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Nanoliter Liquid Patterning in Aqueous Environments for Spatially-Defined Reagent Delivery to Mammalian Cells

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Abstract

Microscale biopatterning allows regulation of cell-material interactions^{1,2} and cell shape³, and enables multiplexed high throughput studies^{4,5,6,7,8} in a cell and reagent efficient manner. The majority of available techniques rely on physical contact of a stamp³, pin⁸, or mask^{9,10} with mainly a dry surface. Inkjet and piezoelectric printing¹¹ is performed in a non-contact manner but still requires a substantially dry substrate to ensure fidelity of printed patterns. These existing methods, therefore, are limited for patterning onto delicate surfaces of living cells because physical contact or substantially dry conditions are damaging to them. Microfluidic patterning with laminar streams^{12,13} does allow non-contact patterning in fully aqueous environments but with limited throughput and reagent diffusion across interfacial flows. Here, we describe a polymeric aqueous two-phase system (ATPS) that enables patterning nanoliters of a reagent-containing aqueous phase, in arbitrary shapes, within a second aqueous phase covering a cell monolayer. With the appropriate media formulation, reagents of interest remain confined to the patterned phase without significant diffusion. The fully aqueous environment ensures high reagent activity and cell viability. Utility of this strategy is demonstrated with patterned delivery of genetic materials to mammalian cells for phenotypic screening of gene expression and gene silencing.

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Author Contributions

H.T. designed and performed experiments, analyzed the data, and wrote the manuscript. A.J. helped optimize transfection experiments. B.M. helped with patterning experiments and imaging microarrays. Q.Y.L. performed image processing of transfected cell microarrays. X.L. prepared collagen and helped with collagen degradation assay. K.E.L. prepared lentiviruses. G.D.L. helped design viral transduction experiments and optimize them. S.J.W. helped design collagen degradation assay. S.T. designed the project and edited the manuscript. All authors commented on the manuscript.

Competing interests statement

The authors declare no competing financial interests.

We selected polyethylene glycol (PEG) and dextran (DEX) as the phase-forming polymers because, first, these polymers form stable ATPSs in a wide range of temperatures¹⁴. This enhances convenience and stability of experiments because the phases maintain segregation under refrigeration at low temperatures as well as during incubation at higher temperatures. Second, high molecular weights of PEG and DEX form ATPSs at low polymer concentrations and ensure that the bulks of both phases remain highly aqueous and non-toxic to cells (Supplementary Fig. 1). Third, due to density differences, DEX always forms the bottom phase and PEG the top phase of the two-phase system¹⁴ (Supplementary Fig. 2).

To generate user-defined shapes of the reagent phase, we load a pipette tip with the DEX phase and lower it into the PEG phase in close proximity (typically <500 μm) to the cell monolayer. Moving the pipette tip horizontally results in the formation of a continuous pattern of the dispensing DEX phase on cells (Fig. 1a). This is demonstrated by patterning “UMICH” on a monolayer of HEK293H cells (Fig. 1b). The resolution of patterns can significantly be improved by using dispensing mechanisms with finer tips (Supplementary Fig. 3). Other shapes, such as triangles and squares, can also be created; the patterns are quite stable over long incubation time periods and are not disturbed by carefully moving the cell culture system (Supplementary Fig. 4). The key to the stability of patterns is an extremely low interfacial energy between the two immiscible phases ($\gamma_{12} \sim 0.003 \text{ mJ/m}^2$)¹⁴ and roughness of the cell monolayer surface and associated cell surface-DEX phase interactions (Supplementary Fig. 5). Thermodynamically speaking, cell monolayer surface gives rise to free energy barriers that prevent the PEG-DEX interfacial tension, γ_{12} , from retracting the three-phase contact line (PEG-DEX-cell surface) of the patterns to a lower energetic state¹⁵, and thus, patterns retain their shapes.

Following, we show utility of this patterning technology for microarray format multiplexed cell-based studies of gene expression and gene silencing. First, we studied partitioning of cell transfection materials in the PEG-DEX ATPS. Complexes of a lipid transfection reagent, Lipofectamine 2000, and 50 nM Alexa fluor-labeled RNA were prepared and suspended in the DEX phase. A droplet of this solution was dispensed into a bath of the PEG phase and imaged every 15 min. The results show that over a period of 4hrs, the fluorescent signal from transfection materials remains quite confined to the DEX droplet (Fig. 1c). To form a microarray, an ATPS is prepared using cell culture media as the solvent. The DEX phase containing the reagent is transferred into the wells of a 1536-well plate. An array of slot pins resting on a commercially-available fixture is dipped into the wells to load. The pins are then lowered close to the cell monolayer in culture in the PEG phase. The DEX phase dispenses and forms droplets over distinct populations of cells (Fig. 2a). This is demonstrated by a microarray of 96 droplets of FITC-labeled DEX solution formed within a lawn of PEG solution covering HEK293H cells (Fig. 2b).

