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High fidelity nanopatterning of proteins onto well-defined surfaces through subtractive contact printing

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Abstract

In the pursuit to develop enhanced technologies for cellular bioassays as well as understand single cell interactions with its underlying substrate, the field of biotechnology has extensively utilized lithographic techniques to spatially pattern proteins onto surfaces in user-defined geometries. Microcontact printing (μ CP) remains an incredibly useful patterning method due to its inexpensive nature, scalability, and the lack of considerable use of specialized clean room equipment. However, as new technologies emerge that necessitate various nano-sized areas of deposited proteins, traditional microcontact printing methods may not be able to supply users with the needed resolution size. Recently, our group developed a modified “subtractive microcontact printing” method which still retains many of the benefits offered by conventional μ CP. Using this technique, we have been able to reach resolution sizes of fibronectin as small as 250 nm in largely spaced arrays for cell culture. In this communication, we present a detailed description of our subtractive μ CP procedure that expands on many of the little tips and tricks that together make this procedure an easy and effective method for controlling protein patterning.

Keywords

soft lithography; micropatterning; self-assembled monolayers (SAMs); cell adhesion; fibronectin

I. Introduction

The ability to precisely control the geometric patterning of surface-bound peptides/proteins down to the micro and nano-scale levels represents a critical area of research with numerous applications in basic science and biotechnology. For example, a seminal paper by Chen *et al.* demonstrated that control of cell geometry through protein patterns regulated induction of apoptosis (Chen, Mrksich, Huang, Whitesides, & Ingber, 1997). Since then, numerous studies have been performed to elucidate how cell shape, mandated by protein patterning, regulates a variety of biochemical pathways, pathophysiology of certain diseases, stem cell fate decisions and more (Guilak, Cohen, Estes, Gimble, Liedtke, & Chen, 2009; Vogel & Sheetz, 2006). The fields of biosensors and diagnostics also require precise patterning of biomolecules through miniaturization of multi-analyte biochemical assays for drug screening and ELISA assays (Gonzalez-Macia, Morrin, Smyth, & Killard, 2010). With the above applications in mind, along with various uses in tissue engineering (Khademhosseini, Langer, Borenstein, & Vacanti, 2006), the evolution of techniques to improve the spatial

resolution as well as the ease of manufacturing of specific protein patterns onto a surface continues to be an active area of research.

Appropriate protein patterning is defined on the basis of two main target outcomes: 1) biomolecules must be immobilized at high densities and bioactivity with precision in user-defined geometries in two or three-dimensional space and 2) the surrounding “non-patterned” areas must be resistant to protein adsorption. In addition, when designing a patterning technique, it is important to consider the resulting resolution (smallest feature size that can be successfully deposited), the scalability of the procedure as well as its reliable reproducibility. To meet these demands, various methods including photo-, scanning probe, and soft lithographies have been explored, each with their own inherent advantages and limitations.

Conventional photolithography remains to be one of the most commonly used techniques for generating protein and biomolecule patterns. In conventional photolithography, a photoresist layer is first spin-coated onto a surface after which a pattern is generated based on the application of UV light through a photomask. Although representing a technique that has been in use since the creation of the microelectronics industry more than thirty years ago, this method heavily relies on the use of clean room facilities which may not be readily available to many researchers in the biomedical field. In addition, many photolithographic methods involve multiple washing steps with the use of harsh chemicals and solvents which can reduce the deposited protein's activity (Patel, Sanders, Shakesheff, Cannizzaro, Davies, Langer *et al.*, 1999). Scanning probe lithography (SPL) techniques have also been explored due to their ability to generate patterns of surface-bound moieties down to nanometer scale resolutions (Kenseth, Harnisch, Jones, & Porter, 2001). Based on technology originally used for scanning probe microscopy, this type of lithography utilizes a physical probe which moves across a surface in a raster scan-like mode. The probe then interacts with the surface through mechanical, thermal, electrostatic or chemical stimuli or a combination among them to ultimately etch a user-defined pattern. Many of these methods including field evaporation, thermomechanical indentation, and anodic oxidation however, require the use of non-physiologically relevant environments such as high vacuum or high electric field conditions and thus are unsuitable for protein and biomolecule deposition (Saavedra, Mullen, Zhang, Dewey, Claridge, and Weiss (2010). For this reason, dip-pen nanolithography (DPN) and mechanical indentation, also known as dynamic plowing, are two of the more widely used biomolecule patterning techniques encompassed within the field of scanning probe technologies (Xie, Chung, Sow, & Wee, 2006). First introduced by Mirkin and colleagues in 1999, DPN utilizes an AFM tip to generate potentially nano-scale patterns through the transfer of biomolecules from the AFM tip to a surface with the aid of a water meniscus (Ginger, Zhang, & Mirkin, 2004; Piner, Zhu, Xu, Hong, & Mirkin, 1999). Unlike other SPL methods, DPN bypasses the need to initially alter the intended surface either physically or chemically before deposition of the biomolecules and instead directly deposits the biomolecule “ink” onto the surface (Salaita, Wang, & Mirkin, 2007). Alternative SPL methods have also been described where regions of a non-fouling surface are “dynamically plowed” off through the use of an AFM tip, thus exposing the scratched off zones to protein adsorption (Muir, Fairbrother, Gengenbach, Rovere, Abdo, McLean *et al.*, 2006). Although these protein-compatible technologies allow for resolutions down to tens of nanometers, the

