High-Resolution Microscopy on Nanostructured Interfaces

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Introduction

Nano- and micro-patterned substrates have become a powerful tool in cell biology in recent years. Although great progress has been made in applying these substrates to cell populations or single cells, visualization of cells in direct contact with nanostructured interfaces remains a challenge. A central question that needs to be addressed by imaging is the number of biomolecules in contact with a single cell.

Over the last few years, we have developed a method for the deposition of Au-nanoparticles less than 10 nm in diameter on different surfaces such as glass and silicon and distributed in a well-defined manner. By decorating these particles with single proteins or bioactive peptides [2] and visualizing them, we can deduce the number of molecules per unit area. Changing the distance between the Au-nanoparticles, on the other hand, provides an elegant method to control the molecule density. Furthermore, by using tag-mediated bioconjugation methods, such as the Nickel-nitrilotriacetic acid (NTA) – His-tag system, we are able to control the orientation of the proteins on a substrate, for example exposing the N- and C-terminus of a transmembrane protein. In contrast to other surface-structuring methods, where self-assembled monolayers (SAM) are used to statistically control the lateral distances between single molecules, we can create regular and homogenous distributions and visualize each molecule indirectly through the conjugated Au-nanoparticles by scanning electron microscopy (SEM). Because conjugation is restricted to the gold Au-nanoparticles on the substrates, this allows for a well-defined number of molecules per surface area. These substrates therefore facilitate studies that examine the correlation between specific cellular behavior and quantitative molecular input. In addition to the biochemical features of the substrate, we can control biophysical attributes of the cellular microenvironment by using hydrogels from different polymers decorated with Au-nanoparticles. By using nanostructured arrays on glass surfaces, functionalized with the extracellular domain of the heparan sulfate proteoglykane (HSPG) Agrin or the immunoglobulin superfamily (IgSF) member DM-GRASP, we were able to investigate neuronal cell adhesion, cell migration, and differentiation in dependence on the structured arrays [3, 4].

One major question still unanswered using this approach is how much of the cell surface is in close contact with the substrate itself. Because standard confocal microscopy is only able to yield a resolution in z-direction of about 500 nm and SEM is not able to visualize the contacts between the cell and the substrate, other approaches are needed. We investigated the use of 4Pi Microscopy, which provides axial (z) super-resolution of about 100 nm to investigate the cell-substrate contact area. 4Pi Microscopy, an advanced laser scanning microscopy technique, utilizes two opposing high numerical aperture objective lenses that focus into a common spot. A coherent combination of the excitation laser light as well as the detected fluorescence light transmitted through both objectives ('Type C' mode of 4Pi Microscopy) create a sharp effective focus of about 250 nm width in x and y directions and about 100 nm axial full-width-at-half-maximum [1]. Axially shifted side-maxima are removed by image post-processing (deconvolution) to yield three-dimensional images at 100 nm axial resolution.

In the following, we discuss the sample preparation and the imaging procedure for CryoSEM and 4Pi Microscopy on nanostructured and functionalized hydrogel and glass substrates.

Sample Preparation

We used substrates based on poly(ethylene glycol) diacrylates (PEG-DA), which are highly hydrated, protein repellent hydrogels, decorated with gold nanostructures on the surface. Scanning electron microscopy (SEM) is a logical choice because the size of Au-nanoparticles lies in the range of several nanometers. However, due to the high water content of the hydrogels, no conventional SEM could be used because of the required vacuum conditions. Instead, we applied low temperature (–160°C) SEM, so-called "cryo scanning electron microscopy" (CryoSEM). The reduced partial pressure of water and ice allows visualization of cellular adhesion organization at the nanometer scale.

For imaging, all samples were first washed with PBS, fixed in 3.7% PFA for 25 minutes at 37°C and washed again with PBS. After fixation, all samples were incubated for 12 hours in aqueous dimethyl sulfoxide (DMSO) solution (14% v/v) at 4°C. DMSO is a widely used cryoprotectant, added to cell media to prevent cell damage and death during the freezing procedure. It prevents crystal formation and protects biological tissues from damage caused by ice crystals. We utilized these properties to conserve the shape of the cells and its organelles. A BAL-TECH VLC 100 shuttle-and-loading system and a BAL-TECH MED 020 preparation device were used to cool down and transfer the PEG-DA hydrogels into the CryoSEM chamber.

