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High-speed atomic force microscopy of membrane and membrane protein dynamics

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High-speed atomic force microscopy (HS-AFM) enables direct nanometer-resolution visualization of single molecules and molecular assemblies in real-time and under physiological conditions, providing unique insights into how membranes and membrane proteins move and interact within native lipid environments. Recent methodological advances and integration with complementary techniques have extended HS-AFM to increasingly complex, physiologically relevant systems, bridging gaps between high-resolution static structural methods and low-resolution functional dynamics. Here, we highlight how HS-AFM has changed our understanding of membrane organization, protein conformational dynamics, and lipid–protein coupling. By capturing transient events inaccessible to ensemble approaches, HS-AFM is transforming our ability to connect structural snapshots with functional behavior, advancing dynamic structural biology.

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Introduction

Despite being the target of over 50% of pharmaceutical drugs, lipid membranes and their proteins remain challenging to study in native-like environments. Membranes are chemically diverse, highly dynamic materials, with a library of lipids that influence thickness, curvature, phase heterogeneity, lateral pressure, and charge [1]. These properties collectively shape membrane-protein function, influencing conformational dynamics, oligomerization, diffusion, and long-range self-assembly.

Traditional approaches such as X-ray crystallography and cryo-electron microscopy (cryo-EM) yield high-resolution structures of membrane proteins [2,3] and, increasingly, of tightly bound lipids [4]. However, these snapshots cannot capture transient conformational states, interactions, or membrane behaviors occurring on millisecond to second timescales. Molecular dynamics (MD) simulations complement structural methods by providing atomistic insight into protein and single lipid dynamics [5], but their accessible timescales (typically nanoseconds to microseconds) and limited system size can restrict their ability to capture slower, larger-scale events. Single-molecule fluorescence approaches, such as DNA-PAINT [6], MINFLUX [7], and smFRET [8], enable the tracking of proteins, lipids and conformational dynamics in a cellular context. However, because they track fluorophores rather than the protein structure, conformational changes must be indirectly inferred. Combined with the limitations of other static and computational methods, this underscores the need for approaches that directly visualize protein and membrane dynamics in real space.

High-speed atomic force microscopy [9] (HS-AFM) bridges this gap by directly imaging individual membrane proteins and their surrounding membrane at nanometer spatial and sub-second temporal resolution under controllable, near-physiological conditions [10]. Depending on the imaging mode, current HS-AFM achieves frame rates of ~1–20 frames per second for typical imaging in amplitude modulation tapping mode and up to 50–70 frames per second with recent developments [11], with line-scanning reaching millisecond resolution and height-spectroscopy extending to ~10 μs temporal resolution [12]. These speeds are primarily limited by cantilever resonance frequency and size, feedback bandwidth, scanner inertia, and tip–sample interaction forces, which constrain stable tracking of soft biological surfaces. Recent applications to complex systems, combined with advances such as localization AFM (LAFM) [13] for sub-nanometer

resolution and integration with cryo-EM and MD data are further enhancing HS-AFM's role in revealing structure–function relationships in dynamic membrane systems.

In this review, we highlight recent key studies over the past 2–3 years demonstrating the use and development of HS-AFM methods for understanding dynamics at membranes including conformational transitions, dynamic assembly processes, and lipid–protein interactions. We also discuss methodological and computational developments that are establishing HS-AFM as a vital tool for studying dynamic membrane systems.

