Comparative study and improvement of current cell micro-patterning techniques†‡

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The original micropatterning technique on gold, although very efficient, is not accessible to most biology labs and is not compatible with their techniques for image acquisition. Other solutions have been developed on silanized glass coverslips. These methods are still hardly accessible to biology labs and do not provide sufficient reproducibility to become incorporated in routine biological protocols. Here, we analyzed cell behavior on micro-patterns produced by various alternative techniques. Distinct cell types displayed different behavior on micropatterns, while some were easily constrained by the patterns others escaped or ripped off the patterned adhesion molecules. We report methods to overcome some of these limitations on glass coverslips and on plastic dishes which are compatible with our experimental biological applications. Finally, we present a new method based on UV crosslinking of adhesion proteins with benzophenone to easily and rapidly produce highly reproducible micropatterns without the use of a microfabricated elastomeric stamp.

Introduction

Micro-fabrication techniques, which have a now long and successful history in biology,[1,2] have often been restricted to bio-engineering. More recently, their applications to cell biology have been developing fast and are getting ever more relevant for basic and applied research in that field.[3,4] One of these techniques, called micro-patterning, allows the control of cell adhesion geometry on a surface, and recently proved an inspiring technique for several questions in cell biology. It has a wide range of application, from size control of single focal adhesion at the micrometre scale,[5] to the confinement of groups of cells coupled with reverse transfection for large scale screens.[6] Confinement of single cells allowed important biological findings, in the fields of apoptosis,[7] control of cell–cell architecture,[8] cell internal organization[9] and division axis.[10] It should also be instrumental in the development of cell based screening strategies like HCS (high content screening).

Many technical papers have been published in the past few years, proposing various protocols for fabrication of adhesive micro-patterns on different substrates and using a variety of methodologies (see references in the Results section). Most of these studies are performed by bioengineering teams which have both expertise and tools that are often hardly accessible to biologists. Current efforts are made to transfer the original microcontact printing (μCP) technique on gold[11] to classical substrates used in cell biology studies (plastic[12] and glass[13]). When non-specialists engage in developing such technologies in their own lab, they have to face several problems often skipped in technical papers. As there is no universal solution to produce micro-patterns, one has to find a compromise between ease, reproducibility and quality of patterning, together with good optical quality of the substrate. Moreover, each cell type or culture conditions will impose specific constraints. We faced and successfully overcame the challenge of adapting micro-patterning techniques in a lab dedicated to cell biology. Here we review the various techniques we assayed with their respective advantages and pitfalls, then expose the solutions we finally chose with, three criteria in mind:

– Fabrication should be as easy as possible for a biology lab without external help.

– Quality should be highly reproducible.

– Techniques should be compatible with as many of our experimental systems as possible.

In particular, our lab is interested in confining single cells, which often proved more discriminating on long term experiments than confining groups of cells.

We will also present a few techniques which have a narrower range of applications but have special advantages, like ease of fabrication. Last, we will present a new method of fabrication based on UV based patterning of proteins which has two main advantages: it requires no special tool that a biology lab could not afford, and it does not involve micro-contact printing which is responsible for most of the lack of reproducibility. This contribution is thus a bench-test, done by biologists in a biology lab, of the various available techniques for micro-patterning. It goes further and proposes a few optimized

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solutions compatible with our experimental systems and hopefully also with many others.

Materials and methods

μCP (micro-contact printing)

The μCP process is applied to directly stamp adhesion proteins onto cell culture substrates, as introduced by Bernard *et al.*[12] Non-printed areas are rendered non-adhesive afterwards.

Photomask manufacturing and stamps fabrication

Stamps were made as previously described.[8] Molds for the stamps were produced with the usual UV lithography technique by illuminating a positive photoresist through a chrome photomask on which micropatterns were designed with an electron beam. PDMS (Sylgard 184 kit, Dow Corning) was finally cast on the resist mold using a 10 : 1 ratio (w/w) of elastomer to hardener and cured over night at 60 °C. The 4 mm-thick cross-linked PDMS layer was peeled-off and stamps were manually cut out of it.

We want to stress that biology labs that do not have access to a micro-fabrication facility can very easily obtain commercial photomasks (Deltamask, Enschede, Netherlands; Advance Reproductions, North Andover, MA, USA; Microtronics, Newtown, PA, USA) and even directly buy customized molds for stamps (Biotray, Lyon, France) on a unit per unit basis at reasonable cost and delivery time.

The design of the patterns is usually made with specialized softwares (*e.g.* L-Edit or Clewin) that produce file formats directly compatible with the manufacturer's machines, but it can also be made with any drawing software, when all the necessary information for size of patterns and arrays are indicated. There will just be an extra cost for the format transfer.

μCP with PEG-malemide backfill

Glass coverslips were first washed in methanol–chloroform (50/50) during 24 h and stored in pure ethanol. After drying (15 min at 60 °C) coverslips were activated in a plasma chamber (Harrick Plasma, Ithaca, NY, USA) during 3 minutes under a weak flow of air. They were then incubated in a closed reactor containing the silanisation mix in methanol, deionised water 4.5%, acetic acid 0.9%, 3 mercapto-propyltrimethoxy-silane (S10475, Fluorochem) 2.5%, overnight at 4 °C. Finally, coverslips were washed twice in methanol and dried under filtered air followed by 15 min at 60 °C.

