

Interdigitated Multicolored Bioink Micropatterns by Multiplexed Polymer Pen Lithography

Falko Brinkmann, Michael Hirtz,* Alexandra M. Greiner, Markus Weschenfelder, Björn Waterkotte, Martin Bastmeyer, and Harald Fuchs*

Multiplexing, i.e., the application and integration of more than one ink in an interdigitated microscale pattern, is still a challenge for microcontact printing (μ CP) and similar techniques. On the other hand there is a strong demand for interdigitated patterns of more than one protein on subcellular to cellular length scales in the lower micrometer range in biological experiments. Here, a new integrative approach is presented for the fabrication of bioactive microarrays and complex multi-ink patterns by polymer pen lithography (PPL). By taking advantage of the strength of microcontact printing (μ CP) combined with the spatial control and capability of precise repetition of PPL in an innovative way, a new inking and writing strategy is introduced for PPL that enables true multiplexing within each repetitive subpattern. Furthermore, a specific ink/substrate platform is demonstrated that can be used to immobilize functional proteins and other bioactive compounds over a biotin–streptavidin approach. This patterning strategy aims specifically at application by cell biologists and biochemists addressing a wide range of relevant pattern sizes, easy pattern generation and adjustment, the use of only biofriendly, nontoxic chemicals, and mild processing conditions during the patterning steps. The retained bioactivity of the fabricated cm^2 area filling multiprotein patterns is demonstrated by showing the interaction of fibroblasts and neurons with multiplexed structures of fibronectin and laminin or laminin and ephrin, respectively.

F. Brinkmann, Dr. M. Hirtz, Prof. H. Fuchs
 Institute of Nanotechnology (INT) and Karlsruhe
 Nano Micro Facility (KNMF)
 Karlsruhe Institute of Technology (KIT), Germany
 E-mail: michael.hirtz@kit.edu;
 fuchsh@uni-muenster.de

F. Brinkmann, Prof. H. Fuchs
 Physical Institute and Center for Nanotechnology (CeNTech)
 University of Münster, Germany

Dr. A. M. Greiner, M. Weschenfelder, Prof. M. Bastmeyer
 Zoological Institute
 Department of Cell- and Neurobiology
 Karlsruhe Institute of Technology (KIT), Germany

B. Waterkotte
 Institute of Organic Chemistry (IOC)
 Karlsruhe Institute of Technology (KIT), Germany

B. Waterkotte, Prof. M. Bastmeyer
 Institute of Functional Interfaces (IFG)
 Karlsruhe Institute of Technology (KIT), Germany

DOI: 10.1002/sml.201203183



1. Introduction

Biofunctional nano- and microarrays^[1] play an increasing role in the fields of diagnostics,^[2,3] drug screening^[4,5] and cell-adhesion studies.^[6–8] Besides commercially available ready-to-use setups for clinical applications, microarrays have their importance in basic research, where new approaches in the mentioned areas are tested. However, the complexity of the conducted experiments is often limited by the microarray fabrication technique. Using only one approach, the researcher has to accept limitations in feature size, pattern complexity, and choice of deposited material or throughput. The development of a strategy that promises flexibility in all these parameters will help in both decreasing the costs of laboratory equipment and increasing experimental performance.

Two molecular printing techniques for the fabrication of micro- and nanoarrays are dip-pen nanolithography^[9] (DPN) and microcontact printing^[10] (μ CP). DPN makes use of atomic force microscope^[11] (AFM) tips, which are coated with

different inks and transfer the material onto the substrate via capillary forces. To enhance throughput and to pattern large areas, arrays of tips are used in parallel DPN. 1D arrays typically have 12 to 50 cantilevers; also 2D arrays for massive parallelization (55 000 cantilevers) are available. DPN is a serial writing procedure,^[12] complex multiplexed structures can be generated.^[13] μ CP, however, is considered as parallel replication lithography.^[12] A polydimethylsiloxane (PDMS) mold of a silicon master is coated with ink and brought into contact with a substrate once. Ink transfer is therefore caused by direct contact. Surfaces in the centimeter-regime can be stamped with pre-defined structures. As this stamping procedure is often carried out by hand, inhomogeneities in feature quality are expected. Each stamp is exclusively fabricated for one pattern and one ink; generation of interdigitated features with multiple inks is hardly possible in a controlled way with this approach. Some variations in pattern from a single stamp can be obtained by dynamic μ CP,^[14] but due to the hand-made printing a lack of precise control limits this patterning strategy, too.

Polymer pen lithography^[12,15,16] (PPL) is a complementary approach in lithography combining aspects of DPN and μ CP. Some 10.000 of 2-dimensionally aligned elastomeric PDMS pens shaped as pyramids are molded from a silicon master. By leveling the stamp relative to a surface, bringing all pens in touch with the surface and applying well-controlled lateral movements of the whole stamp (or the stage respectively) with a piezoelectric system, arbitrary nano-/micropatterns can be readily achieved. The approach partly resembles dynamic microcontact printing,^[14] but is—due to the nanometric lateral and z control gained by the lithography platform—of much higher precision. Coating different pens with different inks by filling the inverted pyramids of the mold by inkjet printing is one approach to generate multiplexed, but non-interdigitated patterns.^[17] Established properties of PPL are variation of feature size by adjusting pen-surface contact area (in the range from below 100 nm to more than 10 μ m)^[12] with a pattern complexity concerning inks and shapes while reaching high throughput with uniform quality (cm² range).

Here, we present strategies in the advancement of PPL using bioinks, which are developed keeping in mind the needs of biochemists and cell biologists, in particular. First, we introduce a novel inking strategy, which enables high throughput multiplexed patterns of variable feature sizes. Second, we demonstrate true multiplexed patterns with different inks within the respective basic patterns, not only chemically diverse repetitions of the basic pattern in different areas of the sample. Finally, we have found a specific substrate-ink-combination that allows the immobilization of covalently bound biotinylated antigens on the generated patterns.

Our developments integrate advantages of other lithographical techniques so that PPL together with high precision positioning of stamps by the DPN system enables a highly flexible technology for microarray fabrication. The focus on bioinks like phospholipids and biotin as well as the proof of bioactivity of our multiplexed fibronectin/ laminin and laminin/ephrin patterns with fibroblasts and neurons,

respectively, will hopefully inspire biologists and chemists to design new experiments in their fields.

2. Results and Discussion

Our strategy for the fabrication of multiplexed bioink patterns is explained schematically in **Figure 1**. Crucial for our approach are the careful deposition of different inks on one polymer stamp and a shift of the stamp between the lithography of sub-patterns. First, the pens coated with ink 1 generate sub-pattern 1 at a region of interest (ROI) on the substrate. Second, the ROI is shifted to the pens coated with ink 2 taking the pen's spacing into account. Generally, an offset is introduced by non-perfect alignment of the stamp that has to be accounted for when shifting the ROI (see method section). Third, sub-pattern 2 is created by the pens coated with ink 2 at the ROI. For additional inks the process is repeated. Precise relative navigation is crucial throughout the process. Therefore, a sophisticated sample positioning system with large movement range, but still at least sub-micron resolution, should be employed. The work presented here was implemented on a commercial DPN system (NLP 2000, NanoInk) with a custom made PPL holder. The system has a piezo-based x-y-z-stage motion (digital closed-loop) with a resolution of less than 100 nm and a travel range of 40 by 40 mm. The symmetrical architecture of a polymer pen array and the large-scale precision of the system enable this novel multiplexed printing technique.

The maximal total area of the multiplexed pattern is in direct relation to the smallest area covered with one specific kind of ink on the tip array, since this will define the area that can be covered with this ink in one approach of the stamp. If only one ink is used for the microstructure, the whole pen array can be coated and all tips contribute to lithography. For two-ink patterns, the pen array is coated with two inks and at most one half of the stamp can contribute to the multiplexed pattern. This principle can be continued and is only limited by the stamp's size. We were able to manually apply nine different inks onto a 4 cm² stamp (see SI Figure 1). A larger number of inks can be easily applied when using a larger stamp or employing more precise ink jet printing for delivery of ink. The largest PPL stamp demonstrated yet with about 11 million pens was molded from a 3-inch silicon-master by the Mirkin group. Applying our strategy to that stamp, even basing our estimations on inking by hand, potentially up to 150 different inks could be deposited in one feature, leaving room to further improvement by invoking e.g. ink jet printing as inking device.

We chose three different bioinks relevant to bioactive surface patterns for our demonstrations: (1) fluorescently labeled phospholipids, (2) biotin-4-fluorescein and (3) a protein-carrier ink that allows the direct deposition of proteins in a similar way to matrix assisted PPL.^[18] Phospholipids, our first demonstration ink, play a major role in biology, their prominently function being the main constituent of cell membranes. It was previously shown, that lipids form 2D membranes that stack from single bi- up to several multilayers when being applied to surfaces by DPN^[3,19–21] or

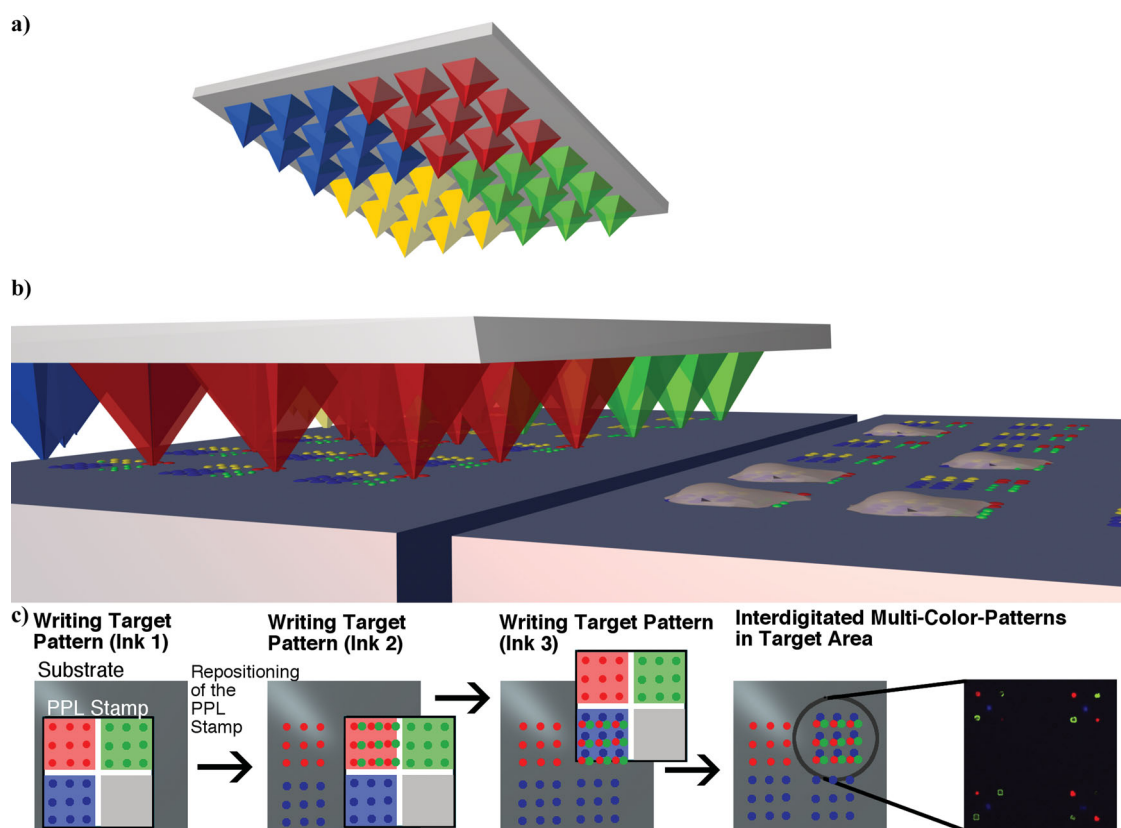


Figure 1. Schematic of multi-ink polymer pens, lithography and application. a) Cut-out bottom view of a polymer pen array with four different inks (for example fluorescently labeled phospholipids). Inks can be precisely deposited on the pen array by hand. A PPL stamp has about some 10^5 pens on a 2 by 2 cm area to fit 9 different inks (see SI Figure 1). b) Multiplexed patterns can be generated by using the differently inked areas of the pen array at the same surface position. This implies high precision of surface/stamp movements, in the range of centimeters. Multiplexed micrometer patterns on the cm^2 scale find applications in cell biology for example (right). A low number of cells seeded on the pattern prevents cell clustering and allows single cell analysis on the pattern. By having a higher number of printed features, it is more likely that cells settle on the desired position. c) Scheme of the writing process for a three ink pattern. The PPL stamp areas with different inks are subsequently positioned over the target area and the different ink subpatterns are created. Around the target area there will be sacrificial areas patterned with only one ink and subpattern. The last frame shows an actual lithography result with three inks.

stamping.^[22] As cell membranes mainly consist of phospholipids, there is a high compatibility between cells and lipids as bioink. By incorporating further material like proteins, small molecule compounds and nanoparticles into lipid formations, cells can be presented with^[4,13] or being made to uptake these materials.^[2,23] Moreover, phospholipids are commercially available with a multitude of modifications, e.g. fluorescent head-groups that allows facile observation by fluorescence microscopy, biotinylated or metal-chelating moieties as specific binding sites,^[13] or linked with bioactive small molecules.^[4]

In our study, we focus on rhodamine- (red), fluorescein- (green) and dansyl-labeled (blue) phospholipids mixed with the native phospholipid DOPC to a concentration of 10 mol% (see the Experimental Section and SI for complete chemical information). Our second demonstration ink, biotin-4-fluorescein, can be used as a potent linker for a variety of substances and proteins not valid for direct application by DPN or PPL. The binding of arbitrary biotinylated molecules to streptavidin is a standard procedure in biology and chemistry.^[3,24,25] Last, we demonstrate direct patterning of the extracellular matrix proteins laminin and fibronectin and the GPI-anchored axon

guidance protein ephrin-A5. Our experiments with 3T3 fibroblasts and retinal neurons show the retained biofunctionality of these proteins after patterning due to specific adhesion and growth on these multiplexed patterns.

