Microstamp patterns of biomolecules for high-resolution neuronal networks

D. W. Branch[1,2] J. M. Corey[2,3] J. A. Weyhenmeyer[3,4]
G. J. Brewer[5] B. C. Wheeler[2,3,6]

[1] Biophysics Program, [2] Beckman Institute, [3] Neuroscience Program,
[4] Department of Cell and Structural Biology, [5] Department of Electrical and Computer Engineering,
University of Illinois at Urbana-Champaign, Urbana, Illinois, USA
[6] Department of Medical Microbiology and Immunology,
Southern Illinois University School of Medicine, Illinois, USA

Abstract—*A microstamping technique has been developed for high-resolution patterning of proteins on glass substrates for the localisation of neurons and their axons and dendrites. The patterning process uses a microfabricated polydimethylsiloxane stamp with micrometer length features to transfer multiple types of biomolecules to silane-derivatised substrates, using glutaraldehyde as a homobifunctional linker. To test the efficacy of the procedure, substrates are compared in which poly-d-lysine (PDL) was physisorbed and patterned by photoresist with those stamped with PDL. Fluorescein isothiocyanate labelled poly-l-lysine was used to verify the presence and uniformity of the patterns on the glass substrates. As a biological assay, B104 neuroblastoma cells were plated on stamped and physisorbed glass coverslips. Pattern compliance was determined as the percentage of cells on the pattern 8 h after plating. Results indicate that the stamping and photoresist patterning procedure are equivalent. Substrates stamped with PDL had an average pattern compliance of 52.6 ± 4.4%, compared to 54.6 ± 8.1% for physisorbed substrates. Measures of background avoidance were also equivalent. As the procedure permits successive stamping of multiple proteins, each with its own micro-pattern, it should be very useful for defining complex substrates to assist in cell patterning and other cell guidance studies.*

Keywords—*B104, Micro, Microstamp, Neural culture, Pattern, Stamp.*

Med. Biol. Eng. Comput., 1998, **36**, 135–141

1 Introduction

SEVERAL RESEARCHERS have investigated techniques for patterning molecules on surfaces which promote localised cell attachment and growth, in order to provide a way of studying cellular activity and development. Patterning and attachment of cells to glass has been accomplished by deposition of lipid films with strips of the lipid removed for cell attachment to the clean glass substrates (IANOVA and MARGOLIS, 1973). LETOURNEAU deposited a thin metal layer (palladium) to promote cell attachment and directed growth to otherwise non-adhesive surfaces (LETOURNEAU, 1975). The deposition of metal gradients on surfaces has been used in migration studies of mouse fibroblasts toward areas of increasing metallisation (CARTER, 1965). Other methods use surface chemical modification using ultraviolet (UV) irradiation through a mask. Hammarback *et al.* inactivated selected areas of laminin with UV irradiation through a mask, allowing dorsal root ganglion (DRG) cells to follow the pattern (HAMMARBACK *et al.*, 1985). Laser ablation of substrates can be used to fabricate high-resolution grid patterns by exposing uniform surfaces through a quartz mask. This method has been used to pattern poly-d-lysine (PDL) (COREY *et al.*, 1991*a*) which confines rat hippocampal neurons to the patterns.

More recently, investigators have made improvements through the application of high-resolution patterning techniques and more careful control of surface chemistry. One approach uses photolithography to produce two-component silane patterns on surfaces for cell growth and adhesion (COREY *et al.*, 1996; KLEINFELD *et al.*, 1988). These methods have also been used to pattern absorbed proteins (LOM *et al.*, 1993) and synthetic peptides (MATSUZAWA *et al.*, 1996). Others have modified organosilane self-assembled monolayers (SAMs) by exposure to deep UV (DULCEY *et al.*, 1991; HICKMAN *et al.*, 1994; STENGER *et al.*, 1992). Aminosilanes coupled to photocleavable linkers are another method for defining surface properties (ELENDER *et al.*, 1996).

Surface topography has been manipulated to control neuronal growth by other investigators; NELSON *et al.* (1989) used scratched grooves to guide axonal extension and to create electrically active circuits of DRG cells *in vitro*. Using controlled anisotrophic etching of silicon, CURUS and CLARK (1990) have investigated the impact of topological factors on cell growth, shape, orientation, and movement. Adhesive pathways and topographic channelling (BRITLAND *et al.*, 1996) have been used simultaneously to align DRG nerve cells in microfabricated channels.

Correspondence should be addressed to Dr. Bruce C. Wheeler, Beckman Institute, University of Illinois, 405 N. Mathews Ave., Urbana, IL 61801; USA; Email: bwheeler@uiuc.edu.

First received 6 May 1997 and in final form 29 August 1997

The most recent alternative method for creating surface patterns is elastomeric stamping (LOPEZ *et al.*, 1993; SINGHVI *et al.*, 1994), in which a microstamp is created by polymerising polydimethylsiloxane (PDMS) in a mould defined by patterned photoresist. The PDMS stamps were used to imprint alkanethiols, forming SAMs on gold substrates. The remaining gold surface was made non-adhesive to proteins by exposure to polyethylene glycol terminated with an alkanethiol. Exposure of alkanethiols to laminin resulted in a thin layer of protein for selective cell adhesion. This microcontact printing method (μCP) has also been applied in the microelectronic industry to selectively deposit oxides for fabrication of devices such as waveguides and DRAM capacitors at low processing temperatures (JEON *et al.*, 1995).

