

Housekeeping notes

- **Second** recording on Mediaspace: issue with screensharing after break, so the video is not available for the second hour. :(My apologies.
- Signup for group assignment closes **tomorrow morning, March 6, 10 am**. Make sure to sign up!
- Next week, we'll have our first debate presentations.

Case study 2

Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy

nature

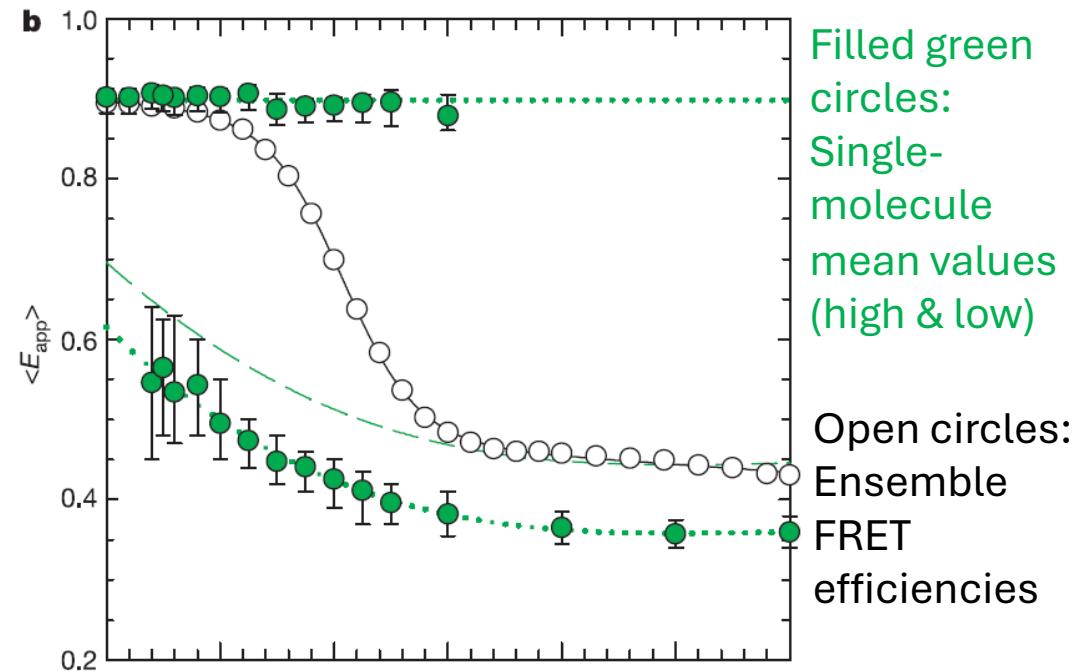
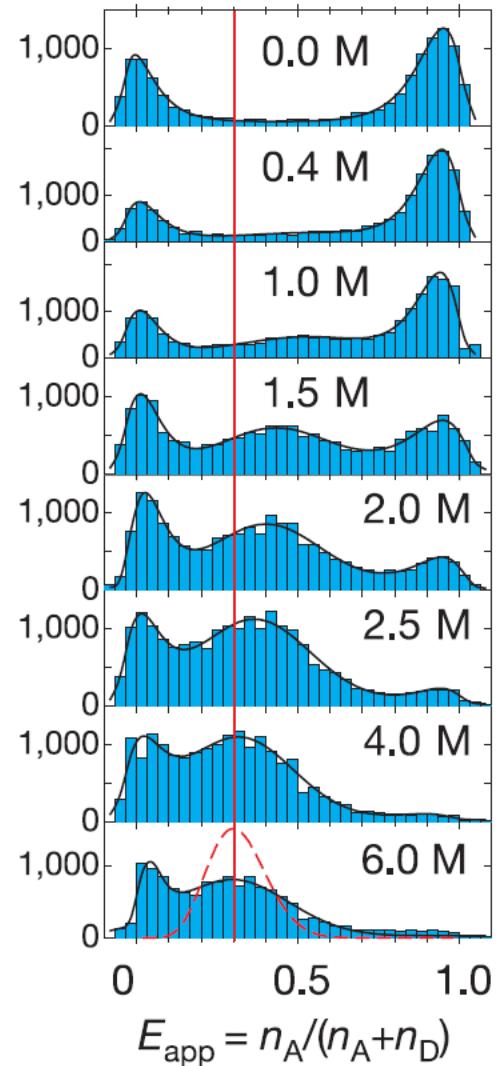
Benjamin Schuler^{*†}, Everett A. Lipman^{*†} & William A. Eaton^{*}

** Laboratory of Chemical Physics, Building 5, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0520, USA*

† These authors contributed equally to this work

For this case study (we didn't have time for last week): I've **uploaded last year's lecture recording** on the Moodle, so you can watch it yourselves (start at 20:42).

Analysis of the protein conformation during unfolding



FRET efficiency of unfolded state shifts to lower values at high denaturant.

- Distance is increased, protein chain more loosely arranged
- n_A and n_D are the average number of detected acceptor and donor photons in the significant bursts

CH-413 Nanobiotechnology

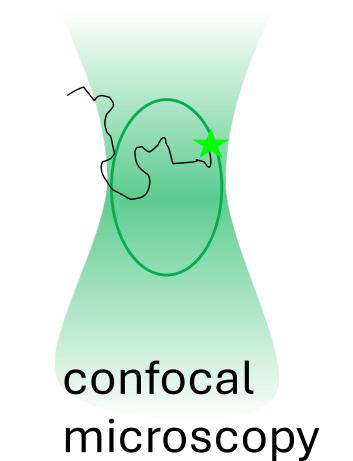
Single Molecule Force Spectroscopy

Angela Steinauer

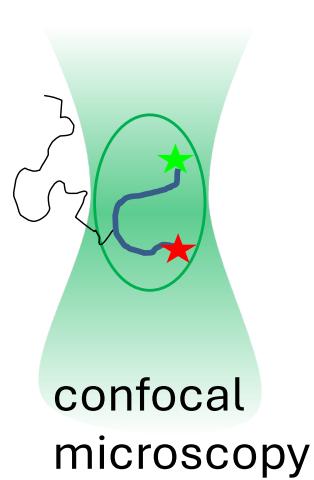
March 6, 2025

Different Single Molecule Approaches

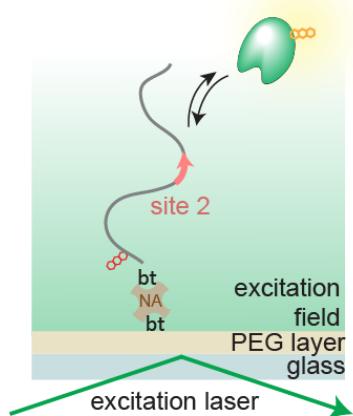
Fluorescence



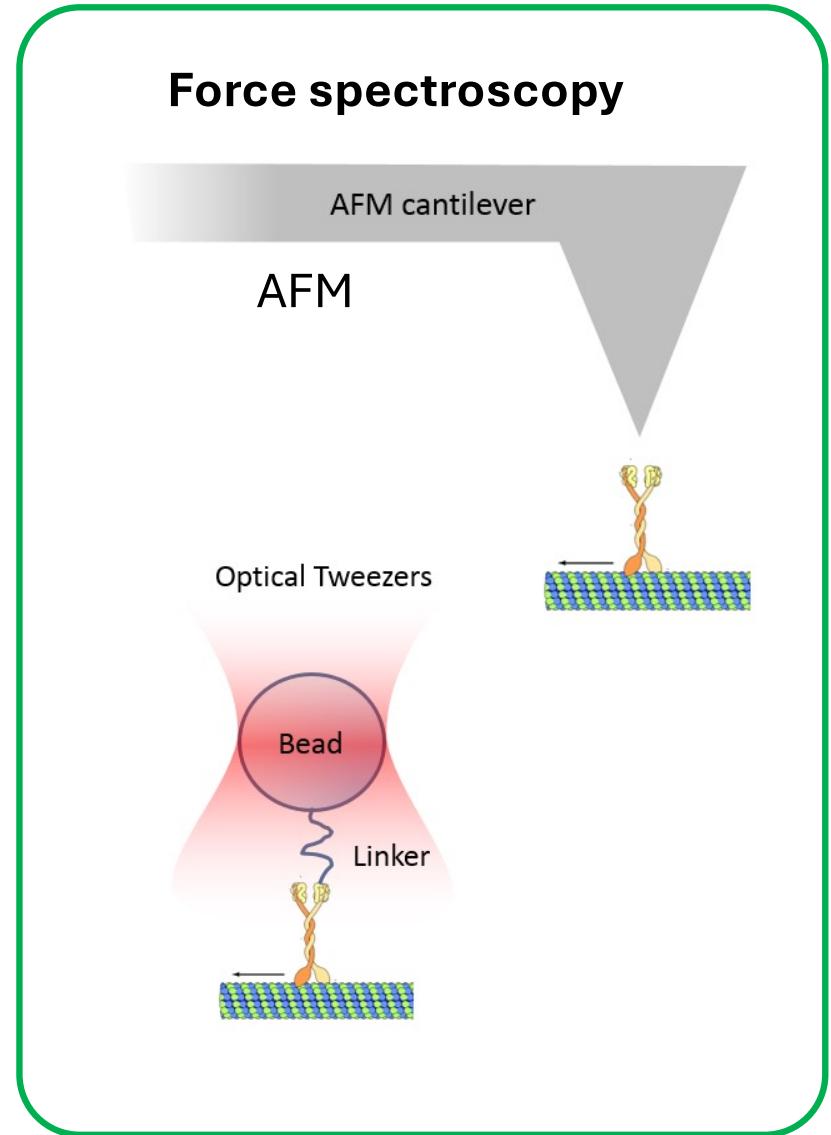
FRET



Total internal reflection
microscopy



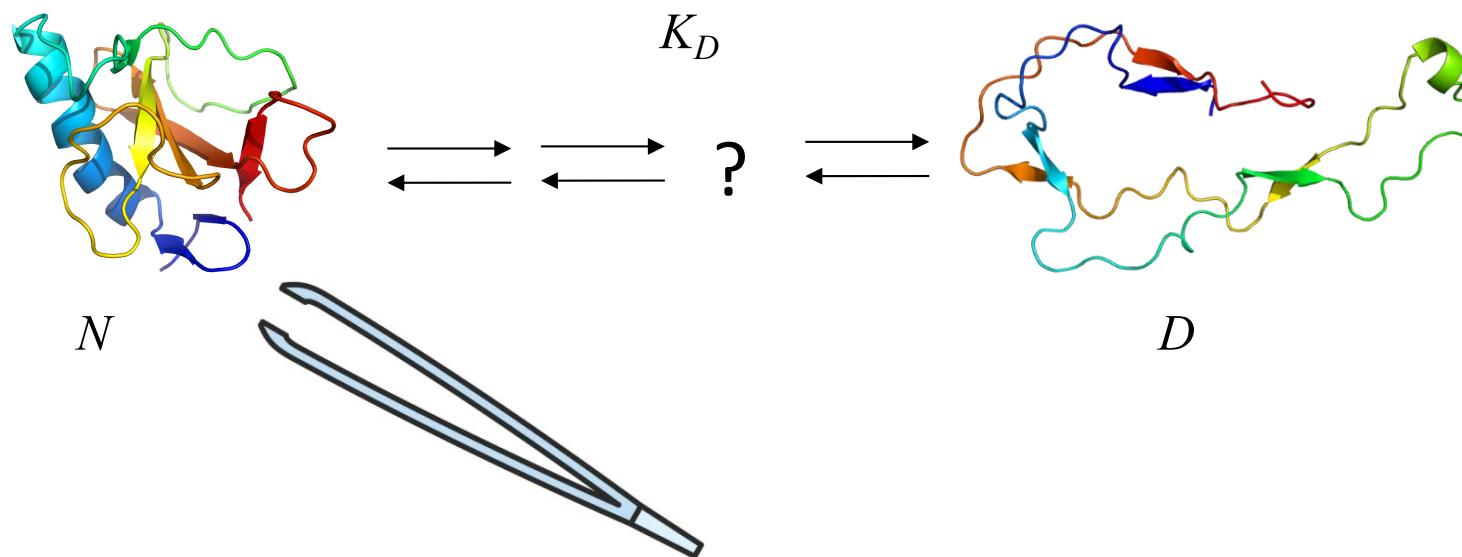
Force spectroscopy



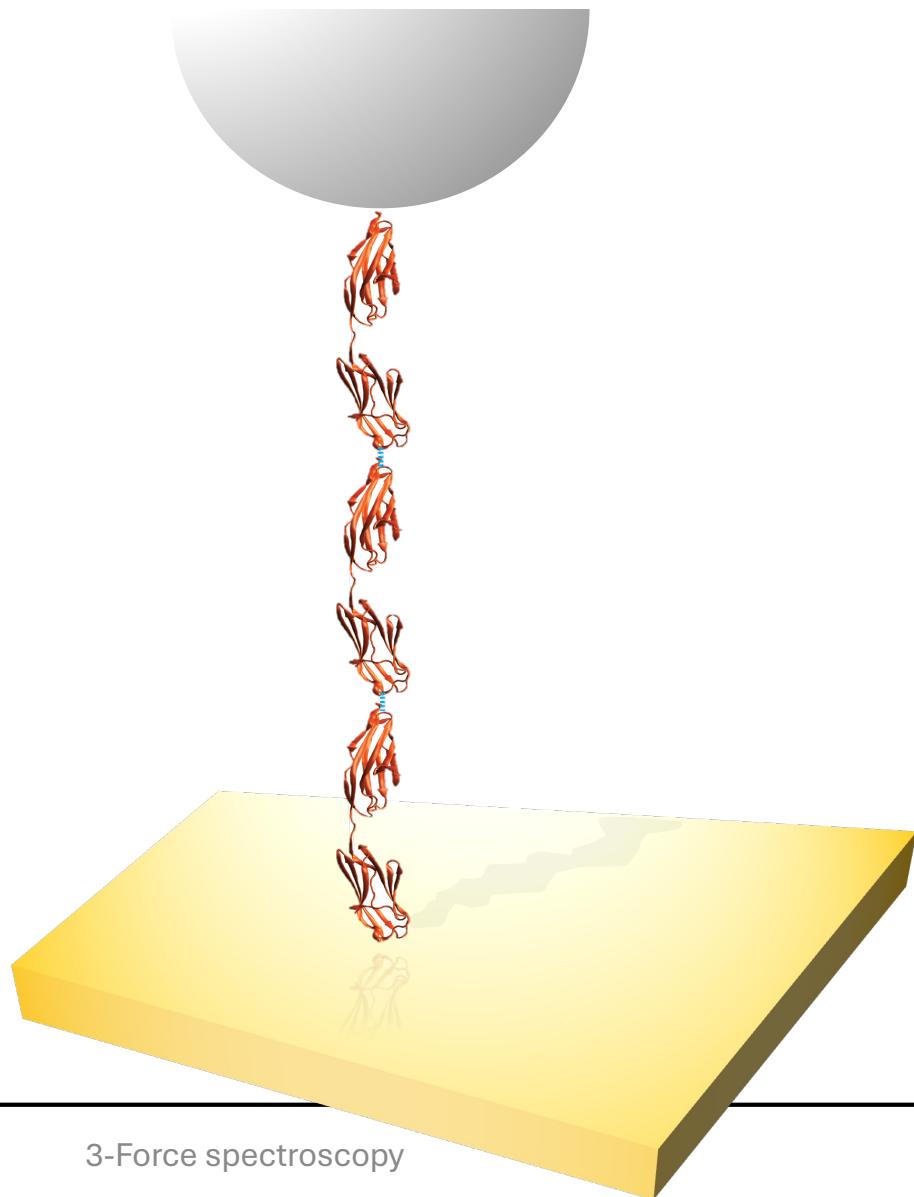
Learning objectives

- 1. Understanding the Principles of Force-Based Techniques:** Acquire a fundamental understanding of the principles behind force spectroscopy, magnetic tweezers, optical tweezers, and atomic force microscopy.
- 2. Exploration of Instrumentation and Hardware:** Identify and understand the key hardware components necessary for conducting experiments using force spectroscopy, magnetic tweezers, optical tweezers, and atomic force microscopy.
- 3. Operational Mechanics of Force-Based Instruments:** Gain insights into how the various hardware components of force-based techniques work collectively to measure and manipulate molecular interactions.
- 4. Analyzing Limitations and Opportunities:** Develop an understanding of the limitations and potential applications of force spectroscopy, magnetic tweezers, optical tweezers, and atomic force microscopy.
- 5. Applying Techniques to Research Design:** Enable students to conceptualize how they might incorporate force spectroscopy techniques into their own scientific research.

Protein folding



Why Single-Molecule Force Spectroscopy?



