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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

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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
82 visualization of cells on microstructures,24,27,28 but the contrast
83 of the resulting samples is still too low to clearly resolve the
84 membrane-to-material interface at the nanoscale. To date, there
85 is no method that can reliably resolve the plasma membrane in
86 proximity to nano- and microstructures and thus to measure
87 the cleft distance between the cell membrane and the material
88 surface. Therefore, the question of how surface topology a
89 ff
90 In this work, we present a FIB-SEM method that can
91 precisely resolve the cell-to-substrate interface with 10 nm
92 resolution. At the core of our FIB-SEM method is a sample
93 preparation method based on controlled thin-resin plasticiza-
94 tion of adherent cells with heavy metal staining. Unlike the
95 usual hard drying methods, this procedure embeds cells in a
96 thin plastic layer, which not only preserves the subcellular
97 structures but also provides a solid support for the subsequent
98 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). Specifi-
cally, 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.29,30 After
fixation, the cells are treated with osmium- and uranium-based
staining series (RO-T-O procedure31,32 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.44 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-embodied HL-1 cell cultured on a quartz substrate with arrays of nanopillars, and Supplementary S1 shows resin-embodied 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.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 sponge-like structures with no discernible subcellular structures,44,27,34 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 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 fibronectin 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 conformally 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.31,32

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 nanotubes 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
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 polyl(3,4-ethylenedioxythiophene):polystyrene sulfonate (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 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 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.
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.
355 resolution at 10 nm and is suitable to investigate the interface
356 between the cell membrane and nonbiological materials. Our
357 study reveals a surprising discovery that the cleft width between
358 the cell membrane and the substrate surface is strongly
359 influenced by the surface topography. As the cell attachment and
360 the membrane-to-material interface strongly influence the
361 performance of medical implants and biosensors, our study
362 suggests that surface topology is a crucial consideration for the
363 development of new materials and devices for biological
364 applications. Furthermore, as the FIB-SEM method is
365 compatible with a variety of substrate materials and top-
366 edges, we expect that this method can be used for more
367 sophisticated in vivo studies such as examining the interfaces
368 between osteoblast and titanium implants. We also expect this
369 FIB-SEM method to be compatible with immunolabeling and
370 genetically encoded EM enhancers.  