Before patterns can be printed, the pens have to be coated with the desired inks. The ideal pen coating or inking procedure, respectively, depends on the ink and solvent. We have tested two ways to deposit ink on the polymer pens. Whereas biotin-4-fluorescein and protein-inks can be transferred to the pens by a stamp pad, it became evident, that in the case of phospholipids it is advantageous to directly deposit the ink on the pen array by pipetting. The stamp pad is a square piece of silicon with a native oxide layer, which is treated with piranha solution before to render its surface hydrophilic. By pipetting few microliters of ink onto the silicon oxide, the surface gets homogeneously coated. After evaporating solvents in vacuum, the stamp pad is ready to use. To ensure a suitable uptake of ink by the tips, the polymer stamp is exposed to oxygen plasma before inking. The inking procedure is carried out after leveling the pen array in the machine. Humidity and contact time are set depending on the ink (see SI video 1).

Phospholipids dissolved in chloroform are directly pipetted on the polymer pen array. As PDMS is insoluble in chloroform, the pens are not dissolved but PDMS stamps might be subject to extensive swelling. Due to the translational symmetry of the polymer pen array in x and y direction, the ink spreads rectangularly leading to a well-defined distribution with perfect square shaped inked areas on the stamp (see SI for an deeper explanation, video and micrographs of this effect). By precisely pipetting inks at different positions on the stamp, the inked areas can be placed with a spacing of approximately one half to one millimeter next to each other. Half a microliter of rhodamine-labeled phospholipids (1 mg/mL DOPC in chloroform) leads to an inked area on the stamp of approximately 5 mm × 5 mm. Remains of solvent should be evaporated prior to lithography by exposing the stamp to a vacuum in a desiccator for at least 5 min. If desired, both the direct inking method and the stamp pad method can be combined. Special attention has to be paid to ensure not to contaminate already inked parts of the stamp with other inks. The order of the inking methods is arbitrary; a stamp pad smaller in size than the stamp itself ensures that already inked pens do not touch the surface. This avoids the loss of ink before lithography.

2.1. Truly Multiplexed Lipid Patterns

PPL can generate patterns from a large complex feature library, due to the different degrees of freedom available during lithography: both dwell time and relative humidity are established parameters in lipid DPN and can be applied in PPL as well. Additionally, the pen-substrate contact area can be utilized for the control of feature sizes in PPL. Using

bioinks like phospholipids and biotin-4-fluorescein, dot diameters of less than 1 μm are as simple to fabricate as square-like features of 15 μm edge length within the same experimental setup. Proteins mixed in a protein-carrier-ink show a more water-like behavior in terms of surface wetting. This results in thicker features about 10 μm in diameter. A variety of truly multiplexed patterns are shown in **Figure 2** that indicate the multiplex strength of PPL using three fluorescently labeled phospholipids. Three different areas of the pen array are coated with one phospholipid ink mixture. Each ink has its own sub-pattern (see Figure 2a). The blue square consists of five by five dots dansyl-labeled phospholipids with 2.5 μm spacing. The tip-surface contact area is set high enough to allow the lipid dots to fuse and become a homogenous layer. The red and green features (rhodamine-, fluorescein-labeled phospholipids) have a spacing of 10 and 20 μm respectively. Each pattern has a total size of 80 μm. With a periodicity of 120 μm, the total array covers an area of approximately 5 mm × 5 mm. The lithography of those 2500 3-ink-features by multiplexed PPL takes about 2 min at a dwell time of 1 s. When using one ink, the total area covered corresponds to the total stamp size, i.e. a 2 cm × 2 cm stamp with a pen separation of 120 μm leads to about 27 000 features (see also **Figure 3e**). In **Figure 2b** different surface-contact areas of the pens were utilized to generate different spot sizes. In combination with multiplexing, this leads to the most complex pattern morphology. **Figure 2c** shows that PPL is suitable for the lithography of lines. By positioning different materials directly next to each other (or even on top, see SI), material concentration gradients can be realized. In comparison to dots, lines have the advantage to fabricate more homogeneous areas.

While we were focusing mainly on patterns with features in the micrometer range, the writing strategy could

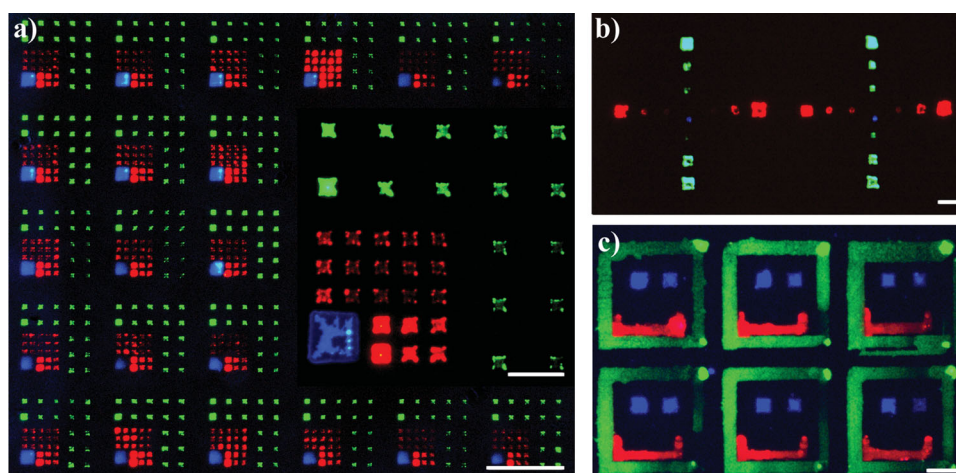


Figure 2. Fluorescence micrographs of multiplex patterns: Fluorescently labeled phospholipids (DOPC mixed with rhodamine- (red), fluorescein- (green) and dansyl-labeled (blue) phospholipids) were deposited on a PPL stamp with a pen spacing of 120 μm. A glass cover slide is used as substrate. The micrographs are overlays of the texas-red, FITC and DAPI fluorescence signals. a) Depending on the chosen pitch and pen-substrate contact area, inks can form uniform layers (see blue squares). Red and green spots do not fuse. Each pattern is realized by three pens that follow a pre-defined sub-pattern design. The total area patterned is about 5 mm × 5 mm. The scale bar equals 20 μm (inlet) and 100 μm (main image), respectively. b) Pattern with different spot sizes and inks. The spot size was varied by adjusting the pen-surface contact area. c) Larger uniform ink layers can also be generated by moving the pens over the surface, i.e. drawing lines. By using different inks and choosing a close feature separation, phospholipid (and other inks) can mix as they are free to move over the surface. Scale bars equal 20 μm in (b) and (c).

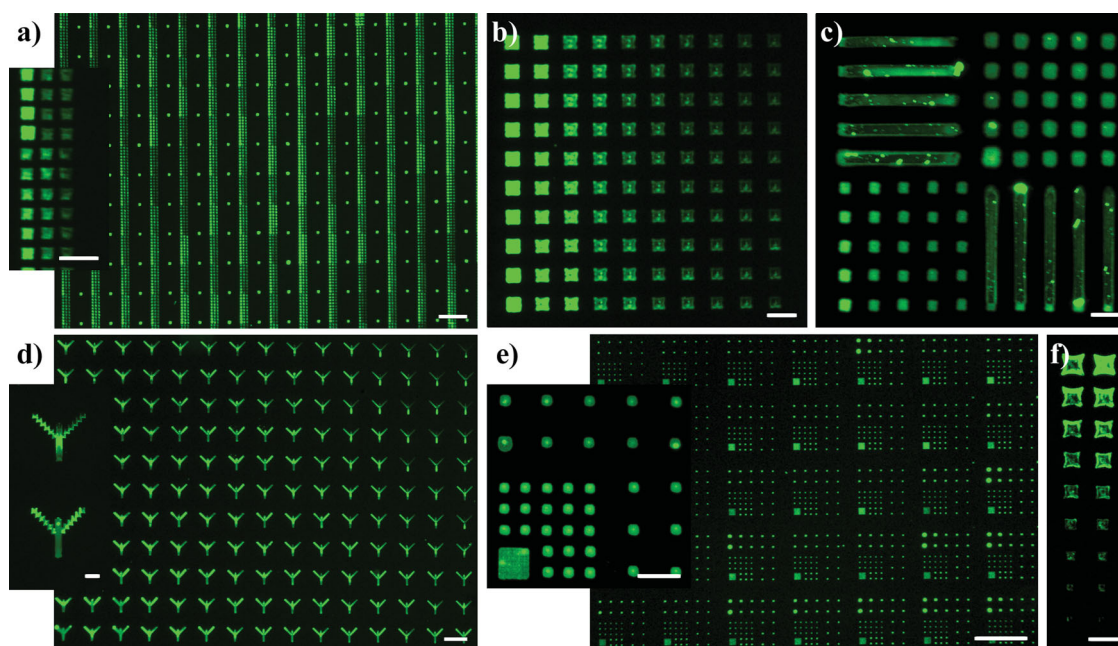


Figure 3. Biotin pattern library: Direct lithography of biotin-4-fluorescein on a layer of bovine serum albumin (BSA) on glass enables a covalent bond between substrate and ink by photobleaching. a) Mosaic pattern consisting of 30 dots by each pen. As the pen spacing is chosen to be a multiple of the dot spacing, a continuous pattern is formed. b) 100 spots generated by one pen. The amount of ink exchanged from tip to surface decreases during lithography. This allows the fabrication of patterns with material gradients. c) Lines and dots generated by one tip. The dwell time is 1 s for the lower sub-array and 5 s the upper sub-array, leading to broader spots at the top. The tip velocity is 1 $\mu\text{m/s}$ for the lines on the right and 5 $\mu\text{m/s}$ for the lines on the left side. d) Y-like patterns consisting of one line and several dots are asymmetrical. e) Same pattern as in Figure 2a, but generated with one ink. f) Different spot sizes were generated by changing the pen-surface contact area. This pattern was fabricated by one tip, starting with the small dot in the bottom left, then approaching the pen 2 additional micrometers and approaching again. After ten spots, the pen-surface contact area is decreased again following the same principle. Scale bars equal 20 μm in all insets and in (b,c,f), and 100 μm for (a,d,e) main images.

potentially be extended into the submicron range, keeping in mind recent advances in controlled submicron patterning by PPL,^[26–28] the limiting factors being the precision of the positioning table (which is well below one micrometer) and the ability to measure the offset between the different colors in a test pattern (which could be done by AFM in much higher precision) to establish the offset correction.

2.2. Covalently Bound Biotin Microstructures

Direct deposition of proteins by PPL has already been presented by Mirkin et al.,^[17] but applications of directly written protein arrays in cell culture were not reported yet, likely because more delicate proteins are easy to lose function during direct writing processes. One way to circumvent this problem is by patterning a robust linker molecule and self-assemble the target protein for cell culture afterwards.^[29] At the beginning of our studies we, too, have found that non-specifically bound proteins on surfaces often do not adhere well enough when immersing the substrate in buffer solution. Especially during washing steps non-covalently bound patterns are prone to fuse, smear or being washed off completely. Even if enough material remains on the surface, this turns into a problem when proteins detach and interfere with interaction partners in the solution, like cells or other proteins and antigens. To alleviate this problem, a stable

substrate with an ink that is strongly, ideally covalently bound is desirable. We found, printing biotin-4-fluorescein directly onto a layer of nonspecifically but strongly adhering bovine serum albumin (BSA) on a glass cover slip fulfills the desired criteria. In order to form a bond between ink and substrate, the biotin-4-fluorescein pattern is bleached with light of 485 nm. The decayed fluorescein interacts with the BSA molecules and adheres covalently.^[30] The excess of ink is rinsed off after bleaching. A uniform biotin pattern of approximately 1 nm height on the BSA layer remains (see SI). This provides the basis for a large number of applications as basically all biotinylated materials can be immobilized on the pattern via the streptavidin linker, which can be fluorescently labeled in addition for better relocation of patterns. This strategy has lately also been used for optical lithography approaches.^[31] Before incubation of streptavidin, the sample can be dried and stored over months while still maintaining pattern fidelity and functionality.

Figure 3 shows a library of biotin-4-fluorescein patterns on a BSA coated cover slip by PPL. By using the same setup, only the pen spacing can be varied, the variety of patterns is only limited by the designers' creativity. Figure 3a shows a mosaic consisting of squares. The larger dots between the belt-like square arrays correspond to each pen of the stamp on a first approach which is needed to define the surface position. To generate the pattern, each individual pen touched the surface only 30 times. This ensures a more equal material

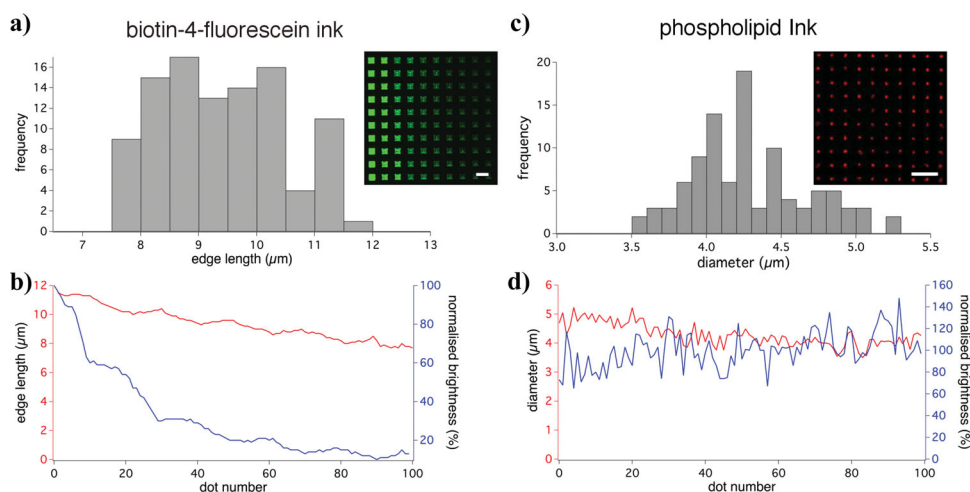


Figure 4. Ink transfer and feature size of biotin-4-fluorescein and a phospholipid ink by one PPL pen: a) The histogram shows the frequency versus edge length (box size $0.5\ \mu\text{m}$) of 100 biotin-4-fluorescein features. The inset illustrates the array observed. The distribution is horizontal relating to linear shrinking effect. b) Edge length (left axis) and normalized brightness (right axis) corresponding to the ink transfer versus the number of pen approaches. The edge length decreases linearly ($12\ \mu\text{m}$ to $8\ \mu\text{m}$) whereas the amount of material transferred decreases exponentially. c) The ink transfer behavior of a phospholipid ink is Gaussian. The variance is narrower compared to the biotin-4-fluorescein ink. Scale bars equal $20\ \mu\text{m}$ in both insets. d) The decay in dot diameter of the phospholipid ink is hardly visible. The graph illustrates a random behavior without a clear de- or increase. The amount of material deposited seems to increase within 100 dots as the blue line in the diagram corresponding to the fluorescent intensity increases slightly.

deposition by each pen (see **Figure 4a**). Figure 3b presents the decreasing ink transfer from one tip to surface during lithography. Printing was done in a zigzag path starting from the bottom left going upwards. It is clearly visible that the squares on the right show less fluorescence intensity than the squares on the left indicating a lower transfer rate. The diagrams in Figure 4 point out the behavior of the ink flow taking feature size and ink transfer into account. The distribution of the first 30 dots shows a co-variance concerning size of 10% and 30% concerning brightness referring to the amount of material on the surface (see SI). Statistical values are comparable to the quality of other dot arrays, for instance fabricated by DPN.

