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Polymer peel-off mask for high resolution surface derivatization, neuron placement and guidance

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Abstract

We present a dry lift-off method using a chemically resistant spin-on plastic, polyimide, to pattern surfaces with high accuracy and resolution. Using well-known lithographic and reactive ion etching techniques, the spin-on polymer is patterned over a silicon dioxide surface. The plastic efficiently adheres to the silicon dioxide surface during the chemical modification and is readily lifted-off following the derivatization process, permitting highly reliable surface derivatization. The verticality of the reactive ion etch enables sub-micrometer features to be patterned, down to 0.8 micrometers. The technique is used to pattern neurons on silicon dioxide surfaces: efficient neuron placement over a 4 mm area is shown for patterns larger than 50 μm while process guidance is shown for 10 μm patterns.

Keywords

Chemical patterning, process guidance, dry lift-off, neural network

1. Introduction

Surface derivatization strategies are an important part of macro- and nano-device engineering for biological analysis. High spatial resolution of micrometer-sized features has permitted the development of highly parallel DNA hybridization and protein assay systems (Barbulovic-Nad et al., 2006). In cell biology, the development of ordered cell systems on a chip has paved the way to a better understanding of cell function and interaction (Falconnet et al., 2006). Several cell culture surface patterning strategies have been presented in recent years. Microcontact printing using poly(dimethylsiloxane) stamps (PDMS) have been widely used (Offenhausser et al., 2007, Charrier et al., 2010); other methods include the use of microfluidic channels (Hwang et al., 2008), ink jet printing (Roth et al., 2004), dry lift-off (or stencil-assisted; Tan et al., 2010; Tourovskaia et al. 2003; Selvarasah et al., 2008; Jinno et al. 2008; Viallet et al. 2008; Frimat et

al., 2009; Atsuta et al., 2007; Hong et al., 2009) and wet lift-off (Diaz-Quijada et al., 2011). While wet lift-off techniques are well-known in the semiconductor manufacturing and MEMS industry, their use of solvent or acid/base for efficient lift-off often precludes the deposition of organic substances, such as those used in cell patterning techniques. The use of the dry lift-off technique pioneered by Craighead (Ilic and Craighead, 2000) overcomes this difficulty; it does however require the use of a parylene coater.

In our work on planar patch-clamp chips, the need for surface derivatization aligned with microfeatures on the planar chip has led to our use of PDMS to stamp poly-D-lysine (PDL) at precise location on the chip surface (Charrier et al., 2010). However, we have found the process to be difficult to replicate in the high-throughput fashion needed for biological applications. Herein, we present a surface derivatization technique based on the dry lift-off of a spin-on polymer, polyimide. Polyimide is well-known in the electronics industry as a flexible substrate for interconnects, and in the MEMS industry as an electrically insulating, chemically inert and biocompatible passivation layer; it has been employed in neurochips (Stett 2003, Martinez 2010), neural implants (Myllymaa 2009) and biochips (Lee 2008; Wang 2009). The efficiency of the derivatization strategy is demonstrated by culturing neurons in patterns on silicon dioxide substrates.

2. Surface derivatization

Figure 1 presents the scheme for the surface derivatization. Silicon wafers (5 cm diameter) having a 3,000 Å SiO₂ layer were pre-cleaned in, successively, acetone, 80 °C isopropyl alcohol and 1% hydrofluoric acid in water (all CMOS grade from J.T. Baker, Phillipsburg, NJ).

Figure 1 - Spin-on strategy for cell patterning

Polyimide is spun onto a Si/SiO₂ substrate (step a); an aluminum mask layer is deposited, followed by photoresist (step b); the photoresist is patterned then Al is wet etched (step c); the exposed polyimide is reactive ion etched (step d); poly-D-lysine is deposited (step e); the polyimide layer is peeled off (step f). Following a sterilization treatment, the substrate is ready for cell growth.

Polyimide (PI 2610 from HD Microsystems, Parlin, NJ) was spun at 2,700 rpm for 30 s, for a final thickness of approximately 3 μm (Figure 1a). The wafers were then heated on hot plates at 100 $^{\circ}\text{C}$ for 90 s and 150 $^{\circ}\text{C}$ for 90 s, followed by a high temperature cure in an inert atmosphere (350 $^{\circ}\text{C}$ for 30 minutes in 200 sccm N_2/H_2 flow). A 50 nm aluminum mask layer was evaporated on the PI film to act as a mask during PI etching (Figure 1b). Lithography followed a standard recipe for Shipley 1813 (Rohm & Haas, Marlborough, MA) positive resist. Following a 6.25 s UV exposure (at 10 $\text{mW}\cdot\text{cm}^{-2}$), both the resist and Al were patterned by the developer solution (Microposit MF-321, Rohm & Haas), exposing the PI (Figure 1c).

