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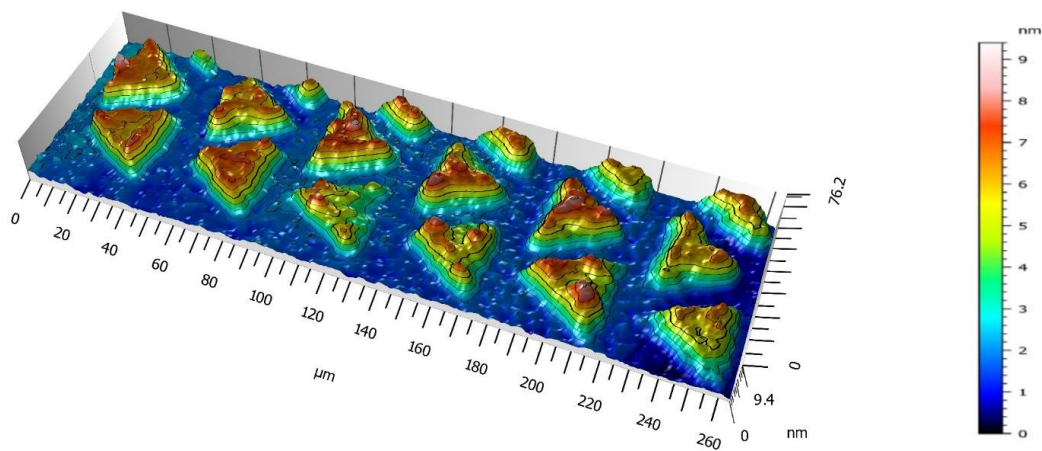
Surface bio-pattern characterization

Nanoscale analysis of surface bio-patterning using SEEC technology

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Abstract

Microcontact printing is a well-known patterning technique that uses polydimethylsiloxane (PDMS) stamps to print micro/nanoscale biomolecule patterns onto sensor surfaces, and is a popular technique to generate low cost protein or cell microarrays. Characterization of such biomolecule patterns is crucial for successful targeted applications. However, methods to perform quality control analysis of these patterns are limited owing to complexity in the analysis of micro-patterned monolayers of biomolecules over a large field of view. In this application note, we demonstrate the capability of SEEC Microscopy to provide rapid and efficient imaging based quality control analysis of microcontact printed fibronectin patterns over large surface areas with nanometric precision.



Introduction

Microcontact printing (μ -CP), a technique developed during early 1980s, is a form of soft lithography that uses polydimethylsiloxane (PDMS) stamps to form patterns of selected molecules, initially dispersed in a liquid inking solution, on a receiving substrate. This technique allows biomolecule printing along micro/ nanoscale patterns, and is widely used today for local bio-functionalization of various substrates. This method has been automated with dedicated instruments in order to process large surfaces (cm^2) in a single step. After printing, a thin layer of proteins (a few nanometers thick) is deposited on the sensor surface, which adopts the exact shapes of the PDMS stamp features (1). Increased demand for such patterned surfaces in academic research and for *in vitro* diagnostics has resulted in an increased requirement for proper characterization techniques to control the quality of such biomolecular prints (2).

Techniques that are employed for quality control of the prints include Atomic Force Microscopy (AFM), immuno-fluorescence, and Fluorescence Resonance Energy Transfer (FRET). Recently, more sophisticated techniques such as Scanning Electron Microscopy (SEM) are being increasingly used. Most of these techniques are probe based e.g. AFM or marker dependent e.g. immunofluorescence techniques. In addition, they are not rapid

and require laborious sample preparation (labeling), which often causes sample deterioration. The surface analyzed are mostly not usable for further experiments. With a large field of view coupled with nanometric axial resolution, SEEC technique allows rapid, label-free, and efficient imaging based quantification from nano- to microscale features. This technology has been successfully exploited to retrieve qualitative and quantitative information on lipid-protein interactions, enzyme-substrate interactions, and biofilm production dynamics (3, 4, 5).

In this application note, we show comprehensive characterization of fibronectin micropatterns printed on SEEC sensor surface and demonstrate the capability of SEEC technology to analyze large areas of biopatterned surfaces with nanometric precision.

