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Application note on Micro-patterning for cell-based assays

Micropatterning for cell-based assays

Application note

Introduction

Cellular heterogeneity is key principle in biology that can be linked to individual health status of humans. To investigate the normal, cancer as well as non-responding cells following exposure to chemicals, nanomaterials and bioactive substances single-cell assays in microfluidic platform are the perfect tool. Cell-based assays such as cell migration, tube formation, chemotaxis and cell culture under microfluidic setup are allowing for quantitative, reproducible and real-time experimental conditions that will facilitate the development of personalized medicine solutions.

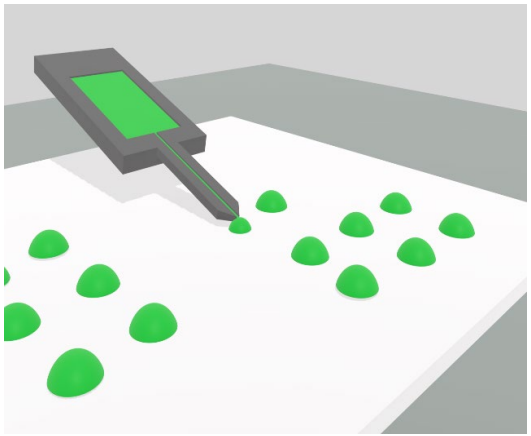
Development of miniaturized assays that allow for large test areas to be created and tested but require only small volumes of test analytes to be used, as often only small test sample sizes are available, is an intensively evolving field. Developing such miniaturized biochips containing arrays of test molecules requires a methodology that allows the user to deposit molecules at high resolution and speed while maintaining functionality. Microchannel Cantilever Spotting (µCS) is an ideal technique to create such biologically active surfaces. It has been successfully applied in the direct deposition of functional proteins as well as in the fabrication of biochemical templates for selective adsorption.

In this Application Note we describe the utilisation of µCS in the generation of protein arrays of the Fibronectin onto glass surfaces modified with GPTMS as a model system. µCS using proteins as binding molecules, enables the decoration of solid supports with tailored patches of proteins, that can be used as *in vitro* models. In addition, such arrays, when prepared with multiple protein can be applied in complex cell culture experiments, but still allowing for the analysis at the single cell level.

Workflow

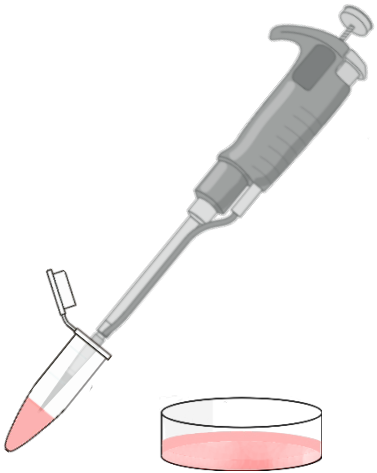
The workflow for preparing the cell arrays is shown below and can be divided into three distinct steps:

Step 1



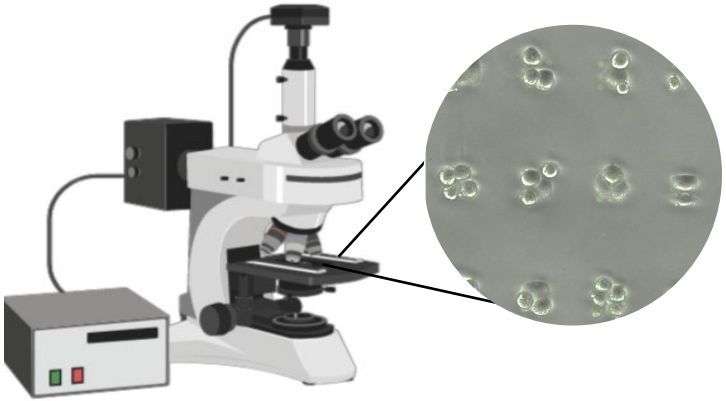
Printing ECM proteins

Step 2



Cell binding

Step 3



Detection and quantification of cells
(e.g. Fluorescence microscope)