Besides creating dot patterns, PPL is also suitable to generate continuous one-stroke lines (Figure 3c). Although being a standard procedure in DPN, this was not used in PPL patterns before due to its focus on printing (in the sense of touching a surface once to transfer a spot of ink then lift up the pen again and shift it for the next approach) instead of writing (bringing the pen into contact with a surface and then move it to deposit the ink without lifting). Given compatible inks, line structures can directly be written by PPL instead of being substituted by repeated printing of overlaying dots. Figure 3c shows a dot and line pattern generated by one pyramid tip. It also indicates the dependency of spot size to dwell time. Figure 3d shows Y-like features that can be used for immobilizing and stretching cells to observe their behavior with different bound adhesion molecules or proteins. Figure 3f illustrates a further degree of freedom in PPL: the pen-surface contact area. The z-position of the PPL stamp is decreased $2\ \mu\text{m}$ before approaching the surface. The area of the square-like features is well reproducible as the left column was designed from bottom to top first, the right column from top to bottom secondly.

As proof of concept, we have bound different biotinylated proteins on the biotin-streptavidin pattern. The presence of immobilized proteins was verified by fluorescently labeled antibodies (see SI). Biotinylated dinitrophenol (DNP) and biotinylated human fibronectin was successfully bound to the pattern. To verify the biological functionality of the bound proteins in a sample experiment, human embryonic kidney (HEK) cells are seeded on an array of biotinylated fibronectin bound on a fabricated biotin-streptavidin-sandwich for 15 min (see SI). Cells only adhere to the spots presenting fibronectin. By exchanging the buffer solution, loosely adhered cells positioned in between dots can be easily washed off the blocked surface. The short processing time for cell immobilization helps to keep cells alive and vivid. Throughout the experiment, the fibronectin pattern is visible due to the cy3-labeled streptavidin linker and can be used to readily locate and relocate areas of interest. However, if further fluorescent staining of the cells with markers of otherwise overlapping spectra is desired, non-labeled streptavidin could be used, too.

2.3. Comparison of Patterning for Biotin and Lipids

Depending on the ink and tip-surface contact area, the polymer pens can be either used as stamp or as pens resulting into a different behavior concerning ink transfer. The flow like behavior seen in our PPL experiments with the low viscosity lipid inks (showing a more constant ink flow from pen to surface) are comparable with DPN processes, where the ink is usually flowing to the surface over a meniscus between tip and surface. PPL printing with the biotin ink (showing stronger decline in transferred material after each printed feature) is more comparable to μCP , where the ink is transferred

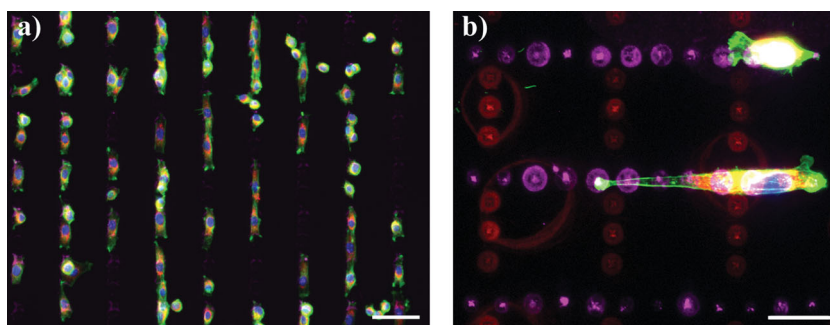


Figure 5. Bioactive multi-protein patterns with interacting cells: a) 3T3 Fibroblasts adhere on a fibronectin pattern fabricated by PPL. It is clearly visible that the cells prefer protein decorated areas and align with the protein pattern. Immunostained fibronectin is shown in magenta, fibroblasts are visualized with actin staining (phalloidin-Alexa488, green) and nuclear staining (DAPI, blue) Scale bar equals 40 μm . b) Multiplexed cross-pattern of immunostained fibronectin (magenta) and laminin (red). The cells align with the fibronectin pattern and do not spread on the provided laminin. Scale bar equals 20 μm .

to the surface by direct contact rather than flowing over a meniscus. We have used both writing and printing for our pattern fabrication depending on the ink. Whereas phospholipids have a low viscosity at humidity higher than 40% and accordingly flow from the polymer pens, biotin-4-fluorescein does not show such a liquid behavior and performs better when printed, though writing can be performed at higher humidity, too (Figure 3c). Although capable of being written, liquid inks can be printed, too. The user can choose between writing or printing upon adjustment of the pen-surface contact area and humidity.

Figure 4 presents the ink properties of biotin-4-fluorescein and a phospholipid ink for PPL. The size distribution for 100 biotin-4-fluorescein squares (Figure 4a) is homogeneous, meaning that the features decrease in size at a constant rate. This is validated as exemplified in Figure 4b, showing the edge length and feature brightness versus the number of contacts between pen and surface. The edge length decays from 12 μm to 8 μm linearly, whereas the corresponding brightness (relating to the material deposition) decreases rapidly to 20% within the first 50 dots. When uniformity is a main concern during patterning with biotin, the stamp should be used on a bleeding pattern for this phase of rapid decrease and the target pattern can be written afterwards with more equal material deposition. The phospholipid ink in PPL behaves like in DPN with a narrow Gaussian distribution on 100 features (Figure 4c). A decline in ink transfer is hardly detectable (Figure 4d) and the dot size remains almost constant over the lithography of 100 dots.

2.4. Direct Multiplexed Patterning of Proteins

As a last example for multiplexed patterning with PPL we chose the application of protein carrier ink for direct protein patterning. To avoid rinsing the proteins off the surface when immersing the pattern in liquid, we added a protein carrier to the protein solution. By printing on a silanized glass surface, the deposited proteins crosslink with the surface and the pattern becomes stable in solution. To check biocompatibility and functionality of direct printed protein patterns we have focused on three well characterized proteins. The

extracellular matrix proteins fibronectin and laminin, commonly used in migration assays with fibroblasts,^[32–34] and the repulsive axon guidance cue ephrin-A5.^[35–37] All proteins have been previously applied for the fabrication of functional surface bound patterns using either microfluidics or microcontact printing.^[34,38,39] **Figure 5a** shows a line-like dot pattern of fibronectin with adhering 3T3 fibroblasts. The cells adhere on the fibronectin and spread in an elongated shape recapitulating the stripe-like protein pattern. **Figure 5b** demonstrates a combined pattern of laminin (red) and fibronectin (magenta) dots. Here, the 3T3 fibroblasts preferentially adhere to the fibronectin dots and not to laminin, indicating a preference of this cell type for fibronectin as a growth substrate.

Large-scale micropatterns consisting of more than one axonal guidance molecule are important for basic research in developmental neurobiology as axon growth *in vivo* is guided by the combinatorial action of several guidance cues.^[40] **Figure 6** depicts axons from chicken retinal ganglion cells labeled in green for actin growing in multiplexed protein patterns. On patterns of alternating laminin (blue) and ephrin-A5 (magenta) lines, axons prefer to grow on laminin (Figure 6a). On patterns consisting solely of laminin lines axon growth is random, indicating that they are forced to grow on laminin by the repulsive action of the neighboring ephrin-A5 lines (Figure 6b). Moving the tips above the surface during the inking process can create thicker features, as can be seen in Figure 6c. This allows the design of broader barriers while maintaining only smaller dots for the guiding tracks (a laminin dot array of about 10 μm spot size and broad bands of ephrin of 50 μm width) while still retaining the guiding properties of the pattern.

3. Conclusion

Polymer pen lithography is a synthesis of dip-pen nanolithography and microcontact printing combining some of the key strengths of both techniques. Highlighting the flexibility in pattern fabrication with high throughput, PPL offers ideal conditions for biological and chemical surface experiments. However, the number of publications containing PPL as keywords is constant in the recent years.^[41] With our strategies

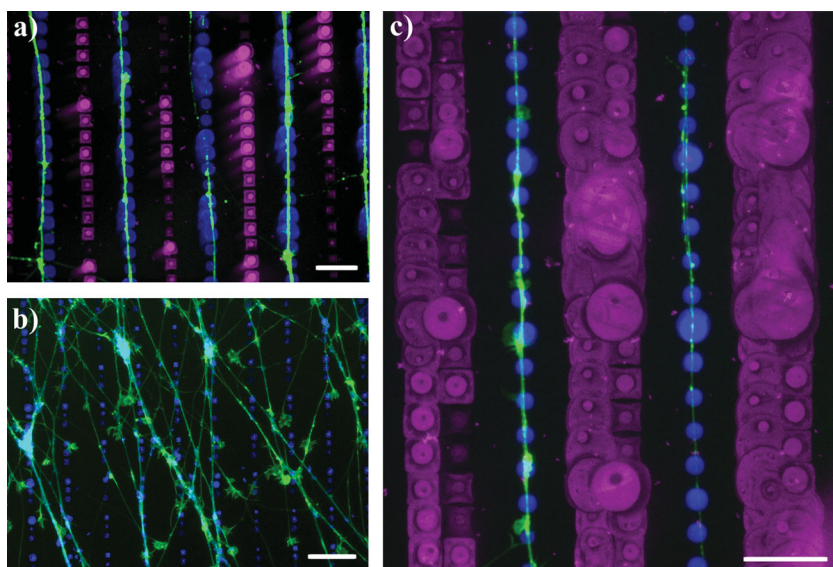


Figure 6. Neuronal outgrowth and axon guidance effects on PPL fabricated protein patterns. a) Axons from retinal ganglion cells (stained for actin, green) grow strongly fasciculated along the dotted laminin lines (immunostaining, blue), guided by the neighboring repulsive ephrin-A5 structures (immunostaining, magenta), compared to laminin only patterns in (b). The guiding effect persists even with occasional disconnected ephrin-A5 lines. c) Additional axonal guidance pattern with large ephrin-A5 barriers separating tracks of small laminin dots. Scale bars equal 50 μm in all images.

presented here, enabling true multiplexed patterns with interdigitated multi-color features, we have opened a new door for a broader target audience in the field of chemical biology, hopefully fostering the adoption of PPL as powerful technique.

We demonstrated the capabilities of PPL for relevant bioinks by demonstrating patterning of phospholipids, a fluorophore conjugated biotin and proteins. The introduction of a simple inking procedure, pipetting bioinks directly on the stamp, combined with precise stamp positioning and an offset correction strategy, leads to a large library of multiplexed patterns, in the cm^2 range suitable for diverse applications in biological experiments. True multiplexing within the basic pattern, not only between the parallel written patterns was demonstrated. By photo immobilization of biotin-4-fluorescein, we have established a new strategy to create covalently bound patterns of arbitrary biotinylated molecules via a streptavidin linker and demonstrated pattern functionality by selective adhesion of HEK cells onto fibronectin immobilized on these patterns. For direct protein patterning by protein carrier inks we showed selective adhesion of fibroblasts and guided axon growth of neurons on multiplexed protein patterns.

The demonstration of the broad capabilities of PPL in particular the integrated interdigitated multiplexed patterning of proteins will hopefully inspire and open up venues for biological experiments to take advantage of the new patterning opportunities presented in this work.

4. Experimental Section

Inks: Biotin-4-Fluorescein (Sigma Aldrich, Germany) is used directly from the bottle. Phospholipid inks are dissolved in

chloroform (Merck, Germany) to a dilution of 1 mg/mL. 18:1 DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, USA) is used as main carrier ink, rhodamine labeled phospholipids (18:1 Liss-Rhod-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl), Avanti Polar Lipids, USA) are admixed to a concentration of 1%, dansyl (18:1 Dansyl-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl), Avanti Polar Lipids, USA) and fluorescein labeled phospholipids (18:1 CF-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein), Avanti Polar Lipids, USA) are admixed to a concentration of 10%.

3 μL human fibronectin (Sigma), natural mouse Laminin (Invitrogen) and recombinant human ephrin-A5-FC (R&D) (1 mg/mL each) are mixed with 0.5 μL protein carrier solution (NanoInk, USA) each.

Substrates: As substrates for the biotin ink, glass cover slips (Menzel-Gläser, Germany) are sonicated in acetone for 30 min, sonicated in nanopure water (18.2 M Ω cm) for 30 min and subsequently blown dry with nitrogen. The samples are immersed in a 3%

(w/V) solution of bovine serum albumin (BSA) (Sigma, Germany) in phosphate buffered saline (PBS) (Sigma, Germany) for 45 min and then dipped into nanopure water (18.2 M Ω cm) ten times and dried with nitrogen.

The silanized substrates used for multiplexed protein immobilization are commercially available as 'SuperChip' aldehyde silane coated microarray substrate (Thermo Scientific, Germany).