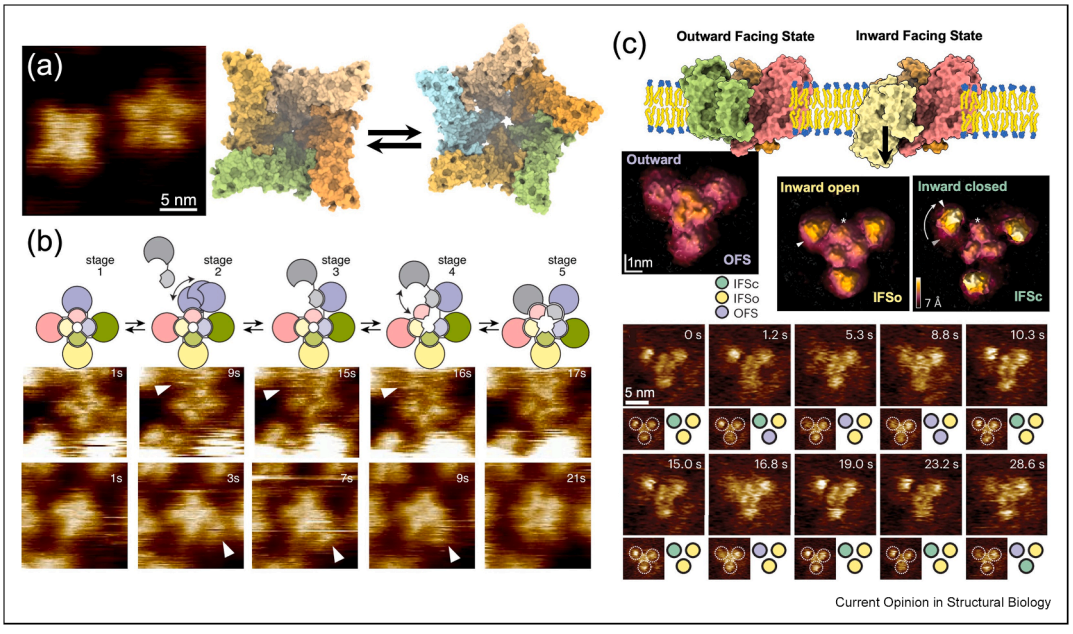
Membrane protein dynamics and conformational changes

A key strength of HS-AFM lies in its ability to capture a continuous structural trajectory of single molecules to reveal previously unknown states and, crucially, the kinetic pathways that link them. This capability enables the identification of short-lived states and oligomeric assemblies while providing the temporal context of these structural changes.

Membrane ion channels depend on rapid structural changes to open and close their pores and regulate ion flow. To date, hundreds of transient receptor potential (TRP) channel structures from over 20 family members have been solved, all adopting a tetrameric assembly [14]. However, recent HS-AFM imaging of TRPV3 revealed that this four-subunit structure is not fixed and that TRPV3 channels can assemble into a five-subunit pentameric structures (Figure 1a) [15]. HS-AFM movies captured pentameric states in equilibrium with tetramers and visualized subunit addition and loss over seconds to minutes via lateral protomer diffusion (Figure 1b). Crucially, the HS-AFM observations also guided the conditions used to stabilize and solve the first pentameric structure of any TRP channel. Building on the initial HS-AFM and cryo-EM observations, the pentameric TRPV3 structure was later refined to resolve side-chain positions and define the expanded pore architecture [16]. This paradigm-shifting work suggests that other TRP and even unrelated ion channels may use similar oligomeric transitions to modulate pore opening.

TRPV2 has also been recently studied by HS-AFM revealing how the intrinsically disordered N and C terminal regions, unresolved in cryo-EM, dynamically extend from the channel core and mediate transient interactions between tetramers [17]. These intrinsically disorded region (IDR)-mediated contacts occur on

Figure 1



Dynamic structural analysis of membrane proteins using HS-AFM and LAFM. (a) HS-AFM topography of a TRPV3 tetramer and pentamer (left) and corresponding structural models (right), illustrating reversible tetramer–pentamer transitions [15]. **(b)** Sequential HS-AFM frames capturing subunit rearrangements over time, with schematic representations above each frame highlighting the stages of tetramer–pentamer interconversion. Arrowheads indicate moving subunits [16]. **(c)** High-resolution LAFM analysis of the *Pyrococcus horikoshii* transporter Glt_{Ph}. Top: structures of outward- and inward-facing states. Middle: 3DLAFM maps of outward-facing (OFS) and inward-facing states (IFSopen and IFSclosed) with structural assignments (OFS: purple, IFSopen: yellow, IFSclosed: green). Bottom: time-lapse HS-AFM frames showing single-molecule conformational dynamics across multiple inward-facing substates, with corresponding schematic representations of subunit configurations below each frame [18]. HS-AFM, high-speed atomic force microscopy; LAFM, localization AFM.

second timescales and across a wide range of angles, suggesting a general mechanism for TRP channel clustering and long-range organization.

