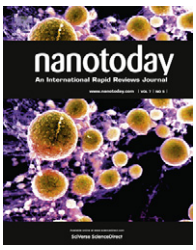
Nano Today (2012) 7, 390—403

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/nanotoday



REVIEW

Recent progress in cell surface nanoscopy: Light and force in the near-field

Yves F. Dufrêne[a,*,1], Maria F. Garcia-Parajo[b,*,1]

[a] *Institute of Condensed Matter and Nanosciences, Université catholique de Louvain, Croix du Sud 2/18, B-1348 Louvain-la-Neuve, Belgium*
[b] *ICFO-Institut de Ciencies Fotoniques, Mediterranean Technology Park, 08860 Castelldefels (Barcelona), Spain, and ICREA-Institució Catalana de Recerca i Estudis Avançats, 08010 Barcelona, Spain*

Received 3 May 2012; received in revised form 2 July 2012; accepted 6 August 2012
Available online 6 September 2012

KEYWORDS
Atomic force microscopy;
Cell surfaces;
Fluorescence correlation spectroscopy;
Force spectroscopy;
Near-field scanning optical microscopy;
Single-molecule localization

Summary A hot topic in current cell biology is to understand the specific nanometer-scale organization and distribution of the surface machinery of living cells and the role these molecular properties play in the spatiotemporal control of different cellular processes. In the past years, near-field nanoscopy — using near-field scanning optical microscopy (NSOM) and atomic force microscopy (AFM) — has enabled key breakthroughs in this fast moving area. These two highly-sensitive surface scanning probe techniques go far beyond nano-imaging, by enabling researchers to localize and manipulate individual molecules on cell surfaces with nanoscale spatial resolution. Specifically, nanophotonic approaches and dynamic measurements in ultra-confined volumes allow nowadays the visualization of dynamic processes on cell membranes with optical spatial resolution down to 30 nm and sub-millisecond time resolution. These NSOM approaches are now exploited to reveal the existence of pre-assembled nanoplatforms of multi-molecular components on mammalian cells, to understand the molecular mechanisms responsible for the well-defined architecture of the cell surface and to decipher the molecular bases of diverse cellular processes, ranging from cell adhesion to pathogen recognition. In parallel, remarkable progress in AFM techniques now provides new opportunities for localizing single molecules in living cells and for analyzing their adhesive and mechanical properties in relation to function. Notably, the ability to observe and force-probe the cell surface machinery at molecular resolution has lead to the discovery of functional protein nanocomplexes that form under mechanical stress to activate cell signaling and cell adhesion.
© 2012 Elsevier Ltd. All rights reserved.

Introduction

Cells communicate with each other and the outside world through a myriad of different receptors located on their surface. It has become increasingly clear that these molecules do not operate separately but are part of well-organized

* Corresponding authors.
E-mail addresses: Yves.Dufrene@uclouvain.be (Y.F. Dufrêne), maria.garcia-parajo@ifco.es (M.F. Garcia-Parajo).
[1] Contributed equally.

1748-0132/$ — see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.nantod.2012.08.002

multimolecular components. This has led to the concept that in addition to receptor expression, their spatiotemporal organization on the cell surface is tightly controlled and crucial for function. In animal cells, many membrane receptors exhibit clear but distinct spatial patterns, which might arise from protein–lipid interactions [1,2], interactions with the cortical cytoskeleton [3–5], and/or with other local organizers of the cell membrane, such as tetraspanins [6,7] or galactins [8,9]. Moreover, recent research is providing evidence that receptors might also exist in pre-assembled nanoclusters prior to ligand activation, in the absence of interactions with other molecular components [10–12]. As such, the overall result is that most plasma membrane receptors distribute heterogeneously in small domains that are diverse in terms of size, composition and stability. Remarkably, microbes such as bacteria and yeasts also exhibit a high degree of heterogeneity in the way their proteins organize and distribute in the plasma membrane and cell wall [13–16]. The complex, heterogeneous arrangement of the cell surface machinery of both eukaryotic and prokaryotic cells is believed to be critical to various physiological processes [10,11,17,18]. Yet, very little is known about the molecular mechanisms leading to cell surface compartmentalization, in particular, prior to cell activation. Elucidating this issue has thus far been hampered by the lack of suitable single-molecule detection techniques in living cells.

For a long time electron microscopy has been the only technique available to observe the nanoscale spatial organization of proteins in plasma membranes and cell walls. However, harsh sample preparations and imaging modes make cell imaging extremely challenging. Therefore, the observation of molecular processes taking place at the cell surface has been mainly obtained by means of fluorescence-based optical techniques, which offer chemical specificity, real-time capability and minimal invasiveness. However, standard lens-based optical techniques are diffraction limited and do not allow optical observation beyond ~300 nm.

An elegant fluorescence based technique that provides information at the nanometer scale is Förster resonance energy transfer (FRET), which exploits a distance-dependent non-radiative energy transfer between two fluorophores to measure molecular distances below 10 nm [19]. Although FRET has been an important tool to demonstrate the existence of nanoclusters on cell membranes [20,21] it is unable to bridge the resolution gap between 10 and 300 nm. Recently, valuable techniques have been devised to achieve this goal by taking advantage of the photophysical properties of fluorescent emitters in combination with standard lens-based microscopes. For instance, stochastic optical reconstruction microscopy (STORM) or the analogous, fluorescent photoactivable localization microscopy (FPALM/PALM) are capable of reconstructing superresolution images of different cellular structures [22–24] and are currently being used to address the organization of the cell membrane at the nanometer scale [25–27]. Another lens-based technique, stimulated emission depletion (STED) microscopy, has also been applied to estimate protein-cluster sizes and their nanoscale distribution on the membrane [12,28].

Besides these far-field techniques, nanoscale spatial resolution on cell surfaces with single molecule detection sensitivity can be obtained using scanning near-field approaches. One of them is near-field scanning optical microscopy (NSOM) in which a subwavelength aperture probe is used as a local illumination source [29–31]. The probe raster scans the cell surface generating simultaneous topography and optical images with a spatial resolution essentially determined by the size of the probe, typically between 50 and 70 nm [32]. Another near-field nanoscopy technique is atomic force microscopy (AFM) where a sharp tip is used to sense the interaction forces between the tip and the sample surface to generate superresolution images of cell surfaces and to probe their biophysical properties such as elasticity and adhesion [33,34]. While NSOM relies on photon-matter interactions in the near-field for optical contrast, AFM is based on force interactions in the near-field. As a result, both techniques share extreme sensitivity for surfaces, being ideally suited for imaging and probing the spatial complexity of living cell surfaces.

In the past few years, rapid progress in near-field nanoscopy has allowed extending the use of these techniques beyond cell nanoimaging to more sophisticated applications, providing unique insights into the spatial organization, dynamics and functions of cell surfaces. Specifically, nanophotonic approaches are being employed to increase further the spatial resolution and combined with other methods, such as fluorescence correlation spectroscopy (FCS), to record dynamic events in living cells with sub millisecond time resolution in ultra-confined volumes. In the force regime, the controlled labeling of the AFM probe with bioligands is used to simultaneously localize and manipulate single molecules in living cells. In this review, we focus on the latest technological developments in this exciting, rapidly growing field and we provide a survey of recent studies in which NSOM and AFM have enabled to answer key questions in cell surface biology.

Near-field cell surface nanoscopy using photons: basic principles of NSOM and recent technical developments

Aperture-type NSOM imaging

Most common NSOM approaches used for biological applications rely on the use of subwavelength aperture probes as illumination nano-sources. The near-field optical probe generally consists of a small aperture (20–100 nm in diameter) at the end of a metal-coated tapered optical fiber [32], which is scanned in close proximity to the specimen under study (Fig. 1a). The lateral resolution, down to tens of nanometers, is essentially determined 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 exponentially away from the probe's end face. Interaction of the near-field with the sample surface induces changes in the far-field radiation, which is collected by conventional optics and directed to highly sensitive detectors to provide an optical image (Fig. 1b) [35]. An independent mechanism is used to control the distance separation between the probe and the sample within 1 to 10 nm. This is commonly achieved using a shear-force feedback based on a tuning fork

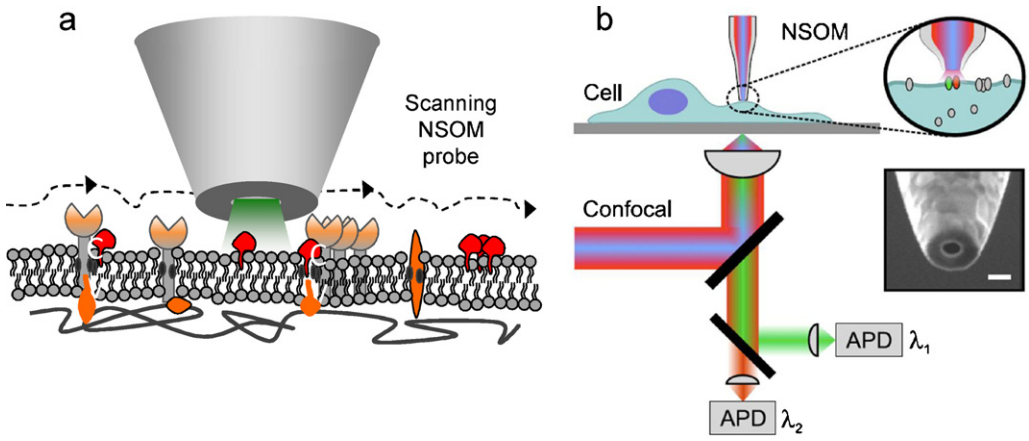


Fig. 1 NSOM based nanoscopy on cell membranes. (a) In the imaging mode the NSOM probe scans the sample in close proximity to the surface (dashed line). Optical resolution depends on the probe diameter and probe-sample vertical distance separation. (b) Schematic set-up of an aperture-type NSOM incorporated on an inverted optical microscope. Excitation of the sample is provided by confocal illumination in the far field or by the nanometric NSOM aperture probe to provide super-resolution optical imaging. Fluorescence from the excited molecules is collected by the lens and sent to sensitive detectors. The inset shows a SEM image of a 70 nm aperture probe.

piezo-electric element that is oscillated at its resonance frequency [36,37]. The probe is attached to one of the prongs of the tuning fork. When the probe is approached to the sample, shear-forces will produce a change on both the amplitude and phase of the oscillation, which is then detected by the feedback loop displacing the scanning piezo in the z direction. The signals sent to the piezo are also used to generate a topographic image of the sample in perfect registry with the optical image. Imaging of biological samples in physiological conditions while preserving efficient distance feedback is obtained by enclosing the tuning fork resonator into an airtight sealed diving bell [38,39].

