Image reversal for direct electron beam patterning of protein coated surfaces

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Electron beam lithography (EBL) is used to create surfaces with protein patterns, which are characterized by immunofluorescence and atomic force microscopies. Both negative and positive image processes are realized by electron beam irradiation of proteins absorbed on a silicon surface, where image reversal is achieved by selectively binding a second species of protein to the electron beam exposed areas on the first protein layer. Biofunctionality at the cellular level was established by culturing cortical cells on patterned lines of fibronectin adsorbed on a bovine serum albumin background for 7 days in culture.

Introduction

The fabrication of biofunctional surfaces represents an enabling technology for fundamental studies in cell biology and biotechnological applications. Cell adhesion, migration, differentiation and cell-to-cell communication are questions which can be addressed with patterned biochips.\textsuperscript{1-3}

Complex surface patterning with biofunctional materials is critical for the development of novel implantable multi-electrode devices for interfacing with neural cells in prosthetic devices.\textsuperscript{4} The most common way to produce two dimensional patterns is to use self-assembled monolayers (SAMs), together with optical lithography or contact printing methods.\textsuperscript{4} However, micro contact printing and photolithography require mask production, which is expensive and adds considerable delay between pattern conception and implementation. For applications such as research and prototyping, where one wants to change the pattern frequently and does not need large numbers of chips, a direct writing technique is favorable. Among direct writing methods, electron beam lithography (EBL) is one with higher speed and resolution, which combined give EBL a very large dynamic range, \textit{i.e.} the ability to pattern very small features and at the same time effectively cover large areas.

Harnett \textit{et al.},\textsuperscript{5,6} have used EBL to pattern SAMs to create templates where fluorescent nanospheres can bind. They have shown that relatively long chain chemicals, such as mercaptohexadecanoic acid and 2-aminopropyltriethoxysilane, can be used as inert and active SAMs, respectively, to create high resolution surface patterns. However, biofunctionality of these templates at the cellular level was not studied. Rundqvist and colleagues\textsuperscript{7} recently showed that EBL can be used to create high fidelity biofunctional protein patterns by direct inactivation with electron irradiation (a negative image process), which, however, does not allow for scaling the patterning down to nanometer scale dimensions, due to the proximity effect caused by backscattered electrons.\textsuperscript{8} In this technical note, we use protein as an active material with EBL to achieve image reversal (negative to positive), fabricating surfaces that are functional at the cellular level, with fewer processing steps compared to conventional lithography. The image reversal process demonstrated here opens up EBL to new applications in nano-biotechnology, by completing the EBL toolbox for biofunctional materials, allowing one to efficiently use EBL exposure time, and minimize problems due to the proximity effect.

Fabrication process

Gold alignment marks were created on the silicon surface using UV lithography and a lift-off process, as previously...
described. The surface was then cleaned and activated by immersion in piranha solution (H\textsubscript{2}SO\textsubscript{4} : H\textsubscript{2}O\textsubscript{2}, 2 : 1, v/v) at room temperature for 15 min. The silicon surface now bearing hydroxyl groups was extensively rinsed with MilliQ water before incubation in 2–3% APTES solution (3-aminopropyl triethoxy-silane SIGMA 3648, in acetone) for 15–20 min. After rinsing, drying with nitrogen, and baking at 110 °C for 10–15 min, the chips were ready for protein adsorption. Depending on the process desired, the chips were incubated in either 1% bovine serum albumin fraction V (BSA) or 0.05 mg ml\textsuperscript{-1} fibronectin (FN) (Sigma F2006) for 2–3 h at room temperature, rinsed with MilliQ water and dried under nitrogen. Chips coated with FN are used in the negative process, while chips coated with BSA are used in the positive process. BSA is a common blocking protein that prevents non-specific binding in immunoassays and FN is an extracellular matrix protein that mediates cell adhesion, the biofunctionality, which we want to pattern.

The FN or BSA-coated chips were patterned by EBL using a Raith 150 system, with a high precision interferometric stage. An accelerating voltage of 5 kV with a 60 µm aperture corresponding to a typical beam current of 0.7 nA was used. The step size was 32 nm, and the area dose was 125 µC cm\textsuperscript{-2}. Exposing an FN coated chip results in loss of the biofunctionality in the exposed regions, so that the desired biofunctional pattern is formed by exposing a negative image of the desired pattern. We can create a biofunctional pattern by exposing a positive image of the desired pattern on a BSA layer, followed by backfilling with FN (Fig. 1, IV). Patterned surfaces were characterized with atomic force microscopy and immunofluorescence microscopy. Further experimental details can be found in the ESI†.

