Lab on a Chip

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COMMUNICATION

Lift-off Cell Lithography for Cell Patterning with Clean Background

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We developed a highly efficient method for patterning cells by a novel and simple technique called lift-off cell lithography (LCL). Our approach borrows the key concept of lift-off lithography from microfabrication and utilizes a fully biocompatible process to achieve high-throughput, high-efficiency cell patterning with nearly zero background defects across a large surface area. Using LCL, we reproducibly achieved > 70% patterning efficiency for both adherent and non-adherent cells with < 1% defects in undesired areas.

High-throughput cell patterning is an important technique for many cytobiological studies and for tissue engineering[1-3]. Much effort has been expended developing efficient and reproducible strategies for cell patterning[4, 5]. Prior studies include active methods that utilize physical phenomena such as dielectrophoresis (DEP)[6-8], optoelectronic tweezers (OET)[9-12], and magnetic[13, 14] or acoustical forces[15, 16]. Passive approaches include cell trapping in a microwell[17, 18] and surface chemical modifications via selective plasma treatment[19, 20], UV light[21, 22], micro-contact printing (μCP)[23-25] and photolithography-based techniques[26-28]. These passive approaches require less specialized equipment and are therefore more practical and user-friendly for typical biology laboratories. However, it still remains challenging to achieve well-defined cell patterning with good pattern filling efficiencies for desired locations and with few cells in unwanted locations across a large surface area[29-31].

Advances in microfabrication technique applications beyond microelectronics have generated opportunities for studies in biology[32, 33]. Lift-off lithography is a traditional wafer-level microfabrication method that can rapidly generate massive array patterns at high resolution for a target material, such as metals[34]. Previously reported micro-stencil methods employed a similar lift-off lithography concept for creating cell patterns[35, 36]. However, micro-stencils are usually fragile freestanding thin membranes that require delicate handling; therefore, they have not been widely used[37]. Here, we demonstrate a novel and simple cell patterning method called Lift-off Cell Lithography (LCL) that utilizes a fully biocompatible process to achieve high efficiency patterning with nearly zero background defects in masked or blocked areas of a surface. A thin film stacked with SU-8 photoresist and water-soluble polyvinyl alcohol (PVA) was used as a sacrificial layer to lift-off un-patterned cells deposited on the substrate. The substrate was precoated with poly-L-lysine (PLL) to anchor cells at the desired locations. Using LCL, we have achieved over 70% cell patterning efficiency for both adherent and non-adherent cells in the target patterned area, with < 1% defect rate in the blocked area. Since the lithography masks are digitally generated, this versatile technique can also pattern cells into custom-shaped colonies.

The major steps of the fabrication process and experimental protocol for patterning cells using LCL are shown in Fig. 1. First, a glass coverslip (2.2 × 2.2 cm^2) is cleaned with 70% ethanol for surface sterilization followed by coating with a poly-L-lysine-FITC labelled solution (Sigma; 0.1 mg mL^{-1}) for 30 minutes at room temperature. PLL is a positively charged cationic polymer that promotes cell adhesion through electrostatic attraction since the plasma cell membrane is negatively charged. Then, an aqueous solution containing 4% (w/w) PVA (Sigma) is spin-coated at 1,000 rpm onto the surface as a sacrificial layer. Next, SU-8 3005 photoresist (MicroChem) is spun onto surface at 3,000 rpm to produce a 5 μm thin film that is then micro-patterned via standard photolithography. Afterwards, an oxygen plasma treatment (Technics Micro RIE 800, 200 W, 300 mTorr) is performed for 2 minutes to etch the exposed PVA. The final fabrication step involves coating the chip with PLL for a second time for another 10 minutes at room temperature in case the first layer PLL is partially etched away by the oxygen plasma in the previous step.

