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Manipulation of 3D Cluster Size and Geometry by Release from 2D Micropatterns

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Abstract—A novel method to control three-dimensional cell cluster size and geometry using two-dimensional patterning techniques is described. Cells were first cultured on two-dimensional micropatterned collagen using conventional soft lithography techniques. Collagenase was used to degrade the micropatterned collagen and release cells from the micropatterns, forming clusters of cells which were then resuspended in a three-dimensional collagen matrix. This method facilitated the formation of uniformly sized clusters within a single sample. By systematically varying the geometry of the two-dimensional micropatterned islands, final cluster size and cell number in three dimensions could be controlled. Using this technique, we showed that proliferation of cells within collagen gels depended on the size of clusters, suggesting an important role for multicellular structure on biological function. Furthermore, by utilizing more complex two-dimensional patterns, non-spherical structures could be produced. This technique demonstrates a simple way to exploit two-dimensional micropatterning in order to create complex and structured multicellular clusters in a three-dimensional environment.

Keywords—Multicellular, Micropatterning, 3D, 2D, Collagenase.

INTRODUCTION

The spatial organization of cells and the surrounding extracellular matrix (ECM) within a tissue is critical for proper tissue function. When cells are removed from their native environment and placed in artificial culture conditions such as on plastic dishes, they typically lose their tissue-specific functions. Mammary epithelial cells, for example, normally have a rounded morphology and exist within acinar structures that are important for milk production, but tend to spread and proliferate when removed from the body and cultured *in vitro*. Defining culture conditions in which cells retain their *in vivo* phenotype has been a major challenge for both studies in cell biology and tissue engineering applications, largely because it is difficult to manipulate the numerous environmental factors that might contribute to cell behavior. Many types of cells *in vivo* exist in a three-dimensional environment, in which cell shape and geometry are confined by ECM and neighboring cells, whereas cells *in vitro* typically spread out on a flat surface and have few interactions with other cells until they are cultured to confluence. Thus, developing methods to control three-dimensional multicellular organization and interactions with surrounding ECM is an important goal for the biomedical engineering community.

Several investigators have cultured cells in three-dimensional multicellular aggregates or spheroids in order to restore cellular function *in vitro*. In one method to form spheroids, cells are cultured on a nonadhesive surface such as polystyrene or agarose, which causes cells to preferentially interact with neighboring cells. Over the course of several hours or days cells form larger aggregates, often only in the presence of specific soluble agents such as growth factors.[7,15] These cultures have been shown to help preserve cellular function in multiple cell types including hepatocytes, osteocytes, neurons, and endothelial cells.[7,9,11,15] Additionally, aggregate size and formation have been found to be critical regulators of stem cell maintenance and differentiation in both embryonic and adult stem cells.[2,20,21] It is possible that aggregation functions by imparting proper dimensionality, cell shape, and cell–cell interactions. Additionally, spheroids establish a barrier to small molecule

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transport resulting in spatial gradients of soluble factors, which have a significant effect on cell behavior.[3] Although spheroid cultures have existed for nearly half a century, the mechanisms by which cells maintain differentiated phenotypes in these structures is not well understood, in part because spheroid formation is difficult to control. The ability to systematically manipulate cluster size may provide a means to organize and regulate complexity within these structures, and hence offer an additional approach to engineer cellular function.

Cultures containing homogenous clusters have been achieved by culturing cells in small nonadhesive round-bottom wells or inverted as a hanging droplet.[8] In this method, each well is seeded with enough cells for a single aggregate, and aggregation is facilitated by gravity pulling cells towards the bottom of the well or droplet. Although the control of cluster size is enhanced when compared to clusters that form spontaneously on nonadhesive substrates, this technique is limited by the fact that only one aggregate forms in each culture well, making it difficult to study populations of spheroids. Microfabrication techniques have recently been used to create micro-scale wells that enable the formation of consistently sized cell clusters, all within a single sample.[4,13,20] Substrates that contain thousands of micro-scale wells with identical geometry are fabricated using techniques such as photolithography or micromilling. Cells are forced to the bottom of the microwells by centrifugation or rotary shaking, and consequently form an orderly array of spheroids. While these microfabrication methods yield consistently sized clusters, it is difficult to obtain more complex structure geometries and configurations, for example structures that contain multiple cell types that are spatially segregated. In contrast to three-dimensional patterning, there are numerous two-dimensional patterning techniques that offer impressive control over the spatial organization and location of multiple cell types.[1,5,6,18] To increase the complexity of three-dimensional structures, one could take advantage of two-dimensional patterning by developing a technique to transform the two-dimensional patterns themselves into three-dimensional structures.