Definition - single-molecule force spectroscopy:

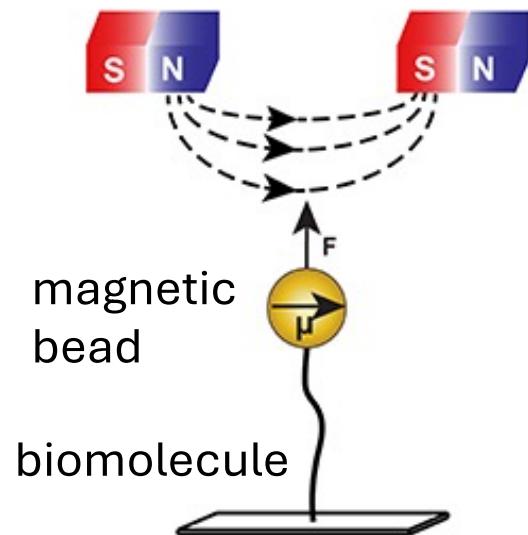
Methods to **probe forces** within or between individual molecules

Analysis of the mechanical properties of single molecules

- Imaging of deformable molecules
- Conformational changes in enzymes
- Rupturing of single bonds up to macromolecular interactions
- Energetics of biomolecular energy landscapes
- Molecular motors that generate translational power or torque

Force spectroscopy – Magnetic tweezers

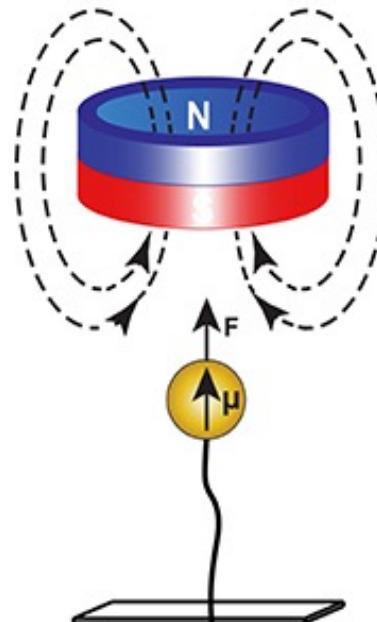
electromagnet



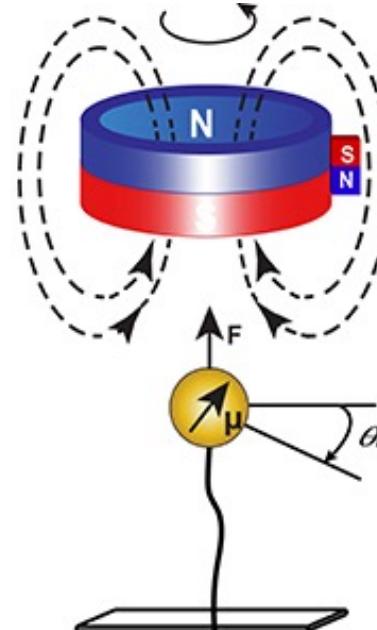
magnetic
bead

biomolecule

**Horizontal
magnetic field**
allows to control
bead rotation

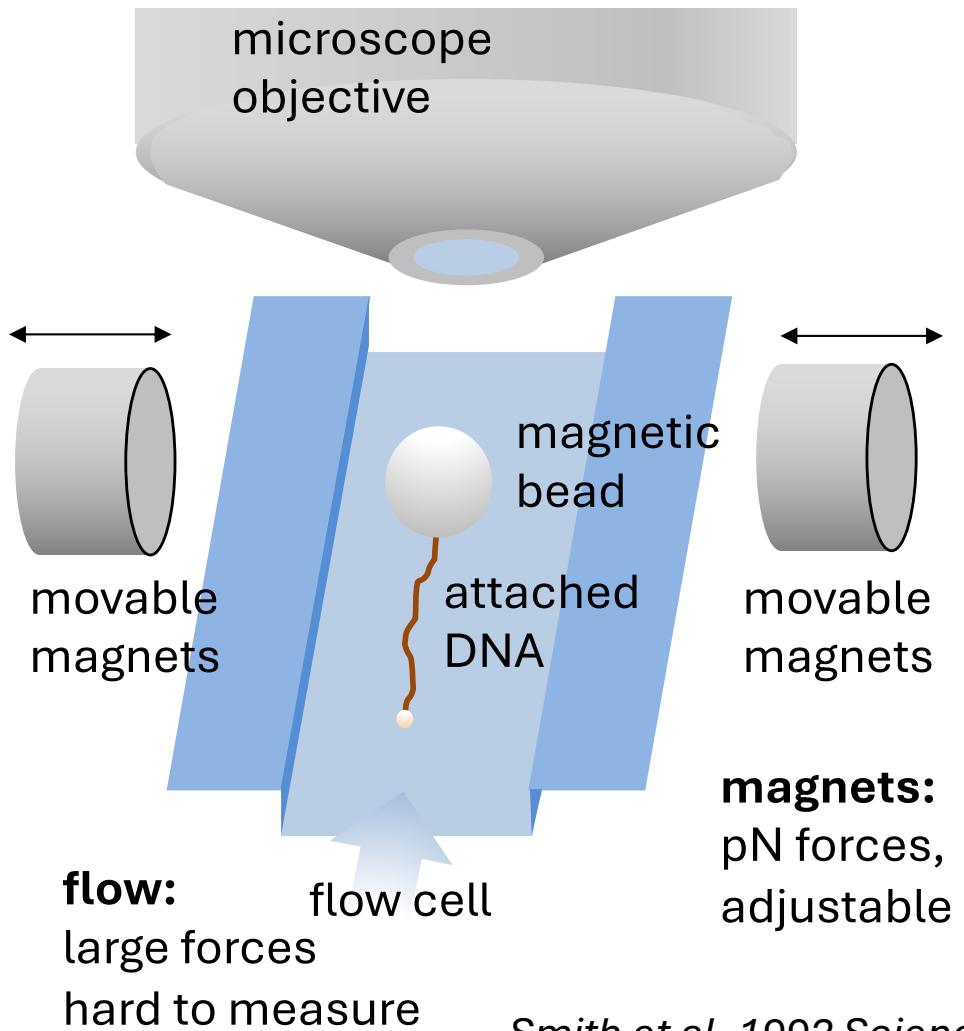


**Vertical magnetic
field**
allows free bead
rotation



**Vertical magnetic field
(strong) with weak
horizontal field**
allows to control bead
rotation (torque tweezer)

Initial Experiments with Magnetic Tweezers: Studying the Behavior of DNA

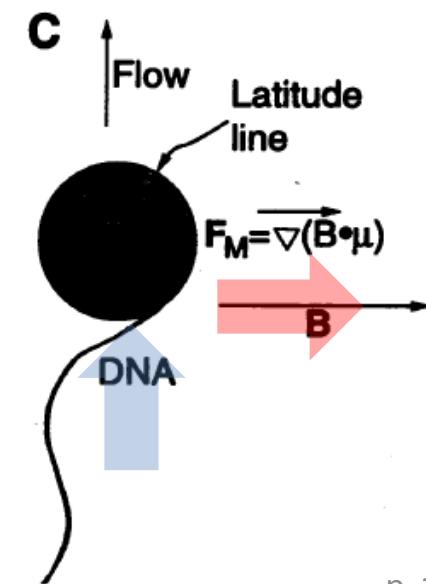
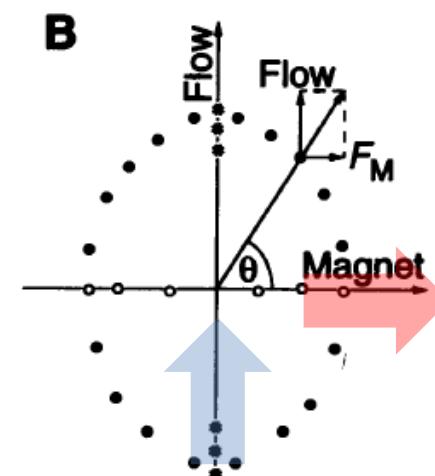
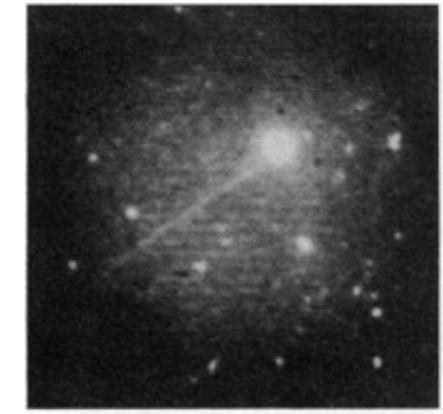


C. Bustamante lab

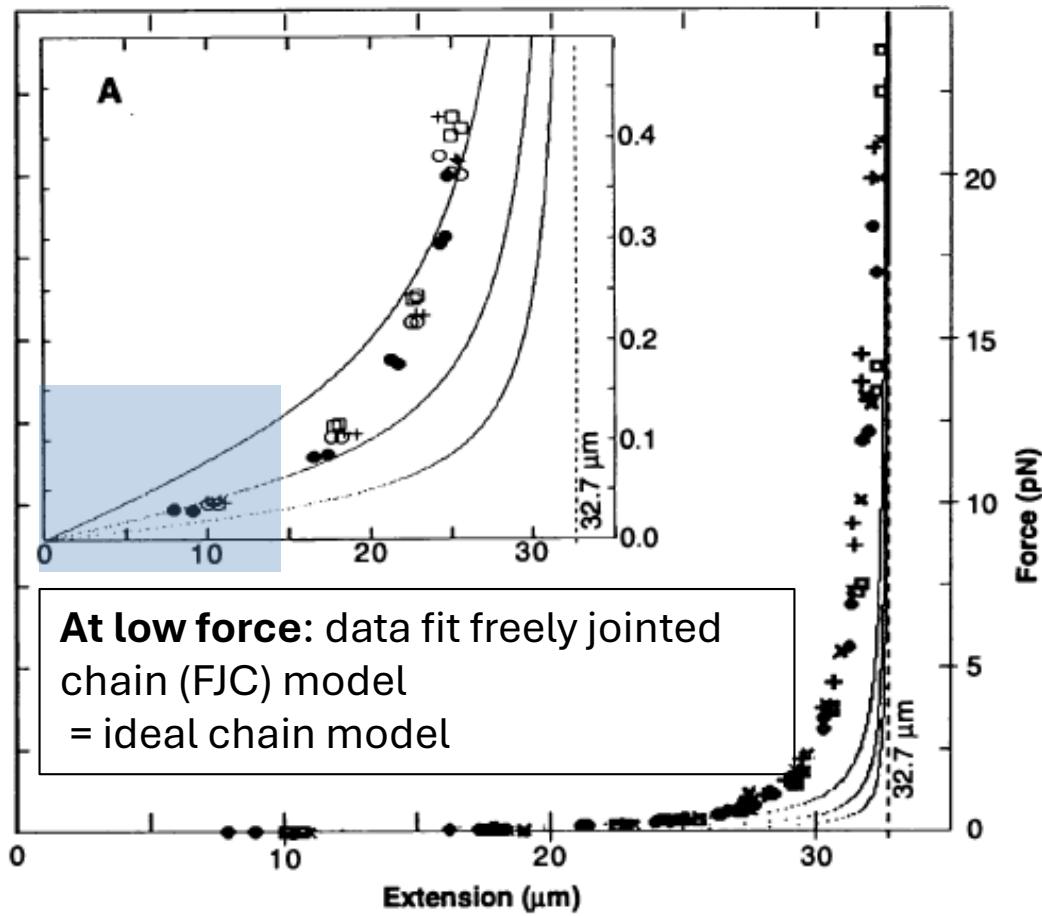
Experimental setup

97kb λ -DNA molecule attached to magnetic bead

Visualization of bead through microscope



Force Dependence of Extension



Behavior measured (in force extension plots):

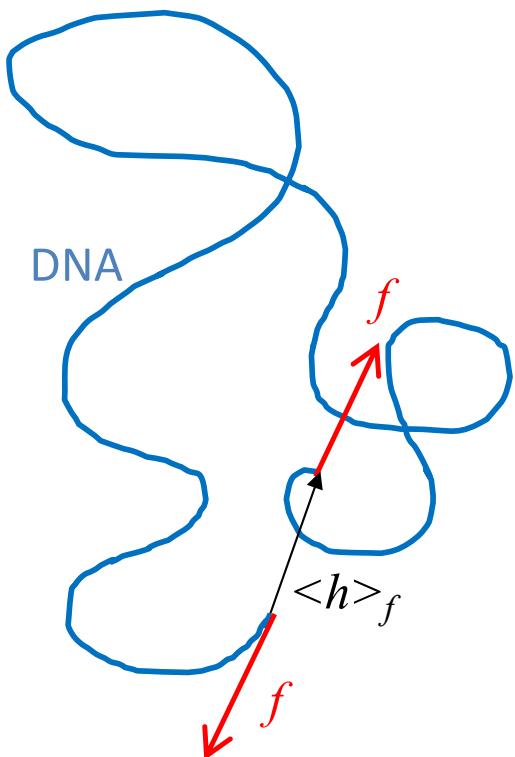
- λ -DNA (48.5 kbp) dimer (model system, easily accessible at the time)
- **Almost linear increase in length at low force**
- **Low force region:** DNA behaves like entropic spring, small changes in force result in large extensions. Elasticity due to entropy.
- Rollover point: rapid increase in force required to get further extension. **Elasticity is structural** rather than entropic.

Smith et al. 1992 Science

DNA Behavior under Tension

Unstructured polymers form a **random coil**

Work required to stretch the polymer : **entropic spring!**



Extension under force for an ideal chain:
Entropic spring following Hooke's law

$$\langle h \rangle_f \cong f \left(\frac{Nl^2}{kT} \right)$$

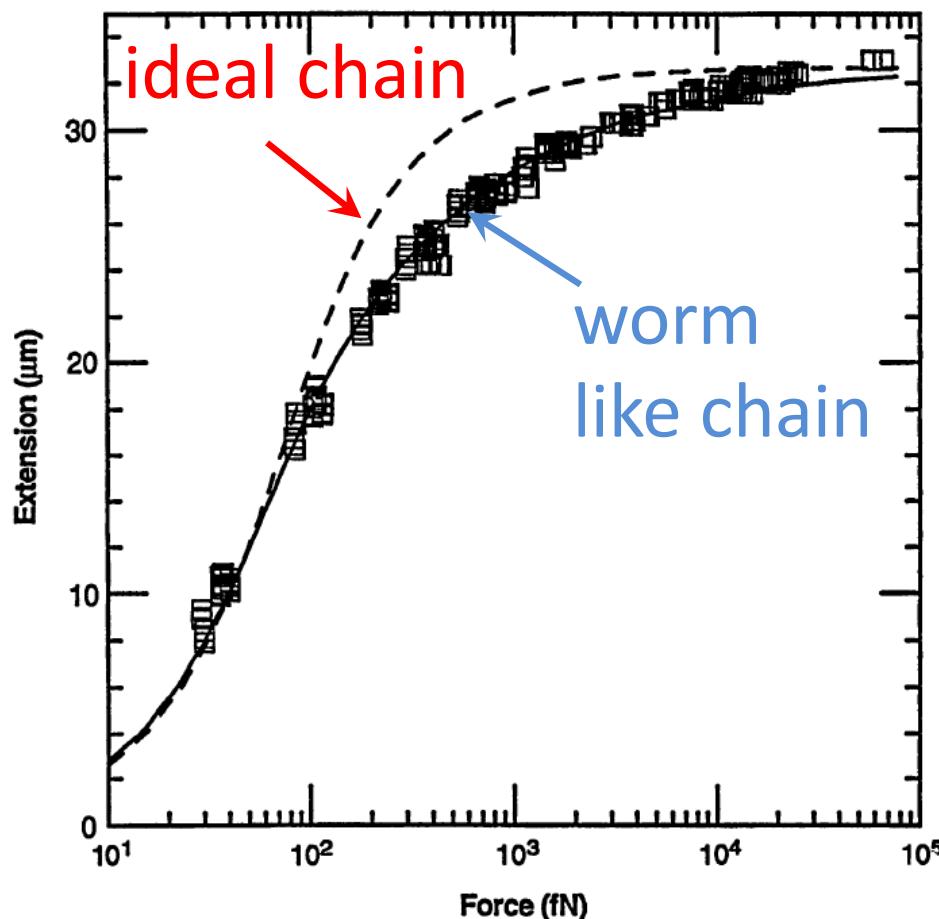
f : force
 $\langle h \rangle_f$: end-to-end distance
 N : Number of segments
 l : Segment length
 kT : Thermal energy

Extension under force for a stiff, wormlike chain:
(Bustamante et al. Science 1994)

$$f = \frac{kT}{L_p} \left[\frac{1}{4} \left(1 - \langle h \rangle_f f / L \right)^{-2} - \frac{1}{4} + \langle h \rangle_f / L \right]$$

L_p : persistence length \rightarrow stiffness of the chain
 L : contour length ($l * N$) \rightarrow length of the polymer

Interpreting DNA Stretching Data



97 kbp λ -DNA (48.5 kbp dimer)

