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Recent advances in the application of atomic force microscopy to structural biology

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A B S T R A C T

The application of atomic force microscopy (AFM) for functional imaging and manipulating biomolecules at all levels of organization has enabled great progress in the structural biology field over the last decades, contributing to the discovery of novel structural entities of biological significance across many disciplines ranging from biochemistry, biomedicine and biophysics to molecular and cell biology, up to food systems and beyond. AFM has the capability to generate high-resolution topographic images spanning from the submolecular to the (sub)cellular range and can probe biochemical and biophysical sample properties in close to native conditions with excellent temporal resolution. Instrumental developments in the past decade enable dynamical structural and conformational studies of single biomolecules and new techniques for structural and chemical modification of the AFM probe have converted the cantilever into a versatile tool to study different biological phenomena, such as the mechanical stability of biomolecular complexes or the force induced dynamic changes of mechanically stressed proteins at the nanoscopic level. To improve the functionality of AFM and approach dynamic processes of complex biological systems *ex vivo*, AFM is combined with complementary microscopy, nanoscopy and spectroscopy tools. These multimethodological approaches provide unprecedented possibilities of probing physical, chemical and biological properties of complex cellular systems with high spatio-temporal resolution, leading to novel applications that correlate structural results with functional biochemical, biophysical, immunological, or genetic data of the system under study.

1. Introduction

Biological molecules, such as carbohydrates, lipids, nucleic acids, and proteins are involved in crucial tasks of cellular life, determining life-sustaining functions. In order to understand these functions within complex biological systems, a quantitative structural and functional characterization of their properties, which are heterogeneous at the nanoscale, is necessary. Moreover, the dynamic nature of biological processes, such as the assembly of lipids and proteins into functional domains in cellular membranes, the remodeling of the actomyosin cortex, or cell reshaping during mitosis, requires the use of highly sensitive techniques that work at high spatial and temporal resolution under physiological conditions. In this context, we will review how recent developments in AFM enabled its use to structurally characterize complex biological systems in their native-like state at (sub-)nanometer resolution and to observe dynamic biological processes in real time. Ideally, it is desirable to structurally and functionally probe proteins within the cell without the need to isolate them. In this regard, AFM offers the possibility to work in liquid conditions similar to physiological environments. We also identify current limitations of AFM operation for a diverse range of structural biology applications and the challenges lying ahead in terms of speed, lateral resolution, interplay between cantilever size and lateral resolution on soft biological samples, as well as integration with correlative techniques to achieve a multimethodological characterization of complex living biological systems.

The AFM was introduced more than three decades ago (Binnig et al., 1986) and key improvements over the past years in terms of force sensitivity (Bull et al., 2014), thermal stability (Churnside and Perkins, 2014), lateral and temporal resolution (Heath et al., 2021; Heath and Scheuring, 2019), as well as imaging modes (Dufrene et al., 2017) have expanded its capabilities from a multifunctional tool into a nanoscopic analytical laboratory (Müller et al., 2020). Fig. 1 shows an overview of significant developments that led to the establishment of AFM as a powerful technique in structural, molecular and cell biology. The operating principle of the first commercially available AFM relies on raster-scanning with a sharp tip at the end of a flexible cantilever spring over a sample, and monitoring the cantilever deflection changes as a result of tip-sample mechanical interactions, in what is widely known as contact mode operation of the instrument. During an experiment, variations in surface topography or material composition are recorded as bidimensional maps. Early on, the development of fluid chambers for imaging in buffer allowed maintaining the native state of the studied

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biological system, leading to applications of AFM to biomolecules, such as for reproducible imaging and dissection of plasmid DNA (Hansma et al., 1992). Although contact mode AFM is widely used to characterize solid substrates, its application to soft biological systems should be carefully evaluated, since imaging forces above 100 pN can cause irreversible deformations. Dynamic mode (DM) AFM in liquids was introduced in the early 1990s to minimize the high imaging and lateral forces applied to biological samples (Hansma et al., 1994). Here the AFM cantilever is oscillated at its resonance frequency, causing the probe to tap on the surface only at the extreme of each modulation cycle, which minimizes frictional forces present when the probe is constantly in contact with the surface. The oscillation amplitude is maintained as feedback, and the tip-sample distance changes to maintain the oscillation constant, while scanning over the surface. The capability of AFM to measure interaction forces was soon exploited in what was initially termed chemical force microscopy and is nowadays is commonly called force spectroscopy mode, by using the cantilever as a force sensor. In this mode, the stylus is cyclically approached and retracted from the surface while monitoring the variation of the force with respect to the tip-sample distance. Recording force-distance curves with pN sensitivity and sub-nanometer vertical resolution makes it possible to extract mechanical and physio-chemical properties of a wide variety of samples. In more advanced approaches, the AFM stylus can also be turned into a nanoscopy laboratory through [targeted (Wildling et al., 2011)] functionalization with chemical groups (Frisbie et al., 1994), ligands (Florin et al., 1994), lipids, proteins, or viruses in force spectroscopy experiments (Müller et al., 2020). Combining single molecule force spectroscopy (SMFS) and imaging through the use of force-distance curve-based