Here, we describe a simple method to form three dimensional cell clusters, whereby cells are first cultured on two-dimensional patterns and then released to form three-dimensional structures. Cluster size and shape is determined by the geometry of the micropatterned islands. We investigate the ability of this method to form uniformly sized clusters within a single sample. Using this technique, we show that proliferation of cells within collagen gels is cluster size-dependent, suggesting an important role for multicellular structure on biological function. Furthermore, by utilizing more complex two-dimensional patterns, we produce non-spherical structures. This technique demonstrates a simple way to exploit two-dimensional micropatterning in order to create complex and structured multicellular clusters in a three-dimensional environment.

MATERIALS AND METHODS

Cell Culture and Reagents

MCF10A mammary epithelial cells (ATCC) were cultured in DMEM:F12 (1:1) supplemented with 5% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, 0.02 μg/mL epidermal growth factor (all from Invitrogen) and 0.1 μg/mL cholera toxin, 10 μg/mL insulin, and 0.5 μg/mL hydrocortisone (all from Sigma-Aldrich). Bovine pulmonary artery endothelial cells (VEC Technologies) were maintained in low glucose DMEM supplemented with 5% bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). NRK-52E rat kidney epithelial cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum.

Formation of Cell Clusters

Micropatterned substrates were prepared as described previously.[17] Briefly, PDMS stamps were fabricated from photolithographically-generated master silicon patterns. Cells were seeded onto patterned substrates, rinsed twice with HBSS (Invitrogen), and then incubated for 10 min with 0.2 % (w/v) collagenase type II (Invitrogen) in HBSS supplemented with 3 mM $CaCl_2$ at 37 °C. Cells were then gently pipetted to facilitate the removal of clusters from the surface, and resuspended in full serum media. 1 μM collagenase inhibitor I (Calbiochem) was added to the cell suspension, which was then centrifuged twice at 800 rpm for 3 min to remove residual collagenase. Cell clusters were resuspended in collagen gels as described below.

Three-dimensional Culture

Collagen gels (2.5 mg/mL) were generated with a solution of acidic collagen (BD), sodium bicarbonate (Sigma), HEPES buffer and 10× M199 (both from Invitrogen), and were neutralized with sodium hydroxide (Sigma). Cells were pelleted and resuspended within the collagen solution, and incubated at 37 °C until the collagen solidified. Full serum media was added on top of the gel, the gel was released from the sides of the well with a pipet tip, and proliferation

was assayed as described in the proliferation assays section.

Quantification of Cell Number

The number of cells per pattern or cluster was determined by nuclear staining. Samples were fixed in 4% paraformaldehyde for 20 min, and stained with Hoechst 33258 to visualize cell nuclei. Cells were manually counted on a Nikon TE200 microscope. Unless otherwise noted, at least 50 patterns or clusters were examined for all conditions reported.

Proliferation Assays

Proliferation assays were performed using the Click-iT Edu cell proliferation assay (Invitrogen) according to manufacturer's instructions. Synchronization in G_0 was achieved in MCF10A cells by serum starvation of confluent cells for 24 h. Cells were then seeded onto micropatterned substrates for 6 h, treated with collagenase, and captured in collagen gels. Cells were cultured in collagen gels in the presence of EdU for 24 h, fixed in 4% formaldehyde for 20 min, and processed for fluorescent visualization of EdU incorporation according to manufacturer's instructions. EdU-positive cells were counted on a Nikon TE200 microscope. Unless otherwise noted, at least 300 cells were examined for all conditions reported.

Microscopy and Image Acquisition

Images of fixed samples were acquired using a TE200 epifluorescence microscope (Nikon) equipped with a mercury arc lamp, a10× Plan Fluor NA 0.3 dry lens, a 60× Plan Apo NA 1.4 oil immersion lens, Spot camera and software (Diagnostic Instruments) or an Axiovert 200 M epifluorescence microscope (Carl Zeiss) equipped with 40× Plan-Neofluar NA 1.3 oil immersion, 63× Plan-Apochromat NA 1.4 oil immersion objectives, Axiocam camera and Axiovision software.

