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## **The microbial adhesive arsenal deciphered by atomic force microscopy**

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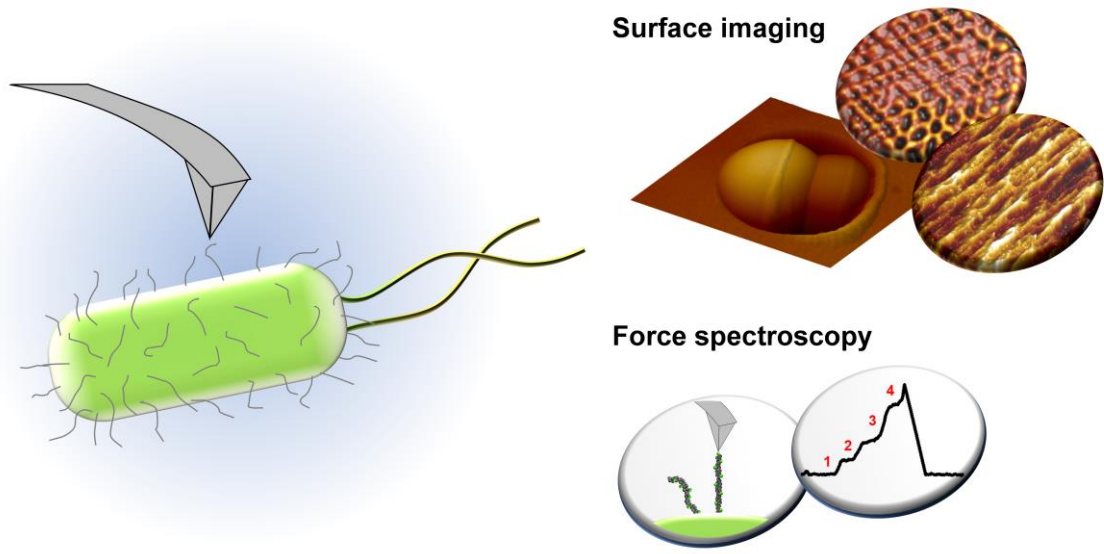
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20 **GRAPHICAL ABSTRACT**



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23 **ABSTRACT**

24 Microbes employ a variety of strategies to adhere to abiotic and biotic surfaces, as well as host  
25 cells. In addition to their surface physicochemical properties (*e.g.* charge, hydrophobic  
26 balance), microbes produce appendages (*e.g.* pili, fimbriae, flagella) and express adhesion  
27 proteins embedded in the cell wall or cell membrane, with adhesive domains targeting specific  
28 ligands or chemical properties. Atomic force microscopy (AFM) is perfectly suited to  
29 deciphering the adhesive properties of microbial cells. Notably, AFM imaging has revealed the  
30 cell wall topographical organization of live cells at unprecedented resolution, and AFM has a  
31 dual capability to probe adhesion at the single-cell and single-molecule levels. AFM is thus a  
32 powerful tool for unravelling the molecular mechanisms of microbial adhesion at scales  
33 ranging from individual molecular interactions to the behaviours of entire cells. In this review,  
34 we cover some of the major breakthroughs facilitated by AFM in deciphering the microbial  
35 adhesive arsenal, including the exciting development of anti-adhesive strategies.

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## 38        **The microbial adhesive arsenal deciphered by atomic force microscopy**

### 39        **Introduction**

40            Microorganisms commonly live in biofilms,<sup>1,2</sup> *i.e.* cell communities embedded in self-  
41        produced extracellular polymeric substances (EPSs) and adhered on substrates.<sup>2</sup> In this  
42        organization, some features typical of microorganisms emerge, such as quorum sensing,  
43        resistance or tolerance to antimicrobials, and cell differentiation. This successful life style has  
44        thus allowed the ubiquitous presence of microorganisms on Earth: they are associated with  
45        biogeochemical cycles, biotechnological processes, and life-threatening infections in plants  
46        and animals.<sup>1,2</sup>

47            Biofilm formation begins with the initial attachment of microbial cells to biotic or  
48        abiotic substrates.<sup>3</sup> This adhesion is mediated by properties of the cell surface, *i.e.* the cell  
49        wall, the ultimate interface between a cell and its environment, and which gives the cell its  
50        shape and protects it from external stresses.<sup>4,5</sup> Bacteria are the microbial kingdom dominantly  
51        involved in biofilms, followed by fungal cells, and are classified into two groups according to  
52        their cell-wall organization: Gram-positive bacteria have cell walls containing a thick  
53        peptidoglycan layer covered by anionic acids such as (lipo)teichoic acids, whereas the cell walls  
54        of Gram-negative bacteria present a thinner peptidoglycan layer coated by an outer  
55        membrane comprising phospholipids and lipopolysaccharides. Fungal cell walls comprise an  
56        inner layer containing chitin and  $\beta$ -glucans and an outermost layer composed of  
57        mannoproteins.

58            Whatever the genus, microorganisms have evolved a plethora of adhesive strategies  
59        to colonize diverse environments. The major effectors for cell adhesion are: i) the  
60        physicochemical properties of the cell surface (*e.g.* charge, hydrophobic balance) and ii)

61 appendages (including pili, fimbriae, flagella) and the expression of adhesins, polymers  
62 embedded in the cell wall or cell membrane that present domains adhering to specific ligands  
63 or chemical properties. Although this adhesive arsenal has been progressively highlighted  
64 since the early 1950s through microscopy, molecular biology, and biochemistry, its role in  
65 microbial adhesion was often only hypothesized or indirectly demonstrated by empirical  
66 methods comparing wild types and mutant strains.

67         Since its invention in 1986 by Binnig *et al.*, atomic force microscopy (AFM) has emerged  
68 as a powerful tool for probing the properties of microbial surfaces at high resolution while the  
69 cell remains alive.<sup>6</sup> The principle of AFM is to scan a sample surface with a sharp tip (radius of  
70 few nanometers) mounted on a flexible cantilever. While scanning, interactions between the  
71 tip and the surface bend the cantilever, and this deflection is monitored using a laser beam  
72 focused at the apex of the cantilever and reflected into a multi-segment photodiode. The  
73 precise positioning of the sample during scanning is controlled by a piezoelectric scanner  
74 moving in x, y and z directions. Because the tip physically touches the sample, AFM can image  
75 sample topography at sub-nanometer resolution and sense interaction forces on the order of  
76 a few piconewtons. The recently developed force-distance-based multiparametric imaging  
77 mode has allowed the imaging of biological structures while simultaneously quantifying and  
78 mapping their physical, chemical, and/or biological properties.<sup>7-9</sup> In addition to imaging,  
79 microbiological prospects have largely benefited from the advances in AFM-based force  
80 spectroscopy methodologies, and more particularly from the development of the single-  
81 molecule (SMFS) and single-cell force spectroscopy (SCFS) techniques, where the apex of the  
82 tip is decorated by a single biomolecule or a single microorganism, respectively.

83

84

85           In this review, we report some of the major breakthroughs in our understanding of  
86 microbial cell surface organization facilitated by AFM.