Fit parameters to approximate formula for wormlike chains:

$$L_p = 53.4 \text{ nm}$$

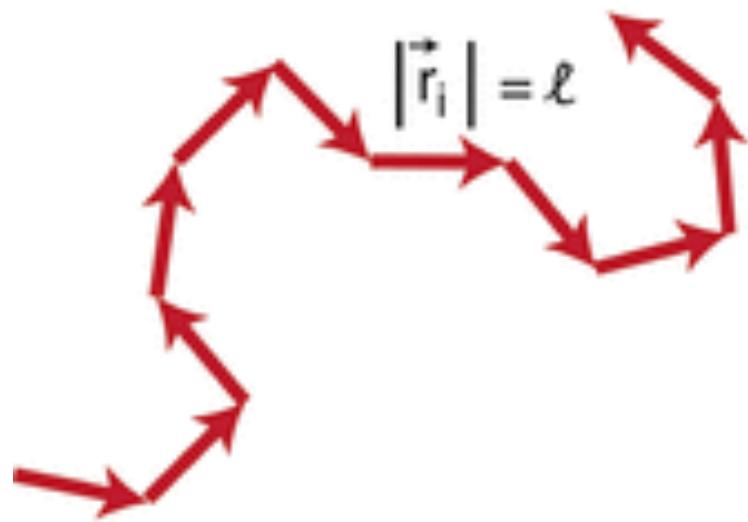
$$L = 33 \mu\text{m}$$

$$(\text{calculated: } 97000 * 0.34 \text{ nm} = 33 \mu\text{m})$$

Persistence length of DNA: 50 nm,
ca. 150 bp

Over length-scales above 100 nm it
behaves as a ideal chain (excluded
volume interactions are neglected)

Worm-like Chain Model

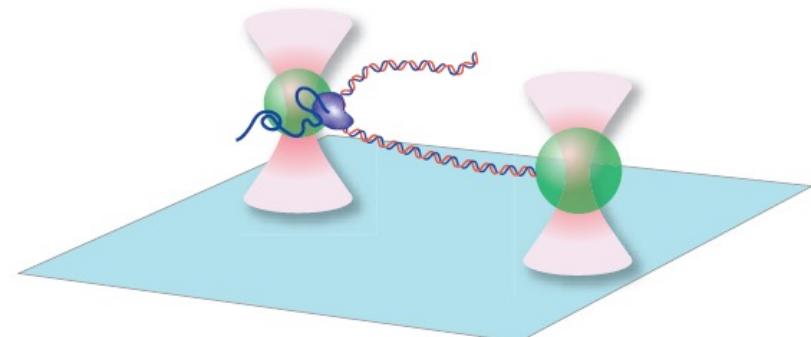


https://doi.org/10.1007/978-3-642-16712-6_502

Major Force Spectroscopy Methods

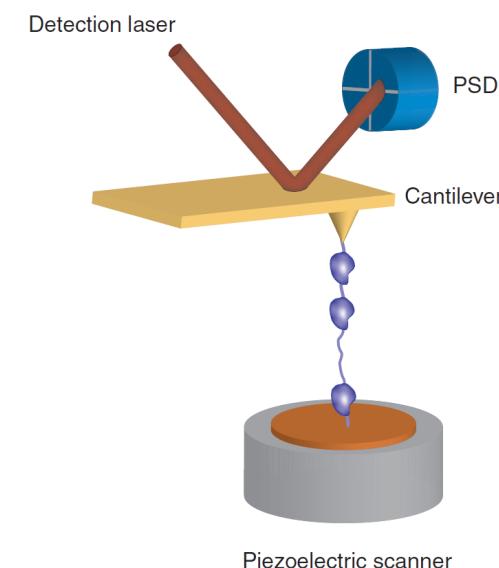
Optical Tweezers

Applying light to trap & manipulate molecules, and study the resulting effects on the system

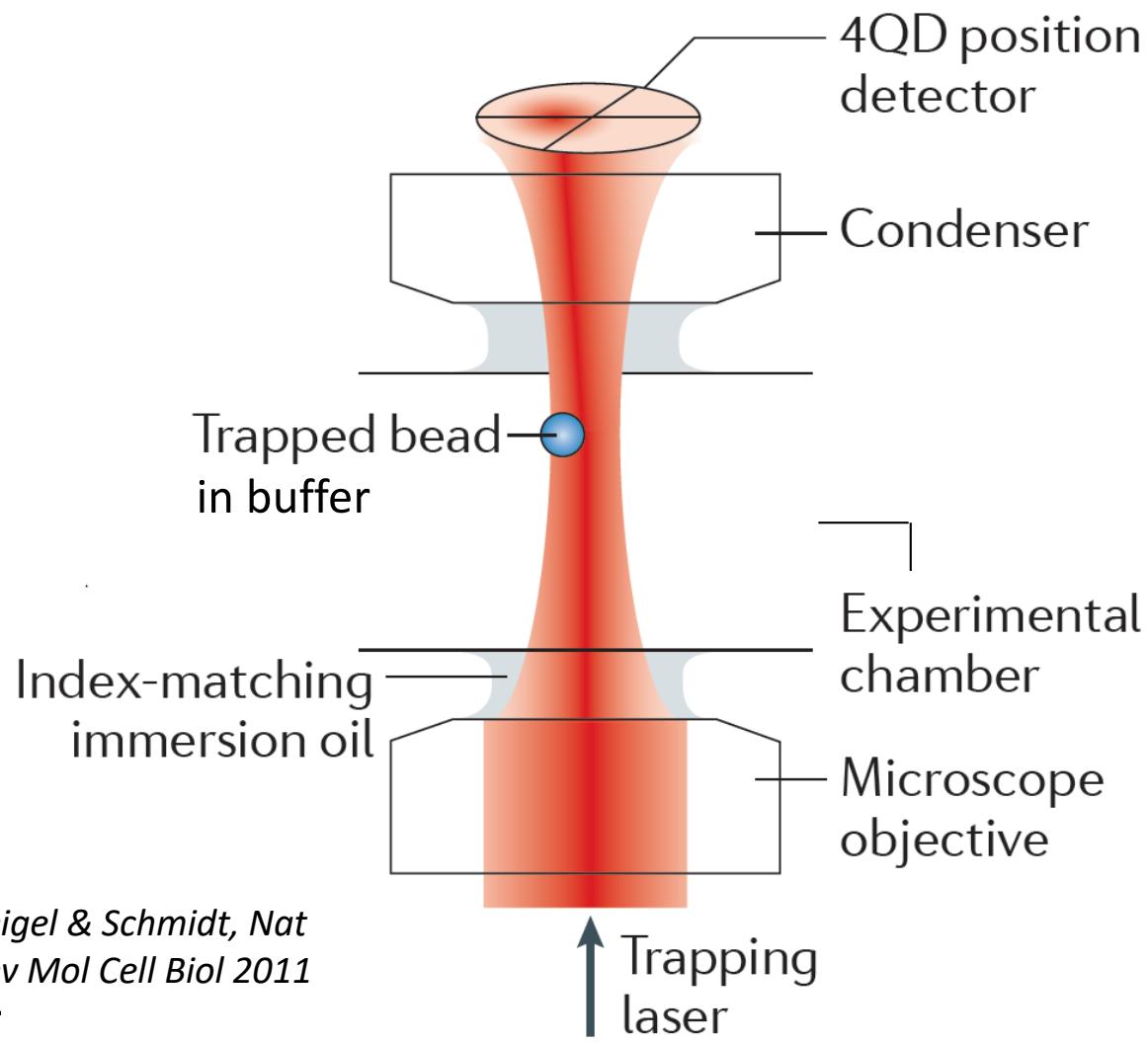


Atomic Force Microscope (AFM) / Molecular Force Probe (MFP)

A surface is scanned by an extremely fine tip, resulting forces are mapped for imaging and force measurements



Optical Tweezers



Veigel & Schmidt, Nat
Rev Mol Cell Biol 2011

3-Force spectroscopy

Detection:

Back-focal plane
interferometer with a 4
quadrant photodiode (4QD)

→ Allows measurements of
bead position with **nm-
resolution** and **μs-time**
resolution (up to 100 kHz)

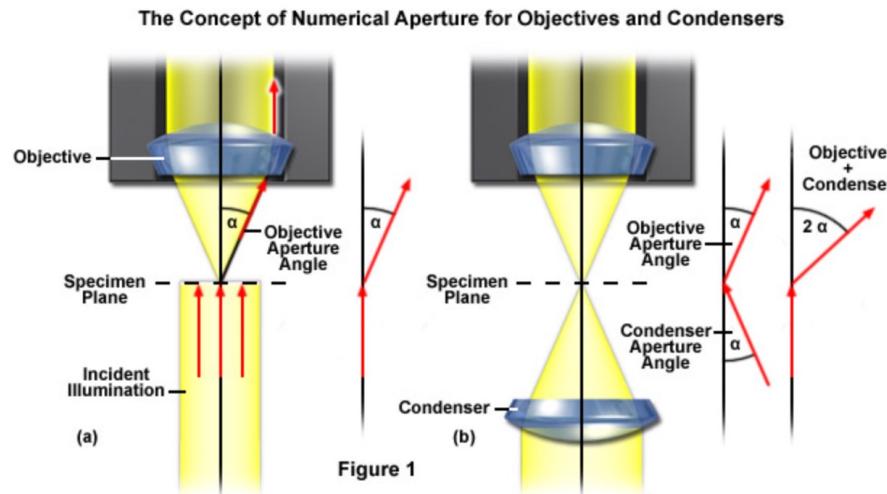
Objective:

High numerical aperture
(NA), water or oil immersion

Trap:

High energy laser
(IR 800-1000 nm, e.g. Nd:YAG)
Forces in excess of 100 pN on
particles from nm to mm

Numerical aperture



In order to enable two objectives to be compared and to obtain a quantitative handle on resolution, the numerical aperture, or the measure of the solid angle covered by an objective is defined as:

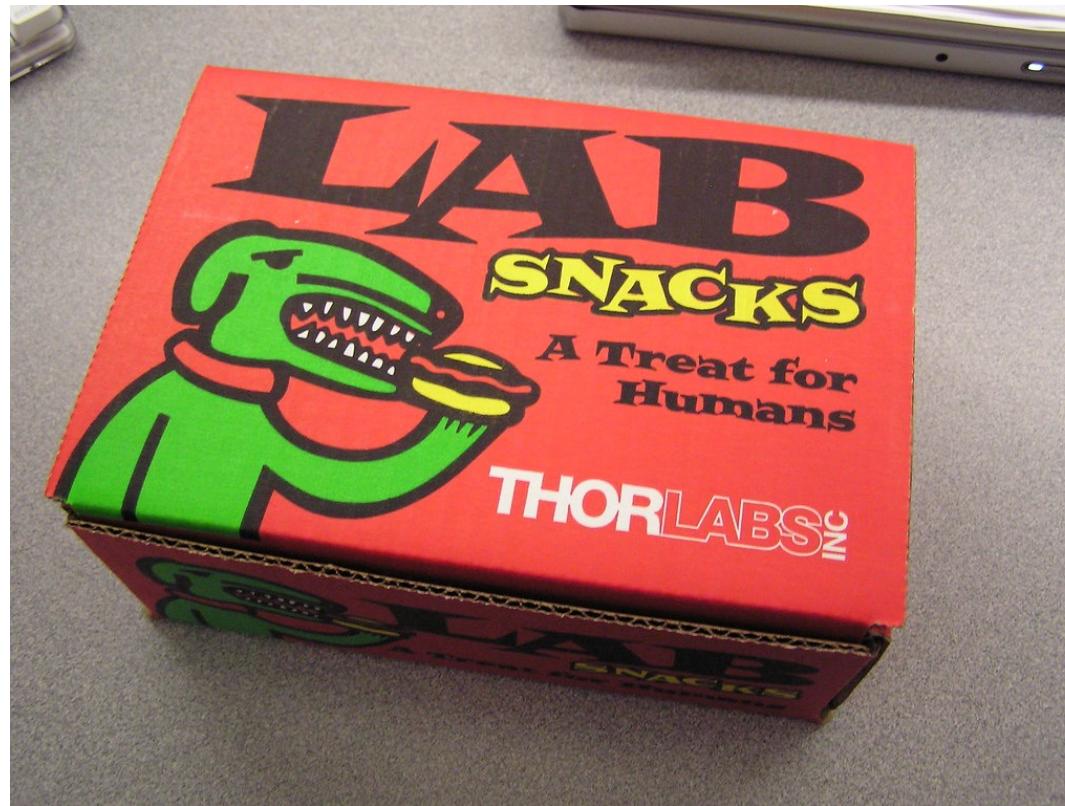
$$\text{Numerical Aperture (NA)} = \eta \cdot \sin(\alpha) \quad (1)$$

where α equals one-half of the objective's opening angle and η is the refractive index of the immersion medium used between the objective and the cover slip protecting the specimen ($\eta = 1$ for air; $\eta = 1.51$ for oil or glass). By examining **Equation (1)**, it is apparent that the refractive index is the limiting factor in achieving numerical apertures greater than 1.0. Therefore, in order to obtain higher working numerical apertures, the refractive index of the medium between the front lens of the objective and the specimen cover slip must be increased. The highest angular aperture obtainable with a standard microscope objective would theoretically be 180 degrees, resulting in a value of 90 degrees for the half-angle used in the numerical aperture equation. The sine of 90 degrees is equal to one, which suggests that numerical aperture is limited not only by the angular aperture, but also by the imaging medium refractive index. Practically, aperture angles exceeding 70 to 80 degrees are found only in the highest-performance objectives that typically cost thousands of dollars.

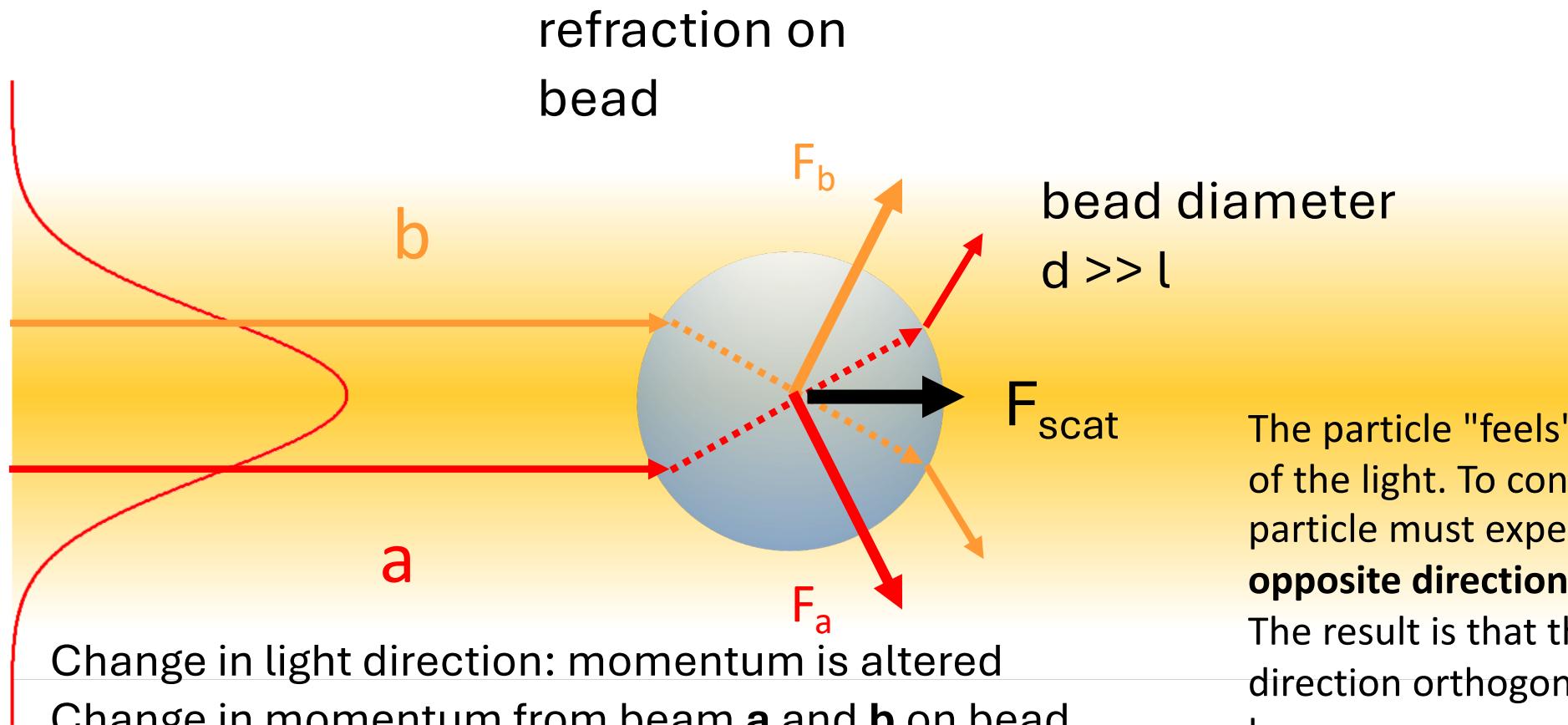
[https://zeiss-campus.magnet.fsu.edu/articles/basics/resolution.html#:~:text=The%20numerical%20aperture%20of%20a,in%20Figure%201\(a\).](https://zeiss-campus.magnet.fsu.edu/articles/basics/resolution.html#:~:text=The%20numerical%20aperture%20of%20a,in%20Figure%201(a).)

