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Application note on Printing Microarrays with Stamps

Printing Microarrays with Stamps

Application note

Introduction

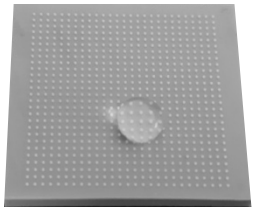
Developed in 1993 by Kumar and Whitesides microcontact printing (μCP) was first used to prepare arrays of self-assembled monolayers (SAMs) of thiols using a stamp on a smooth gold substrate [1]. It represents a micro-scale variation of a classical stamping process and relies on the transfer of a chemically functional ink from a geometrically structured stamp onto a substrate [2]. Since its first demonstration μCP has emerged as a straightforward and cheap bench-top method for the preparation of micro- and nanostructured surfaces. Its ease of handling makes μCP suitable for upscaling to large printing areas (e.g. 4 inches) [3] which is a major advantage in comparison to other techniques for surface patterning such as, dip pen-, [4] electron-, [5,6] ion beam [7,8] or photo-lithography [9], which rely on tedious protocols and require very specialized and expensive instrumentation. And because of that μCP has a great potential in vast selection of applications like (bio)sensing [10-12], microelectronics [13,14], data storage [15,16] or optoelectronics [17,18].

Such vast applications of the surface-patterned substrates impose specific requirements on the how the chemical functionalities can be introduced by μCP. First concern is the printing precision, and this can be a result of few factors. Ideally, the transfer of ink would occur only at contact interface between the stamp and the substrate, transferring the stamp negative on the surface and resulting in a desired pattern layout. The polymer stamp must be flexible enough to make homogenous contact with the substrate yet have sufficient mechanical strength to maintain the topographical features during the printing process. This is especially true for stamps with small, tall and thin features. Furthermore, the interaction of stamp, ink and substrate needs to be optimal to guarantee efficient delivery of ink only in the areas of contact. Complex interplay between factors like: ink rheology, stamp surface chemistry, substrate chemistry and topography, contact time between the stamp and the surface, environmental conditions like temperature and humidity [2] all dd to the pattern quality. And all of those factors contribute also to the success of what will be the smallest size of features that can be transferred during the printing process and how homogenous that transfer will be. The diffusive mobility of the ink may be a difficult factor to control, as it can result in smearing of the ink, which severely limits the resolvable details of the printed patterns. Another, also very important parameter, is the chemical composition of the imprinted area. Particularly important is the facile attachment of functional moieties onto the surface [19]. With the right parameters high quality microarray pattern can be used in a vast selection of subsequent experimental steps in a vast selection of applications [20].

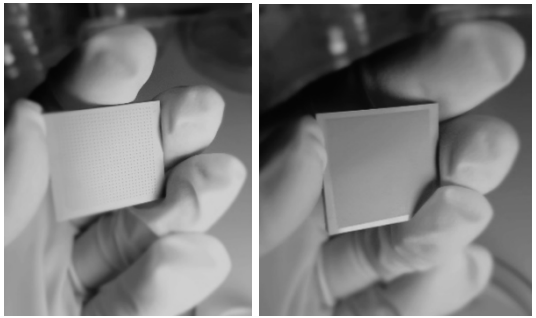
Workflow

Application note

Step 1



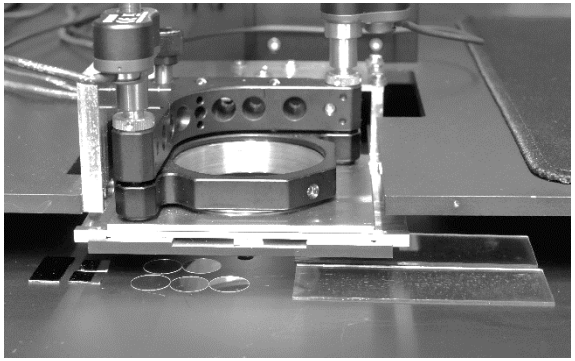
Stamp before activation. The water droplet is demonstrating the surface hydrophobicity.



