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Single-Molecule Imaging of Cell Surfaces Using Near-Field Nanoscopy

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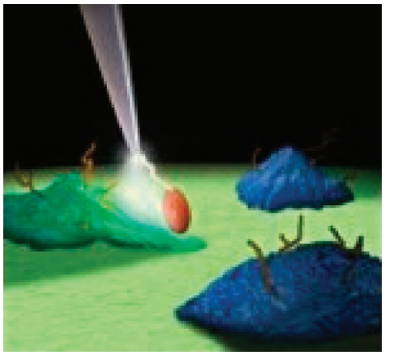
CONSPECTUS

Living cells use surface molecules such as receptors and sensors to acquire information about and to respond to their environments. The cell surface machinery regulates many essential cellular processes, including cell adhesion, tissue development, cellular communication, inflammation, tumor metastasis, and microbial infection. These events often involve multimolecular interactions occurring on a nanometer scale and at very high molecular concentrations. Therefore, understanding how single-molecules localize, assemble, and interact on the surface of living cells is an important challenge and a difficult one to address because of the lack of high-resolution single-molecule imaging techniques. In this Account, we show that atomic force microscopy (AFM) and near-field scanning optical microscopy (NSOM) provide unprecedented possibilities for mapping the distribution of single molecules on the surfaces of cells with nanometer spatial resolution, thereby shedding new light on their highly sophisticated functions.



For single-molecule recognition imaging by AFM, researchers label the tip with specific antibodies or ligands and detect molecular recognition signals on the cell surface using either adhesion force or dynamic recognition force mapping. In single-molecule NSOM, the tip is replaced by an optical fiber with a nanoscale aperture. As a result, topographic and optical images are simultaneously generated, revealing the spatial distribution of fluorescently labeled molecules.

Recently, researchers have made remarkable progress in the application of near-field nanoscopy to image the distribution of cell surface molecules. Those results have led to key breakthroughs: deciphering the nanoscale architecture of bacterial cell walls; understanding how cells assemble surface receptors into nanodomains and modulate their functional state; and understanding how different components of the cell membrane (lipids, proteins) assemble and communicate to confer efficient functional responses upon cell activation.

We anticipate that the next steps in the evolution of single-molecule near-field nanoscopy will involve combining single-molecule imaging with single-molecule force spectroscopy to simultaneously measure the localization, elasticity, and interactions of cell surface molecules. In addition, progress in high-speed AFM should allow researchers to image single cell surface molecules at unprecedented temporal resolution. In parallel, exciting advances in the fields of photonic antennas and plasmonics may soon find applications in cell biology, enabling true nanoimaging and nanospectroscopy of individual molecules in living cells.

1. Introduction

Cellular heterogeneity is a key feature of living systems. At the level of individual cells, cell surfaces are highly heterogeneous systems, whose components are continuously reorganized to achieve specific functions. In higher eukaryotes, a key example of such heterogeneity is the spatiotemporal confinement of proteins and lipids in defined micro- and nanometer-scale regions of the cell membrane.[1,2] Transient associations between lipids and proteins within microdomains can have direct impact on the biological function of

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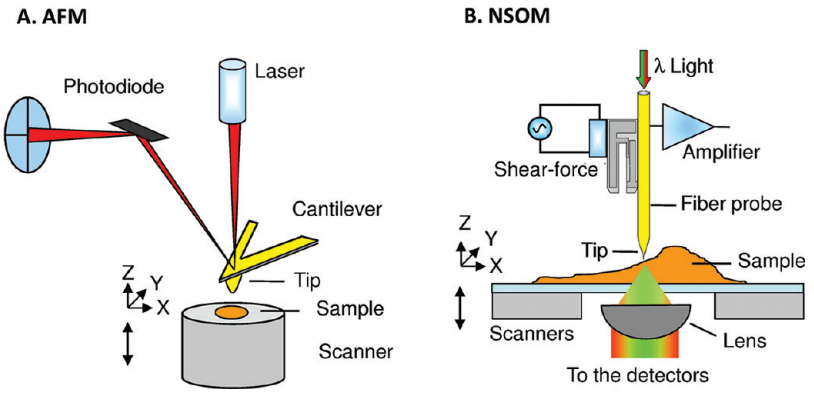

FIGURE 1. General principles of atomic force microscopy (AFM) and near-field scanning optical microscopy (NSOM). (A) AFM imaging is performed by scanning a very sharp tip across the sample surface while the force of interaction between tip and sample is monitored with piconewton sensitivity. Recognition imaging implies labeling the tip with specific antibodies or ligands and detecting molecular recognition signals while mapping the surface (see text for details). (B) In NSOM, the tip is replaced by an optical fiber with nanoscale aperture to simultaneously generate topographic and optical images revealing the spatial distribution of fluorescently labeled molecules (see text for details).

these molecules and therefore in cellular processes such as cell adhesion, signaling, antigen presentation and cell–cell interactions.[3] Yet, the mechanisms orchestrating the formation of membrane domains in eukaryotes remain enigmatic, and are thought to include protein–protein interactions, and cytoskeletal or lipid raft associations.[1,4–6] In particular, membrane microdomains enriched in sphingolipids and sterols, known as "lipid rafts" have been postulated to favor segregation of specific membrane proteins like receptors and glycosylphosphatidylinositol anchored proteins (GPI-APs) and implicated in various biological phenomena, ranging from cell adhesion, pathogen binding, endocytosis, and immune cell signaling. However, because the putative size of individual lipid rafts in higher eukaryotes is estimated to be in the 20–100 nm range and to be transient, their direct visualization in live cells remains very challenging.