Experimental part

Microcontact printing of fibronectin

PDMS patterned stamp inked with a solution of fibronectin (Sigma Aldrich, F1141) 100 $\mu\text{g}/\text{mL}$ in PBS 1X (PBS 1x, pH 7.4: 10 mM Na_2HPO_4 , 137 mM NaCl, 2.7 mM KCl (Sigma Aldrich)) was manually brought in contact with SEEC sensors (toplayer: SiO_2) and left for 1 minute without additional external pressure. Structured PDMS stamp contained four kinds of features (dots,



lines, squares and triangle) of different sizes (from 10 μm to 50 μm) and spacing (10 μm to 100 μm). After the production of a microarray of cell-adhesive protein patterns on the SEEC sensor, the sensor surface was coated with an anti-fouling layer.

Antifouling PLL-g-PEG coating

The patterned sensor was incubated with PLL-g-PEG (100 $\mu\text{g}/\text{mL}$ in PBS 1x, pH 7.4) during 1 hour. The PLL-g-PEG anti-fouling layer prevents cell adhesion in between the adhesive fibronectin patterns. After incubation the sensor was rinsed with PBS 1x (5 min) and dried under a stream of nitrogen.

Prostate Cancer Cell 3 (PC3) immobilization and sample preparation

The patterned SEEC sensor with fibronectin features surrounded by an anti-fouling PLL-g-PEG background, was incubated with fifty thousand PC3 cells at 37°C for 3 hours in RPMI culture medium. The SEEC sensor was then washed to remove non-adherent cells. The cells were then fixed and dehydrated in order to preserve their shapes.

Imaging with N-Lab Station

Experiments were carried out at 21°C \pm 0.1°C. Images of the sensor were acquired after every step of the fabrication process. Labsoft software was used to acquire the images, and MountainsMap® (Digital Surf-France) imaging software was used to perform morphology/ topography analysis and 3D view generation. Background noise is subtracted from the measured pattern thickness to obtain the real thickness of the patterns.

Results

Surface treatment alters fibronectin micropattern thickness

In order to investigate if protein biomolecules can be directly printed on opaque SEEC sensors and to verify if the patterned molecules remain bioactive, Fibronectin, a well-known Extra Cellular Matrix (ECM) protein was printed on SEEC sensor in order to create cell adhesive micropatterns on SEEC sensor. Then, the patterned surface was passivated to avoid unspecific binding and finally, PC3 cells were added in order to verify their attachment to the patterned protein surface. Images were acquired after first and last steps in order to visualize and quantify changes in pattern thickness. SEEC images of triangular patterns before and after cell immobilization are shown in figure 1A, and 1B respectively. Adhesion of PC3 cells to the patterned surface suggests that the fibronectin patterns are bioactive (Fig.1B.). Quantification summary of the analysis of 24 lines, 45 triangles and 128 square patterns is shown in Fig.1C. The results suggest that there is a significant difference in protein pattern thickness when comparing printed patterns (blue bars) and remaining patterns after cell immobilization and sample preparation (red bars). In case of features made of triangles and squares, there is a significant loss of fibronectin material from the patterns (Fig 1C), interestingly, line features exhibit an increase in thickness. These changes that could not be detected by conventional fluorescence characterizations deserve a

dedicated investigation to understand their origin. Lack of adequate characterization methods so far, did not allow such observations before. Thus, this is a clear input from SEEC studies based on the extreme sensitivity of the technique to thickness variations.

