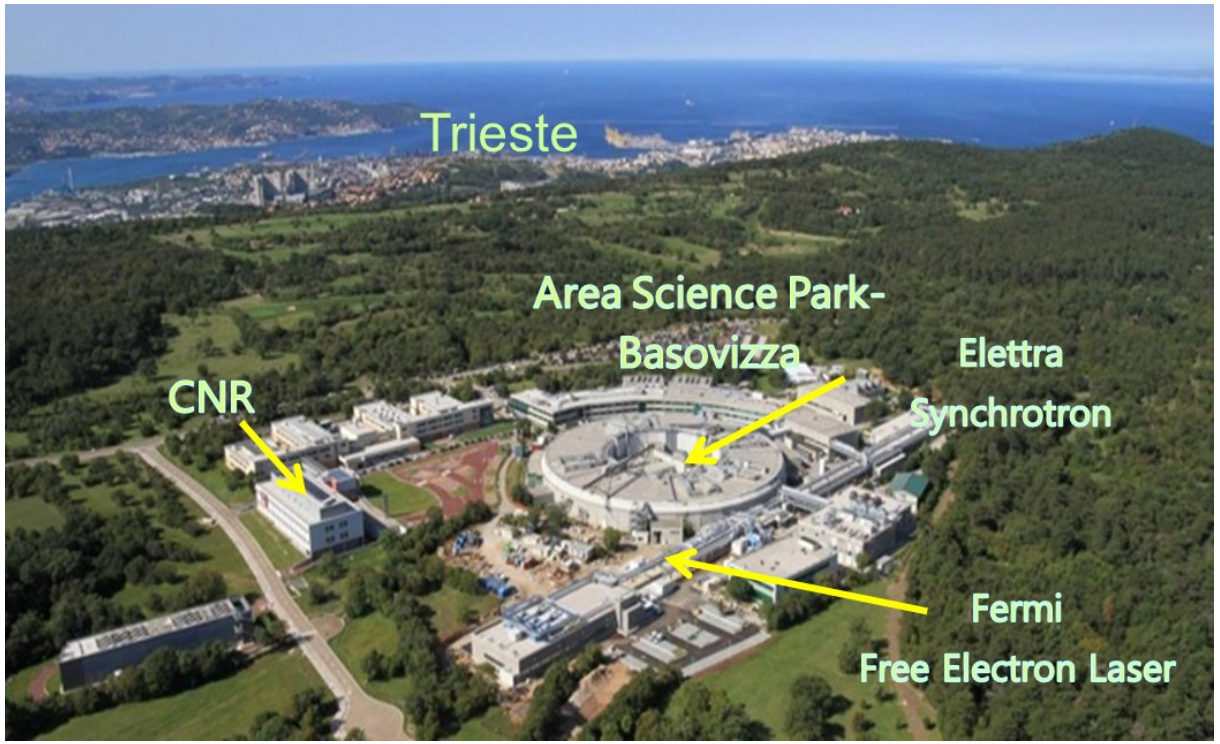
Optical Tweezers Microscopy

and some applications to biological systems

Dan COJOC

Lecture 7



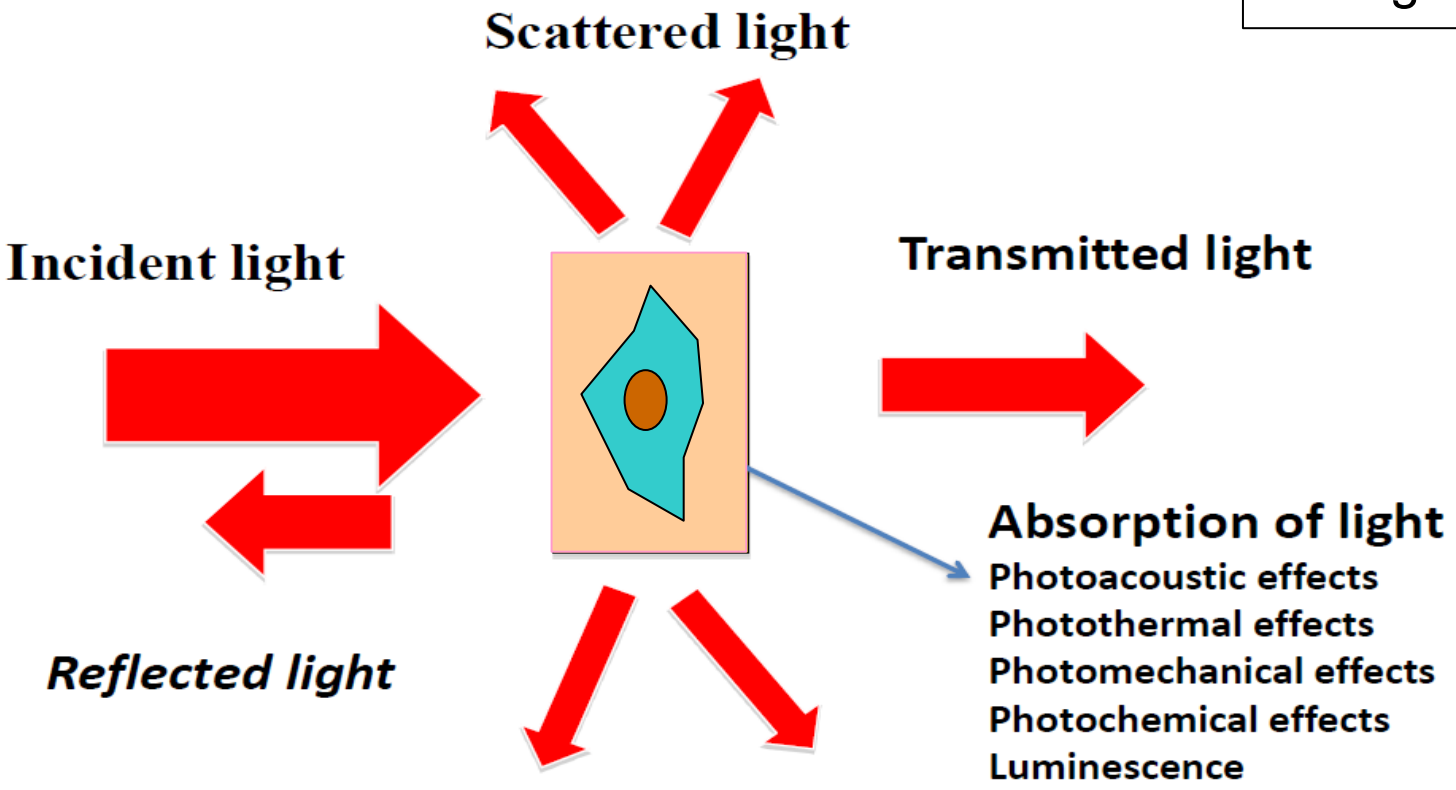
CNR – Consiglio Nazionale delle Ricerche

OUTLINE

1. Can light exert small forces on small objects ?
2. What are Optical Tweezers (OT)?
3. OT application to the manipulation of biological samples
4. OT force spectroscopy for single molecule and cell dynamics
5. Mechanotransduction and cell focal stimulation with OT

Light – Sample interaction

Sample:
Living Cell



is governed by physics laws as

Energy and Momentum conservation

and includes **mechanical effects !**

Could Light exert Force on Objects ?

If Yes, How ?

Light is made by photons and a PHOTHON has MOMENTUM

(even if it does not have MASS):

$$p = \frac{h\nu}{c} = \frac{E_p}{c}$$

Momentum ***p*** and energy E_p of a photon

c - light velocity; v – frequency;
λ = c / v – wavelength.

How big is the photon momentum compared with the momentum of an object with mass m, moving at velocity v << c ?

Momentum and Energy of a single photon:

$p \approx 10^{-27}$ N s $E \approx 2$ eV$= 3.2 \times 10^{-19}$ J

Momentum of a single E-coli bacteria swimming in liquid:

Mass: m= 1 pg = 10^{-15} Kg; Velocity: V= 100 nm/s = 10^{-7} m/s

Momentum: $P_{Ecb} = m\,V = 10^{-22}$ N s (N s = kg m / s)

The momentum of a photon is very small !

Nevertheless, even a low power laser beam, has many photons.

Example: laser beam of power $W_{lb} = 1$ mW (energy $E_{lb} = 1$mJ)

The number of photons is: $N = E_{lb}/E \approx 3\ 10^{15}$ →

→ **Momentum** of the laser beam: $P_{lb} = 3\ 10^{-12}$ N s

$$P_{lb} >> P_{Ecb}$$

Can the laser beam influence the motion of the bacteria ?

We need to consider / remember some laws of mechanics

Newton's three laws of motion:

L1. Every object in a state of uniform motion will remain in that state of motion unless an external force acts on it.

L2. Force equals mass times acceleration: **F= m a**

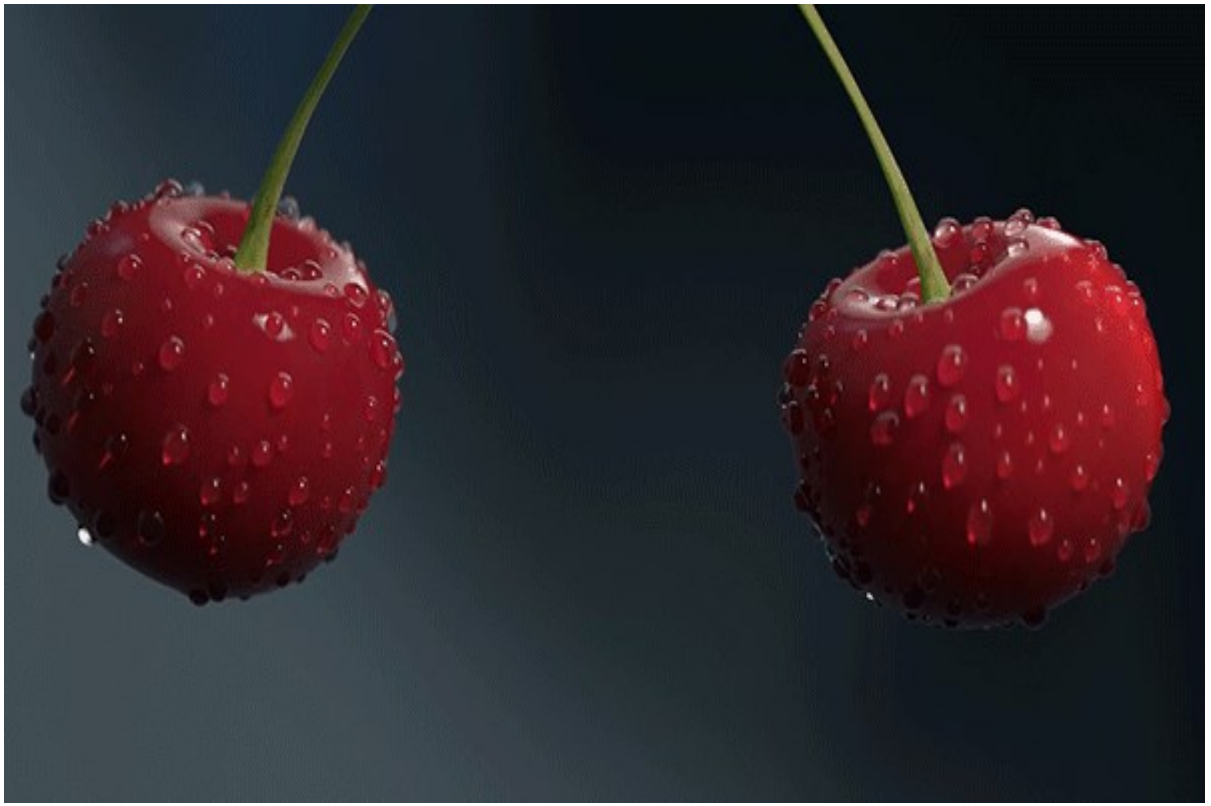
L3. For every action there is an equal and opposite reaction.

+ the laws of conservation of momentum and energy.

If we consider the second law: F= m a ,
and express acceleration a, as a = ΔV / Δt , we get:
F = m ΔV / Δt = Δ (mV)/ Δt = **Δ P/ Δt,**
which means that:

the change of momentum Δ P in a given time Δt produces force F.

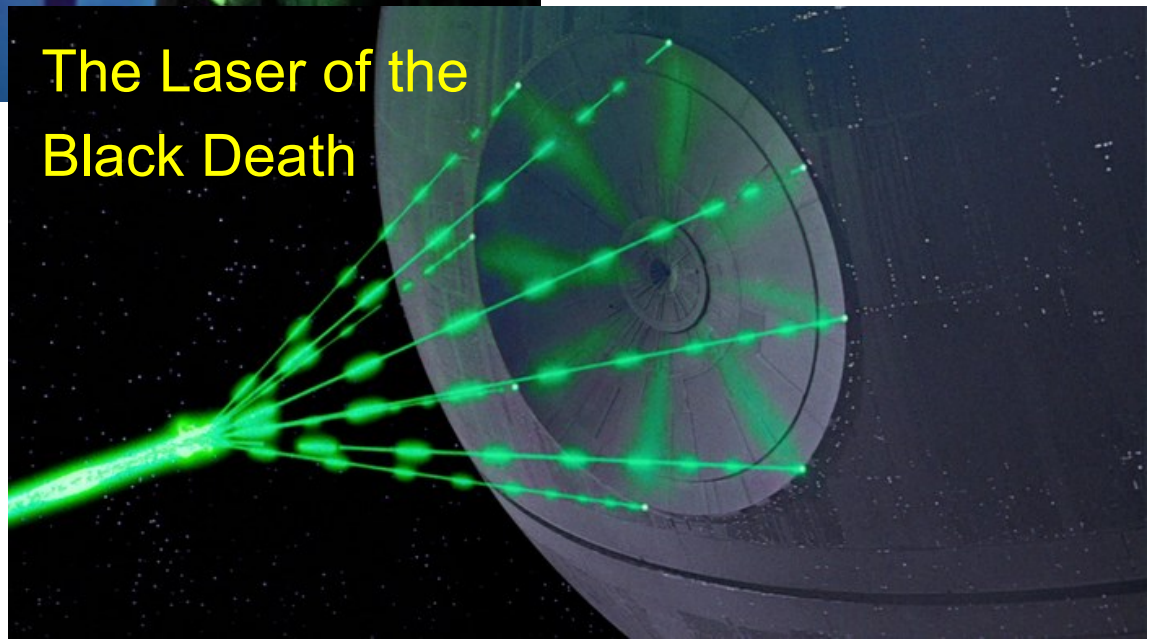
Example of interaction between two objects in motion



Another example (with ellastic and inellastic interaction).

Ellastic: Hammer – Tyre; Inellastic: Hammer-Tom.

Examples of interaction of “object(s)” without mass



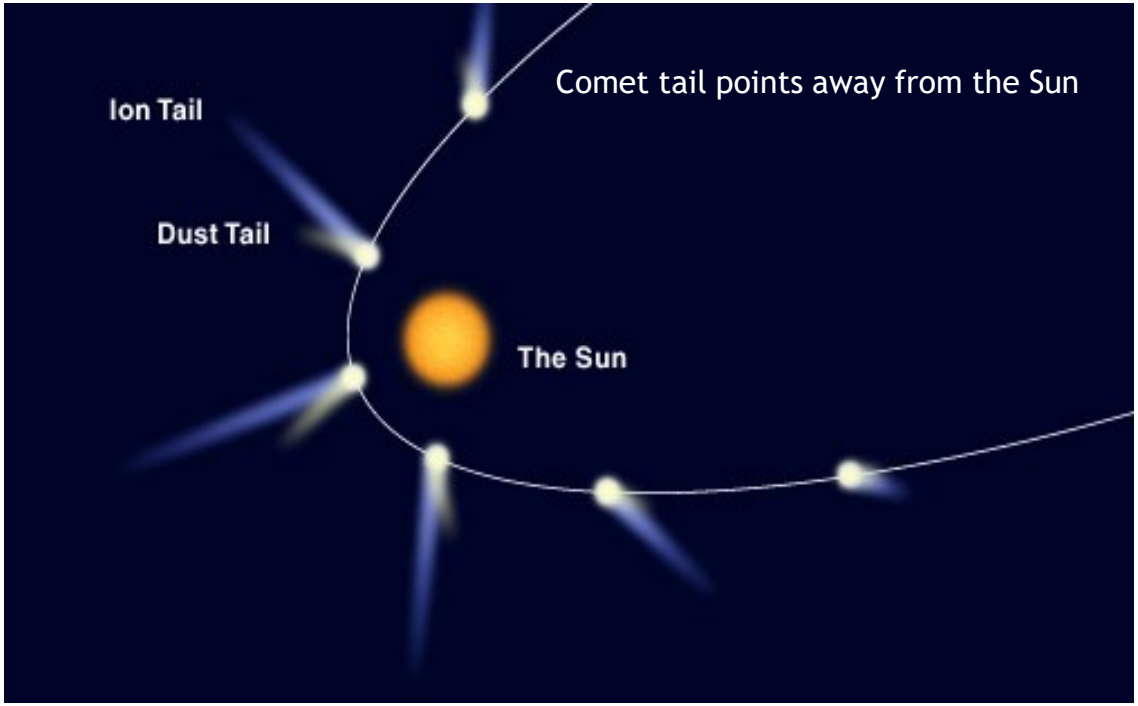
Science Fiction

Star Wars

Light has momentum and can generate force

1619 – Kepler :

Observation of the orientation of the comet tails → suggests that the Sun Light drives the orientation of the comets tail



1873 – Maxwell :

"In a medium in which waves are propagated, there is a pressure in the direction normal to the waves and numerically equal to the energy in unit volume"

1900-1901 Lebedev, Nichols, Hull:

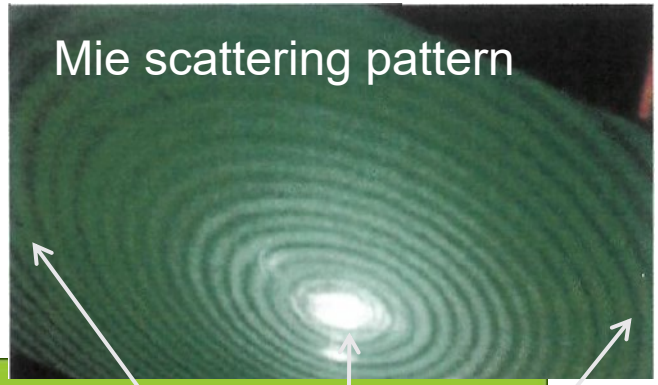
First measurement of the radiation pressure using a torsion balance

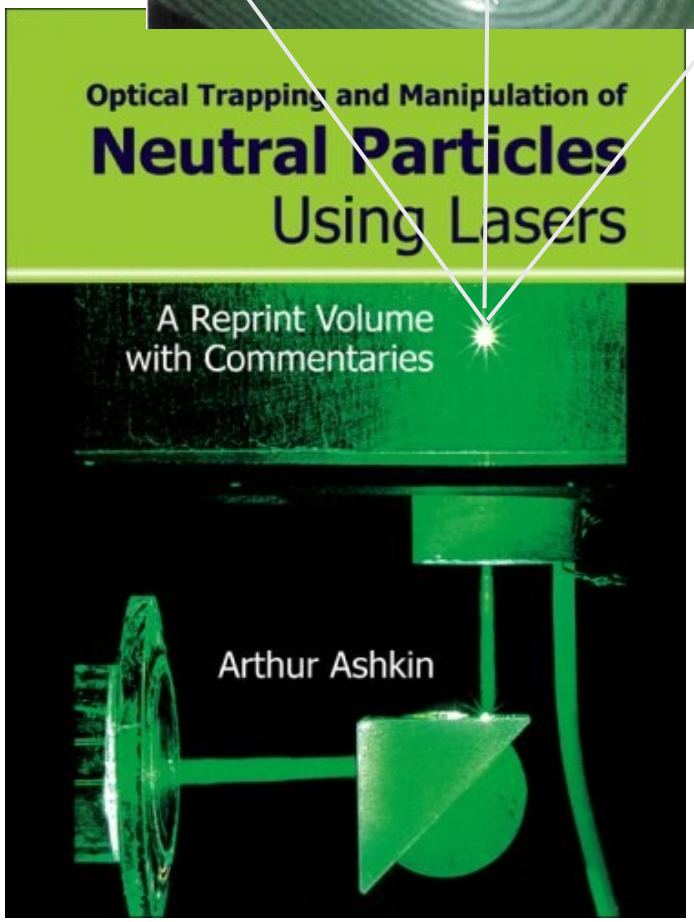
Forces generated by light on objects are in general very small and hence the effect is difficult to be detected

→ **use LASER beam and small objects !**

Newton's second law: $F = m\,a$ or $\mathbf{a = F / m}$

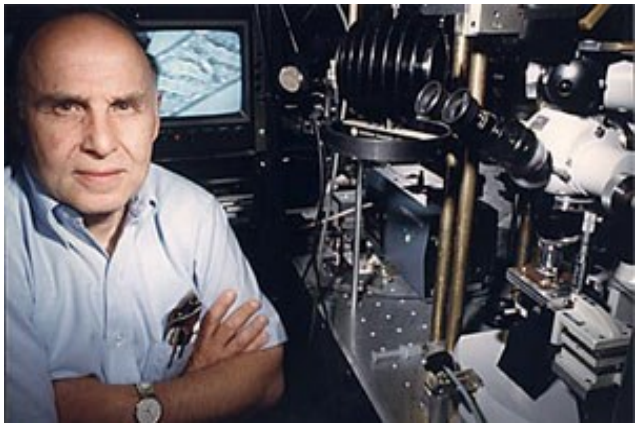
Even if the force F is smal, for small objects of small mass m, the effect (measured by acceleration a) can be considerable (detectable and measurable) !



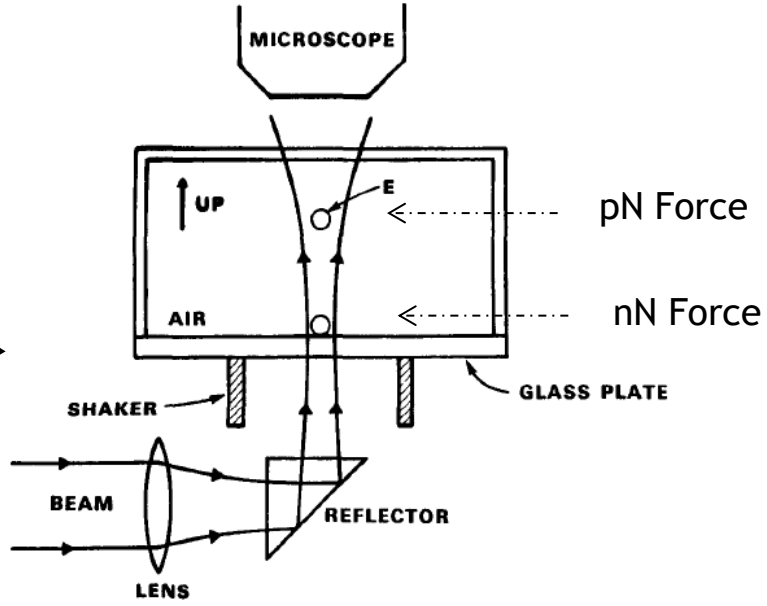


Scientific Publishing 2006

Arthur Ashkin, Bell Labs **(1986)**



Optical levitation of microparticles in air



(hollow silica,beads, diam 50-75 um)

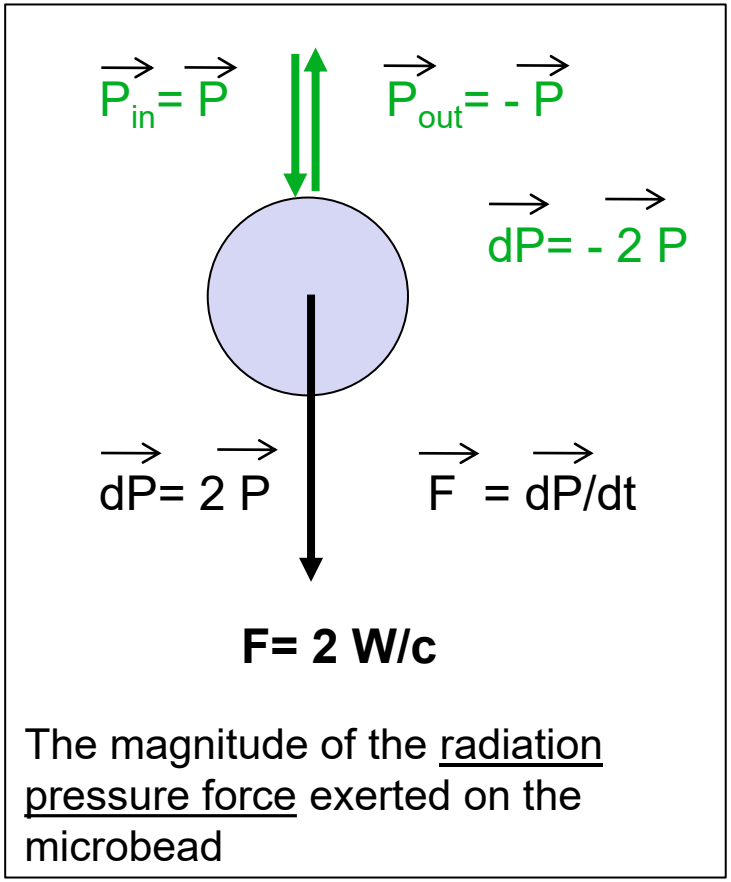
How big is the force exerted by a ray of light reflected perfectly by a microbead ?

Geometrical optics approximation --> light rays

- reflection coefficient R= 1
- (bead diam) d > λ (light wavelength)
- d = 2 μm, λ= 0.5 μm

The magnitude of the momentum associated to the ray of light composed by N photons:

$P = E / c = N h \nu / c$



The magnitude of the radiation pressure force exerted on the microbead

N= 1 photon, -> E≈ 2.5 eV, W≈ 4 x 10^{-19} W -> F≈ 2.7 x 10^{-27} N - very small

N= 10^{15} photons, W ≈ 0.4 mW, F≈ 2.7 x 10^{-12} N = 2.7 pN - **SMALL**

1 pN is the gravitational force of a particle with a mass of 0.1 ng (10^{-10} grams) !

Is the magnitude of this force significant ?

1)

Microbead in free space (vacuum) - no dumping:

F ≈ 2.7 pN - SMALL , but also the mass, m, of the microbead is small

m ≈ 8 pg → acceleration **a**≈ F/m = 3.4 x 10^2 [m/s^2] = **34 g** ,

which is very **BIG** !

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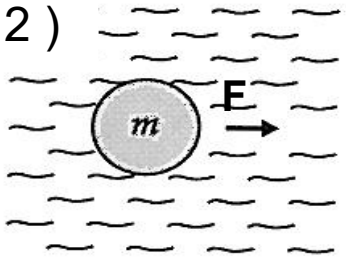
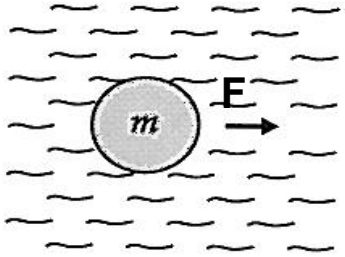
2)



Microbead in liquid - dumping:

F≈ 3.6 pN

refractive index (water) n_m = 1.33; force by light : F= 2 $\mathbf{n_m}$ W/c ;

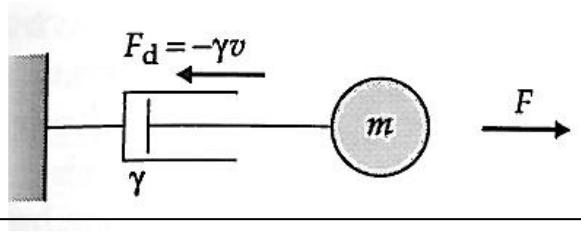


Microbead in liquid - dumping:

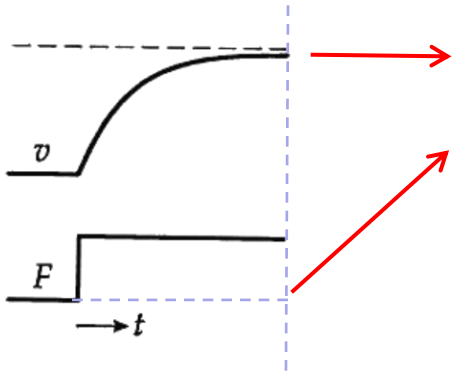
F≈ 3.6 pN

refractive index (water) n_m = 1.33; force by light : F= 2 $\mathbf{n_m}$ W/c ;

mass + dashpot model



$$m\frac{dv}{dt} = F - \gamma v \qquad v(t) = \frac{F}{\gamma}\left[1 - \exp\left(-\frac{t}{\tau}\right)\right]$$



max velocity $v_t = \frac{F}{\gamma}$ **= 360 µm / s**

time constant $\tau = \frac{m}{\gamma}$ **= 0.8 µs**

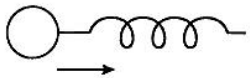
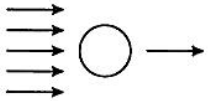
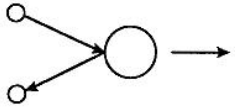
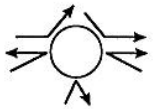
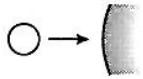
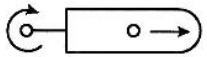
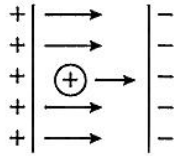
- the max velocity is reached very fast and maintained until the force F is applied.
- When the force is cancelled the particle stops very fast.

For a small particle dumping is dominant over inertia because: *m -> d^3 , γ-> d*

Example from biology: movement of a bacterium in water. The bacterial motor must be able to generate a force > 0.5 pN to swim through water and stops immediately when motor stops.

