Spatially Defined, Multiplexed Arrays Supporting Cell Adhesion

J. Johnson[1], J. Adams[1], M. Pagano[2], K. Radke[1], Z. Ge[2], E. Henderson[1, 2], S. Nettikadan[1]

[1] BioForce Nanosciences, Inc., Ames, IA [2] Iowa State University, Ames, IA

ASCB 2007 Program №. 1180

ABSTRACT: *Spatial control of cell adhesion to surfaces is of critical importance to fundamental studies of cellular biology and tissue engineering. Cell functionality including inter and intra-cellular signaling, the ability to differentiate and to divide are all dependent on spatial organization and interaction. Our understanding of spatial effects on these processes will enable tissue engineering supporting implantation, repair and reconstruction of simple and complex tissues and organs. We used a Nano eNabler (NeN) to create spatially-defined, multiplexed arrays of proteins with domain sizes between <10 and 25 µm in diameter to study cell adhesion. Extracellular matrix (ECM) proteins and IgG were interlaced onto either glutaraldehyde-activated 3-aminopropyltriethoxysilane (APTES-ALD) or hydrogel glass surfaces (HG). In order to obtain consistent printing and prevent evaporation, the proteins were arrayed in 7.5% glycerol in PBS. Freshly trypsinized NIH 3T3 cells were adjusted to ~ 5.0 x 10^6 cells/mL in RPMI 1640 medium containing 7.5% fetal bovine serum and applied to arrayed surfaces in 30 mm Petri dishes. At 7 min intervals the Petri dishes were tilted to resuspend unattached cells. Binding to all of the ECM domains in arrays was achieved within 15 min. No cell binding was observed on the IgG domains or supporting glass surfaces. We found specific cell binding to ECM domains was a function of ECM concentration, time, domain size, blocking reagent and the surface used to immobilize the proteins. On the APTES ALD surfaces, the 3T3 cells initially bound to the ECM domains but cell motility blurred array patterns with time. On hydrogel, however, 3T3 cells remained attached at the location of the ECM for 3 days and underwent 3 divisions to clusters of 8 cells. Our findings illustrate the potential for this method to be applied in tissue engineering studies aimed at identifying important biological structure-function relationships.*

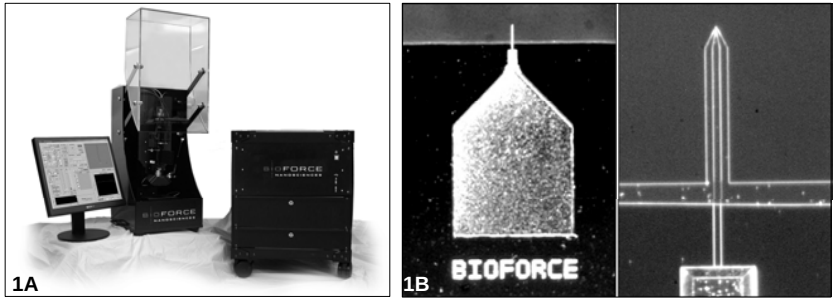

Fig. 1 Instrumentation: We have developed a technology for deposition of liquids in femtoliter to attoliter volumes on solid surfaces and this can be used to print biological materials in intricate and multiplexed, multicomponent patterns. This technology is embodied in the Nano eNabler System™ (NeN) consisting of the Nano eNabler (Fig. 1A) instrumentation (the printer) and the surface patterning tools (SPTs) (Fig. 1B and close up image). Components of the Nano eNabler include precision motion control X and Y axis stages, a coarse Z axis stage, a high-precision fine Z axis stage, a video monitoring system, an environmental control system, a force feedback system used to control and maintain printing force, and a graphical user interface for pattern design and an instrument control.

INTRODUCTION: Cell patterning at the micron-scale will help elucidate communications, control and differentiation in tissues and may act as a segue to construction of artificial tissues. Several microfabrication techniques have been developed to generate controlled patterns of cells to aid the study of attachment, properties, and growth of cells.

We have invented a controllable, contact depositional technology, the Nano eNabler™ System (NeN), to pattern surfaces with a variety of proteins and other molecules. One major advantage of the NeN over conventional microfabrication printing systems is that it accurately places materials in ~1-50 micron domains in user defined geometric patterns. This technology purveys flexibility in printing patterns and deposition of multiple species with micron-scale spatial resolution and can be used to create multicomponent and interlaced multicomponent deposition networks.

We used the NeN to pattern human basal cell extracellular matrix proteins (ECM's) and purified rabbit IgG (IgG) onto 3-aminopropyltriethoxysilane-coated borosilicate glass treated with glutaraldehyde to create an aldehyde silane terminus (APTES-ALD), Acrylamide hydrogel (HG), and polystyrene (PS). Arrayed surfaces were incubated with NIH swiss 3T3 cells and the binding patterns of cells on these arrays were studied.

