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Revealing the Cell—Material Interface with Nanometer Resolution by Focused Ion Beam/Scanning Electron Microscopy

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*Supporting Information

ABSTRACT: The interface between cells and nonbiological surfaces regulates cell attachment, chronic tissue responses, and ultimately the success of medical implants or biosensors. Clinical and laboratory studies show that topological features of the surface profoundly influence cellular responses; for example, titanium surfaces with nano- and microtopographical structures enhance osteoblast attachment and host—implant integration as compared to a smooth surface. To understand how cells and tissues respond to different topographical features, it is of critical importance to directly visualize the cell—material interface at the relevant nanometer length scale. Here, we present a method for in situ examination of the cell-to-material interface at any desired location, based on focused ion beam milling and scanning electron microscopy imaging to resolve the cell membrane-to-material interface with 10 nm resolution. By examining how cell membranes interact with topographical features such as nanoscale protrusions or invaginations, we discovered that the cell membrane readily deforms inward and wraps around protruding structures, but hardly deforms outward to contour invaginating structures. This asymmetric membrane response (inward vs outward deformation) causes the cleft width between the cell membrane and the nanostructure surface to vary by more than an order of magnitude. Our results suggest that surface topology is a crucial consideration for the development of medical implants or biosensors whose performances are strongly influenced by the cell-to-material interface. We anticipate that the method can be used to explore the direct interaction of cells/tissue with medical devices such as metal implants in the future.

KEYWORDS: cell—material interface, nanostructures, scanning electron microscopy, focused ion beam, ultrathin resin plasticization

Many biological applications and biomedical devices require direct contact between cells and nonbiological materials.1 In the case of medical implants, the cell-to-material interface is a key determinant for successful device integration with surrounding tissues, providing mechanical support and minimizing host foreign body responses.2–4 Extensive clinical and laboratory studies have shown that surface topologies of nonbiological materials can significantly affect cellular and tissue responses. For example, titanium implants having a rough surface perform much better than...
those having a smooth surface for osteoblast attachment, host−implant integration, and the overall success of the implant. At the cellular level, surfaces with nano- and micrometer topographical features have been shown to actively affect cell behavior such as stimulating stem cell differentiation, enhancing osteoblast maturation, and regulating macrophage activity. In this context, understanding how cells interact with different features on the material surface is essential to study how surface topologies regulate cell signaling, guide cell migration, and control stem cell differentiation.

The most critical feature of the cell-to-material interface is the cleft between the cell membrane and the material surface, usually in the range of 50−200 nm for flat surfaces. Sophisticated optical techniques have been developed to measure the cleft distance, such as fluorescence interference contrast (FLIC) microscopy, surface-generated structured illumination microscopy, and variable incidence angle FLIC microscopy (VIA-FLIC). However, these interference-based techniques are limited to smooth and reflective surfaces and are not suitable for surfaces with topological features. Transmission electron microscopy (TEM) is the most widely used method to directly visualize membrane structures at the nanoscale. However, TEM requires sectioning the sample into ultrathin slices (<100 nm thickness) with mechanical knives, a procedure not compatible with a variety of substrate materials. For this reason, the support material underneath the cells has to be removed and the removal process by chemical or physical treatment is often not feasible; even if feasible, the procedure is challenging and can induce structural artifacts at the interface.

A combination of focused ion beam (FIB) and scanning electron microscopy (SEM) constitutes an alternative approach for in situ imaging interfaces of any material and any desired location. However, using FIB-SEM to examine the cell-to-material interface is severely limited by the lack of contrast of biological specimens and the sponge-like intracellular defects induced by hard drying procedures. Resin-embedding preparation with heavy metals allows the visualization of intracellular structures even in the proximity of nanostructures, but the resin matrix around the cells does not allow any visualization of the entire cell unless a 3D reconstruction of the whole specimen is performed. Recently, thin-layer resin...
81 embedding methods have been developed to allow the visualization of cells on microstructures, but the contrast of the resulting samples is still too low to clearly resolve the membrane-to-material interface at the nanoscale. To date, there is no method that can reliably resolve the plasma membrane in proximity to nano- and microstructures and thus to measure the cleft distance between the cell membrane and the material surface. Therefore, the question of how surface topology affects the cleft distance remains largely unexplored.