RESULTS

Micropatterning Three-dimensional Cultures

We explored a method to produce uniformly sized cell clusters by bulk detachment of two-dimensional patterns. Cells were first cultured on two-dimensional surfaces that were micropatterned with collagen-coated islands surrounded by Pluronic F127, a non-adhesive polymer that prevents the adhesion of proteins and cells. Cells were seeded for at least 6 h to allow for the formation of stable cell–cell adhesions. To release cells from the micropatterned collagen, the cells were treated with a 0.2 % (w/v) solution of collagenase at 37 °C. After 5–10 min, cells began to detach from the dish while maintaining cell–cell adhesions, forming spherical clusters of cells. Mechanical agitation, either by pipetting or by gentle tapping of the dish, facilitated cluster detachment. Clusters were resuspended in full serum media supplemented with collagenase inhibitor and centrifuged twice to remove residual collagenase. Cells were then resuspended within a 2.5 mg/mL collagen gel. Figure 1 is a schematic depiction of the clustering method and phase contrast images of cells patterned on 10,000 μm^2 islands undergoing the collagenase lift-off process.

Distribution of Cell Number in Micropattern-released Clusters

To manipulate cluster size, we applied this method to cells patterned on different-sized islands. Cells were patterned on 289, 2500, and 10,000 μm^2 islands (Fig. 2a), and treated with collagenase to release the

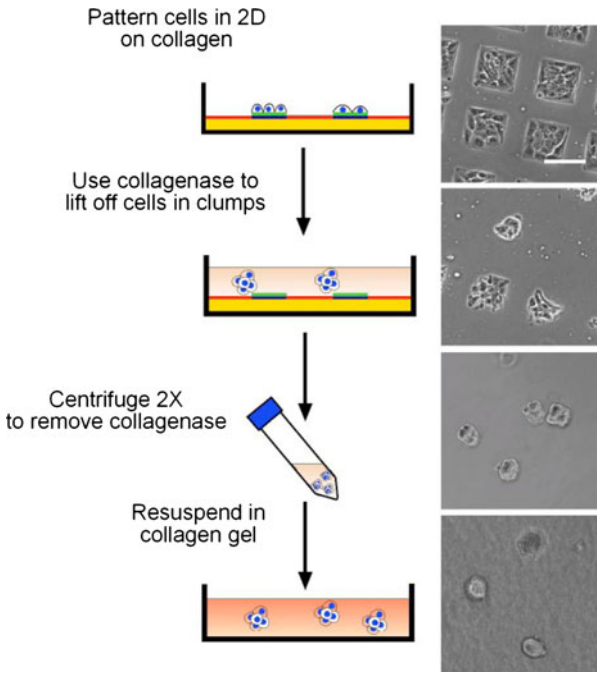


FIGURE 1. Schematic of method to form clusters from micropatterned cells. Cells are seeded onto micropatterned collagen for 6 h. Collagenase is applied to cells for 10–15 min at 37 °C and cells detach as clusters. Clusters are centrifuged to remove collagenase and then resuspended within a 3D collagen gel. Phase contrast images depict MCF10A cells undergoing process (right). Scale bar: 100 μm.