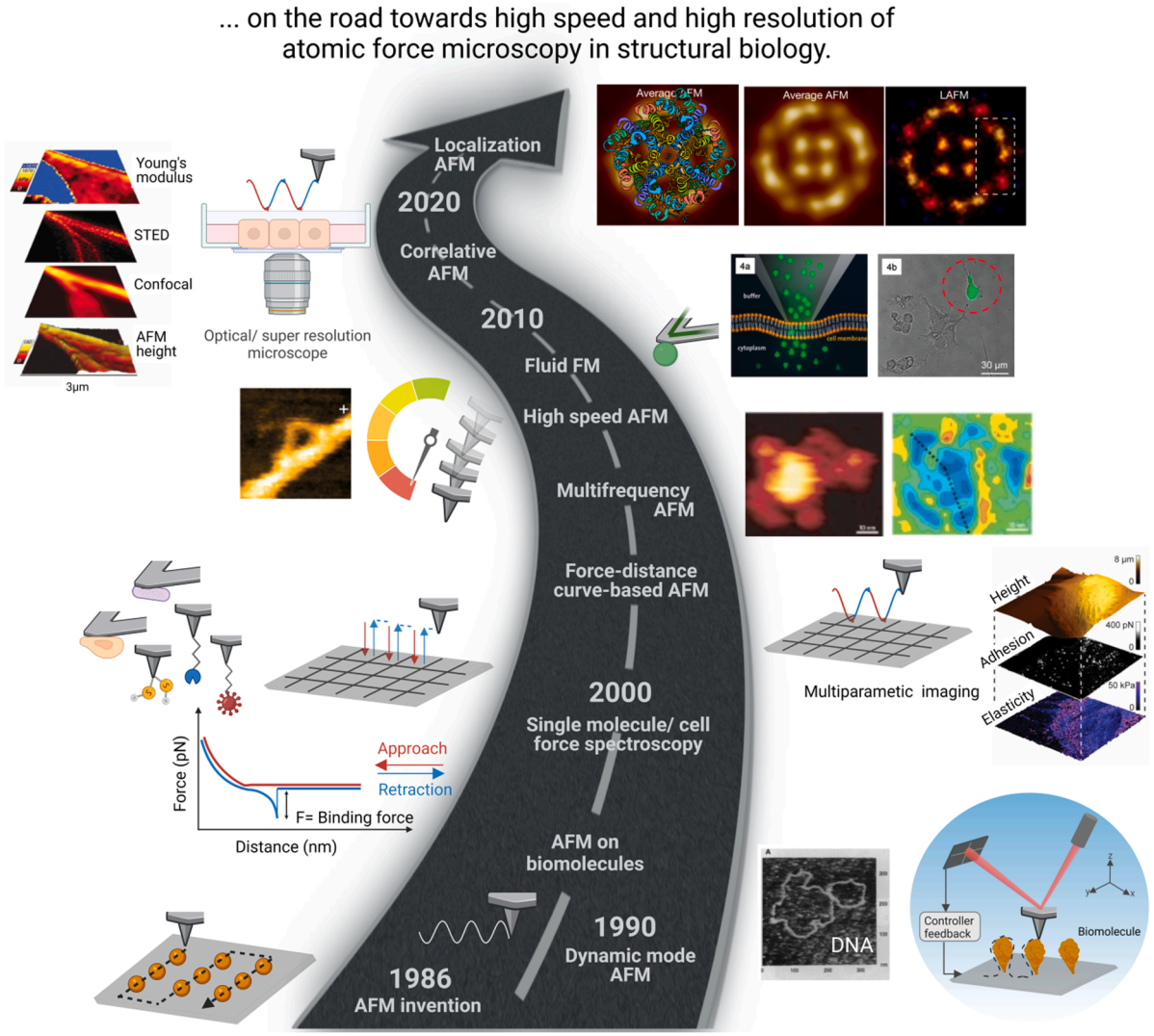

Fig. 1. Timeline of key developments, starting from the invention of AFM in 1986 to the latest AFM developments in the application to structural biology. Key inventions include: (i) Dynamic mode (DM) AFM, which oscillates the AFM tip to reduce friction while contouring the sample; (ii) An optical detection system and fluid cell enabling AFM imaging to be operated in aqueous solution (Hansma et al., 1992); (iii) Single molecule/ cell force spectroscopy, which enables to measure interaction forces between a (biomolecule) functionalized AFM tip and the sample by acquiring single force-distance-curves or pixel-by-pixel across the surface in a linear tip movement; (iv) Force-distance (FD) curve-based AFM which contours the sample while mapping multiple physical or chemical properties including imaging and mapping specific molecular recognition sites in a oscillating tip movement; (v) Multifrequency (MF) AFM, which contours the sample while detecting and/or exciting the cantilever at multiple frequencies, thus mapping various physical parameters (Garcia and Herruzo, 2012); (vi) High-speed AFM, which speeds up the image acquisition time by a factor of ~ 1000, providing access to dynamic processes in structural biology (Kodera et al., 2010); (vii) FluidFM, combining microfluidics and AFM, allowing to manipulate biological samples or to inject or extract a small amount of solutions into cells (Meister et al., 2009); (viii) Correlating advanced optical imaging and AFM for the study of complex biological systems (Miranda et al., 2021); (ix) Localization AFM, which overcomes current resolution limitations, facilitating single-molecule structural analysis with aminoacid resolution (Heath et al., 2021). The development of most modes has been powered by each other, ultimately leading to combinatorial AFM mostly used in structural biology studies nowadays. Cartoons are created with BioRender.com.

AFM (FD-AFM) has opened avenues for quantifying surface and structural properties of biological surfaces from reconstituted membranes to living cells, studying ligand-receptor dissociation dynamics, or localizing specific lipids, drug-binding sites, and bacterial surface-layer proteins (Müller et al., 2020; Lo Giudice et al., 2019). Precedent to FD-AFM, other dynamic (recognition) modes have been developed and applied to biological samples, such as harmonic (Dulebo et al., 2009) and torsional mode (Dong and Sahin, 2011; Husale et al., 2009), with the most prominent example being topography and recognition imaging (TREC) in which topography and molecular recognition signals are extracted from different regions of a magnetically driven cantilever oscillation cycle (Stroh et al., 2004). In order to increase the number of observables and decrease the data acquisition time, multifrequency AFM (M−AFM) methods have emerged [reviewed in (Garcia and Herruzo, 2012)], offering the possibility to use the amplitude, phase or frequency of the different cantilever oscillation modes to quantify and map various physical parameters. In the late 2000s, AFM investigations of biological molecules have turned their focus to mechanistic studies that specifically address structural dynamics and short-lived transition states. High-speed AFM is a particular technique that was invented to directly acquire video-like images of biological processes in 3D at high spatio-temporal resolution and with low scanning forces (Heath and Scheuring, 2019; Kodera et al., 2010). This is the result of numerous instrumental developments such as faster scanners and feedback operation, small ultrafast cantilevers, improved optical beam deflection and environmental control systems. Shortly after, microfluidic devices coupled to hollow AFM cantilevers, also named FluidFM, has been first introduced to manipulate cells or to inject or extract a small amount of solutions into cells (Meister et al., 2009). Around 2010, the advantages of combining microscopy approaches with AFM were acknowledged and implemented, making it possible to retrieve in-depth physical, chemical, and biological information of complex cellular systems (Dufrene et al., 2017; Müller et al., 2020). Combined with optical microscopy, AFM topography images and chemical maps can be correlated with larger cellular structures. However, for visualizing details and composition of smaller cellular compartments/ structures in a minimally invasive manner with high specificity, fluorescence and confocal microscopy are commonly combined with AFM. Just very recently, Heath et al. (Heath et al., 2021) presented a technique, called localization atomic force microscopy (LAFM), which overcomes current resolution limitations, facilitating single-molecule structural analysis with aminoacid resolution. By applying localization image reconstruction algorithms to peak positions in high-speed AFM and conventional AFM data, the authors were able increase the resolution beyond the limits set by the tip radius, and resolve single amino acid residues on soft protein surfaces in native and dynamic conditions.

