



How Microbes Use Force To Control Adhesion

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ABSTRACT Microbial adhesion and biofilm formation are usually studied using molecular and cellular biology assays, optical and electron microscopy, or laminar flow chamber experiments. Today, atomic force microscopy (AFM) represents a valuable addition to these approaches, enabling the measurement of forces involved in microbial adhesion at the single-molecule level. In this minireview, we discuss recent discoveries made applying state-of-the-art AFM techniques to microbial specimens in order to understand the strength and dynamics of adhesive interactions. These studies shed new light on the molecular mechanisms of adhesion and demonstrate an intimate relationship between force and function in microbial adhesins.

KEYWORDS atomic force microscopy, cell adhesion, cell surface

Microbial pathogens (i.e., fungi, bacteria, and viruses) have the ubiquitous ability to adhere to biotic and abiotic surfaces (e.g., fomites, implants, catheters, and host cells) in environmental and clinical contexts (1). Prime examples are *Escherichia coli* and *Staphylococcus aureus* strains involved in nosocomial infections (2) and mycobacteria that cause diseases like tuberculosis and leprosy. These species all rely on adhesion to biomaterials and host factors to initiate infection (3). Moreover, the vast majority of environmental microbial cells rapidly give up their floating planktonic lifestyle in an adhesion-dependent process to assemble into surface-associated communities called biofilms (4). In these structures the cells are protected from various environmental stresses like starvation, desiccation, antibiotics, and xenobiotics (5). So microbial cell adhesion has great medical, environmental, and industrial importance (2, 6).

Microbial cells feature a large variety of adhesion mechanisms involving physical properties of the cell surface, such as charge, hydrophobicity, and stiffness, as well as specific receptor-ligand binding mediated by adhesins and appendages (pili and curli). How these parameters lead to invasion and infection is a fascinating field that has benefited from the recent advent of ultrasensitive technologies (7, 8). Among these, atomic force microscopy (AFM) has proven to be a powerful tool to quantitatively probe the mechanical forces involved in cell adhesion while morphologically characterizing the cells (8). The heart of an AFM consists of a nanometer-sized tip attached to the end of a soft cantilever, which scans the sample in the *x* and *y* directions. Due to interactions between the tip and the sample, the cantilever bends vertically (*z* direction). This deflection is recorded via a laser beam focused on the cantilever and reflected onto a photodiode. Taking into account physical characteristics of the cantilever (sensitivity and spring constant), the exact force (in newtons) exerted by the tip on the sample can be determined. Raster scanning of the AFM tip across the sample, in either constant contact, intermittent contact, or noncontact mode (8), can provide very-high-resolution topographic images of the sample (on microbes, typical lateral resolution is in the range of ~20 nm and vertical resolution is ~0.1 to 1 nm). Moreover, in force spectroscopy, the tip is approached and retracted from the surface and the force-distance (FD) curves generated provide measurements of physical properties of

Citation Viljoen A, Mignolet J, Viela F, Mathelié-Guinlet M, Dufrêne YF. 2020. How microbes use force to control adhesion. *J Bacteriol* 202:e00125-20. <https://doi.org/10.1128/JB.00125-20>.

Editor William Margolin, McGovern Medical School

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Accepted manuscript posted online 6 April 2020