In microbial cells, there is also evidence that the plasma membrane and cell wall are organized and heterogeneous, and that this heterogeneity is important for function. In bacteria, flagella and extracellular components can localize asymmetrically on the cell surface and a number of proteins are localized to the cell poles for specific functions. In yeasts, protein microdomains have been observed within the plasma membrane by the use of specific GFP-labeled marker proteins. Although such protein domains can be observed in yeast membranes using fluorescence microscopy, the distribution and assembly of single proteins within the domains cannot be investigated. Consequently, there is an urgent need for high-resolution imaging tools capable of directly imaging and localizing single molecules on eukaryotic and prokaryotic cell surfaces.

2. Near-Field Nanoscopy: Using Force or Photons?

The advent of scanning probe microscopes (or "near-field nanoscopy") in the 1980s has provided new opportunities for imaging living cells on the nanoscale. Atomic force microscopy (AFM) has proved particularly useful for observing the shape, structure, and organization of mammalian and microbial cells in buffer solution and in real-time.[7,8] AFM works by raster scanning a sharp tip over a biological specimen to contour its surface (Figure 1A). At every location of the specimen surface, the tip locally senses forces that are used for feedback control of the raster scanning. A piezoelectric scanner allows high-resolution, three-dimensional positioning (1 Å) of the tip. The latter is attached to a soft cantilever that deflects and quantifies the force. Cantilever deflection is detected by a laser beam reflected from the free end of the cantilever into a photodiode.

The conventional AFM topographic imaging mode suffers from a major drawback, that is, its lack of biochemical specificity. Therefore, single molecules cannot be identified and localized on live cells, which is a major flaw since organization, assembly, and interactions of the cell surface machinery tightly control its functions. To solve this problem, a first approach is to conjugate the AFM tip with specific antibodies or ligands,[9] thereby enabling detection and localization of single molecular recognition sites. Today, such single-molecule recognition imaging is performed in two ways, adhesion force mapping or dynamic recognition force mapping. In the first modality, molecular recognition sites are mapped using spatially resolved force spectroscopy with modified tips.[10,11] The adhesion (unbinding) force value is estimated for each curve and then displayed as gray

or colored pixels, where the brightness reflects the magnitude of the force. While force mapping provides quantitative analysis of unbinding forces, it is limited by its time resolution (~10−30 min per image), much slower than the time scale at which dynamic processes usually occur in biology.

In dynamic recognition force mapping,[12] topography and recognition images are recorded at the same speed as that used for conventional topographic imaging (0.5−1 min per image). Molecular recognition signals are detected during dynamic force microscopy imaging. To this end, AFM tips carrying ligands are oscillated at very small (5−10 nm) amplitudes while being scanned along the surface to which the cognate receptors are bound. Topography and recognition images are simultaneously obtained (TREC imaging) using an electronic circuit.[13,14] Maxima (U_{up}) and minima (U_{down}) of each sinusoidal cantilever deflection period are depicted and fed into the AFM controller, with U_{down} driving the feedback loop to record the height (i.e., topography) image and U_{up} providing the data for constructing the recognition image.[15]

Near-field scanning optical microscopy (NSOM) is another scanning probe technique that brings more versatile biochemical specificity.[16−18] As in AFM, a sharp probe physically scans the sample surface to generate a topographic image with similar resolution and sensitivity (Figure 1B).[18] However, in NSOM the probe is generally replaced by an optical fiber to simultaneously produce optical images. The probe consists of a small aperture (20−100 nm in diameter) at the end of a metal-coated tapered optical fiber. The lateral resolution, down to tens of nanometers, is given by the size of the aperture and the sample-to-probe distance. The probe illuminates the sample with an evanescent field that is strongly localized at the vicinity of the aperture and decreases very rapidly away from the probe's end face. To keep the probe in the near-field region of the sample (<10 nm), an additional feedback loop is required. This is commonly achieved using a shear-force feedback, which in addition generates a topographic image of the sample in perfect registry with the optical image. Owing to the exponentially decaying character of the illumination field, NSOM is a surface sensitive technique, thus ideally suited for probing cell membranes.[19−23] In addition, the small excitation volume (10^5 nm^3 vs 10^8 nm^3 as obtained in confocal microscopy) reduces dramatically the cytoplasm background fluorescence, enabling single-molecule detection on biological membranes with high signal-to-background ratios.[17,19−22] An important asset of NSOM over AFM is its ability to colocalize multiple molecules simultaneously, using multicolor excitation through the same probe.