Stamp pad fabrication: A piece of silicon (size depending on the stamp's area) is cut from a wafer and cleaned in a 50 mL bath of piranha solution ($\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$ 1:3, Merck, Germany) for 15 min to render its surface hydrophilic. 10 μL biotin-4-fluorescein is directly pipetted on the surface, spread by the pipette tip and dried in low vacuum at 0.1 bar for 15 min. To cover a 5 mm $\times$ 5 mm piece of hydrophilic silicon with a thin layer of protein ink, the amount of 0.5 μL ink is appropriate. The stamp pads are dried in air for 5 min.

Inking: The glass fundament of the polymer pen array is mounted to a microscopy slide, which is then mounted the holder of an NLP 2000 instrument (NanoInk, USA) by two-component epoxy. After leveling the two-dimensional pen array on a flat surface, the tips are ready to be inked. The tips are brought into contact with the stamp pad or ink, respectively, for one second. The relative humidity is set between 60 and 70%. In the case of pipette inking, 0.5 to 2 μL of the lipid mixtures are spotted directly onto the plasma treated polymer pen array. The solvent (chloroform) is evaporated in a desiccator with low grade vacuum of 0.1 bar for 15 min.

Writing Parameters: Biotin-4-fluorescein is deposited at a humidity of 70%. The dwell time is between 1 and 5 s and the velocity, in case of lines, is 1 to 5 $\mu\text{m/s}$. All phospholipids are written at a relative humidity between 40 and 70%. Dwell time and pen velocity are comparable to the biotin-ink.

Offset Correction: As the stamp is glued to the holder of the DPN setup by hand, a non-negligible discrepancy between pen alignment and the stage coordinate system is expected, resulting in a slightly rotated x-y-axis of the stamp in regard to the stage. To mend this mismatch an offset correction after each shift is required. This shift needed to move the stamp area with the next desired ink over the already printed pattern can be determined by performing one approach on the sample surface to pattern it with alignment marks, then conducting the shift and another approach (for a more detailed description of this process see SI). The separation of these two arbitrary approach dots (which would simply overlay given a perfect aligned stamp) corresponds to the offset. This offset has to be taken into account when designing the total pattern with the software for each shift or ink, respectively. However, it should be noted that it is only necessary to determine the offset with the help of two inks, since all additional inks on the stamp will follow the same dislocation, assuming a constant shift. A fresh pristine substrate can be introduced after determining the described procedure to identify the misalignment of a newly mounted PPL stamp.

Biotin Immobilization: Biotin patterns are bleached in a Technotray CU polymerization unit with blue light (wavelength of 488 nm) for 2 h. Nonspecifically bound molecules are then washed off by dipping the substrate into nanopure water ten times. After drying with nitrogen, the sample can be stored over months.

Biotinylation: Human Fibronectin (Invitrogen, Germany) was coupled to Biotin-NHS (Thermo Scientific, Germany) according to the manufacturer's manual. A 50x molar excess was used to leave a fraction of lysine residues in the protein unreacted. The conjugate was purified by size exclusion chromatography with a nominal molecular weight cut-off of 7 kDa (Thermo Scientific, Germany). Biotinylated dinitrophenol (DNP) was purchased (Sigma).

Protein Immobilization: Surface passivation is carried out with a 10% (w/V) solution of BSA in PBS for 15 min. After exchanging the liquid with PBS, the patterned area is covered with a 0.5% (V/V) dilution of streptavidin/cy3 (Sigma, Germany) in PBS for 15 min and washed with PBS afterwards. To avoid the sample from further nonspecific binding, a second blocking step with a 3% w/V concentrated BSA solution is applied. Biotinylated fibronectin is incubated on the pattern at a concentration of approximately 0.2 mg/mL for 60 min. The sample is washed with PBS and kept moist.

Cell Immobilization: Human embryonic kidney (HEK 293) cells were harvested from culture and kept in PBS of 37 °C at a concentration of 10^6 cells per mL. 200 μ L cell suspension was directly pipetted on the pattern. Incubation was carried out on a plate set at 37 °C. After 15 min, the suspension is replaced carefully with warm PBS to remove the non-binding cells from the surface. Micrographs are taken with the phase and texas-red channels of an inverted microscope.

Fabrication of the PDMS Stamps: A published procedure for preparing the PPL stamps was slightly adapted.^[12] 3.4 g of vinyl-compound-rich prepolymer (VDT-731, Gelest, USA), 18 μ L of 2,4,6,8-tetramethyltetravinylcyclotetrasiloxane (Sigma Aldrich, Germany) and one drop of a platinum catalyst (platinumdivinyltetramethyldisiloxane complex in xylene, SIP 6831.1, Gelest, USA) are stirred in a falcon tube and degassed in low grade vacuum of 0.1 bar for 15 min. 0.8 g of hydrosilane-rich crosslinker (HMS-301, Gelest, USA) is admixed, stirred and allowed to degas, before pouring the liquid polymer onto a silicon master. A cut microscopy glass slide is carefully placed on the filled master; air bubbles are

pressed out (less crosslinker gives more preparation time and fills the inverted pyramids of the master more homogeneously). The polymer is baked on a hot plate at 70 °C over night. After cutting residues of polymer at the sides of the glass slide, the glass is carefully detached from the master with a scalpel. To render the surface of the polymer pens hydrophilic, the stamp is treated with oxygen plasma at 10 sccm, 100 mTorr and 30 W for 2 min.

Cell Culture and Immunostaining of Direct Printed Patterns: 3T3 Fibroblasts were cultured on laminin fibronectin patterns for 20 h in DMEM, fixed with 4% paraformaldehyde, treated with TritonX-100 and stained. Chick retinal explants cultures were prepared as previously described in detail.^[39] In brief, embryonic day 7 chicken retinae were dissected, adhered on nitrocellulose filters and cut into 300 μ m wide strips. Explant strips were placed on the protein patterns (natural mouse Laminin (Invitrogen) and recombinant human ephrin-A5-FC (R&D)) and cultured for 36 h in F12 medium containing 0.4% methylcellulose, 2% chicken and 5% fetal calf serum. Cultures were fixed with 4% paraformaldehyde, 0.1% glutaraldehyde in PBS, treated with sodium borohydride, permeabilized with 0.1% TritonX-100 and stained. Antibodies employed for pattern staining: anti-laminin produced in rabbit (Sigma), anti-fibronectin produced in mouse (BD, bioscience), anti-rabbit-Cy3, anti-mouse-Cy5, anti-rabbit-AMCA produced in goat (Dianova) and anti-human-FC-Alexa594 produced in goat (Invitrogen). To visualize the cells on the patterns phalloidin-Alexa488 (Invitrogen) and DAPI were applied with the secondary antibodies. Fluorescence images were acquired with Zeiss Axioimager microscope.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

F. Brinkmann and M. Hirtz contributed equally to this work. This work was carried out with the support of the Karlsruhe Nano Micro Facility (KNMF, www.kmf.kit.edu), a Helmholtz Research Infrastructure at Karlsruhe Institute of Technology (KIT, www.kit.edu). The authors thank Dr. Emmanuel Oppong for the supply of cells and Prof. Dr. Andy Cato, Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, for the access to the cell culture laboratory. Special appreciation goes to Dr. Sylwia Sekula-Neuner for the stimulating discussions. We also thank the Mirkin group, especially Andrew Senesi, for providing the silicon masters used for the first PPL experiments in our laboratory and Dr. Uwe Köhler from the Institute of Microstructure Technology (IMT) at KIT for the fabrication of PPL masters that were used for the main experiments. Special thanks to G. Brinkmann for graphic artwork.

- [1] I. Barbulovic-Nad, M. Lucente, Y. Sun, M. Zhang, A. R. Wheeler, M. Bussmann, *Crit. Rev. Biotechnol.* **2006**, 26, 237.
- [2] A. E. Kusi-Appiah, N. Vafai, P. J. Cranfill, M. W. Davidson, S. Lenhart, *Biomater.* **2012**, 33, 4187.

- [3] S. Lenhert, F. Brinkmann, T. Laue, S. Walheim, C. Vannahme, S. Klinkhammer, M. Xu, S. Sekula, T. Mappes, T. Schimmel, H. Fuchs, *Nat. Nanotechnol.* **2010**, *5*, 275.
- [4] S. Sekula-Neuner, J. Maier, E. Oppong, A. C. B. Cato, M. Hirtz, H. Fuchs, *Small* **2012**, *8*, 585.
- [5] D. Castel, A. Pitaval, M.-A. Debily, X. Gidrol, *Drug Discov. Today* **2006**, *11*, 616.
- [6] M. Théry, M. Piel, *Cold Spring Harb. Prot.* **2009**, DOI: 10.1101/pdb.prot5255.
- [7] J. L. Tan, W. Liu, C. M. Nelson, S. Raghavan, C. S. Chen, *Tissue Eng.* **2004**, *10*, 865.
- [8] C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, D. E. Ingber, *Biotechnol. Prog.* **1998**, *14*, 356.
- [9] R. D. Piner, J. Zhu, F. Xu, S. Hong, C. A. Mirkin, *Science* **1999**, *283*, 661.
- [10] A. Kumar, G. M. Whitesides, *Appl. Phys. Lett.* **1993**, *63*, 2002.
- [11] G. Binnig, C. F. Quate, *Phys. Rev. Lett.* **1986**, *56*, 930.
- [12] F. Huo, Z. Zheng, G. Zheng, L. R. Giam, H. Zhang, C. A. Mirkin, *Science* **2008**, *321*, 1658.
- [13] S. Sekula, J. Fuchs, S. Weg-Remers, P. Nagel, S. Schuppler, J. Fragala, N. Theilacker, M. Franzreb, C. Wingren, P. Ellmark, C. A. K. Borrebaeck, C. A. Mirkin, H. Fuchs, S. Lenhert, *Small* **2008**, *4*, 1785.
- [14] T. Chen, R. Jordan, S. Zauscher, *Small* **2011**, *7*, 2148.
- [15] T. W. Odom, J. C. Love, D. B. Wolfe, K. E. Paul, G. M. Whitesides, *Langmuir* **2002**, *18*, 5314.
- [16] J. M. Hong, F. M. Ozkeskin, J. Zou, *J. Micromech. Microeng.* **2008**, *18*, 015003.
- [17] Z. Zheng, W. L. Daniel, L. R. Giam, F. Huo, A. J. Senesi, G. Zheng, C. A. Mirkin, *Angew. Chem. Int. Ed.* **2009**, *48*, 7626.
- [18] L. Huang, A. B. Braunschweig, W. Shim, L. Qin, J. K. Lim, S. J. Hurst, F. Huo, C. Xue, J.-W. Jang, C. a. Mirkin, *Small* **2010**, *6*, 1077.
- [19] S. Lenhert, P. Sun, Y. Wang, H. Fuchs, C. A. Mirkin, *Small* **2007**, *3*, 71.
- [20] S. Biswas, M. Hirtz, H. Fuchs, *Small* **2011**, *7*, 2081.
- [21] M. Hirtz, R. Corso, S. Sekula-Neuner, H. Fuchs, *Langmuir* **2011**, *27*, 11605.
- [22] O. A. Nafday, T. W. Lowry, S. Lenhert, *Small* **2012**, *8*, 1021.
- [23] J. M. Collins, R. T. S. Lam, Z. Yang, B. Semsarieh, A. B. Smetana, S. Nettikadan, *Lab Chip* **2012**, *12*, 2643.
- [24] A. Holmberg, A. Blomstergren, O. Nord, M. Lukacs, J. Lundeberg, M. Uhlen, *Electrophoresis* **2005**, *26*, 501.
- [25] M. Lehnert, M. Gorbahn, C. Rosin, M. Klein, I. Köper, B. Al-Nawas, W. Knoll, M. Veith, *Langmuir* **2011**, *27*, 7743.
- [26] X. Liao, A. B. Braunschweig, Z. Zheng, C. A. Mirkin, *Small* **2010**, *6*, 1082.
- [27] X. Liao, A. B. Braunschweig, C. A. Mirkin, *Nano Lett.* **2010**, *10*, 1335.
- [28] Z. Xie, Y. Shen, X. Zhou, Y. Yang, Q. Tang, Q. Miao, J. Su, H. Wu, Z. Zheng, *Small* **2012**, *8*, 2664.
- [29] L. R. Giam, M. D. Massich, L. Hao, L. Shin Wong, C. C. Mader, C. A. Mirkin, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4377.
- [30] M. A. Holden, P. S. Cremer, *J. Am. Chem. Soc.* **2003**, *125*, 8074.
- [31] A. Waldbaur, B. Waterkotte, K. Schmitz, B. E. Rapp, *Small* **2012**, *8*, 1570.
- [32] D. S. Rhoads, J.-L. Guan, *Exp. Cell Res.* **2007**, *313*, 3859.
- [33] J. T. Smith, J. T. Elkin, W. M. Reichert, *Exp. Cell Res.* **2006**, *312*, 2424.
- [34] D. Lehnert, B. Wehrle-Haller, C. David, U. Weiland, C. Ballestrem, B. A. Imhof, M. Bastmeyer, *J. Cell Sci.* **2004**, *117*, 41.
- [35] M. Knöll, U. Drescher, *Trends Neurosci.* **2002**, *25*, 145.
- [36] T. McLaughlin, D. D. M. O'Leary, *Annu. Rev. Neurosci.* **2005**, *28*, 327.
- [37] P. Suetterlin, K. M. Marler, U. Drescher, *Sem. Cell Dev. Biol.* **2012**, *23*, 1.
- [38] J. Vielmetter, B. Stolze, F. Bonhoeffer, C. A. Stuermer, *Exp. Brain Res.* **1990**, *81*, 283.
- [39] A. C. von Philipsborn, S. Lang, J. Loeschinger, A. Bernard, C. David, D. Lehnert, F. Bonhoeffer, M. Bastmeyer, *Development* **2006**, *133*, 2487.
- [40] A. L. Kolodkin, M. Tessier-Lavigne, *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, DOI: 10.1101/cshperspect.a001727.
- [41] Number of publications in ISI for the first 5 years after invention of the respective technique (numbers in bracket represent the number of publications by the technique's inventors): PPL 14 (12), DPN 116 (35), μ CP 76 (45), search query used: "keywords PPL", years 2008-2012; "keywords DPN", years 1999-2004; "keywords μ CP", years 1994-1998).