Membrane transporters operate through often complex, sequence-dependent conformational landscapes coupled to substrate binding and energy sources. By resolving the temporal sequence of conformational states, high-speed imaging combined with LAFM analysis of the *Pyrococcus horikoshii* amino acid transporter GltPh (Figure 1c) revealed for the first time that the inward-facing state is a kinetically trapped conformation underlying 'wanderlust' behavior, in which the transporter alternates between active and silent phases [18]. By combining HS-AFM structural and temporal information, transitions and kinetics between six distinct structures were resolved from single molecules. Previous HS-AFM line-scanning had revealed the Glt_{Ph} 'elevator' movement dynamics between outward, inward, and an intermediate state with millisecond time resolution; however, these experiments imaged only the extracellular face [19].

Imaging of membrane proteins reconstituted in vesicles allows access to either the intracellular or extracellular face, but side views can be valuable for proteins with extended extra-membranous regions. Attaching protein-containing nanodiscs to the surface enables this side view by HS-AFM [20]. HS-AFM imaging of GluA2 ionotropic glutamate receptor complexes in nanodiscs revealed state-dependent dynamic behaviors of the receptor's N-terminal domains (NTDs), which mediate synaptic clustering [21]. NTD dimers split into monomers, allowing receptor subunit exchange and more interaction sites with neural pentraxin 1 (NP1). HS-AFM has also been applied to study ATP-driven mechanical cycles and protein translocation. Notably, the first direct visualization of protein translocation across cellular membranes was achieved using HS-AFM of nanodiscs to track, in real time, the movement of an unfolded substrate protein through a single SecYEG—SecA complex [22]. Similarly, HS-AFM has been used to monitor conformational changes in the mitochondrial AAA-ATPase Bcs1's structure and interaction with the Rieske iron—sulfur protein (ISP) during translocation [23]. Using HS-AFM line-scanning, it was possible to observe ISP binding kinetics with 5 ms time resolution showing that ISP attaches precisely to Bcs1's apo state and that its lifetime matches the apo state, enabling substrate translocation.

Extending these insights to other membrane proteins, recent HS-AFM has revealed real-time dynamics within TMEM16F dimers [24]. The two subunits reversibly shift between compact-symmetric and loose asymmetric conformations, swinging apart and together, while each subunit undergoes Ca^{2+}-triggered ~1 nm height changes. These observations highlight HS-AFM's ability to detect transient intermediates and asynchronous subunit activation, which are difficult to capture with ensemble methods.

