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Multi-Protein Microcontact Printing with Micrometer Resolution

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Abstract

Depositing multiple proteins on the same substrate in positions similar to the natural cellular environment is essential to tissue engineering and regenerative medicine. In this study, the development and verification of a multi-protein micro-contact printing (μ CP) technique is described. It is shown that patterns of multiple proteins can be created by the sequential printing of proteins with μ m precision in registration using an inverted microscope. Soft polymeric stamps were fabricated and were mounted on a microscope stage while the substrate to be stamped was placed on a microscope objective and kept at its focal distance. This geometry allowed for visualization of patterns during the multiple stamping events and facilitated the alignment of multiple stamped patterns. Astrocytes were cultured over stamped lane patterns and were seen to interact and align with the underlying protein patterns.

Keywords

microcontact printing; μ CP; surface patterning; laminin; aggrecan; astrocytes

Introduction

Cells in their natural environments maintain contact with each other and with extracellular matrix through molecular recognition events. These events regulate cellular behavior and cell differentiation. Understanding the relationship between the molecular recognition events and their effects is ever more important in tissue engineering and regenerative medicine. With the increasing use of biomaterials in medicine, better ways to interface such materials with cellular systems are being sought using biomimetic strategies; that is, creating an artificial environment similar to the natural environment of cells in a given tissue. One such approach is to deposit precise spatial patterns of proteins on a material that serve to accomplish a biological goal. Recently, protein patterned surfaces have been used to control cell adhesion^{1–5} and to direct the growth of neurons.^{6–10} With the understanding that the cellular interactions with ECM are complex, a method that allows for the precise transfer of multiple independent protein patterns on the same surface becomes necessary. Despite advances towards this goal, no current techniques exist with the resolution necessary to pattern surfaces with multiple proteins at the cellular scale over a sufficiently large area.

There exist numerous ways to pattern surfaces with proteins, each with inherent advantages and disadvantages. The three main methods used today are dip-pen nanolithography (DPN),^{11–13} inkjet printing,^{14–16} and micro-contact printing (μ CP).^{17–20} Dip-pen nanolithography capitalizes on the precision of atomic force microscopy (AFM) to deposit nano-scale patterns of proteins of interest. This system can be multiplexed with many tips, and can be made to deposit multiple proteins in precise registration.¹² Motion of the tips is

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accomplished with piezoelectric actuators while coordinates are programmed into a controlling computer, providing for both accuracy and repeatability. Because of the precise optics and computer controllers required, this system is relatively costly. The main downfall of the DPN technique and its application to biological systems, however, is the small scale over which proteins can be deposited. The x and y range of a typical AFM is limited to a few hundred μm , therefore a large coverage of surface patterns is difficult to achieve in a reasonable amount of time. Multiplexing tips serves to shorten the patterning time, however it results in a pattern repeated across the surface, not a single unique pattern.

Inkjet printing is a rapid surface patterning technique that lends itself to high throughput applications. This method, well established for the printing of inks, has recently been applied to the patterning of proteins. In inkjet printing, the desired protein solution is loaded into a print cartridge. If multiple proteins are to be used, cartridges are selected that allow for the printing of multiple solutions in parallel. High-resolution jet printers are then used to deposit a desired protein pattern onto a surface. While this process is rapid and relatively cheap, the non-uniform drying of spotted solutions can lead to inhomogeneous coverage of the desired patterned regions.²¹ Additionally, the resolution of these printers is limited by the minimum droplet size they can deposit ($\sim 20 - 30 \mu\text{m}$)¹⁴. This resolution limits the application of inkjet printing for cell-pattern interaction studies.

Micro-contact printing (μCP) is a more flexible patterning technique. Taking advantage of established photolithographic methods, master dies are made from etched patterns in photoresist or metals. These patterns are transferred to a soft polymeric material through casting and curing of the polymer in the die. Once the cured polymer is peeled off of the template, it can be used as a stamp with which to pattern surfaces with proteins. Polydimethylsiloxane (PDMS) is a typical polymer used to create these stamps and is widely available. By varying the monomer/crosslinker ratio, stamps with a range of elastic moduli can be created, allowing this technique to be tailored to specific applications. With the exception of the exposure and etching of the master template, all other steps in the stamp generation process can be carried out on the benchtop. This method is limited in minimum achievable stamp feature size ($\sim 100 \text{ nm}$),²⁰ however it provides for any desired pattern of proteins to be deposited over a large area.