Samples used for neuronal cell patterning were etched in a reactive ion etcher in an oxygen-rich environment (Figure 1d, plasma parameters: 35 min, 10 mTorr, 100 W, 10 sccm O_2). The directionality of the etching method means that high resolution patterns can be obtained even in thick polyimide films. Wafers were immersed in poly-D-lysine solution for 2 hours (Figure 1e). After rinsing with deionized water and drying, the PI layer was peeled off (Figure 1f). SiO_2 surfaces were cleaned (successively with acetone, 80 $^{\circ}\text{C}$ isopropyl alcohol and 1% hydrofluoric acid) but not pre-treated with any adhesion promoter as this would result in covalent bonding and make peeling impossible. The films were found to shrink slightly as a result of the high temperature cure, which makes them adherent enough for high fidelity pattern transfer (PDL doesn't creep underneath) yet easy to peel. Figure 2 presents the scanning electron microscope (SEM) image obtained following partial polyimide lift-off. The portion of the chip from which the PI layer is peeled off shows faint circular patterns replicating the mask patterns: they are added, solely for illustrative purposes, by introducing a small amount of CF_4 during the last five minutes of the reactive ion etching process (10 mTorr, 100 W, 20 sccm O_2 , 4 sccm CF_4), thereby slightly etching the SiO_2 surface. The CF_4 treatment is not used in samples plated with neuronal cell cultures. Wafers were cleaved into 10x10mm pieces, sterilized by immersing in 70% ethanol for 5 minutes, followed by a water rinse and drying, after which the samples were ready for cell culture.

Figure 2 – SEM results

Low magnification SEM presenting the polyimide partially lifted off the Si/SiO₂ substrate following the strategy presented in Figure 1 with two modifications permitting scanning electron microscopy (see text for details). Etched apertures in the polyimide allowed sub-micrometer microwells formation in the SiO₂ layer.

3. Neuronal cell culture and imaging

3.1. Neuronal cell culture

Cryopreserved rat cortical neuronal cells (QBM Cell Sciences, Ottawa, ON, Canada) were thawed in a 37 °C water bath for 2.5 min. One milliliter of the aliquot, containing approximately 4×10^6 cells, was resuspended prior to transferring to a 15 mL sterile tube. To avoid osmotic shock, 9 mL of Neurobasal Medium (Introvigen, Carlsbad, CA) containing 2 mM L-glutamine (Sigma, St-Louis, MO), B-27 (Invitrogen) and 5% fetal bovine serum (Gemini, West Sacramento, CA) was added over a period of 2.5 min. Cells were resuspended and a one milliliter aliquot of the cell suspension was added to each well of the 24-well plate containing the patterned sample. Cell cultures were maintained at 37 °C in a 5% CO₂ humidified incubator (NuAire Inc., Plymouth, MN) for a minimum of 14 days. Four hours post-plating, media was replaced with 1 mL of serum-free Neurobasal Medium. Media changes were performed biweekly by replacing half of the media with fresh Neurobasal Medium. No antimitotic drugs were added to reduce glial proliferation.

3.2. Imaging

Immunofluorescence

Cultured patterned cells were rinsed with phosphate buffer saline (PBS, 140 mM NaCl, 4 mM KCl, 0.5 mM Na₂HPO₄, 0.15 mM KH₂PO₄) and fixed for 30 min at room temperature in 4% paraformaldehyde. Samples were incubated in 0.25% Triton-X for 10 min, washed in PBS and a blocking agent (Dako, Mississauga, ON, Canada) was added for 1 hour at room temperature. Cells were incubated overnight at 4 °C with mouse monoclonal Map2 antibody and goat anti rabbit GFAP antibody (both from Sigma, St. Louis, MO). Following a PBS wash, cells were incubated for 1 hour at room temperature with the secondary antibodies, alexa fluor 568 goat anti mouse and alexa fluor 488 goat anti rabbit (both from Introvigen, Burlington, ON, Canada). Samples were washed in PBS prior to imaging.