Thorlabs video

- [Https://www.youtube.com/watch?v=13VXGX2yR3k](https://www.youtube.com/watch?v=13VXGX2yR3k)



Using Light to Trap Objects



Change in light direction: momentum is altered

Change in momentum from beam **a** and **b** on bead

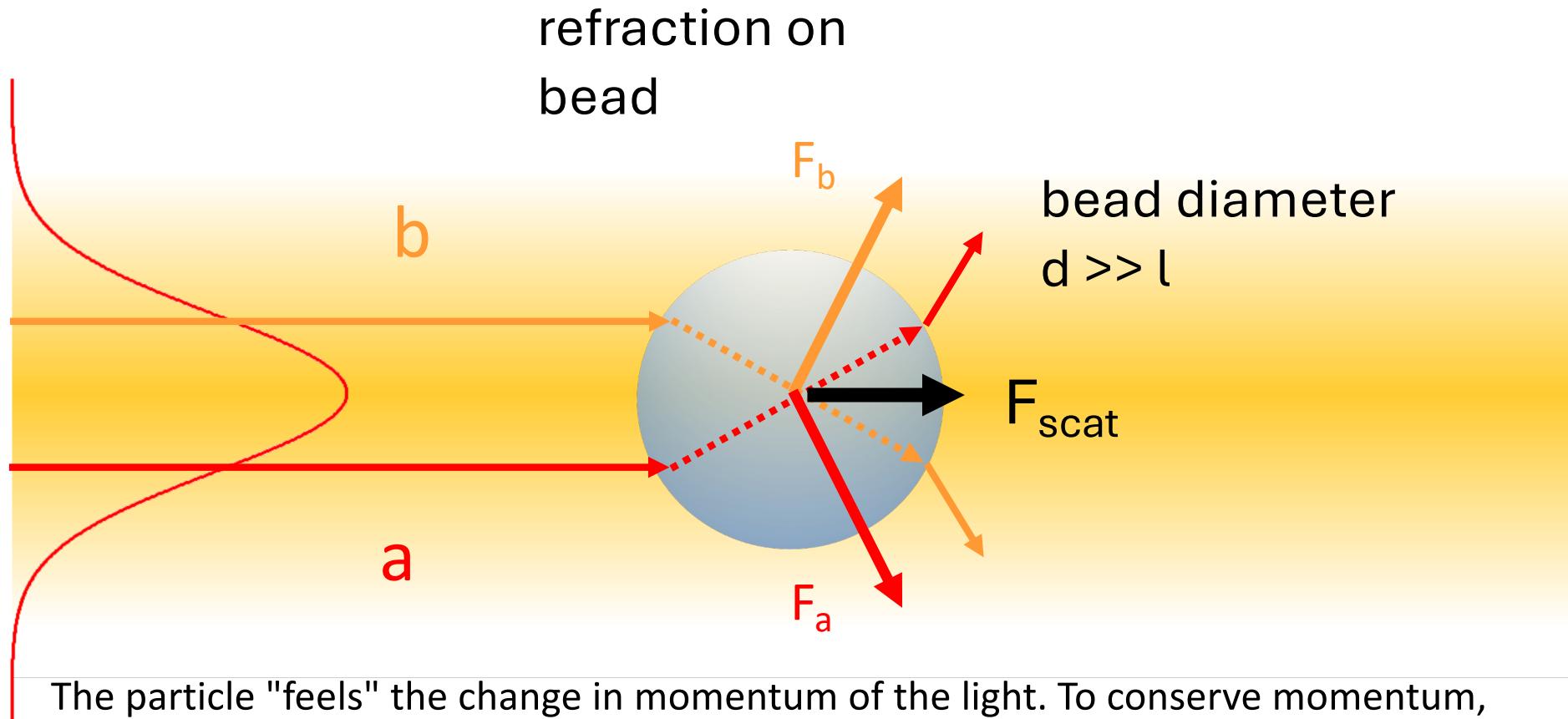
light intensity **a** and **b** equally strong

→ **no net force on bead perpendicular to beam**

→ **force in light direction**: scattering force F_{scat}

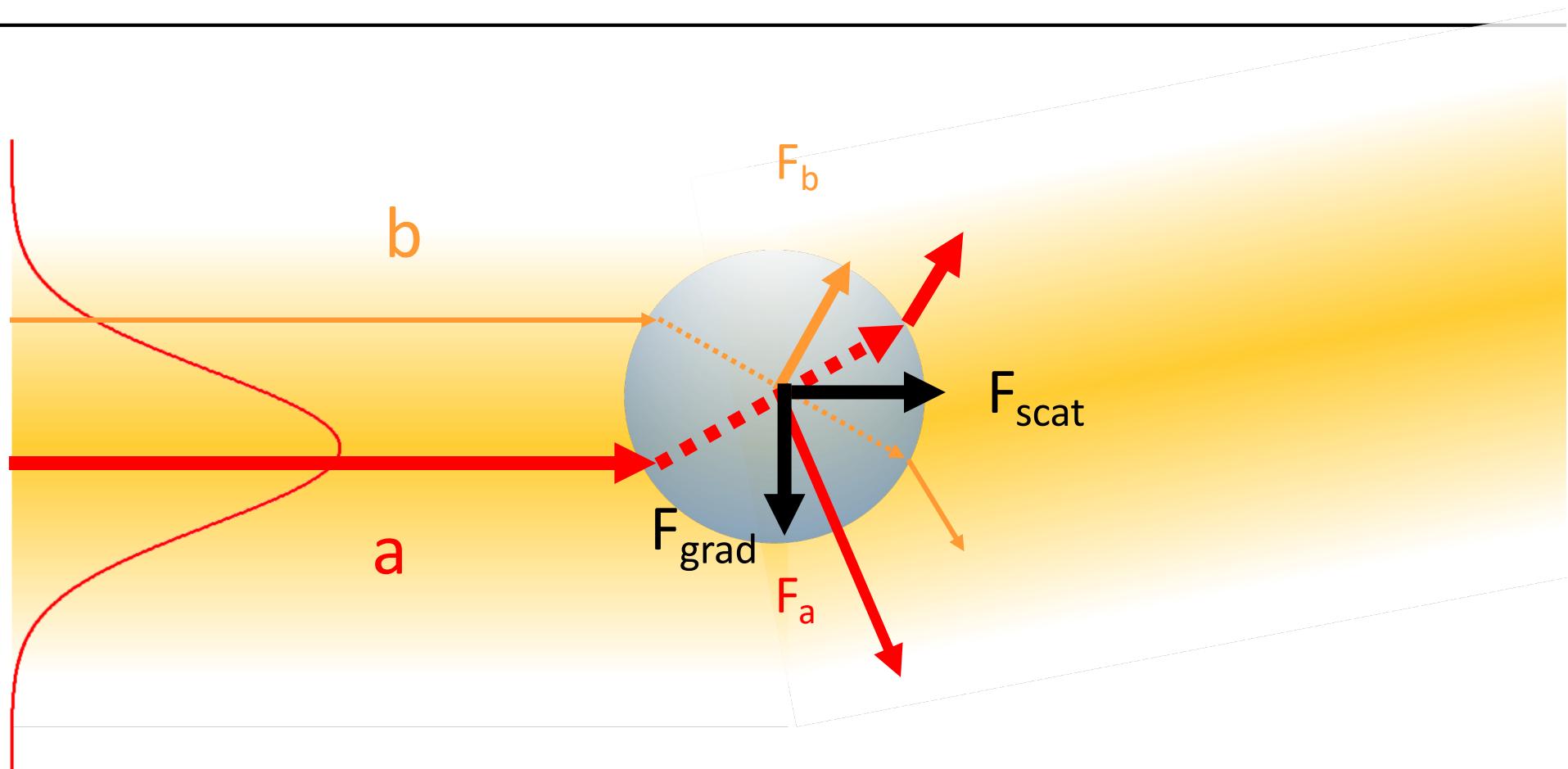
The particle "feels" the change in momentum of the light. To conserve momentum, the particle must experience a force in the **opposite direction** of the change in momentum. The result is that the scattering force is in the direction orthogonal to the direction of the beam.

Using Light to Trap Objects



The particle "feels" the change in momentum of the light. To conserve momentum, the particle must experience a force in the **opposite direction** of the momentum change. The result is that the particle is **pushed** in a direction orthogonal to the refracted light beam.

Using Light to Trap Objects



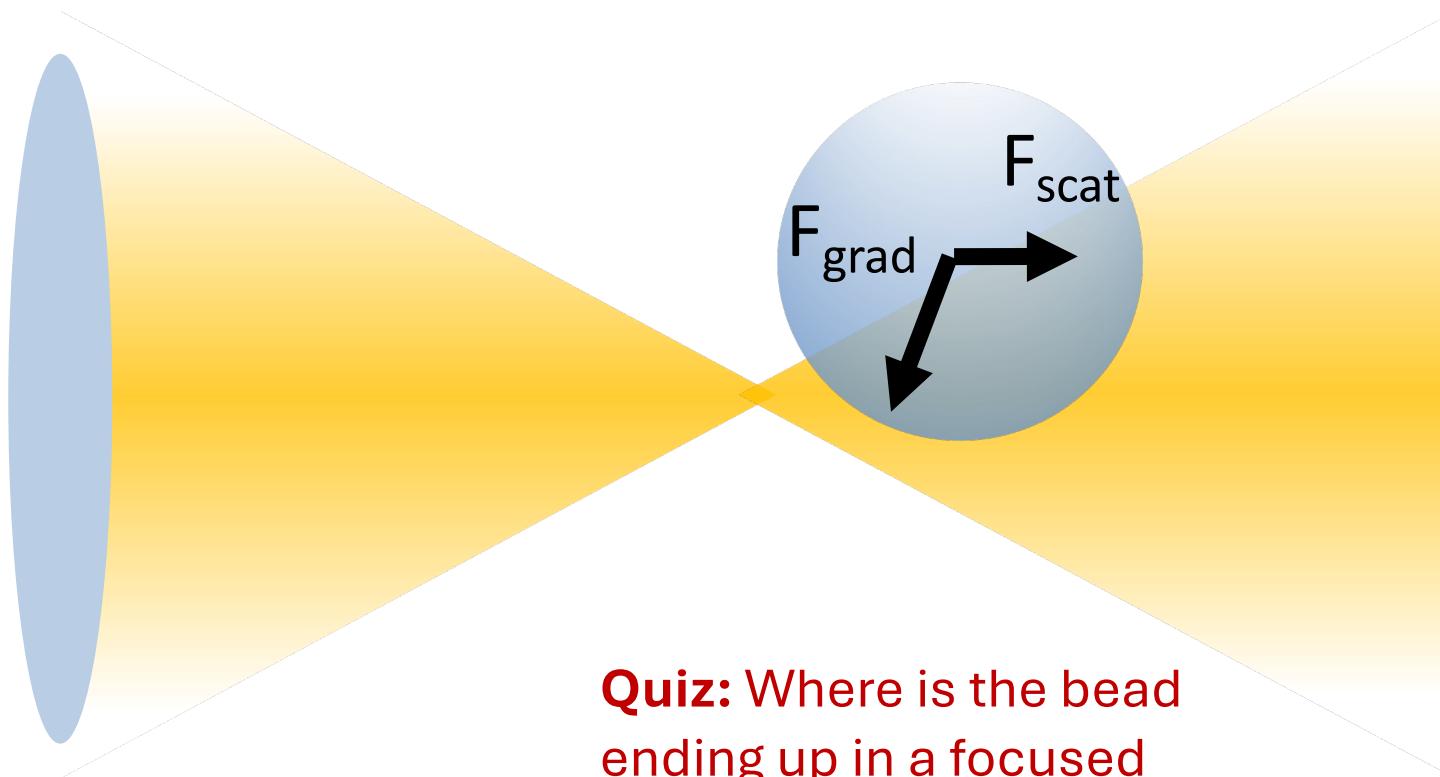
light intensity **a** stronger than **b**

→ **force on bead** perpendicular to beam in **direction of light gradient** F_{grad}

→ **force in light direction**: scattering force F_{scat}

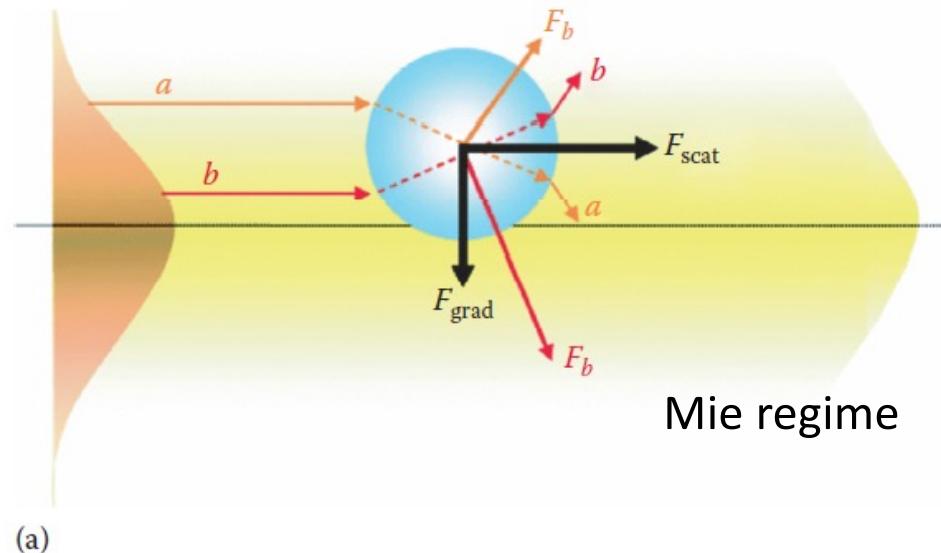
Using Light to Trap Objects

focused laser beam



Quiz: Where is the bead ending up in a focused laser beam?

Using Light to Trap Objects



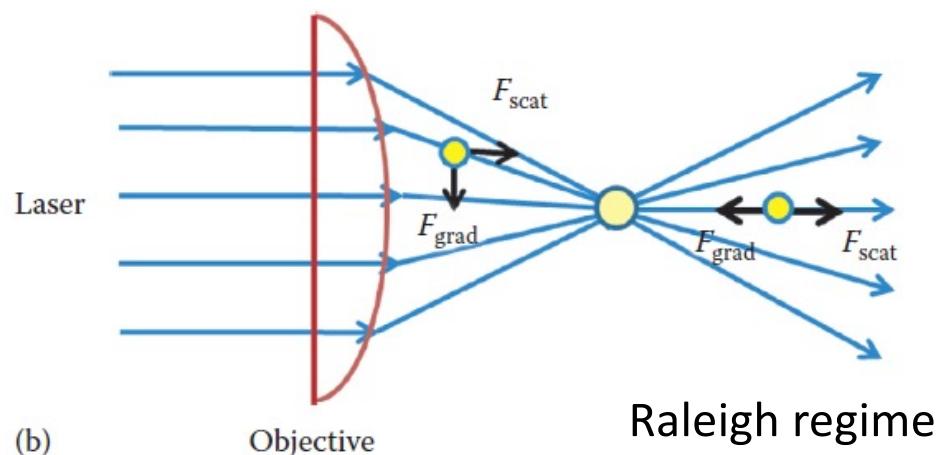
Particle with diameter d in laser beam with gaussian profile

Mie scattering regime ($d \gg \lambda$)

momentum transfer upon bead due to light refraction

Gradient force (Raleigh regime, $d \ll \lambda$)

Dielectric particle (approx. as a point-dipole) \rightarrow Induced dipole \rightarrow Force along optical field gradient



Scattering force (Raleigh regime, $d \ll \lambda$)

Light scattering induces force along light propagation direction

Equilibrium trapping position: just behind focal point of the system

Wait, a photon has momentum?

A photon has no mass, how can it have momentum?

https://www.youtube.com/watch?v=V_fKYrrsVT4

Short answer:

Energy-momentum relation: Photons carry energy, and according to special relativity, energy and momentum are related. Even without mass, the photon's energy results in momentum, consistent with the equation

$$p = \frac{E}{c}$$

Where p is momentum, E is energy, and c is the speed of light.

Why does sunlight not push us up a hill?