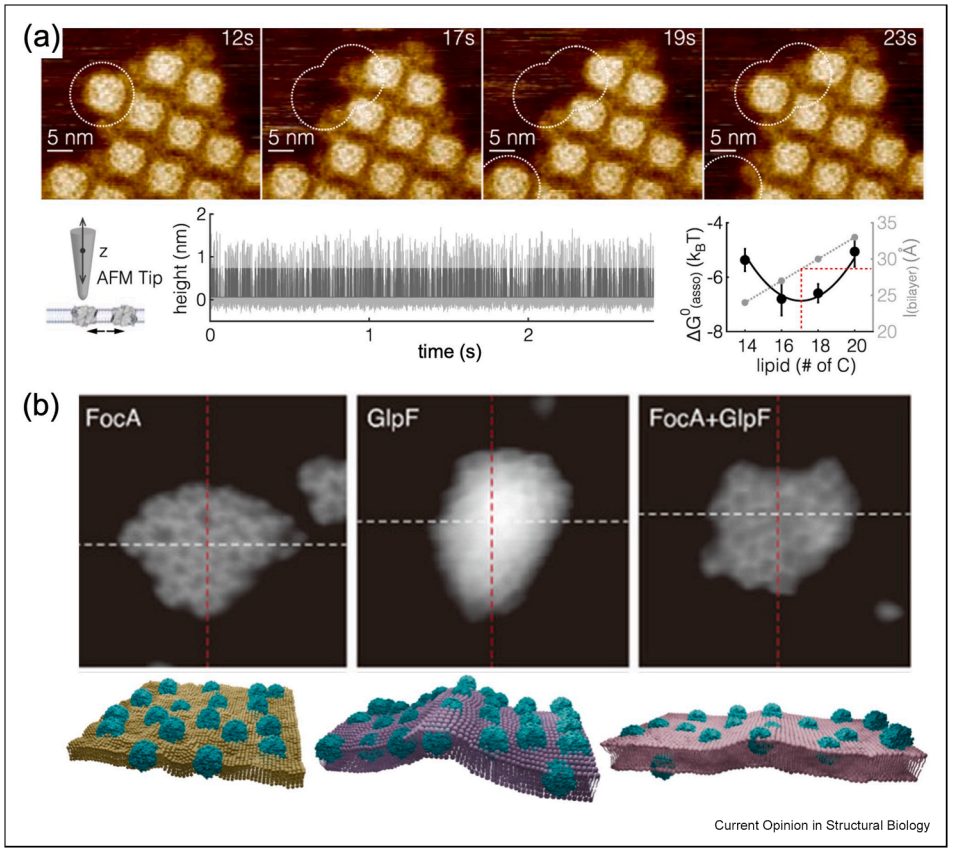
Lipid dependent protein—protein interactions

HS-AFM's μm-scale scanning range with nm-scale local resolution and precise control over membrane composition make it ideal for studying membrane protein—protein interactions. Supported lipid bilayers on mica, formed by vesicle rupture (using proteoliposomes [25], lipid-only vesicles or native membrane extracts), provide a reproducible platform to study how different lipid environments influence lateral interactions, cooperative assembly, and membrane deformation. Protein-containing vesicles typically form discrete membrane patches, which can be backfilled with lipid-only vesicles to alter membrane composition *in situ*. This method was used to study the effect of hydrophobic mismatch on protein—protein interaction using Aquaporin Z (AqpZ) as a model protein by varying the acyl-chain length of the backfilling lipids [26]. HS-AFM imaging of AqpZ after addition of long-chain (C20) or short-chain (C14) lipids showed frequent monomer-dimer and dimer—trimer transitions at the edges of 2D arrays (Figure 2a), whereas the distributions of these states shift predictably for more favorable chain lengths (C16 and C18). HS-AFM height-spectroscopy measurements were used to detect molecules diffusing too fast to be visualized in HS-AFM imaging mode (Figure 2a). The results are consistent with hydrophobic mismatch and the related deformation fields affecting the energy space between unbound, singly bound, and doubly bound states.

An alternative method, termed membrane extension membrane protein reconstitution (MEMPR), mixes lipid-only vesicles, proteoliposomes, and detergent before deposition and was initially created to localize single GltPh transporters (Figure 1c) [18]. The MEMPR method has also been applied to study the effect of lipid species on the supramolecular assembly of membrane proteins and their protein—protein interactions in clusters [27] using FocA and GlpF in *E. coli* lipids. HS-AFM imaging showed that each protein generates characteristic curvature fields, and mixed samples display composite height profiles arising from the superposition of these deformation signatures (Figure 2b). Increasing dioleoylphosphatidylcholine (DOPC) content reduced membrane rigidity, decreased lattice planarity, and lowered packing fraction, consistent with deformation-driven changes in inclusion energies.

