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Generation of Controllable Gradients in Cell Density**

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Gradients in cell types, cell quantities, and extracellular-matrix (ECM) molecules represent a common feature of many biological systems. At the junction of non-mineralized and mineralized tissues in the tendon-to-bone insertion, for example, the cell phenotype gradually changes from fibroblasts in the tendon to fibrochondrocytes at the interface and osteoblasts in the bone.[1] Similar gradations also exist at the myotendinous junction[2] and between various tissue layers that make up the skin.[3] These gradients in cell density regulate both quantity and quality of cell–cell interactions and thus the functions of relevant tissues.[4] In a sense, the gradients determine the number of physical connections that a single cell is able to establish with surrounding cells, which in turn regulates cell behavior by influencing the intracellular signaling pathways.[5] The perturbation of cell–cell interactions, as typically occurs during an injury, triggers a cascade of events that either re-establishes tissue homeostasis or, more often, leads to scar formation.[6] In addition to physical connections, spatial variations in cell density may lead to the establishment of gradations in biochemical or ECM cues, as a result of which cell migration, proliferation, and differentiation can all be affected.[7] As a result, the ability to generate controllable gradients in cell density is critical to the recreation of functionality for many types of tissues.

Various methods have been pursued to generate gradients in cell density. In most cases, the gradient was established as a result of variation in mechanical,[8] chemical,[9] or electrical[10] cues. Despite their different sources of stimuli, these methods all relied on directed cell migration for the development of a gradient in cell density. In general, directed cell migration is plagued by problems such as slow migration rate,[11] low controllability,[12] and limited reproducibility.[13] The unpredictable differences among batches would make the clinical application of these products challenging. As an alternative to these approaches, live cells can be printed with an inkjet printer to deliver cells in a pre-determined pattern.[14] Although this technique overcomes the low reproducibility of directed cell migration and enables the accurate fabrication of gradients in cell density,[15] it tends to suffer from other drawbacks such as frequent blockage of the printer head, limitation of substrates that are both "printable" and biocompatible, and low cell viability owing to the harsh printing process.[16] These and other issues associated with the existing methods for generating gradients in cell density motivated the current study.

Herein, we developed a simple and versatile method for generating gradients in cell density. It involves the insertion of a substrate into a homogeneous suspension of cells at a specific tilt angle (Figure 1). We hypothesized that, because of the varying volumes of cell suspension above the substrate, the number of cells available for sedimentation onto the substrate would vary along the direction of insertion, naturally leading to the establishment of a gradient in cell density on the substrate. Different gradients in cell density could be obtained by altering the tilt angle of the substrate. The capability of this method could also be extended to generate opposing gradients of two different types of cells on the same substrate, which may find use in the regeneration of the aforementioned biological interfaces.

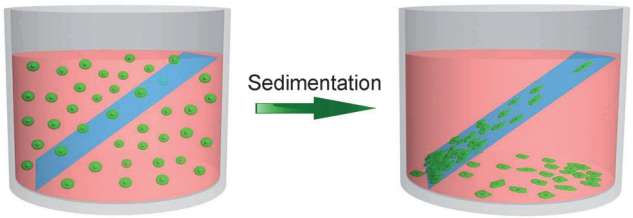

Figure 1. A scheme of the experimental setup. A glass slide is placed at a specific tilt angle in a beaker filled with a homogeneous suspension of cells. Because of the varying volumes of cells available for sedimentation above the slide, the cells are deposited on the slide with a gradient in density.

We first used glass slides and MC3T3 pre-osteoblasts to demonstrate that this technique could generate different gradients in cell density by varying the tilt angle. The experiment was carried out with the glass slides being tilted

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at 15, 30, or 45 degrees. To improve cell attachment, the glass slides were pre-coated with fibronectin and then inserted into homogeneous suspensions of MC3T3 pre-osteoblasts at a concentration of 2×10^5 mL^{-1} (one slide per cell suspension). This concentration of cells was chosen to allow for clear observation of cell density by fluorescence staining. In principle, the concentration of cells can also be varied to alter the gradient of cells. The cells were allowed to sediment for two hours to give them sufficient time to attach to the glass slide. The slides were then extracted and rinsed briefly with phosphate-buffered saline (PBS) to wash off any unattached or loosely-bound cells. All the glass slides were 5 cm in length. The highest point on a slide during the sedimentation process was denoted as the starting point and the lowest point was denoted as the position of 5 cm. Figure S1 of the Supporting Information shows a detailed description of the experimental design. The volume of the cell suspension was set in such a way that the starting point of each slide was barely immersed for each tilt angle. Therefore, the depth of the cell suspension in the beaker was $5\sin(15°)=1.3$ cm, $5\sin(30°)=2.5$ cm, and $5\sin(45°)=3.53$ cm for tilt angles of 15, 30, and 45 degrees, respectively. Similarly, the relationship between the height of any given point on the slide, D, its location along the length of the slide, L, and the tilt angle θ is: $D=L\sin(\theta)$ (Figure S1). In this way, all of the gradient patterns started from a density of zero at the starting point (that is there were no cells at this position available for sedimentation). As an alternative, the volume of the cell suspension could be fixed for all samples, so all of the slides would have the same cell density at the position of 5 cm. However, this scenario would result in variable cell densities at the starting point, and the pattern of the gradient would not stand out as clearly. Therefore, the first method was adopted for this entire study.