Step 1 - Printing ECM Protein

Application note

Ink preparation

To prepare the ink for printing protein arrays the following components were used:

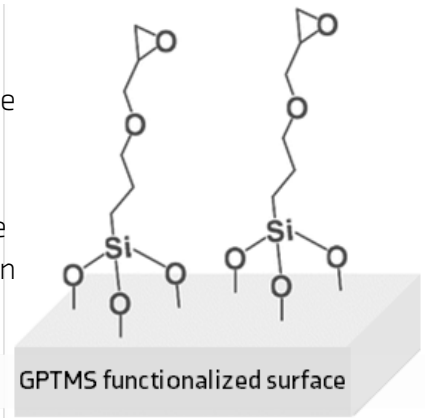
- Fibronectin (1 mg/ml, Sigma Aldrich)
- Glycerol (87%, Sigma Aldrich)

Glycerol is added to prevent ink from drying out. 30 % v/v glycerol can be added (e.g. to 7 µl of protein solution, 3 µl of glycerol).

Prior to spotting, the probe (SPT-S-C10S, Bioforce Nanosciences) was activated in oxygen plasma (100 mbar oxygen pressure, 30W power, 2min.). From the ink mixture 0,5 µl was taken out and applied into the reservoir of the activated probe. Such ink loaded probe is ready for printing the ink onto the target surface.

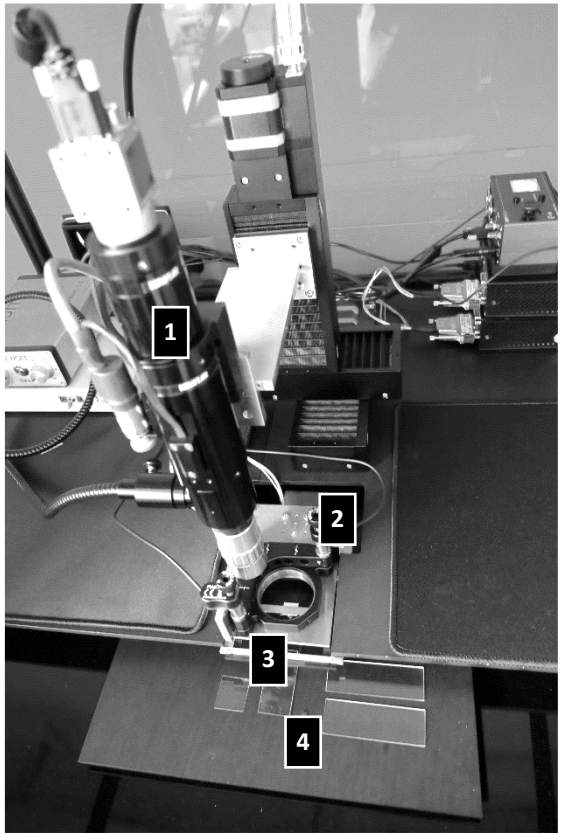
Substrate preparation

Shortly, glass coverslips were cleaned with in an ultrasonic bath with ethanol (Merck, Germany), acetone (Merck, Germany), ultrapure water for 10 min each. Then, substrates were dried under a gentle flow of nitrogen and treated with oxygen plasma for 2 min. (100 mbar oxygen pressure, 30W power, 2min.) to allow surface activation for the subsequent silanization. Next, the cleaned substrates were incubated for 2 h with 1% (3-Glycidyloxypropyl)trimethoxysilane (Sigma Aldrich, Germany) in Toluene (Merck KGaA, Germany). After silinization, the glass substrates were cleaned again with acetone in an ultrasonic bath and dried under nitrogen flow before storing under argon at room temperature.



After printing the protein ink but before loading the cells, sample surface was also blocked with 10% BSA in PBS for 30 min. at room temperature and washed 3x with PBS.

