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Upside and downside views of adherent cells on patterned substrates: Three-dimensional image reconstruction

J.J. Li [a,b], X.T. Zhou [a], J. Shi [a,b], F. Zhang [a,b], X. Li [a,b], L.M. Jiang [a], Y. Chen [a,b,c,*]

[a] Ecole Normale Supérieure, CNRS-ENS-UPMC UMR 8640, 24 rue Lhomond, 75231 Paris, France
[b] Institute for Integrated Cell-Material Science, Kyoto University, Kyoto 606-8507, Japan
[c] Institute for Interdisciplinary Research, Jianghan University, 430056 Wuhan, China

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ABSTRACT

Understanding cell-material interaction is crucial for tissue engineering, wound healing, and new implant manufacturing. By using microfabricated topographic cues, cell-material interaction can be studied in a more systematical way. Here, we describe a simple technique for three-dimensional image reconstruction of cells on a patterned substrate. Whereas the upside morphology of the cell could be easily obtained by atomic force microscopy (AFM) after cell fixation, the downside morphology of the same cell could be retrieved after reversed cell imprinting. Then, the upside and downside AFM images of the same cell could be matched numerically to achieve a 3D view. We show the results obtained with HeLa and NIH 3T3 cells cultured on patterned substrates made of polydimethylsiloxane (PDMS) of different Young's modules. The penetration of the cell membrane into patterned micro-holes could be analyzed quantitatively, indicating a significant difference between the two cell types as well as between different cell adhesion areas.

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1. Introduction

For many applications such as tissue engineering, wound healing, and new implant manufacturing, it is important to understand in detail the mechanisms of cell-material interaction taking into account the surface morphology as well as other properties of the material. To this regard, high resolution imaging of cell morphology on patterned substrates could be one of the most efficient approaches [1–5]. Conventionally, optical microscopy is applied which allows cell imaging and analyses at single cell levels. However, it is not easy to determine for instance the morphology change of the cell membrane at the interface of cells and patterned substrates. Transmission electron microscopy (TEM) can be used to overcome this problem but the scan area of TEM is limited and insufficient for large area cell–substrate observation [6]. The atomic force microscope (AFM) enables cell imaging at high resolution with sufficient size of the observation area [7]. In addition, both upside [8–10] and downside [1] of the cell morphology could be determined by AFM after cell fixation.

The purpose of this work is to demonstrate the feasibility of a three-dimensional image reconstruction of the cell morphology on patterned substrates by using data obtained from both upside and downside AFM measurements. It is known that cell-material interaction strongly depends on the cell type and the stiffness of the substrate [3,11–15]. We therefore considered two types of cells, i.e., HeLa and NIH 3T3, which should have typical characteristics of cancer cells and fibroblasts, respectively. We also varied the stiffness of the substrate by using patterned polydimethylsiloxane (PDMS) of different Young's modules.

2. Materials and methods

2.1. Fabrication of micro-hole substrates

Conventional microfabrication techniques were used to pattern a thin layer of polydimethylsiloxane (PDMS). Firstly, photoresist AZ5214 (Cipec, France) was spun coated on a cleaned silicon wafer and exposed with a photomask containing micro-dots arrays. After development, the resist pattern was transferred into the silicon wafer by reactive ion etching (RIE) with a gas mixture of SF_6 and CHF_3. By controlling the etching time, the depth of the silicon mold could be controlled in the range of 0.1–1.2 μm. A PDMS pre-polymer liquid was prepared by mixing the base polymer and its cross-linker (GE RTV615 kit) at different ratios (5:1, 10:1 and 20:1) with Cyclone MM-103S mixer (UNIX, Japan). To facilitate the subsequent PDMS release, the silicon wafer was exposed in trimethylchlorosilane (TMCS) vapor for 5 min. Then, the prepared PDMS liquid was poured on the patterned wafer. After degassing in a vacuum

* Corresponding author at: Ecole Normale Supérieure, CNRS-ENS-UPMC UMR 8640, 24 rue Lhomond, 75231 Paris, France. Tel.: +33 1 44322421; fax: +33 1 44322402.
E-mail address: yong.chen@ens.fr (Y. Chen).

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chamber, PDMS was cured at 80 °C for 2 h before peeling off. Finally, the PDMS sample was exposed in O_2 plasma for 3 min to increase the wettability of the PDMS surface before cell seeding.

2.2. Cell seeding and culture

HeLa and NIH 3T3 cells were cultured with the following protocol. Firstly, cells were cultured in a tissue culture flask with DMEM (Gibco) supplement consisting of 5% fetal bovine serum, 1% penicillin, 1% streptomycin, 1% L-glutamine, and 0.01% Fungi-zone (Sigma–Aldrich, Australia). They were then dissociated with 0.05% Trypsin–EDTA solution at 37 °C for 3 min. After centrifugation, cells were re-suspended in the medium. The patterned PDMS layers were placed in the wells of a 12-well plate (Fisher Scientific, France). Afterward, cells were seeded to yield a final concentration of about 10^4 cells/cm^2 and then incubated at 37 °C in 5% CO_2 until the next step of the experimentation.