Overall, AFM has shown an extraordinary potential to directly visualize molecular structures at work (Fig. 2), but also to biochemically image, sense and manipulate living biological systems (Müller et al., 2020). Probing out-of-equilibrium and close-to-equilibrium thermodynamic and kinetic parameters of biological bonds is also possible due to the unique ability of AFM to apply directional forces (Fig. 3). When further combined with advanced data analysis and theoretical biophysical models (Merkel et al., 1999; Dudko et al., 2008; Friddle et al., 2012), as well as optical microscopy and spectroscopy techniques (Dehullu et al., 2019; Dumitru et al., 2021; Koehler et al., 2021; Miranda et al., 2021; Odermatt et al., 2015; Rygula et al., 2018; Wood et al., 2011) (Fig. 4), AFM can provide a unique wealth of opportunities to simultaneously quantify structural, functional, and chemical parameters of biomolecular and cellular systems close to their native state.

2. AFM high-resolution imaging applied to structural biology

The main experimental methods widely employed to reveal detailed structural information of proteins are cryo-electron microscopy (cryo-EM), X-ray diffraction and nuclear magnetic resonance (NMR). These well-established approaches provided remarkable insights into inhibitor binding to ion channels or membrane proteins and structures of large or multi-protein complexes at single-digit nanometer resolution [reviewed in (Shimada et al., 2019; Renaud et al., 2018)]. However, these studies require complex sample preparation techniques, large amounts of purified proteins, have limited temporal resolution and usually explore a dominant conformational state among multiple possible states. Fluorescence resonance energy transfer (FRET) and super-resolution optical microscopy also provide high spatial resolution insights into protein structure and binding interactions, with millisecond temporal dynamics. The main limitation is the use of fluorescent labeling molecules that can interfere with function and the semi-quantitative parameters extracted from such experiments.

AFM stands out as a technique with unique capabilities, able to provide ≈1 nm lateral resolution, quantitative and dynamic information of macromolecular structures and protein binding events. Contrary to cryo-EM or X-ray studies, AFM can work in physiological conditions, which allows it to address a central challenge of modern structural biology: relating structural information and dynamic conformational changes to biological function. An additional advantage is that in AFM experiments, environmental conditions, such as buffer composition, temperature, applied force, and light can be altered during acquisition. AFM is however currently bound to scanning the surface of samples immobilized to a solid support, but optimized sample preparation and image acquisition have contributed to structural characterization of biomolecules in conformations close to their native state at sub-molecular resolution (Dufrene et al., 2017; Valotteau et al., 2019). Secreted proteins such as IgM and IgG antibodies are particularly interesting samples to be measured by AFM because their mechanical resilience and supramolecular interactions are directly related to their ability to perform cellular tasks. AFM revealed the interplay between structural features and mechanical properties in antibodies (Perrino and Garcia, 2016), as well as their self-assembly into immunoactive hexamers (Ido et al., 2014). Questions still remain regarding how the interplay between protein-surface interactions, hydration forces and limited conformational freedom of adsorbed proteins could distort their mechanical properties and self-assembly capacity on a given surface.

Studying dynamic processes by AFM had been a long-standing issue in the field due to the limited scanning speed of commercial systems. Several groups achieved key instrumental developments of feedback electronics, scanner and cantilever design that nowadays allow the recording of multiple frames per second (Viani et al., 1999; Picco et al., 2006; Ando et al., 2008). Commercial high-speed AFM systems can achieve sub-millisecond temporal resolution, which allows the technique to fill a gap where routine structural characterization techniques reach their limitations (Dufrene et al., 2017). The potential of high-speed AFM has been exploited to reach a comprehensive understanding of the mechanisms underlying protein conformational, assembly and structural dynamics (Fig. 2A). A few examples are structural and mechanistic studies of molecular motors (Kodera et al., 2010), light-driven proton pumps (Shibata et al., 2010), bacterial outer membranes (Yamashita et al., 2012; Fantner et al., 2010), and CRISPR-Cas9 activity (Shibata et al., 2017). Very recently, high-speed AFM height spectroscopy emerged as a powerful tool for measuring single membrane proteins' conformational dynamics in structural titration experiments (Fig. 2B) (Heath and Scheuring, 2019). Reducing the dimensionality of data acquisition to single line or single point measurements results in an unprecedented increase in temporal resolution in the microsecond range. This novel approach was recently used to directly correlate conformational and functional dynamics of loop-6 in membrane-reconstituted outer membrane protein G (OmpG), bridging an experimental gap for the analysis of fast biomolecular dynamics (Sanganna Gari et al., 2021). Fig. 2B depicts time-lapse high-speed AFM images of OmpG in open and closed states imaged at 5 frames per second. The possibility to probe single-molecules one at a time in AFM experiments allows detailed study of their kinetics. The reaction cycle of

