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User-Friendly Universal and Durable Subcellular-Scaled Template for Protein Binding: Application to Single-Cell Patterning

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A new method for subcellular-sized protein patterning on a SiOx substrate is demonstrated by dip-pen nanolithography printed aldehyde-terminated alkylsilane template. The aldehyde-silane template is stable and durable; for example, subcellular scaled IgG protein array can be obtained using one-year old aldehyde-silane template. Moreover, single cell patterning is successfully carried out by extracellular material (ECM) protein microarray and nanoarray fabricated on an aldehyde-silane template. With more than half of chance, single- or double-cells are successfully attached on fibronectin protein nanoarrays in 21 × 21 μm ^{2} (7 × 7 dot array) and 42 × 42 μm^2 (14 × 14 dot array). The fibronectin nanoarray with small area (21 × 21 μm^2) shows the more rate of single cell attachment. Therefore, it is also demonstrated that cell patterning can be controlled by adjusting the nanostructure of ECM materials.

1. Introduction

The development of micro- and nanofabrication techniques has made protein patterning on the subcellular scale available. Micro- and nanoscale patterns of proteins are in highly demand in various fields of biotechnology, such as tissue engineering,[1–3] biosensors,[4–6] biodiagnostics,[7] and cell-biology,[4,6,8,9], because fully integrated and highly sensitive biochips are manufactured with micro/nanoarrays of "capture proteins" (e.g., antibodies or oligonucleotides).[5,10–12] Another reason for the high demand of protein patterns in cell biology is that cell binding and patterning are made possible by subcellular-scaled patterns of extracellular material (ECM).[8,9] In addition, controllable cell patterning will be a key technique not only for fundamental cell study but also for application as a commercial product. Several factors in protein patterning should be considered for the development of a commercial biochip. (1) Protein patterning on SiO_x surfaces is more useful for applications because a glass substrate is suitable to optically observe biomaterials by transmission microscopy. Some cells are sensitive to metal oxides;[13–16] thus, metal substrates can dominate the behavior of the cell rather than patterned protein features. A substrate that has little effect on biomaterials (non-metallic substrate) is desirable to use. (2) Additionally, a more convenient method of protein patterning should be developed to overcome the limited duration that subcellular patterned proteins can be used; because of protein denaturing by dehydration, the reactivity of subcellular patterned proteins is short-lived.[17–18] Thus, protein patterning and reaction with the protein should be performed within a couple of days. The patterned proteins generally maintain reactivity for a week at most with extreme care. A new method for protein patterning that overcomes this time limitation will encourage the development of commercial biosensors.

Here, we demonstrate a new method of protein patterning on the subcellular scale using a lithographically fabricated silane template on a SiO_x surface. The lithographically fabricated silane template can last for a year; therefore, subcellular-scaled protein patterns can be obtained from silane templates stored for about one year through a simple post-reaction with intended proteins. Moreover, single-cell patterning was demonstrated using subcellular-scaled protein features based upon the silane template.

Generally, protein features on substrates are patterned by either a direct or indirect approach.[19–21] Because proteins are fragile and easily denatured during patterning, they have to be patterned in the liquid phase. A method for direct patterning of proteins should be able to use a liquid-phase material as an ink; for example, micro-stamping,[22] microarray, dip-pen nanolithography (DPN),[23–27] and polymer pen lithography (PPL)[28,29] techniques are available for printing liquid ink. Because of the hydration issue of protein patterning, an indirect approach for protein patterning has also been developed. The indirect approach first patterns protein-adherent molecules at a surface, and then, proteins are attached to the patterned molecules by covalent or noncovalent binding.[21] Because amine groups are abundant in most proteins, covalent binding with amide bond formation is generally used in protein patterning. For noncovalent binding approaches, electrostatic interactions,[30–34] biotin-avidin coupling,[35–38] and coordination chemistry[38–42] have been mainly performed. Subcellular-scaled

