**Conformal Nanopatterning of Extracellular Matrix Proteins onto Topographically Complex Surfaces**

Yan Sun1,2, Quentin Jallerat1, John M. Szymanski1 and Adam W. Feinberg1,3

**SUPPLEMENTARY TEXT AND FIGURES**

**SUPPLEMENTARY NOTE**

**Current Soft Lithographic Methods for Patterning ECM Proteins on Surfaces**

A range of soft lithography techniques have been developed for controlling cell adhesion, structure and function on 2D biomaterial surfaces[1-8](#_ENREF_1" \o "Wang, 2004 #19). In particular, microcontact printing (μCP) has been used extensively to achieve accurate and reproducible chemical patterning[9-11](#_ENREF_9" \o "Tien, 1998 #21) and to control the transfer of biomolecules such as DNA[12](#_ENREF_12" \o "Fredonnet, 2013 #24) and the ECM proteins FN and laminin[13](#_ENREF_13" \o "Feinberg, 2010 #10) to a range of substrates.

The transfer of biomolecules depends in large part on the quality of the conformal contact between the substrate and the raised topographical features of the elastomeric stamp. PDMS (Sylgard 184, Dow Corning) is the most commonly used material for the elastomeric stamp because its relatively low Young’s modulus (*E* ~1.7 MPa)[14](#_ENREF_14" \o "Palchesko, 2012 #2124) enables it to conform to a range of typical cell culture surfaces. Based on mechanical models, PDMS is expected to conform to sinusoidal asperities with a height of 100 nm and a 1 μm wavelength (an approximate depth-to-width aspect ratio of 1:10)[15](#_ENREF_15" \o "Bietsch, 2000 #25). This aspect ratio is predicted to be preserved across several orders-of-magnitude, extending into the 10 to 100 μm range we investigate in our current study. Decreasing the Young’s modulus of the elastomeric stamp and increasing the normal pressure applied during stamping are major factors that decrease the energy threshold required for conformal contact with rough surfaces. However, it has been shown theoretically[15](#_ENREF_15" \o "Bietsch, 2000 #25), [16](#_ENREF_16" \o "Hui, 2002 #2245) and experimentally[17](#_ENREF_17" \o "Sharp, 2004 #27) that taking these steps to improve contact can come at the cost of decreased pattern fidelity due to collapse and/or buckling of the raised features on the PDMS stamp. Our results demonstrate this fact, as decreasing the Young’s modulus of the PDMS stamp (*E* ~50 kPa) enabled patterning of the bottom of trenches between micro-ridges, but at the expense of pattern fidelity on the ridge tops (**Supplementary Fig. 7**). Specifically, the raised features of the stamp will compress and lead to protein transfer from the recessed areas[16](#_ENREF_16" \o "Hui, 2002 #2245). Thus, µCP is limited by the compromise between the need for a stamp to be able to conform to a rough surface and the requirement that the micro and nanoscale features do not deform and cause loss of pattern fidelity.

To address this challenge, many groups have developed modified versions of μCP in order to pattern a wider range of surfaces. For example, researchers have created composite stamps made of a thin layer of stiff PDMS (*E* > 9 MPa) to provide rigid microtopography supported by a layer of softer PDMS so that the stamp can conform to non-planar and rougher surfaces[5](#_ENREF_5" \o "Qin, 2010 #15), [18](#_ENREF_18" \o "Schmid, 2000 #385), [19](#_ENREF_19" \o "Odom, 2002 #29). Patterning of non-planar, curved surfaces has also been achieved by rolling cylindrical substrates over flat stamps[20](#_ENREF_20" \o "Jackman, 1995 #30). Other researchers have devised a two-step process where biomolecules are first μCP onto a sacrificial surface that is then put in contact with a non-planar substrate and dissolved[21](#_ENREF_21" \o "Fernandez, 2011 #31), [22](#_ENREF_22" \o "Yu, 2012 #32). These examples have improved the ability to μCP non-planar surfaces and can adapt to greater surface roughness; however, the surfaces still need to be relatively smooth so that a conformal contact can occur either between stamp and substrate or sacrificial mold and substrate. This requirement prevents the transfer of biomolecules to high aspect ratio topographies.