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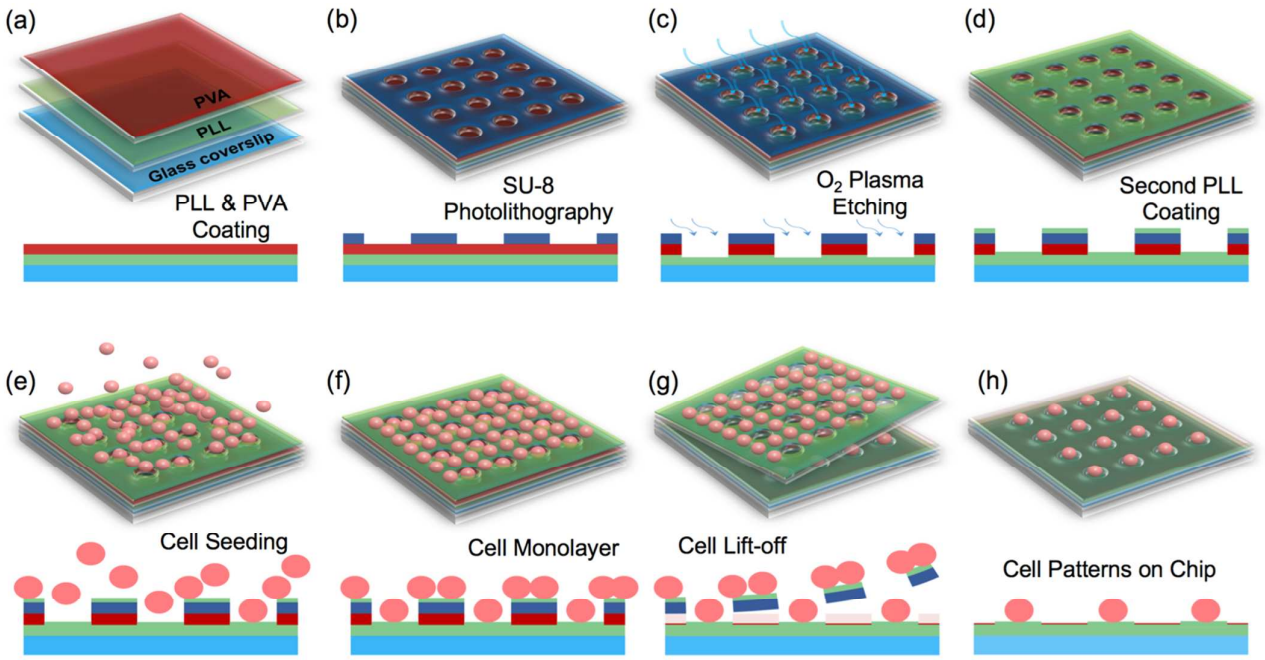

Fig. 1 Illustration of the process flow for the Lift-off Cell Lithography (LCL) patterning method. (a) Multilayers (including PLL and PVA) are coated in sequence on a glass coverslip. (b) SU-8 3005 photoresist is patterned on top of the surface via photolithography. (c) PVA layer is further patterned by O_2 plasma etching. (d) PLL is coated for the second time. (e) Cell suspension medium is loaded on the chip for 10 min at room temperature. (f) A monolayer of cells eventually forms. (g) Cells are further cultured for another 30 min in incubator while PVA gradually dissolves and allows cells adhering on the SU-8 film to be peeled off. (h) The patterned array of cells.

After rinsing and drying the glass substrate, non-adherent Ramos cells suspended in tissue culture medium at a high density of 3.0×10^6 cells mL^{-1} are dispensed onto the substrate for 10 minutes at room temperature where a cell monolayer eventually forms. Ramos suspension cells are free floating in culture media containing RPMI-1640 supplemented with 10% fetal bovine serum and are difficult to attach to the bottom substrate. Serum-free RPMI 1640 medium is found to improve cell adhesion to the glass substrate, possibly due to charged interactions between the hydroxyl groups on the substrate and integrins expressed on the surface of cells[38-40]. Our testing also confirms that we can increase the number of surface-attached Ramos cells by about ten-fold with the serum-free culture medium (Fig. S1). PVA is a water soluble material with good biocompatibility. The dissolution rate increases with temperature and usually cells do not adhere well to PVA[41, 42]. After cell seeding, the substrate is kept in an incubator at 37°C under a 5% CO_2 humidified atmosphere for another 30 minutes, resulting in the partial removal of the PVA layer underneath the SU-8 film. The continuous SU-8 membrane is thin but rigid enough to be peeled-off together with all the cells adhering to it (Fig. S2). This leaves only cells at the desired exposed pattern locations. Since a high concentration of cells can be used in the patterning process of LCL without concerns for background defects, high cell fill-up efficiency and clean background patterning can be achieved at the same time without any trade-off. By contrast, this trade-off often needs to be made in other patterning approaches.