Step 1 - Printing ECM Protein



1 Microscope module for *in situ* process control (image and video aquisition)

2 High precision module for high resolution control over the printing/spotting process

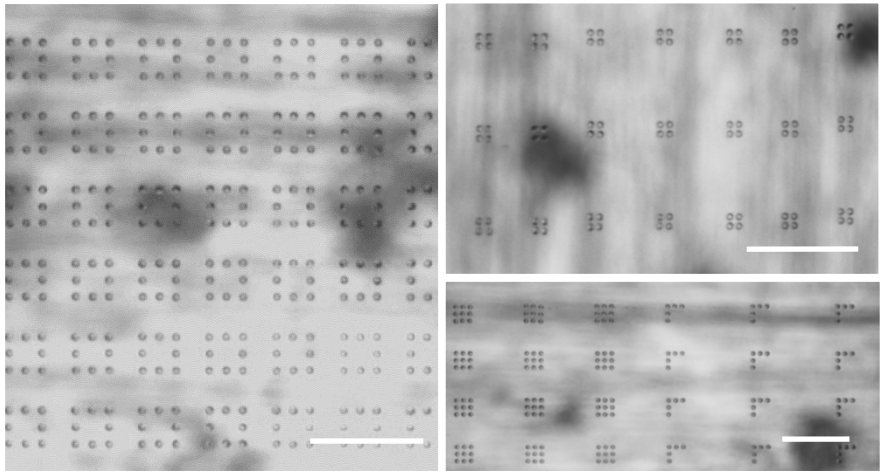
3 Probe holder for mounting probes

4 Sample table for loading the substrates

Molecular Printer Set-up

The probe loaded with ink is mounted onto the probe holder.[3] Spotting of the ink onto the functionalized surface takes place by automated bringing the tip of the probe into contact with the surface for a defined time (0,1 s) at the controlled humidity (45 % R.H.).

After the spotting step, femtoliter sized droplets of ink are allowed to react with the GPTMS on the surface for 2h in the dark at ambient humidity.
Below are examples of various patterns that can be printed for the cell binding experiments (scale bar equals 100 µm).



Step 2 and 3 - Cell binding and Detection (Fluorescence microscopy) Application note

Before loading the cells, sample surface was blocked with 10% BSA for 30 min., and washed with PBS.

For the binding experiments the cells should be prepared based on the cell type and the experimental question.

For the experiment presented here, 3T3 Fibroblasts were first seeded at $1x10^7$ concentration and cultured for 24h in DMEM medium supplemented with 10% FCS. Next, the cells were detached using Trypsin-EDTA (Sigma Aldrich), washed and re-suspended at 1×10^6 cells / ml in DMEM medium supplemented with 10% FCS, but without Phenol Red.

- 10^4 cells were loaded on the sample surface that was pre-warmed at 38°C, and let to adhere for 10, 20 and 30 min at 37°C.
- excess of cells was washed off with pre-warmed PBS.

Immunofluorescence:

- adhered cells were fixed with pre-warmed 3.8% paraformaldehyde in PBS for 20 min. at room temperature
- samples were washed with PBS and blocked with 10% BSA in PBS for 30 min.
- 5 µg/ml of primary antibody (anti-Fibronectin antibody, Millipore) was loaded on the sample and incubated 1h at room temperature
- after washing with PBS, solution of 5 µg/ml of secondary antibody (anti-Rabbit Alexa 488 antibody, Millipore) was loaded onto the samples and incubated 40 min. at room temperature
- after washing with PBS samples were placed on the microscope and imaged in FITC and bright field channel.

Figure 1a shows washed sample where cells were allowed to attach for 30 min. Staining with anti-fibronectin antibody shows that protein was deposited on the substrate and cells adhere exclusively on the dot protein arrays (Figure 1b).

Fluorescence microscopy images were taken with a Nikon upright fluorescence microscope (Eclipse 80i), equipped with a sensitive camera CoolSNAP HQ2 (Photometrics). The broadband excitation light source (Intensilight illumination) is combined with sets of filters (Texas Red, FITC, DAPI) to separate excitation and emission spectra, depending on the dye molecule used.