Physical forces and their magnitudes at the single molecule level

Table 2.1 Examples of forces acting on molecules

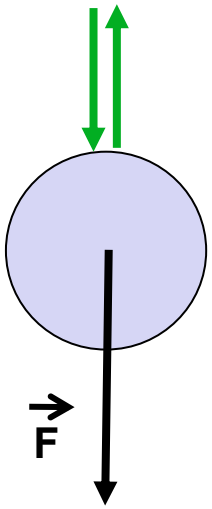
Type of force	Diagram	Approximate magnitude
Elastic		1–100 pN
Covalent		10,000 pN
Viscous		1–1000 pN
Collisional		10^{-12} to 10^{-9} pN for 1 collision/s
Thermal		100–1000 pN
Gravity		10^{-9} pN
Centrifugal		$< 10^{-3}$ pN
Electrostatic and van der Waals		1–1000 pN
Magnetic		$<< 10^{-6}$ pN

pN

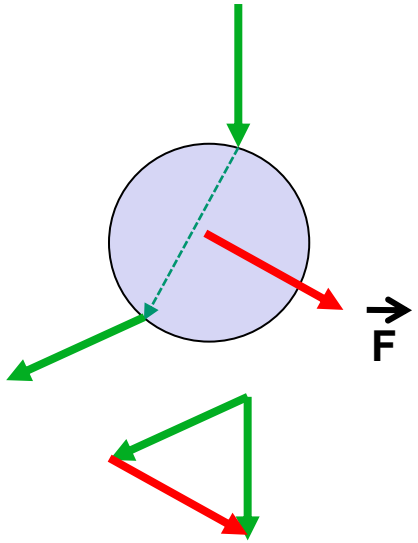
J. Howard, Mechanics of motor protein and the cytoskeleton, Sinauer Associates Inc. , 2001

Force induced by a ray of light by refraction on a bead in water

reflection only
R=1



refraction only,
R=0; $n_b > n_m$



The magnitude of the force:

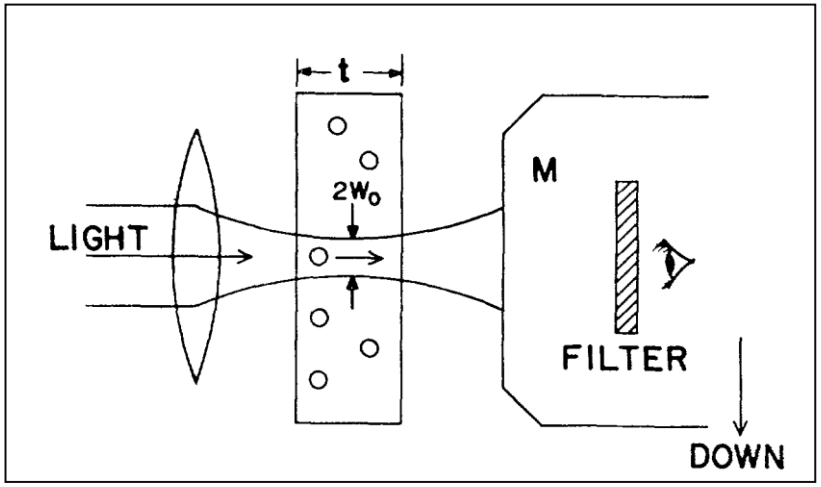
$$F = Q n_m W_{in}/c$$

the incident momentum /s of a ray of power W_{in}

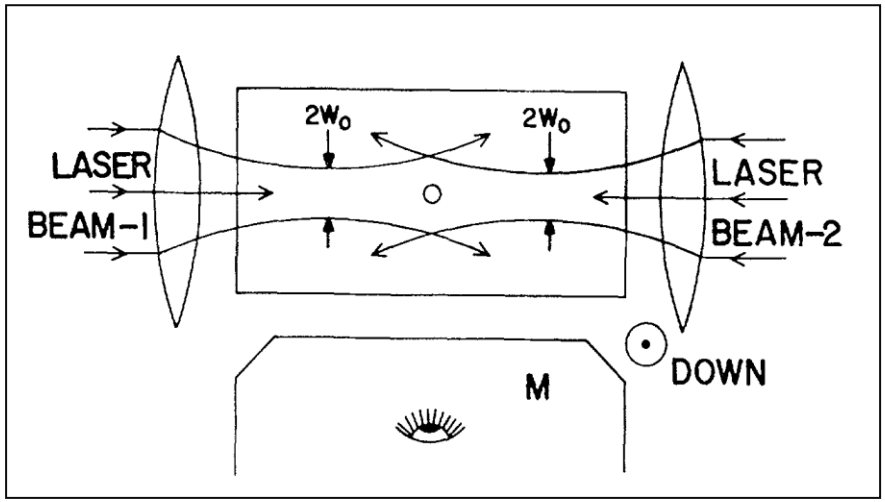
Q - dimensionless factor, $Q \leq 2$
Q - function of shape, material

- If the beam of light is not focused or midly focused, the force always pushes the object forward.
- However, if the beam is tightly focused, there is a force component attracting the object toward the focus → 3D trapping

2D and 3D optical trapping



ONE focused laser beam
2D trapping

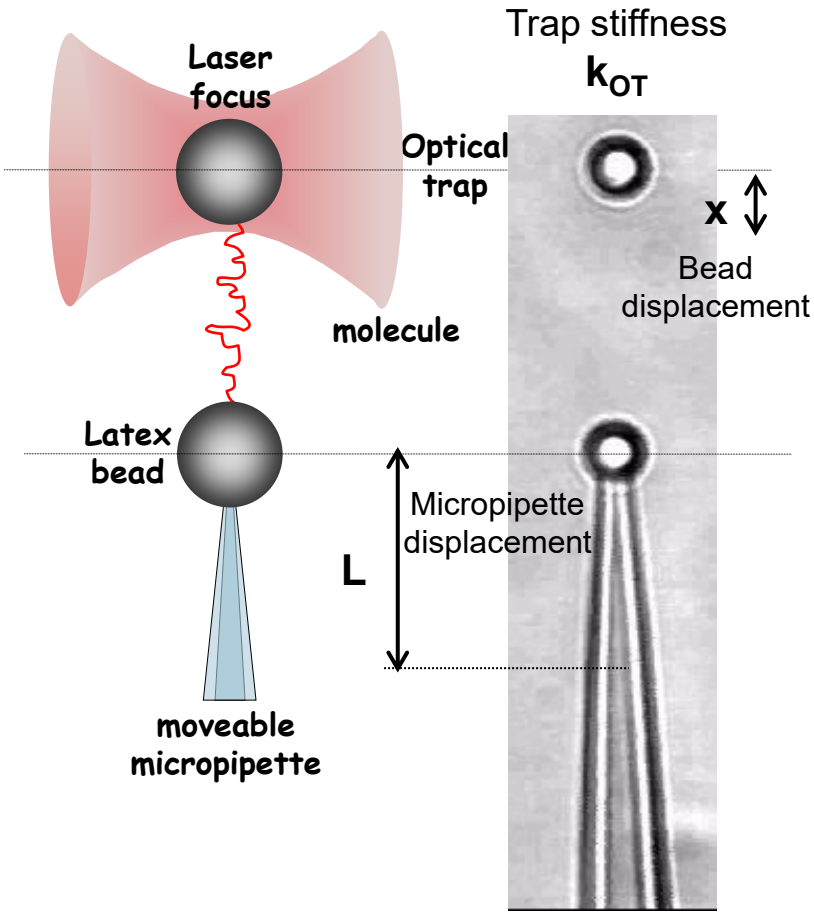


TWO focused laser beams,
(counter propagating)
3D trapping

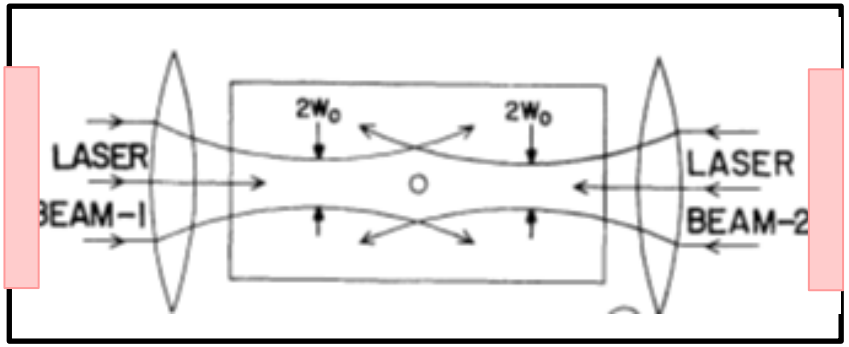
NOTE: focusing through relatively low NA lenses

Acceleration and trapping of particles by radiation pressure
A. Ashkin, *Phys. Rev. Lett.* 24, 156 (1970) >5000 citations

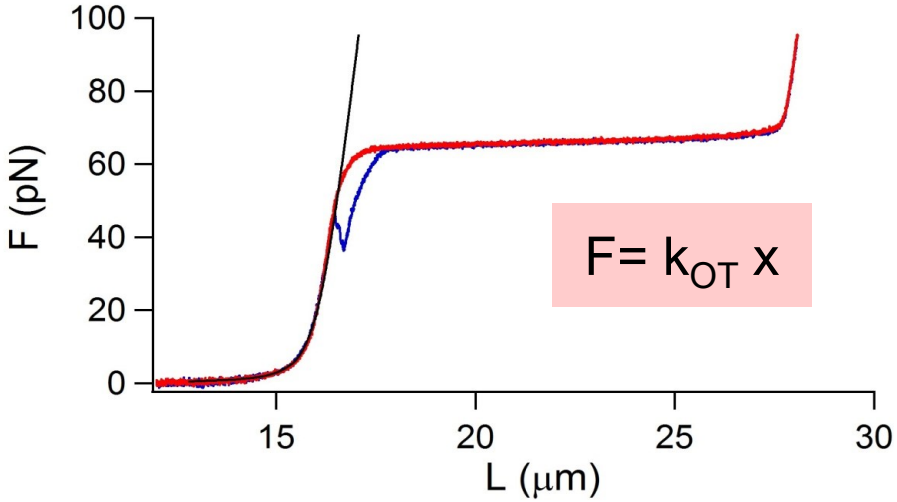
Dual Laser Optical Tweezers DLOT



Counterpropagating beams + detection



stretching λ-phage DNA



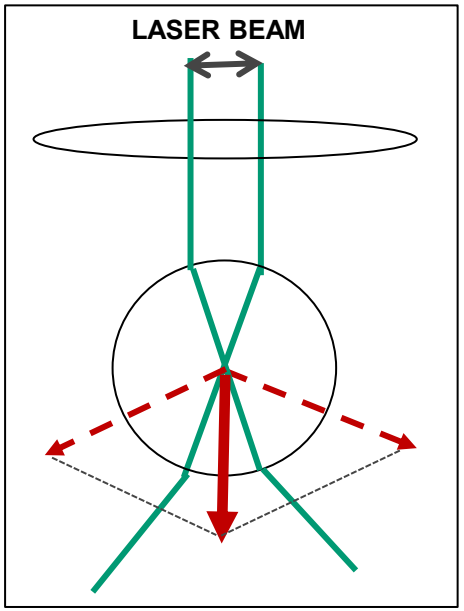
The DNA molecule undergoes a structural change at ~65 pN that implies 70% elongation and is likely involved in the modulation of the access to genetic information

collab with V. Lombardi, P. Bianco, Florence Univ.

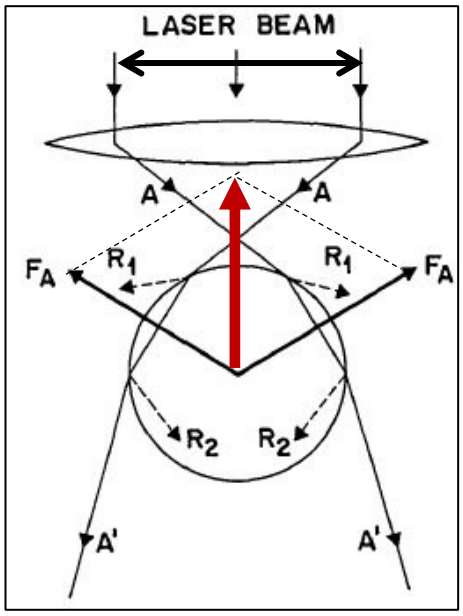
Observation of a single-beam gradient force optical trap for dielectric particles

A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, and S. Chu, *Opt.Lett.* 11, 288 (1986)

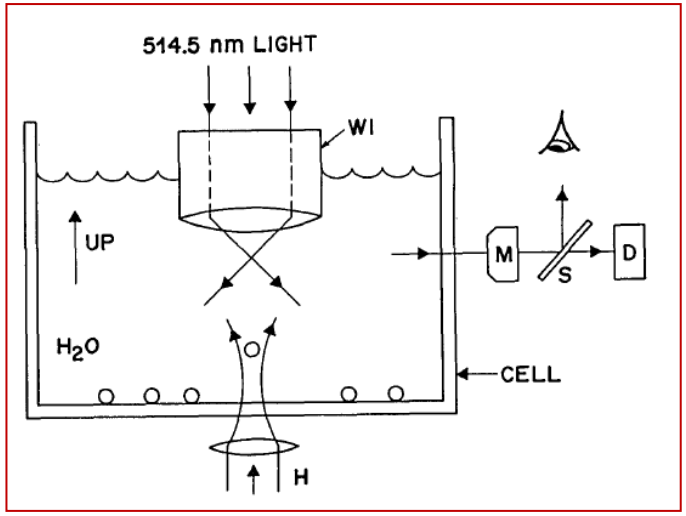
> 6000 citations



Force generated by a **midly** focused laser beam on a transparent microparticle in water.



Force generated by a **tightly** focused laser beam.



Sketch of the basic apparatus.

Size of particles :

10 um (Mie) to 25 nm (Rayleigh)

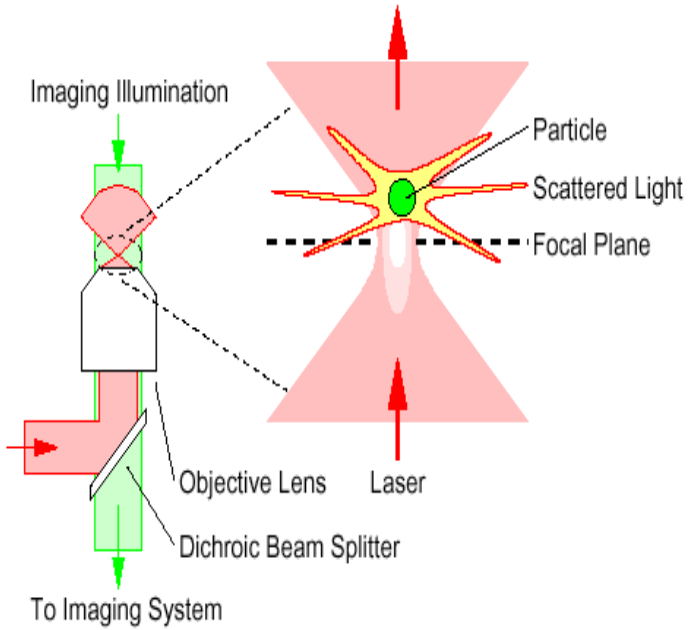
Acceleration and trapping of particles by radiation pressure

A. Ashkin, *Phys. Rev. Lett.* 24, 156 (1970) >5000 citations

What is an Optical / Laser Tweezers ?

A laser beam **tightly** focused through a high Numerical Aperture (NA) objective

A. Ashkin *et al* ***Opt. Lett.*** 1986

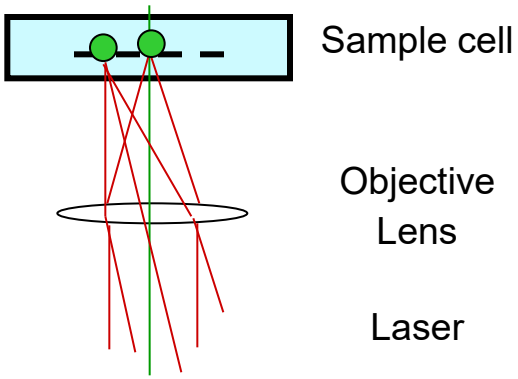


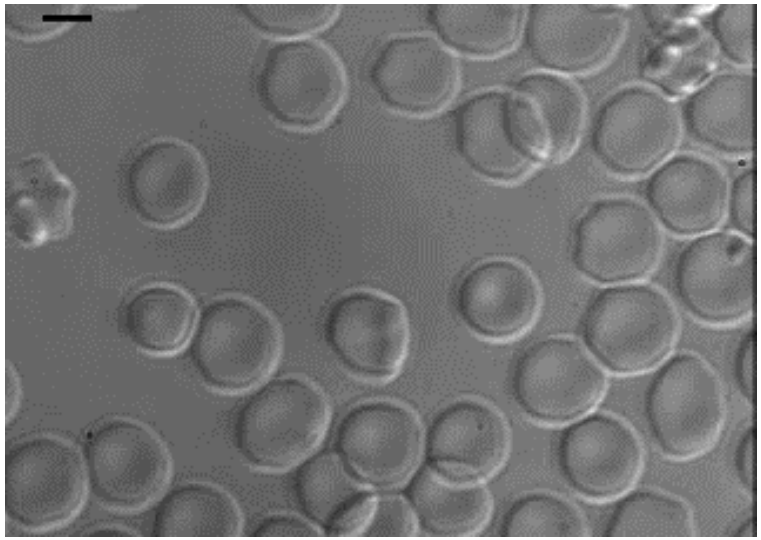
$$F = Q\frac{n_m W}{c}$$

Ex: **F= 1.33 pN** for
W= 1 mW, n_m=1.33, Q=0.3

F – trapping force

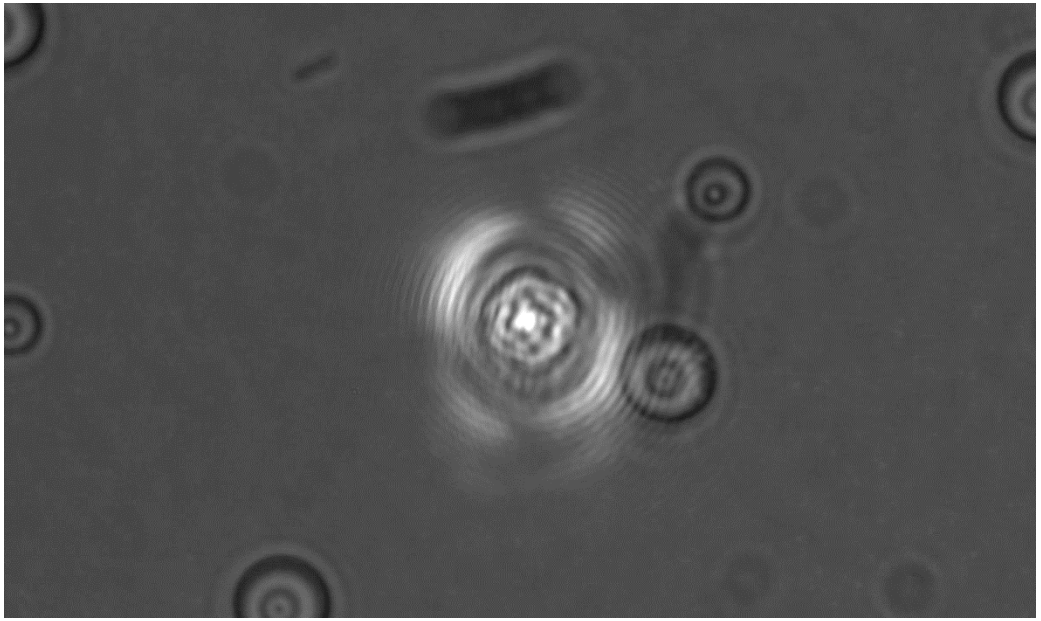
Q – dimensionless efficiency coefficient
W – power of the laser beam
$\mathbf{n_m}$ – refractive index of the medium
c – light speed in vacuum





Example of human erythrocyte trapping
2004 - OM Lab

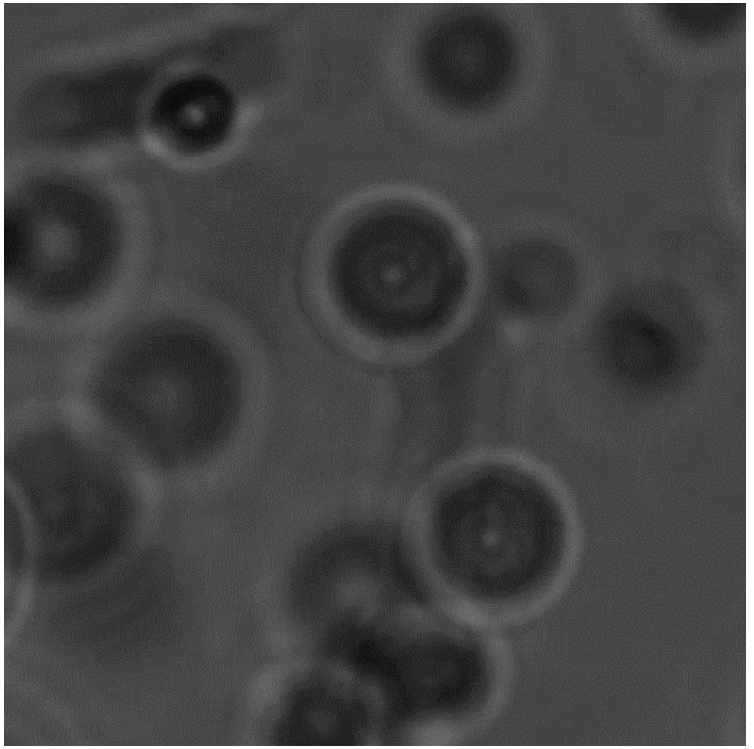
Some examples of trapping from OM Lab



silica microbeads, laser 970 nm,

power at the sample about

P= 5 mW



Optical trap behaves as an

attractor of particles

P= 120 mW

Are there sensitive issues when using optical tweezers to trap biological particles ?

1. The intensity at the trapping position (focal plane) is very high !
   Absorption of light by different components of a biological sample is wavelength dependent !

   Is the laser beam damaging the sample ?

   If yes, which is the level of damage ?

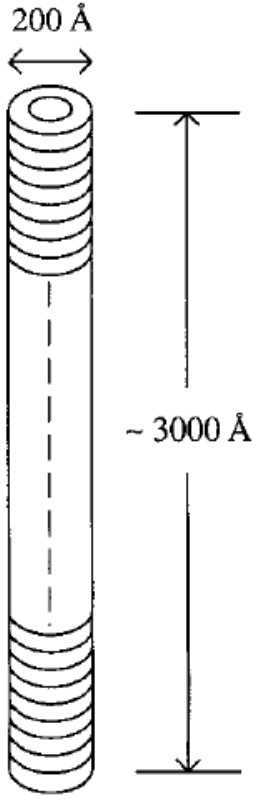
2. Biological samples (e.g. viruses, bacteria, cells) have arbitrary shapes while the laser beam is symmetric.

   Does this mismatch prevent trapping ?

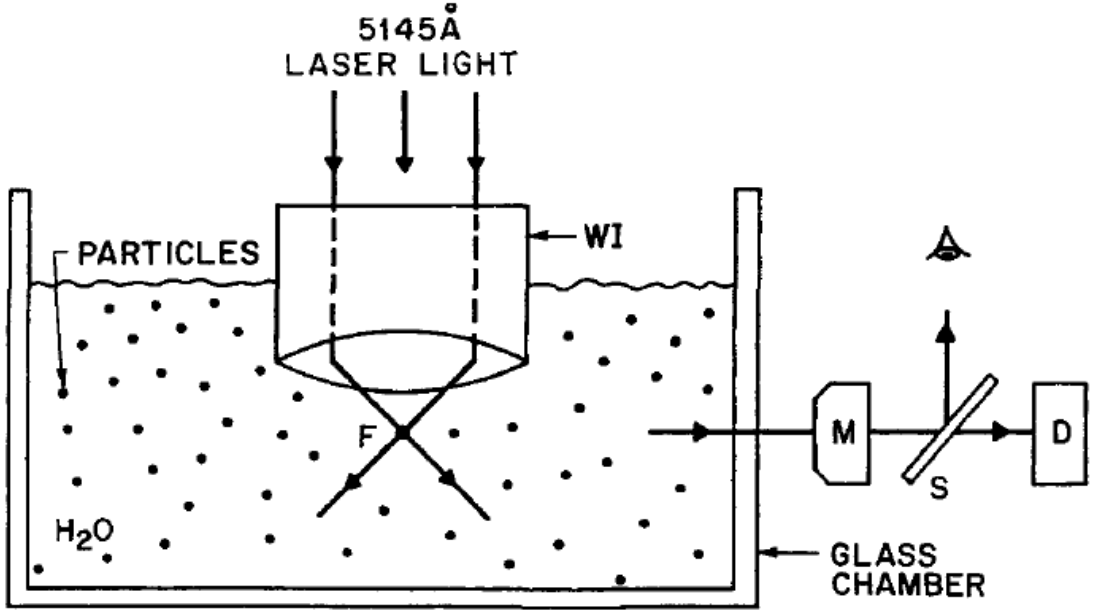
First optical trapping of a biological sample

Tobacco Mosaic Virus (TMV)

TMV
shape & size



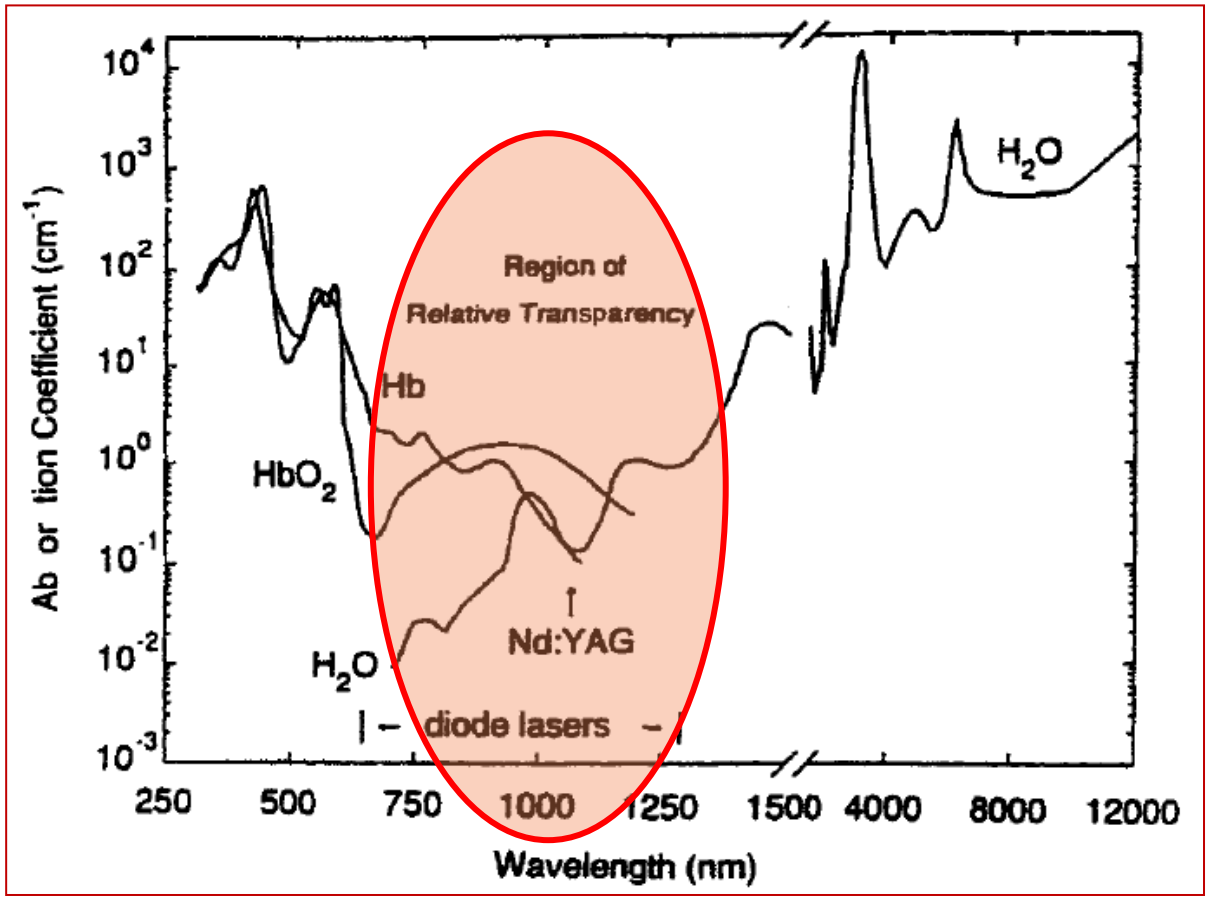
Apparatus used for optical trapping of TMV particles and mobile bacteria



Bacteria (which are slightly larger than Rayleigh particles) trapping was accidentally observed and then rigorously characterized for *E. Coli* in a closed sample cell.

A. Ashkin and J.M. Dziedzic, "Optical trapping and manipulation of viruses and bacteria", *Science* 235, 1517 (1987)

Damage – free trapping of living cells with infrared light



Plot of the optical absorption coefficients of hemoglobin (Hb), oxyhemoglobin (HbCh) and water versus the wavelength.

Damage – free trapping of living cells

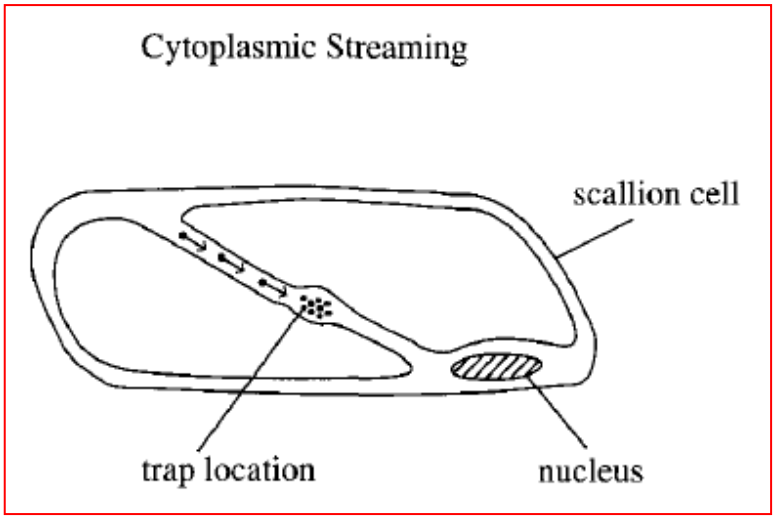
A. Ashkin, J.M. Dziedzic, T. Yamane, "Optical trapping and manipulation of single cells using infrared laser beams", *Nature* 330, 769 (1987)

Ashkin: "We tried red blood cells, plant cells, and the huge number of different types of protozoa, diatoms, and single cells of algae one can find in pond water." **One can trap almost any type of cells with IR beam without, or with limited damage.**

Not only were the cell types quite varied, but also their sizes and shapes. Shape and optical properties of particles are crucial to the trapping process.