Electron Microscopy

Due to the physical and chemical properties of PEG-DA-based hydrogels, no conductive coating could be applied to visualize the gold nanoparticles. To avoid charge artifacts

 doi: 10.1017/S1551929509991155

https://doi.org/10.1017/S1551929510991153 Published online by Cambridge University Press

from the electron beam on the resulting low-conductivity surface in the imaging process, only low-acceleration voltages, 1–2 kV, can be applied. A Zeiss Ultra 55 field emission SEM (FE-SEM) equipped with a secondary electron in-lens detector (in-lens), an Everhart-Thornley detector (SE2) as well as an energy- and angle-selective backscattered electron (EsB) detector, was used for image acquisition. In-lens detectors are mostly used to resolve topographic and charge information, whereas SE2 detectors typically depict surface information, and EsB detectors were developed to enhance compositional contrast. Here, in-lens and SE2 detectors were used to image whole cells and parts of filopodia and lammellipodia of mouse neuroblastoma cells on PEG-DA hydrogels. The substrate was functionalized with a peptide containing the IKVAV sequence from the Laminin 1 chain (Figure 1 and Figure 2). Additionally, the EsB detector was used to differentiate between gold particles (heavy metal) and their surrounding (ice) taking advantage of the high material contrast of this detection mode (Figure 2 C).

4Pi Microscopy

Mouse myoblastoma C2C12 and mouse neuroblastoma N2a cells were plated on Fibronectin- or laminin-coated (each 10 µg/ml in PBS) quartz glass cover slips. Prior to functionalization, quartz glass was acid activated in 1M HCl for four hours at room temperature. Protein solutions were doped with 0.5 µg/ml BSA-fluorescein for visualization of the glass surfaces. Cells were incubated for 36 hours in the incubator and then stained for 1 minute in DMEM without FBS with 1:1000 dilution of DiI (1 mg/ml). After one washing step, cells were fixed in paraformaldehyde (3.7%) for 15 minutes at 37°C, washed, embedded in 87% glycerol, covered with a second quartz cover slip, and transferred into the 4Pi unit of a Leica TCS 4PI microscope, which had been modified for Type C mode operation [1]. Samples were imaged by 100×/1.35 glycerol immersion objectives in regular confocal mode (with one objective blocked) and in Type C mode with one-photon excitation at 488 nm for both fluorescent labels. Fluorescence of fluorescein and DiI was detected by two Avalanche Photo Diodes. Side-maxima in the DiI channel were low enough (~40%) to be removed reliably by linear deconvolution. In the fluorescein channel, the higher side-maxima normally would not allow artifact-free image restoration. In our situation, where the fluorescein signal was only used to identify the position of the flat surface of the substrate, sidelobe removal did not cause any problems. The recorded, deconvolved data was then 3D-rendered by the Amira software program, utilizing as well the "isosurface" mode and the "voltex" mode to visualize the cell membrane and the BSA layer. Figure 3 shows the comparison between regular high-resolution confocal images and 4Pi Type C images of the same regions of interest. 4Pi microscopy is clearly able to resolve the upper from the lower membrane even at the outer edges of the lamellipodium and nicely reveals the uneven membrane facing the cover slip. By employing this method, the cell membrane, which is close to the surface, can be visualized three-dimensionally. Additionally, molecular changes inside the cell, like the organization of the cytoskeleton components or the development of macromolecular complexes like focal adhesion

