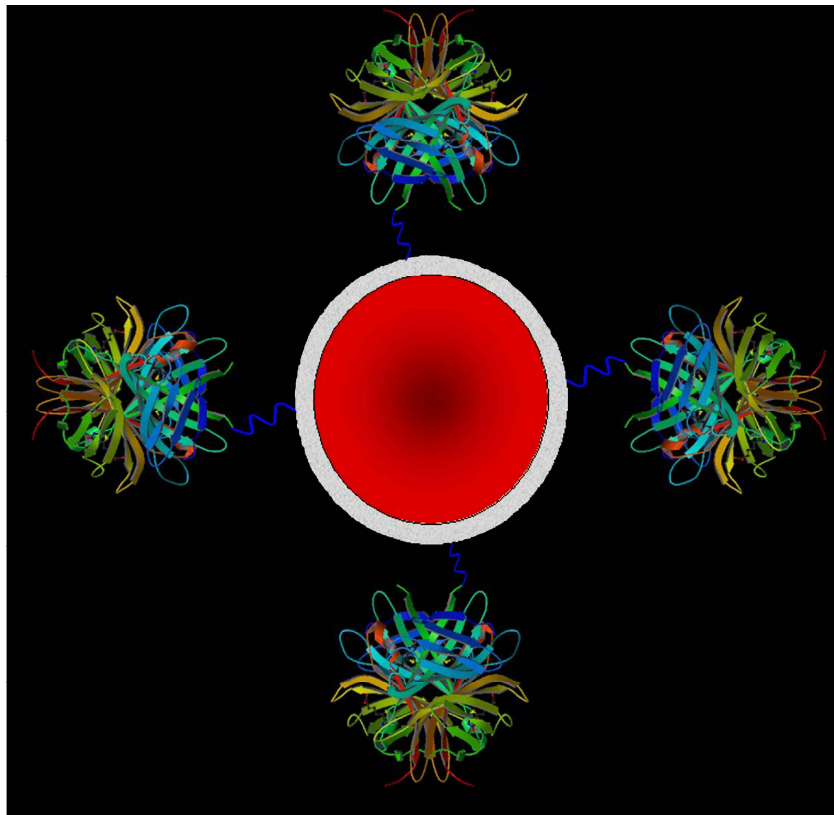


# Single Molecule Studies

Mark Howarth

Lecturer in Bionanotechnology

Department of Biochemistry



# Why study biology at the single molecule level?

## 1. Remove ensemble averaging

- detect rare intermediates,  
no need for synchronization

*F1 ATPase*  
*Junge et al.*  
*FEBS Lett. 2001*



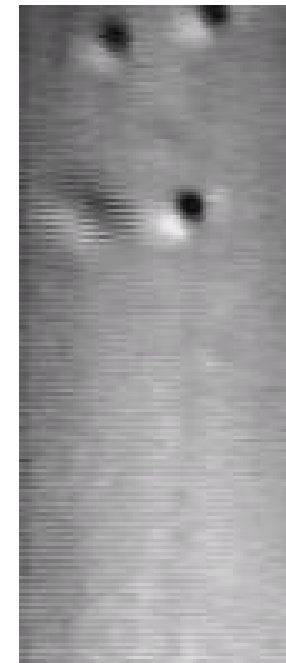
## 2. Many biological events occur at the single molecule level

- DNA replication,  
cytotoxic T cell recognition  
overexpression changes function!

## 3. Image small movements

- kinesin stepping,  
membrane microdomains  
biomolecule conformational changes

*Kinesin steps*  
*Vugmeyster et al.*  
*Biochemistry 98*



## 4. Super-resolution microscopy

*Advances in single-molecule fluorescence methods for molecular biology.*  
*Joo C, Balci H, Ishitsuka Y, Buranachai C, Ha T. Annu Rev Biochem. 2008;77:51-76.*

---

# History of single molecule biology 1

What was the first single molecule experiment in biology?

# History of single molecule biology 1

*4-way patch-clamp  
in brain slice*

(1940/1950s: Avery, Hershey, Chase, Delbruck  
a few atoms changed in single molecule at  
defined time makes heritable mutation)

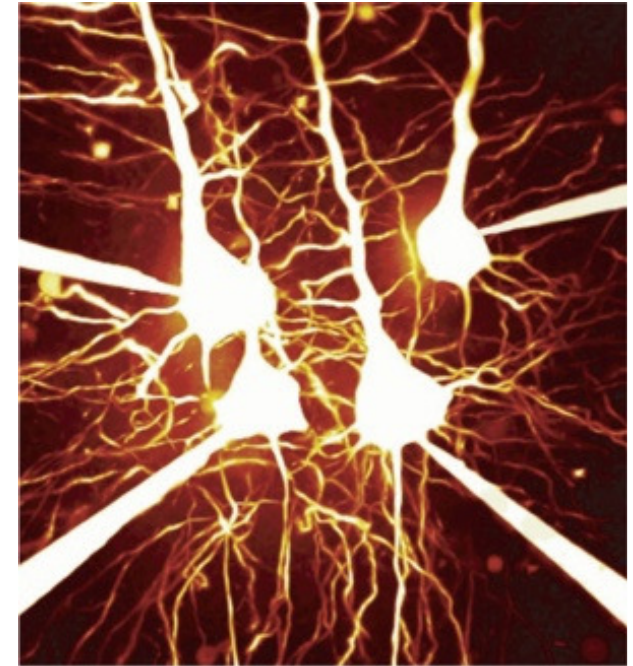
1976 Patch-clamp to watch single ion channels  
(Neher and Sakmann)

1982 Diffusion of dye-labelled LDL bound to single  
receptor at PM (Watt Webb)

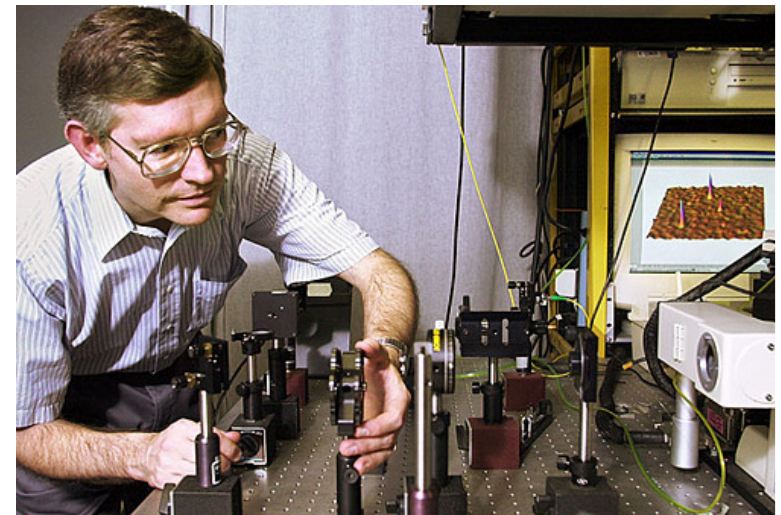
1989 Detection of a single fluorophore in liquid He  
(Moerner and Kador)

1993 Single fluorophore detected at RT  
(Betzig and Chichester)

1998 QDs for biology (Alivisatos, Nie)



*W.E Moerner*



# History of single molecule biology 2

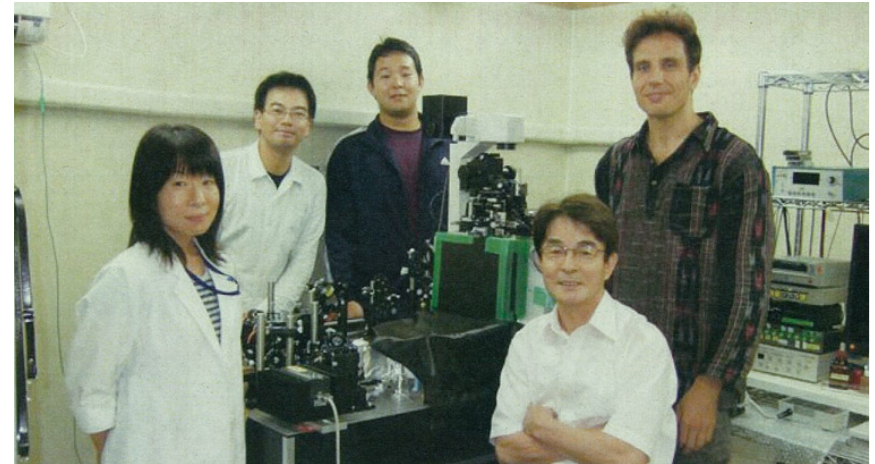
*Toshio Yanagida  
with lab*

2000 Single molecule FRET of dye-EGF on living cells (Yanagida)

2003 1 nm resolution tracking of motor protein (Yildiz, Selvin, Vale)

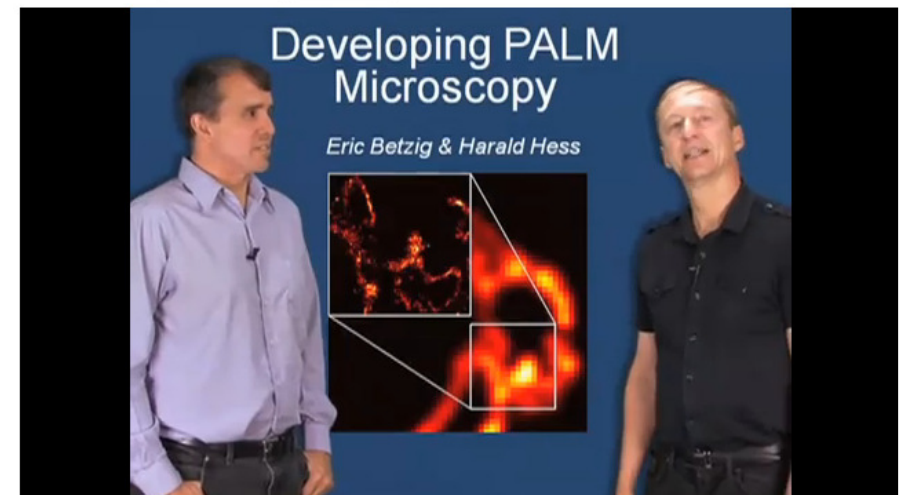
2004 Single molecule imaging in living organism- anti-HER2-QD in mouse tumour (Tada, Ohuchi)

2006 PALM, STORM super-resolution fluorescence microscopy (Betzig, Zhuang, Hess)



Eric Betzig and Harald Hess: Developing PALM Microscopy

biomagazine 82 videos 



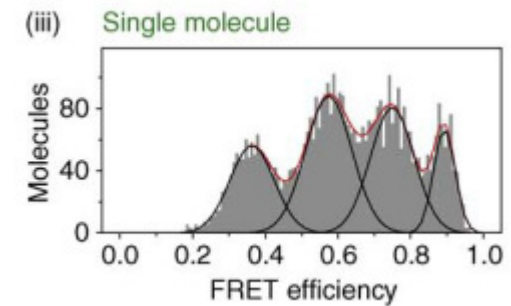
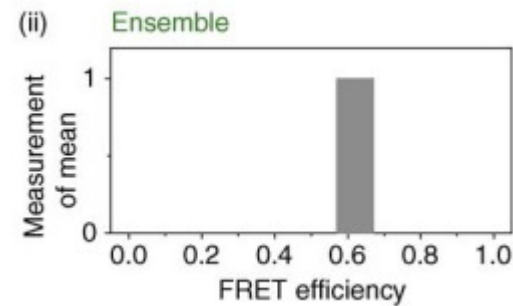
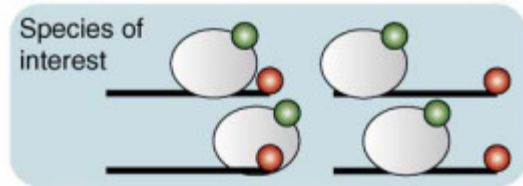


# Heterogeneity seen by single molecule methods

Ensemble averaging hides kinetics, dynamics and mechanism.

