High-Density Cell Microarrays

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Superhydrophobic–Superhydrophilic Micropatterning: Towards Genome-on-a-Chip Cell Microarrays**

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Living cells are extremely complex biological systems, and a variety of cell assays have been developed to study these systems in vitro. Cell microarrays have emerged as a promising technique that enable cell assays in a highly parallel and miniaturized manner.[1–5] However, owing to cross-contamination and cell migration problems, the density of most current cell microarrays is still limited.[6,7] Herein, we describe a facile method for the fabrication of arrays of superhydrophilic microspots separated by superhydrophobic barriers. We show that such arrays provide a great opportunity to solve both the cross-contamination and cell-migration problems of living cell microarrays and to enable fabrication of ultrahigh-density cell microarrays that can be used for genome-wide cell screens using a single array.

The method for the preparation of arrays presented herein is based on creating a grid-like superhydrophobic pattern by UV-initiated photografting on a glass plate coated with a thin layer of superhydrophilic, biocompatible, and transparent nanoporous poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) (HEMA-EDMA). The geometry and size of the produced superhydrophilic spots and superhydrophobic barriers can be precisely controlled by a photomask. The extreme wettability of the microspots guarantees an easy and homogeneous adsorption of the spotting solutions, while narrow superhydrophobic barriers effectively prevent cross-contamination of the spotting solutions between adjacent microspots. Cell experiments carried out with several commonly used cell lines confirmed preferential adhesion and proliferation of cells on the superhydrophilic spots and virtually no cell growth on the superhydrophobic barriers. Finally, the narrow 60 μm superhydrophobic gaps between the spots proved to be highly efficient barriers against cell migration.

The aims of main applications of cell microarrays are to screen chemical[5,8] or genomic[2,4,5,9] libraries or to systematically investigate the local cellular microenvironment.[2,5,10,11] Application of this technique for functional characterization of the genome using the method of reverse cell transfection to perform genome-wide gain- or loss-of-function experiments has attracted exceptional attention.[2–4,6,7,9,12,13] To produce a microarray for reverse cell transfection, solutions of transfection reagents containing gelatin are printed on a solid substrate in an array pattern and dried. Then, cells are seeded on the array and the uptake of nucleic acids by the cells growing on each spot results in an array of locally transfected cells within a lawn of non-transfected cells (Figure 1 A).[2]

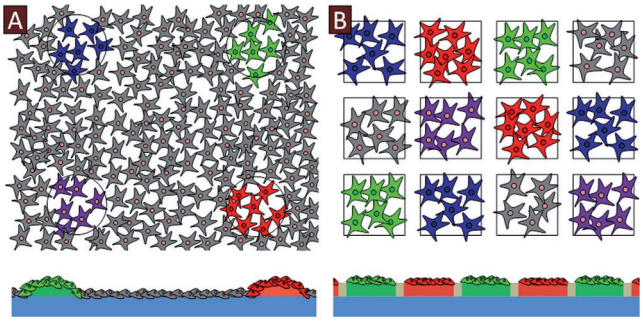

Figure 1. A) Representation of the current state-of-the-art cell microarray. Top: transfected cell clusters are localized in a lawn of non-transfected cells. Cell migration is not controlled; cross-contamination and spot geometry prevent dense spot packing. Bottom: cross-section of the array. The transfection reagents are located in gel pads; cells settle on top of them. B) View of a desired cell microarray. Precise control of spot geometry, size, and density is possible; cells can only settle on microspots containing the transfection reagents; isolated transfected cell clusters are formed; migration and cross-contamination are prevented.

When compared to microwell plates, the cell-microarray approach offers reduced assay volume, increased assay density and throughput, the possibility for long-term storage,[6] and eliminates the need for high-throughput fluid transfer equipment once the spotted array is created.[5] The absence of physical barriers separating the probes facilitates microscopy-based analysis of the results.[6,14]

However, today's cell microarrays have several important limitations. First, the area between spots containing transfection reagents is usually occupied by non-transfected cells, which can easily intrude the spots with transfected cells. In the

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same way, transfected cells can migrate between adjacent spots. Another problem is that the lack of physical barriers between microspots leads to cross-contamination[2,7,14] of the transfection solutions between adjacent spots. Finally, the composition and droplet size of a transfection solution printed on a substrate influence the size and circular shape of the produced microspots, thus limiting the spot-to-spot homogeneity and density of the produced array. These problems have been forcing researchers to keep the distance between microspots relatively large (usually 1 mm or more), thereby limiting the array density and depreciating the potential of the technology.[7,14] Figure 1 A shows the current state-of-the-art cell microarray with the abovementioned limitations, and Figure 1 B shows a desired cell microarray with all the limitations solved.