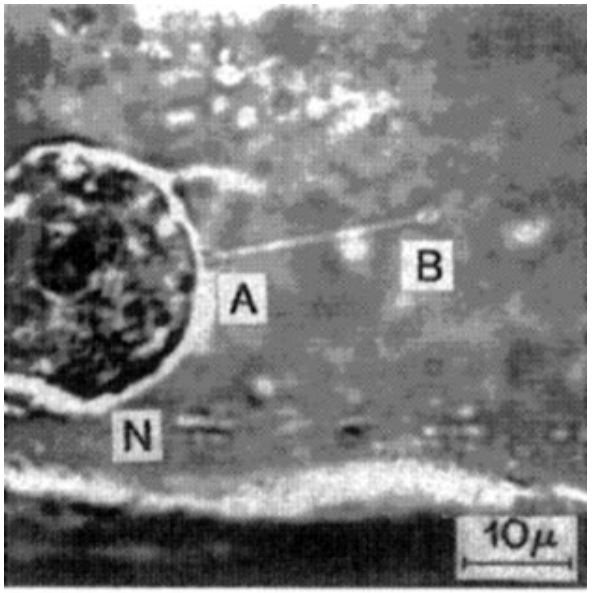
Optical tweezer-type traps are very tolerant of shape particle variation .

Intra-cellular trapping



Internal cell manipulation. Collection of particles and a blob of cytoplasm trapped within a streaming channel of cytoplasm inside a living scallion cell. When released, they simply move on.

A. Ashkin and J. M. Dziedzic, Internal cell manipulation using infrared laser traps, *Proc. Natl. Acad. Sci. USA* ***86,*** 7914 (1989).



Nobel Prize in Physics 2018

Arthur Ashkin invented optical tweezers that grab particles, atoms, viruses and other living cells with their laser beam fingers.

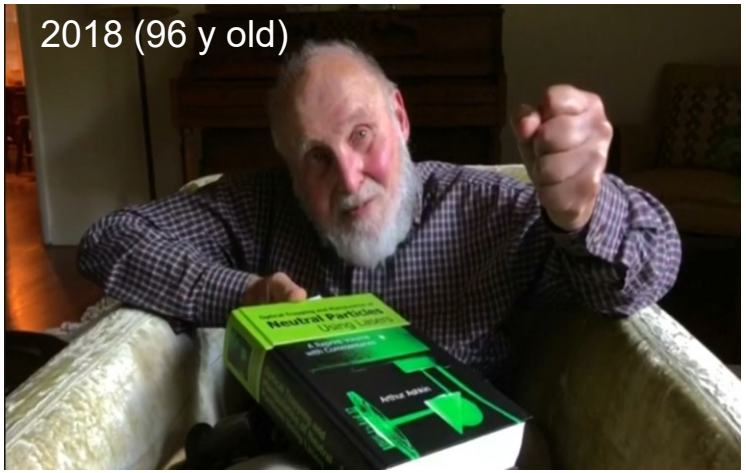
This new tool allowed Ashkin to realise an old dream of science fiction – using the radiation pressure of light to move physical objects.

He succeeded in getting laser light to push small particles towards the centre of the beam and to hold them there. Optical tweezers had been invented.

A major breakthrough came in 1987, when Ashkin used the tweezers to capture living bacteria without harming them. He immediately began studying biological systems and optical tweezers are now widely used to investigate the machinery of life.

Prize motivation :

"for the optical tweezers and their application to biological systems."



Optical Tweezers – some properties

What type of particles can be trapped ?

- **Material:**
  - Dielectric (polystyrene, silica);
  - Metallic (gold, silver, copper);
  - **Biological** (cells, macro-molecules, intracellular structures, DNA filaments);
  - Low index (ultrasound agent contrast); crystal or amorphous material.
- **Size:** 20 nm – 20 μm
- **Shape:** spherical, cylindrical, arbitrary.

Range of forces that can be applied and measured : **0.1 – 100 pN**

Optical tweezers

- Optical trapping and manipulation principles: historical notes / Arthur Ashkin

- Examples of OT applications in biophysics
  - Optical microsample manipulation
  - Piconewton force spectroscopy

Multiple trapping

How can we get multiple optical traps / tweezers?

1. **time-sharing a single beam among several different locations**

   using galvano mirrors (GM), acousto-optic deflectors (AOD)

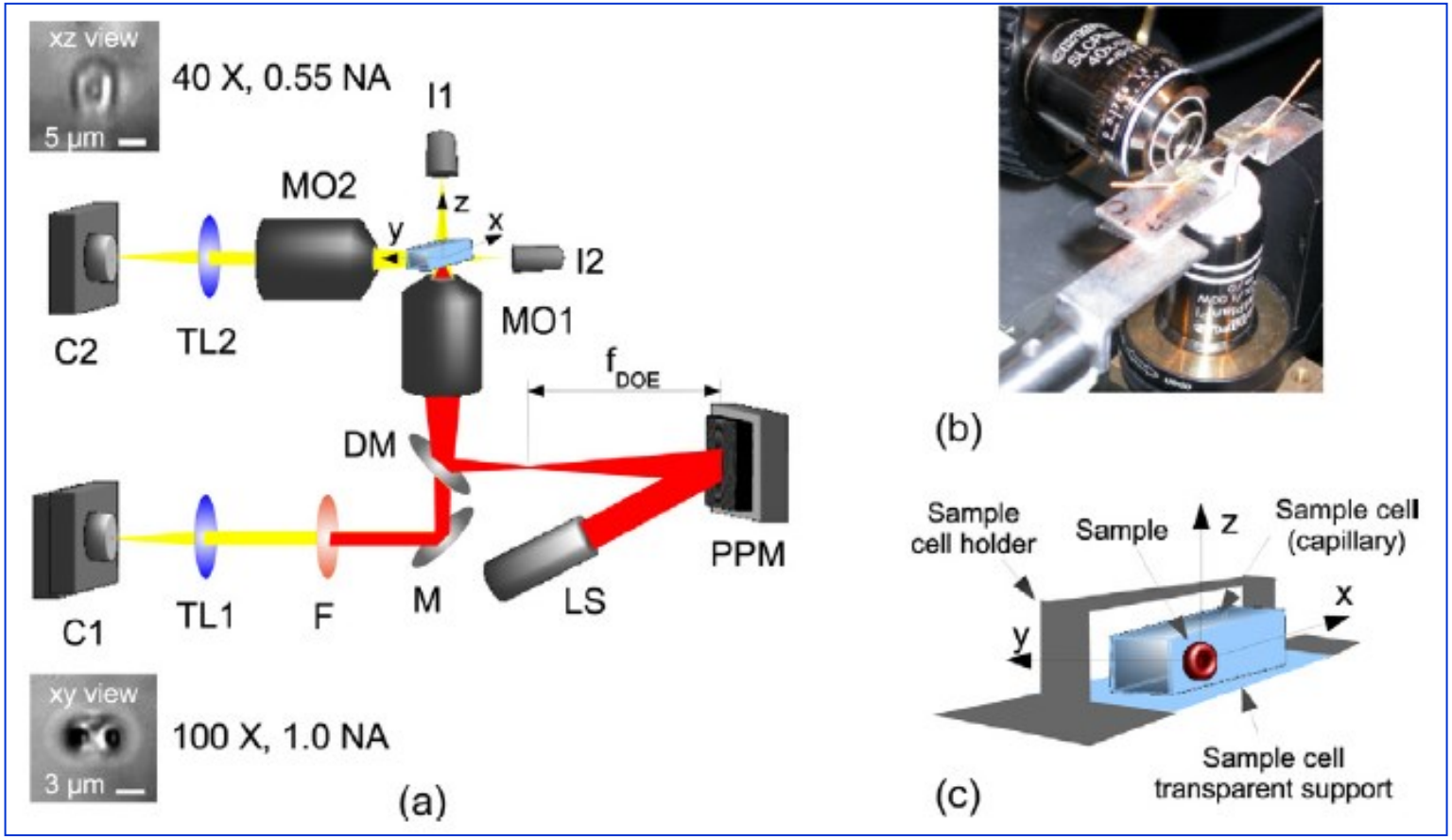
   - Allow to obtain: 2D arrays of dynamic traps; modulate the strength of the traps individually
   - GM are relatively cheap but have a lower frequency (kHz) and hence only few traps can be generated; AOD are more expensive but have a high frequency (MHz) and hence even tens of traps can be generated and controlled.

2. **split the beam into multiple beams**

   using beam-splitter (BS) or spatial light modulators (SLM)

   - BS allow to obtain 2 fixed traps with fixed strengths;
   - SLM allows to obtain: 2D and 3D arrays of dynamic traps; modulate the strength of each trap individually; convert Gaussian beams to Laguerre-Gauss beams (to get helical-vortex beams) or Bessel beams

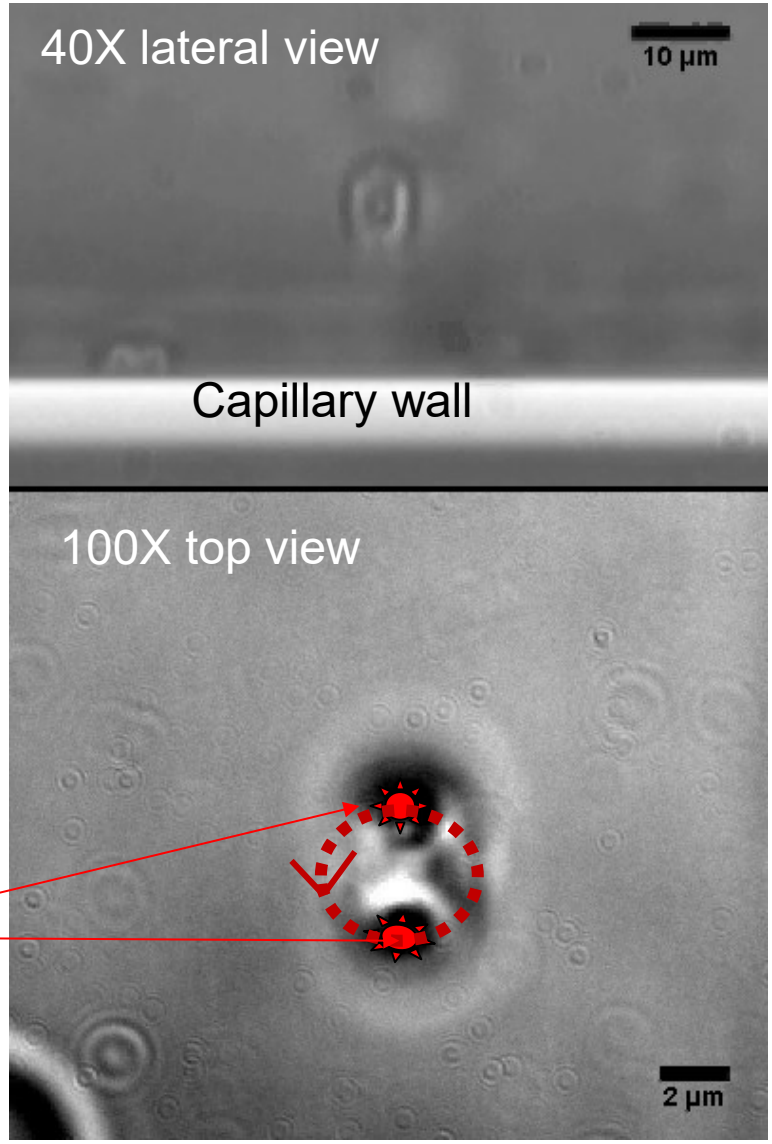
RBC in multiple traps, observed from two sides



(a) Schematic of the two-side imaging setup (not to scale). The red path corresponds to the trapping laser while yellow indicates the imaging paths. I1, I2: illumination, MO1, MO2: microscope objectives, DM: dichroic mirror, M: aluminum mirror, TL1, TL2: tube lenses, C1, C2: cameras, LS: laser source, PPM: programmable phase modulator (generally named Spatial Light Modulator – SLM);
(b) a picture of part of the setup showing the two microscope objectives and the sample cell;
(c) a schematic of the sample cell which allows multi-view imaging of the sample.

L Selvaggi *et al* J. Opt. **12** (2010) 035303

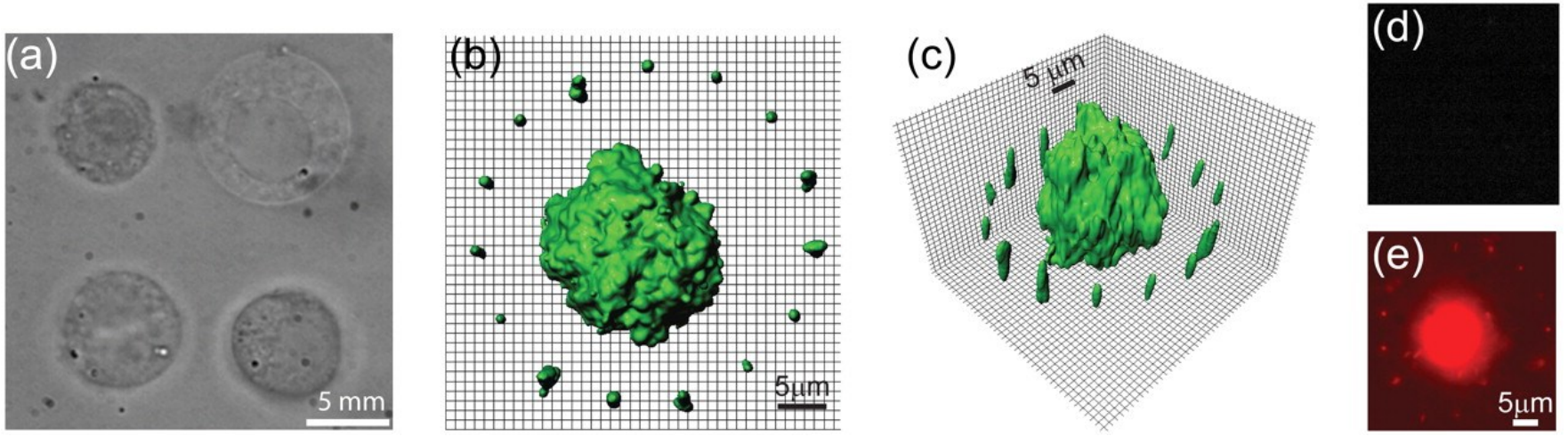
Single RBC manipulated (rotation) by 2 traps and cell rotation monitored by two-side view



two optical traps rotating

Permanent assembly of 3D living cell microarrays

The array is first configured by multiple traps and then the position of the cells is fixed permanently using a photopolymerizable hydrogel



Microarray of **Swiss 3T3 mouse fibroblast** and ***P. aeruginosa* bacteria**.

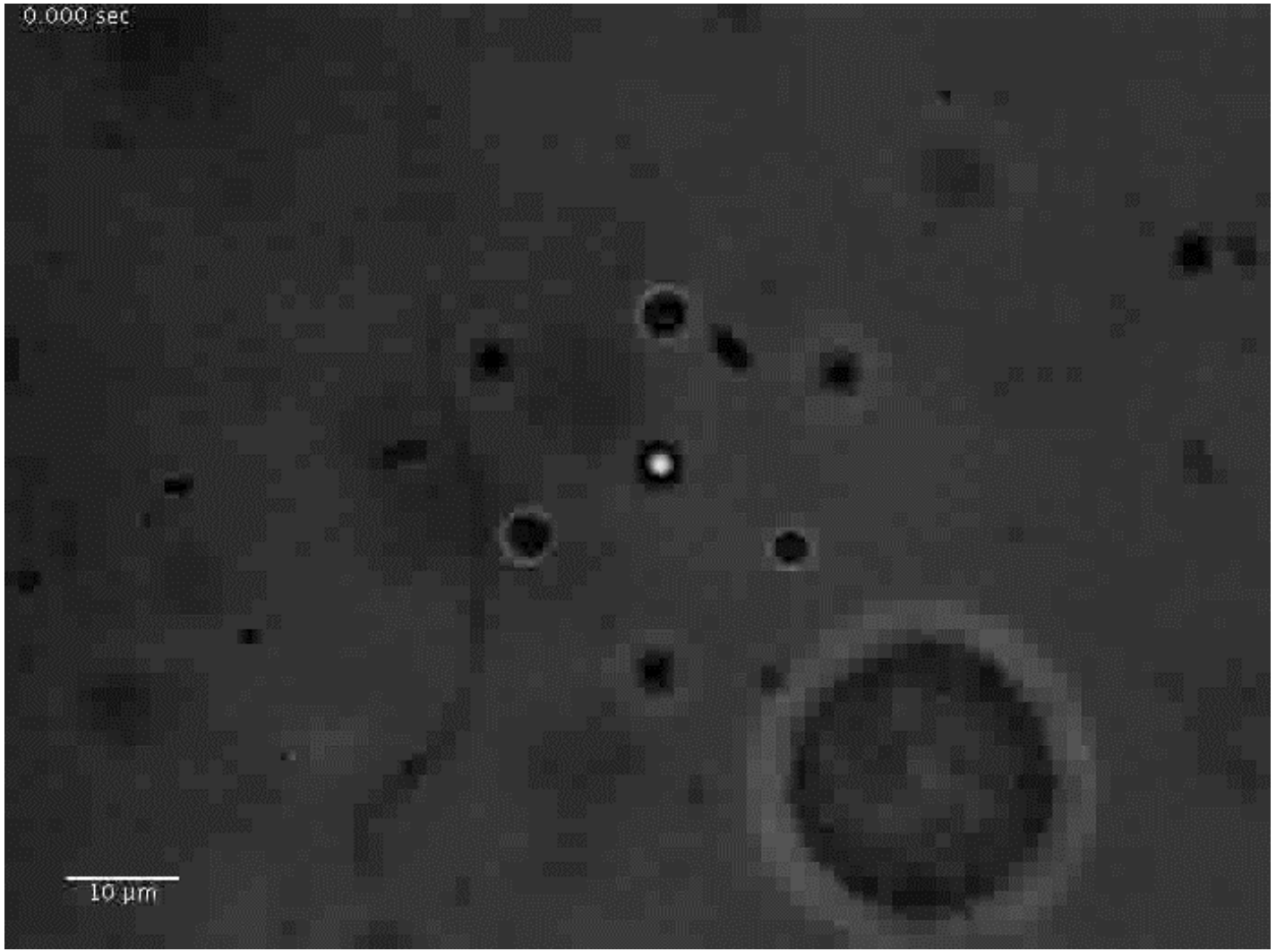
(a) Swiss 3T3 mouse fibroblasts trapped in a 2 x 2 2D array

(b,c) False-color isosurface reconstructions obtained from a confocal image of a Swiss 3T3 cell surrounded by a ring of 16 *P. aeruginosa bacteria.*

(d,e) Viability assay of the same heterotypic microarray showing an image obtained by exciting propidium iodide labels with 488 nm. The lack of red fluorescence in (d) indicates viability, but after killing the cells with ethanol the fluorescence is intensely red (*e*).

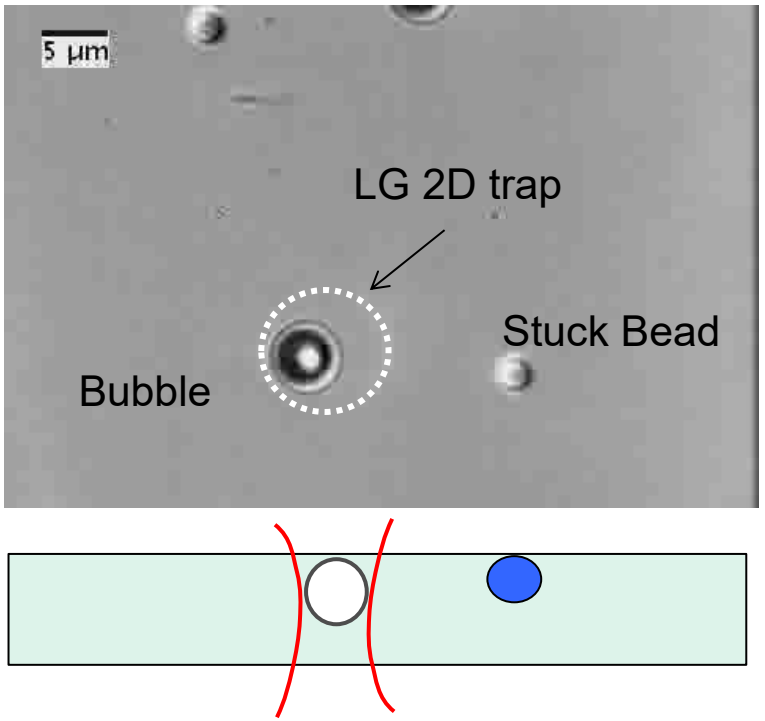
G.M. Akselrod *et al* Biophys J 91, 3465 (2006)

Cell (adherent on substrate) stressed mechanically by a cage of beads

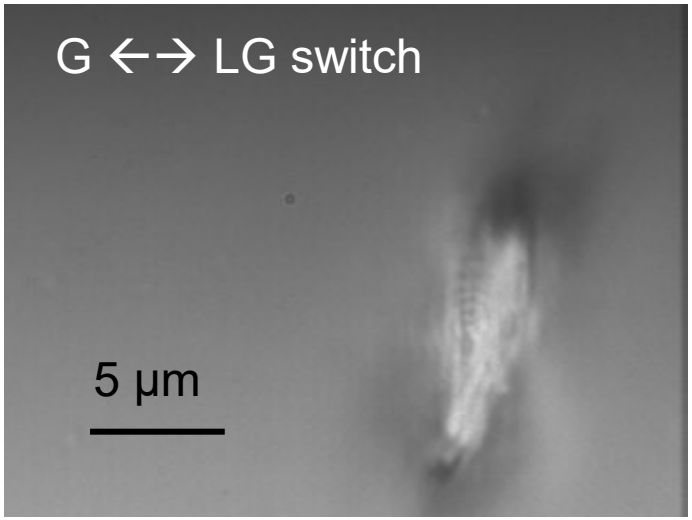


Examples of optical manipulation with Gaussian and LG beams

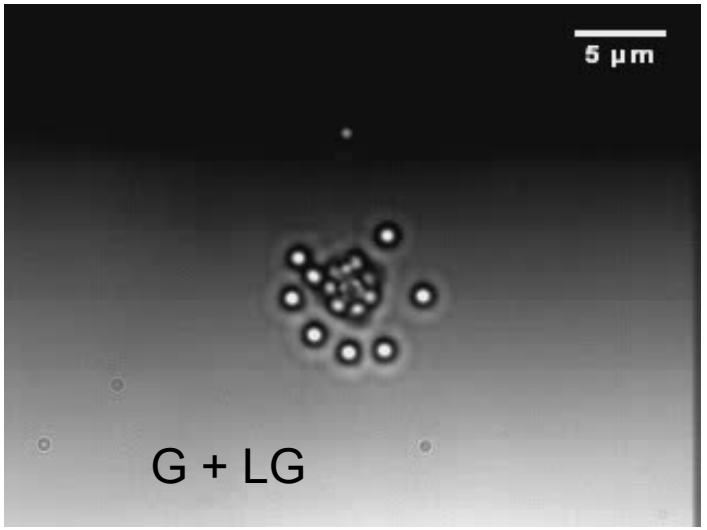
Ultrasound Contrast Bubble – LG 2D trap



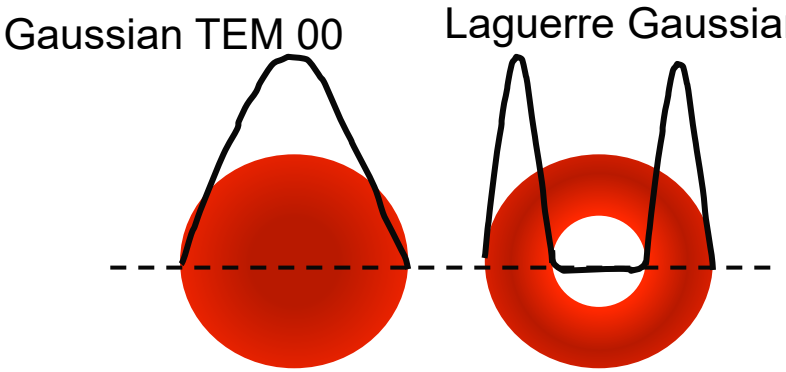
Very simple rotor - piece of glass



LG OAM transfer to silica bead

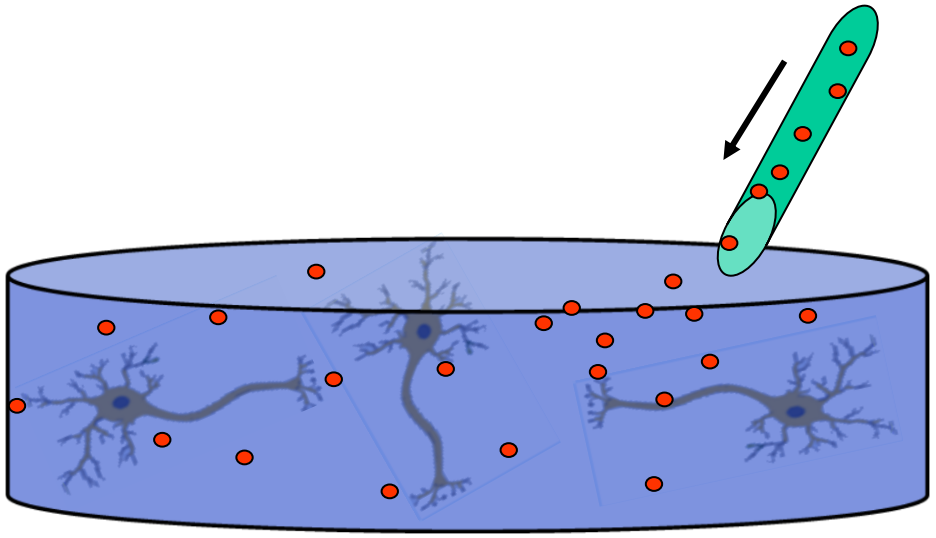


OAM = Optical Angular Momentum



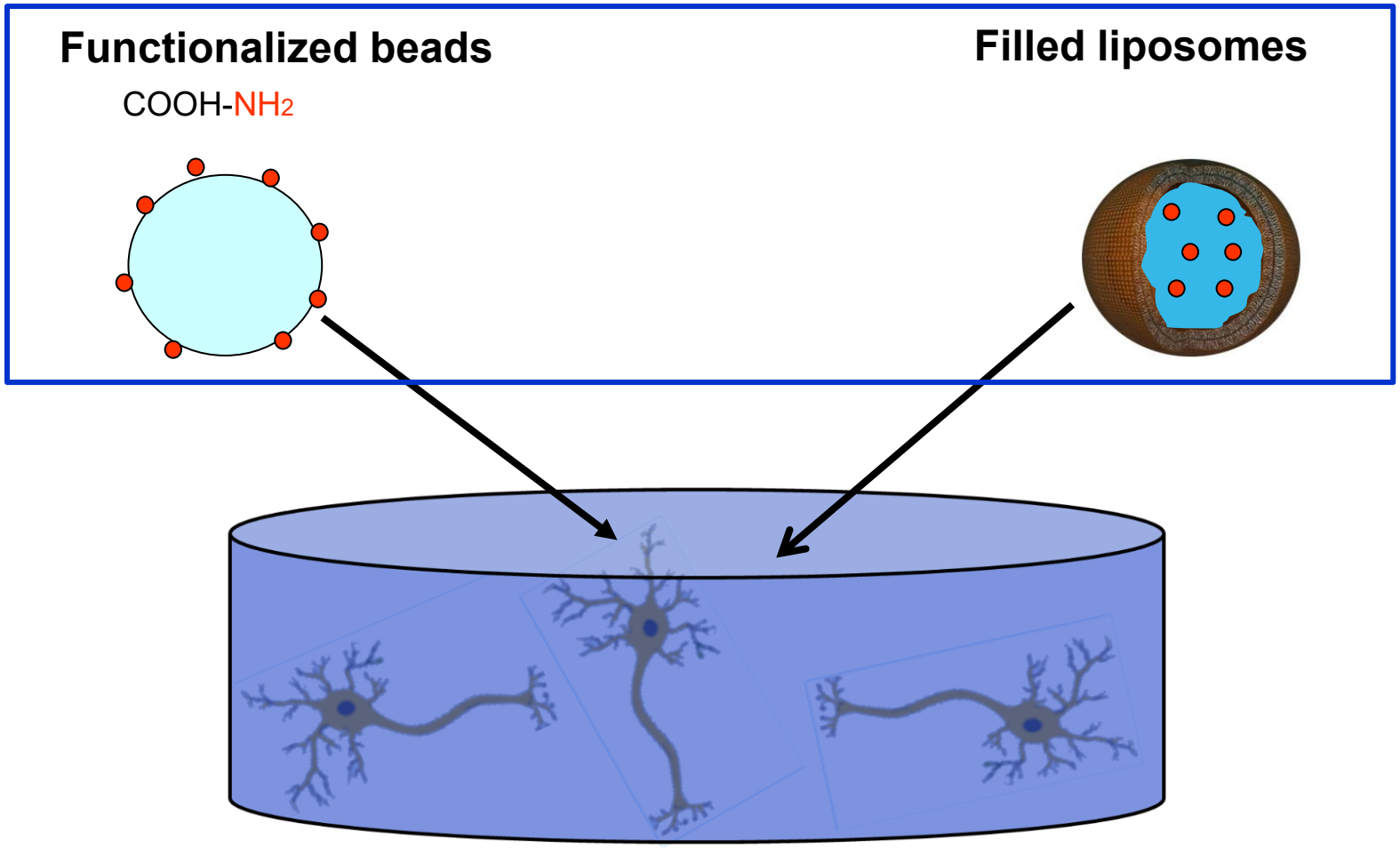
Create physiological inspired experimental conditions !