Results and discussion

In order to determine the localization of proteins on the silicon surface, we performed immunofluorescence microscopy on the substrates stained with FN specific primary and secondary antibodies. When a FN coated surface was patterned with EBL and blocked with BSA, staining for FN showed a strong preference for unexposed areas, as clearly seen by immunofluorescence images (Fig. 2A). This negative process shows that FN exposed to electron irradiation is no longer recognized by FN specific antibodies and indicates that BSA selectively binds to electron beam exposed areas, thereby blocking the adhesion of the antibodies. We examined the positive process where a BSA coated surface was patterned and backfilled with FN. Fluorescent images showed that FN is selectively binding to electron beam exposed areas (Fig. 2B). The high contrast observed in fluorescent images demonstrates that FN affinity to exposed areas is much higher than that to unexposed areas of the BSA layer. The background is uniform in immunofluorescence images, but is detectable at the nanometer scale (Fig. 2, Fig. 3, see below).
Fig. 2 Image reversal: immunofluorescence images of substrates stained with FN specific primary and secondary antibodies. (A) Negative process: FN coated chip patterned with EBL and backfilled with BSA. Exposed areas denoted by asterixes are dark due to exposure to electron irradiation. (B) Positive process: BSA coated chip patterned with EBL and backfilled with FN. Exposed areas denoted by asterixes appear bright due to selective binding of FN.

Fig. 3 Electron irradiation does not change topography, but surface functionality. (A) AFM tapping mode height image of electron beam exposed FN chip shows no change in
topography after exposure. (B) AFM tapping mode phase image of the same area in (A) reveal areas of different functionality. Vertical lines of darker shade are the exposed pattern. (C) AFM contact mode height image of EBL patterned BSA chip after backfilling with FN. The vertical 10 µm wide line is the exposed area. (D) Cross section analysis of the rectangular area in (C) (thin line) and cross section from a chip processed in the same way, but imaged after immunostaining with FN specific primary and secondary antibodies (thick line). Average of the cross sections along all the scan lines enclosed by the rectangular area is plotted. Backfilling with FN (thin line) and subsequent immunostaining (thick line) produced height differences of ~2 nm and ~8 nm, respectively.

In order to gain insight to what type of alterations electron beam exposure causes on protein coated chips, FN coated chips patterned with EBL were imaged using atomic force microscopy (AFM) in air (Fig. 3A and 3B). When protein templates were imaged right after electron beam exposure, tapping mode height images did not reveal the exposed pattern, demonstrating that electron beam irradiation of the first layer of protein did not produce a change in topography. Given that the accelerating voltages and doses used here are sufficient to break covalent bonds (3–4 eV), phase imaging using AFM tapping mode, which shows variations in surface characteristics, such as adhesion and viscoelasticity, was performed in order to determine whether the surface properties change after EBL. Interestingly, phase imaging revealed the EBL pattern, demonstrating that electron irradiation results in a chemical change on the exposed surface.

Contact mode AFM imaging was used to image Si substrates coated with BSA, exposed to electron beam and backfilled with FN (positive process). The exposed areas were ~2 nm higher than the unexposed areas (Fig. 3C, 3D) revealing that the second protein has formed a monolayer on the exposed regions. Both the exposed and unexposed areas after backfilling with FN had an RMS (root mean square) roughness of ~1 nm, suggesting that the surface coverage of both regions is complete and homogenous. Rundqvists and colleagues previously showed that the patterns created by the negative process display high fidelity,7 such that the deviation between the input pattern and the protein pattern is 2% for micron-scale features and the edge quality is superior. The positive process we describe here exhibits high fidelity and sharp edges. Our patterns have virtually flat features. The advantage of such surface patterns is the ability to investigate surface biochemical signals independently of topography. Other lithography methods are inferior in this sense that biochemical moieties are coupled to surface topography induced during the fabrication process.

Substrates fabricated with the positive process and stained with FN specific primary and secondary antibodies that can provide a difference in topography, were imaged with contact mode AFM (Fig. 3D). In this case, exposed regions showed a height difference of ~8 nm, supporting the notion that FN binds to exposed areas on the BSA layer and the antibody binding is specific. However, comparing the RMS roughness of the exposed and unexposed areas (~1 and 2 nm, respectively) suggests that there is a weak background of antibody binding on the unexposed areas.