In this work, we present a FIB-SEM method that can precisely resolve the cell-to-substrate interface with 10 nm resolution. At the core of our FIB-SEM method is a sample preparation method based on controlled thin-resin plasticization of adherent cells with heavy metal staining. Unlike the usual hard drying methods, this procedure embeds cells in a thin plastic layer, which not only preserves the subcellular structures but also provides a solid support for the subsequent FIB milling.

RESULTS AND DISCUSSION

The thin-layer plasticization method includes five major steps: cell fixation, heavy metal staining, resin infiltration, extracellular resin removal, and resin polymerization (Figure 1a). Specifically, mammalian cells cultured on the desired substrate are fixed by glutaraldehyde to cross-link intracellular structures (i.e., proteins) so that they can withstand the subsequent staining and embedding processes without altering the interstitial space between the membrane and the material surface. After fixation, the cells are treated with osmium- and uranium-based staining series (RO-T-O procedure and en bloc staining; see Experimental Procedure for details), a critical step to provide high contrast to membrane and protein structures. Then, cells are infiltrated with liquid epoxy-based resin. Traditional resin-embedding procedures for TEM typically result in a 2-5-mm-thick polymer block, preventing the visualization of the whole-cell morphology. In our method, after resin infiltration and before resin polymerization, a resin-removal step is introduced that strips off the excess extracellular resin by first draining and,
then, flushing the sample with ethanol. This step thins down
the resin coating outside the cell membrane to tens of
nanometers while maintaining a stable intracellular resin
embedding.34 The final step involves curing the liquid resin
to a thin layer of plastic with cells embedded inside. Since
extracellular resin is largely removed, cell topography and
membrane protrusions in contact with the underlying substrate
are clearly visible under SEM. Figure 1b shows a resin-
embedded HL-1 cell cultured on a quartz substrate with arrays
of nanopillars, and Supplementary S1 shows resin-embedded
PC12 cells and primary cortical neurons cultured on flat glass
substrates, where fine features of the cell membrane are well
preserved.

Samples prepared via thin-layer plasticization are directly
mounted on FIB-SEM for in situ examination of the cell-to-
substrate interface. For this purpose, we first examine a large
sample area by SEM to identify locations of interest, such as
places where cell membranes are in contact with topological
features such as nanopillars. Once a desired area is located, it is
coated with a thin layer of platinum to prevent sample damage
during the next FIB milling step (see Experimental Procedure
and Supplementary S2). Then, a high-energy gallium ion beam
(acceleration current of 0.74 nA) is focused on the sample to
cut through the platinum protection layer, the cell-embedded
thin plastic layer underneath, and at least 1 μm deep into the
substrate. This process is repeated to remove material and
opens up a vertical surface (Figure 1c,d). Then, a low-current,
cg., 80 pA, ion beam is used to remove redeposited material
and polish the cross section. This step is critical for limiting the
well-known curtaining phenomena and ion-induced structural
damage at the interface.33 SEM visualization of the cross
section clearly shows intracellular structures as well as the
interface between the cell membrane and the substrate (Figure
1e). Unlike previous FIB-SEM images that usually contain
spoon-like structures with no discernible subcellular struc-
tures,34,27,34 our FIB-SEM images show very clear subcellular
structures such as the cell membrane, the nucleus, nucleoli, the
nuclear envelope, mitochondria, and intracellular membranes.
We note that the resin wash step of the thin-resin plasticization
procedure needs to be carried out gently to avoid over-removal
of the resin, which can cause cracks in the cell membrane and
intracellular space. For the heavy metal staining step, either
overstaining or understaining results in poor structural contrast
and lower resolution, similar to TEM samples. All FIB-SEM
images are black-and-white inverted. Original images are shown
in Supplementary S2.

To determine the resolution of our FIB-SEM method, we
have examined a group of well-characterized cellular compart-
ments using high-magnification SEM imaging. Figure 1f shows
a mitochondrion with clearly resolved inner and outer membranes (~10 nm distance) as well as the cristae structures.
Figure 1g shows the structure of a nuclear envelope with well-
distinguishable inner and outer membranes, which are
separated by an interstitial space of about 20 nm. Endoplasmic
reticulum (ER) structures as parallel running membranes can
be seen in the vicinity of the nucleus, and the associated small
granules attached to the membrane of the ER likely are
ribosomes (Supplementary S3). Other intracellular structures
such as multivesicular bodies and intracellular membrane can
also be resolved in Supplementary S3. Furthermore, a high-
magnification SEM image of the cell–substrate interface clearly
reveals that the plasma membrane is very close to the flat
substrate surface and contours around local nanopillar features
(Figure 1e, inset).