Results

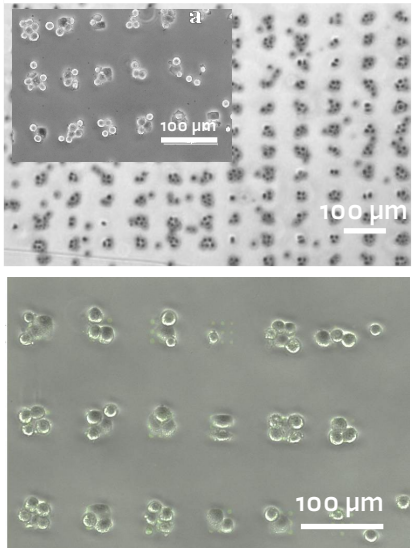


Figure 1. Adhesion of 3T3 Fibroblast cells onto fibronectin dot arrays.
10^4 cells were loaded onto the sample and incubated at 37° for 30 min. Sample was imaged before washing (Top image). After washing and staining with the anti-fibronectin antibody sample was imaged again to visualize the patterned fibronectin protein and adhesion of the cells onto the fibronectin pattern (Bottom image).

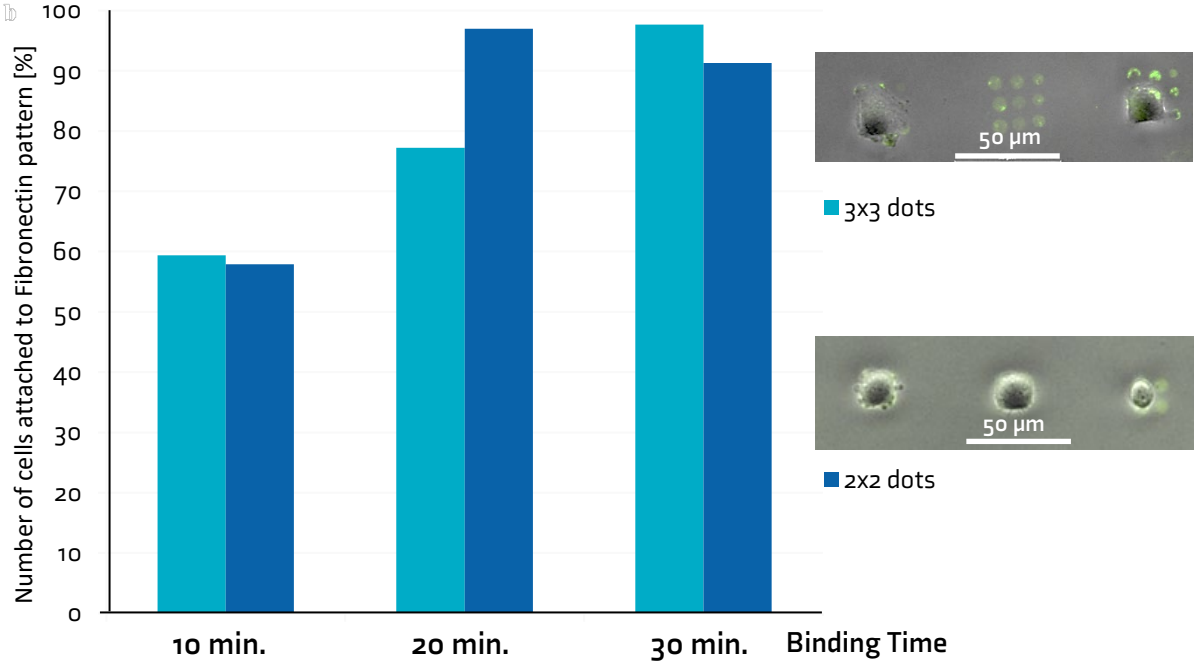


Figure 2. Adhesion of fibroblast cells onto fibronectin dot arrays.
10^4 3T3 fibroblasts cells were incubated on the Fibronectin arrays of 2x2 or 3x3 dots for 10, 20 or 30 min. After washing and staining with the anti-fibronectin antibody sample was imaged to visualize the adhesion of the cells on the patterns. Adhesion was then quantified and is presented in the graph as a number of cells occupying dot arrays in function of time. 2x2 arrays are occupied at almost 100% after 20 min cell binding, whereas 3x3 dots are occupied almost fully by cells after 30 min binding time. The average single dot size in the array is about 5µm in diameter.