Since light of different wavelengths can be simultaneously funneled through the same subwavelength aperture, NSOM allows simultaneous multicolor imaging free from chromatic aberrations. 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 multicolor single molecule detection with high signal-to-background ratios [29,31,40] and localization accuracies better than 3 nm on intact cell membranes under physiological conditions [41,42].

Nano-antenna concepts for near-field nanoimaging

One of the major drawbacks of aperture-type NSOM is the low light throughput (10^{-4}—10^{-6} for a 70 nm aperture) of the probes. This, together with a maximum input power related to the probe damage threshold, limits the practical resolution to about 50 nm for single molecule applications. In recent years, advances in nanophotonics have provided novel routes towards the fabrication of brighter illumination sources using antenna concepts [43,44]. Indeed, creative designs using sharp metallic tips, metal nanoparticles and complex geometrical nanostructures are being currently explored to localize and to boost the optical field to dimensions smaller than 30 nm in size (Fig. 2) [43,44].

While research in photonic antennas is still mostly at the fundamental level, first applications in the field of biology are starting to emerge. For instance, using a gold nanoparticle-based optical antenna excited in the far-field, Hoppener and Novotny imaged single Ca^{2+} channels on erythrocyte plasma membranes in aqueous conditions [45]. The spatial resolution obtained was 50 nm, although improvements on the antenna geometry and illumination schemes that reduce the far-field surrounding background can in principle improve the resolution to 10 nm. Other antenna geometries include the fabrication of a metallic tip on top

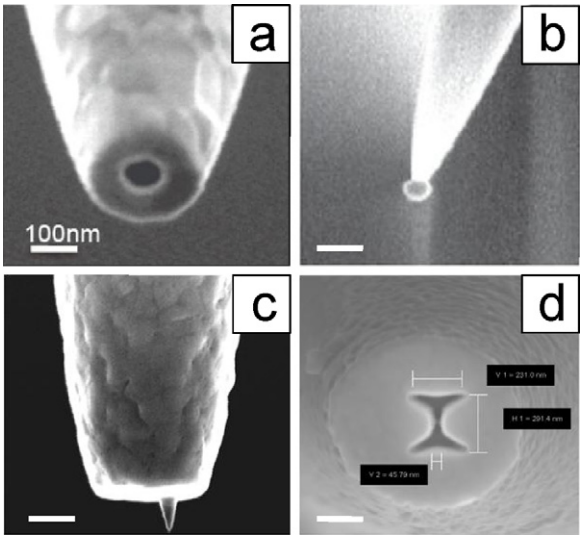


Fig. 2 Different antenna-on-probe geometries. (a) Conventional aperture NSOM probe with a 70 nm aperture diameter. (b) Gold nanoparticle attached at the end of a tapered optical fiber. (c) Metallic tip carved at the end of an NSOM probe. (d) Bowtie nanoaperture carved at the end of a metal-coated tapered optical fiber. Scale bar is 100 nm.
Image in (b) has been adapted from [45].

of the normal nano-aperture. This concept takes advantage of the local illumination properties of the aperture-type NSOM to drive the antenna to resonance, enhancing and confining the optical field at the end of the tip. In the first demonstration of this approach, simultaneous topography and fluorescent imaging of labeled DNA with 10 nm resolution was convincingly demonstrated [46]. More recently, the dimensions of these antenna probes have been tuned to the wavelength of the excitation field and used to detect individual fluorescent molecules embedded in polymer layers [47]. We have explored similar antenna probes to image individual proteins in intact cell membranes with 30 nm resolution and virtually no background, allowing localization accuracies in the order of 1 nm [48]. Achieving higher spatial resolution requires the design of antennas with strongly localized fields by using smaller particles, sharper antenna ends or novel geometries that might include the use of other materials rather than metals, such as graphene and carbon nanotubes [44]. Such efforts are currently being undertaken in the nanophotonic field and hopefully will be soon applied for biological nano-imaging.

Near-field approaches for dynamic studies at the nanoscale

One of the great advantages of light microscopy is the possibility to image living cells. Unfortunately, the diffusion of most proteins and lipids on cell surfaces occurs in the millisecond time scale, posing a challenge to most optical imaging techniques. Therefore, single molecule fluorescence approaches that provide sufficient time resolution do not rely on imaging but rather on following a sub-set of labeled molecules as they laterally diffuse on the cell surface, as used in single particle tracking (SPT) [3,49]. Alternatively, the illumination spot can be parked on a specific region of the cell membrane. Intensity fluctuations resulting from the diffusion of individual components transiting the illumination volume are recorded and autocorrelated in time to provide information on the diffusion behavior, as done in fluorescence correlation spectroscopy (FCS) [50]. Although confocal FCS provides the temporal resolution needed to resolve fast dynamic events, the large illumination volume of the diffraction-limited confocal spot hides diffusion heterogeneities taking place at the nanoscopic length scale [51].

In heterogeneous systems such as the cell membrane, diffusion is influenced by protein confinement and/or interactions. Since these effects might take place on a short spatial and temporal scale, their detection requires an illumination area comparable to the membrane heterogeneities length scale, combined with high temporal resolution. In this context, the reduced illumination size offered by NSOM probes combined with a FCS approach, offers an exquisite tool to monitor single molecule dynamics at the nanoscale, beyond the limited time-resolution associated with the conventional scanning probe approach, thus obtaining high temporal resolution in ultra-confined illumination volumes.

The first demonstration of FCS-NSOM was performed in 2008 by Vobornik et al. by measuring the mobility of fluorescent lipids in supported lipid bilayers [52]. Later on, Naber's group determined the diffusion of ligands transported axially through single nuclear pore complexes using the same approach [53]. Recently, we demonstrated for the first time the feasibility of performing FCS-NSOM on living cells by stably maintaining the NSOM probe in close proximity to the sample, obtaining vertical distance fluctuations below 1 nm [54]. We used this configuration to measure the diffusion of different lipids on living CHO cells (Fig. 3a and b). While the lipid analog phosphatidylethanolamine (PE) showed free,

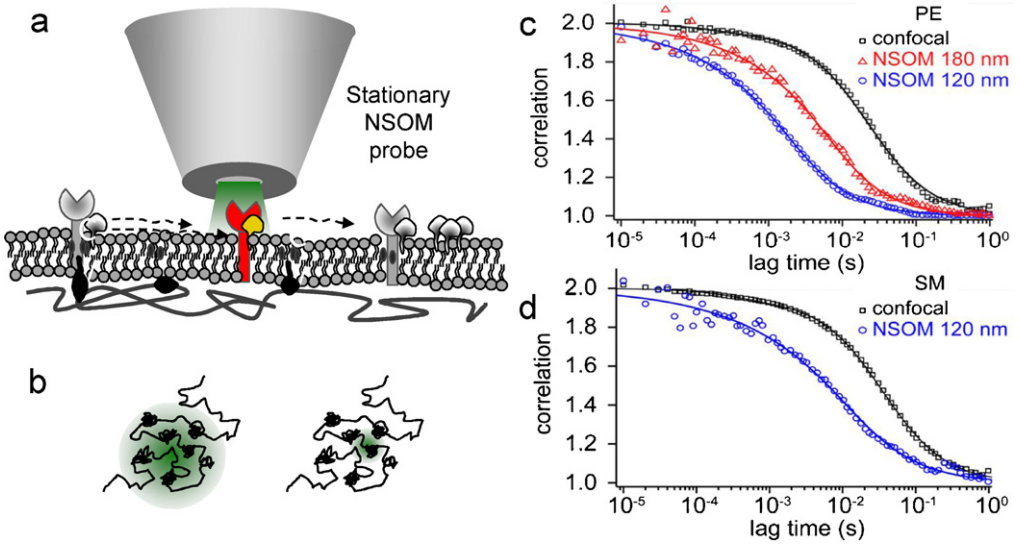


Fig. 3 NSOM-based fluorescence correlation spectroscopy in living cells. (a) In FCS mode, the NSOM probe is kept stationary over the membrane of the living cell so that only molecules laterally diffusing through the small illumination area of NSOM are excited. (b) Schematics showing the comparison between confocal (left) and NSOM (right) illumination areas. Green denotes the illumination spot in both cases, while the black curves illustrate the diffusion of individual molecules. (c) Correlation curves as function of lag time obtained for the lipid PE for different illumination conditions. (d) Correlation curves as function of lag time for SM, obtained with confocal and NSOM illumination.