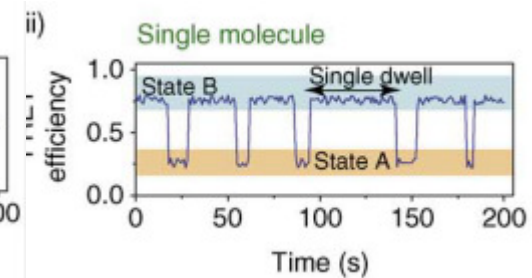
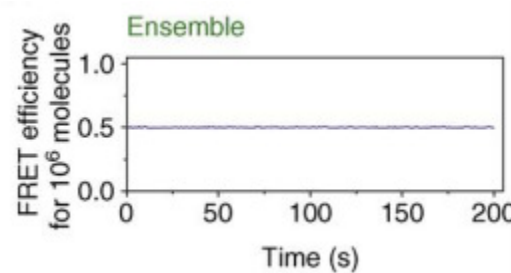
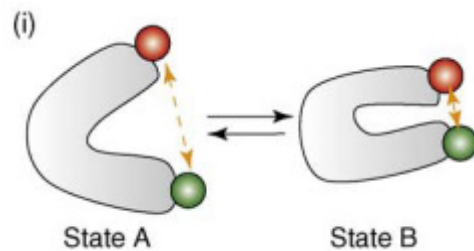
Static heterogeneity:

>1 state, not interconverting on time-scale of measurement



Dynamic heterogeneity:

one type of molecule fluctuating between states on time-scale of measurement

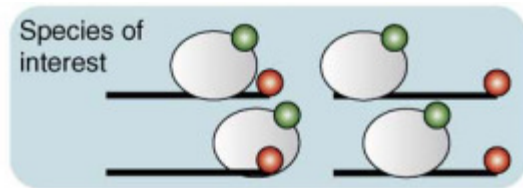


# Heterogeneity seen by single molecule methods

Examples of heterogeneity only seen with single molecule methods:

Static heterogeneity:

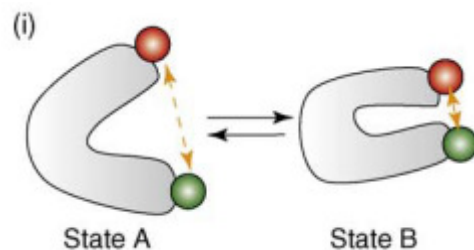
>1 state, not interconverting on time-scale of measurement



some nuclear pores allow better export than others  
(R. Singer, Nature)  
picket-fence model of plasma membrane diffusion  
(A. Kusumi)

Dynamic heterogeneity:

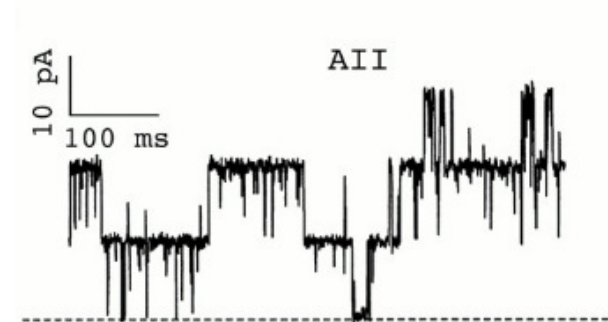
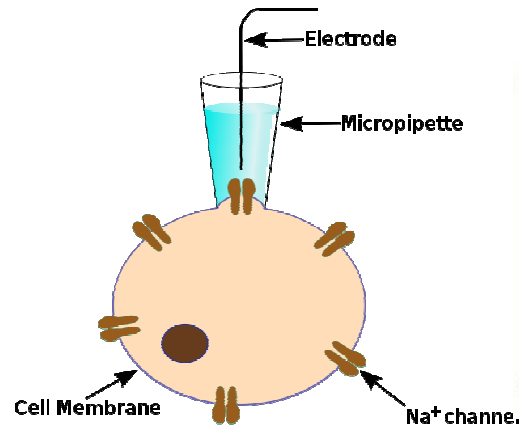
one type of molecule fluctuating between states on time-scale of measurement



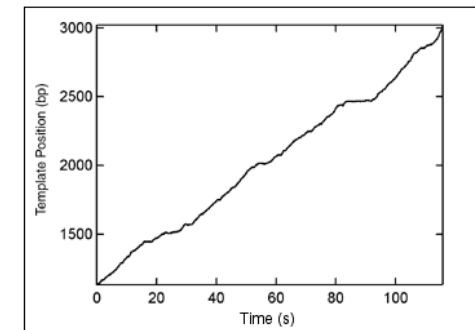
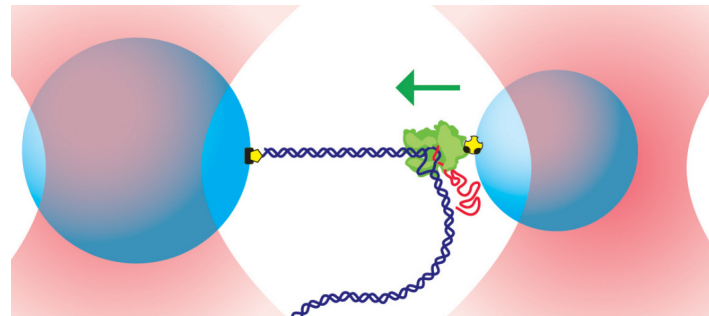
$\beta$ -galactosidase has 2 states with different  $k_{\text{cat}}$   
(S. Xie)

# Non-Spectroscopic Single Molecule methods

Ion flow: patch-clamp,  
nanopores



Using "Touch":  
AFM  
Optical tweezers  
Magnetic tweezers

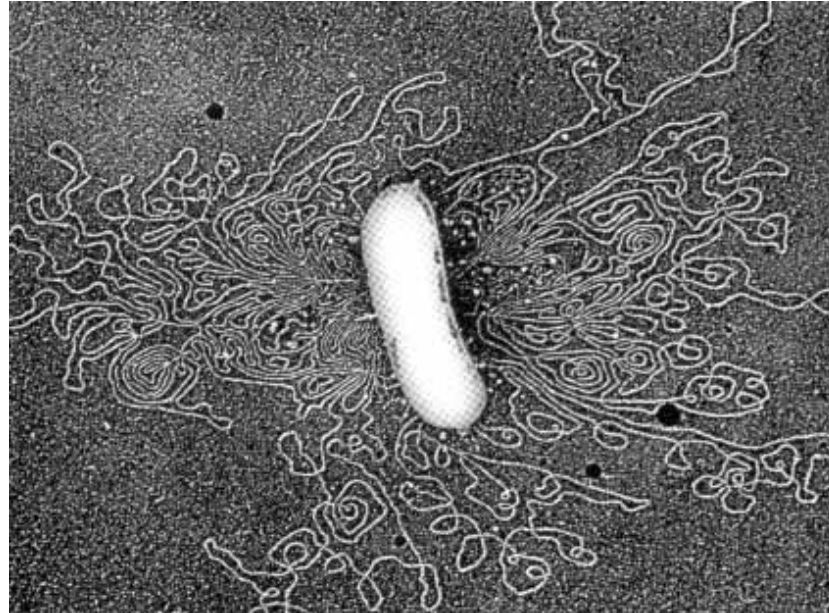


*measuring forces AND exerting forces  
to stretch, twist, cut biomolecules*

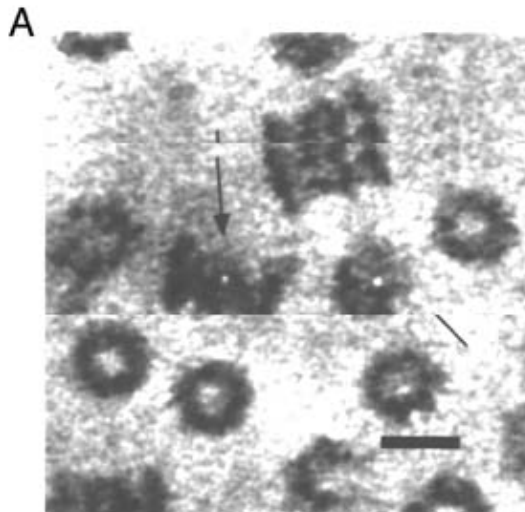


# Spectroscopic Single Molecule Methods

Electron microscopy  
fixing,  
shadowing  
heavy metal staining

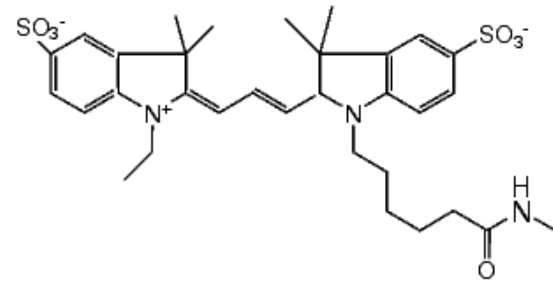


CryoEM tilt-sections

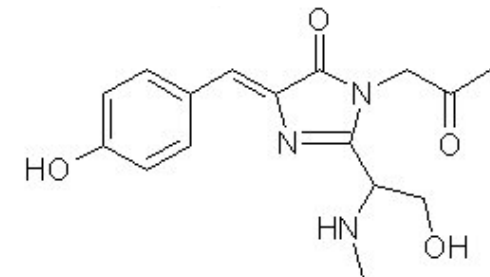
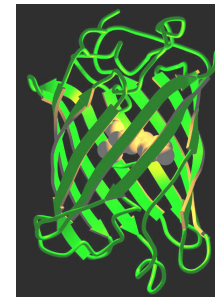


