Reading for lecture 11

1. Optical Tweezers, Myosin
2. Atomic Force Microscopy (AFM)
3. Single-Molecule Fluorescence Microscopy
4. Patch-Clamp
5. Genetic Techniques

- Key references are included *in italics* at the relevant points in the notes.
- Information and tutorials on the techniques in this lecture can be found on the internet.

[reading for lecture 11] (1)

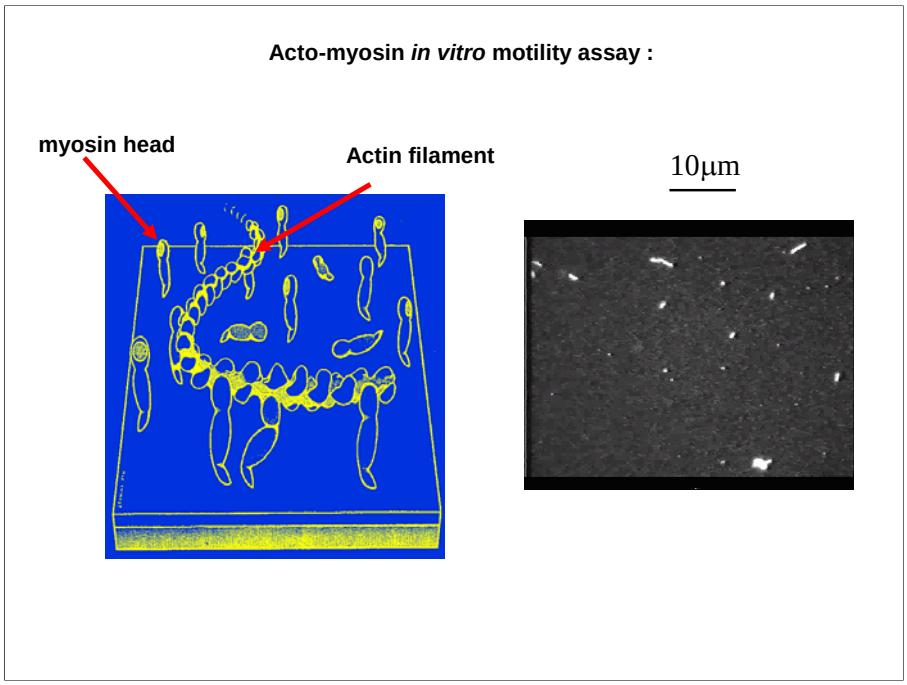
Optical tweezers and Myosin

Myosin and F1 are probably the best understood molecular motors.

Knight AE, Molloy JE. (2000) "Muscle, myosin and single molecules." Essays Biochem 35:43-59

A review of single-molecule experiments on myosin.

Single- molecule experiments with myosin date back to the 1990s.



[Sliding filament assay] (2)

Actin filaments are visible because they have “fluorescent label” molecules attached.

Not really “single-molecule” – several myosin heads propel each actin filament

Direction is determined by the polarity of the actin filament – myosin heads are randomly oriented on the surface.

Each myosin molecule only binds actin intermittently – so the actin and myosin need to be held close together (as in muscle) if single interactions are to be seen “*in vitro*” (“in glass”, as opposed to “*in vivo*”, “in life”)

Make dumbell:
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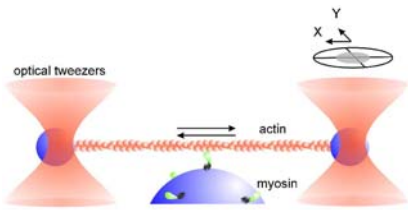
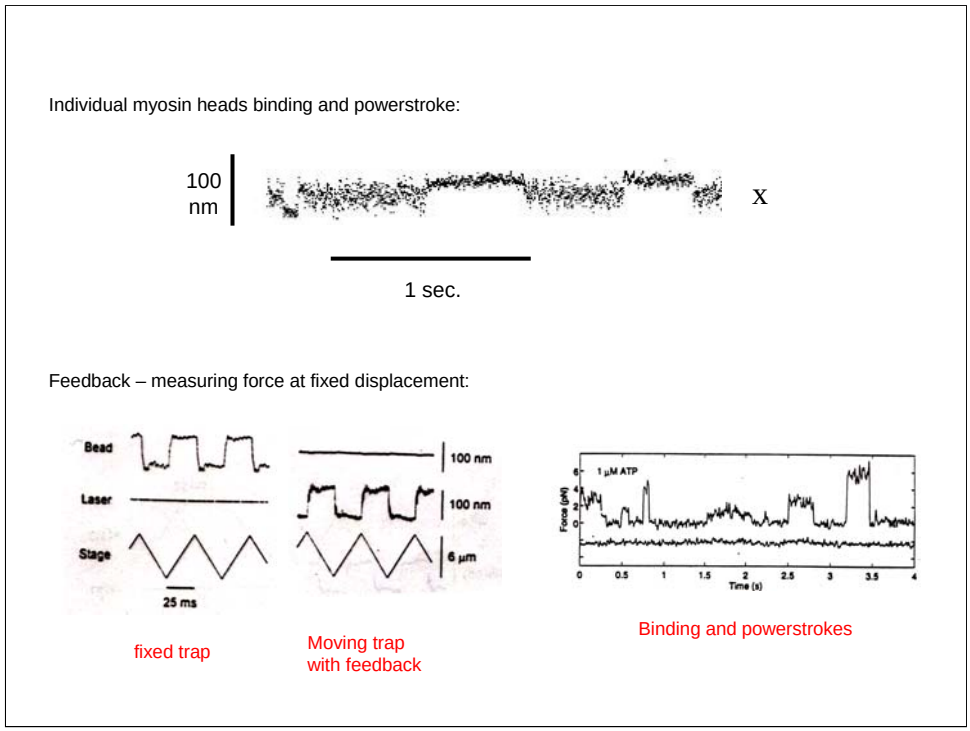