Brownian diffusion on the cell membrane, with transit times that linearly scaled with the illumination area (Fig. 3c), the sphingolipid SM showed a clear deviation from Brownian diffusion when the illumination area was reduced from confocal to NSOM (Fig. 3d). The anomalous diffusion of SM is entirely consistent with a cholesterol-induced confinement of sphingolipids [28,55]. Since this temporal confinement occurs in nanometric membrane domains, their effect on the diffusion dynamics can only be revealed by reducing the illumination area, as achieved by NSOM, to dimensions comparable to the trapping regions.

There is a large potential for the use of FCS-NSOM since it combines membrane (proximal) specificity and high signal-to-noise ratios. Further refinements of the technique will greatly benefit from the design of brighter probes, such as photonic antennas. Combined with multicolor NSOM excitation, it would then be possible to perform dual-color cross-correlation spectroscopy in ultra-small volumes providing exquisite information on the coupling between the inner-outer leaflets of the membrane, signaling complex formation and the intimate relation between membrane nanodomains and the actin cytoskeleton.

Unraveling the nanolandscape of mammalian cell membranes using NSOM

The potential of NSOM for cell surface studies was recognized soon after its first practical implementation more than 20 years ago [35] and initially used for bacterial imaging because of their more rigid surface. However, progress in the field has been slow, mainly hampered by difficulties to operate NSOM under liquid conditions, a necessary condition for fully extending the use of the technique to mammalian cell imaging. The implementation in the last years of different approaches to work under physiological conditions [38,56] has awakened a renewed interest in the community and lately NSOM has been applied to the study of different receptors and lipids on intact cell membranes. We highlight below some of the major contributions of the technique in cell membrane research.

Receptor clustering at a higher resolution

Receptor clustering and organization into membrane microdomains is essential for many different cellular processes, including cell adhesion [11,41,57], pathogen recognition [10] and signal transduction [58,59]. While receptors were initially thought to cluster upon binding multivalent ligands, there is increasing evidence that receptors can also exist in pre-formed nanoclusters that get reorganized and activated upon ligand binding. NSOM has significantly contributed to the discovery of pre-assembled receptor nanoclusters on the membrane of a diversity of cells.

An example studied with NSOM is the pathogen recognition receptor DC-SIGN, expressed on antigen presenting cells, such as immature dendritic cells (imDCs) [10,60]. Single molecule NSOM showed that DC-SIGN organizes in small nanoclusters of ~180 nm in size on the membrane of imDCs, prior to pathogen binding [61]. Interestingly, we found that the molecular density of these nanoclusters is extremely heterogeneous, with some nanoclusters containing only a few molecules while others might contain as much as 20 DC-SIGN molecules per cluster. Together with the broad capability to bind to many different pathogens, these results suggest that the particular heterogeneous packing of DC-SIGN might serve to maximize its binding strength to a large variety of viruses and pathogens having different binding affinities to the receptor. In contrast to DC-SIGN, we found that clusters of IL2R and IL15R (two members of the interleukin family expressed in human T lymphoma cells) had a constant packing density albeit forming clusters of different sizes [62]. The linear increase in number of receptors with domain size suggested a general ''building block'' type of assembly for these receptors on the cell membrane.

In another NSOM study, Vobornik et al. could address whether cluster size of a particular receptor is dependent on its expression level [63]. Although the expression levels of the β_2-adrenergic receptor (β_2AR) were increased by 3-fold, the average cluster size remained similar, and in the order of ~130–150 nm, suggesting that these receptors might have a biological preferred organization in nanodomains. Along these lines, the epidermal growth factor receptor (EGFR) also organizes in nanoclusters and this organization is preserved even after ligand presentation [64].

Aside from cluster size, composition or packing density, the specific distribution of receptor nanoclusters on the cell membrane might also be coupled to function. Taking advantage of the simultaneous topography and fluorescence information afforded by NSOM, Chen et al. could correlate the spatial distribution of the hyaluronan receptor CD44 to specific locations of the cell membrane [65]. Indeed, CD44 was found to form nanoclusters of about 275 nm in size, being particularly enriched on filopodia regions and membrane protrusions, as confirmed by the topography images. The authors speculated that since most of the CD44 is located on filopodia, the receptor enhances its binding properties to the extracellular matrix by concentrating in these regions [65].

Lipid rafts as local organizers of the cell membrane

Lipid rafts are defined as membrane nanodomains enriched in sphingolipids and cholesterol that favor the segregation of specific signaling receptors and glycosyl-phosphatidylinositol-anchored proteins (GPI-APs) [1,20,66]. Although lipid rafts have been implicated in a variety of cellular processes, there is no consensus yet on the spatiotemporal regimes regulating lipid rafts in the resting state. Novel biophysical techniques, including NSOM, have been used to obtain insight on the size and temporal stability of these cholesterol-enriched nanoplatforms on cell membranes. For instance, Chen et al. found that gangliosides GM1 and GM3, two sphingolipids thought to partition in rafts, organized in small nanodomains of comparable size on the membrane of MDCK cells [67]. By taking advantage of the simultaneous topographic information provided by NSOM, the researchers found that GM3 domains were preferentially localized at the peak of microvilli-like protrusions on the cell membrane. In contrast, GM1 lipid domains were localized at the slope or valley of the membrane fluctuations, indicating that the two raft lipids do not spatially colocalize at the nanometer

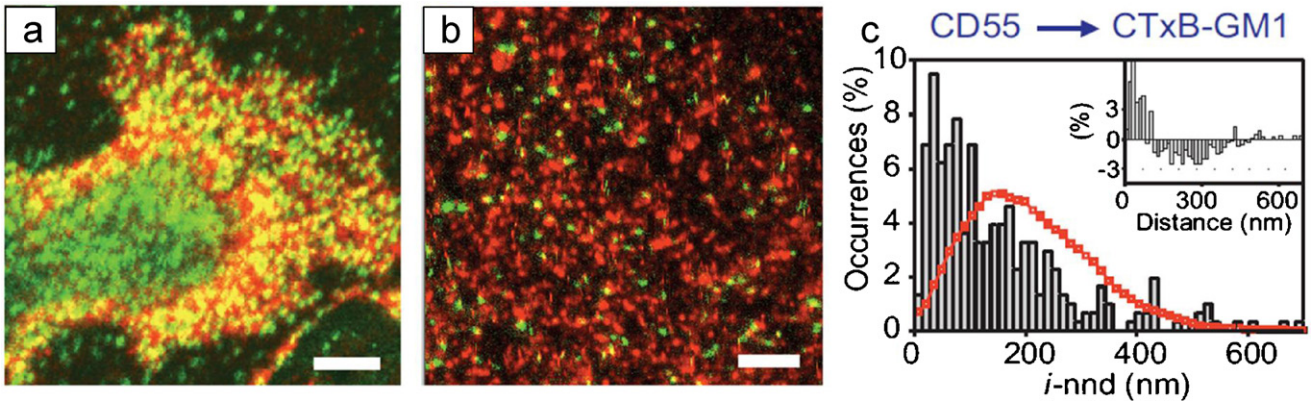


Fig. 4 Nano-imaging of lipid raft nanoplatforms with NSOM. (a) Confocal image of the raftophilic protein CD55 (green) and CTxB-GM1 (red) on monocytes. An apparent high degree of colocalization (yellow) is observed as a result of the limited resolution in confocal. Scale bar: 5 μm. (b) Dual color NSOM image of CD55 (green) and CTxB-GM1 nanodomains (red) on monocytes showing no intermixing (lack of yellow) between these two components at the nanoscale. Scale bar: 1 μm. (c) Interparticle-nearest neighbor distribution (*i*-nnd) of CD55 to its closest CTxB-GM1 nanodomain (bars) together with simulations of random spatial distribution (red). Inset: Residuals between the distribution obtained by experiments and simulations. The increased occurrence of distances <150 nm, as compared to the simulations, demonstrate a spatial proximity, or compositional connectivity, between CD55 and CTxB-GM1 nanodomains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Adapted with permission from [42].

scale. Although such a segregation of distinct raft domains was already observed in polarized T cells and directly correlated with cell migration capacity [68], spatial segregation of different raft constituents on resting cells had not been reported yet.

As an alternative raft marker to GM1, Abulrob et al. examined the membrane partitioning of asialo-GM1, an abundant glycosphingolipid in HeLa cells [69]. NSOM showed that asialo-GM1 is localized in small nanoclusters of about 90 nm in size. In addition to clusters, a second nonclustered and more diffusive population of asialo-GM1 was observed, suggesting that raft components might not necessarily be stably organized in nanodomains, but instead could show differences in their spatial organization [69].