In this work we report on PDMS microstamp method for depositing biomolecules from aqueous solution onto glassy substrates. The technique is additive, in comparison to the subtractive laser and photoresist-based techniques described above. It differs from previous methods of microcontact printing in its use of much higher relief stamps (10–20 μm) and surface linker chemistry, in order to overcome surface tension which causes spreading of aqueous solutions at micrometer scales. Unlike previous photoresist, laser and stamping approaches, this technique is extensible for sequential deposition of several biomolecules on the same substrate, in order to create intricate patterns of biomolecules relevant to neural development as well as biotechnology applications.

To evaluate the stamping process, we chose to compare the response of B104 neuroblastoma cells to stamped against photoresist patterns of poly-d-lysine (PDL). When cultured, the transformed cells possess thin bipolar neurite-like processes (SCHUBERT *et al.*, 1974; 1986), a characteristic which is enhanced by the addition of dibutyrylcyclicAMP (DBcAMP) (COREY *et al.*, 1997). Our previous results showed that PDL is cytophilic (promotes cell adhesion and viability), whereas a silicon dioxide background is cytophobic (discourages cell attachment and viability) for both hippocampal neurons (COREY *et al.*, 1991*a*) and B104 cells (COREY *et al.*, 1997). The B104 cells were plated in serum-free medium to significantly reduce the nonspecific attachment of proteins from solution to the substrate.

2 Materials and methods

2.1 *Stamp fabrication*

Glass slides 2″ × 3″* were cleaned by a 1 min soak in buffered hydrofluoric acid (BHF; 34.6% (w/v) NH_4F/6.8% HF/58.6% H_2O). An adhesion promoter consisting of 95% methanol, 5% de-ionised water and 0.01% VM-651† was spun on the glass substrate at 4500 rev min^{-1} for 30 s, followed immediately by application of polyimide (Pyralin 2611† at 1500 rev min^{-1} 30 s to produce a 10 μm layer. Polyimide thickness was variable up to 10 μm, above which multiple spins and intermediate cures were necessary for thicker layers. Two polyimide layers were used to produce a total thickness of 20 μm for fabricating the grid patterns. SEM§ micrographs verified the thicknesses of the polyimide layers.

After spinning, the polyimide was hard cured for 30 min at 250°C to remove solvents from the resin. The surface of the cured polyimide was cleaned using an oxygen (O_2) plasma, by placing the substrates in a planar plasma etcher** at a pressure of 500 mTorr and RF power of 300 W for 1 min.

To create a masking layer to withstand the subsequent reactive ion etching procedure, a 500 Å thick titanium (Ti) film was deposited on the polyimide layer using a custom-built electron beam evaporator at the Microelectronics Laboratory at the University of Illinois Urbana-Champaign (UIUC). An adhesion promoter, hexamethyldisilazane (HMDS)††, was applied to the Ti layer using a photoresist spinner at 4500 rev min^{-1} for 30 s, followed by application of positive photoresist AZ 5214§§ with the same speed and duration. The substrates were then baked at 110°C for 60 s and exposed for 15 s to a UV lamp (intensity of 11 μW cm^{-2} at 320 nm) from a mask aligner*. Image reversal, mandated by the sense of the original photomask, was achieved by baking at 125°C for 60 s, followed by a flood exposure of 30 s using the same lamp intensity and wavelength. All photoresist substrates were developed for 25 s in Microposit 351 photoresist developer§§ diluted 1 : 4 in de-ionised water. The mask pattern consisted of 27 combinations of internode path length (80, 120, and 160 μm), intersection node diameter (5, 10, and 20 μm), and path width (3, 5, and 10 μm) (COREY *et al.*, 1991*a*).

Fabrication of moulds that reproduce the feature sizes of the mask is critical for creating precise stamps. The sequence of processing steps are shown in Fig. 1. We chose to use a dry etching process, reactive ion etching (RIE), which allows nearly vertical sidewalls and smooth surfaces to be fabricated in the polyimide despite the wide range of feature sizes. The Ti and polyimide layers were etched orthogonally using Freon-14 (CF_4) and O_2 in a Plasma Technology RIE at the Microelectronics Laboratory or a custom-built RIE at the Electrophysics Laboratory both located at the University of Illinois Urbana Champaign (UIUC). The Ti layer was etched using a throttle pressure of 20 mTorr, CF_4 gas pressure of 30 mTorr, and RF power of 70 W for 20 min. The polyimide layer was etched using a throttle pressure of 50 mTorr, O_2 gas pressure of 30 mTorr, and RF power of 175 W for 15 min. The Ti mask was removed by soaking the substrates in a 2% hydrofluoric (HF) acid solution for 20 s. Additionally, the HF solution removed residual polyimide that remained on the substrates after the O_2 etching process.

The polyimide substrates are moulds in which the PDMS stamp (Fig. 1) is cast using a 1:10 (v/v) mixture of Sylgard silicon and Sylgard silicone elastomer 184†. As a release agent for the cured PDMS stamp, a 5% Triton-X solution was applied to the polyimide mould by spinning at 4500 rev min^{-1} for 30 s. Teflon™ spacers (no. 6) were used to separate the mould from a glass back, which was cleaned in buffered HF acid to increase the adhesivity of the Sylgard to the glass. The glass back functioned as a mechanical support for the stamp and allowed a more uniform transfer of protein across the surface. The addition of four Teflon™ spacers to separate the mould from a glass support during the curing process allowed stamps with uniform thicknesses within 1 μm to be produced. Stamps were allowed to cure at ambient temperature for three days to minimise shrinkage.

