



Mission pour les initiatives transverses et
interdisciplinaires (MITI)



Journée thématique

Fonctionnalisation de sondes et analyses de données

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Chimie et Biologie des Membranes
et des Nano-objets
CBMN UMR 5248

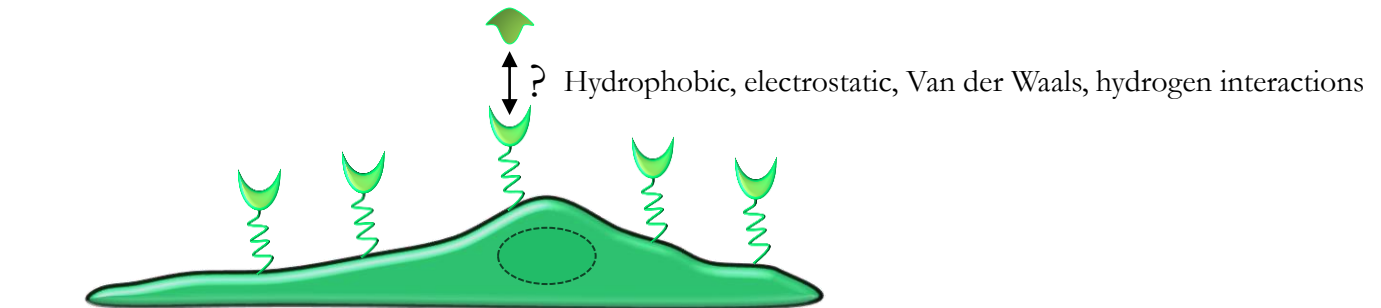


université
de BORDEAUX

Toulouse, 29 novembre 2023

Why measuring bio-interface interactions?

The bio-interface is the extreme limit between the intracellular compartment and the surrounding environment and governs signal transfer, molecular transport, and adhesion.



Probing ligand-receptor interactions and surface chemical properties to:

- Map the distribution of molecules / chemical properties
- Analyze molecular dynamics
- Determine biophysical properties/mechanics of molecules



Probe functionalization: (bio)-probes to scan the properties and organization of bio-interfaces

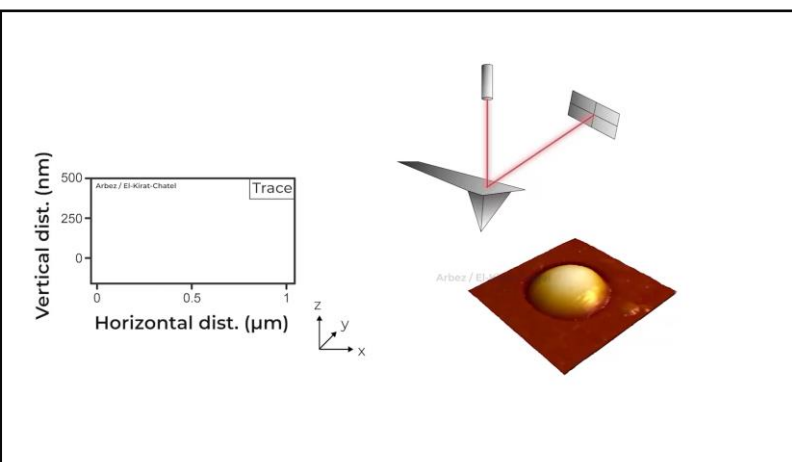


GT Fonctionnalisation

How to measure interaction forces?

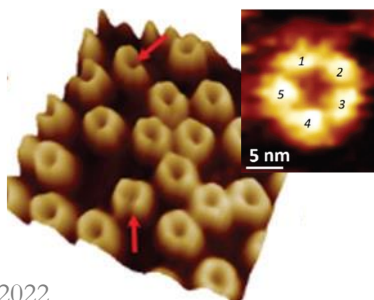
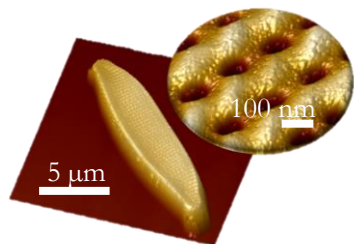
How to measure interaction forces?

AFM: More than an imaging tool → Force spectroscopy to measure interaction forces



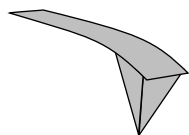
Nitzschia palea

Prx decamer



Laviale *et al.* ACS AMI 2019

Beaussart *et al.* Nanoscale Horiz 2022

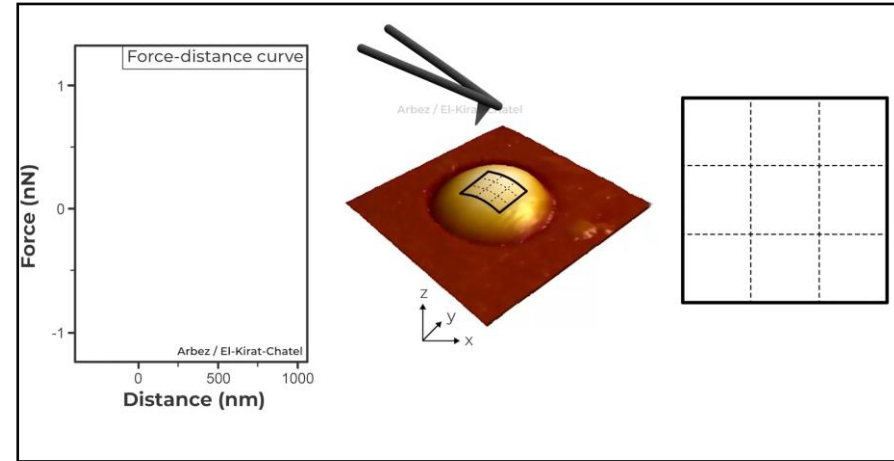
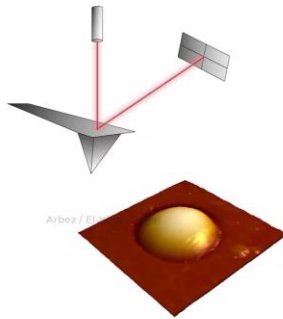
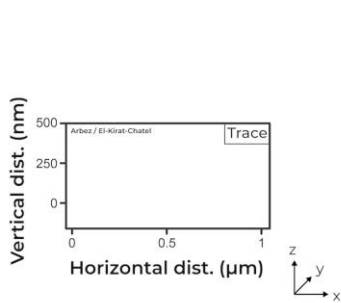


Bare tip

Require strong and reliable sample immobilization

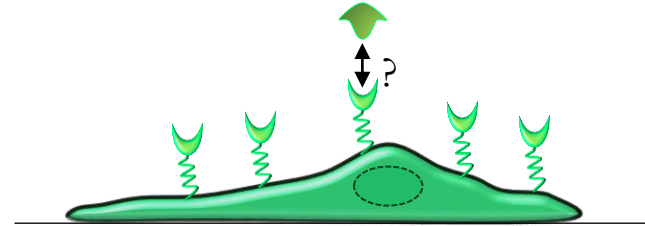
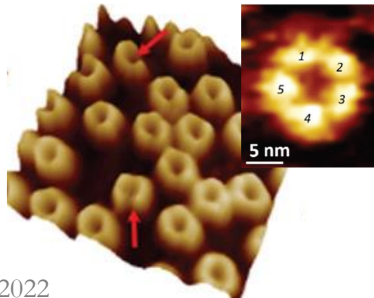
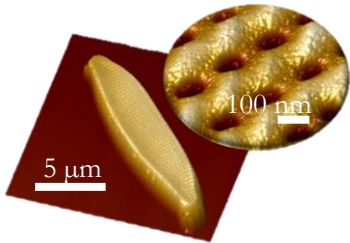
How to measure interaction forces?

AFM: More than an imaging tool → Force spectroscopy to measure interaction forces

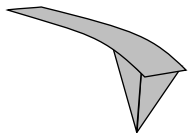


Nitzschia palea

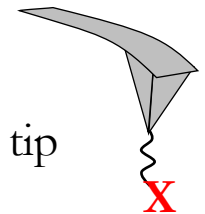
Prx decamer



Laviale *et al.* ACS AMI 2019
Beaussart *et al.* Nanoscale Horiz 2022

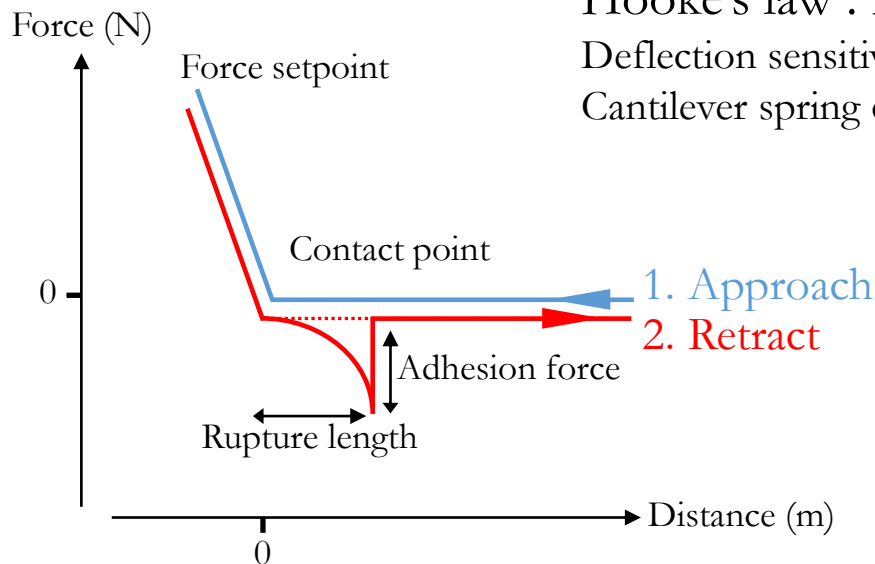
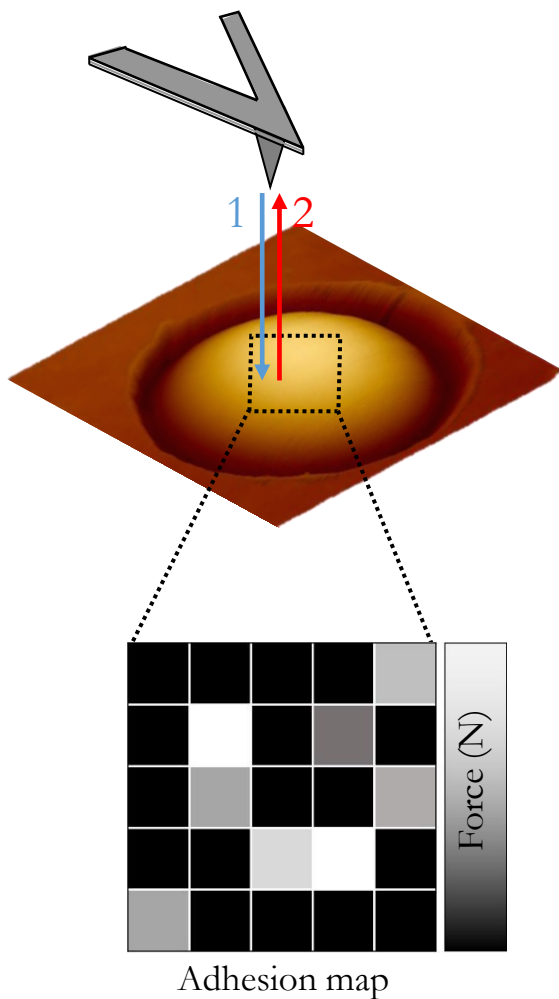