We recently used single-molecule near-field nanoscopy to visualize the nanolandscape of GM1 after tightening by its ligand cholera toxin (CTxB) on intact monocyte membranes [42]. CTxB tightening of GM1 was sufficient to initiate a minimal raft coalescence unit, resulting in the formation of cholesterol-dependent GM1 nanodomains <120 nm in size. This particular arrangement appeared independent of cell type and GM1 expression level on the membrane, since GM1 nanodomains were observed both on monocytes as well as on dendritic cells. Cholesterol depletion abrogated GM1 nanoclustering demonstrating the crucial role of cholesterol for maintaining the integrity of these nanodomains. Simultaneous dual color high-resolution images revealed that the canonical raft component CD55 (a GPI-AP) was recruited to regions proximal (<150 nm) to CTxB-GM1 nanodomains without physical intermixing (Fig. 4), but not the non-raft protein CD71 [42]. These results demonstrated the existence of raft-based compositional connectivity at the nanoscale crucially mediated by cholesterol. Our data further suggested that such a connective condition on resting cell membranes constitutes an obligatory step toward the hierarchical evolution of large-scale raft coalescence upon cell activation. More conventional techniques such as FRET are unable to report on such a spatial proximity at spatial scales >10 nm. On the other extreme, diffraction limited techniques such as confocal microscopy would misleadingly show colocalization between different components located at distances <300 nm (Fig. 4a). As such, NSOM is capable of bridging the gap between 10 and 300 nm providing exquisite information at these important spatial scales.

Molecular interactions and spatial proximity

Cell-signaling events commonly involve a multitude of proteins and/or lipids. Although many of the molecular players involved in specific signaling processes can be readily identified by biochemical means, very little is still known about their tight spatial organization on the membrane. The high spatial resolution of NSOM combined with multicolor excitation/detection has been used to visualize the spatial scale of some of these molecular interactions. For instance, dual color NSOM has been used to investigate the association of β_2AR to small invaginations of the cell membrane known as caveolae [70]. A very modest percentage of β_2AR nanoclusters (15—20%) colocalized in caveolae, suggesting that the diverse functional properties of β_2AR could arise from multiprotein complexes that are not caveolar in nature. Within the context of T cell activation, NSOM combined with dual-color Qdot immuno-labeling has been used to map the nano-spatial relationship between T cell receptor (TCR)/CD3 and CD4

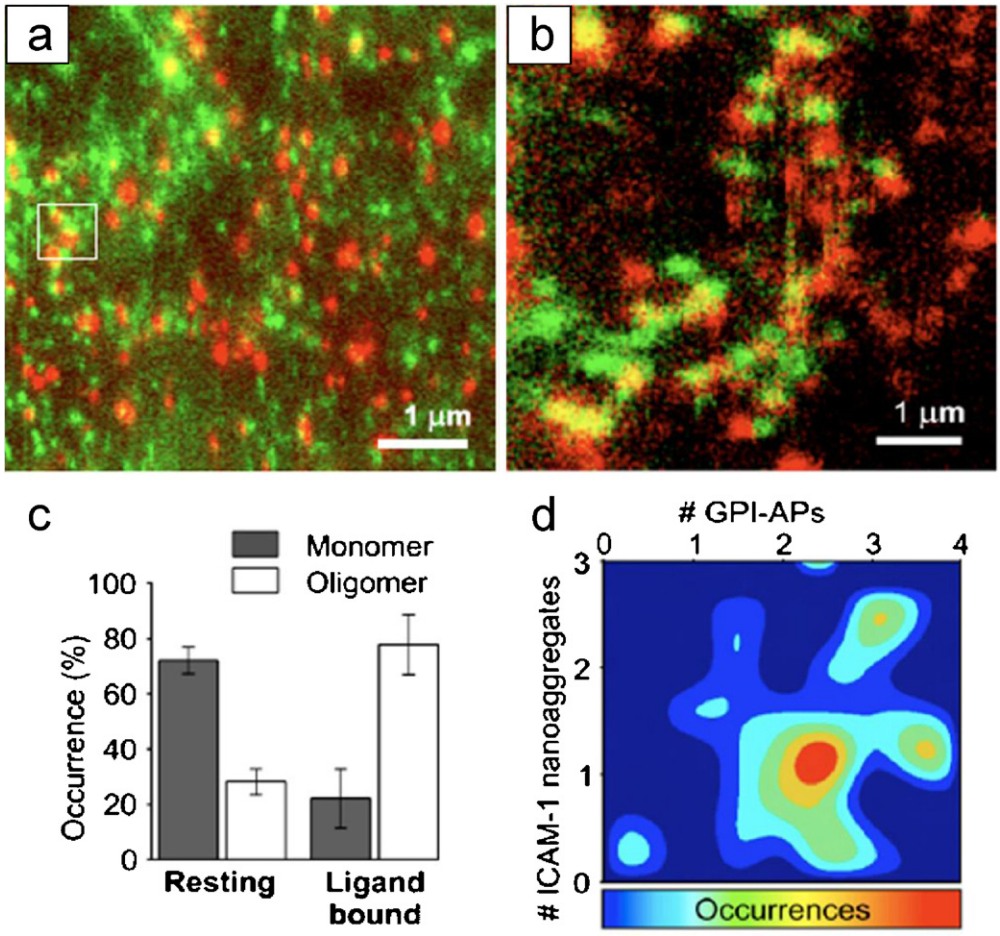


Fig. 5 Molecular interactions at the nanoscale revealed by NSOM. (a) Dual color excitation/detection NSOM image of LFA-1 nanoclusters (red) and GPI-APs (green) in the resting state, i.e., in the absence of ligand. Lack of physical association at the nanometer scale is evidenced by the low occurrence of yellow spots on the images. (b) Dual color NSOM image on a selected region of the membrane after activation of LFA-1 by ICAM-1 nanoaggregates. Increased colocalization between the LFA-l ligand and GPI-APs is observed by the appearance of yellow spots on the images. (c) Monomer and nanodomain percentages of GPI-APs in resting cells and upon LFA-1 activation. (d) Surface correlation plot between the number of GPI-APs per fluorescent spot and its proximal ICAM-1 nanoaggregate. Color code reflects the number of occurrences: blue (low) to red (high). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Adapted with permission from [41].

or CD8 co-receptor before and after activation of primary T cells [71]. In the resting state CD3 and CD4 organized mainly as small nanoclusters made of a few proteins with little interaction between them. T-cell stimulation led to enhanced clustering of the individual proteins and significant co-clustering of the CD3 and CD4 nanoclusters into 200—500 nm size domains on the T cell membrane [71].

Recently, we investigated the spatiofunctional relationship between the integrin receptor LFA-1 involved in leukocyte adhesion and raft components (GPI-APs) using dual color NSOM [41]. While LFA-1 formed nanosclusters of ~85 nm in size on resting monocytes, ~70% of the GPI-APs organized as monomers, and the remaining 30% formed small oligomers containing up to 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. 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 (Fig. 5) [41]. These data demonstrated the existence of nanoplatforms composed by integrins and rafts as essential intermediates in nascent cell adhesion. Since raft association with a variety of membrane proteins other than LFA-1 has been documented, we proposed that hotspot regions enriched with raft components and functional receptors may constitute a prototype of nanoscale inter-receptor assembly and correspond to a generic mechanism to offer cells with privileged areas for rapid cellular function and responses to the outside world.

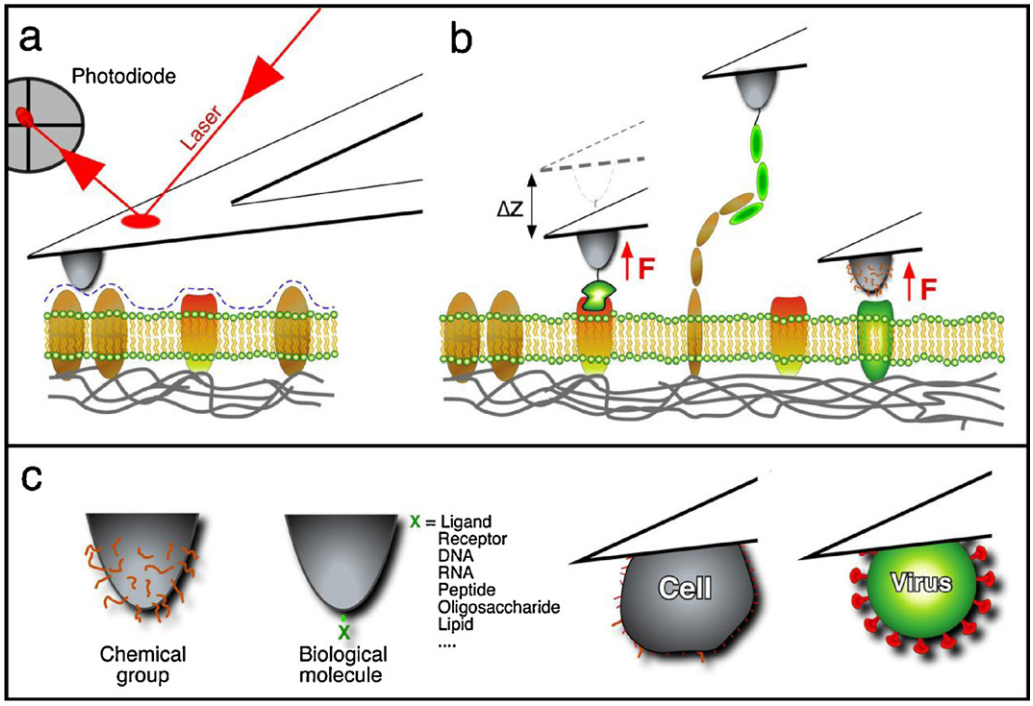


Fig. 6 AFM-based nanoscopy of living cells. (a) In the imaging mode, AFM raster scans the cell surface (dashed line) using a sharp tip. (b) In force spectroscopy, the tip is used to probe interactions of the cell surface to single-molecule resolution. The examples show a tip functionalized with a ligand to probe interactions with its cognate receptor, a tip carrying a cell adhesion molecule to probe heterogeneous interactions with other such molecules, and a tip coated with chemical groups to detect chemical interactions. (c) Advanced protocols are available to functionalize the AFM tip or cantilever to specifically probe chemical, biological, cellular or viral interactions.
Reproduced with permission from [33].