To create a substrate with a superhydrophilic/superhydrophobic pattern, we first prepared a circa 12.5 μm-thin superhydrophilic nanoporous HEMA-EDMA film (Figure 2 A).[15] This film is synthesized by photopolymerization

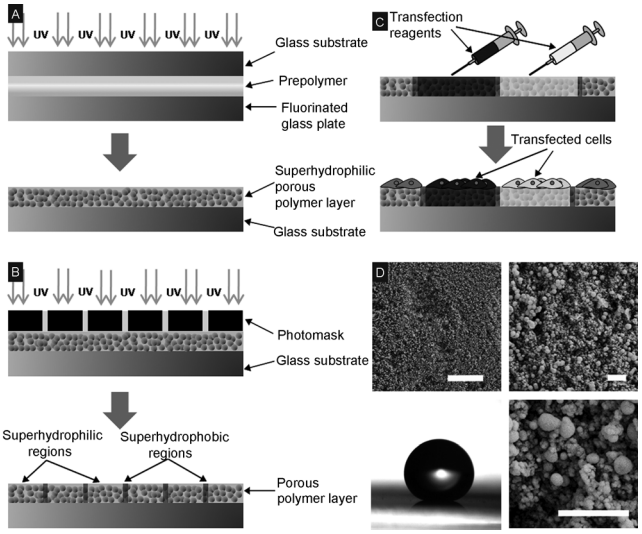

Figure 2. A) The preparation of a superhydrophilic porous polymer film on a glass substrate by UV-initiated free-radical polymerization. B) Fabrication of the superhydrophobic grid-like pattern on the superhydrophilic surface by UV-initiated photografting. C) Representation of an application of the array in a cell-screening experiment (for example, reverse cell transfection): the array is printed with transfection mixtures and dried. Seeded cells are transfected by the reagents incorporated into the superhydrophilic spots. D) Water droplet on the superhydrophobic nanoporous HEMA-EDMA photografted with PFPMA, and SEM images of the same polymer. Scale bars: top left: 10 μm, top right and bottom: 1 μm.

of a prepolymer mixture between two glass plates.[16] The thickness of the polymer film is controlled by two strips of Teflon foil that keep the glass plates apart. The prepolymer mixture consists of the monomer 2-hydroxyethyl methacrylate (24 wt %), the cross-linker ethylene dimethacrylate (16 wt %), a porogen (60 wt %), and the initiator 2,2-dimethoxy-2-phenylacetophenone (1 wt % with respect to monomers). A mixture of cyclohexanol and *n*-decanol (4:1 w/w) is a suitable porogenic solvent, which leads to the nanoporous structure of the polymer with a pore size in the range of 100–500 nm according to the scanning electron microscope (SEM) images (Figure 2 D; Supporting Information, Figure S1). The small size of the pores is important for achieving transparency of the wetted polymer film due to reduced light scattering (Supporting Information, Figure S4). The combination of the high porosity (60 %) of the polymer coated on a glass plate with the hydrophilic hydroxyethyl surface functionality makes the plate extremely wettable with static, advancing, and receding water contact angles (WCAs) close to 0° in the case of a dry surface and a static WCA of 15° in the case of the wetted surface (Supporting Information, Figure S2).