Light carries momentum

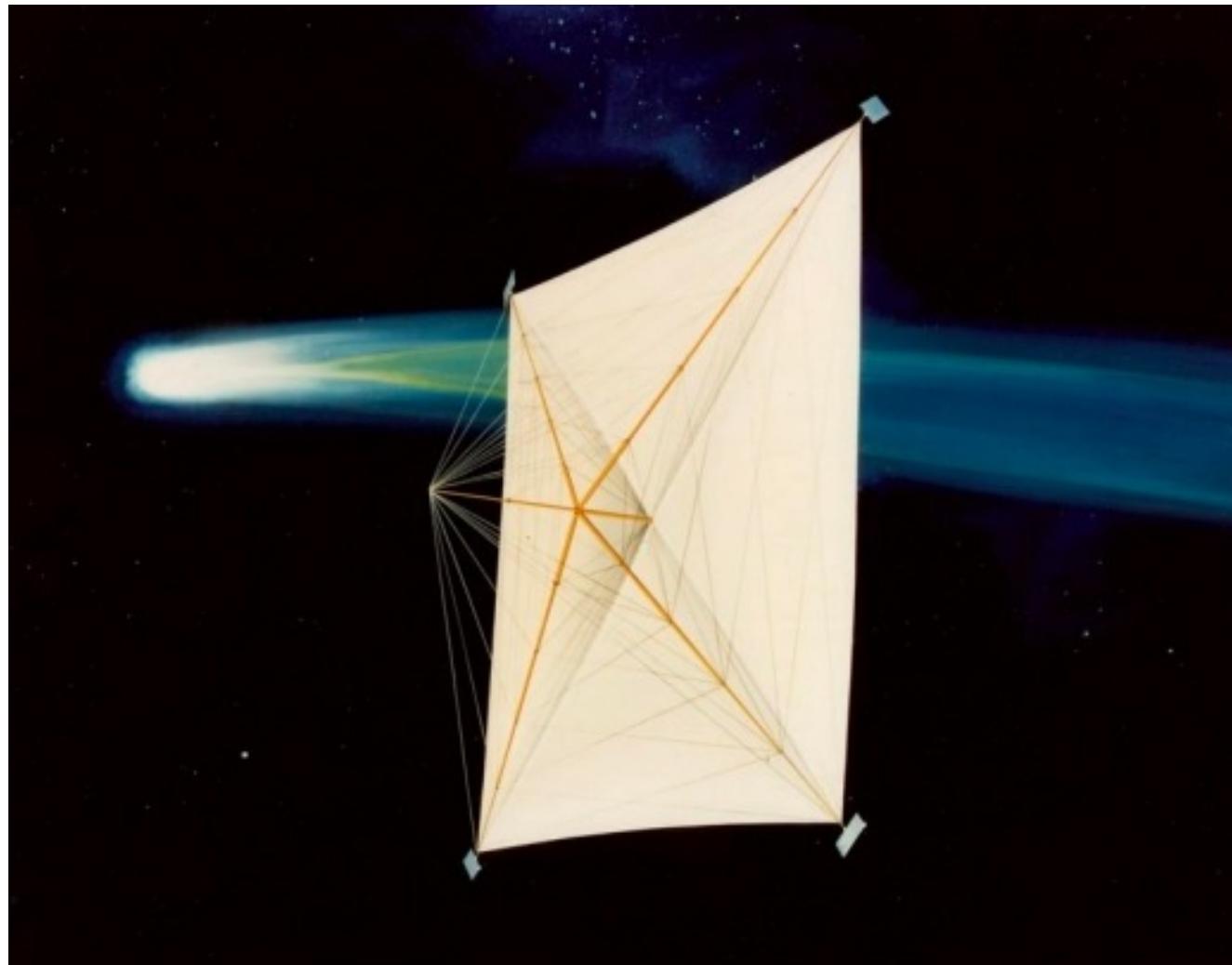


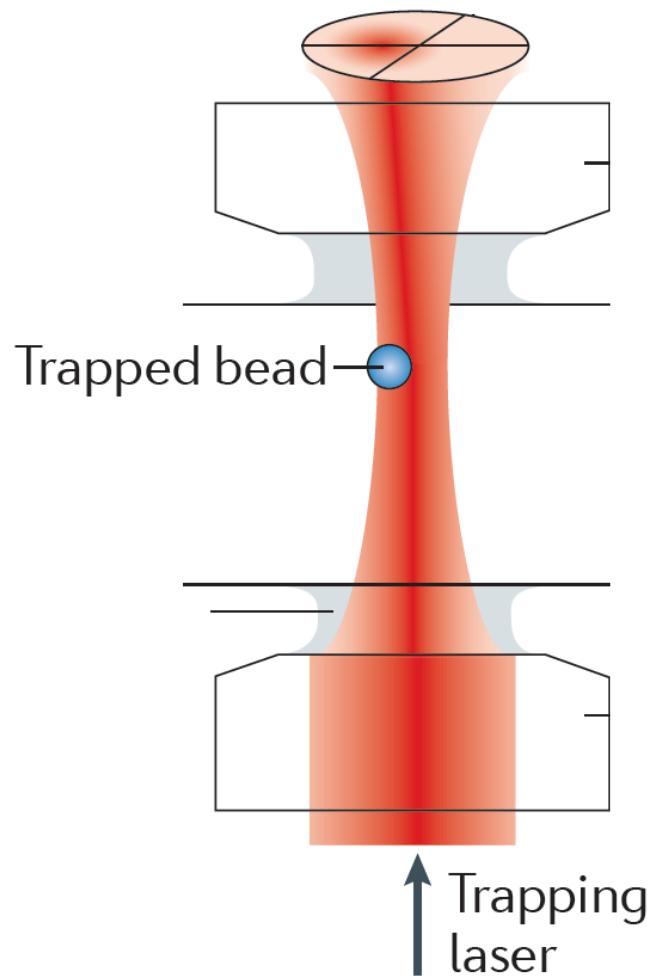
Image: This artist's concept shows an 850-by-850-meter wide solar sail spacecraft approaching Halley's Comet. Credit: JPL-Caltech.

Activity: play with a simulated optical trap

- <https://phet.colorado.edu/sims/cheerpj/optical-tweezers/latest/optical-tweezers.html?simulation=optical-tweezers>

Goal: figure out what you can do to remove the bead from the trapped state

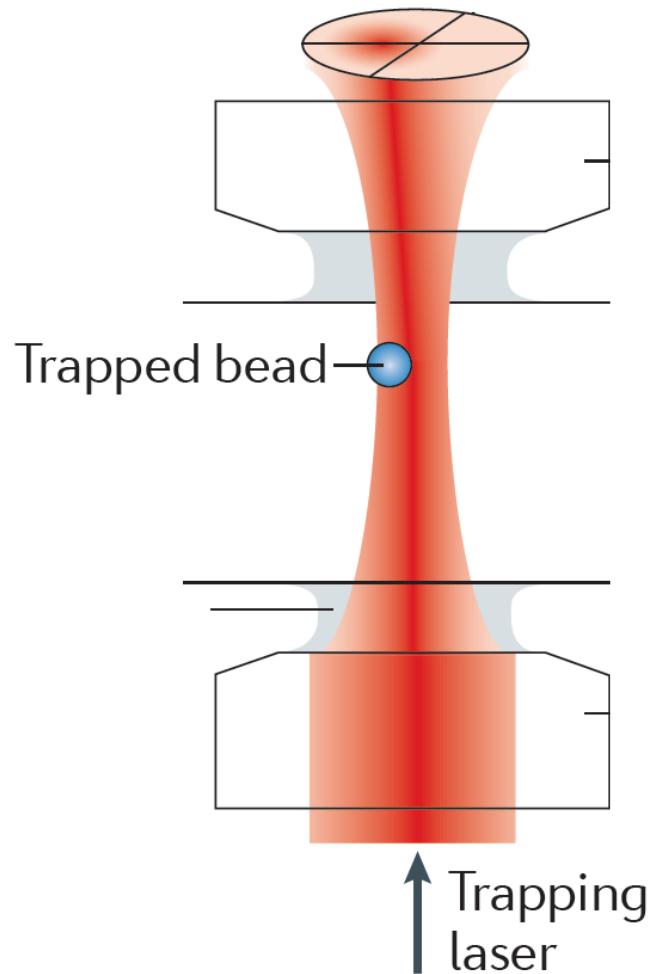
Movement of the trap



Two possibilities to move the trap:

- Movement of laser beam with piezoelectric actuated mirrors
 - Fast, but limited range (micrometers)
- Movement of the microscopy stage (i.e. sample holder) using piezoelectric actuators

Trap Stiffness in Optical Traps

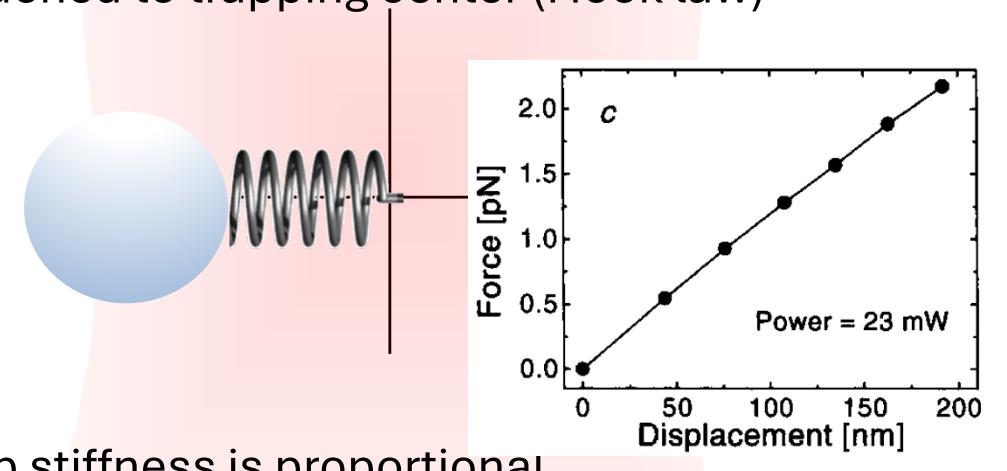


Trap stiffness:

The change in force when the particle (bead) is moved away from the laser beam center
(strong/stiff trap: force increase is steep, weak trap: force stays the same)

For small displacements

Forces acting on bead similar to spring attached to trapping center (Hook law)



Trap stiffness is proportional to **laser power**

Pulling DNA with optical tweezers

A **10 kb DNA molecule** labelled with digoxigenin and biotin is tethered between Streptavidin and anti-DIG coated microspheres held in double-trap optical tweezers.

The DNA is fluorescently labeled using SYBR Gold.

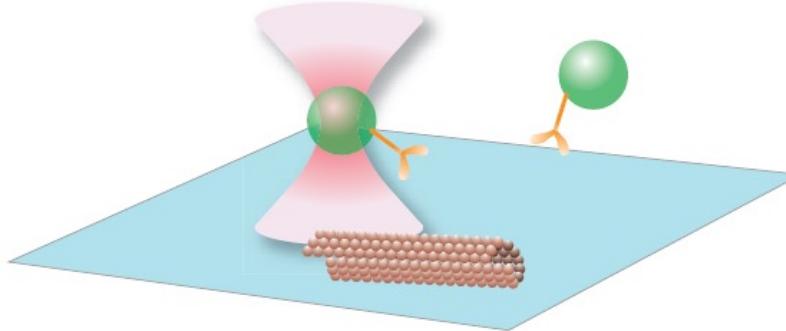


Anders Wallin et al., 2009,
University of Helsinki.

Optical Tweezers Configurations

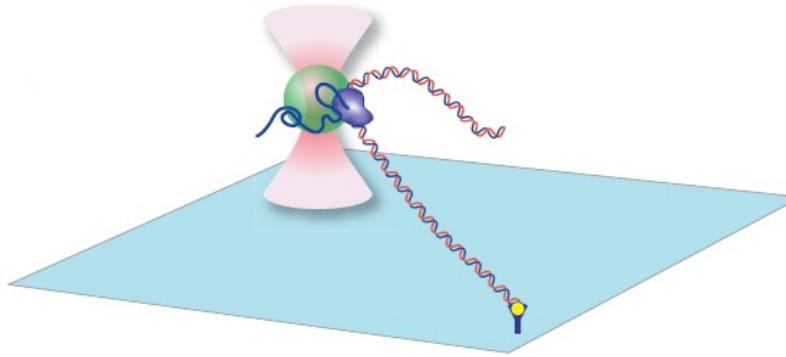
Interaction assay:

E.g. Motor protein coupled beads to immobilized MT



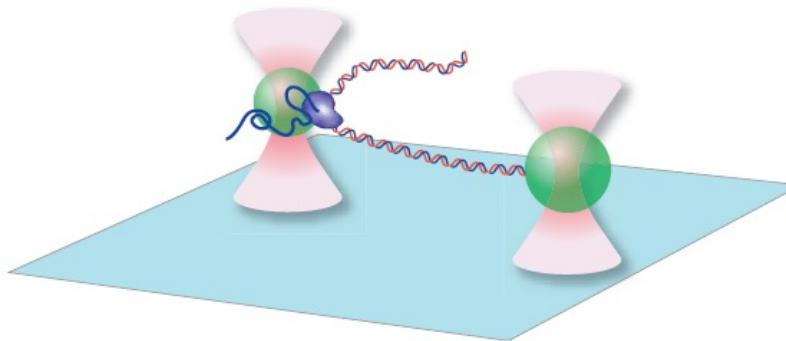
Tethered assay:

E.g. Precise measurements of protein – DNA contacts



Dumbbell assay:

E.g. Polymerase tracking



Limitations of Optical Tweezers

- Optical disturbances degrade trap:
 - Homogenous preparations are required
- No selectivity / exclusivity: any dielectric particle will be trapped → low concentrations are required
- Impurities are trapped (cell extracts etc.)
- Local heating by the laser $10^9\text{-}10^{12}\text{ Wcm}^{-1}$: changes enzyme activity, viscosity, convection streams possible
- Optical damage: minimized by IR laser, but oxygen radicals can still damage (requires scavenger system)
- Range of forces: 0.1-100 pN
- Range of distances with fixed trap: 400 nm

Case study 1: Please watch video on your own time

nature

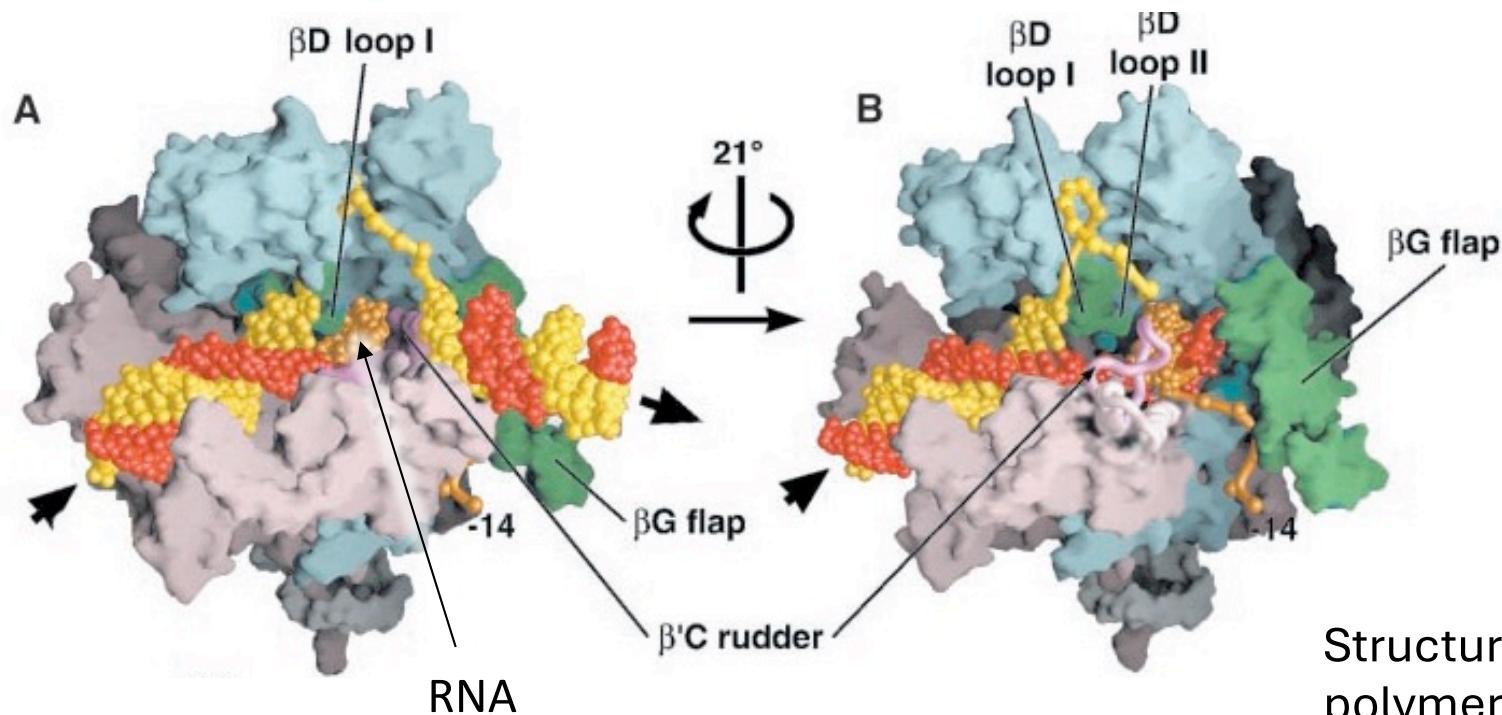
Vol 438 | 24 November 2005 | doi:10.1038/nature04268