Near-field cell surface nanoscopy using force: basic principles of AFM and recent developments

Live-cell imaging

Rather than using light, AFM senses the tiny forces acting between a sharp tip and the sample surface (Fig. 6) [33,34]. Three-dimensional topographic images are generated in liquid by scanning the tip over the sample while sensing the interaction force (Fig. 6a). The sample is mounted on a piezoelectric scanner, which ensures three-dimensional positioning with high accuracy. While the tip (or sample) is being scanned in the (x, y) directions, the force interacting between tip and specimen is monitored with sensitivity in the piconewton range. This force is measured by the bending (deflection) of a soft cantilever, which is detected by a laser beam focused on the free end of the cantilever and reflected into a photodiode. AFM topographic imaging is widely used in life sciences, providing high-resolution views of biomolecules, membranes and cells in buffer and at unprecedented resolution (for reviews see [72,73]).

On mammalian cells, the resolution of topographic images is generally not better than 50 nm because of the soft and dynamic nature of the cell surface. As a result, the scanning AFM tip partially deforms the cell surface, and becomes easily contaminated by loosely bound macromolecules. By contrast, there has been much progress in imaging microbes owing to their very rigid, well-defined structure (Fig. 7) [74,75]. For instance, AFM was able to resolve the nanoscale architecture of crystalline protein layers (rodlets) on the surface of various microbes, including the fungal pathogen *Aspergillus fumigatus* [76]. In the medical context, an interesting feature is the possibility to track cell surface dynamics associated with cell growth or drug interaction, using real-time imaging [77].

Single-molecule imaging

Unlike fluorescence techniques, conventional AFM is unable to detect and localize specific biomolecules, such as a given receptor. This is an important flaw as the organization and interactions of the cell surface machinery are key parameters for controlling its functions. Notably, recent progress in single-molecule force spectroscopy (SMFS) with AFM tips labeled with specific ligands now enables microscopists to detect and localize single functional molecules on cell surfaces (Figs. 6b and c) [78]. The idea is to record spatially-resolved force curves across the cell surface with a bio-labeled tip (Fig. 6c), estimate the specific molecular recognition force value for each force curve and then display it as grey or colored pixels, where the brightness reflects the magnitude of the force. A common strategy for such a functionalization of the tip is to covalently anchor proteins

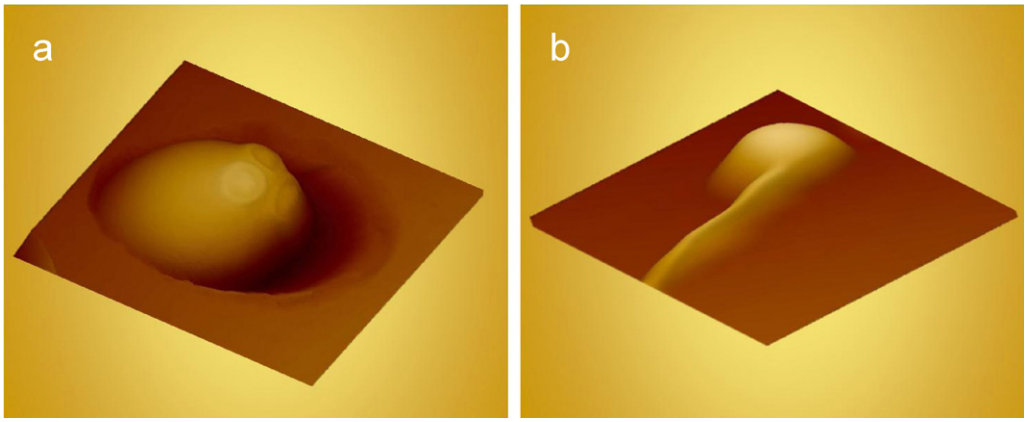


Fig. 7 Live-cell imaging. (a and b) Three-dimensional AFM images of yeast (8 μm × 8 μm) and hyphae (15 μm × 15 μm) of the fungal pathogen *Candida albicans*. The yeast cell shows cell wall heterogeneities in the form of circular bud scars left after detachment of the daughter cell, while the geminating cell clearly shows a germ tube.

via a polyethylene glycol (PEG) crosslinker, which provides motional freedom and prevents their denaturation. Tips are first modified with amino groups, further reacted with PEG linkers carrying benzaldehyde functions, which are then directly attached to proteins through their lysine residues. Another option is to use self-assembled monolayers of alkanethiols on gold tips. Both the thiol and silane approaches can be used to determine the orientation of the attached biomolecules with respect to their C-terminal or N-terminal domains by linking recombinant histidine-tagged proteins onto an AFM tip coated with nitrilotriacetate groups.

Two elegant variations of SMFS are chemical force microscopy (CFM) and single-cell force spectroscopy (SCFS) [33]. Here, the tip is modified with chemical groups (CFM), and cells or viruses are attached to cantilevers (SCFS) in order to image the spatial distribution of chemical interactions and to quantify the forces that govern cell adhesion.

Single-molecule manipulation

In addition to imaging single-molecules, AFM-based nanoscopy can also subject them to force in order to study their adhesion and elasticity (Fig. 6b). These single-molecule manipulations involve continuously approaching and retracting the tip from the biological sample, and quantifying the intra- or inter-molecular interaction forces by measuring the cantilever bending. Pioneering studies have demonstrated that proteins can be subjected to controlled forces yielding details of their unfolding pathways and the forces that anchor them within the cell membrane. Since then, single-molecule AFM, combined with molecular dynamics simulations and protein engineering, has greatly enhanced our understanding of the mechanical behavior of a great variety of proteins [72,79—81].

Because these single-protein manipulation experiments are generally conducted in vitro, the molecules must be removed from the native cellular context that controls their structural assembly and functional state. Hence, understanding the complex functions of the cell surface requires moving these experiments into living cells [82].

Deciphering the nanoscale organization and interactions of microbial cell surfaces using AFM

Today, we know much about the structure and biosynthesis of microbial cell surface constituents. However, the three-dimensional organization, assembly, and interactions of the individual components remain mysterious. What is the spatial arrangement of cell surface receptors, clustered or homogeneous? What are the adhesive and mechanical properties of cell surface proteins and how are they related to function? Traditionally, answering these key questions has been hampered by the lack of high-resolution single-cell and single-molecule probing techniques. With its ability to observe and force probe the cell surface machinery at molecular resolution and under physiological conditions, AFM-based nanoscopy has recently offered new opportunities for localizing single proteins like receptors on living microbes and for analyzing their adhesive and mechanical properties in relation with function.

Protein clustering

By integrating the power of force nanoscopy with the modern tools of molecular genetics (protein design in live cells), it is now possible to image the dynamic clustering of single-proteins on cell surfaces. Recently, this enabled the discovery of the yeast nanosensosome and nanoadhesome, two functional protein complexes that form under stress, thereby activating cell signaling and cell adhesion.

Adhesion molecules that decorate cell surfaces play key roles in mediating cellular processes such as cellular communication, tissue development, inflammation, cancer, and microbial infection. A fascinating feature of adhesion proteins is their ability to form adhesion domains (i.e., focal adhesion complexes) that can grow and strengthen under force [83]. Until the emergence of AFM nanoscopy, such force-dependent clustering had never been reported for microbial adhesion proteins. Adhesion of the pathogenic yeast *Candida albicans* to host tissues, the primary step leading to infection, is mediated by a family of cell

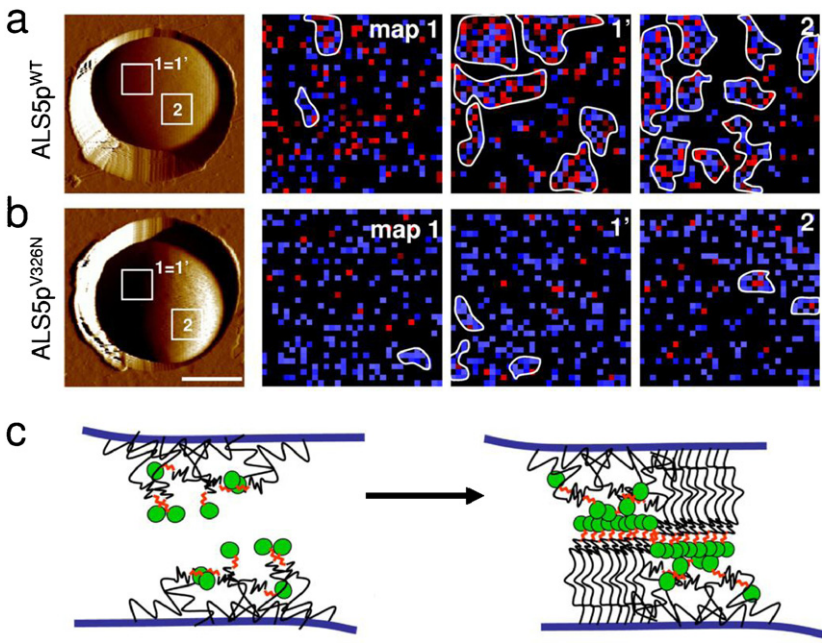


Fig. 8 Force-induced clustering of Als adhesion proteins. (a) Single-molecule imaging of *Saccharomyces cerevisiae* cells expressing V5-tagged Als5p proteins. Left: AFM topographic image (scale bar: 2 μm) of a single cell. Map #1: adhesion force map (1 μm × 1 μm) recorded with an anti-V5 tip on a given target area of the native cell that was never subjected to force (recorded on the square shown in the topographic image). Blue and red pixels correspond to forces smaller and larger than 150 pN, respectively, thus to V5-tagged Als5pWT recognition and unfolding. Map #1′: second adhesion force map recorded on the same target area. The heterogeneous distribution of colored pixels, which represents the detection of single Als5p molecules documents the formation of nanoscale clusters (outlined in white). Map #2: adhesion force map recorded on a remote area localized several hundred nanometers away from the first map. (b) Same sequence of data as in (a) obtained on *S. cerevisiae* expressing the single site mutation Als5pV326N. (c) Cartoon model of force-induced amyloid-dependent clustering of Als5p, strengthening cell—cell adhesion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Adapted from [86].