Increasing interest in designing complex cellular microenvironments has led to the development of several methods to engineer substrates with both chemical and topographical micropatterning. The simplest approach is to use μCP to pattern proteins on the top of raised features in a microtopographically patterned substrate. For example, the tops of micro-ridges can be μCP with FN to increase endothelial cell alignment over microtoprography or micropatterned FN alone[23](#_ENREF_23" \o "Feinberg, 2007 #1418). Multiple research groups have used similar approaches to selectively pattern the tops of microtopographical features with either cell-repellant or cell-adhesive chemicals[24-26](#_ENREF_24" \o "Dusseiller, 2005 #33). In these cases, the recesses in the substrate, such as the bottom and the sides of the trenches between micro-ridges, can be functionalized by adsorption from solution. Thus, the microtopography defines the spatial organization of the chemical pattern. There have also been efforts to engineer chemical micropatterns independent of surface microtopography. For example, photolithography was used to create lanes of aminosilanes across 6 µm tall ridges on etched glass substrates[27](#_ENREF_27" \o "Britland, 1996 #36). Charest *et al.* used µCP instead of photolithography in order to pattern regions of fibronectin across the top of 4 µm tall ridges in polyimide[28](#_ENREF_28" \o "Charest, 2006 #37). However, constrained by the limits of standard µCP, they were unable to get FN within the trenches and concluded that the discontinuity of the chemical pattern was a determinant factor to explain why cells cultured on these substrates aligned with the microtopography for all conditions studied. Morase *et al.* have also shown that micro-ridges with conformal fiber-like proteins can be generated by controlled crack formation in multilayered microfabricated materials under applied strain, removing the requirement for lithographic equipment[29](#_ENREF_29" \o "Moraes, 2014 #2243). However, while all of these techniques improve the ability to engineer micropatterned chemistry and topography on surfaces, none enables independent control.

The PoT printing technique we have developed is unique in its ability to engineer surfaces with largely independent control of surface ECM protein chemical patterning and topographical micropatterning. We use micro-ridges with various width, spacing and height in order to create uniform surfaces to evaluate fidelity and repeatability. Results clearly demonstrate that we can pattern a range of microscale topographic feature dimensions from <1 to >100 µm (**Fig. 1, 2, 3** **and Supplementary Figs. 2, 3, 4, 5, and 6)** with a variety ECM proteins and pattern dimensions. Further, surface features with depth-to-width aspect ratios as high as 1.85 can be patterned with conformal ECM proteins and greater than 2.4 if some loss of pattern fidelity is acceptable. Thus, PoT represents the only viable method to pattern biomolecules in a continuous or near continuous manner across rough and high aspect ratio microtopographies.

**Limitations of the PoT printing technique**

The PoT printing technique has the ability to micropattern ECM proteins on a wide range of surface microtopographies. However, it has inherent limitations based on the way protein transfer is achieved. During PoT the ECM protein patterned on the PIPAAm surface is released and pushed onto the microtopography, which means it must be stretched. There are two factors that determine how much the ECM protein can be stretched and the scale of surface roughness that can be patterned, the thickness of the PIPAAm layer and the mechanical properties of the ~5 nm thick ECM protein layer. The thickness of the PIPAAm layer can be tuned by changing the spincoating parameters and the viscosity and concentration of the PIPAAm/butanol solution (**Supplementary Fig. 1**). The thicker the PIPAAm layer the more material there is to swell and push the ECM protein during the thermal dissolution process (**Fig. 1a**). There is also a limit to (i) how far the ECM protein layer can be strained before it fails and (ii) the mechanical force required to do so. Previous work has demonstrated that the FN nanofibers patterned on the PIPAAm are initially in a pre-stressed state, and that this is ~3.5 times longer than the contracted length if released directly into solution[30](#_ENREF_30" \o "Feinberg, 2010 #1784), [31](#_ENREF_31" \o "Szymanski, 2014 #2226). From this fully contracted state, previous experiments have shown that the FN nanofibers can be stretched up to 9 times[32](#_ENREF_32" \o "Deravi, 2012 #2180). During PoT the regions of topographic surface that touch the PIPAAm pin the FN in these locations and the remaining regions of FN are stretched by the expanding PIPAAm during transfer. For the 20 µm wide, 20 µm spaced micro-ridges the FN on the ridge tops is pinned and only the FN between the ridges (i.e., over the trench) is stretched. Results show that we can PoT print on 37 µm deep micro-ridges to achieve a depth-to-width aspect ratio of 1.85 (**Supplementary Fig. 3**), which equates to straining the section of FN between ridges (initially 20 µm in length) nearly 400%. We also demonstrate that we can PoT print on micro-ridges 48 µm deep at a depth-to-width aspect ratio of 2.4, however at this amount of strain (nearly 500%) some loss of fidelity due to gaps in the transferred FN is observed (**Supplementary Fig. 3**). These results reveal that in PoT printing with FN, the maximum strain that can be achieved before some mechanical failure of the FN occurs is between 400% to 500%. Beyond this failure point PoT printing still works, but it appears that the FN begins to tear apart in places and forms gaps on the vertical sidewalls, though importantly the trench bottoms are still patterned. In terms of applications, the effect these gaps might have on cell behavior is unknown, and will likely be cell type and context dependent.