Classical bath administration of molecules rarely reflects the physiological conditions in which molecules are locally released at low concentrations, creating spatial and temporal gradients.



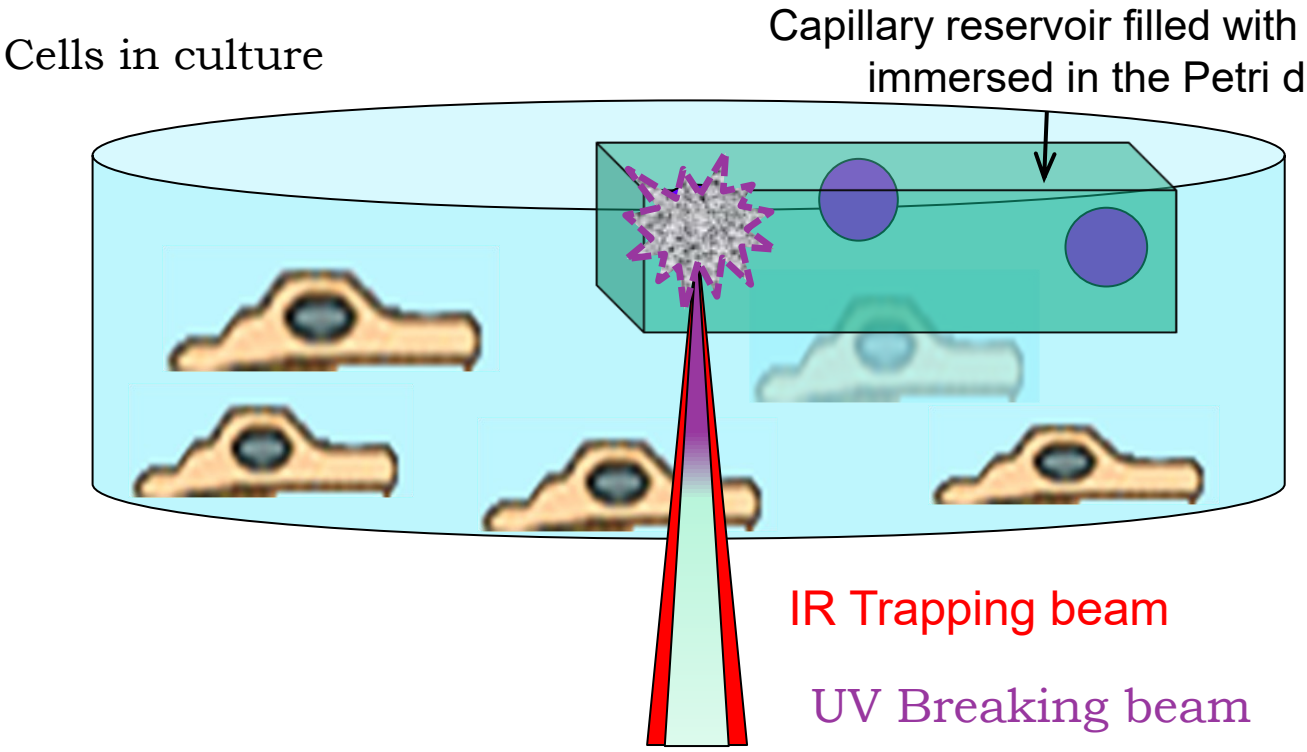
Local stimulation using micro/nano vectors

Active molecules (e.g. guidance cues) are cross-linked to the surface of **microbeads** or encapsulated in **liposomes** (lipid vesicles)



A liposome of 1 µm diameter, filled with 1 nM solution contains 1 MOLECULE (mean value) !!!!!!!!

Vector - Cell Positioning by Optical Manipulation



and delivered by:

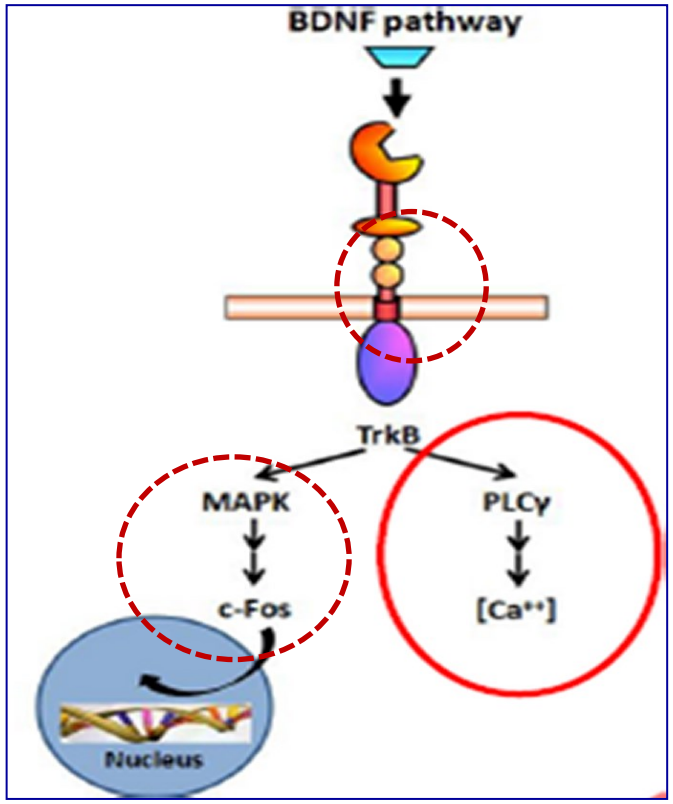
- contact (beads or microsources) – D'Este *et al* Integrative Biology (2011)

- photolysis of liposomes Sun B, Chiu DT , J ACS (2003)

Example 1

Focal stimulation of specific neuronal compartments by optically manipulated microbeads coated with BDNF

Silica beads functionalized with COOH allow cross-linking of any type of proteins on bead surface (beads and kit are commercially available)



A single microbead positioned at about 30 µm from the cell body is enough to:

- increase Ca++ in the cell body and stimulated dendrite
- activate the BDNF receptor TrkB
- Induce c-Fos translocation in nucleus
- increase neurite motility

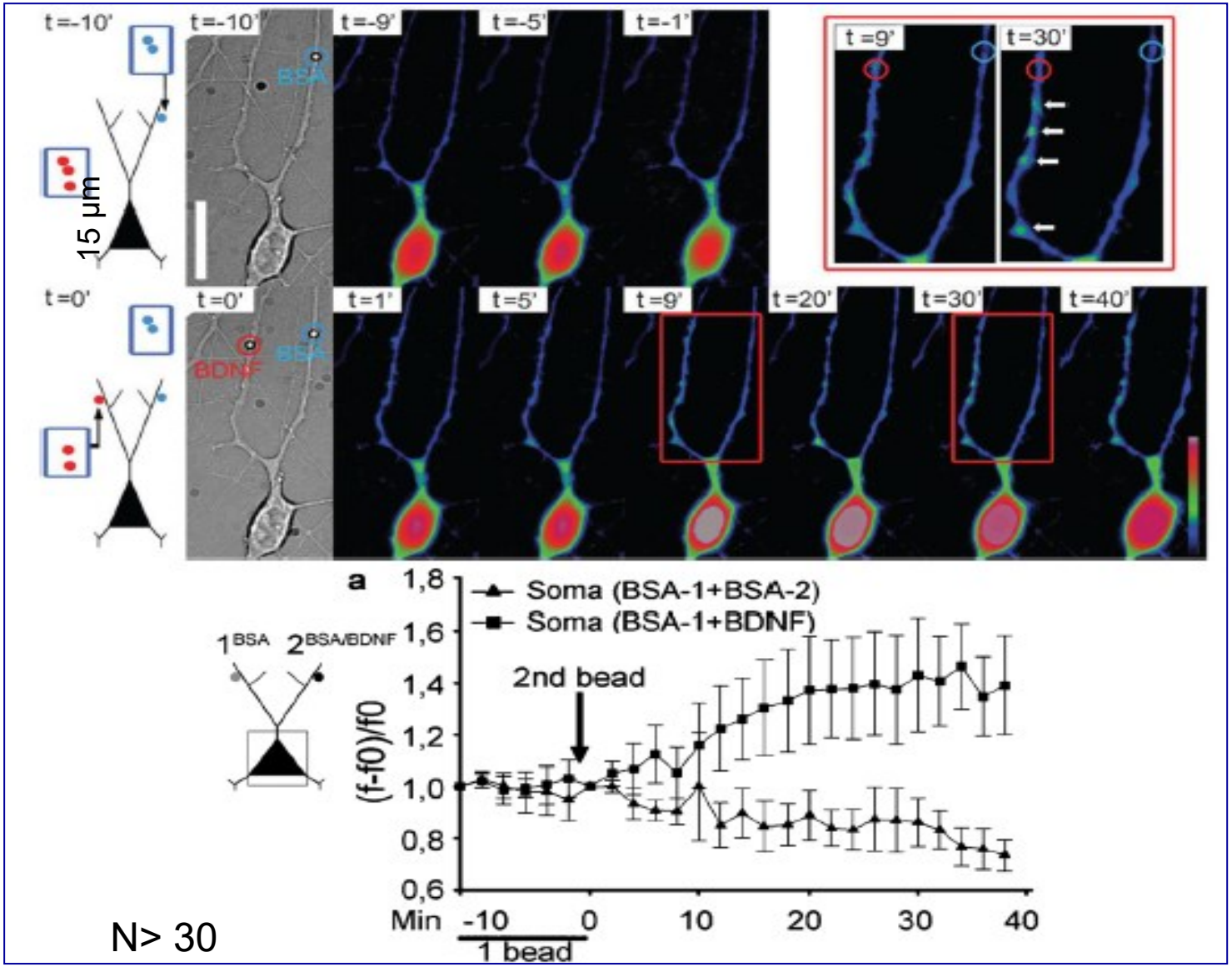
BDNF = Brain Derived Neuotrophic Factor

collaboration with the group of prof. **Enrico Tongiorgi**

BRAIN Centre, University of Trieste

http://www2.units.it/brain/

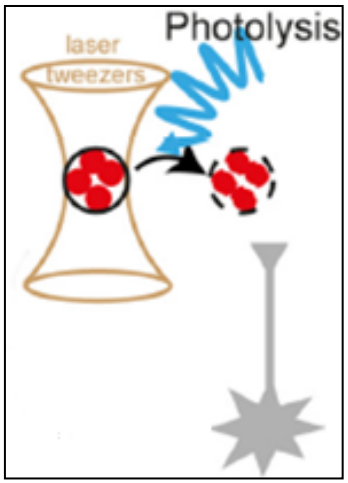
Ca^{++} increases in soma and stimulated dendrite

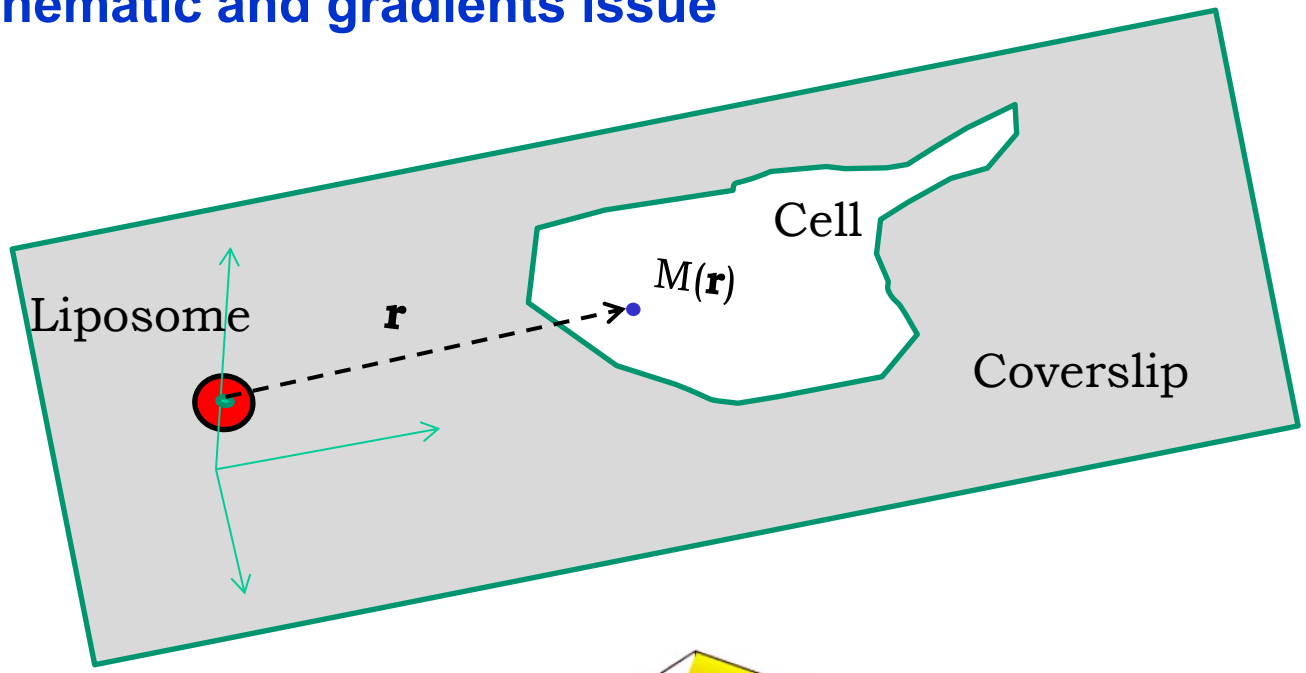


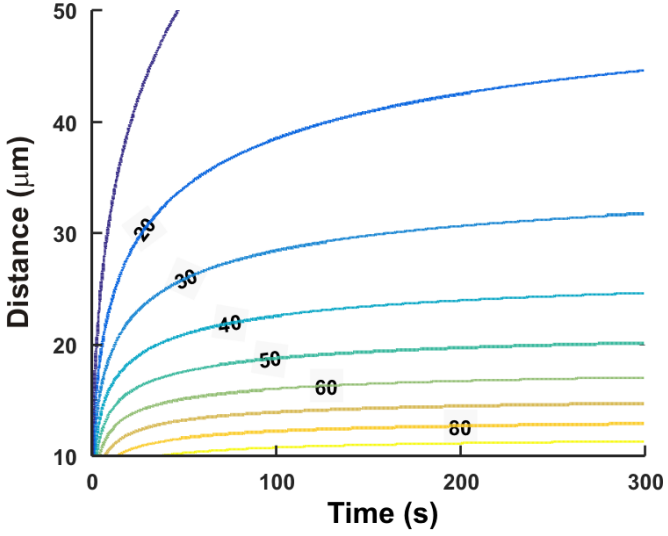
Hippocampal neurons P0-P1 – 1-2 DIV from rats

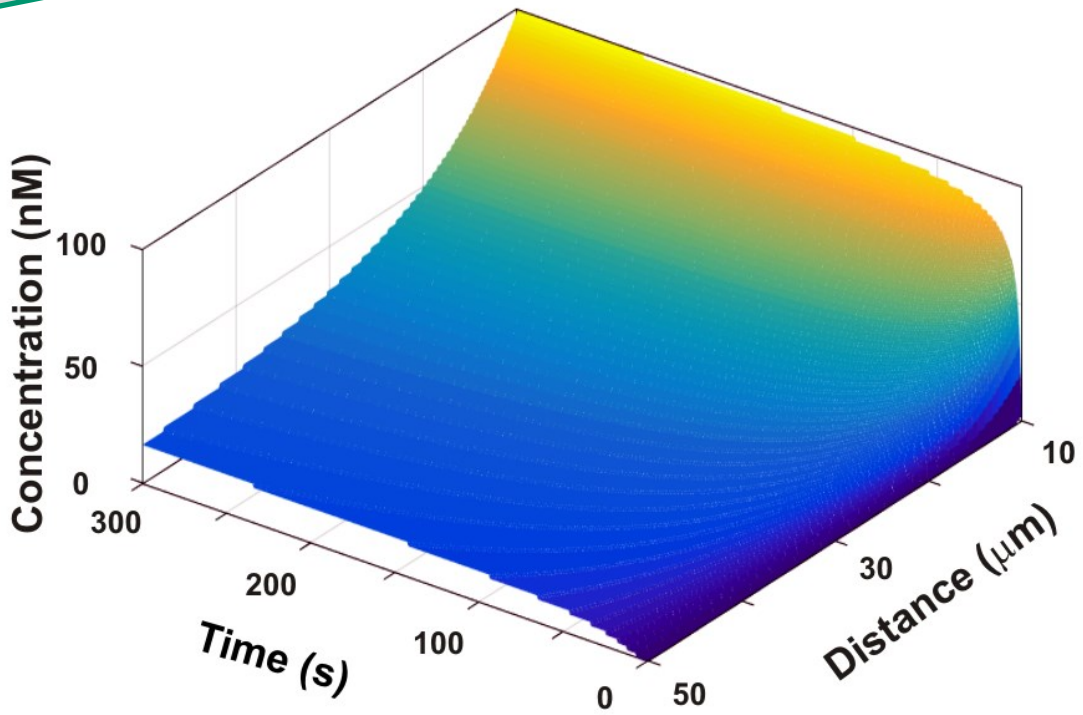
E. D'Este *et al* , Integr. Biol. **3**, 568 (2011)

Release Schematic and gradients issue









Example 2
Focal stimulation of hippocampal neurons by PrP^C

The **cellular prion protein (PrP^C)** is present in all cells, particularly in neurons. PrP^c has been associated with many cellular processes, including the **regulation of ion transport, neuritogenesis, cell survival, cell-to-cell interactions, cell signaling and synaptic transmission** (Linden *et al.* 2008).

Characterization of prion protein function by focal neurite stimulation

Ladan Amin[1], Xuan T. A. Nguyen[1], Irene Giulia Rolle[1], Elisa D'Este[2], Gabriele Giachin[1,*], Thanh Hoa Tran[1], Vladka Čurin Šerbec[3], Dan Cojoc[4,‡] and Giuseppe Legname[1,‡]

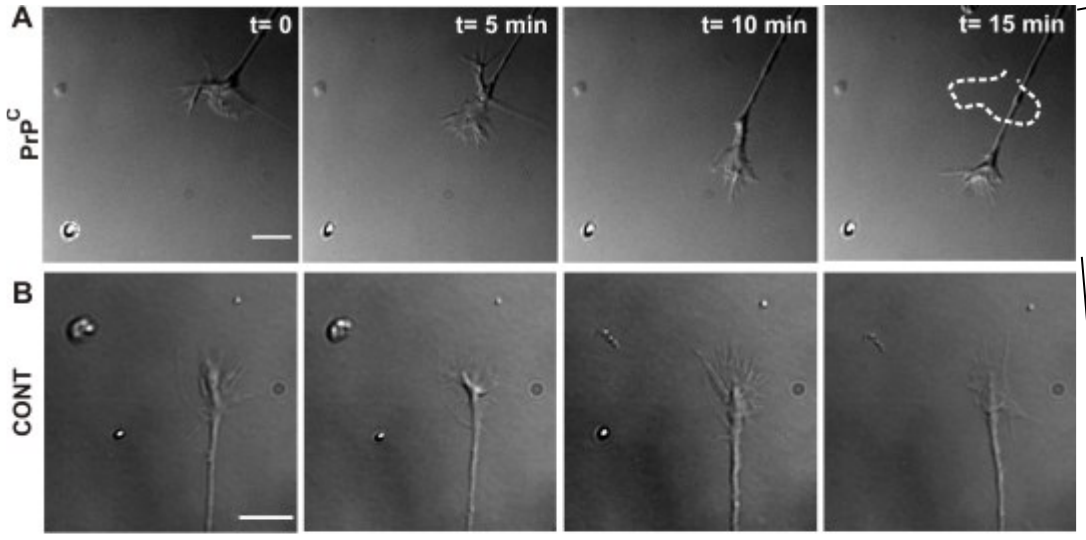
Journal of Cell Science (2016) 129, 3878-3891 doi:10.1242/jcs.183137

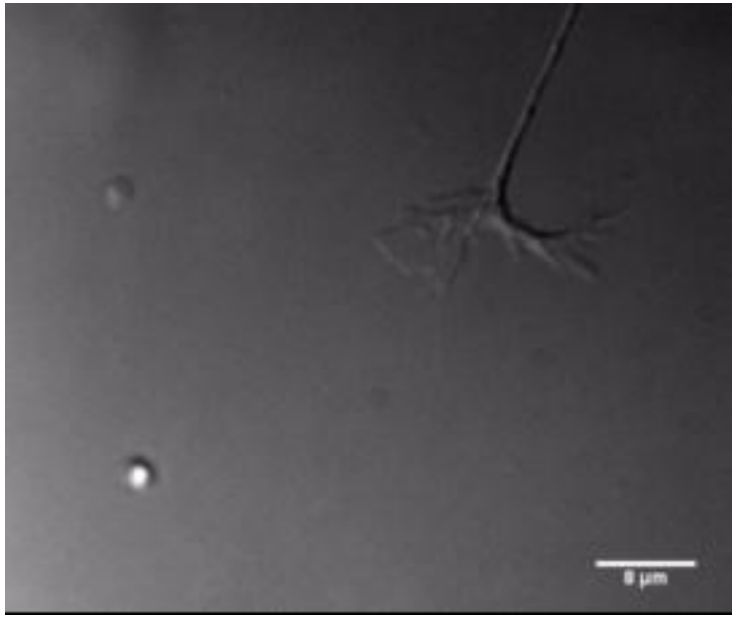
PrP^C encapsulated in lipid microvesicles or cross-linked to the surface of microbeads

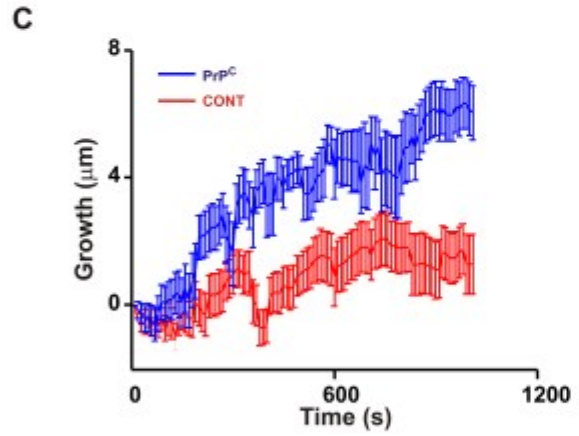
We found:

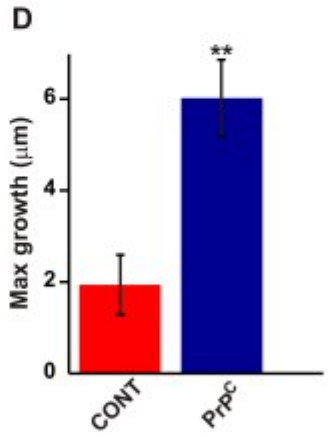
- recPrP^C works as a guidance molecule
- membrane PrP^C is required for the extracellular PrP^C to bind (PrP^C might be the receptor of itself)
- full length PrP^C is required to have the guidance function
- concentration modulates the GC growth

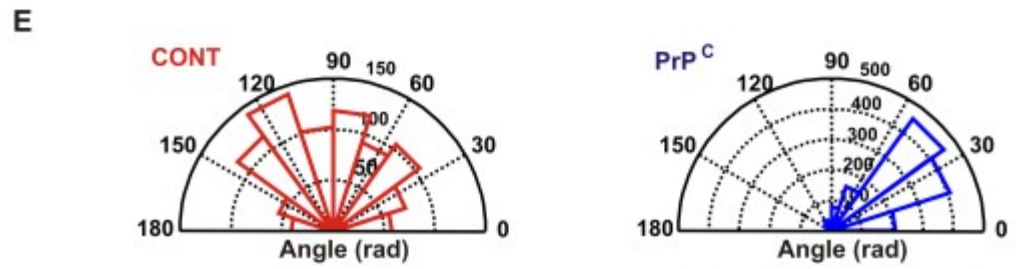
Local delivery of controlled amount of MoPrP^{c} to neurons











Neurite growth is observed in 15 min after local stimulation.

Stimulation by bath administration induced this effect **after 24** h incubation. (Kanaani 2005).

Control liposomes (BSA) do not induce growth or turning. .

PrpC KO neurons do not respond to the stimulation with PrPc

Hippocampal neurons frome mouse P0-1, 1-2 DIV

Amin *et al*, J, Cell Science 2016

Example 3

Signal transduction dynamics

Local stimulation + FRET microscopy

Stimulating the GC with coated beads or liposomes with **Sem3A.**

- Signal transduction makes effective the stimulation effect. This mechanism is very complex and is regulated by many "players" among which the GTPases: Rac1, RhoA and Cdc42, which act together to control cytoskeleton dynamics. [Machacek, M, …& Danuser, G, Nature 461, 99 (2009)].
- **Goal: vizualize the RhoA and Cdc42 activation and their dynamics upon local stimulation with Sem3A**
- **Study case:** Ng 108-15 neuroblastoma cells

Project in collaboration with the group of prof. **Vincent Torre**
Neurobiology Sector, **SISSA, Trieste**

Sem3A = Semaphorin 3A
is a guidance (repellant) molecule released by neurons during their differentiation

GTPase = hydrolyse enzymes that can bind and hydrolyze guanosine triphosphate (GTP)

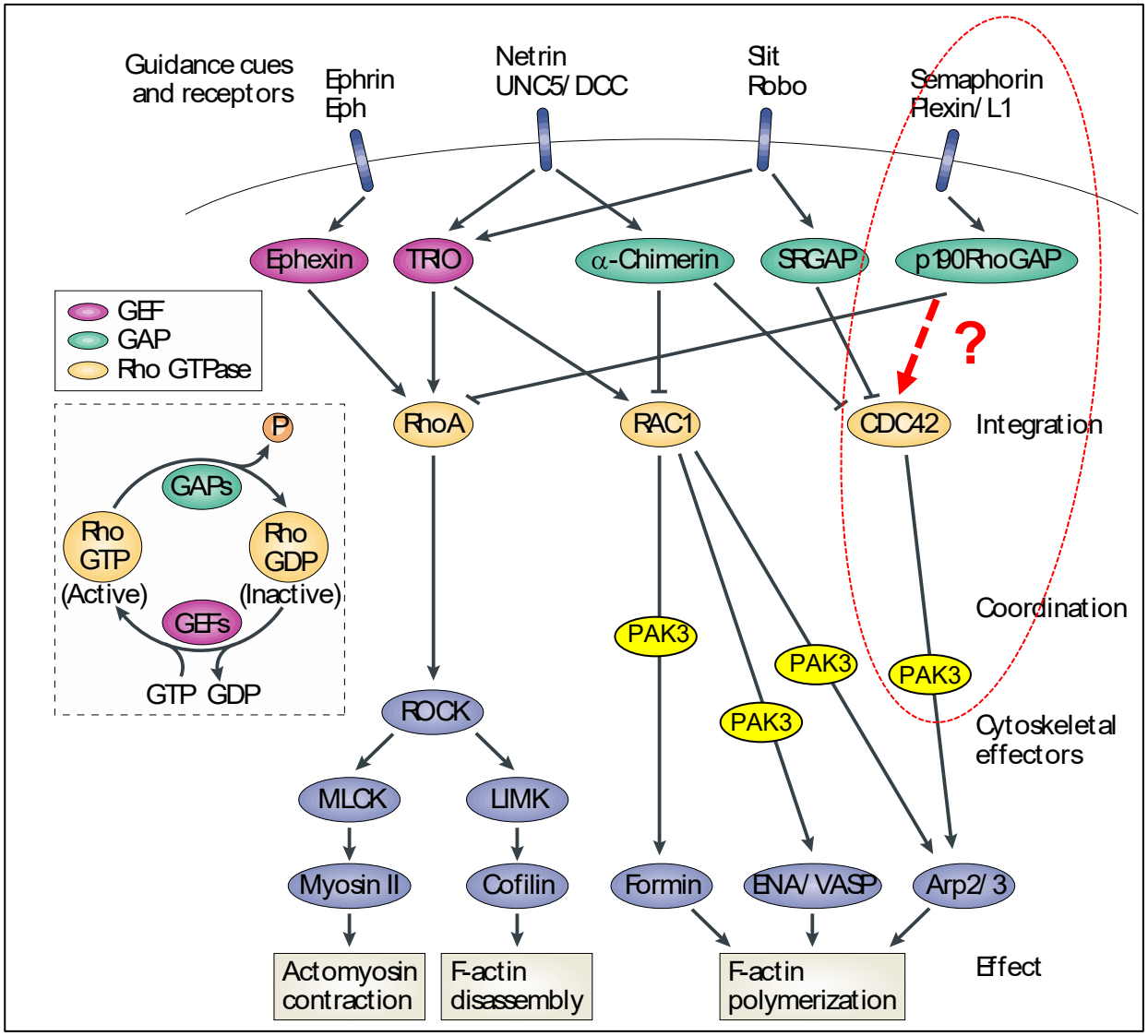
RhoGTPases are signalling nodes that couple upstream directional cues and downstream cytoskeletal rearrangements to either enhance actin polymerization for protrusion or promote disassembly and actomyosin contraction for retraction.

GTPases are a large family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP).

PAK proteins are critical effectors that link Rho GTPases to cytoskeleton reorganization and nuclear signaling.

