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Citation for published version (APA):

DOI:
10.1021/acsnano.7b03494

Document status and date:
Published: 22/08/2017

Document Version:
Typeset version in publisher’s lay-out, without final page, issue and volume numbers

Please check the document version of this publication:

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Download date: 22. Feb. 2019
Revealing the Cell—Material Interface with Nanometer Resolution by Focused Ion Beam/Scanning Electron Microscopy

Francesca Santoro,‡†§ Wenting Zhao,‡† Lydia-Marie Joubert,§ Liting Duan, † Jan Schnitker,∥
Yoeri van de Burgt,‡□ Hsin-Ya Lou,† Bofei Liu,‡ Alberto Salleo,‡ Lifeng Cui,⊥ Yi Cui,‡,#
and Bianxiao Cui*††

† Department of Chemistry, ‡ Department of Material Science and Engineering, and § CSIF Beckman Center, Stanford University, Stanford, California 94305, United States
∥ Institute of Bioelectronics ICS/PGI-8, Forschungszentrum Juelich, Juelich, 52428, Germany
‡ Department of Material Science and Engineering, Dongguan University of Technology, Guangdong 523808, China
# Stanford Institute for Materials and Energy Sciences, SLAC National Accelerator, Menlo Park, California 94025, United States

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

Received: May 18, 2017
Accepted: July 6, 2017
Published: July 6, 2017

Many biological applications and biomedical devices require direct contact between cells and nonbiological materials. 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. 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...
39 those having a smooth surface for osteoblast attachment, host−
40 implant integration, and the overall success of the implant.1,5 At
41 the cellular level, surfaces with nano- and micrometer
42 topographical features have been shown to actively a
43 effect cell
44 behavior such as stimulating stem cell differ
45 entiation, 6
46 enhancing osteoblast maturation,7 and regulating macrophage
47 activity.8 In this context, understanding how cells interact with
48 different features on the material surface is essential to study
49 how surface topologies regulate cell signaling, guide cell
50 migration, and control stem cell differentiation.9
51 The most critical feature of the cell-to-material interface is
52 the cleft between the cell membrane and the material surface,
53 usually in the range of 50−200 nm for flat surfaces.12−14
54 Sophisticated optical techniques have been developed to
55 measure the cleft distance, such as fluorescence interference
56 contrast (FLIC) microscopy,15−17 surface-generated structured
57 illumination microscopy, and variable incidence angle FLIC
58 microscopy (VIA-FLIC18). However, these interference-based
59 techniques are limited to smooth and reflective surfaces and are
60 not suitable for surfaces with topological features. Transmission
61 electron microscopy (TEM) is the most widely used method to
62 directly visualize membrane structures at the nanoscale.13,14,19
63 However, TEM requires sectioning the sample into ultrathin
64 slices (<100 nm thickness) with mechanical knives, a procedure
65 not compatible with a variety of substrate materials. For this
66 reason, the support material underneath the cells has to be
67 removed and the removal process by chemical or physical
68 treatment is often not feasible; even if feasible, the procedure is
69 challenging and can induce structural artifacts at the inter-
70 face.13,20
71 A combination of focused ion beam (FIB) and scanning
72 electron microscopy (SEM) constitutes an alternative approach
73 for in situ imaging interfaces of any material and any desired
74 location.21 However, using FIB-SEM to examine the cell-to-
75 material interface is severely limited by the lack of contrast of
76 biological specimens and the sponge-like intracellular defects
77 induced by hard drying procedures.22 Resin-embedding
78 preparation with heavy metals allows the visualization of
79 intracellular structures even in the proximity of nanostruc-
80 tures,15,26 but the resin matrix around the cells does not allow
81 any visualization of the entire cell unless a 3D reconstruction of
82 the whole specimen is performed. Recently, thin-layer resin

Figure 1. Imaging the cell-to-material interface by FIB/SEM. (a) Schematics of the sample preparation procedure by thin-layer resin
plasticization with contrast enhancement. (b) SEM image of a plasticized HL-1 cell on a quartz substrate with nanopillars showing that
evacuolar resin is removed and the cell morphology is clearly visible. The inset shows that the membrane protrusions in contact with a
nanopillar are well preserved. (c) Schematics and (d) experimental results of using FIB milling to cut trenches through the cell and the
substrate and open up the interface. (e) SEM image of the interface after FIB milling revealing intracellular compartments and organelles such as
mitochondria (1), intracellular membranes (2), nucleoli (3), nucleus (4), and cellular membrane (5). Inset: At the interface between the
cell and the quartz substrate, the plasma membrane is shown to warp around a vertical nanopillar. Intracellular structures and local curvatures
on the plasma membrane resembling clathrin-mediated endocytosis events can be identified. (f, g) Zoomed-in FIB-SEM images of
mitochondria (f) and nuclear envelope (g). The insets clearly resolve the inner and outer membranes and interstitial space. Figures e−g have
been acquired from backscattered detectors (voltage: 5−10 kV, current: 0.64−1.4 nA), tilt is 52°, and original images are black−white inverted.
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-μm-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. 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, e.g., 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.

