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Modulation of Heterotypic and Homotypic Cell–Cell Interactions via Zwitterionic Lipid Masks

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Since the pioneering work by Whitesides, innumerable platforms that aim to spatio-selectively seed cells and control the degree of cell–cell interactions in vitro have been developed. These methods, however, have generally been technically and methodologically complex, or demanded stringent materials and conditions. In this work, we introduce zwitterionic lipids as patternable, cell-repellant masks for selectively seeding cells. The lipid masks are easily removed with a routine washing step under physiological conditions (37 °C, pH 7.4), and are used to create patterned cocultures, as well as to conduct cell migration studies. We demonstrate, via patterned cocultures of NIH 3T3 fibroblasts and HeLa cells, that HeLa cells proliferate far more aggressively than NIH 3T3 cells, regardless of initial population sizes. We also show that fibronectin-coated substrates induce cell movement akin to collective migration in NIH 3T3 fibroblasts, while the cells cultured on unmodified substrates migrate independently. Our lipid mask platform offers a rapid and highly biocompatible means of selectively seeding cells, and acts as a versatile tool for the study of cell–cell interactions.

Cells in vivo face a complex microenvironment, composed of not only a diverse array of biomolecular stimulants and structural cues, but also several homotypic and heterotypic cell populations.[1] As a result, the study of cell–cell interactions has become especially important in fields such as tissue engineering,[1,2] disease studies,[3] wound healing,[4] and developmental biology.[5] Conventional methods for in vitro cell culture, however, are ill-suited to modulate the homotypic and heterotypic cell–cell interactions that occur, which can have drastic effects on parynchemal cell behavior. For instance, the viability, phenotype, and detoxification capabilities of hepatocytes are all dependent on both homotypic and heterotypic support cells.[6]

In order to improve the degree of control over cell–cell interactions in vitro, several "patterned culture" platforms have been proposed.[7] These platforms have utilized methods ranging from microfluidic systems[7c,8] or physical scratchers and stoppers[9] to cell-specific adhesive coatings[10] and stimuli-responsive substrates,[11] to designate the spatial location of cell seeding. One of the early examples of patterned culture platforms includes the pioneering work of Whitesides and co-workers that used the laminar flow in microfluidic channels to seed alternating patterns of chicken erythrocytes and *Escherichia coli*.[8a] The system was also used to spatially localize adhesive-protein flow or trypsin/EDTA flow, which allowed for the selective attachment and detachment, respectively, of different cell types. As an inherent restriction of microfluidics, however, only continuous patterns could be formed, and pattern dimensions were restricted to sizes achievable via laminar flow.[12] Photolithographic and microcontact-printing (μCP)-based strategies have also been prominently employed to selectively seed cells on solid substrates. These lithography-based methods typically featured a cell-repellant component that was manipulatable,[12c–e] reversible,[11a,b] or removable.[9b,13] For example, electroactive self-assembled monolayers were utilized to immobilize cell-adhesive molecules onto previously cell-repellant patterns.[10a,b,11d] Thermally responsive polymers were switched between cell-repellant and -adhesive states at different temperatures to sequentially seed hepatocytes and endothelial cells, or inversely, to locally detach the already adhered cells to expose the areas for seeding of a second cell type.[11a,b] Even patterned stencils were used to mechanically remove adhered cells.[9b,13] While these endeavors have successfully created patterned cocultures, most of the platforms are technically and methodologically complex to produce, or demand severely restrictive materials and fabrication conditions. In this work, we propose a highly simple and biocompatible method for generating patterned cell cultures through the use of lipids, a common biomolecule, as an easily removable, cell-repellant mask. The lipid-mask-based cell culture platform allowed for an extremely facile and rapid means of modulating heterotypic and homotypic cell–cell interactions.