Received: December 18, 2012

Revised: January 28, 2013

Published online:



Supporting Information

for *Small*, DOI: 10.1002/smll.201203183

Interdigitated Multicolored Bioink Micropatterns by
Multiplexed Polymer Pen Lithography

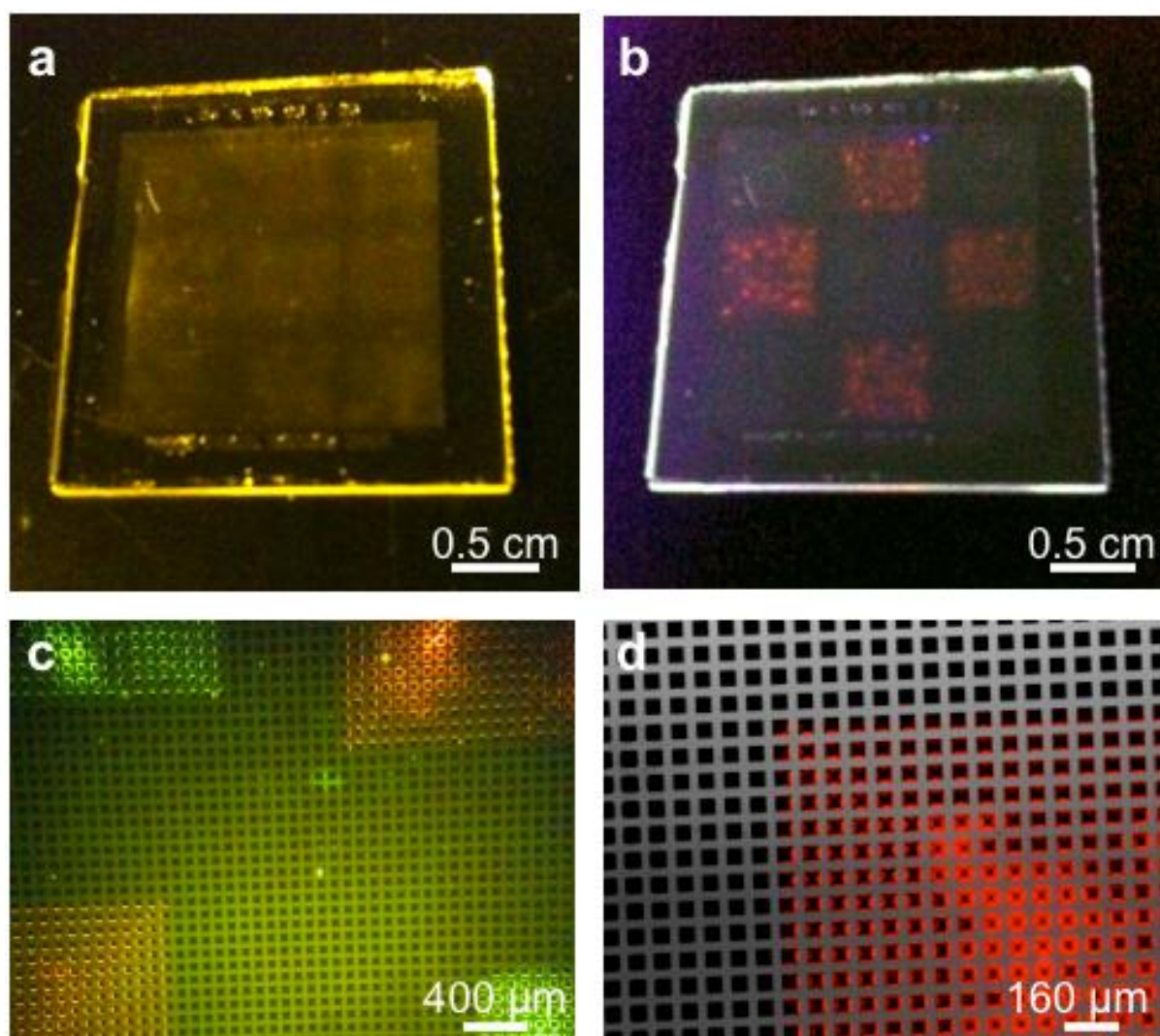
Falko Brinkmann, Michael Hirtz, Alexandra M. Greiner,
Markus Weschenfelder, Björn Waterkotte, Martin Bastmeyer,
and Harald Fuchs**

Supplementary Information

Interdigitated Multi-Color Bioink Micropatterns by Multiplexed Polymer Pen Lithography

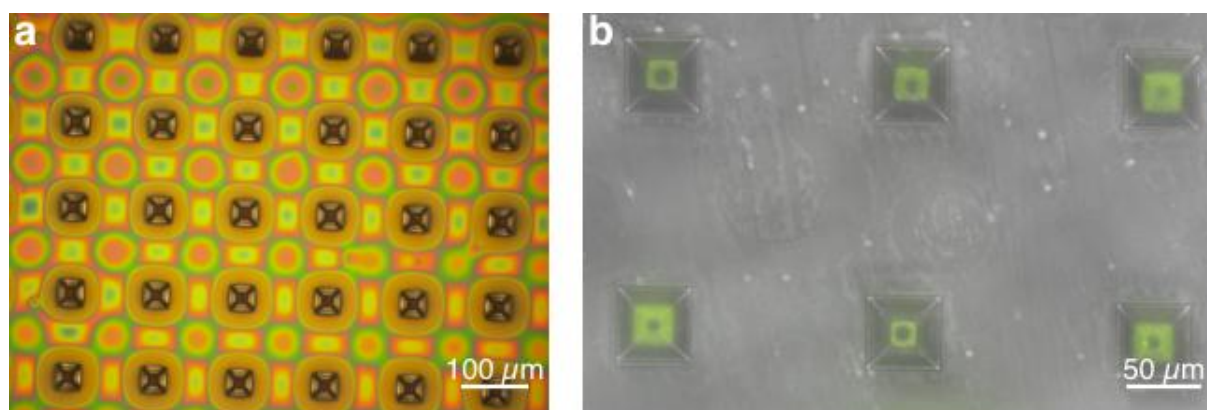
Falko Brinkmann,^{1,5} Michael Hirtz,^{*,1} Alexandra Greiner,² Markus Weschenfelder,² Björn Waterkotte,^{3,4} Martin Bastmeyer,^{2,4} Harald Fuchs^{*,1,5}

- 1 Institute of Nanotechnology (INT) and Karlsruhe Nano Micro Facility (KNMF),
Karlsruhe Institute of Technology (KIT), Germany
- 2 Zoological Institute, Department for Cell- and Neurobiology,
Karlsruhe Institute of Technology (KIT), Germany
- 3 Institute of Organic Chemistry (IOC),
Karlsruhe Institute of Technology (KIT), Germany
- 4 Institute of Functional Interfaces (IFG),
Karlsruhe Institute of Technology (KIT), Germany
- 5 Physical Institute and Center for Nanotechnology (CeNTech)
University of Münster, Germany

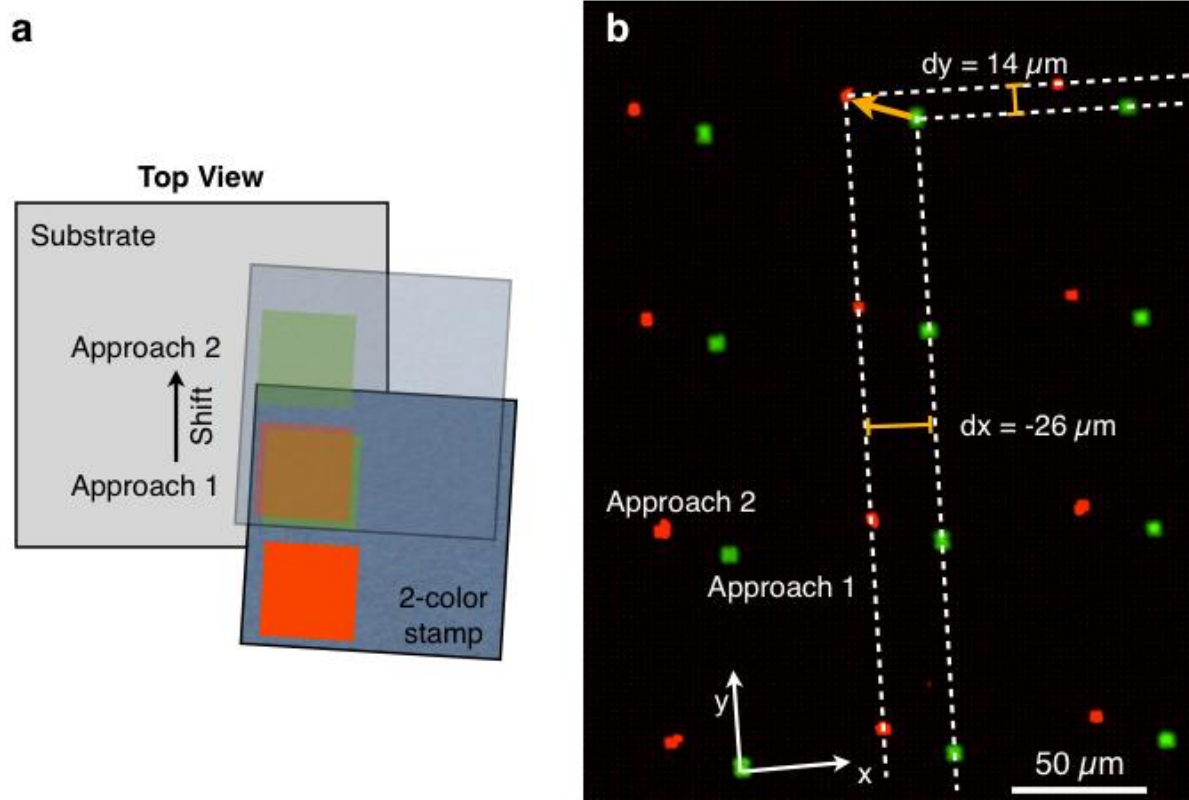


Supplementary Figure 1. (a) Photograph of an ordinary polymer pen lithography (PPL) stamp. The polydimethylsiloxane (PDMS) pens formed from a silicon mold stick to a glass support that was cut from a microscopy glass slide before. The stamp is coated with two phospholipid inks at nine different positions, which are slightly visible. (b) Photograph of the same stamp, which is illuminated with blue light. The areas coated with 0.5 μ l of a rhodamine-labeled phospholipid emit right light. The complementary areas inked with a fluorescein-labeled phospholipid shine in green (visible by eye), but is not detectable with the used camera. (c) Photograph through the ocular of a 4x microscope with fluorescent illumination. The edges of four ink reservoirs appear in green and red. The ink does not spread randomly, but form perfect rectangles when pipetting directly on the oxygen plasma treated stamp. (d), Overlay of the brightfield and texas red channel of a fluorescence microscope. Although the ink does not leave a homogeneous layer on the tips (some parts of the

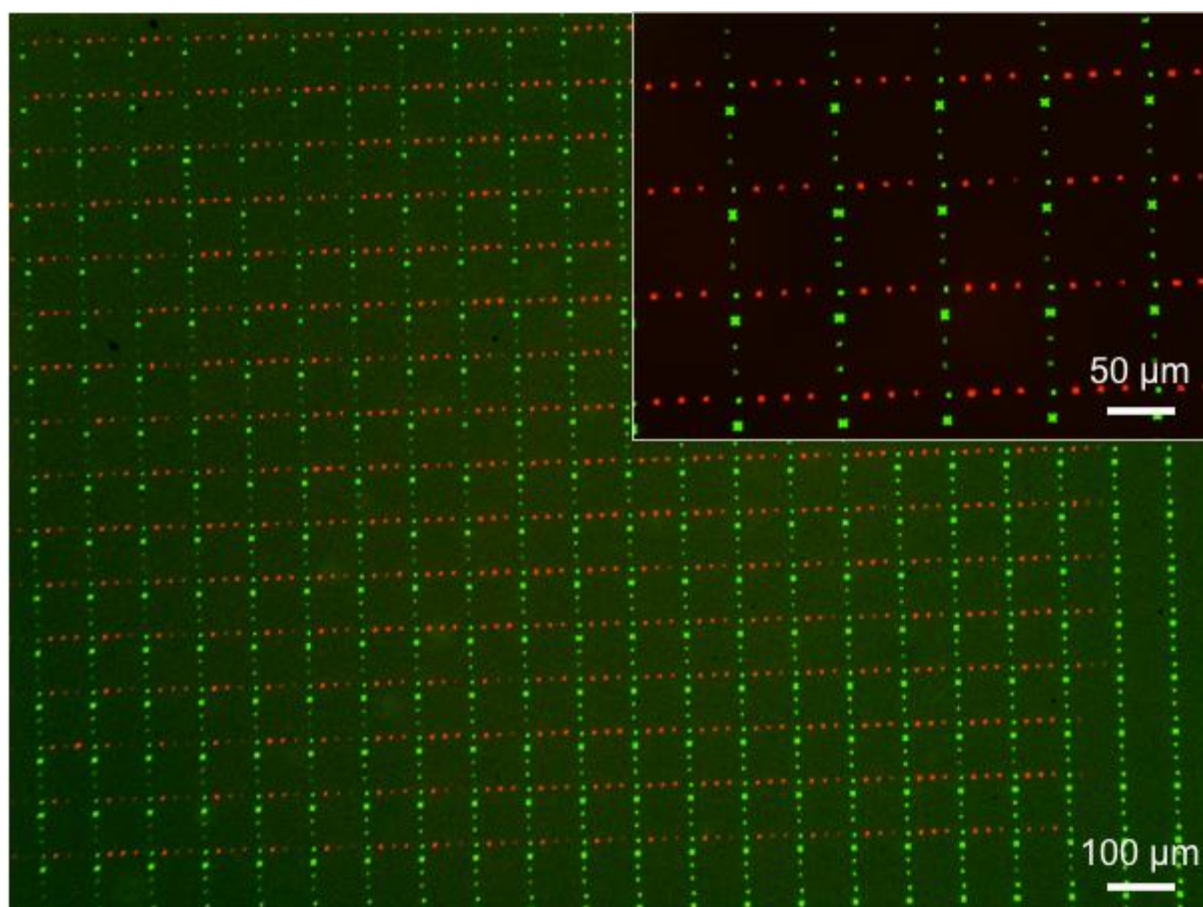
coated areas appear brighter), the total area covered is an exact rectangle. This pinning effect is due to the symmetrical alignment of the pyramidal pens in x/y. When dropping ink onto the pens, the spreading happens to be line-wise from one line or column of pens to the other (see supplementary video 1). This effect occurs as the gaps between the pens' rows is smaller by a factor of $2^{(1/2)}$ compared to the distance between two pens in 45° direction. Therefore, it is more likely that directly neighboring pens get coated with ink than others.