The next step in the creation of the array is the preparation of a grid-like superhydrophobic pattern on the superhydrophilic HEMA-EDMA film (Figure 2 B). To create the superhydrophobic pattern, the HEMA-EDMA surface is modified with brushes of poly(2,2,3,3,3-pentafluoropropyl methacrylate-*co*-ethylene dimethacrylate) (PFPMA-EDMA) by photografting.[17] The method of photografting consists of UV irradiation of the porous polymer wetted with a mixture composed of 2,2,3,3,3-pentafluoropropyl methacrylate, ethylene dimethacrylate, initiator benzophenone, and a water/*tert*-butyl alcohol mixture as solvent. According to SEM, photografting does not alter morphology of the porous structure, while the X-ray photoelectron spectroscopy (XPS) measurements clearly show modification of the HEMA-EDMA surface with pentafluoropropyl functionalities (Supporting Information, Figure S2). The surface modification results in the transformation of the superhydrophilic surface into a superhydrophobic material with static, advancing, and receding WCAs as large as 165°, 167°, and 157°, respectively (Figure 2 D; Supporting Information, Figure S2). As photografting is a photochemical method, we use a standard photomask for creating precise patterns of superhydrophobicity on the superhydrophilic substrate. The patterning process is accurate on a micrometer scale, can be applied to large areas, and takes place throughout the whole thickness of the porous polymer film (Figure 3).

Based on criteria for performing an efficient single-chip genome-wide RNAi cell-screening experiment, we designed a photomask (see the Supporting Information) that was used to create an ultrahigh-density array of 50 400 superhydrophilic square spots separated by narrow 60 μm-wide superhydrophobic barriers (Figure 3).

The surface of an ideal high-density array for reverse cell transfection should fulfill the following biological requirements: 1) It should be biocompatible and nontoxic; 2) cells should adhere to the microspots; 3) cells should not occupy the area between the microspots; and 4) cell migration between neighboring microspots should be minimized. We tested the behavior of several commonly used cell lines on the superhydrophilic/superhydrophobic array that was produced. Fluorescent rat mammary carcinoma cells (MTly-CMV-eGFP-neo and MTly-CMV-mCherry-neo), Hepa, and HEK 293 cells were seeded on the array and incubated for 2 days, which is the time usually required for a reverse cell transfection experiment.[7,12] Cell seeding procedures were optimized to obtain a monolayer of cells on the nongrafted

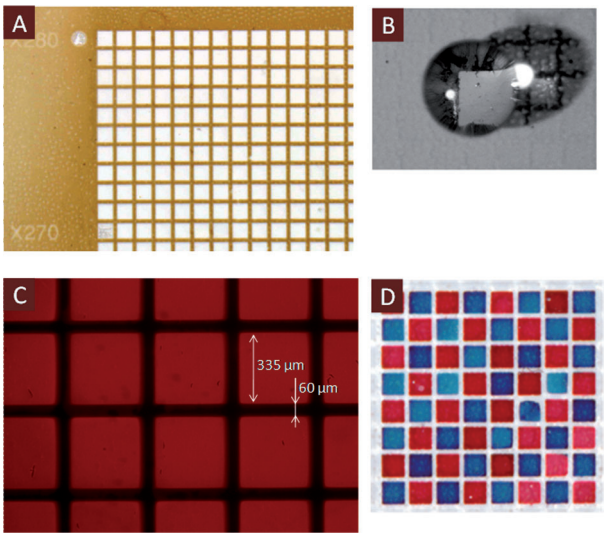

Figure 3. Optical microscope images of the array. A) Part of the array with 50400 superhydrophilic microspots on an 11×7 cm^2 glass slide. B) A water droplet in a single superhydrophilic microspot. The droplet does not spread because of the superhydrophobic barriers. C) Fluorescent microscope image showing the array with spots filled with Rhodamine 6G. D) The array with microspots filled with alternating water solutions of Neutral Red and Methylene Blue dyes.