[Optical Tweezers –two bead assay] (3)

The position of the optical trap can be controlled by computer, using “acousto-optic deflectors” to steer the trap laser beam.

Actin filaments are stretched between two trapped beads and brought up against immobilized myosin heads.

When ATP is present, the actin filament is pulled towards one end by the myosin.



[Myosin, single steps] (4)

When a single myosin head binds an actin filament held loose in the optical traps, the Brownian noise is reduced.

The average position while bound is shifted about 5 nm from the average position when not bound. This is an estimate of the size of the powerstroke

Molloy JE, Burns JE, Kendrick-Jones J, Tregear RT, White DC. (1995) "Movement and force produced by a single myosin head." Nature. 378(6553):209-12.

Detection of binding events and estimate of step-size.

The force generated in the powerstroke can also be measured…

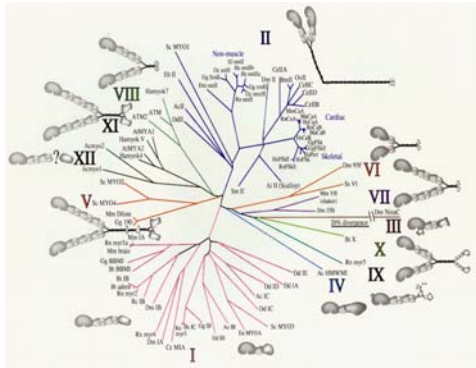
Move the optical traps so as to keep the beads in the same position (feedback). The distance between the trap and the (fixed) beads is then proportional to the force.

Powerstrokes generate about 6 pN under these conditions.

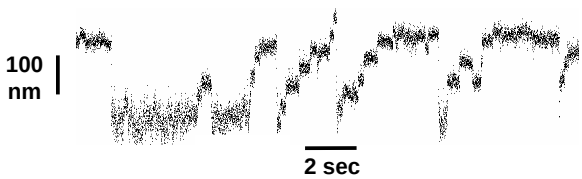
Finer JT, Simmons RM, Spudich JA. (1994) "Single myosin molecule mechanics: piconewton forces and nanometre steps". Nature.368(6467):113-9

The first paper using optical tweezers with myosin. Feedback force measurements.

The myosin family :



Myosin V takes several big steps before letting go:



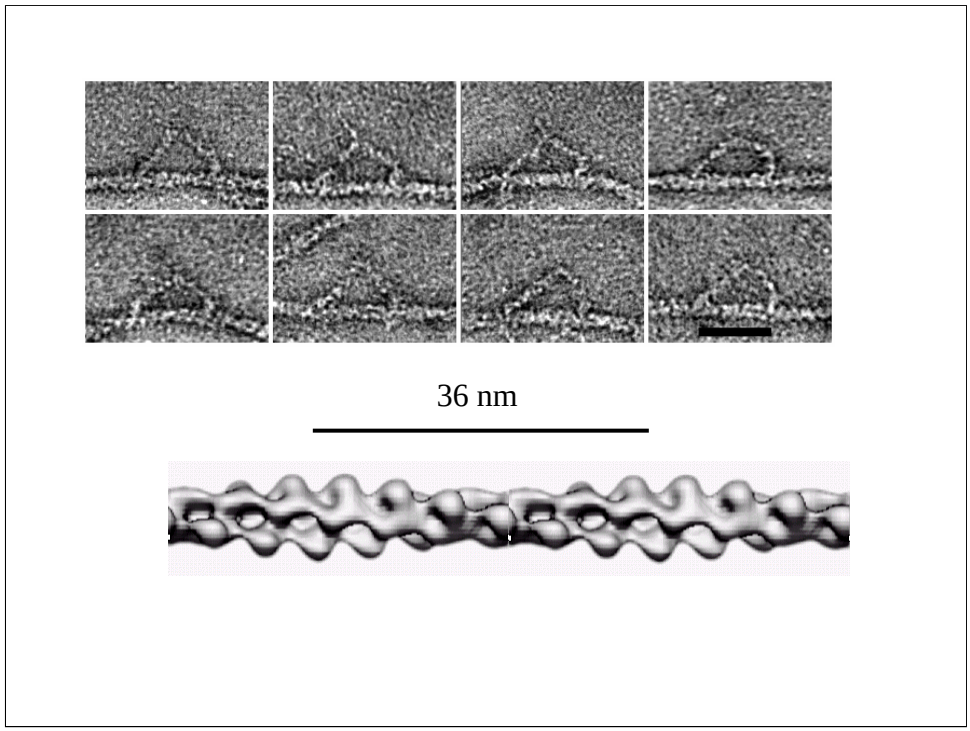
[Other myosins, kinesins] (5)

Muscle myosin is atypical – most linear ATP-driven motors work alone or in smaller groups, not in a muscle.

> "Processivity" is the ability of a single motor to take several steps while remaining attached to the track.
>
> More and more processive motors (myosins, kinesins) are being discovered and studied.

Kinesins are more processive than myosin V. A single kinesin molecule takes hundreds of steps without falling off the microtubule track.

Processive motors usually have two heads – "hand-over-hand" mechanism?

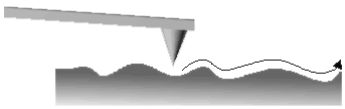


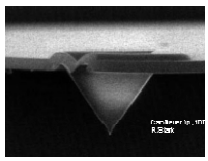
[Myosin V steps –electron microscopy] (6)

The actin filament helical pitch is 36 nm. Myosin V binds once per turn.

Electron microscope pictures are “snapshots” of the motor mechanism.

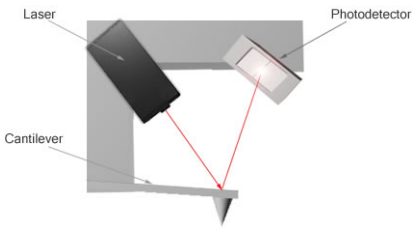
Scanning Probe Microscopy

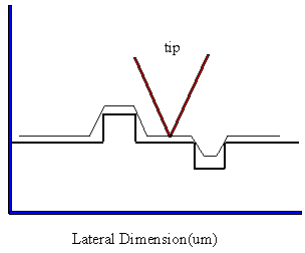




Essential ingredients:

1. **A way to detect the surface**. In "scanning tunelling microscopy" (STM), the tunelling current into a conducting sample is used. In AFM the tip touches the surface and its deflection is measured.
2. **A way to move the tip up and down**, keeping it a fixed distance from the surface. Piezo-electric crystals are used, displacements can be controlled to Angstrom resolution.
3. **A way to move the tip side-to side**, to scan a surface and make a picture based on sample height. Piezo electrics are used for this too.





Atomic Force Microscopy (AFM)

[AFM, the basic idea] (7)

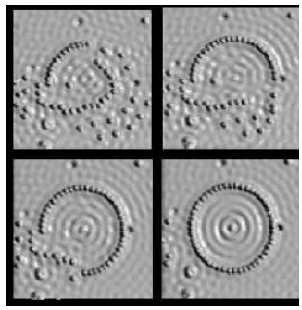
AFM is one type of scanning probe microscopy.

> The idea is to use feedback to control to keep a surface-contact signal constant, by moving the tip up and down as it is scanned steadily from side to side. Thus a map of height is generated.
>
> In AFM the feedback signal is the deflection of a cantilever that physically touches the surface.
>
> The cantilever can also be oscillated at resonance, and either amplitude or phase of the oscillation used as the feedback signal – both are affected in different ways by the closeness and type of the surface.

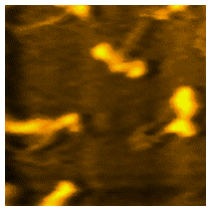
Probe microscopy is possible because piezo-electric crystals give sub-Angstrom control of position, and therefore sub-Angstrom vertical resolution.

On very flat surfaces, single atoms that "stick out" can be seen.

