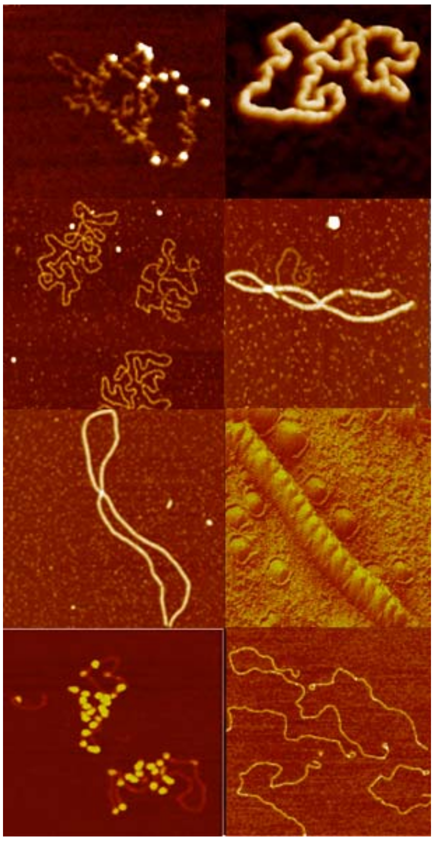


L'AFM pour la biologie: application à l'étude de l'ADN et des complexes nucléoprotéiques

Olivier Piétrement

Maintenance des Génomes et Microscopies Moléculaires

UMR 8126 Signalisation, Noyaux et Innovations en Cancérologie

Institut de cancérologie Gustave Roussy

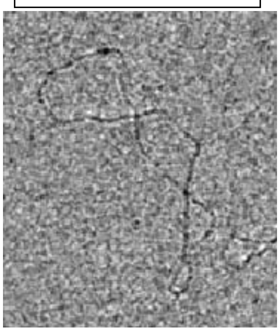
94805 Villejuif, France

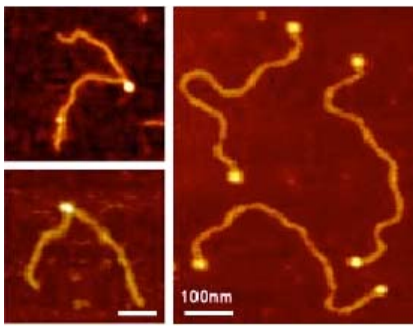
Maintenance des Génomes et Microscopies Moléculaires
Resp. E. Le Cam

TEM

CryoTEM

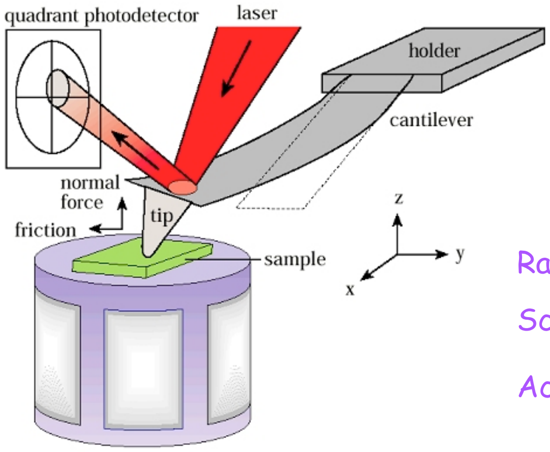
AFM





Atomic Force Microscope (AFM)

- Atomic force microscopy (AFM) uses interatomic forces to sense the surface. It has been proposed in 1986.
- In the simplest form, the system works like a record player: a sharp tip is put “in contact” with a surface and scanned across the surface
- The tip is placed at the end of a cantilever
- The vertical position of the cantilever is monitored as it deflects up and down



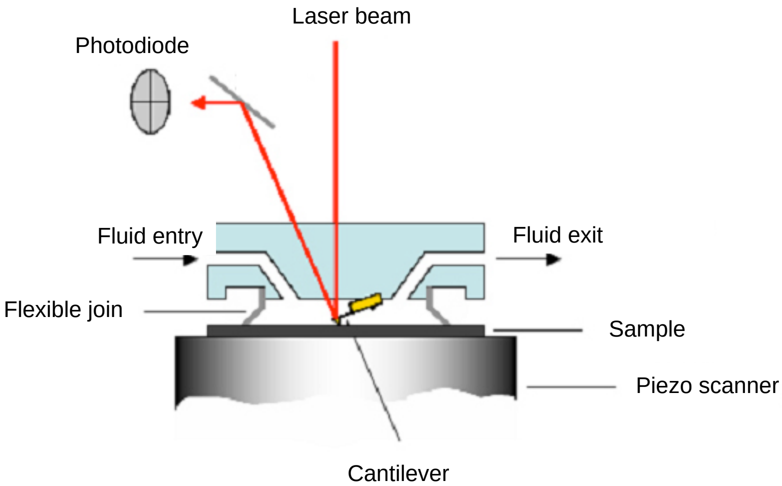
Range of applied force: 40 pN up to 1 μN

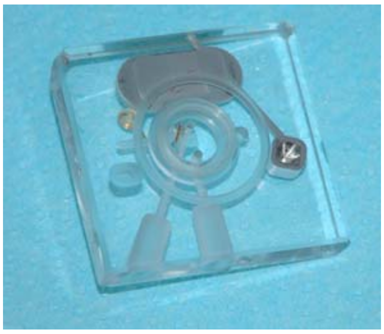
Scanning area: 10×10 nm^2 up to 100×100 μm^2

Acquisition time: 60 sec up to several minutes

Atomic Force Microscope (AFM)

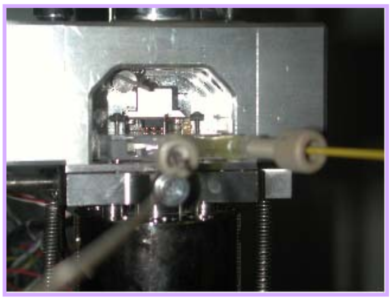
AFM: working in liquid environment





Open the possibility to observe and analyse in real time a biological process

AFM: working in liquid environment



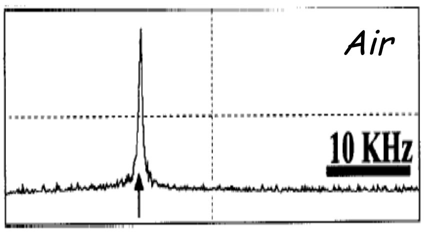
Combination of AFM with a SMART chromatography system

- Precise control of fluid flow (1 up to 1000 μL/min) without excess of overpressure
- Easy change of buffer composition in the fluid cell
- Control of the temperature (between 10 and 40 °C)
- Direct injection of biological sample in the AFM liquid cell

Atomic Force Microscope (AFM)

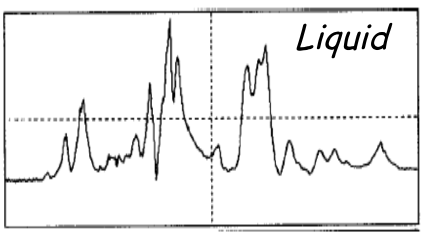
AFM: working in liquid environment

AM-AFM



Q   500-1000

Working points shift with buffer solution



Q   3-50

Scan speed increases

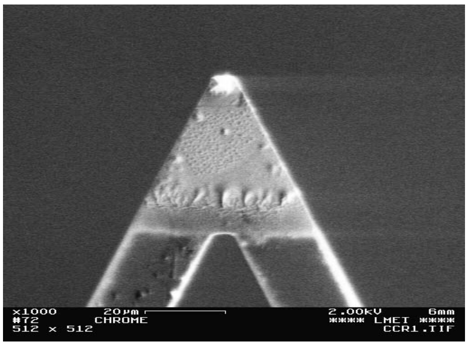
$$\tau = \frac{2Q}{\omega_0}$$

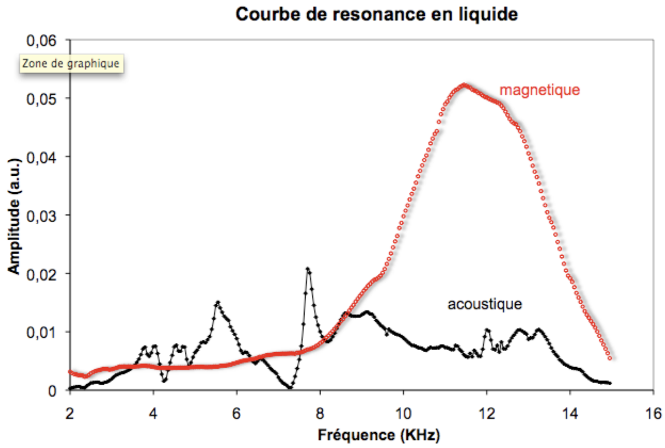
Forum des Microscopies à Sondes Locales 2011

Atomic Force Microscope (AFM)

AFM: working in liquid environment

Magnetic force modulation





Biological application of AFM

At the cellular level:

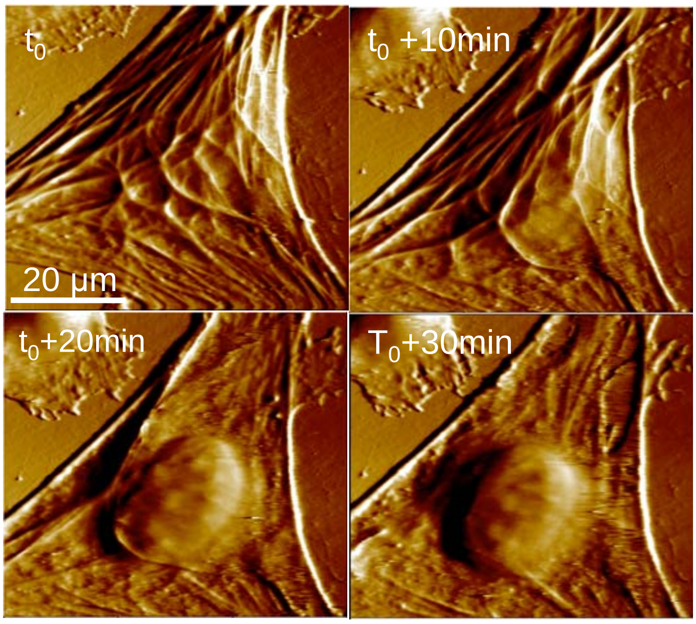
- Cell morphology
- Structure and properties of cell membranes
- Cytoskeleton and cell movements