87

## 88 **Microbial cell surfaces at the nanoscale**

### 89 **Cell wall organization and ultrastructure.**

90           In the last two decades, AFM has provided remarkable insights into the cell wall  
91 architecture of microbes. AFM is advantageous compared to other high-resolution techniques  
92 (*e.g.* electron microscopy, crystallography) because it can be performed in liquid to analyse  
93 samples in physiologically relevant conditions. Yet, AFM microbe analyses require cell  
94 immobilization, *i.e.* attaching the microbes on a solid substrate to allow the cell surface to be  
95 scanned without lateral displacement. As microbial cells are often round and negatively  
96 charged, most immobilization methods rely on electrostatic interactions<sup>10-12</sup> or mechanical  
97 trapping in pores.<sup>13-15</sup> Each immobilization technique can induce a bias and should be carefully  
98 chosen according to the cell properties to be measured. For instance, cell trapping in porous  
99 membrane is very relevant to study cell biomolecules unfolding, as no chemicals are involved,  
100 preventing unwanted interactions with the AFM tips. However, the physical constraint applied  
101 on the cell might influence its nanomechanical properties. Other anchoring methodologies  
102 such as support-coating by chemicals (*e.g.* gelatine, polyethyleneimine, poly-lysine) should be  
103 privileged in this case.

104           For Gram-positive bacteria, the thick layer of peptidoglycan largely contributes to the  
105 cell-wall rigidity and is the most-exposed structure underneath the polysaccharidic surface  
106 layer. On the other hand, the thin layer of peptidoglycan in Gram-negative bacteria is overlaid  
107 by a soft and unstable outer lipid membrane. Therefore, immobilisation methods, imaging and

108 force measurements are usually straightforward for Gram-positive bacteria, while their  
109 application to Gram-negative bacteria is often more delicate.

110 Using the approaches mentioned above, different bacteria have been imaged at high  
111 resolution, revealing diverse cell surface features. The presence of proteinaceous surface  
112 layers (S-layers) give *Viridibacillus arvi*, *Lysinibacillus sphaericus*, and *Corynebacterium*  
113 *glutamicum* textured surfaces with repeated motifs (Fig. 1).<sup>16,17</sup> Single-cell imaging of  
114 *Lactobacillus rhamnosus* and *Lactobacillus plantarum* revealed rough surfaces attributed to  
115 adhesive extracellular polysaccharides and teichoic acids, respectively.<sup>18,19</sup> As peptidoglycan  
116 is a major building block of all bacterial cell walls, major efforts have also been made to  
117 decipher its organisation. However, until recently, its three-dimensional arrangement  
118 remained controversial. AFM imaging has solved this issue for *Lactococcus lactis* mutant  
119 devoid of cell wall polysaccharides, as topographic images revealed 25-nm-wide periodic  
120 ridges running parallel to the cell short axis. Chemical identification was verified by force  
121 spectroscopy using LysM-decorated tips. These results confirmed observations previously  
122 made on purified *Bacillus subtilis* sacculi (exoskeletons).<sup>20,21</sup> Li *et al.* showed that  
123 peptidoglycan organization is more variable than previously thought and changes during cell  
124 growth (Fig. 1A),<sup>22</sup> consistent with observations of *Streptococcus* and *Escherichia coli* (Fig.  
125 1B).<sup>23,24</sup> Nanoscale multiparametric imaging of polysaccharide intercellular adhesin (PIA)-  
126 expressing *Staphylococcus aureus* cells showed the presence of a soft and adhesive  
127 extracellular matrix surrounding the cells.<sup>25</sup>

128 Given its capability to image cell topographies at unprecedented resolution, several  
129 groups have used AFM to study cell wall changes during cell division, exposure to external  
130 stresses (*e.g.* drugs), etc. Eskandarian *et al.* showed that mycobacterial division occurs within  
131 a hollow between two crests on the undulating cell surface, which they linked to the



132 underlying peptidoglycan organization.<sup>26</sup> Similarly, time-series analyses showed that  
133 *Staphylococcus aureus* cells present a hairy surface during the exponential phase and  
134 progressively bald during the stationary phase.<sup>27</sup> AFM has also helped decipher the main steps  
135 of bacterial infection by phages. Protruding phages were detected at the surfaces of *E. coli*,  
136 *Salmonella enteritidis*, and *Bacillus thuringiensis*.<sup>28-30</sup> Using multiparametric AFM, Alsteens *et*  
137 *al.* showed that phages are extruded from densely packed areas corresponding to soft  
138 nanodomains in the cell septum.<sup>29</sup> Surface changes can also occur when cells are exposed to  
139 drugs. The digestion of *S. aureus* cell walls by lysostaphin was directly observed on live cells  
140 by time-resolved imaging.<sup>31</sup> Similarly, Formosa *et al.* revealed that antibiotics affect cell  
141 roughness and remove the capsule of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*,  
142 respectively.<sup>32,33</sup>

143 Yeasts cells have been also investigated by AFM imaging. For instance, AFM analyses  
144 of *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Candida glabrata*, and *Candida*  
145 *albicans* have revealed smooth surfaces that can present bud scars.<sup>34-37</sup> Most fungi undergo  
146 morphogenesis, during which they grow into elongate cells that are difficult to immobilize,  
147 hindering their analysis by AFM. Yet, Beaussart *et al.* immobilized *C. albicans* germ tubes on  
148 hydrophobic surfaces to image and probe the major cell wall components involved during  
149 pathogenesis.<sup>38</sup> El-Kirat-Chatel *et al.* used correlative AFM-fluorescence imaging to decipher  
150 the main steps of *Candida*-macrophage interaction: after 3 h, the macrophage membrane is  
151 pierced by long germ tubes formed by internalized yeasts, on which pieces of the macrophage  
152 are leftover.<sup>39</sup>

153 AFM can also be used to explore changes in bacteria and yeast cells induced by physical  
154 or chemical treatments such as antibiotics. Heat treatment induces the formation of large  
155 circular structures on *S. cerevisiae*,<sup>40</sup> and antifungal treatments have led to abnormally shaped

156 yeasts.<sup>41-44</sup> In particular, caspofungin, an antifungal drug that blocks the synthesis of  $\beta$ -1,3-  
157 glucans, increases the cell surface roughness and adhesiveness of *C. albicans*.<sup>42,43</sup>

158

### 159 **Bacterial appendages.**

160 Besides its basal composition, the bacterial cell wall is often decorated with protruding  
161 proteinaceous structures, *e.g.* flagella and pili, that are involved in motility, adhesion, cell-cell  
162 transfer, and pathogenesis.<sup>3</sup> Such appendages are typically imaged in air as they are too  
163 labile/flexible to be imaged in liquid. Curved structures longer than bacterial cell bodies and  
164 corresponding to flagella have been detected in images of *Pseudomonas fluorescens*, *Bacillus*  
165 *thuringiensis*, and *E. coli*,<sup>45-48</sup> and smaller, rod-shaped appendages corresponding to pili or  
166 fimbriae have been detected in images of *Lactobacillus rhamnosus*, *P. aeruginosa*,  
167 *Corynebacterium diphtheria*, *E. coli*, and *Salmonella*.<sup>46,49-53</sup> Flagella are always longer and  
168 larger than pili and fimbriae. Imaging these structures in physiologically relevant conditions  
169 remains challenging, though we anticipate that the development of new multiparametric AFM  
170 modes will contribute to the acquisition of topographical data on the appendages on living  
171 microbes.

172

### 173 **Detect, pull, unfold: the molecular biophysics of microbial surface-associated** 174 **biomolecules**

175 Biofilm formation depends on the successful adhesion of pioneer cells.<sup>2</sup> Consequently,  
176 microbes have evolved a large arsenal of adhesive mechanisms to stick to substrates and  
177 withstand mechanical shear. Understanding these molecular adhesion mechanisms is key to  
178 developing new antibiofilm strategies and may also inspire new biotechnological concepts.