All current soft lithographic techniques are limited in their ability to pattern multiple proteins on the same surface. Many groups have addressed this problem with innovations such as multiplexing small arrays,²² inkjet printing with multiple inks,²³ and microfluidic deposition.^{24, 25} While these methods do allow for the deposition of multiple proteins on a surface, none allow for large-scale patterning. In order to achieve large area coverage of multiple aligned patterns with small features, stamps need to be used to pattern the same substrate in serial. This method has been used by multiple groups,^{25, 26} but has been limited by only a rough degree of alignment, typically achieved by eye. In order to generate increasingly complex patterns, a more precise and repeatable method is necessary.

One proposed application of multiplexed protein patterns can be found in the field of neuronal regeneration. In healthy tissues, astrocytes guide the growth of neurons through a series of molecular cues. When aligned, they express cellular adhesion and guidance molecules in specific spatial orientations so as to guide developing neurons along the same axis.²⁷ It is thought that by replicating these complex protein patterns, directed neuronal outgrowth can be achieved *in vitro*, an important first step towards neuronal bridging devices. Figure 1 shows the essential steps of creating such multiple protein patterns: a high resolution image of astrocyte expressed laminin (green) and chondroitin sulfate proteoglycan (red) (A) are used to create μCP templates (B) from which the stamps (C) are made to print two proteins (D). In order to replicate these natural patterns, a method is

required that allows for the transfer of multiple complex protein patterns to the same surface in precise registration.

Presented herein is a report on a platform that allows for multiplexed μ CP of independent patterns with μ m spatial registration. The device developed consists of an inverted light microscope modified to accept both stamp and substrate. The substrate is affixed to an optically clear stage at the focal distance of the microscope's objective, providing for real-time imaging of the stamping process. This, along with the use of imaging software, allows the user to overlay a live image of a stamp with a previously transferred pattern, thus enabling accurate registration of two or more patterns.

Methods

Stamping platform

The multi-protein patterning was carried out using a modified inverted light microscope (Diaphot, Nikon) equipped with a CCD camera (Orca, Hamamatsu) and designed to house both a soft polymeric stamp and a glass substrate (Figure 2). The microscope could be run both in bright-field, epi-fluorescence or reflectance interference contrast microscopy (RICM) mode.^{28,29} The stamps were mechanically attached to the microscope stage and could be translated in the x , y and z directions as well as rotated in θ_x , θ_y , θ_z relative to the substrate for alignment and leveling. The stamp holder consisted of an optical two-axis tilt mount (KMM1, Thorlabs) with a custom rotating glass stage. The holder was attached to the microscope stage via vertical sliding rails, providing a rough vertical adjustment and ensuring the stamp holder was level with respect to the microscope stage. The stamp was held on the glass stage simply using capillary forces, allowing for rapid and easy exchange of stamps and eliminating the need for adhesives or clamps. Additionally, the lack of mechanical clamps prevented deformation of stamp features prior to stamping and preserved the integrity of the pattern. The substrate to be stamped was attached to the outer housing of the microscope's objective lens (Nikon, Fluor 10x, NA 0.5) by a custom engineered stage. This stage allowed for the use of glass coverslips or microscope slides as substrates, thus also facilitating simple substrate exchanges. The stage was optically clear and the surface to be stamped could be brought into the focal plane of the underlying objective, thus allowing for real-time in situ imaging during the stamping process.

PDMS stamps

The creation of soft lithographic stamps has been previously described in detail.¹⁸ Briefly, photolithographic techniques were used to transfer template patterns onto chromium-coated fused silica surfaces. A chromium etch was used to provide for relief of features. PDMS (10:1 ratio of base-to-crosslinker by weight) was then cast over the molds and allowed to cure. The PDMS stamps were peeled off of the mold and were cleaned by sonication in a 1% solution of surfactant Alconox (Alconox, New York) for 15 minutes. Stamps were rinsed in ethanol and allowed to dry under nitrogen gas. The stamps were then inked with the desired proteins by depositing 100 μ l of protein solution on the feature-side of the stamp and incubating for 30 minutes. Non-adhered protein was rinsed off with double distilled deionized (DDI) water and the stamps were dried again under a stream of nitrogen.

