Fundamentals in Biophotonics

Single cell quantitative measurements

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26.04.2021.

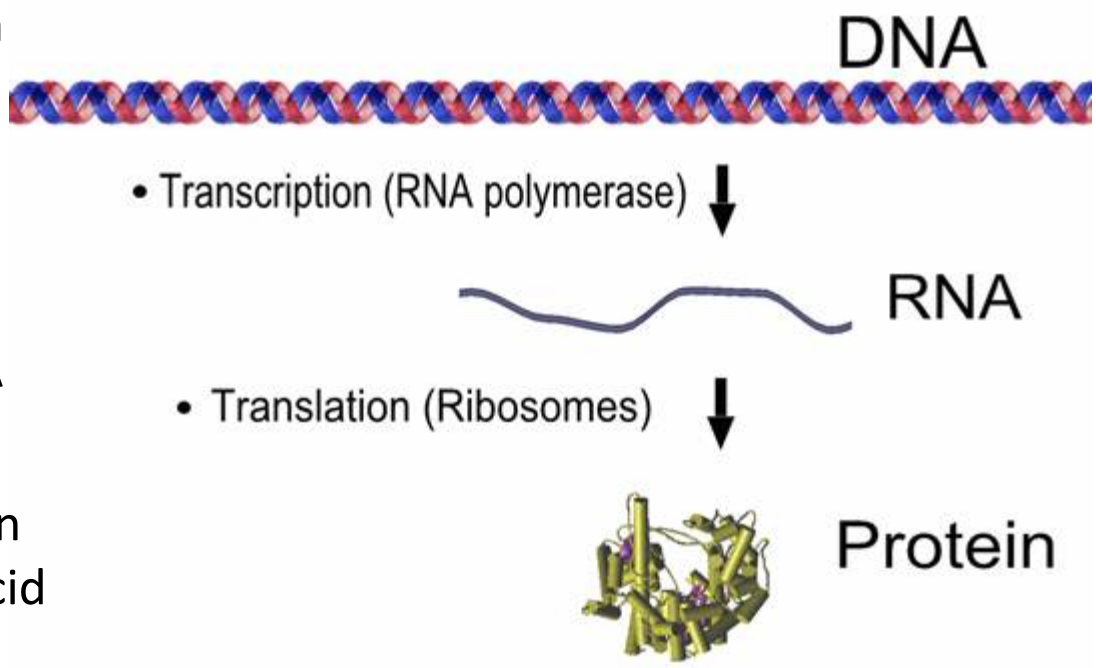
- ONE EXAMPLE OF THE BIOLOGICAL PROBLEM:

- SINGLE PROTEIN EXPRESSION

Monitor gene expression in ***real time*** with ***single protein*** molecule sensitivity in living ***individual bacterial cells***

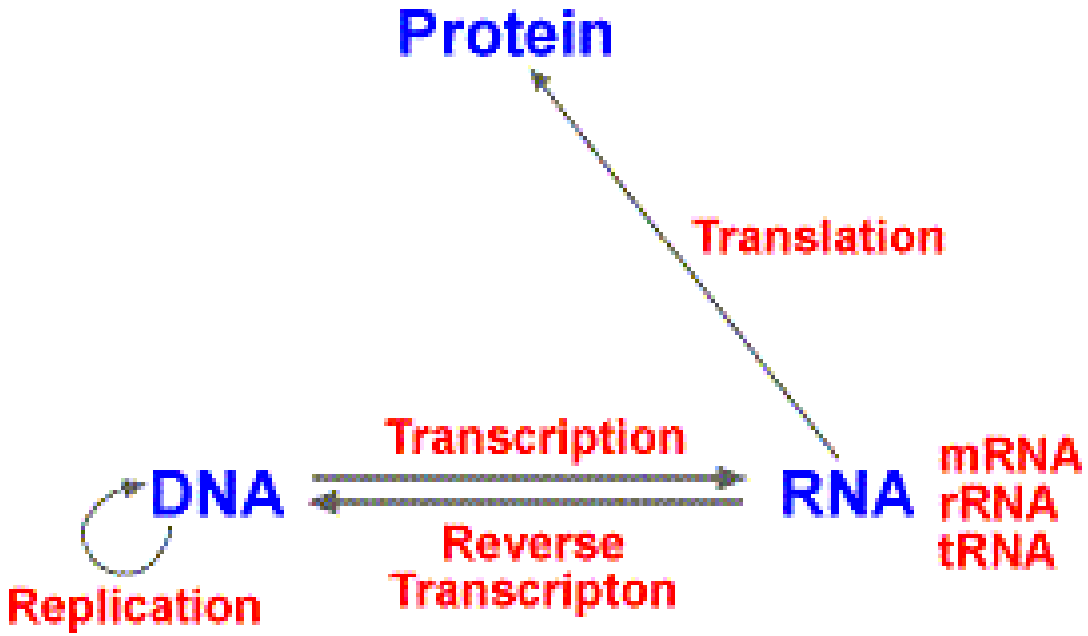
Central Dogma of Molecular Genetics

- The transfer of information from DNA to make a protein
- Transcription of DNA into mRNA RNA splicing (and alternative splicing) to create mature mRNA
- Translation – convert information encoded by mRNA into amino acid sequence



The traditional methods for studying the elements of the central dogma involve bulk experiments involving ensembles of many molecules. The problem with such systems, while they have been very powerful, is that important information is lost due to averaging of all states within the system.

How to measure gene expression



Expression level can be estimated by examining any of the material or processes in red;

Methods Northern Blots, Microarray

None of these provides complete or accurate information – why not?

The only way to solve this is to ***synchronize all molecules*** within the population. Practically, this is very difficult and synchronization would be rapidly lost due to the stochastic nature of these steps; the solution is **to observe the events of the central dogma at the single-molecule level in the cells .**

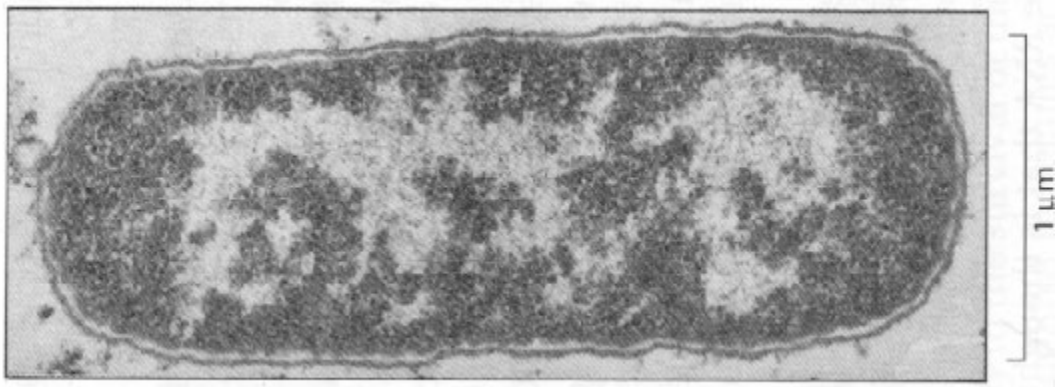
Imaging Gene expression in Living Cells

- "Living cells are the test tubes in the 21st century."

  Jonathan Widom

- Nonequilibrium steady state
- Complex reaction network
- Biomolecules (DNA, mRNA) in low copy numbers

- **Gene Expression Is A Single-Molecule Problem!**



E. Coli has 4,288 genes in the single cell usually one or two copies of the single gene

- Monitor gene expression in ***real time*** with ***single protein*** molecule sensitivity in living ***individual bacterial cells***

Gene Expression Is A Single-Molecule Problem

- A particular mRNA has only a few copies owing to the short cellular mRNA lifetime. Although the copy number for a particular protein varies from 1 to 10000 some important proteins **such as transcription factors and DNA polymerases are** present at **low copy numbers.**

- Due to the low copy numbers of participating macromolecules, cellular processes, such as transcription, translation, gene regulation, and DNA repair, often exhibit **stochastic reaction events**. This means a particular time trace for one cell's behavior is not reproducible and cannot be synchronized with that of another cell, even though the statistical properties are reproducible.

- Cellular biochemical reactions often occur under **nonequilibrium conditions.** Many cellular enzymatic reactions such as transcription, translation, and replication occur with a constant supply of free energy and substrates.

- Their biochemical reactions are stochastic in nature and therefore requires
  - **Real-time measurement in single cells**
  - **With single molecule sensitivity due to the often very low protein copies**

Copy numbers in *Escherichia coli*

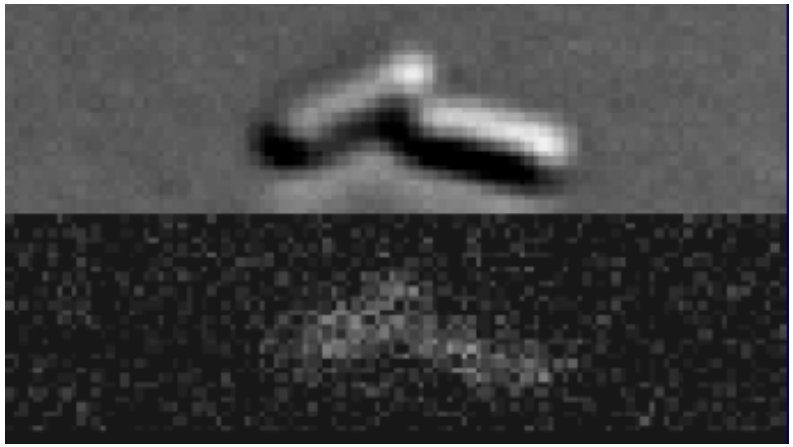
Molecular unit	**Number**
Replication errors per genome	0.002
Double-strand breaks per genome	0.2
Replication forks per cell	1.5–6
Gene copies per cell	1–5
β-galactosidase tetramers per uninduced cell	1
F-plasmids per cell	1–3
Transposon copies per genome	1–15
lac repressor tetramers per cell	5
RNAPs per induced *lac* gene	5–20
DNA polymerase III per cell	10–20
lacZ mRNA per cell	10–30
Ribosomes per *lac* mRNA	20
DnaG primases per cell	50
Actively transcribing RNAPs per cell	200–2000
RecA molecules per cell	1000
Single-stranded DNA binding protein	1000–7000
Total RNAPs per cell	1000–10,000
Ribosomes per cell	7000–50,000
β-galactosidase tetramers per induced cell	10,000
Total nucleoid proteins (e.g., Fis, HU, H-NS) per cell	50,000–200,000

Immobilizing GFP for Single Molecule Sensitivity

- A GFP molecule in cytoplasm undergoes fast diffusion. Its signal is overwhelmed by the strong autofluorescence background
- Problems related to use GFP as gene reporter
  - Long maturation time of the Fluorophore (after the three Fluorophore forming amino acids are brought together by protein folding it normally takes 20 min for them to be oxidized
  - It normally requires 30 copies of fluorescent protein molecules per Escherichia coli cell in thin order to detect the signal above autoluoresce background

DIC Image

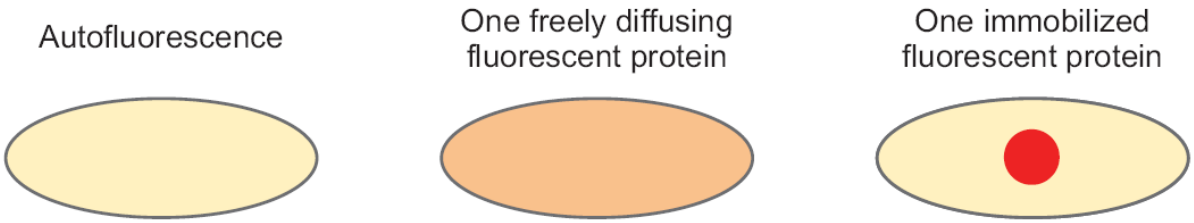
Fluorescence Image



A few diffusing GFP molecule

Single Fluorophore Detection by Localization

- Immobilizing GFP for Single Molecule Sensitivity



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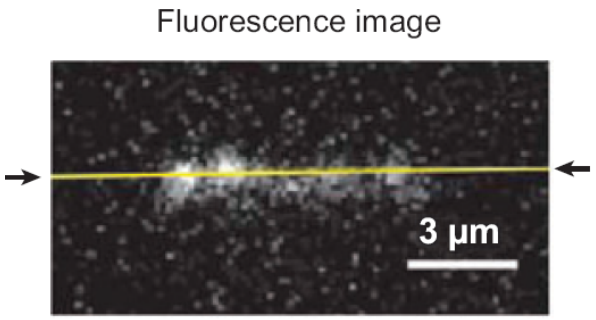
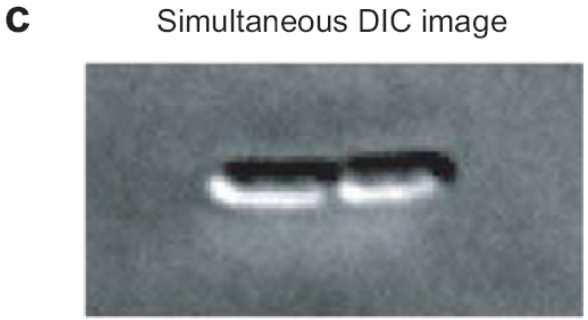
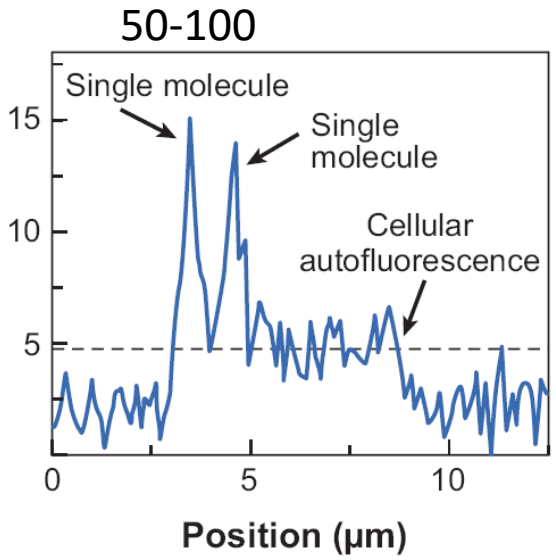
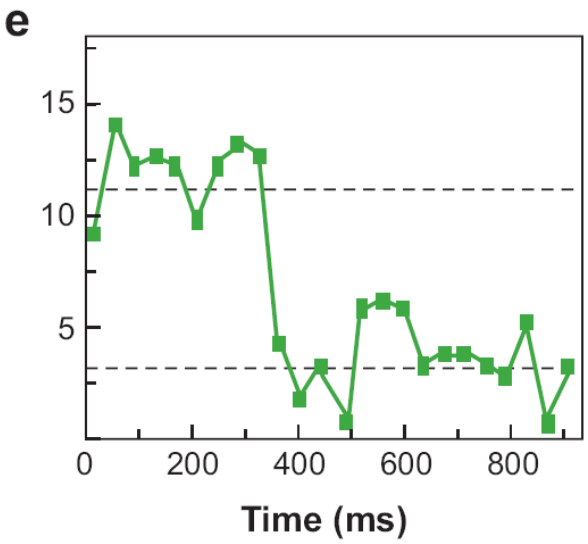




- ***The proof of*** detecting a single molecule is the quantized photobleaching of the fluorescence signal

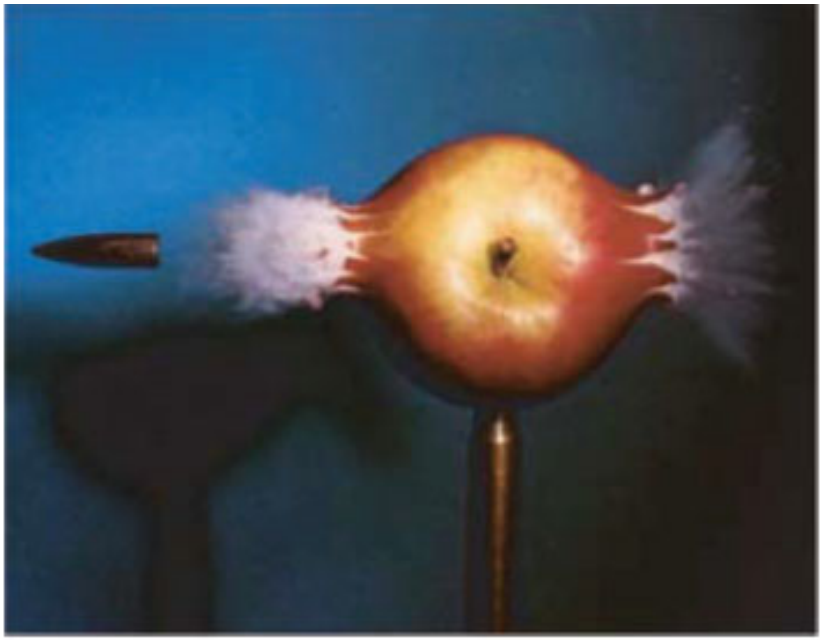


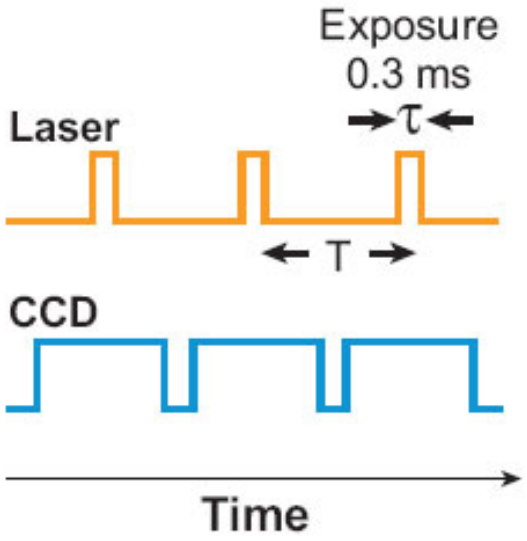


"*Nature*, **440**, 358-362 (2006).

Stroboscopic excitation

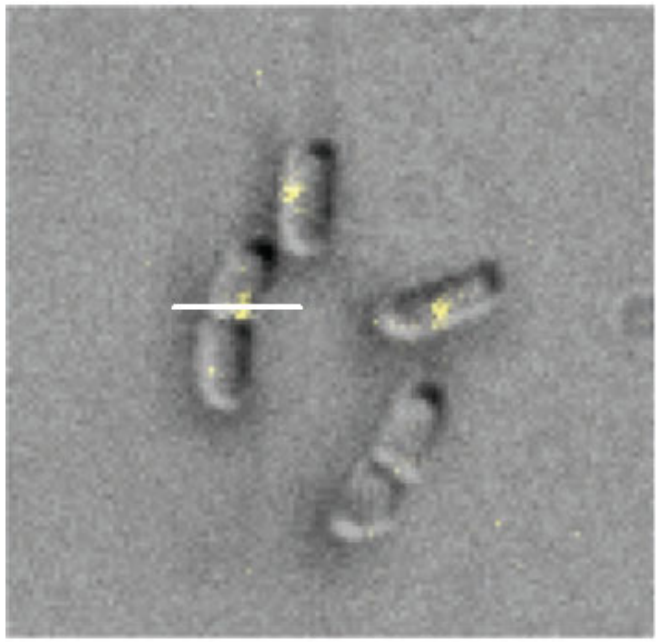
- A short laser pulse can overcome the limitation of the slow shutter or frame rate of the camera. The shutter and the CCD can be left open for longer times . ***An intense laser*** pulse for a short duration (~0.3 ms), during which a protein reporter does not diffuse beyond the diffraction limited spot

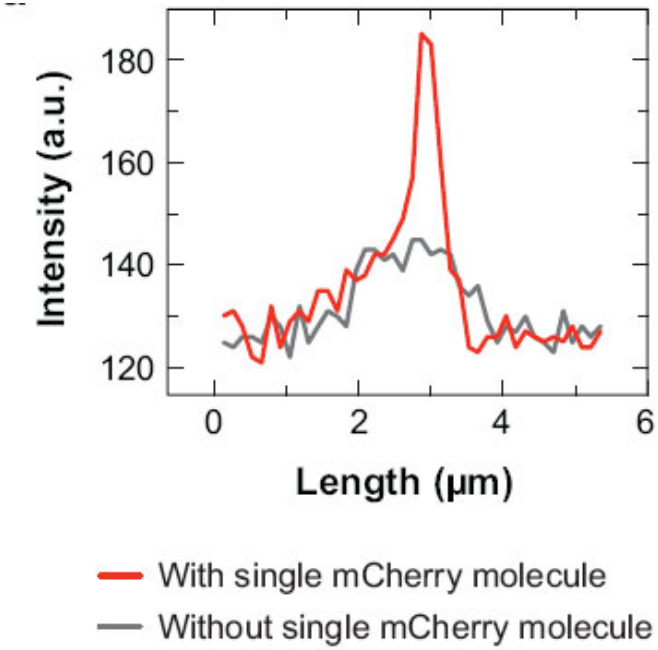




Stroboscopic excitation

- Time resolution of live-cell single-molecule detection can be **submillisecond,** no longer dictated by the shutter speed or frame rate of the CCD, but by the laser pulse width.
- The pulse width and dwell time can be varied to probe dynamical properties such as residence times of weak binding and diffusion constants
- The drawback of the stroboscopic excitation is that single FP molecules are more photolabile under the high pulse intensity.





The fluorescence image was taken with a 300 μs, 50 kW cm^{-2} laser excitation

Choice of FP

- Bacteria exhibit strong **autofluorescence** at short wavelength excitation, monomeric yellow and red FPs are preferred over blue and green variants for single-fluorophore detection

- **color, photostability and brightness of FP**

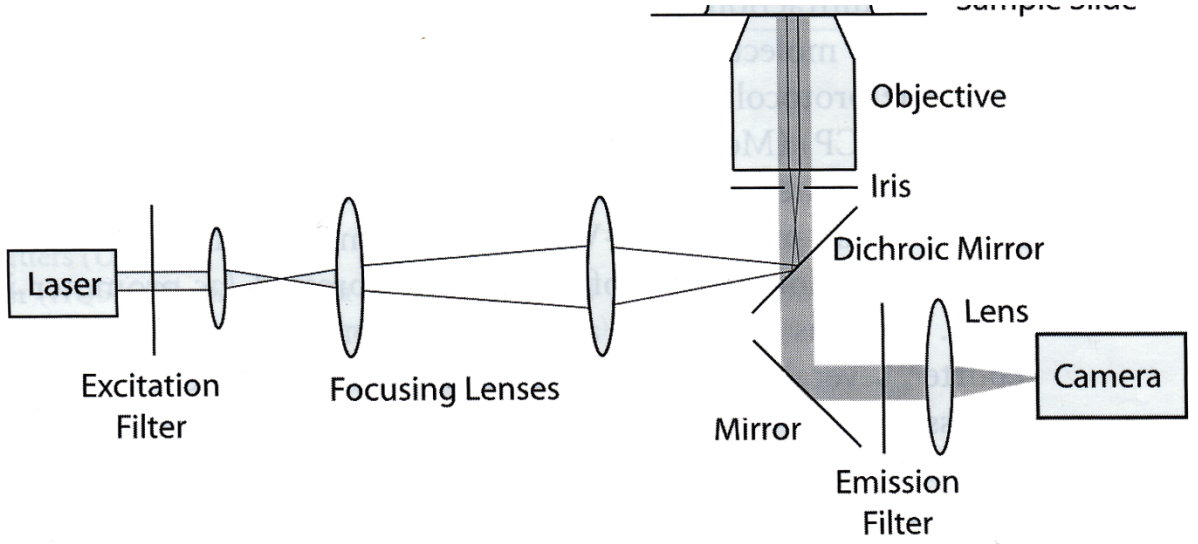
- **Maturation time** of the fluorophore can be the rate-limiting step and determine the time resolution of a live-cell experiment measuring protein production.

- Venus variant (YFP) has a short maturation time **of ∼7 min in bacteria cells.**

- Does a protein fused to an FP have the same activity and structure as the wild-type protein?
- Repeated photoexcitation of a single FP molecule results in intensity fluctuations known as **blinking** making it difficult to quantify fluorophore numbers from the intensity. Under the excitation conditions of a single FP experiment, blinking usually occurs on a ∼1-s timescale, which fortunately does not affect data collection with an exposure time of 0.1 s

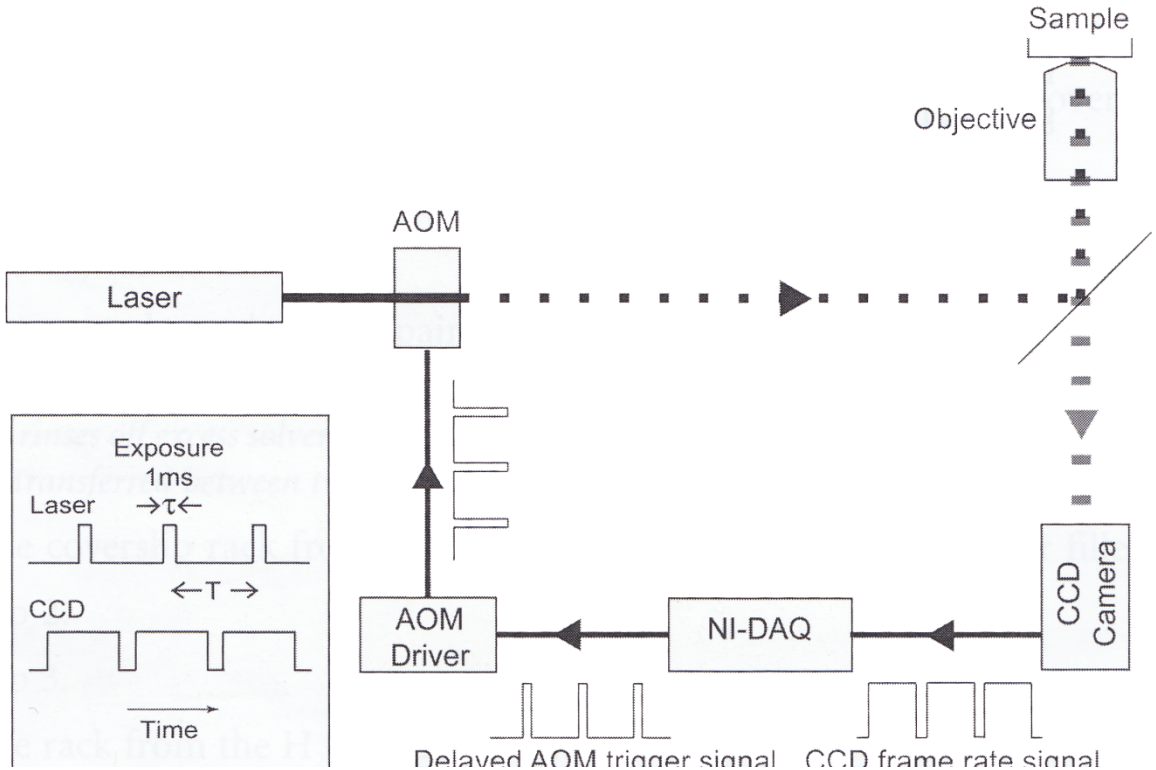
Microscope setup

- An optimized fluorescence microscope with
  - 1. high power argon ion laser >500 mW at 514
  - 2. cooled CCD camera with single photon detection capability
  - 3. microscope with motorized xyz stage allows simultaneous imaging of multiple regions and autofocusing
  - 4. combination of appropriate diachronic mirrors and filters and high NA objective
  - 5. uniform illumination



Microscope setup/ stroboscopic illumination

- To create short laser exposure for stroboscopic illumination, an acousto-optical modulator (40MHz,LiCONix) was placed in the laser beam path as a shutter. A high-speed EMCCD camera (Cascade 512B, Photometrics) was used to achieve fast frame rates up to 100 frames/sec.

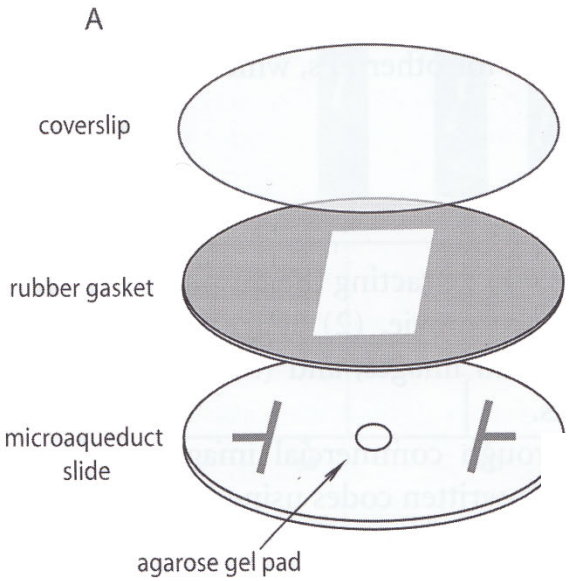


The acousto-optical modulator is synchronized with the CCD frame rate through a Labview based delay generator. At each frame the CCD controller triggers the

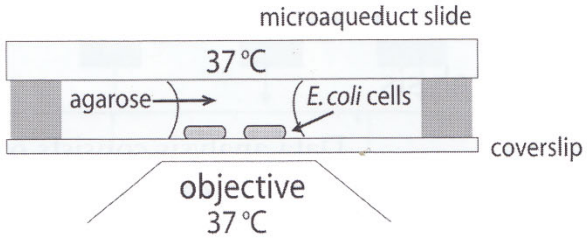
Labview program, which then generates a delayed pulse with controllable delay time and pulse duration. The pulse train is fed into the AOM driver. In this way we created pulsed excitation that is synchronized with the camera

Sample preparation

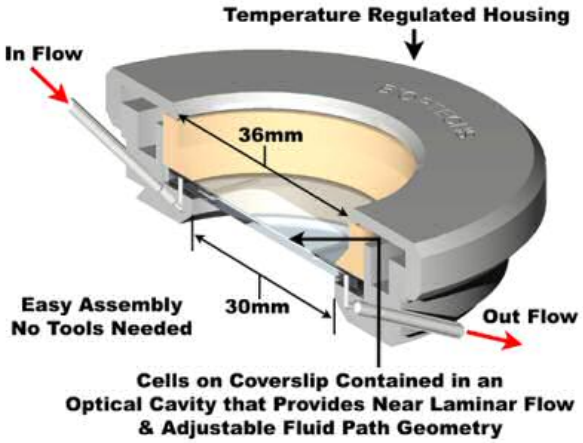
- Requirements for efficient cell growth
  - Constant temperate for several generation without drying out
  - Two number one coverslips sandwiched
  -



B NO FLOW sealed chamber but with enough oxygen for normal cell growth



FLOW



Experimental parameters

- ***Acquisition time***
- Increasing the acquisition time will lead to ***photobleaching*** of the fluorophore for instance Venus YFP variant will photo bleach after 250 ms on average and this leads to decreased signal to noise
- Increasing the acquisition time leads to increased ***variation in the integrated fluorescence intensity*** due to the stochasticity of different fluorophores photobleaching at different times
- Even for the membrane immobilized Venus fluorophore it ***slowly diffuses*** during the long long acquisition time which further contributes to blurred images
- Time interval of 3 min between two consecutive acquisition allows
  - Cells to grow with minimal photodamge
  - If severe photodamage is **induced cells will stop dividing after one or two generations**
  - With optimal conditions cells keep dividing for more than 5 hours
  - If you are about to track only newly generated YFP – use photobleaching strategy
  - Immediately after 100 ms acquisition photobleach for 1000 ms
  - General guideline is that **photobleaching period must be at least 4 times longer than acquisition**

Immobilizing FP for Single Molecule Sensitivity

- PROBLEM how to confine the protein from freely diffusing
- Monitoring protein production by detecting newly generated protein molecules on the cell's membrane by fusing them with the *Venus* gene under the control of lac promoter once expressed the membrane targeted protein Venus molecule quickly folds, mature and translocates onto the cell's plasma ***membrane*** where they can be detected individually

- ***Specific binding*** of Venus labeled transcription factor to its specific site on the chromosome DNA which is stationary on the data collection timescale upon specific binding transcription factor might be detected at single molecule level and in vivo kinetics of IPTG induction

- ***Stroboscopic*** /a short excitation pulses this allow detection of the FP in cytoplasm 0.3 ms pulses during which transcription factor is nonspecifically bound to DNA . Continues stroboscopic illumination can be used to measure protein mobility also by determining the shortest excitation pulse needed to maintain diffraction limited spot size in the image it is possible to estimate upper limit for the mean resident time of the protein on DNA before dissociation

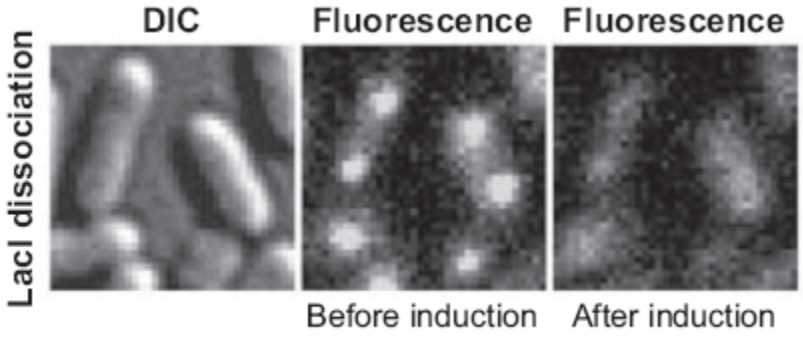
QUIZ TIME

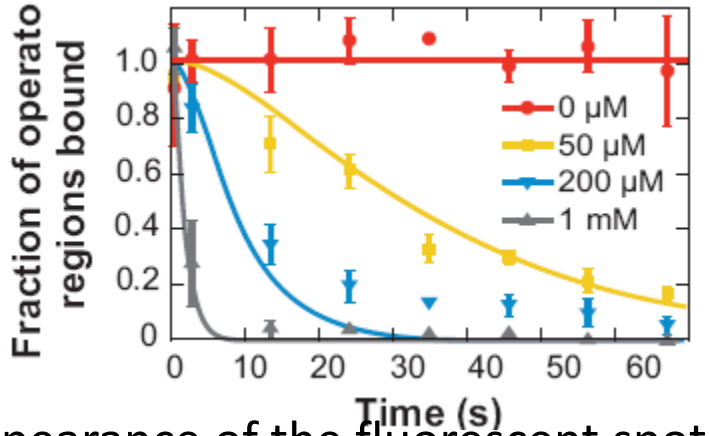
Ready for a short quiz?