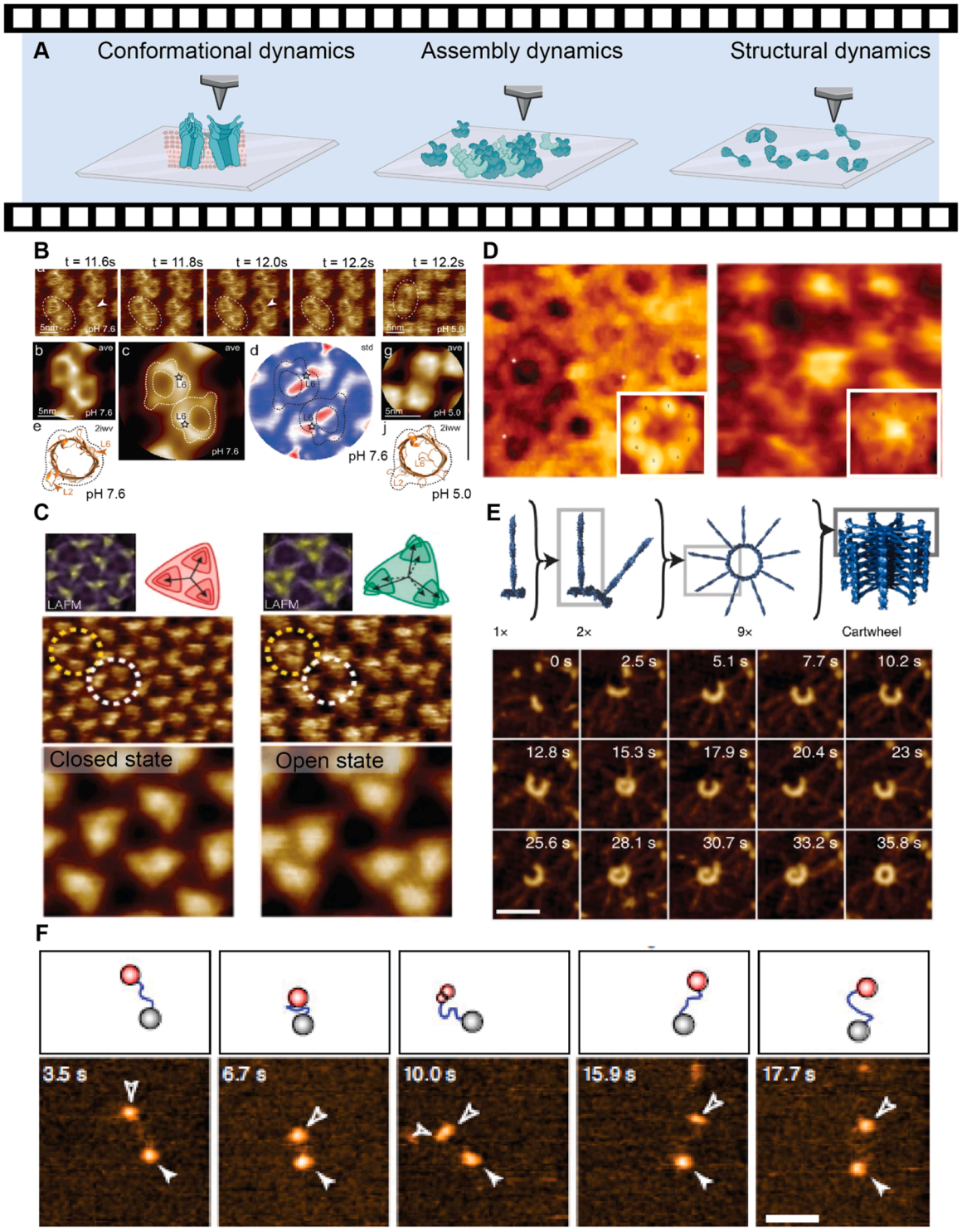

Fig. 2. Direct real-time visualization of proteins with high-speed AFM. (A) High-speed AFM has been applied to analyze the conformational, assembly and structural dynamics of proteins in liquid environment. (B) Time-lapse high-speed AFM imaging of OmpG membrane proteins in lipid bilayers imaged at 5 fps. The molecules are found predominantly in an open conformation at pH 7.6, while at pH 5.0 the channel is observed in a closed state (Sanganna Gari et al., 2021). (C) Structural and kinetic details of photo-activated conformational changes in bacteriorhodopsin D96N observed by high-speed AFM. Closed state dwell-times become shorter with increasing light, while open state dwell-times are of similar length independent of the light intensity (Perrino et al., 2021). (D) Visualization of native NPCs decorating the cytoplasm-facing outer nuclear membrane (left) and nuclear baskets protruding away from NPCs at the nucleoplasmic end (right). Insets represent average projected structures NPC complexes showing eight filaments surrounding a central ring (Sakiyama et al., 2016). (E) Top: sequence of CrSAS-6 assembly process: homodimers interact through their head domains and form intermediates that eventually assemble into nine-fold symmetric rings that form the centriolar cartwheel. Bottom: time-lapse images of in vitro CrSAS assemblies revealing the circular protein ring and the attached spokes (coiled-coil domains) (Nievergelt et al., 2018). (F) Schematics and high-speed AFM imaging of autophagy proteins containing intrinsically disordered regions. Temporarily appearing small globules in Autophagy-related protein 1 correspond to disordered regions (Kodera et al., 2021). Cartoons in A are created with BioRender.com.