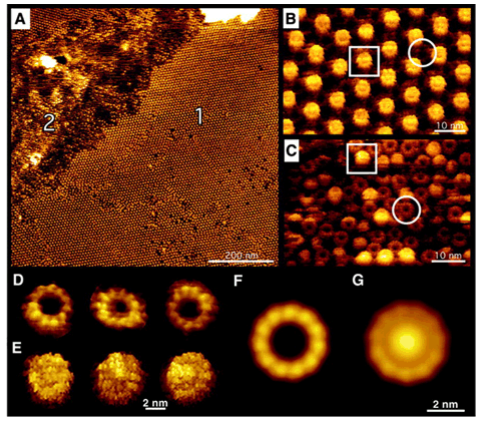
> However, if the surface contours are steeper then the bluntness of the tip limits the lateral resolution.



Atoms and wavefunctions: STM



Moving Myosin heads



Fo rotors from ATP-synthase

[AFM, some pictures] (8)

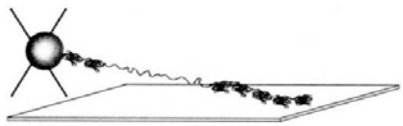
Single bio-molecules can be seen in water in conditions where they are still working.

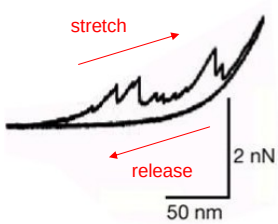
(Single atoms and electronic wavefunctions can be seen in vacuum at low temperatures using STM.)

The tip can be used to manipulate samples as well as to image them…

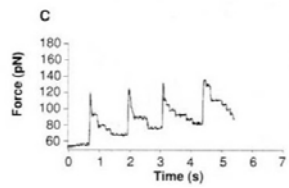
Stretching collagen with AFM

Stretching titin with Optical Tweezers





Collagen Force vs extension



[Force spectroscopy] (9)

Single biological molecules can be pulled with AFM tips or optical tweezers.

Sudden drops in force correspond to unfolding of protein domains.

Force-extension curves:

Unfolding steps during stretch.

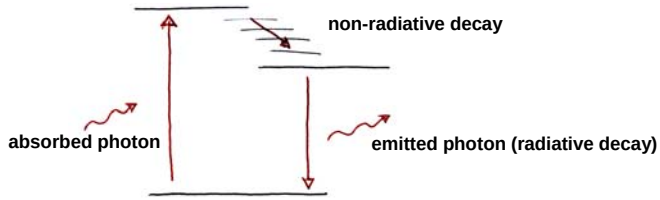
Force-extension curve during release is the classical shape for an entropic spring or “worm-like chain” - the protein is unfolded to its contour length.

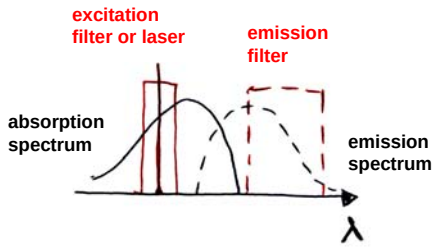
The technique is being used to study protein folding, DNA hybridization and binding of different molecules to each other.

AFM gives higher maximum force. Optical tweezers give higher sensitivity.

Fluorescence Microscopy

Fluorescence, molecular electronic energy levels:





Single-molecule Fluorescence Microscopy

[Fluorescence microscopy] (10)

Fluorescent molecules absorb photons and re-emit longer wavelength photons after a delay lasting typically ns.

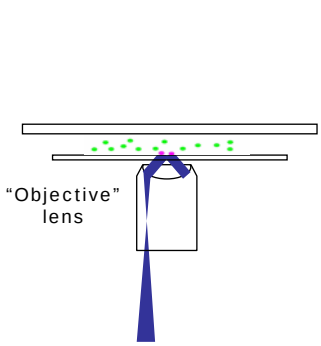
Fluorescence microscopes use filters to ensure that the excitation light is blocked, allowing an image to be formed from the relatively weak emitted light.

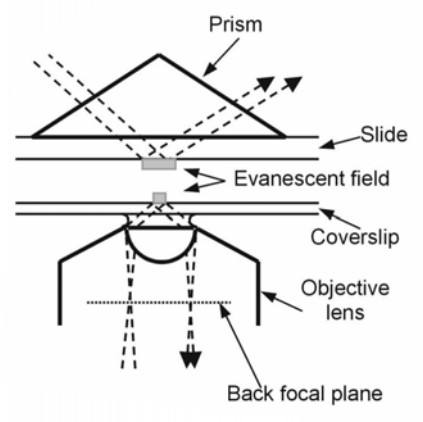
By attaching a fluorescent molecule (“label”) to a molecule of interest, it can be seen even against a large background of non-fluorescent molecules.