surface proteins known as the agglutinin-like sequence (Als) proteins [84]. Macroscopic assays suggested that adhesion-triggered changes in the conformation of Als5p propagates around the cell surface, forming ordered adhesion domains [84]. Recently, we provided direct, single-molecule demonstration for such a mechanism by showing that mechanical force can trigger the formation and propagation of cell adhesion nanodomains in living yeast cells [85] (Fig. 8). The surface distribution of Als5p proteins was probed on live cells using AFM with tips bearing antibodies directed specifically towards the protein. Pulling on single proteins was shown to induce the formation of nanoadhesomes, i.e., adhesion domains of 100—500 nm size (Fig. 8a). In addition, the force-induced nanodomains were found to propagate over the entire cell surface. Remarkably, single-site mutation in the conserved amyloid-forming sequence of the protein revealed that amyloid interactions represent the driving force underlying Als5p clustering (Fig. 8b) [86]. Hence, the strength of yeast adhesion results from the force-activated amyloid-like clustering of hundreds of proteins on the cell surface to form arrays of ordered multimeric binding sites (Fig. 8c) [84]. These results highlight the role that amyloids can play in microbial cell adhesion, both in clustering the adhesins to increase binding avidity, and in the potential to form stable amyloid-type bonds between cells. As functional amyloids may be a general mechanism for activating the adhesion of pathogens, these analyses offer exciting prospects in therapeutics for developing new antimicrobial strategies.

Mechanosensing is another cellular event for which protein clustering is shown to be crucial. In the yeast context, single-molecule AFM was used to map the distribution of single Wsc1 sensors in living *Saccharomyces cerevisiae* cells [87] (Fig. 9). Adhesion maps of 1 μm × 1 μm size were recorded on cells expressing His-tagged elongated sensors using Ni^{2+}-NTA tips. Many sensor molecules appeared to cluster in areas of approximately 0.04 μm^2 and an equivalent diameter of 230 nm (Fig. 9b). This size of Wsc1 clusters matches the range of the 300 nm large patches reported for MCC marker proteins in *S. cerevisiae*, and is clearly larger than the putative size of rafts in higher eukaryotes. Remarkably, both the total amount of wild-type Wsc1 sensors and their tendency to cluster increased when cells where stressed by either heat or in medium with low osmolarity (Fig. 9c and d). This correlates with the observation that stress conditions activate the cell wall integrity pathway, and suggests that clustering is a stress—responsive process, which is intimately connected to signaling. These findings suggest that clustering is a means developed by yeast to locally concentrate Wsc1 sensors and their interacting downstream components. This process ultimately enhances the signal strength and the corresponding cellular response.

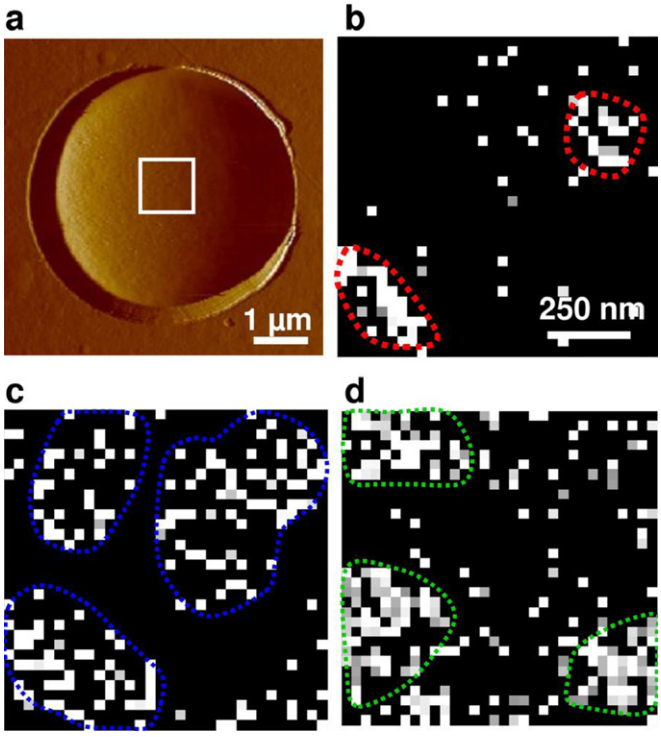


Fig. 9 Stress-induced clustering of Wsc1 sensors. (a) AFM image of a *Saccharomyces cerevisiae* yeast cell trapped into a porous polymer membrane, recorded in buffer solution at 25 °C. The cells express elongated, fully functional Wsc1-Mid2 hybrid sensor bearing a His-tag. (b) Representative adhesion force map obtained by scanning a 1 μm × 1 μm area on the cell surface with a Ni^{++}-NTA-tip. The heterogeneous distribution of the bright pixels, which represent the detection of single sensors, clearly documents the formation of nanoscale clusters (highlighted by dotted red lines). (c and d) Adhesion force maps (1 μm × μm) recorded with a Ni^{++}-NTA-tip either in buffer solution at 37 °C (c, heat shock) or in deionized water at 25 °C (d, hypoosmotic shock). Stressing conditions strongly expand the clusters (highlighted by dotted blue and green lines). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Reprinted with permission from [87].

Protein mechanics

The mechanical properties of cell surface proteins play an important role in defining cellular functions such as mechanosensing and adhesion. A hot topic in cell biology is therefore to understand how such proteins respond to force in living cells, and how this response is related to function.

Single-molecule manipulation by SMFS was recently used to study the mechanical properties of Als5p proteins, both on isolated molecules and on cell surfaces [88]. Als proteins possess four functional regions, an immunoglobulin (Ig)-like region near the N-terminus that initiates cell adhesion, a threonine-rich region (T), a tandem repeat (TR) region that participates in cell—cell aggregation, and a stalk region projecting the molecule away from the cell surface. Soluble Als fragments (Ig-T-TR) were attached on gold surfaces and picked up by their terminal Ig domain using an AFM tip modified with Ig proteins [88]. Force—extension curves showed saw-tooth patterns with well-defined force peaks, each peak corresponding to the force-induced unfolding of an individual TR domain (Fig. 10a). Urea altered the shape of the unfolding peaks, reflecting a loss of mechanical stability of the domains due to hydrogen bond disruption. Remarkably, single Als proteins could also be unfolded directly on live cells [88]. Force curves obtained on cells with proteins having six repeats displayed saw-tooth patterns similar to those found on isolated proteins, while cells not producing any repeats were unable to bind the AFM tip. The unfolding probability increased with the number of repeats and was correlated with the level of cell—cell adhesion, indicating these modular domains might play a role in fungal adhesion. Presumably, the force-induced unfolding of Als proteins leads to extend conformations in which hydrophobic groups are freshly exposed, thus favoring hydrophobic interactions between opposing cells.

In the mechanosensor context, stretching single Wsc1 sensors showed that they behave like nanosprings capable of resisting high mechanical force and of responding to environmental stress [89,90]. His-tagged sensors were picked up with an AFM tip carrying NTA groups. Remarkably, force—extension curves displayed a linear region where force is directly proportional to extension, thus characteristic of a Hookean spring (Fig. 10b). From these data, the sensor spring constant was estimated to be 5 pN nm^{-1}, which is very close to the behavior of ankyrin repeats. For other proteins tested so far, such a nanospring behavior is very unusual in nature and stands in sharp contrast to the properties of modular proteins. In fact, stretching modular proteins leads to non-linear force peaks that are well described by the worm-like-chain model, where each peak

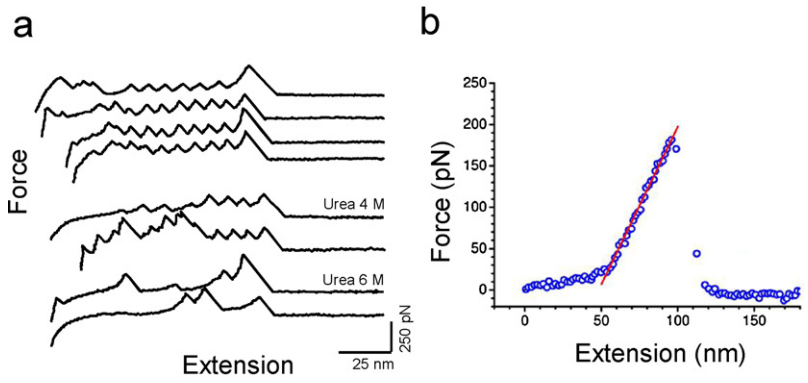


Fig. 10 Unravelling single protein mechanics. (a) Unfolding single adhesion proteins. Force—extension curves obtained by stretching single Als adhesion proteins from the pathogen *Candida albicans*. The curves show periodic features reflecting the sequential unfolding of the tandem repeat domains (upper traces). Addition of urea dramatically altered the unfolding peaks due to hydrogen bond disruption (lower traces). Reprinted with permission from [88]. (b) Pulling on single *Saccharomyces cerevisiae* sensors reveals they behave like nanosprings. Representative force curve obtained upon stretching a single Wsc1 molecule on a yeast cell using a Ni^{++}-NTA-tip. Clearly visible are two extension regimes, reflecting elongation at nearly zero force, followed by a Hookean spring behavior (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Reprinted with permission from [89].