Then open kahoot! ☺

Dynamics of transcription factors

- repressor-Venus fusion proteins
- **DNA protein interaction can be specific or nonspecific in nature.** Specific binding means binding to a specific sequence of DNA, whereas nonspecific binding means transient, weaker binding to DNA regardless of ***the sequence context. Both specific and nonspecific binding are dynamic equilibrium phenomena with stochastic binding and unbinding events, which can now be probed by single-molecule experiments in a living cell.***
- **EXAMPLE *lac repressor*** controls ***gene expression of the lac operon by binding to specific operator sequences of DNA and has been a model system for understanding transcription factor-mediated gene regulation***
- *Confinement due to the stationary chromosomal DNA*

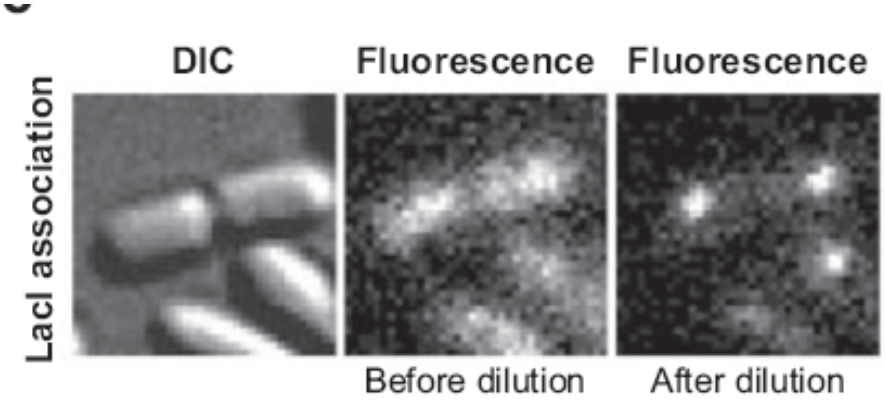


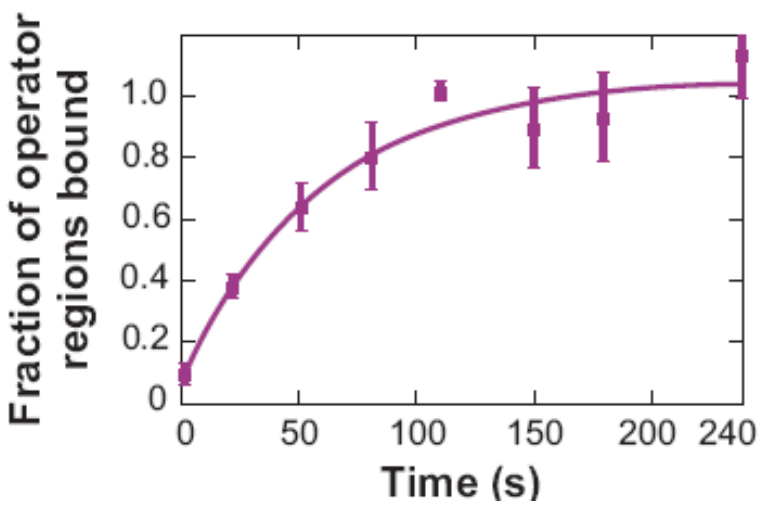


- the repressors dissociate, leading to the disappearance of the fluorescent spots. This loss of fluorescent spots signals the onset of gene expression.

Dynamics of transcription factors

- The dissociation times are stochastic , fraction of operons of a population of cells with at least one bound repressor as a function of time at different IPTG concentrations. This gives **the rate of the repressor dissociation from the operon** upon induction, which **is the rate of IPTG binding to the repressor.** The latter might be limited by the influx of IPTG through the cell membrane. This experiment demonstrates the measurement of live-cell kinetics of transcription factors.
- ***reverse experiment IPTG is diluted***

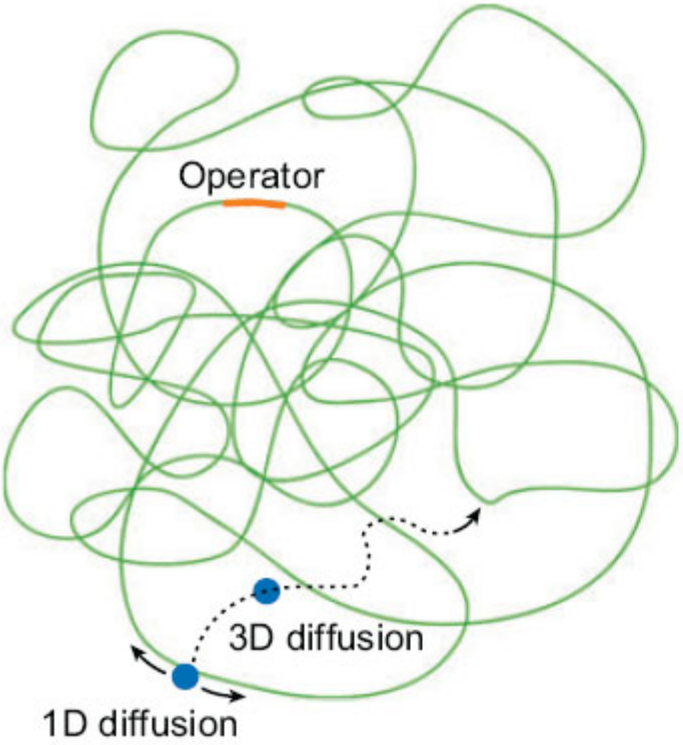


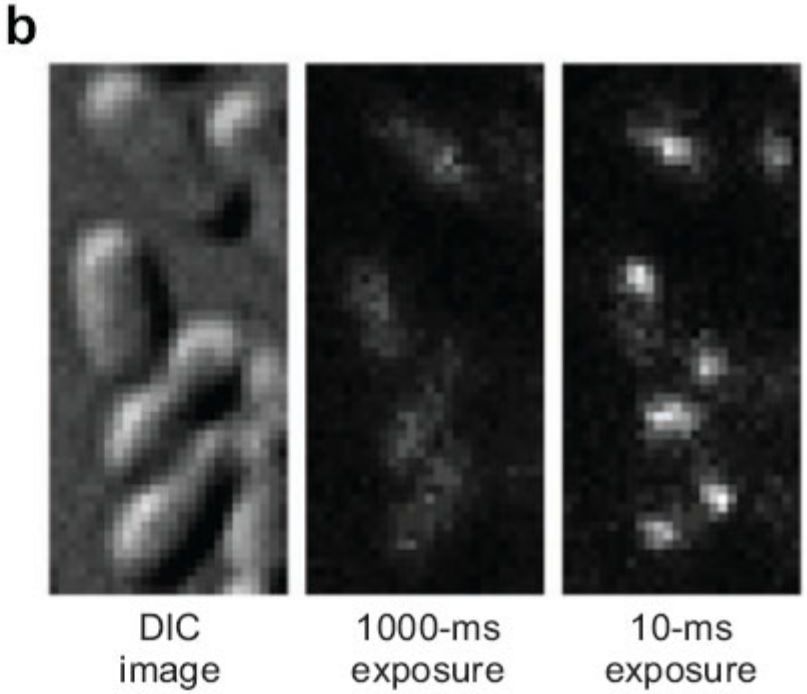


This gives the first experimental measurement of how fast an individual DNA binding protein searches for its target sequence in a living cell.

Searching for specific binding site

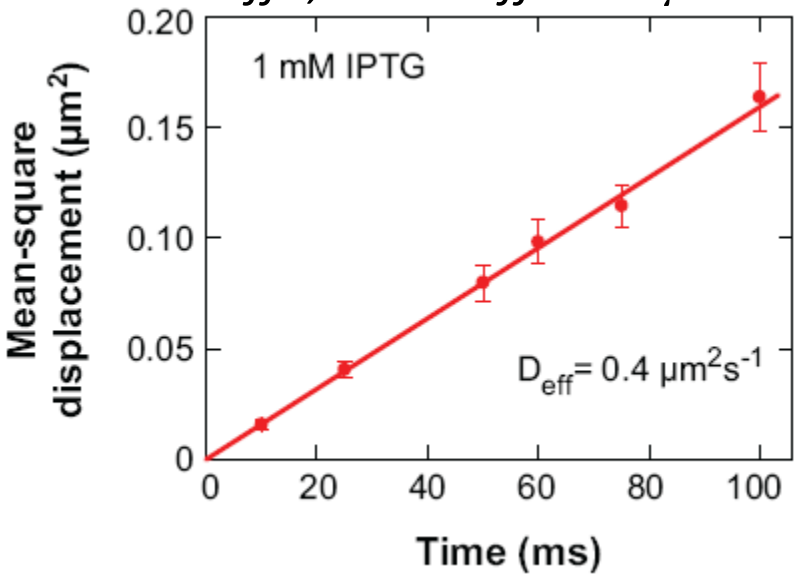
- Von Hippel&Berg studied this subject in the 1980s. Facilitated diffusion
- In searching for a target DNA sequence, a DNA binding protein first nonspecifically binds to DNA and undergoes 1D diffusion along a short segment of DNA before dissociating from DNA, diffusing in 3D through the cytoplasm, and rebinding to different DNA segment.

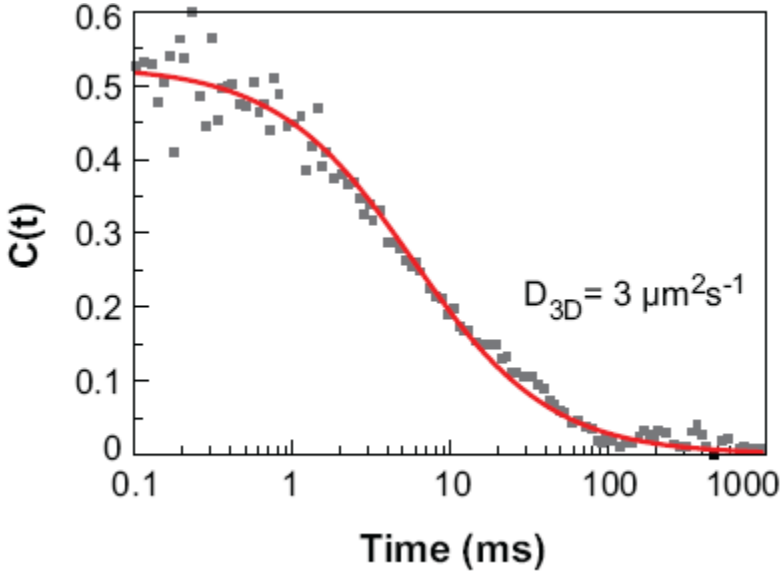




Searching for specific binding site

- Mean-square displacement for nonspecifically bound transcription factors for different time intervals. The red line shows a linear fit of the mean-square displacement. The fitting agrees well with normal diffusion in the imaging plane,
- *<x2> = 4Deff t, with Deff = 0.4 μm2 s−1*





D_{1D} 0.046 μm² s⁻¹

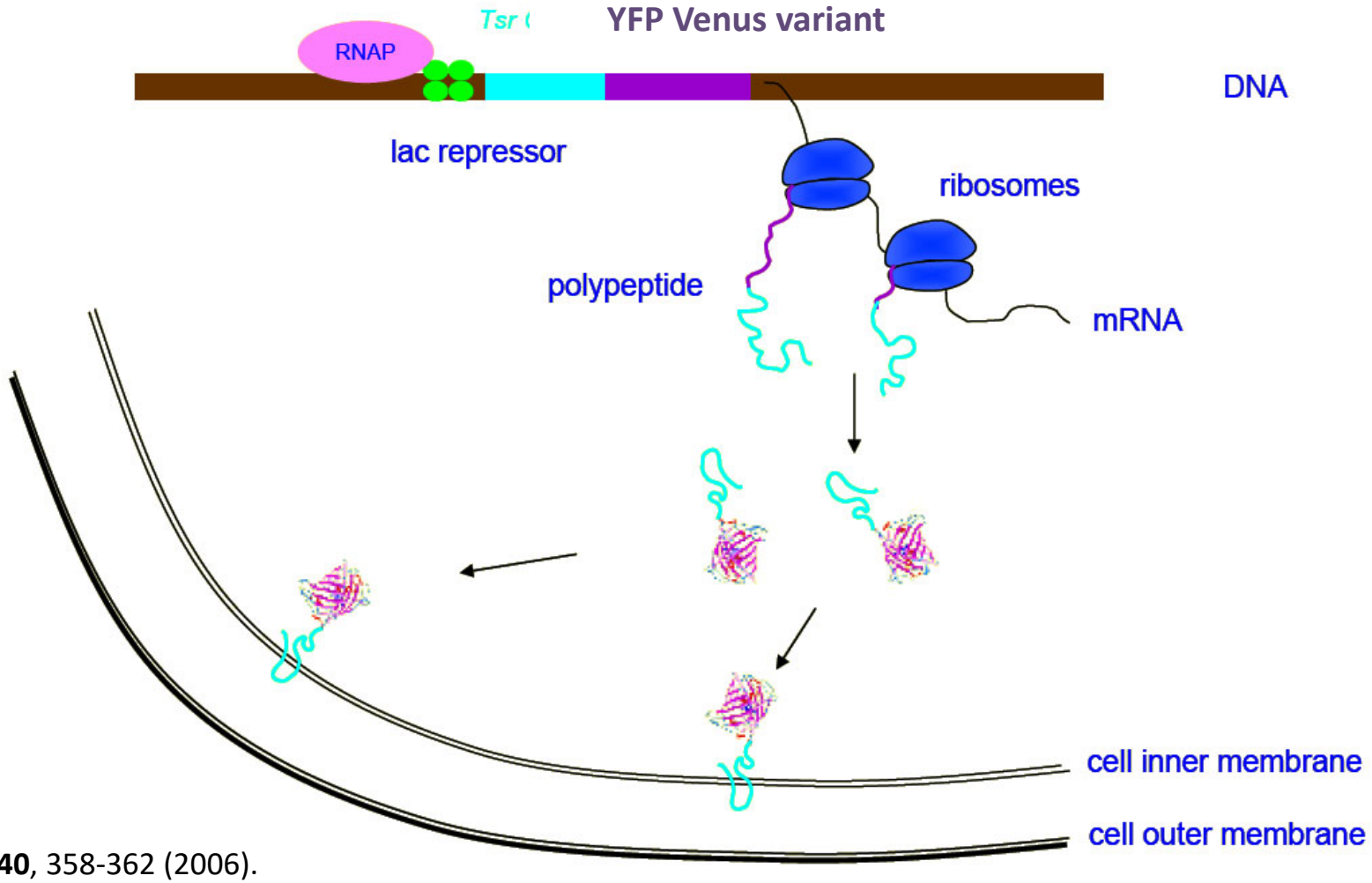
D_{3D} 3 μm² s⁻¹ measured by fluorescence correlation spectroscopy

$D_{eff} = D_{3D}(1 - F) + FD_{1D}/3$, where F is the fraction of time the repressor is nonspecifically bound to DNA, and that the second term is negligible,

F =90%.

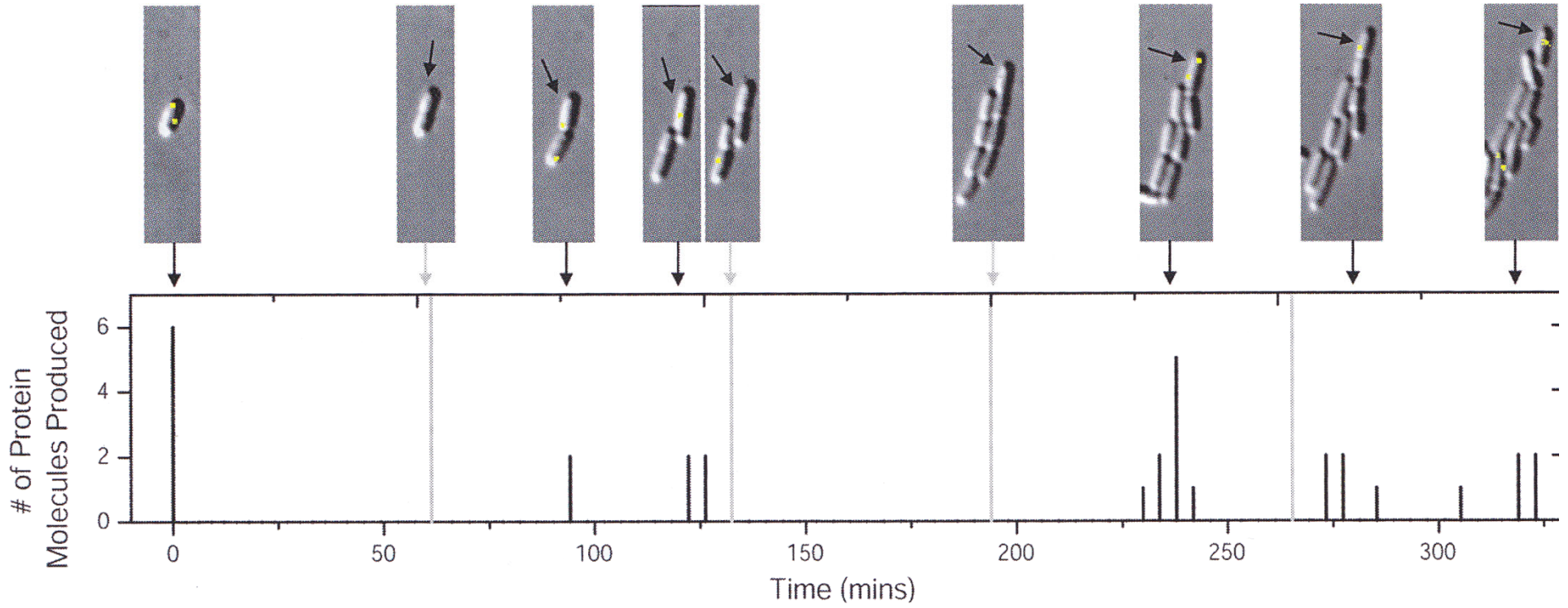
Imaging Gene Expression in a Live E. coli Cell

- Confinement on plasma membrane Tsr gene in place of LacZ under repressed conditions , repressor is tightly bound to its operator sites , ***however infrequent dissociations result in transcription events that generate few copies of the protein molecules***



Nature, **440**, 358-362 (2006).

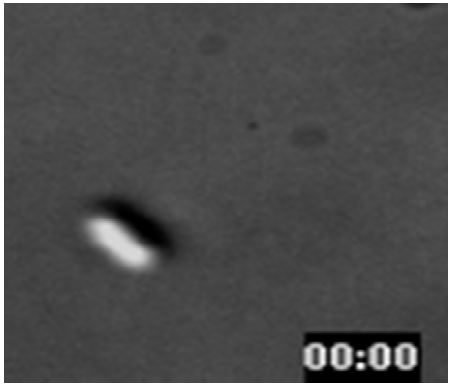
DATA analysis -Creating a cell lineage map with gene activities

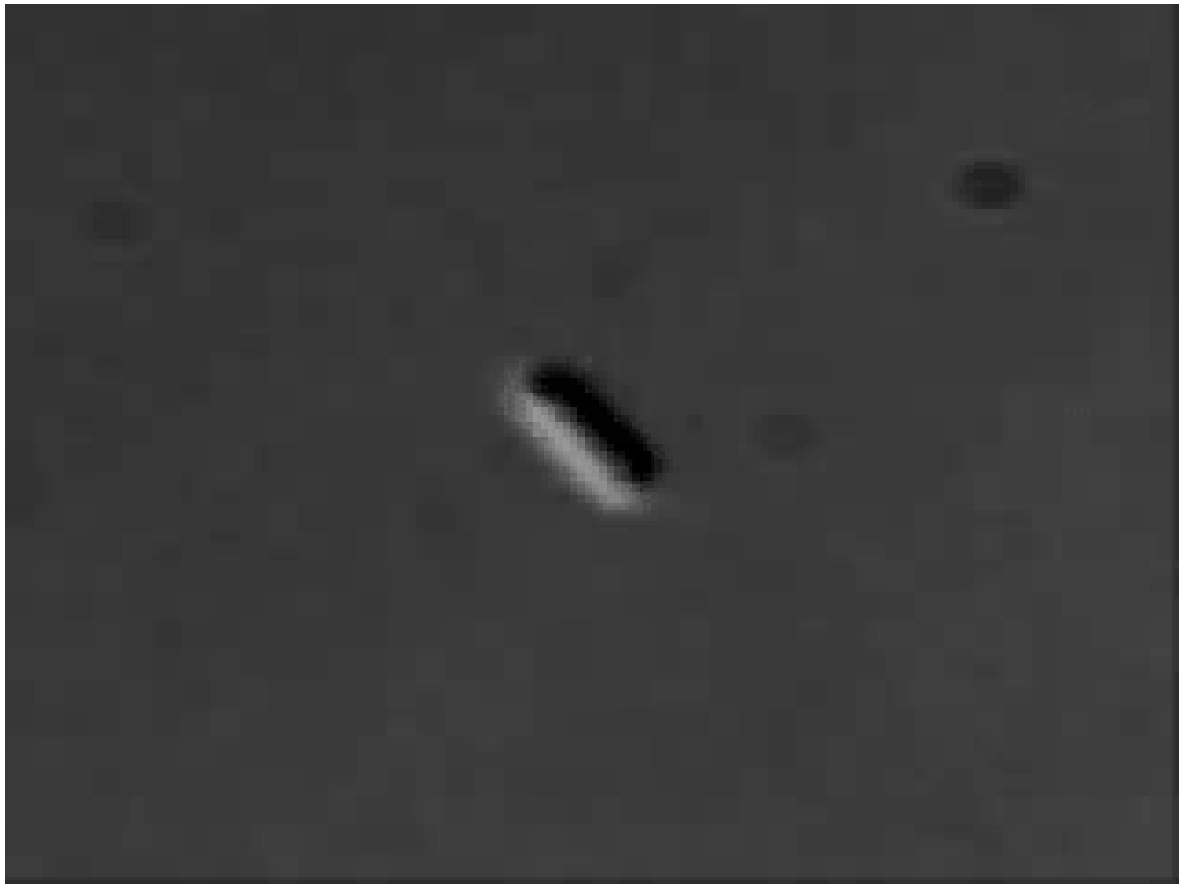


- The last step just simply plots the number of protein molecules generated in each cell lineage along the time axis

FP proteins used as tags +COOH can be purified from the cell culture immobilized on the surface and characterized

Single Molecules of Membrane Immobilized GFP

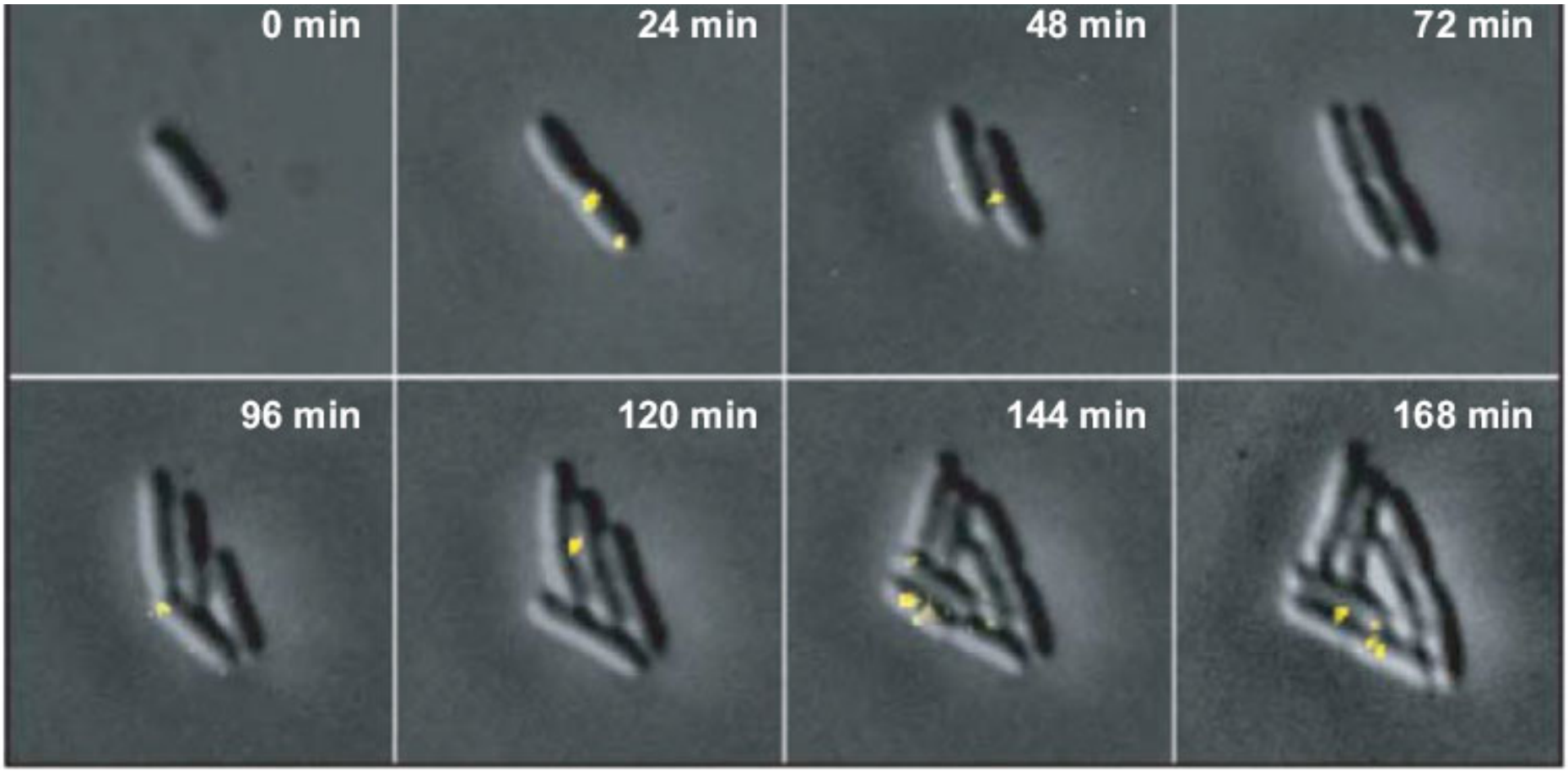




Cell division cycle: 40 min

Stochastic Gene Expression Bursts of Cell Lineages/Translation

- Time-lapse movie of fluorescence images (yellow) overlaid with simultaneous DIC images (gray) of Escherichia coli cells expressing Tsr-Venus fusion proteins under the repressed condition. In the experiment, images are collected every 3 min with a 100-ms exposure immediately followed by a 1-s exposure for photobleaching to prevent accumulation of FPs.