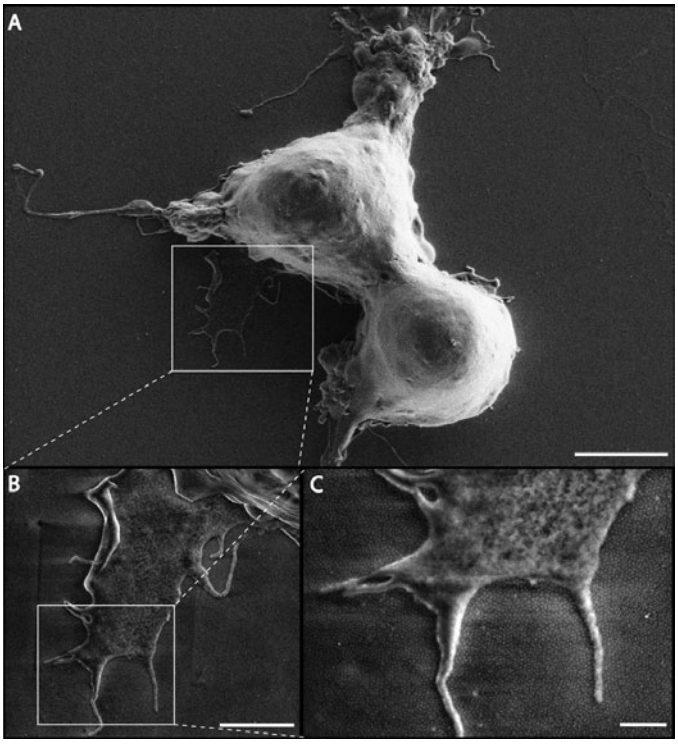

Figure 1: (A) Cryo scanning electron micrograph of dividing mouse neuroblastoma N2a cells on gold nanoparticle patterned PEG-700-DA hydrogel surface. (B) Enlarged area showing part of an adherent lamellipodium as indicated by the square in (A). (C) shows a close-up of the area indicated by the box in (B). The structured gold nanoparticle surface is clearly visible as a dim dot pattern next to the cell. The distance between nanoparticles is about 50 nm. Cellular extensions are attached to biofunctionalized gold nanoparticles. Scale bar in is 9 µm in (A) 2 µm in (B), and 500 nm in (C).

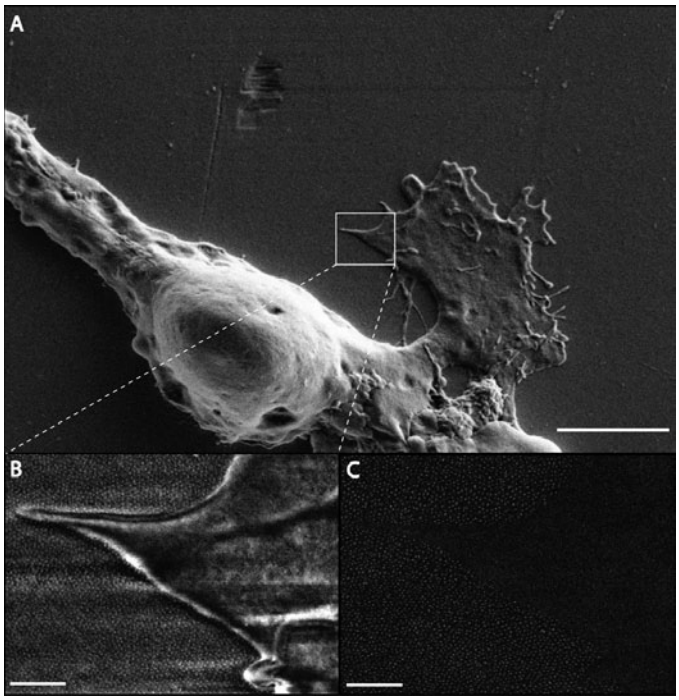

Figure 2: (A) Cryo scanning electron micrograph of a mouse neuroblastoma N2a cell on a gold nanoparticle patterned PEG-700-DA hydrogel surface. (B) and (C) show the area depicted by the rectangle in (A) as seen by an SE2 (B) and an EsB (C) detector, respectively. Whereas topography of the cell membrane is clearly visible in (B), uncovered gold nanoparticles could be observed free of charging artifacts in (C). Scale bar is 4 µm in (A) and is 500 nm in (B) and (C).