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Lipids have been employed as a building block for constructing biotechnological platforms, including those for cell membrane models,[14] protein interaction studies,[15] and cell adhesion studies.[16,17] The popularity stems not only from their high biocompatibility and chemical versatility, but also from their wide availability and ease of handling. In addition, lipids, depending on their chemical composition, possess varying degrees of cell repellency; positively charged lipids, such as 2,3-bis[[(*Z*)-octadec-9-enoyl]oxy]propyltrimethylazanium, are conducive to cell adhesion, while zwitterionic lipids, such as [(2*R*)-3-hexadecanoyloxy-2-[(*Z*)-octadec-9-enoyl]oxypropyl] 2-(trimethylazaniumyl)ethyl phosphate (POPC), are highly cell-repellant.[18] Essentially, these hybrid, patterned substrates possess a cell-repellant backfilling—lipids—that are not only dimensionally versatile and highly biocompatible, but easily removable.[19,20] Previous reports have usually focused on preventing lipid removal.[21] We, however, embraced this latter trait that made them an excellent candidate system to pattern cell cocultures.

We, in this work, utilized POPC as a component of the easily removable, cell-repellant masks, and fabricated micropatterns of fibronectin (FN) and POPC by the μCP of FN and subsequent backfilling of POPC (**Figure 1**). The POPC-lipid masks selectively blocked cell adhesion to the substrate during the initial seeding, and limited cell attachment to the FN patterns. The lipid masks were found to be easily removed in seconds simply by washing the substrate with phosphate-buffered saline (PBS, pH 7.4) (see Figure S1 of the Supporting Information for the data on lipid removal). The entire process did not require any solvents, surfactants, or specialized stimuli.

Prior to the spatio-selective cell seeding, we characterized cell attachment on the lipid mask platform by quantifying the adhesion of NIH 3T3 fibroblasts and HeLa cells on nontreated silicon wafers (Si/SiO_2), silicon wafers coated with FN (FN+) or POPC (POPC+), and silicon wafers after POPC-mask removal (POPC-) (Figure S2, Supporting Information). The cells were also seeded on tissue culture plates (TCP) as a control. Confocal laser-scanning microscopy (CLSM) images showed that NIH 3T3 fibroblasts freely attached to and proliferated on the FN+ substrate (405.7 ± 30.6 cells mm^{-2}), while almost no cells were found on the POPC+ substrate (3.7 ± 0.7 cells mm^{-2}). The NIH 3T3 cells showed similar degrees of adhesion on the TCP, Si/SiO_2, and POPC- substrates, at 200.0 ± 25.6, 223.7 ± 12.4, and 236.7 ± 17.7 cells mm^{-2}, respectively. HeLa cells also exhibited little attachment to POPC-lipid masks (0.7 ± 0.7 cells mm^{-2}), and similar levels of adhesion across the TCP, Si/SiO_2, FN+, and POPC- substrates (130.3 ± 4.9, 133.3 ± 21.2, 116 ± 4.6, and 110.0 ± 8.7 cells mm^{-2}, respectively). The fact that the cells adhered fairly well to the bare silicon wafers (Si/SiO_2 and POPC-) indicated that FN patterns alone would not be enough to pattern cells (**Figure 2**a), and emphasized the need for cell-repellant POPC.

We formed the FN patterns on silicon wafers via μCP, where FN not only promoted cell adhesion, but also acted as a barrier for the lateral diffusion of lipid bilayers in this work.[20,22] Polydimethylsiloxane (PDMS) stamps were used to create FN stripes with 100 μm widths and 200 μm gaps. Successful pattern generation was confirmed by immunostaining the patterned FN with anti-FN antibody (Figure 2b, left). Red-fluorescent