# Spectroscopic Single Molecule Methods

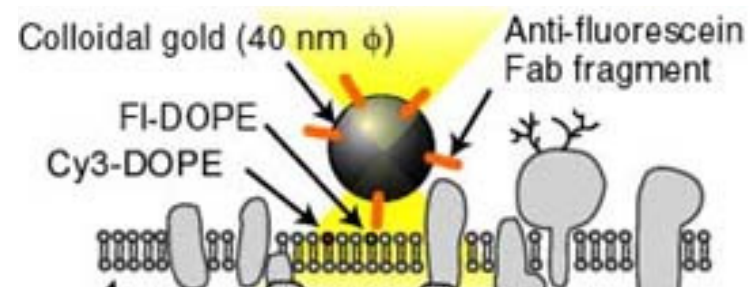
Small molecule dye fluorescence



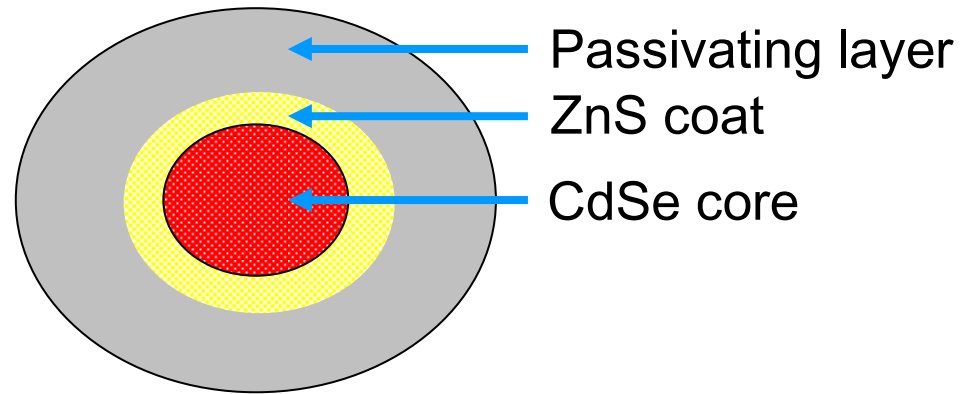
Fluorescent proteins



Gold or latex beads scattering



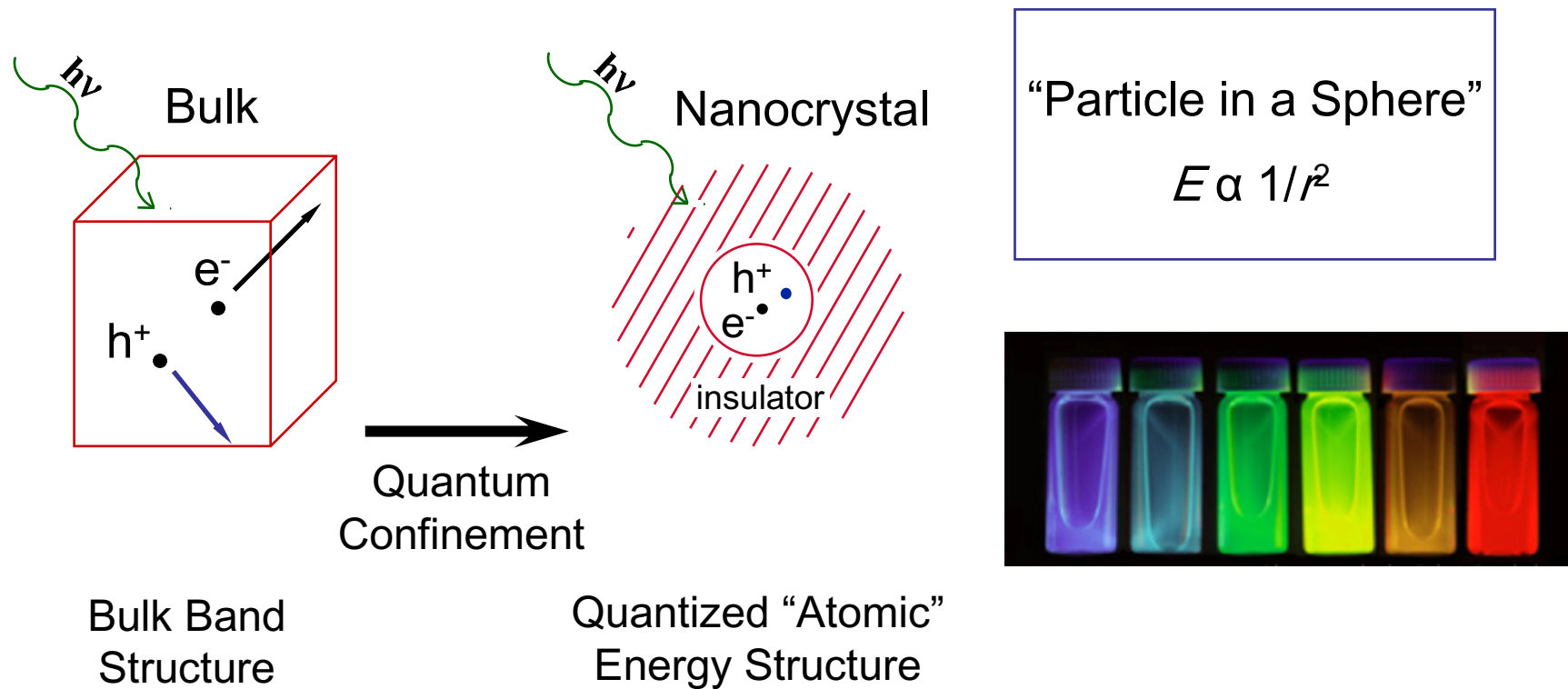
# Quantum dots make single molecule imaging accessible



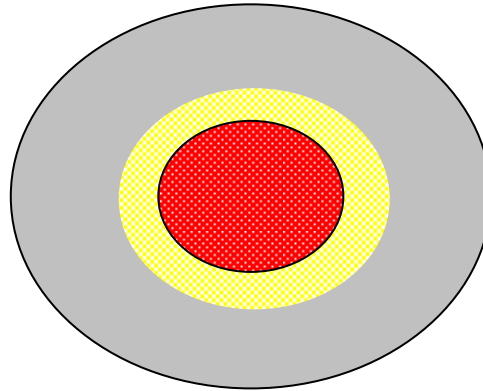
QD structure produces exceptional photophysical properties

*Michalet X et al. Quantum dots for live cells, in vivo imaging, and diagnostics. Science. 2005 Jan 28;307(5709):538-44.*

# Seeing quantum mechanics with the naked eye



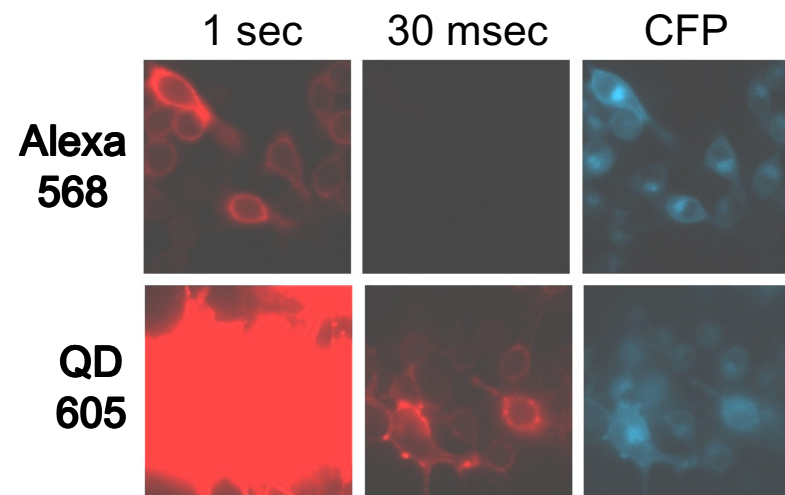
# Quantum dots are exceptionally bright



Quantum dots:

- Are exceptionally bright:  
single molecule imaging much easier

	QY	$\epsilon$ @ 395nm $10^3 \text{ M}^{-1}\text{cm}^{-1}$	$\text{QY} \cdot \epsilon$
QD605	0.4	1450	$580 \times 10^3$
GFP	0.8	30	$24 \times 10^3$

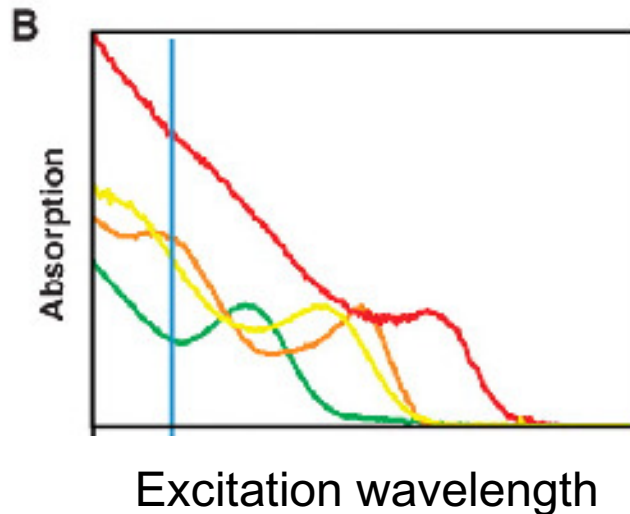


# Quantum dots have narrow emissions

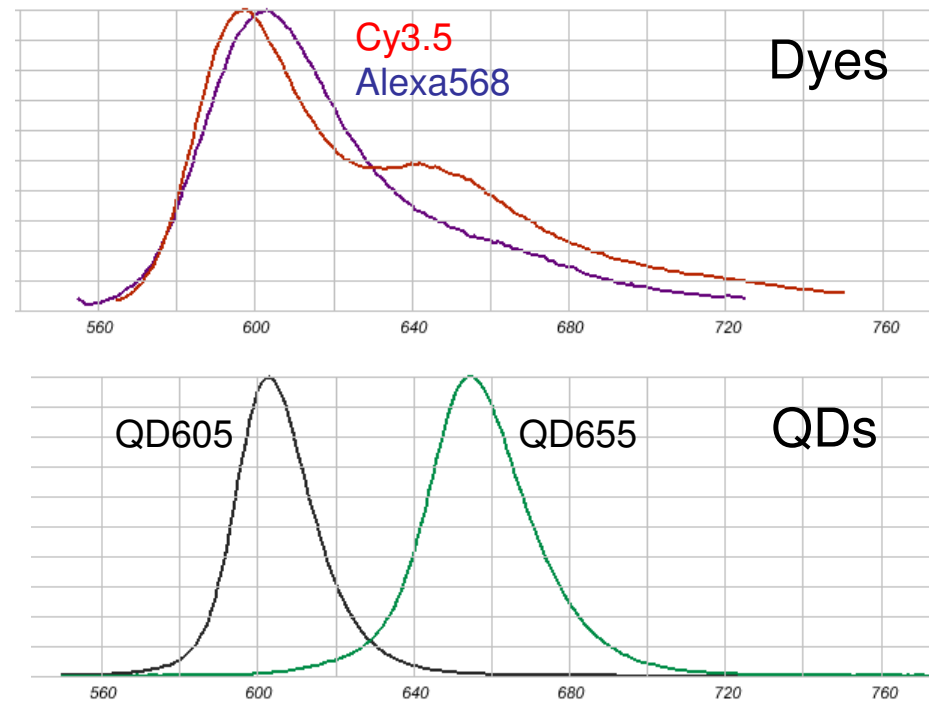
Quantum dot spectra:

- broad excitation spectra
- narrow emission spectra:  
multi-colour imaging easier

Allows 17 colour flow cytometry

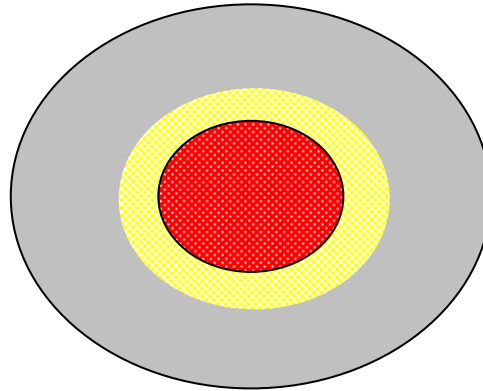


## Emission spectra





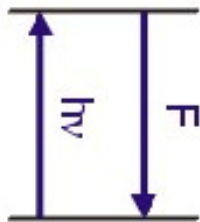
# Quantum dots are bright *in vivo*



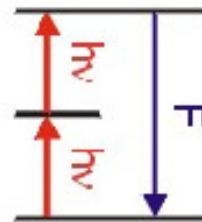
For infra-red emission  
QD core may be InAs,  
not CdSe