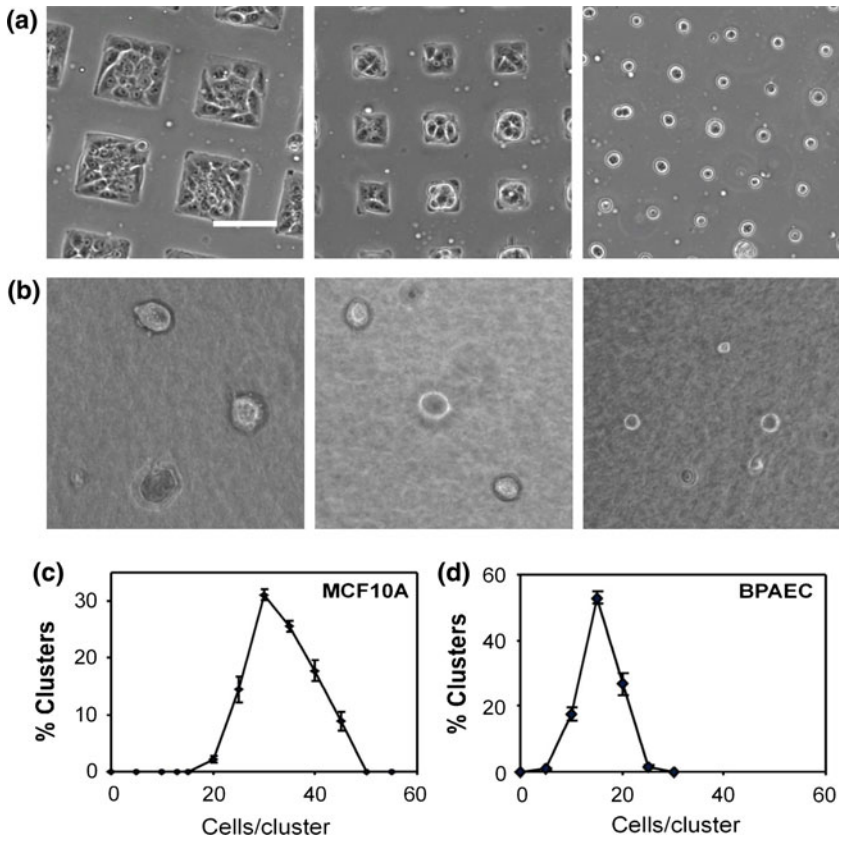


FIGURE 2. Cluster size is controlled by micropatterned island size. (a, b) Phase contrast images of (a) MCF10A cells patterned on 10,000 μm^2 (left), 2500 μm^2 (middle), and 289 μm^2 (right) square islands, released by collagenase, and (b) embedded within collagen gels. (c, d) Graph of percentage of clusters with a given number of cells per cluster of (c) MCF10A and (d) BPAECs patterned on 10,000 μm^2 square islands. Error bars indicate range (the maximum and minimum percentages) between two experiments. Scale bar: 100 μm.

cells as clusters. We found that treatment with collagenase released cells that were patterned on a range of island sizes. We examined cluster size within three-dimensional samples and found that the size of the clusters throughout the gel were fairly consistent (Fig. 2b). The process did not cause cells to dissociate from clusters. Aggregation of clusters was also not observed, likely because the formation of strong cell–cell adhesion requires several hours, a longer time period than the duration of centrifugation and resuspension.

We examined the distribution of cells seeded on the two-dimensional micropatterned substrates overnight. For small islands, typically 1–2 cells landed per island, and a greater fraction of single cells per island could be obtained by decreasing the seeding density (Fig. 3a). For larger islands, we also found that the distribution of cluster size was dependent on the cell type used. Epithelial cells (MCF10A) were not contact inhibited and formed multicellular layers. Therefore, the distribution of these cells was broader. On 2500 μm^2 islands, approximately 9–15 cells land on each island, and on 10,000 μm^2 islands, approximately 30–40 cells land on each island (Fig. 2c). Endothelial cells, however, typically formed monolayers and exhibited a narrow range of cells per island, with approximately 50% of clusters containing 15–20 cells. The distribution of cells per island was also dependent on the seeding density and the culture time on the micropatterned islands (Figs. 3a–3d).

Engineering Three-dimensional Structure

This technique enables the translation of two-dimensional patterns into three-dimensional culture. We explored whether we could obtain clusters of different morphologies by patterning cells on different two-dimensional geometries. Using rectangles with varying aspect ratios, MCF10A cells released from the patterns with increased aspect ratio formed elongated, or rod-shaped, clusters, and these structures were maintained during centrifugation and resuspension in collagen gels, allowing consistent formation of several rod shaped 3D cluster shapes (Fig. 4a). Interestingly,