Bare tip

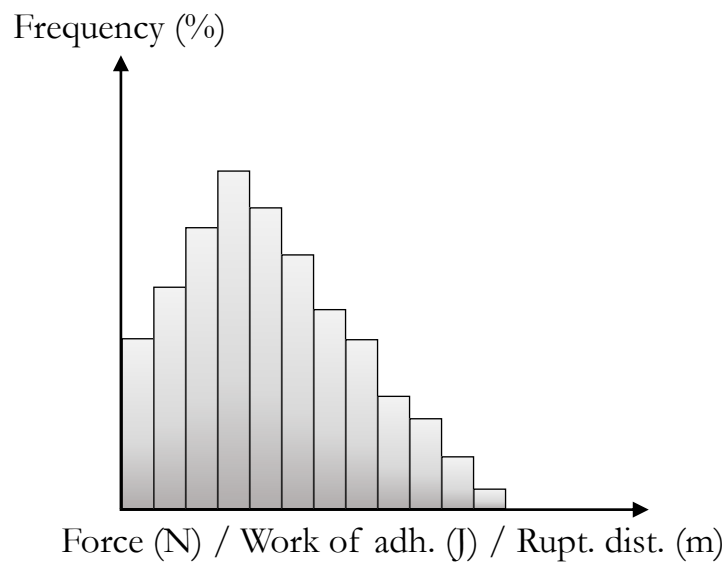


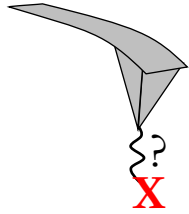
Functionalized tip

Require strong and reliable sample immobilization



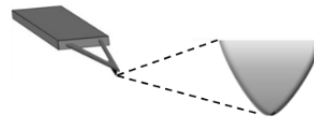
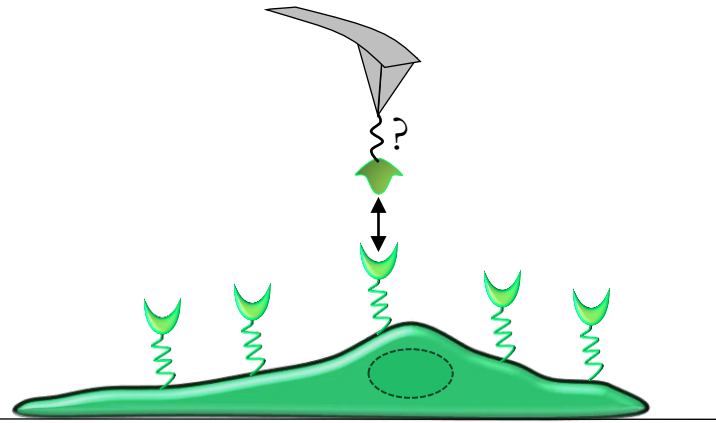
Hooke's law : $F = -k \times d$
 Deflection sensitivity (nm/V)
 Cantilever spring constant k (N/m)





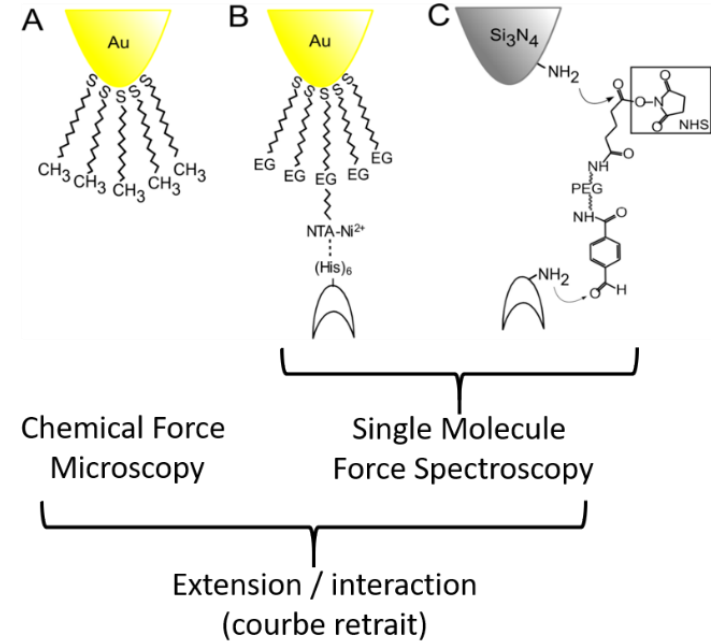
How to functionalize AFM tips?

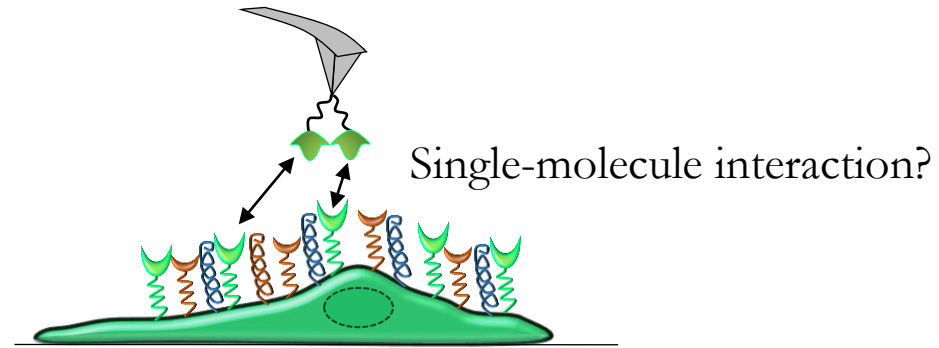
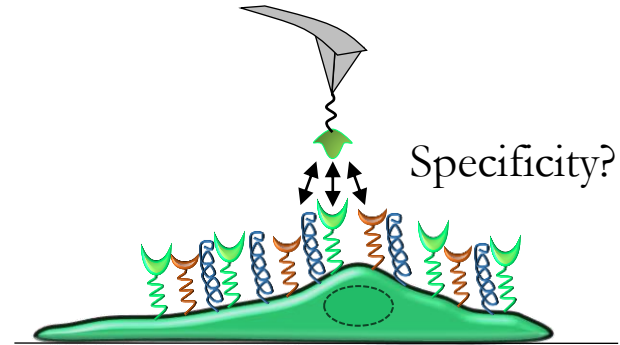
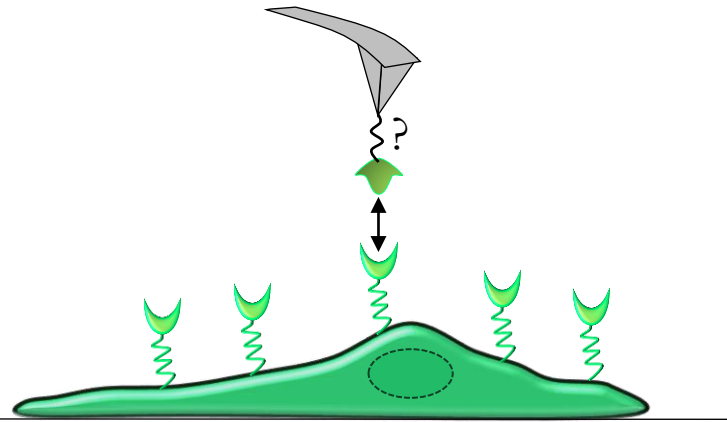
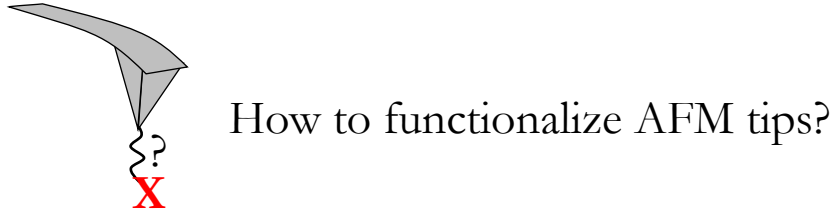
Tip functionalization examples



Nanomechanical properties

Indentation
(courbe approche)



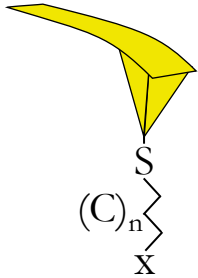


Tip functionalization protocol should ensure:

- Strong and flexible attachment
- Low grafting density for single-molecule
- No unspecific adhesion
- Preserved conformation and properties of the grafted object

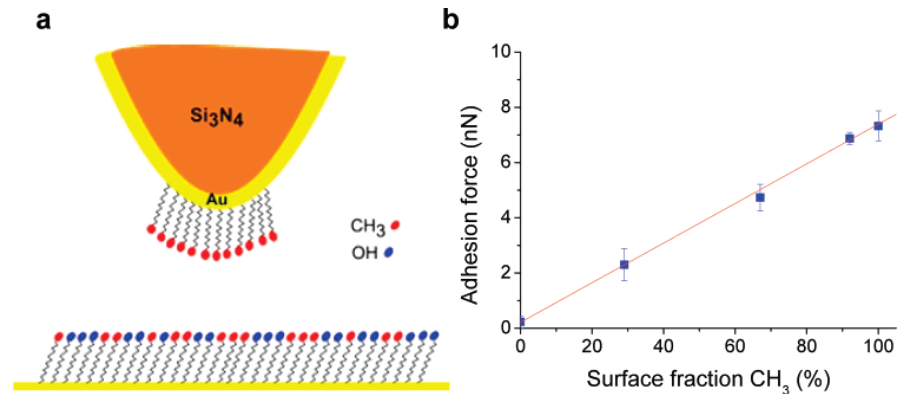
Probing chemical properties of surfaces

Gold coated tip to graft
alkanethiol-terminated molecules



X=functional group

(CH₃, OH, COOH, NH₂...)



Dague et al. Nano letters 2007

Protocol steps:

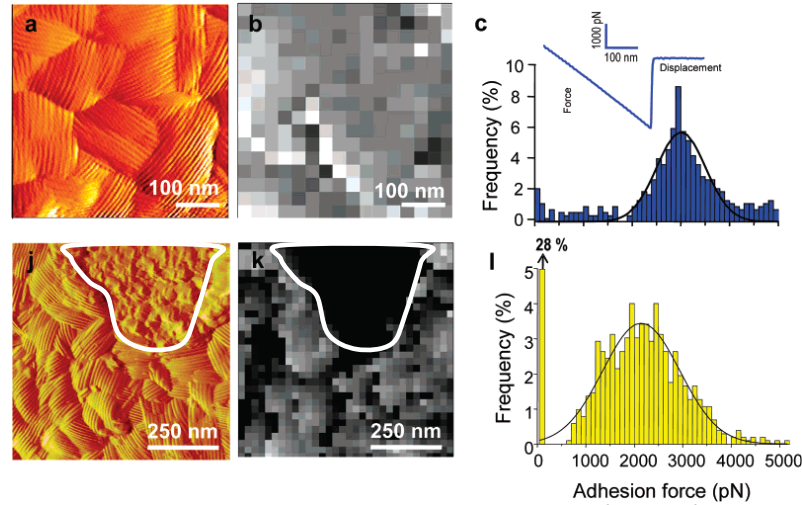
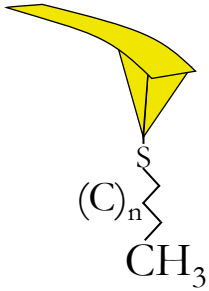
- AFM tip cleaning (UV/O₃ ; piranha...)
- Rinsing with ethanol + N₂ drying
- Immersion 4-12h in 1 mM solution of thiols in ethanol
- Rinsing with ethanol + N₂ drying

Example of thiols from Sigma:

- CH₃: 1-dodecanethiol
- OH: 11-mercapto-1-undecanol
- COOH: 16-mercaptohexadecanoic acid
- NH₂: 1-Amino-1-undecanethiol