3. AFM Unravels the Spatial Organization of Bacterial Cell Walls

Since the pioneering work of Ikai[10] and Gaub[11] in 1997, adhesion force mapping by AFM has been established as a powerful tool for analyzing the distribution of individual molecules on biosurfaces. In recent years, much progress has been made in using the method for imaging the spatial organization of bacterial cell walls. Unlike animal cells, bacteria possess a thick cell wall that fulfils important functions, like controlling cell division and cell adhesion. Today, the structure and biosynthesis of cell wall constituents are well-understood, but how exactly these constituents are organized at the molecular level remains mysterious. In the pathogenesis context, AFM force mapping could visualize the distribution of mycobacterial heparin-binding proteins engaged in host−microbe interactions.[24] Recognition maps obtained on live mycobacteria revealed that the adhesion proteins concentrated into nanodomains, presumably promoting the recruitment of receptors in host cells.

The spatial organization of peptidoglycan, the major constituent of bacterial cell walls, is an important, yet unsolved issue. AFM topographic imaging and adhesion force mapping were used to unravel the nanoscale organization of peptidoglycan in living *Lactococcus lactis* bacteria (Figure 2).[8] While wild-type cells displayed a featureless surface morphology, mutant cells lacking cell wall exopolysaccharides featured 25 nm-wide periodic bands running parallel to the short axis of the cell (Figure 2A). Single-molecule imaging showed that these parallel bands consist of peptidoglycan (Figure 2B). This correlation between structural and recognition images provided direct evidence that peptidoglycan localizes in the form of parallel cables in *L. lactis* (Figure 2C), thus supporting the classical model of peptidoglycan assembly. In another work, AFM tips bearing the antibiotic vancomycin were used to localize single D-Ala-D-Ala peptidoglycan binding sites, revealing that they were essentially located at the cell division site where newly formed peptidoglycan is inserted.[25] Because vancomycin inhibits the growth of pathogens like *Staphylococcus aureus*, this single-molecule approach offers promising perspectives in drug research.

Teichoic acids, another class of cell wall constituents, were recently imaged using fluorescence and AFM imaging to decipher the relationships between their spatial localization and functional roles.[26] AFM topographic images of living *Lactobacillus plantarum* bacteria featured a highly polarized surface morphology, the poles being much smoother than the side walls. Recognition imaging with lectin AFM tips

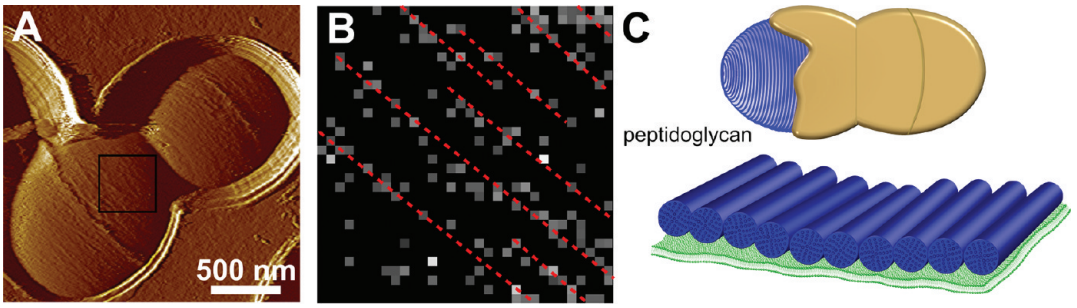

FIGURE 2. Imaging peptidoglycan in living bacteria using single-molecule AFM. (A) Topographic image of two dividing *Lactococcus lactis* cells lacking cell wall exopolysaccharides. (B) Adhesion force map (400 nm × 400 nm) recorded with a LysM probe in the square area shown in the topographic image; peptidoglycan molecules were detected (bright pixels) and found to be arranged as lines running parallel to the short cell axis (red lines). (C) Schematic views of the architecture of the *L. lactis* cell wall: the top cartoon emphasizes the two layers of the cell wall, that is, periodic bands of peptidoglycan (blue) covered by cell wall polysaccharides (brown), while the bottom cartoon is an enlarged view of the peptidoglycan nanocables (blue) lying on the membrane (green). Reprinted with permission from ref 8. Copyright 2010 Nature Publishing Group.

demonstrated that the polarized surface structure correlates with a heterogeneous distribution of teichoic acids, the latter being absent from the surface of the poles. This polarized distribution was found to be important for cell morphogenesis.

4. Discovery of the Yeast Nanoadhesome and Nanosensosome

Understanding how surface proteins dynamically assemble into nanodomains and how such domains reorganize in response to environmental stimuli is a hot topic in current biology. Adhesion force mapping has demonstrated that functional protein domains on yeast cells are able to grow under stress and that this response is important for adhesion and mechanosensing.[27,28] Here, a key feature of AFM was exploited, the ability not only to localize single proteins but also to subject them to force in order to gain insight into their adhesive and mechanical properties.