An array of circular holes, each with a diameter of 20 μm and a center-to-center spacing of 50 μm, repeated over a large 1.2 × 1.2 cm^2 area (Fig. 2a) was used to pattern a Ramos B cell array through LCL (Fig. 2b). Calcein AM/propidium iodide (Invitrogen) staining is used for live versus dead cell recognition at 1h after cell patterning. LCL shows minimal impact on cell viability (Fig. S3). Patterning experiments were repeated multiple times. The number of total spots, spots occupied by cells, cells in the masked background, and the total number of cells were recorded from randomly selected locations on each chip and this data was used to estimate the average values of patterning efficiency and background defect rate. The patterning efficiency is defined as the percentage of spots occupied by cells, and the background defect rate is defined as the number of cells in undesired positions divided by the total number of cells. Fig. 2c shows that LCL can achieve a peak 71% patterning efficiency with a background defect rate as low as 0.31%. For comparison, two other widely used surface chemical treatment approaches, namely micro-contact printing (μCP) and lift-off PLL (LPLL), have also been tested to pattern Ramos cells under the same conditions (Fig. S4). μCP shows no significant effect on non-adherent cell patterning with a low efficiency and high defect rate, and LPLL results in an improved patterning efficiency, however, with the background defect rate an order of magnitude higher than our LCL method (Fig. 2c). With LCL, optimized single-cell array patterning is also possible by reducing the hole diameter further, making it wide enough for one individual cell but not wide enough for multiple cells[29]. The tradeoff is a slightly lower patterning efficiency. In addition, the LCL technique is