179 Force spectroscopy has revolutionized our understanding of the microbial surface  
180 characteristics. In details, the AFM tip is successively approached and pulled away from the  
181 cell surface, resulting in a so-called force-distance curve. During the approach phase, the force  
182 acting on tip starts to increase directly after the contact between the tip and the biosurface is  
183 established. The tip can then be considered as a 'nanoindenter' that allows to sense the  
184 nanomechanics of the microbial surface. In the retract phase, the tip pulling on the surface  
185 biomolecules results in their unfolding. The retract curve can be composed of a single peak or  
186 a succession of peaks corresponding to the unfolding of the different domains forming the  
187 molecules. The shape of this unfolding curve is related to the nature of the biomolecules (*e.g.*  
188 protein, polysaccharide) and to its structure (*e.g.* the presence of repeated domains, the  
189 number of amino-acids in each domains). In SMFS, specific molecular probes are attached to  
190 the AFM tips, which allows mapping and unfolding of adhesive molecules expressed at  
191 microbial cell surfaces. Single-molecule stretching depends on the mechanical response of  
192 biomolecules when subjected to external forces, which can be directly linked to their cellular  
193 function.

194 To adhere to their support, bacteria need to withstand mechanical perturbations such as host  
195 coughing, sneezing or liquid flow. Bacterial adherence is usually maintained by extracellular  
196 protein appendages called pili. Using specific probes, the mechanical responses of pili under  
197 external applied force have been deciphered by SMFS. For instance, pulling individual pili from  
198 the Gram-positive bacteria *Lactobacillus rhamnosus* resulted in stepped force-distance curves  
199 presenting peculiar linear increases and horizontal plateaus, suggesting that these  
200 appendages behave like nanosprings under force stimulation (Fig. 2 A-C).<sup>54-56</sup> In these studies,  
201 pili were pulled with tips bearing pilin subunits or milk proteins, further illustrating their dual  
202 involvement in homotypic interactions with and adhesion to dairy components. The

203 pathogenic Gram-positive bacteria *Streptococcus pyogenes* and *Streptococcus pneumoniae*  
204 produce adhesive pili to colonize host surfaces. Unfolding of *S. pyogenes* pili by SMFS indicated  
205 that the major pilin is an inextensible protein, and that this peculiar mechanical resilience is  
206 attributed to isopeptide bonds strategically located in the pili structure.<sup>57</sup> Further analysis of  
207 the adhesive properties of *S. pyogenes* pili revealed that the reactivity of the pili adhesins -  
208 containing thioester functions- with their ligands is mediated by mechanical force. Indeed, the  
209 adhesion is governed by the pili adhesins folding state, which controls whether the thioester  
210 bond could be cleaved or not.<sup>58</sup> Pili of Gram-positive bacteria are also capable to dissipate  
211 energy through mechanical unfolding and refolding of polypeptides loops contained in the pili  
212 structures. Such an example are the CnaA loops in the pili of *Corynebacterium diphtheriae* and  
213 *Actinomyces oris*, which are highly stable and resist pulling forces of up to 500 pN, acting to  
214 dissipate mechanical energy and help bacteria resist shear forces.<sup>59</sup> SMFS also allowed to  
215 reveal that another Gram-positive bacteria, *S. pneumoniae*, expresses pili that contain two  
216 regions binding simultaneously to host fibronectin, and that this mechanism favours an  
217 efficient scan of the colonized surface, targeting specific cells for interaction and invasion.<sup>60</sup>  
218 The same group has also demonstrated that the backbone protein of this pilus binds to  
219 collagen I in a force-dependent manner, resembling the so-called catch-bond mechanism.<sup>61</sup>  
220 In contrast, the unfolding of the pili and fimbriae of Gram-negative bacteria reveal completely  
221 different force signatures. Pulling on type-1 fimbriae from *E. coli* with mannose-decorated tips  
222 produced force curves with constant force plateaus and long rupture distances (up to 10  
223  $\mu\text{m}$ ),<sup>62</sup> attributed to the uncoiling of the quaternary structure of fimbriae during the catch-  
224 bond interaction with mannose residues, an interaction that is strengthened under tensile  
225 mechanical force. The specific interaction between mannose and *E. coli* fimbriae was  
226 demonstrated by using mannosylated tips to probe wild-type and mutant cells devoid of

227 appendages.<sup>63</sup> This study demonstrated a possible antiadhesive property of mannosylated  
228 fullerenes, which can simultaneously interact with and block multiple fimbriae.<sup>63</sup> Interestingly,  
229 the different Fim domains of *E. coli* fimbriae are organized in a hierarchical mechanical  
230 architecture; domains close to the outer membrane present higher mechanical stability than  
231 those exposed at the apex of the appendage.<sup>64</sup> On *P. aeruginosa*, hydrophobic tips were used  
232 to probe type-IV pili, producing force plateaus similar to those observed for *E. coli* pili.<sup>49</sup> By  
233 comparing different mutant strains, these authors concluded that the pili structure governed  
234 this hydrophobic interaction.<sup>49</sup>

235         In addition to appendages, bacterial adhesion can also be promoted by adhesins, *i.e.*  
236 adhesive proteins anchored at the cell surface. As for pili, SMFS with tips bearing specific  
237 probes can be used to map the distribution and probe the mechanics of individual adhesins at  
238 the surfaces of living cells. Using heparin tips, single heparin-binding hemagglutinin adhesins  
239 were detected on the surface of *Mycrobacterium bovis* and were found to be grouped in  
240 nanodomains.<sup>65</sup> Filamentous hemagglutinin adhesins at the surface of *Bordetella pertussis*  
241 were detected with antibody-bearing tips, which revealed a force-induced reorganization of  
242 adhesins on the cell surface to reinforce bacterial attachment under external forcing.<sup>66</sup> The  
243 specific force signature resulting from molecular unfolding during SMFS also gives crucial  
244 information on the mechanical properties of adhesins. In *Pseudomonas fluorescens*, adhesion  
245 is finely controlled by the Lap regulation system in which LapA is the surface exposed  
246 adhesin.<sup>67</sup> This protein is known to promote adhesion to surfaces of various physicochemical  
247 properties and contains a cell wall-anchoring domain, 37 repeated sequences, and a C-  
248 terminal globular domain (Fig. 2D). The mechanical response of LapA has been deciphered by  
249 SMFS with tips decorated with specific antibodies (Fig. 2E). Unfolding LapA led to force  
250 signatures presenting regular sawtooth patterns that were attributed to the successive

251 unfolding of the 37 repeats (Fig. 2F).<sup>68</sup> High environmental inorganic phosphate  
252 concentrations induce the exposure of LapA to promote cell adhesion, whereas decreased  
253 concentrations lead to the cleavage of LapA to allow cells to explore other environments.<sup>69</sup>  
254 Substrate-adhered LapA presents multiple adhesive peaks with no regular shape, reflecting  
255 that LapA interacts via multiple substrate domains. Moreover, the LapA distribution on the  
256 substrate corresponds to areas where bacteria had previously adhered, indicating that LapA  
257 is left on the substrate as bacterial ‘footprints’.<sup>70</sup>