At the molecular level:

- High resolution imaging of proteins
- Single-molecule spectroscopy
- Molecular motors, actin, microtubulles
- The protein folding problem
- DNA-protein interactions

Cell Morphology

AFM imaging of Cells



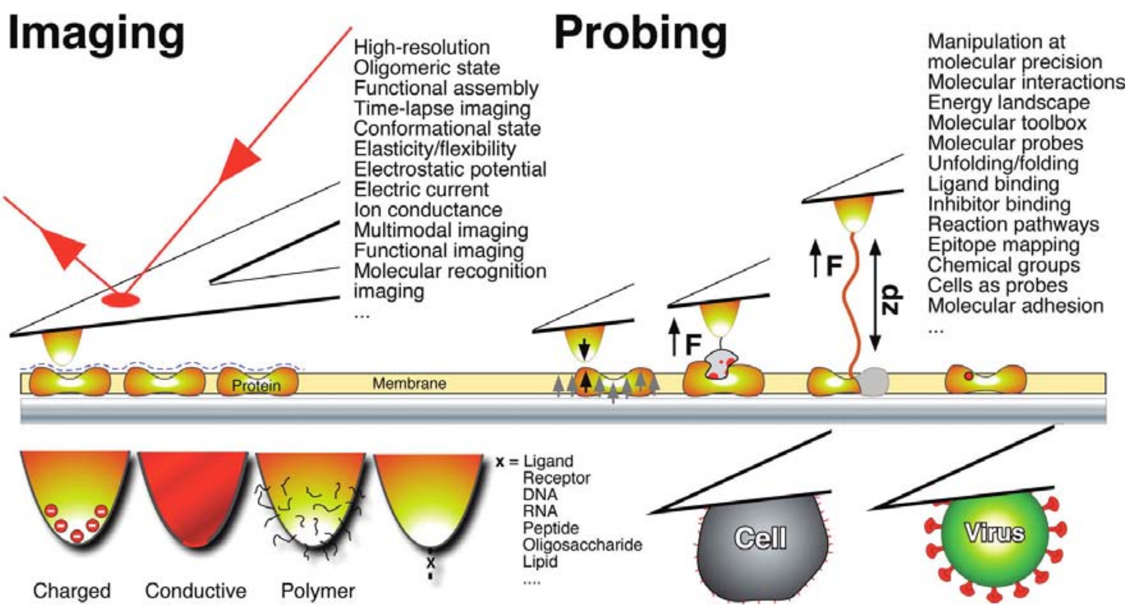
AFM allows the dynamic study of morphology of cells and to follow the changes under the action of a stress.

Effect of Cytochalasine D on neuronal cells

courtesy. F. Héniche

AFM: a nanotool in membrane biology

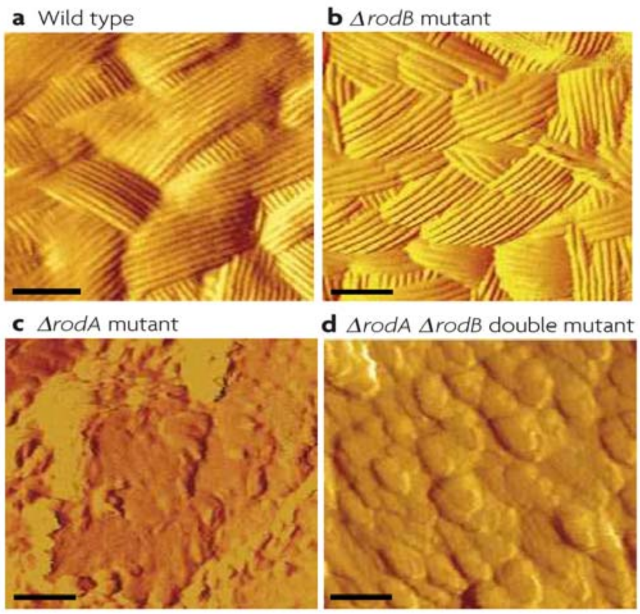
Cellular membranes form cellular contacts and focal adhesions, anchor the cytoskeleton, generate energy gradients, transform energy, transduce signals, move cells…



Muller DJ *Biochemistry* ***2008***

Cell membrane imaging

High resolution imaging of cells outside membranes

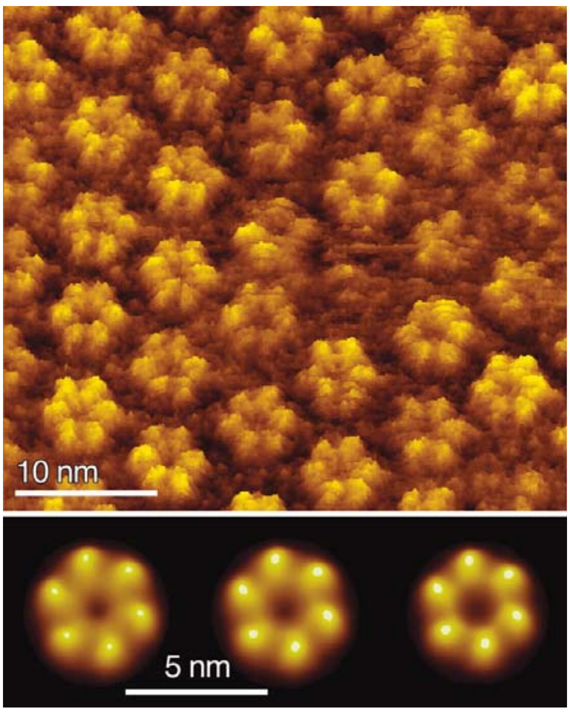


Effect of genes mutation on membrane structure

Image AFM de la surface de cellules d'*Aspergillus fumigatus* sauvages et avec différentes mutations (barre d'échelle 100nm)
(Dague et al. Langmuir 2008).

Cell membrane imaging

High resolution imaging of connexin 26 gap junction channel



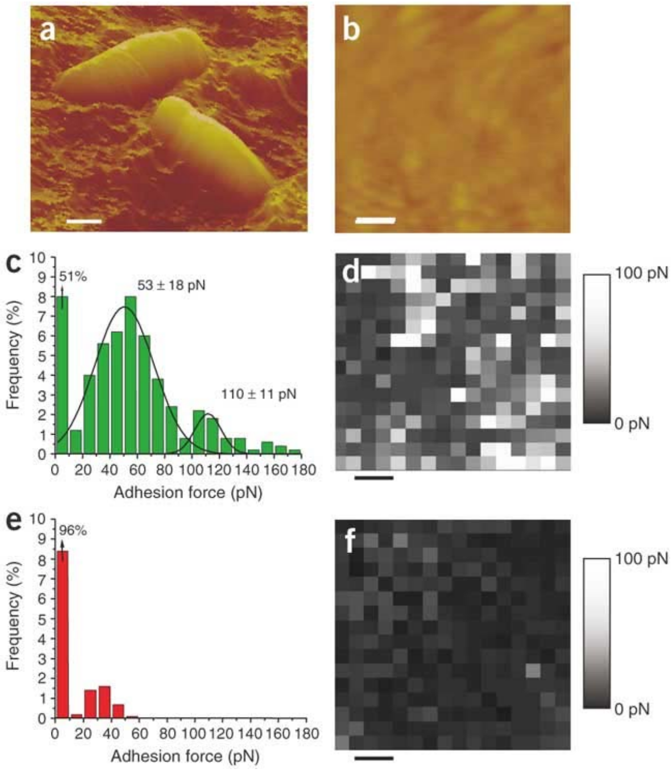
Gap junction channels mediate communication between adjacent cells.

In the presence of Ca^{2+}, the extracellular connexon pore reduces its channel diameter significantly.

This closure was reversed with the removal of Ca^{2+}.

Muller Embo J. 2002

Cell membrane: ligand-receptor binding

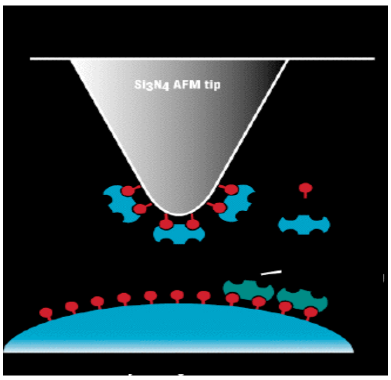


Mycobacterium tuberculosis, adheres to epithelial cells via the HBHA, a 199-residue protein that recognizes heparin molecules on the surface of living cells.

