Supporting Information

Mesopattern of Immobilised Bone Morphogenetic Protein-2 Created by Microcontact Printing and Dip-pen Nanolithography Influence C2C12 Cell Fate

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Materials

Phosphate buffer saline (PBS), Chloroform (HPLC grade), Triton-X, (1-mercapto-11-undecyl)tri(ethylene glycol (PEG-thiol) and Fluoromount were purchased from Sigma Aldrich. Cell culture medium and supplements, as well as Trypsin, Hoechst and Alexa Fluor® 568 goat anti-rabbit IgG antibody were purchased from Invitrogen S.A. Rabbit anti-mouse antibody for Osterix were purchased from Santa Cruz Biotechnology Inc. 4%-Paraformaldehyde was purchased from Merck Sharp & Dohme. The lipid 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids Inc. Carrier free recombinant human BMP-2 was purchased from R&D Systems. Streptavidin (recombinant from Streptomyces avidinii, Texas Red labeled and unlabeled) was purchased from Cultek. Saponin was purchased from Fluka. DPN accessories like tips and inkwells were all purchased from NanoInk Inc. Gold surfaces were purchased from NT Buchs (Switzerland) and consisted of float glass with a 2-5 nm titanium intermediate layer and a gold layer of approximately 45 nm.

Sample preparation

Homogeneous substrates: The gold surfaces were cleaned with piranha acid (7 : 3 v/v solution of H_2SO_4 and H_2O_2; *Caution: piranha acid is a strong oxidiser and a strong acid. It should be handled with extreme care, as it reacts violently with most organic materials*) for 30 min, rinsed with MilliQ water and ethanol. Subsequently, they were immersed for 12h at 4ºC in a 1 mM solution of biotin-thiol in absolute ethanol. Finally, they were rinsed with ethanol to remove excess biotin-thiol, dried with pressurised air and kept sterile.

µCP patterning: A PDMS stamp with 5 µm round posts was fabricated following standard procedures.[1] For printing, the stamp was cleaned with absolute ethanol, incubated in a 1 mM solution of biotin-thiol in absolute ethanol, subsequently dried with pressurised air, placed on the gold surface and left in contact for 1 min. After 12 h, surfaces were rinsed with absolute ethanol, passivated for 2 h in a 2 mM solution of PEG-thiol in absolute ethanol, rinsed with ethanol and finally dried with pressurised air and kept sterile.

DPN patterning: DPN writing was performed using a commercially available DPN writer (NLP 2000, NanoInk Inc. USA) and one-dimensional M-type tip arrays. Tips were inked in corresponding inkwells using the following ink: 20 mol% biotin-thiol in ethanol (8 mg/mL) mixed with 80 mol% of a chloroform solution of DOPC (1 µM). For inking, the humidity was increased to 70% and the tips were brought into contact with the ink during 15 min. For lithography, the humidity was decreased to 35% and the dwell time was set to 0.2 s per dot. After lithography, substrates were incubated overnight at a humidity of 40%, rinsed with a solution of 1% Triton-X in Milli-Q, passivated for 2 h in a 2 mM solution of PEG-thiol in ethanol and finally rinsed with ethanol, dried with pressurised air and kept sterile.

Surface functionalisation

In **Fehler! Verweisquelle konnte nicht gefunden werden.**, the functionalisation steps of the substrates are depicted schematically. In the first step, a biotin-thiol is bound to the gold substrates (using DPN or µCP, or as a monolayer by complete immersion; for the patterned substrates, the remaining free gold is subsequently passivated with PEG-thiol). In the second step, streptavidin is bound to the biotin moieties and in the final step biotinylated bone morphogenetic protein-2 (BMP-2) is bound to the streptavidin.

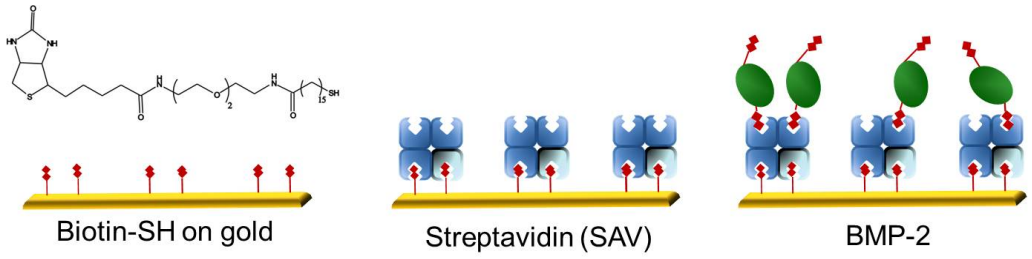


Fig. S1: Schematic representation of the functionalisation steps for the gold substrates: biotin-thiol, streptavidin and bone morphogenetic protein-2 (BMP-2).