The development of this FIB-SEM method allows us to
quantitatively address the question of how different surface
topographies affect the cell–substrate cleft distance. For this
study, we engineer SiO2 substrates (or Si substrates with a SiO2
surface layer) with different surface geometries, including
protrusions, invaginations, flat, and other complex structures
(see Experimental Procedure for fabrication details). The
protrusions are vertical nanopillars with diameters or lengths
varying from 200 to 1500 nm, a height of 1 μm, and spacing of 3–5 μm (Figure 2b,d and Supplementary S4). The
invaginations are pores with diameters varying from 200 to
600 nm, a depth of about 500 nm to 1 μm, and a spacing of 3
μm (20 μm for the largest pore) (Figure 2g,i and Supplementary S5). A cell on a flat surface is shown in Figure
2l. The complex structures include nanotubes, nanobars,
irregular nanocones, nanoletters (CUIO), and grooves, and
they are shown in Supplementary S4 and S6. All substrates
were coated with poly-l-lysine or fibronectine to facilitate cell
adhesion. HEK or HL-1 cells were used for the studies. Cells
cultured on different substrates were processed for FIB-SEM
imaging using the aforementioned preparation method. SEM
images of cells cultured on flat, nanopillar, and nanopore
substrates before FIB milling show healthy and spread cell
morphology (Supplementary S7).

The FIB-SEM imaging reveals drastic differences in how cell
membranes respond to different substrate nanotopologies. For
substrates with protruding structures, the cell membrane
deforms readily and wraps conformably around the surface
topology, as shown in Figure 2c,e and Supplementary S8, for
nanopillars with 400 nm and about 1500 nm diameter, 211
respectively. For nanopillars of all diameters the cell membrane
is usually within 10–30 nm on average from the substrate
cross section. In sharp contrast, for substrates with invaginating
structures, the cell membrane hardly deforms and does not
contour the surface of nanopores or the hollow centers of the
nanotubes (Supplementary S9). For small-diameter pores
(Figure 2h), the cell membrane extends into the pores slightly,
but the cleft distance is usually more than 10 times greater than
that for nanopillars. For nanopores as large as 6 μm in diameter
and 500 nm in depth, the cell membrane is still far away from
the surface (Figure 2j), but some attachment points are created
in the pore. For flat surfaces, the cell membrane remains close
to the surface (Figure 2m). A similar phenomenon is observed
in other complex structures (Supplementary S9). For
protruding structures such as nanobars, CUIO nanoletters, 226
and nanocones, the cell membrane is very close to the substrate
surface, while for invaginating structures such as grooves, the
cell membrane is far away from the substrate surface
(Supplementary S9). For nanotubes, the cell membrane
wraps tightly around the outside wall of the tube (protruding
structure), while it remains far away from the inner wall of the
hollow center (invaginating structure, Supplementary S9). This
is a surprising result, as previous studies suggest that the cell
membrane is highly deformable and can extend into pits as
small as 50 nm.23,30

In order to evaluate the cleft formed between the plasma
membrane and different surface topographies, we systematically
measured the average cleft distance for surfaces with nanopillars
and nanopores with comparable dimensions and flat surfaces
(measurement statistics shown in Supplementary S10). As seen
in Figure 2n, the cleft distance is ~100 nm (stdv 50 nm) for the

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The cleft distance decreases to ~15 nm (stdv 10 nm) for nanopillars, while it increases to >400 nm for nanopores (stdv 300 nm). These dramatic changes in the cleft width suggest that the plasma membrane interacts with protruding and invaginating surface topologies in fundamentally different ways. In addition, we calculated the cleft area between the membrane and the nanostructures for all the investigated nanoholes and nanopillar types. The cleft index measurement confirms that the cleft area increases in the presence of nanopores and decreases in the presence of nanopillars (see Supplementary S10 and S11 for details).