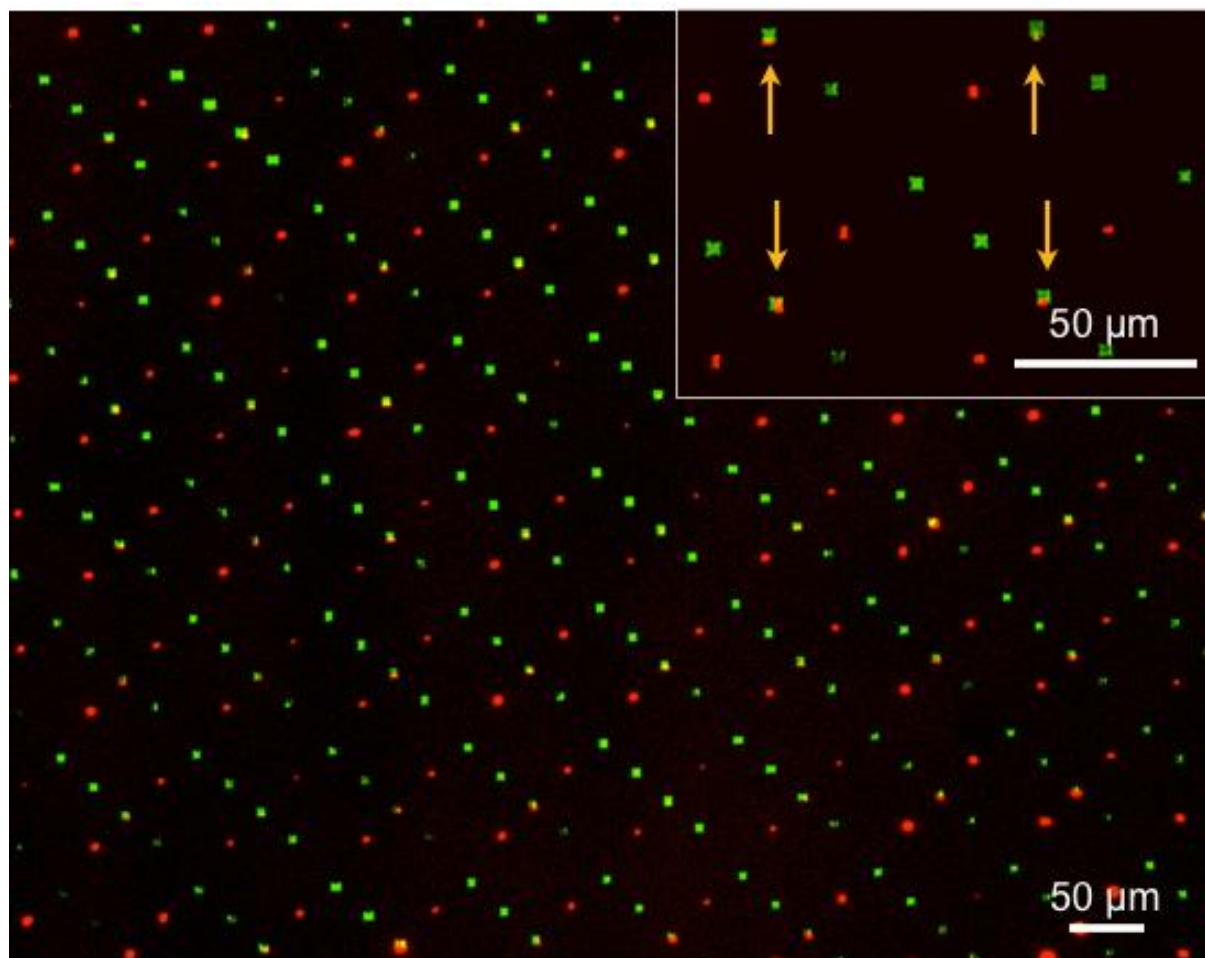
Supplementary Figure 2. Stamp pad inking of biotin-4-fluorescein. The stamp pad is a hydrophilic piece of silicon. 20 μ l of biotin-4-fluorescein was spread and dried. (a) The image shows the polymer pens while being in contact with the stamp pad (see supplementary video). Ink flows up and wets the surface of the pens. (b) Overlay of the phase and fluorescence image of six polymer pens after stamp pad inking. The ink only wets the lower half of the pyramids. Other parts do not get in touch with the ink.



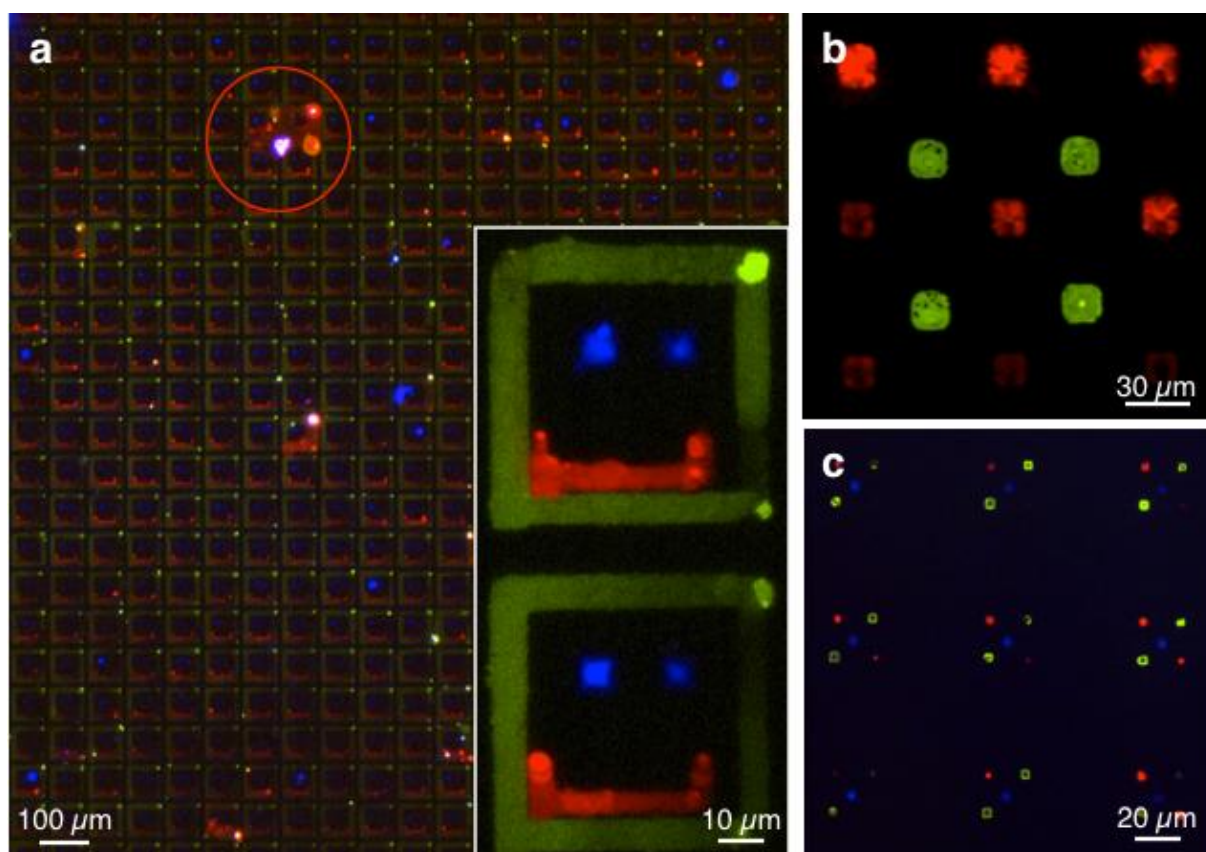
Supplementary Figure 3. (a) Schematic drawing illustrating the strategy to measure the offset caused from a shift between lithography of different parts of the stamp and the misalignment of the stamp concerning the stage movement axis. At first, the area of the stamp coated with ink 1 (green) approaches a sample substrate in one corner. Pens coated with other inks do not touch the surface as they are positioned next to the sample. Second, the stage is precisely shifted to the stamp area coated with ink 2 (red). Last, approach 2 is carried out. (b) Fluorescence overlay of the approach dots explained in (a). The offset in x and y can be determined by measuring the distance of the parallel lines through the red and green approach dots.



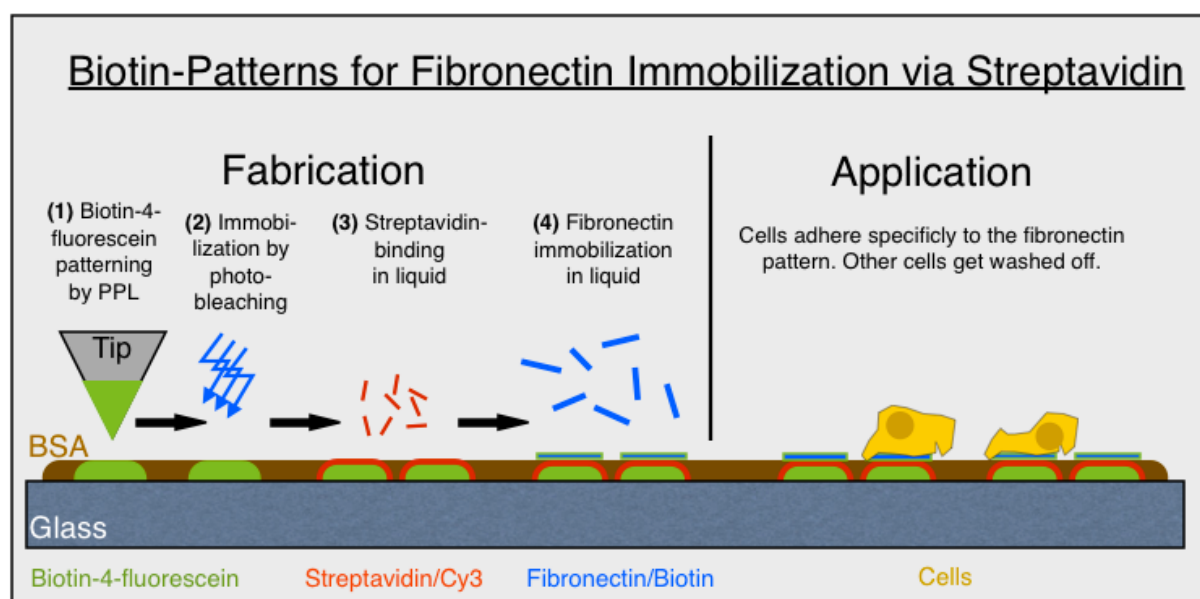
Supplementary Figure 4. The overlay of two fluorescence micrographs shows a two-color mosaic pattern. By taking the offset into account, pens coated with other inks can be precisely added to an existing pattern. The right side of the overlay shows only green dots as the border of the red and green patterns do not perfectly overlay. The larger green dots are a result of a different pen-surface contact area.



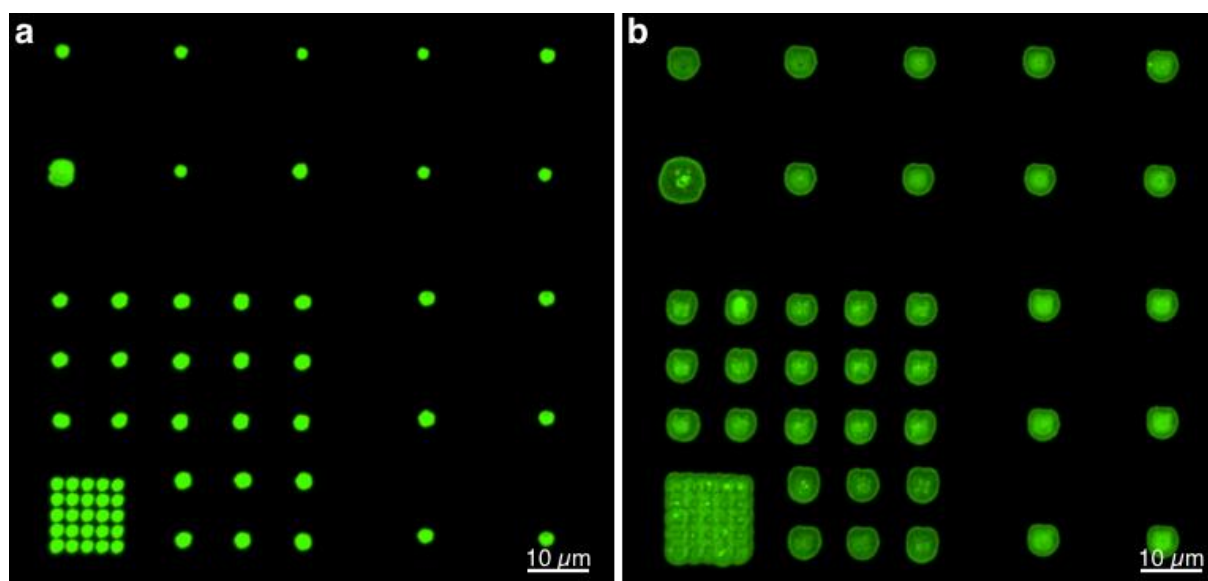
Supplementary Figure 5. Our multiplexing strategy allows interdigitated multi-ink patterns. Furthermore, different materials can be even mixed on the surface by performing pen approaches with different inks at one spot. As pens used for integrating materials may carry ink mixtures after approaches, pens that have not got in contact with other inks on the surface should be used for further lithography.