We examined the efficacy of this strategy for patterned transfection of mammalian cells. Liposomal complexes of an expression construct for eGFP were suspended in the DEX phase and arrayed on discrete groups of HEK293H cells. Imaging cells with a fluorescence microscope 48hrs post-transfection yielded an array of 24 spatially distinct cell clusters expressing eGFP in a lawn of non-transfected cells (Fig. 2c,d). We tested different ratios of the plasmid DNA to the transfection reagent and found that a 1/1 ratio yields optimum eGFP

expression (Fig. 2e). The level of protein expression at various concentrations of plasmid DNA was also examined. Similar to conventional well-based transfections, fluorescent signal intensity proportionally increased with the concentration of the plasmid up to a certain point and saturated thereafter (Fig. 2f). The post-transfection cellular viability was also similar to conventional methods (Supplementary Fig. 6). Next, we showed the possibility of patterning more than one reagent on a single monolayer of cells. Transfection of HEK293H cells with plasmid DNAs for eGFP and dsRed resulted in spatially-distinct groups of cells fluorescing green and red, respectively (Fig. 2g). Due to the fundamental importance of simultaneous expression of multiple genes in a cell to many biological events¹⁶, we induced cells in the same assay to coexpress eGFP and dsRed proteins as well (yellow spots in Fig. 2g).

Each cluster of transfected cells in the above microarrays was exposed to a 500 nl droplet containing only ~10 ng plasmid, which is significantly less than that typically used in microwell-based transfections and is similar to reverse transfection protocols⁸. Choosing dispensing pins with smaller volumes will reduce the size of fluorescent spots and the amount of plasmid. For example, 20 nl pins give spots of ~340 μm . Since the diameter of the DEX droplet approximately varies linearly with the square root of the drop volume, the area of transfected cell clusters can be pre-determined from the pin volume for the range of volumes studied (Fig. 2h). In principle, the resolution of circular patterns can be enhanced by using smaller volumes of the DEX phase or an ATPS containing higher concentrations of phase-forming polymers (Supplementary Fig. 7,8).

Lipid-mediated transfection is a straightforward method to induce transient effects of gene overexpression/knockdown in cells. However, many cell lines and primary cells do not transfect efficiently with lipofection and require infection with viral vectors containing cDNA- or short hairpin RNA (shRNA)-expressing cassettes. We showed that the two-phase patterning approach facilitates lentiviral-mediated transduction of MDA-MB-231 human breast cancer cells. Virus particles encoding eGFP remained confined to patterned DEX droplets and resulted in the localized infection of subpopulations of cells (Fig. 3a,b). The titer of the lentiviral solution in the DEX phase was 1.3×10^7 infectious units (IFU)/ml that corresponded to 10 viral particles per cell. We prepared serial dilutions of the eGFP-encoding lentivirus and arrayed them on the MDA-MB-231 cells at 4, 2, and 1 viral particles per each cell. Subsequent analysis of fluorescent intensity of infected cells showed that similar to conventional well-based lentiviral infections, the level of eGFP expression increases proportionally with the amount of lentivirus in the patterned DEX phase (Fig. 3c). Importantly, the lentiviral solution titer for efficient two-phase patterned infection of cells is 2–3 orders of magnitude less than that required with the reverse transfection approach where the viruses are printed onto a solid substrate and subsequently overlaid with cells¹⁷. This is a major advantage that significantly reduces toxicity to cells and eliminates the need for hard-to-obtain highly concentrated viral solutions.

Next we demonstrated the utility of the two-phase patterning for lentiviral-mediated RNA interference (RNAi)¹⁸. MDA-MB-231 cells permanently expressing CXCR4-eGFP (target gene) and mPlum (red reporter gene) constructs were seeded at a density of 37500 cells/cm². Lentiviruses encoding a shRNA that specifically targets eGFP mRNA were resuspended in

the DEX phase and patterned on the cells. Analysis of cells 72hrs post-infection showed a significant reduction in eGFP expression levels only within targeted cell clusters (Fig. 3d). The fact that cells within the spots actively express the reporter red protein ensures the specificity of the shRNA for the target gene (Fig. 3e).