Adhesion of the pathogen *Candida albicans* to host cells is mediated by a family of cell adhesion proteins known as the agglutinin-like sequence (Als) proteins via molecular interactions that remain largely unknown. Stretching single Als proteins on yeast cells with a modified AFM tip revealed sawtooth patterns with multiple force peaks, each peak corresponding to the force-induced unfolding of individual tandem repeats engaged in cell adhesion.[29] The unfolding probability increased with the number of tandem repeats expressed by the cells and correlated with the level of cell adhesion, suggesting that these modular domains play a role in fungal adhesion. Presumably, the force-induced unfolding of Als proteins leads to extended conformations in which hydrophobic groups are freshly exposed, thus favoring hydrophobic interactions between opposing cells. Combining single-molecule manipulation and imaging enabled tracking of the formation of adhesion nanodomains in response to force.[27] Stretching single Als induced the formation of adhesion domains of 100−500 nm size, referred to nanoadhesomes (Figure 3). Unexpectedly, nanodomains propagated at a speed of 20 nm/min across the entire cell surface. These data indicate that clustering of cell adhesion proteins in response to mechanical stimuli may be a general mechanism for activating cell adhesion in microbial pathogens.

Mechanosensing is another cellular event for which protein mechanics and clustering are crucial. Dupres and colleagues stretched individual Wsc1 sensors on living yeast cells, revealing that they behave like nanosprings capable of resisting high mechanical force and of responding to cell surface stress.[30] In addition, individual sensors were found to form nanoclusters, a process strongly enhanced in stress conditions.[28] These findings show that signaling in yeasts is coupled to the localized enrichment of sensors within membrane patches, for which the term "nanosensosomes" has been proposed.

5. Dynamic Recognition Imaging of Protein Nanodomains in Mammalian Cell Membranes

Mammalian cell membranes represent systems of complex composition, organization, and processing in space and time, containing functional domains enriched in specific transmembrane proteins. Therefore, the application of dynamic recognition force mapping, also known as TREC, to image such membrane domains constitutes a challenge. The first pioneering studies were performed on microvascular endothelial cells from mouse myocardium (MyEnd), with the aim to locally identify vascular endothelial (VE)−cadherin binding sites and assign their positions with membrane topographical features (Figure 4).[31] VE−cadherin is located at intercellular junctions of essentially all types of the

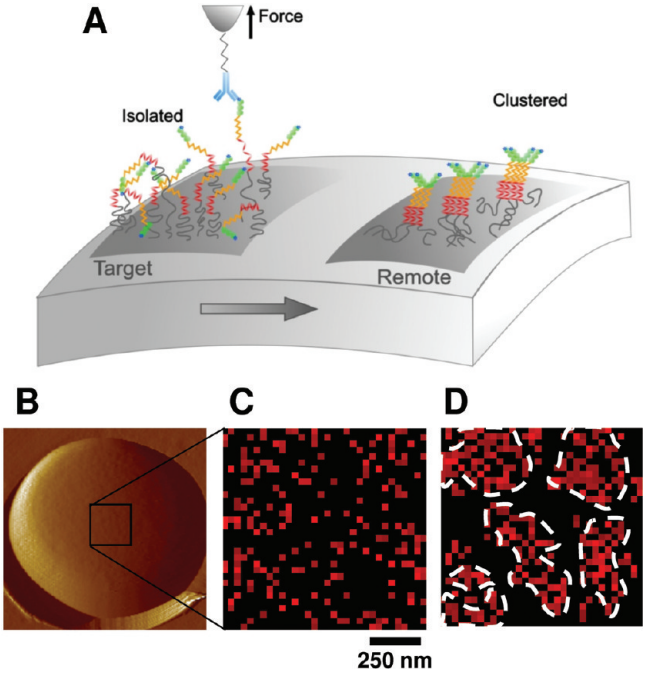

FIGURE 3. Single-molecule AFM imaging unravels the dynamic clustering of cell adhesion proteins on yeast cells. (A) Single Als proteins from the pathogen *Candida albicans* were localized and stretched using an AFM tip bearing specific antibodies. (B) AFM topographic image of a single live cell. (C) Adhesion force map recorded on a cell that was never subjected to force. Red pixels document the detection of single proteins. Most proteins were isolated and evenly distributed, without any clear evidence for clustering. (D) Subsequent map recorded on the same but mechanically stimulated cell. Unlike native cells, cells that had been preactivated by force displayed adhesion nanodomains referred to as "nanoadhesomes" (see drawing in panel A). Reprinted with permission from ref 27. Copyright 2010 National Academy of Sciences.

endothelium, where VE−cadherin molecules are clustered and linked through their cytoplasmic domain to the actin cytoskeleton. The cadherin cis-dimer, which is formed by association of two extracellular domains in physiological Ca^{2+}-concentration (1.8 mM), represents the basic structural functional unit to promote a homophilic bond between cells.[32]