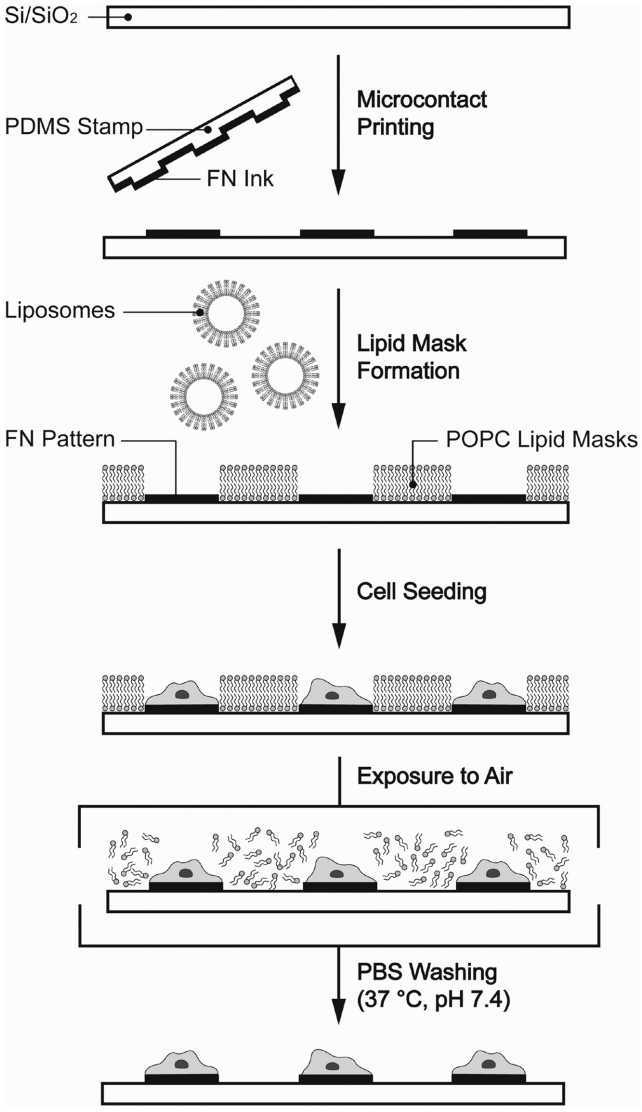

Figure 1. Schematic illustration of the lipid-mask-based platform for modulation of heterotypic (coculture platform) and homotypic (collective migration platform) cell–cell interactions.

rhodamine-conjugated 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine was mixed with POPC to confirm that the stamped FN barriers sufficiently segregated the POPC-lipid masks (Figure 2b, right). Fibroblasts cultured on the FN/POPC-patterned substrates selectively adhered to the FN-stamped areas, which resulted in the distinct patterned stripes of cells (Figure 2c, left). After 3 h of culture, to allow sufficient time for complete cell attachment, the subsequent lipid mask removal was carried out with a gentle PBS wash; fibroblasts on the FN stripes remained firmly attached, while the POPC masks were removed (Figure 2c, right). It is noteworthy that the entire process was performed under physiological conditions (37 °C, pH 7.4). After the lipid masks were removed, the newly exposed areas were coated with FN and prepared for cell attachment. HeLa cells, an immortalized cell line of cervical cancer, were seeded as a second cell line for coculture. CellTracker was used to tag and distinguish the different cell lines prior to cell

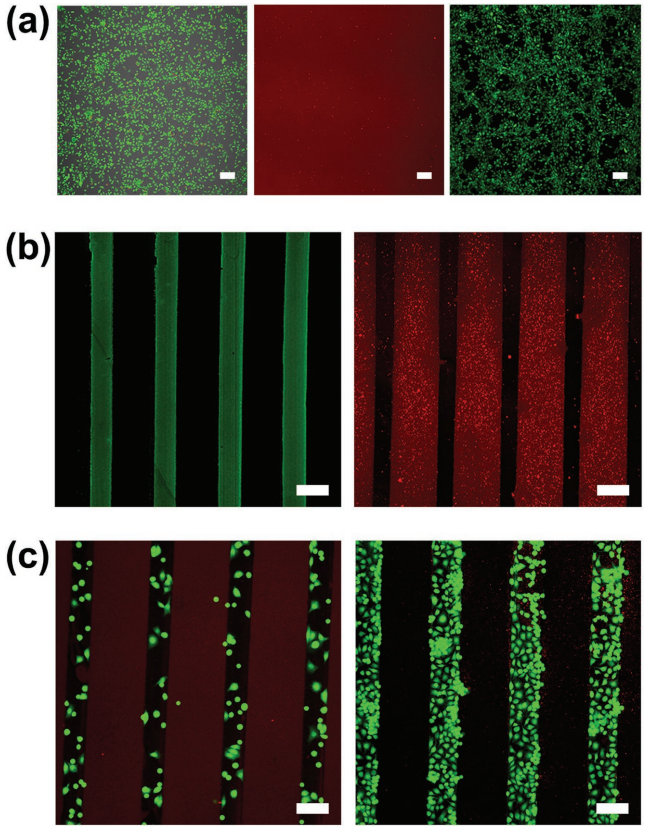

Figure 2. a) Left: NIH 3T3 cells cultured on the fibronectin (FN)-coated substrate. Middle: NIH 3T3 cells cultured on the lipid (POPC) mask substrate (red: lipid; green: cells). Right: NIH 3T3 cells cultured on the FN-patterned substrate without POPC backfilling. b) Left: Microcontact-printed FN lines (immunostained with anti-FN antibody; green). Right: Lipid mask patterns with FN stripes (red: POPC). c) Left: NIH 3T3 fibroblasts cultured on the FN/POPC-patterned substrate (red: POPC; green: cells). Right: NIH 3T3 cells on the FN/POPC-patterned substrate after lipid mask removal. Scale bars: 100 µm.