Published 27 May 2020

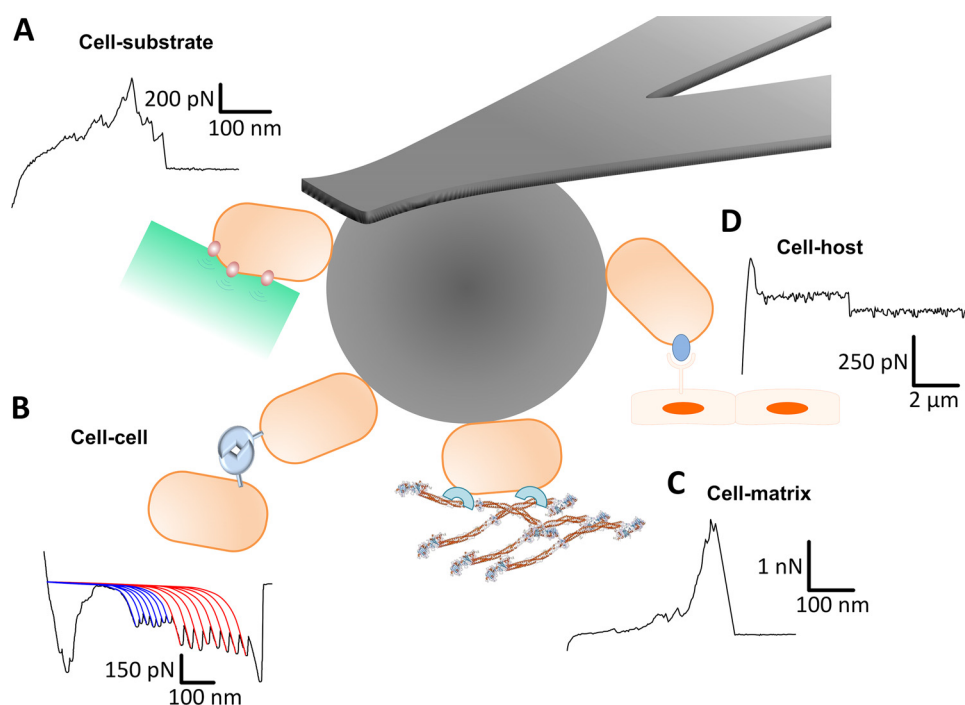


FIG 1 Multifaceted use of single cell force spectroscopy (SCFS) to study microbial cell adhesion. (A) SCFS to measure interactions between cells and abiotic substrates. Shown is a characteristic adhesive curve obtained between a single *Lactococcus plantarum* cell and a model hydrophobic surface (116). (B) Homophilic binding between adhesins promotes cell-cell contacts. A typical force curve shows SasG-SasG interactions displaying sawtooth profiles fitted with the extensible worm-like-chain (WLC) model (49). (C) *Staphylococcus* cells bind with extreme strength to the extracellular matrix protein fibrinogen (Fg). A force curve is shown of the interaction between a single *Staphylococcus epidermidis* HB cell expressing SdrG and an Fg substrate (56). (D) SCFS measuring interactions between pathogens and their host cells. A representative force showing a typical force plateau resulting from pulling on Caco-2 cell plasma membrane tethers by *Lactobacillus rhamnosus* GG cells (41).

the specimen, such as stiffness, elasticity, deformation, and adhesion. FD-based imaging makes it possible to map the spatial distribution of these properties on the nanoscale. Functionalization of the AFM probe with ligands allows probing of molecular interactions with single receptors (single-molecule force spectroscopy [SMFS]), e.g., between a single bacterial adhesin exposed on a living bacterium and a single extracellular matrix protein. Alternatively, attaching a single bacterial cell on the cantilever offers a means to probe force interactions between a single cell and a substrate (single-cell force spectroscopy [SCFS] [Fig. 1]). These various modes have paved the way for a detailed understanding of the interaction strength between microbes and their target surfaces.

CELL ADHESION FORCES

Cell-substrate adhesion. AFM has been instrumental in capturing the adhesive forces between pathogens and medically relevant abiotic surfaces, such as polymeric biomaterials (Fig. 1). SCFS revealed that large cell wall proteins from *S. aureus* are responsible for long-range (50-nm) attractive forces toward hydrophobic surfaces (9, 10). Large forces were measured between *S. aureus* and materials used in the dental practice, such as stainless steel, polyethylene, and polyvinyl chloride (11). The adhesion of *Streptococcus mutans* to different substratum surfaces was correlated with force-responsive changes in gene expression, providing insight into how emergent biofilm characteristics are triggered by quorum sensing (12). Saliva enhanced, but fluoride treatment reduced, the adhesion of streptococci to model enamel surfaces (13, 14). In the fungal context, Valotteau et al. identified three Epa proteins that mediate the adhesion of *Candida* species to hydrophobic and hydrophilic substrates (15). Besides SCFS, FD-based imaging with chemically modified tips was used to quantitatively map