QUIZ TIME

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Then open kahoot! ☺

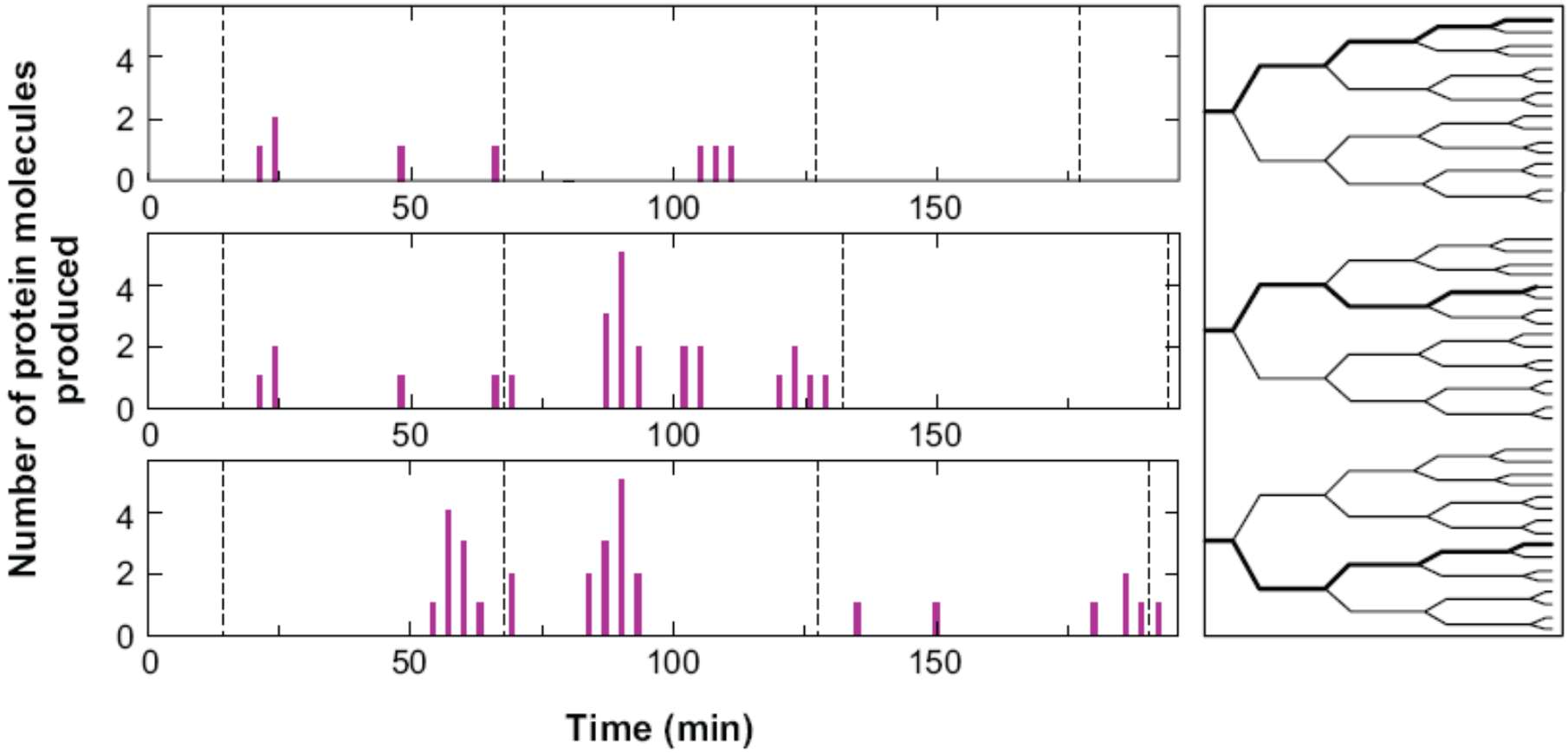
Translation

- Although most studies have been limited to protein expression at high levels because of low sensitivity, it is important to study the **repressed condition** for two reasons.
- many important regulatory proteins, such as transcription factors, have low protein copy numbers
- the analysis of the stochastic time trajectories at a low expression level allows us to obtain quantitative information about the size and shape of **translational bursts** and the cell cycle dependence of the bursts

- What do the translational burst size and shape tell us?

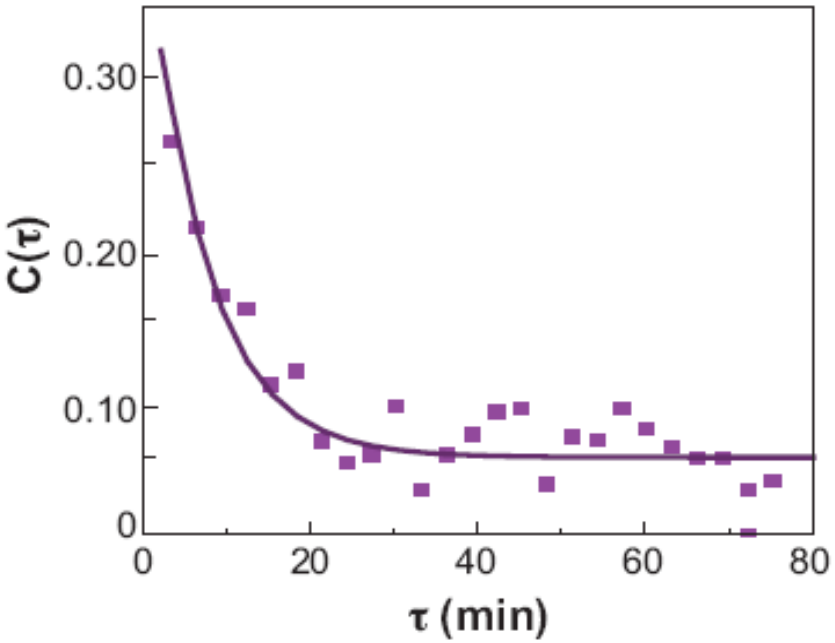
Stochastic Gene Expression Bursts of Cell Lineages

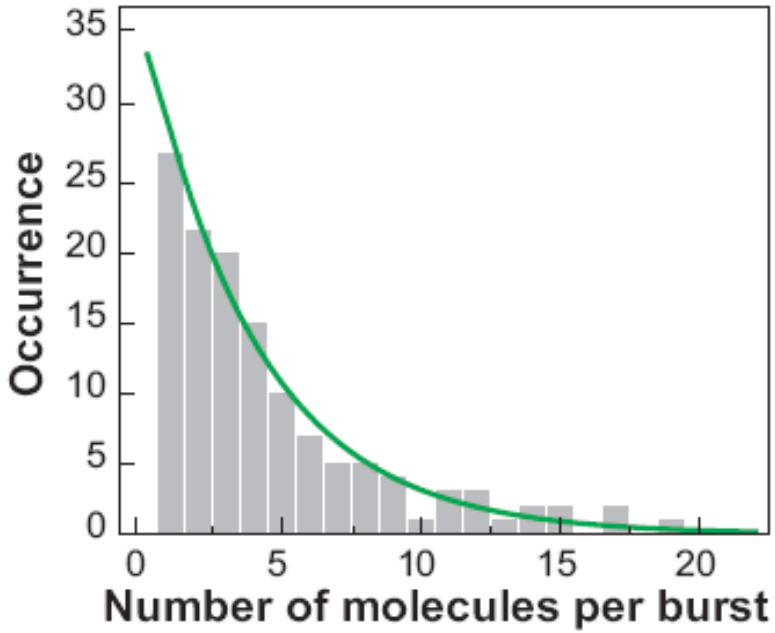
- Time traces of the expression of Tsr-Venus protein molecules (left) along three particular cell lineages (right) extracted from time-lapse fluorescence movies. The vertical axis is the number of protein molecules newly synthesized during the last three minutes. The dotted lines mark the cell division times. The time traces show that protein production occurs **in random bursts**, within which variable numbers of protein molecules are generated.



Distribution of GFP Molecules per Burst

- bursts have a larger characteristic width of 7 min, given by the time constant of the exponential autocorrelation function of ***~7 min time*** constant is consistent with the live-cell maturation time of Venus measured by an ensemble assay.

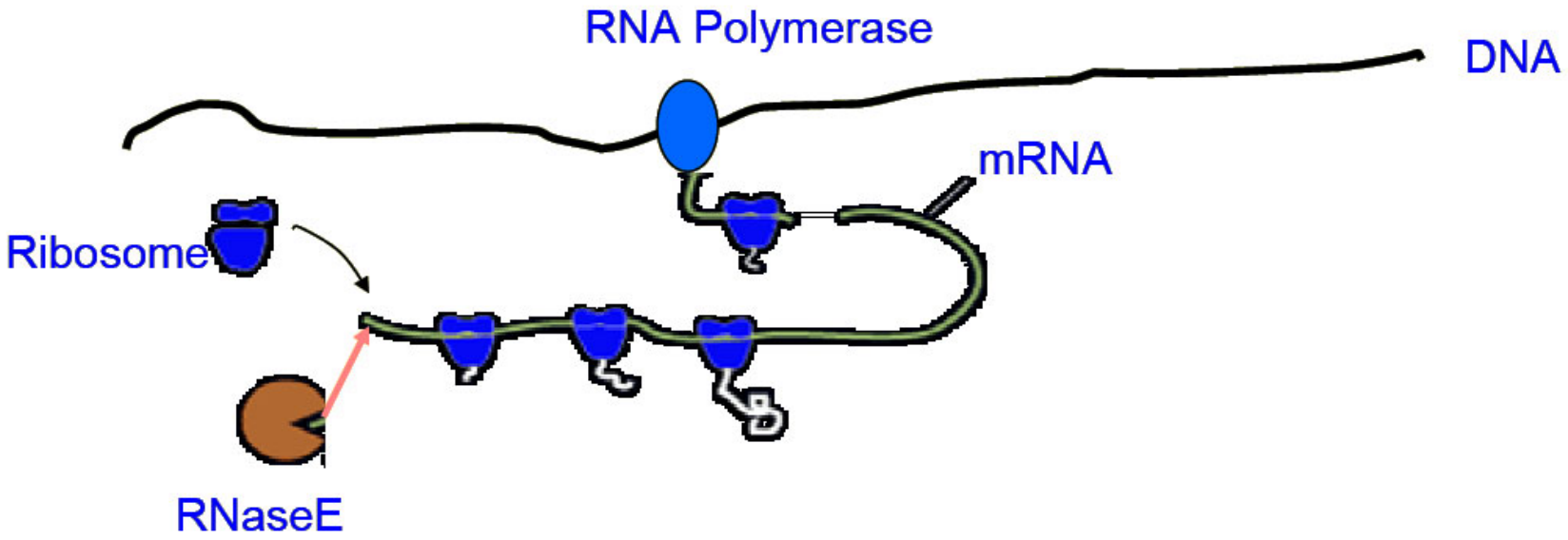


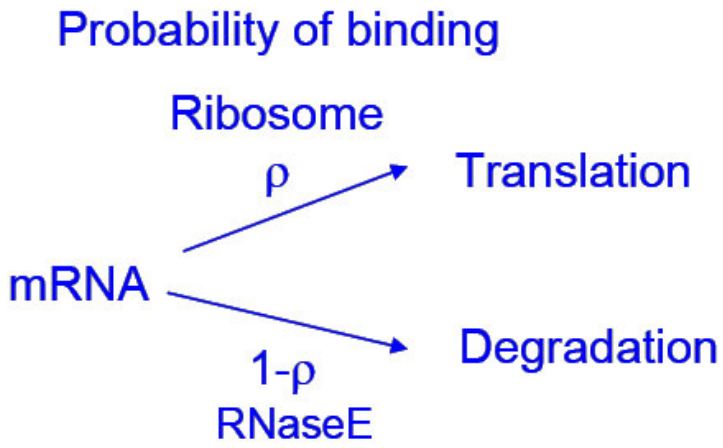


An exponential distribution with an average of burst size b = 4.2 per mRNA molecule

The origin of the exponential distribution is related to the short cellular lifetime of mRNA because of the presence of RNase E, a ribonuclease, which degrades mRNA upon binding to the ribosome binding site.

mRNA Degradation Determines the Burst Size



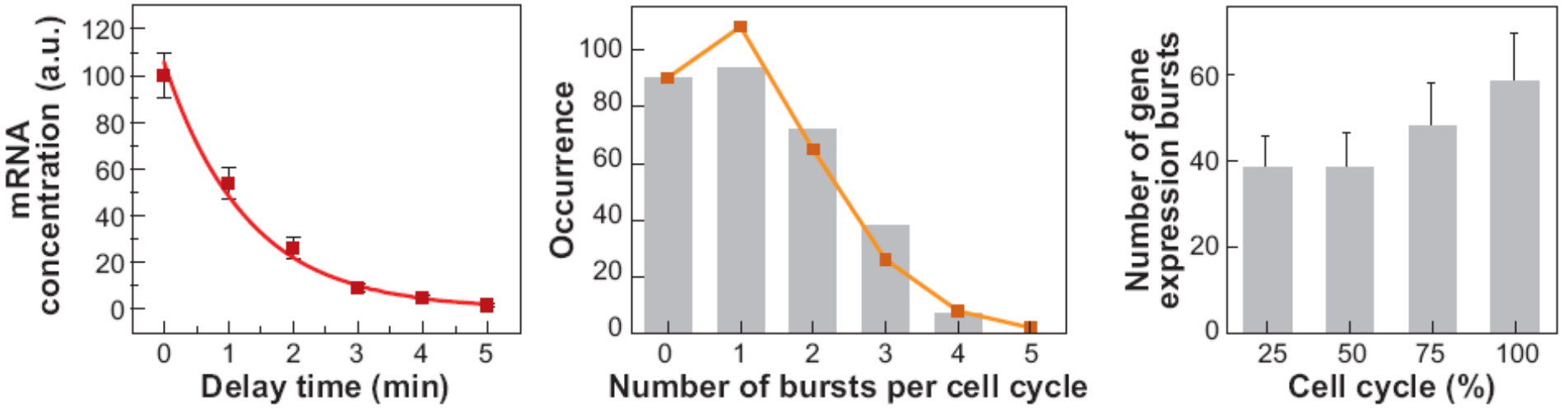


Number of protein per mRNA, N, follows an exponential distribution

$$p(N) = \rho^N (1-\rho)$$

mRNA Degradation Determines the Burst Size

- mRNA lifetime measurement, with a 1.5 min exponential decay of mRNA over time, which means, on a single molecule basis, the probability density of cellular mRNA lifetime also follows an exponential distribution with a 1.5 min time constant



Histogram *of the number of expression events per cell cycle. The data fit well to* Poisson distribution (*solid line), with an average of 1.2 gene expression bursts per cell cycle.*

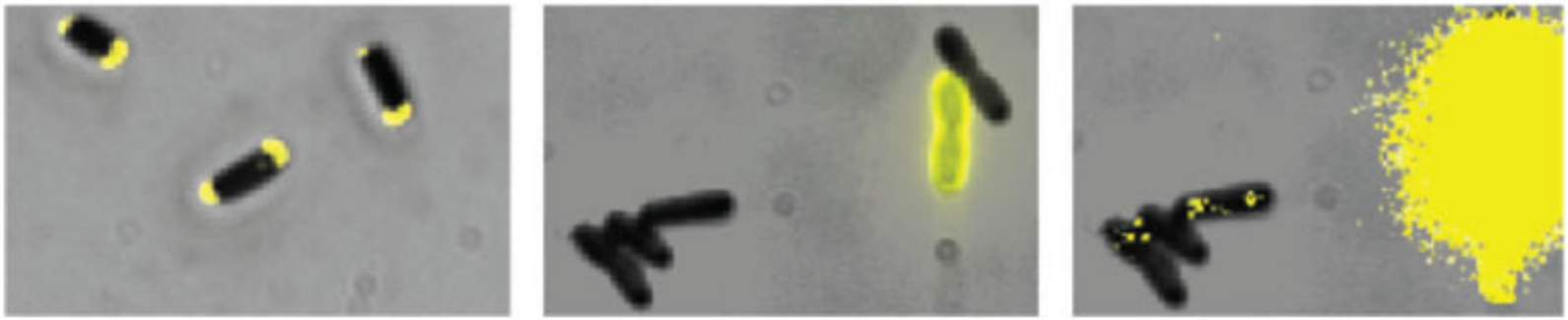
Cell cycle dependence of the gene expression rate for *lac promoter under repressed conditions. The division cycle* of each cell was divided into four time windows of equal length. The result shows that ***more gene expression bursts are observed at the later stage of the cell cycle.***

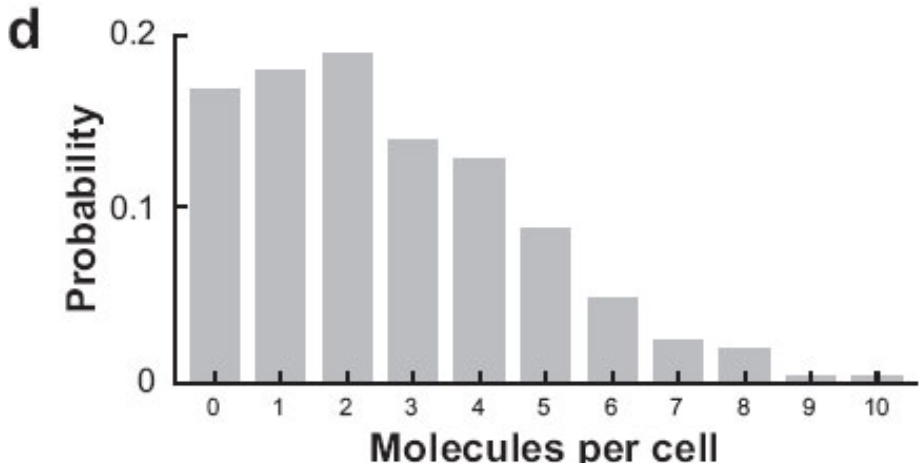
Why *more gene expression bursts are observed at the later stage …*

- replication of the chromosome results in two or more gene copies at later stages of the cell cycle, which increase the protein production rate
- a collision between the replication machinery and the repressor as the replication fork moves through the gene may result in the dissociation of the repressor from its operator and hence a burst of proteins
- the probability of bursts is still well distributed across the entire cell cycle, which suggests that basal level of expression does not result solely from the collision between the replication machinery and repressor. Had that been the case, the bursts would only occur at a specific point during the cell cycle.

Membrane proteins and stochastic nature of protein expression

- Many proteins involved in cell sensing or signaling reside on the membrane
- **Lactose permease,** a membrane transporter for lactose encoded by the lac operon. Expression of the permease is controlled by the lac repressor responding to the level of inducer (IPTG) in the media IPTG-isopropyl-d-1-thiogalactopyranoside
- Under partial induction, genetically identical cells in a population can exhibit two different phenotypes, induced and uninduced, with extremely different expression levels of the permease





Summary What Have We Learned?

- Many important cellular processes, such as transcription, translation, and replication, occur with low copy numbers of macromolecules and hence require single-molecule sensitivity to probe their dynamics.

- The low copy numbers of macromolecules result in the stochastic behavior of biochemical reactions and molecular motions, which cannot be synchronized among a population of molecules or cells.

- Detection by localization allows a single FP to be imaged upon binding on DNA or attaching to the cell membrane. Stroboscopic excitation allows detection of a single FP nonspecifically bound to DNA or freely diffusing in the cytoplasm with submillisecond time resolution. Tandem repeats of fluorophores can be used to visualize single DNA loci and mRNA molecules.

- A transcription factor searches for a target sequence on the genome by repeated nonspecific binding on and 1D diffusion along different DNA segments, with a residence time of less than 5 ms, separated by much faster diffusion through the cytoplasm between two segments in less than 0.5 ms.

Summary What Have We Learned?

- Under repressed conditions, mRNA molecules are generated in a Poisson process owing to random dissociation of the repressor from the operator. Under induced conditions, however, multiple mRNA molecules are generated in pulses of transcriptional activity.

- Protein production occurs in bursts with one mRNA generating a few copies of protein molecules. The number of protein molecules produced per mRNA follows an exponential distribution.

- Stochasticity in gene expression is manifested both in the random events of transcription or translation in time in a single cell, and in the variation of copy numbers of protein or mRNA per cell in a population of cells at a particular time. Both measurements give the same values for the transcription frequency and translational burst size.

Fundamentals in Biophotonics

Single particle tracking

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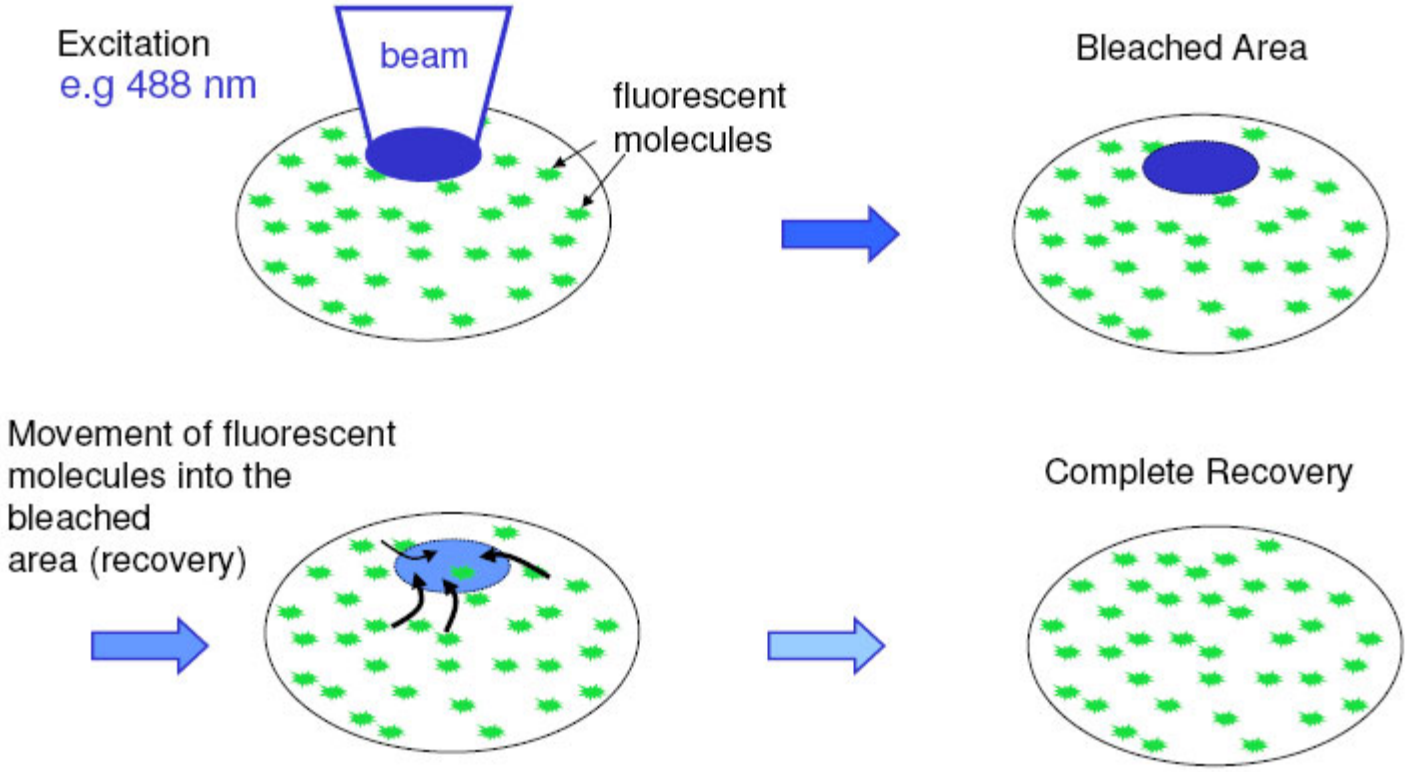
11.05.2020.

Why Single Particle Tracking?

- particle motion in the cells can be studied with **F**luorescence **R**ecovery **A**fter **P**hotobleaching (**FRAP**)
- FRAP is used to measure molecular diffusion and active processes in time.
- Can be Fast or Slow processes – measured in XY:
- To track movement and localization of macromolecules in living cell



Lippincott-Schwartz, et al.

Why Single Particle Tracking?

- **FRAP: Mode of operation**
- 1. **Determination of pre-bleach levels**
- 2. **Photobleaching (**short excitation pulse) of selected cells / areas.
- 3. **Recovery:** diffusion of unbleached molecules into the bleached area and increase of fluorescence intensity. Record the time course of fluorescence recovery at various time intervals, using a light level sufficiently low to prevent further bleaching.
- 4. **Quantification:** graph shows the time course of fluorescence recovery (calculated as average percentage recovery of initial fluorescence

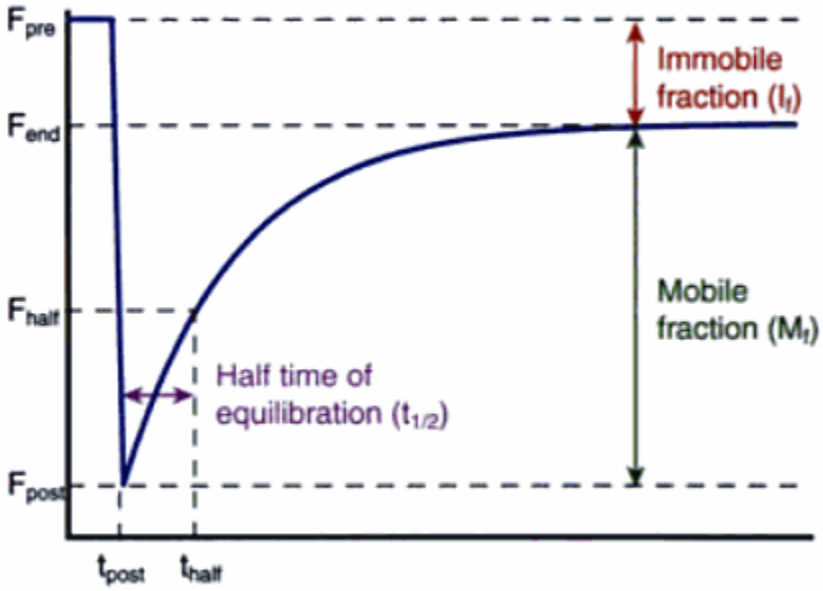
Calculation of recovery normalized

$$\frac{\text{Bleach - Bg}}{\text{Total - Bg}} \times \frac{\text{Total(t0) - Bg(t0)}}{\text{Bleach(t0) - Bg(t0)}}$$

Each fluorophore has different photobleaching characteristics. For FRAP experiments it is important to choose a dye which bleaches minimally at low illumination power (to prevent photobleaching during image acquisition) but bleaches fast and irreversibly at high illumination power.

Why Single Particle Tracking?

- FRAP: mobile fraction vs. Immobile fraction in ER



$$M_f = \frac{F_{end} - F_{post}}{F_{pre} - F_{post}} \qquad I_f = 1 - M_f$$

$$F_{half} = \frac{F_{end} + F_{post}}{2} \qquad t_{1/2} = t_{half} - t_{post}$$

FRAP experiments show that diffusion coefficients for proteins in a cell membrane are **5–100 times lower** than the values for proteins in an artificial bilayer

Many mechanisms may be involved:

- obstruction by mobile or immobile proteins,
- transient binding to immobile or mobile species,
- confinement by membrane skeletal corrals,
- binding or obstruction by the extracellular matrix, and hydrodynamic interactions.

www.nature.com/reviews/molcellbio

Why Single Particle Tracking?

These mechanisms have been difficult to sort out!

- First, some of them may occur simultaneously, and their relative importance may depend on the protein and the cell type
- Second, a significant fraction of proteins and lipids is immobile on the time scale of a FRAP experiment. For artificial bilayers and rhodopsin in the rod outer segment, recovery is close to 100%, but in the plasma membrane, recovery is typically 25% to 80%
- Third, in FRAP experiments, the distribution of observed diffusion coefficients *D* is much broader than expected from experimental error . Values of *D* vary around twofold among different points on a single cell, and tenfold among cells This suggests **significant heterogeneity in the membrane**, a view supported by other evidence
- The increased resolution of SPT ought to make it possible to understand the FRAP immobile fraction.

Single particle tracking-SPT

- In single-particle tracking (SPT), computer-enhanced video-microscopy is used to track the motion of proteins or lipids on the cell surface .
- Individual molecules are observed with a typical spatial resolution of tens of nanometers and typical time resolution of tens of milliseconds .

The technique is addressing following questions

- 1. How do particle move on the cell surface ? To what extent does the motion of various particles deviates from pure diffusion ? How is that motion controlled and what is it function ?
- 2. How is the cell surface organized ? To what extent membranes deviate from the fluid mosaic model? Is a fractal time model a useful description of the cell surface ? How are structures on the cell surface assembled ? Does compartmentation prevent crosstalk of receptors What regional or global control over cell membrane dynamics exists?
- What are the effects of heterogeneous motion in a heterogeneous environment on kinetics and equilibrium?

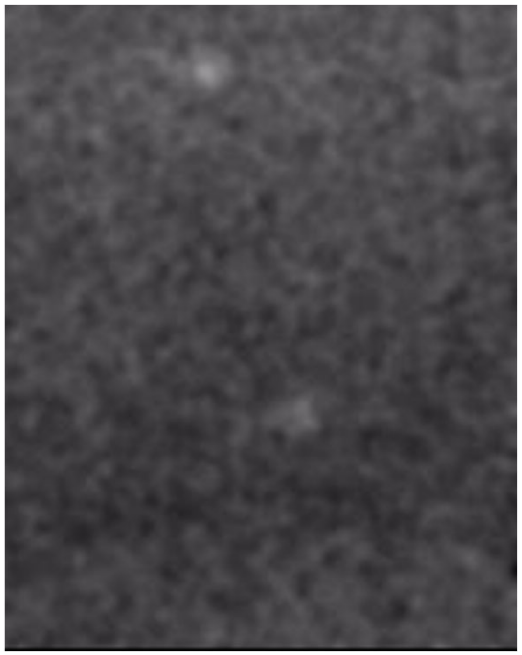
Capabilities of Single particle tracking-SPT

- The spatial resolution is approximately ***two orders of magnitude higher*** than FRAP, so that with sufficient time resolution motion in small domains can be characterized.
- The time resolution ***is similar to FRAP***, so the minimum detectable diffusion coefficient is lowered by approximately two orders of magnitude.
- FRAP averages over hundreds or thousands of diffusing molecules, but SPT ***measures individual trajectories***. Thus, different subpopulations indistinguishable by FRAP can be resolved.
- SPT provides the ultimate specificity in measurement of motion of membrane components, particularly if the individual particle tracked could be characterized in terms of, for example, its phosphorylation state.

- **How do individual molecules move in the cell?**

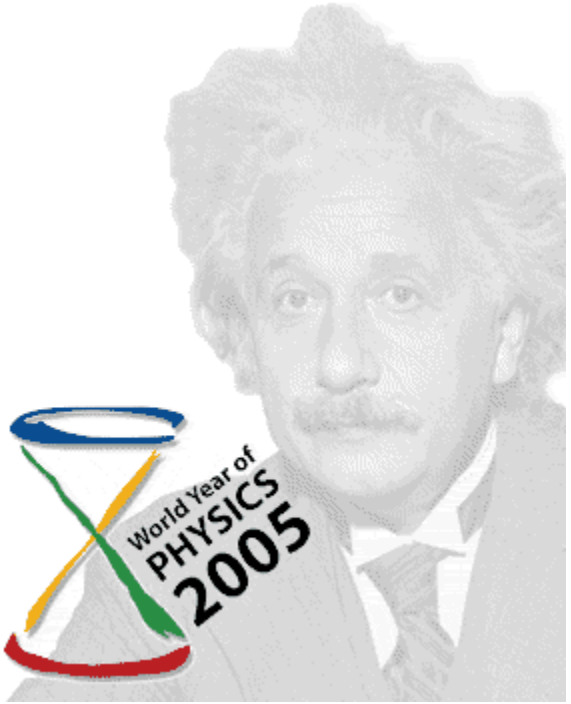
Brownian motion

- **Brown (1828)**
- – Pollen grains (1μm) suspended in water do a peculiar dance
- – The motion of the pollen has never stopped
- – Totally lifeless particles do exactly the same thing



Einstein (1905)

– Quantitative explanation from thermal motion of water molecules



Random Walk Models for Life in Motion

Dynamics in cells is mostly driven by random jiggling of small molecules in solution: diffusion

Because diffusion of molecules is powered by thermal energy, movement is random.