371 EXPERIMENTAL PROCEDURE

372 1. Nanostructure Fabrication, Characterization, and Prepa-
373 ration. Fabrication and Characterization of Quartz Nanopillars,
374 CUKO Structures, Nanobars, and Nanotubes. Nanostructures (NSs)
375 used in this work were fabricated on a 4 in. quartz wafer using
376 electron-beam lithography (EBL). In brief, the wafer was diced into
377 pieces 2 cm x 2 cm square. After sonication cleaning in acetone and 2-
378 propanol, the pieces were spin-coated with 300 nm of ZEP-520
379 (ZEON Chemicals), followed by E-Spacer 300Z (Showa Denko).
380 Desired patterns were exposed by EBL (Raith130) and developed in
381 xylene. The mask was then created by sputter deposition of 100 nm Cr
382 and lift-off in acetone. NSs were generated by reactive ion etching with
383 CHF₃ and O₂ chemistry (AMT 8100 etcher, Applied Materials).
384 Before cell culture, the substrate was cleaned in O₂ plasma and
385 immersed in Chromium Etchant 1020 (Transene) to remove Cr
386 mask. SEM (FEI Nova) imaging was performed on 3 nm Cr
387 sputtered substrates to measure the dimensions of different NSs.
388 Silicon Nanocores. A monolayer polystyrene nanosphere (PS)
389 array, which consists of PSs with an average diameter of 3 μm, was self-
390 assembled on glass-based silicon substrates with the Langmuir–
391 Blodgett method. To control the effective intervals between the
392 formed silicon nanopillars, a reactive ion etching process with oxygen
393 (O₂) as an etching gas was then followed to shrink the PSs (with a
394 final diameter of 1 μm). Silicon nanocores were last formed on glass
395 substrates by introducing chlorine (Cl₂) and hydrogen bromide (HBr)
396 gases to reactive-ion-etch the silicon materials exposed to the plasma.
397 Quartz Nanopillars with PEDOT:PSS Cover Layer. Fused silica
398 glass substrates were cleaned using a standard soap, acetone, 2-
399 propanol sonication sequence. Poly(3,4-ethylenedioxythiophene)
400 polystyrenesulfonate (PEDOT:PSS) (Heraeus, Clevios PH 1000)
401 solution in water was doped with 5 wt % ethylene glycol (EG), 0.1 wt
402 % dodecyl benzzenesulfonic acid (DBSA) as a surfactant, and 1 wt %
403 (3-glycidoxypropyl)trimethoxysilane (GOPTS) as a cross-linking
404 agent to improve film stability. EG, DBSA, and GOPTS were all
405 obtained from Sigma-Aldrich. After spin-coating at 1000 rpm for 2 min
406 the films were baked at 120 °C for 10 min.
407 Furthermore, the nanopillar substrates were cleaned using an
408 oxygen plasma etch and the standard acetone 2-propanol sequence
409 without ultrasonication to protect the pillars. A similar PEDOT:PSS
410 solution was spin-coated at 3000 rpm for 2 min and subsequently
411 baked for 10 min at 120 °C to create a uniform film covering the
412 pillars.
413 Nanopores. A 500 μm thick (100) silicon wafer was used for the e-
414 beam writing. The sample was spin-coated with 300 nm of negative
415 electron-sensitive resist Ma-N 2403 (MicroChem Corp.) and then
416 baked at 100 °C for 4 min. The pattern was written using an e-beam
417 lithography system (NanoBeam nBS) at 80 kV and was developed in
418 Ma-D 525 developer (Microchem Corp.). A 50 nm layer of Cr metal
419 was deposited using e-beam evaporation for mask creation. After lift-off,
420 nanopores were created on the silicon wafer, defined by a Cr mask,
421 and etched using an ICP-GSE200 etcher (North Microelectronics). Finally, the Cr mask was removed by concentrated hydrochloric acid.
422 Silicon Grooves. The samples were manufactured at the Molecular
423 Foundry at Lawrence Berkeley National Laboratory under contract
424 DE-AC02-05CH11231.
425 FIB-Based Procedure. Quartz substrates were coated with a 200 nm
426 thick layer of platinum. Nanopores (1.5–3 μm diameter, 3–5 μm pitch) were etched by focused ion beam (dual beam Helios 600i, at 30
427 kV and a current of 40 pA). Afterward, the platinum layer was removed by aqua regia overnight at room temperature.
428 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.
429 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% polyl-lysine (Sigma Life Science) for primary neurons and HEK cell cultures or with 1 mg/ 
430 mL fibronectin (Life Technologies) in 0.02% gelatin solution for HL-1
431 cells. COS-7 cells were directly plated on the substrate after
432 sterilization.
433 2. Cell Culture and Transfection. Primary Neurons. Cortices
434 were extracted from rat embryos at embryonic day 18 and incubated
435 with 0.25% trypsin/EDTA (Corning) in a 33 nm Petri dish for 5 min
436 at 37 °C. The tissue-trypsin/EDTA solution was transferred into a 2 
437 mL plastic tube. The tissue settled at the bottom of the tube, and
438 leftover trypsin/EDTA was removed. Neurobasal media (Gibco) was
439 supplemented with 1% B27 (Gibco), 0.25% glutaMAX (Gibco), and
440 0.1% gentamycin antibiotic (Gibco). One milliliter of warm media was
441 added, and then the tube was gently swirled by hand. This procedure
442 was repeated five times, and after the last media exchange, the tissue
443 was dissociated until resulting in a cell solution. A total of 80 000 cells
444 were suspended in 3 mL and placed on each substrate. The media was
445 replaced completely 2 h after seeding time. Every second day, half of
446 the media was exchanged with freshly prepared warm (supplemented) Neurobasal media.
447 HL-1 Cells. Confluent HL-1 cells, cultured in a 33 mm Petri dish,
448 were incubated with 1 mL of 0.25% trypsin/EDTA for 5 min at 37 °C. 
449 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 (Sigma Life Science), 0.1 mM norepinephrine (Sigma-Aldrich), and 2 mL glutaMAX were added. The cell solution was placed in a 6 cm dish 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 plated on each substrate in addition to 3 mL of supplemented media.
446 HEK 293 Cells. HEK 293 expressing channels NaV 1.3 and KIR 2.1 were acquired by Adam Cohen laboratory and maintained in DMEM/ 
447 F12 (Gibco), 10% PBS (Gibco), 1% penicillin/streptomycin (100 μg/ 
448 mL, Gibco), Genetin (500 μg/mL, Gibco), and puromycin (2 μg/ 
449 mL, Fisher Scientific). At 80% confluency, cells were divided, 
450 resuspended, and plated on quartz substrates as for HL-1 cells.
451 COS-7 and U2OS Cells. Cells were maintained in DMEM 
452 supplemented with 10% fetal bovine serum, and at 90% confluence 
453 they were divided as for HL-1 cells and plated on the substrates. 
454 CAAx/LifeAct Transfection. U2OS cells were cultured in DMEM 
455 medium (HyClone) with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invivogen) at 37 °C in 5% CO₂. Transfection was preformed using electroporation (Amaza Nucleofector) with the manufacturer’s protocol. U2OS cells were transfected with Lifeact-RFP (transformed bacteria acquired from AddGene and Caa-
456 x GFP and plated on a nanostructured surface for at least 16 h before 
457 examination.