Stamp before (left) and after (right) spin coating the ink: TAMRA-Azide.

Preparing the stamp and substrates
(activation to make the stamp hydrophilic and ink coating (e.g plasma or ozone activation) and subsequent ink spin coating coating)

Step 2



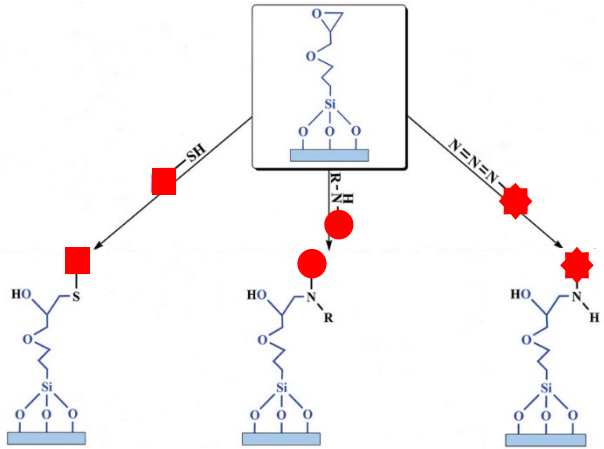
Printing
(with defined contact time between stamp & surface and in controlled environmental conditions (temperature & humidity))

Step 3

Print characterization
(e.g. Fluorescence microscope)

Step 1 - Preparing the stamp and the substrates

As microcontact printing method facilitates patterning with chemically well-defined groups we explored surface functionalization of epoxy-terminated glass. As inks amine and azides were used and resulted with patterns based on amine–epoxy and azide reactions [21]. In this setting both the stamp as well as the substrate, each carry reactive components that will be the basis of the chemical reaction occurring upon contact of the ink coated stamp with the functionalized substrate. Triggered upon their physical contact the formation of chemical bond at the printing area results in substrate patterning with defined chemical groups with varying functionalities. Printing precisions in the low nanometer range can be obtained (~20 nm), assuming that the physical and chemical parameters of ink and surface were met to realize such high resolution pattern[22].



Schematic representation of the chemical immobilization strategies used for functionalization of epoxy-terminated glass resulting in thiol–epoxy, amine–epoxy, and azide–epoxy reactions. Adapted from [21].

Preparing the surface with epoxy-termination and characterization by Contact Angle and Atomic Force Microscopy (AFM)

To obtain epoxy-terminated substrates suitable for various immobilization routes, glass substrates were functionalized with (3-glycidyloxypropyl)trimethoxysilan (GPTMS). Before silanization with GPTMS (2% (v/v) solution of GPTMS in dry toluene for 2 h.), glass slides were cleaned in sonication baths with chloroform, isopropanol and water (5 min. each step) and dried with N2. Next, glass substrates were exposed to an oxygen plasma treatment (10 sccm O2, 0.2 mbar, and 200 W (ATTO system, Diener electronics, Germany) for 2 min.) to ensure a high density of hydroxyl groups on their surfaces. After GPTMS coating was completed, the epoxy-functionalized glass slides were removed from the solution, thoroughly rinsed with acetone, toluene, and deionized water, dried under a stream of nitrogen and stored until used.

Step 1 - Preparing the stamp and the substrates

As a first check of successful functionalization prior to the further experiments, contact angle measurements (OCA-20 contact angle analyzer, DataPhysics Instruments GmbH, Germany) were done on the hydroxyl- and epoxy-terminated glass samples. To prevent ink spreading during spotting of microarrays, the water contact angle (WCA) should not be too low, otherwise the precision of the printing will be jeopardized. Right after plasma treatment, glass surfaces are highly hydrophilic, with hydrophilicity declining during the course of 3 to 4 weeks, approaching that of a nontreated glass surface again.[23] The epoxy-terminated surfaces are less hydrophilic, with an initial WCA of (54.4 ± 1.5)° that stabilizes at around 84.3° (determined by the asymptotic approach of the fit function (Figure 1) [21].