> Labels may be small chemicals (dyes), added to purified proteins by chemical crosslinkers.
>
> GFP (Green Fluorescent Protein) can be genetically engineered attached to a chosen protein, providing a fluorescent label inside a living cell.

Single fluorescent molecules can be detected if the background (light from other sources, particularly other fluorescent molecules) is reduced far enough.

Total Internal Reflection Fluorescence (TIRF) microscopy





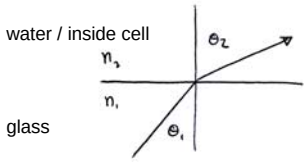
[TIRF microscopy] (11,12)

Total Internal Reflection Fluorescent (TIRF) microscopy reduces the background due to fluorescence of molecules in solution by only illuminating a layer within about 100 nm of the surface.

> This allows single fluorescent molecules to be distinguished if they are close to the surface.
>
> The "evanescent wave" is actually more intense than the incident wave – up to ~5 times more intense, depending on incident angle.
>
> *Duffin, Electricity and Magnetism,*
> *http://www.olympusmicro.com/primer/java/tirf/penetration/index.html*

The excitation light is a laser (high collimation, narrow angle of incidence), and can be from either side of the sample.

Plane wave at a plane boundary, n1 > n2:



E-field in water:

$$E_2 = E_0 e^{i(\underline{k}_2 \cdot \underline{r} - \omega t)}$$

$$= E_0 e^{i(k_2 \sin\theta_2 x + k_2 \cos\theta_2 y - \omega t)}$$

Total Internal reflection:

$$\sin\theta_2 = \frac{n_1}{n_2}\sin\theta_1 > 1$$

$$\cos\theta_2 = (1-\sin^2\theta_2)^{1/2} = \left(1-\left(\frac{n_1}{n_2}\right)^2 \sin^2\theta_1\right)^{1/2}$$

$$= ib \quad (b \text{ real})$$

θ_2 is not real, no wave propagates into water

$$E_2 = E_0 e^{-k_2 b y} e^{i(k_2 \frac{n_1}{n_2}\sin\theta_1 x - \omega t)}$$

$$= E_0 e^{-k_2 b y} e^{i(k^* x - \omega t)}$$

"Evanescent wave"

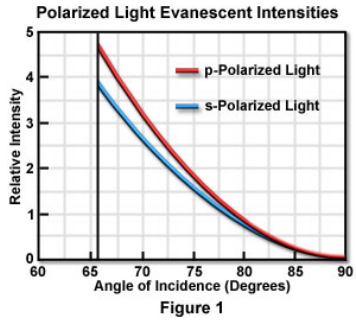
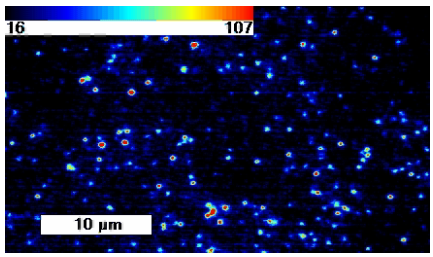
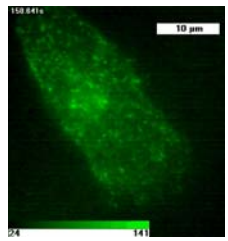


Figure 1

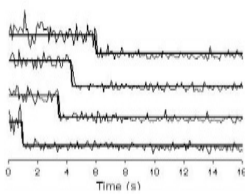
Single Molecule Fluorescence



Single GFP molecules immobilised on glass



GFP-tagged protein inside a living cell



[Single fluorescent molecules] (13)

Most molecules used in biology show “photobleaching”. After about a million photons, they go into a “dark state” and are no-longer fluorescent.

Mostly this is bad news – limited time to observe one molecule.

BUT, it does allow you to be sure that you were only looking at one molecule – if it disappears suddenly rather than gradually.

The arrival of a new fluorescent spot indicates the arrival of a new molecule.

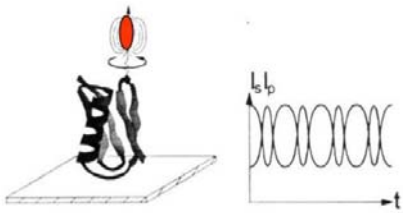
Rate-constants for binding to other molecules at the surface can be measured.

Other examples include detection of the binding of one ATP to myosin simultaneous with the release of actin…

Funatsu T, Harada Y, Tokunaga M, Saito K, Yanagida T. (1995) “Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution.” Nature. 374(6522):555-9.

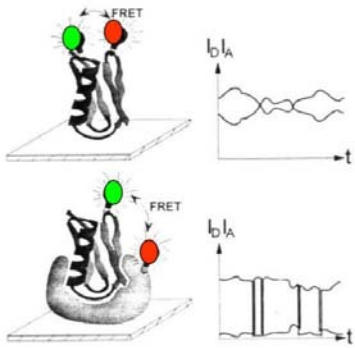
An early single-molecule fluorescence biophysics experiment.