2.3. Sample preparation and cell imaging

After 24-h culture, the cells were washed in physiological phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde in PBS solution for 30 min. Before dehydration procedure, the dish was washed with DI water twice to prevent PBS crystallization. The sample was immersed in 30% ethanol (in DI water) for 30 min and then dehydrated in a graded series of ethanol: 50%, 70%, 80%, 90% and 100% every 10 min. After dehydration, a commercial atomic force microscope (AFM) (Caliber, Veeco Instruments) was used to retrieve the cell upside morphology with tapping modes (Fig. 1a). In order to image the downside of the cell morphology, a drop of UV optical adhesive (NOA81 Norland) was dipped onto the sample surface, followed by a UV exposure (16.2 mW/cm^2) for 60 s to solidify the NOA81 (Fig. 1b). The fixed cells were then peeled off together with the adhesive from the PDMS substrate. Finally, the AFM was used again to obtain the downside image of the cell (Fig. 1c).

2.4. Imaging processing

The upside (Fig. 1d) and downside (Fig. 1e) AFM images of the same area of the sample were combined to form a three dimensional picture (Fig. 1f) of cells by using a Matlab program developed in this work. For each sample, over 8 cells with 20–100 deformation spots were analyzed and the results were reported with standard deviations of the mean. Firstly, the height images of AFM were converted into BMP format. Then, they were respectively loaded into a 256×256 two-dimensional matrix. The color intensity of each pixel is in the range of 0–255, where 0 and 255 correspond to the minimal and the maximal height of the pixel in the image. The two pictures were then adjusted in order to match the upside and downside views of the cell. Afterward, both pictures were cut into the same size and reload into two matrices of the same size. In order to make the surface smoother while maintaining the accuracy, triangle-based linear interpolations were applied to the matrices. Finally, the upside and downside images were fitted into one frame so that 3D-images could be displayed to show the whole cell morphology. The color bar and the axis were also added to indicate the height and the scale of the cell/substrate and the combined 3D image can be dragged and viewed freely in any angle. In addition, the cross-section view of the cell could be displaced by manipulating the cell images.

3. Result and discussion

Cell-material interaction strongly depends on the cell type and the material stiffness. When cultured on a stiffer substrate, cells are generally more stretched. By using our 3D cell imaging technique, we investigated the growth behaviors of both HeLa and NIH 3T3 cells cultured on patterned PDMS substrates of different stiffness.

Fig. 2 shows the results obtained with HeLa cells cultured on a PDMS layer with 2 μm diameter holes. Here, the 3D image (a) was composed by two AFM images, one after cell fixation and another after reversed cell imprinting. The two line profiles (b) were retrieved from the upside and downside AFM images in the selected cross section, showing clearly an ellipsoidal shape of the cell body with *fillopedia*-like extensions. Accordingly, the underneath PDMS layer should be deformed with a dimple-like morphology at a length scale of the cell size downside. Remarkably, the downside cell membrane was also deformed to follow the morphology of the micro-holes due to strong cell-material interaction. Finally, the SEM image (c) of the same cells after fixation and reversed cell

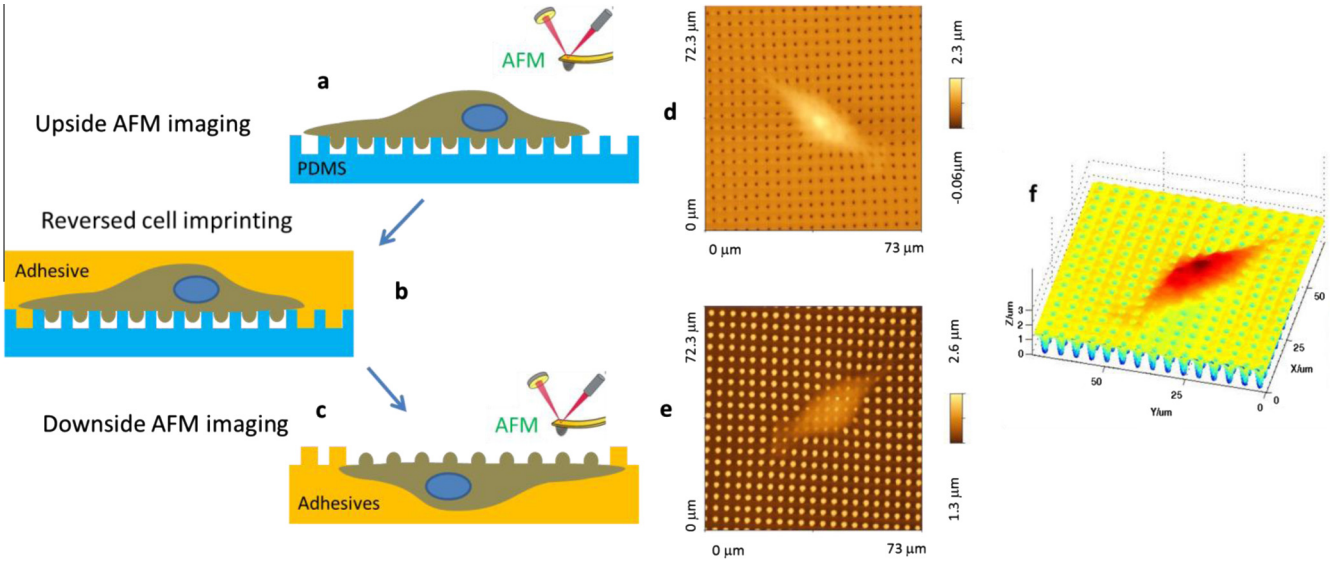

Fig. 1. 3D AFM imaging of adhesion cell on a patterned substrate: (a) schematic of upside cell imaging, (b) schematic of reversed cell imprinting, (c) schematic of downside cell imaging, (d) upside image of a single HeLa cell, (e) downside image of the same cell, and (f) combined 3D image of the cell.

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