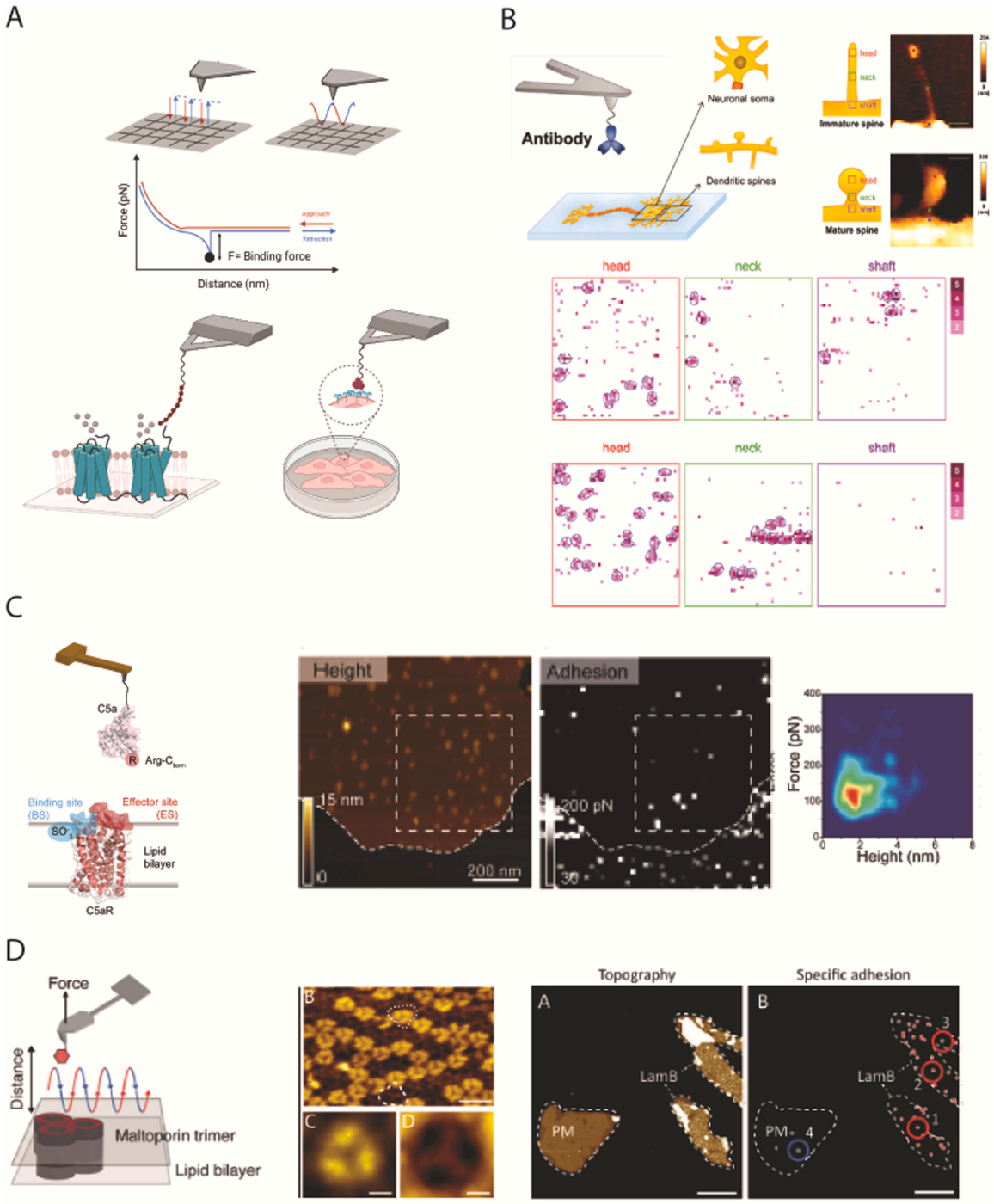

Fig. 3. Probing protein structure and conformation using AFM force spectroscopy. (A) Schematic representation of sample topography and adhesive properties probing using off-resonance methods, where the cantilever deflection is recorded while the tip-sample distance is modulated at frequencies lower than the fundamental one. In force curve AFM, for each pixel of the topography, the tip is approached and retracted using a linear or oscillating movement. Force-distance curves recorded between a ligand-functionalized AFM tip and proteins are used to quantify the binding forces, as the minimum force during the retraction segment. AFM tips functionalized with a ligand of interest can probe single proteins immobilized on a solid support or within the cellular plasma membrane. (B) Anti-LIMK1-tethered AFM tips probe individual LIMK1 proteins in cultured neurons directly using FV-AFM mapping. Mature and immature spines were used to visualize LIMK1 localization at the head, neck and shaft positions. Force-mapping of dendritic spines regions elucidated the spatial distribution of LIMK1 and found that the protein predominantly locates at heads of spines rather than dendritic shafts (Lim et al., 2022). (C) Cartoon representation of an AFM tip tethered with the C5a anaphylatoxin probed against a lipid bilayer embedded C5aR. The interaction between C5a and C5aR is stabilized by an N-terminus binding site (blue shading) and a functionally important effector site located in the extracellular region (red shading). Height and adhesion maps recorded while probing C5aR embedded in a lipid bilayer with a C5a modified AFM tip can be used to correlate the recorded binding forces with receptors orientation within the lipid bilayer, according to their measured height (Dumitru et al., 2020). (D) Schematics of the experimental setup used for probing specific interaction forces between maltoporin trimers reconstituted in a lipid bilayer and a maltodextrin functionalized tip with FD-AFM. Individual LamB trimers reconstituted in DMPC lipid membranes expose the extracellular surface protruding higher from the membrane compared to trimers exposing the periplasmic surface. Overview AFM topography and specific adhesion maps of co-adsorbed purple membrane from *Halobacterium salinarium,* to which maltotriose does not bind, and LamB using a maltotriose–functionalized AFM tip (Mulvihill et al., 2019). Cartoon in A is created with BioRender.com.