TREC measurements were performed on cell surfaces with oscillating AFM tips functionalized with a recombinant VE−cadherin−Fc *cis*-dimer via a soft and long (~8 nm) PEG-linker.[31] Recognition signals correspond to amplitude reduction due to a binding between VE−cadherin molecules on the AFM tip and the cell surface when specific *trans*-interaction occurs (seen as dark red spots in the recognition maps) (Figure 4A′,B′). Cells in the early subconfluent state (1−2 days after seeding) were characterized by a nonuniform distribution of recognition spots with a linear size of 10−100 nm.[31] The shapes and the positions of VE−cadherin domains were correlated with topographical features of MyEnd cell surfaces (compare Figure 4A,A′). The topography of a scanned MyEnd cell surface area represents a complex picture of linear and branched filamentous structures, likely representing filaments of the peripheral actin belt and some globular features as well (Figure 4A). Interestingly, a few VE−cadherin domains were found directly on top of filaments. Nevertheless, most domains were located near and between filaments, indicating that at this stage of cell maturation, clustering of VE−cadherin was incomplete. A closer look at these "hot" spots revealed that they consist of 1−2 rather large domains with the size of 50−80 nm in length, surrounded by 10-20 smaller domains (10−20 nm) or even single molecule spots (Figure 4A″). By contrast, the recognition maps taken on cells treated with nocodazole[33] contained again "hot" pronounced spots but consisted of one big domain of ellipsoidal form (~80 nm or ~180 nm) and a few small domains (10−20 nm) (Figure 4B″). In addition, the number of single molecule spots significantly decreased. Such spots with dimensions of ~8−16 nm (1−4 pixels long, 1 pixel = 4 nm) were attributed to single active VE−cadherin *cis*-dimers, taking into account the size of the *cis*-dimer (diameter ~3 nm) and the free orientation of PEG-linker (~5 nm) leading to binding even before/after the *cis*-dimer position.

TREC has also been introduced as a novel approach to investigate the properties of voltage-gated channels in cells and exploited to locally identify extracellular binding sites of hERG K^+ channels on gently fixed hERG HEK-293 cells.[34] In the medical context, TREC has been used to determine the distribution and the organization of cystic fibrosis transmembrane conductance regulators (CFTRs) in healthy subjects and cystic fibrosis patients.[35]

6. Imaging Recognition Epitopes to Molecular Resolution

What is the highest resolution achievable by TREC? Recognition images obtained on bacterial surface layers (S-layers) have shown a lateral resolution of 1 nm, impressively demonstrating the precision of the technique (Figure 5).[36] S-layers represent a self-assembling system that has been optimized over billions of years of biological evolution. This allows generation of well-defined surface coatings containing high-affinity tags (such as Streptag-II) so as to introduce single reactive binding sites for a functional and addressable nanoarray. For TREC experiments, cocrystallized S-layer proteins of SbpA−Streptag-II with wild-type SbpA proteins were used,[36] resulting in an adjustable density of recognition sites. In the topography image (Figure 5A), the 2D-crystalline