They serve as targets for the small GTP binding proteins Cdc42 and RAC

Guidance cues signaling pathways

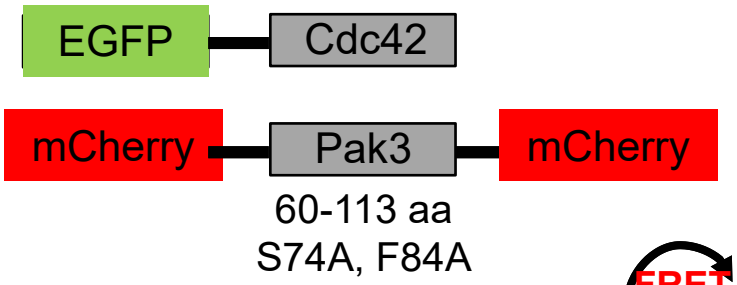


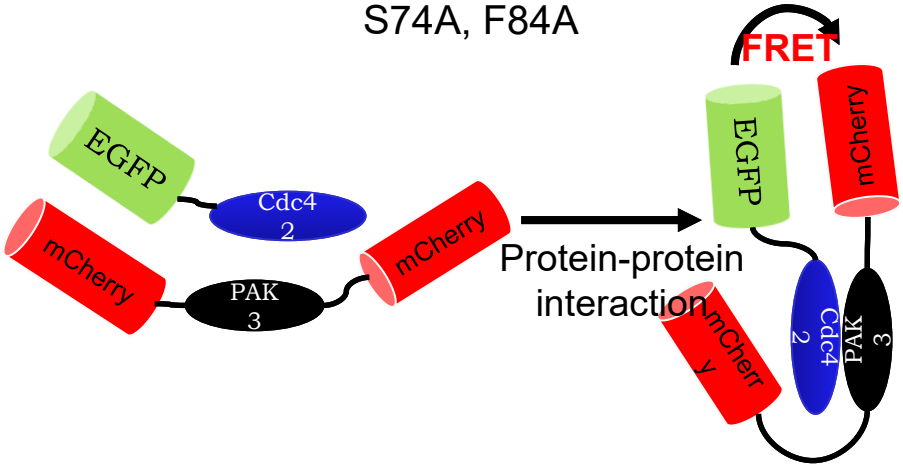
Lawery L.A. Van Vactor D. *Nature Rev-Mol Cell Biol (2009)*

FRET probes

Inter - Molecular

Cdc42 FRET sensor

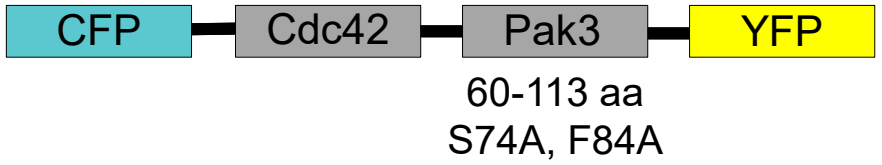


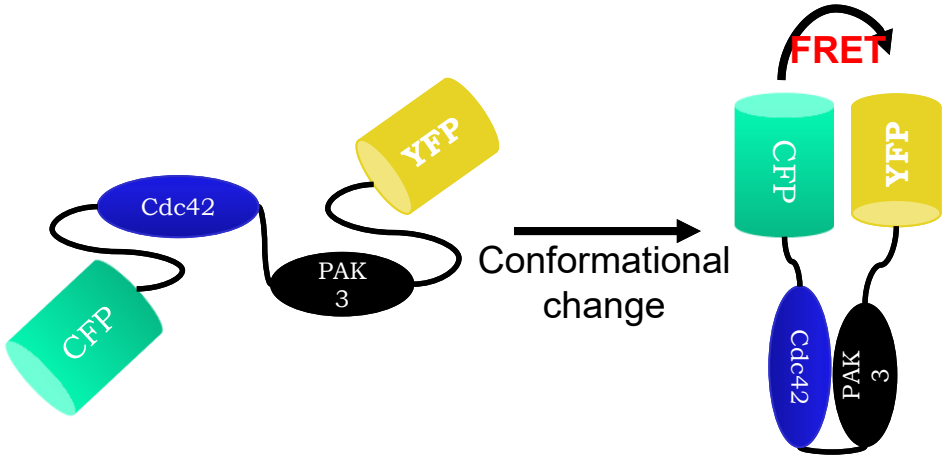


- Suitable for Protein-Protein interaction studies;
- Fluorophore Stoichiometry uncertain.
- Sensitized FRET.

Intra - Molecular

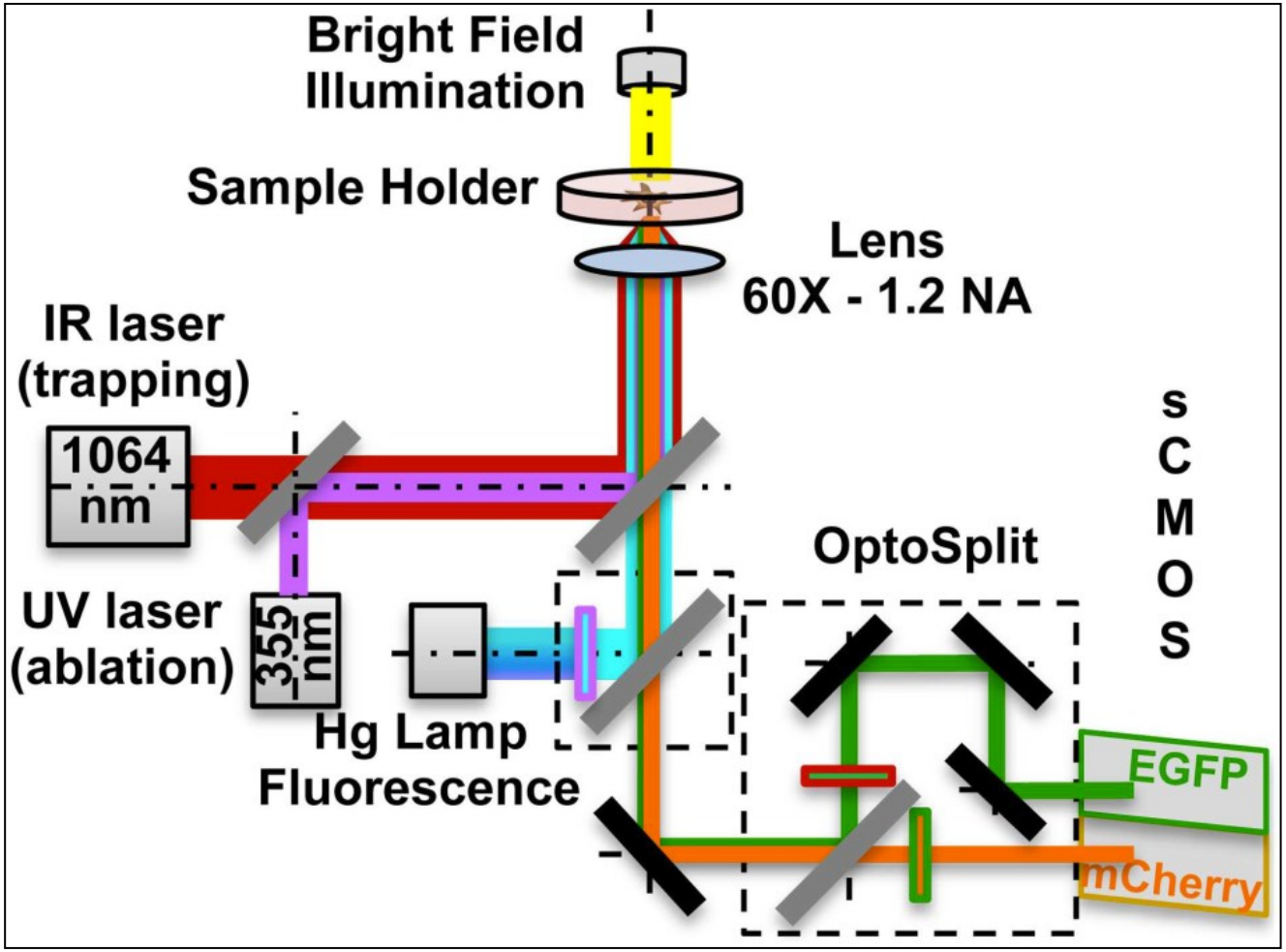
"Raichu" Cdc42 FRET sensor





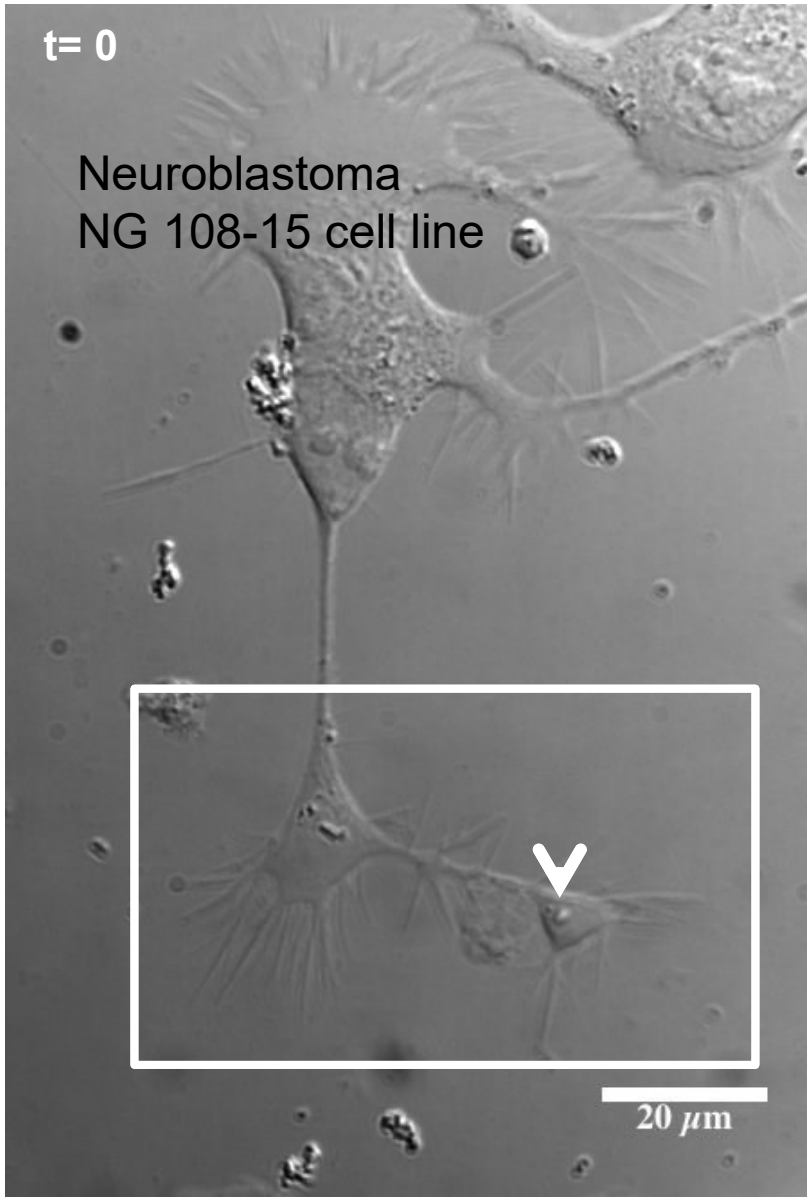
- Suitable for Protein activation studies;
- Fluorophore Stoichiometry 1:1;
- Ratiometric FRET

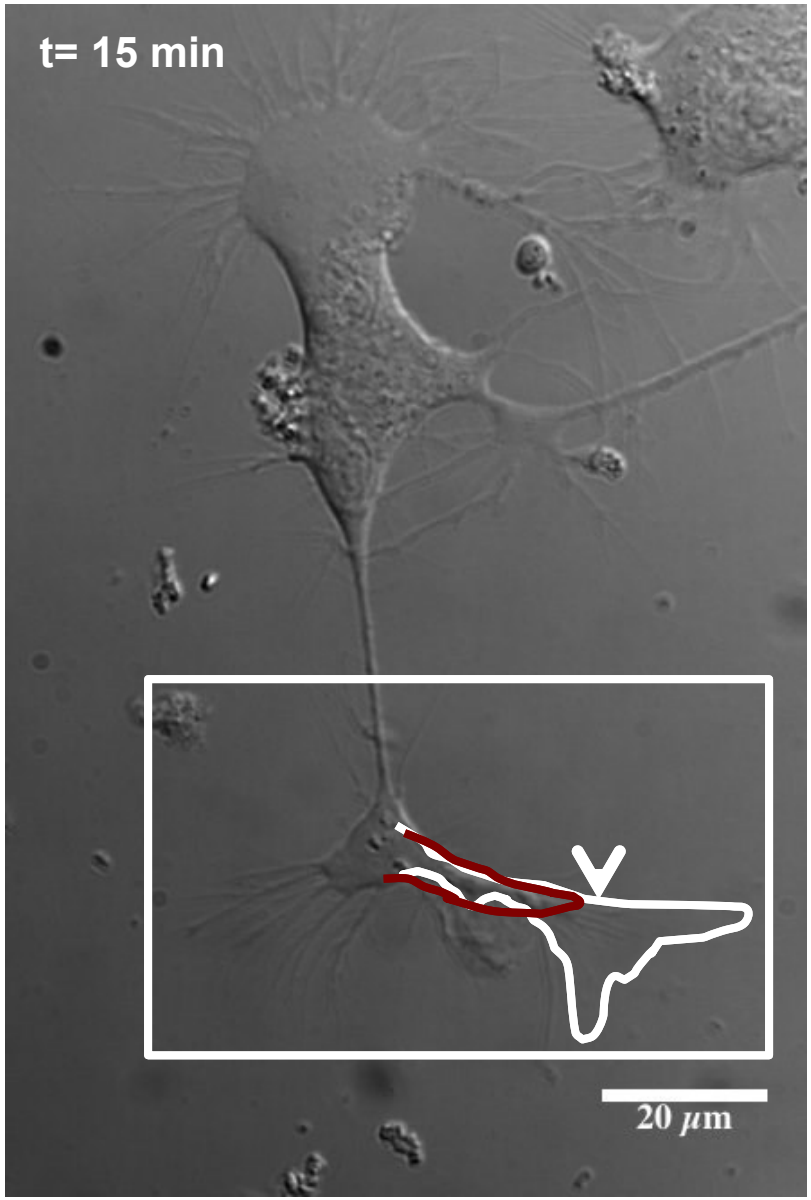
OT local stimulation – FRET imaging setup



Iseppon F *et al* Frontiers Cell. Neuroscience, 2015

Local stimulation: SemA3 bead positioned on the GC and kept in contact for 30 s



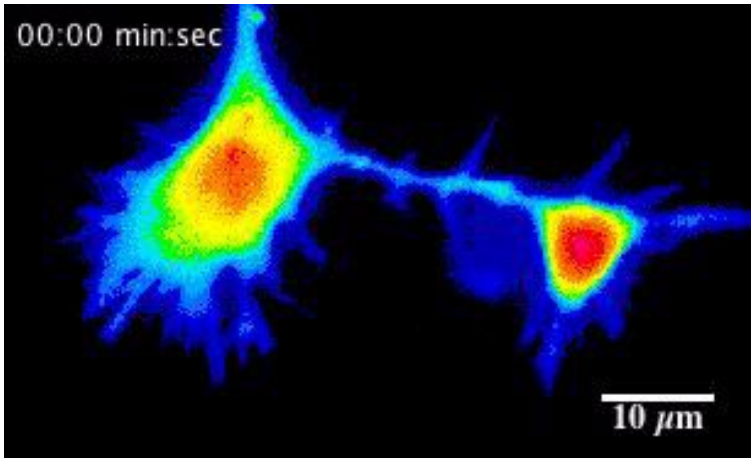


After 30 s the trap is switched off and the bead released.

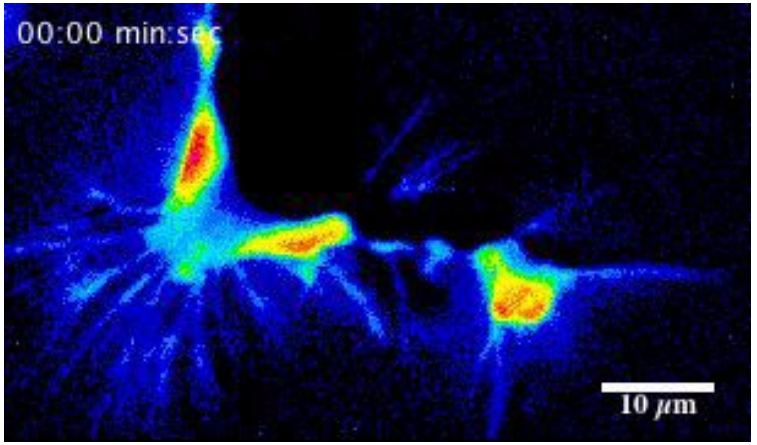
The GC retracts about 15 um after t= 15 min

Dynamics of the Cdc42 activation

using a Cdc42 FRET probe based on mEGFP and mCherry



Spontaneous FRET
before stimulation
(Control)



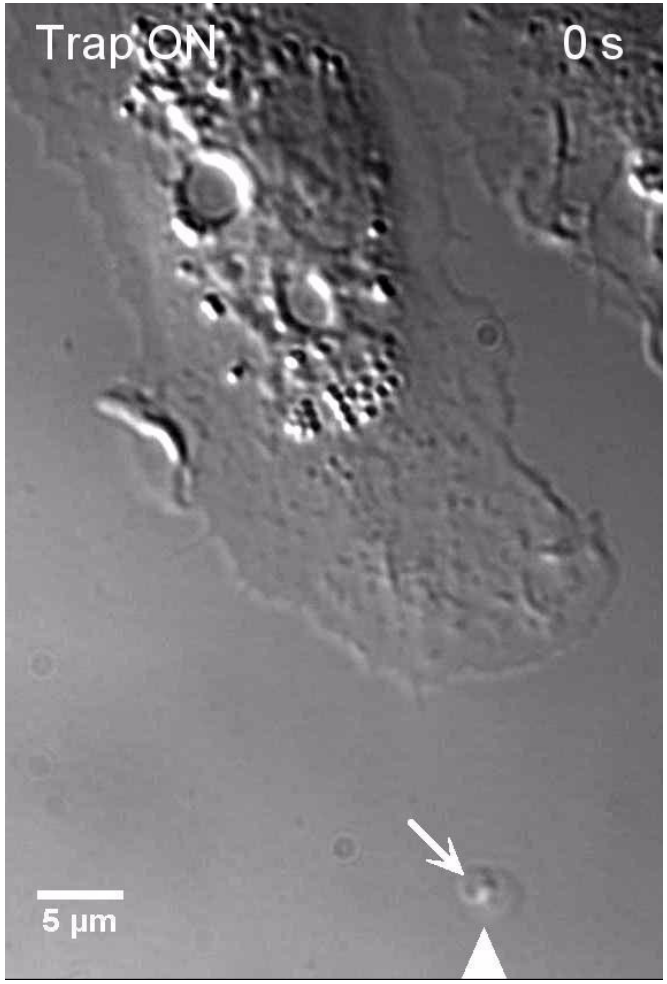
FRET after
stimulation with
SemA3 bead

Iseppon F *et al* Frontiers Cell. Neuroscience, 2015

Iseppon *et al* J. Biol. Meth. 2017

Example 5

Extracellular Vesicles (EV)



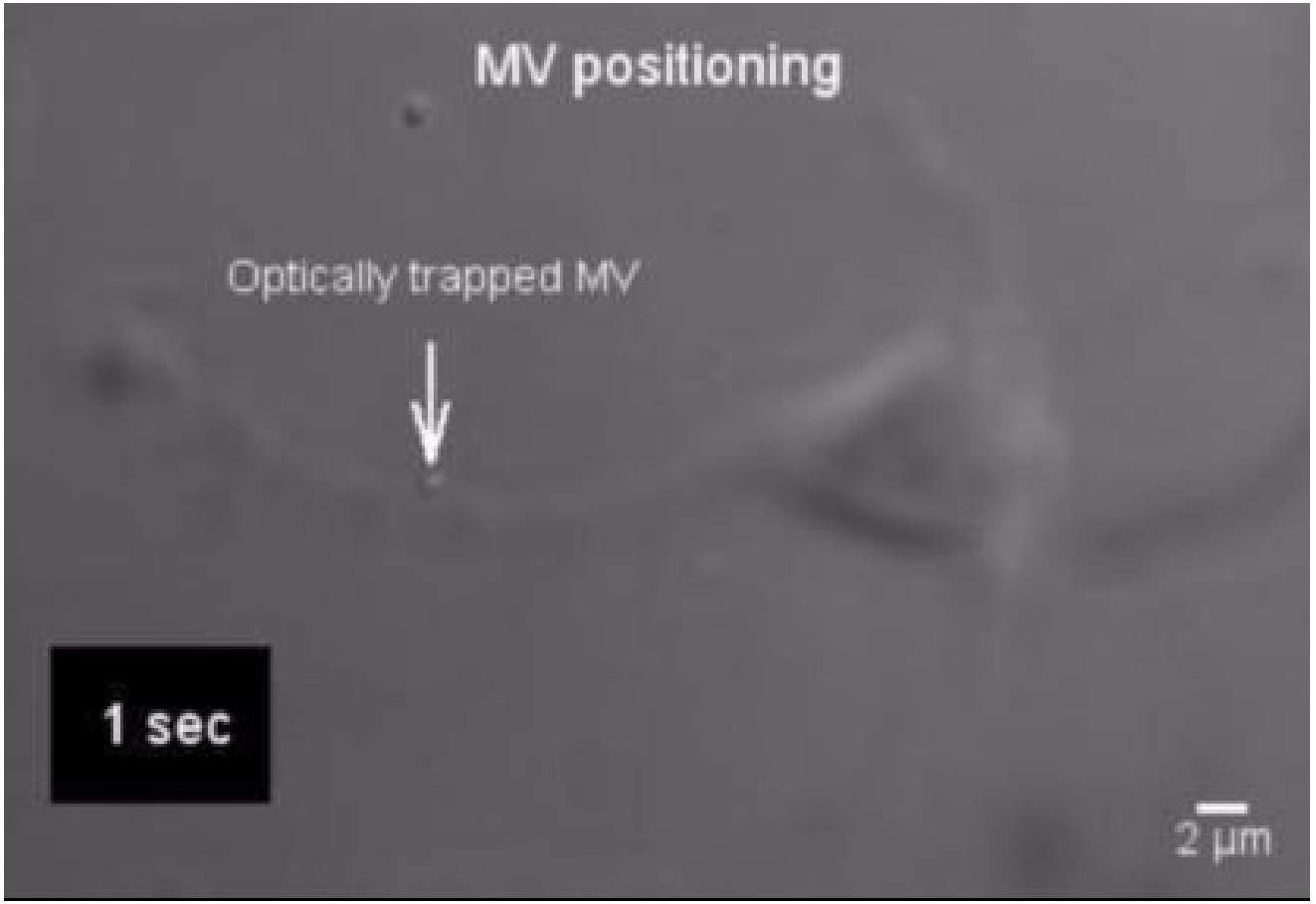
EV from microglial cells on a microglia cell.

- EV are circular membrane structures released by most cells which represent highly conserved mediators of intercellular communication.
- EV carry proteins, lipids and genetic materials and transfer these cellular components between cells by different mechanisms, such as endocytosis, macropinocytosis or fusion.
- Temporal and spatial dynamics of vesicle-cell interaction still remain largely unexplored

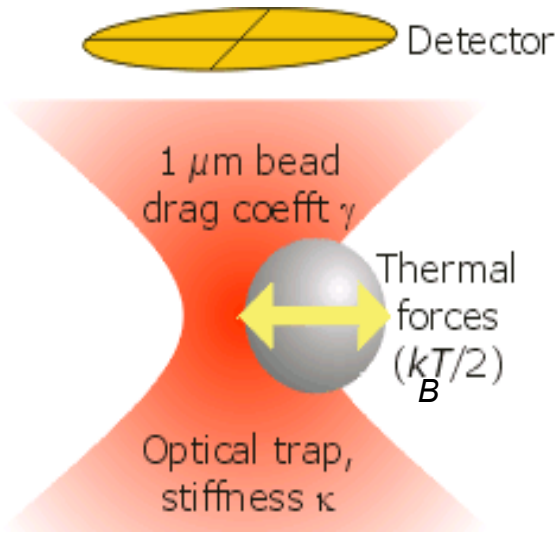
Collaboration:
Claudia Verderio - CNR-Institute of Neuroscience Milan
Roberto Furlan – San Raffaelle, Milan
Giuseppe Legname – SISSA, Trieste

Prada I *et al* BioTehniques, (January 2016)

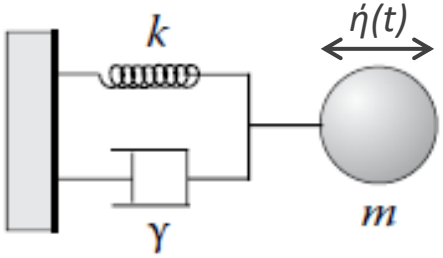
Interaction between a microglial microvesicles (MVs) and a neuron: adhesion and transport



Beside trapping and manipulation OT can measure forces using the trapped bead as probe



Schematic of a microbead in an optical trap



Equation of motion (Langevine) of the overdamped oscillation of a particle in the optical trap for a harmonic potential

$$m\ddot{x}(t)+\gamma_0\dot{x}(t)+\kappa x(t)=(2k_BT\gamma_0)^{1/2}\eta(t)$$

$$\dot{x}(t)+2\pi f_c x(t)=(2D)^{1/2}\eta(t)$$

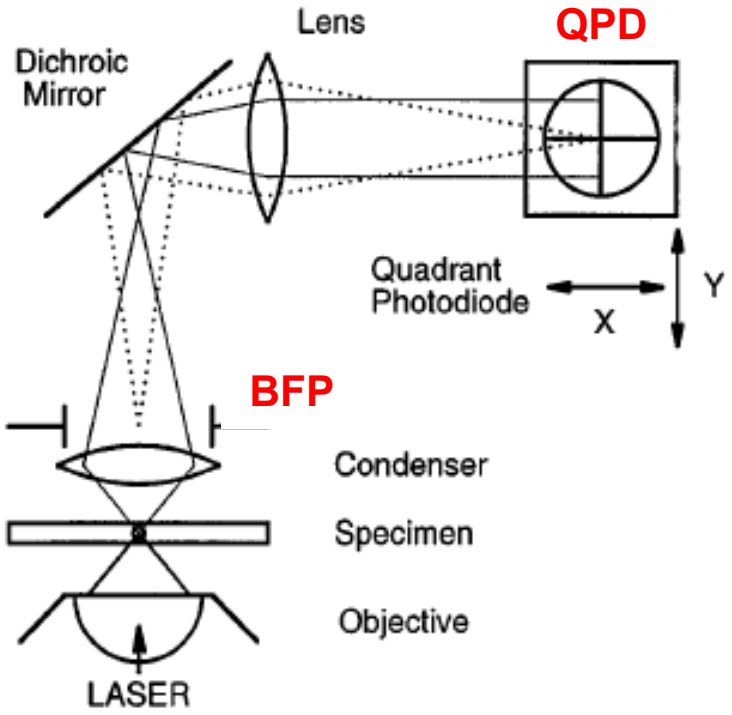
$$f_c\equiv\kappa/(2\pi\gamma_0) \qquad D=k_BT/\gamma_0$$

k – trap stiffness, *fc* – corner frequency,
D - diffusion coefficient, γ_0 - friction coefficient

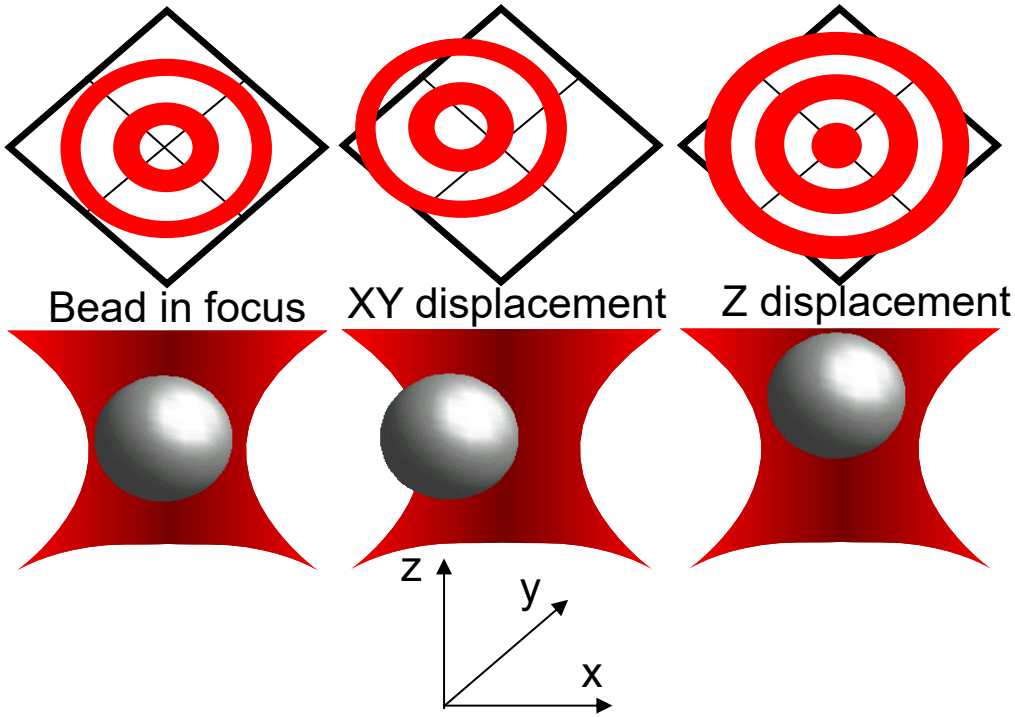
Due to the optical force, the natural Brownian motion of the trapped bead is confined to the trapping region, near the focus of the objective.