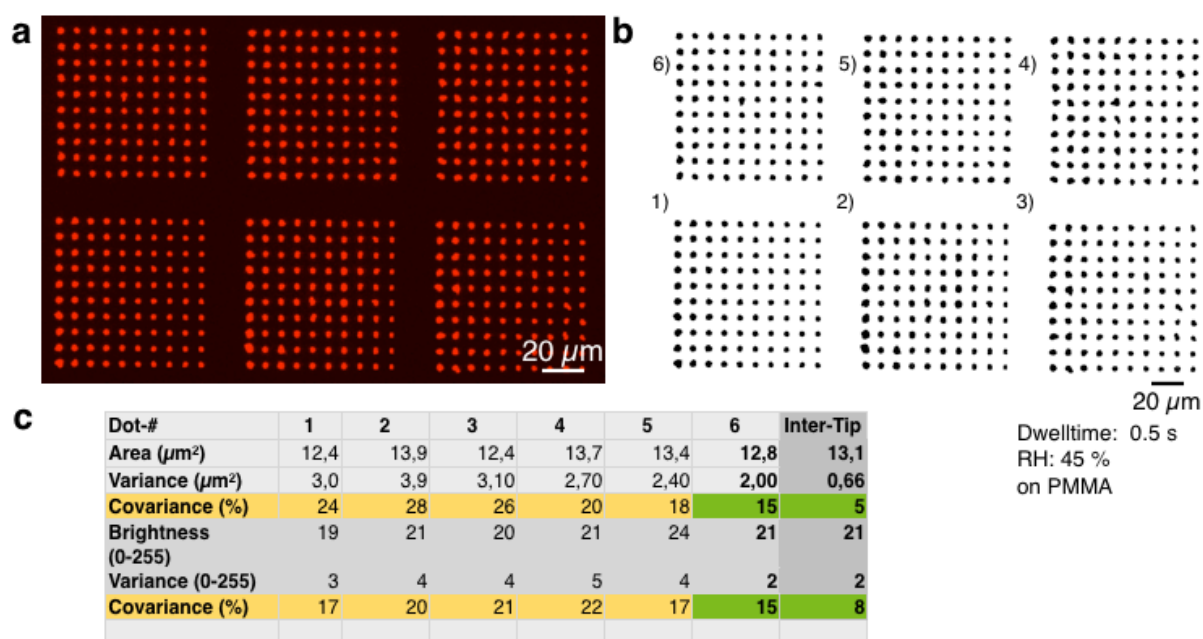
Supplementary Figure 6. Overlays of fluorescence micrographs presenting multiplex patterns. (a) 400 smily-faces of fluorescently labeled phospholipids out of approximately 2.500. The total patterned area is 25 mm^2 . A minority of pens carries too much ink that results into smeared structures (see red circle). (b) Inks of different types, e.g. lipids and proteins, can be integrated in one pattern. Here, greens represents biotin-4-fluorescein and red a rhodamine labeled phospholipid. This micrograph shows one pattern printed by two pens subsequently. The total area is 5 by 5 millimeter. (c) The overlay shows a integrative dot pattern of rhodamine, fluorescein and dansyl-labeled phospholipids.



Supplementary Figure 7. Lithography, immobilization and general functionalization of biotin micropatterns by PPL. (1) The micropattern is generated with biotin-4-fluorescein on a BSA coated glass slide by polymer pens. (2) Immobilization is carried out by bleaching the fluorescein with blue light, followed by a washing step. (3) Streptavidin, possibly labeled with a cy3-fluorophore, is incubated on the biotin pattern. That converts the biotin-pattern into a streptavidin-pattern that offers vacant binding sites for biotin. (4) Arbitrary biotinylated molecules can bound to the streptavidin pattern. In our approaches, we present experiments in that we have created human fibronectin and dinitrophenol (DNP) micropatterns by respective biotinylated molecules.



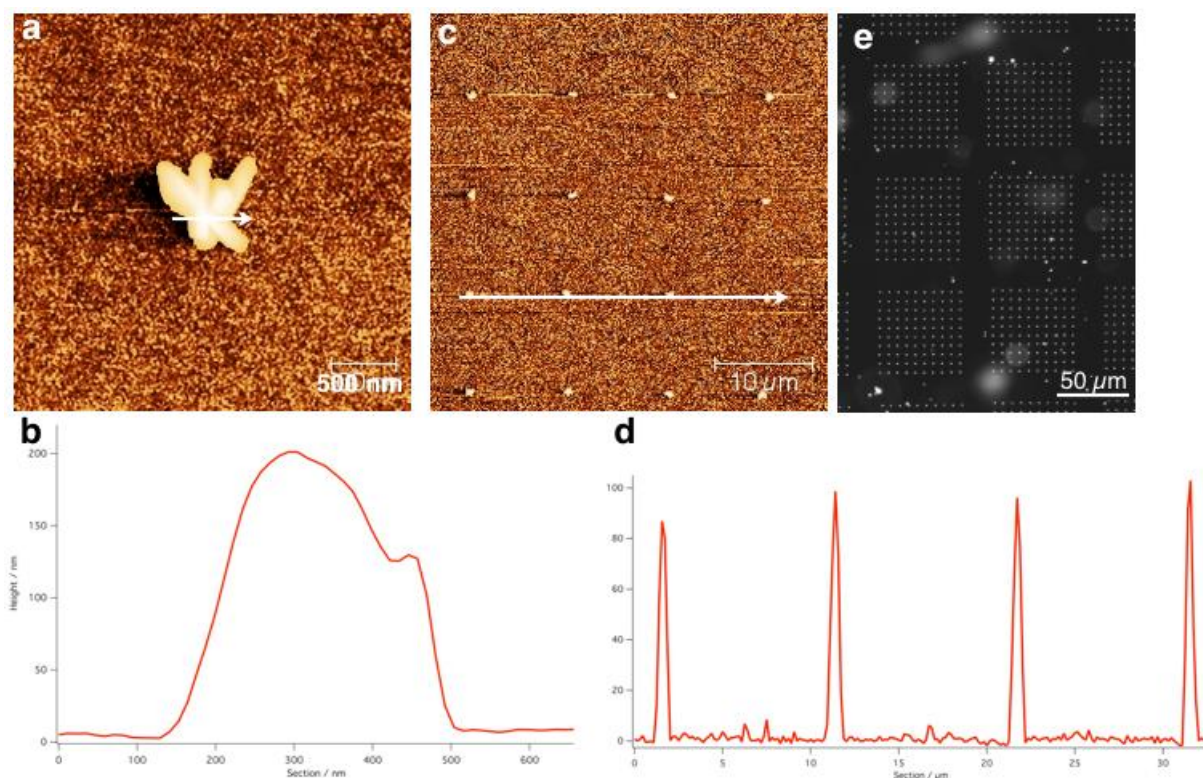
Supplementary Figure 8. These 50x magnified micrographs of a biotin-4-fluorescein pattern on a bovine serum albumin (BSA) coated glass slide show the effect of different pen surface contact areas. Whereas the freshly coated pen hardly touches the surface in (a), the pen surface contact area in (b) is larger resulting into larger and possibly spreading features. The images were taken before washing the sample.



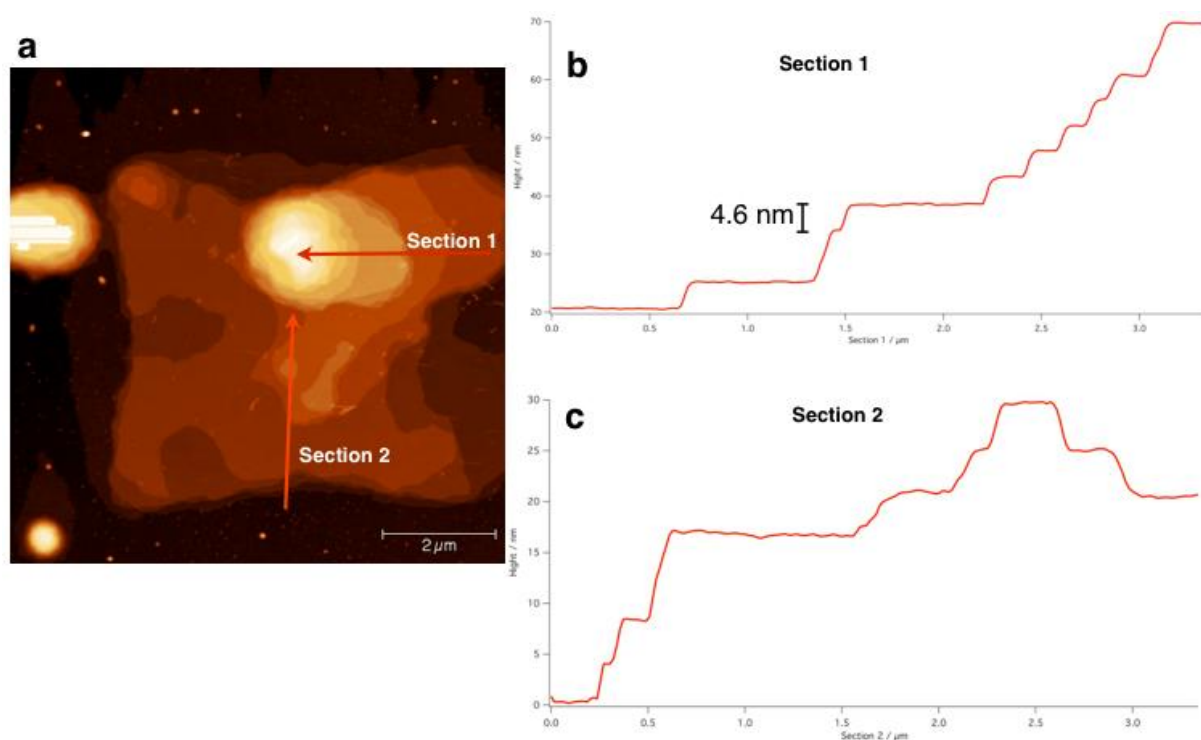
Supplementary Figure 9. Statistical values for the lithography of phospholipids (DOPC + 5 % rhodamin). (a) Arrays consisting of 100 dots patterned by six tips. The image shows a fluorescence micrograph. (b) In order to calculate the area for each dot, a threshold is set. The quantification is carried out with ImageJ. (c) The table presents the mean area of the 100 dots for each tip, the variance and covariance. Furthermore, the outlines of the dots are referred to the original image so that the gray value, meaning the brightness, can be measured. Tip #6 has a covariance of 15 % concerning area and brightness. The average of the average values of the single tips result into the inter-tip statistics. The tips itself differ about 5 % concerning the average area and 8 % concerning brightness.

Biotin Ink	Size (μm^2)	Brightness (GV 0 - 255)	Inter-Tip (Size)	Inter-Tip Bright. (GV 0 - 255)
Measured dots / tips	100 dots	100 dots	3 tips	3 tips
Average	73,8	17,6	77,2	22,0
Std-Deviation	22,1	11,4	16,9	6,1
Co-Variance (%)	30,0	64,8	12,8	27,8
Measured dots / tips	30 dots	30 dots	3 tips	3 tips
Average	97,1	27,5	100,1	35,7
Std-Deviation	9,7	8,2	28,3	11,7
Co-Variance (%)	10,0	29,9	18,3	32,8
Lipid Ink	Size (μm^2)	Brightness (GV 0 - 255)	Inter-Tip (Size)	Inter-Tip Bright. (GV 0 - 255)
Measured dots / tips	100 dots	100 dots	6 tips	6 tips
Average	12,8	21,0	13,1	21,0
Std-Deviation	2,0	2,0	0,7	1,7
Co-Variance (%)	15,2	15,0	5,0	8,0

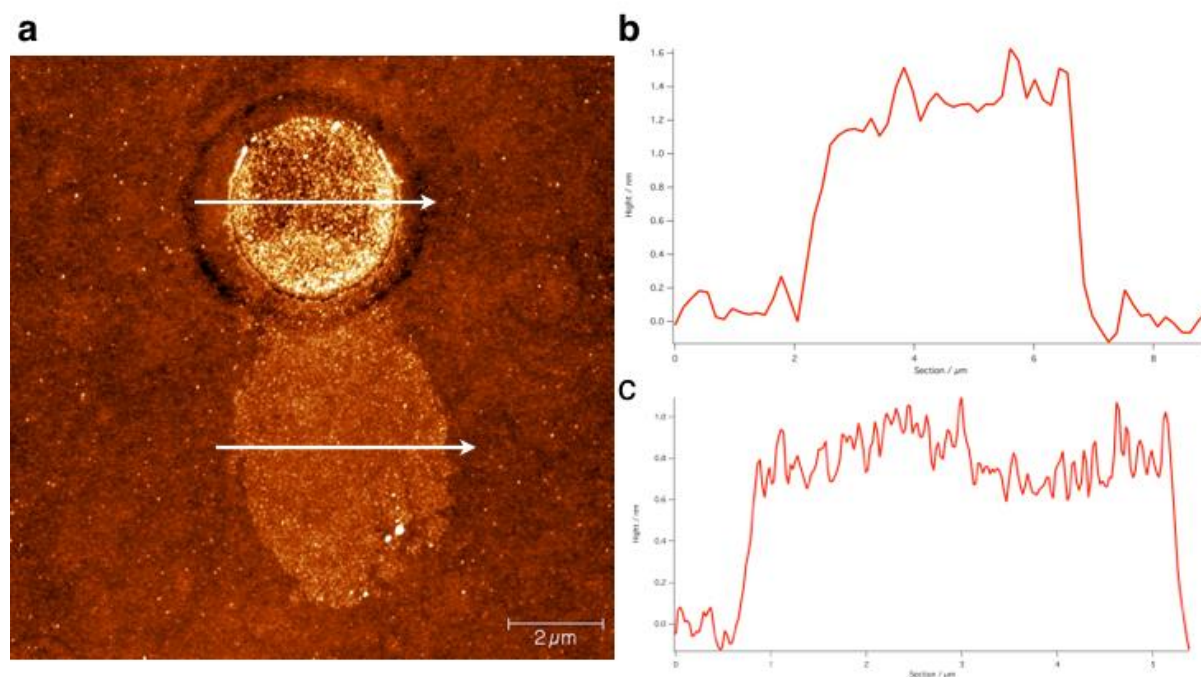
Supplementary Figure 10. This table compares the transfer of biotin-4-fluorescein and a phospholipid ink during 100 pen approaches at the top and the bottom sections. As biotin-4-fluorescein has a strong ink decay (see Fig. 3), the middle section of this table represents the first 30 approaches. The size of 30 biotin-4-fluorescein varies about 10 % while phospholipid dots vary about 15 %. The inter-tip values shows that pens coated with phospholipids write more homogeneously: 5 % variation of 6 tips in contrast to 18 % variation of 6 biotin-4-fluorescein coated tips.