For tissue penetration,  
use near infra-red light and  
2 photon excitation

QDs are much brighter with  
2 photon excitation  
than other fluorophores



1 photon  
excitation  
at 400 nm

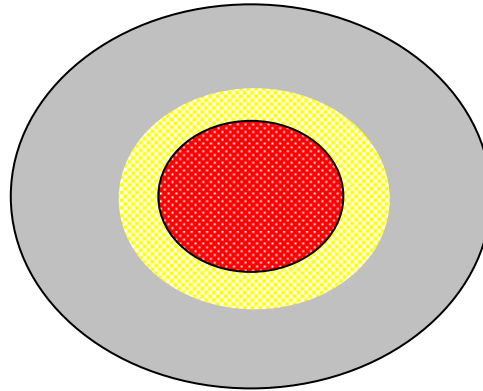


2 photon  
excitation  
at 800 nm

Cross-section (GM) @ 790nm

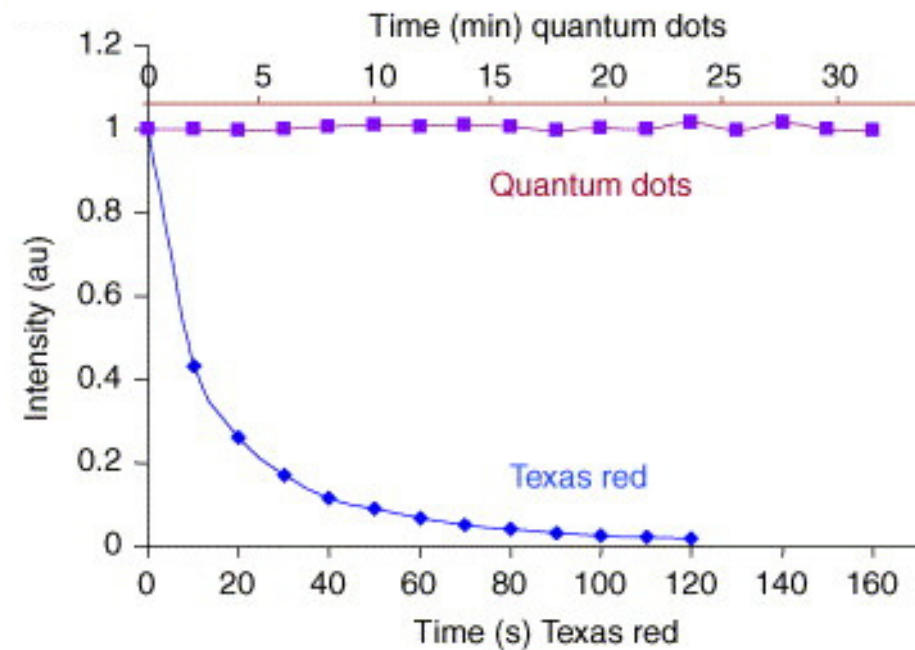
QD	80,000-170,000
GFP	3
fluorescein	100-400

# Quantum dots are photostable

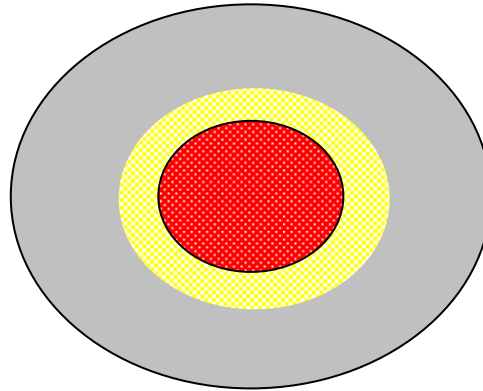


Quantum dots:

- Are exceptionally bright
- Do not photobleach:  
long-term imaging possible  
(bleaching is a major problem  
with dyes and fluorescent proteins)



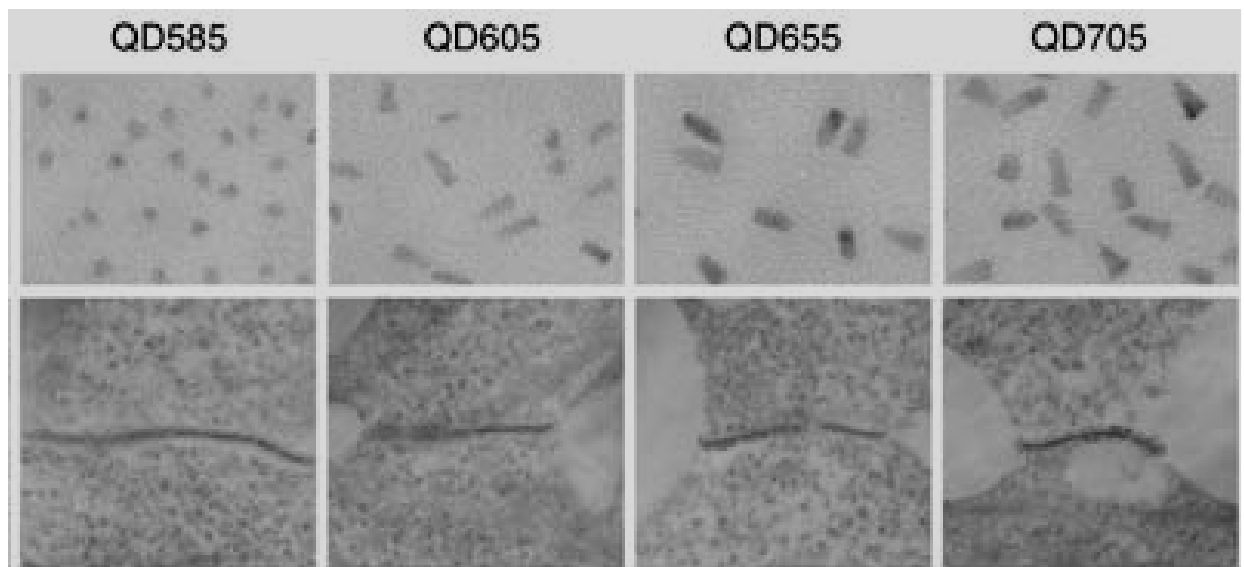
# Quantum dots are visible in the EM



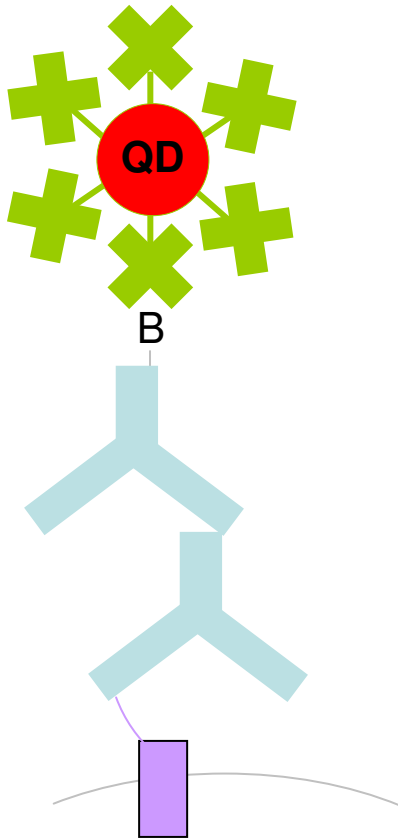
QDs are visible in the electron microscope because they contain heavy atoms. QDs emitting at different wavelength have different shape, as well as different size.

QDs alone

QDs staining gap junction  
between cells  
(zoomed out)



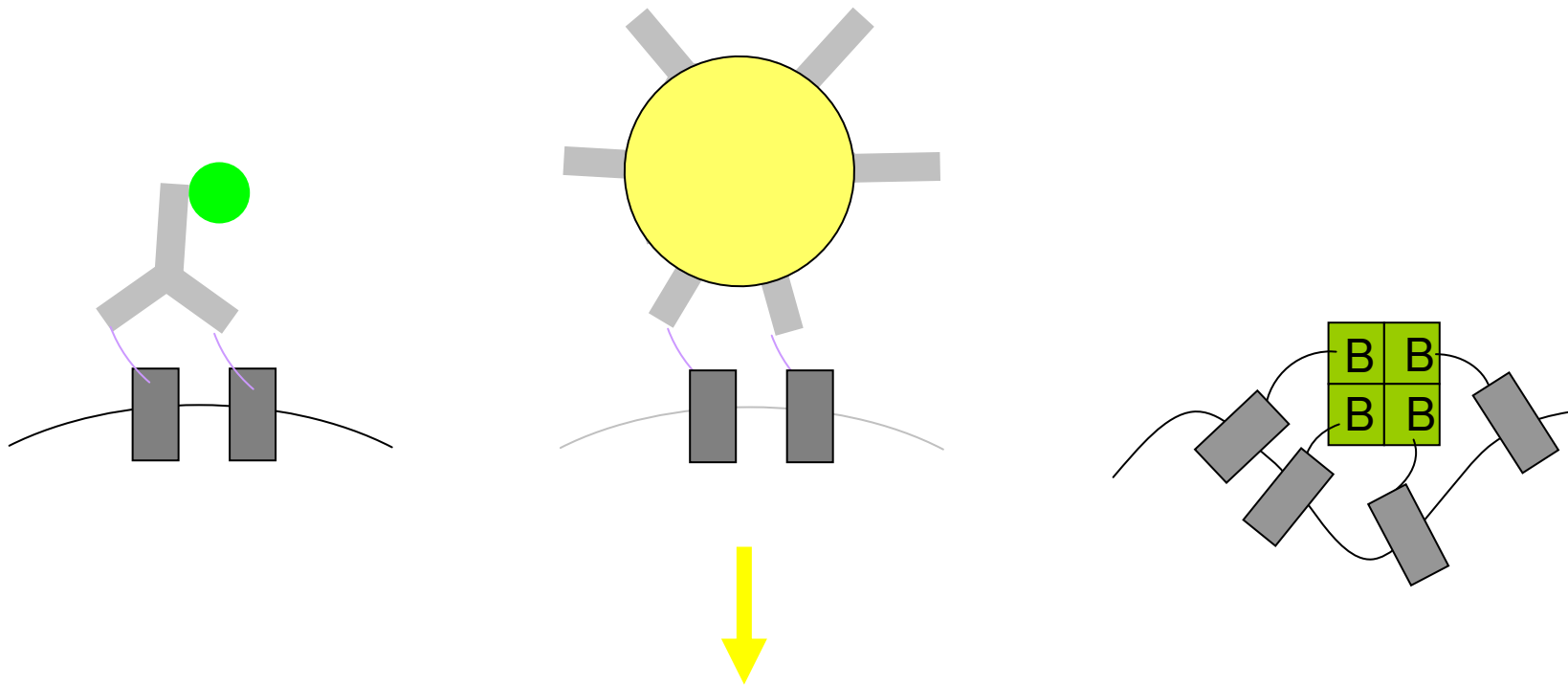
# Disadvantages of Quantum dots



1. Large size:  
of nanoparticle itself  
and antibodies used for targeting
2. Unstable labelling:  
Antibody dissociation
3. Multivalency
4. Hard to use inside cells

