Fabrication of biomolecule microarrays for cell immobilization using automated microcontact printing

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Abstract

Biomolecule microarrays are generally produced by conventional microarrayer i.e. by contact or inkjet printing. Microcontact printing represents an alternative way of deposition of biomolecules on solid supports but even if various biomolecules have been successfully microcontact printed, the production of biomolecule microarrays in routine by microcontact printing remains a challenging task and needs an effective, fast, robust and low-cost automation process. Here, we describe the production of biomolecule microarrays composed of extracellular matrix protein for the fabrication of cell microarrays by using an automated microcontact printing device. Large scale cell microarrays can be reproducibly obtained by this method.

Keywords Biomolecule microarrays, Cell microarrays, Microcontact printing, Micropatterning, Cell architecture

1. Introduction

Microcontact printing (μCP) (1) represents an attractive way of deposition of biomolecules on solid supports. This soft-lithography technique is based on the use of a soft elastomeric stamp, usually made of polydimethylsiloxane (PDMS), which is topographically structured by casting a PDMS prepolymer solution against a silicon master leading to a micro-structured PDMS stamp. The stamp is then inked with the biomolecules of interest and brought into contact with the solid support leading to patterns of biomolecules that are defined by the topographical structures of the stamp. Microcontact printing offers a simple and low-cost surface patterning methodology with high versatility and sub-micrometer accuracy. By this way, it is possible to pattern a large range of biomolecules, from nucleic acids (2-4) to carbohydrates (5) or proteins (6-8), being today the biomolecules of choice to be microcontact printed. Patterning proteins on surfaces have multiple applications in the biomedical field, such as diagnosis using microarrays (9) or biosensors (10) and studies of living cells and tissues (11-12).

Precise patterning of the adhesion landscape of living cells on a surface is an efficient tool for both fundamental and applied research. On one hand, the immobilization of living cell along engineered adhesive patterns can be employed for investigating some fundamental mechanisms of cell biology such as shape control, differentiation, division, polarity or motility (13-21). On the other hand, cell microarrays can be applied to a wide range of assays in drug screening, toxicology, stem cell research. The common point of all these studies is the production of micro-patterns of various shapes and dimensions along which, adherent cells are immobilized in a deterministic way (22-26). The control of cell adhesion is essential to

understand how various living cells (neural, epithelial, tumor and stem cells) respond to various stimuli. Because technology allows reproducing identical precise patterns along well-arranged periodical arrays, these individual observations can be made systematically over large population of cells. Moreover, because the spatial resolution of micro-patterning is well beyond the cell size, it is possible to immobilize cells one by one and ultimately to force the adhesion of each individual cell into a specific and arbitrary shape.

Microcontact printing (27) of proteins of the extracellular matrix (ECM) on conventional glass slides or coverslips turned out to be an efficient method for fabricating micro-patterned cell culture substrates. This chapter presents an automated microcontact printing method enabling to generate arrays over large surfaces where living cells can be immobilized on patterns with a high level of homogeneity and reproducibility.

2. Materials

Reagents

All the solutions were prepared using milliQ water (sensitivity of 18.2 MΩ-cm at 25°C) and solvents (acetone, ethanol and isopropanol) were of analytical grade.

- 4 inches silicon wafer (BT Electronics)
- SU8 3005 photoresist (Micro Chem)
- SU8 Developer (Micro Chem)
- Perfluorododecyltrichlorosilane (FDTS) (Sigma Aldrich)
- Polydimethylsiloxane (PDMS) (Sylgard 184; Dow Corning)
- Iron powder (Sigma Aldrich, ref 209309)

- Glass slides (Thermo Scientific)
- PBS 1x, pH 7.4: 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl (Sigma Aldrich)
- Fibronectin (Sigma Aldrich, F1141, 100 μg/mL in PBS 1x)
- PLL(20)-g(3.5)-PEG(2) (SUSOS AG) (100 μg/mL in PBS 1x)
- Trypsin-EDTA (Sigma Aldrich)
- RPMI culture medium (Gibco, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% geneticin G418)
- Formalin (Sigma Aldrich)
- PC3-GFP cells (prostate cancer cell line modified to produce green fluorescence protein)

Equipment and labware

- Ovens
- Photoresist master
- Mask aligner
- Plasma cleaner
- Spin coater
- Flasks for cell culture
- Petri dish
- Pipettes
- Microscope
- Laminar flow hood
- InnoStamp 40 (Innopsys)
- InnoScan 1100 (Innopsys)