Staining was performed immediately after sedimentation to help visualize the cells, where live cells were stained green and dead cells red (Figure 2). As expected, all the samples had cell viabilities close to 100% following sedimentation. While each of the three substrates showed gradient patterns, the cell density increased faster at a larger tilt angle. There were almost no cells present at the starting point in any of the three groups. Different cell densities were observed at the 5 cm position depending on the tilt angle, with the lowest cell density at 15 degrees (Figure 2a), intermediate cell density at 30 degrees (Figure 2b), and the highest cell density at 45 degrees (Figure 2c). These results suggest that the slope of the gradient can be tuned by simply changing the tilt angle of the glass slide during cell seeding. The relationship between the fluorescence intensity of the cells and their location on the glass slides is plotted in Figure S2. In all cases, a linear regression provided a close fit to the data, indicating that local cell density increased linearly with position on the glass slide. The slopes of the regression lines were calculated to be 4.0, 7.5, and 10.5 for tilt angles of 15, 30, and 45 degrees, respectively. The linear relationship between tilt angle and cell density supported our hypothesis that the density of cells on the slide depends on the depth within the cell suspension.

As expected, the slope of the regression also adopted a linear relationship with $\sin\theta$ (Figure S3). To test the predictive power of this relationship, we calculated the expected cell density for a tilt angle of 75 degrees using the regression equation and seeded cells onto a slide placed in a cell suspension at a tilt angle of 75 degrees. The slope of regression for the cell-density gradient at a tilt angle of 75 degrees was predicted to be 14.5. However, seeding cells at 75 degrees for two hours did not support this prediction, with very few cells adhering to the entire glass slide (Figure 3a). The regression curve from the experimental results had a slope of 1.8, even smaller than the slope for the gradient created at 15 degrees (Figure S4). The likely reason for the low cell density is that, at large tilt angles, most of the cells rolled down to the bottom of the container before they had a chance to adhere to the substrate. Since $2\sin29°$ is roughly the same as $\sin75°$, we performed two sequential seeding procedures at 29 degrees to obtain the gradient in cell density estimated for a tile angle of 75 degrees. Figure 3b shows the gradient after the first seeding process at 29 degrees. Figure 3c shows the cumulative gradient after two sequential seeding procedures at 29 degrees. The fluorescence intensity at each location fits well with the prediction, with a slope of 14.6 (Figure S3). These data indicate that consecutive seeding processes can be used to overcome cell adhesion issues associated with large tilt angles, where cell rolling across the surface becomes an issue.

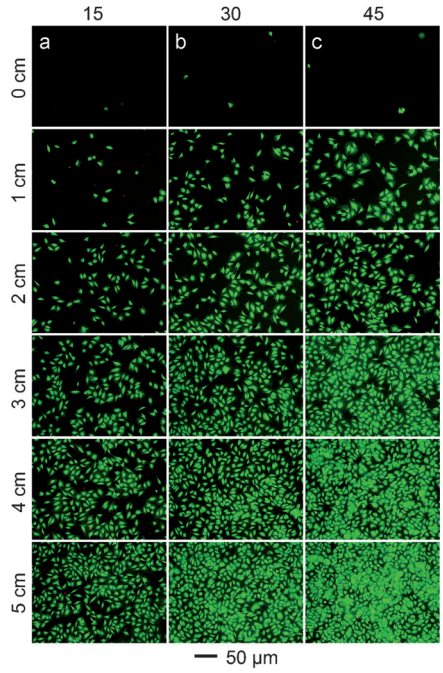

Figure 2. Fluorescence micrographs showing gradients in cell density generated on glass slides at tilt angles of a) 15, b) 30, and c) 45 degrees. The cells were stained with a live/dead kit immediately after seeding; live (green), dead (red).

We also generated opposing gradients in cell density on the same glass slide to demonstrate the power of this method for applications such as tendon-to-bone tissue engineering. Specifically, MC3T3 pre-osteoblasts and tendon fibroblasts (TFB) were used to generate reverse gradients of these two cell types. MC3T3 cells were pre-labeled with DiO (green)

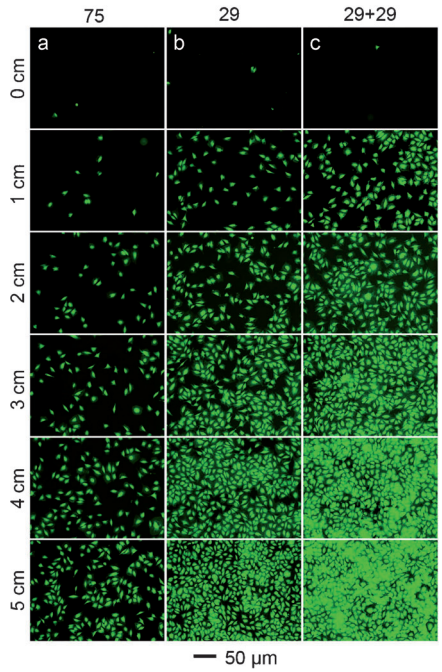

Figure 3. Fluorescence micrographs showing gradients in cell density generated on glass slides at tilt angles of a) 75 and b) 29 degrees. c) Cumulative gradients generated after two consecutive seeding processes at 29 degrees each along the same direction of gradient. The cells were stained with a live/dead kit immediately after seeding; live (green), dead (red).