seeding. NIH 3T3 cells were tagged with CellTracker Green, and HeLa cells with CellTracker Red (**Figure 3**a). The Live/Dead assay confirmed that the viabilities of both cell lines were not diminished by the lipid mask platform (Figure S3, Supporting Information). We also reversed the cell seeding order to demonstrate the flexibility of the lipid-mask coculture platform; HeLa cells were seeded on the FN stripes, followed by a secondary seeding of NIH 3T3 fibroblasts after lipid mask removal. Patterned cocultures were successfully generated, regardless of intial cell–secondary cell seeding order, resulting in NIH 3T3-HeLa and HeLa-NIH 3T3 cocultures, and pattern geometry (Figure 3a). Since adhesion properties differ by cell type, flexibility is a desired trait in an ideal patterned coculture platform. While FN was used exclusively in this study, any cell-adhesion protein, such as neuroadhesive laminin, could be used for the intial cell seeding, or to coat the substrate after lipid mask removal for secondary cell seeding; additionally, the two proteins can be of any combination. Unlike systems that rely on material-specific interactions, which significantly limit the types and combination of proteins that can be used, or stimuli-responsive materials, which can be methodologically

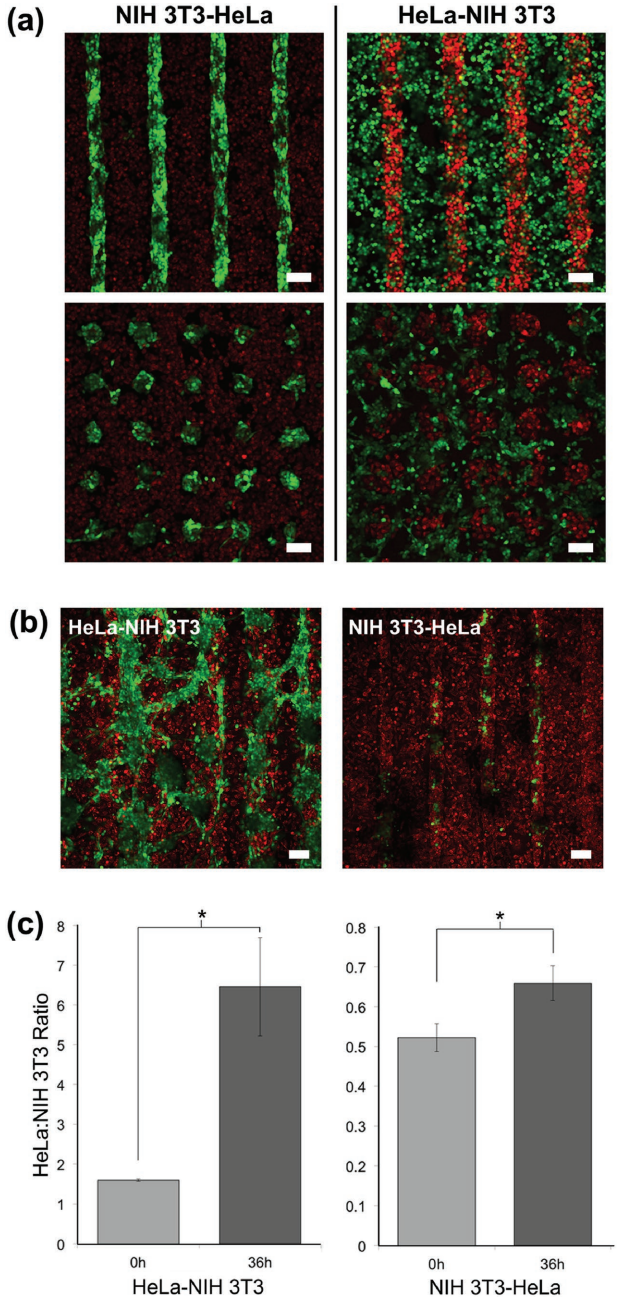

Figure 3. a) NIH 3T3-HeLa and HeLa-NIH 3T3 patterned cocultures created by using the lipid-mask-based platform. Different cell population geometries could be produced depending on the PDMS stamp. b) Long-term behavior of patterned NIH 3T3-HeLa and HeLa-NIH 3T3 cocultures. Patterned cocultures were seeded and cultured for 36 h to observe proliferation behavior. Initially seeded cells covered a smaller area (100 µm stripes) than the secondarily seeded cells (200 µm stripes), yet HeLa cells proliferated more aggressively than NIH 3T3 cells regardless of cell seeding order (green: NIH 3T3 cells; red: HeLa cells). c) Ratio of area coverage between HeLa and NIH 3T3 populations. Scale bars: 100 µm. Statistical significance was determined by using Student's *t*-test ($^*p < 0.05$).

challenging, the lipid-mask-based platform developed offers a quick, flexible, and biocompatible means of generating patterned cocultures.

A primary advantage of patterned cocultures is increased spatiocontrol in the study of heterotypic interactions between two different cell types. After patterned cell seeding, we investigated the proliferation behaviors of NIH 3T3-HeLa and HeLa-NIH 3T3 cocultures for 36 h (Figure 3b). The cell stripe widths were of alternating thicknesses (100 μm widths, 200 μm gaps) so that we could examine the effects of differing population sizes on cell proliferation and migration during coculture. Initially seeded cells covered a smaller area (100 μm stripes) than the secondarily seeded cells (200 μm stripes). In the case of the NIH 3T3-HeLa coculture, HeLa cells, after 36 