The PDMS stamp was activated in the plasma chamber during 10 s under a weak flow of air and inked with a 50 μg ml^{-1} fibronectin solution (Sigma–Aldrich) 10% of which was labelled with Cy3 (Amersham Biosciences, Orsay, France) for 10 min. After aspiration of the fibronectin solution, the stamp was dried with filtered air flow and placed in contact with the silanised coverslip for 5 min. After removal of the stamp the printed coverslip was immersed in a 20 mg ml^{-1} solution of poly(ethyleneglycol)-maleimide (2D2MOH01, Nektar Therapeutics, Huntsville, Alabama, USA) for 1 h at room temperature. The coverslip was then washed in PBS before cell deposition.

μCP with PLL-g-PEG backfill

PDMS stamps were sonicated for 5 min in ethanol. After drying under a hood, they were inked with a fibronectin–collagen solution (50 μg ml^{-1} each in H_2O). 10% of the fibronectin was labelled with Cy3 (Amersham Biosciences). The stamps were incubated for 45 min in the dark. For stamping, the protein solution was removed with a pipette until the stamp surface looked completely dry. The stamps were immediately placed in contact with the substrate and pressed slightly with tweezers for several seconds. After 5 min the stamps were removed, the substrate was washed once with water and incubated in 10 mM HEPES, pH 7.4 containing 0.1 mg ml^{-1} PLL(20)-g[3.5]-PEG(2) (SurfaceSolutionS, Switzerland) for 1 h. The substrate was then rinsed twice with PBS and used for experiments. The stamps were cleaned for one hour in water, dried in EtOH and reused several times.

We tested three different substrates: normal glass coverslips (Marienfeld), tissue culture polystyrene (TCPS) dishes (Falcon and Iwaki) and culture treated Ibidi plastic μ-dishes (Ibidi, Germany, the chemical structure of the plastic is not disclosed by the manufacturer, simple coverslips made of the same plastic can also be purchased). The glass coverslips were sonicated 5 min in EtOH before use. TCPS was used as received without further cleaning. The ibidi plastic dishes were activated directly before use for 30 s in a plasma chamber (Harrick Plasma Ithaca, NY, USA) at maximum intensity under a weak flow of air.

Surprisingly cells weakly attached to the passivated areas of all types of plastic after seeding, but spread only on the printed patterns. However they could not adhere at all to passivated glass.

Non-spread cells could be easily flushed away with a pipette. Otherwise they detached over night.

For unknown reasons passivation did not work on Iwaki glass bottom dishes (commonly used for videomicroscopy).

μCP on polystyrene (PS)

Stamp preparation and printing onto PS dishes (Greiner) were done as described in μCP with PLL-g-PEG backfill, but no PLL-g-PEG backfill was performed. Patterned surfaces were stored in PBS until cell seeding.

Pattern production with UV exposure

18 mm round glass coverslips were uniformly coated with PLL-g-PEG according to manufacturer protocols. The grafting solution was a mixture of the photosensitizer (benzophenone or (4-benzoylbenzyl)trimethylammonium chloride, MW = 289.9 g mol^{-1}, synthesized by LaboTest and available upon request at Sigma–Aldrich, Germany) at 50 mM in PBS, fibronectin (Sigma-Aldrich) at 100 μg ml^{-1} and fibronectin–Cy3 (labelled with the Cy3 labelling kit from Amersham Biosciences) at 6 μg ml^{-1}. A 5 μL droplet of the grafting mixture was deposed on a chromium photomask (homemade but commercially available) on which the patterns to be grafted were transparent. A PLL-g-PEG coated glass coverslip was placed on the grafting solution. UV light was produced by a 400 W UV lamp (Delolux 03 S, Supratec, Bondoufle, France)

mounted with a 310–460 nm filter. The coverslip was placed at 20 cm from the lamp, exposed to UV through the photomask for 60 s and washed in distilled water.

Cell culture

HeLa cells, HeLa-Centrin1-GFP cells (human adenocarcinoma epithelial cells, ATCC-No CCL-2, stably expressing Centrin1-GFP)[14] and Src++ cells[15] (embryonic mouse fibroblasts, ATCC-No CRL-2497) were maintained at 37 °C in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; biowest), 2 mM glutamine (Gibco) and antibiotics (penicilin and streptomycin, PeSt; Gibco) in a humidified atmosphere containing 5% CO_2.

hTERT-RPE1 cells (infinity telomerase-immortalized retinal pigment epithelial human cells, ATCC-No CRL-4000) were cultured at 37 °C in DMEM F-12 (Gibco) supplemented with 10% FCS, 2 mM glutamine and antibiotics (PeSt) in a humidified atmosphere containing 5% CO_2.

Cells were dissociated with PBS containing 0.02% EDTA at 37 °C for 15 min. After centrifugation, cells were resuspended in DMEM for HeLa and Src++ cells or DMEM F-12 for RPE1, supplemented with either 1 or 10% FCS. Cells were seeded on micropatterned surfaces at a density of 1.5×10^4 cells cm^{-2} and were allowed to attach. Unspread and floating cells were removed by extensively washing the surface and gentle flushing. While washing, the substrate surface, often hydrophobic due to passivation, was carefully prevented from de-wetting.

Note that there are many different Hela cell lines. Hela from ATCC and from our lab worked fine, but certain lines from other labs (for example cells used in Walther *et al.*)[16] were not able to spread on fibronectin–collagen patterns.