3. Methods

Bringing microcontact printing at a level where it could become a standard manufacturing method for cell microarray remains a challenge. This involves developing tools to ensure that the printing process leads to uniform and reproducible biomolecular printed patterns of different shape, size and pitch over large areas, typically > 1 cm^2. To this aim, we developed and investigated the ability of an automated microcontact printing device, the InnoStamp40, to produce adhesive patterns for immobilization of living cells. The device assembles different modules corresponding to every step of the microcontact printing process i.e. loading of the micro-structured PDMS stamp, inking, drying and printing of the biomolecules of interest. The microcontact printer utilizes magnetic fields to handle the PDMS stamps (27) and allow a homogeneous printing of defined patterns of biomolecules. The overall process is reported in Figure 1. Structured PDMS stamps contain four kinds of features (dots, lines, squares and triangle) of different sizes (from 10 μm to 50 μm) and separation (10 μm to 100 μm). After the production of microarray of adhesive proteins, the samples were seeded with prostate cancer 3 (PC3) cells. The spreading of the immobilized cells on the different patterns and the selectivity of the immobilization of the living cells on the adhesive patterns compared to antifouling background is also reported.

3.1. Fabrication of the SU-8 photoresist master

This step is the starting point of the fabrication of the micro-structured PDMS stamp. It requires microfabrication facilities. All the steps are illustrated in Figure 2.

1. Clean the 4 inches silicon wafer (BT electronics) by dipping in acetone (5 min), ethanol (5 min) and finally isopropanol (5 min).
2. Dry under a stream of nitrogen
3. Activate the cleaned wafer with oxygen plasma (5 min, 800 W, 1000 mL/min)
4. Place the wafer into an oven (100°C, 30 min)
5. Place the wafer on a spin coater
6. Cover the central part of the wafer with SU-8 3005 photoresist
7. Spin coat the SU-8 3005 photoresist (1000 rpm, 1 min)
8. Soft bake the photoresist layer onto the wafer (95°C, 2 min)
9. Align the optical mask (Figure 3) onto the photoresist layer and illuminate the UV lamp. Adjust time in order to achieve an energy of 150 mJ/cm^2.
10. Post-exposure bake (95°C, 2 min)
11. Develop the resist using SU-8 developer (3 min).
12. Rinse the photoresist master by dipping in an isopropanol bath (2 min) and dry it under a stream of nitrogen.
13. Hard bake the structured photoresist master (125°C, 2 min)
14. Silanize the surface of the photoresist master to prevent the PDMS adhesion during stamp fabrication by SiO2 / FDTS (1H, 1H, 2H, 2H-perfluorododecyltricholorosilane) deposition (5 min, ORBIS TM 1000 platform from MEMSSTAR).
15. Store the photoresist structured master away from dust and handle it with care.

3.2. Fabrication of the magnetic PDMS stamp

1. Mix thoroughly PDMS prepolymer solution and the curing agent in a 10:1 ratio (Sylgard 184 kit from Dow Corning) and degas it under vacuum to remove all of the air bubbles.
2. Pour the degassed PDMS prepolymer mixture onto the silicon photoresist master (thickness between 500 μm to 1 mm)
3. Cure at 60 °C for 45 min
4. Mix PDMS mixture and iron powder (1:1 ratio) and degas it under vacuum
5. Pour the magnetic PDMS mixture on the top of the first PDMS cured layer on the photoresist master
6. Cure (60°C, 6 hours)
7. Gently peel off the reticulated-micro-structured-PDMS magnetic stamp just before protein inking.

3.3. Microcontact printing of extra cellular matrix (ECM) proteins

1. Clean the glass slide to be patterned by dipping in acetone (5 min) and isopropanol (5 min) bathes. Activate the surface of the glass slides with air plasma (3 min, 50 W, 500 mL/min)
2. Place the micro-structured magnetic PDMS stamp on the loading module of the InnoStamp 40
3. Place the activated glass slide on the printing module of the InnoStamp 40
4. Introduce the fibronectin or ECM matrix protein solution (100 μg/mL in PBS 1x, pH 7.4) on the inking module of the InnoStamp 40
5. Proceed a process as follow: inking (1 min), drying (2 min), printing (1 min) using a magnetic pressure of 15 KPa.

6. Incubate the patterned glass slide with PLL-g-PEG (100 μg/mL in PBS 1x, pH 7.4) during 1 hour. The PLL-g-PEG layer prevents cell adhesion in between the adhesive ECM protein patterns.
7. Rinse the micro-patterned glass slides with PBS 1x (5 min) and dry it under a stream of nitrogen.