Dupres et al. Nat Meth. 2005

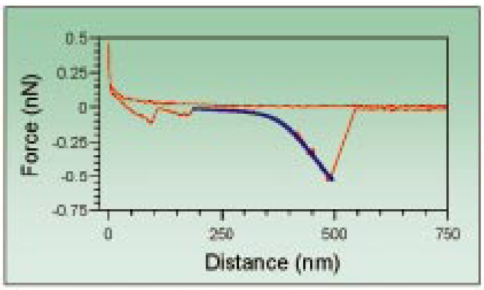
Force spectroscopy

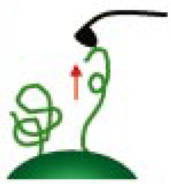
Main Biological applications of Force Spectroscopy



Measurements of specific interaction between a ligand and its receptor

Measurement of the elasticity biopolymers by stretching

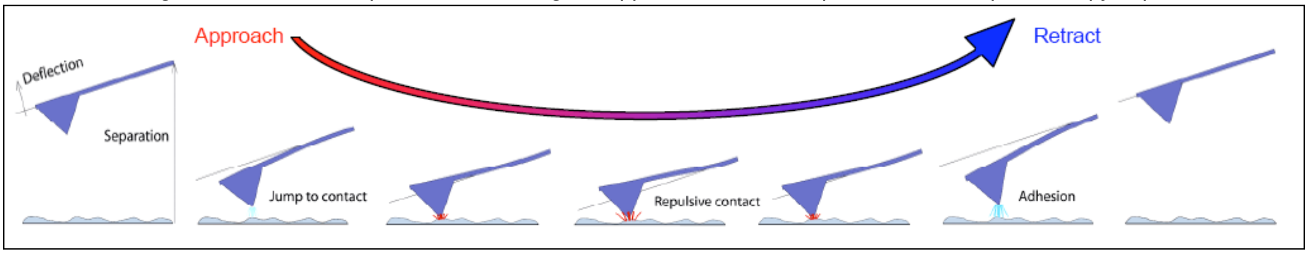




Force spectroscopy

Tip – Sample Interaction

Schematic diagram of the vertical tip movement during the approach and retract parts of a force spectroscopy experiment*



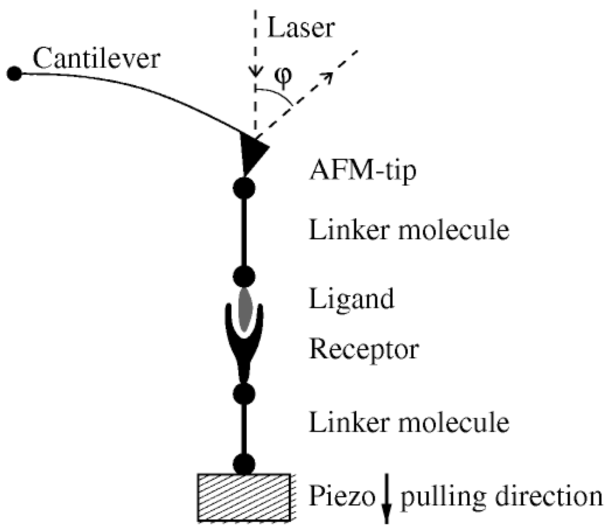
Tip Far Away (10-100 microns)	Tip Approaching (few microns)	Tip Close to Surface	Contact	Lifting Off Surface (few atomic distances to nanometers)	Tip farther away (nanometers to hundreds of nanometers)	Tip Far Away (1-5 microns)
No interactions	Electrostatic Forces	Van der Waal Forces Capillary Forces	Tip Indenting the sample Stiffness Viscoelastic Response		Stretched molecules between tip and surface Protein unfolding, pulling out of membranes	Connection between the tip and substrate is broken. No more interactions

Technical Report Nanowizard – A practical guide to AFM force spectroscopy and data analysis

Force spectroscopy

Working Principle

- Linker molecules are used to functionalize the tip and the substrate
- Ligand and receptor molecules are attached to the linker molecules
- The functionalized substrate holding the receptor molecule is approached with AFM tip
- Ligand recognizes the receptor and fits in
- AFM tip can be retracted to study the response of the ligand-receptor molecule



Substrate Functionalization

Binding of biological molecules to a solid substrate

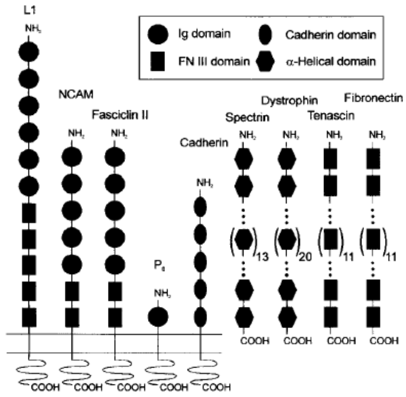
❖poly-L-lysine or poly-L-arginine

❖silanizing a solid surface with 3-aminopropyltriethoxysilane (APTES)

❖Cross-linking group via the amino end of APTES on a glass surface (ANB-NOS).

❖ultraflat Au(111) surface is used as a substrate for N-hydroxysuccinimide terminated self-assembled monolayers

❖For protein adsorption, Interaction forces include dipole and induced dipole moments, hydrogen bond forces and electrostatic potentials



Schematic representations of several proteins attached to the substrate that are exposed to mechanical stress*

*Thomas E. Fisher, Mariano Carrion-Vazquez, Andres F. Oberhauser, Hongbin Li, Piotr E. Marszalek, Julio M. Fernandez. "Single Molecular Force Spectroscopy of Modular Proteins in the Nervous System" Neuron, Vol. 27, Sep. 2000, 435-446

Tip Functionalization

Why do we need to functionalize the tip?

• To alter the surface properties of the tip so that it gains affinity to attach to the required end of the biomolecules

• Helps in selectivity of single molecule

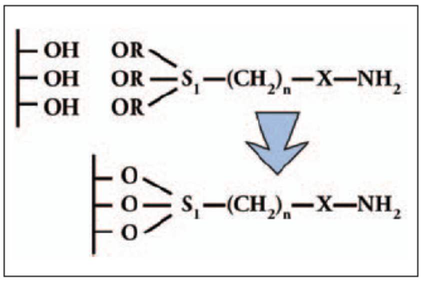
Methods used for Functionalization:

• Plasma Treatment to get desired hydrophobicity

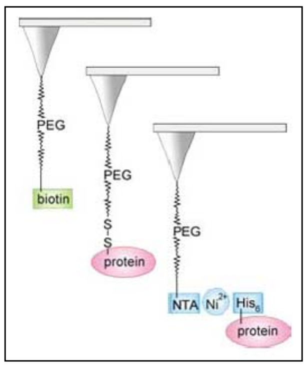
• Silanization

• Using a spacer as a linker molecule in between – eg. PolyEthyleneGlycol (PEG)

• Using appropriate biomolecules for linking – antigen or ligand



Schematic Representation of Silanization Process*



Different ligands tethered to AFM tip via flexible PEG linker **

*C. Tolksdorf, I. Revenko "Choosing AFM Probes for Biological Applications"
**Cordula M. Stroh et. al. "Tools for single molecule Recognition Force Microscopy and Spectroscopy"

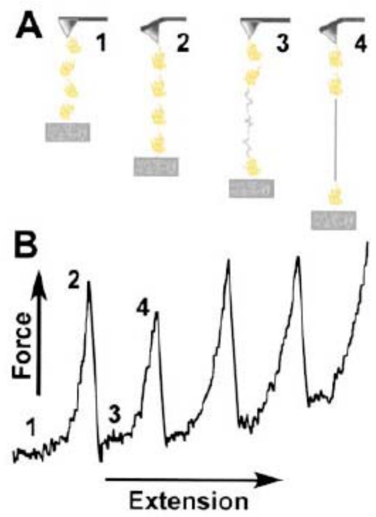
Typical Force Curve

The curve shows a typical force characteristics of a biomolecule (typically a folded protein):

• Each drop in force (2-3) corresponds to one unfold in the molecular structure

• Similar order of peaks show that same force is required to unfold it every time

• Number of peaks tell about the total number of folds in the molecule



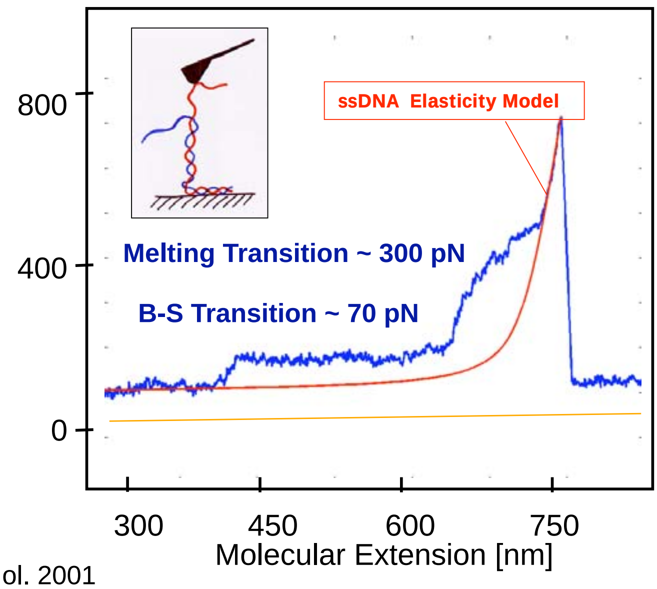
The Forced extension of Modular Proteins exhibits a Saw-Tooth Pattern*

**Thomas E. Fisher, Mariano Carrion-Vazquez, Andres F. Oberhauser, Hongbin Li, Piotr E. Marszalek, Julio M. Fernandez. "Single Molecular Force Spectroscopy of Modular Proteins in the Nervous System" Neuron, Vol. 27, Sep. 2000, 435-446*

DNA Structural Transitions

AFM Force Spectroscopy in TRIS Buffer

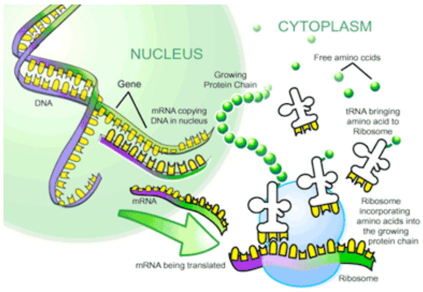
Duplex poly(dG-dC)



Anselmetti et al. Single Mol. 2001

The DNA molecule

- DNA is the fundamental carrier of the genetic code
- The genetic material must be able to:
  - → Replicate (when cells divide)
  - → Express information