techniques suffer from very low throughput and thus present a multitude of challenges when considering the scalability of the process. Even with the massive parallelization of DPN systems where multiple “dip-pens” are utilized at once, this method still produces a daunting task for researchers to rapidly and inexpensively create surface-bound protein patterns (Bullen, Chung, Wang, Zou, Mirkin, & Liu, 2004; Kisiday, Hale, Almodovar, Lee, Kipper, McIlwraith *et al.*, 2011). With that being said, it is crucial for researchers to develop novel patterning techniques that can combine high resolution sizes with ease of manufacturing and scalability.

Soft lithography, on the other hand, offers an enticing set of properties including its simplicity, ability of rapid prototyping and inexpensiveness which make its application to biological systems incredibly useful for researchers (Whitesides, Ostuni, Takayama, Jiang, & Ingber, 2001). Pioneered by Whitesides and others (Balmer, Schmid, Stutz, Delamarche, Michel, Spencer *et al.*, 2005; Delamarche, Geissler, Wolf, & Michel, 2002; Delamarche, Schmid, Michel, & Biebuyck, 1997; Kumar & Whitesides, 1993; Weibel, Diluzio, & Whitesides, 2007), soft lithography encompasses a large set of techniques which utilize elastomeric polymers, mainly polydimethylsiloxane (PDMS), for fabrication of micropatterns and microstructures. Though a variety of soft lithographic approaches exist, the basis of all techniques lie in the same general concept of creating a stamp or mold by curing a prepolymer solution over a master containing relief features. Once cured, the polymerized block can be peeled off and used depending on the specific approach. Microcontact printing (μ CP) specifically has become one of the more highly used soft lithography techniques in biotechnology due to its heightened compatibility and ease of use with proteins and biomolecules as compared to other soft lithography techniques such as micromolding in capillaries (MIMIC) and solvent-assisted micromolding (SAMIM) (Xia & Whitesides, 1998). μ CP relies on the simple idea that the relief pattern transferred to the polymer upon curing can be used to “stamp” or transfer an adsorbed layer of molecules from the surface of the polymer to another surface with a pattern resembling an exact negative of the master. In 1993, the Whitesides group first capitalized on this technology by μ CP a self-assembled monolayer (SAM) of an alkanethiol onto a gold substrate and showing that a variety of geometries could be patterned based on the master through which the PDMS stamp was cured on (Kumar & Whitesides, 1993). Shortly thereafter, other types of materials were used and have since led to applications seen today which include patterning proteins, cells, DNA, and polymers (Lange, Benes, Kern, Horber, & Bernard, 2004; Mrksich, Dike, Tien, Ingber, & Whitesides, 1997; Ruiz & Chen, 2007). Like other soft lithographic techniques, μ CP benefits from facile preparation, inexpensive means of fabrication and rapid prototyping.