Cell seeding

1. Wash the PC3-GFP cells in PBS 1x and unstick them with trypsin-EDTA (3 min, 37°C). Check with a microscope if all the cells are unstuck. If not, tilt the dish to spread the trypsin-EDTA.
2. Seed the micro-patterned slide with 42000 cells/cm^2.
3. Cultivate the cells in RPMI culture medium containing 10% of fetal bovine serum, 1% of geneticin G418 and 1% of penicillin/streptomycin (3h, 37°C).
4. Rinse with RPMI medium (5 min)
5. Fixate the cells with 10% of formalin in PBS 1x (20 min)
6. Rinse (PBS 1x pH 7.4, 3 x 5 min)
7. Dehydrate the cells by incubation in successive baths of ethanol: 50% in water, 75% in water and 100% (3 min each)
8. Dry under a stream of nitrogen

4. Notes

Silicon master fabrication

- For UV insolation, we recommend to use the vacuum contact.
- An alternative for mask fabrication is the internet based mask supplier: JD Photo Data (www.jdphoto.co.uk)

Magnetic stamp fabrication

- The prepolymer PDMS solution could be stored at -20°C for 3 months. Before using it, wait until the mixture returns to room temperature.
- It is possible to use a razor blade to cut the PDMS stamps. Do not use the razor blade when the stamp is in contact with the master. First, unmold it from the master and then cut the stamp at the desired dimensions.

ECM protein printing and antifouling

- In order to reduce the time between plasma activation and printing, it is recommended to launch the InnoStamp40 protocol before the plasma activation. It will be stopped at the drawer opening command. The InnoStamp40 will wait the activated glass slide, the stamp and the ink.
- The antifouling step can be made into droplets in order to reduce the volume of expensive PLL-g-PEG. To this aim, place of droplet of PLL-g-PEG onto the microcontact printed area on the glass slide. Chambers could also be cut into a thin (1 mm) reticulated PDMS film. After applying them around the adhesive micro-contact printed area a droplet of PLL-g-PEG is deposited on the adhesive area. Antifouling incubation should be done with a minimal volume of 150 μL for a PDMS chamber of 1.5 cm*1.5 cm.
- Do not touch the surface with the pipette tip during incubation, it could damage the patterned surface.
- After antifouling incubation, remove the PDMS chamber before rinsing and drying active adhesive surface.

Cell seeding

- When using formalin solution, use nitrile gloves and manipulate under hood. All the consumables used during the formalin step (gloves, pipette tips, petri dish, …) should be covered before throwing them away.
- A PDMS chamber could be used to limit the volume of cell solution used for the seeding. Before use, the PDMS chamber should be cleaned by dipping in ethanol, then in sterilized milliQ water (10 min) before drying them under a stream of nitrogen.

5. Results

The InnoStamp 40 microcontact printer allows an automation of the full microcontact printing process, a precise control of the pressure applied during the printing step and a high reproducibility of the microcontact printing process. As shown in Figure 4, a large printed area of fluorescent Cy5-streptavidin could be produced as a first proof of concept of the automated printing protocol.

Adhesive microarrays of ECM protein were then produced according to the different steps depicted in Figure 1 and following the layout of Figure 3. Four different shapes of micro-patterns with five different sizes (from 10 μm to 50 μm) using four gaps (from 10 μm to 100 μm) were printed on activated glass slides. An anti-fouling treatment using PLL-g-PEG was then applied, followed by the seeding of PC3-GFP cancer cell on fibronectin microarrays, rinsing and dehydration. As shown in Figure 5, the cells were selectively immobilized on the fibronectin patterns. The quantification of the cell immobilization selectivity, calculated as the number of cells counted inside the printed

patterns divided by the number of cells counted in the PLL-g-PEG surface, indicates that, for all shapes, more than 75% of the cells are specifically attached to the fibronectin patterns (Table 1). For triangle, the selectivity is the lowest and it can be observed that the highest is the spatial confinement produced by the patterns, the lowest is the selectivity.

Finally, a cell microarray can be produced after fibronectin patterning, anti-fouling treatment and seeding of PC3-GFP cells. As shown in Figure 6, more than 95% of the cells have adhered on the fibronectin patterns whatever the pattern size and pitch.

Acknowledgements

This work was supported by the national research agency 'LABCOM' program (ANR-13-LAB2-0009-01) and partly supported by LAAS CNRS micro and nanotechnology facilities platform (member of the French RENATECH network). We thank Charline Blatché for her assistance in the cell microarray fabrication.