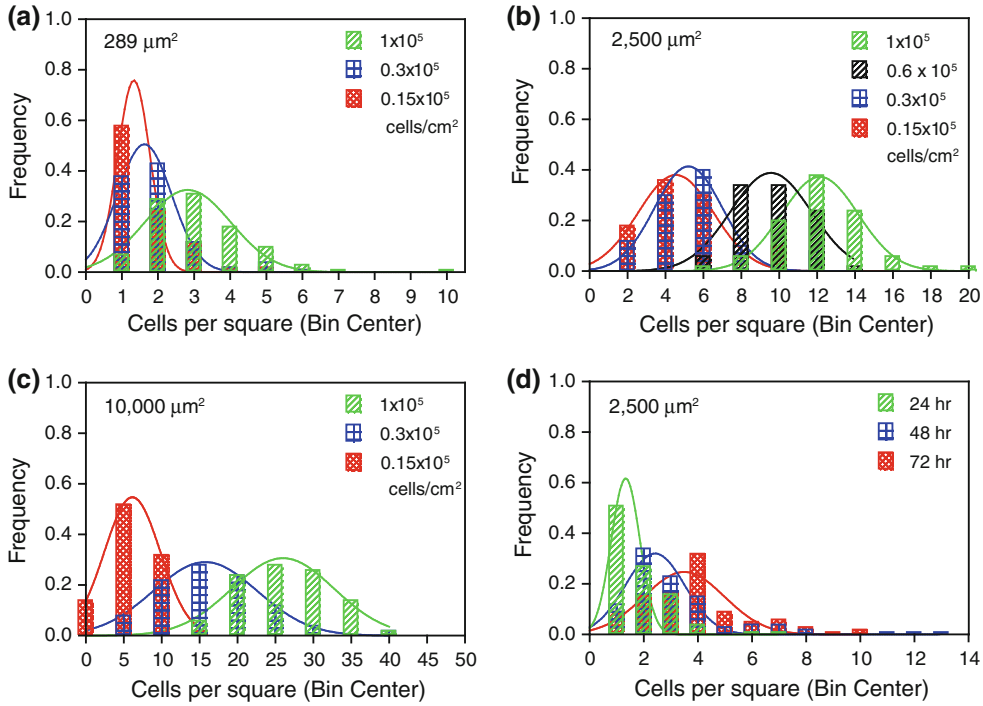


FIGURE 3. Effect of pattern size and seeding density on cells per pattern distribution. (a–c) Histograms of number of NRK-52E cells per pattern. Cells were seeded on micropatterned collagen of several areas (289, 2,500, and 10,000 μm^2) at different seeding densities ranging from 0.15 to 1 × 10^5 cells/cm^2 for 24 h. (d) BPAECs were seeded at 0.1 × 10^5 cells/cm^2 on 2,500 μm^2 patterns for 24–72 h. 100 patterns per condition were counted and histograms were fit with a normal Gaussian curve.

NRK-52E cells seeded on rectangle patterns did not form rod shapes in 3D, but curled and formed spiral shaped clusters in 3D (Fig. 4b). To achieve these elongated shapes, longer culture time (24–48 h) on the 2D micropatterns was required to maintain cluster integrity as compared to the square micropatterns (6–8 h).

Patterned Clusters Exhibit Cluster-dependent Proliferation

We and others have previously observed that cell proliferation is dependent on cluster size.[9,12] However in these studies, clusters were randomly formed and each sample typically contained clusters of various sizes, causing data acquisition to be tedious and time consuming. Measurement of cell proliferation within samples containing uniformly sized clusters would facilitate high throughput data acquisition. We examined whether proliferation within clusters formed by this micropatterning technique was dependent on cluster size. MCF10A cells were synchronized by serum starvation for 24 h, and then trypsinized and seeded onto micropatterned substrates ranging from 625 to 62,500 μm^2. Cells were seeded for 6 h to allow the formation of stable cell–cell adhesion, followed by treatment with collagenase to release the cells into clusters. Clusters were centrifuged to remove collagenase and resuspended within 2.5 mg/mL collagen gels. Full serum media was added on top of the gels. We observed that cells in three-dimensional culture remained in spheroids over the course of this experiment (Fig. 5a). Cells on 625 μm^2 islands typically contained 1–2 cells, while cells on 10,000 μm^2 islands contained approximately 35 cells and cells on 62,500 μm^2 islands contained approximately 90 cells (Fig. 5b). Cells were fixed and assayed for proliferation by EdU incorporation at 24 h after seeding. We found that cell proliferation was indeed dependent on cluster size (Fig. 5c). Cells seeded on small islands, which were predominantly single cells, had low levels of proliferation. Proliferation was also low in the largest cluster size formed from cells seeded on the largest islands (62,500 μm^2). The highest levels of proliferation were observed in intermediate cluster sizes formed from cells seeded on 10,000 μm^2 islands. Interestingly, we observed that EdU incorporation in the largest cluster size was typically located in the cells towards the outer edge of the sphere, similar to what we had observed in unpatterned clusters (data not shown).

