effective in solubilizing numerous proteins by disrupting non-covalent bonds within and between proteins. Non-ionic detergents, often called non-denaturing detergents, like Triton X-100 are potentially useful since they break lipid-lipid and lipid-protein interactions and leave more protein-protein interactions intact. For this reason, Triton X-100 is often used to isolate biologically active membrane proteins. Following our work with SDS, we examined the effects of non-ionic detergent, Triton X-100. We used 3% Triton X-100 as an etchant to etch single layers in the epithelium to expose a broad swath of mesenchymal cells (Figure 4A, Movie S4). Triton X-100 did not appear to remove the epithelial layer as well as SDS. We noticed that the removal of epithelial and mesenchymal cells within the etched regions took much longer than with SDS.

Next, we used base sodium hydroxide (NaOH) as an etchant, since strong bases can affect ECM mechanical properties and NaOH is known to disrupt tissue architecture³⁴. Using 0.1N NaOH with the previously described approach for single layer etching removed a single cell layer from the EL within 80 seconds (Figure 4B, Movie S5). Other potential etchants are proteolytic enzymes such as trypsin, which provide high specificity for removal of cell residues and undesirable ECM constituents. Complete cell removal by enzymatic treatment alone is difficult, however, and residual enzymes and partially denatured proteins as well as highly charged nucleic acids may impair recellularization³⁵. We used trypsin as an etchant, which is commonly used in cell culture protocols to dissociate cell monolayers from substrates. The ability of trypsin to etch cells locally from a substrate was not known. We found that trypsin did not remove cell layers even when the tissue was exposed for much longer time periods (> 15 minutes) (Figure 4C, Movie S6).

4 Conclusion

We demonstrated the ability to create and shape 3D composite tissues with subtractive manufacturing or bio-etching using a microfluidics based approach. While many approaches use 2D environments with monolayers, our unique microfluidic technique provided the ability to control the 3D environment and study the interactions of individual epithelial and mesenchymal layers. We used the animal cap ectoderm of the *Xenopus laevis* embryo as a model system for the study of tissue bio-etching in embryonic cell layers as they are multi-layered with both an epithelial and a mesenchymal layer, and are well suited to microscopy analysis. The cells of the animal cap are considered to have similarities to mammalian embryonic stem cells, which may provide insight into stem cell response in the future as well. Since our method introduced a new chemical bio-etching method with a precise spatial and temporal control of the etched area, this accurate positioning allowed us to construct novel epithelial and mesenchymal tissue architectures. Through deep layer etching and single layer etching we are able precisely shape 3D tissues. The deep cells below the EL removed by etchants were not exposed unless the stream remained after the cells dissociated and were removed by flow. Also, short exposures to etchants were quickly washed away with fresh media, which replaced the etchant in the microfluidic chamber. Unlike decellularization approaches, this technique leaves most cells viable. Additionally, this bio-etching method can be used with other tissues. The only potential limitation would be the thickness of the tissue. A thick tissue sample could disrupt the the flow profile and require

more extensive feedback control to maintain the precise position of the etchant stream. As long as the profile is not disrupted, any 3D tissue can be studied using bio-etching technique. Thus, this 3D tissue-etching assay will be useful in the future to study biological composite materials, mechanical responses, collective cell sheet migration, and explore processes that are used to coordinate composite tissues during morphogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

3D bio-etching of a complex composite-like embryonic tissue. A) Schematics and images of two layer animal cap tissue with bottom and side view. B) Experimental set up for 3D tissue chemical etching/subtractive biomanufacturing and images of the microfluidic channel C) Long exposure of AC to a narrow stream of etchant removed all layers of cells from the exposed regions of the AC. (Movie S1) D) 3-inlet microfluidic channel schematic and interface control over the time. Center flow width (green) changed over the time with resistances. Resistances of left and right flows (pink) stayed the same but interface between green and pink flows changed according to resistance change in center green flow. Center inlet was set to Case 1 (C1): lower resistance, Case 2 (C2): Higher resistance, Case 3 (C3): shut off

Fig. 2.

Regulation of the width and depth of the tissue etching: Single layer etching of a 3D AC tissue. A) Schematics of a single epithelial layer etched animal cap tissue with top and side views. B) Experimental time sequence showing removal of a single layer embryonic tissue by etching over 180 seconds using 0.3% SDS etchant (Movie S2). C) Interface control over the time with intensity profile during etching. Green center flow was set by R-30 during etching. After the epithelial layer was etched, the center inlet was closed off so that the etchant was no longer on the AC. Side pink flows were set by R-10

Fig. 3.

Refined Multi-step etching created novel 3D tissues. A) Schematics of double layer subtractive manufactured animal cap tissue with top and side view. 0.3% SDS was used as the etching chemical. B) The center inlet resistance was lowered to create a wider interface. The AC exposed to a wider stream etched a wide swath of the epithelial layer. Second step of the experimental technique etched a narrower swath of ML. The center inlet resistance was increased to produce a narrower stream. C) Time lapse images of multiple etching show the removal of wide swath of epithelial layer and then removal a narrow swath of ML over 100 seconds (Movie S3). D) Interface control over the time with intensity profile during etching. Center flow (green) is set by R-30 and switched to R-60 during etching. After etching the center inlet was closed. Side pink flows were set by R-10.

Fig. 4.

Etching of the tissue using different etchants. The single layer etching approach was used to test the effectiveness of a) nonionic detergent, 3% Triton X-100 (Movie S4), b) alkaline base (Movie S5), 0.1N NaOH, and c) biological agent, trypsin (Movie S6)