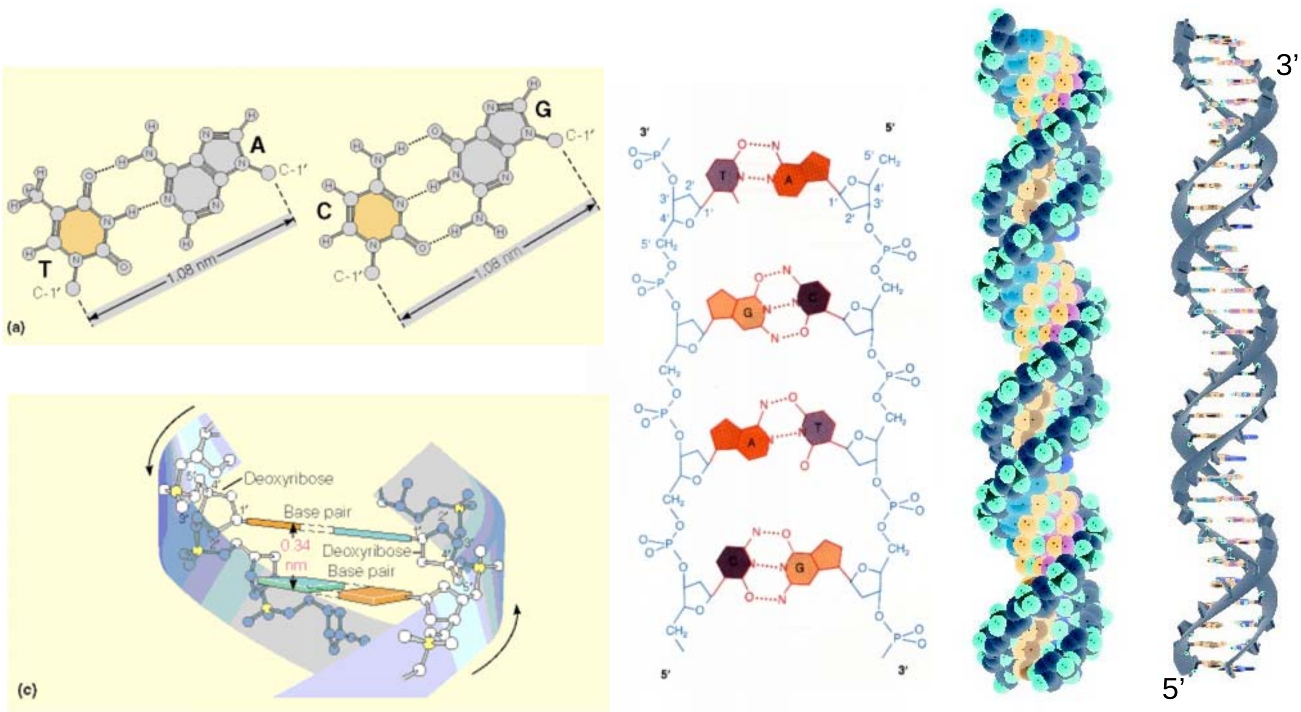


Through transcription and translation DNA is expressed in proteins

  - → Mutate at a low frequency (less than 1 in a million)
- DNA is a molecule that is very well suited to doing all 3 of these

The DNA molecule

The structure of DNA

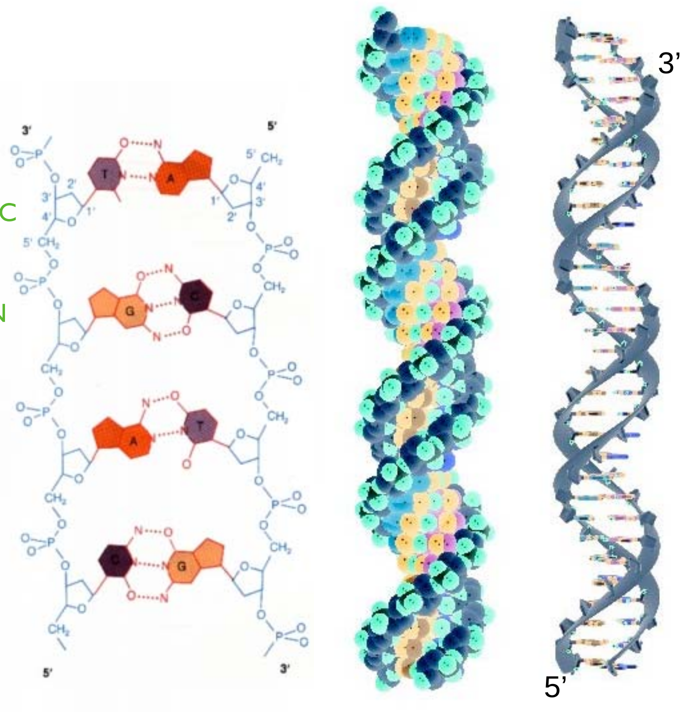


Figures : Mathews & van Holde, “Biochemistry”

The DNA molecule

The structure of DNA

- A POLYMER OF DEOXYRIBONUCLEOTIDES
- DOUBLE-STRANDED
- INDIVIDUAL deoxyNUCLEOSIDE TRIPHOSPHATES ARE COUPLED BY PHOSPHODIESTER BONDS
  - → LINK 3' CARBON OF ONE RIBOSE WITH 5' C OF ANOTHER
  - → TERMINAL ENDS : 5' AND 3'
  - → BASES ARE STACKED IN PARALLEL FASHION
  - → CHARGAFF'S RULES
    - A = T
    - G = C
  - → "COMPLEMENTARY" BASE-PAIRING
- A "DOUBLE HELICAL" STRUCTURE
  - → COMMON AXIS FOR BOTH HELICES
  - → ANTIPARALLEL RELATIONSHIP BETWEEN 2 DNA STRANDS



The DNA molecule

The structure of DNA

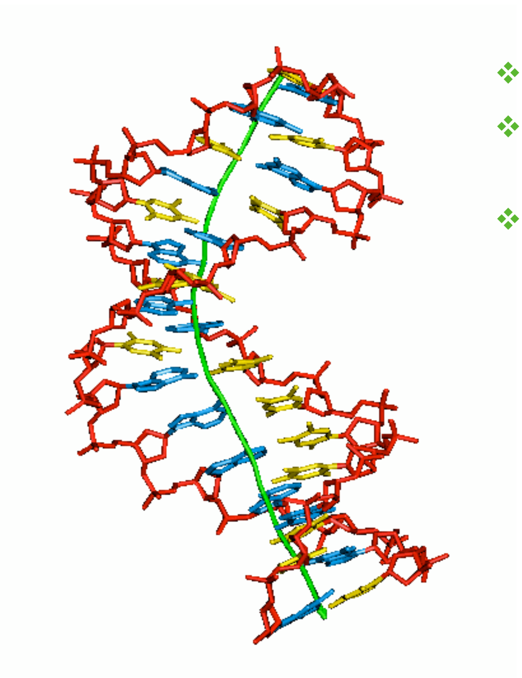
FORCES THAT STABILIZE NUCLEIC ACID STRUCTURES

- SUGAR-PHOSPHATE CHAIN CONFORMATIONS
- BASE PAIRING
- BASE-STACKING, HYDROPHOBIC
- IONIC INTERACTIONS
  - → THE DOUBLE HELIX IS ANIONIC
    - One negative charge per phosphate on each helix
  - → DIVALENT CATIONS INTERACT SPECIFICALLY WITH DNA
    - Bind to phosphate groups
  - → MAGNESIUM (2+) ION STABILIZES DNA STRUCTURES

DNA is a highly charged biopolymer which interacts greatly with multivalent cations present in solution

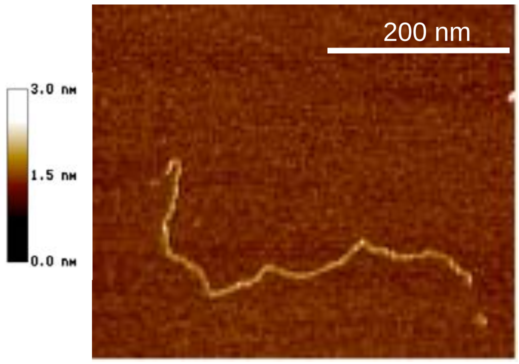
The DNA molecule

DNA Polymorphism



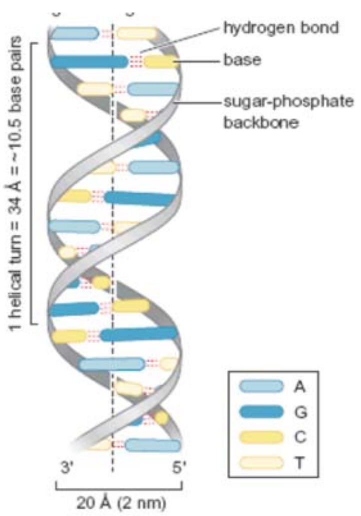
R. Lavery and H. Sklenar J. Biomol. Struct. Dyn. 6, 1989, 655.

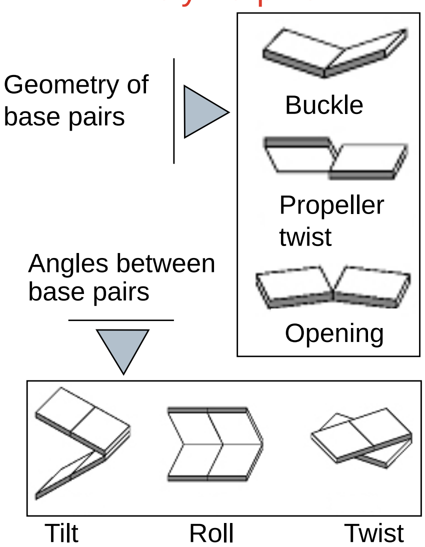
- The axis of the double helix is not straigth
- Its trajectory in 3D space depends on the sequence of base pairs.
- DNA is considered as a biopolymer with own mechanical properties (curvature, flexibility, persistence length…) depending on base-pair sequence and ionic environment.