458 3. Ultrasound Plasticization and RO-T-O Procedure. Substrates 
459 with cells were rinsed with 0.1 M sodium cacodylate buffer (Electron 
460 Microscopy Sciences) and fixed with 3.2% glutaraldehyde (Sigma-
461 Aldrich) at 4 °C overnight. Specimens were then washed (3 × 5 min) 
462 with chilled buffer and quenched with chilled 20 mM glycine solution 
463 (20 min). After rinsing (3 × 5 min) with chilled buffer specimens were 
464 postfixed with equal volumes of 4% osmium tetroxide and 1%
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% thio-carbohy- 494 draze (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 at 497 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,44 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 Ion Beam Sectioning. Sample Preparation. Each sample was glued with 516 colloidal silver paste (Ted Pella Inc.) to a standard stub 18 mm pin 517 mount (Ted Pella Inc.). A very thin layer of gold−palladium alloy was 518 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 μs, 528 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− 533 assisted deposition of a 0.5 μm double platinum layer and ion-assisted 534 deposition of a (nominal) 1 μm thick coating. First, trenches were 535 created with an etching procedure fixing an acceleration voltage of 30 536 kV and currents in the range of 9.1−0.74 nA depending on the effective 537 area to remove. A fine polishing procedure of the resulting cross 538 sections was carried out on the sections, with a voltage of 30 kV and 539 lower currents in the range of 0.74 nA to 80 pA so that redeposition 540 phenomena in the cross section are very limited.

Image Analysis and 3D Reconstruction. All images were 541 preprocessed with ImageJ (National Institutes of Health, USA, 542 http://imagej.nih.gov/ij/). The images of the sequential cross sections 543 shown in Figure 2 were collected as a stack, analyzed, and processed 544 with an open source tool chain based on Python (Python Software 545 Foundation, USA, http://www.python.org) scripts and tools. The 546 image stack was cropped, filtered, and down-sampled. The isotropic 547 resolution in x, y, and z amounts to 38.5 nm. The reconstructed data 548 are visualized with Blender (Blender Foundation, The Netherlands, 549 http://www.blender.org).

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.