Tracking the probe/bead with nm resolution

Bead position is determined by back focal plane (BFP) interferometry



Interference pattern – position detection in X-Y and Z



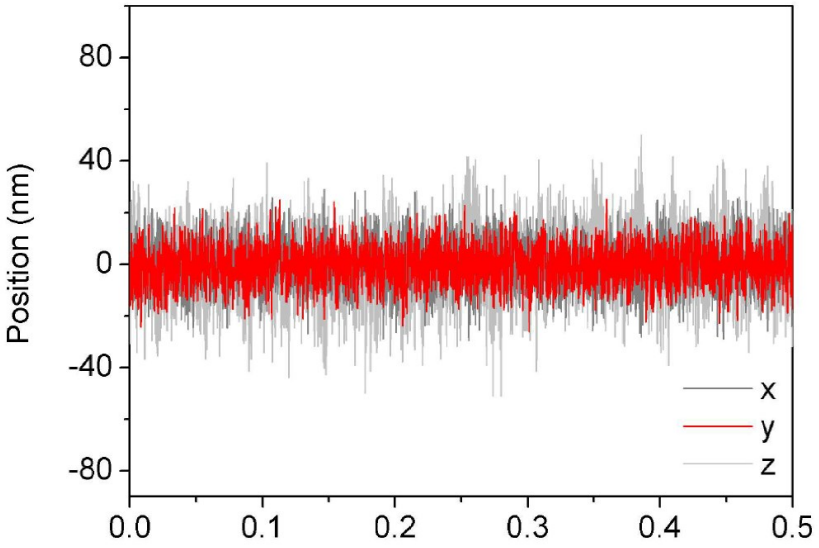
The interference pattern formed by the interference of the laser light scattered by the bead in the BFP is imaged onto a QPD

Sensitivity of the QPD is measured using a stuck microbead on the coverslip and a piezo to move the coverslip in controlled nm steps .

F. Gittes, Optics Letters, 1998

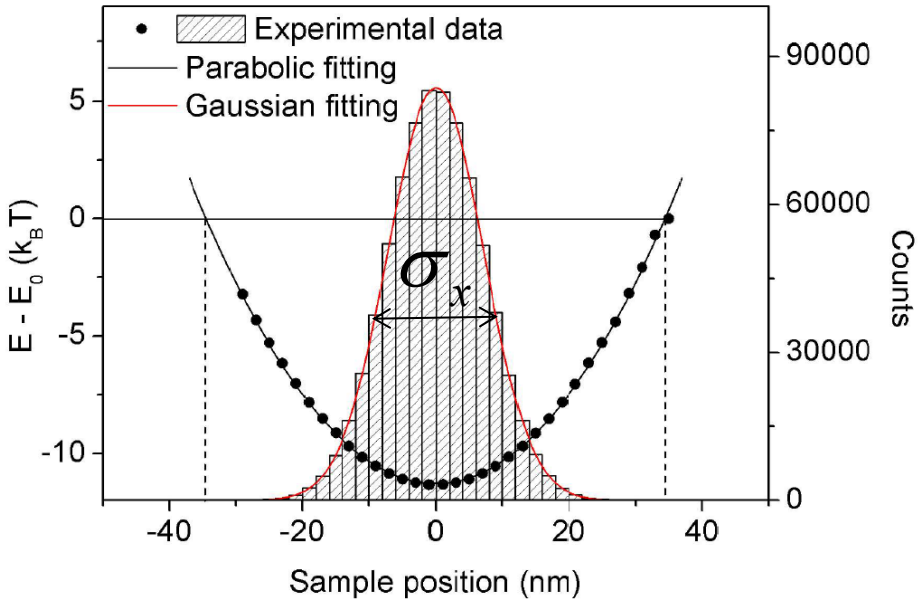
Determining the trap stifness, k

Track the bead position in the trap



X, Y, Z - bead in trap

Position histogram, potential energy



Probability density of the bead position (Boltzmann distribution)

$$\rho(x,y)=C\exp\left(\frac{-U(x,y)}{k_B T}\right)$$

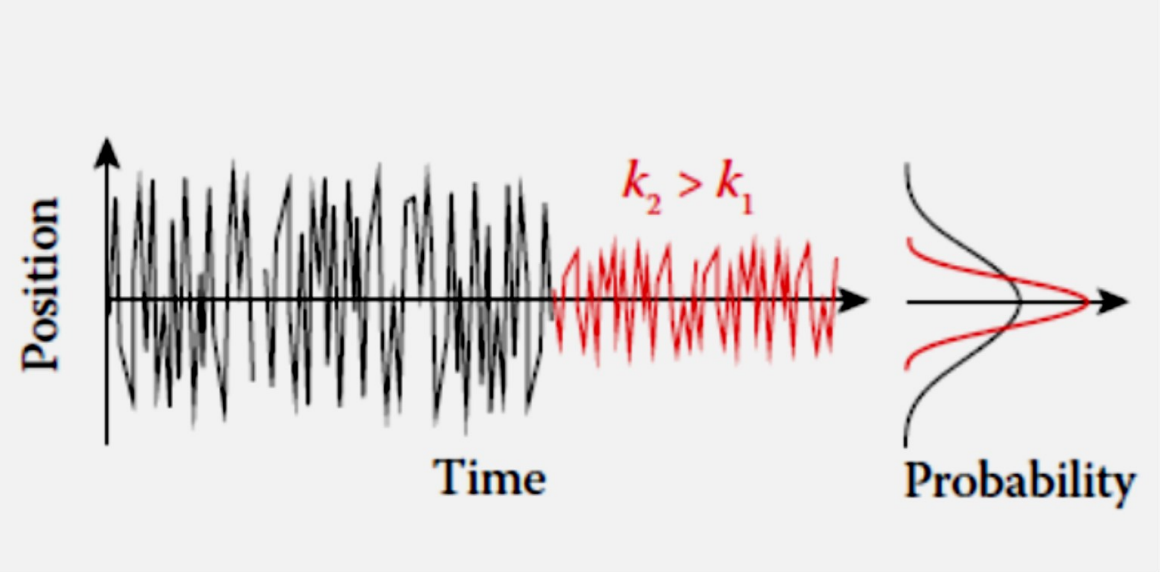
C- Constant

$$\rho(x,y)=C\,e^{\frac{-k_x x^2}{2k_B T}}\,e^{\frac{-k_x y^2}{2k_B T}}$$

$$k_x=\frac{k_B T}{\sigma_x^2}$$

$$k_y=\frac{k_B T}{\sigma_y^2}$$

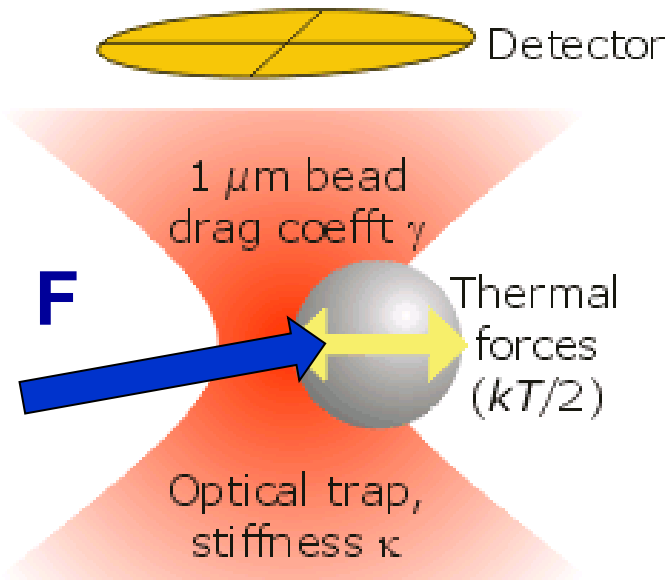
Example of two tracking traces of a trapped bead , with different stiffnesses



$$\frac{1}{2}k_BT = \frac{1}{2}k\langle x^2\rangle \quad \Rightarrow \quad \langle x^2\rangle = \sigma_x^2 = \frac{k_BT}{k} \qquad p(x) = \frac{1}{Z}\exp\left[-\frac{U(x)}{k_BT}\right] = \frac{1}{Z}\exp\left(-\frac{x^2}{2\frac{k_BT}{k}}\right)$$

Measuring an external force exerted on the bead

Measuring the displacement Δ of the particle and knowing the stiffness of the trap K we get F:



$$F = K \Delta$$

F = (Fx, Fy, Fz) Force

K = (Kx,Ky,Kz) stiffness of the trap

Δ = (Δx, Δy, Δz) Displacement

OT allows measuring forces in 3D !

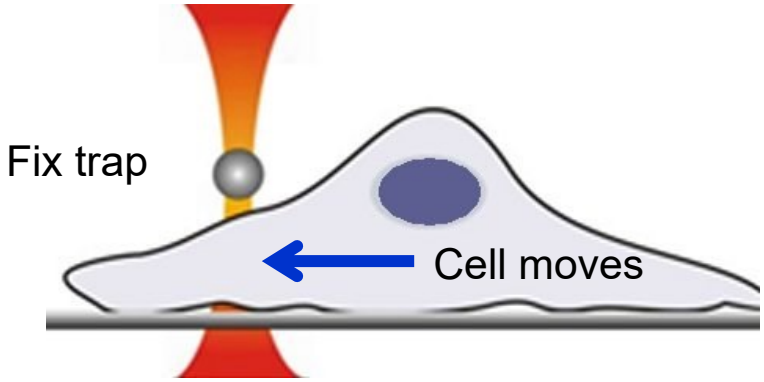
Typical values for **OT : K_{OT} = 0.001 – 0.5 pN/nm**

Typical values for **AFM: K_{AFM} = 1 – 1000 pN/nm**

OT and AFM are complementary Techniques

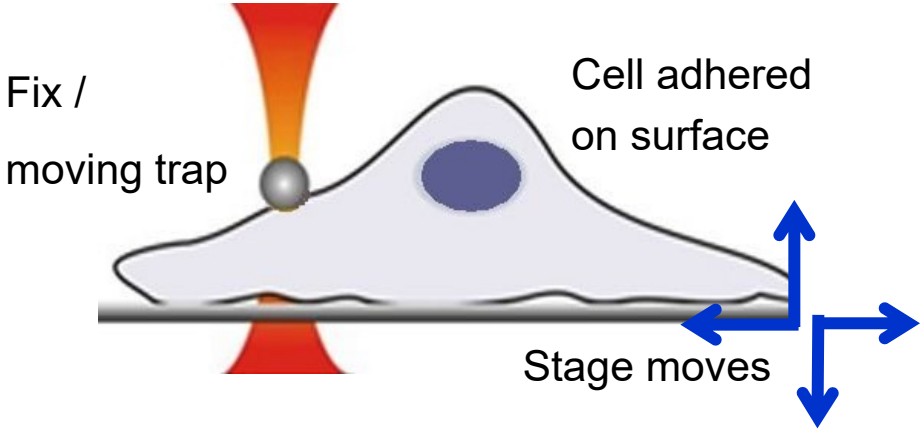
Optical Tweezers to locally probe living cells

(experimental approaches)



Touch / intercept

Measure forces when the cell or part of the cell moves



Pull / Push

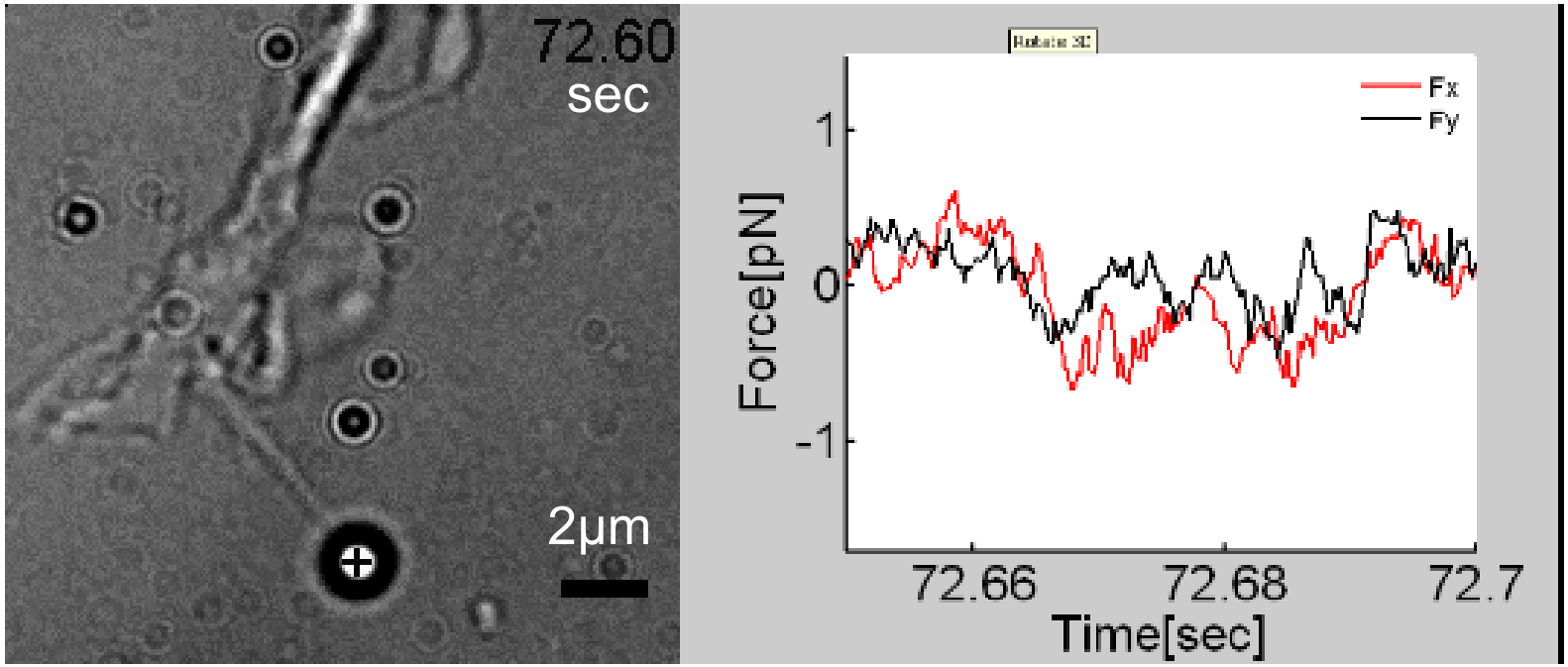
Local adhesion / binding

Local viscoelasticity (tether membrane, indentation)

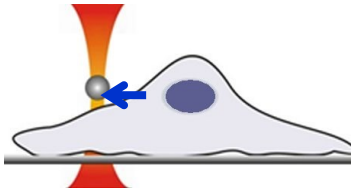
Local mechanical stimulation - mechanotransduction

Force exerted by Filopodia of Growth Cone during Protrusion

2 Days In Vitro hippocampal neuron from mouse



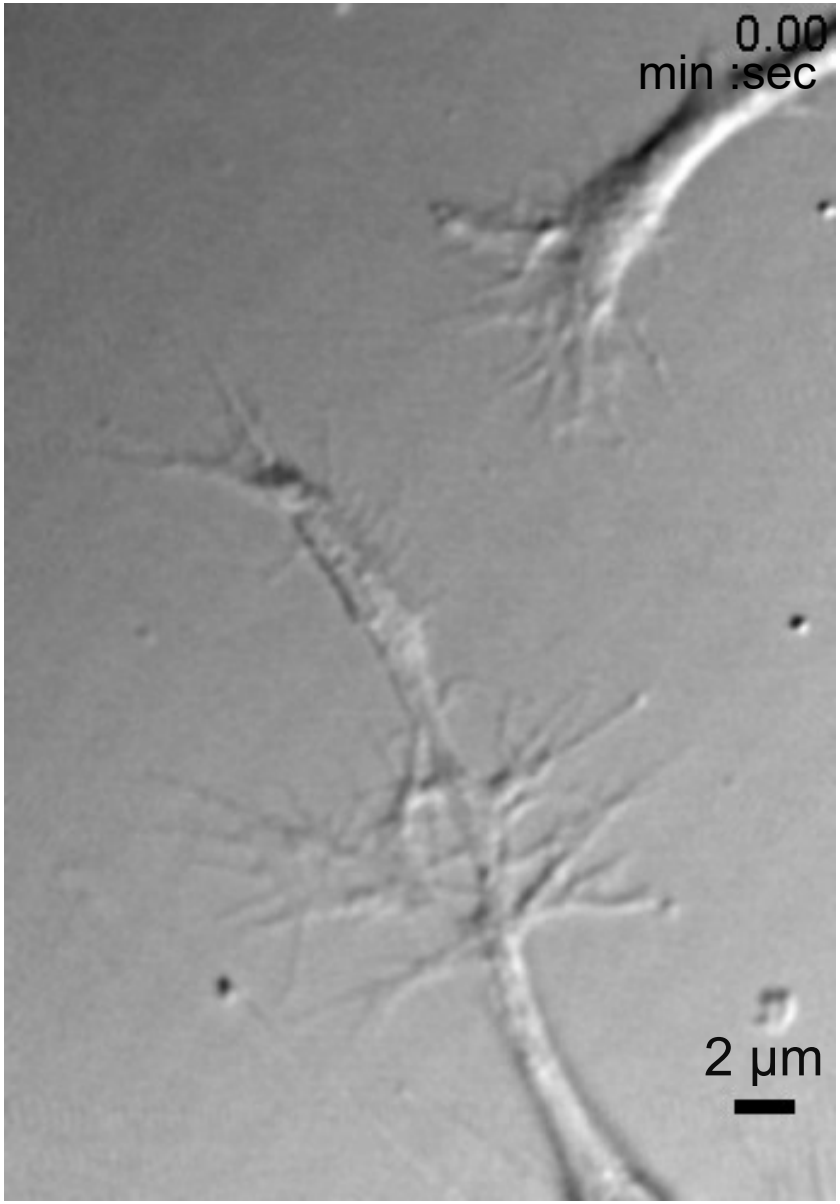
The force and protrusion due to actin polymerization of the bundle of actin filaments in the filopodia is observed.



Cojoc, D, … & Torre, V, PLoS One 2 (10), e1072 (2007)

Difato, F, Pinato, G & Cojoc, D, *Int. J. Mol. Sci.* **14**, 8963 (2013) - REVIEW

Neuronal development (pre and post natal)



Neurons release biochemical cues which are intercepted and interpreted by their nearby neurons but

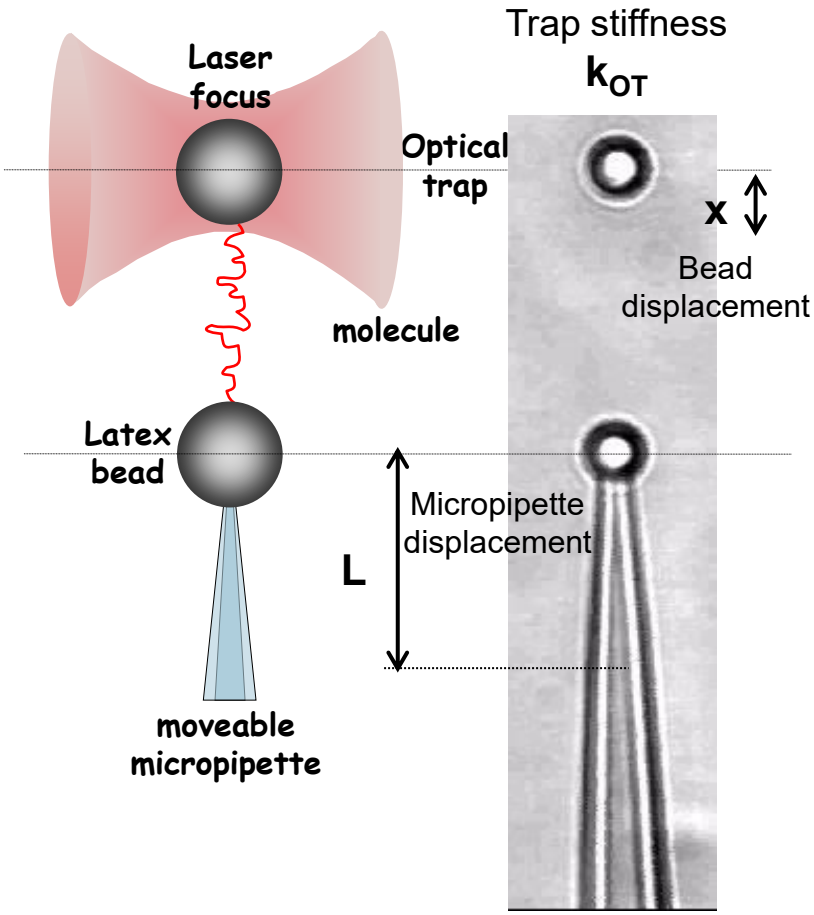
they interact also mechanically

➢ The **Growth Cone (GC)** searches and detects molecular signposts that are displayed by the nearby developing neuron and the environment.

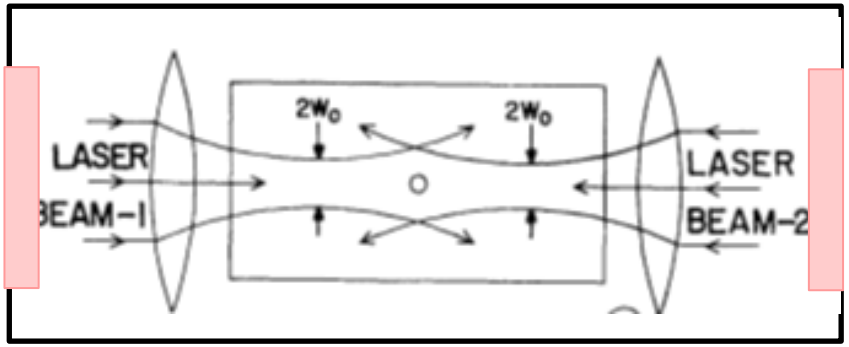
➢ **GC** responds to these signs by advancing, pausing and turning until it reaches its proper destination

F. Difato *et al* (2006) OM-Lab & SISSA

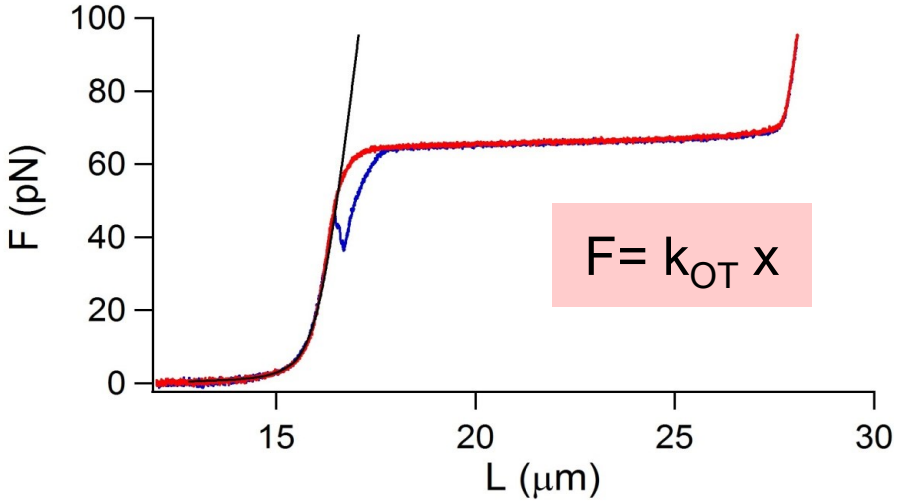
Dual Laser Optical Tweezers DLOT



Counterpropagating beams + detection



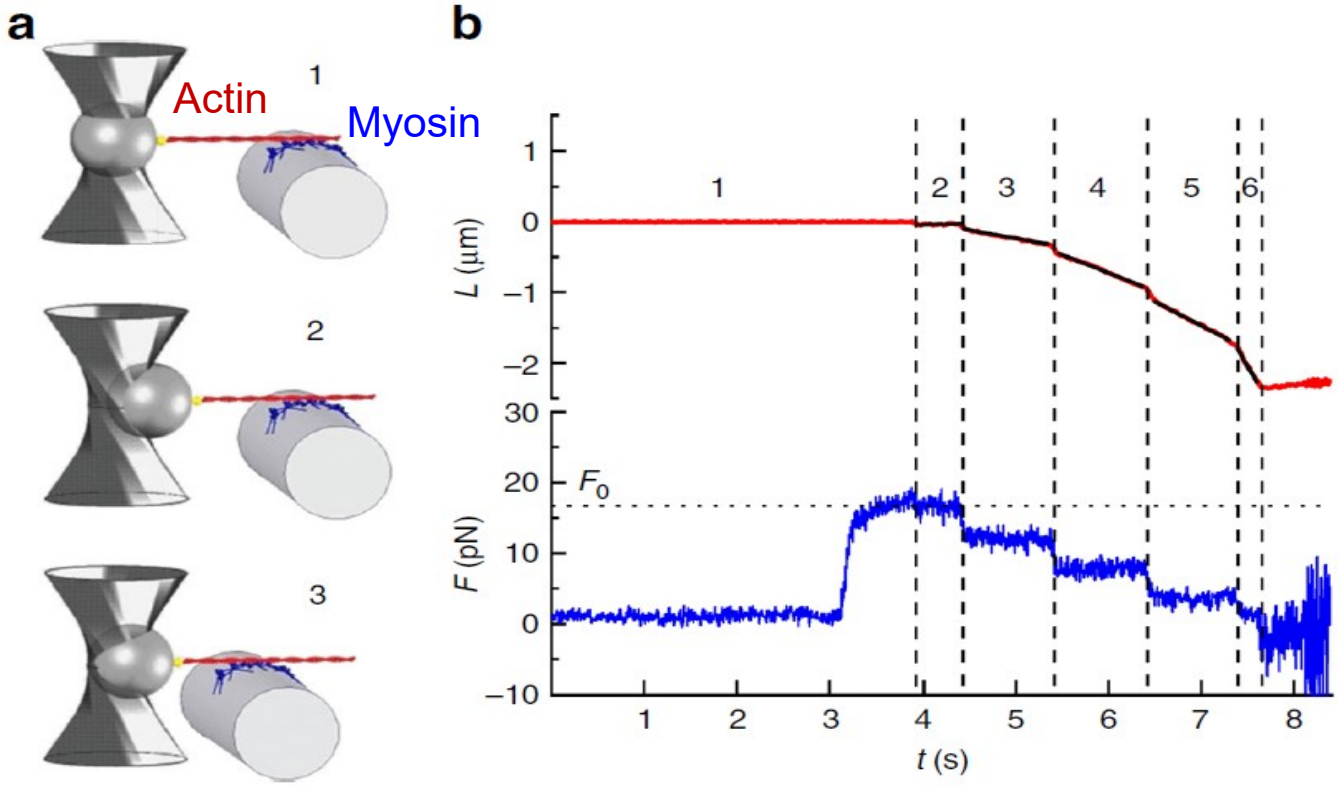
stretching λ-phage DNA



The DNA molecule undergoes a structural change at ~65 pN that implies 70% elongation and is likely involved in the modulation of the access to genetic information

collab with V. Lombardi, P. Bianco, Florence Univ.

A myosin II nanomachine mimicking the striated muscle,



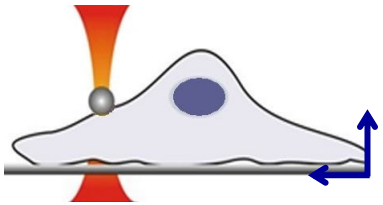
a. Schematic representation of three snapshots during the phases of the interaction between the actin filament and the motors.
b. Recording of the relative sliding (red) and force (blue) during interaction. Phase 1, following the formation of the first bonds between the actin filament and myosin motors, the force rises in position feedback to the maximum isometric value ~17 pN.

Pertici et al., *Nature Communications*, (2018) 9:3532.