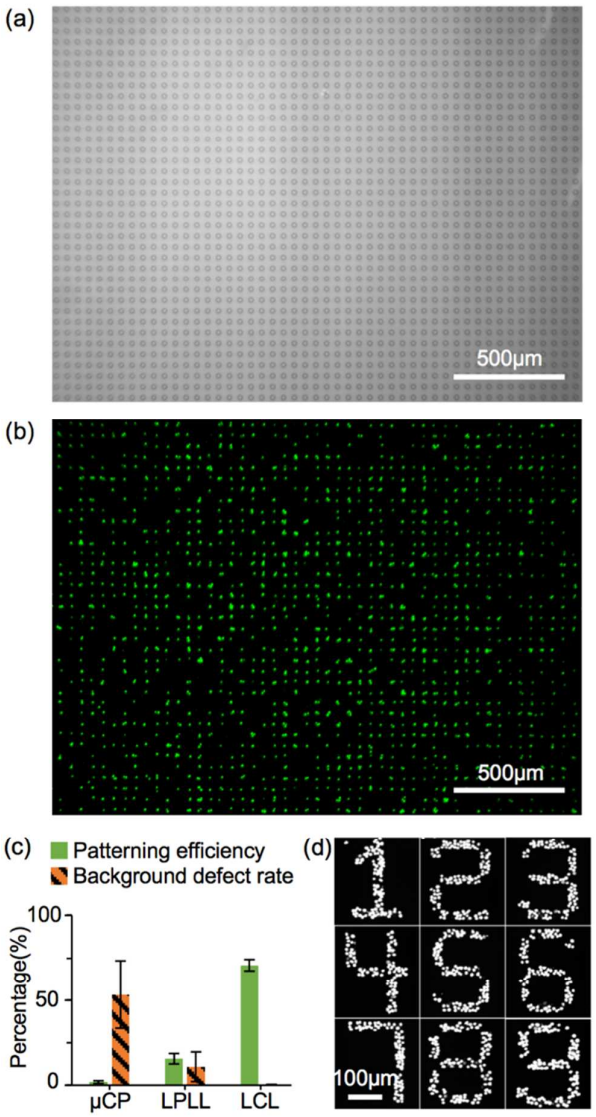

Fig. 2 Non-adherent Ramos cell patterning through LCL. (a) Image of a SU-8 microwell array with 20 μm diameter holes. (b) Fluorescence image of patterned cells stained with calcein AM. (c) Results of cell patterning efficiency and background defect rate via μCP, LPLL and LCL. (d) Fluorescence image of cells patterned to form number characters from "1" to "9".

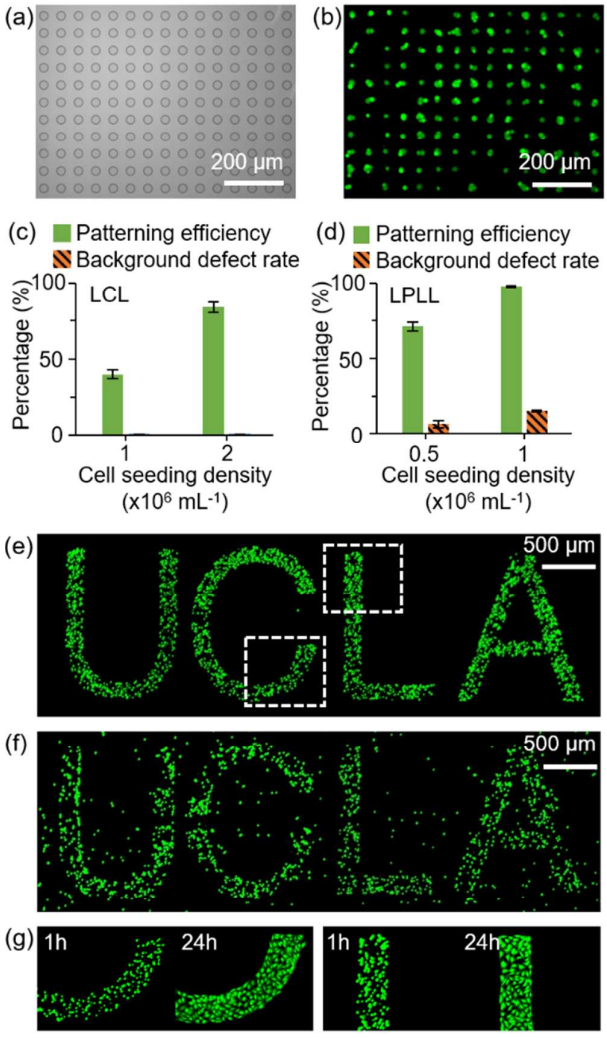

Fig. 3 Adherent HeLa cell patterning through LCL. (a) Image of SU-8 microwell array with a diameter of 30 μm on the chip. (b) Fluorescence image of cell array patterning stained with calcein AM. (c) & (d) Results of cell patterning efficiency and background defect rate via LCL and LPLL, respectively, with different cell seeding densities. (e) & (f) Fluorescence images of large-scale cell patterns arranged in the letters of "UCLA" by LPLL and LCL, respectively. (g) Fluorescence images of patterned cells marked in the white dashed boxes in (e), after culturing for 1h and 24h.

also effective for patterning cells to form arbitrary shapes. As demonstrated in Fig. 2d, cells can be arranged into alphanumeric shapes from "1" to "9" with a clean background.

LCL also works well with adherent cells (Fig.3a and b). In order to pattern HeLa cervical carcinoma cells whose average size is larger than Ramos B cells, the circular hole size on LCL is increased to 30 μm. The second PLL coating step is also skipped to prevent HeLa cells from adhering tightly to the PLL-coated SU-8 sidewall, which can result in unexpected removal of cells from desired locations during peel-off.