To corroborate the FIB-SEM studies, we also examined how the plasma membrane interacts with different surface topologies by fluorescence imaging. At the same time, we simultaneously probed the distribution of actin filaments, which are well known to participate in the dynamics and the formation of protrusions or invaginations on the cell membrane. Cells were cotransfected with two plasmids, CAAX-GFP, which serves as a marker for the plasma membrane, and LifeAct-RFP, which is widely used to visualize F-actin in cells. Fluorescence imaging of CAAX-GFP confirms that the cell membrane wraps around nanopillars (bright spots due to projection of the vertical membrane in Figure 2a) but not nanopores or flat surfaces (Figure 2f,k). LifeAct-RFP imaging shows that F-actin accumulates strongly on nanopillar locations, but is absent at nanopores (Supplementary S12) and flat surfaces (data not shown). This preliminary result suggests that actin filaments might be involved in forming the close contact between the cell membrane and the nanopillars.

Next, we examine whether the topological effect for the interface cleft depends on the chemical composition of the material. Considering that our FIB-SEM method is applicable to materials with diverse composition and stiffness, we compared flat and nanopillar surfaces made of quartz (Young’s modulus ~80 GPa) and conductive polymer blend poly(3,4-ethylenedioxythiophene):poly(styrenesulfonate) (PEDOT, Young’s modulus ~1 GPa). Unlike quartz (shown as the gray bottom layer in Figure 2f–i), PEDOT is conductive and scatters electrons strongly (shown as the black bottom layer in Figure 3b), which reduces the effective contrast of the biological sample. Despite this, the FIB-SEM image in Figure 3b (cells before cut shown in Figure 3a) still clearly resolves the cell membrane–surface gap, achieving the first cross section visualization of cells on the PEDOT surface. Here, we measured the effective distance of the plasma membrane from the surface. The cell membrane is seen in close contact with the flat PEDOT surface, and the average cleft distance is measured to be 89 ± 73 nm (stdv), similar to the cleft distance for the flat quartz surface at 98 ± 52 nm (stdv). Next, we compared the cleft distances for nanopillar substrates made of quartz and covered with a thin layer of PEDOT (Figure 3c,d). Our measurements show that the average cleft distances for quartz nanopillars and PEDOT nanopillars are similar (15 ± 2.7 nm and 11 ± 4.1 nm, stdv) but much smaller than that for the flat surfaces. The statistical details of these measurement are shown in Supplementary S10.

Finally, we explored the capabilities of the FIB/SEM method for volumetric imaging and multiangle imaging. FIB-SEM allows repetitive milling and imaging, allowing the investigation of a volume of interest (Figure 4a). We used low current (e.g., 80 pA) for sequential FIB milling, which achieves a slice thickness of about 20–40 nm and well beyond the capability of mechanical slicing by means of ultramicrotomes (70–200 nm). Figure 4bc show two representative cross sections of the same cell (shown in Figure 4a) interacting with two different lines of nanopillars. By sequentially imaging a set of 72 sequential sections, we reconstructed a 3D intracellular space and its interaction with nanopillars using a segmented 3D reconstruction method (Figure 4d, Supplementary Movie 1). In particular, we modeled the 3D morphology of the nuclear envelope, nucleoli, and the nonadherent cellular membrane domain, which were individually constructed and overlaid on the remaining structures, as shown in Figure 4e. The nuclear envelope appears to be bent upward on top of a nanopillar by as much as 800 nm (Figure 4f), agreeing well with our previous observation by TEM.

Unlike the ultramicrotome sectioning method, which slices materials sequentially in only one direction, the FIB-SEM method is highly versatile and allows sectioning of the same sample with different directions at multiple locations. This capability is often important for cells with protrusions such as neurons. Primary cortical neurons from embryonic rats were cultured on a quartz substrate with arrays of solid nanopillars. After 5 days of culturing in vitro, neurons were fixed and processed for FIB-SEM imaging as described earlier. The SEM image in Figure 4g (inset) shows a neuron cell body together with multiple neurites growing out from the cell body. We first identified four regions of interest from the SEM image: the cell body, neurite-1, neurite-2, and neurite-3. Then, after coating a layer of Pt, FIB milling was used to cut open the interfaces.
along six connecting lines (yellow arrowed lines corresponding to four regions of interest and green arrowed lines being the connecting lines in Figure 4g). FIB-SEM imaging of the cell body shows the nucleus, a large number of intracellular organelles, and the plasma membrane wrapping around the nanopillars (Figure 4i). By multiangle milling, FIB-SEM also offers the advantage of examining a location from multiple directions, as shown by the 90-degree intersection between neurite-2 and the cell body (Figure 4h). The cross section of neurite-3 is shown in Figure 4j, which illustrates a neurite attached to the top and the side of two nanopillars. A magnified image of a neurite reveals multiple longitudinally oriented microtubules parallel to the direction of the neurite (Figure 4k), comparable in morphology to those investigated by TEM. 