Collisions in chemical reactions
Ligand binding/unbinding
Distribution of ions or newly made proteins
Homogeneous distribution of abundant molecules
Equilibrium of pressure and equipartition of energy

- Opening of ion channel & diffusion of ions

When channel is open, ions diffusion inward--
Passive transport (no energy input)

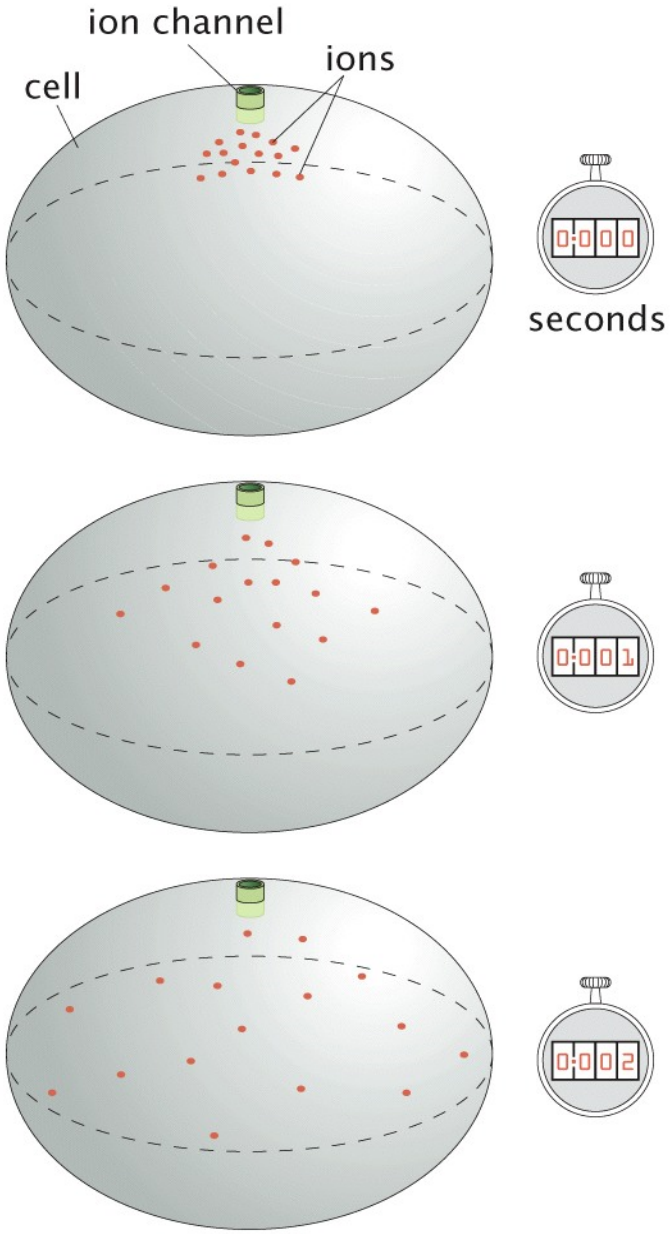
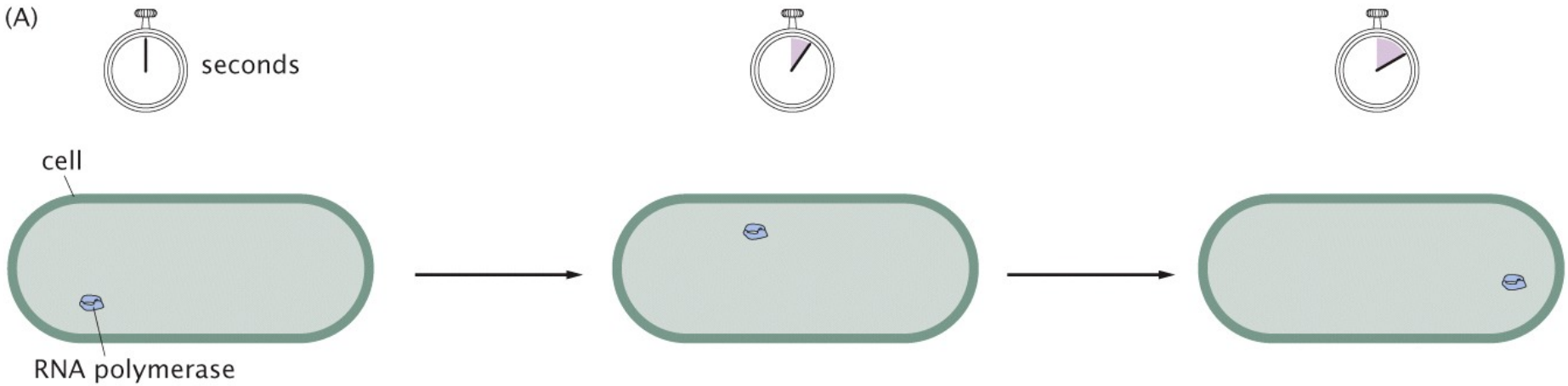


Figure 13.1 Physical Biology of the Cell, 2ed. (© Garland Science 2013)

Diffusive & directed motions of RNA polymerase

- **Passive:** Free RNA polymerase molecule diffusing in a bacterial cell



Active: One-dimensional motion of RNA polymerase along DNA

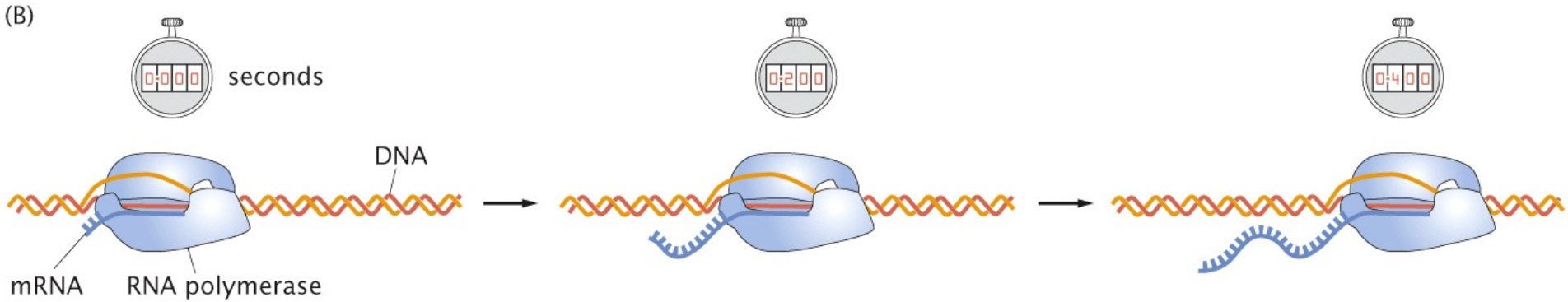


Figure 13.2 Physical Biology of the Cell, 2ed. (© Garland Science 2013)

Energy source: NTP

Patterns of *E. coli* swimming at different scales

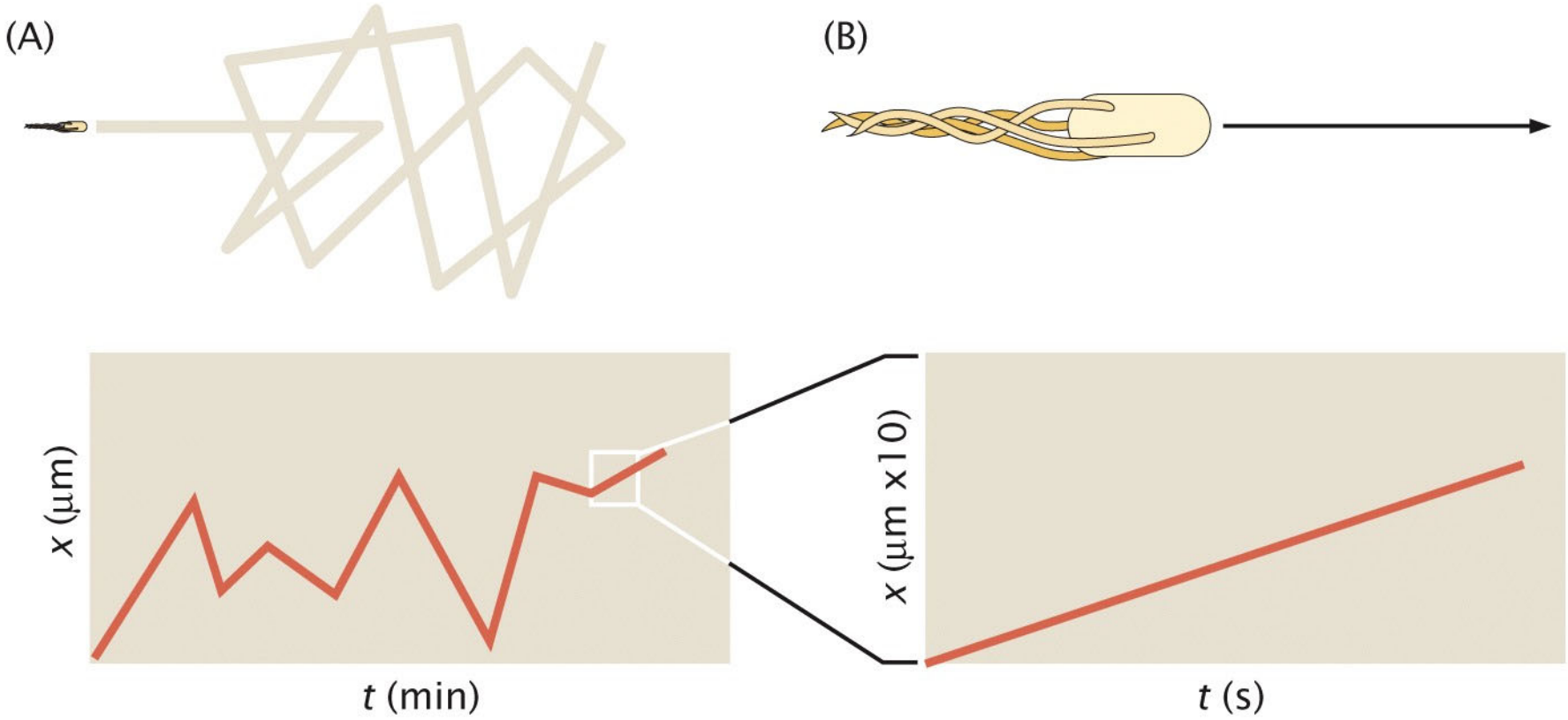


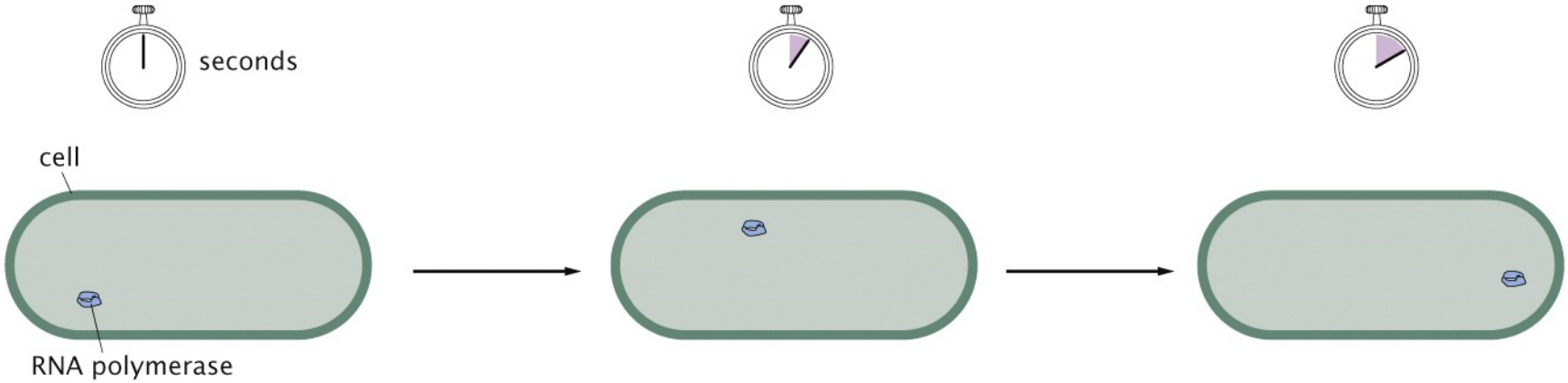
Figure 13.3 Physical Biology of the Cell, 2ed. (© Garland Science 2013)

At low magnification, the swimming movement of a single bacterium appears to be a **random walk**

At higher magnification, it is clear that each step of this random walk is made up of very straight, **regular movements**

Biological distances measured in diffusion times

How long does it take to move across the bacterial cell?



$$< r^2 > = 6Dt \quad and \quad r = 1\,\mu m\ (assuming\ bacteria\ as\ a\ sphere)$$

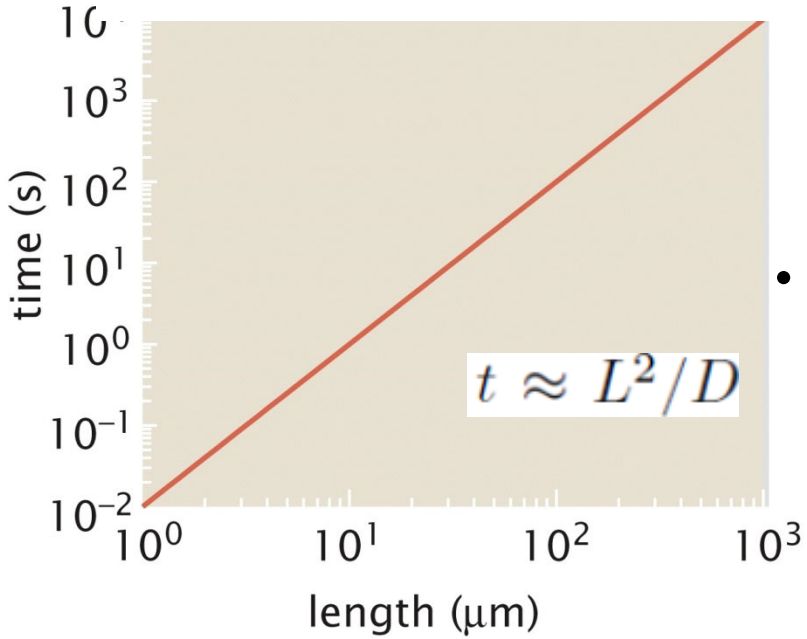


Figure 13.4 Physical Biology of the Cell, 2ed. (© Garland Science 2013)

$$D = 100\mu m^2 s^{-1}$$

$$t = 2\ ms$$

- Diffusion time as a function of the length

$$L \approx 10^6\,\mu\text{m}\ (1\,\text{m})$$

$$\Downarrow$$

$$t = 10^{10}\text{s} = 300\text{y}$$

Diffusion is not effective over large cellular distances

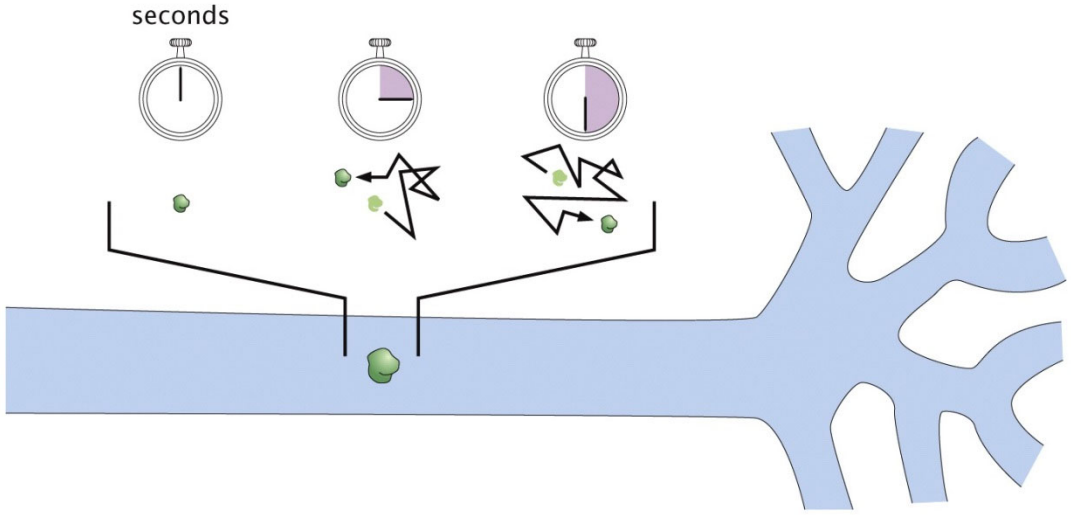


Figure 13.5a Physical Biology of the Cell, 2ed. (© Garland Science 2013)

- Nerve cell

$$D = \frac{kT}{6\pi\eta R}$$

In reality, it takes much longer than 12 days, because viscosity of cytoplasm is much higher for large particles and the crowding effect slows down diffusion.

$$for\ an\ organelle\ 500\ nm\ in\ radius, D \approx 0.5\ \mu m^2 s^{-1}$$

$$travelling\ across\ a\ nerve\ cell\ 1\ m\ in\ length\ in\ 1D,$$

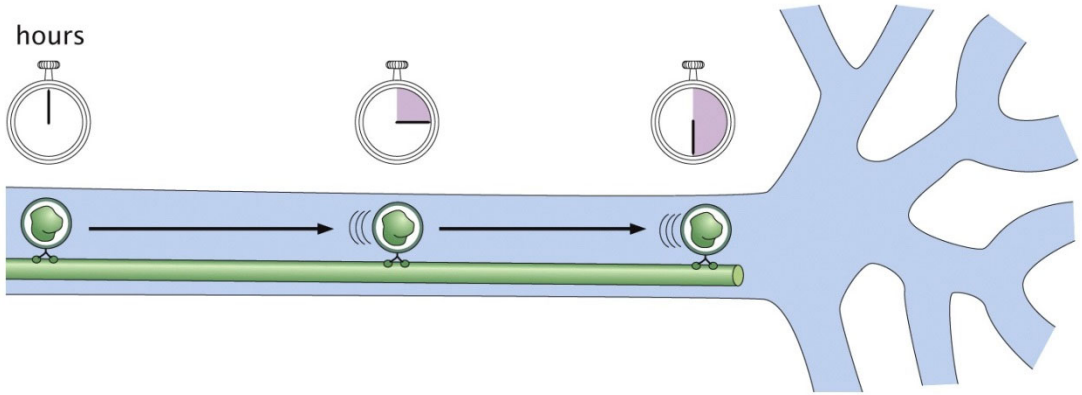
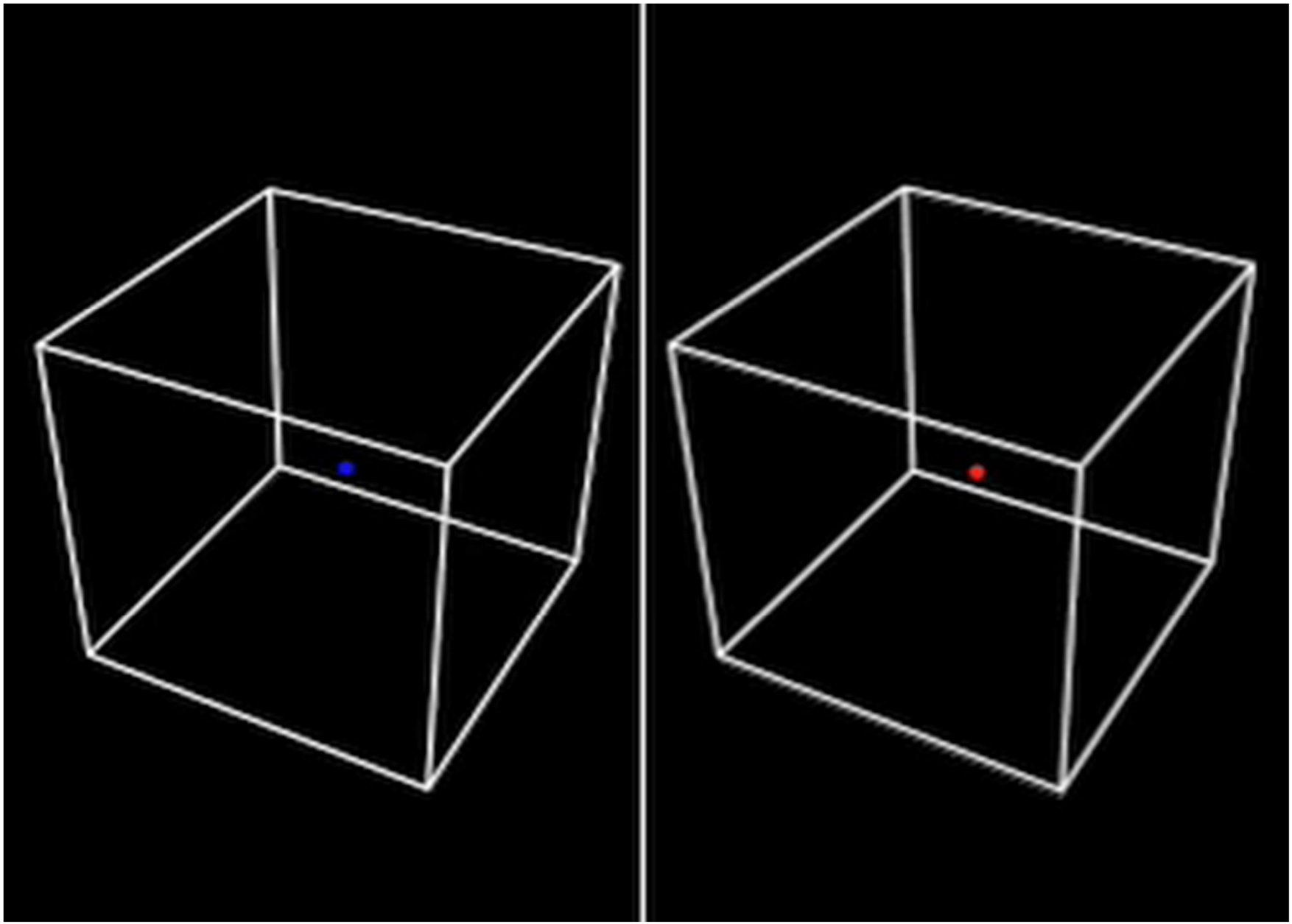


Figure 13.5b Physical Biology of the Cell, 2ed. (© Garland Science 2013)

Time scale of the **active** transport by virtue of **molecular motors** is shortened by many orders of magnitude than **passive** diffusion

Random Walk



Experimental techniques: measuring diffusive dynamics

- Fluorescence recovery after photo-bleaching

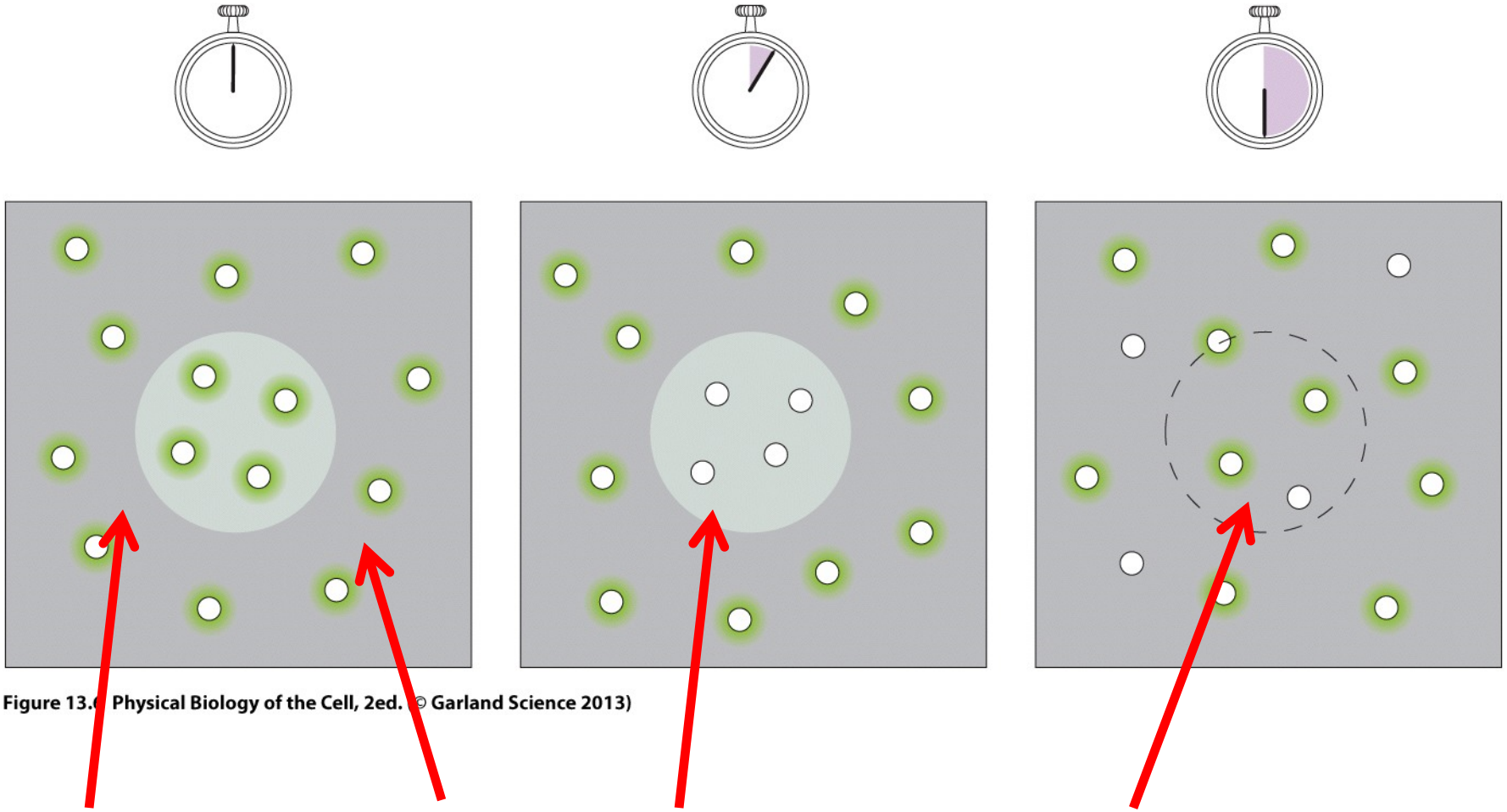


Figure 13.(Physical Biology of the Cell, 2ed. © Garland Science 2013)

Fluorescently labelled molecule

Photobleached region
by laser pulse

Fluorescently labelled
Molecules diffuse into the reg

FRAP experiment

- showing recovery of a GFP-labeled protein confined to the membrane of the endoplasmic reticulum

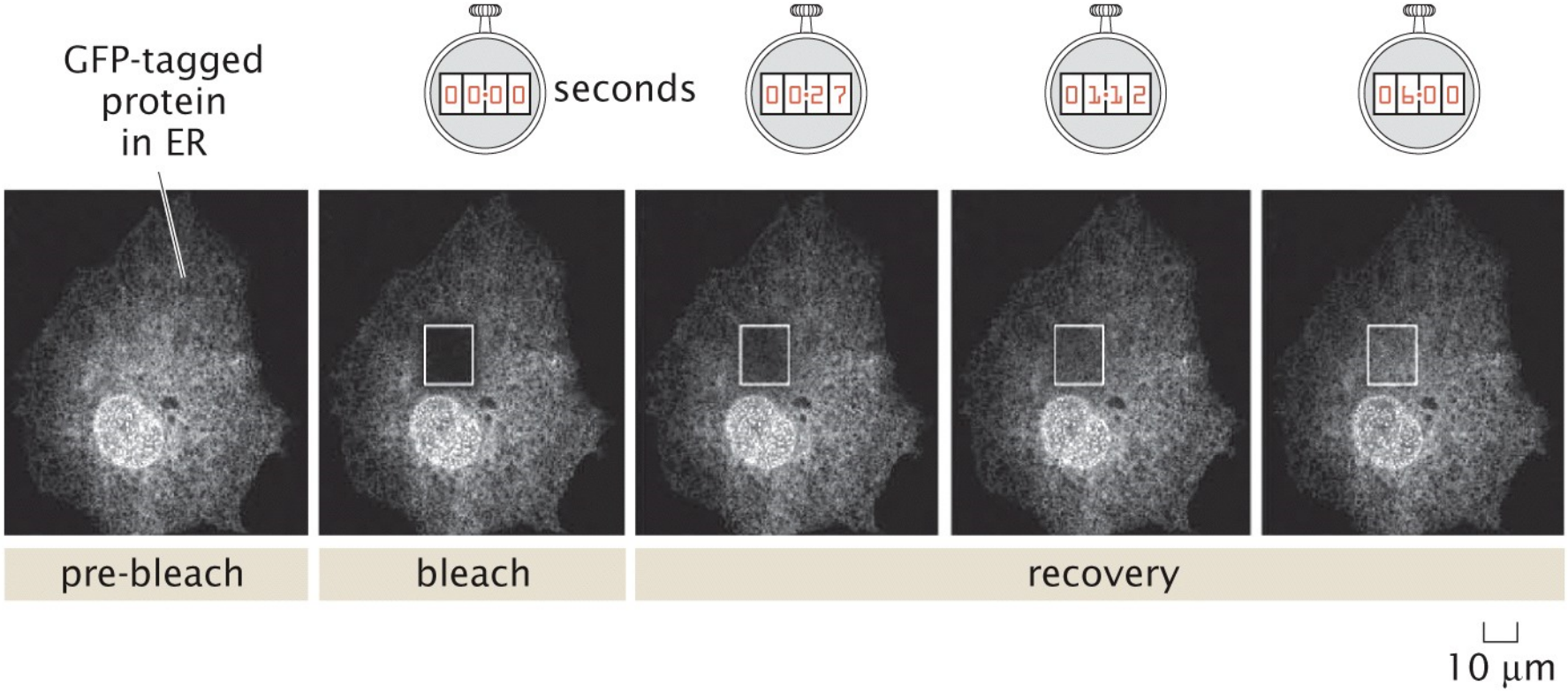
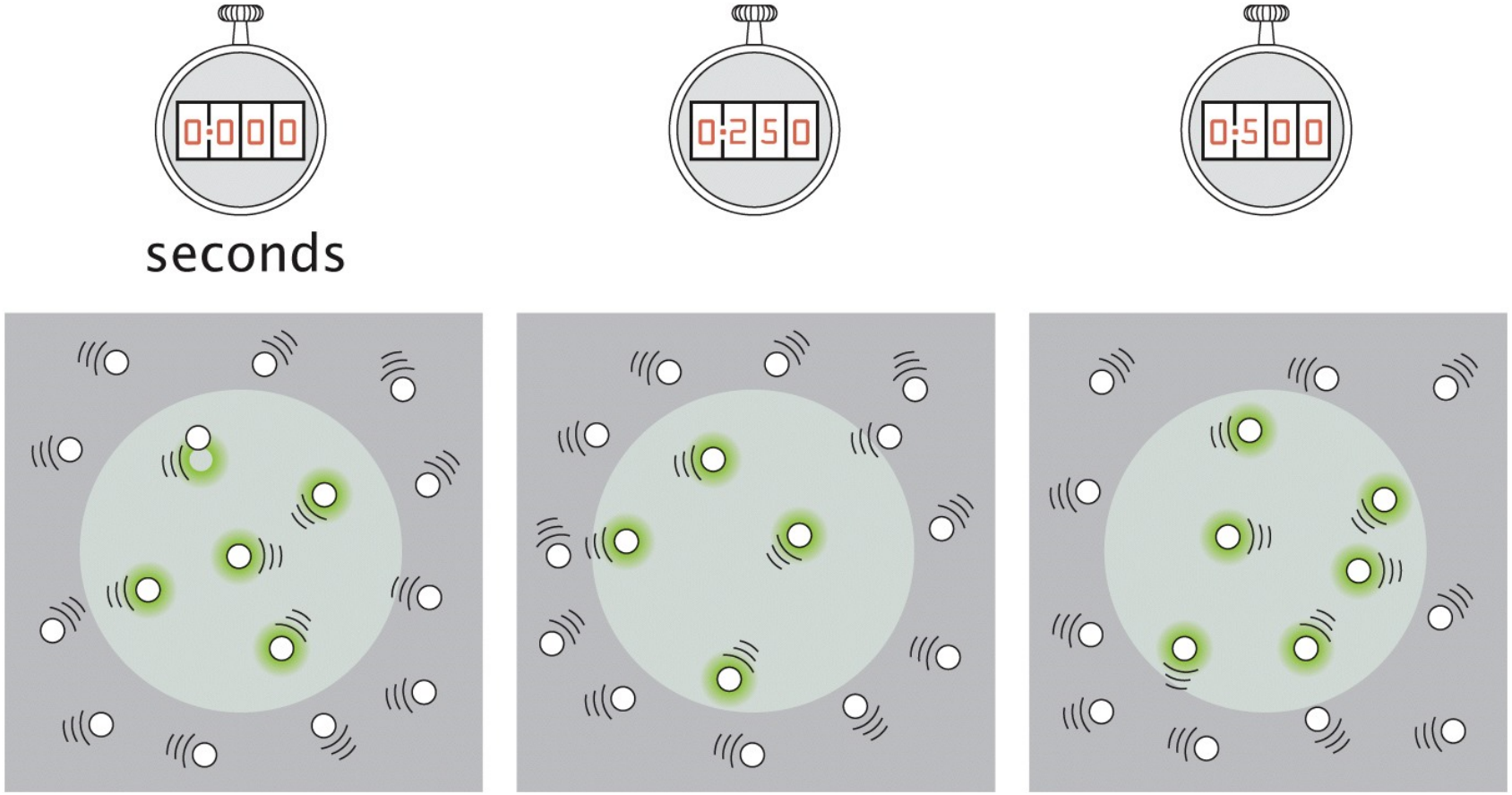


Figure 13.7 Physical Biology of the Cell, 2ed. (© Garland Science 2013)

The boxed region is ***photobleached*** at time instant, t = 0. In subsequent frames, fluorescent molecules from elsewhere in the cell diffuse into the bleached region.