h, were found to completely cover NIH 3T3 populations, which did not proliferate beyond their original striped patterns. As for the HeLa-NIH 3T3 coculture, at 36 h NIH 3T3 cells were more successful in proliferating beyond their initial seeding areas compared with the NIH 3T3-HeLa coculture, mostly likely due to the larger seeding area. Quantification of the areas covered by HeLa and NIH 3T3 populations revealed a statistically significant increase in the HeLa:NIH 3T3 cell ratio after 36 h for both NIH 3T3-HeLa and HeLa-NIH 3T3 cocultures (Figure 3c). These results indicated that HeLa cells, presumably because of their identity as cancerous cells,[23] aggressively proliferated to the extent of inhibiting and covering the already established NIH 3T3 populations, even when the latter began with a greater seeding area.

We also used the lipid mask platform to modulate homotypic cell–cell interactions, which is a critical component of collective cell migration. Collective cell migration is loosely defined as the concerted movement of physically and functionally connected cells. Unlike in single cell migration, the physical intercellular dynamics of neighboring cells at cell junctions largely influence overall population behavior. Platforms for collective migration study, like those for coculture studies, require the ability to selectively seed dense cell populations in a simple and precise manner; additionally, the unpopulated areas must still be conducive to cell migration and attachment.[24] Therefore, we envisioned that our lipid mask platform was well-suited to provide these conditions. In order to observe the effects of biochemical surface characteristics on collective cell migration, we compared the behavior of cell populations encountering FN-coated (FN+) or uncoated (FN–) surfaces. After cell seeding and lipid mask removal, the samples were either incubated in cell media containing an FN solution for 30 s, or left unmodified.

We observed a drastic difference in collective cell behavior between the FN+ and FN– substrates. At 6 h, the migration of NIH 3T3 fibroblasts on the FN+ substrate was highly polarized (**Figure 4**a). The fibroblasts moved in unison to form narrow bridges across the gaps between the patterned cell stripes, rather than migrate uniformly, which would be more reminiscent of "sheet" migration.[25] The cells eventually proliferated to form confluent cell populations on the entire substrate. Contrastingly, the fibroblasts cultured on the FN– substrate showed little collective migration and polarity. While the overall progression and directionality of cell movement was uniform in nature, intercellular connectivity was low. At 6 h, the cells that had migrated onto the gaps displayed little to no physical contact, and appeared to move independently rather than collectively. The cells on the FN– substrate, however, also formed confluent cell populations, eventually.