ARTICLES

Direct observation of base-pair stepping by RNA polymerase

Elio A. Abbondanzieri^{1*}, William J. Greenleaf^{1*}, Joshua W. Shaevitz^{2†}, Robert Landick⁴ & Steven M. Block^{1,3}

RNA Polymerase DNA:RNA Complex

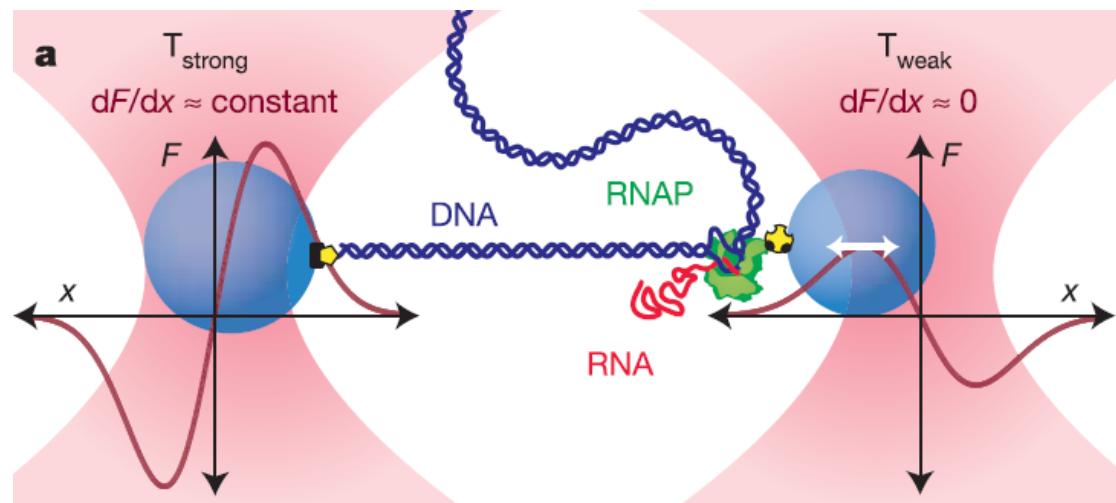


Structure of a bacterial polymerase
Korzheva et al., Science 2000

Taq core RNA Polymerase: $\alpha_2\beta\beta'$, 400 kDa

RNA:DNA hybrid (unstable) highly stabilized by protein

Single Molecule Experiments: Dumbbell configuration



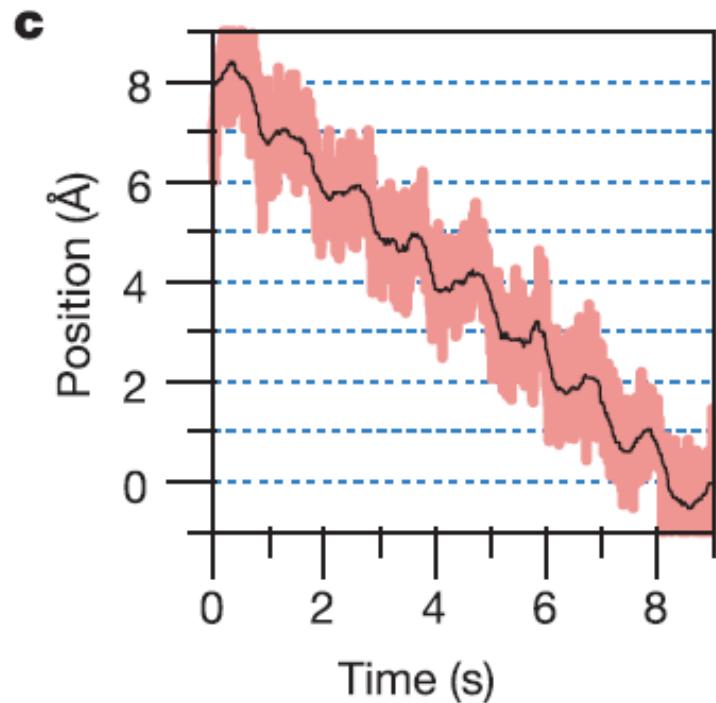
Strong trap: Controls position

Weak trap: bead kept at flat part of force potential \rightarrow constant force

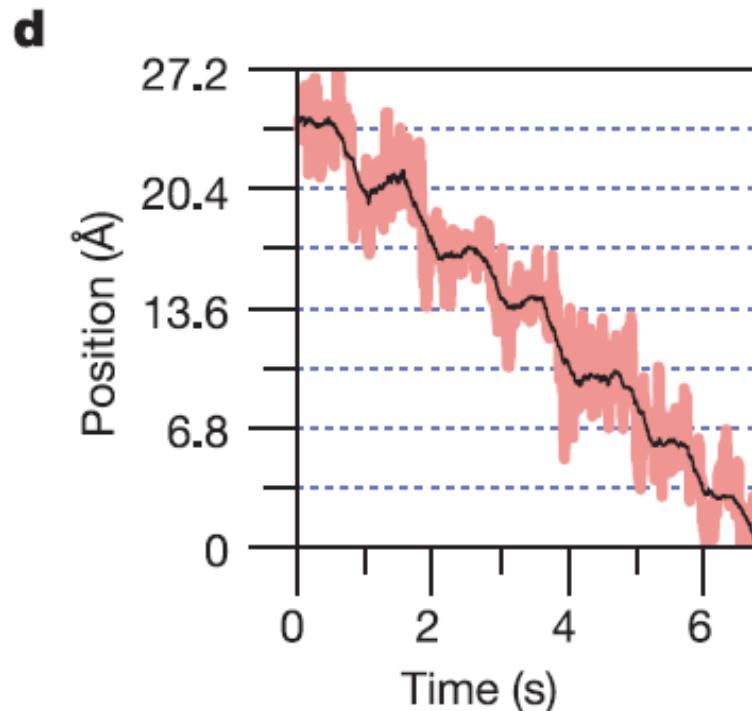
Dual-optical traps allow Å-level resolution of transcription elongation

RNAp linked to bead at β' subunit

Resolution achieved

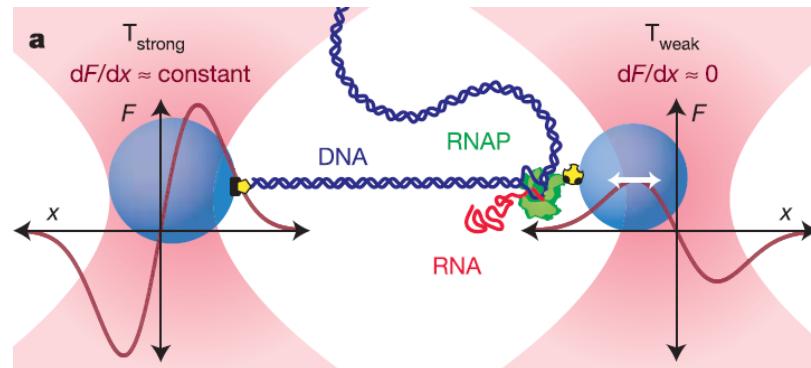


Bead moved in 1 \AA increments
using an acousto-optical deflector



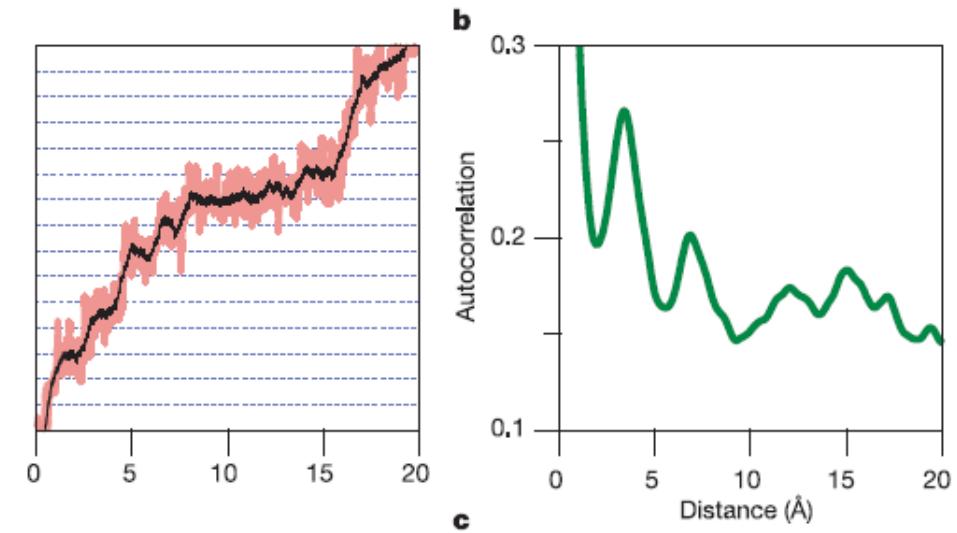
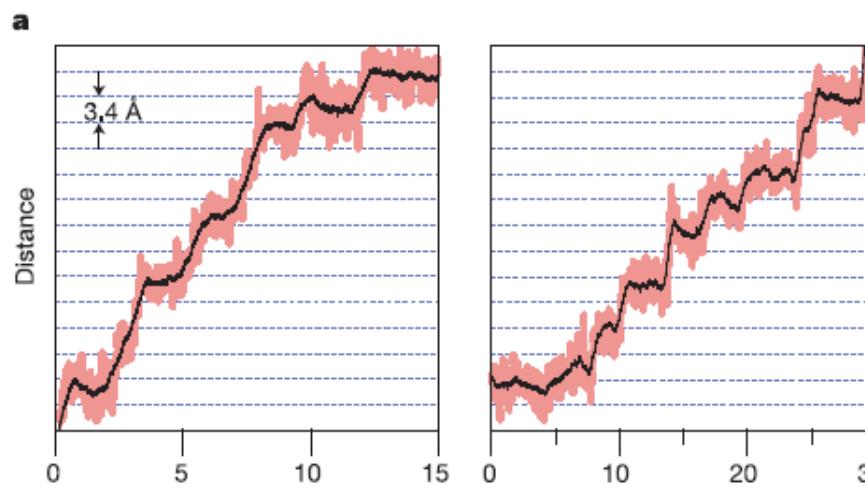
movement of T-strong in 3.4 \AA
increments at 1 Hz and measuring the
displacement in T-weak

Measuring RNA polymerase

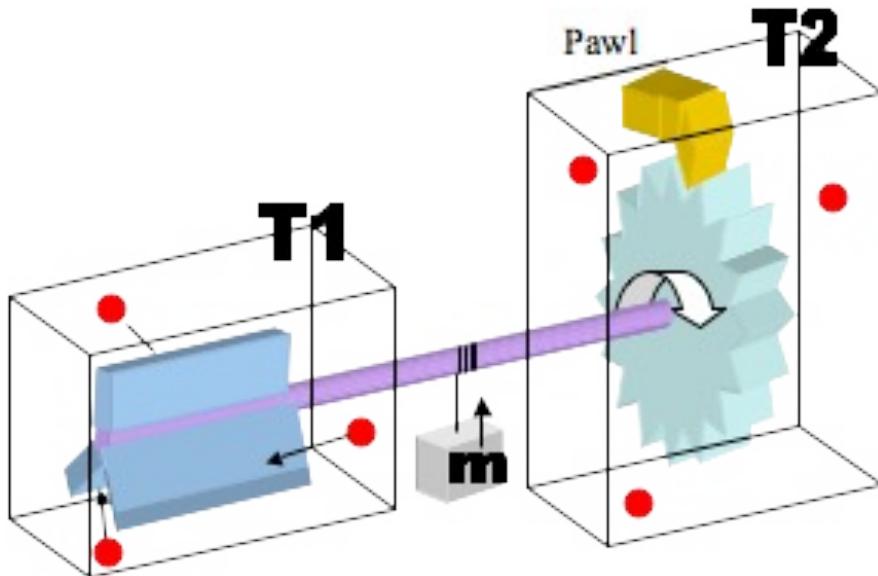


addition of dNTPs:
steps of 3.4 Å (corresponding to
DNA basepair) are observed
RNA pol pausing observed

Quiz: From where is the energy that
moves the motor? Why are there
pauses?



Power stroke or brownian ratchet



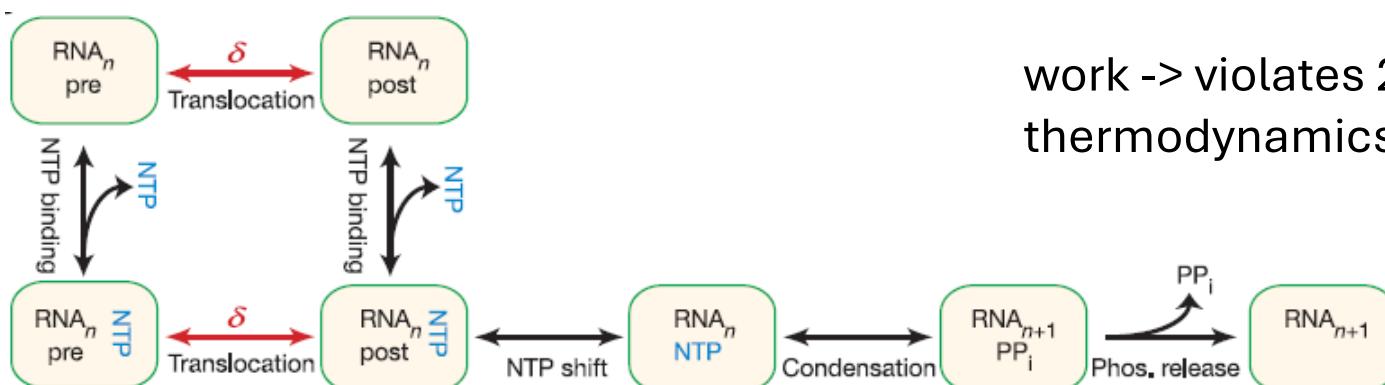
Brownian Ratchet

Original concept:

Temperature reservoirs T1 & T2
at same temperature

brownian motion rotates paddle,
pawl forces rotation in one
direction only

work \rightarrow violates 2nd law of
thermodynamics

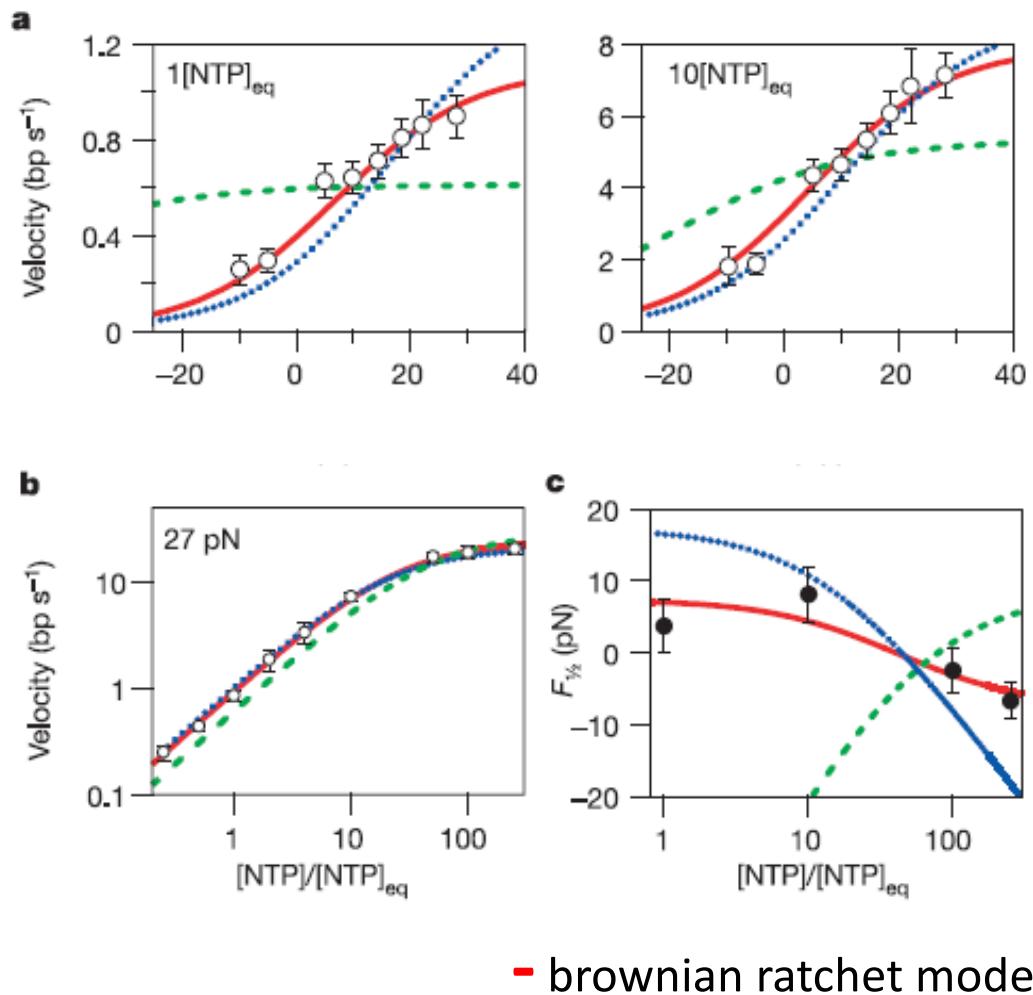


Force – Velocity relationship in mechanoenzymes

$$v(F) = \frac{v_{\max}}{1 + \exp\left[-\frac{(F-F_{1/2})\delta}{k_B T}\right]}$$