CONCLUSIONS

We demonstrate a FIB-SEM method for imaging the cell-to-material interface in situ, without removing the substrate. The FIB-SEM method has the advantages of examining a large sample area, opening up cross sections at any desired location, achieving volume reconstruction, and performing multidirectional milling. This method achieves a high contrast and...

Figure 4. FIB-SEM for sequential volumetric imaging and multiangled imaging. (a) SEM image of a plasticized HL-1 on nanopillars where yellow dashed lines indicate the region of interest for the sequential milling. (b, c) SEM images of two exemplary slices from a stack of 78 slices at two different pillars’ lines. (d–f) Images collected in the stack were assembled, segmented, and analyzed. Automated 3D reconstruction of the top membrane and the nuclear envelope overlaid on the SEM background image. Reconstruction shows that the nuclear envelope is deformed upward by a nanopillar. (g) FIB milling of a neuron where yellow arrows indicate the regions of interest and green lines indicate the connecting regions (the inset shows a SEM image of the same neuron before FIB milling). (h) FIB-SEM image of the body–neurite 2 connecting region opened at a 90-degree angle. (i) FIB-SEM image of the neuronal body on a line of nanopillars. (j) FIB-SEM image of neurite 3 on top of nanopillars. (k) Zoomed-in image of neurites revealing multiple longitudinally oriented microtubules parallel to the direction of the neurite.
EXPERIMENTAL PROCEDURE

1. Nanostructure Fabrication, Characterization, and Preparation. Fabrication and Characterization of Quartz Nanopillars, CUIO Structures, Nanobars, and Nanotubes. Nanостructures (NSs) used in this work were fabricated on a 4 in. quartz wafer using electron-beam lithography (EBL). In brief, the wafer was diced into pieces 2 cm × 2 cm square. After sonication cleaning in acetone and 2-propanol, the pieces were spin-coated with 300 nm of ZEP-520 (ZEON Chemicals), followed by E-Spacer 300Z (Showa Denko). Desired patterns were exposed by EBL (Raith150) and developed in xylene. The mask was then created by sputter deposition of 100 nm Cr and lift-off in acetone. NSs were generated by reactive ion etching with CHF₃ and O₂ chemistry (AMT 8100 etcher, Applied Materials). Before cell culture, the substrate was cleaned in O₂ plasma and immersed in Chromium Etchant 1020 (Transene) to remove Cr masks. SEM (FEI Nova) imaging was performed on 3 nm Cr sputtered substrates to measure the dimensions of different NSs.

Silicon Nanocores. A monolayer polystyrene nanosphere (PS) array, which consists of PSs with an average diameter of 3 μm, was self-assembled on glass-based silicon substrates with the Langmuir–Blodgett method. To control the effective intervals between the formed silicon nanopillars, a reactive ion etching process with oxygen (O₂) as an etching gas was then followed to shrink the PSs (with a final diameter of 1 μm). Silicon nanocores were last formed on glass substrates by introducing chlorine (Cl₂) and hydrogen bromide (HBr) gases to reactive-ion-etch the silicon materials exposed to the plasma. Quartz Nanopillars with PEDOT:PSS Cover Layer. Fused silica glass substrates were cleaned using a standard soap, acetone, 2-propanol, the pieces 2 cm × 2 cm square. After sonication cleaning in acetone and 2-propanol, the pieces were spin-coated with 300 nm of ZEP-520 (ZEON Chemicals), followed by E-Spacer 300Z (Showa Denko). Desired patterns were exposed by EBL (Raith150) and developed in xylene. The mask was then created by sputter deposition of 100 nm Cr and lift-off in acetone. NSs were generated by reactive ion etching with CHF₃ and O₂ chemistry (AMT 8100 etcher, Applied Materials). Before cell culture, the substrate was cleaned in O₂ plasma and immersed in Chromium Etchant 1020 (Transene) to remove Cr masks. SEM (FEI Nova) imaging was performed on 3 nm Cr sputtered substrates to measure the dimensions of different NSs.