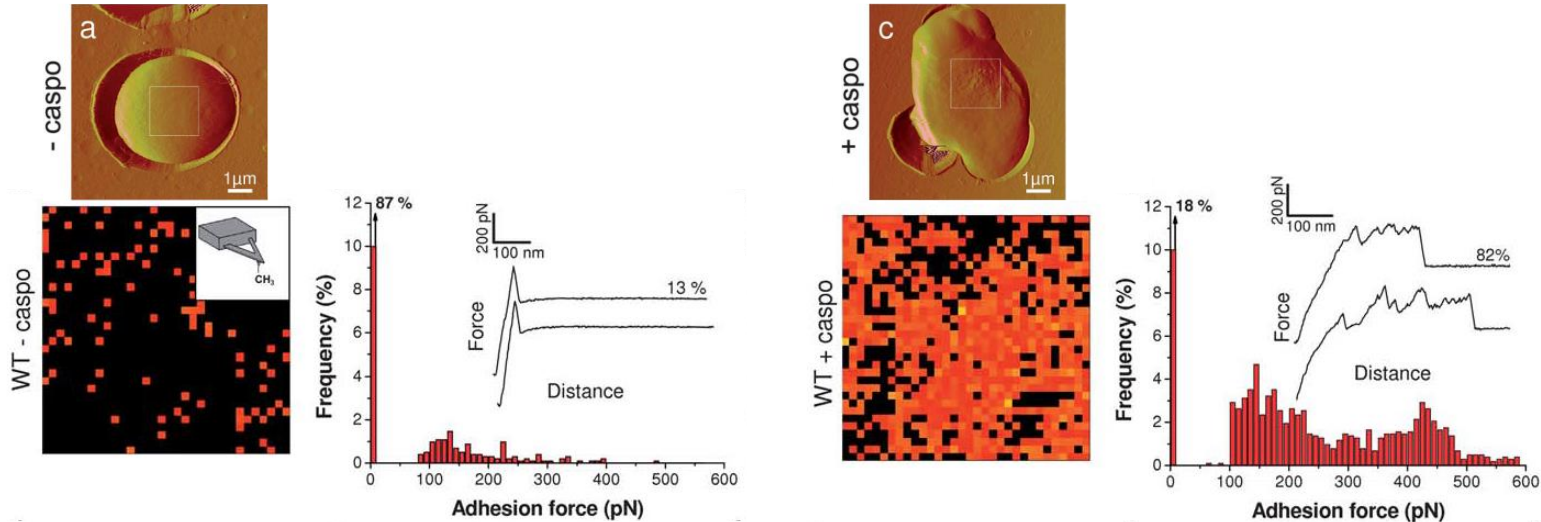
➤ Surface hydrophobicity of fungal germinating spores

Hydrophobic tips coated with 1-dodecanethiol



Dague et al. Nano letters 2007

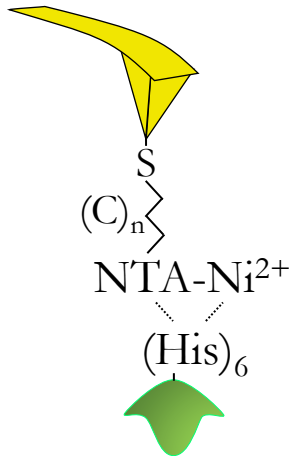
➤ Effects of antifungal drugs on yeast surface hydrophobicity



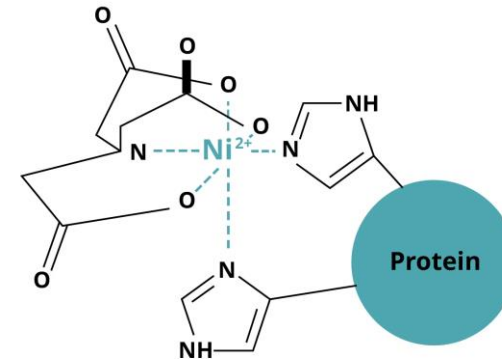
El-Kirat-Chatel et al. Nanoscale 2012

Limit: No molecular specificity

Gold coated tip to graft thiol-terminated molecules



Detection or oriented grafting of His-tagged proteins



Protocol steps:

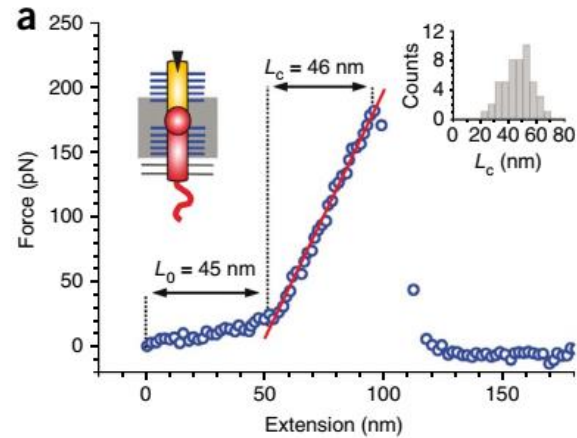
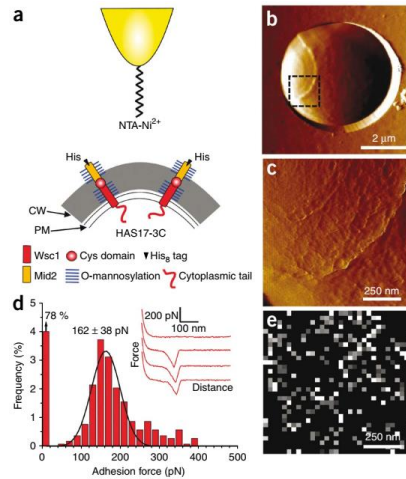
- AFM tip cleaning (UV/ O_3 ; piranha...)
- Rinsing with ethanol + N_2 drying
- Immersion 4-12h in 0.01 mM NTA-terminated alkanethiols (or NTA-PEG alkanethiols) in ethanol (+spacers?)
- Rinsing with ethanol + N_2 drying
- Immerse 30 min in 80 mM $NiSO_4$
- Rinse gently with pure water (avoid drying)

For protein oriented grafting

- Add 100-200 μ L of His-tagged proteins (200 μ g/mL) 1-2h before gentle rinsing in buffer

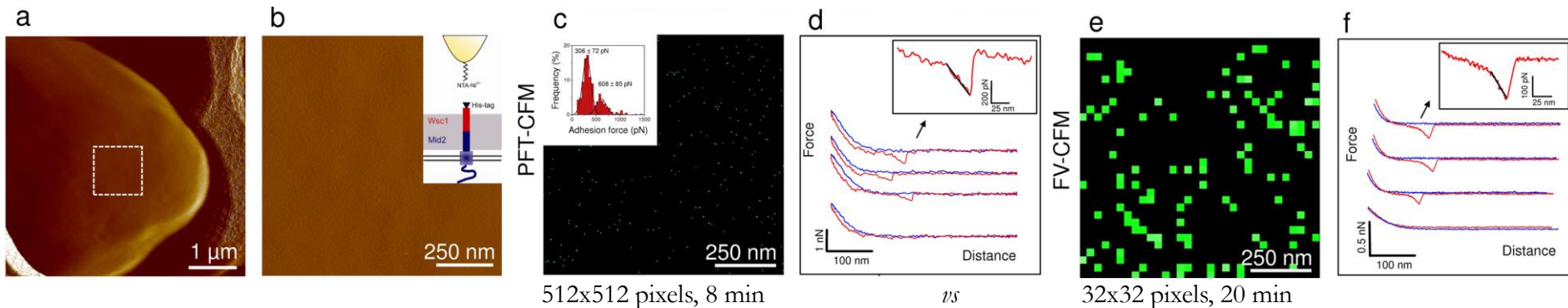
NTA-Ni²⁺ tips to detect His tagged proteins on live cells

- Revealing the spring behavior and clustering of Wsc1 in the yeast cell wall



Dupres *et al.* Nat. Chem. Biol. 2009

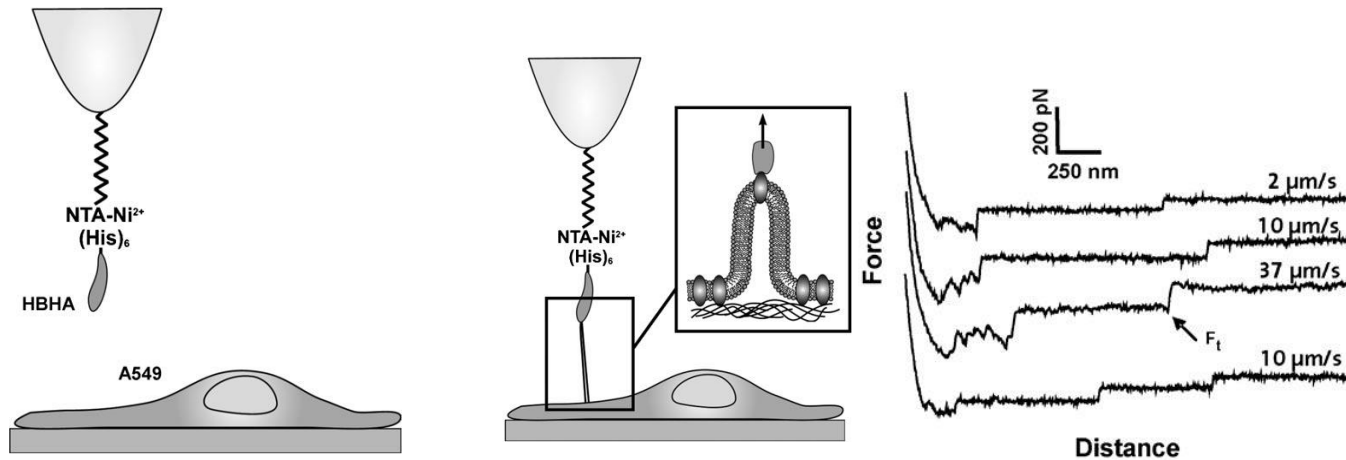
- High resolution detection of proteins using PFT-QNM



Alsteens *et al.* Langmuir 2012

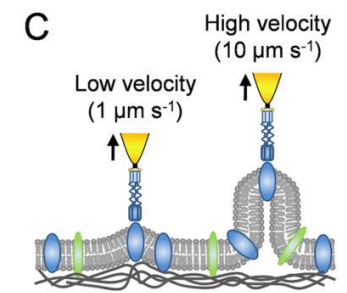
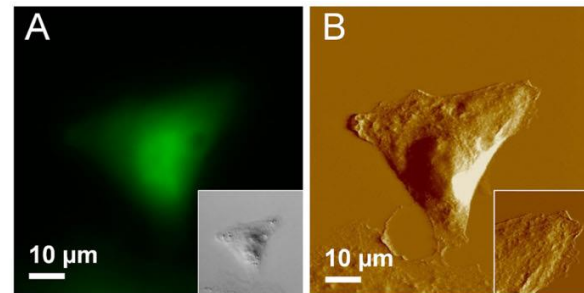
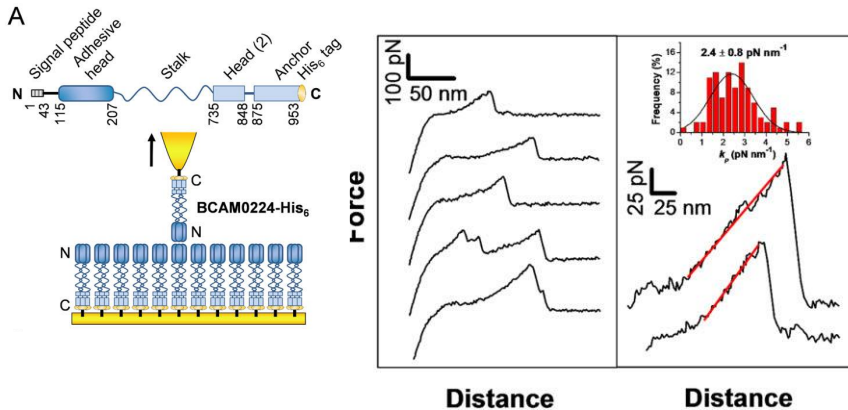
NTA-Ni²⁺ tips to graft oriented proteins and probe their interaction