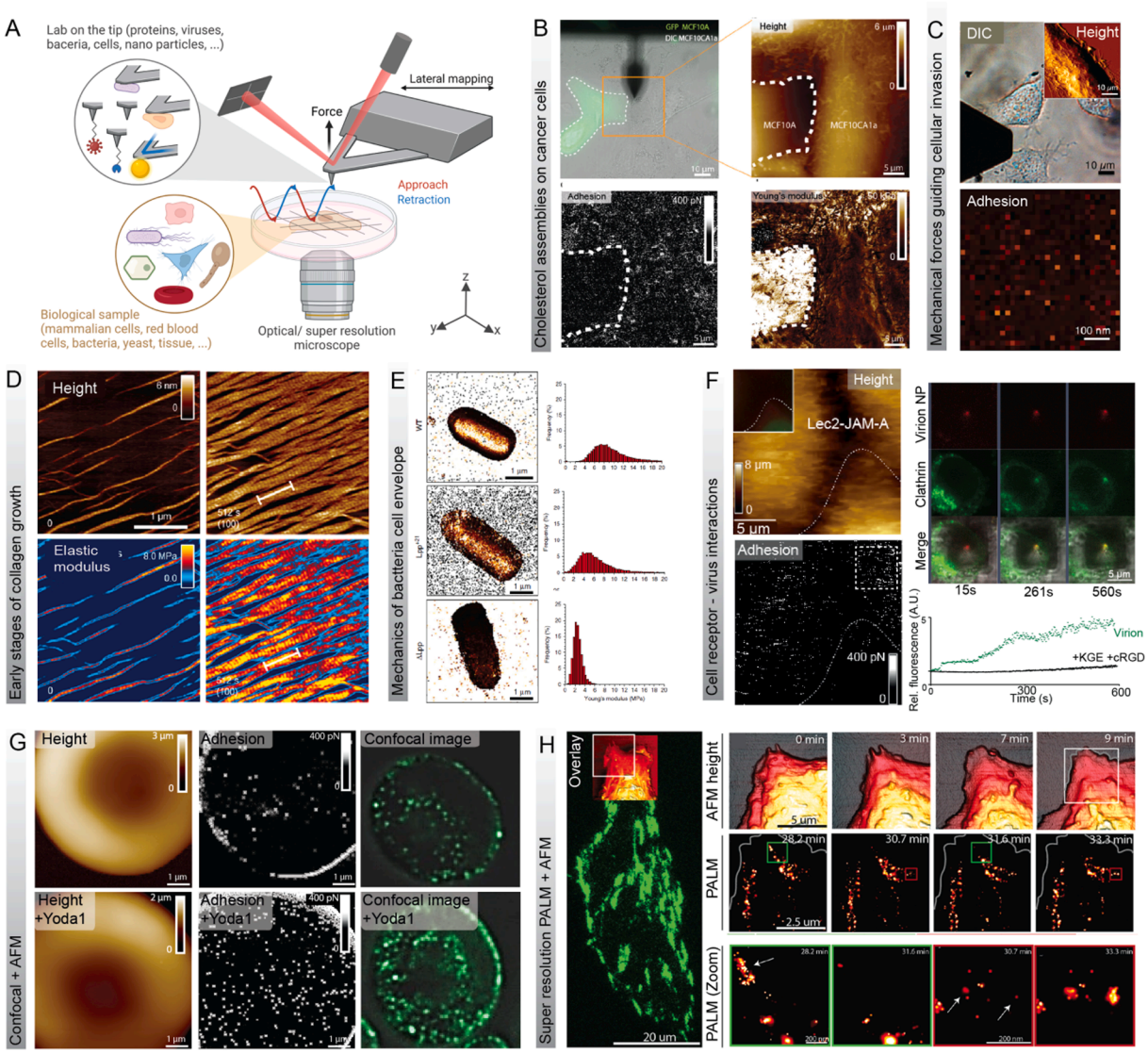

Fig. 4. From multiparametric imaging of cellular systems to combining (FD-based) AFM with other techniques for multimethodological characterization of biological structures. (A) FD-AFM can be used to contour the sample topography while measuring adhesive and mechanical (e.g. elasticity) properties. For each pixel of the sample, at least one FD curve is recorded. Additionally, the AFM can be combined with confocal or super resolution (PALM, STED) microscopy. Upgrading the bare cantilever to a lab on tip via (bio)chemical functionalization or using microfluidic hollow cantilevers (FluidFM) opens new avenues for correlative, multi-methodological characterization of biological samples. (B) Optical microscopy and FD-AFM height, adhesion and Young's modulus maps of cholesterol-enriched domains on adjacent healthy MCF10A and malignant MCF10CA1a cells probed with a θ-toxin functionalized AFM tip (Dumitru et al., 2020). (C) Forces between AFM probes functionalized with bacterial fibronectin (Fn) binding proteins FnPBA$^{(+)}$ and HUVEC monolayers are measured and quantified (histogram) at the nanoscale, showing that FnBPA mediates bacterial adhesion to soluble Fn via strong forces. Color scale (adhesion) = 400 pN (Prystopiuk et al., 2018). (D) The early stages of collagen microribbon growth and self-assembly are monitored by nanomechanical mapping (elastic modulus) at different time points. Over time, the physical properties changed, making it possible to identify 4 different growing stages (Gisbert et al., 2021). (E) The Young's modulus of the *E. coli* cell envelope is quantified at different Lipoprotein Lpp expression levels, conforming its role in regulating the outer membrane stiffness. Color scale (elasticity) = 10 MPa (Mathelié-Guinlet et al., 2020). (F) A single virion is covalently linked to the AFM tip, and interaction forces are probed on living cells expressing virus-specific JAM-A receptors. The fluorescently labelled cell (JAM-A deficient) in the lower right corner serves as an internal control, showing almost no binding events (Koehler et al., 2019). Right panel: Application of FluidFM in combination confocal microscopy to study time dependent clathrin recruitment upon landing of a virus decorated nanoparticle on the cell surface (Koehler et al., 2021). (G) Combining AFM with confocal microscopy reveals PIEZO1 mechanoreceptor localization on living red blood cells after activation. Upon interaction with Yoda1, an allosteric modulator, PIEZO1 clusters increase in abundance in regions of higher membrane tension and lower curvature (Dumitru et al., 2021). (H) Time-resolved, super resolution live-cell PALM in combination with AFM shows the reorganization of paxillin-mEos2 clusters on CHO-K1 cells (PALM channel) while simultaneously recording the samples topography (AFM height). The AFM sequence of the leading edge of the cell, showing the normal cell behavior of filopodia protrusion with subsequent lamellopodia extension. While the AFM images show the dynamics of the cell membrane, the PALM series monitors the changes in the paxillin-mEos2 clusters (Odermatt et al., 2015). Cartoon in A is created with BioRender.com.

bacteriorhodopsin, a light-driving proton pump had been studied by spectroscopic analyses, X-ray, cryo-EM and NMR based methods. However, the identification of all reaction intermediates and monitoring individual proteins conformational changes had not been possible using the ensemble averaged data acquired with these techniques. To measure single molecule kinetics of bacteriorhodopsin upon light activation, a green laser was integrated into a high-speed AFM to stimulate the protein during image acquisition (Perrino et al., 2021) (Fig. 2C). Open and closed conformational states of bacteriorhodopsin proteins were observed during and after light stimulation, which allowed to resolve

single molecule rate constants at different pH. Due to the very fast dynamics of wild-type bacteriorhodopsin proteins (≈ 10 ms), high-speed AFM line scanning was used to obtain kymographs reporting structural changes of contoured molecules with a temporal resolution of 1.6 ms. Despite the impressive high-resolution studies of membrane proteins using high-speed AFM, the particular sample preparation methods used for these experiments can be a limiting factor. Proteins embedded in solid-supported lipid bilayers have lower mobility and could display different conformational and structural features than in their native environment.

Nucleocytoplasmic transport is a process that proceeds through nuclear pore complexes (NPC) in a matter of milliseconds in vivo. This process involves a series of intrinsically disordered proteins called phenylalanine-glycine nucleoporins (FG Nups). The barrier mechanism mediated by NPCs had been elusive for structural analysis studies due to the lack of proper techniques that can visualize and probe the time-dependent behavior of FG Nups inside the pores. Stable tapping-mode and minimally disturbing high-speed AFM measurements provided insights into the nanoscopic spatiotemporal dynamics of FG Nups inside *Xenopus laevis* oocyte NPCs at timescales of ~100 ms (Fig. 2D), approaching coarse-grained computer simulations (Sakiyama et al., 2016). This study shows that FG Nups that rapidly elongate and retract in the cytoplasmic orifice and do not cohere into a tightly crosslinked meshwork, which is consistent with the diffusive motion of tethered polypeptide chains.

Studying the assembly dynamics of proteins is usually a challenging task due to the weak interactions formed in the process, which are characterized by dissociation constants in the micromolar range. Commercial AFM instruments still display certain limitations in terms of how accurate and fast the cantilever deflection can be regulated. This is a key factor in preventing force-induced conformational changes or sample damage when studying proteins assembly dynamics. Thanks to better force control, photothermal off-resonance tapping, a new approach gentle enough to monitor self-assembly reactions driven by weak interactions, was able to follow the assembly kinetics of ring formation of SAS-6 proteins. This study resolved the nine-fold symmetrical structure in real-time and revealed how distinct biogenesis routes can be followed to assemble the complex (Fig. 2E) (Nievergelt et al., 2018).

The characterization and understanding of intrinsically disordered proteins have been particularly difficult tasks in structural biology. The structure of these highly dynamic proteins samples a multitude of conformational states, which combined with their thin nature renders them inaccessible for most structural biology techniques. High-speed AFM is one of the few techniques versatile enough to provide a simultaneous and temporal assessment of both local, and overall structures of such proteins, even with semiresidue resolution (Kodera et al., 2021). Fig. 2F displays structural features of Autophagy-related protein 1 and the appearance of transient small globules. This study showed that high-speed AFM can identify both fully disordered regions and the border region with folded domains at the near residue level, without fragmentation of the entire chain. The dynamic nature of the acquisition process allowed to extract information of the structural nature of the temporarily folded structures, their transition dynamics and order propensity.