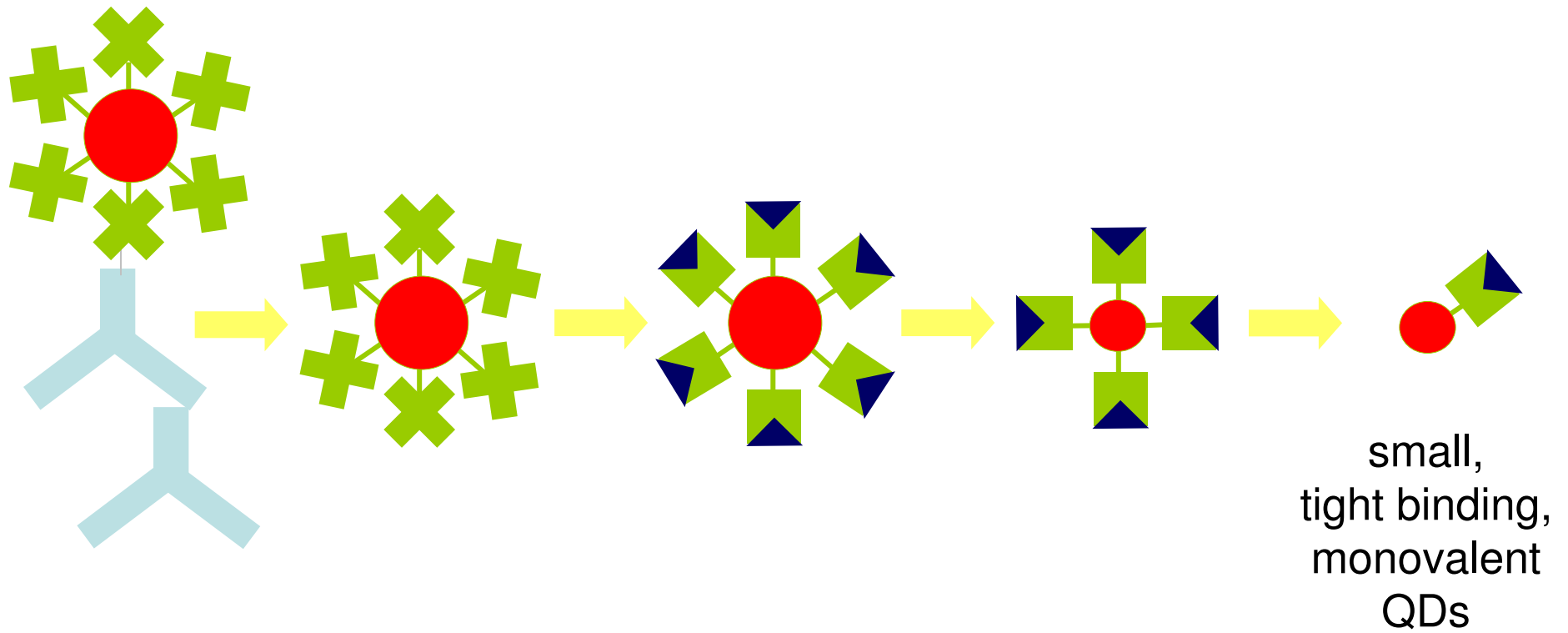
---

# Cross-linking is a key challenge for cellular labeling



Cross-linking reduces protein mobility  
and triggers cell signaling

# The Descent of Quantum dots



*Howarth M, et al. Monovalent, reduced-size quantum dots for imaging receptors on living cells. Nature Methods 2008 May;5(5):397-99.*

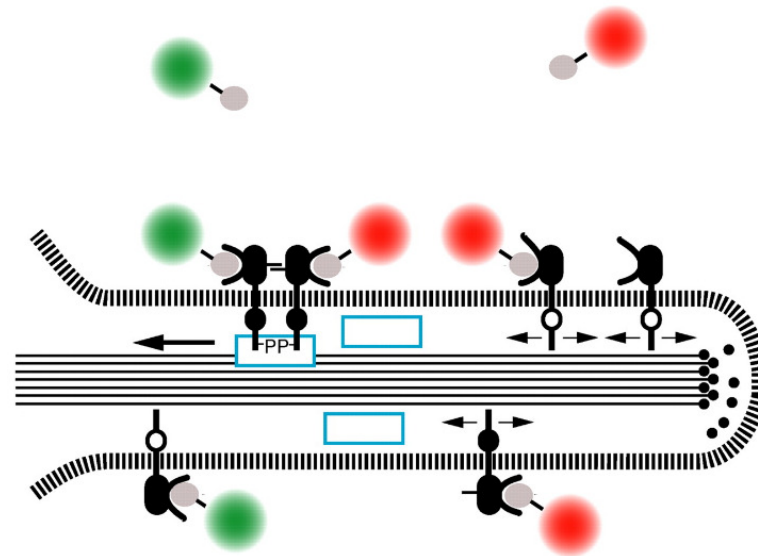
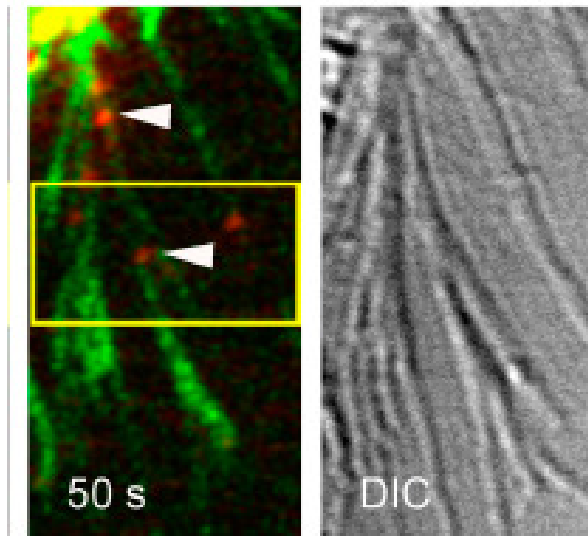


# QDs to study cancer cell biology at the single molecule level

Epidermal Growth Factor Receptor overexpressed or mutated in many cancers.  
Imaged at the single molecule level with QDs-

1. EGFR diffusing on filopodia (thin processes extending out from the cell)
2. “Reaches out” for EGF, EGF binds and receptors dimerise
3. Actin-dependent directional transport to the main body of the cell
4. Endocytosis at the base of the filopodia

Green actin-GFP, Red QD



See video 4 (<http://www.jcb.org/cgi/content/full/jcb.200503140/DC1>)

*Lidke DS et al. Reaching out for signals: filopodia sense EGF and respond by directed retrograde transport of activated receptors. J Cell Biol. 2005;170(4):619-26.*

---

# Optimising single molecule data

One molecule does not give a huge signal:

1. Maximise signal
2. Minimise background
3. Process data cleverly

# Optimising single molecule data

## 1. Maximise signal

EM-CCD

strong excitation (but not so that damage cell)

ideal filters

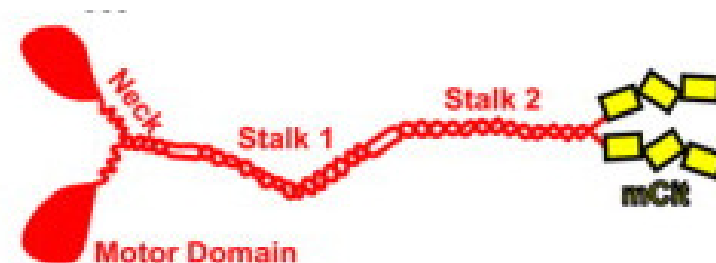
good fluorophore- brightness, photostable, non-blinking

maximized fluorophore conjugation: tandem FP (Cai Meyhofer 2007)

dyes per Ab

*single molecule imaging is not always single fluorophore imaging!*

optimal exposure time according to biology ( $\geq$ double the highest freq. event)



Kinesin with 3 Citrine on each tail

# Maximising signal: beware fluorophore blinking

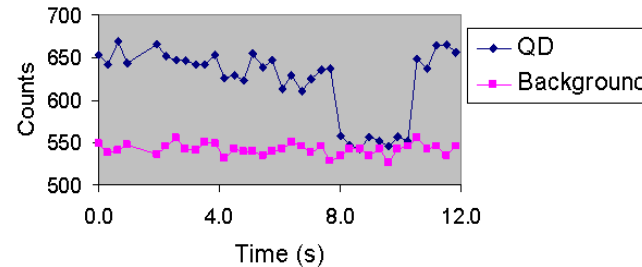
Single molecule imaging shows that most fluorophores can enter a dark state-

can be a problem for tracking. To avoid:

(i) do not over-excite

(ii) reducing agent, antioxidant sometimes helps

QDs:



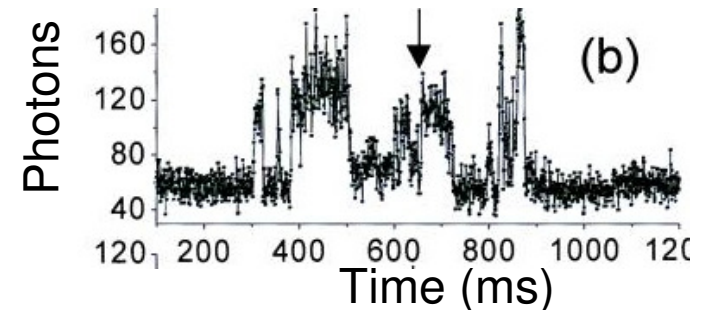
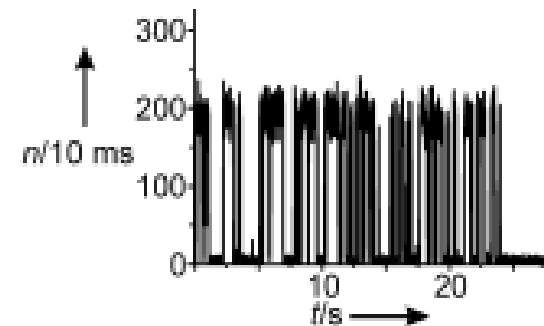
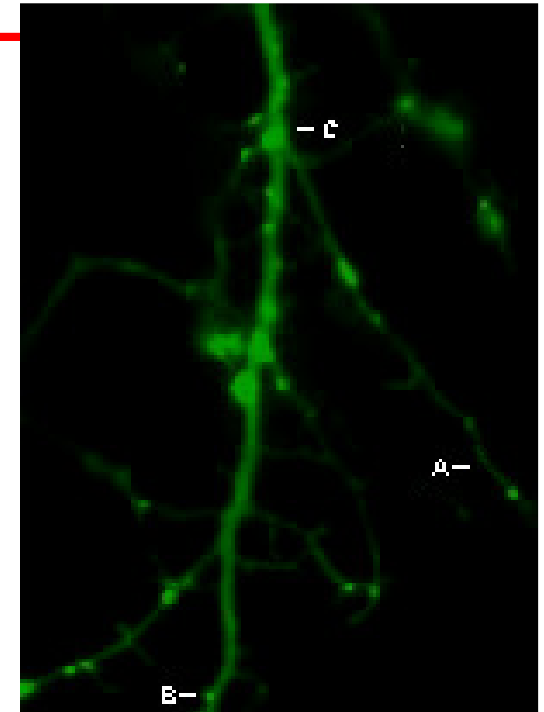
Atto647N dye:

S65T GFP:

Garcia-Parajo PNAS 2000

as fluorescent intensity increases

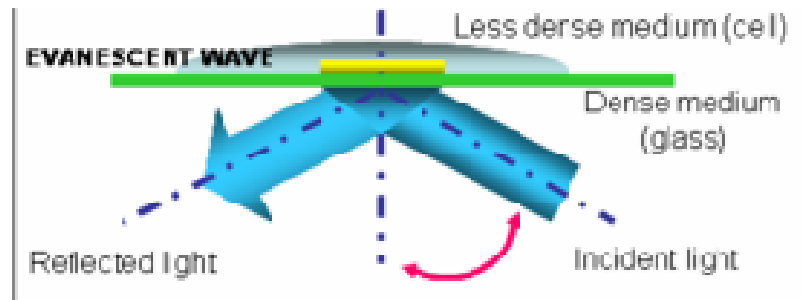
on-time reduced, off-time unaffected, ~1s



# Optimising single molecule data

## 2. Minimise background

non-specific probe binding- PEG, BSA, casein passivation  
fluorophore elsewhere in cell (control expression levels)  
minimize excitation volume- TIRF, slimfield



reduce stray light

minimize autofluorescence- high-grade reagents, 0.2  $\mu\text{m}$ -filtered,  
no phenol-red with cells, serum also autofluorescent

NaOH-cleaned or quartz coverslips

minimize vibration-

work in basement on vibration table

have fiduciary marker/ reference point

temperature fluctuation/ air-flow:

drift  $\sim 1\mu\text{m}/^\circ\text{C}$ , so 10nm per  $0.01^\circ\text{C}$ !