Microscopy

We recorded cell behaviour on patterns using a 10× objective on an inverted IX71 Olympus microscope or a 20× objective on an Axiovert 200 M Zeiss microscope with a Coolsnap HQ Roper camera and a temperature control chamber from Life Imaging Services (The Cube) and a CO_2/hygrometry control from either Oko Lab or LIS (The Brick). The acquisition was controlled by Metamorph (Universal Imaging). For live imaging of centrioles with centrin-GFP, a 40× oil-immersion objective was used, together with a piezo device (Piezosystem Jena from National Instruments) to record Z-stacks, and Uniblitz shutters.

Results and discussion

Originally, μCP was used to print self-assembled monolayers of alkanthiolates on gold surfaces.[11] Although this technique works very efficiently, it is expensive, not accessible to most of the biology labs and not optimal for videomicroscopy. Therefore use of μCP was soon extended to the stamping of proteins and peptides on a variety of different substrates, such as glass, polystyrene (PS) and tissue culture polystyrene (TCPS). Besides choosing the appropriate substrate and adhesion molecules for printing, the passivation of the non printed areas is crucial to avoid cell spreading outside the patterns. Here we tested several of the already published techniques (see below) on several substrates with three different cell lines: HeLa cells, RPE1 cells and mouse embryonic fibroblasts.

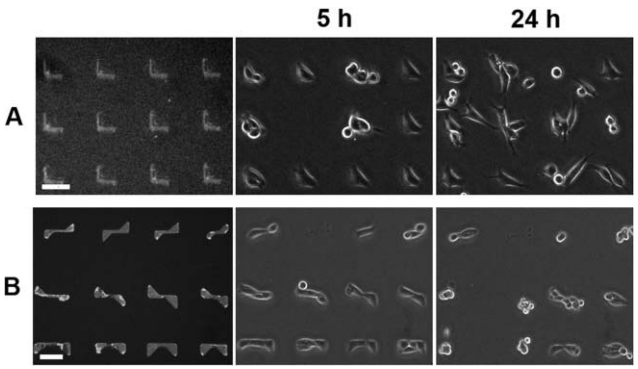

Fig. 1 Undesired behavior of cells on micropatterns. Fluorescent images of the micropatterns (fibronectin–Cy3, left panels) and cell behavior after 5 and 24 h (phase contrast) are shown. (A) HeLa cells on L shapes. Cells were restrained to the patterns after 5 h, but had escaped after 24 h. (B) HeLa cells on dumbbell shapes. Cells were nicely spread in the beginning (5 h) but had rounded up after 24 h. Finally they detached. Scale bars represent 40 μm.

In our experiments we were generally facing two major problems: firstly, cells could escape from the patterns and spread over the passivated areas (Fig. 1A, 24 h). Secondly, cells could round up and detach from the patterns (Fig. 1B, 24 h). This behaviour could be explained by the observation that the patterned proteins were peeled off by the cells (Fig. 2, see below) resulting in pattern degradation. Throughout this paper we assessed qualitatively the stability of passivation and the stability of printed areas upon cell adhesion by observation of cell behaviour. Passivation stability was judged by the ability of cells to escape from the patterns and spread inside the passivated areas. Stability of printed areas was evaluated by the occurrence of cells rounding up and detaching from the patterns.

The tested cell types varied from each other in terms of their ability to escape from the patterns and to rip off the patterned proteins. The Src++ mouse fibroblasts showed the highest potential to escape and to tear off the proteins, followed by RPE1 cells. HeLa cells were the easiest to work with. Therefore appropriate solutions had to be found for the different cell types and cell biological applications.

Passivation by functionalized PEG on silanized glass

We silanized glass with mercaptosilane, printed fibronectin and backfilled the non-patterned areas by covalently coupling a PEG-malemide to the silane.[17] Unfortunately passivation efficiency was not very reproducible, probably due to the instability of the maleimide function and the quality of silanization. Often cells escaped overnight (Fig. 1A). Compared to HeLa and RPE1 cells, the fibroblasts were always the first to leave the patterns after a few hours, probably due to their high motility. HeLa cells on single cell sized patterns stayed until having divided. When the passivation procedure was successful, this method provided very high quality patterns. It unfortunately had too many drawbacks:

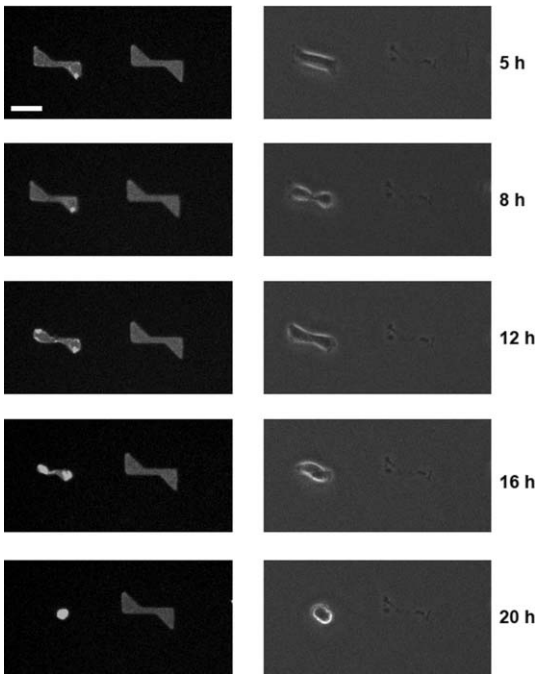

Fig. 2 Pattern degradation on glass. Fibronectin–collagen patterns were printed on untreated glass. After PLL-g-PEG backfill, HeLa cells were seeded on the patterns in medium containing 10% FCS and allowed to spread. Pictures of the fluorescent micropatterns (fibronectin–Cy3, left column) and cell behavior (phase contrast, right column) were taken at different times as indicated. Whereas the right pattern, on which no cell was attached, was not degraded, the left pattern was peeled off by the cells. After 20 h only a little spot of fibronectin remained. This spot was located where retracted cells were still adhering to the glass. The scale represents 40 μm.