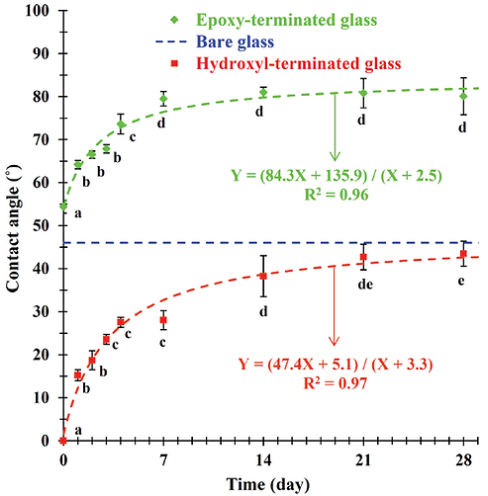


Figure 1. Water contact angles over time on hydroxyl- and epoxy-terminated glass samples. Adapted from [21].

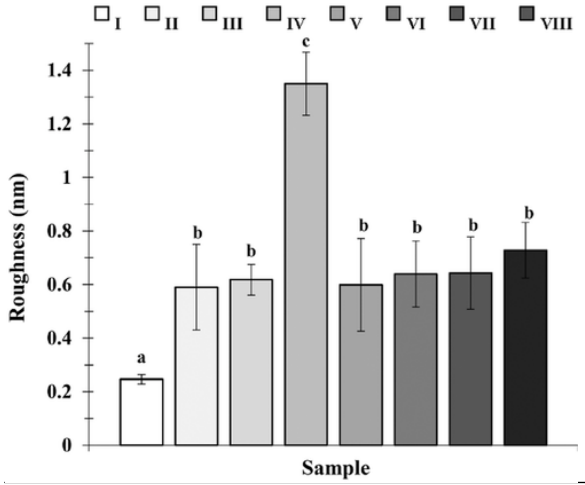


Figure 2. Roughness values as determined by AFM measurements for the I) hydroxyl-terminated and II) epoxy-terminated glass surfaces, as well as for the epoxy-terminated glass surfaces after coating with III) Cy5 thiol, IV) R6G, V) TAMRA-azide, VI) biotin-thiol, VII) biotin-amine, and VIII) biotin-azide, respectively. Significant changes in roughness ($p < 0.05$) are marked by different letters. Adapted from [21].

As next measurement of surface quality, the roughness of the hydroxyl- and epoxy-terminated glasses as well as of the epoxy-terminated glasses was monitored by AFM (Dimension Icon, Bruker, Germany) (Figure 2) .The hydroxyl-terminated glass features have a roughness of (0.25 ± 0.02) nm, while the silanization leads to significantly higher roughness ((0.59 ± 0.16) nm). This difference in roughness can be understood due to the possibility of crosslinking between silanes, leading to a less even surface. Further functionalization with TAMRA-azide and biotin-amine only minimally increases the roughness to (0.60 ± 0.17) nm, (0.64 ± 0.12) nm respectively [21].

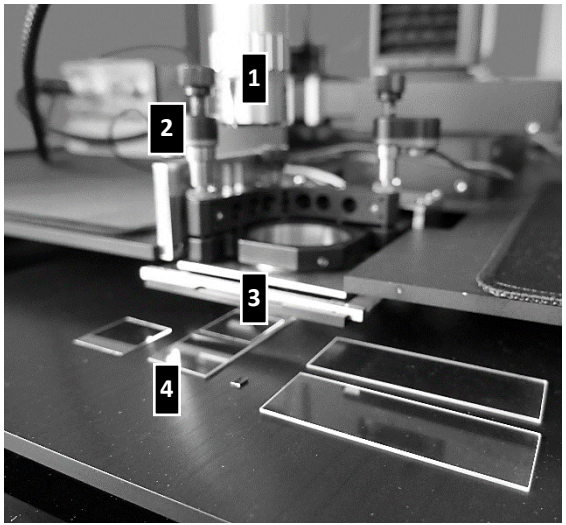
Step 2 - Printing microarrays

Application note

Ink preparation

The inks were prepared by using:
- 100 µm/ml of Tamra-Azide
- 100 µm/ml of Cy5-Azide
- 10 mM $CuSO_4$ and 20 mM sodium ascorbate as catalysts for azide binding
- 1 mg/ml GFP- His6x protein