surface adhesive properties on living bacteria. Patchy hydrophobic nanodomains were observed on *Acinetobacter venetianus* and *Rhodococcus erythropolis* (16), while *Mycobacterium bovis* BCG and *Aspergillus fumigatus* exhibited homogenous hydrophobic surfaces (17–19). Mature cell walls of *Aspergillus nidulans* were less hydrophilic than growing tips and branch point junctions (20), consistent with variations in surface hydrophilicity as a function of cell wall composition (21). In the same line, *Rhizobium leguminosarum* showed an inverse relationship between surface hydrophilicity and mature biofilm formation (22). AFM has also shown its potential to assess antifouling and antiadhesive coatings, with the goal of preventing the initial step of biofilm formation on implanted biomaterials. For instance, polyzwitterionic polymer brushes were found to drastically reduce the force needed to detach *Yersinia pseudotuberculosis* (23). Insertion of cationic nanoclusters in such brushes also enhanced the removal of *S. aureus* (24). Two studies identified the antiadhesive properties of negatively charged polymer brushes against *E. coli* (25) and that of sophorolipid biosurfactants against both *E. coli* and *S. aureus* (26). AFM also demonstrated how the herbicide 2,4-dichlorophenoxyacetic acid, which induced remodeling of the cell wall of *Rhizobium leguminosarum*, led to alterations in its surface hydrophobicity (27). Exposure of *E. coli* to 2,4-dichlorophenoxyacetic acid led to an oxidative stress response accompanied by increased surface hydrophilicity (28, 29), which showed a time dependency (30).

Gram-positive and Gram-negative appendages, such as pili and fimbriae, also adhere through nonspecific and specific interactions with surfaces (31). This confers functions like promoting interactions between bacteria of the same or different species, between bacteria and abiotic surfaces, or between bacteria and host tissues (Fig. 1) (32). Pili in some organisms also play a role in motility within biofilms, which is dependent upon interactions between them and the substratum (33). In Gram-negative bacteria, type I, type IV, and P pili elongate under force, giving rise to characteristic constant force plateaus (34–37). These pili, of which the subunits are held together through noncovalent interactions, can bear forces in the range of 250 pN (34), and multiple pili often work together to confer their force-dependent functions (38). In Gram-positive bacteria, pilus subunits are usually bound to each other via covalent bonds, allowing them to resist forces in excess of 500 pN (39, 40). Due to the covalent bonds, these pili behave like nanosprings under force (41, 42).

Cell-cell adhesion. Microbial cells can adhere to each other using homophilic bonds between adhesins (Fig. 1). Examples are the aggregation mediated by the mycobacterial heparin-binding hemagglutinin adhesin of mycobacteria (43, 44), by the *Burkholderia cenocepacia* trimeric autotransporter adhesin (45), and by the *Candida albicans* Als adhesins (46, 47). In *S. aureus*, FnBPA proteins bind to each other in a Zn^{2+} -dependent way with moderate forces, on the order of 150 pN (48), while SasG forms much more stable homophilic bonds, resisting forces of 500 pN (49). This suggests that bacteria have evolved specialized intercellular adhesion mechanisms that allow them either to detach from biofilms or to resist detachment when subjected to shear forces. *Saccharomyces cerevisiae* flocculins (50) and *C. albicans* Als adhesins (46, 51) use β -sheet interactions to support cell aggregation.

Cell-host interactions. Adhesion to host tissues is the first step of infection for many pathogens (52). Several studies have brought detailed molecular insights into how adhesins specifically bind to host proteins, sometimes in a mechanoresponsive manner under shear stress (7, 53) (Fig. 1). Staphylococcal adhesins bind their target ligands with extremely strong forces (~ 1 to 2 nN) that largely outperform classical binding forces. These include the collagen-binding protein Cna (54) and fibrinogen (Fg)-binding proteins SpsL (55) and SdrG (56–58). Such extreme forces structurally originate from the “dock, lock and latch” mechanism (or variations of it), which involves locking of the docked target peptide sequence by a N-terminal segment of the adhesin followed by latching of this complex on to a neighboring domain in the adhesin (59, 60). Steered molecular dynamics simulations in combination with AFM experiments showed that the extreme mechanical stability of the SdrG-Fg complex results from an