HS-AFM has also revealed how membrane architecture shapes interactions between soluble proteins and membranes. Imaging antibody binding to extracellular vesicles

Figure 2



Lipid-modulated membrane protein–protein interactions. (a) Upper: HS-AFM frames showing reversible encounters at AqpZ array edges following continuous bilayer formation. Lower: HS-AFM height-spectroscopy trace used to detect transient binding events with a schematic of the fixed-tip configuration, representative height–time trace, and plot of association energy (ΔG^0asso), defined as the energy difference between the unbound and bound states [26]. **(b)** HS-AFM images of FocA, GlpF, and mixed FocA + GlpF in 100% *E. coli* lipids, with corresponding deformation renderings illustrating protein-specific curvature fields and their combined effects in the mixed system [27]. HS-AFM, high-speed atomic force microscopy.

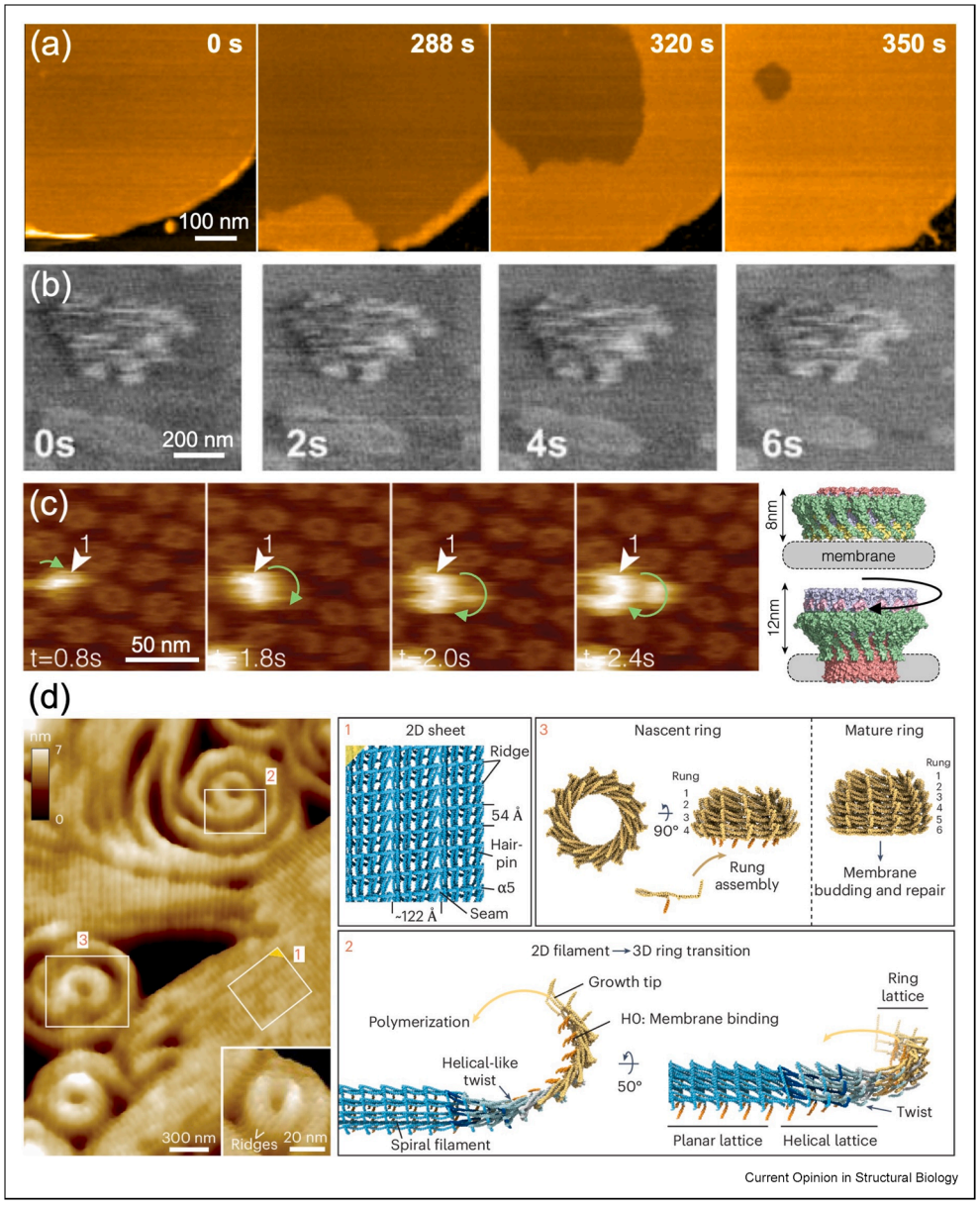
showed that Fab conformational states correlated with vesicle size and curvature, indicating that local membrane geometry strongly influences binding behavior [28].