258 SMFS was also used to decipher the nanobiophysics of *Staphylococcal* adhesion,  
259 especially for the two major pathogens *Staphylococcus epidermidis* and *Staphylococcus*  
260 *aureus*. During infection, *Staphylococci* express different adhesins grouped as microbial  
261 surface components recognizing adhesive matrix molecules (MSCRAMMs) to bind to their  
262 host surfaces.<sup>71</sup> To characterize the contribution of SdrF adhesins in the adhesion of *S.*  
263 *epidermidis* to collagen, recombinant *Lactococcus lactis* expressing the *S. epidermidis* SdrF  
264 gene was probed with collagen-decorated tips.<sup>72</sup> This revealed that regions A and B of SdrF  
265 form strong and weak bonds with collagen, respectively, and both interactions present high  
266 dissociation rates.<sup>72</sup> *L. lactis* also served to demonstrate the specific recognition of fibrinogen  
267 by SdrG.<sup>73</sup> On *S. epidermidis*, probing SdrG with fibrinogen tips resulted in a remarkably strong  
268 interaction of ~2 nN;<sup>72,74</sup> such strong forces were previously only reported for covalent  
269 bonds.<sup>75</sup> Although the fibrinogen-SdrG interaction withstands high forces, it does not correlate  
270 with high affinity ( $K_d \approx 400$  nM).<sup>76</sup> This high mechanical stability is thus attributed to the N2  
271 and N3 domains of SdrG, which operate the so-called “dock, lock, and latch” (DLL) mechanism  
272 on the target and allow strong mechanical attachment.<sup>76</sup> Further SMFS analysis coupled with  
273 all-atom steered molecular dynamics (SMD) simulations confirmed this high interaction force

274 and identified that a minimal six-residue sequence in the fibrinogen is sufficient for SdrG DLL  
275 binding.<sup>77</sup>

276 Similarly high interaction forces (~1.5 nN) were reported for the collagen-binding  
277 protein Cna of *S. aureus* using collagen tips.<sup>78</sup> In this case, SMFS was used to decipher the  
278 nanospring role of the CnaB region in the collagen hug mechanism, whereas the A region is  
279 involved in initial ligand binding and can be blocked by monoclonal antibodies. The capability  
280 of Cna to bind to other host proteins was also established with tips decorated with laminine  
281 or the complement system protein C1q.<sup>79</sup> This study demonstrated that Cna is a  
282 multifunctional adhesin that can bind several host proteins through different mechanisms to  
283 increase the probability of *S. aureus* adhesion. In the dual binding modes of the *S. aureus*  
284 clumping factors A and B (ClfA and ClfB) to the fibrinogen and loricrin proteins of the host,  
285 respectively, mechanical tension can convert an initial weak interaction into a strong one  
286 involving a DLL mechanism with N2N3 subdomains.<sup>80,81</sup> The dual binding mode of ClfA to  
287 fibrinogen was further confirmed by injecting blocking agents *in situ* during AFM experiments:  
288 only strong interactions were blocked by a short Fg  $\gamma$ -chain peptide.<sup>80</sup> Strong adhesion was  
289 also reported between *S. aureus* protein A and the plasma glycoprotein vonWillebrand factor.  
290 Analyses of this interaction in flow chambers revealed that strong interactions are induced by  
291 high shear forces.<sup>82</sup> Furthermore, the DLL conformation of *S. aureus* fibronectin-binding  
292 proteins (FnBPs) adopted when bonded to fibrinogen dramatically increases the plasminogen  
293 interaction.<sup>83</sup> Similarly, the interaction of FnBPA with host integrins is strongly enhanced in  
294 the presence of fibrinogen, forming a triple interaction involving FnBPA, the host fibrinogen,  
295 and  $\alpha 5\beta 1$  integrin.<sup>84</sup> These results have revealed the cryptic role of FnBPs and showed that  
296 bacterial adhesins have evolved towards multipotent effectors for successful cell adhesion.

297 During infection, biofilm formation requires not only adhesion to host surfaces, but  
298 also cohesion between neighbouring bacteria. In *S. aureus*, this second function has been  
299 highlighted for FnBPA and SdrC cell wall proteins.<sup>85,86</sup> In both cases, the interaction involves  
300 homophilic bonds between proteins of two adjacent cells.

301 SMFS has also revealed the molecular mechanisms governing the surface properties  
302 of fungal cells. Probing the mechanical response of individual WSC1 proteins at the surface of  
303 *Saccharomyces cerevisiae* revealed that this protein behaves like a nanospring, acting as a  
304 mechanical force sensor by forming force-induced clusters on the yeast surface.<sup>87</sup> Exploiting  
305 this typical mechanical response, the authors used truncated and elongated versions of WSC1  
306 to demonstrate that the yeast cell wall is ~200 nm thick.<sup>88</sup> In the fungal pathogen *Candida*  
307 *albicans*, cell adhesion is governed by proteins of the Agglutinin-like sequence (Als) family.<sup>89</sup>  
308 These proteins share a common structure with an immunoglobulin (Ig)-like region, followed  
309 by an amyloid-forming region, a tandem repeat (TR) region, and a stalk region anchored in the  
310 cell wall. SMFS was used to unravel the mechanisms by which Als proteins promote yeast  
311 adhesion. The unfolding of single Als proteins at the surfaces of living cells produced force  
312 signatures presenting sawtooth patterns, in which each peak corresponded to the quantum  
313 force required to unfold one repeat of the TR region.<sup>90,91</sup> Consecutive Als mapping on the same  
314 cell resulted in the remarkable formation of adhesive patches, documenting the capacity of  
315 Als to form clusters upon mechanical stimulation.<sup>90</sup> The role of Als in yeast cell adhesion was  
316 also demonstrated during the cell stress response to caspofungin and during  
317 morphogenesis.<sup>38,42</sup> The presence of Als in these conditions was correlated with increased cell  
318 surface hydrophobicity by chemical force microscopy (CFM), where AFM tips are decorated  
319 by methyl-terminated thiols to decipher the sample hydrophobicity with a few-nanometres  
320 lateral resolution. This increased hydrophobicity was attributed to the exposure of TR domains



321 by unfolded Als proteins.<sup>38,42,43</sup> The amyloid-forming region was shown to be involved in  
322 homophilic zipper-like interactions and the formation of adhesive nanodomains.<sup>92,93</sup>

323 In *Candida glabrata*, the second major human fungal pathogen, Epa adhesins are  
324 known to bind to host cells through lectin-like interactions.<sup>94</sup> In addition, it was recently shown  
325 by CFM that Epa6 confers strong surface hydrophobicity to *C. glabrata* and may promote  
326 adhesion to abiotic surfaces.<sup>37</sup> In *S. cerevisiae*, analysis of the lectin function of Flo1 with a  
327 mannose-decorated tip revealed that Flo1 presents mechanical properties similar to Als in *C.*  
328 *albicans*, with a repeat region leading to sawtooth force-distance curves.<sup>95</sup>

329 As demonstrated above, the ultrasensitivity of AFM to force changes can be used to  
330 decipher adhesive mechanisms at molecular resolution. Another challenge is to understand  
331 how adhesive molecules work together at the scale of the entire cell, in both the contexts of  
332 cell-substrate and cell-cell interactions. To address single-cell adhesion, single-cell force  
333 spectroscopy (SCFS) was recently adapted to microbial cells.