Rotation by polarized fluorescence:



Fluorescent molecules are dipoles. Their orientation can be determined by measuring the polarization of fluorescence.

Molecular distances by Forster Resonant Energy Transfer:



Resonant Energy Transfer between fluorescent molecules depends sharply on distance over a range of several nm.

If the molecules are far apart, most fluorescence comes from the donor (green).

If the molecules move closer together, more fluorescence comes from the acceptor (red)

[other modern fluorescence techniques] (14)

As a single molecule rotates, its absorption and emission in a given polarization changes. Example of what has been observed this way…

Rotation of the lever arm of myosin.

Rotation of F1 g-subunit

FRET can be used to measure the binding of two molecules if one is labelled with the donor and the other with the acceptor.

OR, donor and acceptor can be at either end of the same molecule.

Protein folding

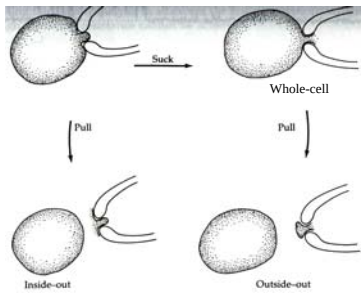
DNA hybridisation

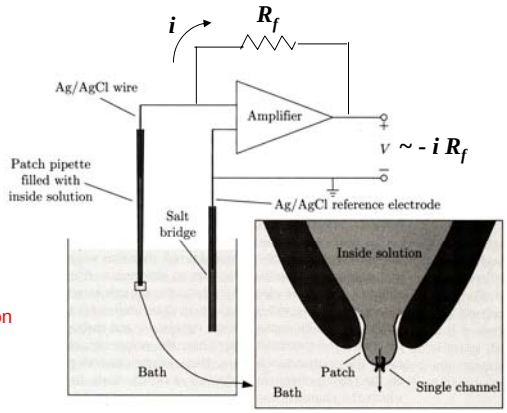
Yurke B, Turberfield AJ, Mills AP Jr, Simmel FC, Neumann JL. (2000) "A DNA-fuelled molecular machine made of DNA". Nature. 406(6796):605-8.

FRET as an indicator that a man-made molecular motor really works.

FRET can be seen between single molecules as well as lots of molecules.

Patch Clamp ion-channel recording





Electrode chemistry:

dissolved chloride — Conduction electron

$Ag + Cl^- \rightleftharpoons AgCl + e^-$

silver metal — silver chloride (salt)

Patch Clamp Current Recording

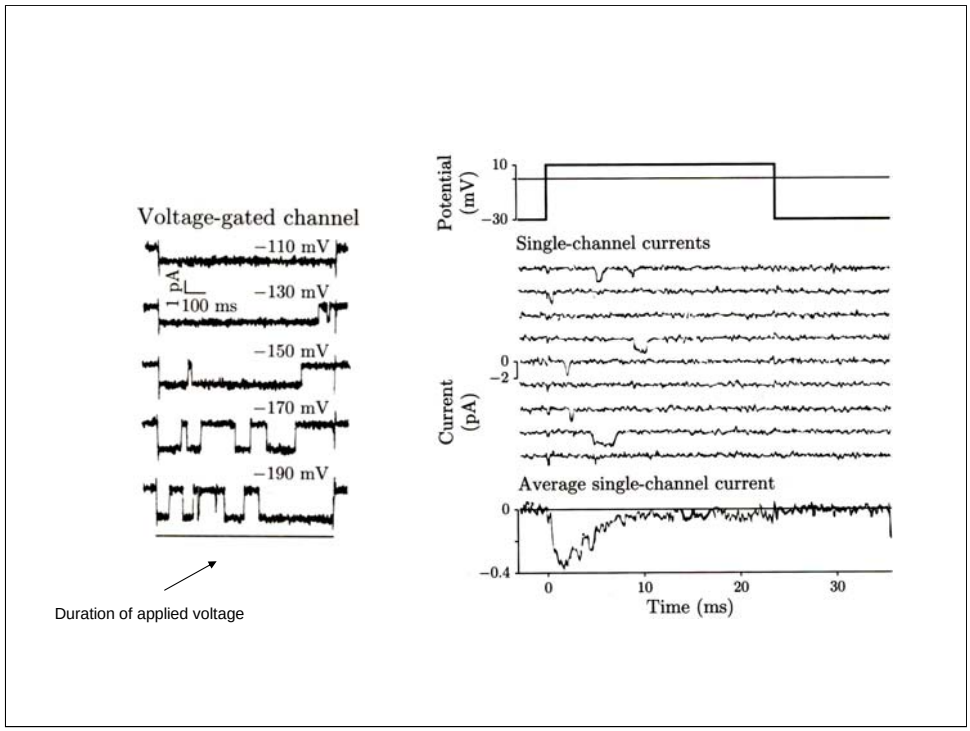
[Patch clamping with pipettes] (15)

Current is picoamps (10-12 A), feedback resistor ~gigaohm (109 W).