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Table 1: Selectivity of the cells for adhesive patterns according to their shape

Shape	Selectivity (inside/outside %)
Triangle	75
Square	85
Dot	91
Line	99

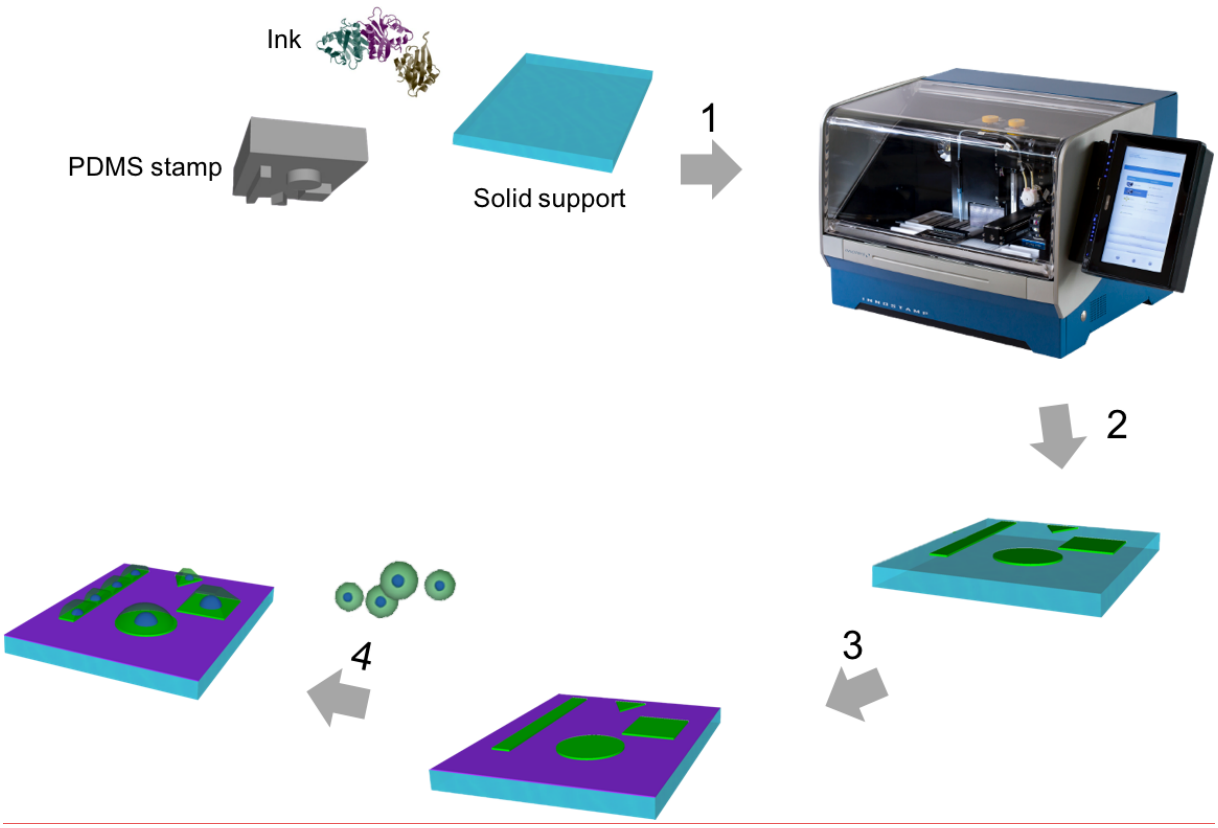

Figure 1: Overall microcontact printing of adhesive patterns using InnoStamp 40. 1/ Loading of solid support, ink and structured-PDMS stamp into the InnoStamp 40. 2/ Automated microcontact printing leading to the deposition of ink (adhesive protein) on the surface of the solid support. 3/ Anti-fouling treatment (PLL-g-PEG, BSA, pluronic…) preventing the adhesion of living cell in between the adhesive patterns. 4/ Cell seeding.