the technique included several time consuming steps and required specialized equipment for activation of the glass by plasma treatment. Additionally, the PEG maleimide is expensive and sensitive to air and humidity which makes it difficult to handle and thus a source of variability in pattern quality.

Passivation by PLL-g-PEG on glass

This technique was first introduced by Csucs *et al.*[18,19] Passivation is based on backfilling of the non printed areas by a polycationic graft polymer, poly-L-lysine-g-poly(ethylene glycol) (PLL-g-PEG). PLL-g-PEG has been shown to adsorb rapidly and strongly through electrostatic interactions to negatively charged surfaces, rendering them highly protein and cell resistant.[20] PLL-g-PEG can be stored in aqueous solution at 4 °C for months without loss of passivation capacity. The backfill simply consists of incubating the substrates (untreated glass or tissue culture activated polystyrene) in the PLL-g-PEG solution (0.1 mg ml^{-1} in HEPES 10 mM pH 7.4). Furthermore, this method is cost effective, since only low concentrations of this polymer are needed.

We printed a mixture of fibronectin–collagen on untreated glass followed by PLL-g-PEG backfill. Cells could not adhere or spread outside the printed areas and spread nicely on the patterns. They were, nevertheless, rounding up after a few hours (Fig. 1B) and were finally detaching from the patterns. This behaviour was not due to cell death since these detached cells were able to respread on neighbouring patterns on which no other cells had spread before (see movie S1 in ESI‡). Additionally, cell toxicity studies indicated that the PLL-g-PEG did not lead to enhanced cell death of fibroblasts cells, even after 75 min incubation of cells with a 0.2% solution of the polymer.[20] The observed cell rounding was rather due to cells tearing off the patterned proteins as shown by fluorescent imaging of fibronectin–Cy3 over 20 hours using HeLa cells (Fig. 2). This effect was not linked to general pattern degradation in the medium, since patterns without cells were not degraded.

Using medium with 1% FCS slowed down this process remarkably, making it a worthy technique for Hela cells. The cells neither rounded up nor escaped from the patterns (see Fig. 3A). They even stayed confined after cells had divided on single cell sized patterns. We observed this behaviour for at least 3 days (not shown). Note that little pressure had to be applied on the stamp with the tweezers during the first 20 s of stamping, which resulted in increased protein transfer efficiency. Additionally, the mixture of fibronectin–collagen provided more stable patterns than fibronectin or collagen alone (not shown). Glass coverslips are easy to handle and are optimal for high resolution microscopy making them the substrate of choice when using HeLa cells in low serum conditions.

Nevertheless, mouse fibroblasts and RPE1 cells on single-cell sized adhesive patterns started peeling off the pattern proteins as soon as 3–4 hours even in low serum conditions, and detached overnight (not shown). With this technique, proteins are not covalently bound to the glass surface. It is therefore understandable that cells could rip off the proteins. Moreover, it has been shown that cells exert high forces in the corners of small patterns,[21] and indeed, they ripped off the patterns more rapidly on single cell sized patterns than on larger adhesive areas accommodating several cells (not shown).

In conclusion, this technique met our criteria for HeLa cells in low serum. It is simple and robust in these conditions and is currently used in our lab for this cell line. But HeLa cells in 10% serum and RPE1 or primary fibroblast in any conditions could not be efficiently constrained for more than 3 hours.

The resistance of the printed molecules to cell tearing could be reinforced by heating the printed glass coverslip. After the printing step, and before the washing step, the printed coverslips were put on a hot plate at 140 °C for 30 sec. The coverslips were then passivated with 0.1 mg mL^{-1} of PLL-g-PEG during one hour. RPE1 cells were plated in 10% containing medium on these coverslips. Whereas they detached rapidly from the coverslips which had not been heated, they stayed perfectly spread for at least 10 h on coverslips which had undergone heating (Fig. 4). Compared to cells on non-heated coverslips, cells on heated coverslips were almost not rounding up after 24 h. This additional heating step allowed the use of glass coverslips for one day long experiments in classical 10% serum conditions. Alternatives still need to be developed to maintain the adhesive and non-adhesive areas efficiency of single-cell sized micropatterns for longer experiments, such as the one dedicated to study cell division.