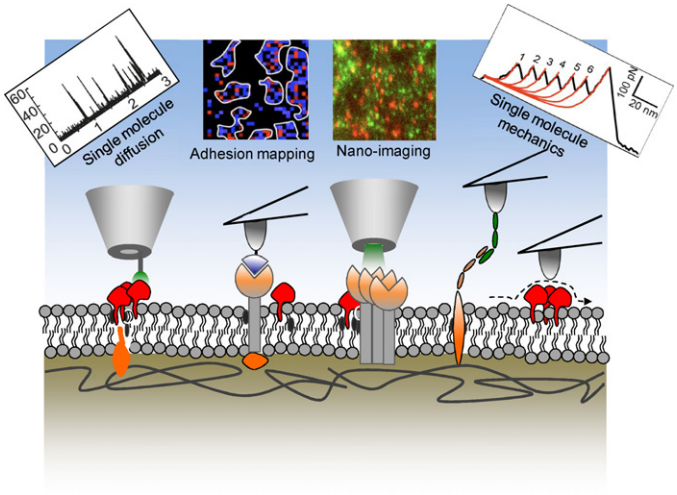


Fig. 11 Different possibilities for combining AFM and NSOM to measure and probe complex biochemical and mechanical properties in living cells. Multicolor fluorescence imaging, adhesion mapping, single molecule fluorescence diffusion and single molecule mechanics could in principle be integrated in one single instrument.

reflects the force-induced unfolding of secondary structures [81]. The use of mutants with reduced glycosylation resulted in severe alterations in protein spring properties, indicating the important role of glycosylation at the extracellular serine/threonine-rich region. At low salt concentration or elevated temperature, the sensor spring constant was substantially reduced, demonstrating that Wsc1 is sensitive to cell surface stress.

Conclusion

In this review, we surveyed recent technological developments in near-field nanoscopy using either light or force, and discussed recent studies in which NSOM and AFM have been applied to address key questions in the field of cell membrane biology and microbiology. Owing to its surface sensitivity and excellent localization accuracy, NSOM has been able to map molecular interactions and their spatial nanoscale proximity on intact mammalian cell membranes in relation to function. We have highlighted first exciting results where optical antennas have been already extended to biological applications to quantitatively image, with exceptional resolution, intact cell membranes in physiological conditions. Concepts from FCS implemented in ultra-confined volumes as afforded by NSOM probes allow nowadays measurements of ultra-fast dynamics at the nanoscale in living cell membranes. In addition to nano-imaging, the single molecule manipulation capabilities of AFM have been exploited to measure the adhesive and

mechanical properties of individual proteins in their cellular context and to probe mechanosensitive responses in living cells.

Whereas NSOM provides optical contrast, single molecule specificity and localization accuracy down to the nanoscale, AFM can exquisitely probe the adhesive and mechanical properties of single molecules in living cells. Hence, combining both techniques in a single instrument could certainly provide unprecedented possibilities to measure and probe complex biochemical and mechanical properties in living cells (Fig. 11). Progress in this direction has already been demonstrated through the functionalization of NSOM probes with specific ligands in a similar way as done with AFM [91]. Further refinements on the technology would make it possible to simultaneously record topography, biochemical recognition, mechanical properties and fluorescence imaging at the nanometer scale, and all at the single molecule level. Alternatively, AFM can be easily combined with other forms of superresolution optical nanoscopy such as STED, PALM and/or STORM.

Acknowledgements

Work in the M.F.G.-P. team has been mainly performed by T.S. van Zanten, C. Manzo and M. Meville and supported by the European Commission (FP7-ICT-2011-7, under grant agreement n° 288263), Spanish Ministry of Science and Agencia de Gestion d'Ajuts Universitaris i de Recerca (AGAUR). Work in the team of Y.F.D. was supported by the National Foundation for Scientific Research (FNRS), the Foundation for Training in Industrial and Agricultural Research (FRIA), the Université Catholique de Louvain (Fonds Spéciaux de Recherche), the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Program), and the Research Department of the Communauté française de Belgique (Concerted Research Action). Y.F.D. is a Senior Research Associate of the FNRS.

References

[1] D. Lingwood, K. Simons, Science 327 (2010) 46—50.
[2] K. Simons, M.J. Gerl, Nat. Rev. Mol. Cell Biol. 11 (2010) 688—699.
[3] A. Kusumi, C. Nakada, K. Ritchie, K. Murase, K. Suzuki, et al., Annu. Rev. Biophys. Biomol. Struct. 34 (2005) 351—378.
[4] A. Cambi, D.S. Lidke, ACS Chem. Biol. 7 (2012) 139—149.
[5] D. Goswami, K. Gowrishankar, S. Bilgrami, S. Ghosh, R. Raghupathy, et al., Cell 135 (2008) 1085—1097.
[6] M.E. Hemler, Nat. Rev. Mol. Cell Biol. 6 (2005) 801—811.
[7] M. Yanez-Mo, O. Barreiro, M. Gordon-Alonso, M. Sala-Valdes, F. Sanchez-Madrid, Trends Cell Biol. 19 (2009) 434—446.
[8] P. Lajoie, J.G. Goetz, J.W. Dennis, I.R. Nabi, J. Cell Biol. 185 (2009) 381—385.
[9] L. Belanis, S.J. Plowman, B. Rotblat, J.F. Hancock, Y. Kloog, Mol. Biol. Cell 19 (2008) 1404—1414.
[10] A. Cambi, F. de Lange, N.M. van Maarseveen, M. Nijhuis, B. Joosten, et al., J. Cell Biol. 164 (2004) 145—155.
[11] A. Cambi, B. Joosten, M. Koopman, F. de Lange, I. Beeren, et al., Mol. Biol. Cell 17 (2006) 4270—4281.
[12] J.J. Sieber, K.I. Willig, C. Kutzner, C. Gerding-Reimers, B. Harke, et al., Science 317 (2007) 1072—1076.
[13] B.F. Brehm-Stecher, E.A. Johnson, Microbiol. Mol. Biol. Rev. 68 (2004) 538—559.
[14] J.R. Maddock, M.R.K. Alley, L. Shapiro, J. Bacteriol. 175 (1993) 7125—7129.
[15] A. Janakiraman, M.B. Goldberg, Trends Microbiol. 12 (2004) 518—525.
[16] G. Ebersbach, C. Jacobs-Wagner, Trends Microbiol. 15 (2007) 101—108.
[17] S. Mayor, R.E. Pagano, Nat. Rev. Mol. Cell Biol. 8 (2007) 603—612.
[18] S. Manes, A. Viola, Mol. Membr. Biol. 23 (2006) 59—69.
[19] E.A. Jares-Erijman, T.M. Jovin, Nat. Biotechnol. 21 (2003) 1387—1395.
[20] R. Varma, S. Mayor, Nature 394 (1998) 798—801.
[21] P. Sharma, R. Varma, R.C. Sarasij Ira, K. Gousset, et al., Cell 116 (2004) 577—589.
[22] E. Betzig, G.H. Patterson, R. Sougrat, O.W. Lindwasser, S. Olenych, et al., Science 313 (2006) 1642—1645.
[23] S.T. Hess, T.P. Girirajan, M.D. Mason, Biophys. J. 91 (2006) 4258—4272.
[24] M.J. Rust, M. Bates, X. Zhuang, Nat. Methods 3 (2006) 793—795.
[25] B.F. Lillemeier, M.A. Mortelmaier, M.B. Forstner, J.B. Huppa, J.T. Groves, et al., Nat. Immunol. 11 (2010) 90—96.
[26] L. Balagopalan, E. Sherman, V.A. Barr, L.E. Samelson, Nat. Rev. Immunol. 11 (2011) 21—33.
[27] D.J. Williamson, D.M. Owen, J. Rossy, A. Magenau, M. Wehrmann, et al., Nat. Immunol. 12 (2011) 655—662.
[28] C. Eggeling, C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, et al., Nature 457 (2009) 1159—1162.
[29] F. de Lange, A. Cambi, R. Huijbens, B. de Bakker, W. Rensen, et al., J. Cell Sci. 114 (2001) 4153—4160.
[30] T.S. van Zanten, A. Cambi, M.F. Garcia-Parajo, Biochim. Biophys. Acta 1798 (2010) 777—787.
[31] P. Hinterdorfer, M.F. Garcia-Parajo, Y.F. Dufrene, Acc. Chem. Res. 45 (2012) 327—336.
[32] J.A. Veerman, A.M. Otter, L. Kuipers, N.F. van Hulst, Appl. Phys. Lett. 72 (1998) 3115—3117.
[33] D.J. Muller, J. Helenius, D. Alsteens, Y.F. Dufrene, Nat. Chem. Biol. 5 (2009) 383—390.
[34] D.J. Muller, Y.F. Dufrene, Trends Cell Biol. 21 (2011) 461—469.
[35] E. Betzig, J.K. Trautman, T.D. Harris, J.S. Weiner, R.L. Kostelak, Science 251 (1991) 1468—1470.
[36] K. Karrai, R.D. Grober, Appl. Phys. Lett. 66 (1995) 1842—1844.
[37] A.G.T. Ruiter, K.O. van der Werf, J.A. Veerman, M.F. Garcia-Parajo, W.H.J. Rensen, et al., Ultramicroscopy 71 (1998) 149—157.
[38] M. Koopman, B.I. de Bakker, M.F. Garcia-Parajo, N.F. van Hulst, Appl. Phys. Lett. 83 (2003) 5083—5085.
[39] M. Koopman, A. Cambi, B.I. de Bakker, B. Joosten, C.G. Figdor, et al., FEBS Lett. 573 (2004) 6—10.
[40] N.F. van Hulst, J.A. Veerman, M.F. Garcia-Parajo, L. Kuipers, J. Chem. Phys. 112 (2000) 7799—7810.
[41] T.S. van Zanten, A. Cambi, M. Koopman, B. Joosten, C.G. Figdor, et al., Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 18557—18562.
[42] T.S. van Zanten, J. Gomez, C. Manzo, A. Cambi, J. Buceta, et al., Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 15437—15442.
[43] M.F. Garcia-Parajo, Nat. Photonics 2 (2008) 201—203.
[44] L. Novotny, N. van Hulst, Nat. Photonics 5 (2011) 83—90.
[45] C. Hoppener, L. Novotny, Nano Lett. 8 (2008) 642—646.
[46] H.G. Frey, S. Witt, K. Felderer, R. Guckenberger, Phys. Rev. Lett. 93 (2004) 200801.
[47] T.H. Taminiau, R.J. Moerland, F.B. Segerink, L. Kuipers, N.F. van Hulst, Nano Lett. 7 (2007) 28—33.
[48] T.S. van Zanten, M.J. Lopez-Bosque, M.F. Garcia-Parajo, Small 6 (2010) 270—275.
[49] M.J. Saxton, K. Jacobson, Annu. Rev. Biophys. Biomol. Struct. 26 (1997) 373—399.
[50] S.A. Kim, K.G. Heinze, P. Schwille, Nat. Methods 4 (2007) 963—973.