Despite its advantages however, μ CP is certainly not without limitations and is mainly hindered by the mechanical instability of relief areas in the stamp. Because of this, the resolution size of most μ CP methods remains above 0.5 microns (Perl, Reinhoudt, & Huskens, 2009). Recently, our group developed a novel method for protein patterning through which traditional μ CP could be modified in order to attain resolution sizes in the nanometer range (Coyer, Delamarche, & Garcia, 2011). In this communication, we discuss important aspects of our method to provide readers with greater knowledge of the procedure.

Accordingly, we present here our subtractive contact printing method which consists of the following steps:

- A. Nanotemplate fabrication
- B. PDMS stamp fabrication
- C. Coverslip surface activation with self-assembled monolayers
- D. “Inking” of the PDMS stamp with an adsorbed protein layer
- E. Subtractive contact printing
- F. Nanotemplate cleaning

II. Materials

1. Sylgard 184 silicone elastomer kit (Dow Corning Corporation, Midland, MI).
2. Alkanethiols for self-assembled monolayer, two different thiols are required: a) Alkanethiol for non-fouling background (EG₃): HS-(CH₂)₆-(ethylene glycol (EG))₃ (Prochimia Surfaces, Poland, Cat No. TH 003-m11.n6) b) Alkanethiol for protein or biomolecule tethering (EG₆-COOH): HS-(CH₂)₁₁-(EG)₆-OCH₂-COOH (Prochimia Surfaces, Poland, Cat No. TH 001-m11.n3). Aliquot out stock solutions into separate vials under complete nitrogen or argon atmosphere and store at -20°C. Ensure that the vials used for storage are secure and prevent the exchange of gases in order to prevent excessive oxidation of the solutions. When time for use, dilute stock with absolute ethanol to a final concentration of 1.0 mM. For EG₃, these steps correspond to aliquoting 6.12 µL stock EG₃ solution into vials followed by dilution with 18 mL of absolute ethanol. For EG₆, these steps correspond to aliquoting 1 µL into vials followed by dilution with 1.9 mL of absolute ethanol.
3. Untreated polystyrene dishes (100 mm diameter).
4. Blocking buffer: 0.1% heat-denatured bovine serum albumin (BSA, Sigma, St. Lois, MO). Pre-heat a water bath to 50-55°C. Make a solution of 0.1% (w/v) solution of BSA in Dulbecco's phosphate buffered saline (PBS, calcium/magnesium free, Gibco-BRL Life Technologies) and ensure complete mixing. Incubate the solution in the 50-55°C water bath for one hour. Do not leave the solution in the water bath for longer than an hour.
5. Freshly prepared 2-(N-morpholino)ethanesulfonic acid (MES) buffer-0.1M 2-(N-morpho)-ethanesulfonic acid (Sigma-Aldrich, St. Louis, MO. Cat No. M3058) and 0.5 M NaCl in deionized water. In 50 mL of deionized water, add 976 mg of MES and 1.461 g of NaCl. Adjust pH of this solution to 6.0 with 1M NaOH.
6. Freshly prepared 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) (Sigma-Aldrich, St. Louis, MO. Cat No. E7750 (EDC) & Cat. No. 130672 (NHS)) activation buffer: 200mM EDC and 100mM

NHS in fresh MES buffer. For 30 mL of MES buffer, add 931 mg of EDC with 345 mg of NHS.

7. 2-propanol (C_3H_8O , Anhydrous, 99.5%)
8. 25- millimeter diameter coverslips (#1.5 thickness) (Warner Instruments, Hamden CT. Cat No. 64-0715)
9. Tweezers (both curved and flat-tipped tweezers, TDI International, Pima Tucson, AZ)
10. (Optional) 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO. Cat No. M6250): 20 mM. To 50 mL of deionized water, add approximately 70.1 μ L of 2-mercaptoethanol.
11. (Optional) Alexa Fluor 555NHS ester (Molecular Probes, Eugene OR. Cat No. A37571).

III. Methods

A. Nanotemplate fabrication

The basis of this protocol lies in the removal or “subtraction” of adsorbed proteins from a flat surface of a PDMS stamp through contact transfer to a high-resolution nanotemplate. In order to create a user-defined geometry on the final stamped surface, the wafer must be processed in such a way that patterned indentations in the template correlate with the structures and geometries desired in the final patterned surface. In essence, once the stamp is brought into contact with the template, the layer of adsorbed protein on the surface of the stamp is removed in areas that have not been patterned while the relief regions allow for adsorbed proteins to remain on the stamp. A review of this process can be found below (Figure 1).