Immobilisation of streptavidin and biotinylated BMP-2

All substrates (homogeneous, µCP and DPN) were incubated with 100 µM/mL streptavidin (µCP) or streptavidin Texas Red® (DPN) in PBS for 1 h at room temperature and rinsed with PBS. Subsequently, 14 µL of biotinylated BMP-2 in PBS (biotinylation protocol available in the article by Lagunas *et al.*)[2] were incubated on the substrates for 1 h at room temperature. After rinsing the samples with PBS, they were transferred into culture well plates.

Cell culture

C2C12 mouse myoblast cells (kindly provided from Prof. Francesc Ventura, Hospital de Bellvitge, Spain) from passage 4 were expanded for 1 day at 37 ºC and 10% CO_2 in growth

medium prior to the experiments. Growth medium consisted of: Dulbecco's Modified Eagle Medium liquid high glucose (D-MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin and 1% sodium pyruvate. After treating the cells for 1 min with Trypsin, they were centrifuged, resuspended and seeded on the different samples at a density of 30.000-35.000 cells/cm². All surfaces were prepared triplicate. The expression of osterix (OSX) was evaluated after 24 h of cell culture. Therefore, cells were fixed with a solution of 4% paraformaldehyde in PBS for 30 min, rinsed with PBS and permeabilised with a solution of 0.1% saponin in PBS with 1% BSA for 10 min at room temperature. Subsequently, cells were stained with rabbit anti mouse antibody for OSX (1:500 dilution in PBS with 1% BSA; 200 µg/mL) and detected with Alexa Fluor® 568 goat anti-rabbit IgG (2 mg/mL, diluted 1:400) and Hoechst (10 mg/mL, diluted 1:1000) in PBS with 1% BSA. After incubation, samples were mounted with Fluoromount and examined with fluorescence microscopy (green excitation G-2A long pass emission filter for OSX and UV emission filter for Hoechst). For cell counting, Image J software was used.

DPN calibration of the biotin-SH compared to MHA

Figure S2 shows the two calibration curves for dots with MHA (16-mercaptohexadecanoic acid, blue line) and biotin-thiol (red line).[3] It is obvious that the slope of the red line is very low, when compared to the blue. This means that the dot diameter increases only very slow with increasing dwell time and, in turn, in order to get bigger dots, the dwell time has to be increased dramatically and the overall patterning time will become too long.

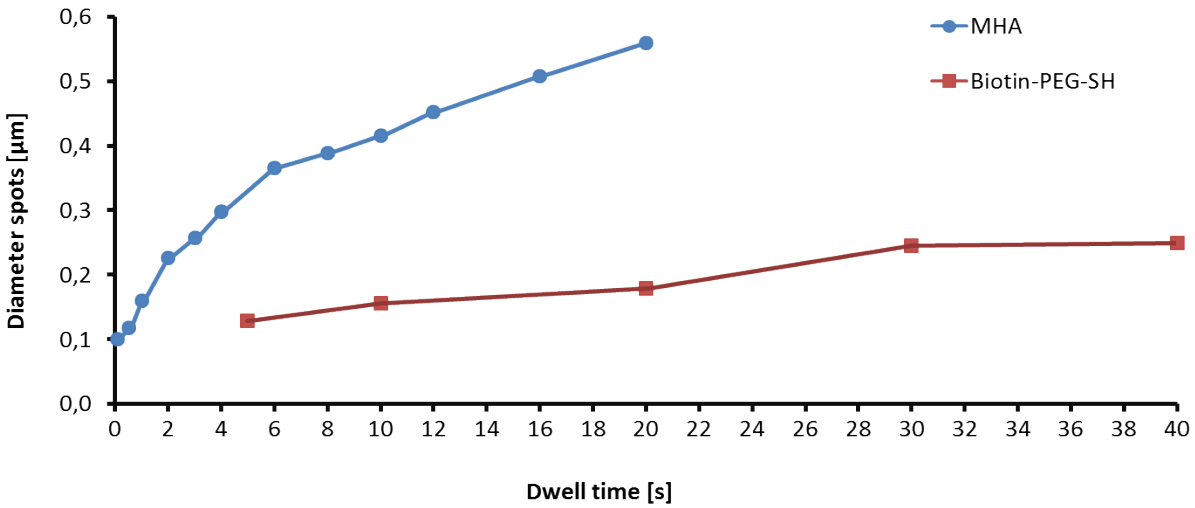


Figure S2: Calibration curve for biotin-thiol (red line). The values were obtained by measuring the calibration pattern dots with the InkCad (Nanoink Inc.) software. As a reference, the calibration for MHA is given (blue line).