# Optimising single molecule data

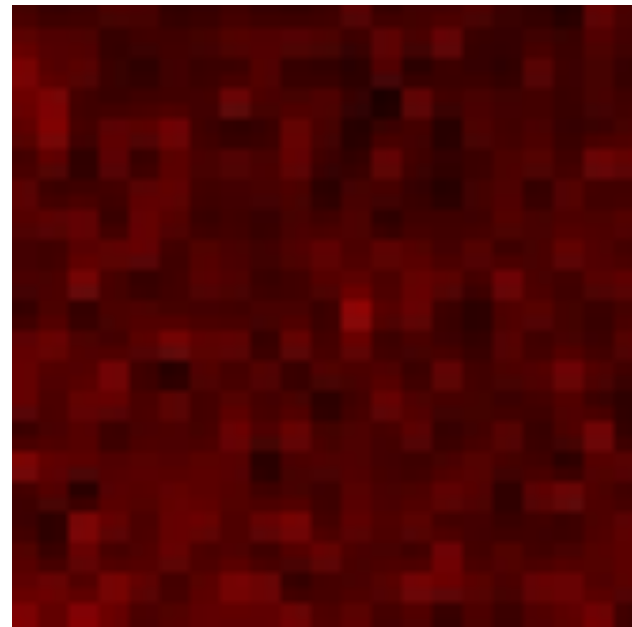
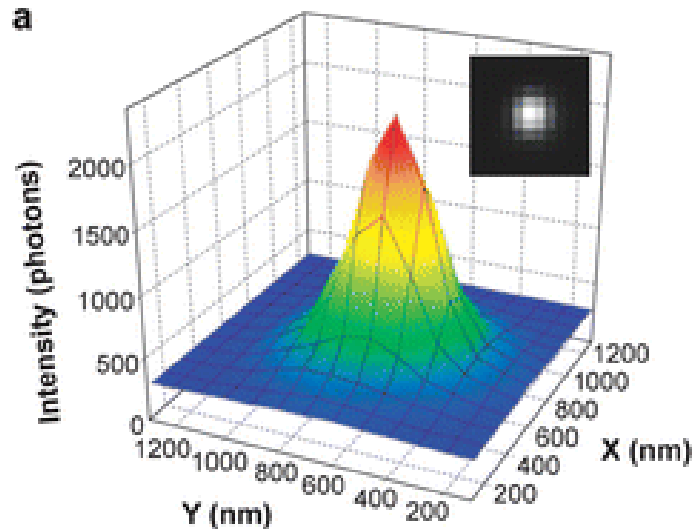
## 3. Analyse data cleverly

centroid fitting to get sub-pixel resolution

800 nm spot, 100 nm pixels, down to 1 nm resolution

frames are not independent- bkg will not fluctuate in same way as signal

CCD noise uncorrelated from pixel to pixel- filter to isolate features with size of diffraction limited spot

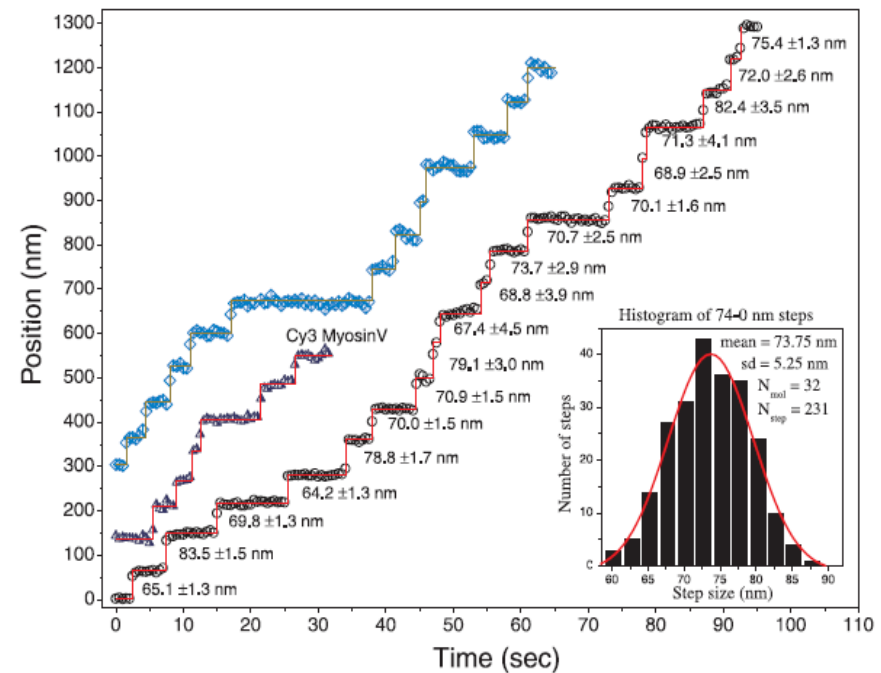
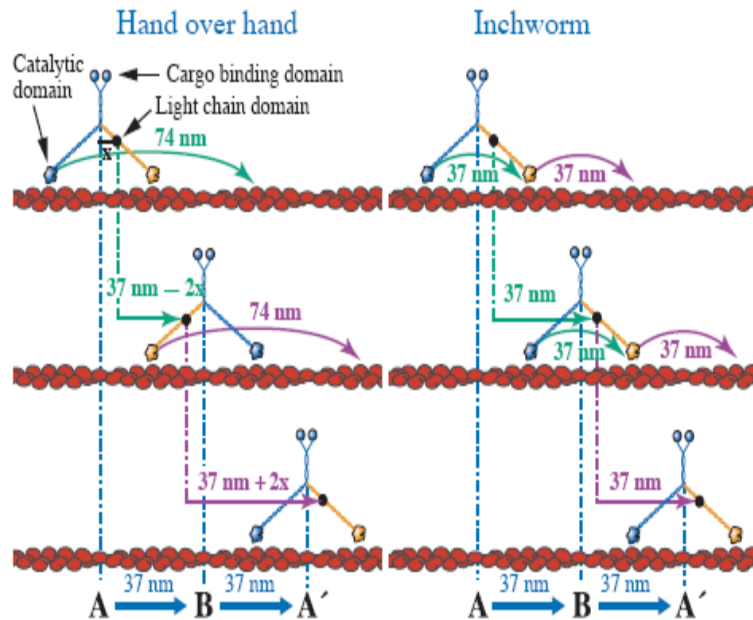




# How does myosin V walk?

## Single-molecule fluorescence investigation

Myosin V is not involved in muscle contraction, but in transporting cargo along actin cytoskeleton. Two models of motion:



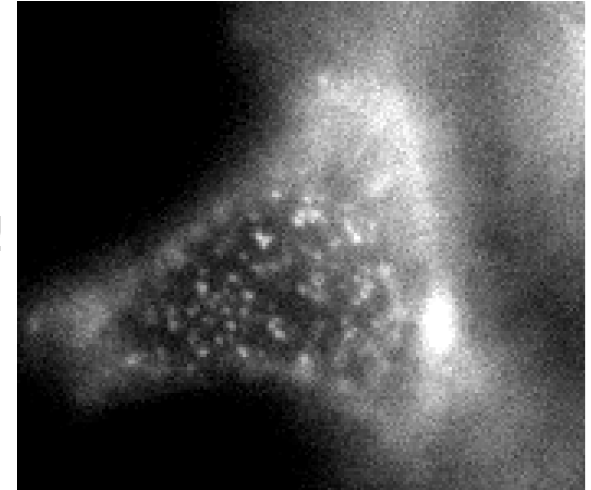
Steps of 3 different myosin V molecules  
all around 70nm

TIRF on myosin fluorescently labeled on one leg,  
with record-breaking spatial resolution  
(1nm, rather than 200nm with conventional microscopy)

# Controls that imaging single molecules

**Look at a cell and see bright dots  
(sometimes only after image processing):  
Do the spots correspond to your target protein?**

There is always autofluorescence and background binding!  
Image cell without FP-fusion  
Image cell lacking protein to which probe should bind  
no primary Ab control, RNAi,  
peptide to block antibody



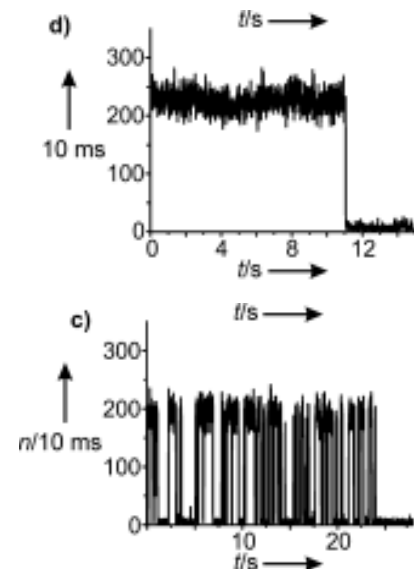
**Do the spots represent 1 copy of your target protein or 2,3...20?**

Diffraction limited spot

Step-wise photobleaching

Step-wise blinking

Uniform spot intensity - calibrate to known  
fluorescence of single GFP/QD



# Controls for biological relevance

## Not too much light

(UV toxic, blue can be damaging, even red can be damaging if enough of it)

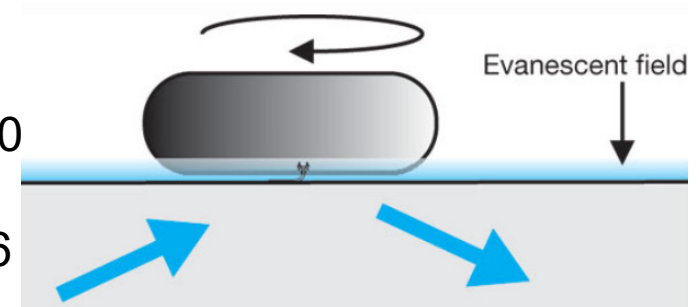
cell still divides

*E. coli* Reyes-Lamothe et al. Science 2010

yeast Carlton, Sedat PNAS 2010

flagellum still rotates, Leake et al. Nature 2006

NB imaging bursts at exocytosis...



## Fluorophore modified biomolecule still functional:

goes to right cellular location/ protein complex  
complements knockout cell

put in genomic locus and control expression level too  
ideally **quantitatively** the same

e.g. division time by FACS,  
(for wt is 111 min)

Reyes-Lamothe et al.