Nanotemplates should be manufactured based on patterning requirements of the final application. For the purposes of this communication, we recommend creating the nanotemplate using electron-beam (e-beam) lithography of silicon wafers as it provides smaller resolution sizes when compared to conventional photolithographic methods. To do so, begin by spin-coating a positive tone resist (an example of a high resolution resist would be ZEP520A and is the one we recommend for this procedure) onto a silicon wafer (100-125 nm film thickness) followed by baking the resist at 180°C for 180 seconds (Matsui, 1997). Standard e-beam lithography procedures can then be followed to create the resist geometry after which the pattern can be transferred into the silicon substrate using an oxide etcher system. Due to the fact that this template works as a “master”, only a limited amount of templates are needed based on user-needs which cuts down on the amount of clean-room time required. Although the principles of e-beam lithography are similar across platforms, clean-room facilities should have their own procedures and protocols depending on the specific system that is in place. If one does not have access to a clean-room facility, these services can be readily outsourced.

B. Fabrication of PDMS stamp

The first step in our subtractive contact printing protocol is the fabrication of the PDMS stamp. To adequately create the stamp, it is crucial to formulate a prepolymer PDMS solution with appropriate ratios of the base polymer and curing agent. Excessive amounts of curing agent will cause the stamp to be highly rigid which, although leads to greater stability of the stamp, will cause minor defects in the stamped surface that prevent proper protein transfer during later steps. Too small amounts of curing agent on the other hand, will cause deformations and instability to arise over time. For the purposes of this procedure, a 10:1 base polymer to curing agent ratio was utilized but it is worthy to note that some alterations to this ratio may be required depending on the specific application. Once this solution is made, pour the PDMS into untreated polystyrene dishes and allow it to cure at 60°C overnight under vacuum. The polystyrene should create a uniformly flat surface from which the stamp can be extracted. After allowing the PDMS to cure overnight, cut and remove the PDMS from the polystyrene dish and transfer to a clean dish ensuring that the PDMS is flipped in the process such that the flat side (the one in contact with the polystyrene dish during the curing process) is facing up. Cut and separate the PDMS into square stamps with dimensions appropriate for the specific derived pattern at hand. Due to the numerous washing steps that follow, it is important to somehow denote on the stamp itself which side is the flat side to be used for later stamping procedures. A simple way of doing so is to cut a small piece of PDMS off the bottom corner of the stamp while remembering the side opposite to the cut is the one used for stamping. Although the size of these PDMS squares can be altered based on user-preference and throughput, it is important to keep in mind the ergonomics of the process as too large or small of PDMS stamps may be unwieldy for users to stamp with.

Clean each stamp through sonicating (bath sonicator) in a 1:3 solution of isopropanol:deionized water for 5-10 minutes, followed by rinsing in solutions of deionized water and ethanol. From the ethanol wash, immediately blow dry at 45° angle under a stream of compressed nitrogen for 15 seconds or until completely dry. Once dry, pre-stamp the flat side of the squares on clean glass coverslips to remove unreacted PDMS. After 30 seconds the stamp should be removed, inverted (so that the flat side is face up) and placed in a clean polystyrene dish. Throughout this preparation process, it is important to keep the dishes containing the stamps covered whenever possible to prevent dust and other particulates from accumulating on the surface.

C. Substrate Monolayer Surface Preparation

Self-assembled monolayer (SAM) systems have been extensively studied and utilized in biological systems to create highly defined surfaces for applications in biosensors and basic life science studies (Hodneland, Lee, Min, & Mrksich, 2002; Mrksich & Whitesides, 1996; Ostuni, Yan, & Whitesides, 1999). The basis of monolayer formation in our application lies in the binding of alkanethiols to a gold coated coverslip in order to create a densely packed monolayer of defined chemical structures. To allow for biomolecule tethering as well as for creation of a non-fouling background in non-printed areas, two different SAM solutions are utilized: a) carboxylic acid-terminated alkanethiol (EG₆-COOH) for surface activation and protein tethering and b) tri(ethylene glycol)-terminated alkanethiol (EG₃) for a non-fouling

background. It should also be noted that although we utilize a carboxylic acid-terminated alkanethiol due to its ability to ultimately be conjugated to an amine group on a protein, other alkanethiol SAMs with different activating functional groups are also able to be used based on the intended applications. It is also possible to use silane-terminated groups on a glass surface for monolayer preparation rather than using thiol terminated groups on gold-coated surfaces if readers do not have available access to electron-beam evaporators for gold deposition.