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patterns of protein adherent molecules can be stored for a certain time period until just before reaction with a target protein. Because the protein is attached to the patterned precursor molecule at the final step of the entire process (the exposure time of the protein during the whole patterning procedure is much shorter than that in the direct patterning approach), the subcellular-patterned proteins are not easily denatured and can maintain their reactivity for a long time. This indirect approach will be useful for biochip and sensor manufacturers. For instance, a chip with subcellular-scaled patterns of precursor protein-adherent molecules can be shipped to customers, and the customers will then perform the final step (target protein attachment) in their laboratory. Therefore, the subcellular-scaled protein chip can be used with high reactivity without severe denaturing of the protein. The chip will be safe during shipping because dehydration is not a matter for reactivity of the precursor pattern. Moreover, the chip can be stored for a long time until the moment of utilization.

Submicrometer-sized protein patterns on SiO_x substrate have been reported with tip-based lithography methods.[43] Because the AFM tip has a submicrometer size, nanomter-scale patterning of an ink material is available by tip-based lithography.[23–25] Protein patterning with DPN has been mostly performed using an alkylsilane-molecule-modified surface of a SiO_x substrate.[44–48] Protein patterns can be obtained by direct printing on a SiO_x substrate with complete coating on top by an alkylsilane layer (direct method) or the protein attachment on a submicron alkylsilane-patterned SiO_x substrate (indirect method). Submicrometer alkylsilane molecule patterning on SiO_x substrate is more suitable for applications than the entire coating of the SiO_x substrate by alkylsilane molecules. For protein printing on a complex substrate such as an electrical device with interdigitated electrodes, the substrate would be less exposed to chemicals by alkylsilane molecule patterning than by the entire coating. Although DPN printing of silane molecule on a SiO_x substrate is challenging, there are a few reports regarding the direct printing of silane molecules on a bare SiO_x substrate by DPN.[49,50] Jung et al. demonstrated direct DPN printing using mercaptosilane (thiol-group-functionalized silane) on glass substrate.[49] The thiol functional group of DPN-printed patterns was covalently bound with a biotin-maleimide molecule, and streptavidin molecules were finally attached to biotinylated sites of DPN-printed submicrometer-wide line patterns. Even though reactivity of the directly printed mercaptosilane molecule was demonstrated, it was noted that with mercaptosilane DPN printing, it is difficult to produce regular patterns in a humid environment,[49] which is probably why direct DPN printing of saline on Si-based substrates has been scarcely reported.

2. Results and Discussion

In this study, we conducted direct printing of aldehyde-terminated alkylsilane (11-triethoxylsilylundecanal) on a SiO_x substrate by DPN for use as a template for protein subcellular-scaled patterns. To obtain stable and regular DPN printing of alkylsilane on a SiO_x substrate, we executed "wet printing" using a liquid-phase ink material (see the Supporting Information). Because 3-mercaptopropyltrimethoxysilane (MPTMS), which was the ink material of Jung et al., is volatile under room conditions (it has a high vapor pressure), we used aldehyde-terminated alkylsilane with a longer alkyl chain (potentially lower vapor pressure) than MPTMS as an ink material. A uniform aldehyde-terminated silane subcellular-scaled patterned array was obtained by a one-dimensional (1D) parallel DPN printing method.[51] A subcellular-scaled protein array was acquired by covalent attachment using an aldehyde-amine reaction on the DPN-printed aldehyde-terminated silane pattern array (**Figure 1**). Before protein attachment on the aldehyde-terminated silane pattern array, passivation by poly(ethylene glycol) (PEG)-silane was conducted to minimize non-specific binding of protein on the non-patterned area on the surface. An important advantage of this strategy is that the aldehyde-terminated silane pattern array on a SiO_x substrate is durable and can be stored for a relatively long period of time of one day to ~ one year (353 days). This characteristic can be utilized in a universal template concept for a user-friendly subcellular-scaled protein chip array. A submicron array based on an aldehyde silane template is prepared and shipped to users by the manufacturer, and then, the user attaches proteins of interest to the template without using any tools for submicron array fabrication. Users can store the template for a long time (approximately one year) and prepare the protein array whenever they want by using the stored silane template.