- HBHA Mycobacterial adhesin recognize HSPG receptors on pneumocytes



Duprès *et al.* ChemPhysChem 2009

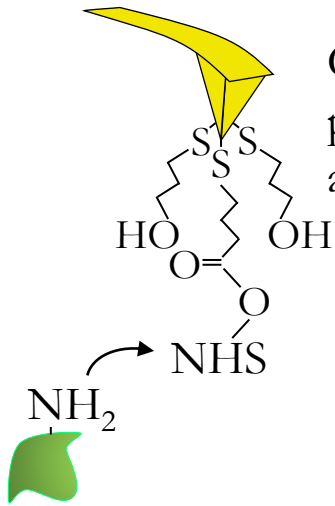
- BCAM *Burkholderia* TAA adhesin is involved in homotypic interaction and host recognition



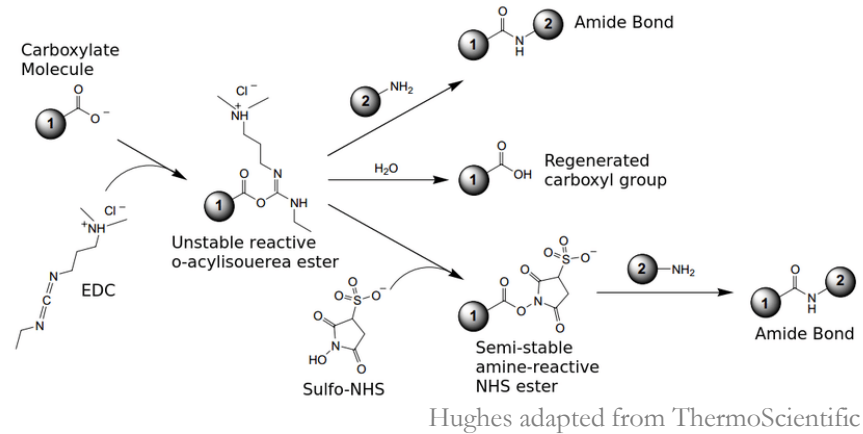
El-Kirat-Chatel *et al.* Molec. Microbiol. 2013

Limit: Functionalization reliable only if interaction force < 300 pN

Gold coated tip to graft thiol-terminated molecules



Covalent grafting of protein with primary amines

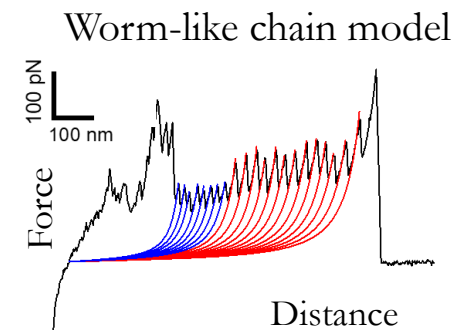
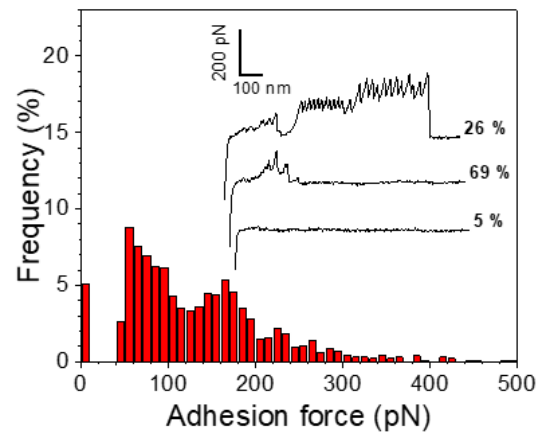
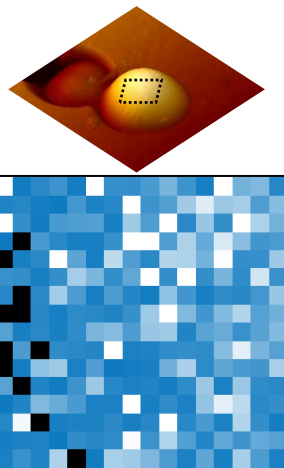
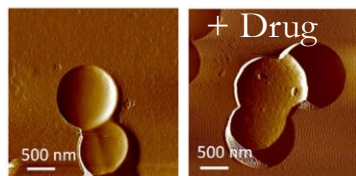
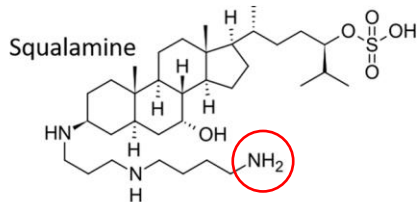


Requires highly pure proteins for reproducible results

Protocol steps:

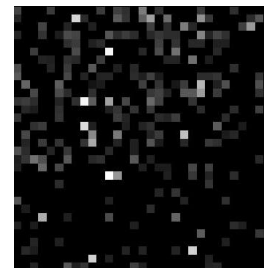
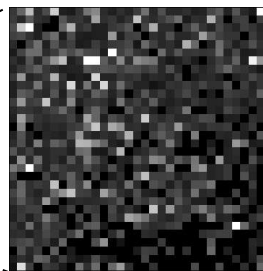
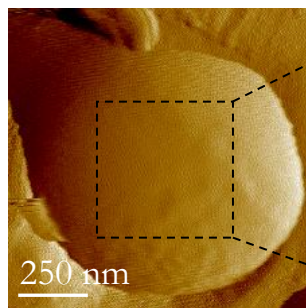
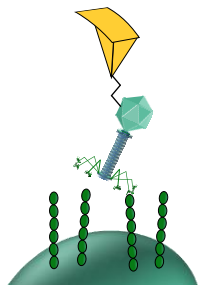
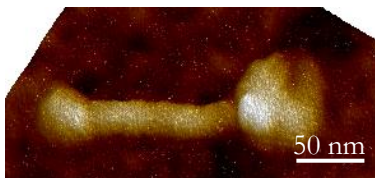
- AFM tip cleaning (UV/O₃ ; piranha...)
- Rinsing with ethanol + N₂ drying
- Immersion 4-12h in 0.1 mM 1-dodecanethiol and 0.9 mM SH-undecanol (=spacers)
- Rinsing with ethanol + N₂ drying
- Immerse 30 min in NHS-EDC (10mg/mL-25mg/mL in water)
- Rinse gently with pure water (avoid drying)
- Add 100-200µL of purified proteins (200µg/mL) 1-2h before gentle rinsing in buffer.

➤ Revealing nanoscale adhesion of antimicrobial molecules



El-Kirat-Chatel *et al.* Col. Surf. B 2023

➤ Measuring phage-bacteria interaction

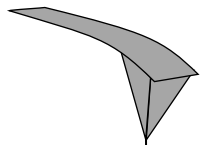


+ *GlcNAc*

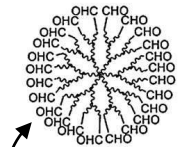
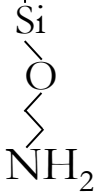
Arbez *et al.* Nano Res. 2022

Limit: Low molecular flexibility

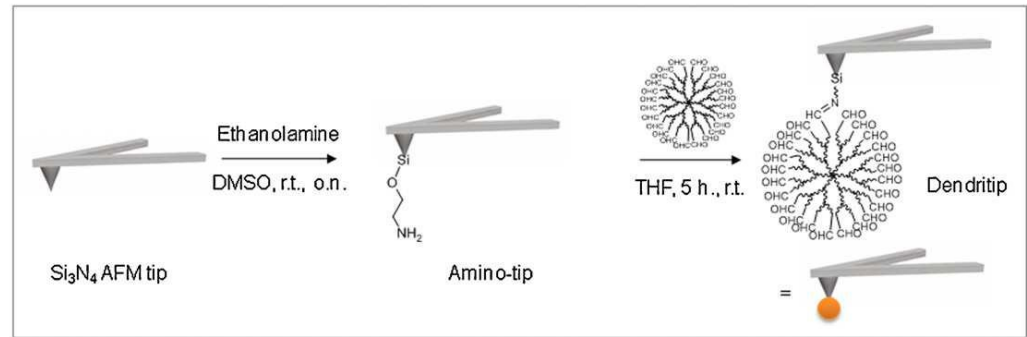
Si₃N₄ tips decorated with amino groups



Covalent grafting of protein with primary amines



Aldehyde dendrimer



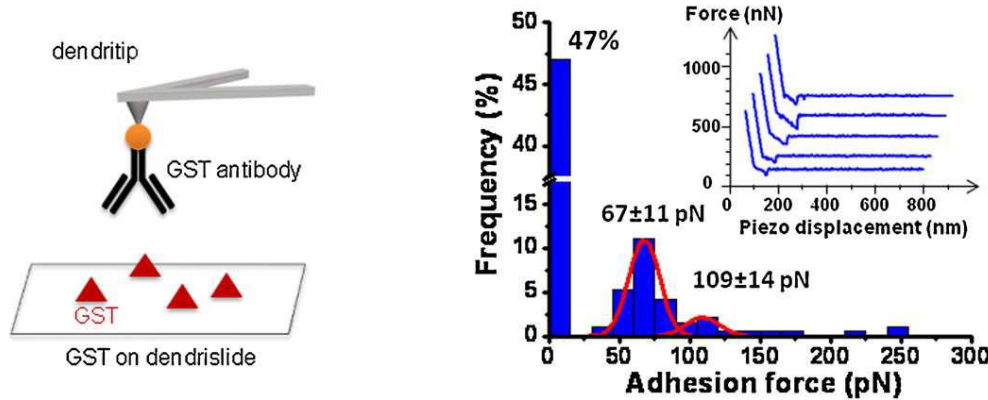
Jauvert *et al.* Sens. Actuators B Chem. 2012

Requires highly pure proteins or mAb

Protocol steps:

- AFM tip cleaning (Dichloromethane, acetone, ethanol, UV/O₃)
- Immerse o/n in 0,5 mg/mL ethanolamine in DMSO+molecular sieve beads then rinse with DMSO and ethanol and dry with N₂
- Immerse 5h tetrahydrofuran containing dendrimer at 58 μM and rinse with DMSO and ethanol and dry with N₂
- Rinsing with ethanol + N₂ drying (possible to store in desiccator)
- Immerse 1h in 0,5mg/mL of proteins + 20 mM NaCNBH₃
- Rinse and store in buffer