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 sponge-like structures with no discernible subcellular structures, 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 compartments 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 6000 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, respectively. For nanopillars of all diameters the cell membrane is usually within 10−30 nm on average from the substrate surface. 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 nanotubes 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, 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.

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
flat surface, which agrees with previous studies. The cleft distance decreases to \(\sim 15\) 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 nano-holes 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 \(\sim 80\) GPa) and conductive polymer blend poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PEDOT, Young’s modulus \(\sim 1\) 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 \(89 \pm 52\) nm (stdv), similar to the cleft distance for the flat quartz surface at \(98 \pm 52\) 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 (\(15 \pm 2.7\) nm and \(11 \pm 4.1\) 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 multilayer 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 4b,c 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.
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 comparable in morphology to those investigated by TEM.\textsuperscript{40,41}

\section*{CONCLUSIONS}

Along six connecting lines (yellow arrowed lines corresponding to four regions of interest and green arrowed lines being the connecting lines in Figure \ref{fig:4}g), 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 \ref{fig:4}i). By multangle 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 \ref{fig:4}h). The cross section of neurite-3 is shown in Figure \ref{fig:4}j, 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 \ref{fig:4}k), comparable in morphology to those investigated by TEM.\textsuperscript{40,41}

Figure 4. FIB-SEM for sequential volumetric imaging and multangled 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.

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Method} & \textbf{Advantages} & \textbf{Disadvantages} \\
\hline
FIB-SEM & High contrast & Requires removal of substrate \\
\hline
SEM & Easy to perform & Limited depth of field \\
\hline
\end{tabular}
\caption{Comparison of FIB-SEM and SEM methods.}
\end{table}
resolution at 10 nm and is suitable to investigate the interface between the cell membrane and nonbiological materials. Our study reveals a surprising discovery that the cleft width between the cell membrane and the substrate surface is strongly influenced by the surface topology. As the cell attachment and the membrane-to-material interface strongly influence the performance of medical implants and biosensors, our study suggests that surface topology is a crucial consideration for the development of new materials and devices for biological applications. Furthermore, as the FIB-SEM method is compatible with a variety of substrate materials and topographies, we expect that this method can be used for more sophisticated in vivo studies such as examining the interfaces between osteoblast and titanium implants. We also expect this FIB-SEM method to be compatible with immunolabeling and genetically encoded EM enhancers.42

**EXPERIMENTAL PROCEDURE**

1. **Nanostructure Fabrication, Characterization, and Preparation.** Fabrication and Characterization of Quartz Nanopillars, CUIO Structures, Nanobars, and Nanotubes. Nanostructures (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 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 was 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 sonication sequence. Poly(3,4-ethylenedioxythiophene) polystyrenesulfonate (PEDOT-PSS) (Herceus, Clevios PH 1000) solution in water was doped with 5 wt % ethylene glycol (EG), 0.1 wt % dodecyl benzenesulfonic acid (DBSA) as a surfactant, and 1 wt % (3-glycidoxypropyl)trimethoxysilane (GOPTS) as a cross-linking agent to improve film stability. EG, DBSA, and GOPS were all obtained from Sigma-Aldrich. After spin-coating at 1000 rpm for 2 min the films were baked at 120 °C for 10 min.

Furthermore, the nanopillar substrates were cleaned using an oxygen plasma etch and the standard acetone 2-propanol sequence without ultrasoundication to protect the pillars. A similar PEDOT:PSS solution was spin-coated at 3000 rpm for 2 min and subsequently baked for 10 min at 120 °C to create a uniform film covering the pillars.

**Nanopores.** A 500 μm thick (100) silicon wafer was used for the e-beam writing. The sample was spin-coated with 300 nm of negative electron-sensitive resist Ma-N 2403 (MicroChem Corp.) and then baked at 100 °C for 4 min. The pattern was written using an e-beam lithography system (NanoBeam nBS) at 80 kV and was developed in Ma-D 525 developer (Microchem Corp.). A 50 nm layer of Cr metal was deposited using e-beam evaporation for mask creation. After liftoff, nanopores were created on the silicon wafer, defined by a Cr mask, and etched using an ICP-GSE200 etcher (North Microelectronics). Finally, the Cr mask was removed by concentrated hydrochloric acid.

**Silicon Grooves.** The samples were manufactured at the Molecular Foundry at Lawrence Berkeley National Laboratory under contract DE-AC02-05CH11231.

**FIB-Based Procedure.** Quartz substrates were coated with a 200 nm thick layer of platinum. Nanopores (1.5–5 μm diameter, 3–5 μm pitch) were etched by focused ion beam (dual beam Helios 600i, at 30 kV and a current of 40 pA). Afterward, the platinum layer was removed by aqua regia overnight at room temperature.