Cell membrane indentation

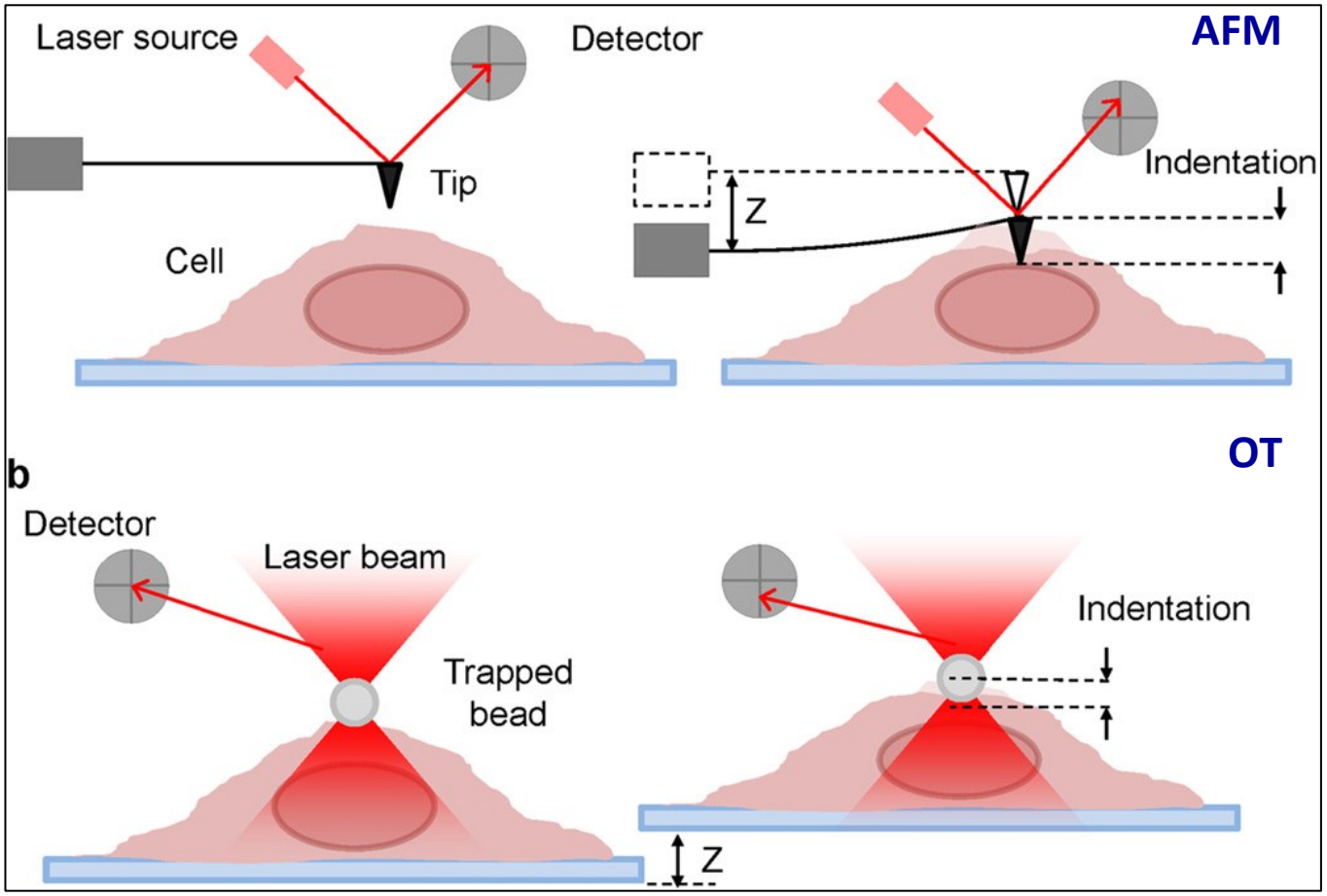
AFM

k = 150 pN/nm,
A_{PF}= 1um,
f_{PF}= 200 Hz,
F_{SP}= 1 nN



OT

k = 0.015 pN/nm,
A= 1 um,
f= 0.2 Hz,
F= 5-20 pN

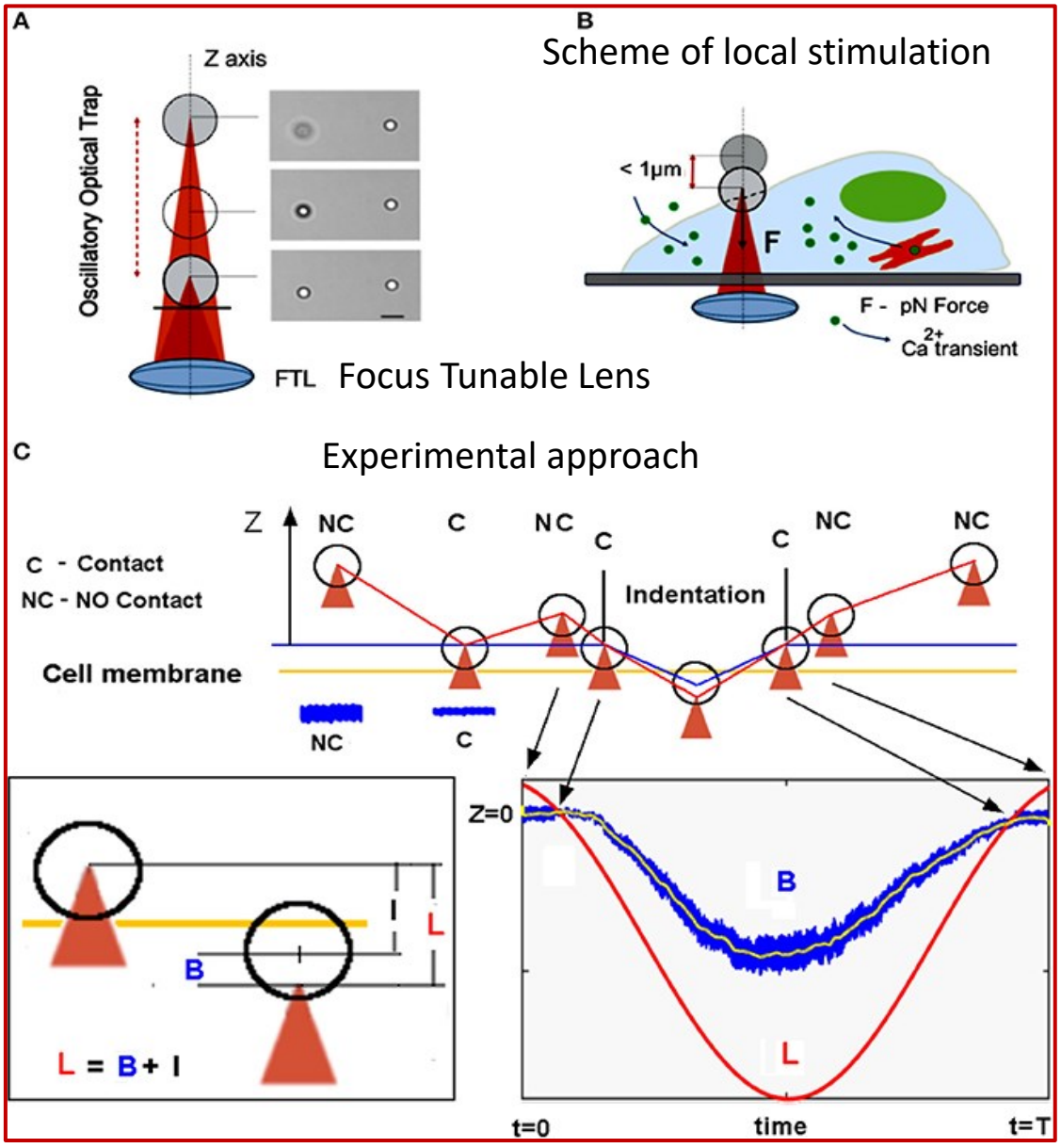


Young's modulus is extracted from the Force – Indentation curve using the Hertz model

Coceano *et. al.* 2016, *Nanotechnology*

Nawaz S, et al. (2012) doi:10.1371/journ

Cell mechanotransduction with piconewton forces



Forces expressed by neurons during development, cell-cell and cell ECM interaction are in pN-nN range.

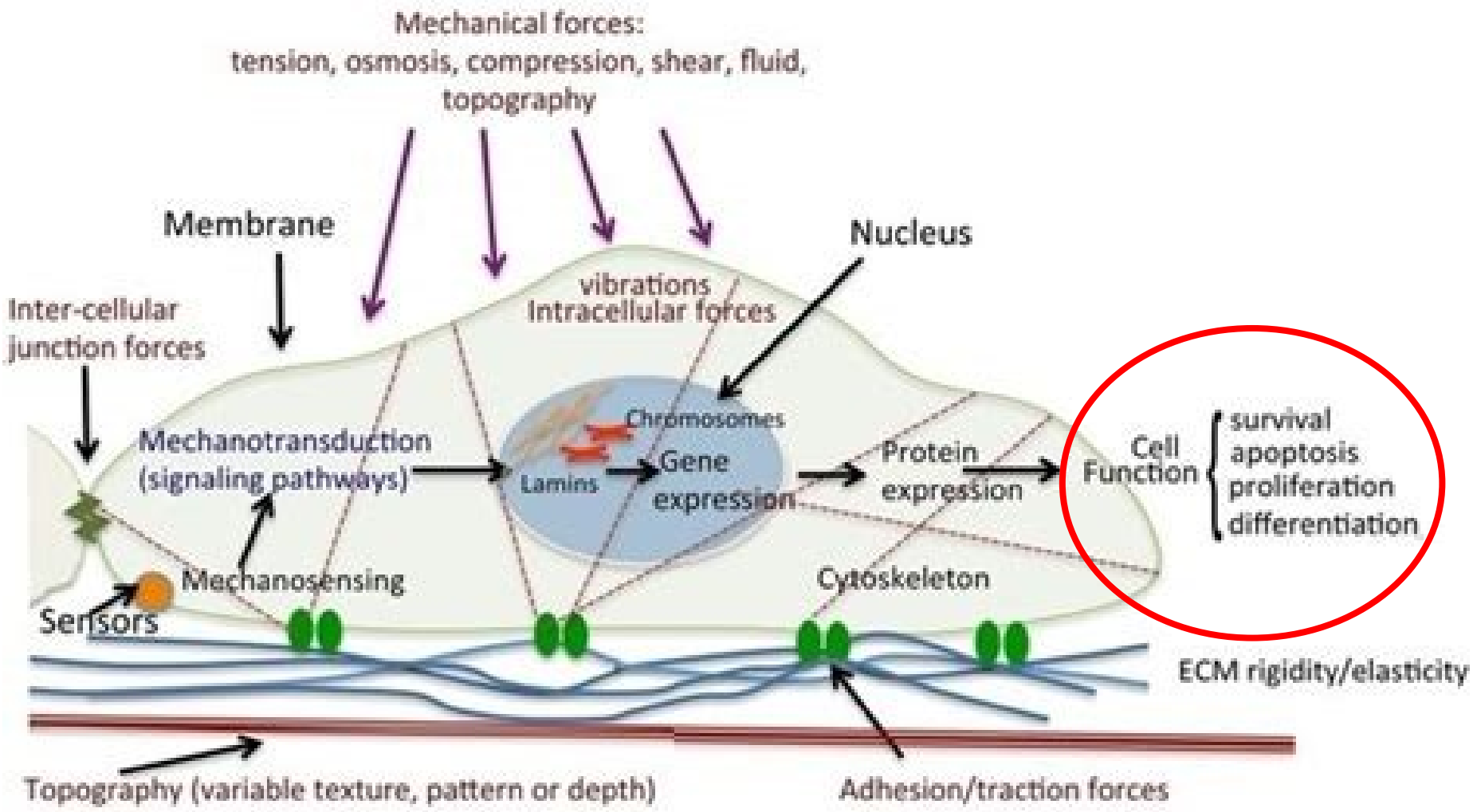
We study the effect of mechanical stimulation of neuronal cells with controlled piconewton forces .

- Mouse neuroblastoma NG108-15
- Rat hippocampal neurons (1-2 days postnatal)

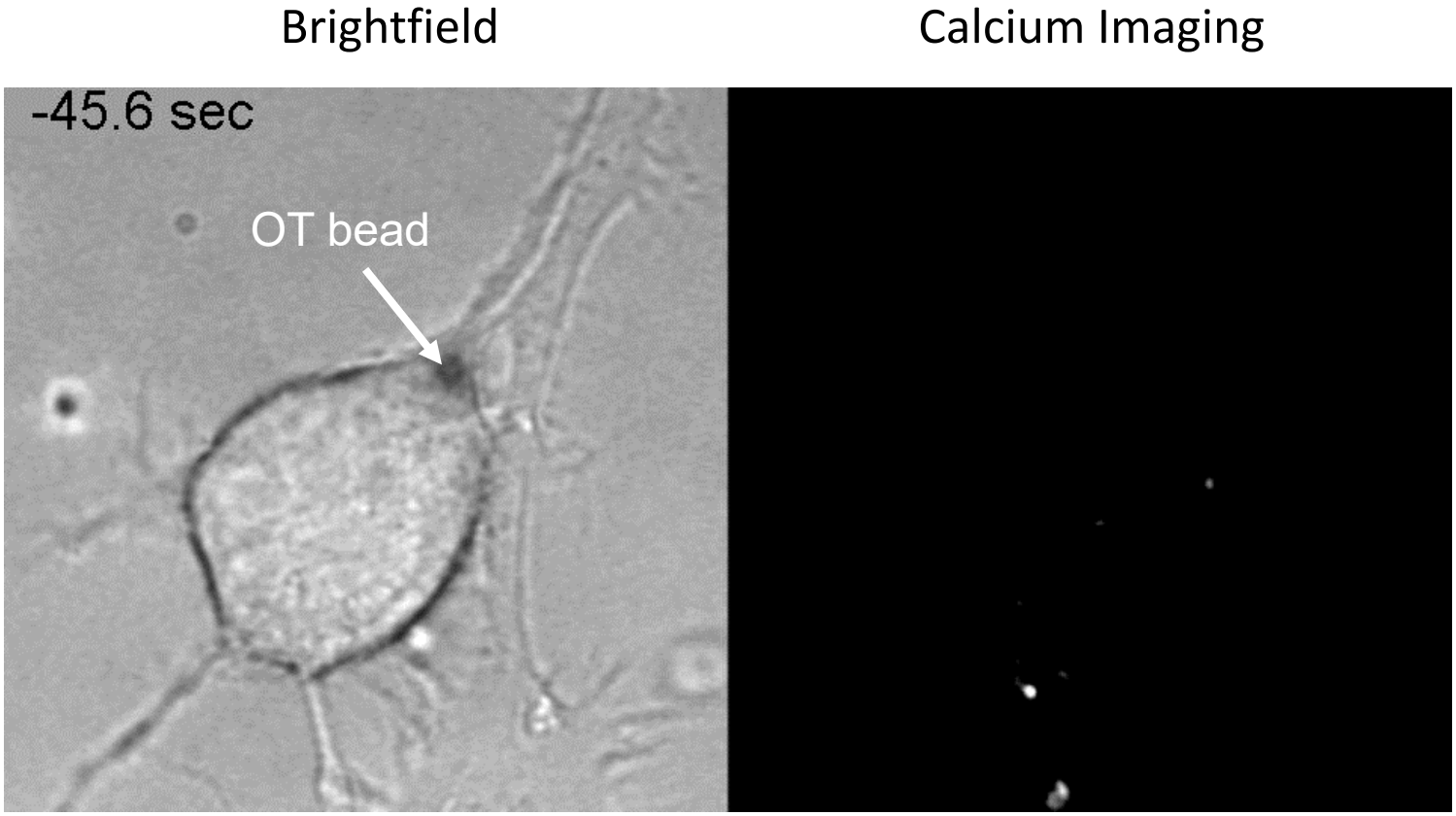
F. Falleroni *et al*,
Frontiers Cell Neurosci, 2018

F. Falleroni *et al*, submitted

Cell mechanotransduction – cell function



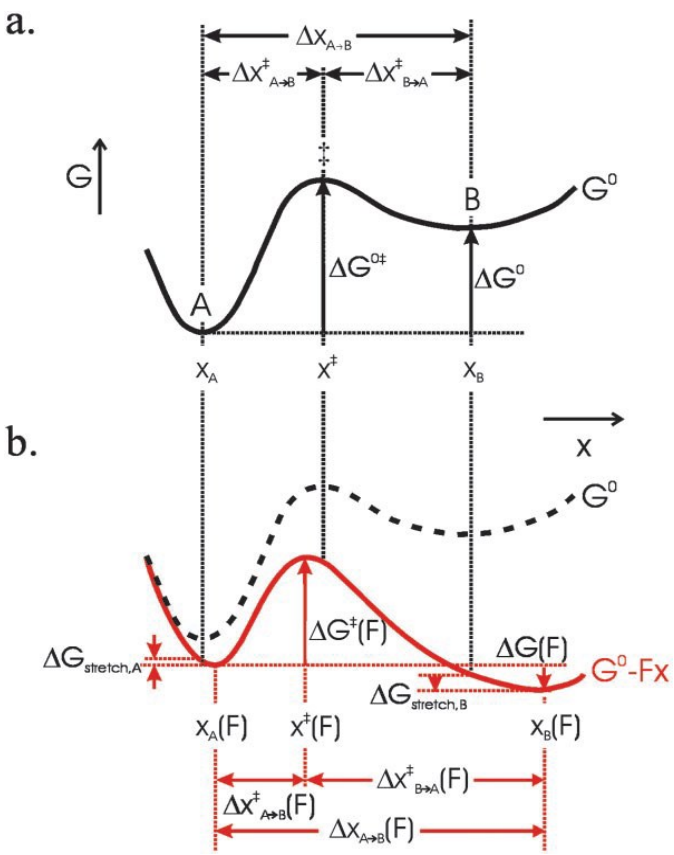
Ca^{2+} transients evoked by calibrated mechanical stimulations

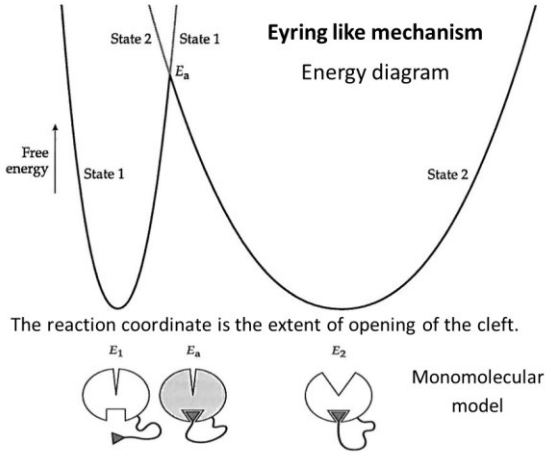


mouse neuroblastoma NG108-15

F. Falleroni *et al*, Frontiers Cell Neurosci, 2018

Single Molecule Dynamics





The effect of force on the free energy of a two-state system, where x represents the mechanical reaction coordinate.

(a) No applied force.

(b) Red curve: positive applied force.

The application of force lowers the energy of both the transition state ‡ and state B relative to state A, which increases the rate of the forward reaction and the population of state B, respectively.

The positions of the free energy minima (x_A and x_B) and maximum ($x‡$) shift to longer and shorter x, respectively, with a positive applied force. Their relative shifts in position depend on the local curvature of the free energy surface.

The free energy change of states A and B upon stretching is $\Delta G_{stretch\ A,\ B}$

2020 Annual Review of Biochemistry, Single-Molecule Studies of Protein Folding with Optical Tweezers, Carlos Bustamante et all Review

$$\frac{k_1}{k_{-1}} = \frac{[E_2]}{[E_1]} = K_{eq} = exp\left[-\frac{\Delta G}{KT}\right]$$

K_1 , K_{-1} the forward, backward reaction rate constants (association and dissociation constants) s^{-1}

$$k_1 = A\ exp\left[-\frac{\Delta G a_1}{KT}\right]$$

A – frequency factor

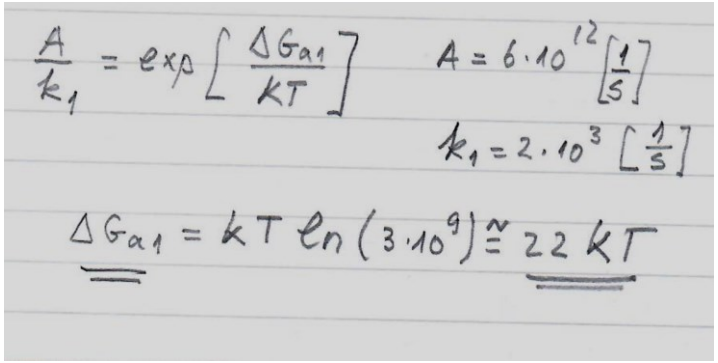
Eyring theory

In the **Eyring rate theory,** the reaction is assumed to correspond to the breakdown of a single quantum-mechanical vibration of the protein.

Therefore the frequency factor is considered ***A~ KT/h*** ≈ 6 x10^{12} [s^{-1}], where h is the Planck constant.

E.g. A reaction with a rate constant k_1= 2 x 10^3 s^{-1} , would have an activation energy : ΔGa_1 = 22 *KT*

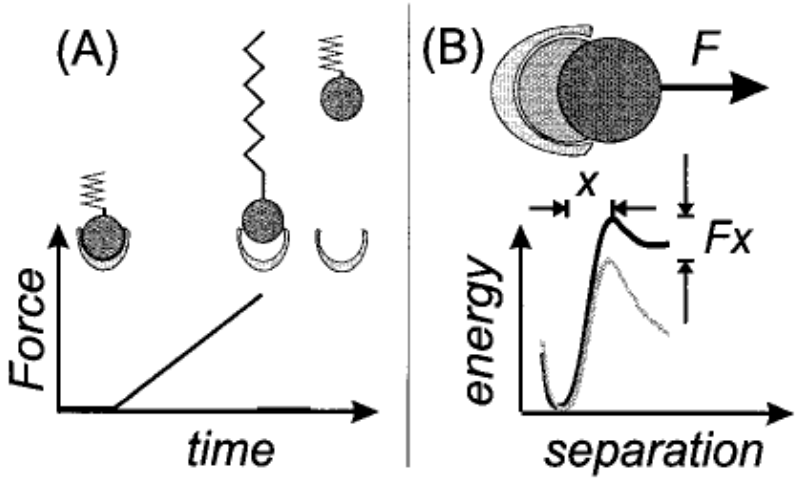
$$k_1 = A\, exp\left[-\frac{\Delta Ga_1}{KT}\right]$$



The **Eyring theory** is expected to apply to **covalent changes of proteins and their ligands** but it is not expected to apply to global conformational changes of proteins in which a large number of bonds are made and broken, because in this case the reaction does not correspond to a single mode of vibration of the protein.

How to determine the dissociation rate of a ligand – receptor bond using force

Model and design experiment – single molecule vs bulk



(A) Direct observation of the dissociation under a mechanical force. The force on a single complex increases until it dissociates. The dissociation is monitored by an abrupt relaxation of the macroscopic spring of a force probe.

(B) The dissociation over a sharp energy barrier is characterized by a decrease of the barrier, giving rise to a characteristic length scale *x*.

$F = r\,t$ where r – loading rate

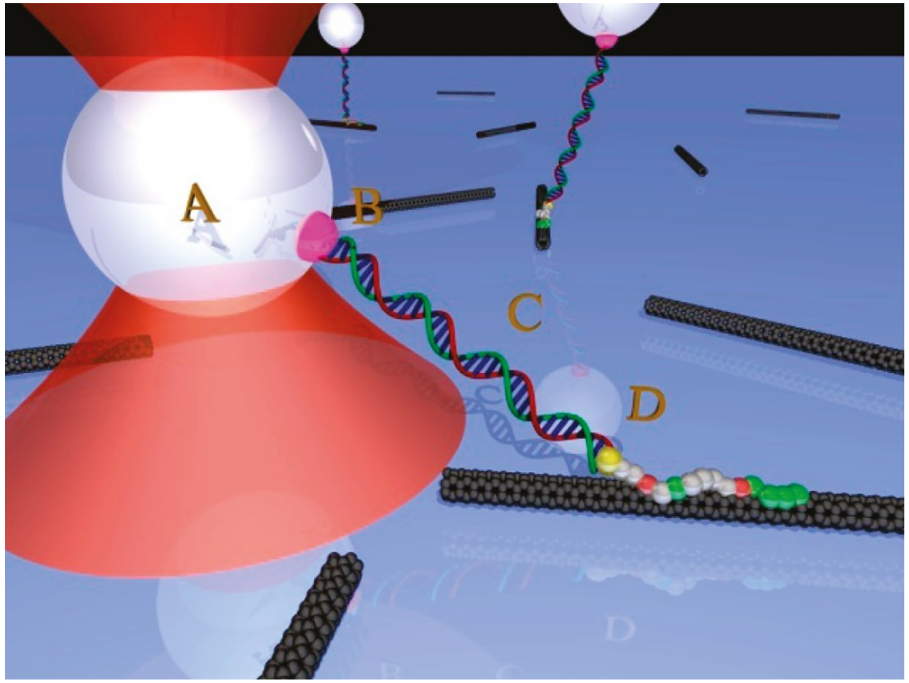
Strunz et al, Model Energy Landscapes and the Force-Induced Dissociation of Ligand-Receptor Bonds, Biophys.J 79 (2000) - model

Eyring theory → Reaction rate without force

$$k_1^0 = \frac{KT}{h}\, exp\left[-\frac{\Delta G a_1}{KT}\right]$$

The goal is to determine the dissociation rate k_{off} (k_1^0)

How the experiment is developed



Schematics of optical tweezers pulling on a single peptide aptamer molecule linked to a carbon nanotube. The optical trap captures a bead (A) that is linked to an aptamer (D) via a DNA molecule (C) and a biotin/streptavidin linkage (B).

Adhesion through Single Peptide Aptamers

Aptamers are biomolecules with specific binding affinity, enabling applications in sensing, diagnostic, drug delivery, imaging, and therapy.

Peptide aptamers typically contain 8-20 amino-acids and bind materials or biomolecules.

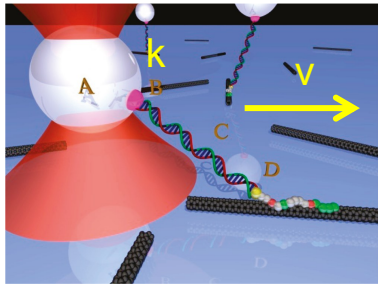
They can be engineered via selection from large libraries of random sequences ($\sim 10^{10}$) by directed evolution techniques such as phage display.

Aubin-Tam et al, Adhesion through single peptide aptamers

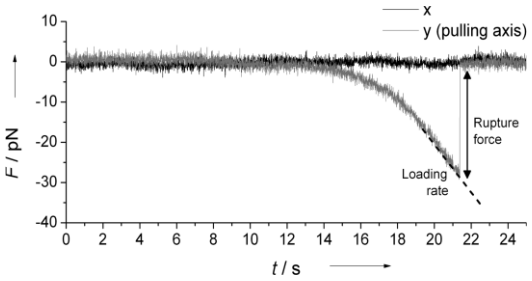
dx.doi.org/10.1021/jp1031493 |J. Phys. Chem. A 2011

Use the force to measure unbinding forces and dissociation rate k_{off} of Ligand to Receptor

How it works in practice / experimentally ?



1. We need a tool to exert force (F= k_{probe} x): AFM, OT, MT, AT; the choice depends on the strength of the bond. The probe exert force on the ligand –receptor bond.
2. We need linkers to connect the ligand with the probe (e.g. OT bead, AFM tip) because the probe is much bigger than the ligand molecule.



* Measurement procedure:

1. Pull the "construct" (ligand + linker) with a force F:

$F = r \cdot t$, r - load rate $\left[\frac{pN}{s}\right]$, t - time [s]

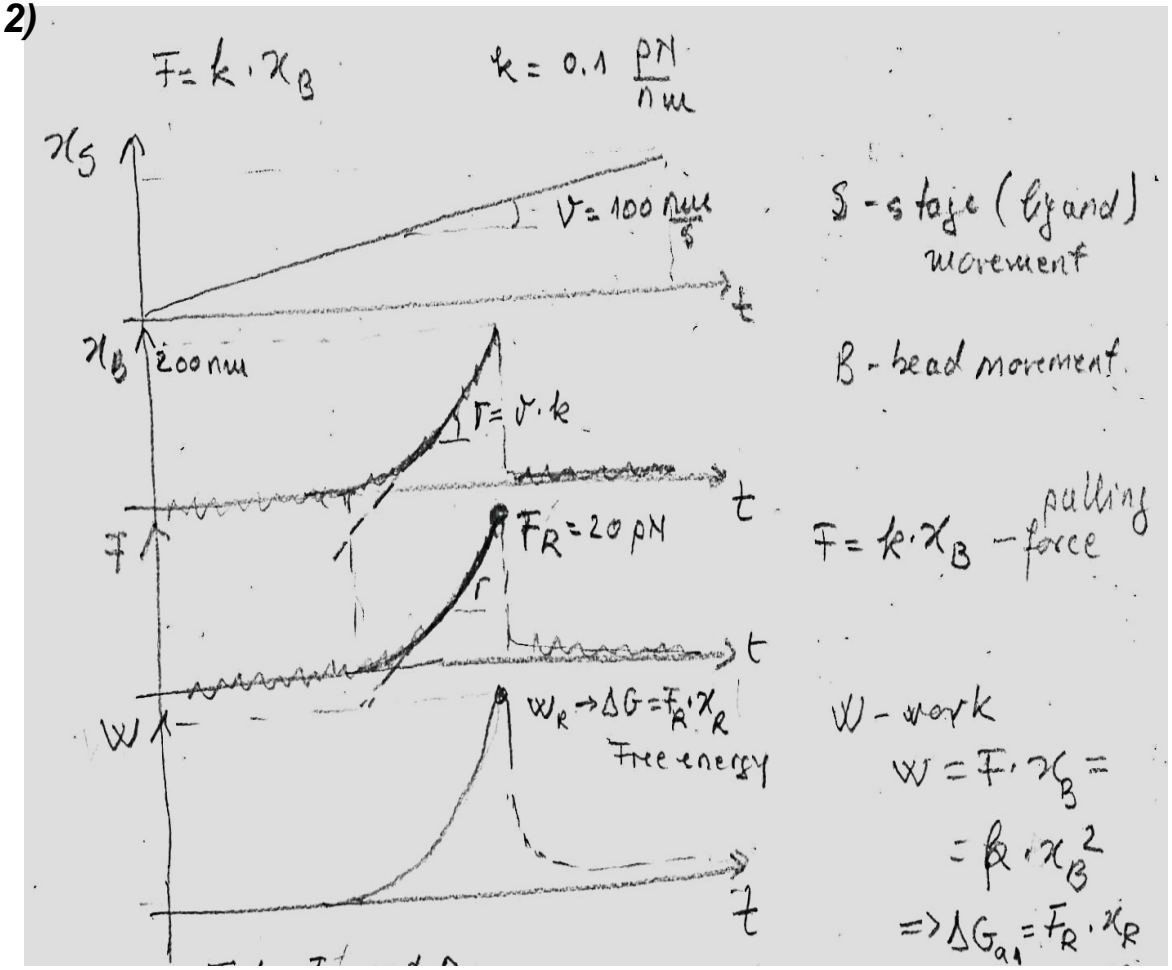
eg. for OT: stiffness of the trap $k_{OT} = 0.001 - 0.5 \left[\frac{pN}{nm}\right]$ $F = k \cdot x_B$

Force is applied by moving the ligand (fixed on substrate) with a constant velocity $\vec{v}$; $v = 20 - 2000 \left[\frac{nm}{s}\right]$

The load rate, r, will be then $r = \vec{v} \cdot k$ *r= 0.02 – 1000 [pN/s]*

Aubin-Tam et al, Adhesion through single peptide aptamers
dx.doi.org/10.1021/jp1031493 |J. Phys. Chem. A 2011

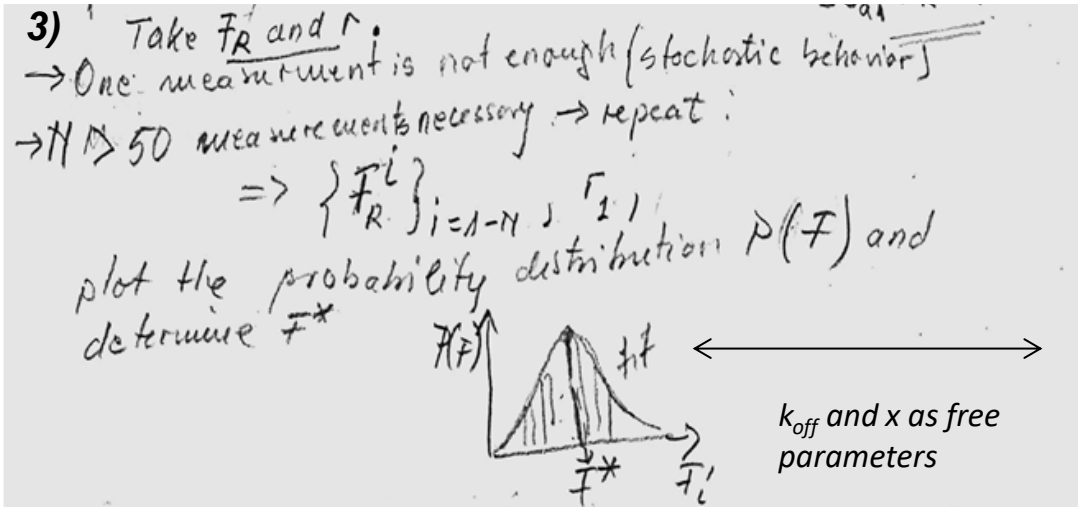
2)



Force ramp approach

The force F increases with time t:
$F(t) = r\ t$
Force is calculated measuring the displacement x_B: $F(x_B) = k\ x_B$

The load rate $r = v\ k$ and is the slope of the tangent to the measured displacement of the bead.



$$P(F) = \frac{k_{off}}{r} \cdot \exp\left\{\frac{Fx}{kT} + k_{off}\frac{kT}{R}\left(1-\exp\frac{Fx}{kT}\right)\right\}$$

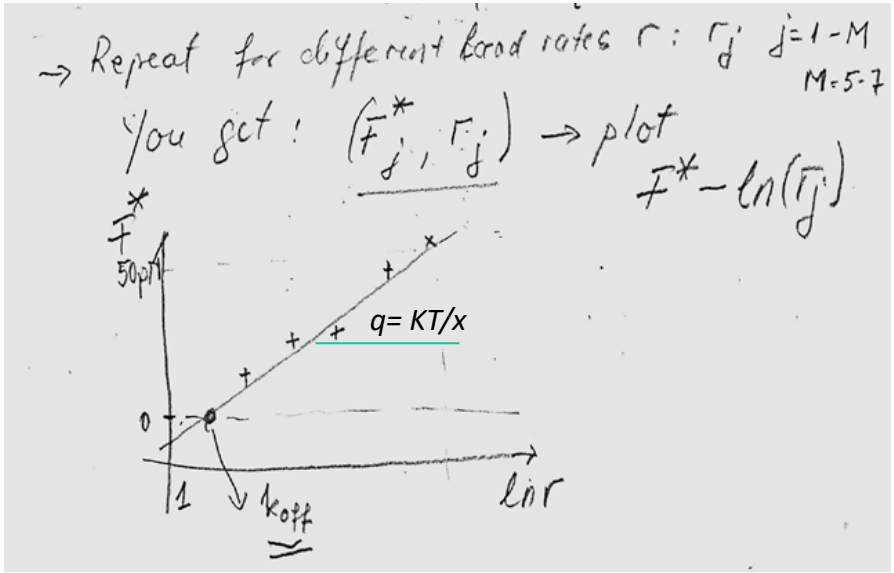
The most probable unbinding/dissociation force

$$F^* = \frac{kT}{x}\ln\left(\frac{x}{kT}\frac{r}{k_{off}}\right) \qquad \frac{kT}{x} = 2$$

$k_{off}(F^*) = k_{off} \cdot \exp(F^* x/KT)$

Having more than one dataset at different load rates ***r*** is crucial to extrapolate the value of $\boldsymbol{k_{off}}$ in absence of load F=0, (i.e. natural thermal off-rate), that is the most relevant parameter the assay can return.