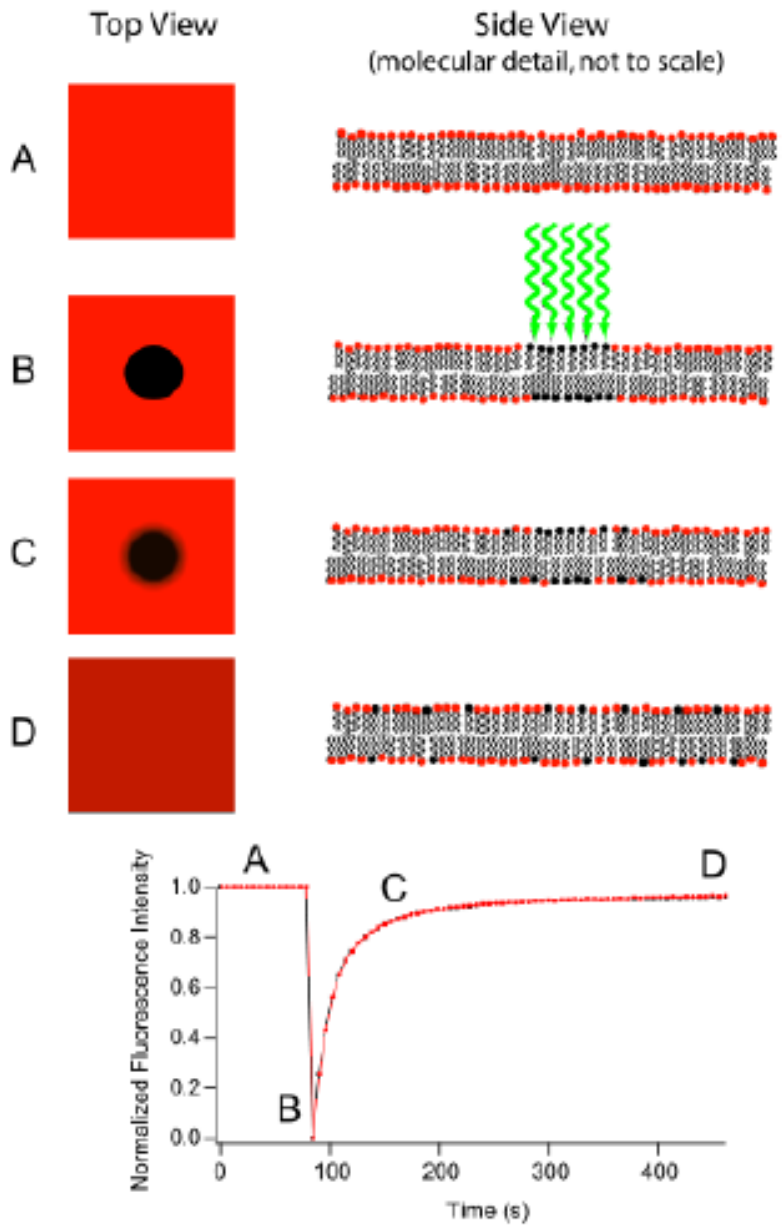
Fluorescence correlation spectroscopy



1) Measure the fluorescence intensity in a small region of the cell as a function of time

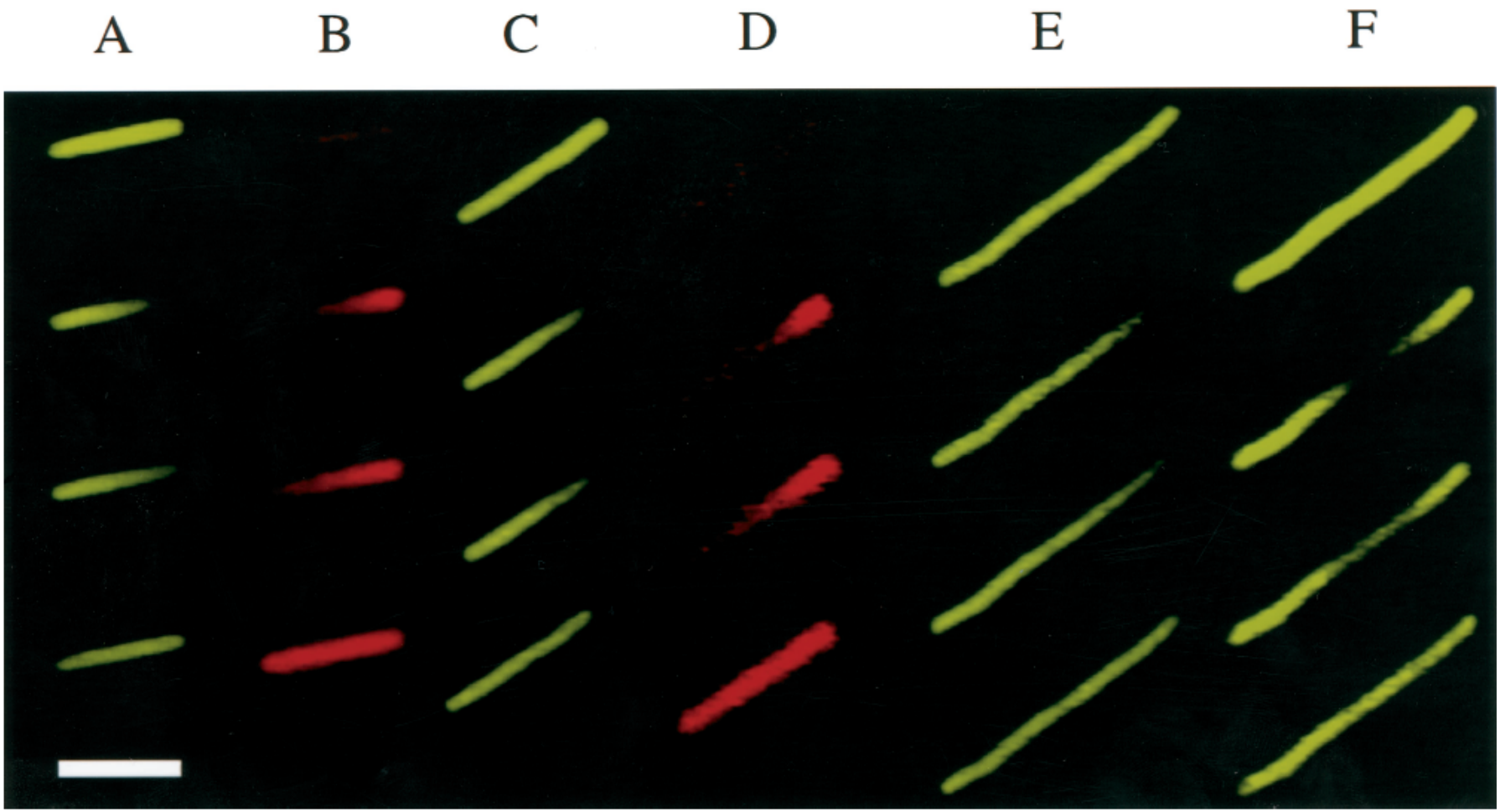
(2) By analyzing temporal fluctuations of the intensity through the use of time-dependent correlation functions, the diffusion constant and other characteristics of the molecular motion, such as concentration, can be uncovered

FRAP



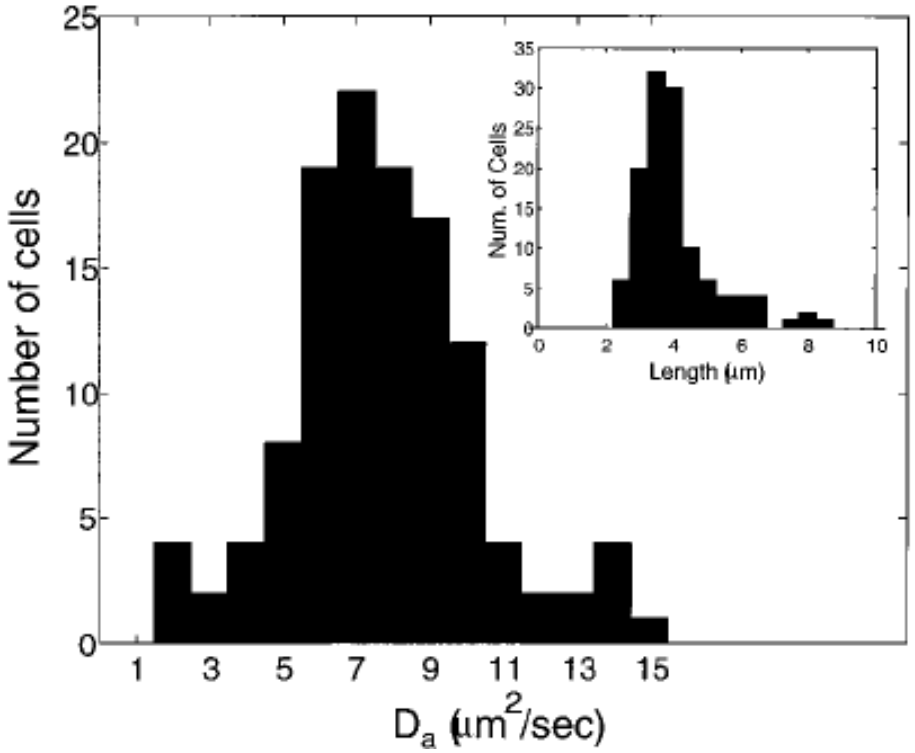
- FRAP denotes an optical technique capable of quantifying the diffusion of fluorescently-labeled biological molecules.
- Diffusion can be in 1-D in long processes (e.g. flagellum), 2-D on membrane proteins in a lipid bilayer or 3-D inside cytoplasm.
- (B) High power laser pulse is sent to photobleach all of the fluorescent probes in certain region.
- (C) Recovery of the fluorescent from the neighboring area is recorded as a function of time.
- (D) The half life of recovery is a function of diffusion constant.

Snapshots from photobleaching and photoactivation experiments



Snapshots from photobleaching and photoactivation experiments

- FRAP and photoactivation measurements must be interpreted with caution; in particular, one cannot assign an effective viscosity to the cytoplasm which would be applicable to all proteins inside it. Mobility depends sensitively on the protein under consideration, on its concentration, and on any genetic modification it may have undergone, such as His tagging.



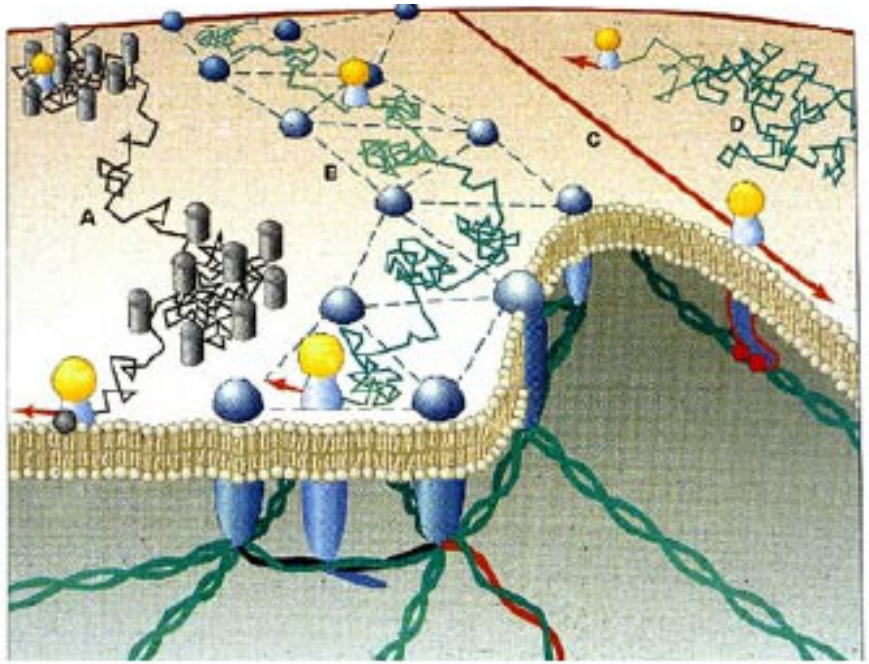
Thus, protein mobility must be seen not only as a property of the geometrical structure of cytoplasm and the background macromolecular concentrations alone but also as a characteristic of the diffusing species

QUIZ TIME

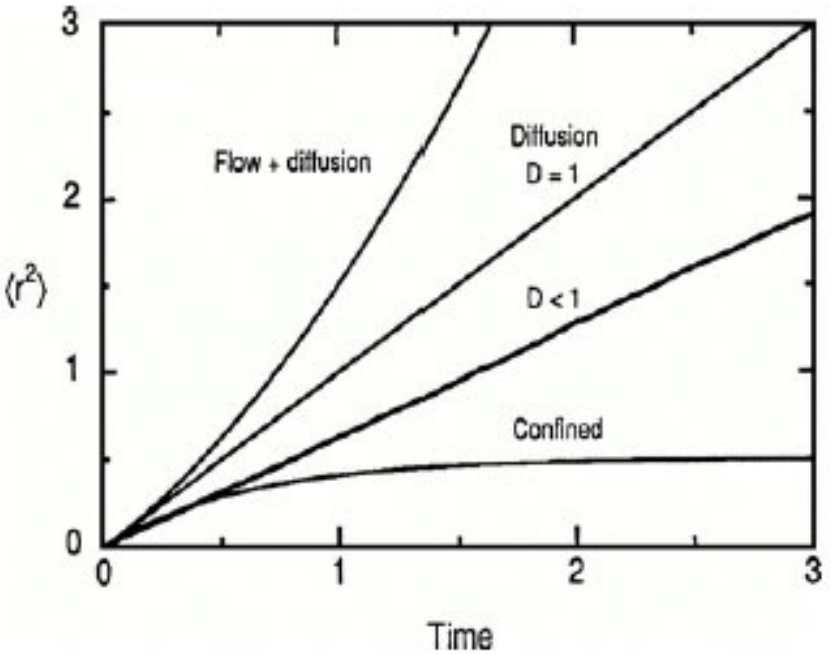
Ready for a short quiz?

Then open kahoot! ☺

Single-particle tracking



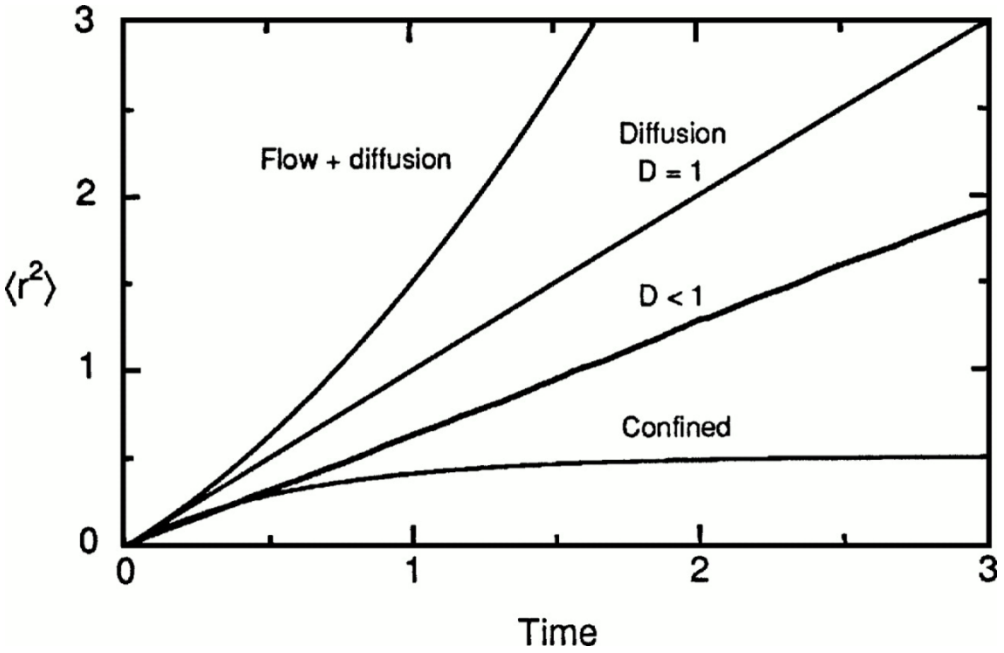
Record trajectory {x(t),y(t),z(t)} of a particle



Calculate Diffusion constant

Modes of Motion

- SPT can resolve modes of motion of individual molecules, and a major result of the technique is that motion in the membrane is not limited to pure diffusion.



Several modes of motion have been observed:

immobile,

directed,

confined,

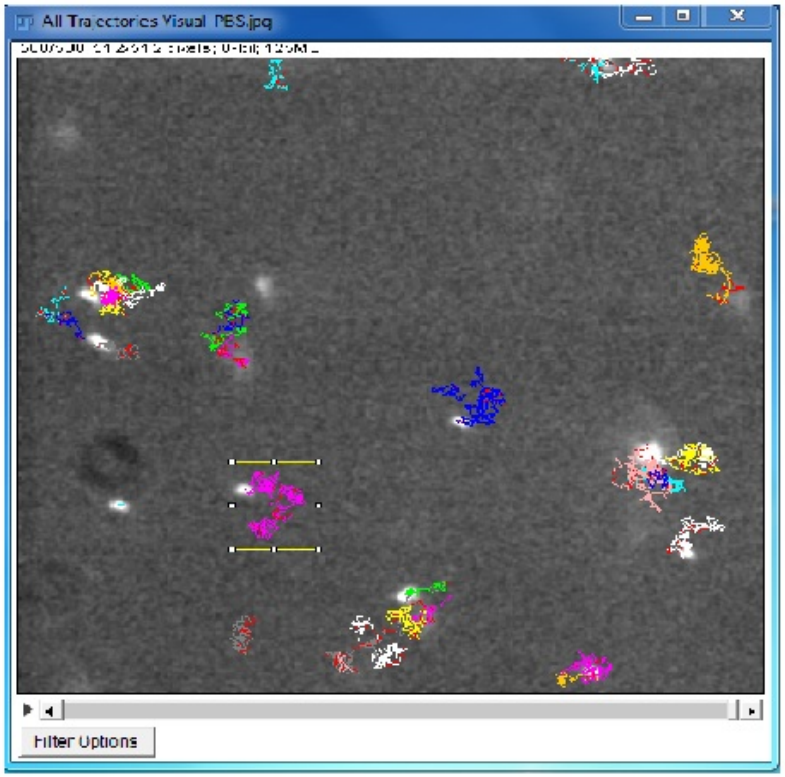
tethered,

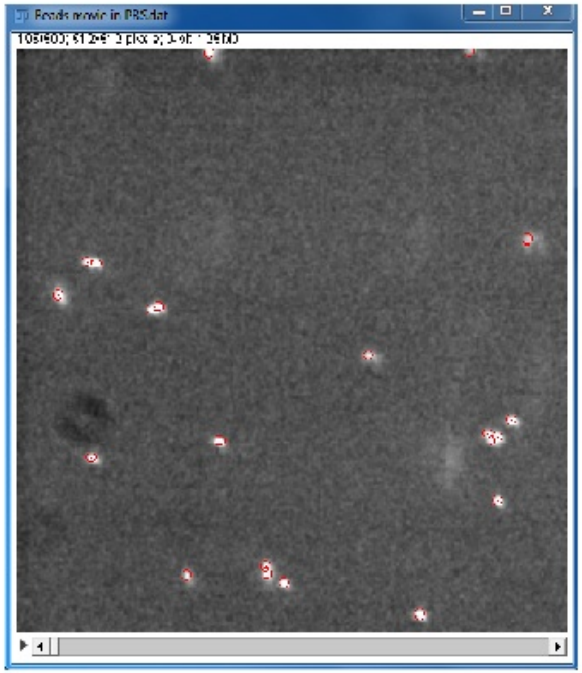
normal diffusion,

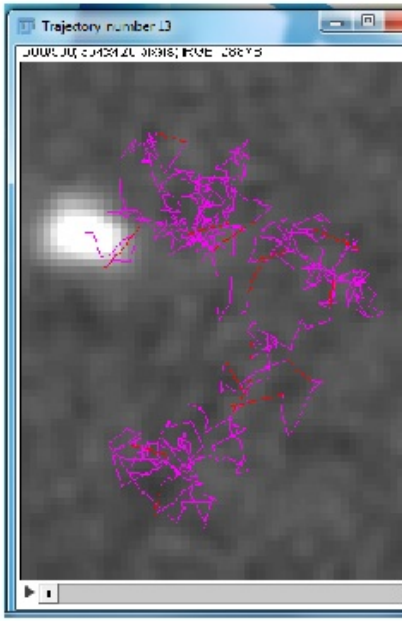
and anomalous diffusion

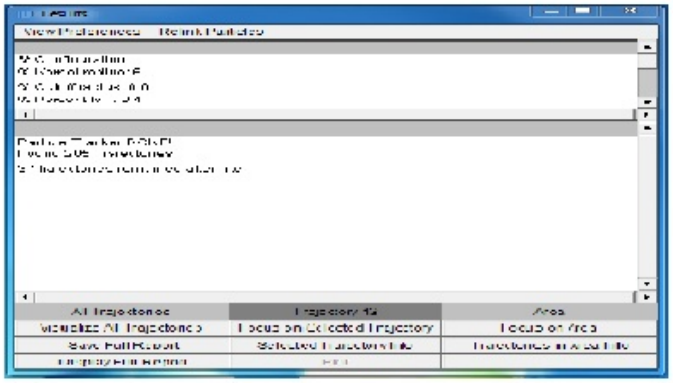
- The mean-square displacement r^2 as a function of time t for simultaneous diffusion and flow, pure diffusion, diffusion in the presence of obstacles, and confined motion.
- By checking MSD we can easily classify the modes of motion

Diffusive dynamics: Diffusion & Random Walk



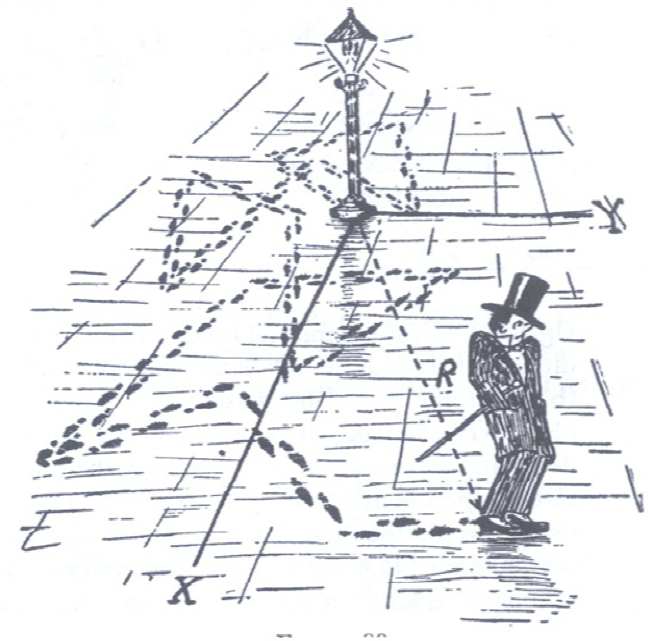






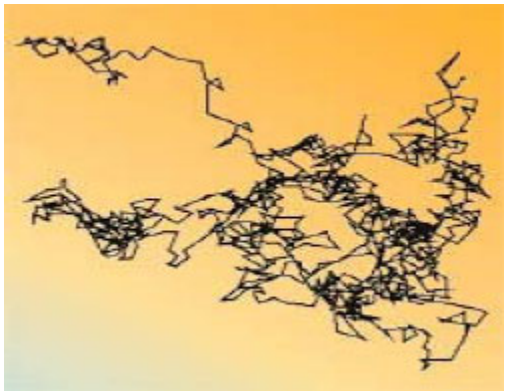
Random walks, diffusion

- **Key themes:**
  - **Brownian motion**
  - **Random walks**
  - **Diffusion laws**
  - **Biological applications of diffusion**



- ***Biological question:***

  If everything is so random in the nanoworld of cells, how can we say anything predictive about what's going on there?



- ***Physical idea:***

  The collective activity of many randomly moving actors can be effectively predictable, even if the individual motions are not

Friction

- **Friction ?**
  - **It is characterized by dissipation that reduces order**

Diffusion of ink in wet paper erases order

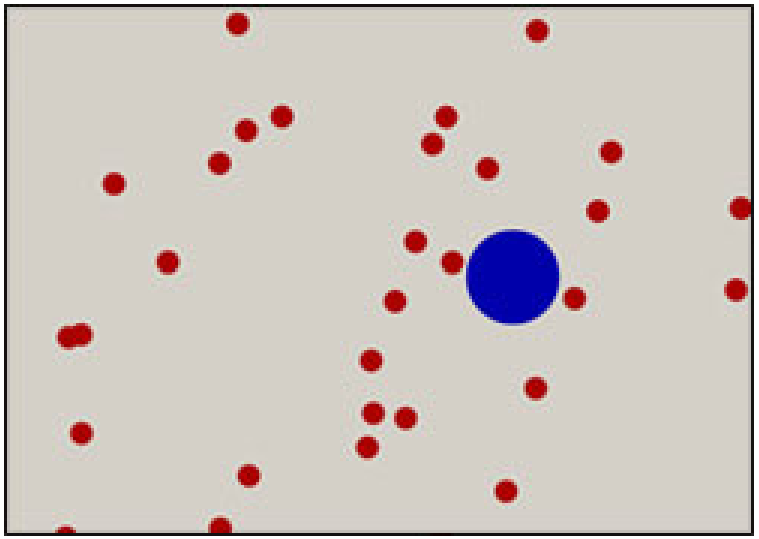
Friction erases order in the directed motion of a particle

Electrical resistance makes heat as it runs down flashlight batteries

That is, organized kinetic or potential energy gets degraded into disorganized motion. That in turn is characterized by the **random walk**

The random walk is an example of diffusion, which in cells ***turns out to be the dominant form of material transport on submicrosecond scales*** – *we will soon see why the scale is* important

Brownian motion



Robert Brown: observed in 1827

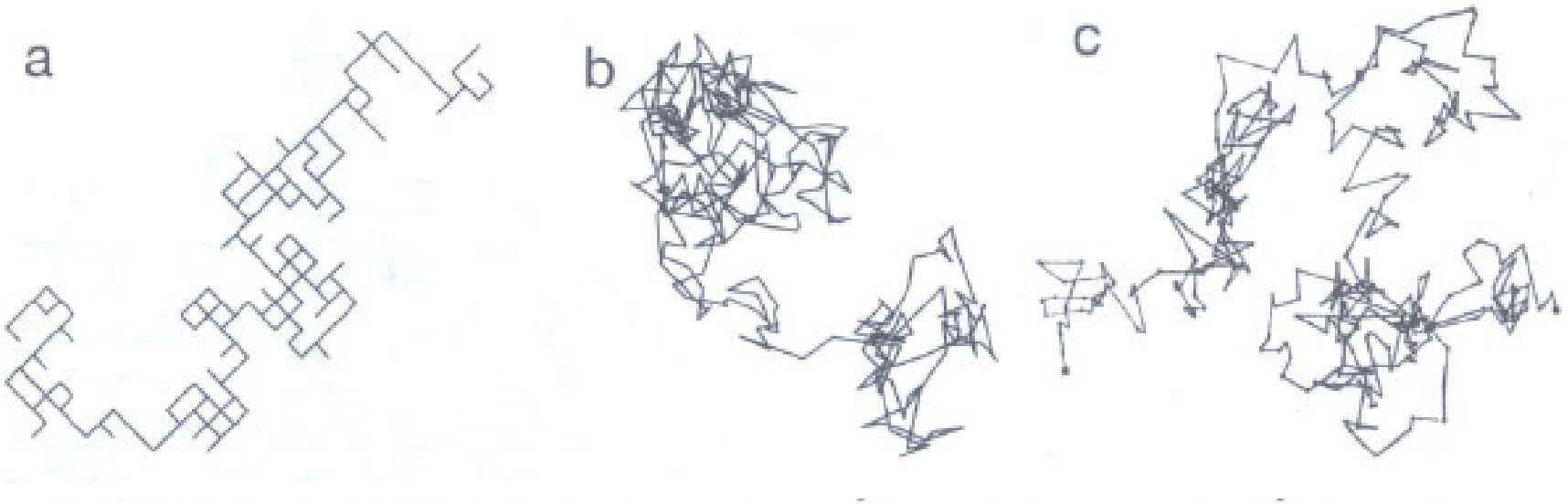
Tiny solvent molecules interacting with the colloid through collisions

Colloid mass much larger than the mass of solvent molecules

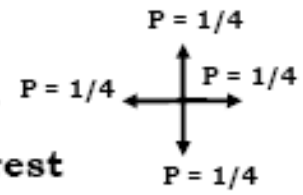
While the effect of each single collision is almost negligible, the large number of them gives rise to macroscopic observable motion

Brownian motion is thus driven by thermal energy

Brownian motion & random walk



Un-biased Random Walk on a 2D square lattice



1. Start from the origin
2. Choose one of 4 nearest neighbors randomly
3. Make a jump to the chosen nearest neighbor site
4. Go to 2.