Two-protein stamping protocol

For illustration, a two-protein stamping protocol is described here, however it should be noted that this technique could be easily used to create higher order protein patterns. In a two-stamp protocol, the first stamp was placed on the optical two-axis tilt mount and fastened to the stage of the inverted light microscope with its inked side facing towards an objective lens. A substrate to be stamped was attached to the custom stage on the top of the

objective (Nikon, Fluor 10x, NA 0.5) (Figure 2) and was brought into the focal plane of the objective. The microscope turret was then rotated to retract the substrate stage and to advance the second, so-called “leveling” objective (Nikon Fluor 20x, NA 0.75) below the mounted stamp. Stamp leveling with respect to the substrate was accomplished by translating and imaging each corner of the stamp and ensuring they are in focus by adjusting the tilt mount (θ_x , θ_y) as necessary. Through the use of an objective lens with a shallow depth of field, very precise leveling could be achieved which was important as features on the elastomeric stamp can deform differently under a non-uniformly applied load. If one portion of the stamp is incident upon the sample first, it will ultimately experience a higher load than the rest of the stamp, which may lead to deformed features or stamp collapse.

The microscope turret was then rotated back to advance the substrate below the stamp. The stage was lowered to bring the stamp into contact with the surface for five minutes, depositing the protein on the substrate. Using the CCD camera, real-time images of the stamping process were generated. This allowed for recording of the stamping process and, in the case of RICM images, provided quantitative information as to the actual area of the stamp in contact with the surface. When multiple stamped patterns were desired on the same substrate surface, an image of the first stamp was recorded using bright field while the stamp was in contact with the substrate. The first stamp was then retracted and a second stamp was loaded. The microscope turret was again rotated to advance the leveling objective. The second stamp was leveled in the same manner as the first. The turret was rotated back to again bring the substrate into place. Using the “overlay” function in ImageJ,³⁰ the recorded image of the first stamp in contact with the surface was layered over the live image from the microscope. Since the substrate remained stationary in the field of view of the camera, the image of the first stamp was representative of the first transferred protein pattern on the substrate. The pattern on the second stamp could therefore be pre-aligned with the pattern transferred from the first stamp prior to second stamp-substrate contact. The second stamp was then brought into contact with the surface for five minutes and retracted, resulting in the transfer of the second protein pattern. In this way, surfaces with multiple precisely aligned patterns of proteins were generated. Figure 3 shows a schematic of the two-step protocol highlighting the different steps of this process.

Feature deformation and collapse are two inherent problems in microcontact printing²⁰ and vary based on the physical distribution of pillars used to pattern the substrate. Since the substrate stage was mounted to an objective lens, real time *in situ* imaging was made possible. This provided for both qualitative and quantitative information during the stamping process. The use of RICM greatly assisted in calibrating the system for each stamp. Using RICM mode, the contact area of the stamp was measured and the effect of various loading forces on stamp feature deformation were determined. Figure 4a shows an RICM image of a random gradient stamp touching a substrate, with contacting features appearing black. Using fluorescently labeled proteins in the stamping process, the resulting patterns on the substrate were visualized using epifluorescence microscopy. Figure 4b shows an example of a transferred AlexaFluor 488-labeled laminin (Invitrogen) from the same gradient stamp. Using proteins conjugated with different fluorophores, multiple patterns of proteins on the same surface were viewed independently and alignment of the patterns was verified.

Cell adhesion experiments

Primary P1 CD rat astrocytes were seeded onto patterned coverslips at a sub-monolayer density of approximately 4000 cells per cm². The cells were cultured at 37°C and 5% CO₂ for 48 hours in DMEM/F12 media (Gibco) supplemented with 10% FBS (Sigma). Astrocyte cultures were fixed with 4% paraformaldehyde for 15 minutes. Fixed samples were treated with DAPI (Invitrogen) in a 1:100 dilution in PBS for 15 minutes to stain for cell nuclei. Samples were then rinsed in DDI water, dried, and mounted with Fluoromount-G (Southern

Biotech) for imaging using a fluorescence microscope (Nikon Eclipse E600, Plan 20x, NA 0.75). Cell alignment with respect to alternating patterns of laminin (Invitrogen) and aggrecan (Sigma-Aldrich) was calculated by taking the angle of the longest chord drawn through the nucleus of each cell relative to the patterned lanes using Image Pro Plus (Media Cybernetics). Astrocytes cells present in aggregates were excluded to eliminate cell-cell interactions from two-protein pattern effects.