4)



$$F^* = 2 \ln\left(\frac{r}{2 \cdot k_{off}}\right) = 2 \ln r - 2 \ln(2 \cdot k_{off})$$

F^* linear with ln r

The slope is q= KT/x

$F^*=0 \rightarrow \quad \mathbf{k_{off} = r_0 / q}$

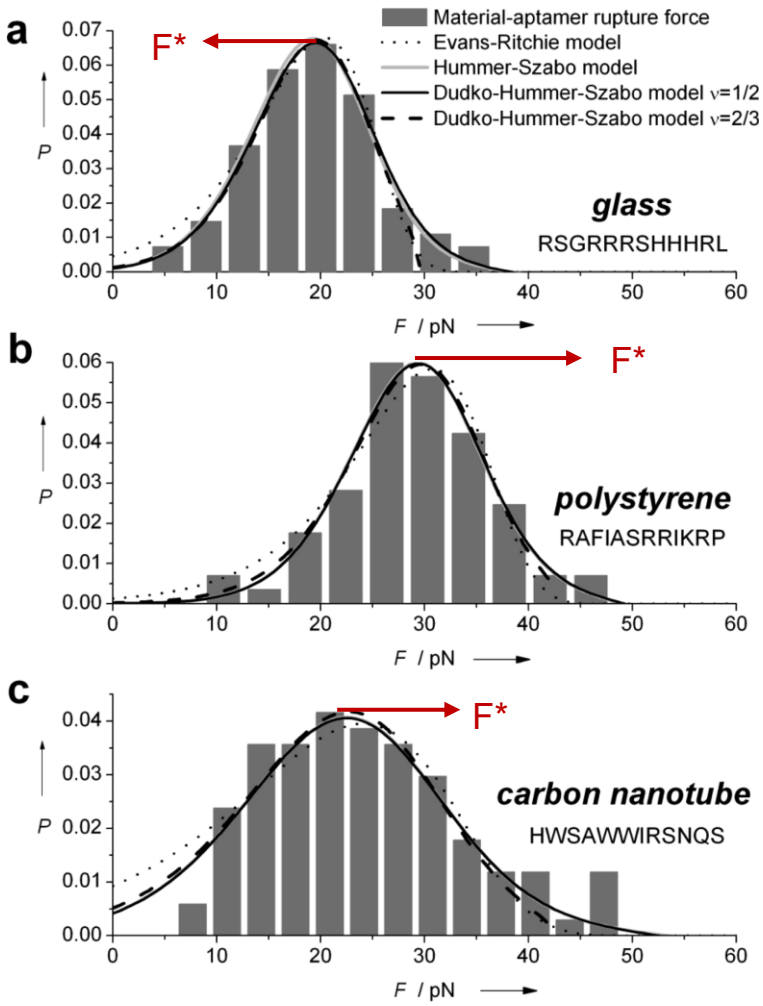
r_0 *– extrapolated load rate r for F*=0*

$k_{off}(F^*) = k_{off} \cdot \exp(F^* x / KT)$

model prediction

Dataset	r [nN/s]	F^* [pN]	x_{a1} [nm]	k_{off} (F) [1/s]
1	10	20	0.3	0.13
⋮	⋮	⋮	⋮	⋮
j	40	50	0.2	0.21
⋮	⋮	⋮	⋮	⋮
M	80	70	0.15	0.34

fit F^* parameters k_{off}, x_{a1}



Rupture-force probability *P(F)* distributions for peptide aptamer binding to :

(a) glass,
(b) polystyrene, and
(c) carbon nanotubes

$\tau_0^a = {}^1/_{k_{off}}$	Evans–Ritchie model	
interaction	$\tau_0{}^a$	$x^{\ddagger\, b}$
glass/aptamer	96.9	0.747
polystyrene/aptamer	109.5	0.652
CNTs/aptamer	20.7	0.404

[a] Units are s. [b] Units are nm. [c] $\Delta G^{\ddagger}$ is in $k_B T$ units.

Example of application : Calmodulin folding-unfolding energy landscape

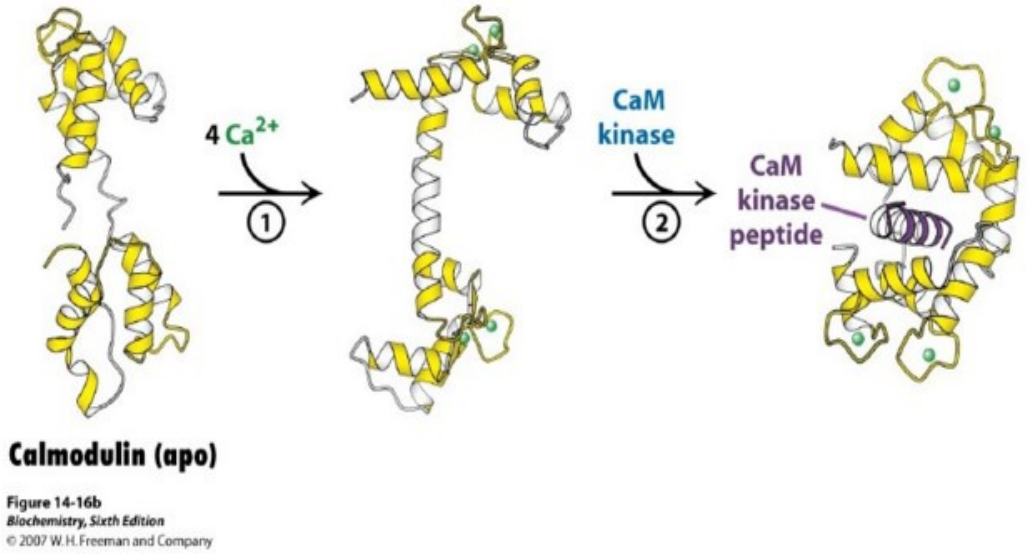


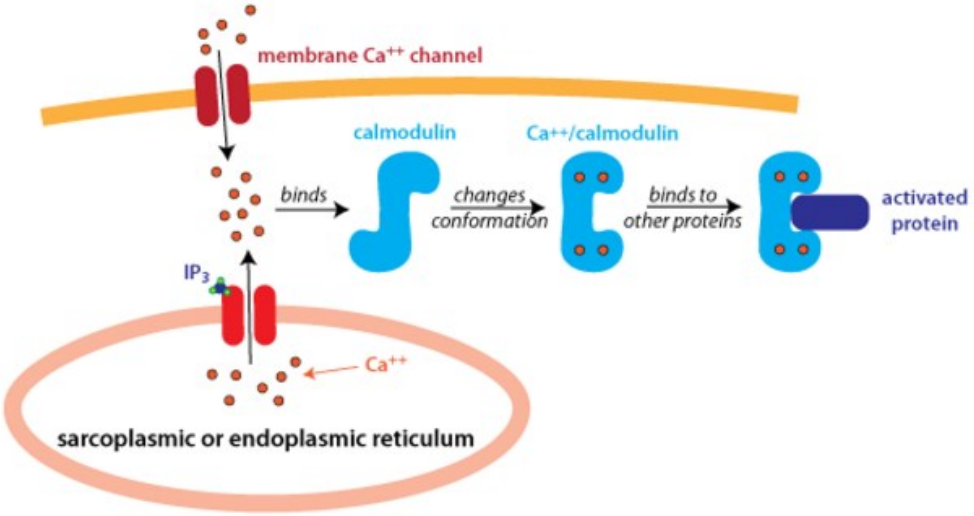
Figure 14-16b
Biochemistry, Sixth Edition

Calmodulin CaM is a protein with a molecular mass of 16 kDa, consisting of 148 amino acid residues and is characterized by a helix-loop-helix binding motif, also known as the E-F hand motif.

CaM has one subunit with a distinct dumbbell shape in which a linker region joins two globular domains.

- CaM is known to undergo a conformational change upon binding with a calcium ion in which each lobe transitions from a closed conformation to an open conformation.
- CaM has four major, high-affinity binding sites.
- The CaM binding region is a series of hydrophobic amino acids (such as Trp or Leu), hydrophilic amino acids (such as Glu or Asp), and basic amino acids (such as Arg or Lys). 12 - Ionic bonds – about 100 KT strength
- CaM typically wraps around its target, with the two globular domains gripping either side of it.

Calmodulin Pathway simplified



Ca++ cell exterior – interior influx, or release from the Endoplasmatic Reticulum

Calmodulin binds to 4 Calcium Ions and undergoes conformational changes characterized by different states in the free energy landscape

→ activates other proteins

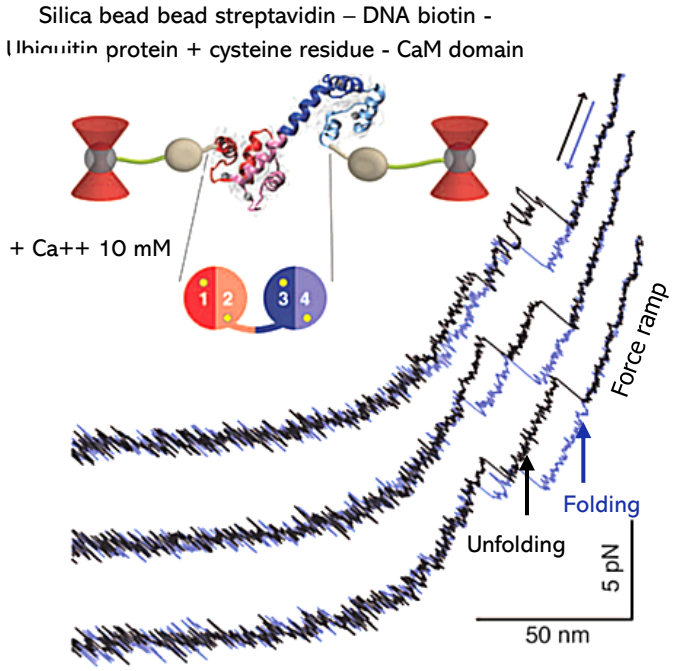
Calmodulin folding-unfolding energy landscape

Ref: The complex folding network of single calmodulin molecules, Stigler, J., et al., Science, 334, 512–516, 2011

The work shows that between the unfold and folded states there are also other (intermediate) states

Calmodulin folding-unfolding energy landscape – Optical Tweezers

Sketch of the experimental setup with
the protein linked with ubiquitin-DNA handles



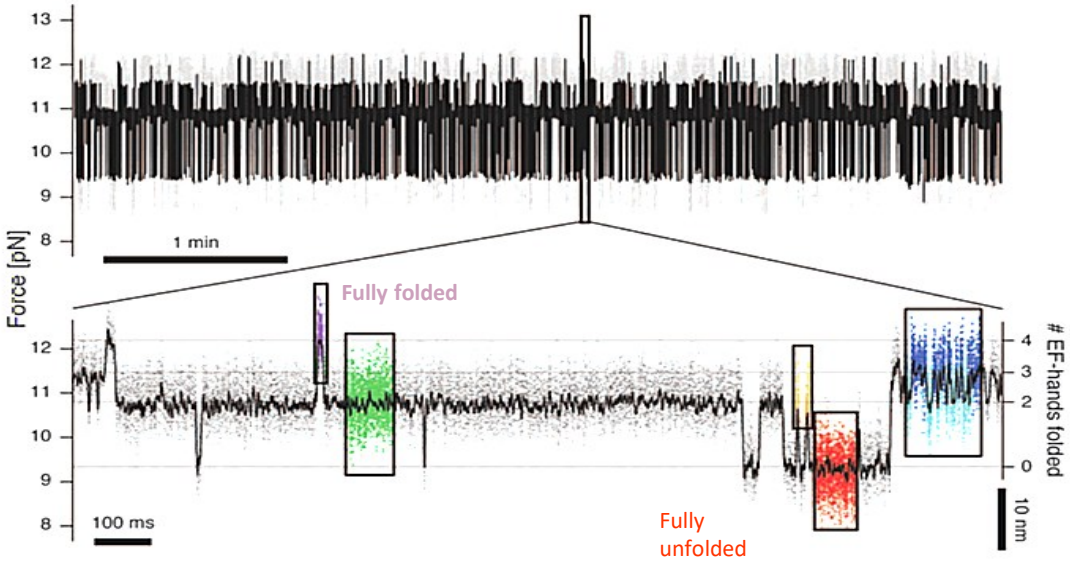
Dual OT
k= 0.25 pN/nm; v= 500 nm/s; r = 12.5 pN/s
Data collection 100 kHz, averaged to 20 kHz before storage

Representative stretch-relax cycles for CaM showing unfolding - folding of the two globular domains of the CaM

The rapid oscillations in the upper traces provide indications for deviations from a simple two-step unfolding behavior

The complex folding network of single calmodulin molecules, Stigler, J., et al., Science, 334, 512–516, 2011

Sample trace during 5min of the fluctuations of a singleWT-CaM molecule at a pretension force set at 11 pN



The vertical scale denotes the force acting on the molecule as measured by the deflection of the beads from the trap center.

Six different states can be identified

See suplem info for details on states classification using Markov analysis, concluding that at least 6 states are required.

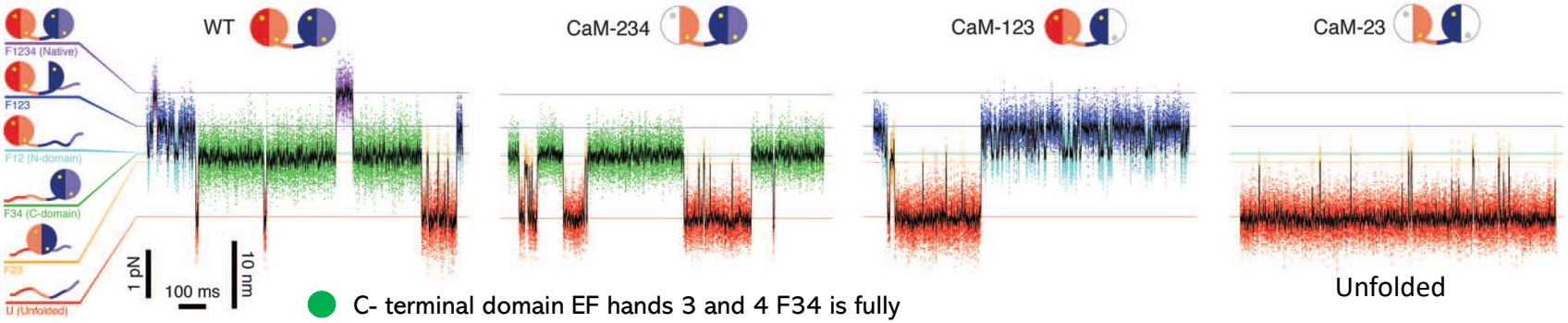
Length expected for a state with 2 EF hands folded and 2 EF hands unfolded. However, they have different kinetics.

The extension corresponds to 3 folded EF hands

Unfolded Folded

Molecular deletion constructs were used to decipher the structures underlying the various states

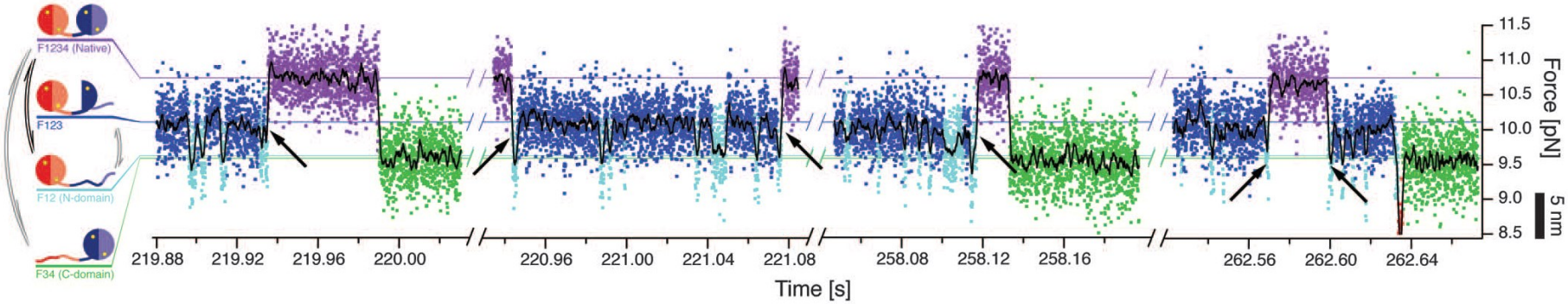
State assignment and comparison of WT traces with truncation mutants



- C- terminal domain EF hands 3 and 4 F34 is fully folded

Unfolded

Transitions to and from the native state F1234

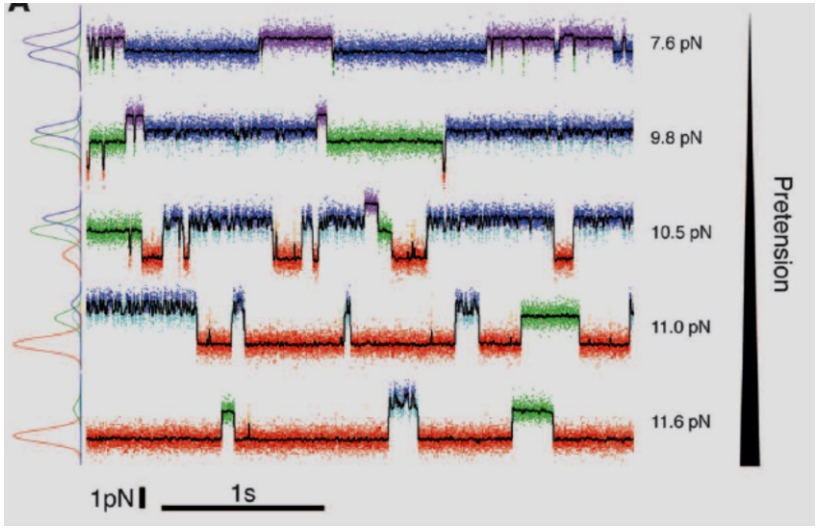


Direct transitions to and from the native state (purple) can only occur to and from states F34 (green) or F12 (light blue).

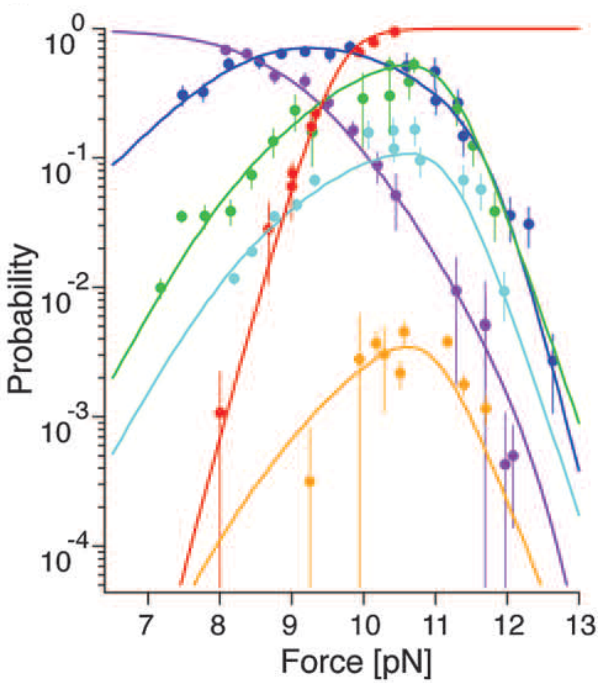
Transitions of F123 (dark blue) to and from F1234 always occur through F12 (see arrows), identifying F123 as an off pathway intermediate.

Observing the transitions between the six states at constant tension / force

Traces of WT-CaM at different pretensions



Probabilities of the states vs Force / Tension



At low pretensions of 7.6 pN folded or largely folded states dominated. The more tension applied, the more unfolded states were populated, until, at 11.6 pN, the unfolded state prevailed.

Observing the transitions between the six states at constant tension / force

→ from the probabilities the free energy of all states at zero force can be calculated

The free energy data

	Difference from U state, ΔG_0 (k_BT)									
	10 mM Ca^{2+}						100 µM Ca^{2+}			
State	WT	CaM-12	CaM-34	CaM-23	CaM-123	CaM-234	WT	CaM-23	ΔL (nm)	ΔL_{calc} (nm)
U									52.2 ± 0.6	50.6
F_{23}	13	–	–	12	13	13	4	4	27.4 ± 0.7	27.4
F_{34} (C domain)	21	–	18	–	–	20	11	–	23.8 ± 0.5	25.7
F_{12} (N domain)	20	19	–	–	20	–	11	–	23.3 ± 0.4	25.3
F_{123}	30	–	–	–	28	–	14	–	13.0 ± 0.3	13.2
F_{1234} (native)	36	–	–	–	–	–	17	–	0	0

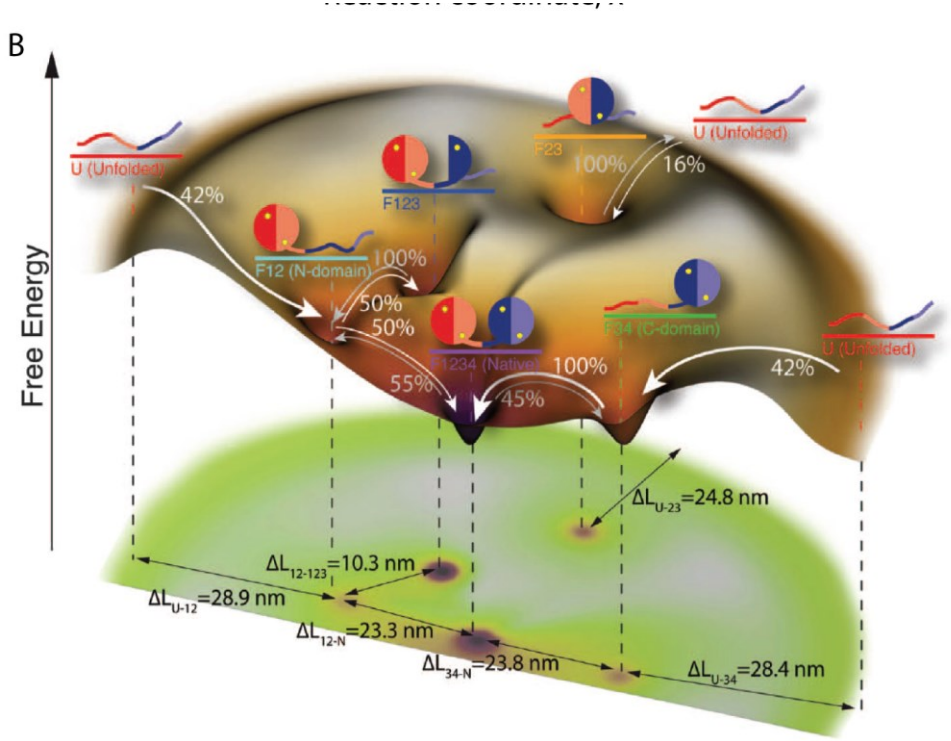
The data reveals a significant anti cooperativity between the folding of the two CaM domains.
From the unfolding state, folding of either N terminal (F12) or C terminal (F34) domain results in an energetic gain 20 KT.
However, folding of a second domain to the native state provides 15 KT.
Apparently, the presence of one folded domain prevents the other domain from reaching its energetically optimal state.

Transition constants

Transition	$\log_{10}(k_{0,unf})$ (s^{-1})	$\log_{10}(k_{0,fold})$ (s^{-1})	Δx_{unf} (nm)	ΔL_{fold} (nm)
$F_{1234} \rightleftharpoons F_{12}$	−0.7 ± 0.9	5.0 ± 0.4	1.3 ± 0.8	14.4 ± 1.3
$F_{1234} \rightleftharpoons F_{34}$	−0.8 ± 0.3	5.6 ± 0.2	1.7 ± 0.3	16.9 ± 0.8
$F_{123} \rightleftharpoons F_{12}$	−0.13 ± 0.04	5.0 ± 0.2	1.92 ± 0.04	7.2 ± 0.6
$F_{12} \rightleftharpoons U$	−5.0 ± 0.7	5.8 ± 0.5	5.0 ± 0.6	18.2 ± 1.5
$F_{34} \rightleftharpoons U$	−4.1 ± 0.5	5.8 ± 0.5	4.1 ± 0.4	17.3 ± 1.5
$F_{23} \rightleftharpoons U$	−1.4 ± 0.3	5.4 ± 0.3	3.7 ± 0.3	15.6 ± 1.0

Dx unf is the change in the length required to reach the transition state of unfolding.
DL fold is the contour length change required to reach the transition state of folding.

Full kinetic network of WT-CaM folding and unfolding at zero load



Arrows show all observed transitions. The percentage values provided for each transition give the fraction of transitions along the respective pathways out of each state. Distances in the lower part are differences in contour length.

The complex folding network of single calmodulin molecules, Stigler, J., et al., Science, 334, 512–516, 2011

OT for single molecule spectroscopy; spatial and temporal resolution.

- measure conformational changes and displacements produced by single biological molecules.

Such movements range from several nanometers (molecular motors) down to one base pair (0.35 nm, for DNA and RNA processing enzymes) -- > **high spatial resolution** detection based on Interferometry (see next section).

Actually, position detectors do not set a limit on spatial and temporal resolution of OT.

Thermal noise sets instead fundamental limits on displacement and force measurements with single molecules.

Temporal resolution limit due to relaxation time for bead position.

When a single bead trapped in optical tweezers is perturbed from equilibrium, for example, by protein conformational changes or by trap displacements, it moves exponentially to a new equilibrium position with a time constant (relaxation time) $\tau = \gamma/k$, where γ is the viscous drag coefficient and k the stiffness of the system.

Therefore, systems with higher stiffness attached to small probes exhibit faster responses to perturbations.

If the perturbation develops faster than τ, the bead moves with the same relaxation time τ, filtering out all the movements that occur on shorter time scales.

AFM : k = 1 pN /nm → τ – microseconds , OT : k= 0.001 pN/nm → τ - milliseconds

Optical Tweezers Microscopy

- Light has momentum, change of momentum generates force
- Optical Tweezers (OT): Laser beam tightly focused on micro/nano objects in liquid
- Forces applied and measured by OT: 1 - 200 pN
- OT with IR laser can be applied to living cells and biomolecules without damaging them
- OT is implemented on a microscope platform → trap and manipulate what you see and see what you manipulate (ex. Mechano transduction)