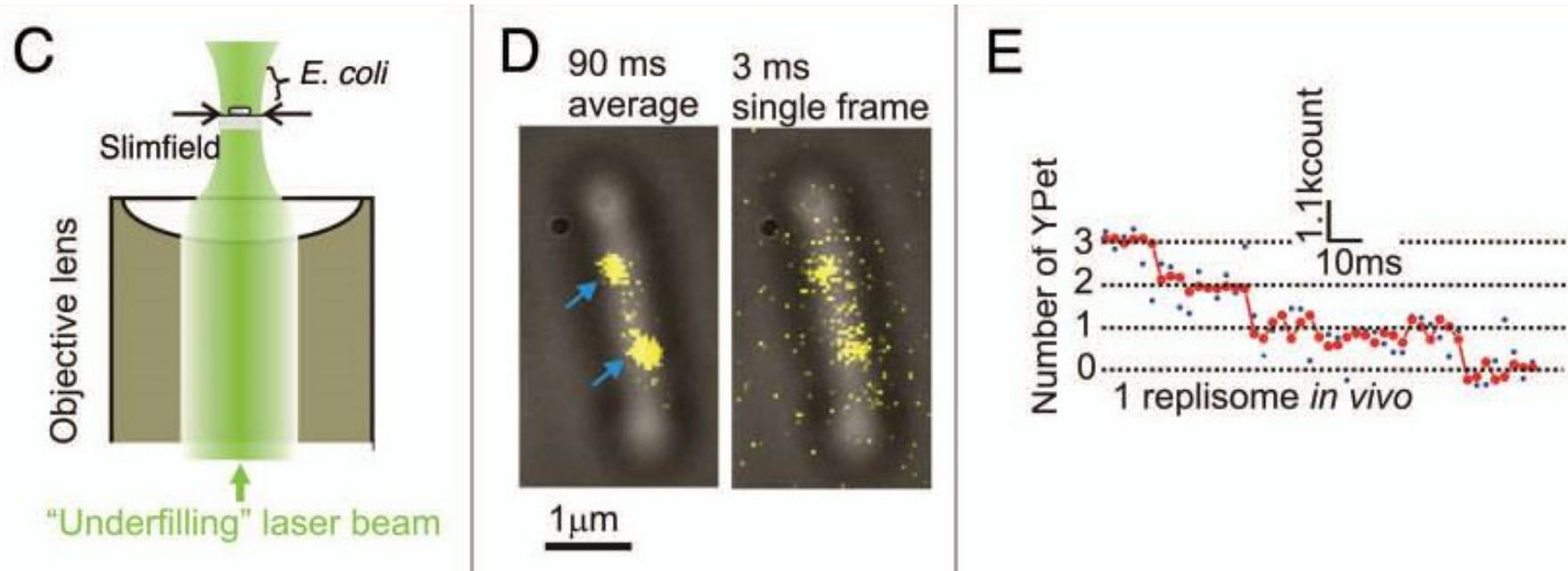
flagellar fusions swim as fast

possible that some properties intact but others  
different

Table S1

Protein	YPet fusion/terminus	Generation time/ min <sup>a</sup>
DnaB	C	121
$\alpha$ (DnaE)	C	119
$\beta$ (DnaN)	N	119
$\epsilon$ (DnaQ)	C	111

# Step-wise photobleaching



Sensitive imaging of YPet-tagged protein in complex in cell by concentrating excitation to small volume- "Slimfield" microscopy. See discrete changes in fluorescence as individual YPet bleaches. Tag multiple species in complex e.g. *E. coli* replication fork and obtain stoichiometry... unexpectedly 3 DNA polymerases per fork

Science. 2010;328(5977):498-501.

Stoichiometry and architecture of active DNA replication machinery in Escherichia coli.

Reyes-Lamothe R, Sherratt DJ, Leake MC.

---

# From single molecule, back to population!

In medicine, one patient can be meaningful if the phenotype and cause is clear enough.

# From single molecule, back to population!

In medicine, one patient can be meaningful if the phenotype and cause is clear enough.

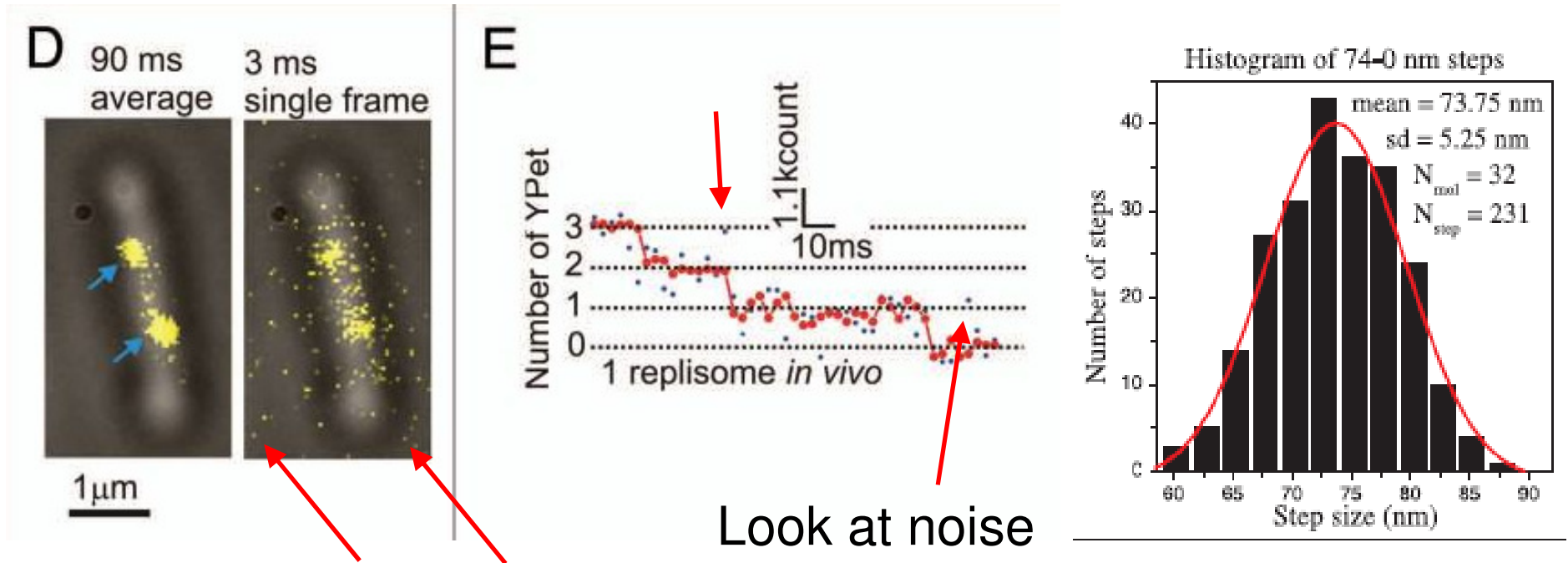


A single mutation can be informative because it persists and can be transferred.

A single molecule fluorescent measurement is still too noisy to be confident from one observation.



# From single molecule, back to population!

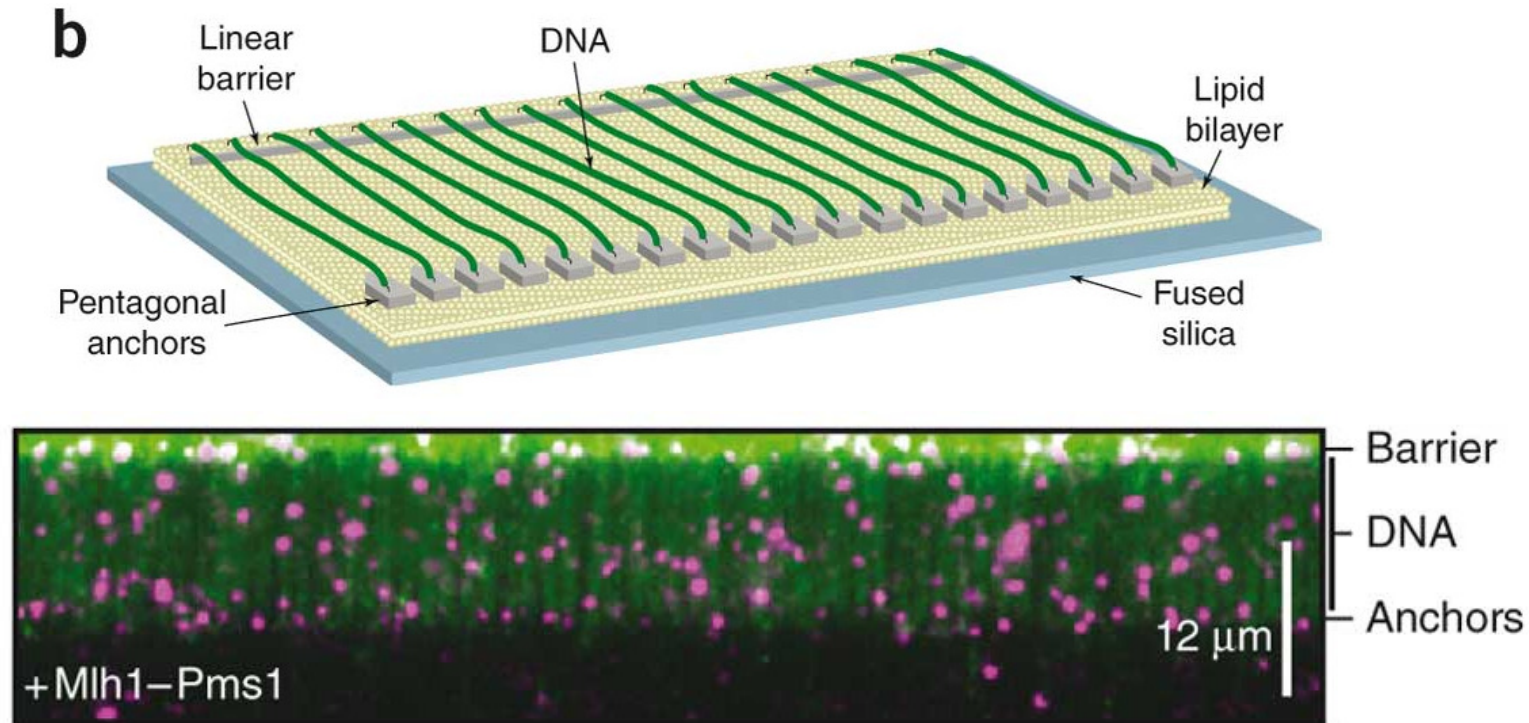


Look at noise

Know what confidence one can have in the data.  
If “raw” data is not presented, it is very hard to say.

Stochastic events in experiments- fluorophore bleaching, dust, camera noise, proteins 99 and not 100% pure  
and in biology- mistranscription, mistranslation, misfolding, misassembly...