Conclusions

Cell adhesion is a complex process involved in migration / invasion, embryogenesis, wound healing and tissue remodelling. Cells adhere to the extracellular matrix by cell junctions, forming complexes with cytoskeleton components that can affect cell motility, differentiation, proliferation and survival. Various extracellular matrix (ECM) component proteins are continually being examined for their in effects on the adhesion, migration, and invasion on many diverse cell types.

Adjusting the biochemical and mechanical cues of the cell microenvironment *in vitro* ,allows to generate more physiologically-relevant cell models. Micropatterns for cell adhesion are a perfect tool for designing *in vitro* systems for quantitative, reproducible and real-time experimental conditions. Such models allows to easily study the behaviour and development of living cells in a broad range of applications such as: cell adhesion for single cell screens and adhesion force measurements, cytoskeleton dynamics, cell confinement, cell migration, tissue engineering, spheroid formation. Isolating single cells under reproducible conditions is fundamental for *in vitro* cell assays. Micropatterning of adhesion proteins at a cellular and subcellular scales with µCS allows for a flexible fabrication of models for cell-based assays.

However, micropatterning is not restricted to single cells. With the use of bigger or multiplexed (more then one adhesion protein printed) micropatterns, high content screening assays on cell populations can be easily conducted. Increasing the complexity of the *in vitro* models brings it closer to physiologically-relevant conditions. And that in turn brings it closer to accurate design of assays that can be used in personalized medicine solutions.

Selected literature:

- Fabrication of 3-D Reconstituted Organoid Arrays by DNA-Programmed Assembly of Cells (DPAC).
  Todhunter et al., 2016, Current Prot. in Chem.Biol
- Micropatterned ECM Substrates Reveal Complementary Contribution of Low and High Affinity Ligands to Neurite Outgrowth.
  Fereol et al., 2011, Cytoskeleton 68, 373–388
- The extracellular matrix at a glance.
  Frantz et al., 2010, J Cell Sci. 15, 123(24): 4195–4200.
- A matrix micropatterning platform for cell localization and stem cell fate determination.
  Huang et al., 2010, Acta Biomaterialia 6,12,4614
- Cell-Compatible, Multicomponent Protein Arrays with Subcellular Feature Resolution.
  Mei et al., 2008, Small 4, 10, 1600–1604

Microchannel Cantilever Spotting (µCS) in literature:

- Phospholipid arrays on porous polymer coatings generated by micro-contact spotting.
  Sekula-Neuner et al., 2017, Beilstein J. Nanotechnol. 8, 715–722
- Click-Chemistry Immobilized 3D-infused Microarrays in Nanoporous Polymer Substrates.
  Hirtz et al., 2016, Adv. Mater. Interfaces 3, 1500469
- Attoliter Chemistry for Nanoscale Functionalization of Graphene.
  Hirtz et al., 2016, ACS Appl. Mater. Interfaces 8, 33371-33376
- Click-Chemistry Based Multi-Component Microarrays by Quill-Like Pens.
  Hirtz et al., 2014, Adv. Mater. Interfaces 1, 1300129
- Porous polymer coatings as substrates for the formation of high-fidelity micropatterns by quill-like pens.
  Hirtz et al., 2013, Beilstein J. Nanotechnol. 4, 377–384
- Immobilization Mechanisms of Deoxyribonucleic Acid (DNA) to Hafnium Dioxide (HfO2) Surfaces for Biosensing Applications.
  Fahrenkopf et al., 2012, ACS Appl. Mater. Interfaces 4, 5360–5368

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