The DNA molecule

DNA Polymorphism

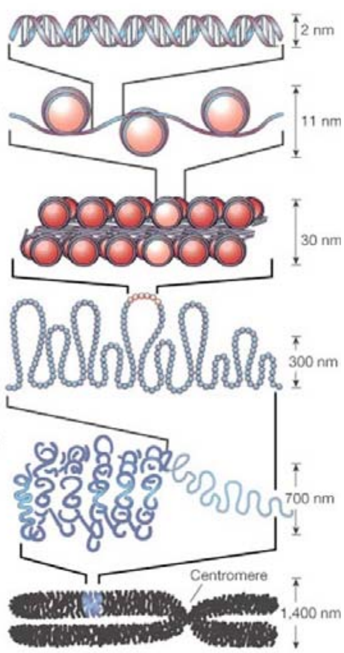




DNA structural and conformational polymorphism

Local properties depend on DNA sequence

Local curvature and flexibility



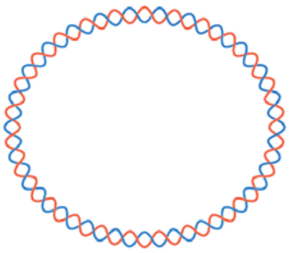
DNA compaction and folding in eukaryotic cells

The DNA molecule

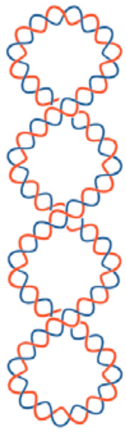
DNA Polymorphism

Most DNA in its natural state, including chromosomal and plasmid DNA in bacteria as DNA in eukaryotes cells, is circular and negatively supercoiled.

A superhelix is formed when the double helix is further coiled around an axis and crosses itself. Supercoiling not only allows for a compact form of DNA, but the extent of coiling also affects the DNA's interactions with other molecules by determining the ability of the double helix to unwind.



"relaxed" double-helical segment of DNA



negative supercoiling

The DNA molecule

DNA Polymorphism

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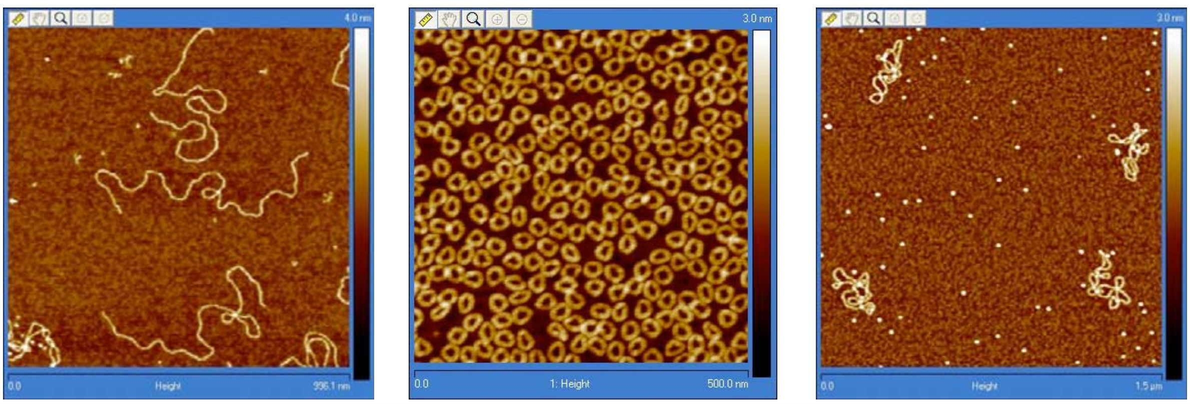
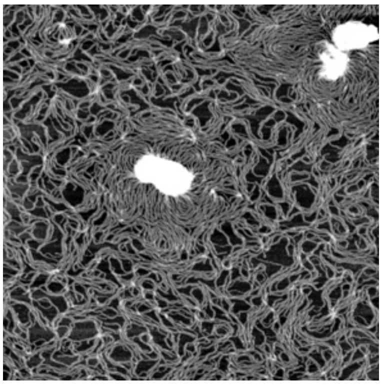
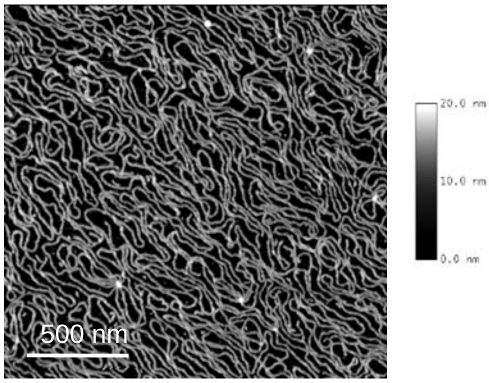


DNA condensation

DNA condensation refers to the process of compacting DNA molecules. Understanding the factors that govern the condensation of nucleic acids (DNA and RNA) will have fundamental implications in cell biology and virology. Furthermore, the ability to control DNA condensation has been identified as a necessary step towards the development of more efficient protocols for gene therapy.

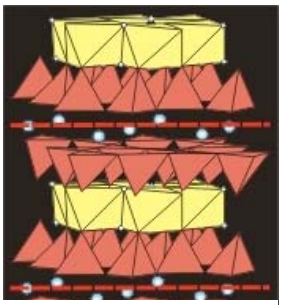


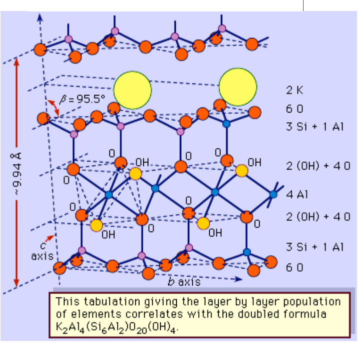


Pastré et al. Biomacromolecules 2007

DNA imaging with AFM

What substrate used for AFM imaging?





$KAl_2[AlSi_3]O_{10}(OH)_2$

- Bare mica: use of multivalent cations for DNA adsorption
- Mica surface pre-treatment

  Polylysine, silanes (AP-mica, APTES-mica), lipids…
- HOPG Graphite
- Ultraflat Gold
- Silicon with silanes
- Langmuir-Blodgett films

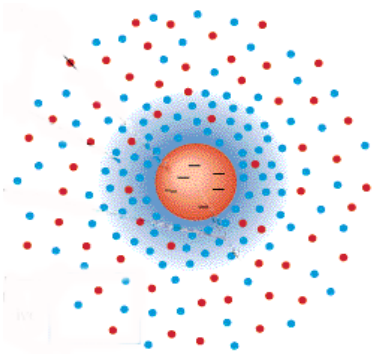
Bare mica is the only substrate for which a reversible binding is possible

(Piétrement et al. Langmuir 2003)

DNA imaging with AFM

DNA adsorption

DNA is a highly charges molecules

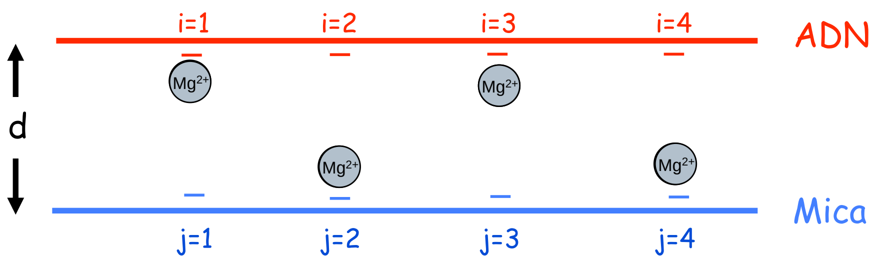


	DNA adsorption	DNA/protein specific interaction
Monovalent cations	NO	YES
Multivalent cations	YES	YES

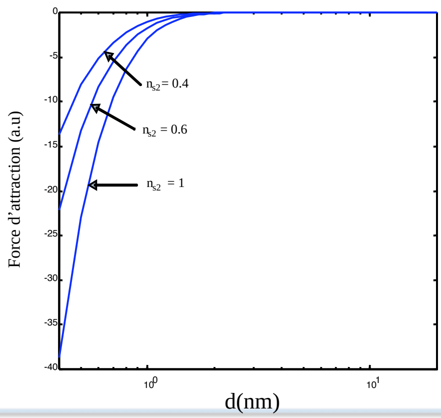
Mono and multivalent cations are in competition for the neutralization of DNA backbone in solution

DNA imaging with AFM

Mechanism of adsorption



The attractive force comes from counterions correlation between mica and DNA surfaces



$$F_c(d) = \frac{e^2}{\varepsilon}\sum_{i,j}\frac{(1-z_j\phi_j)(1-z_i\phi_i)d}{\left(x_{i,j}^2+d^2\right)^{\frac{3}{2}}}$$

- Very short distance attractive force (<*nm*)
- Competition between mono and divalent cations decreases the attractive force

Pastré et al., Biophys J 2003

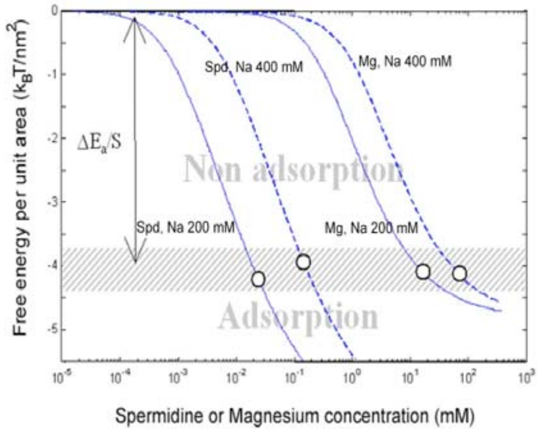
DNA imaging with AFM

DNA adsorption on a mica surface

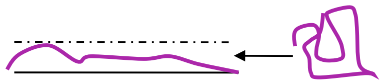
- Energetic gain from counterions correlation:

$$\frac{d(\Delta F_{WC})}{dN} \approx \mu_{WC}(n_i) - \frac{n_s}{n_i}\mu_{WC}(n_s) - \frac{n_p}{n_i}\mu_{WC}(n_p)$$