DISCUSSION

We developed a technique to form three-dimensional multicellular structures by detaching cells from two-

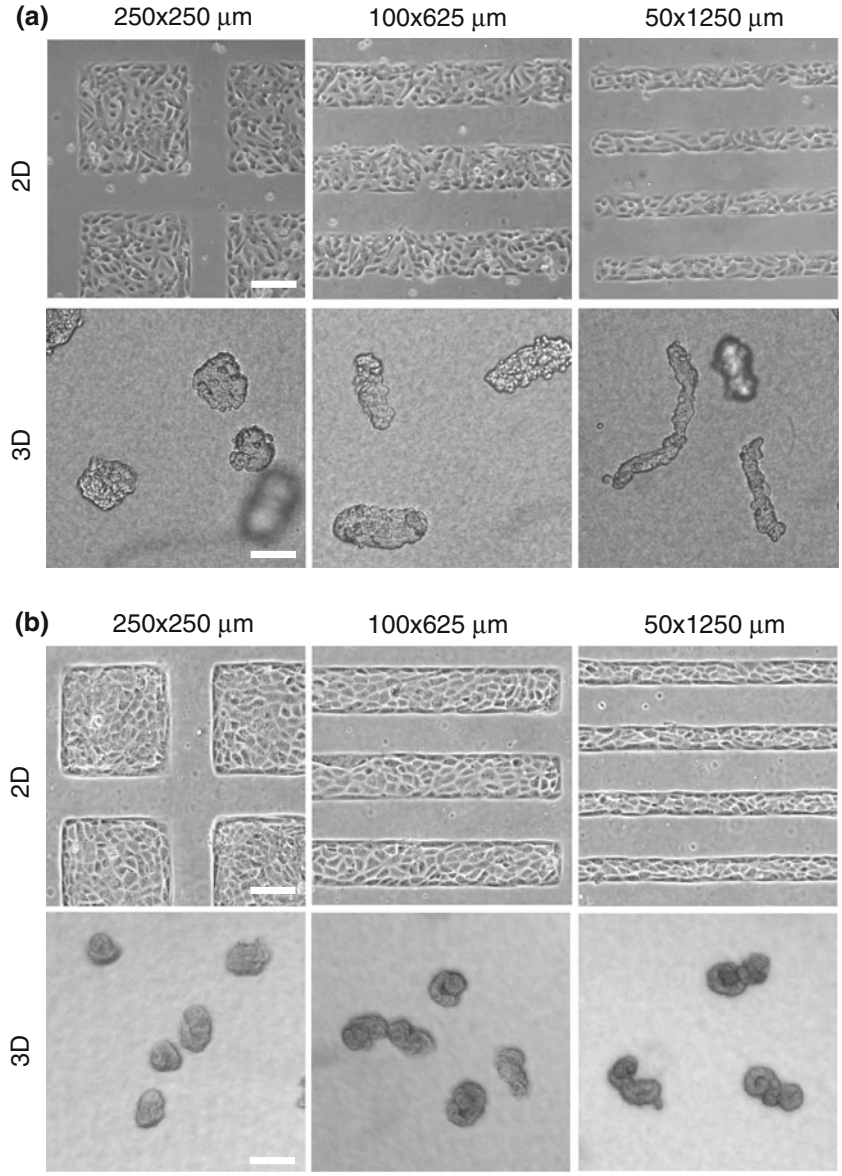
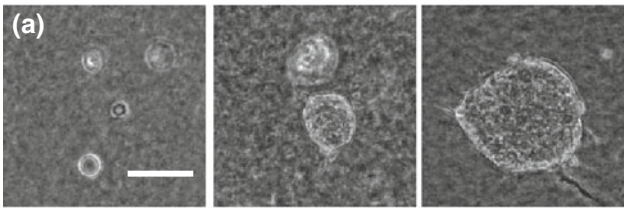


FIGURE 4. Use of 2D micropattern geometry to control 3D cluster shape. (a) Phase contrast images of MCF10A cells on micropatterned collagen rectangles and the resulting 3D clusters formed after collagenase detachment and 3D embedding. (b) Phase contrast images of NRK-52E cells on micropatterned collagen rectangles and the resulting 3D clusters formed after collagenase detachment and 3D embedding. Scale bar: 100 μm.