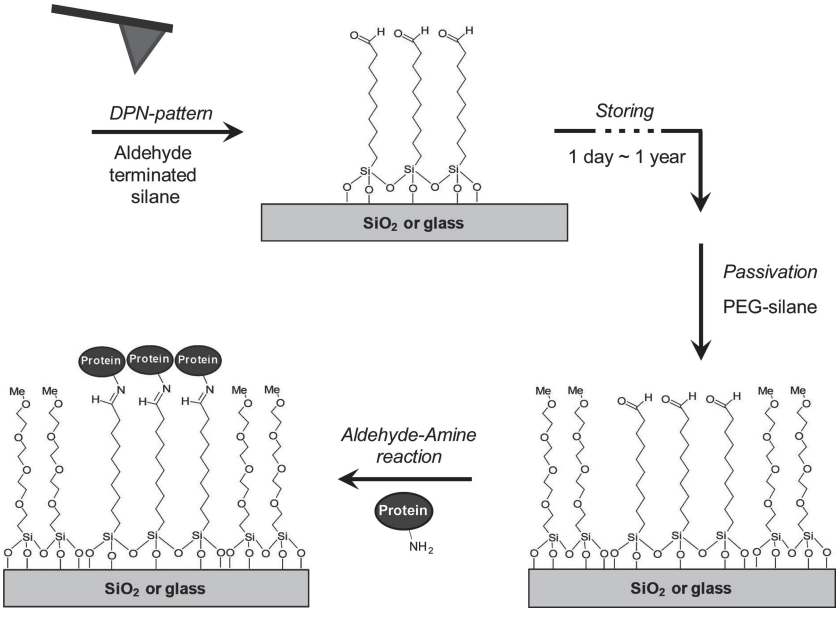

Figure 1. Schematic representation of template fabrication for subcellular-scaled protein patterns.

The protein binding ability of the aldehyde-terminated silane pattern array on the

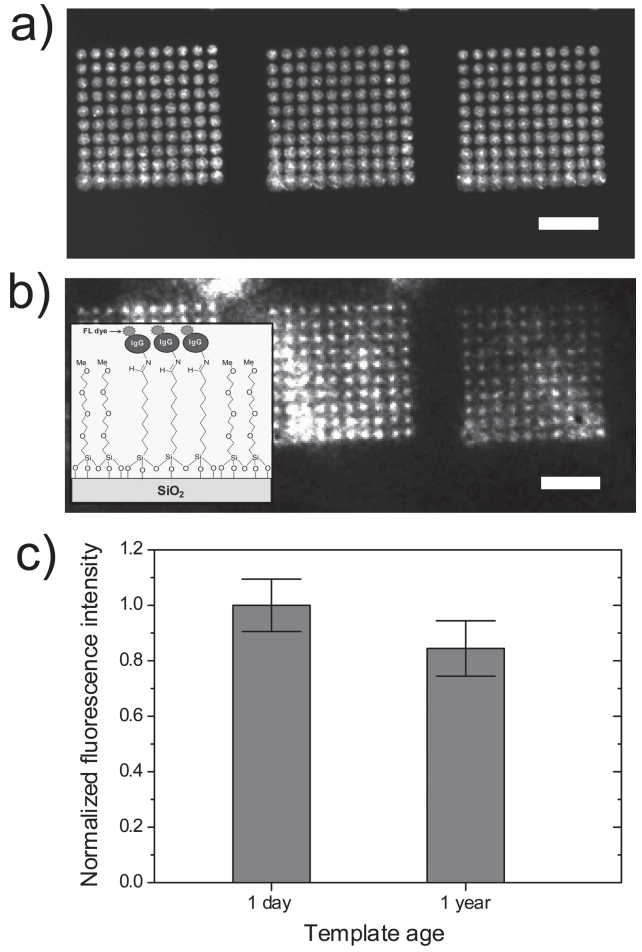

Figure 2. a,b) Fluorescence (FL) microscopy image of IgG protein patterns of a) one-day-old and b) one-year-old aldehyde-silane template. Scale bars are 20 μm. Inset of (b) Schematic representation of the IgG protein patterns shown in (a) and (b). c) Normalized FL intensity of the samples.