2.2 *Preparation of physisorbed substrates*

Glass coverslips (22 × 22 mm, no. 1 thickness) were obtained from VWR Scientific§. Each coverslip was coated with 1200 Å of silicon dioxide (oxide) using the plasma

* Fisher Scientific, St. Louis, Missouri

† DuPoint, New Jersey

§ Cambridge Stereo Scan

** Texas Instruments

†† United Chemical Technologies, Bristol Pennsylvania

§§ Hoechst Celanese, Corp., Brandeburg, New Jersey

* Carl Suss MJB3

† Dow Corning Corporation

§ Chicago, Illinois

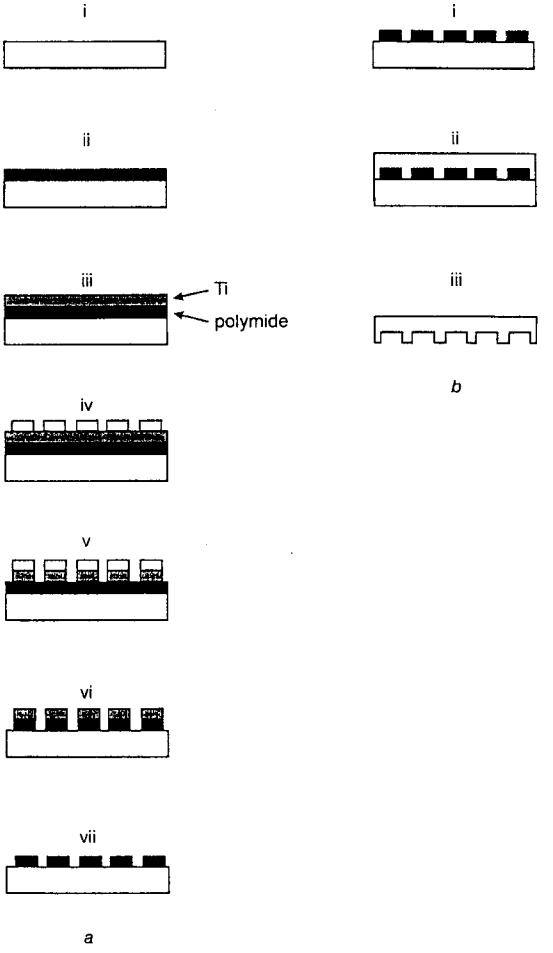


Fig. 1 *Stamp microfabrication process: (a) mould fabrication; (i) substrate; (ii) polyimide spun on glass surface followed by cure; (iii) plasma clean and deposit Ti layer; (iv) spin on photoresist, expose through mask and develop; (v) RIE Ti using Freon-14*

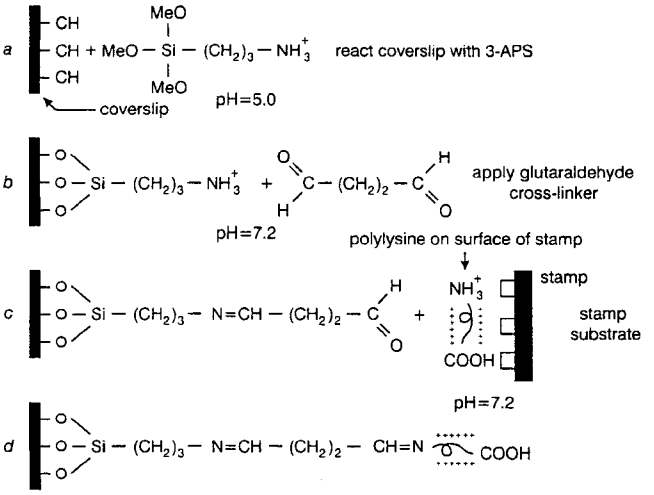


Fig. 2 *Silanisation, cross-linking, and stamping process: the microstamp transfers polylysine from the places of contact to the glass coverslip: (a) aminosilane linked to glass; (b) glutaraldehyde linked to aminosilane; (c) polylysine stamped onto glutaraldehyde; (d) final polylysine pattern linked to coverslip*

enhanced chemical vapour deposition (PECVD) process at the UIUC Microelectronics Laboratory. The PECVD process uses a Plasma Technology PECVD at a gas pressure of 1 Torr, growth temperatures of 300°C, RF power of 23.5 W, 96 sccm of nitrous oxide (N_2O), and 1.92 sccm mixture of 5.3% silane gas (SiH_4) in pure nitrogen (N_2). The growth rate for this process was 220 Å min^{-1}.

Standard photolithography was used to pattern the physisorbed substrates with the 27 combinations of internode path length, intersection node diameter and path width. Sterile PDL 130,000 MW and FITC labelled poly-l-lysine (PLL-FITC) 45,700 MW were obtained from Sigma Chemical Company** and reconstituted at 100 μg ml^{-1} using 18.2 MΩ cm sterile water. Both were stored frozen at −80°C. PDL and PLL-FITC were allowed to adsorb on the oxide not protected by the photoresist for a period of 2 h at 4°C. The coverslips were then dried in a nitrogen stream and sonicated in acetone for 10 min to remove the photoresist. The acetone residue was removed by drying in a nitrogen stream. Whenever acetone failed to remove all the photoresist, there was obvious cell death where the photoresist remained, and the coverslips were discarded.