This finding is in good correspondence with a work published by Rakickas *et al.* who reported that, using a biotin-disulfide as ink on gold substrates, spot diameters of 200 nm were

achieved with dwell times of 60 s.[4] As example, the substrates designed for cell experiments should have ~5000 dots each, resulting in a total patterning time of 83 h (almost 3,5 days) for only one substrate!

Expansion of the final pattern area

As stated by Derda et al.,[5] substrates suitable for cell experiments have to have a patterned area of minimum 0.25 mm².

Figure **S3** explains how a sufficiently large patterned area can be obtained by pattern expansion. After completing the pattern in y-direction, the whole set of cantilevers is shifted a specific distance to the left or right, where the patterning is continued. If all distances are calculated well, the resulting pattern will be homogeneous.

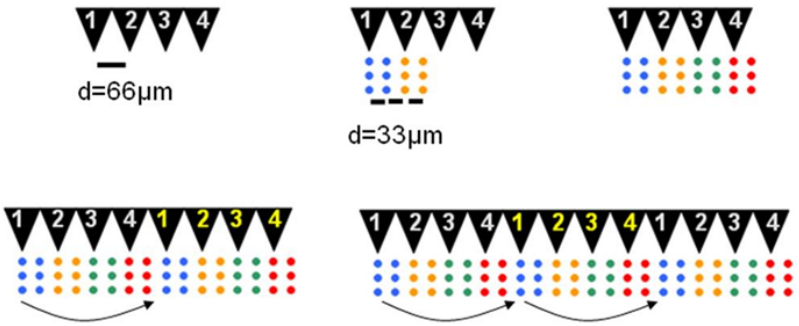


Figure S3: Schematic explication of the pattern expansion in x-direction by moving all cantilevers to the right (arrow). (Distance between tips is 66 µm, between spots 33 µm.)

Applying said strategy, a pattern is obtained as shown in Figure S4. The patterning process went on the left side from bottom to top. Then, the whole cantilever array was moved to the right and the patterning continued from bottom to top. Numbers indicate the different tips (12 cantilevers on a chip). For distinguishing better between the area first patterned (blue numbers) and the second (green), the first area has double y-spacing between the dots.

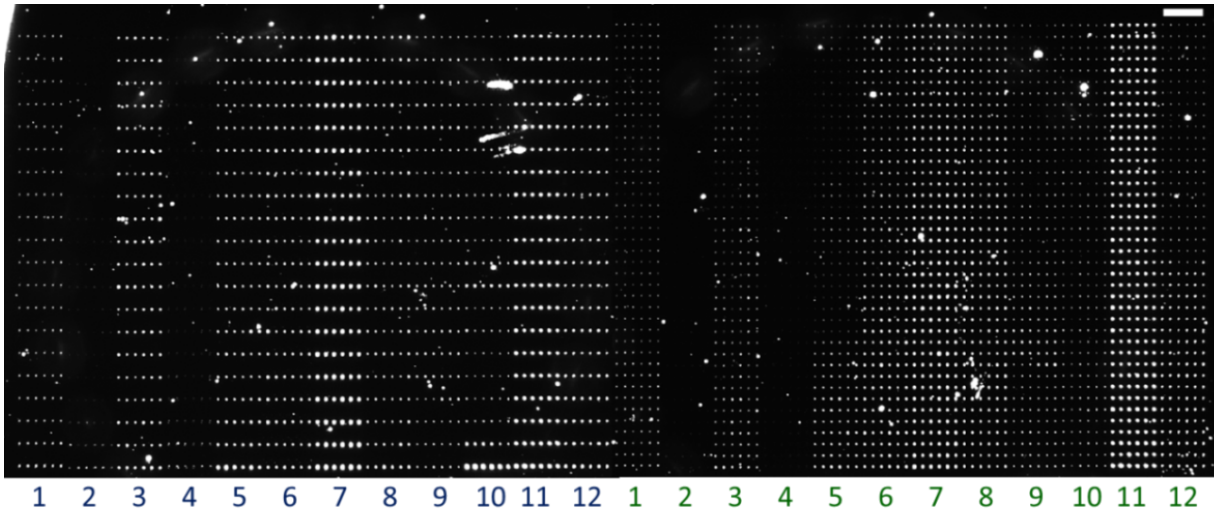


Figure S4: Darkfield microscopy image of DOPC with biotin-thiol on gold after patterning. The numbers indicate the different cantilevers; the writing direction is from bottom to top. Blue numbers

indicate where the first area was patterned and the green where the whole cantilever array was moved to the right and the pattern area thus amplified. The scale bar equals 50 µm.

