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Research paper

Easy alignment strategy for multi-level micropatterning

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ABSTRACT

In recent years, there has been a strong demand in the biological community for multiplexed protein patterns for the control and study of cellular growth at subcellular and cellular length scales. Here, an easy alignment strategy for multi-level micropatterning is proposed enabling the broadening of patterning possibilities and the increase of architecturing complexity. In practice, our method consists of a set of custom-made machined holders for standardized fabrication of polymeric stamps and for controlled alignment of successive printing steps. With this approach, three interdigitated protein patterns of 5 μm width were successfully obtained with an angular alignment precision below 0.55° and a lateral alignment accuracy of less than 35 μm over areas of 1 cm^2. After growth of primary neurons onto these multiplexed substrates, guided neurite extension was observed along the patterns.
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1. Introduction

Microcontact printing is a simple, versatile and efficient technique to pattern biomolecules onto a variety of supports. This method developed in the 90's by A. Kumar and G. M. Whitesides [1] has received particular attention in cell biology, as there is a strong demand in the biological community for multiplexed protein patterns for the control and study of cellular growth at subcellular and cellular length scales. In particular, protein micropatterning is a powerful technique that has been widely applied with up to three-component patterns in neuroscience [2,3], *e.g.* for the *in vitro* generation of neuronal networks [4,5] and for the study of axon guidance [6,7] and branching [8,9]. However depositing multiple biomolecular inks in an easy manner and with controlled alignment is still a technological challenge to overcome.

Alignment techniques usually require optical visualization and controlled displacements of the sample for reference marks of both components to overlap. Several research groups have developed techniques with this concept, adapting a photolithography mask aligner [10], a laser displacement sensor [11] or a microscope [12] coupled with an automated stage. Although these techniques provide an accurate alignment, they require sophisticated setups rendering sample preparation complex and time-consuming.

Other methods have been developed to subvert alignment involving the loading of several inks onto the same polymeric stamp and their transfer in a single printing step [13] or variants with subtraction of inked regions [14,15]. These are suitable for providing discrete regions of distinct biomolecular inks with a high degree of alignment and architecture complexity, but continuity between patterns of different biomolecules remains challenging, the latter being an important requirement for applications in neurobiology for instance. Other groups have reported a well-thought self-aligned system [16] for multi-level microcontact printing but requiring engineered substrates that match with their developed stamps. Alternatively, the use of parylene stencils in combination with inkjet printing has been reported as a two-step replacement for microcontact printing. Although multiplexing can be obtained even at sub-micron scales, this approach requires, for each sample, the use of equipment and infrastructure for the deposition of parylene and its structuration using photolithography [17,18].

In this paper, we present a simple strategy to align successive patterns using microcontact printing without the need for any specific equipment. The key elements, namely an appropriate design, the standardization of polydimethylsiloxane (PDMS) stamp fabrication and a guiding system for controlled positioning of the inked stamps during printing were optimized to generate multiplexed protein patterns in a fast and easy manner. Cell adhesion and growth on the generated patterns was performed for studies in neurobiology.

2. Easy alignment strategy

2.1. Pattern design and silicon mold fabrication

The design is conceived so that the overlay of the different mask levels generates the targeted patterns. The desired number of proteins

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to deposit dictates the number of stamps to be used, thus the number of mask levels and silicon molds to fabricate. Fig. 1 depicts a schematic representation of 2-level interdigitated stripes where features of silicon mold #2 are placed with an offset distance with respect to the edge of the mold compared to the stripes of silicon mold #1. Glass chromium photolithography masks were fabricated using Direct Writing Lithography (DWL 200, Heidelberg instruments MickroTechnik). Various features were included, among them arrays of 50 μm × 50 μm squares spaced by 300 μm and 5 μm width stripes spaced by 75 μm, all arranged in 15 mm × 15 mm areas delimited by demarcation lines. Silicon wafers (p-type, 4 in. diameter, 525 μm in thickness) were plasma cleaned with oxygen plasma at 800 W for 5 min and dehydrated under hexamethyldisilazane (HMDS) to promote resist adherence. A 1.1 μm thick layer of ECI resist (Clariant ECI 3012,) was spin-coated and baked for 1 min at 90 °C. The resist layer was then exposed for 8 s (UV 405 nm) through the glass chromium mask. A post-bake at 110 °C was performed for 1 min before dipping the wafers for 25 s in a developer solution (Microposit MF-CD-26 developer, Shipley) and rinsing them in DI water. Etching of the silicon wafers was achieved by inductively coupled plasma - Reactive Ion Etching (ICP-RIE) (Alcatel, ASM4200) to obtain 5 μm or 25 μm pattern depth depending on pattern dimensions. After removing resist leftovers, structured silicon wafers were cut using a diamond saw into 15 mm × 15 mm squared pieces, following the demarcation lines. Finally, silicon molds were coated with Perfluorodecyltrichlorosilane (FDTS) using Spray Pyrolysis Deposition (SPD MEMSSTAR) to render their surface antiadhesive.