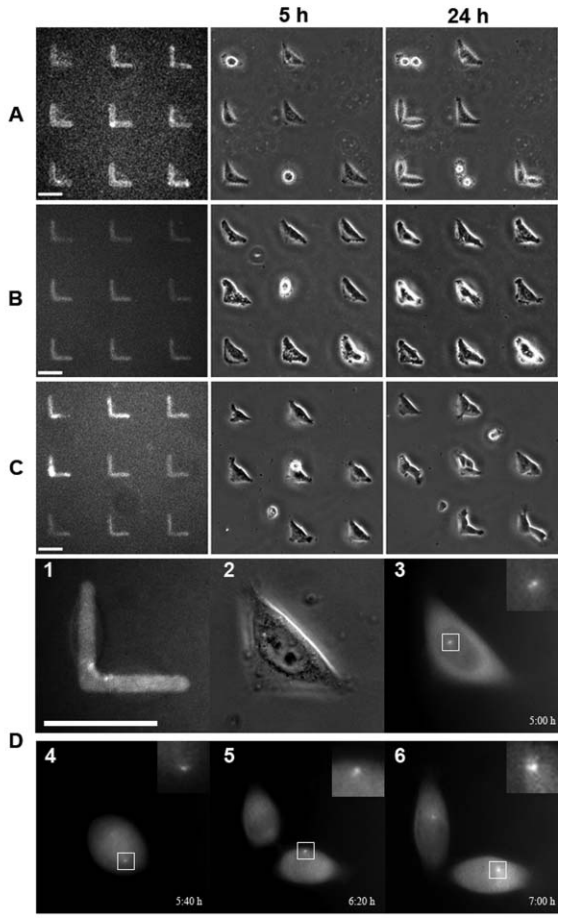

Fig. 3 Cell behavior on different substrates with PLL-g-PEG backfill. L-shape patterns of fibronectin–collagen were stamped onto different substrates. The printing efficiency was controlled using fibronectin–Cy3 (left column). After printing, backfill was performed with PLL-g-PEG and cells were seeded. (A–C) Cell behavior was observed over time (phase contrast; middle and right columns). (A) HeLa cells were seeded in medium containing 1% FCS on patterns that had been printed on untreated glass. Cells stayed nicely on the patterns. After division, daughter cells respread on one side of the L. (B) Patterns were printed on TCPS and RPE1 cells were allowed to spread in medium containing 10% FCS. Even after 24 h cells were still nicely restrained on the patterns. (C) Src++ fibroblasts were plated on patterns that had been printed on ibidi plastic, in medium containing 10% FCS. After 24 h cells still could not leave the patterns. (D) HeLa-Centrin1-GFP cells were allowed to spread in medium containing 10% FCS on L- patterns that had been stamped on Ibidi plastic. Pictures of the fluorescent pattern (1) and of the spread cell (2, phase contrast) were taken using a 40× magnification oil objective. Movement of the centrosomes during interphase and mitosis could be monitored thanks to Centrin1-GFP expression (3–6). Scale bars represent 40 μm.

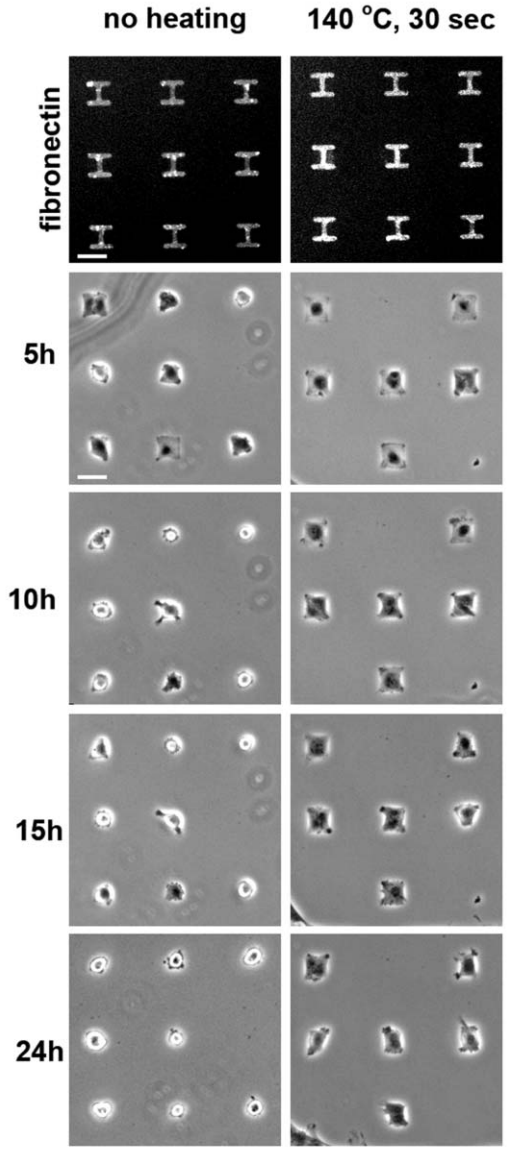

Fig. 4 Heat stabilized micropatterns on glass. Fibronectin–collagen patterns were printed on glass. Printed slides were either not heated (first column) or incubated on a hot plate at 140 °C for 30 s (second column). Fibronectin–Cy3 patterns were imaged after heating (first row), then backfilled with PLL-g-PEG. RPE1 cells were plated on the printed slides in medium containing 10% FCS. Cells on patterns were imaged after 5 h (second row), 10 h (third row), 15 h (fourth row) and 24 h (fifth row). Whereas cells contracted and rounded up rapidly on non-heated slides, they stayed much longer on slides heated at 140 °C. However, after 24 h cells started contracting even on the heated slide. Scale bars represent 40 μm.