In LCL, high cell patterning efficiencies can be achieved with higher cell seeding densities and no significant increases in background defect rates (Fig. 3c). This is a unique feature of LCL that differs from traditional surface chemical modification approaches, in which a higher cell seeding density usually adversely results in a corresponding higher background defect rate (Fig. 3d).

Using LCL, large-scale custom arrays of HeLa cells can also be patterned. As demonstrated in Fig. 3e, cells can be patterned into the letters of "UCLA" with nearly zero defects in the background. By comparison, Fig. 3f shows the result of cell patterning via the LPLL method with many cells outside of the desired patterning areas. In Fig. 3g, the magnified regions exhibit normal cell attachment and proliferation at 1 hour and 24 hours, respectively, after LCL. Since there is undissolved PVA residue in the background, its anti-adhesion property helps to form sharp and well-defined pattern edges as well as clean un-patterned surroundings. The cells can keep growing

for days within the defined areas until the PVA dissolves completely, after which patterned cells gradually spread out of the pattern boundaries (Fig. S5).

In conclusion, our LCL technique can produce high-throughput and high-efficiency cell patterning across a 1.2 x 1.2 cm^2 area. A bilayer stack of SU-8 and PVA is used as a sacrificial layer to remove cells deposited at un-patterned locations to realize cell patterning with a low background defect rate. The whole process is biocompatible and easy-to-fabricate. Using LCL, over 70% cell patterning efficiency with a nearly zero background defect rate has been achieved for both adherent and non-adherent cells. This approach can also be applied for patterning cells into arbitrary shapes with clean surroundings on large scales. Therefore, the LCL technique has potential for wide use in cell biology and related fields.

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Conflicts of interest

There are no conflicts of interest to declare.

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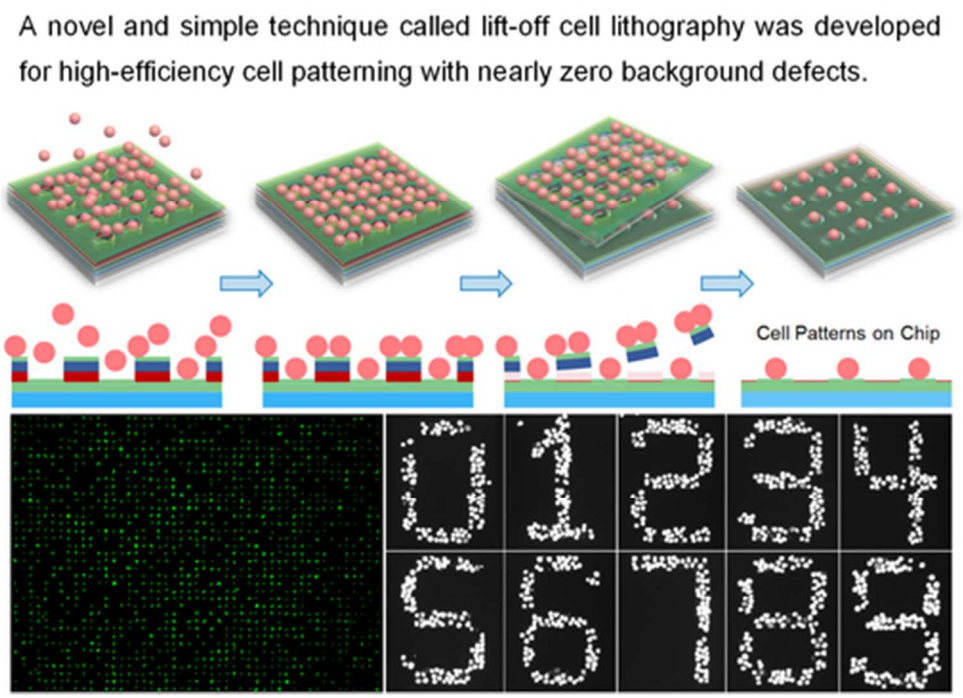

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