Prior to printing, stamps (various geometries, Research Micro Stamps, USA) were activated in oxygen plasma (100 mbar oxygen pressure, 200W power, 30 sec., ATTO system, Diener electronics, Germany). From the ink mixture 5 µl was taken out and applied onto the freshly activated stamp and spin coated to homogenously deposit ink over the hydrophilic stamp surface (3000 rpm, 30 sec,spin coater type). Such coated stamp was used directly for printing.



Molecular printer set-up

The printing was performed on a Molecular Printer, (n.able, Germany). The stamp coated with ink was mounted onto the probe holder **3**.
Printing of the ink onto the GPTMS functionalized samples occurs upon contact of the ink coated stamp with the subastrate. The contact time between the stamp and the substrate should be defined each time using a new ink fomulation. In case of the inks used here 5s contact time at 50% relative humidity (regulated by the humidity module) was sufficient to create homogenous microarray patterns. After printing, ring-opening reactions were allowed to take place at room temperature (22°C) over night before imaging.

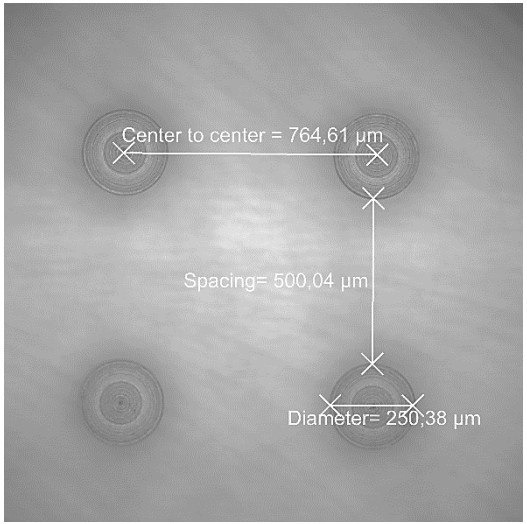
1 Microscope module for *in situ* process control (image and video aquisition)
2 High precision module for high resolution control over the printing/spotting process
4 Sample table for loading the substrates
(Molecular Printer, n.able, Germany)

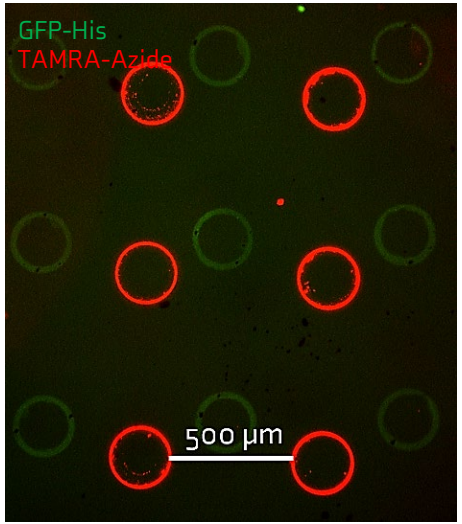
Step 3 - Pattern characterization

Application note

Characterization by Fluorescence Microscopy

The fluorescently-labeled patterns were imaged using a Nikon Eclipse 8oi upright fluorescence microscope (Nikon, Japan) equipped with an Intensilight illumination (Nikon, Japan), a CoolSNAP HQ2 camera (Photometrics, USA), and a set of BrightField, EGFP, Texas Red and Cy5 filters (Y-2E/C, Nikon, Japan). Average fluorescence intensity per feature was measured with the onboard software (NIS-Elements, Nikon, Japan) by appropriate masking of features.