**Supplement-only References**

1. Wang, Y.C. & Ho, C.C. *FASEB J.* **18**, 525-+ (2004).

2. Whitesides, G.M., Ostuni, E., Takayama, S., Jiang, X. & Ingber, D.E. *Annu. Rev. Biomed. Eng.* **3**, 335-373 (2001).

3. Kane, R.S., Takayama, S., Ostuni, E., Ingber, D.E. & Whitesides, G.M. *Biomaterials* **20**, 2363-2376 (1999).

4. Kim, H.N. *et al.* *Ann. Biomed. Eng.* **40**, 1339-1355 (2012).

5. Qin, D., Xia, Y.N. & Whitesides, G.M. *Nat Protoc* **5**, 491-502 (2010).

6. Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M. & Ingber, D.E. *Biotechnology Progress* **14**, 356-363 (1998).

7. Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M. & Ingber, D.E. *Science* **276**, 1425-1428 (1997).

8. Singhvi, R. *et al.* *Science* **264**, 696-698 (1994).

9. Tien, J., Xia, Y. & Whitesides, G.M. in Thin Films (Academic Press, 1998).

10. Quist, A.P., Pavlovic, E. & Oscarsson, S. *Anal. Bioanal. Chem.* **381**, 591-600 (2005).

11. Perl, A., Reinhoudt, D.N. & Huskens, J. *Advanced Materials* **21**, 2257-2268 (2009).

12. Fredonnet, J. *et al.* *Microelectronic Engineering* **111**, 379-383 (2013).

13. Feinberg, A.W. & Parker, K.K. *Nano Lett.* **10**, 2184-2191 (2010).

14. Palchesko, R.N., Zhang, L., Sun, Y. & Feinberg, A.W. *PLoS ONE* **7**, 1-13 (2012).

15. Bietsch, A. & Michel, B. *J. Appl. Phys.* **88**, 4310-4318 (2000).

16. Hui, C.Y., Jagota, A., Lin, Y.Y. & Kramer, E.J. *Langmuir* **18**, 1394-1407 (2002).

17. Sharp, K.G., Blackman, G.S., Glassmaker, N.J., Jagota, A. & Hui, C.Y. *Langmuir* **20**, 6430-6438 (2004).

18. Schmid, H. & Michel, B. *Macromolecules* **33**, 3042-3049 (2000).

19. Odom, T.W., Love, J.C., Wolfe, D.B., Paul, K.E. & Whitesides, G.M. *Langmuir* **18**, 5314-5320 (2002).

20. Jackman, R.J., Wilbur, J.L. & Whitesides, G.M. *Science* **269**, 664-666 (1995).

21. Fernandez, J.G., Samitier, J. & Mills, C.A. *J. Biomed. Mater. Res. A* **98**, 229-234 (2011).

22. Yu, H., Xiong, S., Tay, C.Y., Leong, W.S. & Tan, L.P. *Acta Biomater.* **8**, 1267-1272 (2012).

23. Feinberg, A.W. *et al.* *Journal of Biomedical Materials Research Part A* **86A**, 522-534 (2007).

24. Dusseiller, M.R., Schlaepfer, D., Koch, M., Kroschewski, R. & Textor, M. *Biomaterials* **26**, 5917-5925 (2005).

25. Embrechts, A. *et al.* *Langmuir* **24**, 8841-8849 (2008).

26. Martinez, E. *et al.* *Nanomedicine-Uk* **4**, 65-82 (2009).

27. Britland, S. *et al.* *Exp. Cell Res.* **228**, 313-325 (1996).

28. Charest, J.L., Eliason, M.T., Garcia, A.J. & King, W.P. *Biomaterials* **27**, 2487-2494 (2006).

29. Moraes, C. *et al.* *Lab on a Chip* **14**, 2191-2201 (2014).

30. Feinberg, A.W. & Parker, K.K. *Nano Letters* **10**, 2184-2191 (2010).

31. Szymanski, J.M., Jallerat, Q. & Feinberg, A.W. *Journal of Visualized Experiments*, e51176 (2014).

32. Deravi, L.F. *et al.* *Nano Letters* **12**, 5587-5592 (2012).