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Sample Preparation for Cell Culture. Quartz substrates were treated with piranha solution with sulfuric acid and hydrogen peroxide (Fisher Scientific), in a 7:1 dilution at room temperature overnight. Samples were washed with distilled water, dried, and placed in 70% ethanol in a sterile hood. Samples were washed with sterile distilled water and allowed to dry. After a 15 min UV light exposure, samples were incubated overnight with 0.01% poly-l-lysine (Sigma Life Science) for primary neurons and HEK cell cultures or with 1 mg/mL fibronectin (Life Technologies) in 0.02% gelatin solution for HL-1 cells. COS-7 cells were directly plated on the substrate after sterilization.

2. Cell Culture and Transfection. Primary Neurons. Cortices were extracted from rat embryos at embryonic day 18 and incubated with 0.25% trypsin/EDTA (Corning) in a 33 mm Petri dish for 5 min at 37 °C. The tissue-trypsin/EDTA solution was transferred into a 2 mL plastic tube. The tissue settled at the bottom of the tube, and the trypsin/EDTA was removed. Neurobasal media (Gibco) was supplemented with 1% B27 (Gibco), 0.25% glutaMAX (Gibco), and 0.1% gentamycin antibiotic (Gibco). One milliliter of warm media was added, and then the tube was gently swirled by hand. This procedure was repeated five times, and after the last media exchange, the tissue was dissociated until resulting in a cell solution. A total of 80 000 cells were suspended in 3 mL and placed on each substrate. The media was replaced completely 2 h after seeding time. Every second day, half of the media was exchanged with freshly prepared warm (supplemented) Neurobasal media.

HL-1 Cells. Confluent HL-1 cells, cultured in a 33 mm Petri dish, were incubated with 1 mL of 0.25% trypsin/EDTA for 5 min at 37 °C. The cell–trypsin solution was transferred into a 15 mL tube, and 2 mL of Claycomb media (Sigma Life Science) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 μg/mL penicillin/streptomycin (Gibco) (Sigma Life Science), 0.1 mM norepinephrine (Sigma-Aldrich), and 2 mM glutaMAX were added. The cell solution was placed in a 2 mL centrifuge for 3 min with a rotation of 1300 rpm. The cell pellet was resuspended in 1 mL of media, and 50 μL of the resuspension was placed on each substrate in addition to 3 mL of supplemented media. HEK 293 Cells. HEK 293 expressing channels NaV 1.3 and KIR 2.1 were acquired from AddGene. Cells were maintained in DMEM/F12 (Gibco), 10% FBS (Gibco), 1% penicillin/streptomycin (100 μg/mL, Gibco), Geneticin (500 μg/mL, Gibco), and puromycin (2 μg/mL, Fisher Scientific). At 80% confluency, cells were divided, resuspended, and plated on quartz substrates as for HL-1 cells.

COS-7 and U2OS Cells. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, and at 90% confluence they were divided as for HL-1 cells and plated on the substrates. CAXX/LifeAct Transfection. U2OS cells were cultured in DMEM medium (HyClone) with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in 5% CO₂. Transfection was performed using electroporation (Amazix Nucleofector) with the manufacturer’s protocol. U2OS cells were transfected with Lifeact-RFP (transformed bacteria acquired from AddGene) and CAXX-GFP and plated on a nanostructured surface for at least 16 h before examination.