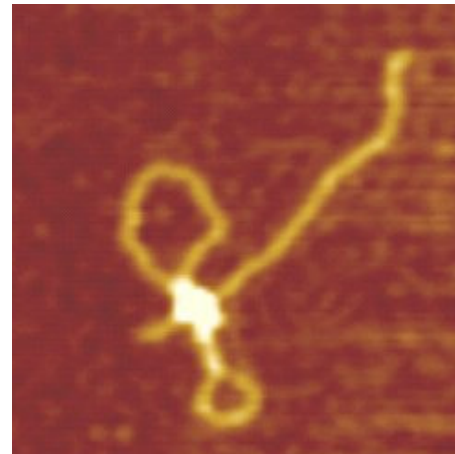
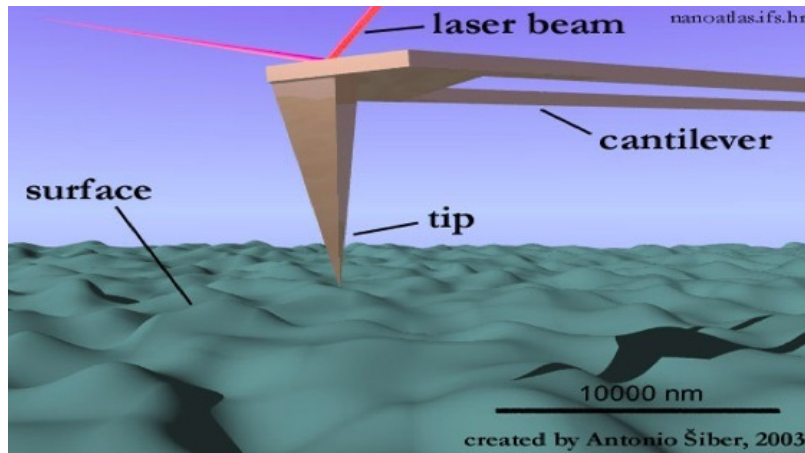
# From single molecule, back to population!



Eric Greene: DNA curtains to collect several hundred single molecule tracks in same microscope field of view on DNA motors/ repair proteins (usually tagged with QDs).

Rapid data collection  $\longrightarrow$  Good statistics  $\longrightarrow$  Clearer findings, not anecdotes!

# Principle of atomic force microscopy



AFM image of DNA looped by restriction enzyme

Atomic Force Microscopy (AFM) depends on:

(i) a sharp tip vibrating up and down to measure the height of a surface  
(height precisely measured by bouncing laser of top of the tip)

(ii) raster scanning the tip across the surface to create a picture of surface topography.

Gives unique pictures of orientation of single molecules and complexes

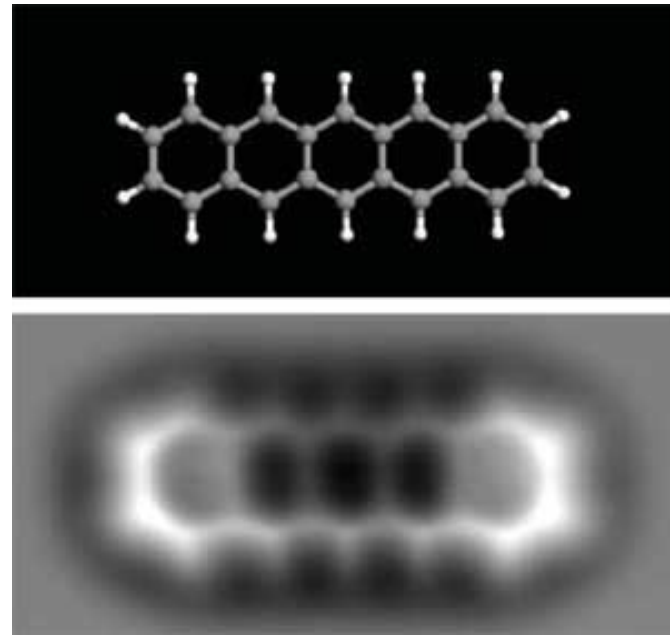
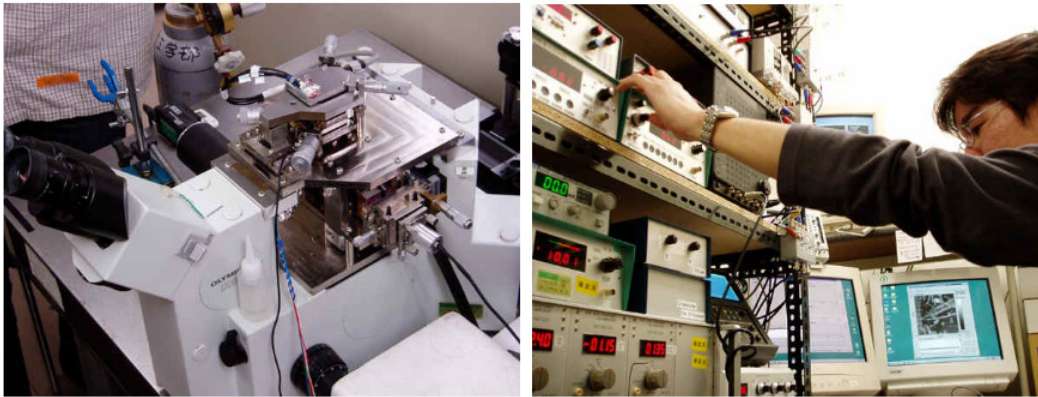
BUT

Moderate spatial resolution

Slow

Concern that tip is disturbing sample

# A vision of the future: a new generation of AFM



Decades of optimization- vibration, electronic noise, data processing

Tip has 4 nm radius (other people have got a single molecule at the tip)

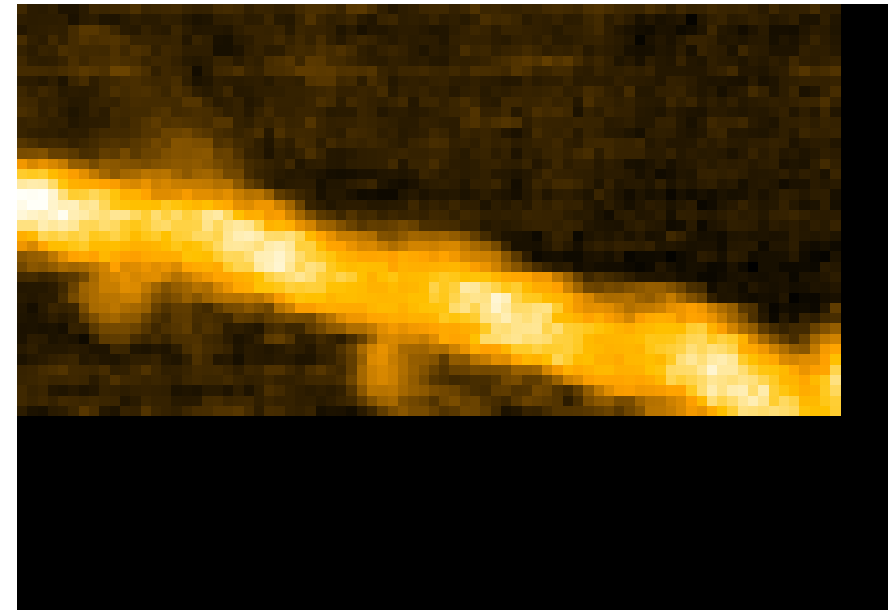
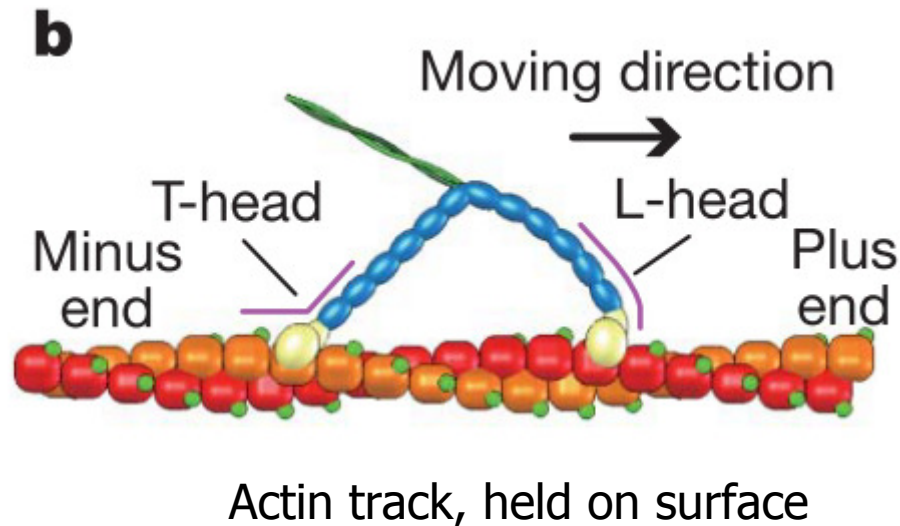
Effect on sample depends on Force x Time. Time kept down to 100ns.

Scan in  $\sim 0.1$ s so can image motor dynamics

# A vision of the future: kinetics and structure at the same time

Fragment of myosin V walking  
powered by ATP hydrolysis

Snapshot from high-speed  
AFM movie



36 nm steps.

Hand-over-hand movement driven by rotation of the L head,  
as soon as T head detaches.

Does prodding the motor affect its movement? No! Max. speed seen by AFM  
comparable to that measured by fluorescence tracking.

N. Kodera et al. Nature 2010



# Conclusions

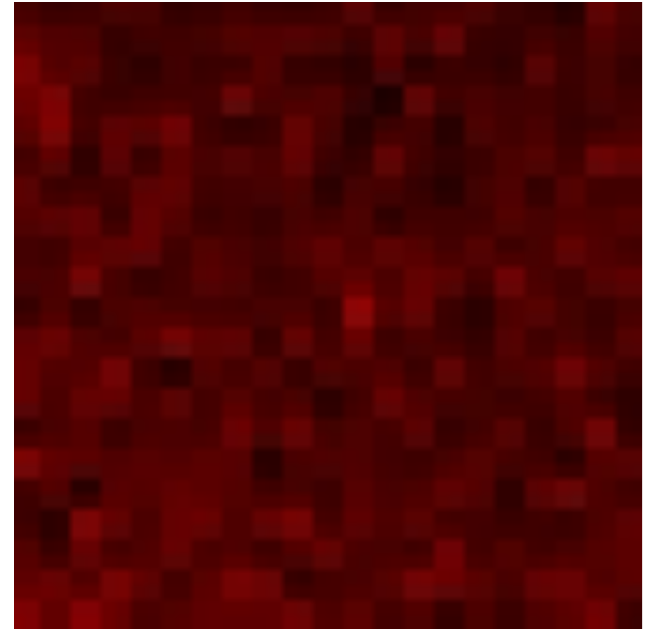
Single molecule biology is advancing rapidly.

Single molecule approaches reveal:  
small movements,  
heterogeneity of biomolecules and cellular assemblies  
rare events

Single molecule methods include patch-clamp, AFM, EM  
-not just fluorescence microscopy

Many single molecule approaches require good imaging set-ups and careful data analysis.

Quantum dots make single molecule imaging accessible,  
with high brightness (TIRF not required, high spatial/temporal resolution)  
and photostability (no need to scavenge oxygen)  
but at the cost of large size (12-25 nm) and a delivery challenge



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# Further reading



Advances in single-molecule fluorescence methods for molecular biology.  
Joo C, Balci H, Ishitsuka Y, Buranachai C, Ha T.  
Annu Rev Biochem. 2008;77:51-76.

Single Molecule Techniques: A Laboratory Manual  
by Paul R. Selvin and Taekjip Ha 2007

Review on Quantum Dots:  
Probing cellular events, one quantum dot at a time.  
Pinaud F et al. Nat Methods. 2010 Apr;7(4):275-85.