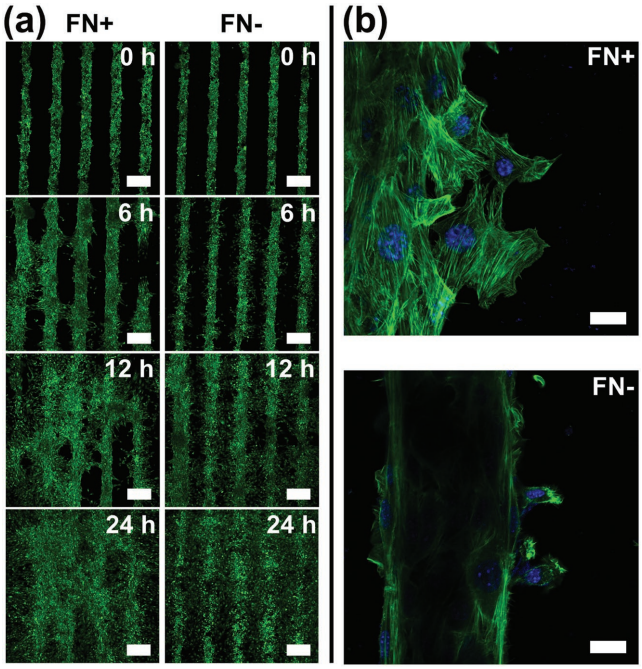

Figure 4. a) Migratory behavior of NIH 3T3 fibroblasts. The cells were tagged with CellTracker Green. Scale bars: 200 μm. b) High-magnification CLSM images of NIH 3T3 cells on FN+ and FN– substrates. The cells were stained with Alexa Fluor 488 phalloidin (for filamentous actin (F-actin); green) and 4′,6-diamidino-2-phenylindole (DAPI) (for nuclei; blue). Scale bars: 20 μm.

Since FN is a cell-adhesive, extracellular matrix protein, the NIH 3T3 fibroblasts on the FN+ substrate were presented with gaps that were highly conducive to cell adhesion. We, therefore, believed that the cells on the border could readily attach to and probe unexplored territory, promoted by strong integrin interactions on the FN-coated surfaces. The focal adhesions formed on the FN-presenting surfaces would not only stimulate an intracellular signal cascade, but also enable the formation of a stable cytoskeletal structure necessary for the mechanically intense pulling forces involved in collective cell migration.[24,26] The high-magnification CLSM images of fibroblasts on the FN+ substrate showed that nascent leader cells exhibited a highly spread morphology with clearly visible actin stress fibers, while maintaining tight cell–cell junctions with follower cells (Figure 4b). By contrast, the fibroblasts on the FN– substrate were morphologically circular, and exhibited less spreading. Quantification of cell areas showed that the average area of migrating NIH 3T3 cells on FN+ was over twice that of cells on FN– (Figure S4, Supporting Information). Additionally, intercellular interaction between pioneering cells and their neighbors was very low, and there was no indication of physical pulling by leader cells in the case of the FN– substrate. While the gaps on the FN– substrate were in no way cell-repellant based on our data, the exposed Si/SiO_2 area, we believed, would not be as conducive to integrin binding as the FN-coated area, resulting in weaker focal adhesions and a less stable foundation for cytoskeletal involvement. We further corroborated the importance of cytoskeletal dynamics via biochemical inhibition studies; cytochalasin D, a known inhibitor of actin polymerization, eliminated any form

of collective fibroblast migration on both FN+ and FN– substrates (Figure S5, Supporting Information).

In summary, we created a lipid-mask-based platform for spatio-selectively seeding cell populations to modulate cell–cell interactions. We demonstrated its abilities as a tool to observe both heterotypic and homotypic cell–cell interactions through cocultured cell proliferation studies and collective cell migration studies, respectively. The platform developed in this work has several advantages over previous selective seeding methods: (1) high biocompatibility: no cytotoxic materials or components are required, and all experimental conditions are physiological; (2) rapid and facile experimentation: the cell-repellant (zwitterionic) backfilling (in the form of a lipid mask) is easily applied, and can be removed in seconds with a simple washing step; (3) universality: previous reports have relied on cell-specific adhesives, and are therefore limited to specific cell types and/or seeding order. This platform, on the other hand, can be applied to most adhesive cells.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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