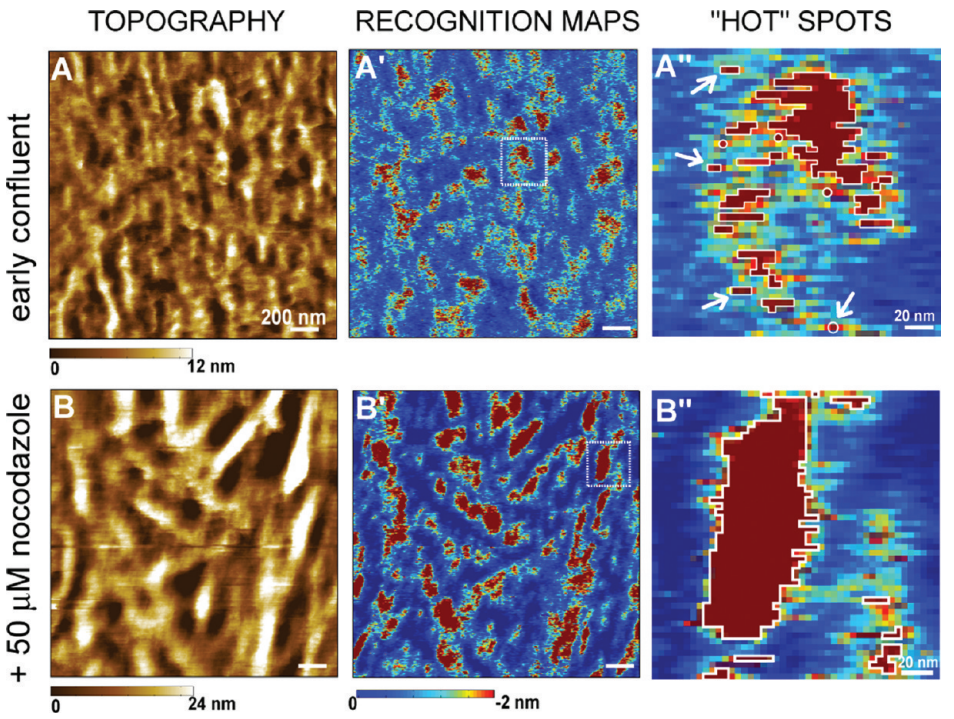

FIGURE 4. Dynamic recognition imaging (TREC) on MyEnd cells either in the early state of cell maturation (upper A panels) or treated with nocodazole (lower B panels). Simultaneously recorded topography (A, B) and recognition (A′, B′) images on MyEnd cells acquired with VE−cadherin−Fc functionalized AFM tip. The reduction of the oscillation amplitude was used as a recognition signal. (A″, B″) Examples of typical spots enriched in active VE−cadherin *cis*-dimers. The domains were magnified from the corresponding recognition maps (A′ and B′). VE−cadherin domains were bordered by white lines. Single VE−cadherin *cis*-dimers can be easy detected (arrows in A″). Reprinted with permission from refs 31 and 33. Copyright 2007 Elsevier and Copyright 2010 John Wiley & Sons, Ltd.

surface layer of p4-symmetry with a center-to-center spacing of 14 nm is shown. In the simultaneously acquired recognition image (Figure 5B), the tip-bound ligand (Strept-Tactin) on the AFM tip recognizes only the S-layer proteins fused with the Streptag-II. Thus the dark spots in the recognition image indicate the location of the recognition events between Streptag-II and Strept-Tactin and obviously reflect the distribution of the fusion protein rSbpA−Streptag-II in the cocrystallized S-layer lattice of rSbpA−Streptag-II and wild-type SbpA (molar ratio of 1:7). For the investigation of the obtainable limit of the lateral resolution of TREC imaging a smaller scan area was chosen. This allowed precise localization of the Streptag-II position at the grid using a "center of mass" approach with 1 nm accuracy demonstrating the lateral precision achievable in recognition imaging. The overlay of the topographical image (Figure 5A) with the recognition spots of the simultaneously acquired recognition image (Figure 5B) showed that 88% of the Streptag-II positioned on the corner of the square lattice (Figure 5C; red spots). The positions of the affinity tag of a mixed (wild-type and Streptag-II modified) SbpA S-layer crystal are indicated in Figure 5D. This study demonstrates the tremendous potential of TREC for imaging the localization of single cell surface epitopes at nanometer resolution.

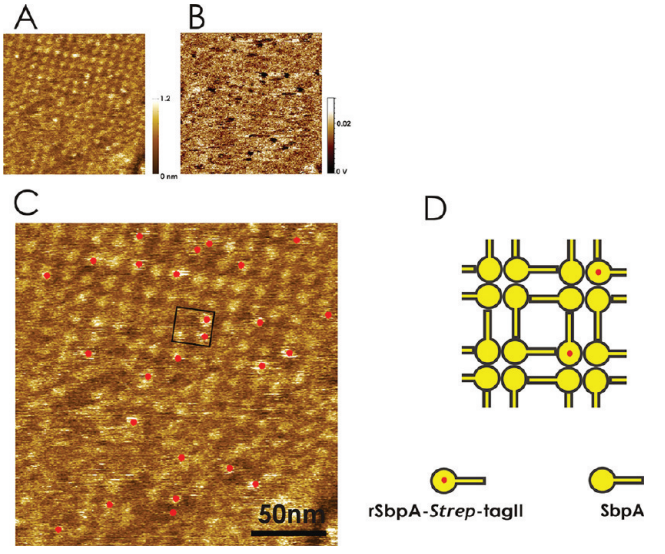

FIGURE 5. TREC imaging localizes surface layer proteins to a resolution of 1 nm. (A) Topography and (B) recognition images of a S-layer protein crystal (cocrystallized proteins wild-type SbpA and rSbpA−Streptag-II in a molar ratio of 7:1). The superimposition (C) allows direct localization of the recognition sites on the topography as schematically drawn in panel D. Reprinted with permission from ref 36. Copyright 2008 American Chemcial Society.