2.3 *Preparation of stamped surfaces*

Substrates were coated with 1200 Å of oxide (see above). Some substrates had Ti alignment patterns deposited on their surfaces using e-beam evaporation prior to PECVD oxide deposition. Substrates were cleaned with concentrated nitric acid for 15 min, then transferred to boiling water for 10 min prior to the silanisation procedure. The substrates were silanised by immersing them in a mixture of 1% 3-aminopropyltrimethoxysilane (3-APS)** in absolute methanol : acetic acid 99.9 : 0.1 by volume for 15 min after boiling. Substrates were transferred immediately from the boiling water to the 3-APS solution (COREY *et al.*, 1996). Substrates were rinsed in methanol for 1 min, dried with nitrogen and baked at 120°C for 5 min to complete the hydrolysis reaction (KLEINFELD *et al.*, 1988). A 2.5% solution of glutaraldehyde** in de-ionised water was used as a cross-linker and applied to the silanised substrates for 30 min. Excess glutaraldehyde solution was removed from the substrates by rinsing twice in the de-ionised water. The fabrication procedure is illustrated in Fig. 2.

2.4 *Stamping and alignment procedure*

Stamping the silane-derivatised and cross-linked substrate was performed on a mask aligner to allow visual inspection of stamp deformation upon surface contact. The treated coverslips were suspended from a glass plate using 1 μl of water and mounted in the mask aligner. The microstamps were soaked in a 2 ml solution of PLL-FITC or PDL solution at 100 μg ml^{-1} using 35 mm Petri dishes†† for at least 2 h before stamping the substrates. A nitrogen stream was used to dry the surface of the microstamp until no visible signs of solution remained on the surface. The microstamp was placed on a translational stage with 4 degrees of freedom in x, y, z, and ϕ (rotational). The stage was gradually raised until contact and visually checked to avoid any microstamp distortion upon contact with the substrate. Initial trials showed that excessive contact pressure deforms the micron resolution features of the stamp, causing poor pattern reproduction and possibly insufficient protein transfer to the substrate. Contact time was 2 min for each substrate.

** St. Louis, Missouri

†† Fisher Scientific, St. Louis, Missouri

2.5 *Imaging PLL-FITC*

Before patterning B104-neuroblastoma cells on the glass coverslips, we stamped several substrates using PLL-FITC with a procedure identical to that for preparation of PDL substrates. PLL-FITC was used to verify successful protein transfer from the PDMS stamp to the glass substrates. Using an Olympus BH-2 microscope with epillumination attachment, fluorescence microscopy showed that using stamps without a glass back prevented uniform transfer of PLL across the stamped surfaces.

2.6 *Cell culture*

The B104 neuroblastoma cell line was supplied by David Schubert of the Salk Institute, La Jolla, California. Cells were cultured in 75 cm^2 canted tissue flasks§§. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% v/v fetal bovine serum (FBS), 1.0% penicillin (10 000 units ml^{-1})/streptomycin (10 mg ml^{-1})* and fungizone (2.5 μg ml^{-1})† at 37°C in a humidified atmosphere containing 10% CO_2. Cells were cultured until 80–90% confluent, then passed. Cells were harvested for plating onto coverslips by scraping and centrifuging for 10 min at 1000 rev min^{-1}. The pellet containing the cells was resuspended in 2 ml serum-free DMEM containing penicillin/Streptomycin and fungizone using the above concentrations.

Cells were plated at 5000 cells cm^{-2} on physisorbed and microstamped substrates in multiple-well 35 mm culture dishes in 2 ml medium§. Cell plating was done 40 min after substrates were stamped. After plating, 80 μl of 25 mM dibutyrylcyclicAMP (DBcAMP) was added to the medium to induce neurite outgrowth. After 8 h, cells were fixed for 30 min using Karnovski's fixative, rinsed twice in phosphate buffered saline, stained with 0.25% Coomassie blue in ethanol : acetic acid : water 45 : 10 : 45 by volume, and rinsed in 10% acetic acid. Cell photomicrographs were taken using a Nikon Diaphot microscope.

2.7 *Evaluation of cell patterning and survivability*

The quality of cell patterning was evaluated with three compliance measures: the fraction of somata adhering to the nodes; the fraction of somata adhering to the pattern (nodes and paths); and the fraction of the 81 background squares within the grid pattern which were free of attached cells and neurites (COREY *et al.*, 1996). To assist the counting procedure, a cell counting mask was created by evaporating 300 Å of Ti on a glass coverslip containing the 27 grid patterns, then placed over the mounted substrate and aligned to the cell patterns to score the location of the somata on the substrates using a Nikon Diaphot microscope.

To test the hypothesis that cells survived better on the physisorbed patterns, the total number of cells adhering to the substrate (including cells on and off the patterns) at 8 h were counted for the 27 grid patterns for both patterning techniques. The cell counts for the 27 grid patterns were averaged together to compose a single trial. Four trials, each on a different substrate, were evaluated for each condition. A chi-square test was performed to test the null hypothesis that cell survivability is identical for both techniques. Cells counted in the off-pattern areas were also used to compare survival for cells on bare glass and on unlinked glutaraldehyde. In this case, an average for all the patterns was computed from cells in the off-pattern areas for both patterning methods. Paired means were then tested for the null hypothesis that survival is the same for cells in the off-pattern areas of the substrates.