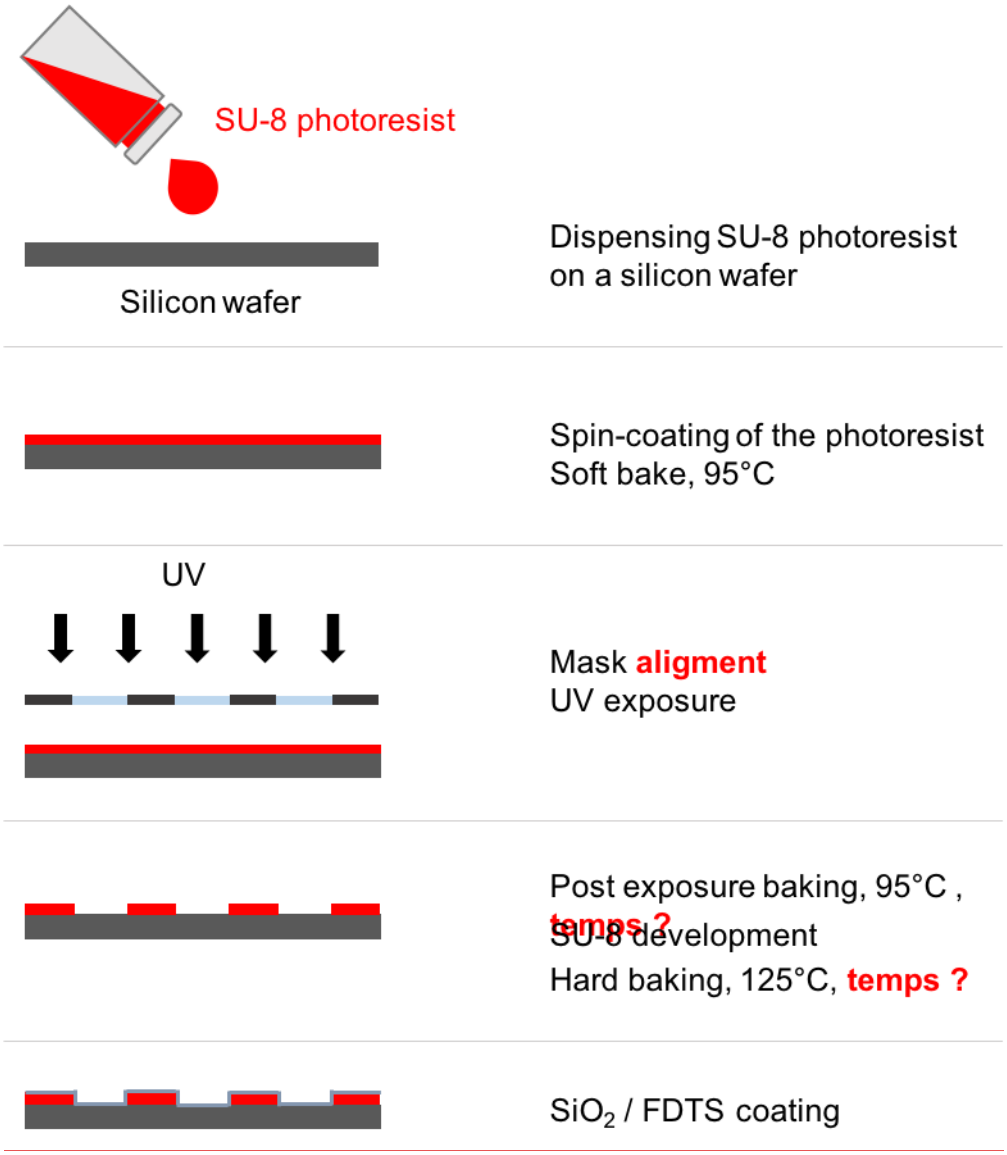

Figure 2: Production of the photoresist mold by photolithography.