334

### 335 **SCFS methodologies to study microbial cells at the single-cell level**

336 In SCFS, the AFM probe is decorated with a single cell and used to measure the  
337 interaction forces between this cellular probe and target surfaces or cells. Initially described  
338 for mammalian cells,<sup>96</sup> different protocols have emerged to adapt SCFS to smaller cells, *i.e.*  
339 microbes. Tip-less cantilevers are usually coated with poly-L-lysine, Cell-Tak, or polydopamine  
340 to attach a single microbial cell at the apex (Fig. 3A).<sup>97,98</sup> Whereas the toxicity of poly-L-Lysine  
341 is uncertain, polydopamine—a bio-inspired adhesive—is reported to preserve cell viability.<sup>99-</sup>  
342 <sup>101</sup> However, when probing small cells such as bacteria (~1  $\mu\text{m}$ ), the precise positioning of the  
343 cell at the apex of the tip-less cantilever is complicated, and unwanted contact between the

344 tip-less cantilever and the probed surface may occur. In this context, the use of colloidal  
345 probes is an attractive alternative, ensuring precise positioning of the bacteria while  
346 maintaining its viability.<sup>102</sup> Another elegant technique is fluidic force microscopy (FluidFM)  
347 adapted for SCFS of microbial cells which uses hollow cantilevers to apply an under-pressure  
348 and trap single cells without the need for chemical fixation, thus allowing the screening of tens  
349 to hundreds of cells within hours.<sup>103-105</sup> Although well-suited to yeast cells (Fig. 3B), the  
350 application of FluidFM to bacteria remains limited because hollow cantilevers with apertures  
351 sufficiently small to immobilize bacteria are not yet commercially available. Nonetheless,  
352 focused ion beam milling can be used to mill the aperture of a hollow cantilever to obtain 300–  
353 900 nm holes, suitably small to immobilize bacteria.<sup>105</sup> A recent study ingeniously reversed  
354 the system, using a commercial hollow cantilever to immobilize a C30-functionalized colloidal  
355 silica bead, mimicking the hydrophobic surface of a leaf.<sup>106</sup> This functionalized bead is then  
356 used to probe bacterial cells immobilized on a surface at a higher throughput than traditional  
357 SCFS methods.

358

### 359 **Microbial adhesion revealed by SCFS**

360         Since its development, several studies have demonstrated the importance of the SCFS  
361 technique. For instance, *L. rhamnosus* pili interactions with hydrophobic and mucin surfaces  
362 produce force signatures indicating nanospring behaviour, whereas those with Caco-2 cells  
363 produce force plateaus reflecting membrane tethering.<sup>107</sup> This suggests that the interaction  
364 force between pili and Caco-2 cells is not strong enough to induce pili unfolding or that the  
365 interaction involves basal regions of the pili (*i.e.* close to the bacterial surface). Similarly, it was  
366 demonstrated that type IV pili of *P. aeruginosa* are involved in interactions with both abiotic

367 surfaces and host pneumocytes.<sup>49</sup> In this case, the force signatures revealed that interactions  
368 with abiotic surfaces lead to pili unfolding whereas adhesion to host cells is mediated by both  
369 cellular membrane tethers and bacterial pili.

370         The role of surface adhesins can also be deciphered by SCFS. Analyses of different *P.*  
371 *fluorescens* mutants revealed that LapA promotes adhesion to both hydrophilic and  
372 hydrophobic substrates via specific domains of the protein, *i.e.* the C-terminal globular domain  
373 for adhesion to hydrophilic substrates and the repeated sequence for hydrophobic  
374 interactions.<sup>68</sup> The function and specificity of *Staphylococci* adhesins described at the single-  
375 molecule level (see above) were confirmed at the single-cell level by measuring the  
376 interactions between individual cells and surfaces coated with different host proteins or  
377 chemicals (Fig. 4).<sup>25,72,78,85,86,108</sup> Compared to SMFS, SCFS allows the forces between two living  
378 cells to be probed, providing insight into the role of surface adhesins during cell-cell or cell-  
379 host interactions. For instance, the cationic PIA was shown to promote *S. aureus* cell-cell  
380 adhesion through electrostatic interactions with polyanionic teichoic acids, strengthening the  
381 cohesion of the bacterial biofilm.<sup>25</sup> *S. aureus* cellular aggregation is also promoted by the  
382 adhesin SasG, and the underlying molecular mechanism was determined by SCFS  
383 measurements of the forces between two partner cells (Fig. 4B).<sup>109</sup> This mechanism requires  
384 Zn<sup>2+</sup> to activate cell surface reorganization and unmask SasG proteins from the other cell wall  
385 components. Once protruding, SasG promotes cell aggregation through homophilic  
386 interactions.<sup>109</sup> This is reminiscent of the roles of SdrC (Fig 4C)<sup>86</sup> and FnBPA,<sup>85</sup> two other  
387 surface adhesins in *S. aureus*, for which their role in cell aggregation was demonstrated  
388 through homophilic interactions. FnBPA is also implicated in the adhesion of *S. aureus* to host  
389 endothelial cells.<sup>84</sup>

390           Among the bacterial adhesive arsenal, some surface proteins involved in cell-cell  
391 adhesion also facilitate bacterial conjugation, or horizontal gene transfer. In the conjugation  
392 process, a donor cell comes in contact with a receptor cell, and transfers part of its genetic  
393 material, for instance a plasmid. Recently, SCFS has been used to study cell-cell adhesion and  
394 the transfer of the pXO16 plasmid in the Gram-positive bacterium *Bacillus thuringiensis*, which  
395 is accompanied by macroscopic cellular aggregation.<sup>110</sup> In that study, a donor or receptor cell  
396 was immobilized on a colloidal probe, and put in contact with a mating partner long enough  
397 for the plasmid to transfer. Their results show that the donor-receptor interaction is very  
398 strong and relies on surface proteins encoded by the pXO16 plasmid. In a minimal medium  
399 and with enough time for the transconjugant cells to express the plasmid, major changes in  
400 the surface adhesive properties were observed. This work demonstrates that SCFS not only  
401 allows studying the role of surface proteins at the scale of a single cell-cell pair, but can also  
402 be used to induce local gene transfer and study the resulting cell surface changes.

403           In mixed biofilms, several species interact and SCFS has been used to decipher these  
404 interactions. Notably, *S. epidermidis* and *C. albicans* are frequently associated in polymicrobial  
405 biofilm infections with enhanced antibiotic resistance. SCFS measurements probing the *C.*  
406 *albicans* surface with single bacterial cells revealed that *S. epidermidis* adhere strongly (~5 nN)  
407 on *C. albicans* germ tubes and poorly on yeast cells.<sup>111</sup> This adhesion was attributed to Als and  
408 O-mannosylated proteins of the fungal cell wall, as demonstrated with different mutant  
409 strains. FluidFM experiments on *C. albicans* cells have recently measured strong Als5-  
410 mediated intercellular adhesion with homophilic bonding between Als5 proteins and amyloid-  
411 like bond formation (Fig. 3B).<sup>112</sup> In *C. glabrata*, the role of EPA adhesins in adhesion to  
412 hydrophobic and hydrophilic abiotic substrates was demonstrated by SCFS.<sup>37,113</sup> The  
413 mechanism by which the Flo1 adhesin induces cell aggregation in *S. cerevisiae* was also

414 demonstrated by measuring interactions between two partner cells:<sup>95</sup> by injecting EDTA or  
415 free mannose as blocking agents in the AFM liquid cell during force measurements, they  
416 showed that Flo1 adhesion is calcium-dependent and that cell aggregation is governed by the  
417 lectin part of the protein, which recognizes mannosides on the partner cell.