First, wash 25mm diameter coverslips extensively by sonicating in ethanol for five minutes followed by blow drying with nitrogen air and plasma treating for three minutes. Place the coverslips in an electron beam (e-beam) evaporator and sequentially coat with 100 Å titanium and 200 Å gold at a rate of 1 Å/s and a pressure of 2×10^{-6} Torr. It is essential that coverslips are plasma etched prior to placing in the e-beam evaporator to ensure proper adhesion and coating of the titanium and gold. After successful metal deposition, remove the coverslips from the evaporator and store under vacuum to minimize possible environmental contaminants on the surface. When ready for SAM application, remove gold-coated coverslips from vacuum and place in untreated polystyrene dishes with the gold-coated surface face up. Remove aliquots of the EG₃ and EG₆-COOH solution from storage and dilute each stock down to 1.0 mM in absolute ethanol. Immerse the coverslips in a mixed solution of EG₃/EG₆-COOH thiols (100 parts EG₃ to one part EG₆-COOH, final concentration 1.0 mM), cap with nitrogen air, and seal the polystyrene dish with Parafilm. Allow the reaction to continue overnight at room temperature. Although we recommend using a 1:100 ratio of EG₆-COOH/EG₃, higher ratios of EG₆-COOH can be utilized if the biomolecule concentration intended for printing is particularly low. Too high EG₆-COOH ratios, however, will cause the loss of a non-fouling background due to insufficient amounts of EG₃. If a 1:100 ratio of EG₆-COOH/EG₃ does not exhibit enough biomolecule signal, it is recommended to attempt to optimize the ratio of mixed SAM solution.

Following overnight incubation, remove the Parafilm seal and wash each coverslip in ethanol for 15 minutes followed by three washes in deionized water and one wash in fresh MES buffer. Then, transfer coverslips to 6-well plates and incubate in fresh activation buffer (200 mM EDC and 100 mM NHS in MES buffer at pH 6.0) for 25 minutes. After incubation, rinse the coverslips quickly in deionized water and blow dry with nitrogen air for approximately five seconds. This washing step is crucial as salts from the MES buffer as well as any EDC or NHS left on the coverslip may reduce the efficiency of protein tethering. We recommend either applying a quick stream of deionized water to the coverslips for about four seconds or quickly dipping the coverslips into a Petri dish with deionized water to rinse the EDC/NHS buffer off but prevent hydrolysis of the now activated carboxylic acid group of the EG₆-COOH. In addition to washing, it is also possible to quench the EDC/NHS activity by stabilizing the remaining carbodiimides through the addition of 2-mercaptoethanol (optional). To incorporate this step, following the 25 minute incubation in activation buffer, immediately immerse the coverslips in a 20 mM solution of 2-mercaptoethanol followed by quick rinsing under deionized water and drying with nitrogen air. Although this step would help quenching any residual EDC moieties, it is equally important to wash off all remaining 2-mercaptoethanol from the coverslip surface as residues may cause unwanted redox reactions with the immobilized proteins. It is important to note

that the step involving 2-mercaptoethanol is optional and equivalent results were shown with and without this step so long as the proper washing protocols are followed. After activation, washing and drying of the SAM surface, subtractive contact printing can be performed.