Printing on non-treated polystyrene (PS)

PS is an appealing substrate, as it is very cheap and compatible with low magnification microscopy and immunofluorescence staining. It prevents cell adhesion when untreated, thus allowing single-step production of adhesive patterns.[12]

However, stamping efficiency onto PS was often bad in our hands (only small amounts of protein were transferred, not shown). When the protein transfer was bad, cells spread well in the beginning, but started rounding up after few hours. Nevertheless, using non plasma-activated PDMS as a stamp and applying soft pressure on the stamps when printing improved the transfer (not shown) and provided more satisfactory results. When protein transfer was good, all three cell types stayed at least 3 days on the patterns and did not round up and detach (Table 1). However controlling the right pressure for PS stamping was difficult and not easily reproducible in our hands. Previous studies have shown that

Table 1 Comparison of different patterning techniques. Different patterning techniques and substrates were tested in terms of passivation stability (as judged from the ability of cells to spread over the passivated areas) and stability of printed areas upon cell adhesion (as judged from cell rounding and detaching from the patterns). To obtain these parameters HeLa cells, RPE1 cells and Src++ fibroblasts were put on single cell sized patterns and their long term behavior was observed[a]

	Glass PLL-g-PEG/1% FCS		Glass + 140°C PLL-g-PEG/ 10% FCS		Untreated Polystyrene/ 10% FCS		TCPS PLL-g-PEG/10% FCS		Ibidi plastic PLL-g-PEG/ 10% FCS		UV exposure on glass/10% FCS	
	1	2	1	2	1	2	1	2	1	2	1	2
HeLa	>72 h	>72 h	n.d.	n.d.	>72 h	>72 h[b]	>72 h	>72 h	>72 h	>72 h	n.d.	n.d.
RPE1	~24 h	~3–4 h	~24 h	~10 h	>72 h	>72 h[b]	>72 h	>72 h	>72 h	>72 h	~24 h	~24 h
Src++ fibroblasts	~24 h	~3–4 h	n.d.	n.d.	>72 h	>72 h[b]	>72 h	>72 h	>72 h	>72 h	n.d.	n.d.

[a] 1: Passivation stability; 2: Stability of printed areas upon cell adhesion; n.d. = no data. [b] Except when protein transfer is insufficient.

protein transfer is more efficient when the substrate is more hydrophilic and the stamp more hydrophobic and suggested that it is in fact the differential in wettability between the substrate and the stamp that matters.[22] We tried to render the stamp even more hydrophobic than PS by coating the stamp with fluorosilane[23] but we could not improve the reproducibility of transfer on PS enough to make it a method of choice. It still remains an interesting approach due to its simplicity and low cost.

Passivation by PLL-g-PEG on tissue culture polystyrene (TCPS) and Ibidi μ-dishes

To prevent RPE1 cells and primary fibroblasts from peeling off the printed proteins from glass, we printed fibronectin on TCPS, which is treated to strongly promote protein adsorption, and backfilled with PLL-g-PEG.[18] RPE1 and primary fibroblasts stayed constrained on the patterns for at least 3 days in 10% FCS (see Table 1) and were able to divide on them. We could not observe any rounding up and detaching (see Fig. 3B). TCPS is derived from PS by plasma treatment which creates negatively charged groups on the plastic surface.

Lussi *et al.* already tried to explain the high passivation stability on TCPS by the strong interaction of PLL-g-PEG with the surface. This interaction could be dependent on the highly negative ζ-potential of TCPS, which is related to charge densities.[19] The same could be true for protein–surface interactions and would therefore account for the good pattern stability.

But the thickness of the TCPS bottom is a limitation for cell imaging. It can be used for classical immunofluorescence stainings of fixed cells, since it shows only little autofluorescence and since cells are accessible from the top without the need for the fluorescence light to pass through the plastic bottom. TCPS can also be used for low magnification inverted microscopy. But the plastic bottom of most TCPS dishes prevents high resolution inverted microscopy often used for fluorescence live cell imaging.

To overcome this limitation we stamped proteins on Ibidi tissue culture plastic μ-dishes whose bottom is only 180 μm thick and has almost the same optical quality as glass. IbiTreat dishes promoted good protein adsorption. They had to be further activated in a plasma activation chamber directly before stamping for passivation to be efficient enough. We backfilled as usual with PLL-g-PEG. Stability of passivation and of printed areas upon cell adhesion were comparable to using TCPS (Fig. 3C, with fibroblasts). Ibidi μ-dishes are suitable for immunofluorescene staining and high resolution microscopy. Using this plastic we were able to follow GFP-centrin labelled centrosomes during mitosis in live cell videomicroscopy and even distinguish the two centrioles (objects which are about half a micron in size and often less than a micron apart from each other, see Fig. 3D).

TCPS and Ibidi μ-dish thus appear as efficient and cost-effective solutions to produce resistant micropatterns and successful long term substrate passivation. All tested cell types in all serum conditions were perfectly constrained on the patterns for at least 3 days (Table 1). The former substrate is usually available in cell biology labs. The latter, less common but commercial, also allowed us to perform high resolution live cell microscopy. In our hands, this protocol was the most efficient and easiest to produce micropatterns routinely.