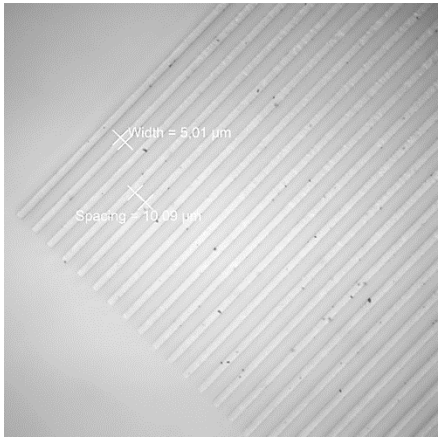
Stamp with pillars (Research Micro Stamps, USA) was coated with two inks: one half with GFP-His protein and another half with TAMRA-Azide. When spotting two inks onto the hydrophilic stamp one needs to leave sufficient spacing, so the inks will not mix. Before spin coating inks should be allowed to air dry on the stamp surface. That should prevent inks mixing as well.

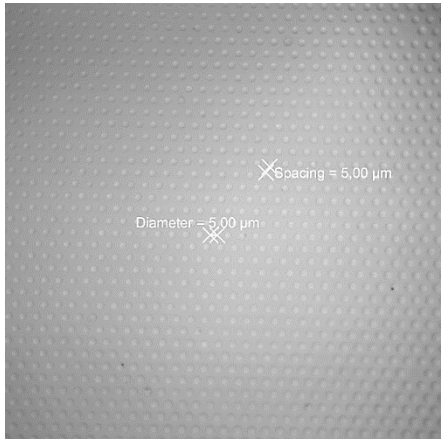
Spin coated stamp was then mounted onto the stamp holder and the GPTMS functionalized surface was brought in contact with the stamp by moving the stage up along the Z axis. Pressure that was applied onto the stamp could be easily controlled by regulating how high the substrate will move along the Z axis. If the pressure is small enough, the resulting pattern will not be a full dot but a ring, like in a presented fluorescent image.

The movement along the Z axis should be first established on test-samples, that will be used as a reference to realize a desired pattern. After the first contact, the stage was moved down along the Z axis and shifted in X and Y by a defined distance, dependent on the stamp size and the periodicity of the features. Next, the sample was brough again in contact with the stamp section coated with second ink by simply moving the stage up along the Z axis. By keeping the same value of the movement of the Z axis and keeping the humidity at the defined 50%, the reproducibility of patterning was assured.