Membrane remodeling

Reorganization of the membrane's structure and composition is often required in response to cellular needs, external stimuli or therapeutic interventions that disrupt pathogen membranes. HS-AFM, with ~1 Å vertical resolution, can capture nanoscale changes in curvature, thickness, and lipid phase organization, as well as perturbations including lateral domain reorganization, detergent-like solubilization, and supramolecular assembly.

Because many antimicrobials disrupt membrane integrity, HS-AFM can directly visualize their real-time effects on membrane structure and lipid organization. This has been demonstrated across a range of antimicrobial molecules; for example, studies of the antimicrobial peptide plectasin have shown how it oligomerizes on lipid II-containing membranes, forming dense sheets [29]. Oligomerization was observed with nucleation occurring at membrane edges (Figure 3a) and progressing inward, producing tightly packed sheets in the presence of calcium or disordered assemblies without it. Similarly, the small cationic agent N-alkylamide 3d assembles into dense, carpet-like structures on membrane surfaces while inducing domain reorganization and partial bilayer breakdown [30]. Movies of the lipopeptide daptomycin, show binding to membranes, oligomerization, followed by pore and tubule formation, linking supramolecular assembly directly to membrane deformation and permeabilization [31]. HS-AFM and complementary MD simulation have shown that antimicrobial agents such as AMC-109 and teixobactin self-assemble into supramolecular and fibrillar structures on membranes, driving lipid reorganization and membrane destabilization without classical pore formation [32,33].

Figure 3



Membrane remodeling processes detected by HS-AFM. (a) HS-AFM movie frames capturing the oligomerization of plectasin on supported DOPC/DOPG bilayers containing 1% lipid II in the presence of 1 mM Ca^{2+} [32]. **(b)** HS-AFM images of Aβ oligomers interreacting with lipid domains in phase-separated high-cholesterol containing membranes [35]. **(c)** Left: time-lapse HS-AFM images (t = 0.8 s to t = 2.4 s) showing how acidification changes the shape of a PFN2 ring on a supported lipid bilayer, from pre-pore to pore in a clockwise manor. Right: PFN2 pre-pore and pore cryo-EM structures (PDB 6SB3 and 6SB5) show increased thickness from ~8 nm to ~12–14 nm after membrane insertion and pore formation [39]. **(d)** The Vipp1 HS-AFM image on the left shows planar sheets, spiral filaments, and ring-like assemblies; white boxed (1–3) correspond to the schematic models displayed on the right (1–3), which show Vipp1 assembly from 2D lattices to 3D rings [40]. cryo-EM, cryo-electron microscopy; HS-AFM, high-speed atomic force microscopy; PFN2, Perforin-2.

Lipid domains can be identified in AFM imaging by the thickness differences between phases, typically ranging between 0.5 and 1.5 nm [34]. Recently a custom contact-mode HS-AFM platform was employed to visualize amyloid-β interactions with lipid domains [35]. The study shows that $A\beta_{1\text{-}42}$ monomers preferentially bind to liquid-ordered domains, while oligomers cause pore formation and with less specificity to a particular lipid phase (Figure 3b) [35]. Similarly, HS-AFM imaging of septins on phase-separated membranes revealed

preferential binding and assembly on liquid-disordered domains, underscoring the role of nanoscale lipid organization in protein–membrane interaction [36].