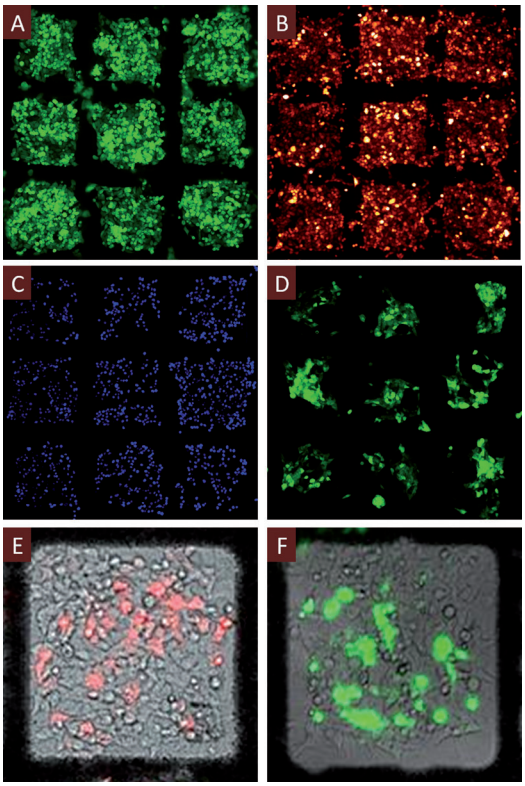

Figure 4. Fluorescent microscope images of four different cell lines after growing for 48 h on the array. A) MTly-eGFP cells. B) MTly-mCherry cells. C) HEK cells, DAPI-stained. D) Hepa cells, eGFP expressing. HEK cells transfected with different plasmids sitting on two superhydrophilic spots. E) mCherry plasmid. F) eGFP plasmid.

areas after 48 h of incubation. Figure 4 shows the results of these experiments. The superhydrophilic microspots were populated by all tested cell types. At the same time, superhydrophobic gaps separating the microspots were significantly less occupied. Moreover, cells sitting on the superhydrophobic gaps were apparently apoptotic, as was testified by round cell shapes and the absence of their motility. Thus, this cell behavior leads to the formation of arrays of isolated cell colonies in each superhydrophilic microspot separated by superhydrophobic barriers.

For HEK 293 cells, we quantified the difference between cell occupation of the superhydrophilic spots and the superhydrophobic areas. After two days of culturing, 79 cells on average occupy one superhydrophilic spot, and less than 2 cells can be found on a superhydrophobic barrier between two spots. Similar experiments with MTly-mCherry cells at three different time points showed that there was almost no difference immediately after the initial precipitation of cells, and the produced difference in cell occupation constantly increased during the cell growth and proliferation (Supporting Information, Figure S5). This process is clearly seen from the time-lapse videos recorded for MTly-eGFP and HEK 293 cells (Supporting Information, Videos S1–3). These videos also confirm that there is virtually no cell migration between neighboring microspots, despite the distance of only 60 µm.

Superhydrophobicity is a result of combination of micro- and nanoscale roughness and the intrinsic hydrophobicity of a material. A rough, superhydrophobic surface usually consists of small asperities. Water can either penetrate these and wet the whole surface (Wenzel state), or remain on top of them owing to its surface tension (Cassie–Baxter state).[18,19] We hypothesized that the surprising cell-resistant properties of the superhydrophobic barriers was the result of air trapped inside the porous polymer surface, that is, the Cassie–Baxter state. To investigate this hypothesis, we analyzed cell behavior on arrays transformed into the Wenzel state. The difference in cell occupation between microspots and the wetted barriers was significantly decreased for the MTly cells (Supporting Information, Figure S6B). In the case of HEK 293 cells, no pattern at all was formed on the array in the Wenzel state and the cells could freely migrate and proliferate (Supporting Information, Figure S6C).

To show the applicability of the array for reverse cell-transfection experiments, we filled several microspots with two different plasmid-based mixtures for reverse cell transfection.[13] The array was dried, seeded with HEK 293 cells, and incubated for 48 h. The resulting expression of mCherry and green fluorescent proteins in HEK 293 cells is shown in Figure 4 E,F.

In conclusion, we have developed a facile method for creating ultrahigh-density cell microarrays based on the photochemical preparation of superhydrophilic spots separated by thin superhydrophobic barriers. We envision that this technology will enable fabrication of "genome-on-a-chip" cell microarrays and will transform genome-wide cell-screening experiments into a significantly more affordable and convenient biological method. Finally, the new technique can be used for patterning cell clusters in a predetermined spatial order and for creating high-density cell microarrays for testing other classes of biologically active compounds. Experiments for

creating cell microarrays for screening proteins and drug candidates are now underway in our laboratory.

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