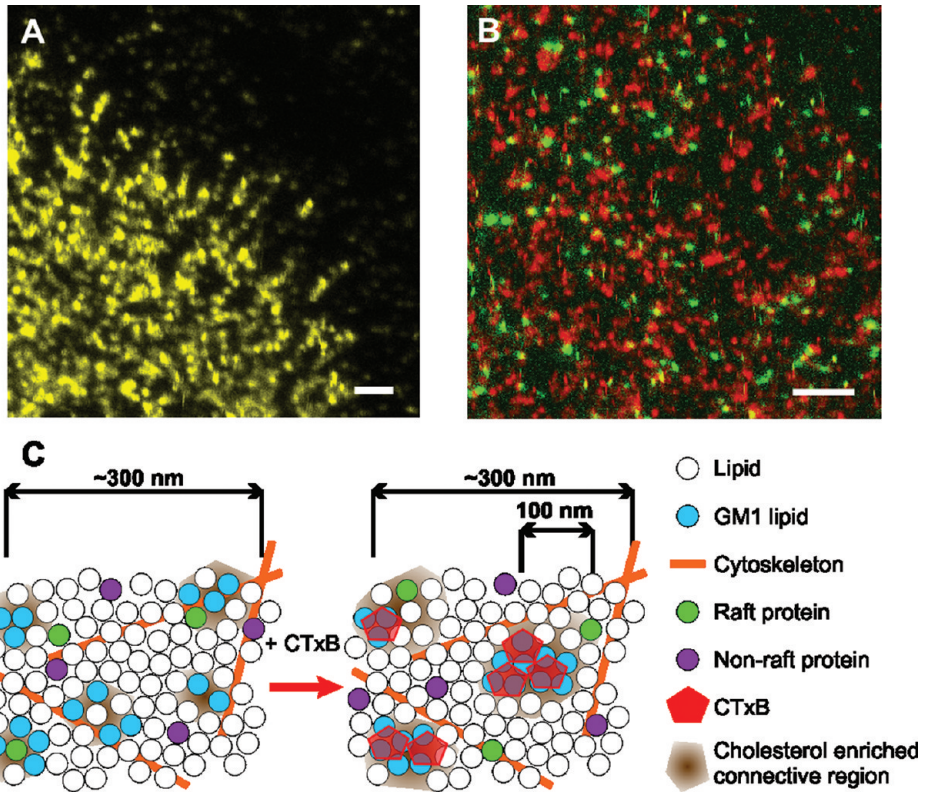

FIGURE 6. Imaging nanoscale compositional connectivity with NSOM. (A) Representative NSOM fluorescence image of CTxB−GM1 nanodomains at the cell surface of a fixed monocyte in liquid conditions. (B) Dual color NSOM image of the GPI-anchored protein CD55 (green) and CTxB−GM1 nanodomains (red). Although CD55 does not colocalize with GM1 nanodomains (lack of yellow spots), both components organize proximal to each other. Proximity can be thoroughly quantified by determining the nearest neighbor interdomain distances between red and green spots and contrasting the experimental results with a similar analysis performed on simulated images of random organization.[22] Scale bars: 1 μm. (C) Schematic representation of underlying nanoscale connectivity on fully intact cell membranes. Distribution of raft-like and non-raft-like lipids and proteins in the unperturbed state (left). Upon GM1 tightening by CTxB, a connective region of ~100 nm is formed, with raft-like components being selectively recruited (right). Reprinted and adapted with permission from ref 22. Copyright 2010 National Academy of Sciences.

7. Visualizing the Spatial Organization of Lipids Rafts Using NSOM

The feasibility of using NSOM to image nanoscale features in mammalian cells was already demonstrated in 1992 by the pioneering work of Betzig and Trautman.[18] A few years later, Enderle et al. used for the first time dual-color NSOM to directly measure the association of a host and parasite proteins in malaria (*Plasmodium falciparum*) infected erythrocytes.[37] Since then NSOM has been applied to the study of many different receptors and lipids on mammalian membranes. In the lipid rafts context, single-molecule NSOM has been recently used to visualize the nanolandscape of ganglioside GM1 after tightening by its ligand cholera toxin (CTxB) on intact fixed monocytes.[22] CTxB tightening of GM1 was sufficient to initiate a minimal raft coalescence unit, resulting in the formation of cholesterol-dependent GM1 nanodomains smaller than 120 nm in size (Figure 6A). These CTxB−GM1 nanodomains were further capable of recruiting certain types of transmembrane and GPI-APs without physical intermixing (Figure 6B), but not the transferring receptor CD71, a classical nonraft marker. These results demonstrated the existence of raft-based compositional connectivity at the nanometer scale crucially mediated by cholesterol. The data further suggested that such connective condition on resting membranes constitute an obligatory step toward the hierarchical evolution of large-scale raft coalescence upon cell activation (Figure 6C).

The recruitment of specific receptors to lipid raft regions has been also studied using near-field nanoscopy. For instance, single-molecule NSOM has been recently exploited to capture the spatiofunctional relationship between the integrin receptor LFA-1 involved in leukocyte adhesion and raft components (GPI-APs).[21] While LFA-1 formed nanoclusters of ~85 nm in size on resting monocytes, ~70% of the GPI-APs organized as monomers, and the remaining 30% formed small oligomers containing two to four molecules. Surprisingly, whereas GPI-AP monomers distributed

randomly on the cell surface, the oligomers resided in regions proximal to each other (within ~250 nm). In the resting state, that is, prior to LFA-1 activation, ~50% of the GPI-AP oligomers were found close to LFA-1 nanoclusters, whereas GPI-AP monomers exhibited no particular spatial correlation with respect to LFA-1. Interestingly, ligand-mediated LFA-1 activation not only resulted in a spatial interlocking of the integrin and GPI-APs generating nascent adhesion sites but importantly resulted in an interconversion from monomers to nanodomains of GPI-APs. These data demonstrated the existence of nanoplatforms composed by integrins and rafts as essential intermediates in nascent cell adhesion.