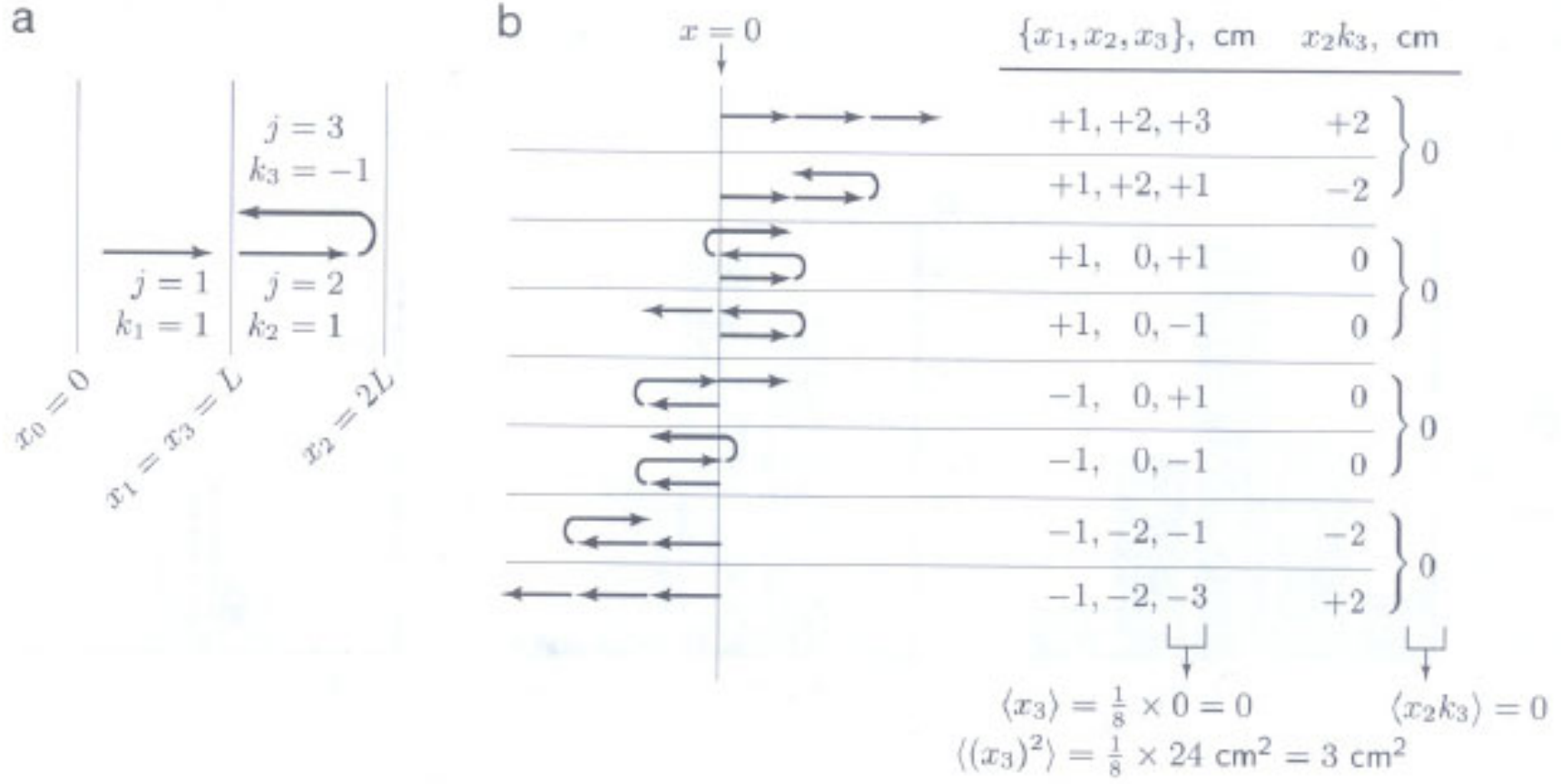
Self-Avoiding Random Walk on a 2D lattice

1. Start from the origin
2. Choose one of 4 nearest neighbors randomly
3. Make a jump to the chosen nearest neighbor site provided that it has never been visited before
4. Go to 2.

Coupling to polymer models...

Random walks leading to diffusion

- Random walks leading to diffusion



a) 1D random walk through three steps *j = 1, 2, 3. Step j makes a* displacement of *kj = ± 1.*

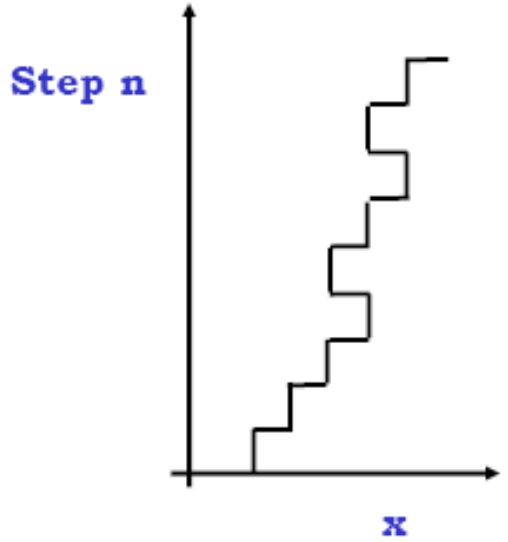
b) Examples of eight distinct 3-step walks with step length *L = 1 cm.*

Random walk leading to diffusion

- **Random walk in d = 1 dimension:**
- For simplicity, consider a random walk in 1D. Position after n steps xn,
- $x_0 = 0$, δ is constant, and jumps take place with an interval of τ .

$$x_1 = \pm\delta$$
$$x_2 = x_1 \pm \delta$$
$$x_N = x_{N-1} \pm \delta$$

Clearly < xn > = 0 for all n .



The second moments, however,

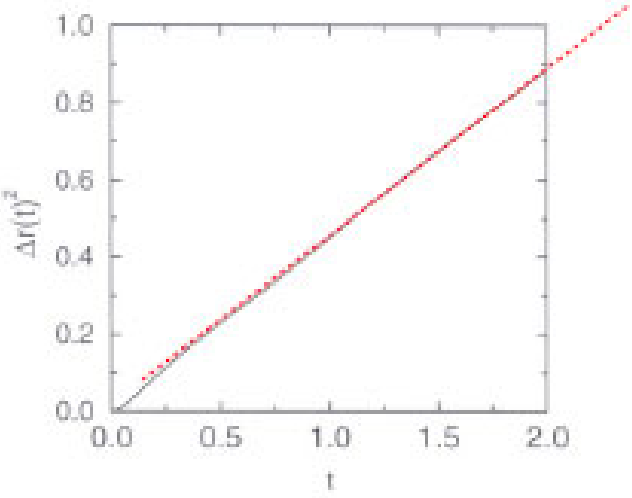
$$\left\langle x_1^{\,2} \right\rangle = \delta^2$$
$$\left\langle x_2^{\,2} \right\rangle = \left\langle x_1^{\,2} \right\rangle \pm \left\langle x_1\delta \right\rangle + \delta^2 = 2\delta^2$$
$$\left\langle x_N^{\,2} \right\rangle = N\delta^2$$

Here the discrete step number N relates to time t, thus we have

$$\left\langle x^2(t)_1 \right\rangle = \frac{t}{\tau}\delta^2$$

$$D_T = \frac{1}{2d}\lim_{t\to\infty}\frac{\langle x^2\rangle}{t}$$ **Diffusion coefficient by Einstein's definition**

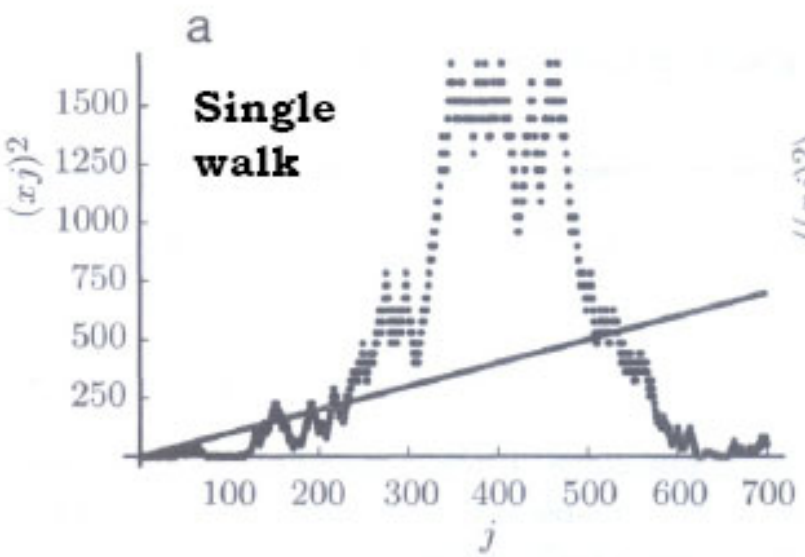
Random walk and diffusion

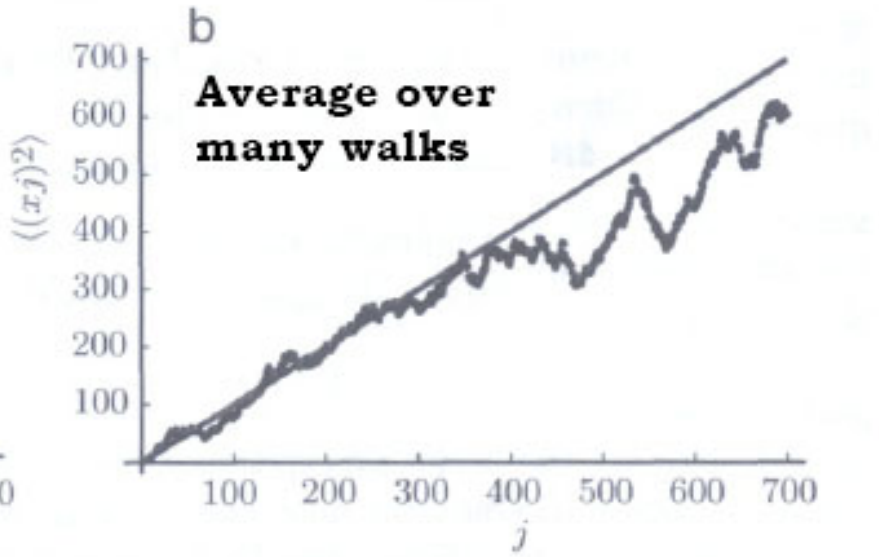


- **Slope of the mean-squared displacement at long times is proportional to the diffusion coefficient.**

$$D_T = \frac{1}{2d} \lim_{t \to \infty} \frac{\langle x^2 \rangle}{t}$$

Diffusion coefficient by Einstein's definition





Friction coupled to diffusion

- Consider a time period Δt
- Suppose only one collision to occur during Δt
- Between collisions, there is free flow subject to force f (e.g. gravitation).
- Then

$$\mathbf{dv_x / dt = f / m , \quad thus \ v_x(t) = v_{0x} + f\,t / m}$$

- The resulting uniformly accelerating motion of the particle is then

$$\mathbf{\Delta x = v_{0x}\,\Delta t + (1/2)(f/m)\,(\Delta t)^2}$$

- The average

$$\mathbf{< \Delta x > = (f / 2m)\,(\Delta t)^2}$$

- This implies that there is **a *net drift velocity*** *equal to (Δx/Δt), which is*

$$\mathbf{v_{drift} = f / \zeta, \qquad \zeta = 2m / \Delta t.}$$

The ***friction coefficient ζ*** *hence depends on e.g. the inter-particle* collision rate (the period of free flow). The well-known relation is the ***Stokes formula***

$$\mathbf{\zeta = 6\,\pi\,\eta\,R,}$$ Where R is a particle size and η viscosity

Friction coupled to diffusion

- To couple diffusion and friction, Einstein further used the equipartition theorem

$$< (v_{0x})^2 > = k_B T / m$$

- Then combining the equipartition theorem with the above equations

$$D = L^2 / \Delta t$$

$$\zeta = 2m / \Delta t , \qquad \Delta t = 2m / \zeta$$

- gives us

$$D = (L / \Delta t)^2 \Delta t$$

$$= < v_{0x}^2 > \Delta t$$

$$= (k_B T / m)(2m / \zeta) .$$

- Einstein therefore found

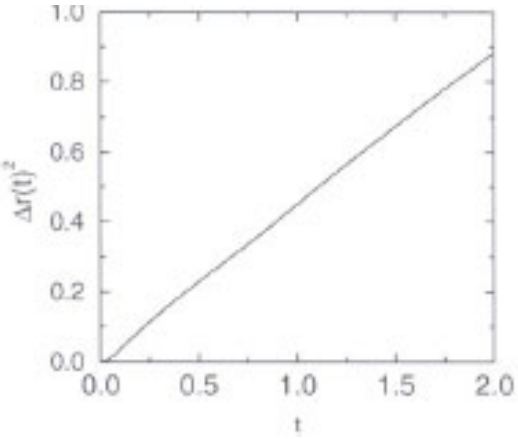
$$D \zeta \propto k_B T.$$

- This is an example of the famous ***fluctuation-dissipation theorem,*** *which* couples microscopic fluctuations with macroscopic transport coefficients.

Friction & Diffusion: Langevin equation

- Brownian diffusion for a colloidal particle, using the Langevin equation

$$m\frac{d^2x}{dt^2} = -\eta_0\frac{dx}{dt} + F(t)$$ **Langevin's form**



$$\Rightarrow \quad m\left\langle x\frac{d^2x}{dt^2}\right\rangle + \eta_0\left\langle x\frac{dx}{dt}\right\rangle = 0$$

$$m\left\langle\left(\frac{dx}{dt}\right)^2\right\rangle = k_BT$$ **Equipartition theorem**

White noise

$$\langle F(t)\rangle = 0$$

$$\langle F(t)F(t')\rangle \propto \delta(t-t')$$

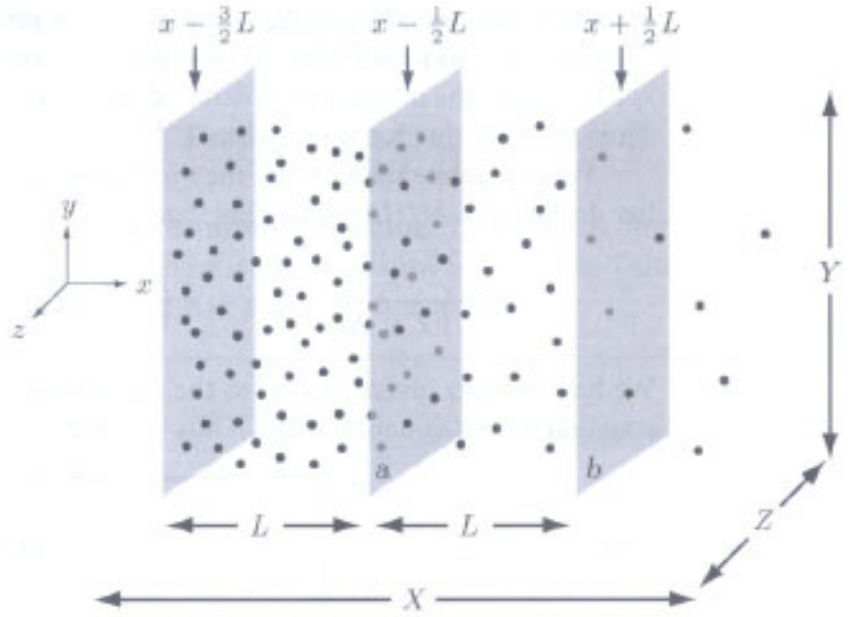
$$\langle F(t)x\rangle = 0$$

$$\left\langle F(t)\frac{dx}{dt}\right\rangle = 0$$

$$x\frac{dx}{dt} = \frac{1}{2}\frac{d}{dt}x^2$$

$$x\frac{d^2x}{dt^2} = \frac{1}{2}\frac{d^2}{dt^2}x^2 - \left(\frac{dx}{dt}\right)^2$$ **Useful tips**

Diffusion equation



- **How to determine D?**
- **1. Follow a single moving particle**
- **2. Follow the distribution of many (many!) diffusing particles**

Assume non-uniform distribution in x, but uniform in y and z.

N(x) Total number of particles in a slot centered at x

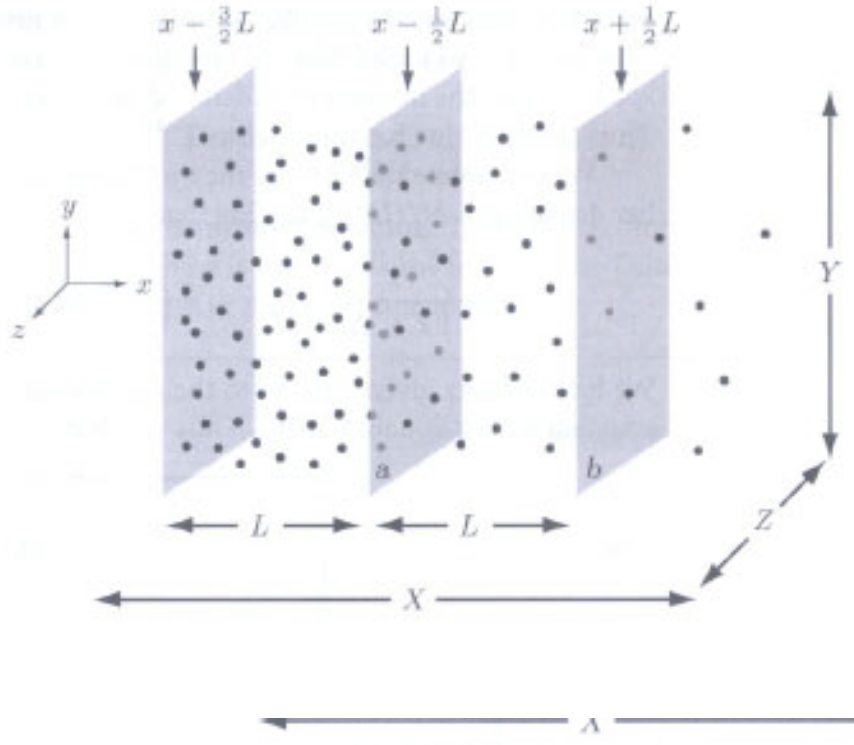
Assume that at every time step Δt every particle moves a distance L to the left or the right, at random. In the slot represented by (N – L), there is an excess of particles. This leads to a flow to the right.

The net number of particles crossing the boundary ”a” from left to right

is then

$$[\ N(x - L) - N(x)\ ] \ / \ 2$$

Diffusion equation



- Considering L to be very small, we get

$$N(x - L) - N(x) = - L \, (dN/dx).$$

- Dividing N(x) by the volume of the slice
- (V = XYZ), we are given the number density of particles, c(x).
- Then the flux (number of particles crossing "a" (from left to right) per unit area per unit time) is

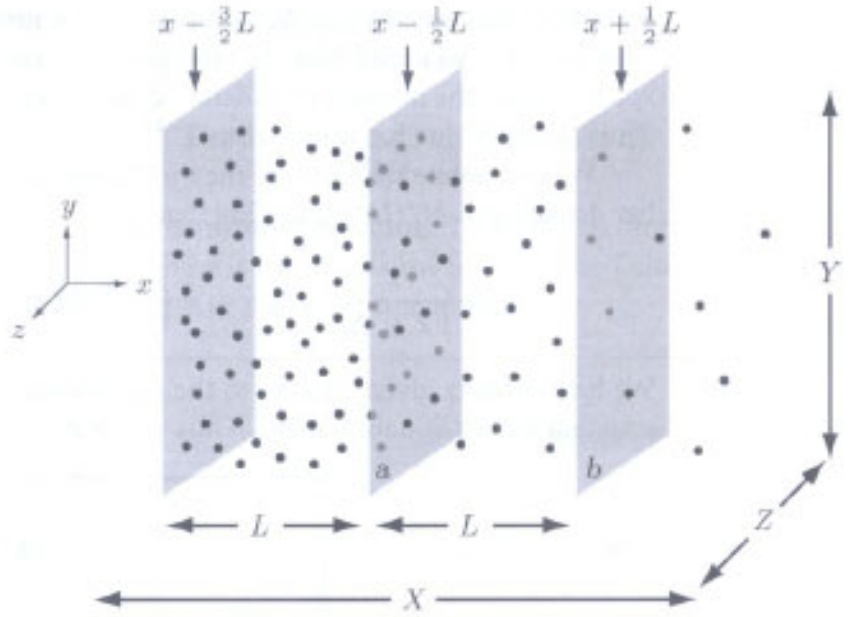
$$j = \frac{1}{YZ\,\Delta t} \frac{1}{2} L \left[\frac{-d}{dx} LYZ\, c(x) \right] = - \frac{1}{\Delta t} \frac{L^2}{2} \frac{dc}{dx}$$

This corresponds to Fick's first law

$$j = - D \frac{dc}{dx}$$

The flux is hence driven by the density gradient

Diffusion equation



- This corresponds to Fick's first law

$$j = -D \frac{dc}{dx}$$

- For practical purposes, this is easier to measure in a different form using the continuity equation

Net change of particles across the surface "a" is

$$d\,N(x) / dt = [\, YZ\, j(x - (L/2)) - YZ\, j(x + (L/2)) \,]$$

In the limit of small L, this leads to known as the ***continuity equation***.

$$dc/dt = -d\,j/dx$$

Combining the above two yields the diffusion equation

$$dc/dt = d/dx\,(\,D\,dc/dx)\,)$$

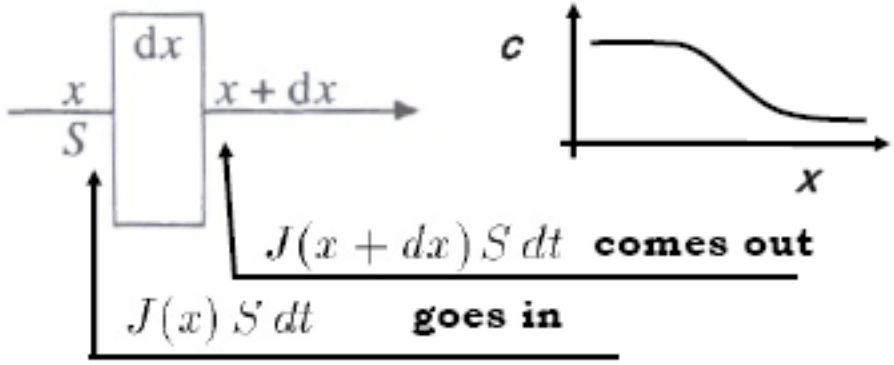
$$dc/dt = D\,d^2\,c/dx^2$$

Diffusion equation & Fick's laws

- **Macroscopically the diffusion of particles (mass) is traditionally described in terms of the diffusion equation and Fick's laws. Let the flow of matter be J**

Assume linear response, when c is the concentration, A some constant, and $S\,dx$ **a volume element.**

$$J = -A\,\partial c/\partial x$$ **Fick's 1. law**



$$J(x+dx) = J(x) + (\partial J/\partial x)\,dx$$

$$dm = -(\partial J/\partial x)\,S\,dx\,dt \quad \Rightarrow \quad \frac{\partial c}{\partial t} = -\frac{\partial J}{\partial x}$$ **Continuity equation**

$$\frac{\partial c}{\partial t} = A\frac{\partial^2 c}{\partial x^2}$$ **Fick's 2nd law**

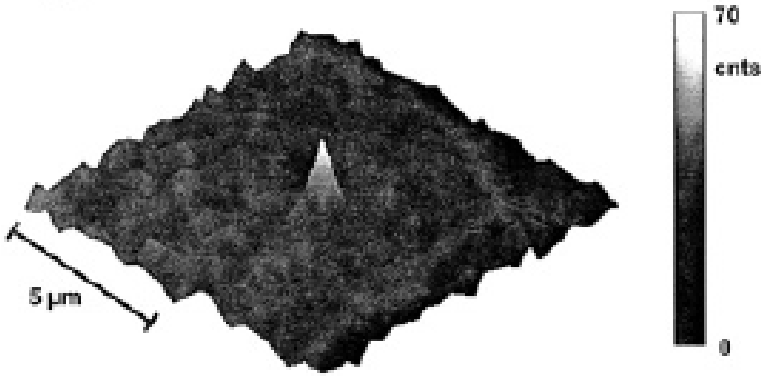
$$A = D$$

Usually this is not the tracer (single-particle) diffusion coefficient

Biological applications of tracer diffusion

T. Schmidt et al. Biophys. J. 77, 2638 (1999).

A

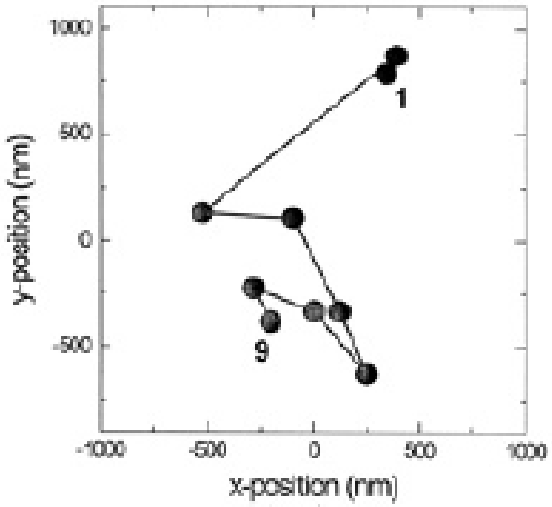


Lateral diffusion of lipids in the plane of the membrane

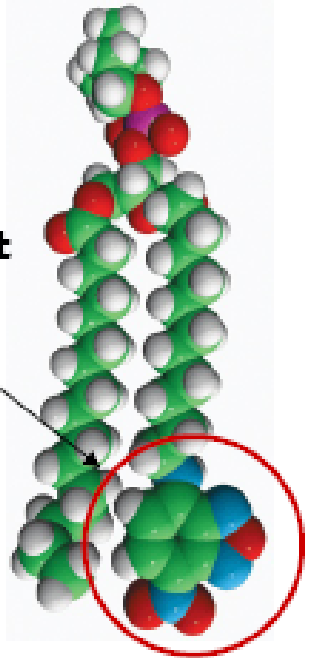
Single-particle tracking

Fluorescence image of an individual labeled lipid.

B



Trajectory of the lipid in the plane of the membrane (from above, every 5 ms).



Do fluorescent probes perturb the structure and dynamics of lipid membranes?

See:
J. Repakova et al. Biophys. J. (2005).

Diffusion and diffusion length

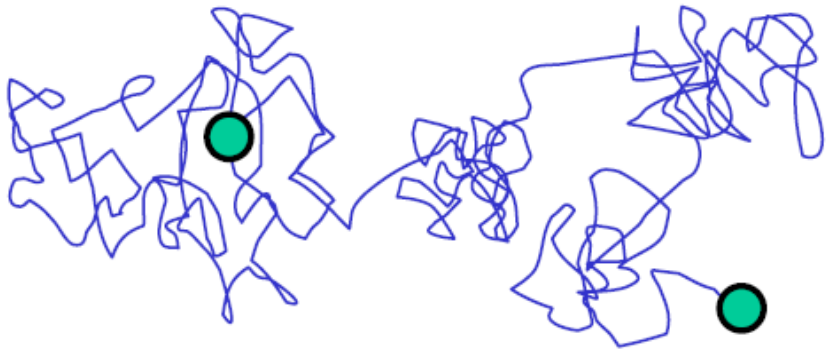
- ***Random walk in D dimensions:***
- Generally in d dimensions,

$$< r^2(t) > \; = \; 2d \;\; D \; t$$

- and one can define the diffusion coefficient via the Einstein's relation

$$D = (1 \; / \; 2d) \lim_{t \to 0} < r^2(t) > \; / \; t \; .$$

- Importantly, there is the limit of long times there are reasons for the prefactor of 2d the above coefficient describes the motion of single particles only those who are interested, can recall how the random walk gives rise to a Gaussian distribution in time and space



Diffusion length:

To characterize the length scale crossed by a diffusing particle during a time scale t, define the diffusion length

$$l_D = (2d \; t \; D)^{1/2} \; .$$

For typical solvents D ≈ 10^{-5} cm^2 / s.
Thus in 3D they cover
2.4 nm in 1 ns
7.7 nm in 10 ns
24.5 nm 100 ns
77.5 nm in 1000 ns .
The cell diameter would be crossed in about ***0.1 seconds***.

Single particle tracking (SPT)

- ***Single particle tracking (SPT)*** is a powerful method for studying the movement of individual or small groups of proteins or lipids in the plasma membrane of live cells or in model membranes.

- ***Labeling strategies***
  - Secondary antibody fluorophores as Cy3, Alecxa etc..
  - antibody-coated sub-micron colloidal gold particles
  - antibody-coated sub-micron colloidal quantum dots
  - Florescent proteins
  - latex beads

- ***The time resolution*** *of* SPT depends on the frame rate of the camera being used. Video cameras using the NTSC standard have a sampling frequency of 30 frames/s, while those using the PAL standard have a frame rate of 25 Hz. However, SPT has been performed with specialized video cameras that have time resolution of 25 μ

Labelling bottelnecks

- 1. most labels are large, so that drag from the interaction of the label with the extracellular matrix may be significant

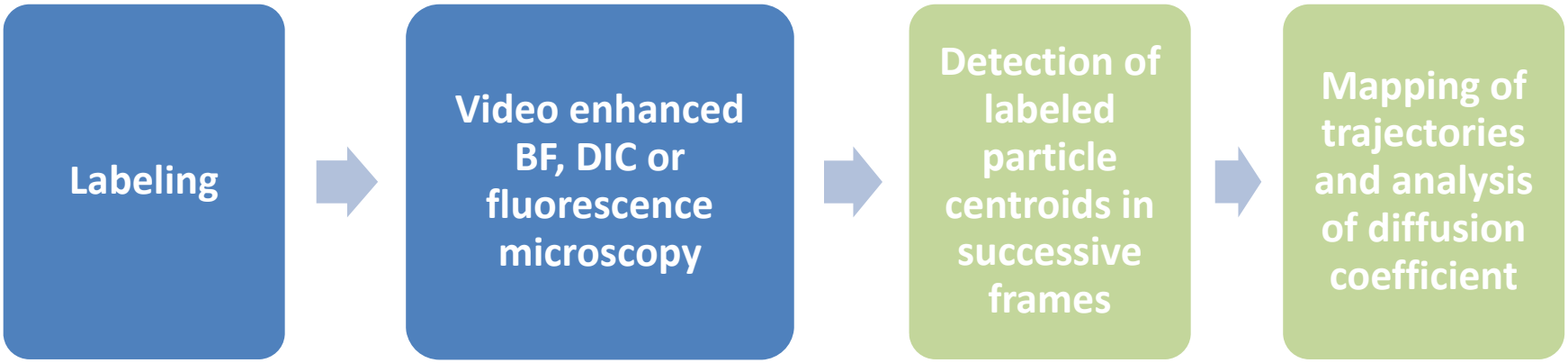
- 2. labels are often multivalent and can crosslink binding sites. Crosslinking lowers *D* through hydrodynamic effects and may trigger biological responses such as transmembrane signaling and interactions with the cytoskeleton. if diffusion is restricted by corrals, crosslinking yields aggregates less likely to cross corral walls.