intricate hydrogen bond network between the ligand peptide backbone and the adhesin (57, 61). When a strong mechanical load is applied in a direction that occurs under natural conditions (pulling force is applied at the C termini of both SdrG and Fg), an unbinding pathway is followed with a high energy barrier (58). Conversely, the unbinding pathways that would be followed under thermal unbinding (at equilibrium) or when weak mechanical loads are applied exhibit lower energy barriers (58). Such “catch” bonds (62) are proposed to exhibit longer lifetimes than their antonymous “slip” bonds, which is very useful to pathogens that need to stay adhered to their host-target tissues under hydrodynamic forces (58). AFM has provided evidence for the existence of catch bonds in ClfA, ClfB, and SpA (63–65), revealing that their binding strength is considerably increased by mechanical tension. The proposed mechanism involves force-induced conformational changes in the adhesins, from a weakly binding state to a strongly binding state, and explains how staphylococci can resist physical stresses such as hydrodynamic shear forces. Similarly, force-induced conformational changes in yeast flocculins (50) and Als adhesins (46, 51) trigger β -sheet interactions between amyloid sequences that strengthen cell aggregation.

Adhesive interactions between single microbes and host cells have also been studied (Fig. 1). Early examples include the heparin-binding hemagglutinin adhesin, which contributes to adhesion of mycobacteria to pneumocytes (66), and the *Listeria monocytogenes* invasion protein InlB, which supports adhesion to the Met receptor tyrosine kinase (67). More recently, strong FnBPA-Fn-integrin interactions between living *S. aureus* and endothelial cells were shown to involve an activation mechanism in which FnBPA binding to Fn stimulates the exposure of cryptic integrin-binding sites in Fn (68). In the skin context, it was found that decreases in the levels of natural moisturizing factor in cultured corneocytes increased *S. aureus* adhesion via the ClfB adhesin (65). Viela et al. investigated the Fg-dependent interaction between *S. aureus* ClfA and the endothelial cell integrin $\alpha_v\beta_3$ (63) and revealed that the ClfA-Fg- $\alpha_v\beta_3$ ternary complex sustains very strong forces (~ 800 pN).

Adhesin clustering. Another pertinent question is whether microbes express their adhesins homogenously or whether these form nanodomains capable of enhancing adhesion. FD-based imaging detected such clustering in *S. aureus* for various adhesins, including FnBPs, SdrG, ClfA, ClfB, SpA, and SpsL (48, 64, 69, 70). It has been suggested that protein clustering on the cell surface may favor adhesion through multivalency. In the case of FnBP-Fn interactions, cluster bonds involving ~ 10 or ~ 80 proteins in parallel were reported for bloodstream isolates from patients with infected cardiovascular implants (71). Heterogenous clusters of rough lipopolysaccharide were observed on *Brucella abortus* cells (72), evidence that bacteria compartmentalize their surfaces for function. Notably, *C. albicans* was observed to redistribute Als5p adhesins into nanodomains in response to mechanical stimuli (46), suggesting that the pathogen is capable of actively responding to physical stresses in favor of adhesion.

Cell adhesion and antimicrobial therapy. The effect of antimicrobials on cell surface properties has also been investigated. Exposure of *C. albicans* to the antifungal drug caspofungin induced overexpression of Als adhesins, which correlated with increased cell surface roughness, decreased stiffness, and increased hydrophobicity (73, 74). In the case of mycobacteria, treatment with several classes of antibiotics that target the synthesis of different components of their cell envelope also lead to dramatic alterations in their surface hydrophobicity (18). More recently, Laskowski et al. showed that although ampicillin increased surface roughness in the Gram-positive *Bacillus subtilis* and Gram-negative *E. coli*, only *B. subtilis* had altered adhesion (75). The effect of antibiotics on bacterial adhesion may thus be considered in future AFM studies focusing on antimicrobial mechanisms. Another exciting avenue for AFM is in the detection of antibiotic-resistant bacterial strains, especially in the climate of increased drug resistance. Longo et al. demonstrated that bacterial adhesion to sensitive cantilevers causes their deflection to fluctuate, allowing the ability to distinguish between

metabolically active and dead bacteria for the detection of antibiotic-resistant strains within minutes (76).