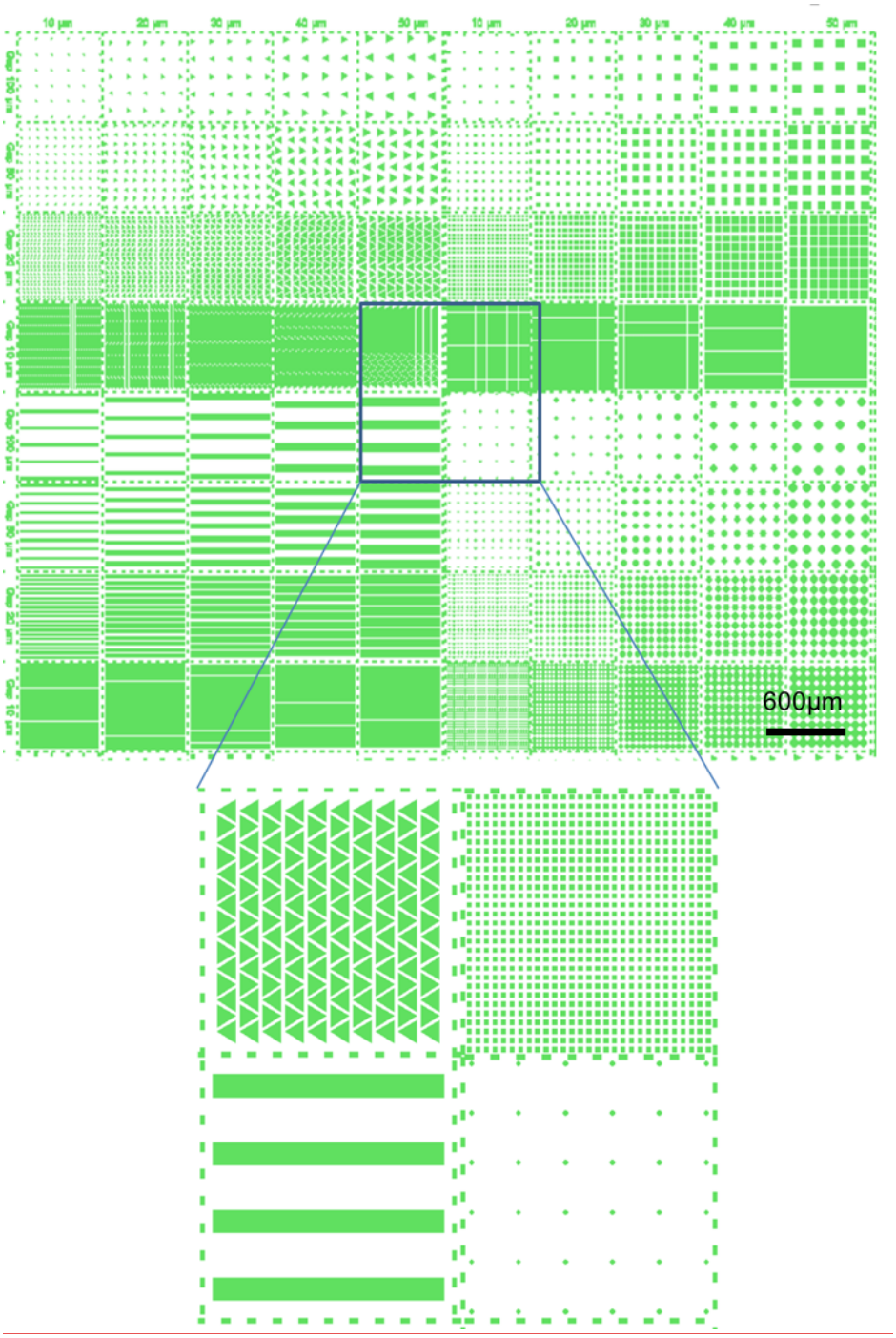

Figure 3: Layout of the micropatterns of different shapes (squares, dots, triangles and lines). Micropattern sizes range from 10 μm to 50 μm, gaps: 10 μm, 20 μm, 50 μm and 100 μm. Scale bar 600 μm.