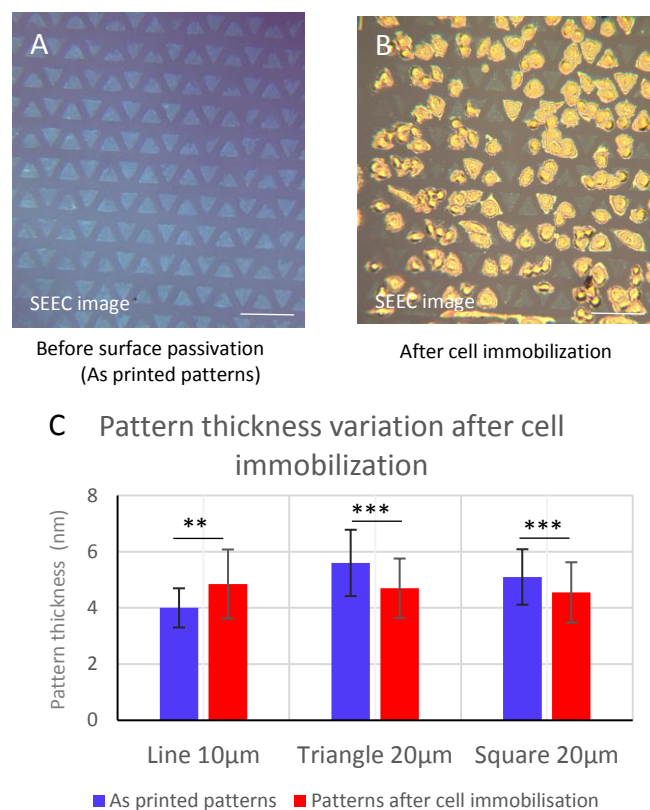


Figure 1. Effect of passivation process and cell immobilization on pattern thickness: SEEC images of triangular patterns (x,y: 20 μm) on SEEC sensor, A. before surface passivation, B. after PLLg passivated and PC3 cell culture C. Quantitative summary of printed patterns (blue bars) and patterns after cell immobilization (red bars). 24 linear patterns, 45 triangular patterns and 128 square patterns were analysed. Paired t tests were performed to derive statistical significance. $p < 0.05$ is considered significant (as indicated by *). Scale bar: 50 μm .

Characterization of Fibronectin printed patterns: 3D topo-morpho analysis

Next, in order to investigate the quality of the printed fibronectin patterns, 3D topographical and morphological analysis of a selected region of interest were performed. Figure 2A shows a representative 3D image of the triangular fibronectin patterns. Figure 2B shows the colormap of the region indicating the potential distribution of the fibronectin molecules in the printed pattern. Colormap analysis reveals the 3D arrangement of the printed molecules which seems to fluctuate from pattern to pattern. Table below figure 2B shows the summary of computed thickness, volume and area for the selected region with 12 triangular patterns.

Characterization of Fibronectin printed patterns: Contour analysis

The large field of view offered by SEEC provides imaging based analysis of over 320 patterns in a single shot. Contour analysis for the patterns such as median length of the triangle sides



(sides of the Δ) and median distance between two triangular patterns (DBT) were performed.