Beyond peptide and small-molecule-induced membrane perturbations, HS-AFM has recently been applied to dissect the mechanisms of protein-mediated plasma membrane rupture. A recent study used HS-AFM to capture, in real time, how NINJ1 transitions from metastable oligomeric states to arc-shaped assemblies that nucleate nanoscale pores and drive progressive membrane disintegration, revealing multi-stage dynamics of membrane rupture that integrate structural transitions with membrane deformation [37]. HS-AFM imaging of membrane attack complex (MAC) visualized the assembly kinetics of MAC pore as they form, enlarge, and reach completion within seconds to minutes [38]. Likewise, imaging of Perforin-2 (PFN2) captured a complete morphological transition from prepore to pore states in a clockwise manner, accompanied by height changes from ~8 nm to 14–16 nm (Figure 3c) [39].

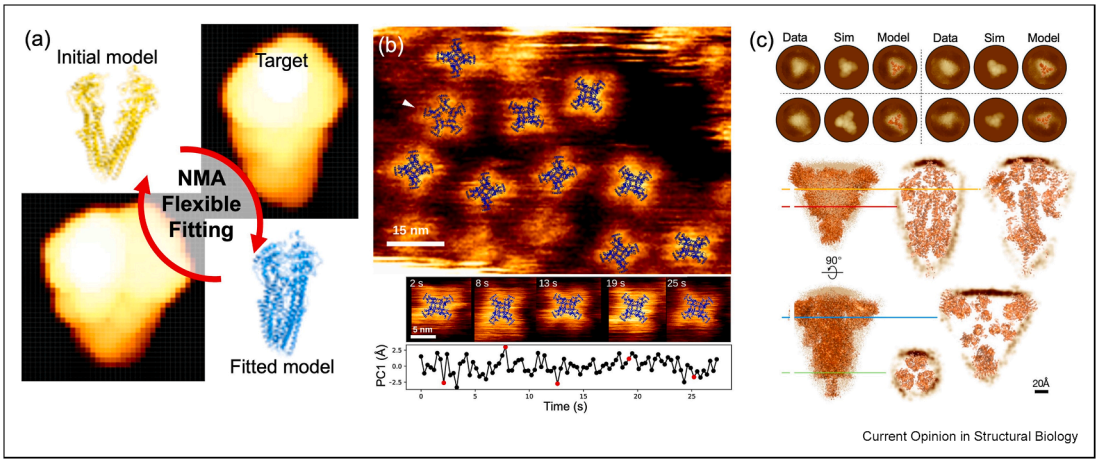
In contrast to pathogen- and peptide-driven membrane attack, cells deploy mechanisms that actively remodel membranes. Vesicle-inducing protein in plastids 1 (Vipp1) can be observed assembling into filaments and expanding into empty membrane areas, covering the whole lipid surface in minutes, with networks of planar sheets, spiral filaments, and rings (Figure 3d) [40].Two recent HS-AFM studies provide insight into both the dynamics and mechanistic pathways of this process. Work by Pan et al. demonstrates that Vipp1 self-assembles on membranes through distinct growth modes, with spirals nucleating from central hubs and polygons expanding from their edges, revealing ESCRT–III–like polymerization behavior governed by directional growth and filament remodeling [41]. In contrast, the study by Low et al. focuses on the formation of discrete structural intermediates, showing how spirals mature into rings while progressively deforming the underlying membrane, with increasing height differences between inner spiral turns and the bilayer as assemblies develop [40]. Together, these HS-AFM observations bridge mesoscale self-organization with functional membrane remodeling, while complementary cryo-EM structures provide high-resolution insight into the underlying filament and ring architectures.

Advances in computational methods

In parallel with improvements in imaging speed, advances in computational methods are enabling improved spatial resolution and interpretation, quantitative analysis, and interoperability between techniques, taking advantage of the rich 3D and multi-dimensional information that exists in AFM and HS-AFM datasets. These advances span flexible fitting, localization-based super-resolution methods, volumetric reconstruction, and open computational pipelines that increase reproducibility and facilitate integration with mainstream structural biology.