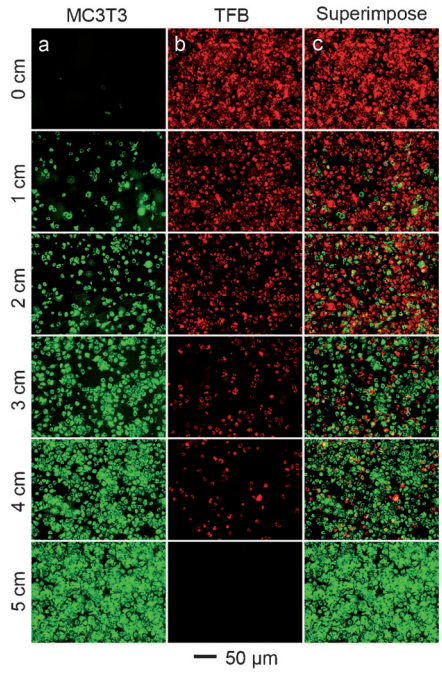

Figure 4. Fluorescence micrographs showing the generation of reverse gradients in cell density for MC3T3 pre-osteoblasts (green) and TFB cells (red) on the same glass slide after two consecutive sedimentation processes along opposite directions. The two types of cells were labeled with different membrane dyes prior to seeding.

and TFB cells with DiI (red) prior to sedimentation. The MC3T3 pre-osteoblasts were seeded onto the glass slide at a tilt angle of 30 degrees for two hours. The glass slide was briefly rinsed with culture medium to wash off any loosely attached cells and observed under a fluorescent microscope (Figure 4a). The glass slide was then horizontally rotated 180 degrees and inserted into a suspension of TFB cells at a tilt angle of 30 degrees for two hours. After washing off loosely attached TFB cells, the glass slide was imaged again. As shown in Figure 4b, a similar gradient in the density of TFB cells was generated along the glass slide but in the reverse direction of the MC3T3 pre-osteoblasts. Figure 4c shows a superimposed view of the images, indicating a transition zone at the center of the scaffold where MC3T3 pre-osteoblasts and TFB cells were brought into close proximity.

The gradient in cell density could also be fabricated on a scaffold of electrospun nanofibers using the same procedure (Figure S5). The nanofibers were fabricated according to our previously published methods.[17] The scaffold was attached to a glass slide prior to seeding and the tilt angle was set to 30 degrees. The scaffold was designed to have two different types of structures with uniaxially aligned nanofibers on one region and random nanofibers on the other. When placed in the cell suspension, the random nanofibers were kept at the bottom whereas the aligned end was kept at the top. A clear gradient in cell density can be observed on the scaffold with no obvious difference in cell attachment between the aligned region and the random region. This observation indicates that both aligned and random nanofibers are suitable substrates for the generation of gradients in cell density using this simple method.

There are several advantages associated with this method for generating gradients in cell density as compared to previously published approaches. First, the experimental setup is simple and cell-friendly compared to the printing techniques for live cells. Maintaining sterility is trivial for the method presented in this study, but can be difficult for the cell printing approaches. Because of minimal cell processing, it is expected that the cells will display similar behaviors to the cells plated using the conventional methods. Maintaining cell function is especially critical for studies involving stem-cell differentiation or primary-cell types that must maintain their phenotypes. Techniques involving chemical, mechanical, or electrical stimulation for the formation of cell gradients may result in unwanted or uncontrollable differentiation of stem cells or de-differentiation of primary cells. A second advantage of the method presented herein is the predictability of the gradient based on the tilt angle. A simple linear relationship can be used to reproducibly generate cell gradients in a particular density. A third advantage of the present method is the ability to easily produce complex gradients in terms of patterns and cell types using sequential seeding processes, many of which would be difficult or impossible to be fabricated using the previously reported techniques.

In summary, a simple and robust method for generating gradients in cell density has been demonstrated. In this method, a substrate is inserted into a homogenous suspension of cells at a pre-set angle and a cell density gradient will naturally be created owing to the variation in the number of

cells available for sedimentation above the substrate. By adjusting the tilt angle, various gradient patterns can be easily produced in a controllable and reproducible fashion. Reverse gradients of different cell types can also be fabricated on the same substrate by taking advantage of multiple sequential seeding processes.

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