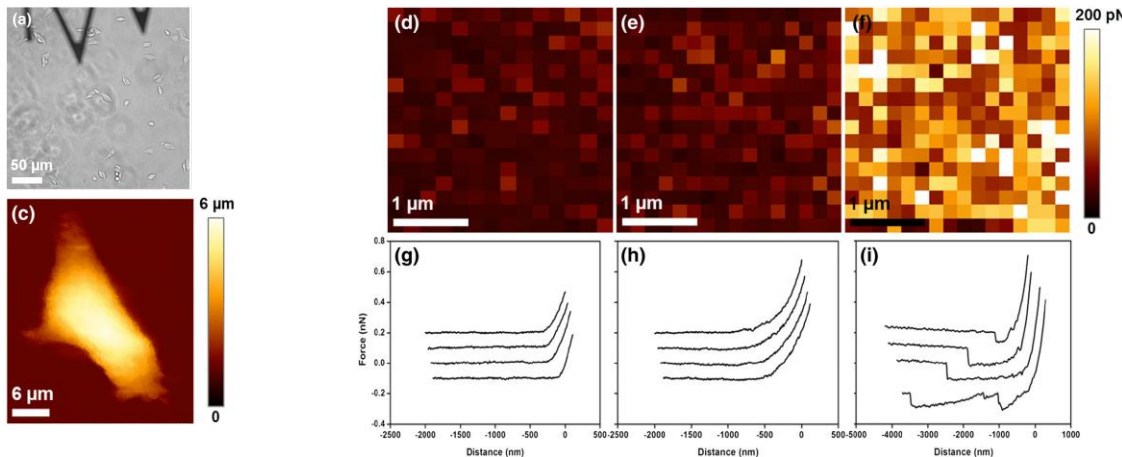
- Probing the interaction between glutathione-S-transferase (GST) and anti-GST mAb



→ Proof of concept

Jauvert *et al.* Sens. Actuators B Chem. 2012

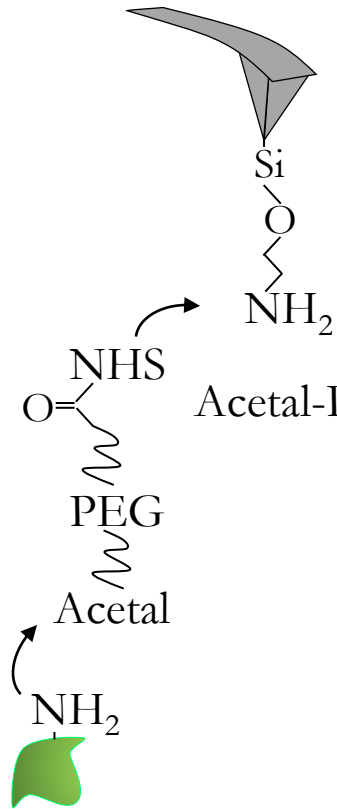
- Detecting HA-tagged $\beta 2$ -adrenergic receptors on CHO cells



Flexibility, low unspecific binding BUT non oriented and NaCNBH_3 needed.

Si₃N₄ tips decorated with amino groups

Covalent grafting of protein with primary amines

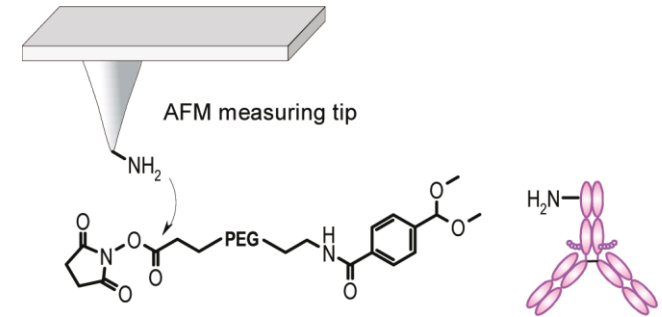


Acetal-PEG-NHS

Requires highly pure proteins or mAb

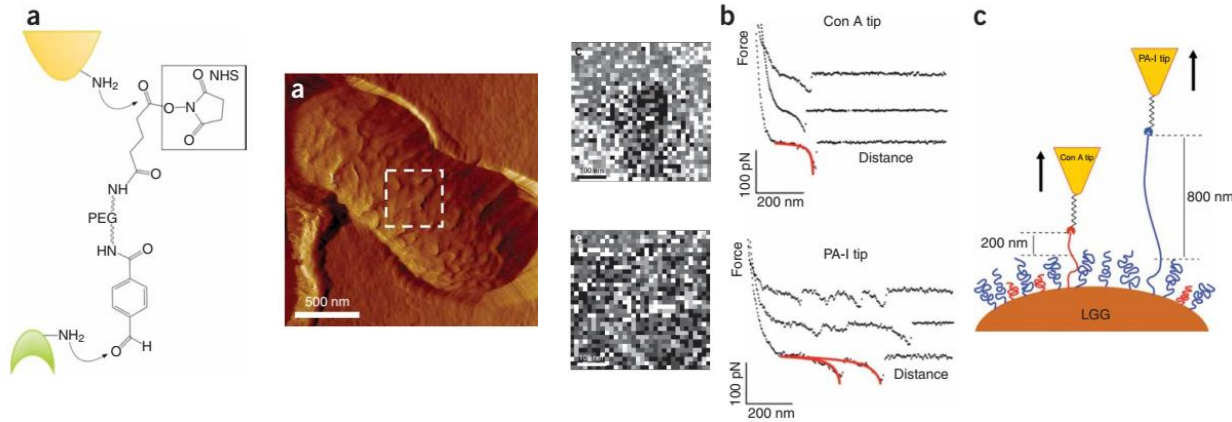
Protocol steps:

- AFM tip cleaning (Piranha?, UV/O₃, 3x chloroform)
- Immerse o/n in 0,5 mg/mL ethanolamine in DMSO+molecular sieve beads then rinse with DMSO and ethanol and dry with N₂
- Immerse 2h in 0,5mL chloroform + 1 mg Acetal-PEG-NHS
- Rinse with chloroform and dry with N₂ (possible to store in desiccator)
- Immerse 10 min in 1 % citric acid and rinse with water and dry with N₂
- Place cantilevers on parafilm
- Add ~100 μL of protein (~2μM) + 2μL of NaCNBH₃ for 1h
- Add 5 μL of ethanolamine (1 M, pH 8.0) for 10 min
- Wash with buffer and store at 4°C



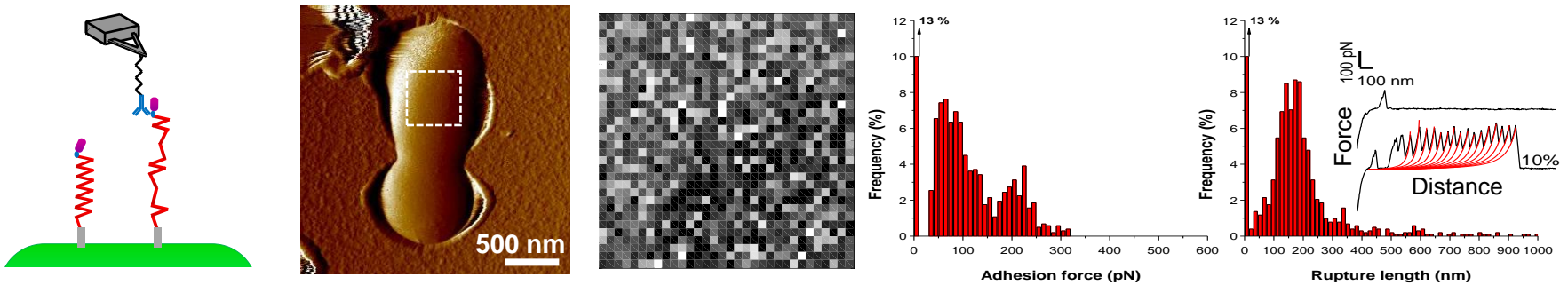
Wildling *et al.* Bioconjug. Chem. 2011

➤ Probing polysaccharides on bacteria



Francius *et al.* Nat. Protocol 2009

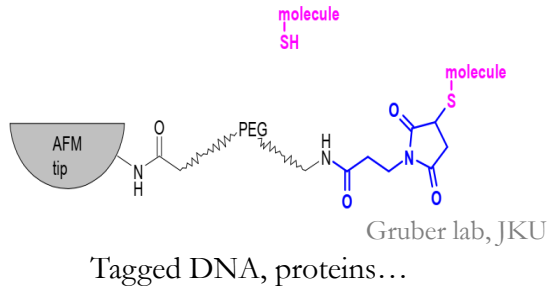
➤ Molecular biophysics of single bacterial adhesins



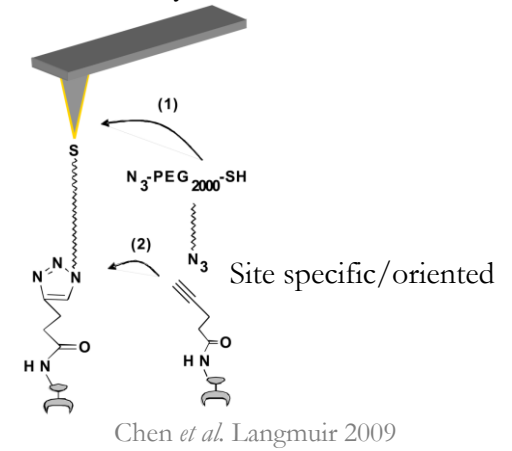
El-Kirat-Chatel *et al.* ACS Chem. Biol. 2013

Flexibility, low unspecific binding, specific FD signature BUT non oriented and NaCNBH₃ needed.

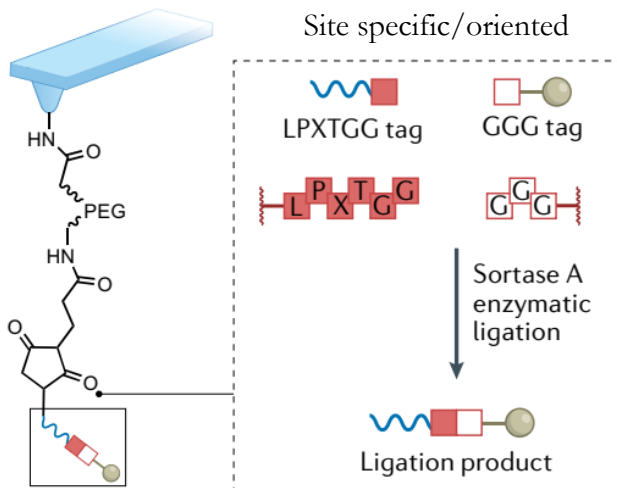
➤ Maleimide to graft –SH molecules



➤ Click chemistry

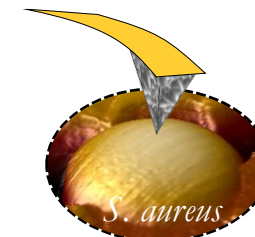
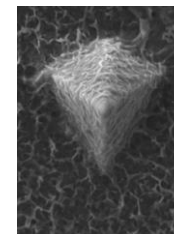
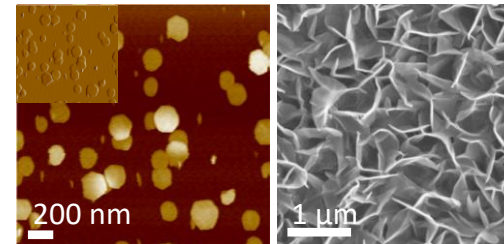


➤ LPXTGG/GGG/SortaseA from *S. aureus*



Theile *et al.* Nat. Protoc. 2013
 Viljoen *et al.* Nat. Rev. Methods Primers 2021

➤ Particles: Layered double hydroxides

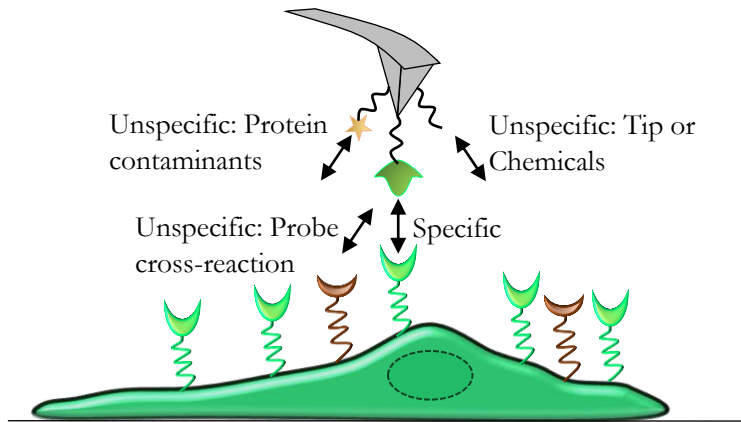


Probing antimicrobial LDH vs cells

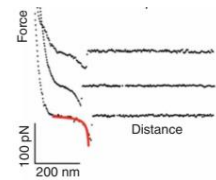
Awassa *et al.* Nanoscale 2022

➤ What are the control experiments?