AFM topographies of proteins can be simulated by virtually scanning model AFM tip geometries across three-dimensional atomic coordinates from the protein

Figure 4



Simulation-based extraction of structural and dynamic information from AFM and HS-AFM data. (**a**) Normal-mode analysis (NMA) based flexible fitting of simulated AFM images: an initial atomic model of the ABCB1 transporter is iteratively deformed to match a target AFM topograph, generating a fitted structure consistent with a closed-state conformation [45]. (**b**) Application of AFMfit to HS-AFM movies of TRPV3, showing particle identification and flexible fitting across frames (top), and the corresponding principal component (PC1) time trace revealing dominant in-plane conformational fluctuations (bottom) [47]. (**c**) Reconstruction of 3D structure from single AFM topographs using libraries of simulated images: comparison between experimental data, simulated topographs and fitted models (top), and the resulting 3D point clouds from multiple orientations for the SARS-CoV-2 spike protein shown at different planar slices through the structure (bottom) [48]. HS-AFM, high-speed atomic force microscopy.

data bank [42] or AlphaFold-predicted structures [43], providing a means to validate membrane protein orientation and predict expected static views [44]. Building on these simulations, flexible fitting using normal-mode analysis (NMA) has been introduced to infer atomistic-scale conformational dynamics from AFM and HS-AFM images [45–47]. Initial validation using different conformations of various proteins including the ABCB1 transporter (Figure 4a) showed that the method can accurately fit from an open to a closed state [45]. NMA has been implemented into the BioAFMViewer software platform and the python package AFMfit [47]. Applied to HS-AFM movies of TRPV3, AFMfit distinguished tetrameric and pentameric states and resolved their dominant motions by Principal component analysis (PCA), with in-plane deformations of ~6.3 Å (RMSD) and rapid, uncorrelated equilibrium fluctuations in the tetrameric state faster than the 300 ms frame rate (Figure 4b) [47].

Simulated AFM images can also be used to determine protein orientation and reconstruct 3D models from single topographs containing multiple particles in different orientations [48]. By comparing experimental images to libraries of simulated AFM images, 3D structures can be built from multiple viewpoints analogously to single-particle cryo-EM, but using a reconstructed point cloud of AFM tip contact points, as demonstrated for the SARS-CoV-2 spike protein (Figure 4c).

To support the integration of AFM structural analysis into standard structural biology workflows, the 3D localization AFM (3DLAFM) pipeline has been developed to generate density maps within the volumetric domain [49]. The 3DLAFM approach supports data sharing, cross-method comparison, and deposition alongside conventional structural datasets, enabling direct comparison of AFM data with cryo-EM, NMR, and X-ray crystallography, as well as the ability to guide MD simulations. This approach allows AFM to bridge the gap between ensemble-averaged structural information and dynamic single-molecule observations, as demonstrated for Glt_{PH} (Figure 1b).

Parallel progress in computational tools has improved the accessibility of HS-AFM data analysis. Open frameworks such as NanoLocz [50] which provide end-to-end pipelines for drift correction, particle tracking, simulation AFM, segmentation, and LAFM, are enabling reproducible and high-throughput processing of both HS-AFM movies and LAFM datasets. By lowering barriers to adoption across laboratories, these tools are fostering a more interoperable and robust AFM data ecosystem.

Conclusions

High-speed AFM is rapidly transforming our ability to visualize membrane protein dynamics, lipid–protein interactions, and membrane remodeling in real time. Methodological innovations, such as MEMPR, use of nanodiscs, curved or deformable substrates [51,52], and extending to live cell membranes [53,54] are broadening the range of systems accessible to AFM. Future advances in computational analysis, data sharing, and integration with complementary structural methods will enable even more quantitative, reproducible, and high-resolution interpretation of dynamic molecular events. These developments promise to reveal mechanisms underlying disease processes, including anti-microbial resistance, protein aggregation, and the function of intrinsically disordered regions, positioning HS-AFM as a central tool for connecting structure, dynamics, and function in native membrane environments.

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.

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