Results

To determine the positional error introduced by the leveling procedure, the reproducibility of the microscope turret was investigated. The turret was rotated between the sample stage objective and leveling objective 20 times and an image of a reticle was taken each time. These images were used to calculate the variation in set position inherent to the microscope turret. The variation was found to be $\pm 1.96 \mu\text{m}$ in the x direction and $\pm 1.23 \mu\text{m}$ in the y direction.

The registration capability of the device was demonstrated by aligning two stamps: the first stamp was based on a randomized 20% coverage pattern generated using Mathematica's (Wolfram) random function with the smallest feature size being $1 \mu\text{m}^2$, while the second stamp was its inverse.³¹ Figure 5a represents a subsection of the template used to generate the 20% coverage stamp and Fig 5b shows a bright field image of the corresponding portion of the stamp. Figures 5c and 5d show the same for the complementary 80% coverage stamp. Figure 5e shows an overlay of an image of the 20% stamp with the live CCD feed of the 80% stamp during alignment. Colors are added to provide contrast between the features of each stamp.

The precision in printing two different proteins was demonstrated through the use of stamps that were patterned with $15 \mu\text{m}$ lanes spaced $25 \mu\text{m}$ apart. Figure 6 shows the printing of AlexaFluor 488-labeled laminin using the first stamp and AlexaFluor 592-labeled aggrecan using a second stamp with identical features on the same substrate surface at various inter-lane spacings. Figure 6a shows lanes printed with an intended space of $0 \mu\text{m}$ between them, while Figure 6b shows lanes printed with an intended space of $5 \mu\text{m}$ between them. Actual distances were $1.18 \pm 0.25 \mu\text{m}$ (Figure 6a) and $4.76 \pm 0.24 \mu\text{m}$ (Figure 6b), calculated by taking the average intensity profile across the gap between lanes and finding the full-width half-maximum value for each.

Astrocytes were cultured over a two-protein pattern of laminin and aggrecan lanes and over appropriate controls (laminin only or aggrecan only patterns). Cells were observed to interact with the underlying protein layers and preferential adhesion and alignment to the laminin lanes was observed. Figure 7 shows the alignment of astrocytes cultured on laminin and aggrecan lanes. Alignment along the axis of the laminin lanes was observed in samples that contained laminin patterns either with or without aggrecan, while this alignment was absent for samples containing only aggrecan lanes (Figure 7b). Figures 7c and 7d show histograms of astrocyte alignment relative to the axis of the underlying protein patterns.

Discussion

When aligning multiple stamps, it is important to eliminate motion in the system to ensure the image taken of the first stamp in contact with the surface continually represents the distribution of the first transferred protein species. In printing multiple proteins, the greatest source of alignment error is introduced in the leveling process. During leveling, the microscope turret is rotated between the attached substrate and a separate objective lens. This mechanical system has an intrinsic amount of deviation at each of these set positions.

Any deviation in the position of the turret will cause an offset between the image of the first pattern and the actual location of the transferred protein pattern. This will cause the second protein pattern to be deposited slightly out of alignment with the first. The amount of error introduced in this step is within the tolerance of the designed application of the system. It is important to note, however, that for certain stamps, leveling is less of a concern. Depending on the distribution of features on the stamp, variations in pressure across the stamp can have either profound or little effect on the fidelity of pattern transfer. Additionally, the elastic modulus of PDMS can be tailored to reduce the need for stamp leveling. For stamp systems in which pressure variations have little effect, the leveling step can be skipped and thus reduce error in pattern transfer.

As illustrated in Figure 5, this technique can be used to precisely register multiple patterns of proteins. The image in Figure 5e shows what the user observes while aligning a second stamp with a previously transferred pattern. The first stamp is loaded and brought into contact with the substrate. An image of this stamp in contact with the substrate (Figure 5b) is used in conjunction with the live image of a second stamp (Figure 5d) to ensure the two patterns are in registry before stamping. In this example, the features from the 20% random coverage stamp can be seen through the holes in the live image of the complementary stamp (Figure 5e). Once the patterns are aligned, the user brings the second stamp into contact with the surface, thus transferring the second pattern of proteins. This demonstrates the precision afforded by this method in the x, y, and rotational degrees of freedom. Higher order patterned systems can be easily created with the same degree of accuracy.