  → More important for close charged surfaces
  → Increases with Z² tand $\sigma^{3/2}$



- Energetic costs to DNA adsorption:
  → The entropy cost of DNA confinement

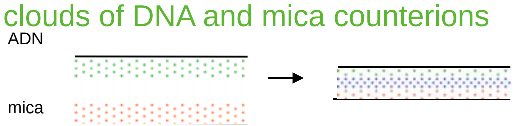


$$\frac{\Delta E_{conf}}{k_B T} \approx -\frac{2}{3}\cdot\frac{L}{C^{2/3} l_p^{1/3}} \ln(l_p / C)$$ 1 -0.1 kT/nm²

  → Electrostatic repulsion

$$\frac{\Delta E_{el}}{S k_B T} \approx -(0.25\frac{\sigma_p}{e})4\pi\left(0.15\frac{\sigma_s}{e}\right)d.l_b$$

  → Energy barrier due to the interpenetration of the clouds of DNA and mica counterions



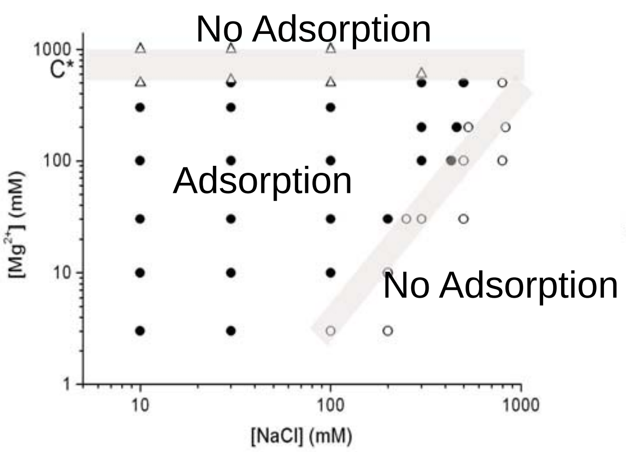
$$\frac{\Delta E_{thermal}}{S k_B T} \approx -\left(\frac{\sigma_s}{e}\right)\ln\left(\frac{c_{si}}{c_{ss}}\right) - \left(\frac{\sigma_p}{e}\right)\ln\left(\frac{c_{si}}{c_{sp}}\right)$$ environ 3-4 kT/nm²

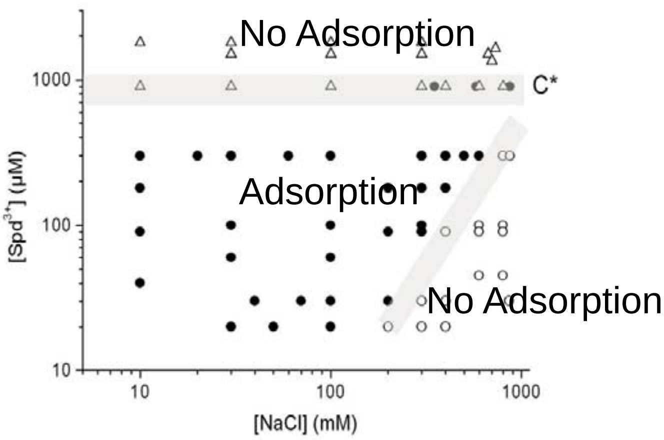
Pastré et al., Langmuir 2006

DNA imaging with AFM

AFM analysis of DNA adsorption

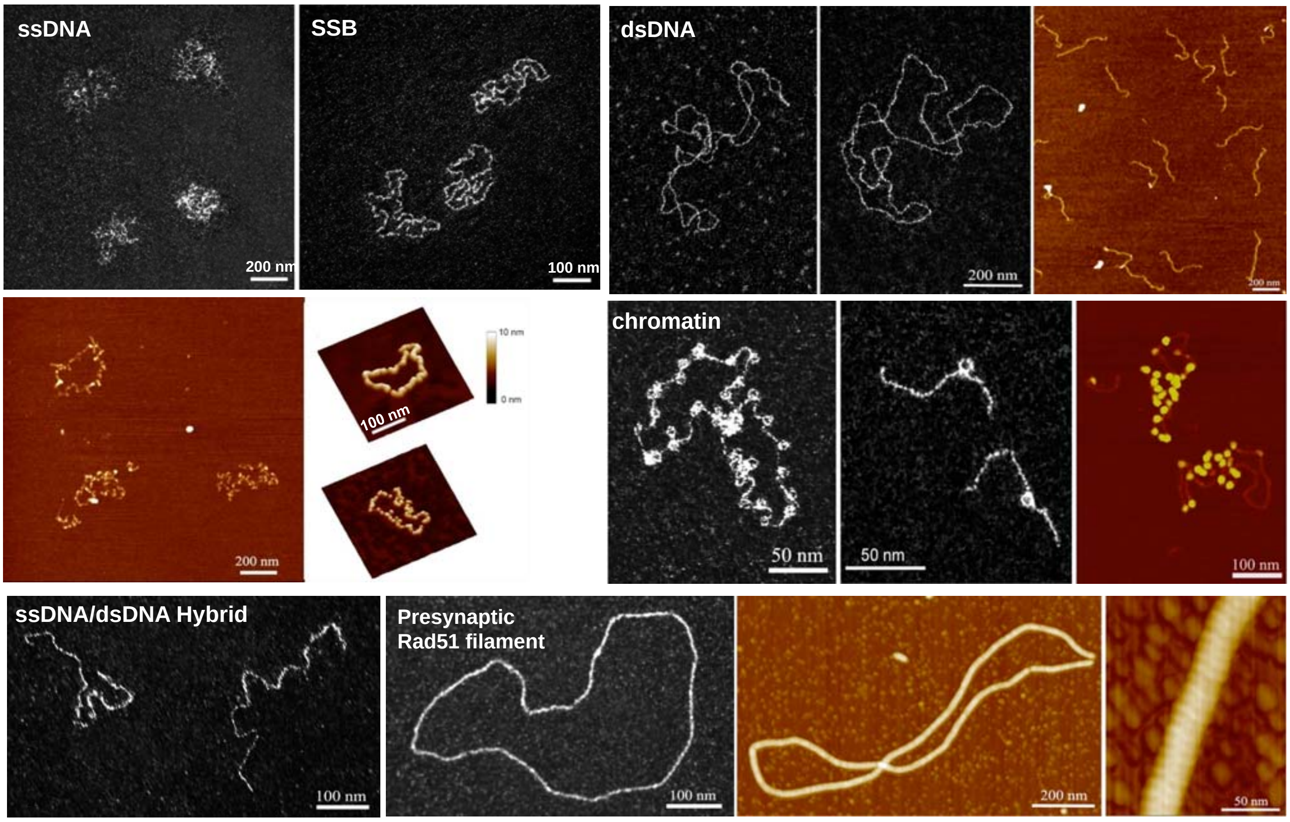
Competition between monovalent, divalent and trivalent counterions for AFM imaging





Spermidine is more efficient than magnesium for DNA adsorption and allows AFM imaging with higher monovalent salt concentrations

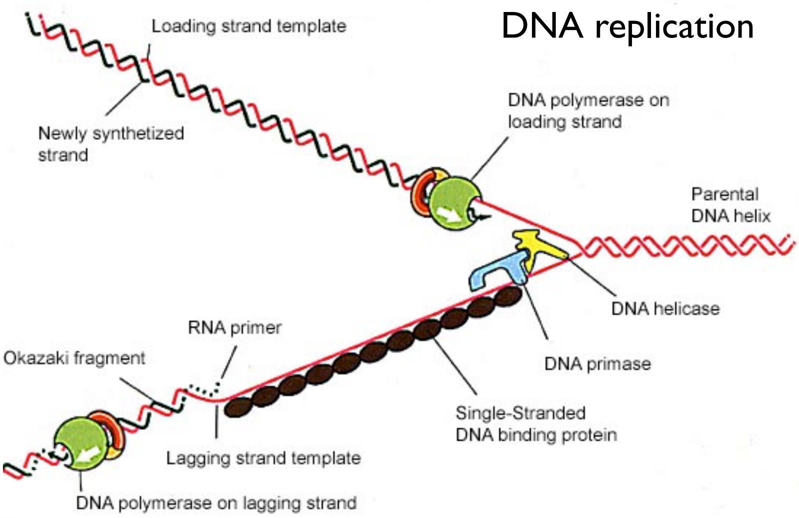
DNA imaging with AFM / TEM



AFM studies of nucleoproteic complexes

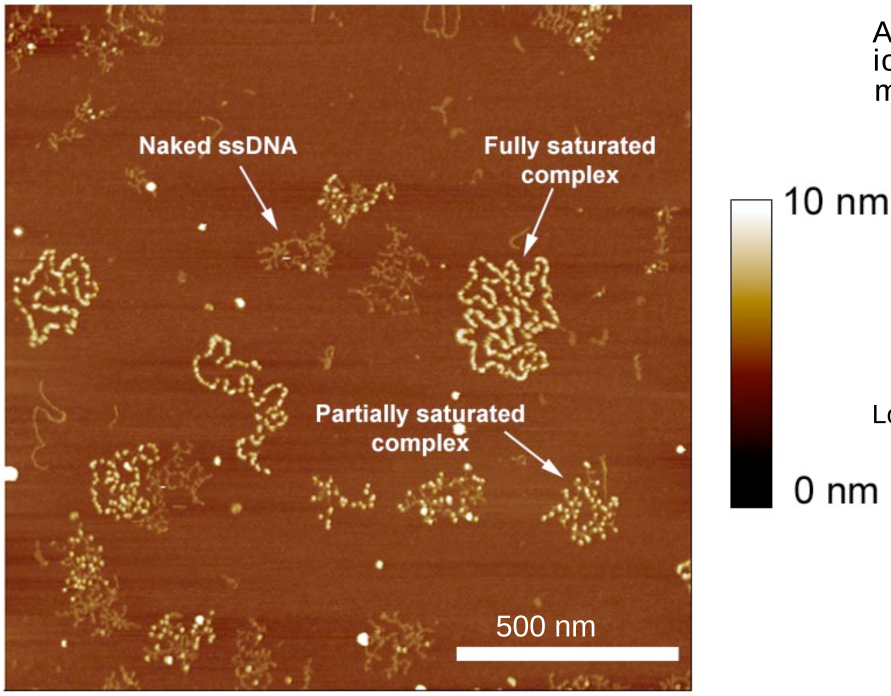
Study of ssDNA/ SSB proteins complexes

- SSB proteins bind with a high affinity to single-stranded (ss) DNA and this formed complex plays a central role in all DNA metabolism (reparation, recombination, replication and transcription)