Cell viability

Normal Bath Media (NBM) was prepared as follows: 140 mM NaCl; 3.5 mM KCl; 0.4 mM KH₂PO₄; 20 mM HEPES, 5 mM NaHCO₃; 1.2 mM MgSO₄, 1.3 mM CaCl₂; 15 mM glucose. Calcein-AM (green fluorescence, Molecular Probes, Carlsbad, CA) was used as a cell viability indicator. A 5 mM stock solution was prepared in DMSO and further diluted to 40 µM in NBM. Cells were subsequently stained using 5 µM Calcein-AM. RH-237 (red fluorescence, Molecular Probes) was used to stain cell membranes. A 10 mM stock solution was prepared in ethanol and further diluted in NBM to 50 µM. Cells were subsequently stained using 50 µM RH-237. Prior to imaging, a 500 µL aliquot of 5 µM calcein was placed in each well and incubated in the dark at 37 °C for 30 min. Calcein was then replaced by the same volume of 50 µM RH-237 solution and left in the dark at room temperature for 10 min. Finally, chips were placed in NBM solution for imaging. Fluorescence images of cell cultures were obtained using a LSM-410 Zeiss (Thornwood, NY) confocal microscope equipped with a krypton/argon laser (Melles Griot, Carlsbad, CA) and a LSM Tech Inc. (Etters, PA) objective inverter. For each dye, excitation wavelength and emission filter were appropriately selected and images of both dyes were collected sequentially. Calcein was excited at 488 nm and emitted fluorescence collected using

an emission filter with a bandwidth of 515 to 540 nm. For RH-237, the excitation wavelength of 488 nm and a long-pass emission filter with a cut-off at 590 nm were used. Images were merged using a Northern Eclipse Software (Empix, Mississauga, ON, Canada).

4. Results and Discussion

In our previous work on PDMS stamping, we showed that glass surfaces coated with poly-D-lysine (PDL) resulted in random cell growth; stamping PDL at defined location on the surface enabled organized growth over PDL islands (Charrier et al., 2010). We found drawbacks to the technique: reusing the stamp requires thorough cleaning and soaking (which limits throughput); design is limited by the fact that PDMS sags between patterns, and finally precise alignment between the stamp and device is challenging (in our case requiring a flip-chip bonder). The method presented here addresses these issues. First, the pattern is defined directly on the surface to be derivatized by standard lithography, not transferred by stamping. The fabrication process could easily be scaled up in production; for wafers above 150 mm in diameter, the polyimide film formation process may have to be switched from spinning to doctor-blading to preserve thickness homogeneity. This means that cells can be aligned to existing features on the chip with the high precision of semiconductor lithography in a highly parallel and economical manner. Peeling the polyimide is itself a manual process that might be impractical on samples larger than 5 cm, but it is performed on singulated chips, which for the applications we envisage are likely to be smaller. Second, by defining the structure to be derivatized on the surface of the sample, there are no non-uniformities due to sagging.

Figure 3 – Efficiency of brain cell positioning over large areas

a) Low magnification (2.5x) fluorescence imaging of a 4 mm area patterned following the dry lift-off method at 14 DIV. The long-range patterning of 100 and 50 micrometers squares is shown, as indicated on the image. High magnification (20x) fluorescence imaging of results presented in (a) is shown for b) 50 µm and c) 100 µm patterns. Process formation is visible and highlighted by arrows in (b). The sample was labeled with RH-237 (red fluorescence, membrane dye) and Calcein-AM (green fluorescence, cell viability dye).

As in Charrier et al. (2010), the following patterns were used for cell placement: arrays of 100 µm side-length squares repeated every 200 µm; 50 µm squares every 100 µm; 25 µm squares every 50 µm; 10 µm squares every 20 µm; and 5 µm squares every 10 µm.

Cortical cells were cultured for 14 Days In Vitro (DIV) on patterned substrates and labeled using cell viability (green fluorescence) and cell membrane (red fluorescence) dyes: Figure 3 presents the fluorescence images for 100 and 50 μm square patterns. As expected, neuronal cells were confined to PDL-derivatized regions of the substrate. In Figure 3a, the long-range uniformity of the technique is shown: efficient cell patterning is observed over the entire 4.5 mm field in the image. From the 20x images of Figure 3b (50 μm) and Figure 3c (100 μm), controlled poly-D-lysine physisorption enables cell placement. From these figures, independent neural islands (aggregate of cell soma) are predominantly created using the 100 μm squares separated by 200 μm , while axonal processes are observed (some highlighted by arrows in Figure 3b) connecting neural islands in the 50 μm patterns. This will enable the study of synaptic connectivity in a controlled environment (Vogt et al., 2005; Wiyart et al, 2002), for example on Multi-Electrode Arrays (Jones et al, 2010) or patch-clamp array neurochips (Py et al 2011). These observations are in-line with our previous study (Charrier et al. 2010).