The resulting pattern is more or less homogeneous for the blue numbers. For the green numbers, a slight gradient in y-direction can be seen, especially for tips number 5 and 12, which were slowly running out of ink. In order to increase the overall homogeneity of the patterned area, changes were made to the patterning routine as indicated in Figure S5. The image on the left side shows the pattern of the first tip (red dots). The red arrows correspond to the way each tip makes during lithography, meaning that the tip will fist pattern the left column of dots (blue number 1), move to the right and then pattern the right column (green number 1). For all further experiments, the tip described the way as indicated in the image on the right side, where each column is written in fractions in order to minimise the gradient in y-direction.

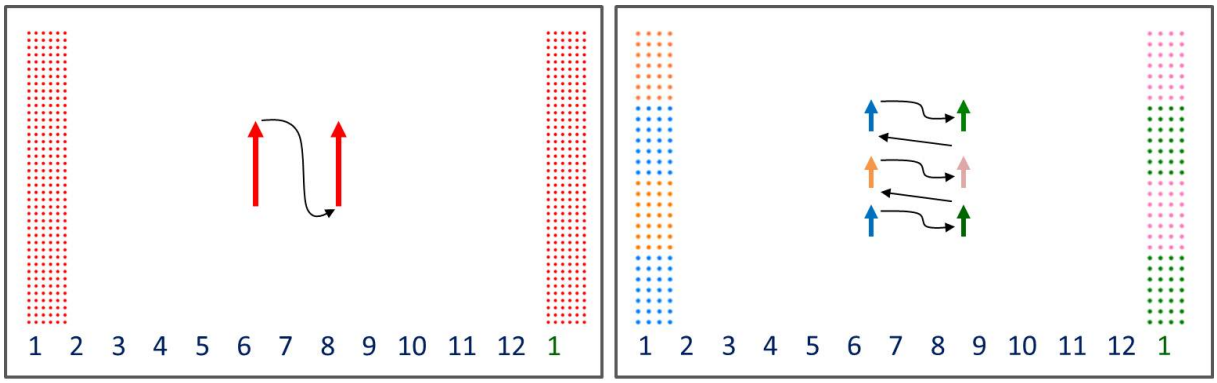
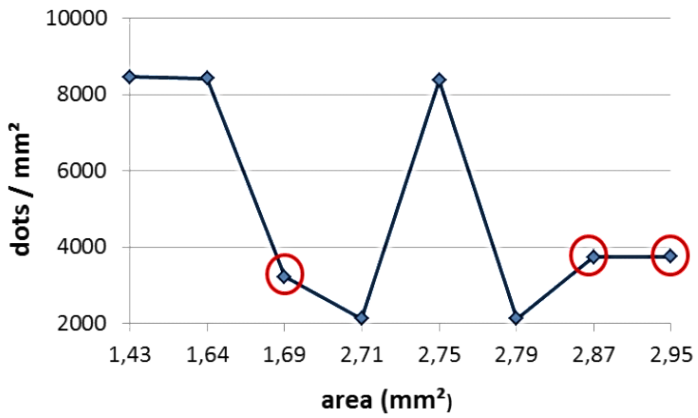


Figure S5: Schematic set-up of the improved patterning conditions (right side) for a big area pattern with one or two shifts in order to amplify the area in y-direction. Numbers indicate the different cantilevers; blue is the first patterning area, green the second after the shift. For simplification, the pattern of only one cantilever is shown but all cantilevers perform equally.

Low- and high-density pattern via Dip-pen Nanolithography

Further improvement of the pattern homogeneity was achieved by testing different dot densities of the pattern. For high dot densities, the pattern is less likely to be homogeneous since the cantilevers can run out of ink faster. Figure S6 shows a table with different values for the dot density and different pattern sizes.



area (mm²)	dots	dots/mm²
1,43	12096	8459
1,64	13824	8429
1,69	5445	3222
2,71	5760	2125
2,75	23040	8378
2,79	5940	2129
2,87	10752	3746
2,95	11088	3759

Figure S6: Graph and table for different dot densities. The dot density was calculated with the pattern area and the total number of dots written. The red circles indicate the – as determined – optimal dot densities for further experiments.