Cells inside the body reside in a tissue specific environment where cell-extracellular matrix (ECM) interactions are a major regulator of cellular behavior¹⁹. Therefore, screening functions of genes in cells cultured on ECM substrates such as soft proteinoous gels, rather than solid substrates, may elicit more physiological cellular responses^{20,21}. Direct patterning of reagents on soft aqueous gels is another area where conventional contact-mediated patterning methods would face challenges but the non-contact, aqueous nature of the ATPS patterning technique should be ideal. To establish this point, we showed patterned degradation of collagen I fibrils by matrix metalloproteinase (MMP)-expressing cells. Degradation of collagen is implicated in the physiological remodeling of connective tissue during growth and development²² as well as in cancer invasion and metastasis^{23,24} where malignant cells cleave their subjacent matrix proteins and initiate invasiveness. We cultured HEK293H cells on Alexa fluor-labeled collagen I substrates and patterned transfection complexes of full-length membrane-type1 matrix metalloproteinase (MT1-MMP) and MMP2 cDNAs and eGFP plasmid DNA as a control. Imaging 72hrs post-transfection showed degradation of type I collagen only by MT1-MMP-expressing cells (Fig. 4a,b). The loss of collagen appears as black pits under the fluorescent light (Fig. 4b) and immunostaining of cells with anti-HA.11 antibody shows that degradation of the matrix correlates well with the expression of this epitope-tagged MT1-MMP protein (Fig. 4c). This observation is consistent with recent findings that show the pro-form of MT1-MMP undergoes intracellular processing to its active form prior to its display on the cell surface and highlight MT1-MMP as the major regulator of the collagenolytic activity of normal and neoplastic cells^{24,25,26}. On the other hand, the MMP-2 zymogen is unable to degrade type I collagen directly under these conditions. Two-phase reagent micropatterning on soft substrates enabled detection of the mechanistic role of MT1-MMP in ECM invasion. This type of strategy may generally be useful to study how expression or knockdown of different genes affects proteolytic activities of cells during events such as tissue remodeling and cancer metastasis.

In summary, we described a new approach for spatially-defined delivery and retention of nanoliters of biological reagents over living cells. We demonstrated broad utility of this strategy with patterned gene expression/knockdown in mammalian cells cultured on soft and solid substrates using both lipid- and lentiviral-mediated gene delivery techniques. The method enables delivery of reagents to cells in arbitrary geometrical shapes as well as in standard high-throughput 1536 array formats using only nanoliters of reagents. Although reagents of interest are confined over cells within only nanoliter volumes of the reagent phase –a volume which would typically compromise cell viability over substantial period of culture– the reagent-excluding phase is also aqueous and abundant in nutrients supporting high cellular viability. With slight media adjustments, patterning other reagents including proteins, antibodies, and small molecule drugs on cells is also envisioned. This reagent patterning strategy is straightforward to implement, economically sound requiring only off-

the-shelf equipment, and conveniently accessible to researchers without a need for fabrication expertise or complicated equipment.

Methods

Cell culture

HEK293H cell line was obtained from Invitrogen (11913-019). Cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco) at 37°C in a humid incubator with 5% CO₂ and passaged every 3–4 days. For transfection experiments, we used cells with passage number between 5 and 30. MDA-MB-231 breast cancer cell line was obtained from ATCC (HTB-26) and maintained in DMEM supplemented with 10% heat-inactivated FBS (Hyclone), 1% glutamax (Gibco), and 0.5% penicillin/streptomycin (Gibco). MDA-MB-231 cells stably expressing CXCR4-eGFP were previously described²⁷.

Plasmids and lentiviral vectors

eGFP (PT3148-5) and dsRed (6924-1) plasmid DNAs were obtained from Clontech. mPlum plasmid DNA was provided by R.Y. Tsien and described before²⁸. Full-length MT1-MMP cDNA was prepared as described before²⁵. Full-length MMP2 cDNA was obtained from Origene (SC117323). The lentiviral vector for eGFP (pSico) has been described before²⁹. The eGFP shRNA Control Vector (SHC005) was obtained from Sigma. Lentiviruses were generated and titered according to the protocol described previously³⁰.