> The seal between the membrane and the pipette must also be >~ gigaohm.

Electrodes are made of silver coated with silver chloride, so that electronic currents in the wires can become ionic currents in the solutions.

The technique is mature – dates back to 1976



[example of single-channel currents] (16)

Many things can be measured

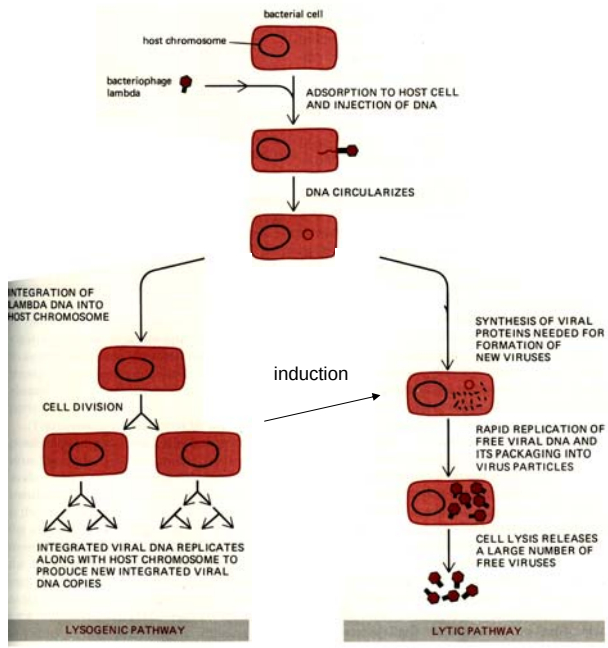
Gating kinetics: transient and steady-state rate constants.

Ion selectivity, I-V curves

Effects of voltage, gating molecules, mutations etc.

Many ion channels are drug targets.

Lambda phage:
a bacterial virus



Modern Genetic Techniques

Alberts Chapter 5

[viruses – lambda bacteriophage] (17)

Many types of viruses (including retroviruses)

Various life-cycles, infect all types of life.

Plasmids:

Like viruses, encode genes required to replicate themselves and to pass between bacterial cells.

Circular DNA, don't encode proteins to pack themselves and destroy host cell.

Often carry resistance to antibiotics – pass from cell to cell even across species.

Viruses and plasmids can be used as "vectors" for genetic engineering…They deliver foreign DNA into cells.

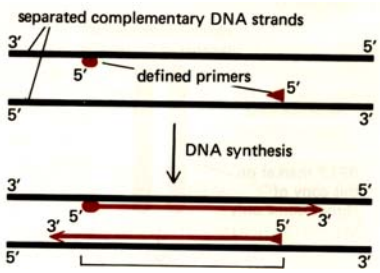
Finding and altering genes for disease etc. Gene therapy.

Transgenic crops and animals.

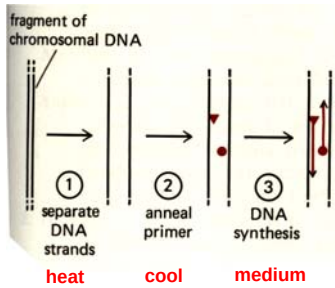
Production of proteins in bacteria – for science, medicine.

Genetic engineering of proteins – for science, medicine.

PCR – polymerase chain reaction



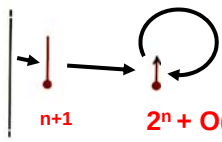
Section between primers
is to be amplified



heat cool medium

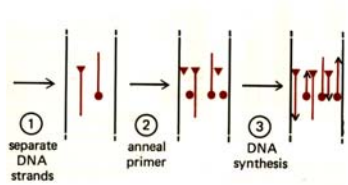
One cycle

After ***n+2*** cycles:



n+1 $2^n + O(n)$

one



second cycle

Some important genetic techniques

[PCR] (18)

PCR depends upon DNA polymerase from a thermophilic bacterium, which stays properly folded at the high temperatures needed to separate DNA strands.