SiO_x substrate is demonstrated in **Figure 2**. Fluorescence (FL) dye-tagged IgG proteins (AlexaFluor 546-labeled donkey anti-mouse IgG) were attached on a one-day-old and one-year-old subcellular-scaled 10 × 10 array based on a 1D parallel DPN-printed aldehyde-silane template after PEG-silane passivation (Scheme is shown in Figure 2b; inset). The aldehyde-silane dot arrays with a 5 μm spacing were printed by 1 s of tip-dwell time. A series of 10 × 10 arrays with high-contrast FL signal were well established for the one-day-old (Figure 2a) and one-year-old (Figure 2b) samples. Protein binding was judged according to the averaged FL intensity of the 10 × 10 arrays. Error bars of the FL intensity in Figure 2c were propagated from FWHM of intensities at background and the arrays in Figure 2a,b. The error bars of one-day-old and one-year-old samples are 9.5% and 9.9%, respectively. Figure 2c shows 85% FL intensity for the one-year-old sample relative to the one-day-old sample. Thus, the one-year-old aldehyde-silane subcellular-scaled array still had sufficient protein binding ability. Non-uniform FL intensity of arrays in one-year-old sample would result from non-specific binding and poor cleaning of the sample.

Subcellular-scaled ECM protein pattern arrays were prepared from 1D parallel DPN-printed aldehyde-silane templates for application to single-cell patterning (**Figure 3**). Fibronectin (FN) was attached to the aldehyde-silane microarray and nanoarray after PEG-silane passivation of the surface. FN dot size of the arrays was controlled by changing size of the aldehyde-silane template pattern. Because aldehyde-silane ink is liquid, a certain amount of volume of wet-ink is loaded on a tip cantilever (Supporting Information Figure S1b). In order to pattern aldehyde-silane nanoarray, excessive ink on a tip cantilever should be removed by a bleeding step (Supporting Information Figure S3a). After the bleeding of excessive ink, aldehyde-silane ink shows a typical ink diffusion behavior in tip-dwell time dependent dot patterns (Supporting Information Figure S4).

FN protein microarrays and nanoarrays are shown in Figure 3b,c, respectively. The FN microarray patterns in Figure 3b were obtained from aldehyde-silane template printed by 1D pen array after 3 times of excessive ink bleeding steps, while the FN nanoarray patterns (Figure 3c) were obtained from sub-micrometer aldehyde-silane template printed by 1 s of tip-dwell time after more than 10 times of excessive ink bleeding steps. FN binding on the aldehyde-silane template was confirmed by FL microscope images. Anti-FN IgG proteins were attached to the FN-bound silane array, and FL-dye-tagged anti-IgG proteins were adhered to the anti-FN IgG proteins; the scheme is shown in Figure 3a. FN binding on aldehyde-silane template can be surely assured by a negative control experiment (Supporting Information Figure S5). The size distribution of FN microarray shown in Figure 3b is 11.2 ± 0.9 μm in

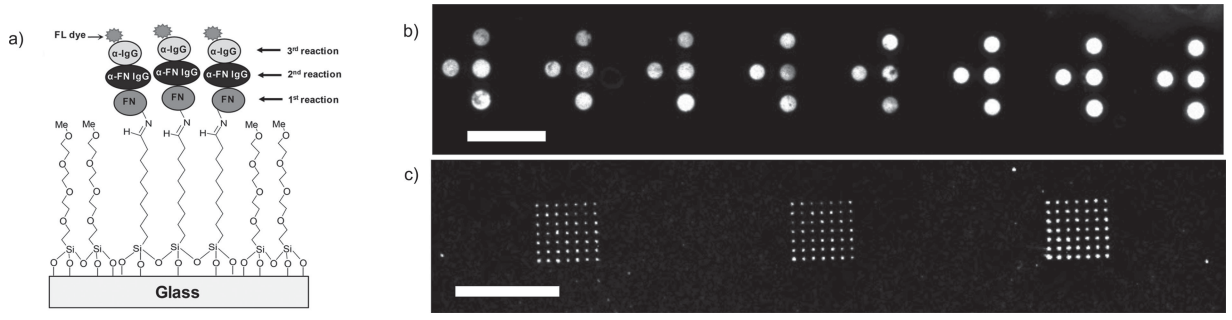

Figure 3. a) Schematic representation of fibronectin (FN) protein patterns using fluorescent dye labeling. b,c) FL images of FN (b) microarray and (c) nanoarray with scale bars of 50 μm.