Dual color NSOM has also been used to investigate the association of β-adrenergic receptors (β-AR) and caveolae on the surface of cardiac myocytes.[23] Caveolae are small (~ 60 nm) flask-shaped invaginations of the plasma membrane and constitute a special type of lipid raft enriched in proteins, cholesterol, and sphingolipids. The study showed that ~15−20% β_2AR clusters colocalized in caveolae while the rest appeared proximal to it, indicating that β_2AR clusters are preassembled in or near caveolae.[23]

8. Mapping Protein Nanoclustering and Multimolecular Interactions in Cell Membranes

In addition to lipid rafts, other mechanisms are responsible for orchestrating the organization of the cell surface at different spatial scales. In particular, homophilic protein−protein interactions could also lead to the formation of protein nanoclusters. One example is the receptor DC-SIGN, a transmembrane tetrameric protein expressed on antigen presenting cells and involved in the recognition of several pathogens.[38] Single molecule NSOM showed that ~80% of the receptors form clusters of 185 nm in size on the membrane of immature dendritic cells (DC) (Figure 7).[19] Interestingly, these nanoclusters showed a remarkable heterogeneity in their packing density, suggesting that this particular arrangement might serve to maximize binding strength to a large variety of viruses and pathogens having different binding affinities to DC-SIGN.

At a different level of hierarchical organization, interactions between clusters of different proteins have been also predicted and resolved using simultaneous dual color excitation/detection NSOM.[20] As single receptors, IL2R and IL15R (two members of the interleukin family expressed in human T lymphoma cells) did not spatially colocalize and randomly scattered on the cell membrane. However, in their clustered form, both receptors significantly colocalized in the same nanocompartments.

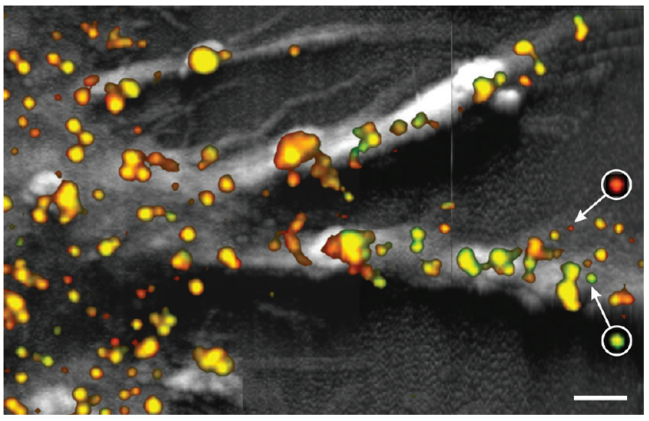

FIGURE 7. Simultaneous topographic (gray) and near-field fluorescence image (color) of DC-SIGN expressed on immature DCs. The finger-like extensions on the topographic image are characteristic of DCs. Spots of different sizes and intensities reflect the organization of DC-SIGN in nanoclusters. Arrows point to individual molecules that have been enlarged to facilitate their visualization. Scale bar: 1 μm. Reprinted and adapted with permission from ref 19. Copyright 2007 Wiley-VCH.

Recent technical developments in near-field optical nanoscopy aiming at increasing the spatial resolution to a few tens of nanometers exploit the concept of nanoantennas to enhance and localize the electric field at the end of a tiny antenna.[39] The technique has been recently applied to image single Ca^{2+} channels on erythrocyte plasma membranes and LFA-1 organization of monocytes at 50 nm and 30 nm optical resolutions, respectively.[40,41] In the near future, these exciting experiments will undoubtedly contribute to increase our understanding of many biological processes occurring at cell surfaces, where spatiotemporal interactions of individual molecules at the nanometer scale play a crucial role for cellular function.

9. Conclusions

Understanding how individual constituents of cell surfaces localize and assemble is a crucial question since the spatial organization and interactions of the cell surface machineries tightly control their functions. Conventional AFM techniques are not suited to address this issue because they lack biochemical specificity. By contrast, we have shown here that recognition imaging by AFM and NSOM offer unprecedented possibilities for imaging the distribution of single molecules on cell surfaces, providing new insight into the highly sophisticated functions of the cell surface. It is worth mentioning that far-field optical techniques have recently enabled breaking the diffraction limit barrier of light microscopy,

having also great potential for probing the nanoscale localization of biomolecules on cell surfaces. In contrast to these newly developed techniques, NSOM allows truly quantitative nanoscale imaging and correlated topography or force information and enables researchers to colocalize multiple molecules at once using multicolor excitation and detection. In addition, single-molecule AFM force spectroscopy provides a means to measure not only the localization, but also the intra- and intermolecular interactions of cell surface molecules.

An important technological challenge remaining to be addressed is to improve the temporal resolution of AFM and NSOM imaging, which is currently much slower than the time scale at which dynamic processes usually occur in biology. Interestingly, remarkable advances have been made in developing high-speed instruments, providing access to unprecedented time scales (millisecond resolution). On the one hand, high-speed AFM imaging opens up fascinating new perspectives to explore membrane dynamics and to elucidate how membrane molecules function. For instance, dynamic molecular processes induced by light could recently be observed in photoactivated bacteriorhodopsin within 1 s.[42] On the other hand, NSOM can now be combined with other methods, such as fluorescence correlation spectroscopy (FCS), to record dynamic events in living cells with submillisecond time resolution in ultraconfined volumes.[43]

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FOOTNOTES

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