dimensional micropatterned substrates. This method yielded uniformly sized clusters within a single gel sample. We demonstrated that this method was compatible with different two-dimensional micropatterned cell configurations, including squares and rectangles, resulting in three-dimensional structures with different geometries. The fidelity of two-dimensional patterning techniques has been greatly improved in recent years such that a single substrate contains hundreds of thousands of cells with controlled adhesive microenvironments.[17] Furthermore, two-dimensional micropatterning techniques have advanced in sophistication such that multiple cell types can be patterned in a spatially segregated and defined manner, for in depth review see,[6] and though here we used enzymatic methods to release cells from the pattern, the approach would be compatible with multiple other elegant release methods that have been developed.[19,22] By taking advantage of the control one has in organizing cells using two-dimensional patterning, we can increase the level of control of cell organization in three dimensions.

Cluster size was directly manipulated by the island size, and the distribution of cells per cluster corresponded to how uniformly cells distributed onto the islands. Thus, heterogeneity of cell spreading within a population of cells, seeding density and time of culture



(b)

Patterned area (μm^2)	Cells/cluster
625	1 ± 0.6
10,000	34 ± 5
62,500	90 ± 12

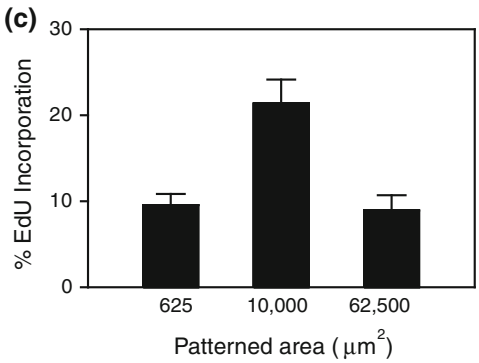


FIGURE 5. Proliferation of MCF10A cells is dependent on cluster size. (a) Phase contrast images of MCF10A cells patterned on 625 μm^2 (left), 10,000 μm^2 (middle) and 62,500 μm^2 (right) islands, lifted off with collagenase, and embedded within collagen gels. (b) Table of number of cells/cluster in each of the patterned areas. (c) Graph of EdU incorporation of MCF10A cells synchronized, seeded on indicated island sizes, lifted off, and embedded within collagen gels. Error bars indicate standard deviation of five independent experiments. Scale bar: 100 μm.

can affect the distribution of cluster sizes. Aggregation of cell clusters rarely occurred for the cell types that we used, likely because the formation of strong cell–cell contacts requires hours (longer than the duration of centrifugation). The consistency of cluster size obtained using this method significantly improved on other methods that depend on spontaneous formation of cellular aggregates. Therefore, this technique provides a reliable way to form a homogeneous population of clusters useful for many biological studies. For example, homogeneous cluster populations may be beneficial for investigating diffusion gradients across spheroids as a model for tumor biology.[3] In such studies, examining many clusters with identical geometries would greatly enhance the robustness of the study, making it possible to validate and or develop theoretical models.[16]

In the current study, we found that the size of the clusters formed by this method correlated with the degree of cell proliferation in 3D, confirming previous reports using randomly formed cell aggregates.[9,12] Previous studies have also suggested that interactions with neighboring cells within spheroids promote survival of endothelial cells.[9] More specifically, cell–cell interactions were required for cells within spheroids to respond to survival factors such as VEGF, since disruption of cell–cell interactions caused spheroid disaggregation and apoptosis.

Precise control of proliferation patterns within three-dimensional constructs may be important for multiple aspects of engineering artificial tissues. First, controlling proliferation by culturing cells in clusters may be used to expand cells, including stem cells, for use in tissue engineering. Inadequate supply of cells has been one of the major practical challenges during development of stem cell technologies, and clustering-induced proliferation might provide a way to continuously renew these valuable cells. Secondly, although we did not specifically examine the ability of spheroids to maintain differentiated cell phenotypes, multiple groups have demonstrated its potential.[4,7] Using clusters to build differentiated tissue micromasses could be a first step in creating functional constructs. Furthermore, vascularization of small multicellular structures such as spheroids has been successfully demonstrated and may provide a simple way to vascularize engineered tissues.[10,14] Finally, many tissues such as the liver are composed of thousands of units, called sinusoids, which are composed of hepatocytes, fibroblasts, and blood vessels organized in a particular spatial arrangement to form functional liver tissue. Greater control of the spatial arrangement of multiple cell types and tissue micromasses could potentially improve the functionality of the entire tissue construct.

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