.Determining if the measured interaction is specific



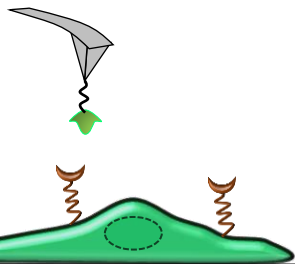
- Use highly pure protein preparation and avoid cross-reactive compounds
- Block unlinked linkers and add unreactive spacers
- Use poorly adhesive linkers (PEG...)
- Use linkers with specific extension signature (PEG → WLC)



Control experiments

Negative control sample (mutants):

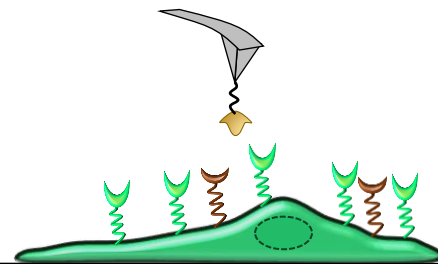
No adhesion



- No tip/chemical interaction
- No probe cross-reaction
- Reactive protein contaminant?
- Specificity

Aberrant probe:

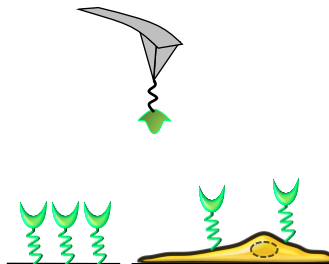
No adhesion



- No tip/chemical interaction
- No sample adhesion

Model surface/cell:

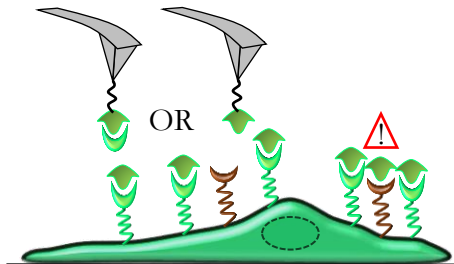
Adhesion



- Specificity
- FD shape

Blocking with free molecules:

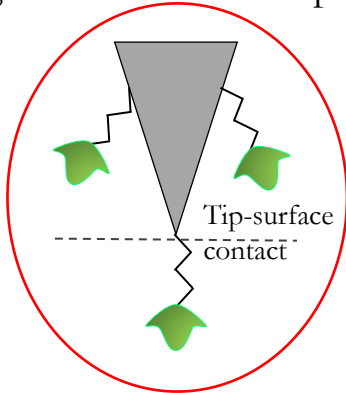
No adhesion



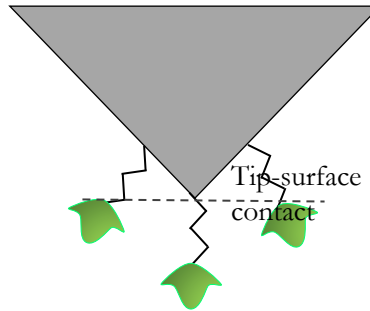
- No tip/chemical interaction
- Specificity (only with receptor injection, not with probe)

➤ How to determine if single-molecule interaction are probed?

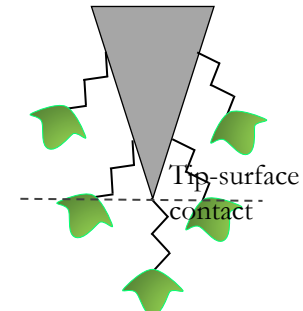
. Single-molecule force spectroscopy relies on the grafting density of molecules and the tip radius/sharpness



Only 1 molecule in contact with the surface

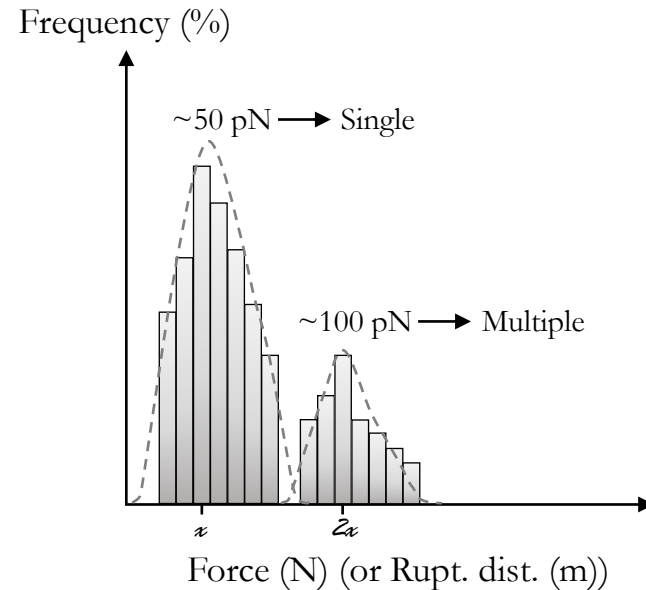
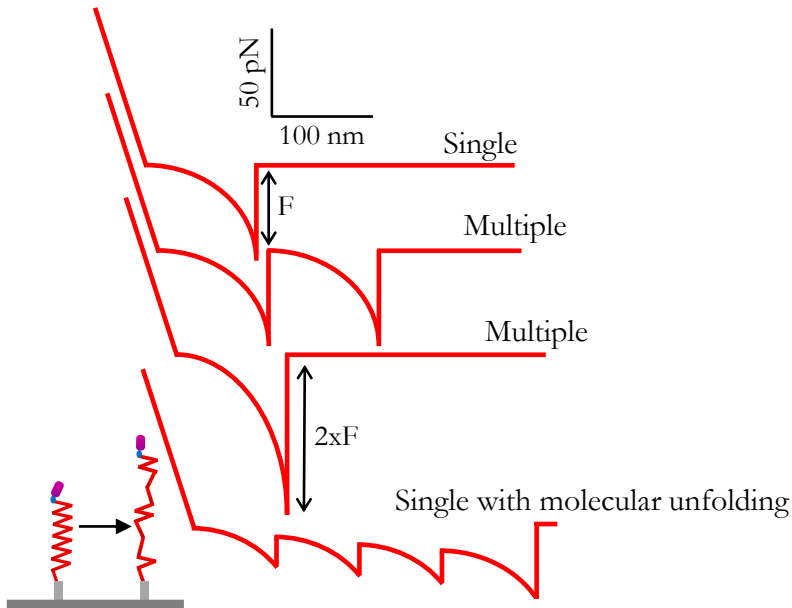


Tip too large



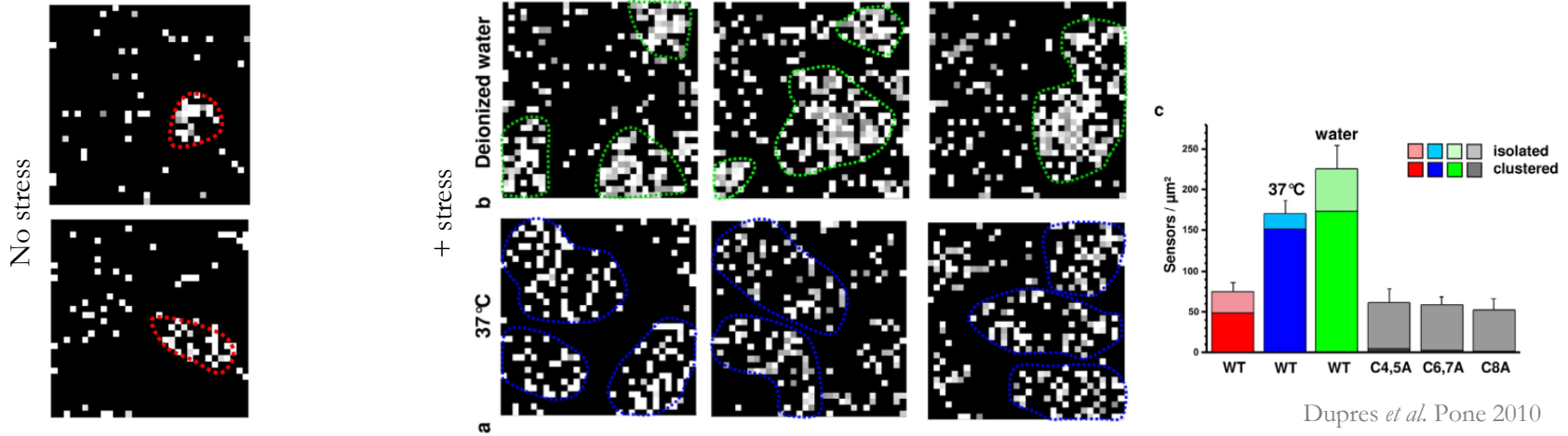
Density too high

. The force-distance curve signatures and the distribution of forces



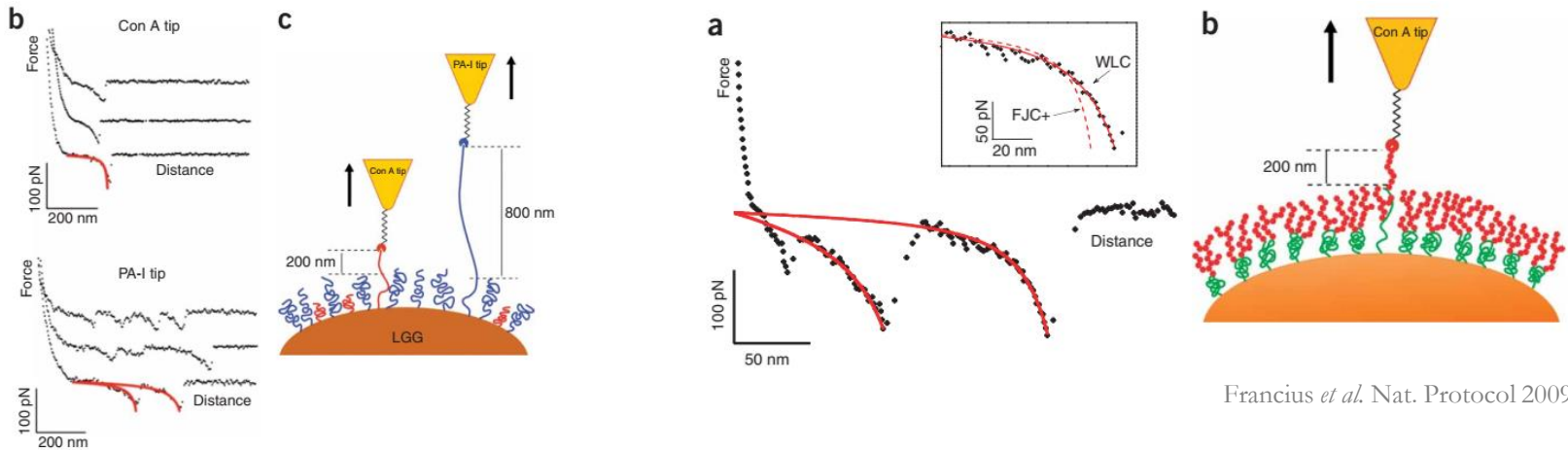
➤ What can be extracted from single-molecule force spectroscopy data?