The precise control of alignment allows for the transfer of multiple protein patterns to the same surface in registry. Figure 6 demonstrates the control over spacing between printed lane patterns. Both laminin (AlexaFluor 488) and aggrecan (AlexaFluor 592) were used as they are two proteins of interest in the study of neuronal guidance in spinal cord injury bridging devices. Images of each species were obtained separately using appropriate filters then combined to form the final image. Precision in alignment is demonstrated by the proximity of stripes in the x-direction and the rotational orientation. Spacing between lanes was controlled with an accuracy of $\sim 1 \mu\text{m}$ at two different spacings, $0 \mu\text{m}$ and $5 \mu\text{m}$.

This pattern is fundamentally different from current two-protein patterns in that it is continuous over a large area (1 cm^2) and the two species were precisely deposited in register with one another. Conventional large area, two-protein patterns were previously generated by backfilling one of the protein, which might have resulted in non-specific binding and interactions with previously deposited protein layers.³ Other multi-protein patterns have been generated through the stamping of a single substrate in serial, however no known current methodologies have the precision and repeatability demonstrated by this method. The current method allows for the presence of non-patterned space within a multi-protein system, which conventional methods can not produce.

Astrocytes cultured over laminin and aggrecan lanes were seen to interact with the underlying protein patterns. Cells were observed to preferentially adhere to laminin lanes (versus non-stamped and aggrecan lanes), and aligned along the axis of the pattern. This was expected, as laminin lanes have been previously shown to induce alignment in cultured astrocytes²⁷. Aggrecan, typically implicated in inhibiting neuronal guidance, was not seen to play a significant role in modifying astrocyte behavior versus non-stamped controls. The interaction of cells with the underlying protein patterns demonstrates the transfer of an active protein species to the surface in a controlled pattern and validates this method as a surface-patterning tool.

Conclusion

Presented here is the description of a technique developed for the deposition of multiple independent protein patterns on the same surface. The integration of live-imaging capabilities makes possible the precise alignment of multiple stamps and introduces valuable imaging tools *in situ*. The capabilities of this method to align and print multiple protein patterns in precise registration have been demonstrated, as has the bioactivity of these transferred patterns. The verification of this technique addresses a previously unmet need in the field of μ CP.

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Figure 1.

Essential steps of creating multiple protein patterns. (A) A high resolution image of astrocyte expressed laminin (stained green), chondroitin sulfate proteoglycan (stained red) and cell nucleus (stained blue) (courtesy of F. Meng, Tresco's lab, University of Utah). (B) A smaller region of astrocyte membrane is used to create μ CP templates for two proteins. (C) These templates are used to prepare two μ CP stamps with the same repeating patterns. (D) The stamps are used to print two proteins in registration.

Figure 2. Schematic of the multi-protein μ CP device. A light microscope was modified to accept stamps and substrates while providing real-time imaging capabilities.

Figure 3. Schematic of the multi-protein μ CP process. A stamp is inked and dried (1–2) then loaded and leveled using the leveling objective. The substrate stage is advanced with the microscope turret and the stamp is aligned with a previous pattern (3). The stamp is then brought into contact with the surface and the protein pattern is transferred (4–5). This process is repeated to deposit multiple patterns of proteins.

Figure 4. Examples of *in situ* microscopy available in multi-protein μ CP process. (A) Reflectance interference contrast microscopy (RICM) image of a gradient patterned stamp in contact with a substrate. (B) Fluorescence microscopy image of a transferred laminin (AlexaFluor 488-labeled) protein pattern from a gradient stamp.

Figure 5.

The registration capability of multi-protein μ CP process. (A) A subsection of a random 20% coverage pattern. (B) Bright field image of a randomized 20% coverage stamp. (C) A subsection of the complementary 80% coverage pattern. (D) Bright field image of a complementary 80% coverage stamp. (E) Overlay of the two stamps as seen by operator during the alignment step. False color was added for contrast.

Figure 6. Lane patterns printed with laminin (AlexaFluor 488-labeled) and aggrecan (AlexaFluor 592-labeled). (A) Intended lane spacing of 0 μm . (B) Intended lane spacing of 5 μm .

Figure 7. (A), (B) Fluorescent images produced from the sequential stamping of laminin (green, AlexaFluor 488-labeled) and aggrecan (red, AlexaFluor 592-labeled) using two lane stamps with bright field overlay of cultured astrocytes. Astrocytes were seen to interact with the underlying laminin pattern but not the underlying aggrecan pattern. (C) Histogram showing the alignment of astrocytes cultured on aggrecan lanes relative to underlying pattern ($n = 97$). (D) Histogram showing the alignment of astrocytes cultured on laminin and aggrecan lanes relative to underlying pattern ($n = 179$).