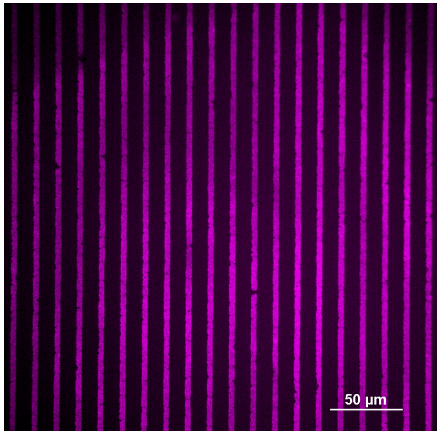
Step 3 - Pattern characterization

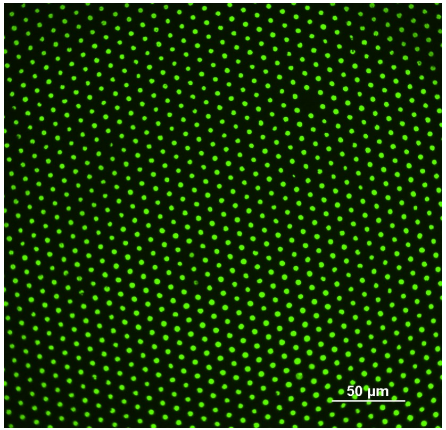
Application note

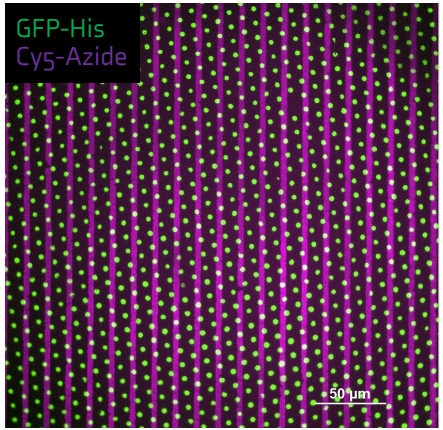




Similarly, one can use two stamps coated with different inks to create one or two ink pattern.
In this example GFP-His protein and Cy5 –Azide were used to create micro-patterns. Again, the stamp with lines or pillars (Research Micro Stamps, USA) was mounted onto the stamp holder, and the GPTMS functionalized surface was brought in contact with the stamp by moving the stage up along the Z axis. After the test-print on sacrificial samples it was defined by what Z distance sample should be moved. This is important in this example, as each stamp varies in thickness. So doing test printing is critical to establish the Z movement values to realize the micropatterns with 2 different stamps used one after another.
After the first print the stage was moved down along the Z axis and stamp was exchanged on the stamp holder. Next, the sample was brought again in contact with the stamp coated with second ink by simply moving the stage up along the Z axis by the value pre-defined in test-prints. By keeping the defined value of the movement of the Z axis and keeping the relative chamber humidity at 50%, the reproducibility of patterning was assured.