**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.
491 potassium ferrocyanide (Electron Microscopy Sciences, RO step) (1 h
492 on ice). Samples were then washed with chilled buffer (3 × 5 min),
493 and the solution was replaced with freshly prepared 1% thiocarbohy-
494 drazine (Electron Microscopy Sciences, T step) (20 min at room
495 temperature). After rinsing with buffer (2 × 5 min), the samples were
496 incubated with 2% aqueous osmium tetroxide (O step) (30 min
497 at room temperature). Cells were again rinsed (2 × 5 min) with distilled
498 water and then, finally, incubated with syringe-filtered 4% aqueous
499 uranyl acetate (Electron Microscopy Sciences, en bloc step) (overnight
500 at 4 °C). Cells were rinsed (3 × 5 min) with chilled distilled water,
501 followed by gradual dehydration in an increasing ethanol series (10%−
502 30%−50%−70%−90%−100%, 5−10 min each on ice). The last
503 exchange with a 100% ethanol solution was performed at room
504 temperature. Epoxy-based resin solution was prepared as previously
505 described,24 and samples were infiltrated with increasing concen-
506 trations of resin in 100% ethanol, using these ratios: 1:3 (3 h), 1:2 (3
507 h), 1:1 (overnight), 2:1 (3 h), 3:1 (3 h). Infiltration was carried out at
508 room temperature and in a sealed container to prevent evaporation of
509 ethanol. Samples were then infiltrated with 100% resin overnight at
510 room temperature. The excess resin removal was carried out by first
511 draining away most of the resin by mounting the sample vertically for
512 1 h and, then, rapidly rinsing with 100% ethanol prior to
513 polymerization at 60 °C overnight.

4. Scanning Electron Microscopy Imaging and Focused lon
514 Beam Sectioning. Sample Preparation. Each sample was glued with
515 colloidal silver paste (Ted Pella Inc.) to a standard stub 18 mm pin
516 mount (Ted Pella Inc.). A very thin layer of gold–palladium alloy was
517 sputtered on the sample before imaging.

SEM Imaging. Samples were loaded into the vacuum chamber of a
520 dual-beam Helios Nanolab600i FIB-SEM (FEI). For selecting a region
521 of interest, an (electron) beam with an accelerating voltage of 3−5 kV
522 and current of 21 pA to 1.4 nA was applied. For image acquisition of
523 whole cells (i.e., Figure 1b) a secondary electron detector was used.
524 For cross section imaging, a beam acceleration voltage of 2−10 kV
525 was selected, with the current ranging between 0.17 and 1.4 nA, while
526 using a backscattered electron detector (immersion mode, dynamic
527 focus disabled in cross section, stage bias zero), a dwell time of 100
528 μs, and 3072 × 2048 pixel store resolution. For the sequential sectioning,
529 the function iSPI was enabled in order to slice and acquire an image of
530 the stack every 38.5 nm with 5 kV voltage, 1.4 nA current, and 1024 ×
531 884 resolution. FIB Sectioning. Regions of interest were preserved by electron-
532 assisted deposition of a 0.5 μm double platinum layer and ion-assisted
533 deposition of a (nominal) 1 μm thick coating. First, trenches were
534 created with an etching procedure fixing an acceleration voltage of 30
535 kV and currents in the range 9.1−0.74 nA depending on the effective
536 area to remove. A fine polishing procedure of the resulting cross
537 sections was carried out on the sections, with a voltage of 30 kV and
538 lower currents in the range 0.74 nA to 80 pA so that redeposition
539 phenomena in the cross section are very limited.

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

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

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the

ACS Publications website at DOI: 10.1021/acsnano.7b03494.

Movie (AVI)

Ultrathin 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, nanopilar, and nanopore surfaces, cleft visualization for cells on complex structures, cleft distance, cleft index, CAAX/ACTIN cotransfection (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: santorof@stanford.edu.
*E-mail: bcui@stanford.edu.

ORCID

Yoeri van de Burgt: 0000-0003-3472-0148
Bianxiao Cui: 0000-0002-8044-5629

Present Addresses

Department of Mechanical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, 5612 AZ, Eindhoven, The Netherlands.
Italian Institute of Technology—Center of Advanced Biomaterials and Healthcare, 80125, Naples, Italy.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the Stanford Nano Shared Facility (SNSF) for a seed grant to a complementary use of the Helios 600i and Dr. Juliet Jamtgaard and Dr. Richard Chin for the useful discussions. The authors also acknowledge the Heart Rhythm Society for F.S.’s research fellowship, the National Science Foundation for the grants NSF 1055112 and NSF 1344302. Y.V.B was a user project at the Molecular Foundry, Lawrence Berkeley National Laboratory, all supported by the Office of Science, Office of Basic Energy Sciences, U.S. Department of Energy, under contract DE-AC02-05CH11231.

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