To determine the biofunctionality at the cellular level, cortical cells isolated from the embryonic rat brain were cultured on FN line patterns created with the positive process. Embryonic cortex, dissected from timed-pregnant Sprague-Dawley rats (B&K) on embryonic day 15, consists of neural precursor cells, immature neurons and glial cells.9 Cells were cultured on patterned chips for 7 days in DMEM/F12 differentiation media with supplements, but lacking the mitogen, fibroblast growth factor 2 (FGF2), to allow differentiation into more mature phenotypes and process formation. In the absence of FGF2, cortical precursor cells spontaneously differentiate into mature neurons and glial cells. Cultures on patterned surfaces were inspected using differential interference contrast microscopy and immunofluorescence microscopy. The cortical cells preferentially grew on and sent out processes along the FN patterned lines for 7 days in culture (Fig. 4A). In comparison with cell adhesion assays on patterned surfaces, which are frequently carried out for only a few hours, our results clearly demonstrate the biofunctionality of the surface patterns and the effectiveness of BSA as a blocking agent. Counting the total number of cells attached to the FN lines from 2 mm × 2 mm areas in four different experiments, we found that more cells attached to 10 and 50 µm lines (100 and 93) than to the 20 and 30 µm lines (55 and 35). This could be due to seeding methodology, random variations in the population or FN line width. To further examine whether different populations of cells show a different preference for the widths of FN lines, we stained a neuronal marker βIII tubulin (Fig. 4B and C). βIII tubulin is expressed by cells of the neuronal lineage, including immature and mature neurons, but it is not expressed by glial cells. Although the total cell number changed between different width FN lines, the 10 µm and 50 µm lines had almost the same total number of cells, making it possible to reliably compare variation in cell type as a function of the surface pattern: 96 (96%) cells on the 10 µm FN lines were βIII tubulin positive, compared to only 10 (11%) on 50 µm FN lines, showing a clear trend for βIII tubulin positive and
negative cells to prefer narrower and wider FN lines, respectively. This observation can have implications for cell fate determination by the surface patterns, but the cell population used here was a mixed culture, and thus we cannot distinguish if βIII tubulin expression was induced by the surface pattern or if cells already positive for βIII tubulin preferred the narrow FN lines. Further experiments with purer stem cell populations are required to investigate this very interesting aspect.

Fig. 4 Cultures of primary cells from embryonic rat cortex on fibronectin patterned surfaces. (A) Differential interference contrast image of cortical cells strictly following the FN lines during 7 days in culture. The arrow shows a typical cell, arrowheads show gold alignment marks. (B) Almost all cells on the 10 µm FN lines (green) stained positive for the neuronal marker βIII tubulin (red). The inset shows only βIII tubulin and DAPI staining (blue) for the cell indicated by an asterix. The arrows show cellular processes. (C) On the wider lines (30 and 50 µm) most cells were βIII tubulin-negative. The green staining on cells is due to endogenous FN production. (D) For 4 independent experiments, average percentages of βIII tubulin positive cells decreased as the FN line width increased. The bars represent standard deviation.

In conclusion, we have demonstrated that proteins absorbed on silicon surfaces can be patterned using EBL through both positive and negative processes to create substrates that are functional at the cellular level. In both immunofluorescence and AFM images, the edges of exposed (backfilled) and unexposed areas were clearly defined, due to the high resolution of EBL. The ability to make two tones of pattern transfer reduces exposure times significantly. The positive process greatly reduces exposure times for patterns were the biofunctional area is much smaller than the non-functional area, which is usually the case. Overcoming the proximity effect through a positive process opens up the possibility of scaling our technique to smaller dimensions. The limiting factor is the electron scattering in the protein layer, which will depend on the acceleration voltage and the backscattering characteristics of the substrate. Experiments are underway for accurate determination of these parameters for our system, but it is already clear that a sub 100 nm feature size is achievable. The surface patterns had high fidelity and were practically flat, enabling the study of biochemical signals independent of topography. Here we have tested two proteins (BSA and FN) which have different sizes (67 kDa, 550 kDa), structures (globular, heterodimer) and functions (blocking, cell adhesion). In principal, most protein of interest can adhere preferentially to electron irradiated areas. However, verification of this technique with proteins, such as proteoglycans, need to be performed. Finally, we show that FN line patterns on a BSA background support cortical cell adhesion and localization on silicon surfaces for 7 days in culture, and different cell populations prefer to adhere to different patterns.

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Footnote

† Electronic supplementary information (ESI) available: Materials and methods. See DOI: 10.1039/b710991a

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