Because pathogens are becoming more and more resistant to antibiotics, it is becoming more and more urgent to discover new antimicrobial therapies. Antiadhesive agents capable of blocking the adhesion of pathogens to host tissues and biomaterials are an attractive alternative to classical antimicrobials (77). In early work, the Camesano team unraveled the antiadhesive activity of cranberry juice that targets *E. coli* fimbriae, effectively inhibiting their adhesion to abiotic surfaces and uroepithelial cells (78–80). AFM has been used to assess the blocking activity of antiadhesion compounds, such as peptides and antibodies. The capacity of glycofullerenes to interact with *E. coli* fimbriae and block their adhesion to mannose surfaces was quantified and the underlying mechanism revealed (81), opening new avenues for further studies into innovative treatments of intestinal colonization. Likewise, plant-based antifungals increased the surface hydrophilicity of *C. albicans* (82). AFM studies have also emphasized the antiadhesion potential of peptides and antibodies. Monoclonal antibodies acting as competitive inhibitors against Cna showed great efficiency in blocking *S. aureus* adhesion to collagen surfaces (54). A similar competitive inhibition was observed for a peptide derived from β -neurexin, which efficiently blocked homophilic SdrC interactions in *S. aureus* (83).

NEW TECHNOLOGICAL DEVELOPMENTS

Fast single-cell manipulations. Fluidic force microscopy (FluidFM) uses microchanneled AFM cantilevers connected to a pump through a microfluidic system (84). Such hollow probes offer possibilities like extracting or injecting material from or into mammalian cells and capturing single microbial cells by applying negative pressure to the aperture of the tip (85, 86). Hence, the technology makes it possible to prepare single cell probes for SCFS in a faster way than classical approaches (87). Therefore, FluidFM was used to detect hydrophobic adhesive forces as high as 50 nN among a large collection of bacteria, demonstrating its ease of use and applicability, especially when very large forces are of interest (88). As adhesion between the cell and the probe is reversible, a single bacterial cell can be picked up, transferred to a region of interest within the experimental setup, and released. This was successfully applied to isolate single bacteriochlorophyll-producing bacteria from a mixed population of leaf microbiota (89). Dehullu et al. used FluidFM to study cell-cell adhesion in *C. albicans* and found that amyloid sequences of the Als adhesin play an important role (47, 90). Although it shows great promise, we point out that SCFS-based FluidFM is still in its infancy and some caveats exist, such as the high stiffness of the cantilevers, which precludes precise measurements of smaller forces in the range of ~ 100 pN.

High-speed molecular and cellular imaging. During the past decade, advances have been made in the development of imaging modes with enhanced spatial and temporal resolutions. Classical topographic imaging is slow, meaning that it takes minutes to record an image (91). However, high-speed AFM (HS-AFM) instruments now enable researchers to reach millisecond resolution, owing to the use of small cantilevers and improved electronics (92). Even if it has not yet been established for studying cell adhesion, HS-AFM has potential in microbiology, allowing the filming of biological processes with molecular resolution without any staining (93). By way of example, HS-AFM was used to film real-time conformational changes in several bacterial integral membrane proteins, including the F_1 -ATPase rotary motor protein (94), an aspartate-sodium symporter (95), a pH-sensitive ion channel (96), and an ATP-dependent protein disaggregation machine (97). Recently, HS-AFM tracked the dynamic oligomerization of immunoglobulin G (IgG) on lipid membranes (98). Until now, the technology has been mainly limited to studying proteins in flat two-dimensional (2D) crystals or in model membranes because of technical limitations related to the large curvatures and roughness of living cells. However, several recent studies have addressed these issues, leading to the visualization of outer membrane proteins in curved membranes, including on living bacterial cells (98–101). Nievergelt et al. developed a new type of HS-AFM, called photothermal off-resonance tapping AFM (102),

which allowed real-time, simultaneous nanomechanical and topographic imaging of *B. subtilis* cells as their walls were degraded by lysozyme (103). This strongly suggests that HS-AFM might soon be able to capture dynamic molecular events on pathogens, in relation with adhesion functions.