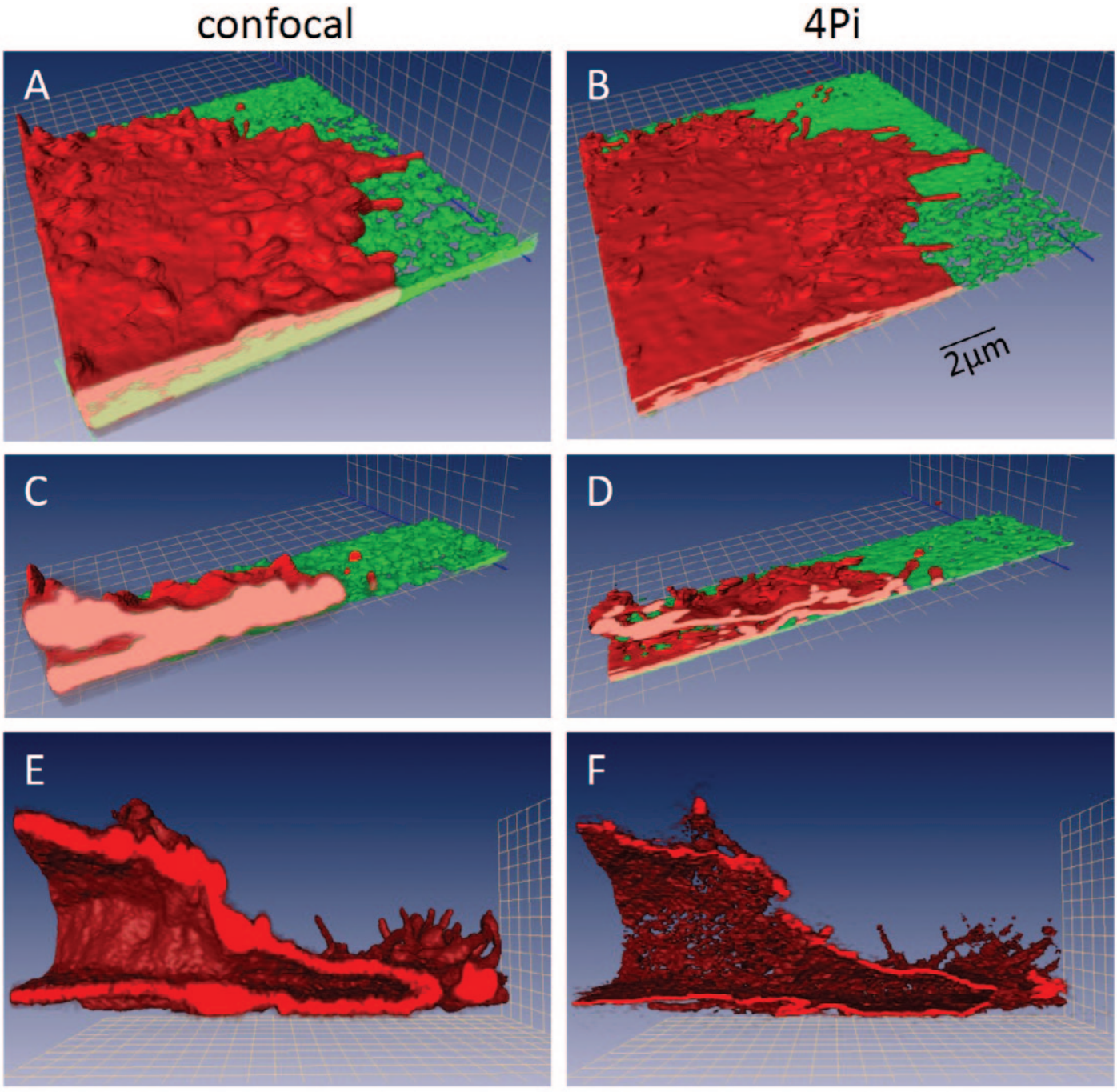

Figure 3: 3D reconstruction of the same part of a lamellipodium of a C2C12 cell (A-D) and of an N2a cell (E, F) as seen by confocal (A, C, E) and 4Pi microscopy (B, D, F). (C) and (D) show subsets of the data displayed in (A) and (B), respectively, visualizing features hidden in the other view. The 3D rendering was created with Amira, showing the plasma membrane labeled by DiI in red and the BSA-covered substrate labeled by fluorescein in green (A-D). Grids have a 1-µm spacing.

complexes, could be investigated and correlated with changes of structural composition of the substrate.

Conclusion

In summary, we demonstrate the combination of high-resolution SEM images with the 3D information of cellular structures resolved by 4Pi microscopy on defined nanostructured and functionalized hard or soft substrates. This approach provides new investigative tools for biomedical engineering and gives new insights into basic cell biology on 2-dimensional interfaces.

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