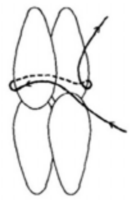
AFM studies of nucleoproteic complexes

E. Coli SSB binding to ssDNA at low ionic strength



AFM imaging under low ionic strength (NaCl 20 mM & Spd 20 µM)

Low Salt Binding mode $(SSB)_{35}$



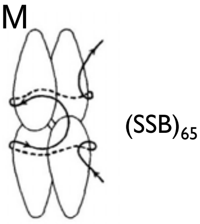
Hamon, et al. NAR 2007

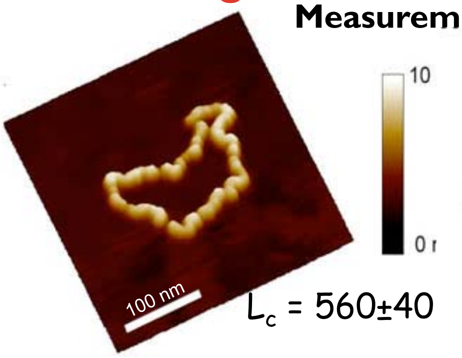
AFM studies of nucleoproteic complexes

NaCl concentration-dependence of *E. Coli* SSB binding to ssDNA

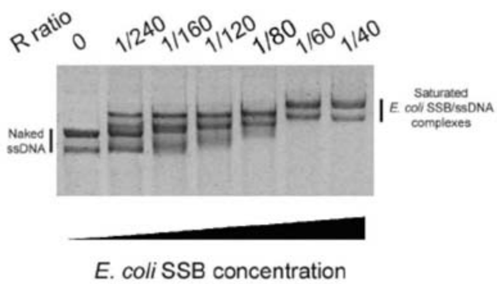
Measurement of the contour length L_c

- NaCl 300 mM

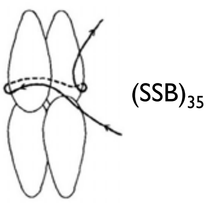


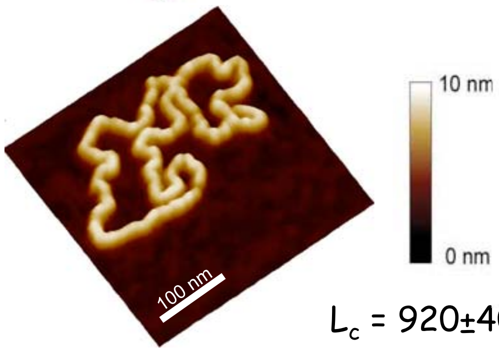


L_c = 560±40

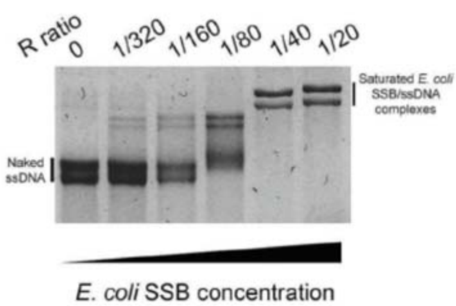


- NaCl 20 mM





L_c = 920±40 nm

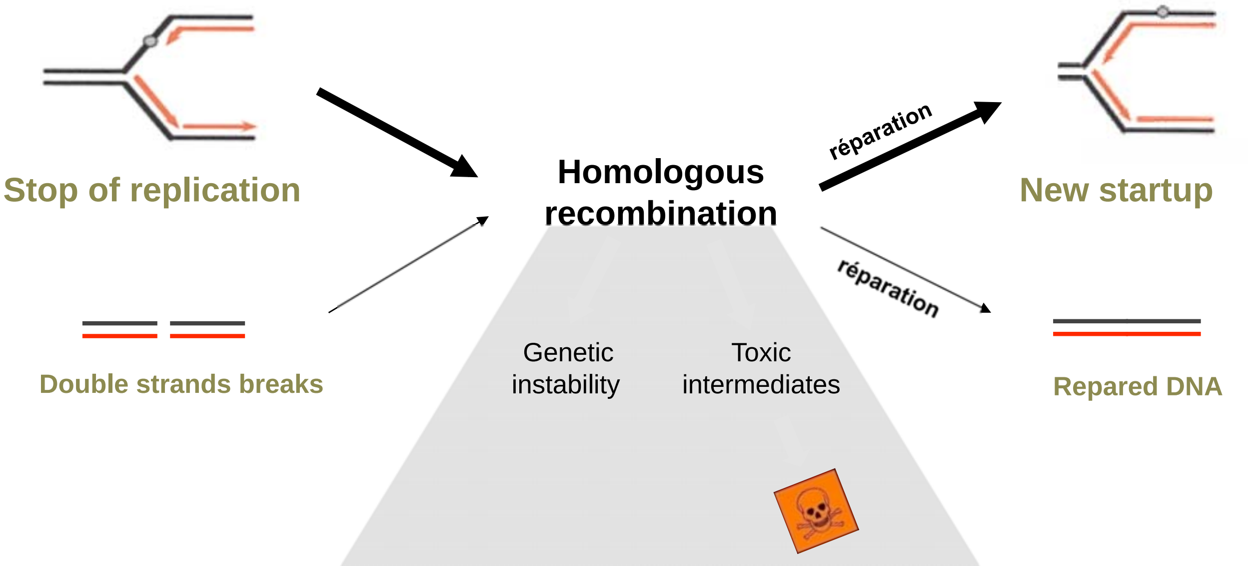


Hamon, et al. NAR 2007

AFM studies of nucleoproteic complexes

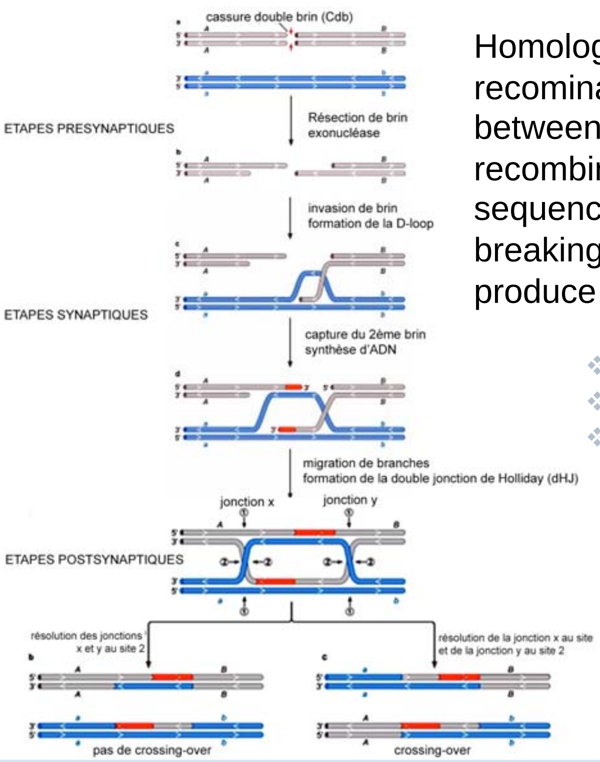
The homologous recombination

It is a mechanism allowing the repair of DNA breaks and new starup of replication



AFM studies of nucleoproteic complexes

The homologous recombination



Homologous recombination is a type of genetic recomination, a process of rearrangement occurring between two strands of DNA. Homologous recombination involves the alignment of similar sequences, formation of a Holliday junction, and breaking and repair, known as resolution, of the DNA to produce an exchange of material between the strands.

- Extremly conserved mechanism during evolution
- Many intermediates state with various structures
- Control of recombination

Formation of the nucleofilament (RecA, Rad51, RadA)?
Role played by SSB proteins?
Role played by helicase in the regulation of intermediate states?