D. Inking of PDMS elastomer & subtractive contact printing

Although the inking and stamping of the PDMS elastomer onto the SAM-activated coverslip represents the final step of the protocol, there are some preliminary steps that need to be taken prior to incubation of the SAMs in EDC/NHS buffer. First, a solution of the peptide to be printed must be dissolved in PBS at an appropriate concentration. For our purposes of studying cellular adhesion, we usually utilize fibronectin as the immobilized protein at a concentration of 100 µg/mL in PBS. Depending on the specific protein along with its activity, the concentration may need to be optimized. If users would like to verify the patterning of the protein onto the coverslips, fluorescently tagging the protein prior to stamping can be performed using a variety of techniques and dyes. Once stamped, a fluorescent microscope can be used with appropriate filters to visualize the pattern. Once this protein solution is made, take the PDMS stamps that were previously cut and ink the flat side that was in contact with the polystyrene dish during curing (flat side should be facing up if stored properly) with approximately 800 µL of the biomolecule solution. When depositing the 800 µL, ensure that the solution spreads over the entire surface area of the stamp in order to get complete printing. This can be done by taking the pipette tip and dragging the solution to each of the four corners of the stamp. Allow this layer of biomolecule solution to incubate on top of the stamp for 30 minutes at room temperature.

Immediately prior to the end of the 30 minute incubation, oxygen plasma treat the nanotemplate made in Part A of the protocol for three minutes. This treatment will improve the transfer of the biomolecule from the stamp surface of the PDMS to the template and is essential for the subtraction step of the protocol. After plasma treatment, the stamps should be nearing the end of their incubation period. Take each stamp and rinse with PBS and deionized water followed by drying with nitrogen air. A thin layer of adsorbed biomolecule will remain on the PDMS and be used in the stamping process.

Take the now dry PDMS stamp and, with a pair of curved-tip tweezers, bring the elastomer into contact with the nanotemplate ensuring that the flat side with the adsorbed protein layer is the side being stamped. A good strategy for making contact is to angle the PDMS before stamping such that only one side comes into initial contact with the nanotemplate. After this first contact is made, gently lower the rest of the stamp onto the silicon wafer. Apply a slight pressure to the top of the stamp with tweezers to achieve intimate contact between the PDMS and the nanotemplate. Through this contact, proteins from the homogeneously inked elastomer are removed in patterned areas due to the affinity for proteins to adhere to the less hydrophobic nature of the silicon wafer compared to the PDMS generating a negative pattern on the stamp. After 15 seconds, remove the PDMS from the silicon wafer by gently lifting off one side of the stamp first. Transfer the protein pattern from the elastomer to the activated SAM-coated coverslip through a stamping step similar to the one used before with initial contact being made with one side followed by gently lowering the rest of the stamp onto the coverslip. Again, apply a slight pressure with the tweezers and allow the stamp to

remain on the coverslip for 30 seconds. After this time, gently remove the PDMS stamp. Ideally, this final stamping process between the elastomer and coverslip should occur immediately after the SAM-coated coverslip has been incubated in the EDC/NHS buffer for the specified 25 minutes. If left out for too long, the hydroxysuccinimide group now present on the SAM-activated surface will hydrolyze and the efficiency of protein immobilization will decrease. We recommend that users begin the incubation of the protein on the PDMS stamp first (30 minute incubation) followed by addition of the SAM-coated coverslips into the EDC/NHS activation buffer (25 minute incubation) a short time after. Timed correctly, the printing process should occur right when the coverslips have been washed and dried.

After the PDMS elastomer has been removed from the activated SAM surface, block the non-adhesive areas by incubating the coverslips in 0.1% heat denatured BSA for 30 minutes followed by incubation in PBS for two hours to remove any biomolecules that failed to tether. At this point, the coverslips should be patterned with the specified biomolecule and further protocols utilizing such a pattern can be carried out.

E. Nanotemplate cleaning

Routinely, nanotemplates should be cleaned due to the build-up of adsorbed proteins and biomolecules after every stamping process. To do so, treat the nanotemplate with oxygen plasma for three minutes. If dust particles or the like are present on the wafer, users can also place the template in a solution of 30% isopropanol in deionized water and sonicate for five minutes. Afterwards, rinse with absolute ethanol and 30% isopropanol followed by blow drying with nitrogen air. Both sonicating and plasma treating will remove particles from the template; however, residues may still be visible on surface. Although the subtraction printing will still proceed at good efficiencies with residues present, it may be necessary to perform a more extensive clean by washing the wafer in a high percent solution of hydrofluoric acid. We recommend that this step be reserved only for extreme circumstances as working with this reagent poses considerable safety hazards and is most likely to be solely used in a clean-room facility.