418 In the context of the host-pathogen relationship, SCFS has been used to decipher *C.*  
419 *albicans* interactions with immune cells. It was shown that DC-SIGN receptors on dendritic  
420 cells recognize a specific N-mannan structure in the yeast cell wall, and that N-glycans chains  
421 of these receptors are essential for the clustering that induces pathogen internalization.<sup>114,115</sup>  
422 On macrophages, it was shown that yeast recognition mostly involves mannose receptors, and  
423 that *C. albicans*-macrophage interactions lead to the formation of long tethers (up to 100  
424  $\mu\text{m}$ ).<sup>116</sup> SCFS has also been used to measure and image the interactions between *S. aureus*  
425 and human skin cells.<sup>117-119</sup> The ClfB staphylococcal adhesin has been evidenced as a major  
426 actor in such interactions, as it is responsible for mechanically strengthening interactions,  
427 notably through the ClfB-loricrin interaction.<sup>81</sup>

428

## 429 **Anti-adhesive strategies screened by AFM**

430 Increased bacterial resistance to antibiotics makes blocking bacterial adhesion an  
431 attractive alternative to antibiotics in order to prevent bacterial infections linked to biofilm  
432 formation. AFM-based force spectroscopy techniques are useful for studying the potential of  
433 anti-adhesive strategies and molecules, and several strategies are being pursued to block both  
434 non-specific adhesion (*e.g.* hydrophobic interactions) and specific interactions involving  
435 different adhesion proteins. In particular, SCFS has been instrumental in deciphering anti-  
436 adhesives strategies illustrated through several examples given below.

437 For example, the capacity of glycofullerenes, as compared to glycomonomers, to block  
438 *E. coli* adhesion was quantified and attributed to the enhanced antiadhesive activity of  
439 mannofullerene compared to that of heptylmannoside.<sup>63</sup> Similarly, the anti-adhesive effects  
440 of charged polymer brushes on *E. coli* were investigated by SCFS, with anionic and neutral  
441 brushes leading to decreased *E. coli* adhesion.<sup>120</sup>

442 Valotteau *et al.* studied the anti-adhesive properties of sophorolipids on the  
443 pathogenic bacteria *S. aureus* and *E. coli*.<sup>121</sup> They demonstrated an overall decrease of  
444 bacterial adhesion forces and probability on sophorolipid-coated surfaces. Mutant *S. aureus*  
445 with no surface adhesins showed further reduced adhesion to the surface, indicating that  
446 sophorolipids not only block adhesins, but also other cell wall components such as teichoic  
447 acids. The specific targeting of adhesin-ligand interactions has been successful for several  
448 staphylococcal adhesins. For instance, a  $\beta$ -neurexin-derived peptide partially blocks SdrC-SdrC  
449 interaction (Fig. 4C),<sup>86</sup> and anti-CNA antibodies inhibit the adhesion of *S. aureus* on collagen-  
450 coated surfaces.<sup>78</sup>

451 Anti-adhesion is also being pursued to block the first step of bacterial invasion in the  
452 context of host cell-bacteria interactions. The invasion of human lungs by *P. aeruginosa* is  
453 facilitated by the interaction of the lectin LecA with the host epithelial cell's  
454 globotriaosylceramide.<sup>122</sup> A new therapeutic approach relies on synthetic glycoclusters  
455 targeting LecA with higher affinity than its natural ligand. Measurements of the interactions  
456 between a human bronchial epithelial cell immobilized on a tip-less AFM cantilever and a  
457 bacterial monolayer of *P. aeruginosa* demonstrated promising anti-adhesive properties, with  
458 the LecA lectin-glycocluster binding weakening bacteria-cell adhesion.<sup>123</sup>

459

## 460 Perspectives

461 In addition to traditional AFM imaging, single-cell and single molecule force  
462 spectroscopy, new AFM-based approaches have emerged and provide information on  
463 microbes and their surface properties. Among them, high-speed AFM has allowed imaging  
464 dynamic processes on living cells, helping to decipher, for instance, the mechanism of action  
465 of antimicrobial peptides on *E. coli* individual cells in real time.<sup>124,125</sup> Recently, high-speed AFM  
466 combined to tapping mode with high-resonance-frequency small and soft cantilevers has also  
467 enabled the imaging of intact bacterial photosynthetic organelles with very low applied forces,  
468 providing access to single *Rhodobacter sphaeroides* photosystem-protein complexes and  
469 intact ATPases.<sup>126</sup> To summarize, high-speed AFM is an increasingly valuable method to  
470 characterize microbial envelopes and their proteins in physiologically relevant conditions.

471 In parallel, AFM has also recently been combined to infrared spectroscopy,<sup>127,128</sup>  
472 leading to an exciting method of chemical characterization and mapping with a spatial  
473 resolution of ~20-30 nm. Applied to microorganisms, AFM-IR has so far allowed the chemical  
474 mapping of intra-cellular nanogranules of polyhydroxybutyrate in *Rhodobacter capsulatus*,<sup>129</sup>  
475 and of triacylglycerol in *Streptomyces* bacteria, *Yarrowia* yeast, and *Parachlorella Kessleri*  
476 microalgae.<sup>128</sup> Rapid progress has been reported with this technique, leading to label free  
477 mapping of thin phospholipid membranes,<sup>130</sup> access to molecular orientation<sup>131</sup> or light-  
478 induced conformational changes of bacteriorhodopsin in native purple membrane patches.<sup>132</sup>  
479 If so far, AFM-IR is limited to dried samples, it has the potential to become a great tool to  
480 better understand the structure and organization of membranes and bacterial envelopes, as  
481 well as their interactions with their environment.

482

## 483 **Conclusions**

484           The various AFM-based techniques developed in recent years are perfectly suited to  
485 deciphering microbial surface properties. AFM imaging has revealed morphological features  
486 and cell wall topographical organization in live cells at unprecedented resolution. Single-  
487 molecule and single-cell force spectroscopy are powerful tools for unravelling molecular  
488 mechanisms by probing the mechanics of individual molecular interactions or the adhesion of  
489 entire cells. The dual capability of AFM to probe adhesion at the single-cell and single-  
490 molecule scales makes it an attractive tool for the development of anti-adhesive strategies,  
491 for which the mode of action must be demonstrated at multiple scales. Indeed, the molecular  
492 mechanisms revealed by SMFS can inform the design of new anti-adhesive molecules, and  
493 SCFS is suitable for the subsequent screening required to identify the best candidates.

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## 498 **Author contributions.**

499 AB, CF and SEKC wrote the paper.



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754

## 755 **Figure legends**

756 **Figure 1. High resolution AFM imaging reveals bacterial S-layer and peptidoglycan**  
757 **structures.** A) AFM images of side-wall peptidoglycan from *Bacillus subtilis* at different growth  
758 phases. Scales bars = 200 nm (a-d) and 100 nm (e,f). (B) AFM images of *Escherichia coli*  
759 peptidoglycan organization (left) together with gradient organisation map (right) indicating  
760 the tendency of the structure to order. Scales bars = 50 nm (C-D) Images of *Viridibacillus arvi*  
761 (C, D bottom) and *Lysinibacillus sphaericus* (D, top) depicting S-layer envelopes. (E) S-layer is  
762 visible at the surface of *Corynebacterium glutamicum* grown in specific growth medium.  
763 (A) has been reproduced from ref<sup>22</sup>. (B) has been reproduced from ref<sup>24</sup>, with permission from  
764 Springer Nature. (C-D) have been reproduced from ref<sup>17</sup>, with permission from RSC Publishing.  
765 (E) has been reproduced from ref<sup>16</sup>, with permission from the American Chemical Society.