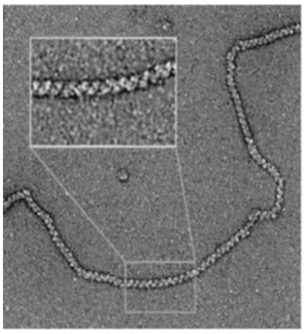
AFM studies of nucleoproteic complexes

Structural properties of Rad51 nucleofilament

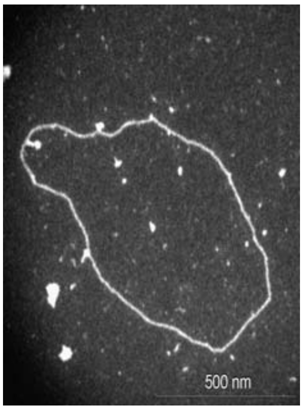
Rad51Filament

92 Å

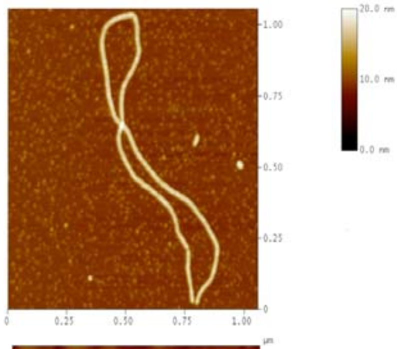
Egelman & Col.; PNAS, vol. 98, July 17 2001

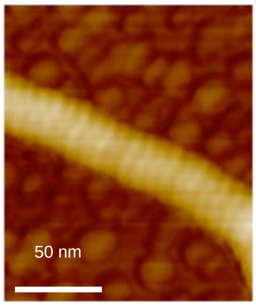


Brigth field TEM



Dark Field TEM

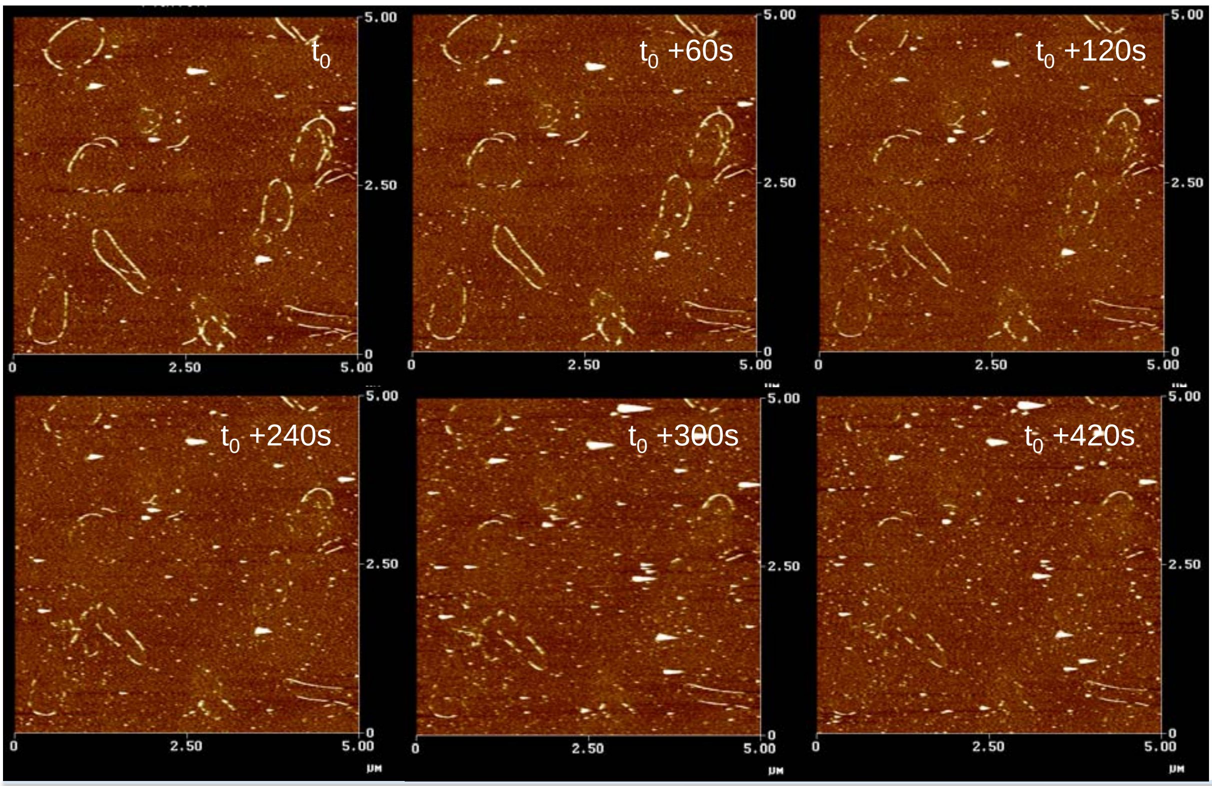


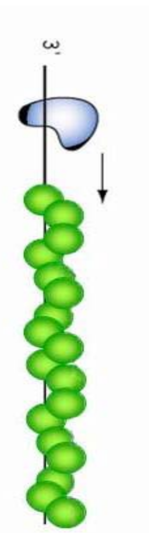


AFM

AFM studies of nucleoproteic complexes

Destabilization of Rad51 nucleofilament



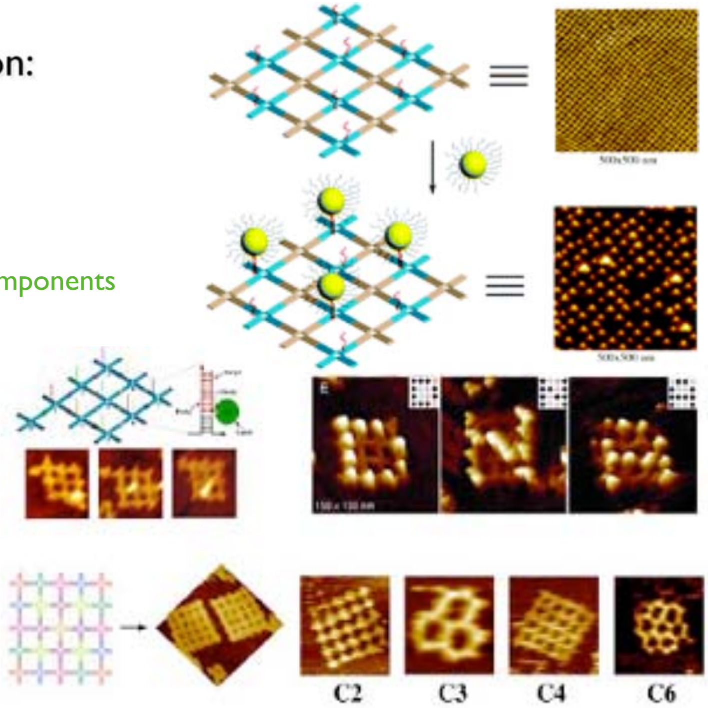


Self-assembly of DNA nanostructures

DNA Nanobiotechnology

Current Research and Application:

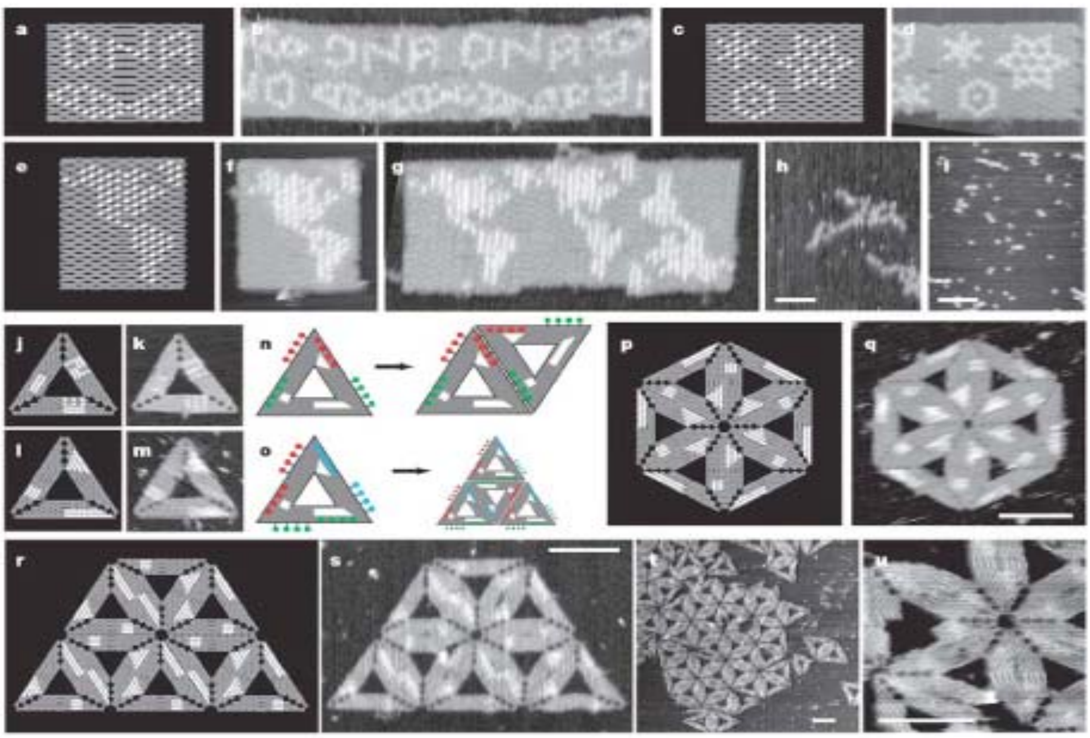
- Applications of DNA sequences:
  - → Sensitive molecular detection
  - → DNA-templated synthesis
  - → Parallel reaction discovery
  - → Computing with DNA
  - → Organization of nano-electronic components
  - → and other materials, etc.
- Applications of DNA structures:
  - → Molecular detection
  - → DNA scaffolding
  - → Nanoelectronic assembly
  - → Algorithmic self-assembly, etc.
- Applications of DNA-folding pathways:
  - → Molecular detection
  - → Diagnosis and drug release
  - → Binding and release of a protein target, etc.



Hao Yan's lab http://www.biodesign.asu.edu/

Self-assembly of DNA nanostructures

« DNA Origami » ***P. Rothemund, Nature 2006***



1 pixel = 1 tour = 3.6nm

Hauteur des lettres : 30nm

q, s-u: 100nm
h, i: 1µm

Conclusion

AFM Applications In Life Science

- High resolution imaging of biomolecules under physiological conditions *in situ*

    Nucleic Acids, Actin, Proteins, Membranes, Cells, Tissues, Biomaterials

- Single molecule measurements of inter-/ intra-molecular interactions and nanomechanical properties

    Protein-Protein / Ligand-Receptor Binding, Molecular (Un)Folding, Surface Elasticity / Adhesion

Perspectives

Main developments for biological applications:

- High speed AFM
- Association with fluorescence microscopy (TIRF, STED, PALM, STORM…)
- Use of KFM and SICM for studying electrostatic properties of membrane cell

Merci de votre attention!