[51] H.T. He, D. Marguet, Annu. Rev. Phys. Chem. 62 (2011) 417—436.
[52] D. Vobornik, D.S. Banks, Z.F. Lu, C. Fradin, R. Taylor, et al., Appl. Phys. Lett. 93 (2008) 163904—163906.
[53] M. Herrmann, N. Neuberth, J. Wissler, J. Perez, D. Gradl, et al., Nano Lett. 9 (2009) 3330—3336.
[54] C. Manzo, T.S. van Zanten, M.F. Garcia-Parajo, Biophys. J. 100 (2011) L8—L10.
[55] P.F. Lenne, L. Wawrezinieck, F. Conchonaud, O. Wurtz, A. Boned, et al., EMBO J. 25 (2006) 3245—3256.
[56] C. Hoppener, D. Molenda, H. Fuchs, A. Naber, J. Microsc. 210 (2003) 288—293.
[57] G.J. Bakker, C. Eich, J.A. Torreno-Pina, R. Diez-Ahedo, G. Perez-Samper, et al., Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 4869—4874.
[58] J.T. Groves, J. Kuriyan, Nat. Struct. Mol. Biol. 17 (2010) 659—665.
[59] M.L. Dustin, J.T. Groves, Annu. Rev. Biophys. 41 (2012) 1—24.
[60] T.B. Geijtenbeek, R. Torensma, S.J. van Vliet, G.C. van Duijnhoven, G.J. Adema, et al., Cell 100 (2000) 575—585.
[61] B.I. de Bakker, F. de Lange, A. Cambi, J.P. Korterik, E.M.H.P. van Dijk, et al., ChemPhysChem 8 (2007) 1473—1480.
[62] B.I. de Bakker, A. Bodnar, E.M.H.P. van Dijk, G. Vamosi, S. Damjanovich, et al., J. Cell Sci. 121 (2008) 627—633.
[63] D. Vobornik, Y. Rouleau, J. Haley, M. Bani-Yaghoub, R. Taylor, et al., Biochem. Biophys. Res. Commun. 382 (2009) 85—90.
[64] A. Abulrob, Z. Lu, E. Baumann, D. Vobornik, R. Taylor, et al., J. Biol. Chem. 285 (2010) 3145—3156.
[65] J.A. Chen, Y. Pei, Z.W. Chen, J.Y. Cai, Micron 41 (2010) 198—202.
[66] S. Chatterjee, S. Mayor, Cell. Mol. Life Sci. 58 (2001) 1969—1987.
[67] J. Chen, Y. Wu, C. Wang, J. Cai, Scanning 30 (2008) 448—451.
[68] C. Gomez-Mouton, J.L. Abad, E. Mira, R.A. Lacalle, E. Gallardo, et al., Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 9642—9647.
[69] A. Abulrob, Z. Lu, E. Brunette, D. Pulla, D. Stanimirovic, et al., J. Microsc. 232 (2008) 225—234.
[70] A. Ianoul, D.D. Grant, Y. Rouleau, M. Bani-Yaghoub, L.J. Johnston, et al., Nat. Chem. Biol. 1 (2005) 196—202.
[71] L.Y. Zhong, G.C. Zeng, X.X. Lu, R.C. Wang, G.M. Gong, et al., PLoS ONE 4 (2009).
[72] A. Engel, H.E. Gaub, Annu. Rev. Biochem. 77 (2008) 127—148.
[73] D.J. Muller, Y.F. Dufrene, Curr. Biol. 21 (2011) R212—R216.
[74] Y.F. Dufrene, Nat. Rev. Microbiol. 6 (2008) 674—680.
[75] G. Andre, S. Kulakauskas, M.P. Chapot-Chartier, B. Navet, M. Deghorain, et al., Nat. Commun. 1 (2010) 27.
[76] E. Dague, D. Alsteens, J.P. Latge, Y.F. Dufrene, Biophys. J. 94 (2008) 656—660.
[77] G. Francius, O. Domenech, M.P. Mingeot-Leclercq, Y.F. Dufrene, J. Bacteriol. 190 (2008) 7904—7909.
[78] P. Hinterdorfer, Y.F. Dufrene, Nat. Methods 3 (2006) 347—355.
[79] E.M. Puchner, H.E. Gaub, Curr. Opin. Struct. Biol. 19 (2009) 605—614.
[80] H. Li, Y. Cao, Acc. Chem. Res. 43 (2010) 1331—1341.
[81] P.E. Marszalek, Y.F. Dufrene, Chem. Soc. Rev. 41 (2012) 3523—3534.
[82] Y.F. Dufrene, E. Evans, A. Engel, J. Helenius, H.E. Gaub, et al., Nat. Methods 8 (2011) 123—127.
[83] V. Vogel, M. Sheetz, Nat. Rev. Mol. Cell Biol. 7 (2006) 265—275.
[84] P.N. Lipke, M.C. Garcia, D. Alsteens, C.B. Ramsook, S.A. Klotz, et al., Trends Microbiol. 20 (2012) 59—65.
[85] D. Alsteens, M.C. Garcia, P.N. Lipke, Y.F. Dufrene, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 20744—20749.
[86] M.C. Garcia, J.T. Lee, C.B. Ramsook, D. Alsteens, Y.F. Dufrene, et al., PLoS ONE 6 (2011) e17632.
[87] J.J. Heinisch, V. Dupres, S. Wilk, A. Jendretzki, Y.F. Dufrene, PLoS ONE 5 (2010) e11104.
[88] D. Alsteens, E. Pesavento, G. Cheuvart, V. Dupres, H. Trabelsi, et al., ACS Nano 3 (2009) 3063—3068.
[89] V. Dupres, D. Alsteens, S. Wilk, B. Hansen, J.J. Heinisch, et al., Nat. Chem. Biol. 5 (2009) 857—862.
[90] J.J. Heinisch, V. Dupres, D. Alsteens, Y.F. Dufrene, Nat. Protoc. 5 (2010) 670—677.
[91] M. Hofer, S. Adamsmaier, T.S. van Zanten, L.A. Chtcheglova, C. Manzo, et al., Ultramicroscopy 110 (2010) 605—611.

Yves Dufrêne received a PhD in bioengineering in 1996, at the Université catholique de Louvain (UCL, Belgium). After a post-doc at the Naval Research Laboratory (USA) with Gil Lee, he became Research Associate at the National Foundation for Scientific Research (UCL, Belgium). He is interested in understanding the nanoscale properties and interactions of biosurfaces, from single molecules to live cells, using atomic force microscopy. The ultimate goal is to elucidate the molecular bases of crucial cellular events such as cell adhesion and mechanosensing.

Maria Garcia-Parajo received a PhD in physical electronic in 1993 at Imperial College, London, UK. After a two-years postdoc at the CNRS-Bagneux, she spent 10 years at the Optical Techniques group of the University of Twente in the Netherlands. Since 2005 she is an ICREA Research Professor (Barcelona, Spain) and joint the Institute of Photonics Sciences, ICFO (Barcelona, Spain) in 2011 as leader of the Single Molecule Biophotonics group. Her team focuses on the development of nanophotonics and antennas approaches for the study of biological processes at the single molecular level on living cells. The group exploits fluorescence correlation spectroscopy in ultraconfined volumes, and multi-color single particle tracking to gain access to dynamic processes in living cells down to the μs time resolution.