2.2. Fabrication of standardized magnetic PDMS stamps

For the overall printing strategy to be successful, it is important that the offset positions of the features are kept constant throughout the process. To fulfill this goal, the first step is to standardize the fabrication of the PDMS stamps. For that purpose, a set of custom-made machined parts (Fig. 2) was fabricated in polyurethane (Tufset rigid polyurethane, Tufnol). It is composed of both a holder with notches dimensioned so that squared silicon molds can be placed inside, and an upper part with thru-openings. Both parts are screwed together to ensure proper sealing. A volume of 500 μl of PDMS mixture (Sylgard 184, Dow Corning) prepolymer and curing agent at 10:1 weight ratio was poured onto each silicon mold through the openings, then degassed under vacuum and pre-cured at 60 °C for 1 h leading to a native PDMS layer of about 1 mm in thickness. A layer of magnetic PDMS composed of a mixture of PDMS and iron powder (Sigma Aldrich, hydrogen reduced, 50 μm in diameter) at 1:1 weight ratio was added on top of the first native PDMS layer. After degassing, a stamp holder was placed and partially immersed in the uncured magnetic PDMS. The whole system was then put into an oven at 60 °C overnight. As the stamp holders are polymerized along with the stamps, the latter can be easily demolded, ready to be used. The thickness of the magnetic PDMS layer was set to about 5 mm.

2.3. Microcontact printing process using a guiding system for successive printing steps

The different stamps were inked (Fig. 3a) by dipping each one of them in a different protein solution for 1 min, rinsed with PBS 1× (Gibco) and dried under nitrogen flow. Another machined holder was then employed to guide the inked magnetic stamp and hold the substrate in place during successive printing steps (Fig. 3b). Following the same concept as the custom-made machined system for the fabrication of standardized PDMS stamps, this holder is composed of a first part equipped with a notch for the substrate to be placed and a second upper part with thru-openings having the same dimensions as the stamps. After placing the substrate, namely a plasma cleaned coverslip (Fisherbrand™, Thermo fisher scientific, 18 mm in diameter), the two parts were screwed together for the substrate to be hold in place. The inked stamps were sequentially brought into contact with the substrate through the guiding system for 1 min. The magnetic PDMS layer of the stamps is intended so that magnetic forces can be employed to ensure a uniform and conformal contact between the stamp and the substrate by placing magnets under the substrate [19]. The distance between the magnetic PDMS layer of the stamp and the magnets under the substrate was experimentally set around 3 mm. This distance was identified as optimal for providing a uniform contact without collapsing the features of the stamps. The magnetic force generated was not evaluated but estimated around few hundreds of Pa given the similarities with the configuration in the work of J-C. Cau et al. [19]. This process was sequentially repeated to deposit each protein level. The whole process is simple and fast. A three-step patterning is performed in less than 10 min: the first stamp is inked for 1 min, rinsed in 30 s, dried under nitrogen flow for 30 s and printed for 1 min. These steps are repeated for the two subsequent stamps.

3. Evaluation of the alignment accuracy

3.1. Alignment angle evaluation

To evaluate the accuracy of our multilevel patterning approach, several 2-level and 3-level patterned protein depositions were generated using different combinations of 5 μm-width stripes. Fluorescence images (Fig. 4a, b and c) of the substrates were acquired with a fluorescence reader (InnoScan1100AL, Innopsys) at a resolution of 0.5 μm/px. Proteins used were Laminin-Rhodamine (Cytoskeleton Inc.) at 20 $\mu g \cdot ml^{-1}$, Cy5-labelled Streptavidin (Invitrogen™, Thermo Fisher Scientific) at 1 $\mu g \cdot ml^{-1}$ and a mix of Cy3- and Cy5-labelled streptavidin at 1 $\mu g \cdot ml^{-1}$. In the case of the 3-level interdigitated stripes, we observe the three stripes of different proteins are not crossing each other and have constant interdistance over several millimeters and over an overall area of 14 mm^2 (3.5 mm × 4 mm). To evaluate angle accuracy in a quantitative manner, angles between two adjacent lines of different proteins from two different printed stamps were measured in the case of crisscross patterns as

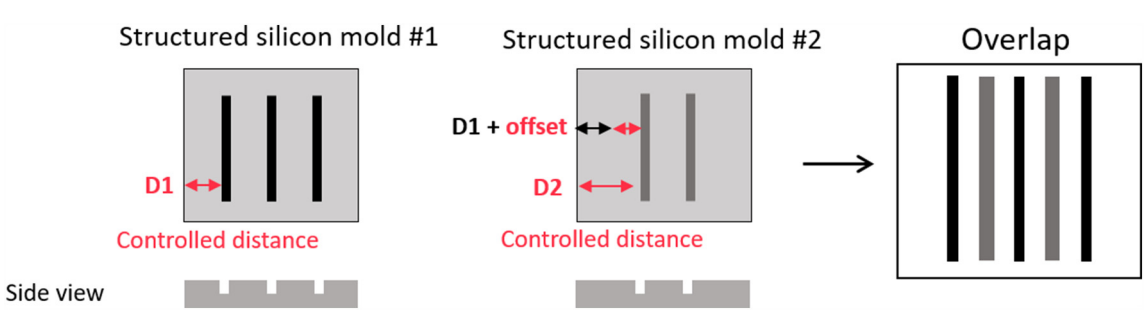

Fig. 1. Schematic representation of the silicon molds on which features are positioned with controlled offset distance with respect to the edge.

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