Another grand challenge is the application of FD curve-based multiparametric imaging for the fast structural and physical mapping of living cells (104). Force volume mode, in which arrays of force curves are recorded across the sample, has been extensively used to study protein distributions on bacterial cell envelopes. They have provided valuable insights on adsorption of bacteriophages (105, 106), on docking of motorized extracytoplasmic appendages such as pili (34, 41, 42) and on clustering of adhesins (63, 64, 69). Nevertheless, FV suffers from low imaging speed and resolution; i.e., it takes about 30 min to obtain a 32- by 32-pixel image. The advent of multiparametric imaging, such as peak force tapping (PFT) and quantitative imaging (QI), has enabled simultaneous imaging of the structural, chemical, and biophysical properties of living cells at higher spatiotemporal resolutions (91, 104). The multiparametric imaging toolbox has been instrumental in probing ligand-receptor bonds and mapping epitopes. A nickel-functionalized tip was used to locate and number the binding events of His-tagged bacteriorhodopsin on purple membranes of halobacteria at subnanometer resolution (107), to characterize its folding-unfolding state, and to draw a cartography of molecular interactions (75). QI and PFT have revealed the scaffolding nanostructures that cohere cable bacteria into millimeter-scale filaments (108), the physicomolecular damages electromagnetic stresses inflicted on cell walls (109), the localization of bacteriophage extrusion sites on infected bacteria (110), the organization of *C. albicans* mannoproteins into nanodomains (111), and the presence of hydrophobic nanodomains defined by glycopeptidolipids in mycobacteria (112) (Fig. 2A). QI was also used in the context of biofilm formation and showed the effect of metal ions on cell wall stiffness and adhesin pattern (49). Zn^{2+} cations enhanced the cohesion of the *S. aureus* cell wall, which promoted the extension of SasG, an adhesin that mediates homophilic interactions and cell-cell contact in biofilms, beyond masking surface components. As biofilms are dense bacterial communities highly resistant to antibiotics and chemical treatments, these results might help find new therapies to dismantle the biofilm matrix or prevent cell-cell junctions to form. An exciting approach for adhesion studies is to perform SCFS using a multiparametric mode. Formosa-Dague et al. used QI with single bacterial probes to study *S. aureus* adhesion to human skin cells (corneocytes) allowing mapping of adhesin-ligand contacts all over these cells with high spatiotemporal resolution (113) (Fig. 2B). Lastly, PFT allowed nanomechanical mapping of viral envelope glycoprotein binding to cognate receptors on host mammalian cells within the first millisecond of contact (117) (Fig. 2C). The data obtained allowed contouring the free energy landscape of the interaction. From this, the dynamics of the interaction could be determined, which showed that positive allosteric modulation led to rapid occupancy of the three binding sites of the glycoprotein. Using a similar approach, the mucin-like region of viral envelope glycoproteins was shown to play an important role in regulating the affinity, type, and number of glycoproteins that participate in viral interactions with cellular glycosaminoglycans (114). Similarly, a new mechanism was discovered whereby the herpesvirus glycoprotein negatively regulates initiation of viral binding to heparan sulfate through its effect on the valency of the interaction (115).

CONCLUSION

AFM has demonstrated that adhesins are engaged in a wealth of molecular interactions, with mechanical strengths ranging from a few dozen piconewtons to several nanonewtons. Perhaps the most exciting discovery of the recent years is that staphylococcal adhesins engage in ultrastrong catch bond interactions that are much stronger than any noncovalent biological interaction studied so far. These interactions are activated by mechanical force, offering the pathogens an elegant means to withstand physiological shear forces during adhesion and colonization. Other major achievements are the measurements of homophilic binding forces involved in cell aggregation, of the