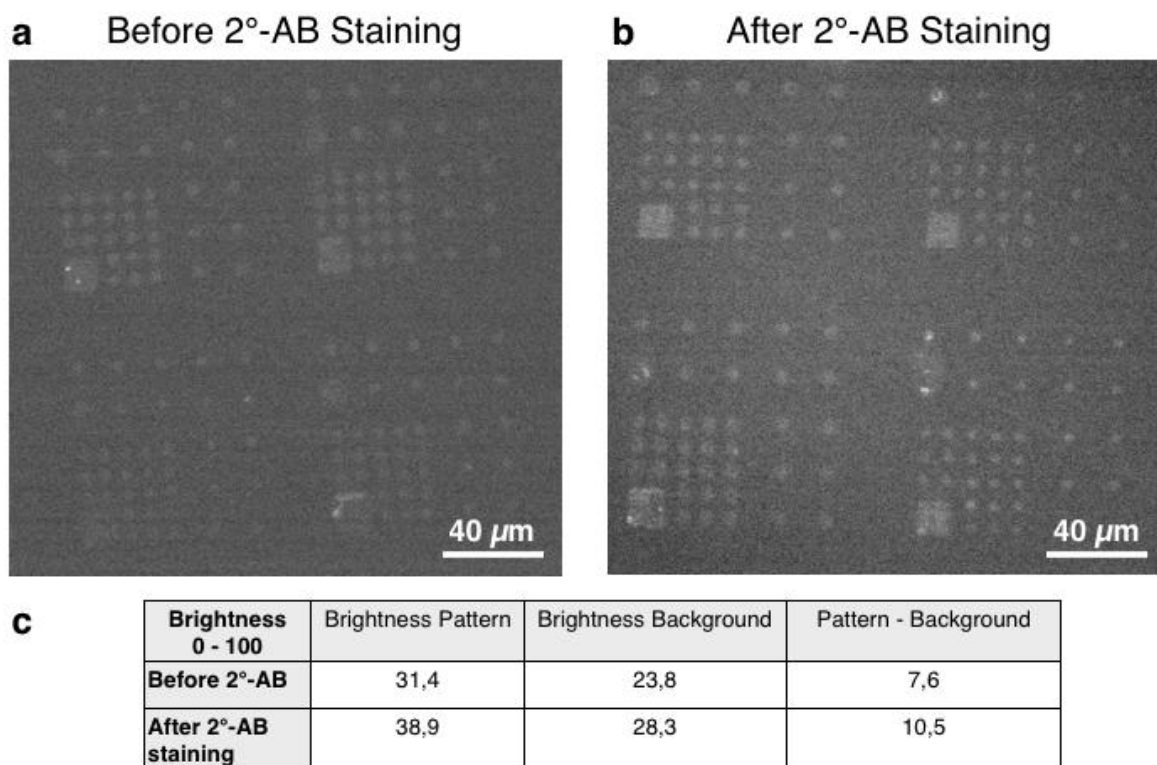
Supplementary Figure 11. Atomic force microscopy images of a phospholipid dot array presented in SI Fig. 9. (a) Topography of one dot. (b) Section of the marked line (see a). The maximum height is 200 nm. The minimum diameter of this dot is 400 nm. (c) Topography of 20 dots to illustrate their homogeneity. The section in (d) shows a height between 90 and 100 nm. (e) Darkfield micrograph of the analyzed pattern.



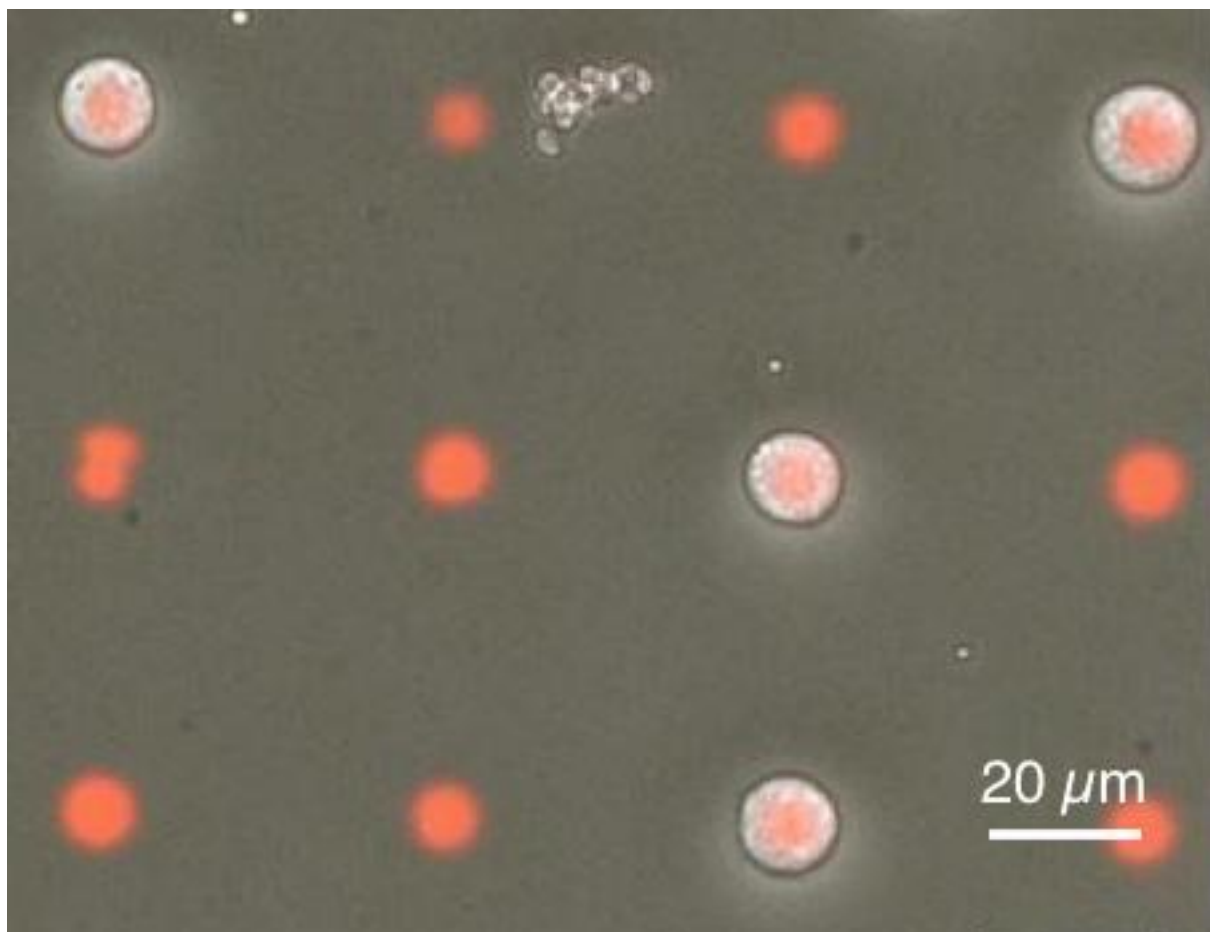
Supplementary Figure 12 (a) AFM topography image of one approach dot of a phospholipid coated PPL tip. Phospholipids form multilayers that are clearly visible in the sections (b, c). The single bilayer layer height is 4.6 nm.



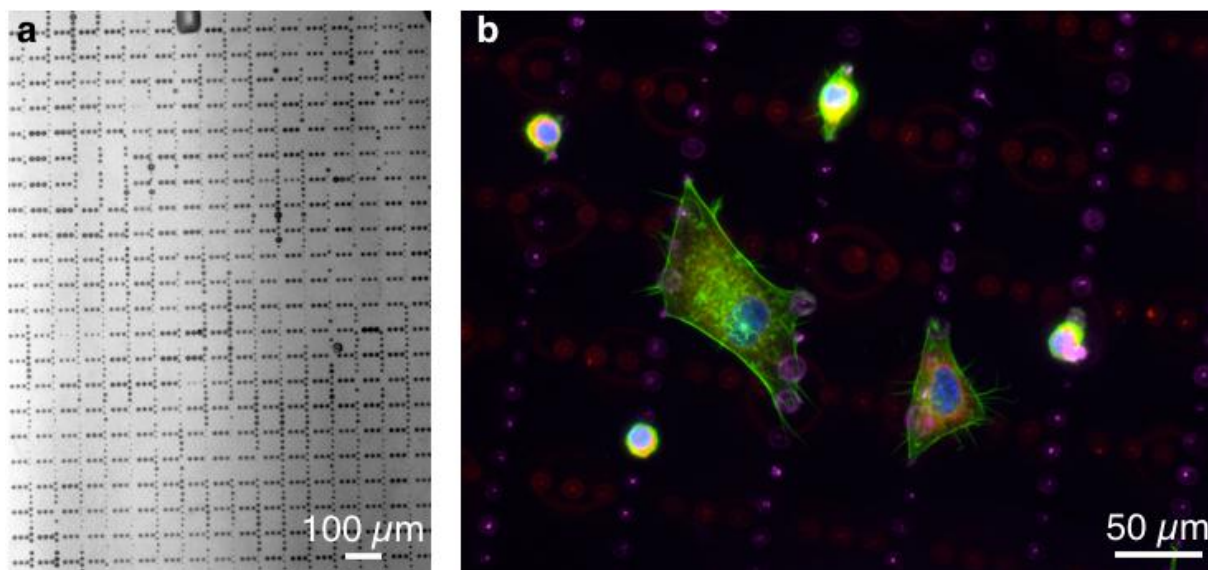
Supplementary Figure 13. AFM topography image of one approach dot of a biotin-4-fluorescein coated PPL tip. The squared darker area in the middle of the top round feature corresponds to the pen surface contact area. The section in (b) verifies a height of 1.5 nm; the tail in (c) a height of approximately 0.8 nm.



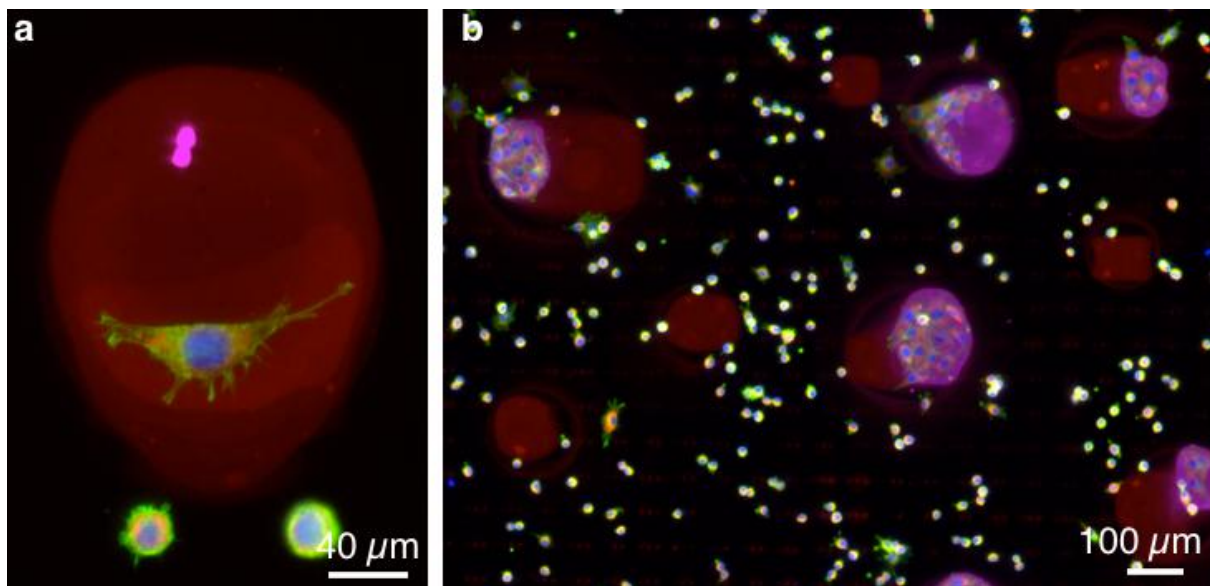
Supplementary Figure 14. Binding of biotinylated dinitrophenol (DNP) to a biotin-streptavidin pattern (see SI Fig. 8). The active binding of DNP is proven with a secondary antibody staining. (a) Fluorescence micrograph showing the remaining signal of the initial biotin-4-fluorescein pattern. (b) After secondary antibody binding, the intensity of the pattern increases. This is underlined by the normalized brightness values in the table in (c).



Supplementary Figure 15. Human embryonic kidney (HEK) cells immobilized on biotinylated fibronectin that was bound to a biotin-streptavidin-micropattern (see SI Fig. 7 for detailed description). HEK cells are incubated on the pattern for 15 minutes and rinsed with warm PBS to wash off not adhering cells. The image is an overlay of the phase channel and texas red channel of a fluorescence microscope.



Supplementary Figure 16. Large scale fibronectin / laminin pattern with adhering fibroblasts. The two proteins mixed with a protein carrier ink were printed in a direct method. (a) Brightfield micrograph of the fabricated multiprotein pattern. (b) Overlay of different channels to visualize the multiplexed pattern and the fibroblasts. The affinity to the fibronectin pattern (magenta) is dominant over laminin (red).



Supplementary Figure 17. Direct protein lithography. (a) An adhering fibroblast spreads well on a large spot of fibronectin. The cells in the bottom stay spherical as they have no affinity to the substrate. (b) Large spots of fibronectin (purple in this micrograph) and laminin (red). The laminin areas do not show any adhering cells whereas the fibronectin areas are covered with cells showing their affinity to fibronectin.