§§ Corning Inc., Corning, New York

* Sigma Chemical Company, St. Louis

† Life Technologies, Gaithersburg, Maryland

§ Costar, Cambridge, Massachusetts

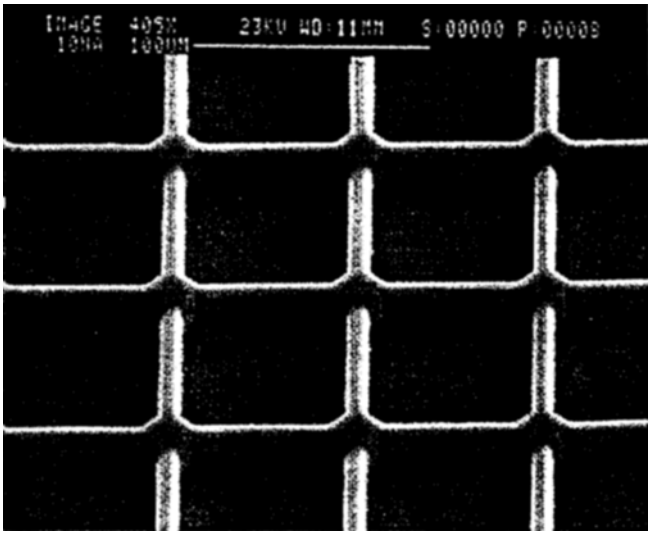


Fig. 3 *SEM of a PDMS microstamp; internode path length = 80 μm, node diameter = 15 μm, and line width = 5 μm, relief = 20 μm*

3 Results

3.1 *Stamp creation and PLL transfer*

Fig. 3 shows a PDMS stamp made using the procedures described above. High-resolution, vertical sidewall molds and stamps have been made with vertical heights ranging from 5 to 20 μm and with line widths (ridges) as small as 3 μm. Inverse patterns have also been made, in which the stamp consists of plateau regions criss-crossed by narrow channels.

Fig. 4 is a fluorescence image, indicating that PLL was transferred successfully from the PDMS stamp to the glass/3-APS/glutaraldehyde substrate. This argues that PDL, and by extension almost any protein, can be transferred by the same process and may be available for interaction with cells plated on the substrate, as tested below.

3.2 *Comparisons of stamped and physisorbed PDL patterns of B104 cells*

After 8 h, B104 neuroblastoma cells showed compliance (Fig. 5) to both the stamped substrate and physisorbed, photoresist patterned substrates. Visually, the two techniques appear to have roughly the same pattern quality. The physisorbed pattern appears to have a slightly greater number of somata and a greater density of neurites.

Quantitative measures confirm the visual impression that compliance is the same for both the stamped and control substrates for $N = 4$ trials. The combined compliance results for all 27 patterns are summarised in Table 1, indicating essentially identical compliance for both patterning techniques. With both stamped and physisorbed substrates, the compliance of somata to nodes increased slightly with node diameter, which ranged from 5 to 20 μm (data not shown), consistent with previous results (COREY *et al.*, 1991*a*). Cell survival for each trial was compared for both patterning methods in Table 2. Results indicate there was no statistically

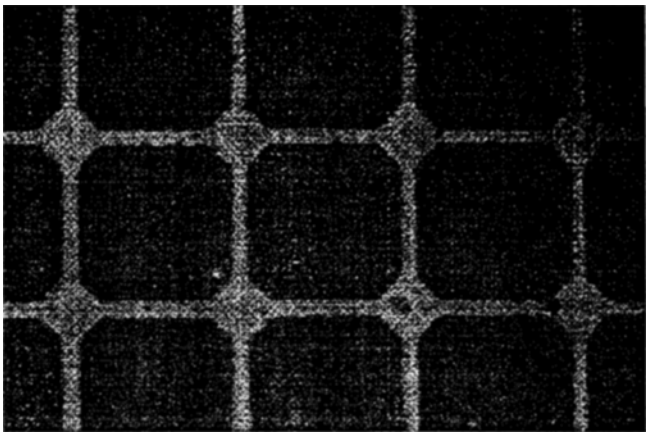
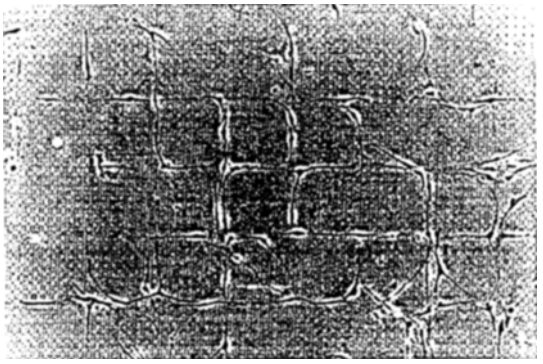
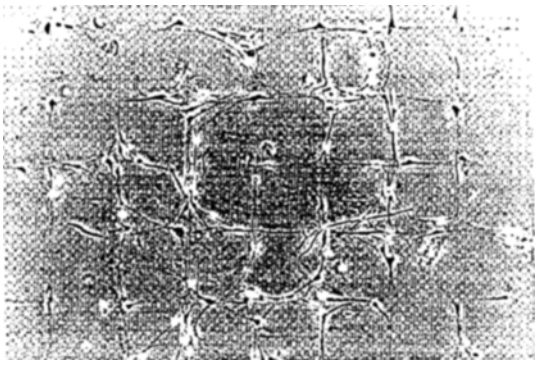


Fig. 4 *Stamped PLL-FITC coverslip visualised using epifluorescence microscopy; lighter areas are stamped with PLL-FITC, illustrating the high-resolution capability of the stamping process; darker area are unstamped, and have residual aminosilane and glutaraldehyde cross-linker; internode path length = 80 μm, node diameter = 15 μm, and line width = 5 μm*



a



b

Fig. 5 *B104 cell patterning 8 h after plating: (a) typical compliance to pattern using the stamping process: (b) typical pattern compliance to physisorbed substrate; (a, b) pattern dimensions are line width 10 μm, internode path length = 160 μm, and node diameter = 20 μm*

significant difference ($p < 0.4$) for cell survival on physisorbed against stamped substrates. The B104 neurons survived in patterns for 40 h, although we did not measure compliance at this time point.