IV. Discussion

Microcontact printing, a subset of soft lithography, remains to be one of the more accessible and utilized modes of biomolecule patterning due to its relatively inexpensive nature, high throughput capacity and experimental simplicity. In addition, its wide compatibility with organic and biological materials along with the lack of diffraction limit constraints together make μ CP an ideal technique that has been used to pattern for a variety of applications. Despite its many advantages however, a majority of traditional μ CP methods suffer from certain key limitations, the most critical being that of stamp deformation, that cause resolution size to remain in the micron-meter range (Hui, Jagota, Lin, & Kramer, 2002). The majority of μ CP applications begin by pouring uncross-linked PDMS over a patterned silicon master and allowing the polymer to cure. The stamp is then peeled off, inked with a biomolecule solution and then used for printing purposes with the stamp ideally being an exact negative replica of the master. Upon peeling the stamp from the master however, due to the low modulus of PDMS, deformations can easily occur in the pattern, especially when

considering nanometer-scale structures (Delamarche *et al.*, 1997; Perl *et al.*, 2009). During actual ink transfer as well, the weight of the PDMS along with small resolution sizes can cause a multitude of failures including buckling, lateral collapse and roof collapse, all of which can detrimentally affect the final desired pattern (Sharp, Blackman, Glassmaker, Jagota, & Hui, 2004). Although certain groups have attempted to develop deformation-proof stamps through the strengthening of stamp walls with thin films of polymers and metals, these methods may be inaccessible for certain laboratories and represent a significant number of additional steps for users to employ (Phan, T Waldron, & Springer, 2006; Trimbach, Feldman, Spencer, Broer, & M., 2003).

The modification to traditional μ CP described in this communication bypasses the limitations posed by the mechanical instability of the elastomeric material by utilizing a completely flat stamp surface rather than one containing ridges and corrugations. Free from the constraints of mechanical stamp failure, we have been able to utilize this technique to achieve resolution sizes superior to conventional μ CP methods. The only caveat is that the ink must be able to be transferred from the PDMS to the nanotemplate in an efficient manner. In other words, the ink must be hydrophilic enough that the hydrophobicity of the PDMS surface will force the ink to adhere preferentially to the nanotemplate upon stamp contact. Since many of these intended systems utilize hydrophilic inks, this provision is not projected to pose much of a problem for prospective users. With that being said, Coyer et al. successfully demonstrated high-contrast patterning of fibronectin at micrometer- and nanometer- scale lengths while still maintaining the non-fouling and protein resistant background in non-patterned areas (Figure 2) (Coyer *et al.*, 2011). Using fluorescent polyclonal antibodies specific for certain fibronectin epitopes, we also show the binding sites and biological activity of the tethered protein remains unaffected by this technique thus improving its applicability in the biotechnology field.

In addition, we have also put this tool to practice in order to answer fundamental cell biology questions related to cell mechanics and specifically to the generation of focal adhesion complexes. A recent study published by our group investigated whether a threshold for minimal area of integrin-FN clusters was needed for appropriate force transmission to occur between the cell and the underlying substrate (Coyer, Singh, Dumbauld, Calderwood, Craig, Delamarche *et al.*, 2012). After nanopatterning areas of fibronectin of differing size (from 1 μ m squares down to 250 nm squares) and geometries, NIH3T3 fibroblasts were seeded and allowed to generate focal adhesions on the patterned fibronectin. Immunostaining for fibronectin-bound integrins showed that on geometries exhibiting a large center square pad of fibronectin (2 μ m $\times$ 2 μ m) surrounded by 250 nm $\times$ 250 nm fibronectin “islands” surrounding the pad, cells could not form stable integrin clusters on the islands and instead, formed clusters exclusively on this center pad. Conversely, cells that were seeded onto geometries exhibiting the same center square pad but with 333 nm $\times$ 333 nm islands exhibited enhanced integrin clustering on the islands as compared to the cells seeded on the 250 nm islands. Through further characterization of intracellular focal adhesion proteins vinculin and talin in the cell, along with our findings using the patterning tool described above, our group found that there exists a surface area threshold needed for stable integrin-fibronectin complexes and that this threshold is not constant but rather is

controlled through the assembly of intramolecular proteins that regulate the balance between adhesive forces and cytoskeletal tension (Figure 3).