766

767 **Figure 2. Single-molecule force spectroscopy allows to decipher adhesion and**  
768 **nanomechanics of bacterial appendages.** A) Primary structure of the subunits constituting pili  
769 of the Gram-positive bacteria *Lactobacillus rhamnosus* GG. B) AFM image of *Lactobacillus*  
770 *rhamnosus* GG living bacteria trapped in a porous membrane, with pili structures imaged in  
771 air depicted in the inset (left). Pulling single pili with pilin-subunits-decorated tips (right)  
772 results in various force-distance curves profiles. C) Representative force-distance curves of pili  
773 unfolding revealing single adhesion force peaks with linear, spring-like shapes and  
774 characteristic steps; red numbers correspond to linear segments of increasing slopes. Such  
775 patterns helped to understand the nanomechanical response of pili under external force. D)  
776 Primary structure of LapA, an adhesin from the Gram-negative bacteria *Pseudomonas*  
777 *fluorescens*. E) AFM image of *Pseudomonas fluorescens* living bacteria trapped in a porous  
778 membrane (left). AFM tips functionalized with anti-HA antibodies were used to detect HA-

779 tagged LapA molecules at the surface of living cells. F) Representative force-distance curves  
780 of LapA protein unfolding. The sawtooth patterns observed on the curve correspond to the  
781 sequential unfolding of repeated patterns in the protein sequence. Red lines correspond to  
782 worm-like-chain model fit.

783 (A-C) have been modified from ref<sup>56</sup>, with permission from the American Chemical Society. (D-  
784 F) have been reproduced from ref<sup>68</sup>, with permission from the American Chemical Society.

785

786 **Figure 3. Methodologies used to attach a single-cell to the AFM cantilever.** (A) Single-cell  
787 force spectroscopy consists in approaching a biological-glue (polydopamine) coated tip-less  
788 cantilever towards a living cell (here yeast) to attach it to the cantilever and then probe its  
789 adhesion on different (bio)-surfaces. (B) With the FluidFM set-up, a single cell is immobilized  
790 on the cantilever aperture by applying a negative pressure. The cell probe is then moved  
791 toward a target cell immobilized in a porous membrane and force-distance curves are  
792 recorded.

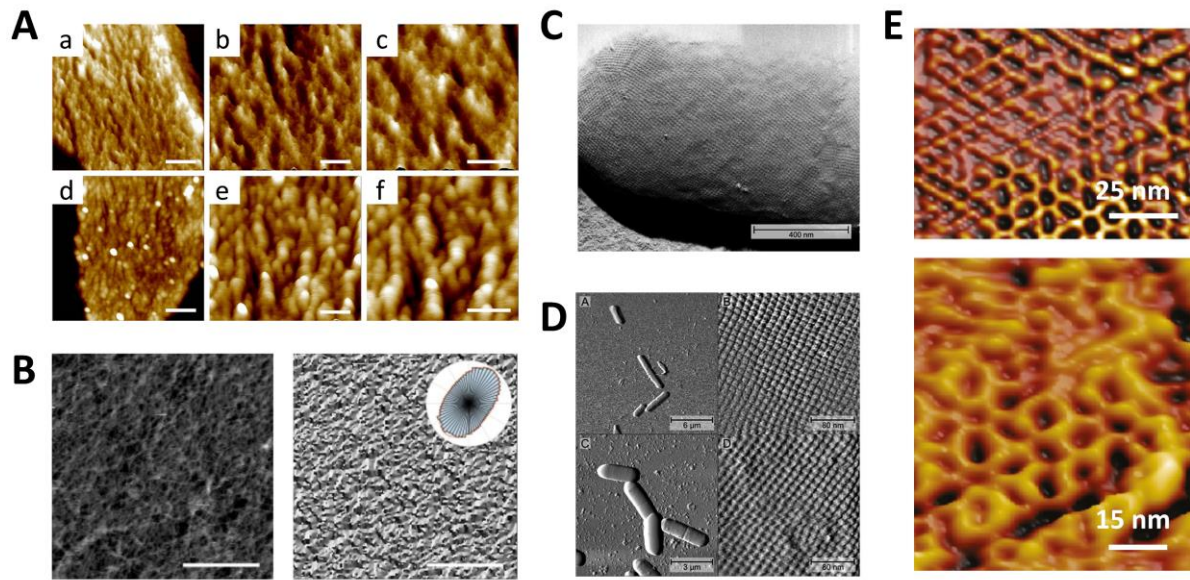
793 (A) has been reproduced from ref<sup>100</sup>, with permission from the Royal Society of Chemistry. (B)  
794 has been reproduced from ref<sup>112</sup>, with permission from the American Chemical Society.

795

796 **Figure 4. Deciphering the binding mechanism of different *Staphylococcal* adhesins by single-**  
797 **cell force spectroscopy.** SCFS with *Staphylococcus* bacteria used as a bioprobe revealed that  
798 (A) its adhesin SdrG binds to fibrinogen following a 'dock, lock and latch' model with strong  
799 forces of  $\sim 2$  nN; (B) Zinc activates the adhesive function of the adhesin SasG in homophilic  
800 cell-cell interactions with representative sawtooth patterns specific of SasG protein unfolding  
801 and (C) the adhesin SdrC is engaged in low affinity intercellular adhesion below 500 pN and  
802 strong cellular interactions with hydrophobic surfaces.