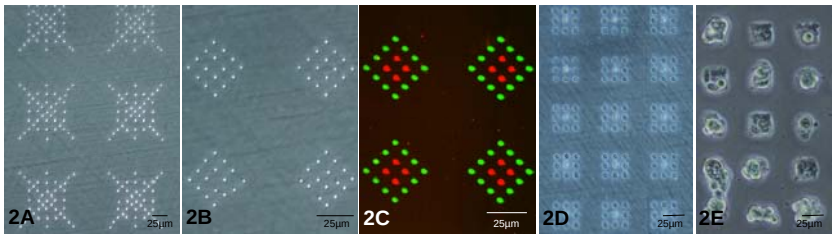

Fig. 2 Flexibility & Multiplexing: Complex patterns of ECM's (2A) and multiplexed, multicomponent patterns of interlaced ECM and IgG in diamonds (2B) as demonstrated by fluorescent labeling where the ECM labeled with a Mo anti-fibronectin primary (green) and goat anti-rabbit IgG (red). False colors applied with Meta Vue (2C). Nine-spot (7µm dia.) patterns of ECM's (clear) with a center deposition of IgG (opaque) (2D) to bind 3T3 cells (2E). PS, 2A and 2D. On APTES-ALD, 2B and 2C.

METHODS: A single lever SPT was oxidized by UV-ozone in a ProCleaner™ to enable flow and loaded with a human basal membrane ECM extract (Becton-Dickinson, Bedford, MA) (0.2 mg/mL in PBS buffer, 5% glycerol) or rabbit IgG (0.1 mg/mL in PBS buffer, 5% glycerol). The SPT was mounted and incubated (20 min at 70% RH) prior to arraying. The SPT cantilever was brought into contact with the substrate under controlled applied force and environment to transfer the ECM's from the SPT to the substrate. Repetition with X,Y translocation created the pattern. The deposition volume depended chiefly on SPT geometry, composition of deposition material, SPT environment, contact time, applied force, and surface and surface chemistry.

ECM's and IgG were printed on APTES-ALD , HG, and PS. The printing conditions were optimized to print spots from 7 to 25 µm. Arrays of ECM's were printed in different geometric patterns consisting of tens to several hundreds of spots per array. Following a 2 h incubation at RT in sealed, humidified Petri dishes, the APTES-ALD chip surfaces were blocked 2.5 h at RT using a filter sterilized solution of 4% pluronic F-127 containing 15 mg/mL BSA in PBS; PS surfaces were blocked with 10% pluronic F-127 in PBS for 15 min followed by draining.

Cultures of subconfluent NIH 3T3 cells were harvested by Trypsin-EDTA, collected, and suspended in RPMI-1640 culture media containing 7.5% fetal bovine serum (CM). The surfaces were incubated with sufficient amounts of cell suspensions (1.0 x 10^6/mL in CM) to cover the surface. Incubation was for 7-8 min in the CO_2 incubator. Chips and surfaces were rinsed twice with CM to remove the unbound and loosely bound cells. The chips were incubated for 2 to 12 days.

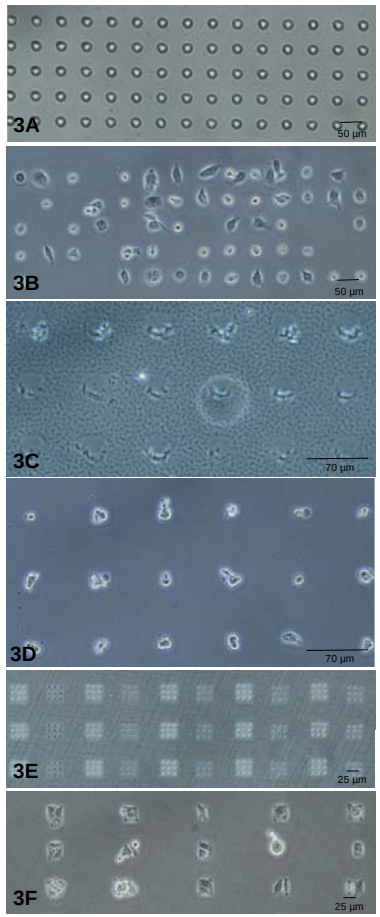

Fig. 3 Patterns and binding of NIH 3T3 on different substrates: The NeN is able to print different size depositions in different patterns of ECM's on different substrates such as 25 µm dia. spots with a 50 µm pitch on APTES-ALD (3A), depositions with a 70 µm pitch on HG (3C), and 7 µm dia. spots in clusters on PS (3E). After incubation, NIH 3T3 cells were found to be bound exclusively to ECM depositions on each of the three surfaces: APTES-ALD (3B), HG (3D), and PS (3F). Surface images taken 30 min post free and unbound cell rinsing.