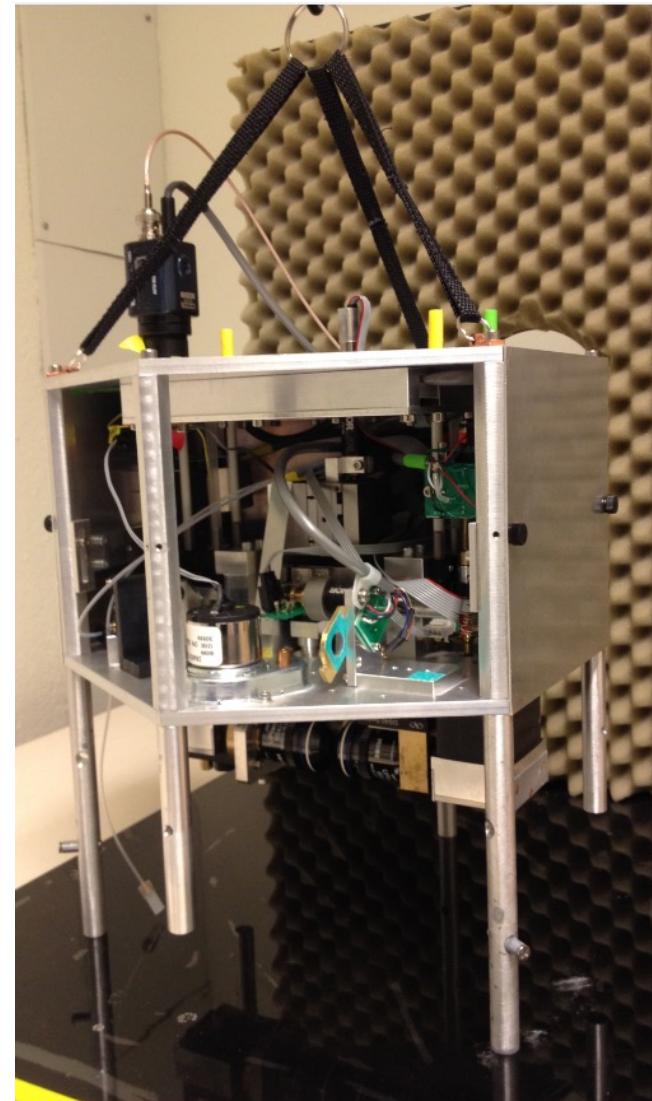
Boltzmann term:

- v_{\max} : velocity at large assisting load
- $F_{1/2}$: force at half maximal velocity
- δ : effective distance of force, e.g. power-stroke distance or translocation distance



Conclusions

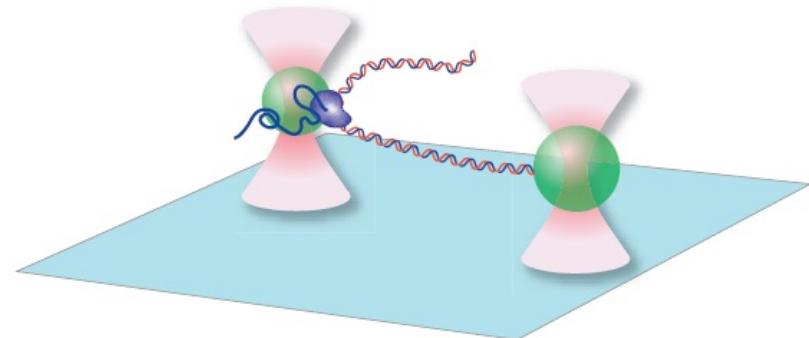
- Dumb-bell geometry of optical traps under helium results in very low noise
- Å sized motions are measured
- RNA polymerase performs 3.4 Å steps
- works as a **brownian ratchet** with a secondary NTP binding site



Major Force Spectroscopy Methods

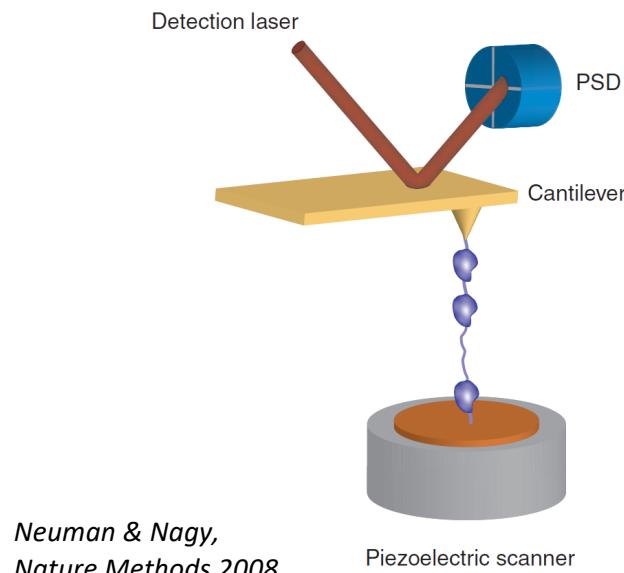
Optical Tweezers

Applying light to trap & manipulate molecules, and study the resulting effects on the system



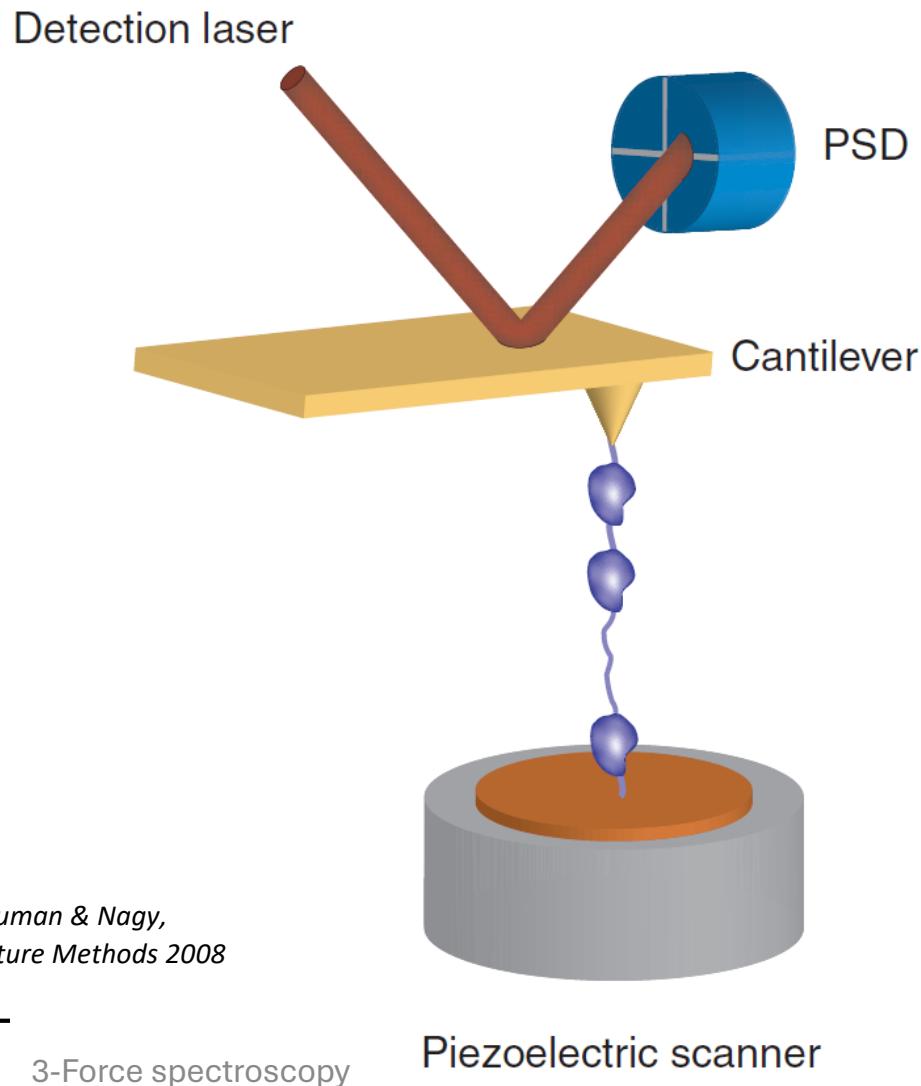
Atomic Force Microscope (AFM) / Molecular Force Probe (MFP)

A surface is scanned by an extremely fine tip, resulting forces are mapped for imaging and force measurements



*Neuman & Nagy,
Nature Methods 2008*

Atomic force microscopy (AFM)



Setup of an atomic force microscope

A cantilever with a nanometer tip is scanned over the sample using a piezo-scanner. A detection laser is reflected on the cantilever and detects movements using a quadrant photodiode (PSD).

Uses:

- AFM is a version of a scanning probe microscope → imaging
- Mapping of a surface with sub-nanometer resolution
- Imaging and manipulating individual molecules
- Ability to conduct experiments under near-physiological conditions (solution)

Atomic force microscopy: analogy to Braille

Braille is a tactile writing system used by people who are visually impaired.

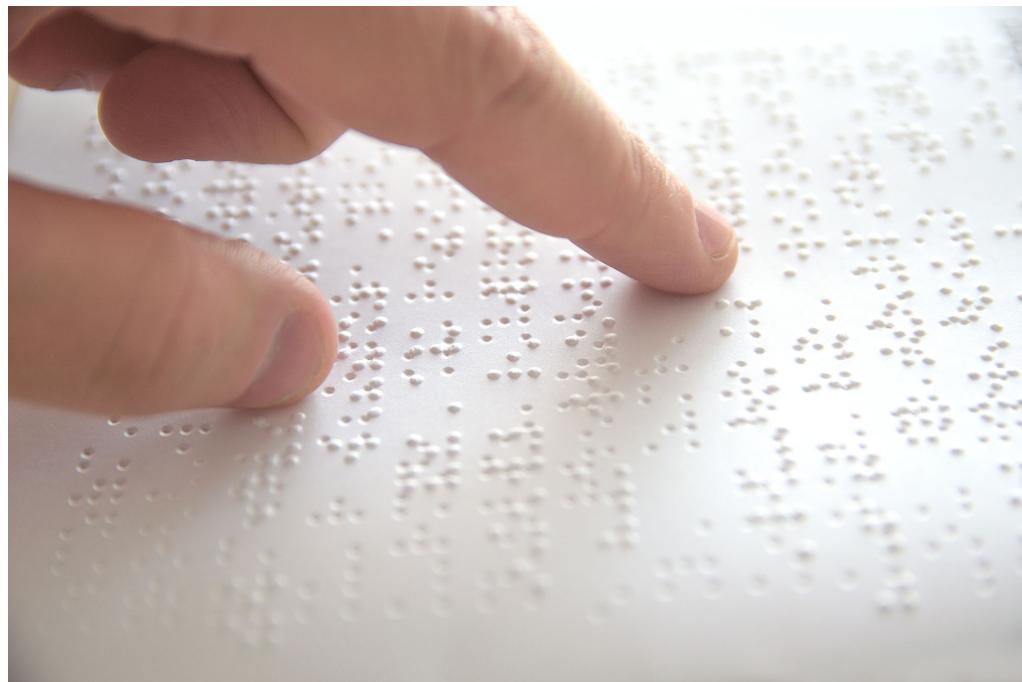


Image: <https://lhbblind.org/the-importance-of-braille-literacy/>

1. **Tactile Sensing:** AFM also uses a physical probe (the cantilever with a sharp tip) to 'feel' the surface of a sample
2. **Spatial Resolution:** In Braille, information (letters and numbers) is conveyed through patterns of raised dots within a small area. Similarly, AFM achieves high spatial resolution by detecting minute variations in height and texture at the nanoscale.
3. **Interpretation of Signals:** As a finger moves over Braille, the brain interprets the patterns into meaningful information. In AFM, the movements and deflections of the cantilever as it interacts with the sample surface are translated into a topographical map of the surface.
4. **Direct Interaction:** Both Braille reading and AFM imaging rely on direct physical interaction – the finger with the Braille dots, and the AFM tip with the sample surface.
5. **Limitation in Depth Perception:** Just as Braille does not provide information about the color or visual appearance of letters, AFM primarily provides surface topography and lacks the depth perception or internal structure imaging provided by some other forms of microscopy.

Piezoelectric scanner

A piezoelectric scanner is a critical component in an Atomic Force Microscope (AFM) and plays a central role in the precise manipulation and imaging of samples at the nanoscale. It operates based on **the piezoelectric effect, which is the ability of certain materials to generate an electric charge in response to applied mechanical stress.**

Conversely, these materials can change shape or size when an electric field is applied to them. In the context of a piezoelectric scanner in an AFM:

1. **Function:** The piezoelectric scanner moves the sample or the cantilever in very precise, small increments. This movement can be in the x, y, and z directions, allowing for detailed scanning of a sample's surface or for precise positioning of the cantilever for force measurements.
2. **Precision and Control:** Piezoelectric materials can produce extremely fine movements, often in the order of nanometers or even picometers. This makes them ideal for the high-resolution requirements of AFM.
3. **Applications in AFM:**
 1. **In Imaging Mode:** The scanner moves the sample in a raster pattern beneath the stationary cantilever. As the sample moves, the cantilever tip scans across the surface, allowing for the collection of topographical data.
 2. **In Force Spectroscopy Mode:** The scanner can precisely control the position of the cantilever relative to the sample, facilitating accurate measurements of the forces between the cantilever tip and the sample.
4. **Advantages:** The use of a piezoelectric scanner in AFM provides a high level of control over the position and movement of the sample or cantilever. This is essential for the kind of detailed, nanoscale analysis that AFM is capable of.
5. **Limitations:** While piezoelectric scanners offer high precision, their range of movement is limited. This limitation is generally not a problem for AFM, given the small scale at which it operates.

Two imaging modes

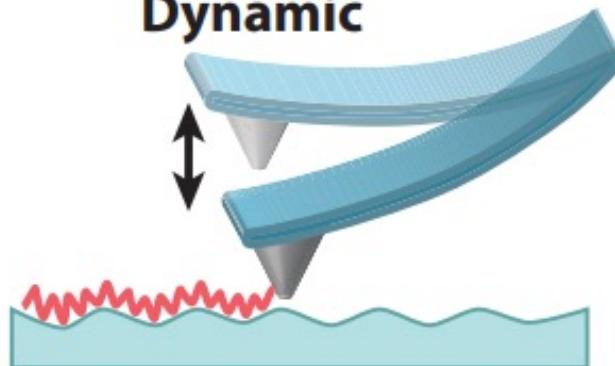
Contact



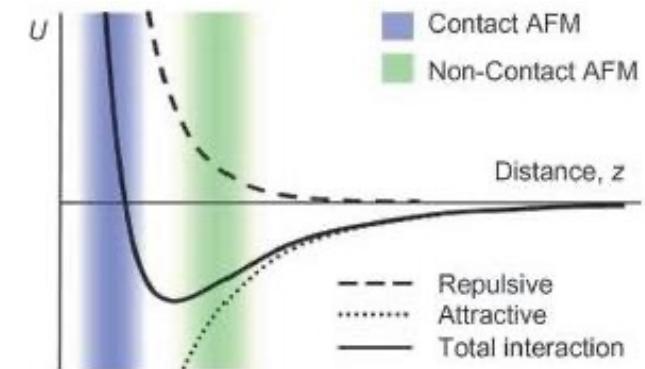
Contact mode

- Cantilever scans surface line by line
- Deflection of the cantilever is recorded