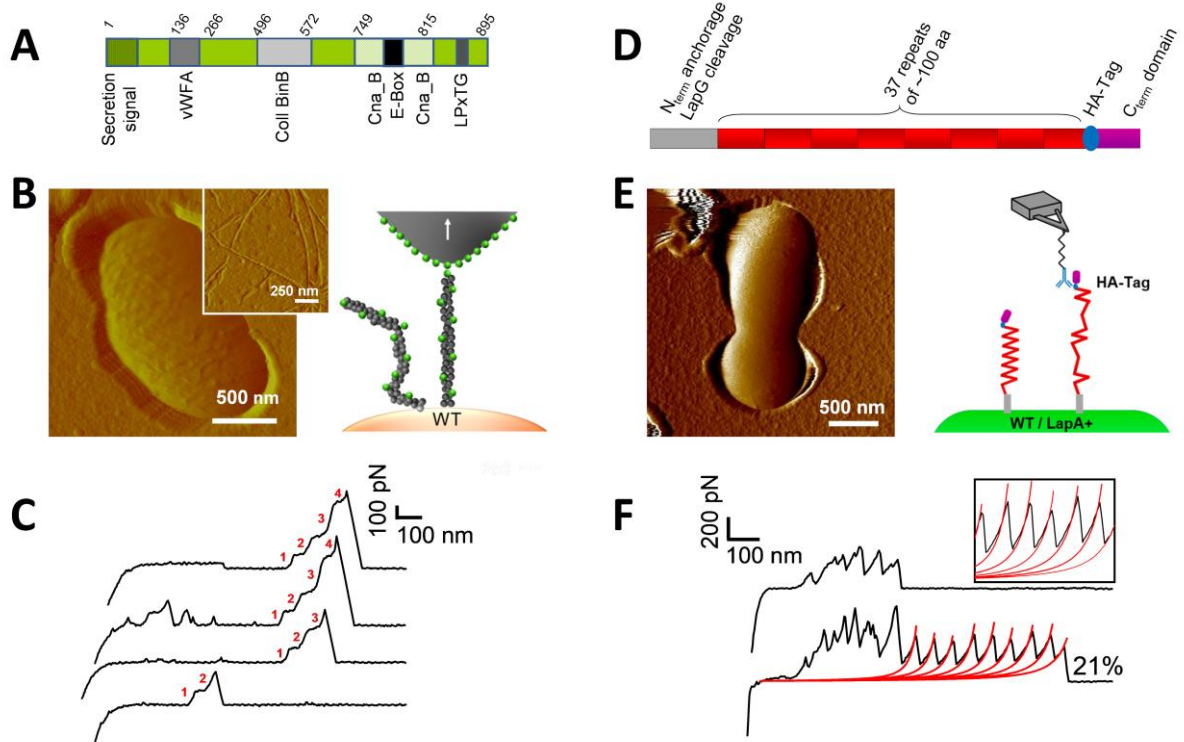


803 (A) has been modified from ref<sup>72</sup>, with permission from John Wiley and Sons, (B) from ref<sup>109</sup>  
804 and (C) from ref<sup>86</sup>, with permission from the National Academy of Science.  
805

806 **Figure 1**

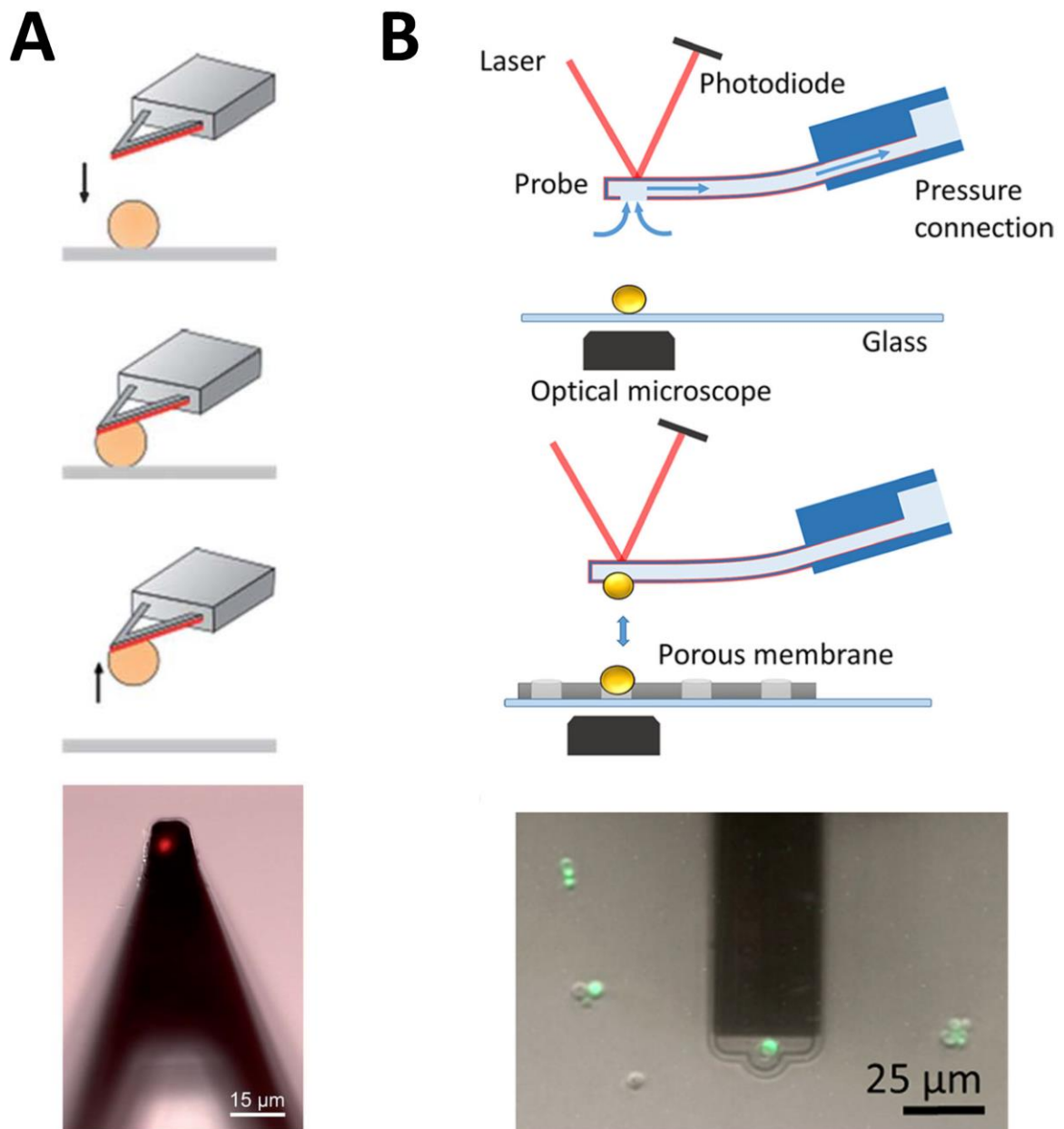
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809 **Figure 2**

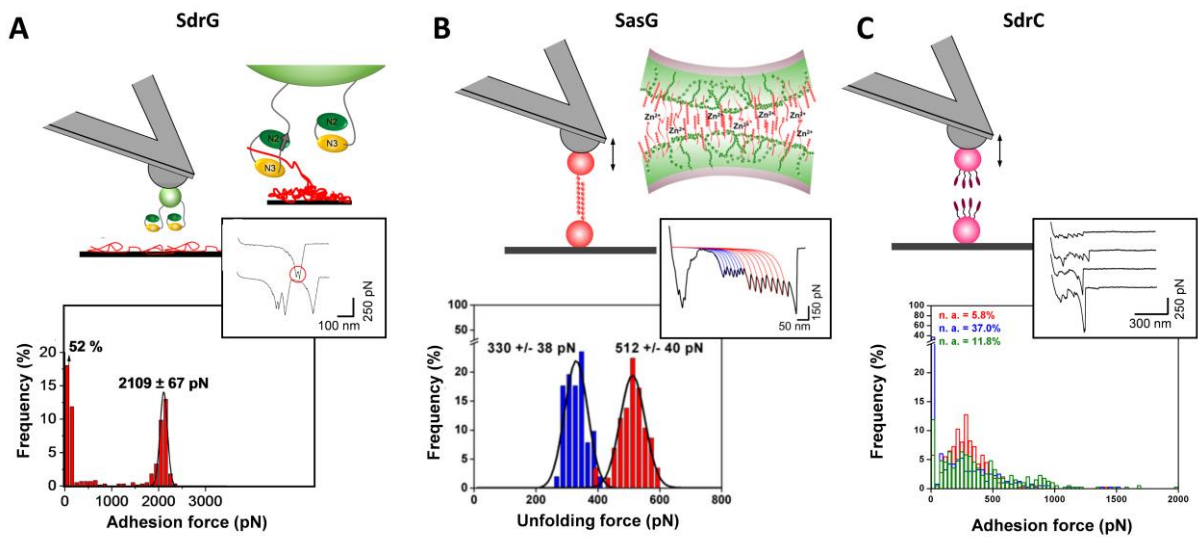
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812 **Figure 3**

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815 **Figure 4**

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