Pattern production with UV exposure

The quality of the contact between the PDMS stamp and the substrate and therefore the quality of adhesive molecules transfer depends on many parameters which are difficult to control. The stamping step is thus responsible for a large part of the variability in production of micro-patterned substrates. Moreover, it is not convenient, and sometimes not even possible, to print molecules in micro-fluidic devices using μCP. Alternative techniques based on UV directed light micropatterning have been described.[24,25] They rely on the ability of UV light to either oxidize surface coatings and thus destroy their anti-adhesive properties[26,27] or to deprotect[28] or activate photosensitive linkers.[29,30] However, these methods usually require the development of dedicated chemistry that can not be easily transferred to a biology lab. Other UV-based techniques are much easier but have to be performed on polystyrene which prevents high quality imaging.[27,31] New photosensitizers such as benzophenone could be linked to polyethylene glycol coated glass upon UV activation and allowed subsequent protein grafting.[32] More recently, UV activated benzophenone was used in solution to produce free radicals in the presence of alkene groups and thereby stimulate their binding to the mercaptopropyl groups attached to the glass.[33] The generation of free radicals by benzophenone upon UV excitation and in the presence of proteins could thus be used to directly graft proteins onto polyethylene glycol coated glass in a one-step procedure (patent WO2006/084482). Based on this, we propose a new technique to produce micropatterns of

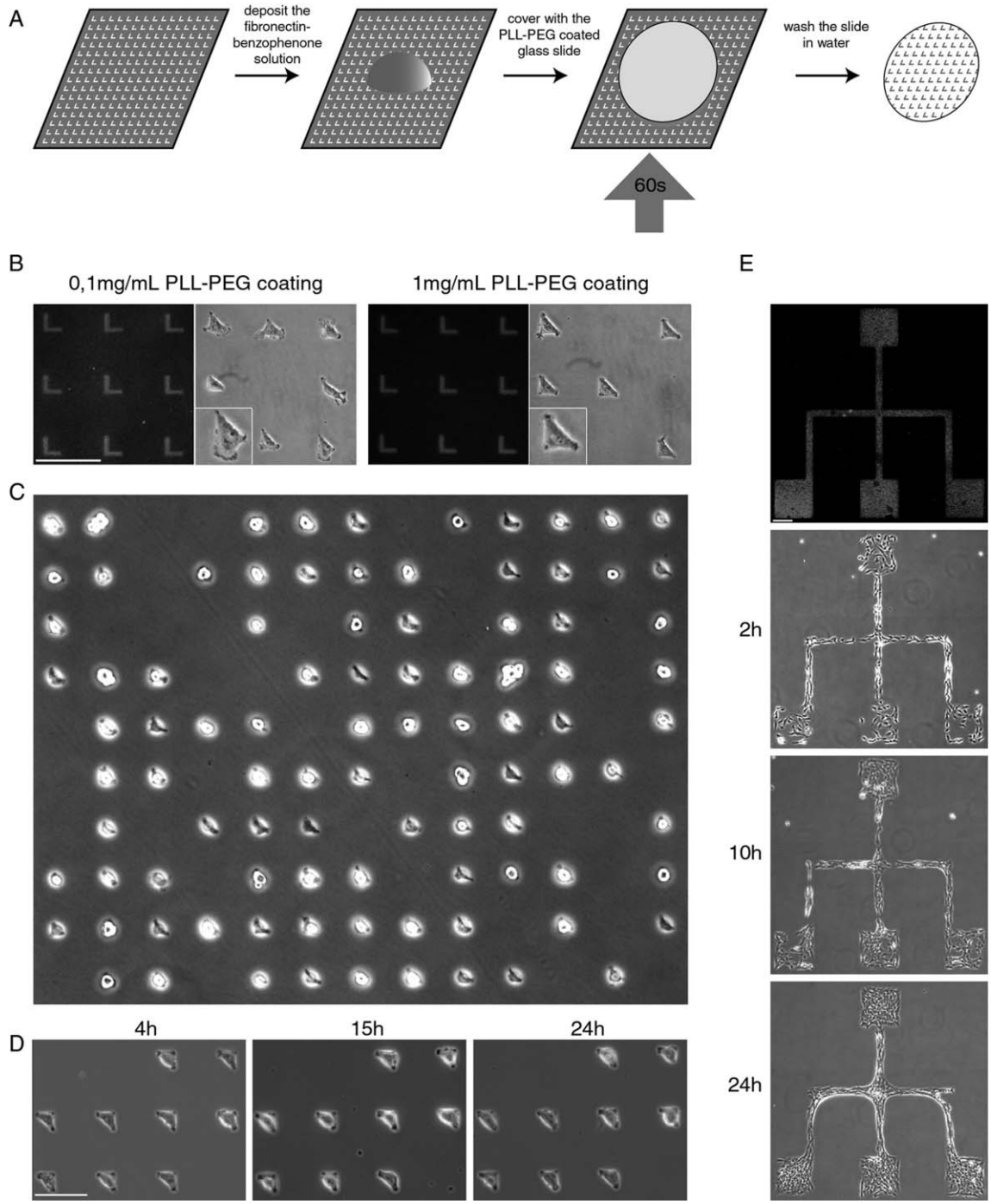

Fig. 5 UV-directed light micro-patterning. (A) Fabrication method. A droplet of the fibronectin-benzophenone grafting solution was deposed on an optical chromium photomask. A PLL-g-PEG coated glass slide was placed on the droplet. UV light (310–460 nm) was sent on the slide through the photomask for 60 s. The glass slide was washed twice with distilled water. (B) Resistance of the PLL-g-PEG coating to non-specific fibronectin grafting. UV-exposure was performed with the same grafting solution either on a 0.1 mg mL^{-1} PLL-g-PEG coated glass slide (left) or on a 1 mg mL^{-1} PLL-g-PEG coated glass slide (right). In the first case, the fluorescence background was quite high and RPE1 cells managed to spread out after a few hours (left). In the second case, fluorescence background was lower and cell spreading out of the pattern was significantly reduced (right). (C) Large and regular arrays of cells could be produced with this technique. (D) Individual cell pattern resistance to cell detachment. RPE1 cells were plated on patterns produced with this UV-directed light patterning techniques in 10% serum containing medium. Cells were monitored in time-lapse phase contrast microscopy. Cells remained well constrained for at least 24 h. (E) Large pattern resistance to cell detachment. The same procedure was used to produce large patterns allowing the spreading and migration of several cells. Fibronectin was grafted on a PLL-PEG coated glass slide (top image). Cells nicely spread specifically on the patterns. 24 h after cell plating, patterns were still efficiently constraining cell spreading. Cells could not escape the patterns. Scale bars represent 100 μm.