Microarray formation

Solutions of 4% (w/w) PEG (Mw:8000, Sigma) and 8% (w/w) DEX (Mw:500000, Pharmacosmos) were prepared in Optimem (Gibco) and mixed. After adding 5 mM KH₂PO₄ salt to adjust the media composition, the mixture was shaken thoroughly and kept at 4°C overnight to equilibrate and form an ATPS. The two phases were carefully separated and centrifuged at 3500 rpm for 45 min to remove counter polymers excessively dissolved in each phase. The stock solutions of PEG and DEX phases were stored at 4°C.

Dilutions of 2.5 µg plasmid DNA in 12.5 µl Optimem and 2.5 µl Lipofectamine 2000 (Invitrogen) in 12.5 µl Optimem were prepared and incubated for 5 min at room temperature. The solutions were mixed and incubated for 5–20 min at room temperature. The resulting solution was mixed with 90 µl of the DEX phase stock solution, incubated for 5 min at room temperature, and transferred to a 1536-well plate (Corning).

Prior to experiments, slot pins (V&P Scientific, FP3S500H) were mounted on a pin tool fixture (V&P Scientific, AFIX1536FP3), which itself was assembled with a micromanipulator. Pins were dipped into a cleaning solution (V&P Scientific, VP110), DI water, and isopropyl alcohol, as indicated by the manufacturer. Clean pins were dipped three times into the wells and filled with the solution containing transfection complexes. Final retraction of pins from the solution was done slowly to minimize residue on the outer surface of the pins. Next, pins were lowered into the close vicinity of the cell monolayer covered with the PEG-Optimem solution and were allowed to dispense the transfection

complexes-containing DEX-Optimem solution. After formation of droplets on cells, pins were slowly retracted and moved out of the culture dish.

For viral transduction experiments, solutions of 4% (w/w) PEG and 15% (w/w) DEX were prepared separately in MDA-MB-231 culture medium. 40 μ l lentiviral solution was suspended in 20 μ l of the DEX solution to a final titer of 1.3×10^7 (IFU)/ml. To enhance viral infection of cells, a cationic agent, polybrene (Sigma), was added to the resulting solution at a final concentration of 10 μ g/ml. Droplets of this solution were arrayed on cells as described above.

Preparation and labeling of collagen substrates

Type I collagen was prepared from rat tail tendons and dissolved in 0.2% acetic acid to a final concentration of 2.7 mg/ml. To induce gelling, collagen was mixed with 10 $\times$ MEM (Gibco) and 0.34 N NaOH in an 8:1:1 ratio at 4°C and 2 ml of this mixture was added to each chamber of a 2-well chambered slide (VWR). To obtain a thin film, collagen was immediately removed and the slide was kept at 37°C for 45 min to allow gelling to complete. The collagen film was then labeled with Alexa fluor 594 carboxylic acid, succinimidyl ester (Molecular probes) for 1h at room temperature. After removing the dye, the film was incubated three times with PBS PH7.4 (Gibco) at room temperature for a total of 30 min. Then, 2 ml PBS was added to each chamber and the slide was wrapped in aluminum foil and stored at 4°C.

Immunostaining

Anti-HA.11 mAb was used to detect epitope-tagged MT1-MMP protein expression in transfected HEK293H cells. After fixing in -20°C methanol for 6 min, cells were washed three times with PBS and twice with PBS containing 5% BSA. The mouse anti-HA.11 mAb (Covance, MMS-101P) in PBS/5% BSA at a concentration of 1 μ g/ml was added to fixed cells for 1h. After washing, the primary antibody was visualized with fluorescently labeled goat anti-mouse IgG (Molecular Probes, A-11001) at a concentration of 1 μ g/ml.

Imaging and fluorescence microscopy

We imaged transfected microarrays section by section using an inverted fluorescence microscope (Nikon, TE300). After removing the background intensity of images and uniformly enhancing brightness and contrast in Matlab R2007a (MathWorks), images were pseudocolored, merged, and superimposed in Photoshop 10.0 CS3 (Adobe). We used SimplePCI (Compix) for fluorescence intensity measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Polymeric aqueous two-phase systems generate user-defined patterns of a reagent on a cell monolayer

(a) Schematic representation of patterning aqueous DEX phase (blue) on a cell monolayer covered with the PEG phase (pink).
 (b) Bright-field and fluorescent images of patterned DEX phase on HEK293H cells spelling “UMICH”. The shapes were generated by continuous horizontal movement of a pipette tip filled with FITC-labeled DEX solution over cells covered with the PEG phase.
 (c) Complexes of genetic materials and the transfection reagent, Lipofectamine 2000, partition well to the DEX phase and remain within the dispensed drop over a 4hrs imaging period. Scale bar, 1 mm in a,b, 500 μ m in c.