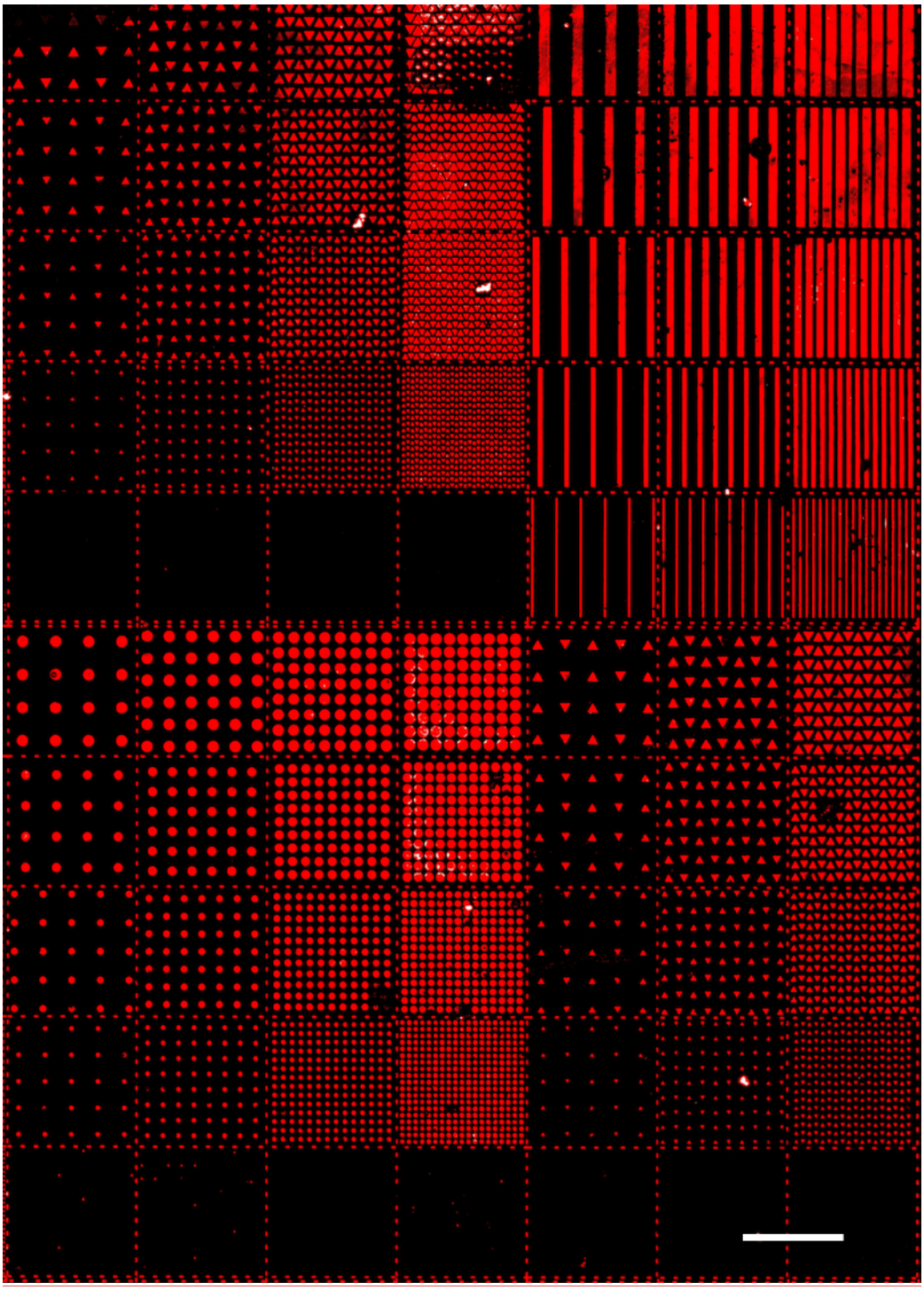

Figure 4: Printing of Cy5-streptavidin. Scale bar 600 μm.