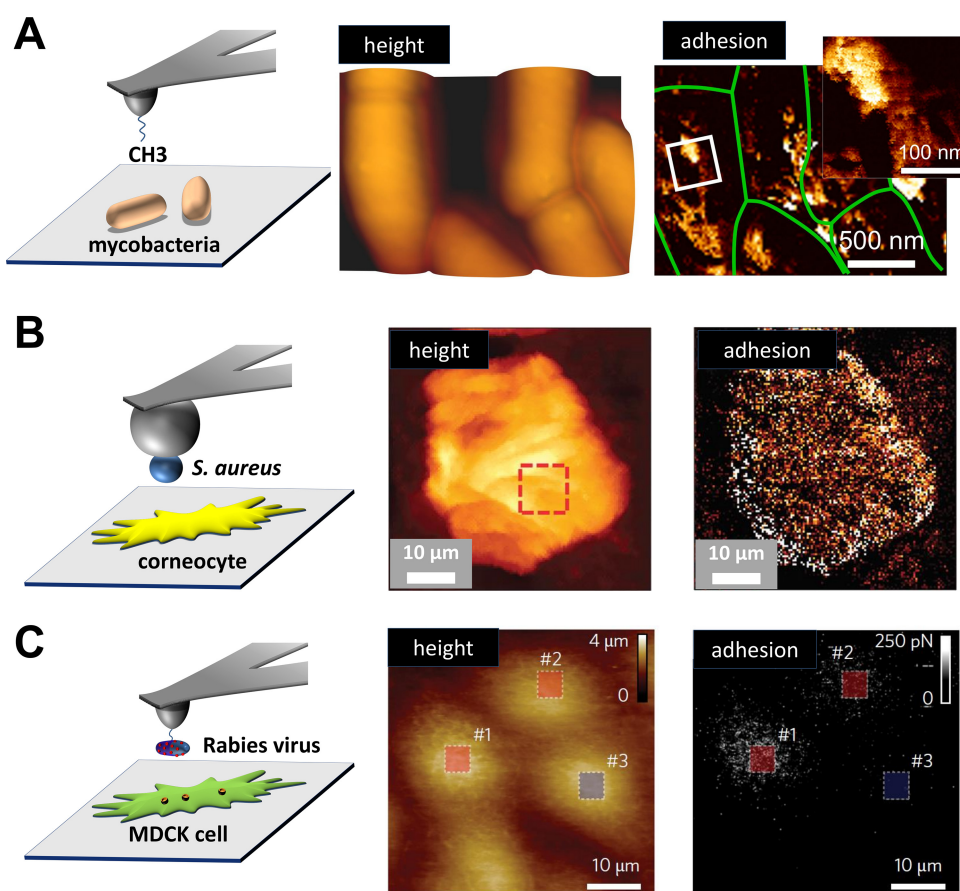


FIG 2 Fast multiparametric imaging leads the way. (A) Fast chemical microscopy (QI with hydrophobic tips) reveals hydrophobic and hydrophilic nanodomains with unprecedented resolution on mycobacterial cells. Adapted from reference 112 with permission of the publisher. (B) QI used in combination with SCFS and single *S. aureus* cell probes versus corneocytes cells. Adapted from reference 113 with permission of the publisher. (C) PFT mode with a virus-functionalized tip probing a host cell. Adapted from reference 117 with permission of the publisher. For all panels, on the left are cartoons of the different multiparametric imaging approaches used. The middle portions show height images, and the right portions show corresponding adhesion maps. In panel A, a zoom of a high-resolution adhesion map recorded on one cell is also shown.

adhesive and mechanical responses of bacterial pili when interacting with a substrate, and of the interaction forces between pathogens and either host proteins or host cells. These novel insights open up new avenues for the identification of small inhibitors to prevent pathogen adhesion and invasion.

ACKNOWLEDGMENTS

Work at UCLouvain was supported by the Excellence of Science-EOS program (grant number 30550343), the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement number 693630), the FNRS-WELBIO (grant number WELBIO-CR-2015A-05), the National Fund for Scientific Research (FNRS), and the Research Department of the Communauté française de Belgique (Concerted Research Action). Y.F.D. is Research Director at the FNRS.

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