High resolution patterning: effect on cell placement and process guidance

Figure 4 – High resolution patterning: effect on cell placement

High magnification (20x) fluorescence imaging of brain cells at 14 DIV on a) 25 μm , b) 10 μm and c) 5 μm square patterns. Samples were labeled with RH-237 (red fluorescence, membrane dye) and Calcein-AM (green fluorescence, cell viability dye). While single viable cell soma (green fluorescence) per PDL island are seen in (a), cells predominantly form aggregates straddling several islands. In (b), cell membranes (red fluorescence) are shown to be patterned on the substrate with neuronal cells depositing on the surface seemingly randomly. Both cell body and membranes appear random in (c).

The high efficiency of our new spin-on polymer lift-off technique permitted the derivatization of small patterns in a highly regular fashion: the effect on cell placement was studied for features approaching the size of neural soma and processes. Cortical cells were cultured to 14 DIV on 25 μm , 10 μm and 5 μm patterns, labeled and imaged using the same technique as above. The fluorescence images obtained are shown in Figure 4 for a) 25 μm , b) 10 μm and c) 5 μm patterns. While few islands having a single viable cell (green fluorescence) are observed on the 25 μm patterns, the results show a propensity for cells to amalgamate over several PDL islands. For 10 μm patterns, membranes (red fluorescence) are shown to be patterned on the substrate with cells seemingly depositing randomly over the islands; membrane patterns are not distinguishable on 5 μm islands. While patterning of membranes is evident on 25 and 10 μm , patterning of neurons is rendered inefficient for islands less than 25 μm apart.

*Figure 5 – High resolution patterning: effect on process guidance
Combined GFAP and MAP-2 fluorescence images of brain cells at 14 DIV following immunostaining on 10 μm patterns. Processes are shown to follow PLL patterns.*

Figure 5 shows the combined green and red images for a 10 μm pattern following cell growth to 14 DIV and immunostaining with GFAP (green fluorescence) and MAP-2 (red fluorescence). GFAP and MAP-2 selectively stain astrocytes and neurons, respectively. The 10 μm pattern readily guides axonal process elongation along a patterned path, sometimes over a length of 200 μm (left side). The guidance does not yield perfectly straight processes, as the dense 10 μm pitch makes it possible for processes to “jump” from one line to the next -this would not happen with a single dot line. The technique can therefore be used to direct process elongation in a controlled manner from one placed neuronal cell to another, thereby fostering controlled neuronal communication. As cells do not attach on surfaces that have not been derivatized, we infer that a low-density of small dots will not result in preferential placement, but our results show that it may still guide the growth of axonal processes. One can therefore envision a pattern of large pads, big enough to attract neuronal cells as demonstrated in Figure 3, connected by dotted lines to direct processes, as seen in Figure 5. This would circumvent the possible problem of cell soma sticking on tracks meant only for process guidance (Pfleging 2007), as well as minimize the possible problem of mask tearing during peel-off. The result would be an organized neural network.

5. Conclusion

The new spin-on polymer lift-off technique presented can efficiently derivatize glass surfaces under aqueous conditions. Patterned cell growth in culture was observed for 100 μm and 50 μm patterns, while 10 μm patterns guided the long-range extension of neural processes. We demonstrated that sub-micrometer derivatization is readily achieved with the new technique, thus paving the way to a new surface derivatization strategy for highly parallel DNA hybridization and protein assays, and guided process growth. Because of its ease of use and well-know microfabrication, the spin-on strategy presented here can be readily used by researchers having access to a clean room environment or be manufactured by industry for chemical derivatization in a high-throughput fashion. Furthermore, the simple physical peeling technique could be

carried out in any cell biology facility, allowing the end users rather than the chip producer to customize the chip by applying their own cell adhesion protocol to a cell chip fitted with the patterned polymer. This feature provides maximum flexibility to the user without forcing costly customization for the chip manufacturer.

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