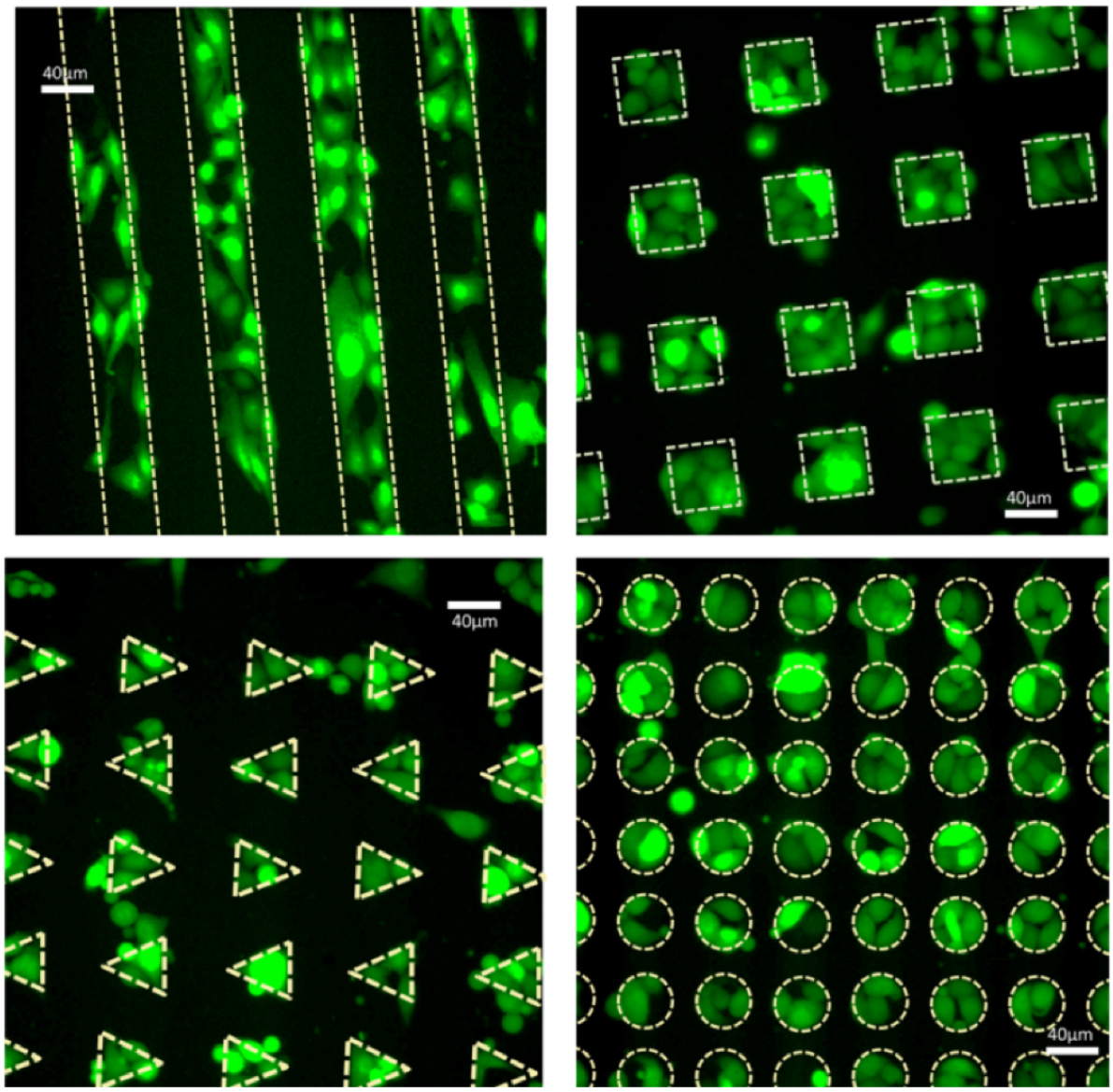

Figure 5: Fluorescence images of PC3-GFP cells immobilized on fibronectin micro-patterns of various shapes (patterns are depicted in dashed lines). Images were recorded using InnoScan 1100 (Innopsys, France). Scale bar 40 μm.

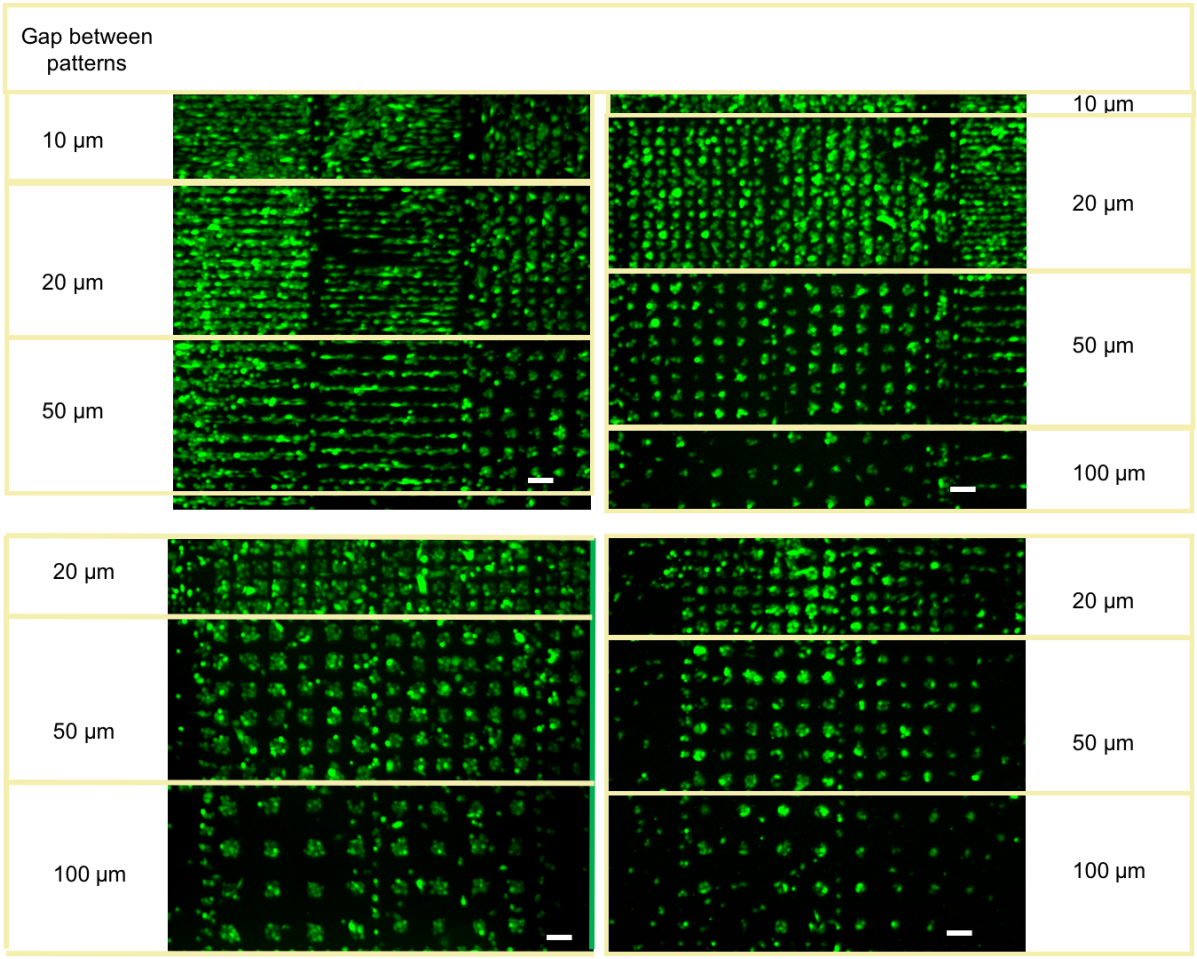

Figure 6: PC3-GFP cell microarrays obtained on fibronectin patterns of different shapes and gaps: line (up left), triangle (up right), square (down left) and dot (down right). Scale bar 100 μm.