adhesion proteins on glass coverslips by exposing the coverslip to UV light through a photo-mask. It allowed the rapid production of highly reproducible patterns.

A 5 μL droplet of fibronectin, fibronectin–Cy3 and benzophenone solution in PBS was deposited on a regular photomask (Fig. 5A). The droplet was then covered by a PLL-g-PEG coated 18 mm glass coverslip. This resulted in an approximate 20 μm distance between the glass surface to be grafted and the photomask. The coverslip was then illuminated with UV light through the photomask. Coverslips coated with a 0.1 mg mL^{-1} solution of PLL-g-PEG displayed a fluorescent background revealing unwanted adsorption of protein on the entire surface (Fig. 5B). This allowed cells to escape from the patterns. Coverslip coated with 1 mg mL^{-1} of PLL-g-PEG displayed an intense and homogeneous grafting of fibronectin only in the UV-illuminated regions. In this case, cells were nicely constrained in the fibronectin pattern. Large and regular cell arrays were produced by this technique and observed in a time-course experiment (Fig. 5C). Individual cells were correctly constrained for at least 24 h (Fig. 5D) but started to detach or spread out of the patterns after 48 h (not shown). Larger patterns containing many cells also constrained cell attachment for at least 24 h (Fig. 5E) but appeared to remain efficient for more than three days (not shown). Therefore, this simple and rapid one-step patterning technique allowed us to produce homogeneous, reproducible and stable patterns for large cell population as well as individual cells.

Conclusions

Successful micro-patterning implies that cells show both a good adhesion on the patterns and long term confinement. We found this to be more difficult to achieve on single cell sized patterns than on large areas and that some cell types are more demanding than others. Nevertheless optimisation based only on these parameters leads to techniques which are often too difficult to handle on a daily basis in a biology lab. They frequently require the use of specialized material and non-trivial chemistry. One has thus to find compromises to minimize specialized devices and techniques and keep a good micro-pattern quality.

Since molds made from customers' designs can be easily purchased (see Materials and Methods) no specialized material is needed for the most reproducible and easiest solutions we assayed using μCP (for a summary of the tested conditions see Table 1). All proposed chemicals can be bought at affordable prices and are neither unstable nor toxic. Furthermore, the methods do not require expertise in chemistry. Nevertheless there are still limits to the feature size of the commercially available molds whose resolution is 5 μm. So μCP will therefore still sometimes require access to a clean room facility for the fabrication of smaller features on molds (wafers).

Photomasks, on the other hand, are easily available even for small pattern sizes (see Materials and Methods), and we thus developed techniques allowing fabrication of adhesive micro-patterns directly from photomasks.

We showed a proof of concept for a new patterning technique based on UV exposure easily accessible to all biology labs. Other non-adhesive substrates could be envisaged to improve UV protein grafting and allow a more efficient initial spreading of the cells while still preventing cell attachment in non patterned areas. A promising perspective for this technique would be to allow the patterning of closed microfluidic devices which are not accessible for printing. This would require the filling of the channels with the protein and benzophenone mixture and focusing the UV light with a lens on the surface to be patterned.

We are still in the process of exploring and improving techniques to achieve the highest possible reproducibility, which is the most important criterion for biological studies, and we believe the improvements described in this paper will be of great value to biologists.

In this context, we are currently exploring also other techniques, such as the production of micropatterns on cell-repellent hydrogels,[25] which are very attractive as they meet most discriminating criteria for biologists. Another appealing technique, due to its low cost, is the use of Pluronic described by Tan *et al.*[23] Unfortunately this low cost method requires precise control of the substrate wettability, which implies an additional surface treatment. Moreover the anti-adhesive properties of Pluronic were reported to be strongly impaired after drying of the substrate, thus preventing long term storage of the stamped substrates.

We believe that when an optimal technique is achieved, micro-patterning will become a basic technique for cell biology labs. Such an optimal technique should achieve the following points:

(1) Ease, low cost and reproducibility. This might allow micro-patterned substrates to become commercially available at reasonable prices, an ideal solution for most biology labs.

(2) High filling efficiency of the micro-patterned arrays. Ideally one cell per pattern. This might be achieved by techniques such as single cell dispensers with micrometric precision,[34] or microfluidic devices.

(3) Compatibility with any type of cell culture substrate, and in particular multi-well plates meant for automated analysis in high content cell based screening.

Because single cell sized micro-patterns place cells in a regular lattice, it allows the automation of image acquisition at high magnification and makes the technique ideal for high throughput screens. Moreover, the reduced inter-cellular variability induced by the control of cell adhesion geometry allows easy cell normalization, a major advantage for cell based screens.[9]

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