3. UltraThin Plastification and RO-T-O Procedure. Substrates with cells were rinsed with 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) and fixed with 3.2% glutaraldehyde (Sigma-Aldrich) at 4 °C overnight. Specimens were then washed (3 × 5 min) with chilled buffer and quenched with chilled 20 mM glycine solution (20 min). After rinsing (3 × 5 min) with chilled buffer specimens were prefixed with equal volumes of 4% osmium tetroxide and 2% glutaraldehyde.
potassium ferrocyanide (Electron Microscopy Sciences, RO step) (1 h on ice). Samples were then washed with chilled buffer (3 × 5 min), and the solution was replaced with freshly prepared 1% thiocarbohydrazide (Electron Microscopy Sciences, T step) (20 min at room temperature). After rinsing with buffer (2 × 5 min), the samples were incubated with 2% aqueous osmium tetroxide (O step) (30 min at room temperature). Cells were then rinsed (2 × 5 min) with distilled water and then, finally, incubated with syringe-filtered 4% aqueous uranyl acetate (Electron Microscopy Sciences, en bloc step) (overnight at 4 °C). Cells were rinsed (3 × 5 min) with chilled distilled water, followed by gradual dehydration in an increasing ethanol series (10%—30%—50%—70%—90%—100%, 5—10 min each on ice). The last exchange with a 100% ethanol solution was performed at room temperature. Epoxy-based resin solution was prepared as previously described, and samples were infiltrated with increasing concentrations of resin in 100% ethanol, using these ratios: 1:3 (1 h), 1:2 (3 h), 1:1 (overnight), 2:1 (3 h), 3:1 (3 h). Infiltration was carried out at room temperature and in a sealed container to prevent evaporation of ethanol. Samples were then infiltrated with 100% resin overnight at room temperature. The excess resin removal was carried out by first draining away most of the resin by mounting the sample vertically for 1 h and, then, rapidly rinsing with 100% ethanol prior to polymerization at 60 °C overnight.

**SEM Imaging.** Samples were loaded into the vacuum chamber of a dual-beam Helios Nanolab600i FIB-SEM (FEI). For selecting a region of interest, an (electron) beam with an accelerating voltage of 3—5 kV and current of 21 pA to 1.4 nA was applied. For image acquisition of whole cells (i.e., Figure 1b) a secondary electron detector was used. For cross-section imaging, a beam acceleration voltage of 2—10 kV was selected, with the current ranging between 0.17 and 1.4 nA, while using a backscattered electron detector (immersion mode, dynamic focus disabled in cross section, stage bias zero), a dwell time of 100 μs, and 3072 × 2048 pixel store resolution. For the sequential sectioning, the function iSPI was enabled in order to slice and acquire an image of the stack every 38.5 nm with 5 kV voltage, 1.4 nA current, and 1024 × 1284 resolution.

**FIB Sectioning.** Regions of interest were preserved by electron-assisted deposition of a 0.5 μm double platinum layer and ion-assisted deposition of a (nominal) 1 μm thick coating. First, trenches were created with an etching procedure fixing an acceleration voltage of 30 kV and currents in the range 9.1–0.74 nA depending on the effective area to remove. A fine polishing procedure of the resulting cross sections was carried out on the sections, with a voltage of 30 kV and lower currents in the range 0.74 nA to 80 pA so that redeposition phenomena in the cross section are very limited.

**Image Analysis and 3D Reconstruction.** All images were preprocessed with ImageJ (National Institutes of Health, USA, http://imagej.nih.gov/ij). The images of the sequential cross sections shown in Figure 2 were collected as a stack, analyzed, and processed with an open source tool chain based on Python (Python Software Foundation, USA, http://www.python.org) scripts and tools. The image stack was cropped, filtered, and down-sampled. The isotropic resolution in x, y, and z amounts to 38.5 nm. The reconstructed data are visualized with Blender (Blender Foundation, The Netherlands, http://www.blender.org).

**Cleft Distance.** The average cleft distance has been calculated by selecting 10 equally distributed points on the part of the plasma membrane that surrounds the nanostructures. For each point, the distance is measured as the shortest distance between the membrane and the material surface. The number of points, the number of nanostructures, and the number of cells that are used to calculate the average number (and the standard deviations of the mean) are listed in Supplementary Table S10. The measurements have been performed with ImageJ.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.7b03494.

**Movie (AVI)**

Ultra thin plasticization of cells on planar substrates, sectioning procedure, ultrastructure resolution, substrate geometry of nanopillars and nanocones, substrate geometry of nanopores, substrate geometry of CUI, nanobars, nanotubes, and grooves, SEM of cells on a flat surface and diverse nanostructures, cleft between cells and flat, nanopillar, and nanopore surfaces, cleft visualization for cells on complex structures, cleft distance, cleft index, CAAX/ACTIN cotransfection (PDF)

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**Notes**

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