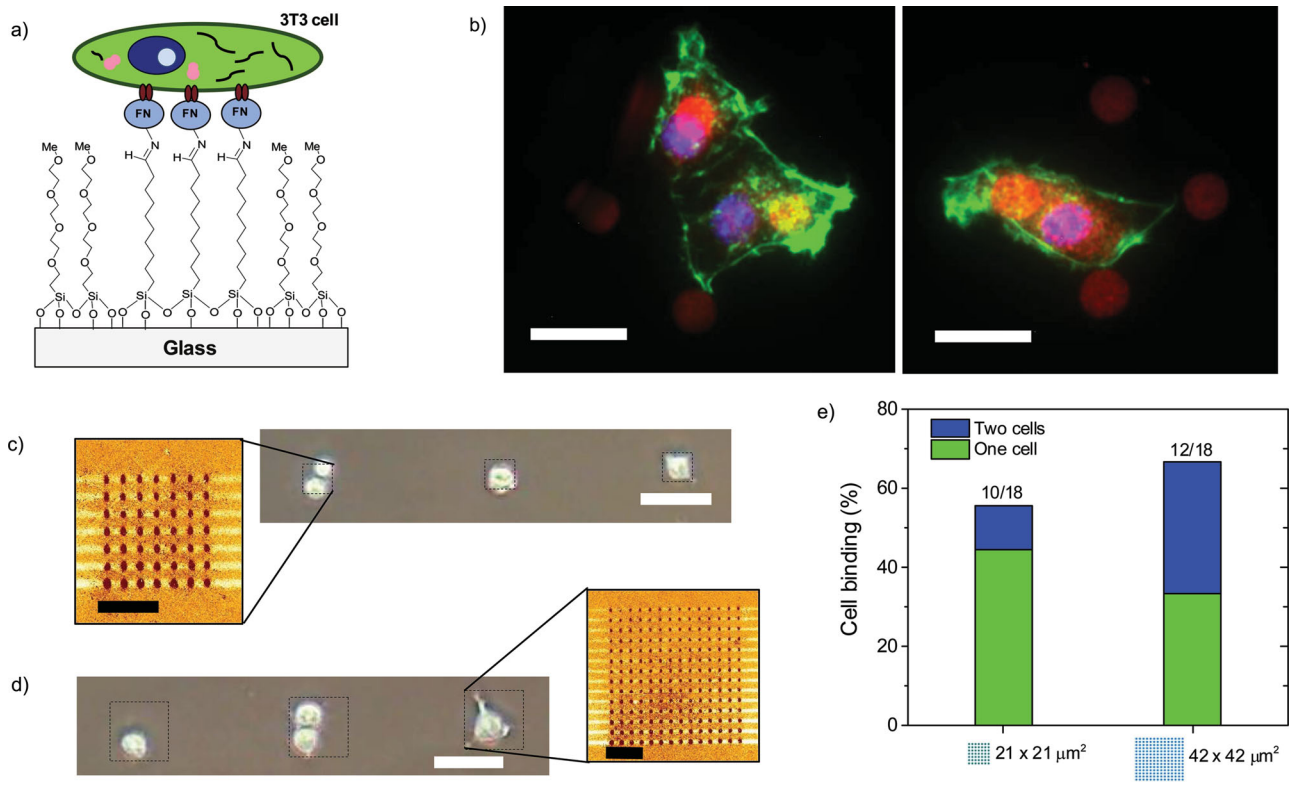

Figure 4. a) Schematic representation of 3T3 fibroblast cell patterning using FN protein pattern. b) FL images of patterned cells on FN microarray. FN, actin, and nuclei of the cell are labeled red, green, and blue, respectively. Scare bars are 10 μm. c,d) Optical microscope (bright field) images of cells on FN nanoarrays (dotted squares) with scale bars of 50 μm. The size of FN arrays was (c) 21 × 21 μm^2 (7 by 7) and (d) 42 × 42 μm^2 (14 by 14). The magnified dotted squares are LFM images obtained after cell detachment; scale bars are 10 μm. e) Ratio of cell binding in FN nanoarrays; 10 cells and 12 cells were bound on 18 nanoarrays of each size.