4 Discussion

We have shown that it is possible to fabricate microstamps with high (20 μm) vertical sidewalls and that these stamps can

Table 1 Compliance of B104 neurons on patterned polylysine 8 h after plating

	combined pattern compliance (mean ± SD)		
method	node (percent of somata on nodes)	pattern (percent of somata on pattern)	background (percent of squares free of neurites and somata)
stamped	21.0 ± 2.9%	52.6 ± 4.4%	55.8 ± 1.1%
physisorbed	22.0 ± 1.0%	54.6 ± 8.1%	60.0 ± 9.3%

Table 2 Total number of somata found on and off the patterns for each trial

	stamped pattern			physisorbed pattern		
trial number	on	off	total	on	off	total
1	574	596	1170	631	830	1461
2	748	634	1382	812	672	1484
3	682	706	1388	1068	663	1731
4	740	530	1270	604	426	1030
mean	686	617	1303	779	648	1427
SD	80	74	104	214	167	291

be used to transfer easily detected amounts of biomolecule (PDL) from aqueous solution onto a glassy substrate (Fig. 3). The biomolecule can be transferred without apparent degradation of its biological functionality, as indicated by the patterned growth of the B104 cells (Fig. 5*a*). Furthermore, quantitation of compliance (Tables 1 and 2) indicate that stamping is functionally equivalent to previously demonstrated physisorption/photoresist patterning techniques, and can achieve micrometer resolution patterning on glassy surfaces.

Although we have demonstrated both a successful fabrication process (Fig. 1) and a stamp process (Fig. 2), much remains to be optimised at the physical, chemical and biological levels. Physical and process variables, including stamp dimensions, application pressure, deposition time and stamp surface pretreatment, can be optimised for maximally efficient transfer of biomolecules, both onto the stamp and subsequently onto the substrate. The 3-APS/glutaraldehyde system is perhaps the simplest of a variety of linker chemistries, including both homobifunctional and heterobifunctional linkers which are broadly applicable to immobilising almost any protein to a surface. Similarly, there is an increasingly broad selection of trophic biological agents to choose from, such as extracellular matrix molecules, cell adhesion molecules, and growth factors, in order to achieve desired control of cell growth.

Stamping proteins offers several advantages over existing patterning methods in which the scientist is restricted to the use of a single mask and hence two materials (foreground and background). For example, photoresist methods preclude the use of a second temperature cycle after one protein is absorbed to the substrate, and subtractive laser ablation removes all previously deposited materials in each area exposed. The gold/protein linker stamping method of SINGHVI *et al.* (1994) allows a second protein to be applied by a flood procedure, but not by stamping. In contrast, our stamping method allows sequential deposition of multiple patterns of biomolecules

without affecting previously deposited materials. As in other methods, a final flood procedure could cover the background.

Our compliance results are weaker than those previously achieved (COREY *et al.*, 1991*b*; 1996) and those apparent in images of hepatocyte patterning (SINGHVI *et al.*, 1994). The B104 cells show signs of fasciculation (Figs. 5*a* and *b*). Our preliminary experiments indicated that fasciculation became worse when higher plating densities were used (10 000 cell cm^{-2}) or when the cells became highly confluent (90%) prior to plating. However, we have shown previously that hippocampal pyramidal primary neutrons can be patterned with high compliance and low fasciculation, especially when the background is highly controlled (COREY *et al.*, 1996). We expect that stamped patterns, when used against an optimised background, will result in the same high degree of compliance that we have previously shown with hippocampal neurons.

Although the initial fabrication of moulds involves sophisticated processes and equipment, the models can be used to make multiple stamps. Additionally, stamps can be used repeatedly to define protein patterns with minimal loss of transfer capability. The lifetime of the polyimide moulds depended on the feature size. Our experience suggests that polyimide moulds with thick lines, which are used to produce thin line stamps, can be used at least 100 times without noticeable damage to the polyimide mould. However, if the moulds have thin lines, then adhesion of the polyimide to the glass substrate fails after a number of stamps are produced. Optimisation should again improve the reusability of both moulds and stamps.

We have also experimented with moulds produced by anisotropic etching of silicon using a potassium hydroxide (KOH) solution (KENDALL, 1975; UENISHI *et al.*, 1995). This technique allows high aspect ratios (1 : 200) with micron level features to be etched in silicon. However, preferred etching along the crystal planes limits the patterns that can be made.

During stamping with the mask aligner, suitable contact pressure achieved adequate protein transfer. However, the grid patterns with a relief of 15 μm deform sufficiently upon contact, with the result that both raised and unraised areas of the stamp transfer protein to the glass coverslip. This was most noticeable for grid patterns with internode path lengths of 120 and 160 μm. Owing to large internode path lengths separations relative to the relief of the stamp, all the moulds were fabricated with a relief of 20 μm.