3. Probing protein structure and conformation using FD-AFM force spectroscopy

The AFM stylus can also be turned into a nanoscopy laboratory through functionalization with chemical groups, ligands, lipids, proteins, or viruses in force spectroscopy experiments. This is usually achieved with quasi-static or off-resonance methods that combine single molecule force spectroscopy (SMFS) and imaging. Force-distance AFM (FD-AFM) is an off-resonance method where in each pixel of the scanned area, the tip is approached and retracted using either a linear or oscillating movement. Force-distance curves recorded in FD-AFM provide the variation of the tip-sample interaction force with respect to the tip-sample distance on each point of the surface (Dufrene et al., 2017; Müller et al., 2020). The use of functionalized tips in combination with FD-AFM has opened avenues for quantifying surface and structural properties of biological surfaces from reconstituted membranes to living cells, studying ligand-receptor dissociation dynamics, or localizing specific lipids, drug-binding sites, and bacterial surface-layer proteins (Müller et al., 2020; Lo Giudice et al., 2019) (Fig. 3A).

Most techniques used to detect and quantify ligand binding to cell membrane proteins rely on signal amplification or immunolabeling. However, these techniques either require harsh experimental treatments like cell lysis or require gene editing to increase the abundance of the studied protein. Force curve nanoscale mapping provides quantitative information regarding the spatial distribution and expression levels of membrane proteins, as recently shown for individual LIMK1 proteins in cultured neurons (Lim et al., 2022) (Fig. 3B). LIMK1 is a protein involved in the growth of neurons, especially of dendritic spines. High-resolution AFM force maps were obtained at neuronal somas and dendritic spines using AFM tips functionalized with anti-LIMK1 antibodies. This work reveals that LIMK1 proteins are more abundant at the head than at the shaft in both immature and mature spines, and proposes a model for the molecular mechanism associated with LIMK1 localization in dendritic spines.

FD-AFM proved to be a powerful and highly sensitive tool able to measure the ligand-binding free-energy landscape of G protein-coupled receptors (GPCRs), their supramolecular assembly in native membranes, and the interactions governing their structural properties (Sapra et al., 2019; Alsteens et al., 2015). Studying the mechanism and quantifying the kinetic parameters of ligand binding to GPCR is not straightforward, especially when dealing with large endogenous ligands in the kDa range or multiple binding sites. A recent study of the C5a anaphyltoxin binding to its receptor showed for first time a cooperativity between the two orthosteric binding sites, where the N-terminal site serves as a kinetic trap and the transmembrane domain acts as the functional site (Dumitru et al., 2020) (Fig. 3C). Since the AFM stylus is a force transducer that can probe bond dissociation, SMFS measurements can also elucidate fundamental aspects of thermally activated bond ruptures under load to provide insights into their energy landscapes (Müller et al., 2020). This is achieved by converting rupture forces measured by SMFS into the equilibrium energy or dissociation constant using appropriate biophysical models (Merkel et al., 1999; Dudko et al., 2008; Friddle et al., 2012) [reviewed in (Müller et al., 2020)].

Applied to other membrane proteins, FD-AFM proved to be a promising nanotechnological approach to detect and map sugar binding and transport of LamB maltoporins (Mulvihill et al., 2019) (Fig. 3D). These outer membrane proteins of Gram-negative bacteria have the role of facilitating the diffusion of maltosaccharides and other hydrophilic molecules across membrane. Despite the fact that crystal structural studies provide detailed descriptions of maltodextrins bound to the LamB, basic aspects of the sugar-uptake and -diffusion mechanism remain unclear. FD-AFM was used to address a contradiction regarding the symmetry of the sugar-binding, -uptake and -transport mechanism by maltoporins by imaging single LamB trimers in a lipid bilayer at sub-nanometer resolution and simultaneously quantify the binding of different malto-oligosaccharides. FD-AFM probed the adhesion of maltotetraose to wild-type LamB trimers exposing either their higher-protruding extracellular or lower-protruding periplasmic surface to the liquid interface. This study concluded that an asymmetrical two-barrier model describes sugar binding and -uptake of LamB, since periplasmic and extracellular surfaces of LamB expose different affinities for the sugar.

Despite the advantages of higher scanning speeds in some FD-AFM approaches, this can also be a drawback when probing biomolecular interactions far from equilibrium. Very short, sub-microsecond contact times between the tip and the sample could limit the formation of stable bonds or shift the energy landscape towards certain states with low

association rates, while the high retraction speed may elude intermediate states that require conformational rearrangements prior to dissociation.

4. From multiparametric imaging of cellular systems to combining (FD-based) AFM with other techniques for multimethodological characterization of biological structures.

Over the last decade, efforts have been made to investigate cellular systems in physiologically relevant conditions, obviating the use of phototoxic buffers. This was achieved by carefully controlling the working environment with living cells in terms of pH, salts, CO_2, and humidity, as well as tailoring cell immobilization techniques for different applications (Müller et al., 2020; Alsteens et al., 2017). For example, FD-AFM can now be used with living cells and many studies focus on measuring their mechanical properties (Young's modulus and stiffness) under different conditions. However, biological samples are intrinsically viscoelastic, meaning that their mechanical response combines the elastic response of a solid and the viscous flow of a liquid. A plethora of recently developed descriptions of viscoelastic materials now allow the quantification of viscoelastic parameters such as loss and storage moduli, viscosity coefficient or loss tangent from AFM data (Garcia, 2020; McCraw et al., 2021). Additionally, by functionalizing the tip with a specific binding partner (e.g., proteins, viruses, nanoparticles or whole cells and bacteria) interaction sites within complex biological samples can be structurally mapped at molecular resolution (Fig. 4A). This methodology can be used to combine molecular recognition and nanomechanical measurements to detect cholesterol assemblies on breast cancer cells in a label-free manner (Fig. 4B) (Dumitru et al., 2020). Results from such studies demonstrated that the presence of altered levels of plasma membrane-associated cholesterol levels leads to stiffening of the membrane that is uncoupled from cytoskeletal elasticity. In another study, single bacterial AFM probes were used to quantify mechanical forces guiding *Staphylococcus aureus* cellular invasion, revealing that fibronectin (Fn)-binding adhesins FnBPA mediates bacterial adhesion to soluble Fn via strong forces (Prystopiuk et al., 2018) (Fig. 4C). This activation mechanism emphasizes the importance of protein mechanobiology in regulating bacterial-host adhesion, as revealed by AFM. However, the relationship between mechanical properties of proteins and their function is still emerging. The dynamics of proteins on surfaces and their self-assembly has an impact on a variety of biological processes, such as e.g. polymerization of the cellular actin cytoskeleton or the amyloid fibrils involved in neurodegenerative diseases, which have to be followed in real time and with high spatial resolution. HS-AFM is an optimal tool to follow such processes in real time, but a key is limitation that the data does not provide information about the mechanical properties of the sample, such as elastic modulus or loss tangent. Introducing bimodal AFM and instrumental developments in scanning speed of commercial AFMs, enabled the acquisition of high-spatial resolution maps of topography and the corresponding Young's modulus at imaging rates of as high as 5 fps (Gisbert et al., 2021). As a first proof-of-concept, this approach was used to identify the four stages collagen structures self-assembly (Fig. 4D), where earlier studies failed to resolve the temporal and mechanical properties. In another study, AFM-based indentation experiments on *E. coli* were used to capture the interplay between their structural and mechanical properties, enabling to quantify the mechanical properties of whole bacterial cells and isolated cell walls. Focusing on the role of lipoprotein Lpp in regulating cell envelope mechanics (Mathelié-Guinlet et al., 2020) (Fig. 4E), the authors revealed that the length and structural properties of Lpp have evolved in such a way that this protein is an important factor controlling envelope mechanics.