Dynamic



Ann. Rev. Anal. Chem.
2018. 11:329–50



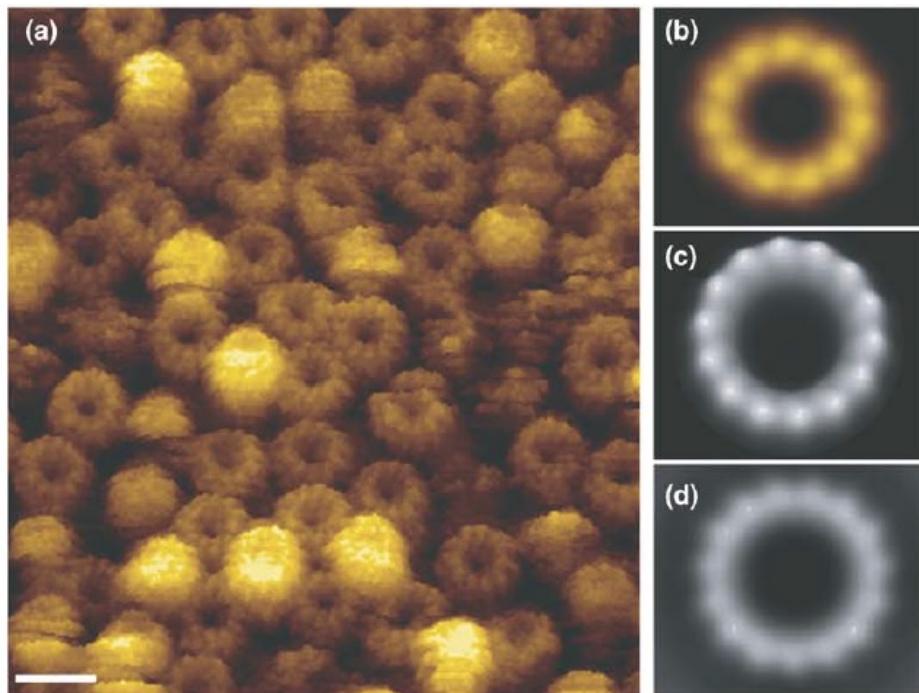
Intermittent-contact / tapping mode

- The **cantilever oscillates** near or at its resonance frequency — that is, the frequency at which it naturally vibrates due to its inherent physical properties.
- During tapping, changes in cantilever frequency (FM) or amplitude (AM) are monitored
- Can measure attractive features of the sample's surface forces, e.g. Lennard-Jones-interatomic potential

An AFM tip at work (combined SEM/AFM)



Imaging with sub-nm Resolution



Cantilever scans the surface of a specimen line after line

Sample adsorbed on a mica layer

Very high contrast, no staining

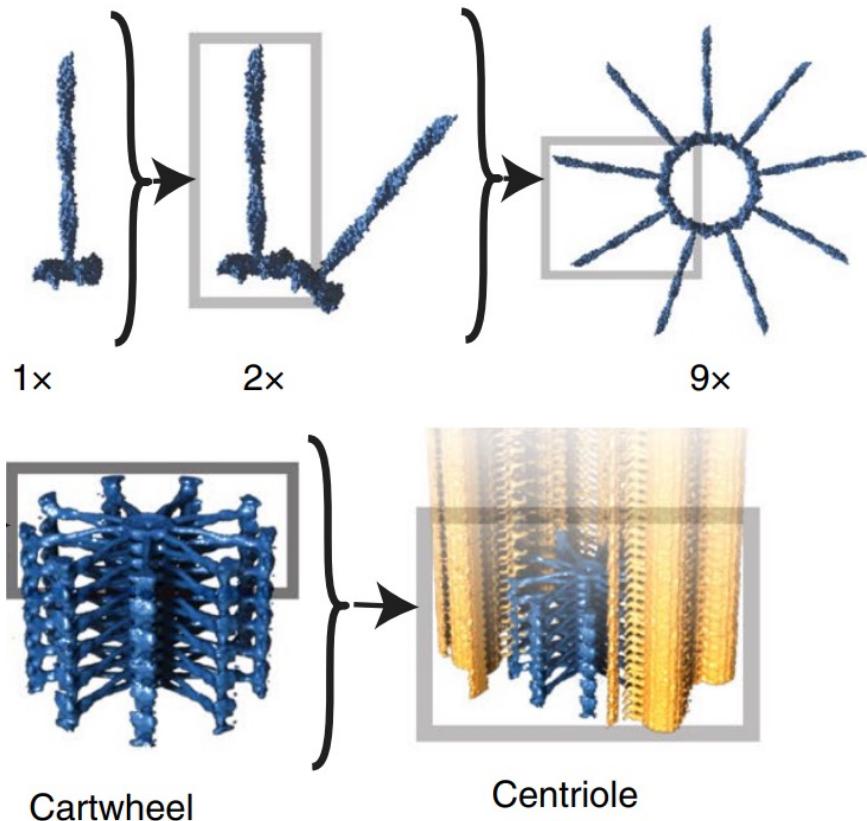
Topography mapped and displayed (z-accuracy: 0.1 nm)

Imaging times long (seconds)

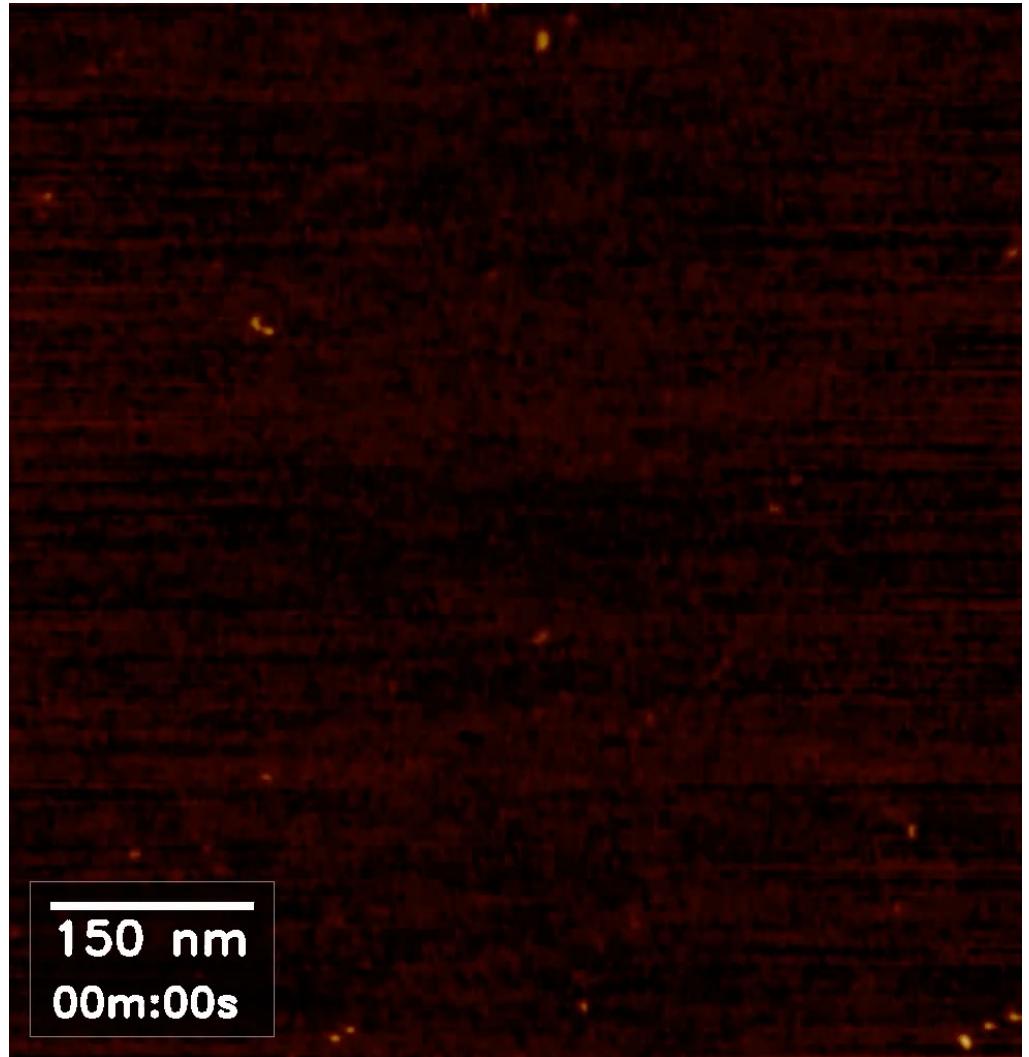
Image: Topography (raw data) of Na-driven rotors from F_0F_1 -ATP synthases from different species (a-c)

Stahlberg et al. Embo Rep, 2001

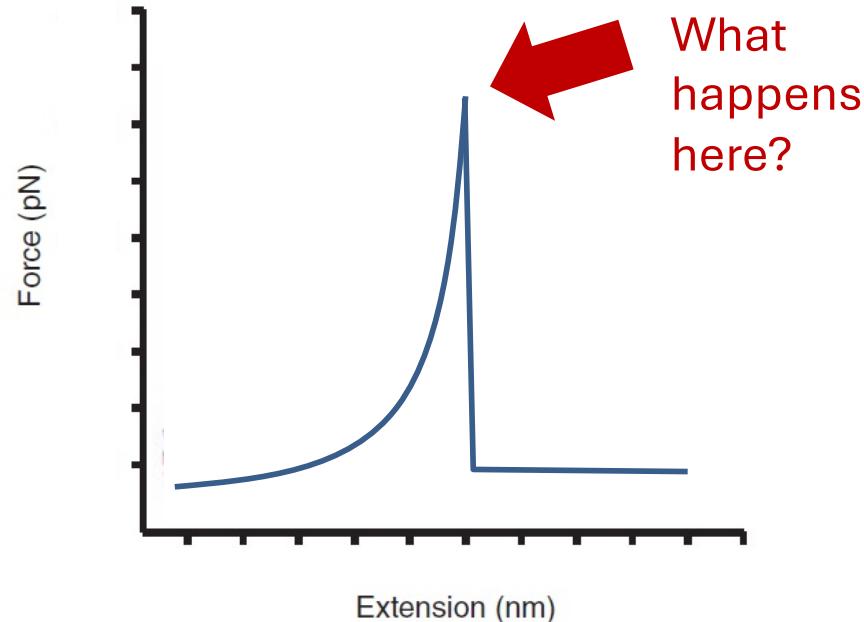
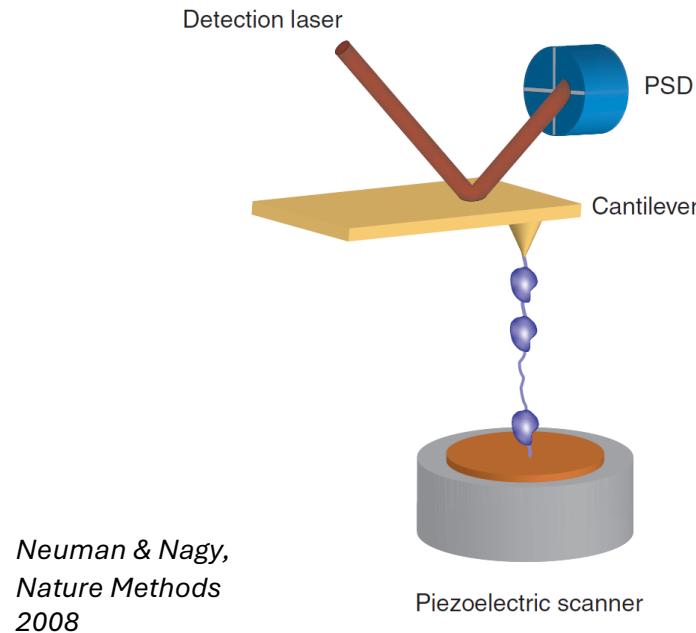
Dynamic imaging of centriole assembly



Fantner & Gönczy labs, EPFL
Nature Nanotechnology 13, 696–701(2018)



AFM as a molecular force probe

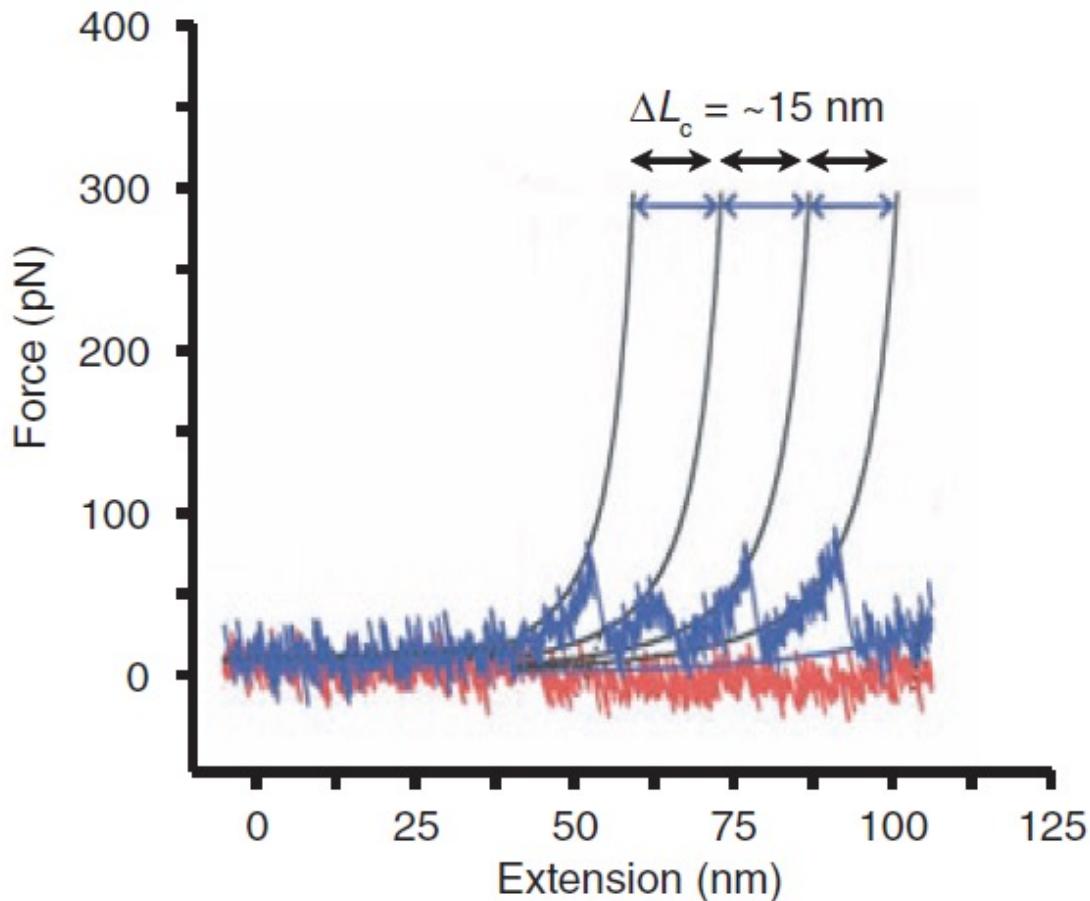


Biomolecules or polymers are attached at one end to the stage and the other to the cantilever.

- **Cantilever Manipulation:** The Z position of the cantilever is adjusted to apply force, causing deflection.
- **Force Measurement:** Cantilever deflection alters the laser reflection angle, detected by a 4-quadrant photodiode.
- **Force Quantification:** Using the known spring constant of the cantilever, the force exerted on the molecule is measured.

- **Extension Curves:** Plots of force vs. extension (distance) are generated, showing the mechanical properties of the molecule.

Stretching curves of proteins



A protein is attached to cantilever and stage. Then the cantilever is moved upward, stretching the protein. The force is recorded as the bending of the cantilever.

- Force-extension-relationship
- Model for data analysis: **WLC** entropic elasticity model
- **Fitting:** persistence length, contour length
- Multidomain proteins: "sawtooth" pattern due to unfolding of individual protein domains

AFM limitations

- AFM can only image the surface of objects
- Large size of cantilevers
- large stiffness of cantilevers
 - Imposes lower bound on force range
 - reduced bandwidth under aqueous conditions
- Specificity may be an issue, as the cantilever by itself will interact with all biomolecules
 - This can be fixed by adding an engineered adaptor (e.g. antibody or other receptor)

Comparison of Single-Molecule Force Spectroscopy Techniques

	Optical tweezers	Magnetic (electromagnetic) tweezers	AFM
Spatial resolution (nm)	0.1-2	5-10 (2-10)	0.5-1
Temporal resolution (s)	10^{-4}	10^{-1} - 10^{-2} (10^{-4})	10^{-3}
Stiffness (pN nm ⁻¹)	0.005-1	10^{-3} - 10^{-6} (10^{-4})	10 - 10^5
Force range (pN)	0.1-100	10^{-3} - 10^2 (0.01- 10^4)	10 - 10^4
Displacement range (nm)	0.1- 10^5	5 - 10^4 (5- 10^5)	0.5- 10^4
Probe size (μm)	0.25-5	0.5-5	100-250
Typical applications	3D manipulation Tethered assay Interaction assay	Tethered assay DNA topology (3D manipulation)	High-force pulling and interaction assays
Features	Low-noise and low-drift dumbbell geometry	Force clamp Bead rotation Specific interactions	High-resolution imaging
Limitations	Photodamage Sample heating Nonspecific	No manipulation (Force hysteresis)	Large high-stiffness probe Large minimal force Nonspecific
	works <i>in vitro</i>	works in cells	works on cells

Neuman & Nagy,
Nature Methods 2008

Next week: super-resolution microscopy

Dr. Milena Schuhmacher

Laboratory of Chemical and
Membrane Biology @ EPFL



Connections

Nanobiotech_week03

by AS

Share Puzzle 

HIGH ENERGY LASER

ELASTIC

ENTROPIC SPRING

MAGNETIC

NANOMETER TIP

FOUR-QUADRANT
PHOTODETECTOR

PIEZOELECTRIC SCANNER

MECHANICAL

CANTILEVER

ELECTROMAGNETIC

RANDOM COIL

FLUIDIC

DETECTION LASER

HIGH NA OBJECTIVE

CONDENSER

WORMLIKE CHAIN