diameter, while the FN nanoarray shown in Figure 3c represents averaged diameter of 670 ± 6 nm.

FN protein arrays on the subcellular scale can be used for single-cell patterning. **Figure 4**a,b show single-cell patterning on FN protein micron patterns; 3T3 fibroblast cells selectively adhere to FN micron dot patterns, which are shown as red-colored diamond shape dot arrays. In Figure 4b, actin and the nuclei of the fibroblast are colored by green and blue dyes, respectively. The fibroblasts appear to be anchored well on the FN micron dots. Cell adhesion to FN protein was confirmed by a negative control experiment (Supporting Information Figure S6). In addition to cell patterning by FN micron patterns, single-cell patterning was conducted using the FN nanoarray. FN nanodot arrays were prepared from a 1D parallel DPN-printed aldehyde-silane nanoarray template. The size and uniformity of FN array is examined by AFM (Supporting Information Figure S7). Figure 4c,d show fibroblasts binding to FN nanoarrays, represented by dotted squares, with a size of 21 × 21 μm^2 (7 by 7 squares) and 42 × 42 μm^2 (14 by 14 squares), respectively. Cell binding to each size of the FN nanoarray is shown in Figure 4e. Fibroblasts attached more to the FN nanoarray with a size of 42 × 42 μm^2 (≈67%) than to the FN nanoarray with a size of 21 × 21 μm^2 (≈56%). However, single-cell binding was higher in the FN nanoarray with a size of 21 × 21 μm^2 (75%) than in the FN nanoarray with a size of 42 × 42 μm^2 (50%). This finding shows that the number of cells patterned on a FN nanoarray can be finely controlled by adjusting the size of the FN nanoarrray.

3. Conclusions

In summary, we demonstrated that subcellular-scaled protein patterns can be generated in an indirect manner by utilizing an aldehyde-terminated silane template on a SiO_x substrate. Patterns of aldehyde-silane on the SiO_x substrate were easily achieved by wet 1D parallel DPN printing. Because the aldehyde-silane template is stable and durable, it was able to maintain its reactivity for up to one year. In addition, single-cell patterning was also demonstrated using an ECM protein microarray and nanoarray fabricated on an aldehyde-silane template. The method of protein binding to SiO_x described in this report will be useful for researchers and biochip manufacturers because of the ability to store the protein-binding template for a long period of time.

4. Experimental Section

Aldehyde-Terminated Silane DPN Printing: 11-(Triethoxysilyl) undecanal (Gelest Inc.) was used as an ink material for DPN printing of

subcellular-scaled aldehyde-terminated silane on clean SiO_2-deposited silicon or glass substrates. 1D parallel DPN printing was conducted using commercially available nanolithography platforms (DPN5000, NLP2000, NanoInk Inc.) with 1D pen array cantilevers (M-Exp type, NanoInk Inc.). The liquid-phase aldehyde-silane ink material was loaded on the 1D pen array tip by using DPN Inkwell, which is a series of microchannels for liquid ink dipping. Excessive ink on the pens was removed by bleeding the tips on the substrate in a non-interesting area prior to printing. The initial DPN printing spot size can be controlled by the time of bleeding. All DPN printing was conducted with 0.1–3 s of tip dwell time on the surface of the substrate under room conditions (25 °C, 30% RH). The subcellular-scaled aldehyde-terminated silane templates on the SiO_2 substrate were able to be stored under room conditions without any special treatment just before protein binding.