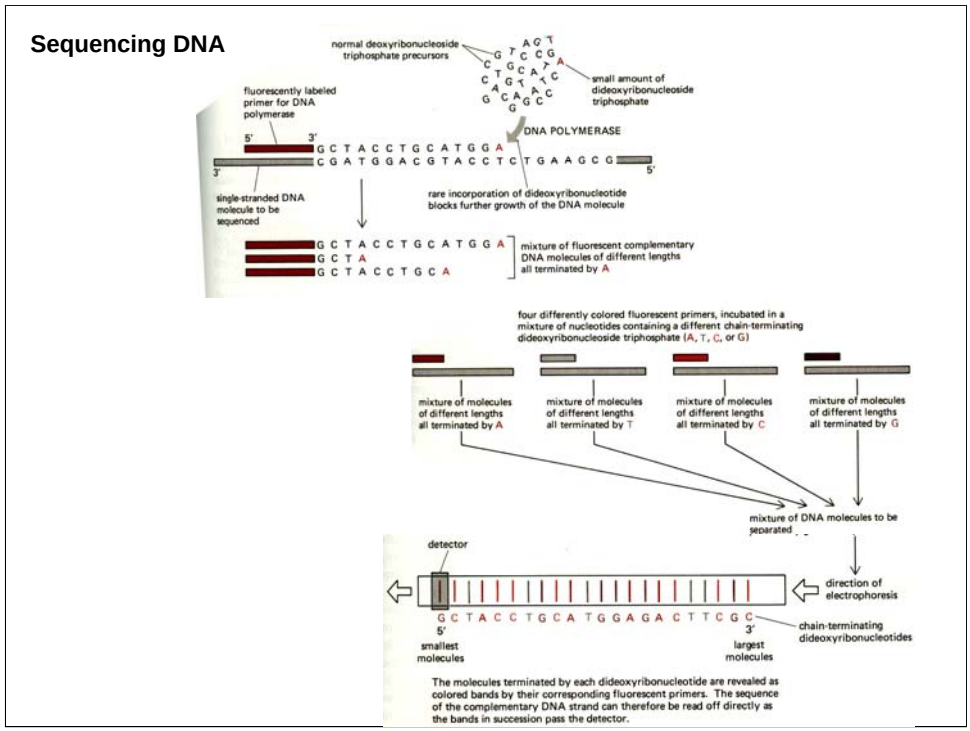
Can think of it as artificial “natural” selection – the DNA strand between primers copies itself, so it very quickly dominates in the very unnatural conditions of PCR

Macroscopic quantities of DNA from single molecule

- For sequencing
- For cloning

Techniques develop all the time – eg) PCR on a chip, Nature 13/3/03

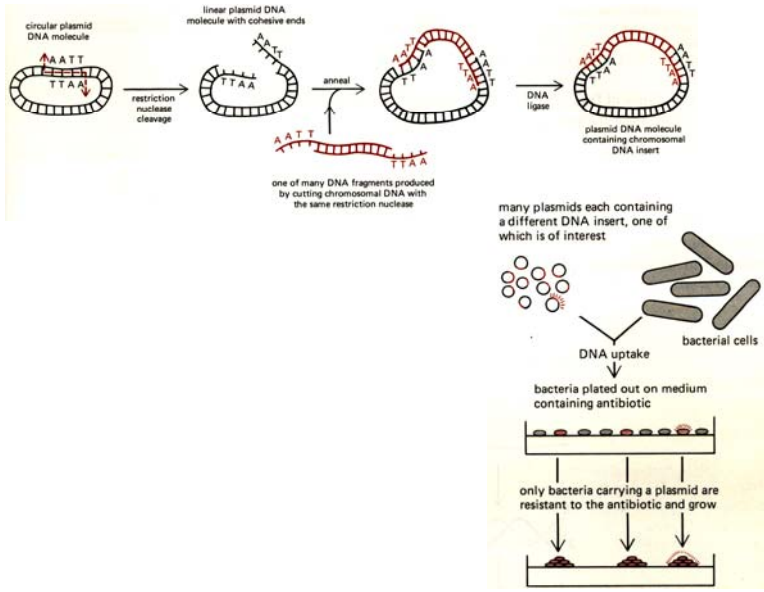
Sequencing DNA



[sequencing] (19)

Modern sequencing is automated – high throughput sequencing machines.

Cloning with plasmids in bacteria



[cloning] (20)

Restriction enzymes cut DNA at specific sites, leaving “sticky-ends”

They can be used to insert DNA into plasmids.

Plasmids are taken up by bacteria under the right conditions.

Usually the plasmid carries a “marker”, eg. a gene for resistance to some antibiotic. This allows bacteria which got the plasmid to be selected.

The plasmid can also carry other inserted genes of interest.

Use bacteria to produce inserted DNA or proteins in large quantities

Proteins for drug use (biotech bubble - only insulin so far)

A toolbox for whatever you can think of…