A current limitation of AFM is that it can only probe the sample surface and the precise identification of morphological and structural details becomes increasingly challenging with complex biological samples. However, resolving subsurface structures and providing analytical information of proteins and their assemblies is possible with variants of the AFM, such as scanning thermal microscopy (Zhang et al., 2020), electric and Kelvin probe force microscopy (Gachon and Mesquida, 2021), magnetic force microscopy (Hsieh et al., 2010) and ultrasonic AFM excitations (Sharahi et al., 2021). Nevertheless, the advantages of combining microscopy approaches with AFM were soon acknowledged and implemented, making it possible to retrieve in-depth physical, chemical, and biological information of complex cellular systems (Fig. 4A). Combined with optical microscopy, AFM topography images and chemical maps can be correlated with larger cellular structures. However, for visualizing details and composition of smaller cellular compartments/ structures in a minimally invasive manner with high specificity, fluorescence and confocal microscopy are commonly combined with AFM. In virology, AFM tips bearing single virions in combination with FD-AFM and confocal microscopy emerged as a useful tool to investigate the first binding steps to cell surface receptors and has been applied on a variety of viruses (Lo Giudice et al., 2019; Koehler et al., 2020). For instance, the direct interplay between attachment factors of reoviruses binding to entry cell surface receptors was observed for the first time by AFM (Koehler et al., 2019). Additionally, using the FluidFM technique enabled elucidating time-dependent clathrin recruitment upon landing of a virus decorated nanoparticle on the cell surface (Fig. 4F) (Koehler et al., 2021). Very recently, the combination of AFM with confocal microscopy was applied to probe PIEZO1 mechanoreceptor localization on living red blood cells upon activation (Dumitru et al., 2021) (Fig. 4G), showing PIEZO1 microcluster formation, which is regulated by membrane tension and cytoskeleton anchoring. Applying AFM in combination with confocal microscopy on mechanosensitive proteins (*i.e.* ion channels) is an elegant way to study their response upon targeted, controlled mechanical stimulation under different environmental conditions (Gaub and Müller, 2017), shedding light into basic receptor properties such as their gating mechanism, the interaction with extracellular matrix proteins, and the response to mechanical stimulation. Combining AFM with super resolution microscopy techniques, such as Photo-Activated Light Microscopy (PALM, Fig. 4H) (Odermatt et al., 2015) or STimulated Emission Depletion (STED) microscopy (Miranda et al., 2021) has opened new avenues of studying complex biological samples and dynamic processes ranging from bacterial cells in aqueous conditions to living mammalian cells. Another field of multimethodological characterization of complex cellular systems includes spectroscopic AFM-based approaches, such as tip-enhanced infrared (IR) spectroscopy, scanning near field microscopy (SNOM) and tip-enhanced Raman spectroscopy (TERS). In SNOM, the tip is replaced by an optical fiber with a nanoscale aperture to simultaneously record topographic and optical images, making it possible to not only visualize structural but also chemical differences (Müller et al., 2020; Rygula et al., 2018). Going even further, TERS combines the spatial resolution of AFM with the chemical selectivity of Raman spectroscopy, as has been shown for hemozoin crystals within a sectioned, malaria parasite-infected erythrocyte cell (Wood et al., 2011).

However, combining AFM with other microscopy techniques usually requires a trade-off between spatial and temporal resolution, and especially with living samples, illumination intensity and labelling methods are critical issues. In particular, for hybrid AFM – superresolution systems additional compromises need to be considered, such as e.g. synchronization of data acquisition in the different imaging modes or spatial overlap of the field of view.

5. Perspectives

In the recent years, the use of different microscopy techniques has enabled an increases in knowledge regarding the structural biology of different biomolecular and cellular specimens. Multimethodological approaches have filled the gaps of information that a single technique could not provide. In the future, using AFM with complementary methods such as SNOM, single molecule fluorescence, magnetic

resonance imaging, crystallography, ultrasonic imaging, light sheet florescence microscopy or optical/ magnetic tweezers is expected to open new avenues for enhancing AFM-based applications for structural biology. During the next few years, *in silico* techniques are also expected to experience enhancements in computing speeds, increasing the use of molecular dynamics experiments as a complementary tool. This will enhance our understanding about molecular mechanisms underlying protein functions or interactions after certain stimuli with great atomistic detail and closer to real experimental time scales. As we have highlighted, AFM-based spectroscopic methods (TERS, photothermal induced resonance) offer new possibilities for chemical and spectroscopic imaging of complex biosystems at the nanoscale and future efforts for the integration with biologically relevant environmental conditions will allow the analysis of dynamic cellular compartments. Machine learning has already permeated several areas of AFM, such as autonomous operation, image, particle and force spectroscopy data analysis. We expect further dialogue between the two communities to foster further progress in the analysis of large datasets, dynamic cellular processes or image-based noninvasive detection of different pathologies. We also predict AFM-based (bio)-chemical imaging will go beyond its initial purpose of sensing and localizing specific interactions between biological partners, and proceed to triggering reactions that induce structural conformations of proteins and cellular elements. This will be achieved by implementing the use of advanced functionalization protocols, photoswitchable linkers or fluorescently labeled ligands, which will open avenues for groundbreaking experiments in structural biology studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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