Our stamping technique reported here should be applicable to a number of cellular guidance problems. For example, developing growth cones are known to utilise numerous topological, cellular and chemical cues in the cellular environment for proper guidance (GOODMAN and SHATZ, 1993). Elucidating these cues is crucial for the construction of neural circuits, as well as the investigation of developing neural circuits. Reducing the complexity of the cellular environment, followed by the sequential addition of further guidance information, may allow more complex neuronal circuits to be grown *in vitro*, as well as the determination of minimal information for proper neurite guidance. A test for rank ordering the power of chemical cues could be created, by inducing a cell to extend a neurite along a narrow path to a junction where a choice is made between two (or more) different stamped molecules. If the technique is combined with electrical recording and stimulation cellular models of learning (e.g. long-term potentiation) could be investigated on small, visually identifiable networks of neurons. More speculative is the use of axonal growth and guidance cues to direct the regrowth to assist of spinal cord injury. To do so, it will be necessary to understand how the surface changes over longer durations. More generally, precise geometric control of an arbitrary number of chemical cues may assist investigations in cellular biology, artificial organs and biotechnology.

Cell and surface patterning approaches to date establish the initial conditions in the culture, but are unable to control those conditions over time. B104 cells will maintain patterns for at least 72 h (COREY *et al.*, 1997) and hippocampal neurons show robust patterning at four days (COREY *et al.*, 1991*a*; 1996). Not much is known, however, about how long patterned growth can be maintained. Further progress in the creation of biological networks would be significantly aided by characterisation of the culture environment over long periods of time.

Acknowledgments—This project has been supported by a US Department of Education GAANN grant, PHS grant HD07333, Life Technologies, Inc., and the University of Illinois.

The authors would like to thank Mr. James Frame and Professor Thomas Dtemple of the UIUC Electrical and Computer Engineering Department, and the UIUC Microelectronics Center staff.

References

BRITLAND, S., PERRIDGE, C., DENYER, M., CURTIS, A. and WILKINSON, C. (1996): 'Morphogenetic guidance cues can interact synergestically and hierarchically in steering nerve cell growth,' *Exp. Biol. Online*, **1**, (2)

CARTER, S. B. (1965): 'Principles of cell motility. The directionality of cell movement and cancer invasion,' *Nature*, **208**, pp. 1183–1187

COREY, J. M., BRUNETTE, A. L., CHEN, M. S., WEYHENMEYER, J. A. and WHEELER, B. C. (1997): 'Differentiated B104 neuroblastoma cells are a high-resolution assay for micropatterned substrates,' *J. Neurosci. Meth.*, **75**, pp. 91–97

COREY, J. M., WHEELER, B. C. and BREWER, G. J. (1991*a*): 'Compliance of hippocampal neurons to patterned substrate networks,' *J. Neurosci. Res.*, **30**, pp. 300–307

COREY, J. M., WHEELER, B. C. and BREWER, G. J. (1991*b*): 'Localization of hippocampal neurons to chemically modified siliton nitride surfaces.' *Ann. Meeting of the Society for Neuroscience*. Vol. 17, 210, New Orleans, USA

COREY, J. M., WHEELER, B. C. and BREWER, G. J. (1996): 'Micrometer resolution silane-based patterning of hippocampal neurons: Critical variables in photoresist and laser ablation processes for substrate fabrication,' *IEEE Trans.*, **BME-43**, pp. 944–954

CURTIS, A. S. G. and CLARK, P. (1990): 'The effects of topographic and mechanical properties of materials on cell behaviour,' *Crit. Rev. Biocomput.*, **5**, pp. 343–362

DULCEY, C., GREGOR, J., KRAUTHAMER, V., STENGER, D., FARE, T. and CALVERT, J. (1991): 'Deep UV photochemistry of chemisorbed monolayers: Patterned coplanar molecular assemblies,' *Science*, **252**, pp. 551–554

ELENDER, G., KHNER, M. and SACKMANN, E. (1996): 'Funtionalisation of Si/SiO2 and glass surfaces with ultrathin dextran films and deposition of lipid bilayers,' *Biosens. Bioelectron.*, **11**, pp. 565–577

GOODMAN, C. S. and SHATZ, C. J. (1993): 'Developmental mechanisms that generate precise patterns of neuronal connectivity,' *Cell*, **72/Neuron**, **10**, *Suppl.*, pp. 77–98

HAMMARBACK, J. A., PALM, S. L., FURCHT, L. T. and LETOURNEAU, P. C. (1985): 'Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin,' *J. Neurosci. Res.*, **13**, pp. 213–220

HICKMAN, J. J., BHATIA, S. K., QUONG, J. N., SHOEN, P., STENGER, D. A., PIKE, C. and COTMAN, C. W. (1994): 'Rational pattern design for in vitro cellular networks using surface photochemistry,' *J. Vac. Sci. Technol. A*, **12**, pp. 607–616

IVANOVA, O. Y. and MARGOLIS, L. B. (1973): 'The use of phospholipid film for shaping cell cultures,' *Nature*, **242**, pp. 200–201