- 3. perturbations caused by antibody binding can affect interactions of the labeled protein with other proteins

- Finally, during a measurement, a particle **may disappear** as a result of moving out of the focal plane, endocytosis, detachment from the membrane, or photobleaching

Correlated walks are a special case of random walks, in which the moving particle has a retention of the directional memory over a certain number of trajectory steps

Overall schematic of an SPT experiment



SPT is being used increasingly in living cells in which particle diffusion is often complex because of the presence of barriers such as

lipid rafts

intermolecular interactions

molecular crowding

heterogeneity in membrane physical properties

as combinations of obstacles such as barriers and rafts

SPT trajectory classification

- How to sort out trajectories !
- Be careful do controls for data analysis using a pure random walk as a reference. The minimum test for a classification algorithm is to try it on pure random walks of the appropriate number of time steps.
- The analytical forms of the curves of MSD versus time for the different modes of motion form the basis of various classification methods.

$$\langle r^2 \rangle = 4Dt \qquad \text{normal diffusion}$$

$$\langle r^2 \rangle = 4Dt^{\alpha} \qquad \text{anomalous diffusion}$$

$$\langle r^2 \rangle = 4Dt + (Vt)^2 \qquad \text{directed motion with diffusion}$$

$$\langle r^2 \rangle \simeq \langle r_C^2 \rangle \left[1 - A_1 \exp\left(-4A_2 Dt / \langle r_C^2 \rangle\right)\right] \qquad \text{corralled motion}$$

If a <1 the motion is classified as subdiffuison . rc is corral size and A_1 and A_2 are constants determined by corral geometry

SPT trajectory classification

- The probability density ***p*(r,t)dr** is the probability that a particle at the origin at time zero is at position **r** at time *t*. For pure diffusion in two dimensions

$$p(\mathbf{r}, t)d\mathbf{r} = \frac{1}{4\pi Dt}\exp(-r^2/4Dt)2\pi r dr,$$

- and for diffusion with simultaneous flow along the *x-axis with velocity V,*

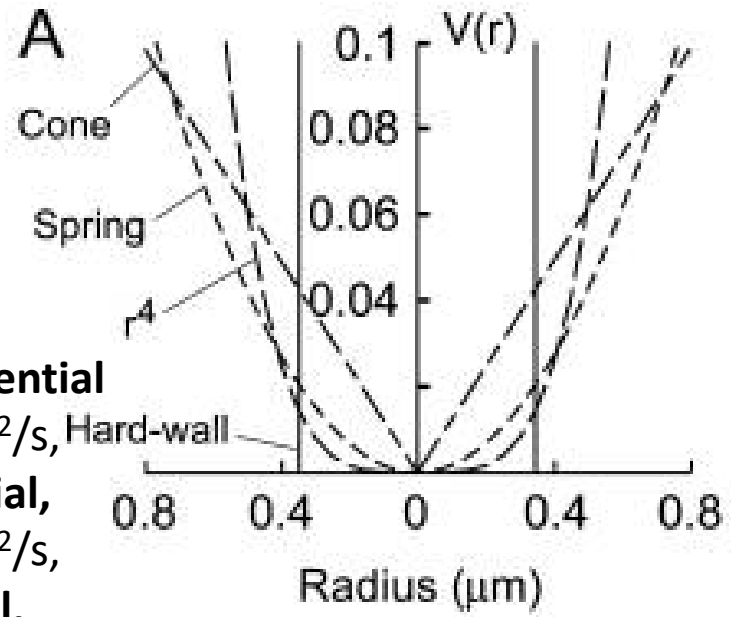
$$p(x, y, t, V)dx\ dy = \frac{1}{4\pi Dt}\exp([-(x - Vt)^2 + y^2]/4Dt)dx\ dy.$$

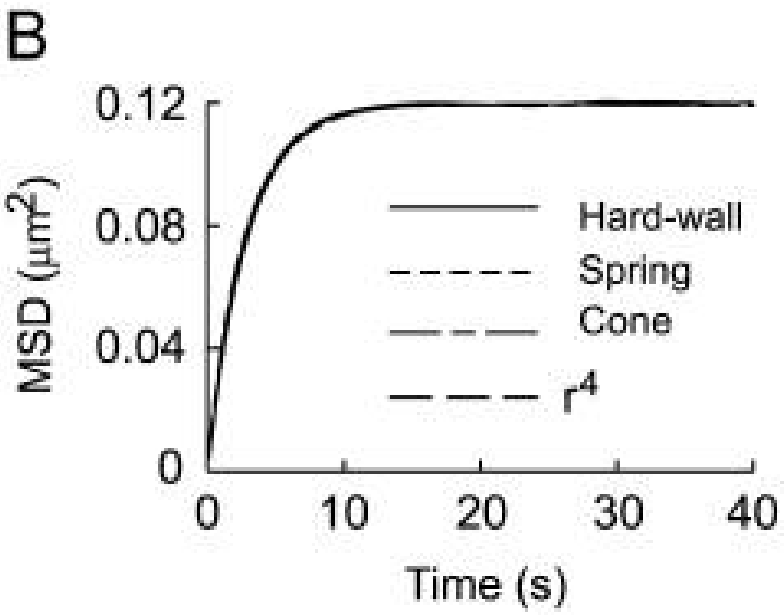
One of the most important results of SPT to date is the observation and measurement of anomalous diffusion in cell membranes. Anomalous diffusion can be used as a **probe of membrane organization**.

Anomalous diffusion implies slow diffusional mixing and therefore affects reaction rates in the membrane

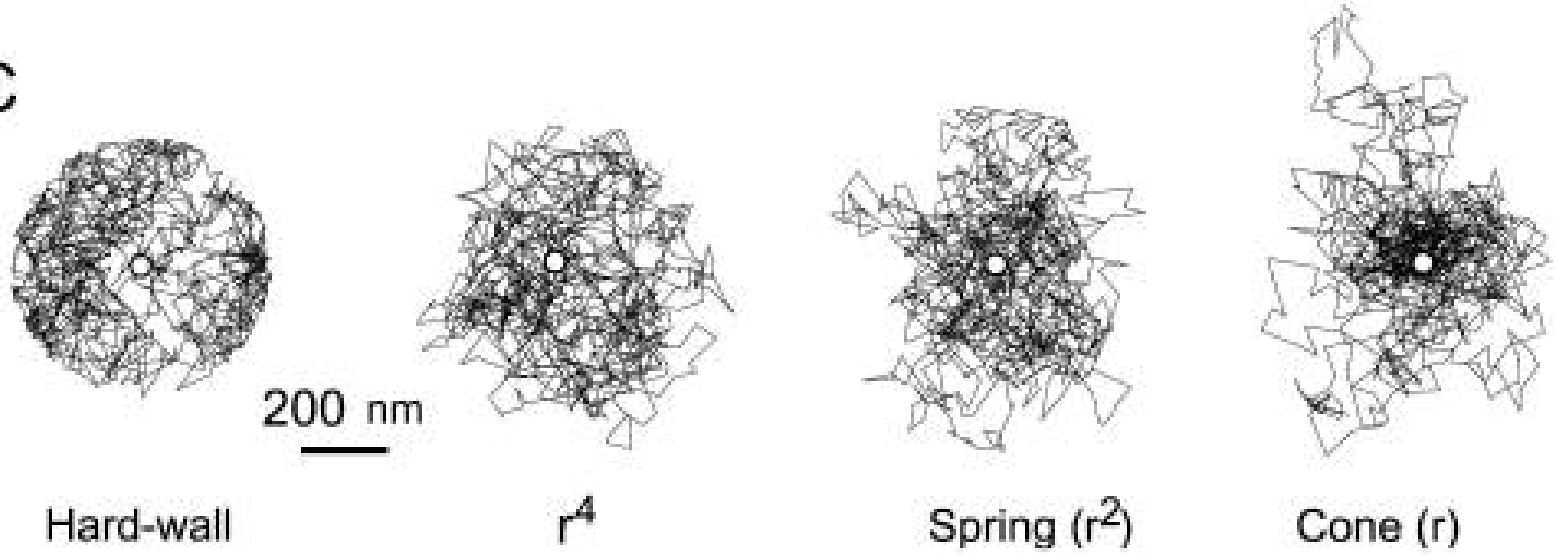
In cell membranes, anomalous diffusion is most likely the result of both obstacles to diffusion and traps with a distribution of binding energies or escape times.

Simulation of confined diffusion in a potential, $V(r)$





hard-wall potential
D = 0.012 μm^2/s,
spring potential,
D = 0.010 μm^2/s,
cone potential,
D = 0.012 μm^2/s
***r*^{4} potential,**
D = 0.011 μm^2/s



Jin S Biophys J.93(3): 1079–1088. (2007)

Simulation of confined diffusion in a potential, $V(r)$

Simulated single-particle diffusion in two dimensions in an arbitrary potential, $V(r)$

Confined diffusion has been seen for a variety of membrane proteins

hard-wall potential

spring potential

Confinement resulting from physical barriers within membranes would be describable by a "hard-wall" potential, whereas "softer" potentials would describe confinement resulting from springlike or viscoelastic-like particle tethering to relatively immobile structures such as the cytoskeleton

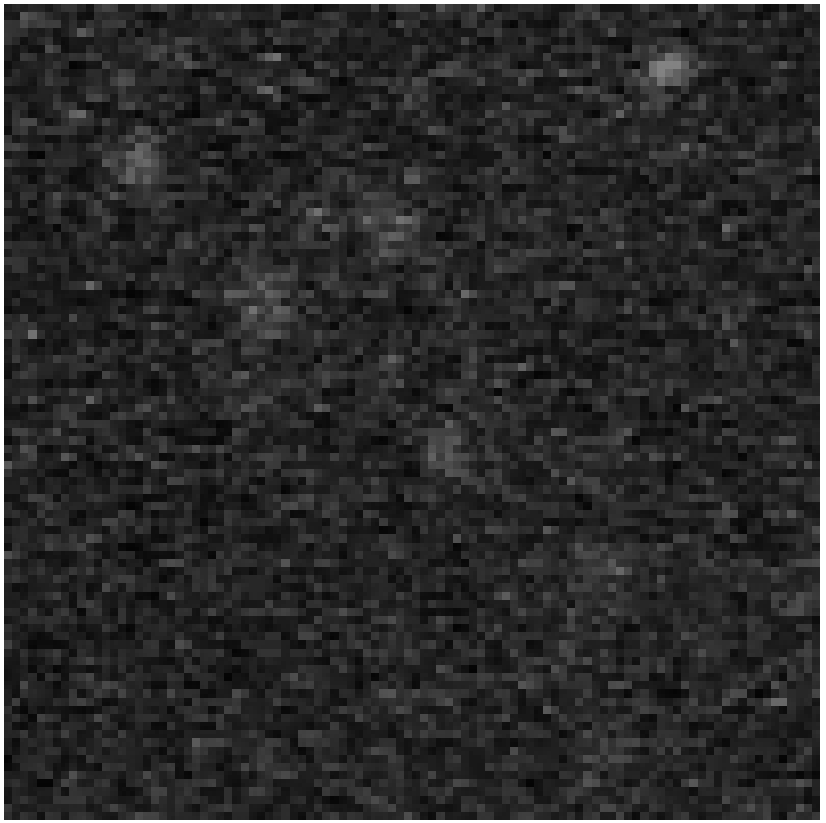
Jin S Biophys J.93(3): 1079–1088. (2007)

Example: motion of individual proteins membrane

- diffusion of cystic fibrosis transmembrane conductance regulator (CFTR) Cl**.**

This approach was validated and applied to the analysis of confined diffusion ***of CFTR Cl^- channels*** in cell membranes.

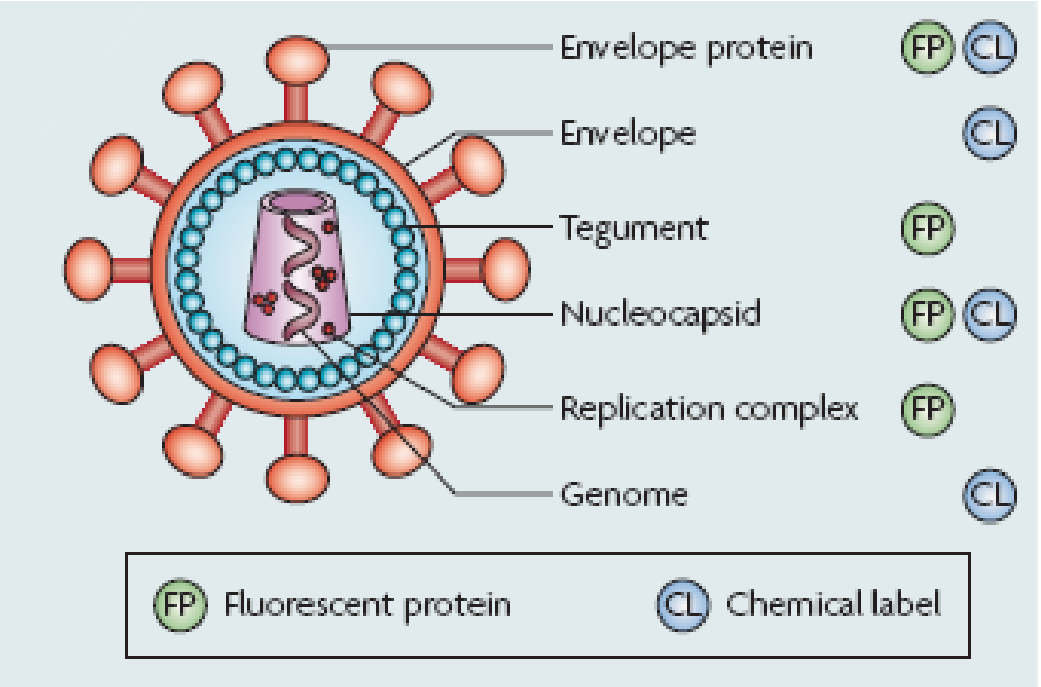
Data demonstrate the ability to distinguish barrier from tethering mechanisms using experimental SPT data, and they indicate springlike tethering of CFTR by the actin cytoskeleton.



Jin S Biophys J.93(3): 1079–1088. (2007)

Strategies for labelling various viral components

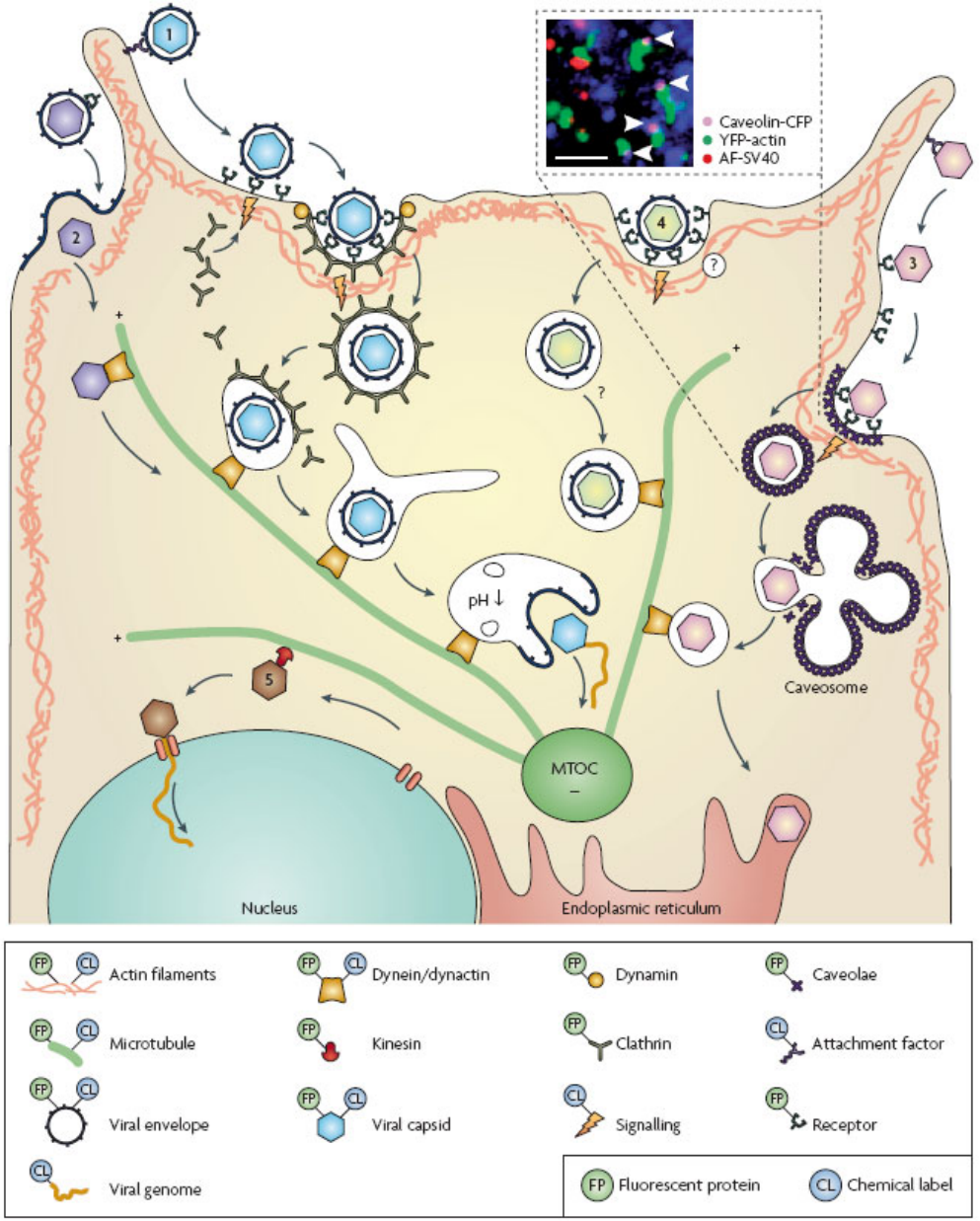
- Two general strategies exist for labelling viruses: the fusion of a target viral protein
- with a fluorescent protein (FP), or direct chemical labelling with small dye molecules.



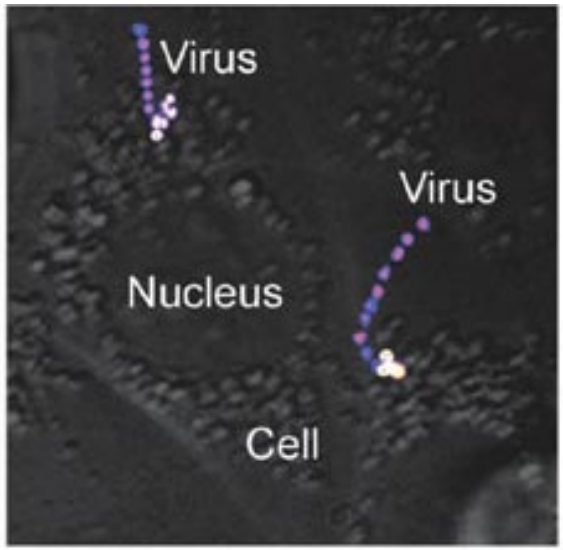
- ***FP advantages***
  - broad range of colours,
  - pH sensitive
  - photo-switchable
- ***FP disadvantages***
  - Labelling occurs during
  - virus replication in host cells
  - Requires several FP proteins
- ***CL advantages***
  - labelling can be at different time-points during the viral life cycle
  - covalently attached or non-covalently associated with the target protein
- ***CL disadvantages***
  - Cant use antibodies block the function of viral proteins after binding.

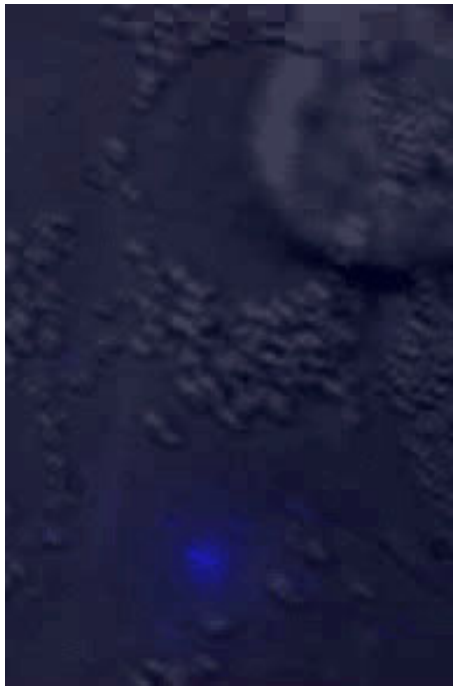
Viral entry and transport

- Viruses attach to the plasma membrane, surf on the cell surface or along the filopodia (and bind to specific receptors before entering the cell.
- (1) clathrin-dependent caveolin-dependent
- (2) directly fuse with the plasma membrane
- (3) caveolin-dependent
- (4) clathrin- and caveolin-independent
- (5) viruses can be transported by dynein or dynactin along microtubules. From the MTOC, capsids can be transported by kinesin towards the replication
- site of the nucleus

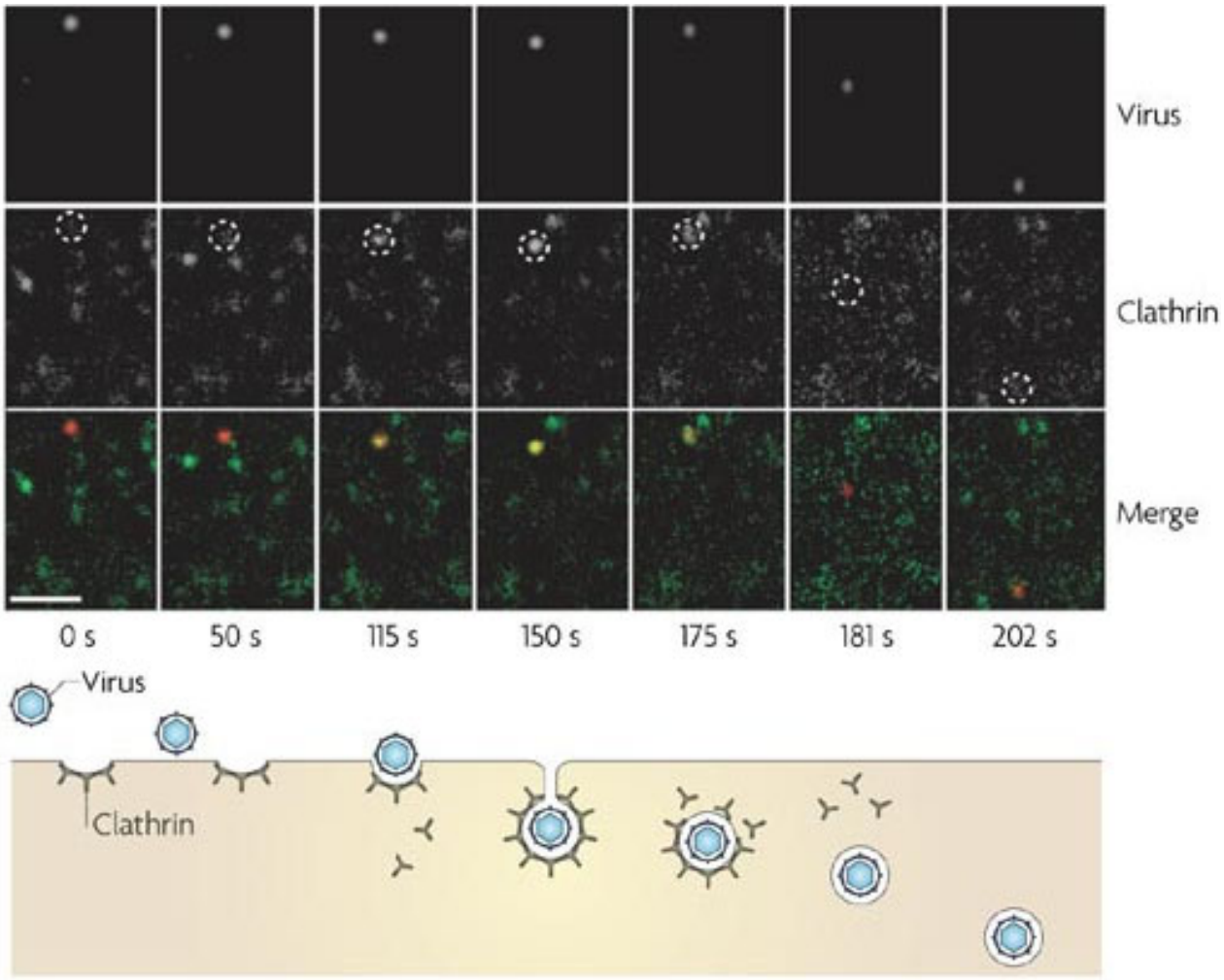


Time-lapse images of influenza viruses in live cells



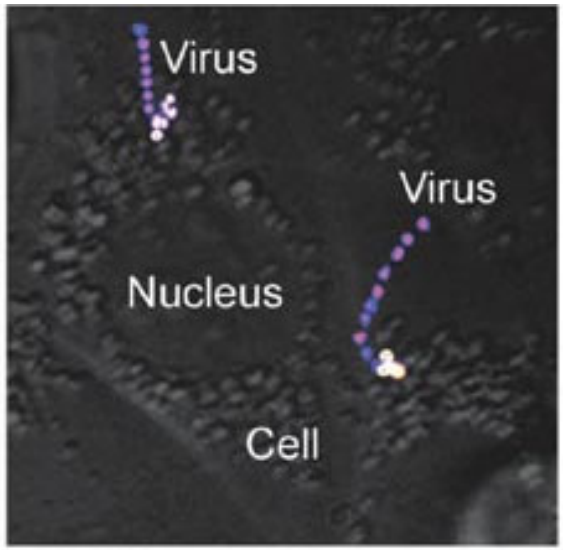


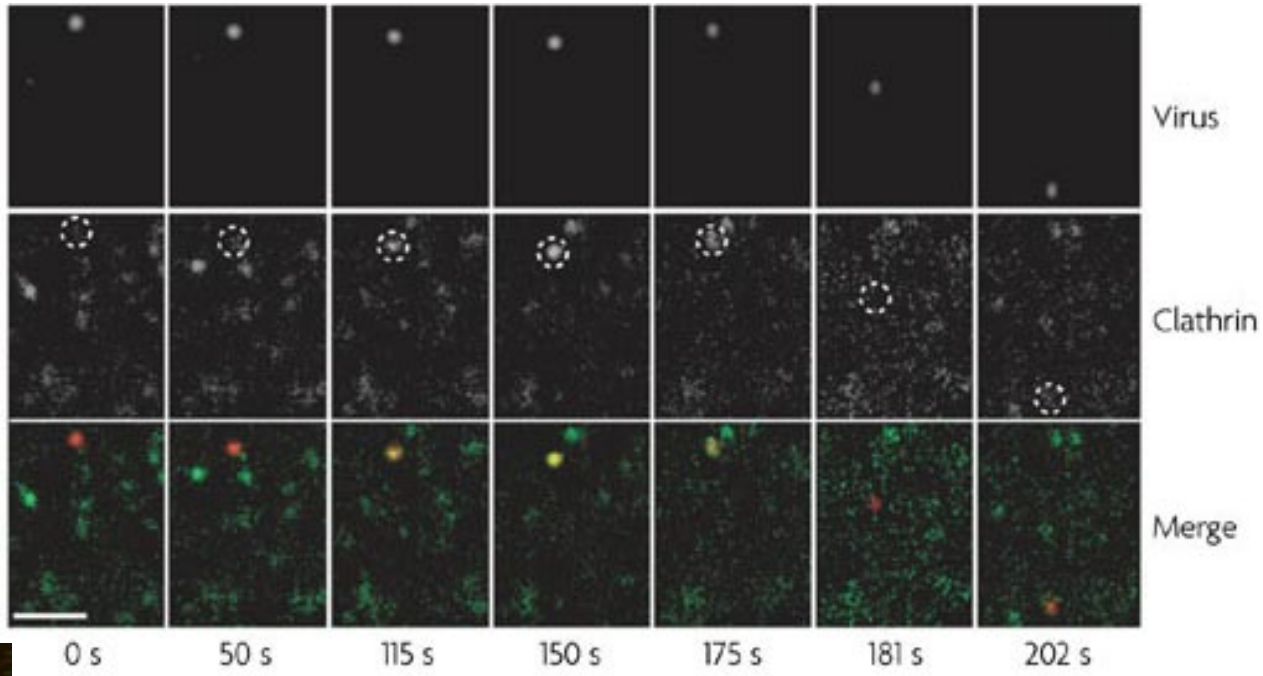
Lakadamyali, M PNAS ***100, 9280–9285***(2003)

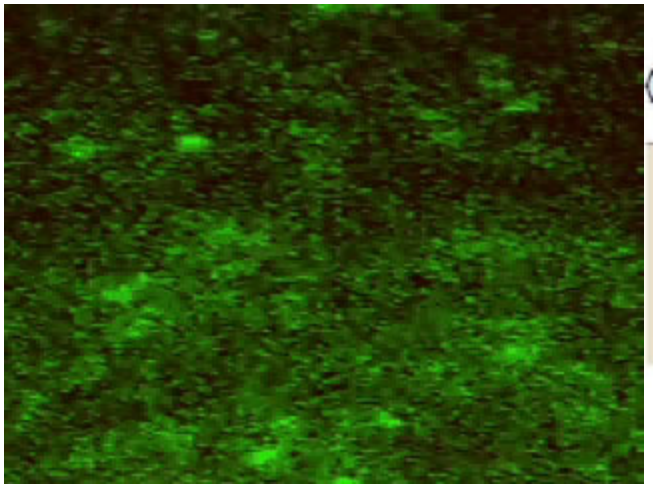


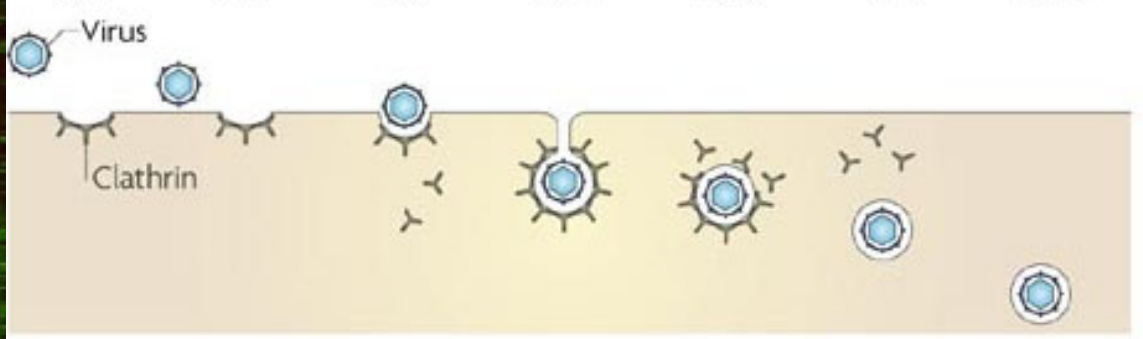
Simultaneous images of a DiD-labelled virus and fluorescent protein-labelled clathrin (middle panels and green in lower panels) in a cell show the internalization of the virus by a clathrin-coated vesicle.