. Mapping: distribution, dynamics



Dupres *et al.* *Pone* 2010

. Nature of the ligands: curve fitting



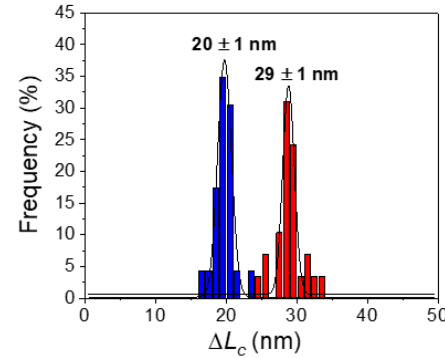
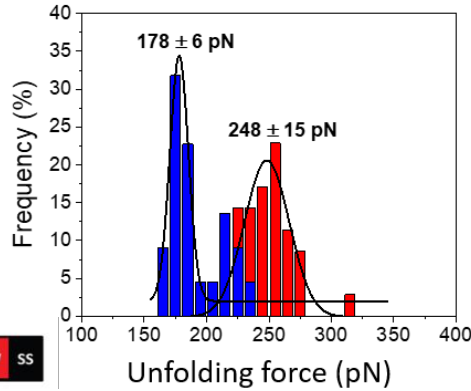
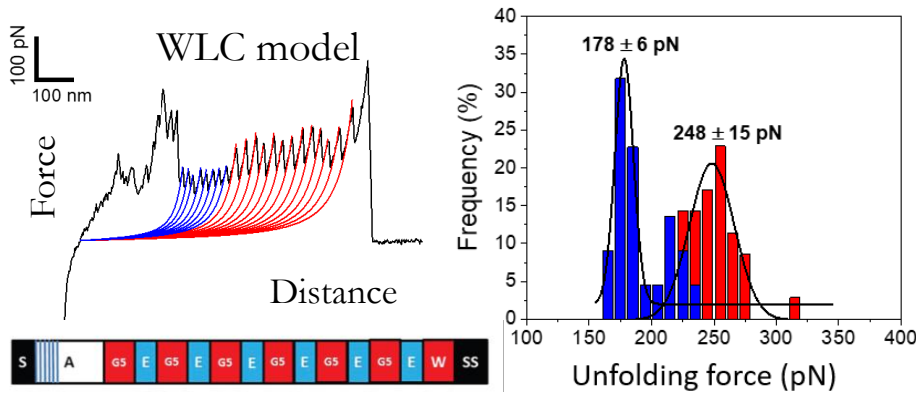
Francius *et al.* *Nat. Protocol* 2009

FJC and FJC⁺ for polysaccharides extension
(ideal chain with flexible joints)

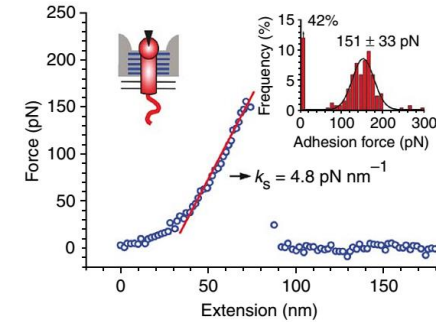
WLC and WLC⁺ for proteins and PEG extension
(continuously flexible chain irregularly curved)

➤ What can be extracted from single-molecule force spectroscopy data?

. Molecular mechanics: Unfolded domains? Adhesive domains? Nanosprings (linear fits)?

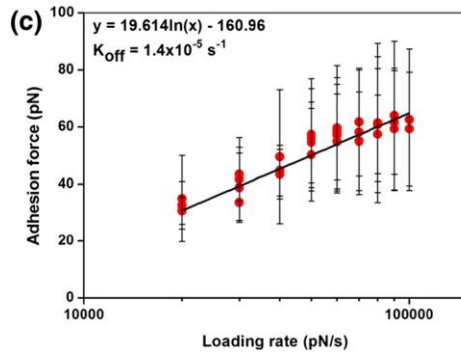


El-Kirat-Chatel *et al.* Colloids Surf. B 2023

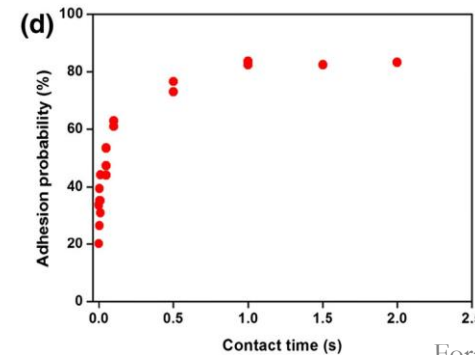


Dupres *et al.* Nat. Chem. Biol. 2009

. Dynamic force spectroscopy (Bell-Evans; Dudko; Friddle *etc* models)



Increased loading rate by increasing pulling velocity
the dissociation constant (k_{off})

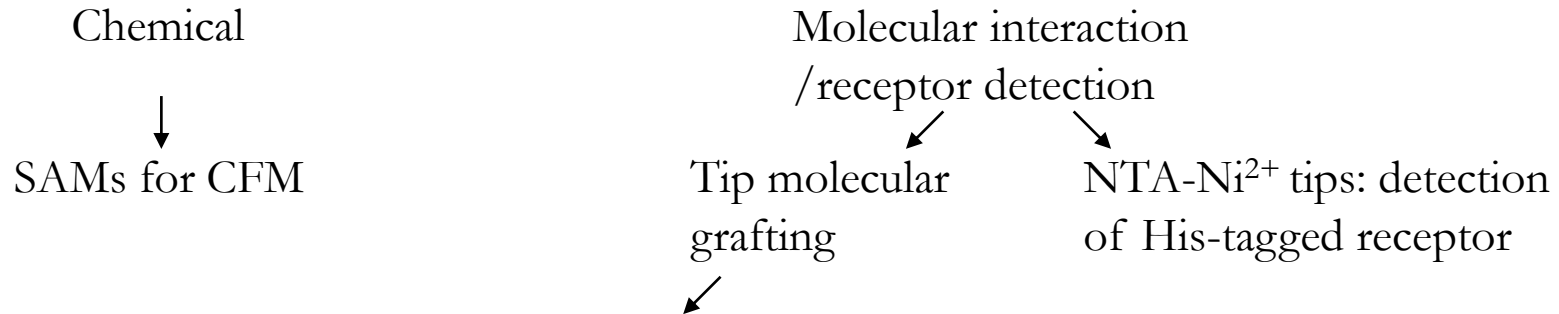


Increased contact time to determine the association
constant (k_{on})

Formosa *et al.* JMR 2014

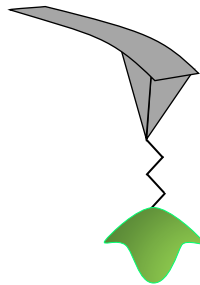
→ Affinity of the interaction k_d

1. What info I want to get?



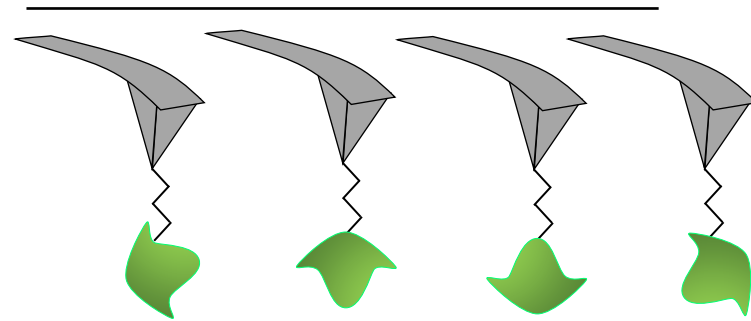
2. Orientation needed?

Yes



- Ni²⁺-NTA ($F < \sim 300$ pN)
- LPXTGG/GGG/SortaseA from *S. aureus*
- Click chemistry

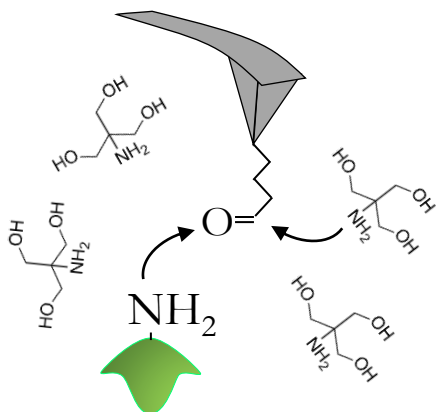
No



- All other covalent grafting through NH₂ groups (N-terminus and Lys side chains): NHS-EDC, PEG-linker, Dendritips... depending on the molecular flexibility and density required

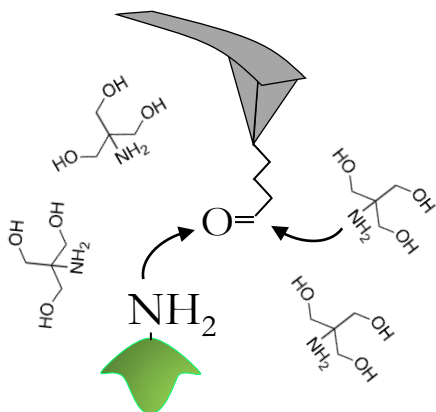
- + Maleimide linkers for -SH tagged molecules (Proteins, DNA). Oriented if only 1 -SH/molecule
- + Mineral chemistry for particles...
- + ...

3. Possible competition/cross-reactions?

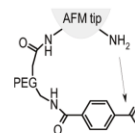


Do not use buffers containing the coupling reactive group (e.g. Tris for amine reactions)

3. Possible competition/cross-reactions?

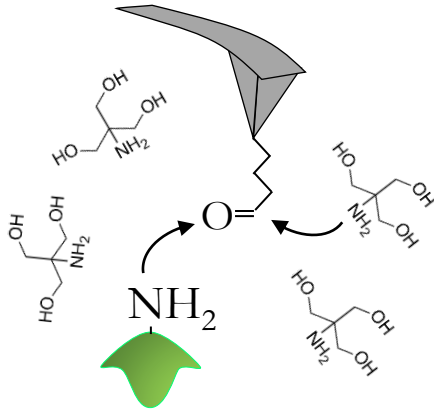


Do not use buffers containing the coupling reactive group (e.g. Tris for amine reactions)

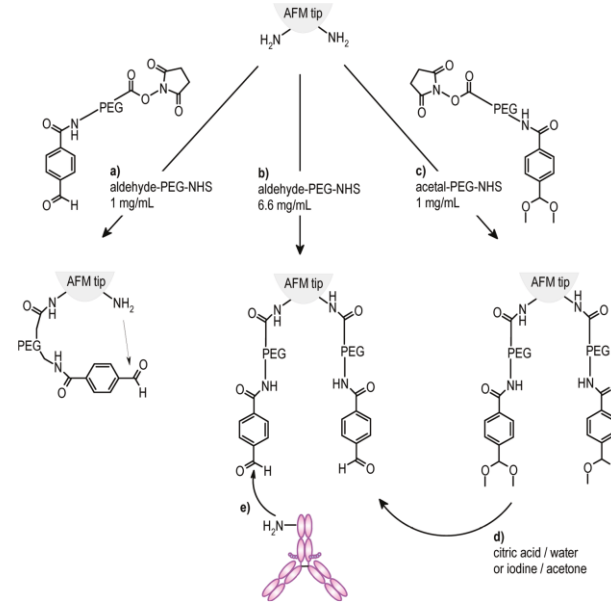


Blocking linker loops

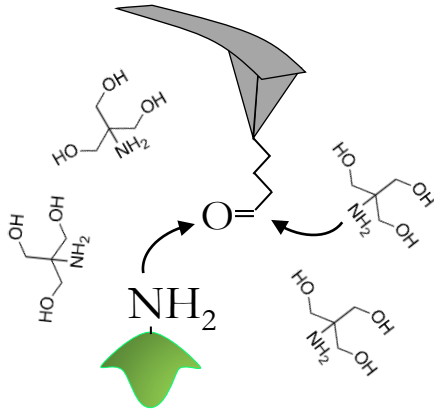
3. Possible competition/cross-reactions?