After evaluation of all patterns by darkfield microscopy, concerning the homogeneity and integrity of the complete patterned area, the optimal dot density value was determined for around 4000 dots/mm² (red encircled data points in the graph). This decision was taken due to personal evaluation of all created pattern with microscopy, considering the highest density possible (the smallest dot-dot distance) without losing pattern quality. This approximate density was used for all further experiments. Figure S7 shows the darkfield microscopy image of a mesopattern of DOPC containing biotin-thiol on gold, with a total pattern area of 2.36 mm². The homogeneity of the pattern is satisfying, because all dots have similar diameters with a narrow distribution. The mean dot size was determined to be 4.01 ± 0.90 µm in diameter.

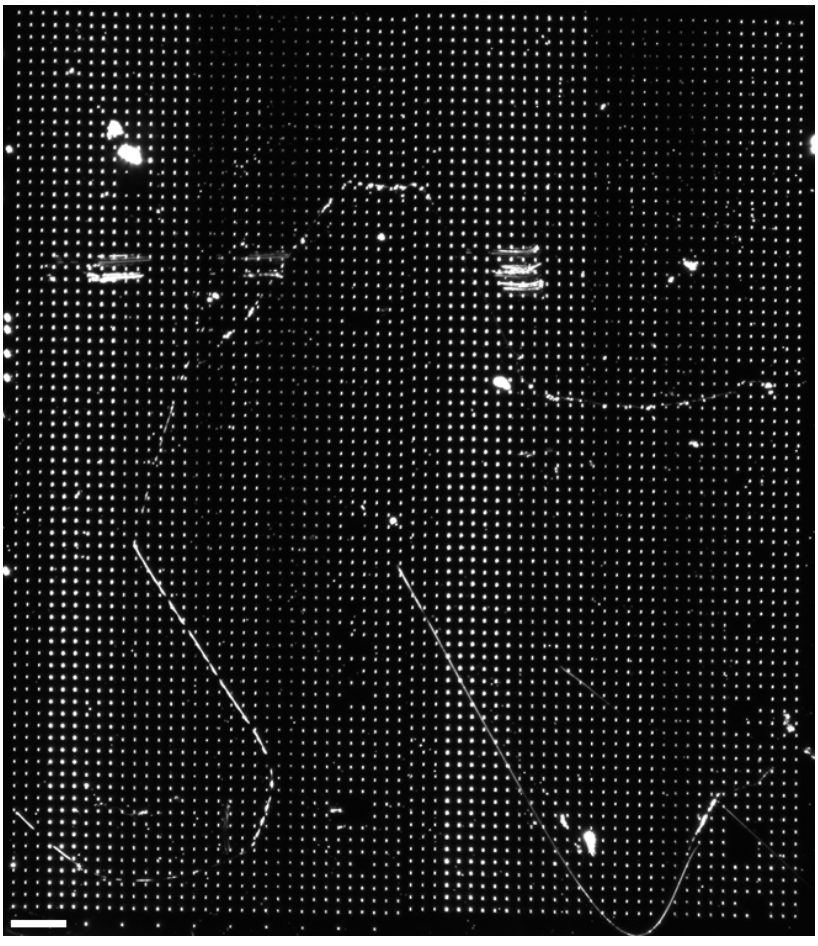


Figure S7: Darkfield microscopy image of a pattern of DOPC with biotin-PEG-thiol on gold. The homogeneity of the pattern was increased by changing the patterning routine towards stepwise patterning of small fractions. The scale bar equals 100 µm.

[1] Y. Xia, G.M. Whitesides, *Ang. Chem. Int. Ed.* 1998, **37**, 550.
[2] A. Lagunas, J. Comelles, S. Oberhansl, V. Hortigüela, E. Martínez, J. Samitier, *Nanomedicine NBM* 2013, **9**, 694.
[3] S. Oberhansl, *PhD Thesis*, University of Barcelona (Spain), October, 2012, pp. 153-155.
[4] Rakickas, M. Gavutis, A. Reichel, J. Piehler, B. Liedberg, R. Valiokas, *Nano Lett.* 2008, **8**, 3369.
[5] R. Derda, L. Li, B. P. Orner, R.L. Lewis, J.A. Thomson, L.L. Kiessling, *ACS Chem. Biol.* 2007, **2**, 347.