Time-lapse images of influenza viruses in live cells







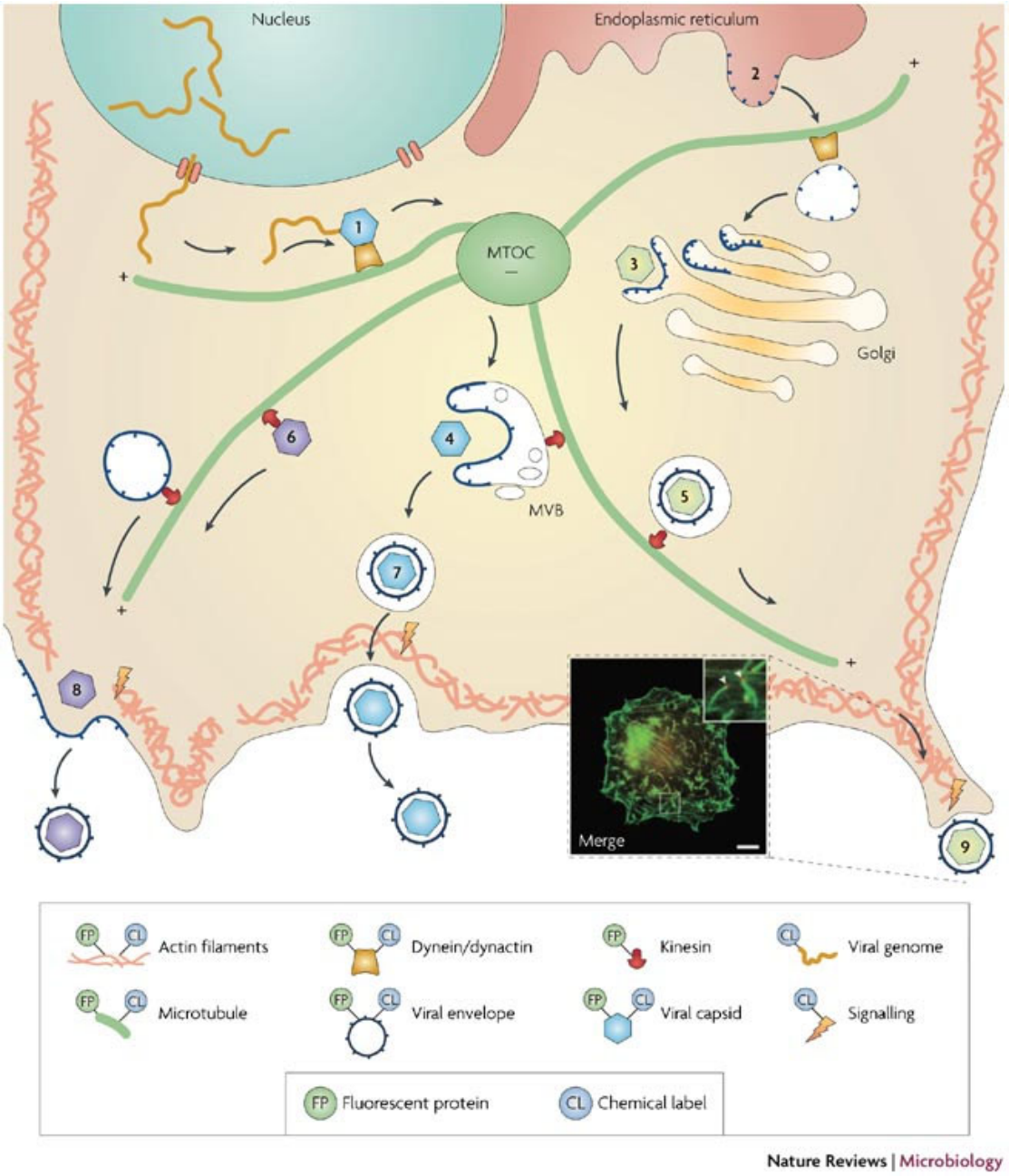


The virus is labelled with the lipophilic dye, DiD

Lakadamyali, M PNAS ***100, 9280–9285*** 2003

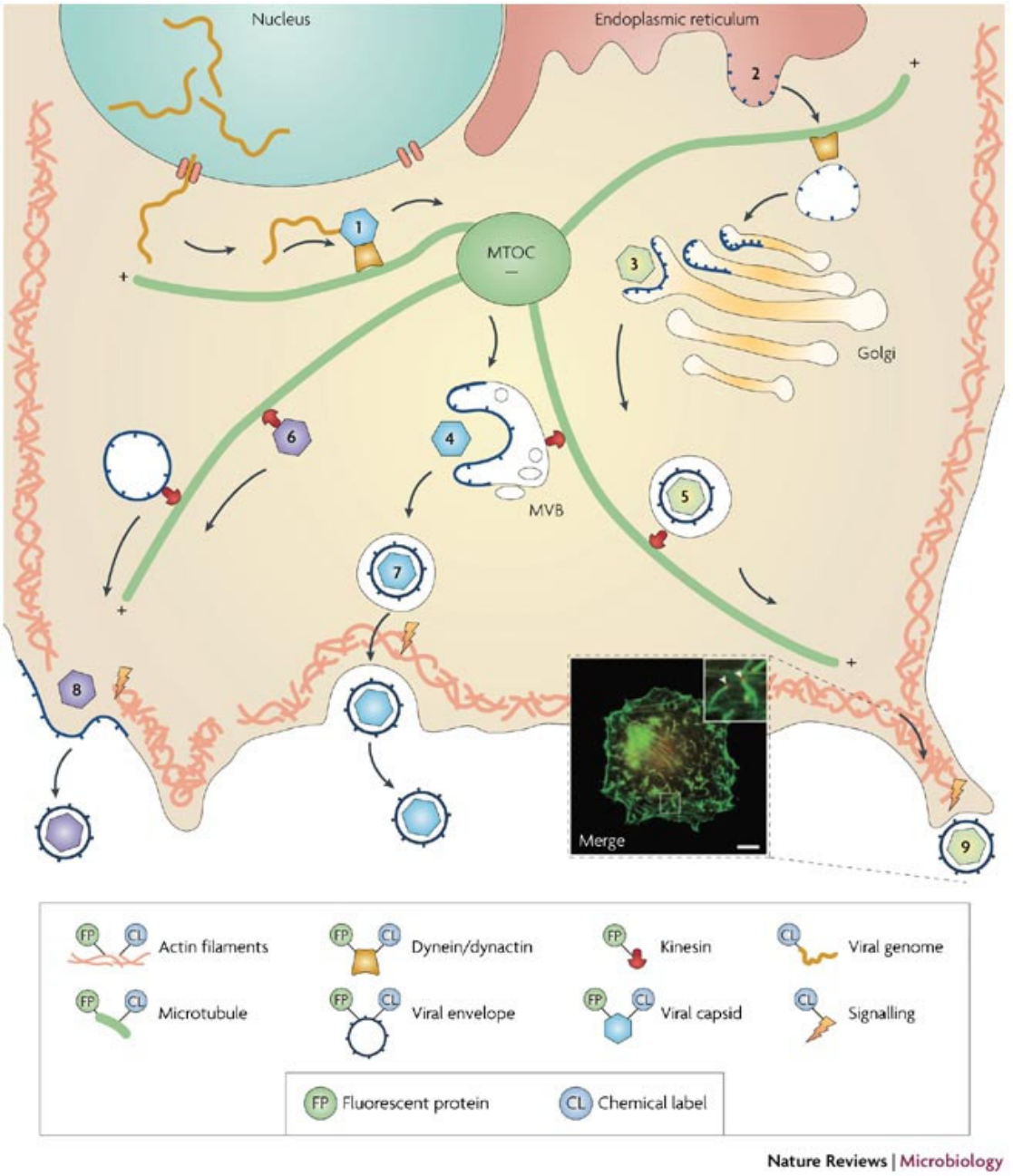
Viral assembly and exit

- (1) Viral membrane proteins are translated at the endoplasmic reticulum membrane
- (2) and transported along microtubules to the Golgi apparatus, where capsids can bud into an envelope
- (3). Viruses also bud into the multivesicular bodies (MVB)
- (4). Complete virions inside transport vesicles
- (5), or subviral particles
- (6), are transported by kinesin on microtubules towards the plasma membrane, and exit the cell by exo

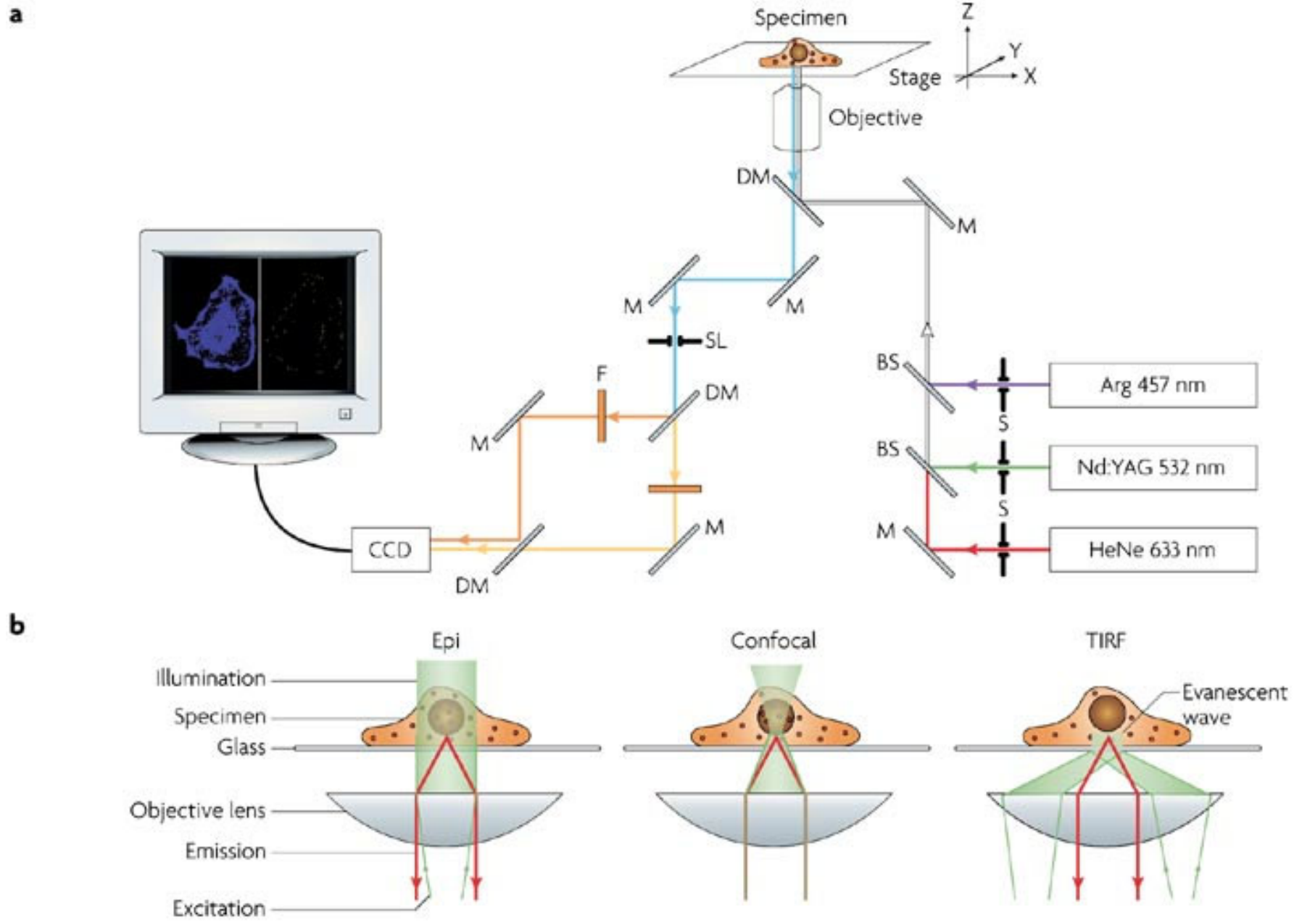


Viral assembly and entry

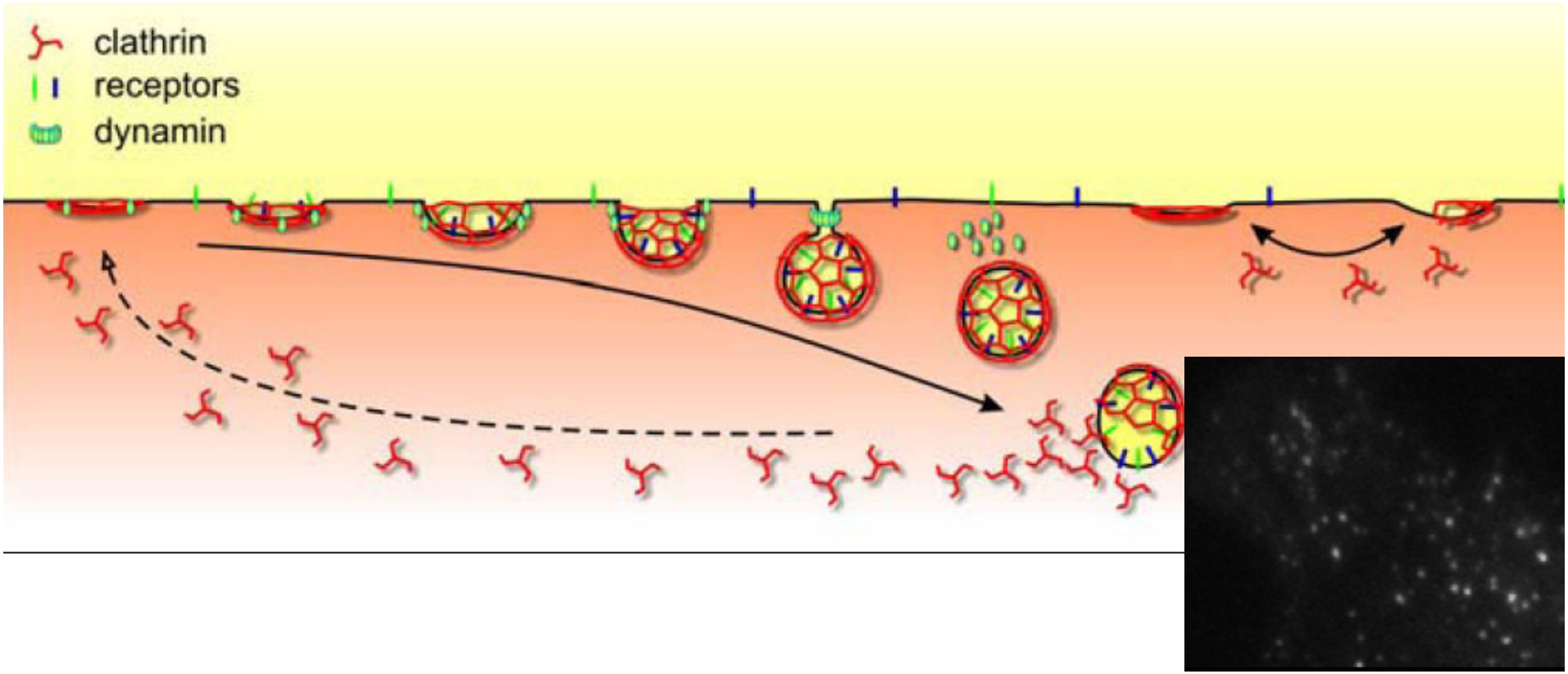
- (7) or budding
- (8) at the plasma membrane. During egress, the actin cortex might propel viruses towards neighbouring cells through a dynamic actin tail (9)



Single-virus tracking setup



Kinetics of clathrin-mediated endocytosis



Clathrin is a trimer composed of three heavy chains and three light chains, each monomer projecting outwards like a leg; this three-legged structure is known

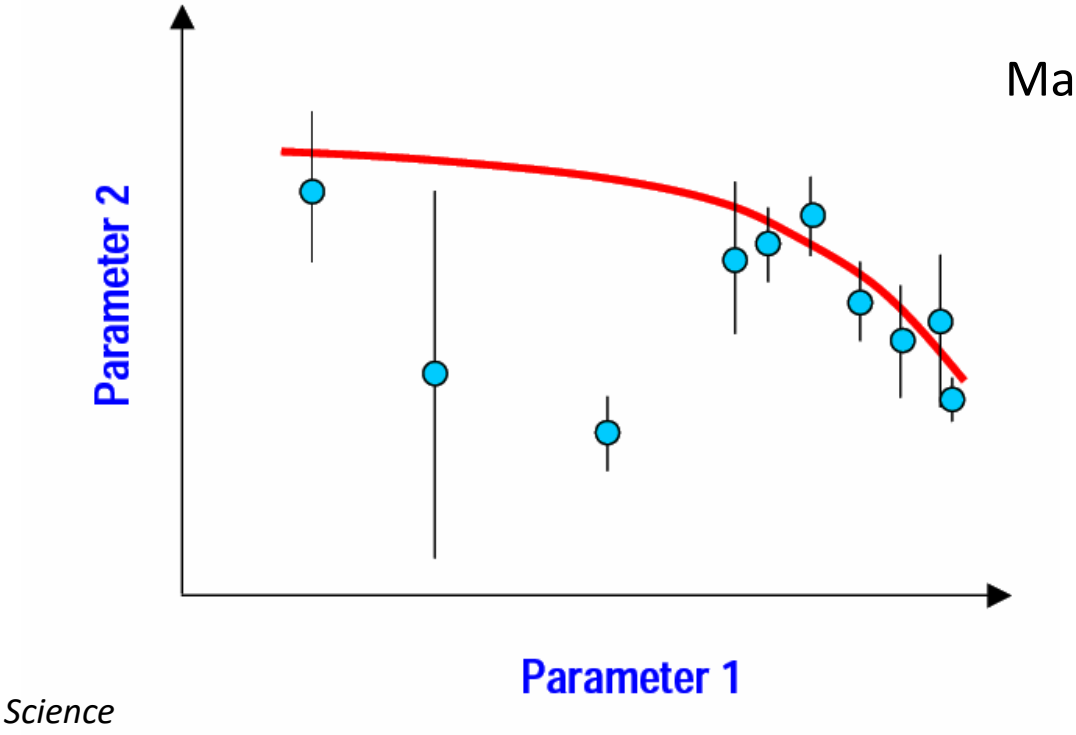
Particle Tracking -

- *The squared distance between all particles in consecutive frames was minimized, constrained to a maximal speed of 8.3 nm/ms (10 pixels/frame). Tracking is not possible, if the average particle distance is smaller than the displacement of individual particles from one frame to the next. Therefore excessively dense parts of the image series were excluded from tracking analysis.*

Stefan Hell, Science 2008

- *You can fit any model to your data if you add sufficient parameters*

Many anonymous



Anonymous data from *Science*

Objectives of the good tracking algorithm

- analysis of ***low labeling density*** samples that does not allow probing of the interactions between particles . The amount of data collected per experiment is low, limiting the observation of spatially and temporally heterogeneous particle behavior and hindering the capture of infrequent event

- Even with low particle density, low signal-to-noise ratio (SNR) and probe flicker complicate the search for particle correspondence.

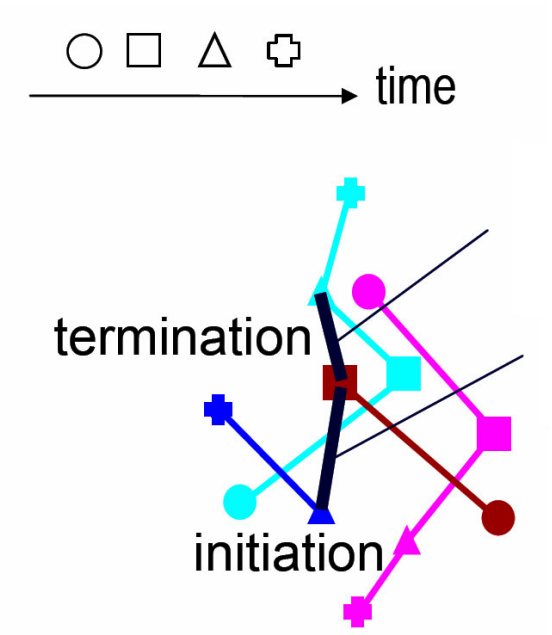
- Obtain a complete and accurate representation of the movement of *all particles from appearance to disappearance independent of their heterogeneous images, dynamics, and neighborhood relations.*

- No a priori selection of particle behaviors
- Measure as much as possible simultaneously
- **DO NOT KILL DISCOVERY UPFRONT !**

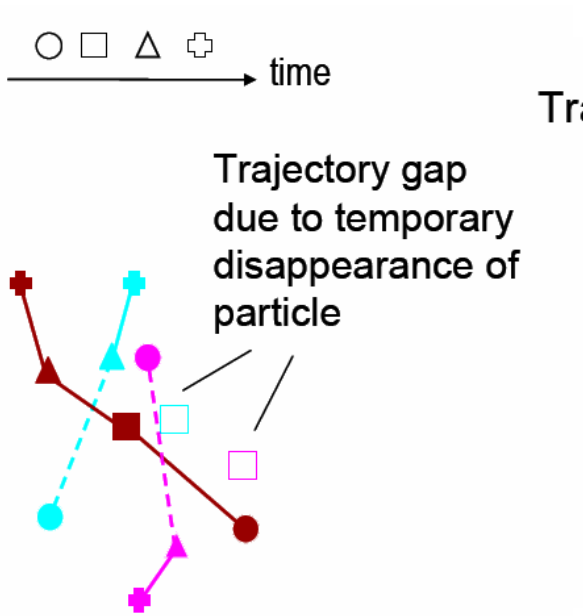
Additional issues: Track termination and initiation, gaps and merges

- How to avoid false continuation on another existing track?
- How to avoid false continuation on an initiating track in the next frame?

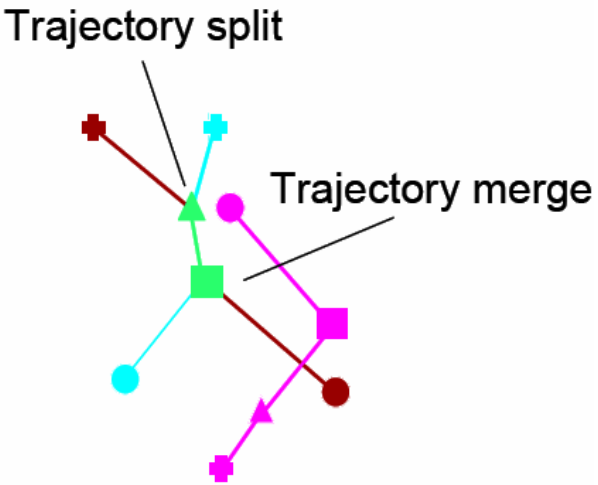
In general terms: Initiation and termination of tracks violate the constrain that every source has a target and every target has a source.



GAPS



MERGE

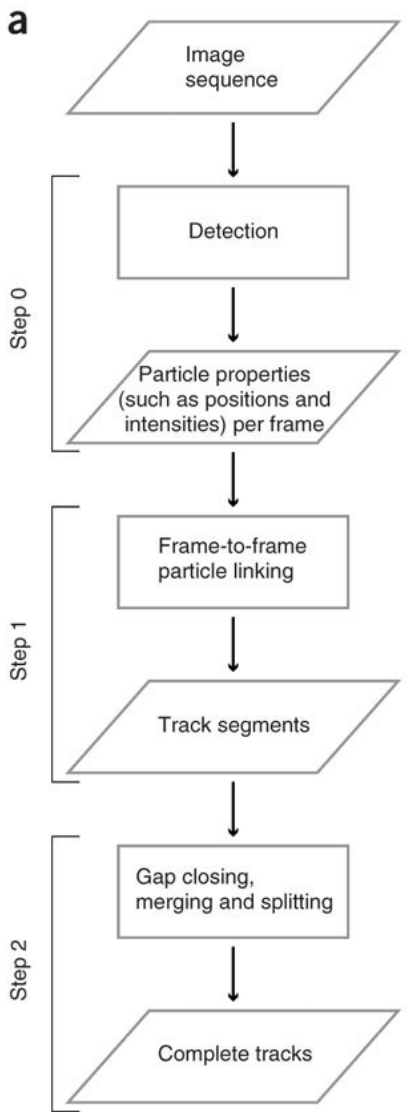


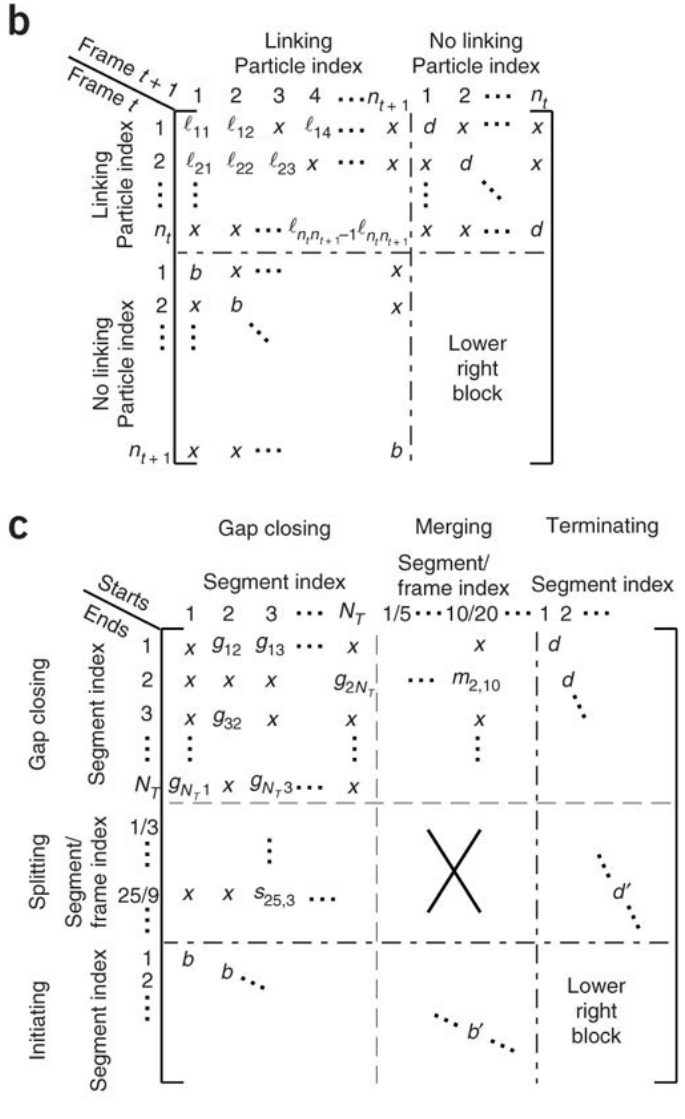
Tracking particles via spatially and temporally global assignments

Find the combination of links which connects every source to a target, and every target to a source, so that the overall ***cost of the links is minimal.***

- linear assignment problem (LAP)
- Cost matrix controlling particle assignments between frames
- Cost matrix controlling gap closing, merging and splitting
- Globally optimal in space *and in time* Computationally tractable approximation t o MHT

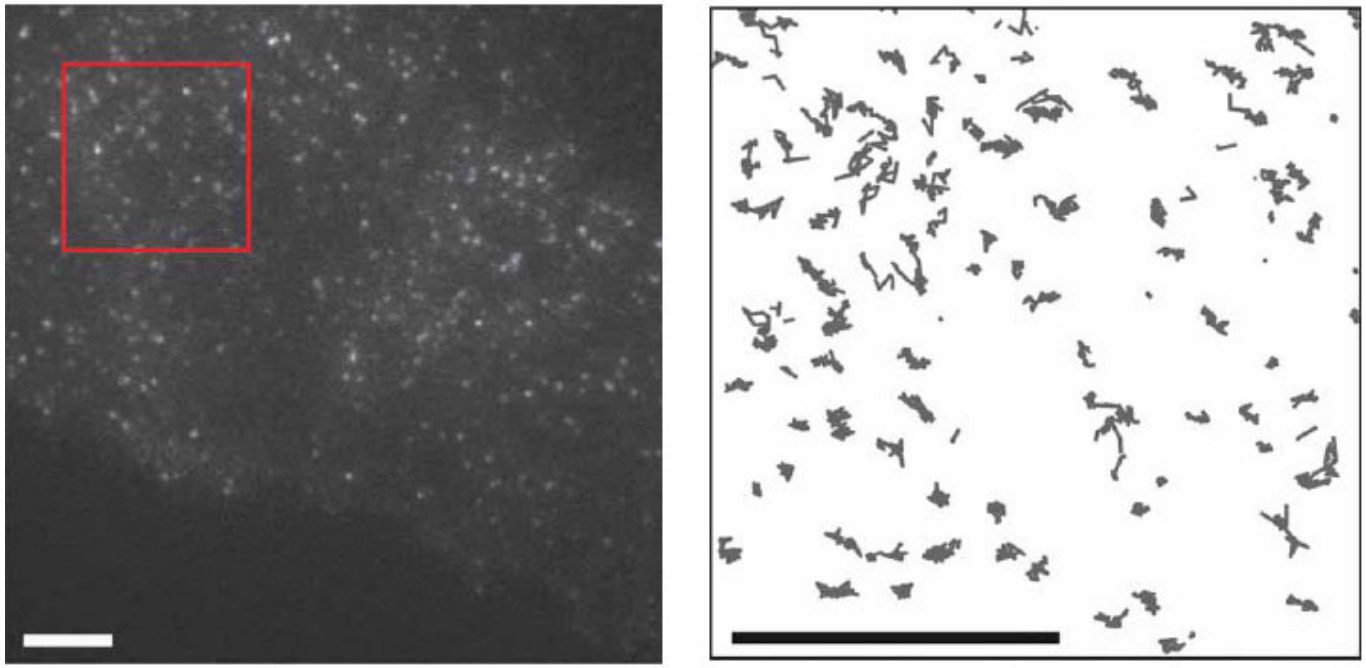
Jaqaman et. al. *Nature Methods,* ***(2008)***





Dynamics of EGFP-clathrin light chain

- Clathrin is a trimer composed of three heavy chains and three light chains, each monomer projecting outwards like a leg; this three-legged structure is known

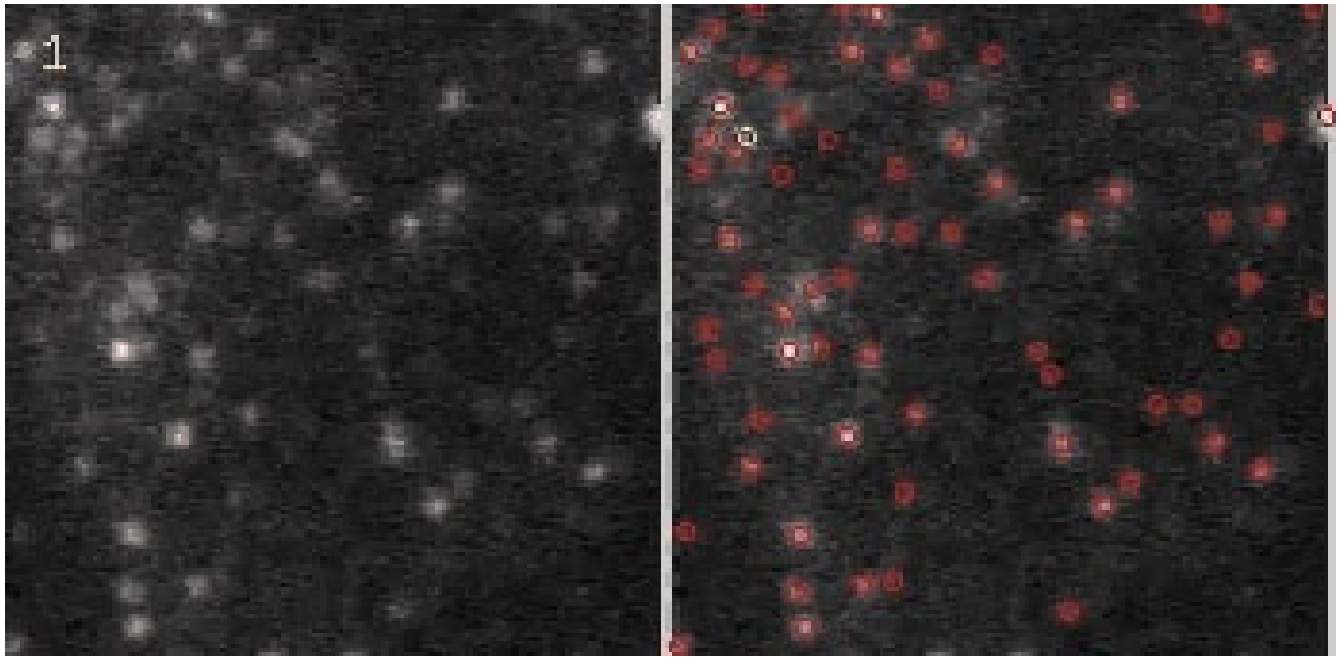


TIRF microscopy image ofa BSC1 cell fluorescently labeled with clathrin light chain–EGFP. Scale bar 5 μm.

CCP trajectories in the 10 10 mm area indicated by a red box

Jaqaman et. al. *Nature Methods,* ***(2008)***

Dynamics of EGFP-clathrin light chain



Ongoing track

Track initiation

Track termination

* Gaps closed

Jaqaman et. al. *Nature Methods,* ***(2008)***