V. Summary

The need for reducing the resolution size of patterned biological moieties while retaining a high throughput capacity and low cost signifies a tremendous obstacle for current scientists to overcome. Numerous advantages underscore the ability to reduce patterned scales down to tens of nanometer resolution including the ability for higher density protein and DNA arrays, more in-depth studies into fundamental cell biology, and the integration of circuits into BioMEMs devices exhibiting advanced functionality. To achieve this goal, new technologies and modifications to existing ones must be made. The protocol outlined here provides a detailed guide for users to perform the many steps involved in subtractive contact printing including creation of a nanotemplate master, fabrication of PDMS stamps, SAM preparation and final contact printing procedures. We believe that the lack of need for highly specialized equipment, along with the ease and robustness presented by this system will allow researchers to utilize this technique for other biological applications as well as for continuous improvement on μ CP methods.

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Fig 1. Fabrication of cell adhesive nanopatterned substrates

(A) Schematic representing nanofabrication of protein nanopatterns. Protein inked PDMS stamps are brought into contact with a high-precision nanotemplate to selectively subtract protein of interest from the PDMS stamps. Stamps with subtracted protein patterns are next pressed against a functionalized gold coated glass coverslip to transfer the nanopatterns. (B) Functionalized gold coated substrates are prepared by exposing the coverslips to EG3/EG6 self- assembled monolayers. Carboxylic acid anchoring groups on the EG3/EG6 self- assembled monolayers are further activated with EDC/NHS to produce amine-reactive NHS- esters that react with the amine groups on any protein of interest (e.g. fibronectin, FN). (C) Layout of a typical nanopattern (500nm×4 shown) with adhesive zone of nanoislands. Each adhesive zone consists of a center square (2×2 μm) surrounded by 8 radially distributed adhesion pads that are made of 1, 4, or 9 square nanoislands. Four adhesive pads are projected at 45° and four at 90° with respect to the center pad. [Adapted with permission from(Coyer *et al.*, 2011)].

Fig 2. Protein tethered nanopatterns

A) Fluorescence micrograph represents an array of eight adhesive pads with 1000×1 nm islands around a center square (2×2 μm), produced over large area of 490 mm². **B)** AlexaFluor 555-conjugated fibronectin printed into 1000×1 nm, 500×4 nm, 333×9 nm, and 250×4 nm configurations using the subtractive nanopatterns. scale bar 1 μm. [Adapted with permission from(Coyer *et al.*, 2011; Coyer *et al.*, 2012)].

Fig 3. Nanoscale adhesive geometry reveals an adhesive ECM area threshold for stable FN-integrin clustering and adhesion

(A) Fluorescence microscopy analysis of integrin (green) recruitment to AlexaFluor 555-labeled FN nanopatterns (red) reveals that head domain of focal adhesion protein talin drives integrin-FN clustering at nanoscale area formed by 250×4 nm islands. Control cells with no over expression of talin head or with mutations in in talin head domain failed to form stable adhesion complexes on these 250×4 nm islands. No differences in adhesion was observed on 500×4 nm islands. (B) Cell adhesive force analysis reveals stringer ECM-binding force response in cells overexpressing talin head. Bar graph represent fold change in adhesive force response over control talin1(A/E)-transfected cells adhering to 250nm×4 patterns (mean ± SD) (* p < 0.05). (C) Nanoscale adhesion of vinculin mutant cells reveal that vinculin regulates nanoscale assembly of adhesive clusters by balancing integrin-ECM adhesive force and cytoskeletal tension. Expression of a wild type vinculin (VinWT) in vinculin null mouse embryonic fibroblasts or expression of VinT12 that has a mutated head-tail interface that reduces the head-tail affinity 100-fold and thus exposes binding sites for talin and actin failed to form stable FN-integrin clusters on 250×4 nm islands. Expression of only the head domain of vinculin (V_H) resulted in stable FN-integrin clusters on 250×4 nm islands, indicating a role of tail mediated actomyosin contractility in formation of stable adhesion complexes. (D) Inhibition of actomyosin contractility with blebbistatin (20 μM) in VinT12 expressing cells restored the ability of the cell to form stable FN-integrin clusters on 250×4 nm islands. Scale bar 1 μm. [Adapted with permission from (Coyer *et al.*, 2012)].