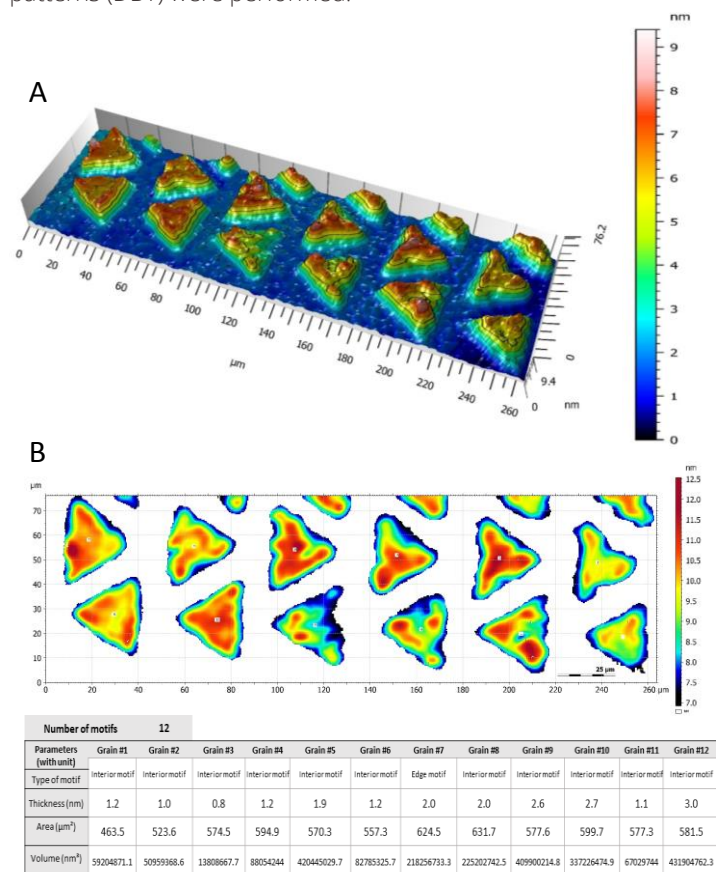
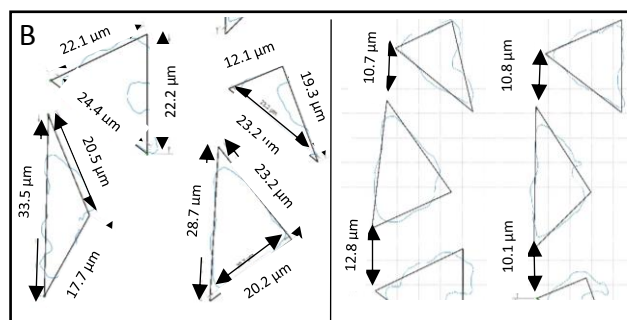
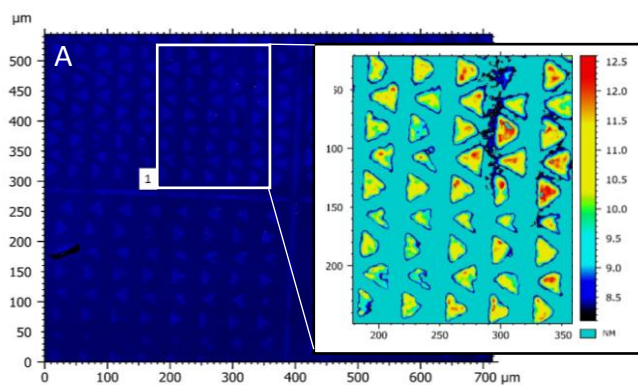


Figure 2. Qualitative analysis of the triangular patterns: A. 3D topographical view of the selected region of the pattern. B. Top. Colormap of the selected region, with pattern (motif) numbers labelled. Bottom. Table: Quantitative summary indicating the thickness, area and volume calculated for the patterns indicated as grains.

Figure 3 shows the summary of contour analysis of a region with 45 triangular patterns. Figure 3A shows the pseudo-SEEC image (processed SEEC-image obtained using MountainsMap[®]) of the patterns with a selected region containing 45 triangular patterns. Figure 3B shows the representative images of the analysis performed. The quantification summary of the analyzed parameters is listed in table below (Fig 3C).

The median pattern length in the four regions calculated are 24.7 μm while the median distance between two triangular patterns is 11.5 μm . The coefficient of variation (CV) calculated as the percentage of the ratio between standard deviation and median value, for the two parameters suggests some variation in the inter-pattern distance as well as the triangular length. Overall, these results indicate that the manual printing of biomolecules creates some variation in the printed patterns.



Contour analysis of triangular patterns			
	Med. Length (μm)	std.dev.(μm)	CV (%)
Sides of the Δ	24.7	2.4	9.6
DBT	11.5	2.8	24.1

Figure 3. Contour analysis of the triangular patterns: A. Pseudo SEEC image of the sensor with micropatterns with a magnified region showing 45 triangular micropatterns B. Contour analysis: measurements of triangular sides (sides of the Δ) and median distance between the two micropatterns (DBT). Bottom: Table: Quantification summary showing the median values for all the calculated parameters with computed coefficient of variation (CV).

Conclusion

In the present work, we show that fibronectin patterns generated by manual microcontact printing on opaque SEEC sensors remain bioactive for cell immobilization but that the pattern thickness is altered upon surface treatments. Overall, this work exploits the ability of SEEC technique to offer imaging based surface analysis and demonstrates a novel application of SEEC for rapid visualization, characterization and quantification of biomolecule patterns over large surfaces.

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