Figure 2. Addressable delivery of nucleic acids to cells using two-phase patterned microarrays
 (a) Cells cultured to desired confluence are covered with the PEG phase (pink). Slot pins filled with transfection complexes suspended in the DEX phase (blue) are slowly lowered into this solution using a micromanipulator that controls the vertical motion. Pins dispense their contents onto the cell monolayer and small droplets of transfection complexes form on discrete groups of cells.
 (b) Fluorescent micrograph of a 12×8 microarray of FITC-DEX solution droplets patterned on a cell monolayer covered with PEG solution. All droplets were formed in one printing step using 500 nl dispensing slot pins that rest on a 1536-well plate format fixture.
 (c) Only cells confined to the DEX droplets become transfected and exhibit the corresponding phenotype.
 (d) Fluorescent micrograph of a 6×4 microarray of HEK293H cell clusters expressing eGFP.

(e) The highest eGFP transfection efficiency as measured by fluorescence intensity in the cell clusters is obtained using a complex preparation with a 1/1 ($\mu\text{g}/\mu\text{l}$) ratio of plasmid DNA/transfection reagent. Each bar represents the mean value of five sets of data and the error bars represent $\pm\text{SD}$.

(f) The level of protein expression in the microarray of transfected cells initially increases with the amount of plasmid DNA and then levels off. Each bar is the mean value of five different data sets and the error bars represent $\pm\text{SD}$.

(g) Fluorescent micrograph of arrays of cells transfected with plasmid DNAs for eGFP, dsRed, or both. Green and red cell clusters correspond to the eGFP and dsRed transfected cells, respectively. Co-transfected cells are shown in yellow and are obtained by superimposition of green and red fluorescent signals.

(h) Assuming that each droplet is a spherical cap, the drop volume follows a quadratic relation with the contact diameter of the drop ($V \propto D^2$). Therefore, the contact diameter of DEX drops and hence the size of transfected cell clusters approximately change linearly with the square root of the drop volume according to the following relation that represents a trend line obtained from experimental diameter and volume data: $D = 43.2V^{0.5} + 211.2$. The goodness of fit, R^2 , is 0.992. This is useful to pre-determine the number of treated cells from the dispensing pin volume within the drop volume range 20–1000 nl. Scale bar, 700 μm in b,d,g,h.

Figure 3. Patterned microarrays of lentiviral-mediated gene expression and gene knockdown
 (a) Fluorescent image of a 3×4 array of MDA-MB-231 human breast cancer cells transduced with a lentiviral vector containing eGFP gene. The infected cells are well-contained to patterns of DEX droplets during transduction process.
 (b) A magnified view of the boxed spot in (a).
 (c) Dose-dependent infection of cells with lentiviral vectors. Increasing the number of lentiviruses from 1 to 10 per each cell results in increase in the intensity of the eGFP signal.
 (d) A 3×4 microarray of localized eGFP gene knockdown obtained with patterned infection of cells with lentiviruses encoding eGFP shRNA.
 (e) A 3×4 microarray of localized eGFP gene knockdown obtained with patterned infection of cells with lentiviruses encoding eGFP shRNA.

(e) Cells in the spots express similar levels of the mPlum (red) gene compared to cells outside the spots. This confirms that silencing of the eGFP gene is due to the specificity of the eGFP shRNA and not due to interference of shRNA with gene expression. Scale bar, 500 μm in a,c,d,e.

Figure 4. Patterned microarrays facilitate phenotypic screening of function of different genes in cell cultures on soft substrates

- (a) Schematic representation of localized degradation of collagen I only by MT1-MMP-expressing cells. Cells expressing MMP2 or control eGFP are incapable of collagenolysis.
- (b) Fluorescent micrographs of HEK293H cells cultured on Alexa fluor 594-labeled collagen I and transfected with expression constructs for MT1-MMP (bottom row), MMP2 (middle row), and eGFP (top row). MT1-MMP expressing cells showed collagenolytic activity and degraded their subjacent ECM whereas cells expressing MMP2 pro-enzyme or eGFP lacked this invasive phenotype.
- (c) Fluorescent micrograph of cells stained with antibody for MT1-MMP (green) grown on fluorescently labeled collagen (red). MT1-MMP protein expression correlates with the degradation of collagen. Scale bar, 700 μm in b,c.