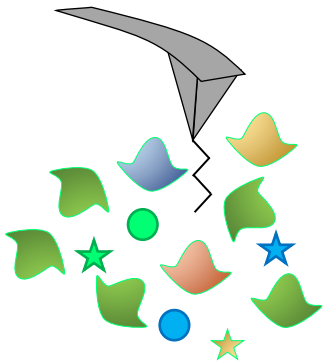
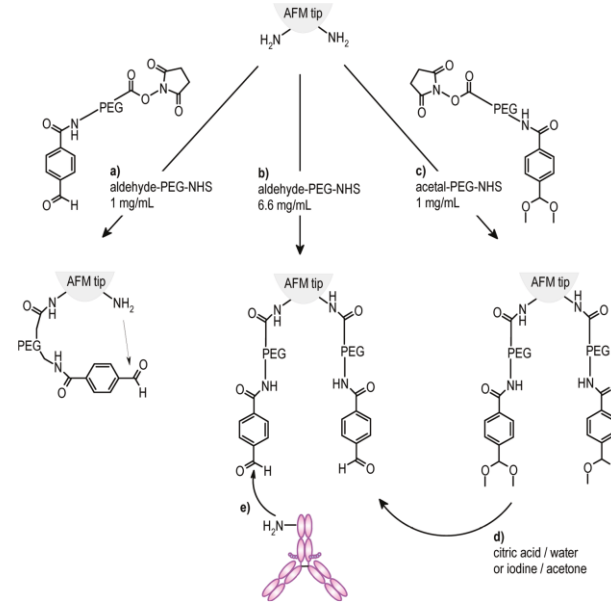
Do not use buffers containing the coupling reactive group (e.g. Tris for amine reactions)



3. Possible competition/cross-reactions?

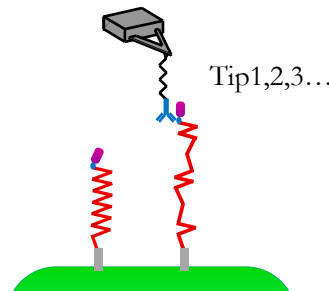


Do not use buffers containing the coupling reactive group (e.g. Tris for amine reactions)

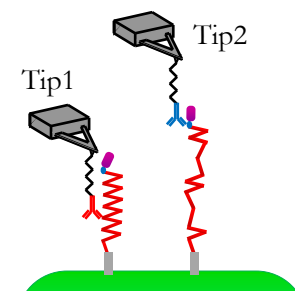


Always use highly pure proteins for reproducible results (purified proteins, mAb...)

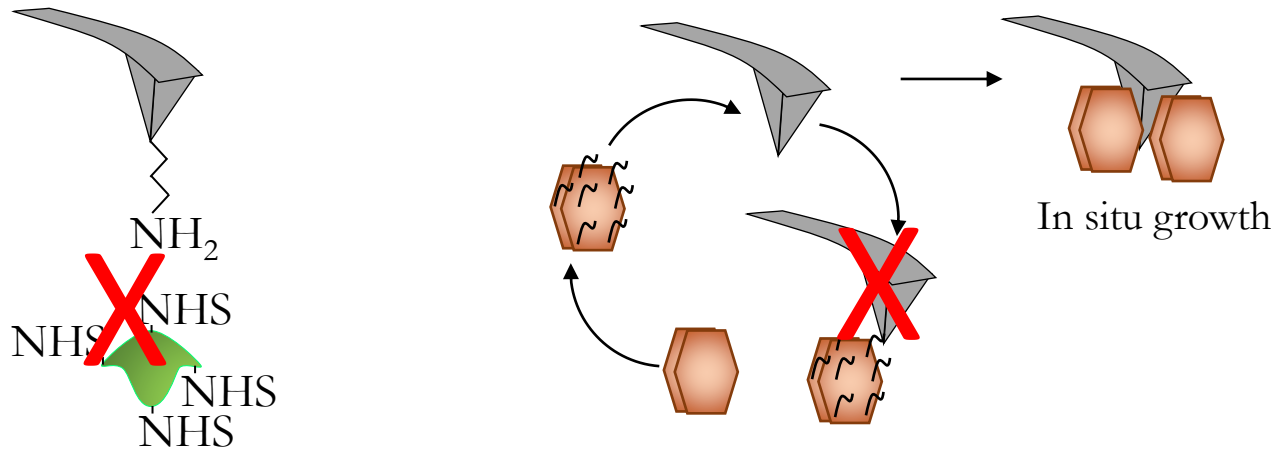
Monoclonal Ab:
Reproducible results and force signatures



Polyclonal Ab:
Inconsistent results and variable force signatures



4. Probe modification/denaturation by the grafting?

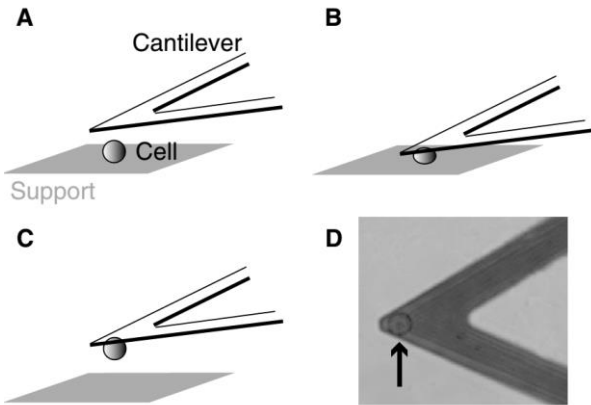


The protocol should modify the tip to add pure proteins. Avoid protein chemical modification/activation

Single-cell force spectroscopy: Attaching a single-cell to AFM cantilevers

- Tipless cantilevers coated/functionalized with lectins or polydopamine OR FluidFM

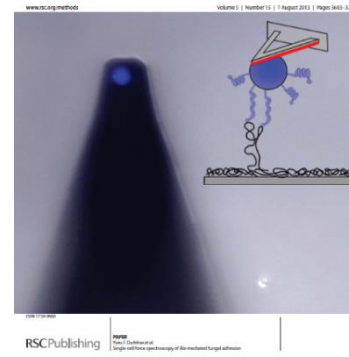
.Mammalian cells



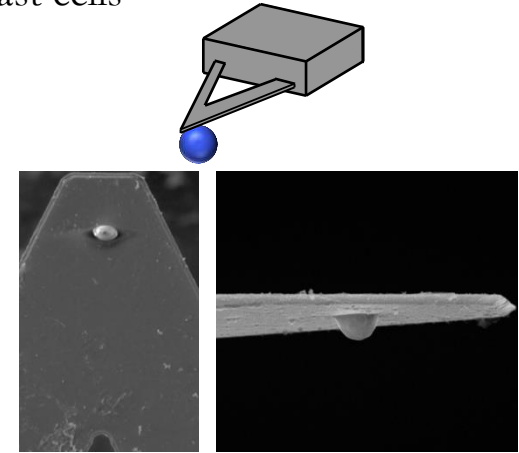
Helenius *et al.* J. Cell Sci. 2008

.Yeast cells

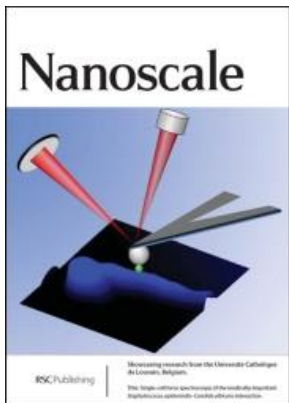
Analytical Methods



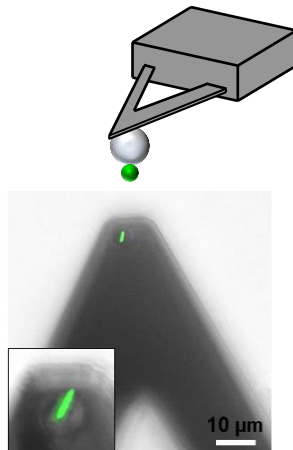
Alsteens *et al.* Analyt. Methods 2013



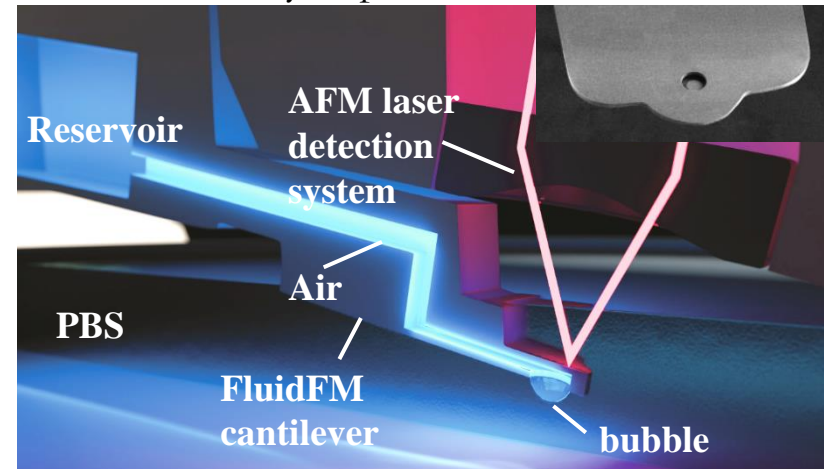
.Bacteria



Beaussart *et al.* Nanoscale 2013

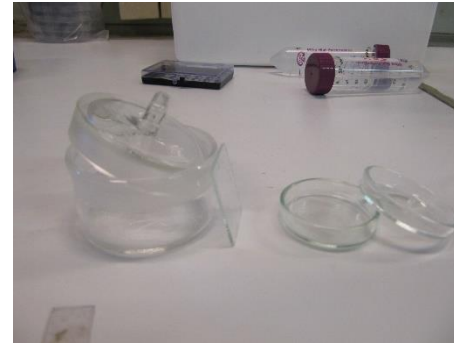
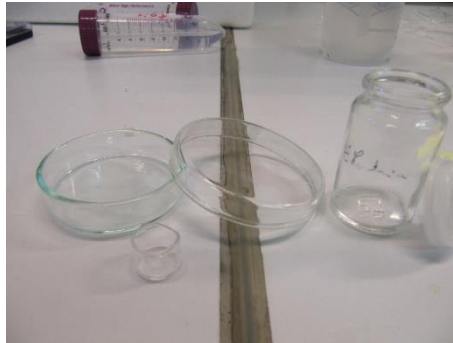


.Hydrophobic bubbles



Demir *et al.* J. Coll. Interf. Sci. 2021

- For single-molecule force spectroscopy cantilevers should be sharp and soft enough to detect single-molecule interactions: $0,01 < k < 0,06$ N/m ; MLCT, SNL, NPG...
- Few suppliers for thiols, PEGs...: Sigma, Prochima, Broadpharm...
- Most protocols require delicate handling of probes for immersion in several small volume solutions poured in ultra clean glasswares.



- Few refs:
 - jku.at for pdfs on aminofunctionalization, acetal-PEG, maleimide-PEG...
 - Viljoen *et al.* Nat. Rev. Methods Primers 2021: Review on SMFS
(doi: 10.1038/s43586-021-00062-x)
 - Lostao *et al.* Internat. J. Biol. Macromolec. 2023: Review on SMFS
(doi: 10.1016/j.ijbiomac.2023.124089)
 - Bizzarri and Cannistraro Chem. Soc. Rev. 2010: Review on loading rate
(doi: 10.1039/b811426a)

Thank you for your attention



Enjoy your molecular fishing