IgG Protein Binding on Aldehyde-Silane Templates: To prevent non-specific binding of protein on the surface of the SiO_x substrate, PEG-silane passivation was conducted by dipping the aldehyde-silane template-patterned sample in 10 mM 2-methoxy(polyethyleneoxy) propyltrimethoxysilane (PEG-silane) ethanolic solution for 1 h. Immediately after PEG-silane passivation, the sample was sequentially rinsed by ethanol and 1 × phosphate-buffered saline (PBS) solution. The aldehyde-silane template region in the specimen was then exposed to AlexaFluor 546-labeled donkey anti-mouse IgG (Invitrogen) in 1 × PBS solution (20 μg/mL) for 2 h. After the IgG binding reaction, the sample was rigorously rinsed by 0.1 vol% Tween 20 in 1 × PBS solution, followed by deionized (DI) water.

Protein Binding Ability Characterization: Fluorescence optical microscope images were measured using an optical microscope (Axio Imager Z1m, Carl Zeiss). The same exposure times for FL imaging were used for the one-day-old and one-year-old aldehyde-silane template samples. The difference in FL intensity between the IgG-patterned region and the background of the samples was measured using an identical imaging process (i.e., same gamma value and bandwidth of contrast). The average FL intensity was obtained by subtracting the background intensity from intensity of the region of interest for the 10 × 10 array. The average FL intensity for the one-day-old aldehyde-silane template sample was set as 1.

FN Binding to Aldehyde-Silane Template: Subcellular-scale aldehyde-silane arrays were prepared on a clean glass substrate (SuperClean2, ArrayIt Corporation) by 1D DPN printing. The aldehyde-silane array-patterned sample was passivated using a PEG-silane toluene solution (10 mg/mL) for 1 h. After the passivation, the sample was sequentially washed by toluene, ethanol, and DI. The washed sample was exposed to FN (human plasma fibronectin, Millipore) in 1 × PBS solution (600 μg/mL) for 1 h. To remove non-specifically bound FN, the sample was washed with 0.1 vol% Tween 20 in 1 × PBS solution. For fluorescent dye labeling of the FN pattern, the FN-patterned sample was sequentially exposed to α-FN IgG (monoclonal anti-human fibronectin, Sigma Aldrich) in 1 × PBS solution (1:200 dilution) and FL dye-labeled α-IgG (AlexaFlour 568-labeled donkey anti-mouse IgG antibody, Invitrogen) in 1 × PBS solution (20 μg/mL). Each step was followed by rigorous washing with 0.1 vol% Tween 20 in 1 × PBS solution.

AFM Characterization: Topography and lateral force microscopy (LFM) images of the FN nanoarrays were obtained using a DPN5000 device (NanoInk, Inc.) using a SiN tip cantilever (A-type, NanoInk, Inc) under room conditions. To ensure FN binding on the aldehyde-silane template, AFM measurements were conducted by detaching 3T3 fibroblast cells as shown in Figure 4c,d.

Cell Binding on FM Subcellular-Scaled Arrays: FN subcellular-scaled arrays were obtained from a 1D DPN-printed (6-pen, 133 μm pitch) aldehyde-silane template. For cell binding on the FN array, 3T3 fibroblast cells were added at 2.5×10^4 cells/cm^2 in Dulbecco's modified Eagle medium (DMEM) supplemented with ITS+1 (Invitrogen). Cells are allowed to bind for 30 min.

Optical Microscope Characterization: The patterned cells were visualized using fluorescence microscopy (Axio Imager Z1m, Carl Zeiss) to image FN, actin filaments, and nuclei. Labeling of FN was conducted by α-FN IgG loading followed by attachment of FL-dye labeled α-IgG as described above. The actin filaments and nuclei of the cells were labeled using phalloidin-488 (Invitrogen) and DAPI (4′,6-diamidino-2-phenylindole), respectively. FL microscope images of the labeled FN, actin filaments, and nuclei were obtained using HE-DsRed, FITC, and DAPI filters, respectively. The FL microscope images obtained using each filter were merged for clear visualization (Figure 4b).

Supporting Information

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

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