Furthermore, we were able to adjust the depositional diameter and assay conditions such that each deposition (or set of depositions as in 3E) would affix one single cell

Additionally, we were also able to print multiplexed, multicomponent ECM patterns and rabbit IgG in alternating columns of nine-spot arrays (3E) and demonstrate that, on PS, only the ECM bound NIH 3T3 cells (3F). Neither the surface nor the IgG was found to bind cells.

Single cells spread to the shape of the nine-spot array (Squares).

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1615 Golden Aspen Dr. Ste. 101
Ames, IA 50010
www.bioforcenano.com

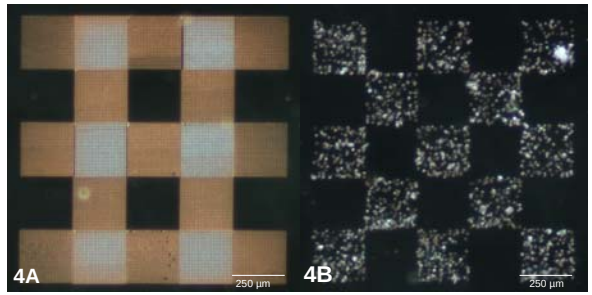

Fig. 4 Multicomponent protein patterns of ECM and IgG - Large Array Format: Multiplexed 7µm spots with 12 µm pitch in 20 x 20 patterns of ECM (4120 spots) (orange) and IgG (2400 spots) (white) on PS (4A). NIH 3T3 cells on blocked surface using standard assay (4B). No 3T3 cells were found bound to either the large patterns of IgG or to the blocked PS substrate (dark squares-4A). With outgrowth, cells occupied the available ECM surface but did not enter the IgG or the blocked PS surface.

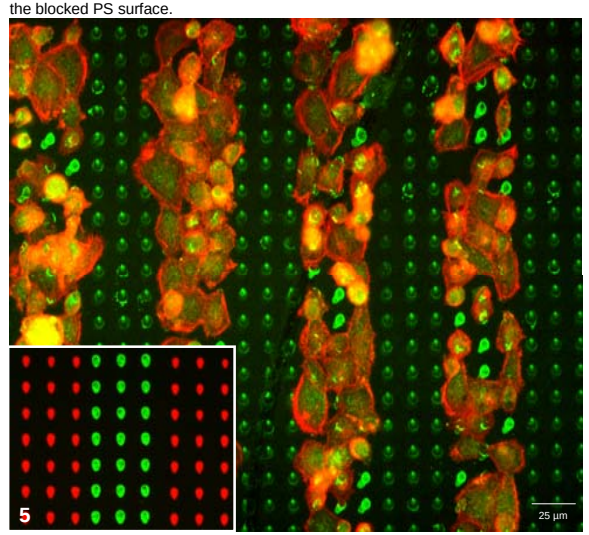

Fig. 5 Restriction of NIH 3T3 cells to ECM patterns on an APTES-ALD surface with multicomponent proteins: Multicomponent patterns of ECM (red flurorescence) and IgG (green) were created on APTES-ALD glass surfaces (3 spots x 20 spots – 7 µm dia. x 12 µm pitch) in alternating patterns (see inset). 3T3 cells were applied in standard assay to these chips and following removal of the free cells and were incubated for 30 min in the incubator. Chips were immersed in formalin (3.7%) and the fixed cells permeabilized with Triton x100. ECM and IgG were detected with biotin anti-fibronectin and biotin goat anti-rabbit IgG following reaction with Alexifluor 488 streptavidin. Cells were labeled with phallotoxin. Living 3T3 cells initially bound to ECM then moved elsewhere.

CONCLUSIONS: Patterns of ECM spots on APTES-ALD, HG, and PS surfaces were found to support NIH 3T3 adherence while depositions of IgG spots did not. Cells did not bind to the substratum. The number of cells bound to a given spot was a function of spot size and the NeN could be tuned to produce spots of a size capable of binding single cells on each of these surfaces. Outgrowth and cell migration was a function of the surface. 3T3 cells migrated from attachment sites on APTES ALD surfaces but did not on either PS or the acrylamide HG surface used. On these latter two surfaces cells multiplied to form small aggregates of cells that remained attached. Large patterns on PS resulted in precisely copied cell patterns without outgrowth into non-patterned surfaces. With this technology it is possible to interrogate the ventral surface of living cells with differentiation-inducing, signal transductants.