JEON, N. L., CLEM, P. G., NUZZO, R. G. and PAYNE, D. A. (1995): 'Patterning of dielectric oxide thin layers by microcontact printing of self-assembled monolayers,' *J. Mat. Res.*, **10**, pp. 2996–2999
KENDALL, D. L. (1975): 'On etching very narrow grooves in silicon,' *Appl. Phys. Lett.*, **26**, pp. 195–198
KLEINFELD, D., KAHLER, K. H. and HOCKBERGER, P. E. (1988): 'Controlled outgrowth of dissociated neurons on patterned substrates,' *J. Neurosci.*, **8**, pp. 4098–4120
LETOURNEAU, P. (1975): 'Cell-to-substratum adhesion and guidance of axonal elongation,' *Dev. Biol.*, **44**, pp. 92–101
LOM, B., HEALY, K. E. and HOCKBERGER, P. E. (1993): 'A versatile technique for patterning biomolecules onto glass substrates,' *J. Neurosci. Meth.*, **50**, pp. 385–397
LOPEZ, G. P., ALBERS, M. W., SCHREIBER, S. L., CARROLL, R., PERALTA, E. and WHITESIDES, G. M. (1993): 'Convenient methods for patterning the adhesion of mammalian cells to surfaces using self-assembled monolayers of aklanethiolates on gold,' *J. Am. Chem. Soc.*, **115**, pp. 5877–5878
MATSUZAWA, M., LIESI, P. and KNOLL, W. (1996): ' Chemically modifying glass surfaces to study substratum-guided neurite outgrowth in culture,' *J. Neurosci. Meth.*, **69**, pp. 189–196
NELSON, P. G., YU, C., FIELDS, R. D. and NEALE, E. A. (1989): 'Synaptic connections in vitro: modulation of number and efficacy by electrical activity,' *Science*, **244**, pp. 585–587
SCHUBERT, D., BRASS, B. and DUMAS, J. P. (1986): 'Protein complexity of central nervous system cell lines,' *J. Neurosci.*, **6**, pp. 2829–2836
SCHUBERT, D., HEINEMANN, S., CARLISLE, W., TARIKAS, H., KIMES, B., PATRICK, J., STEINBACH, J. H., CULP, W. and BRANDT, B. L. (1974): 'Clonal cell lines from the rate central nervous system,' *Nature*, **249**, pp. 224–227
SINGHVI, R., KUMAR, A., LOPEZ, G. P. STEPHANOPOLOUS, G. N., WANG, D. I. C., WHITESIDES, G. M. and INGBER, D. E. (1994): 'Engineering cell shape and function,' *Science*, **264**, pp. 696–698
STENGER, D. A., GEORGER, J. H., DULCEY, C. S., HICKMAN, J. J., RUDOLPH, A. S., NIELSEN, T. B., MCCORT, S. M. and CALVERT, J. M. (1992): 'Coplanar molecular assemblies of amino- and perfluorinates alkylsilanes: characterizatiopn and geometric defintion of mammalian cell adhesion and growth,' *J. Am. Chem. Soc.*, **114**, pp. 8435–8442
UENISHI, Y., TSUGAI, M. and MEHREGANY, M. (1995): 'Micro-optomechanical devices fabricated by anisotropic etching of (110) silicon,' *J. Micromech. Microeng.*, **5**, pp. 305–312

Authors' biographies

Darren W. Branch was born in San Diego, California in 1970. He received his BS in Physics from the University of California at San Diego, in 1994. Since 1995, he has been working for his PhD in Biophysics at the University of Illinois at Urbana-Champaign. His research interests are micropatterning neural cells, cell adhesion, and neuronal guidance and mechanisms.

Joseph M. Corey was born at Great Lakes, Illinois in 1965. He received his BA in Chemistry and Mathematics/Physics from Albion College, Albion, Michigan, in 1987, and his MS in Biomedical Engineering from Case Western Reserve University, Cleveland, Ohio, in 1990. Since 1989, he has been working for his MD and PhD in the Medical Scholars Program and Neuroscience Program at the University of Illinois at Urbana-Champaign. In 1997, he was awarded his PhD in Neuroscience. His research interests are neurology, neural prostheses, tissue engineering and developmental biology.

James A. Weyhenmeyer was born in Hazleton, Pennsylvania, in 1951. He received his BA from Knox College, Galesburg, Illinois, in 1973 and his PhD in Cell Biology from Indiana University, Bloomington, Indiana, in 1977. He undertook postdoctoral training at the University of Iowa from 1977 to 1979. Since 1979 he has been with the University of Illinois at Urbana-Champaign, where he is Professor of Cell & Structural Biology and Pathology, and is a member of the Neuroscience Program. He is currently a Fellow in Academic Administration. His research interests include the cellular mechanisms underlying neuronal growth, survival and death as they relate to the pathogenesis of neurodegenerative disorders.

Gregory J. Brewer was born in Los Angeles, California, in 1947. He received his BS from Caltech, Pasadena, California, in 1968, and his PhD in Biology from the University of California, San Diego in 1972. He did postdoctoral training at the Massachusetts Institute of Technology, Cambridge from 1972 to 1974. From 1974 to 1980, he was Assistant Professor of Microbiology at the University of Southern California School of Medicine, Los Angeles. Since 1980 he has been with the Southern Illinois University School of Medicine, Springfield, where he is Professor of Medical Microbiology. His research interests include neuron cell culture. Alzheimer's disease and creation of living neuronal networks.

Bruce C. Wheeler was born in Schenectady, New York, in 1948. He received his BS from the Massachusetts Institute of Technology, Cambridge, in 1971, and his MS and PhD in Electrical Engineering from Cornell University, Ithaca, New York, in 1977 and 1981, respectively. Since 1980 he has been with the University of Urbana-Champaign, where he is Associate Professor of Electrical and Computer Engineering, of Bioengineering, of the Beckman Institute, and he is a member of the Neuroscience Program. He is the past Chair of both Neuroscience and Bioengineering, and currently serves as Associate Head of the ECE Department for Undergraduate Affairs. His research interests are micropatterning of neural cells and electrode arrays, biomedical signal processing, and the use of computer conferencing to enhance the delivery of education.