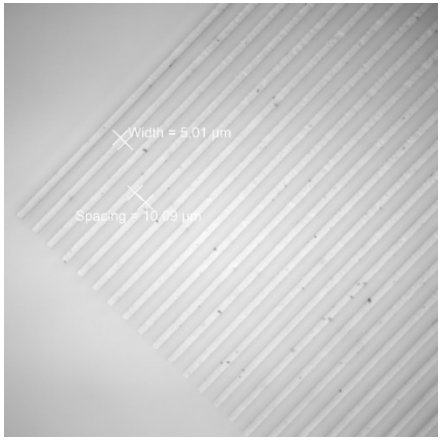


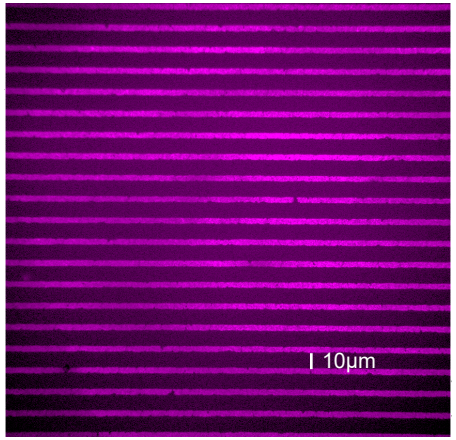


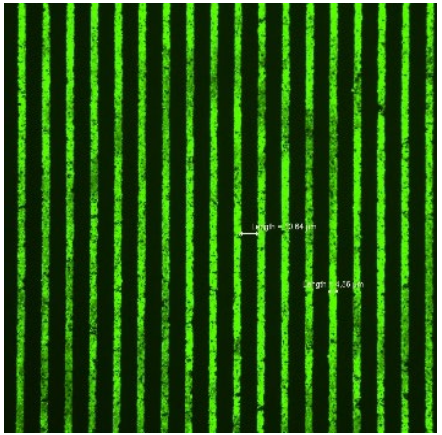


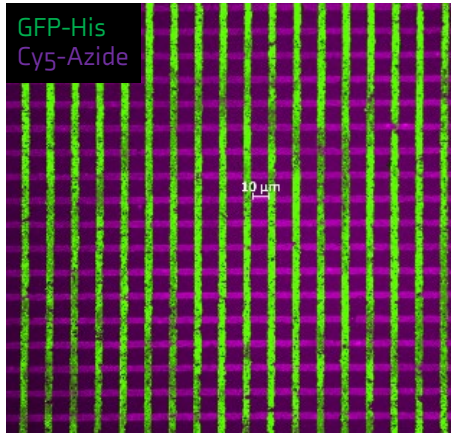
Step 3 - Pattern characterization

Application note





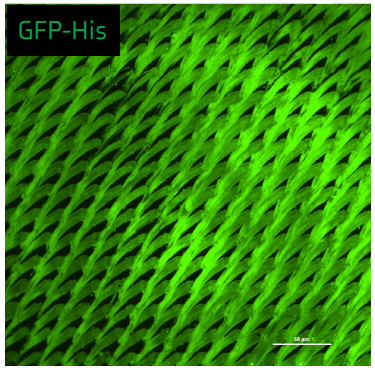




In the same way one can use two stamps coated with different inks to create one or two ink pattern.
In this example GFP-His protein and Cy5 –Azide were used to create various micro-patterns. Again, the stamp with ridges (Research Micro Stamps, USA) was mounted onto the stamp holder, the GPTMS functionalized surface was brought in contact with the stamp by moving the stage up along the Z axis. After the test-print on sacrificial samples it was defined by what Z distance sample should be moved. Doing test printing is critical to establish the Z movement values to realize the desired patterns with 2 different inks. After the first print the stage was moved down along the Z axis and stamp was exchanged on the stamp holder. Next, before the sample was brought again in contact with the stamp coated with second ink, the sample was rotated by 90° using a rotation module built into the sample table. Once the rotation was accomplished the sample was moved up by simply moving the stage up along the Z axis by the value pre-defined in test-prints. By keeping the same value of the movement of the Z axis and keeping the humidity at the defined 50%, the reproducible patterns were printed.

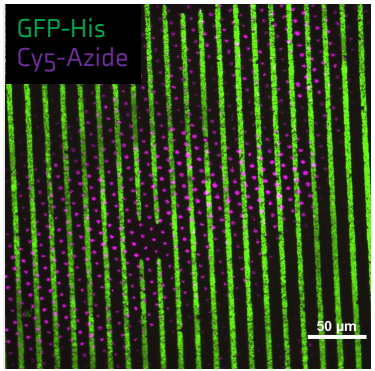
Troubleshooting

Every ink should be tested on the chosen stamp geometry in a thoughtfully designed test print experiment series, where all the important parameters of the ink, substrate and printing process will be explored. That enables to establish a protocol with optimal parameters for homogenous and reproducible printing.



Ink smearing

To avoid smearing of the ink caused by the movement of the sample when in contact with stamp or upon separation of the stamp from the substrate, the simplest solution is to attach the sample to the sample table. It can be done by spotting a drop of water under the sample and have water work as a glue to hold the sample in place. This approach is very useful especially when working with thin coverslips, which are light and often get stuck to the the large stamp area and are difficult to remove without destroying the pattern.
Secondly, the sample can be placed in a specially designed sample holder built into the table.
Important is that the sample is immobilized and also easy to remove from the table after the printing is completed. So glue and double sticky tape can be used only if it does not obstruct the removal of the sample from the table.



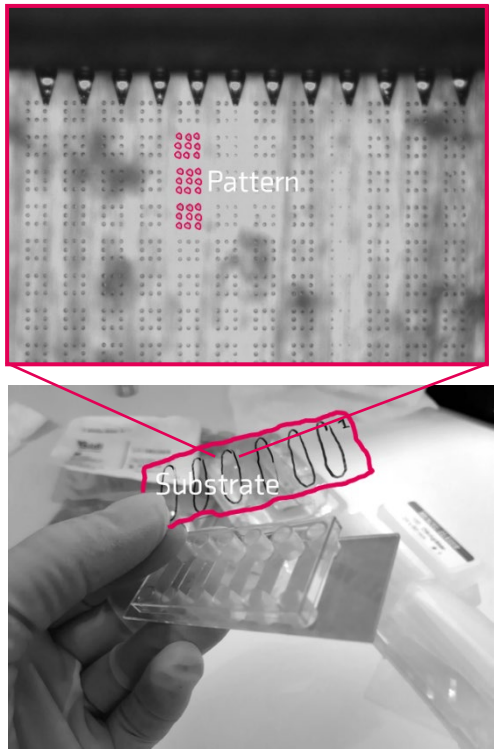
Unhomogeneous patterning

Printing two inks over the same area is not trivial and needs to be explored before the right parameters can be established. Factors that can be especially important to create homogeneous features are:

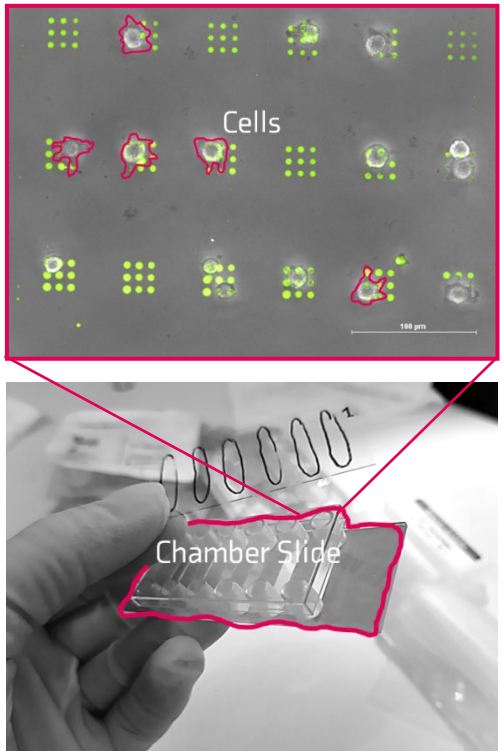
- Ink concentration
- Coating on the stamp with ink
- Stage movement up in Z axis to optimize the pressure and contact time between the substrate and the stamp
- Humidity
- Surface chemistry (in case of functionalization, concentration of the terminal groups might have big impact on forming bonds with the ink molecules)

Next steps

Step 1 – Printing & encapsulation



Step 2 – Imaging living cells



Once the sample with the defined pattern is ready, it can be processed in multiple secondary experiments. It can be either imaging by fluorescence, Atomic Force Microscopy (AFM) or X-ray photoelectron spectroscopy (XPS) .
It can also be used as a platform for live cell imaging.
In the example on the left multi chamber sticky-slides available from www.ibidi.com can be used for flushing the sample with the solution of cells, as well as with suitable buffers or other analytes.
After the desired interaction takes place such samples can be easily imaged under inverted microscope.

Conclusions

The functionalization in form of bonding the material to the surface, is creating a microarray that when exposed to the right environment is key in a wide range of applications ranging from microelectronics, sensing, photovoltaics, catalysis, and many others. Using a micro-structured stamp, which delivers the ink exclusively onto the area of contact between stamp and substrate one can easily and flexibly create a vast array of patterns and chemistries. In contrast to most types of lithography techniques, microcontact printing does not require expensive equipment. Other benefits include very short patterning times, low consumption of ink molecules, high resolution and large area patterning. Stamps can be purchased commercially and printing can be done even by hand. However, for more precise and reproducible patterning a Molecular Printer proves useful and easy to use. Having not only the pressure control over the stamp- surface contact, but a rotation capabilities and environmental control, creating reproducible and complex microarrays is hugely enabled by the printer.

We have demonstrated here microcontact printing approach to successfully immobilize fluorescent GFP-His6x, Cy5-Azide and TAMRA-azide into arrays on epoxy-terminated glass through ring-opening reaction. Using variety of stamp geometries even only those 3 inks can be printed in various microarray patterns, creating a library of features that can be studied in secondary experiments. From cell culture to simple analyte binding, such arrays are a immensely valuable tool in basic research.

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Application note

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Dr. Sylwia Sekula-Neuner

Marketing, Customer Relations, Innovation Projects

ssn@n-able-innovation.com

Dr. Uwe Bog

R&D, Process Development

ub@n-able-innovation.com

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