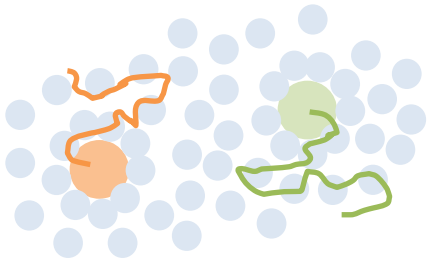
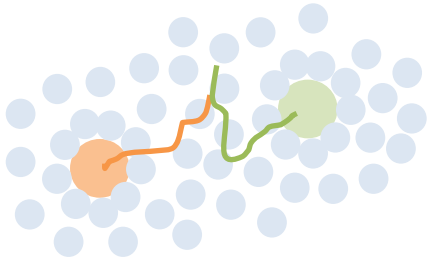
Reactions in solution

Steps for a reaction to occur:

- molecular diffusion
- results in random collisions
- every molecule is surrounded by a solvation shell
- solvation shell is shared → encounter complex, repeated collisions
- **reaction?** not every encounter complex is productive → depends on the reaction
- dissociation of the complex



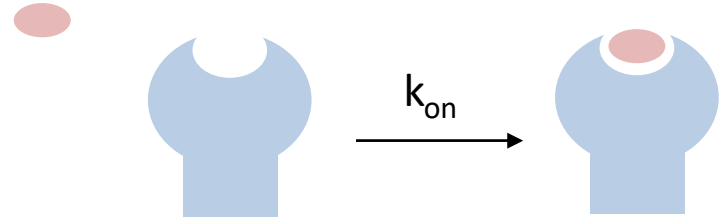
10^{-10} – 10^{-8} s for small molecules



Bimolecular kinetics – irreversible

$$A + B \xrightarrow{k_{on}} AB$$

k_{on} ($M^{-1}s^{-1}$) association rate constant



differential equation

$$v = -\frac{d[A]}{dt} = k[A][B]$$

integrated:

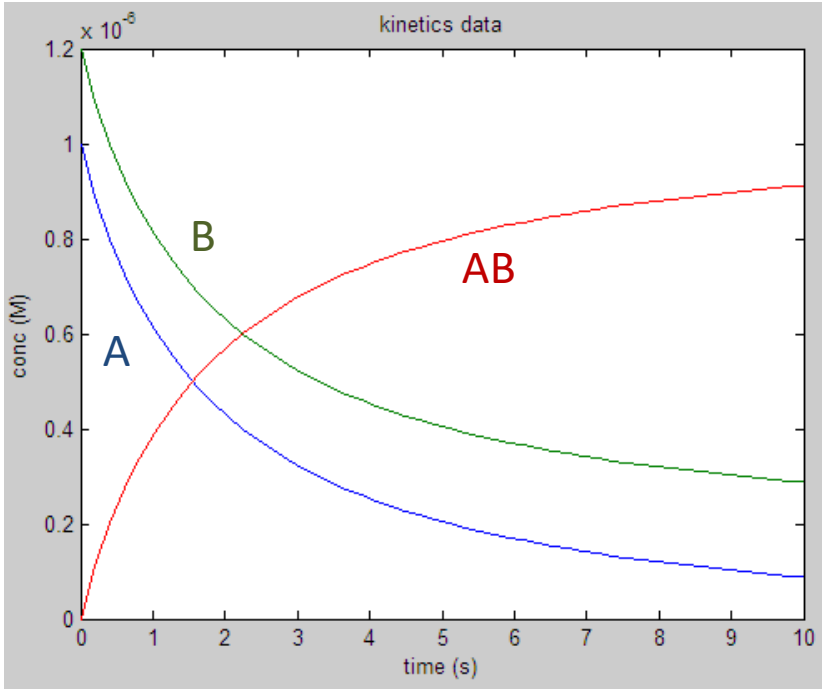
$$\frac{1}{[B]_0 - [A]_0} \ln \frac{[B][A]_0}{[A][B]_0} = kt$$

simulated for

$[A_0] = 1 \times 10^{-6}$ M

$[B_0] = 1.2 \times 10^{-6}$ M

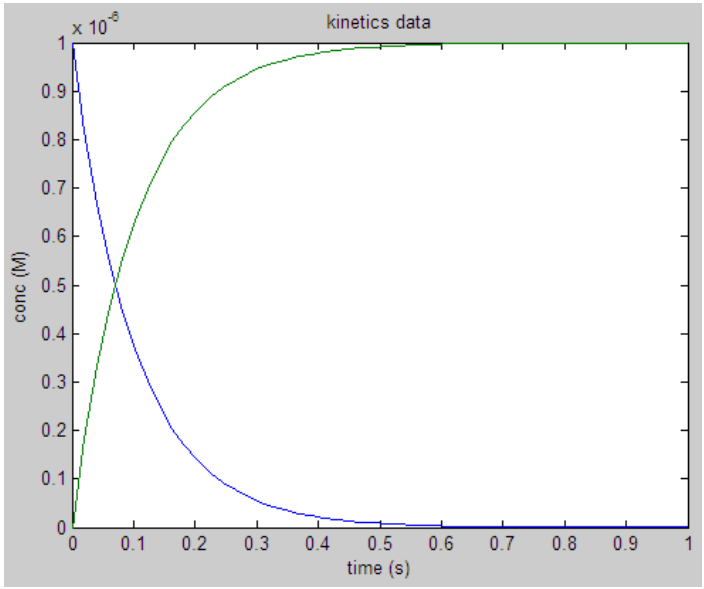
$k_{on} = 5 \times 10^{5}$ $M^{-1}s^{-1}$



Reaction under pseudo-first order

$$A + B \xrightarrow{k_{on}} AB$$

one partner in large excess, its concentration does not change throughout the reaction, e.g. [B] ~ $[B]_0$



pseudo 1st order

$$-\frac{d[A]}{dt} = k[B]_0[\mathrm{A}] = k_{app}[\mathrm{A}]$$

integrated:

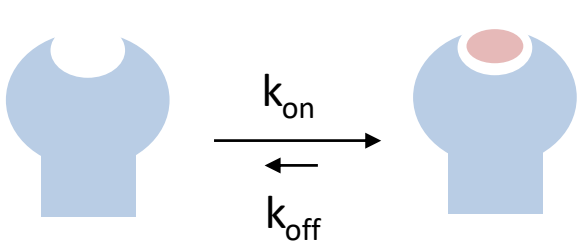
$$[A](\mathrm{t}) = A_0 e^{-k_{app}t}$$

$$[AB](\mathrm{t}) = A_0\left(1 - e^{-k_{app}t}\right)$$

Bimolecular kinetics – reversible

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

k_{on} ($M^{-1}s^{-1}$) association r. c.
k_{off} (s^{-1}) dissociation r. c.



$$K_D = \frac{[A][B]}{[AB]} = \frac{k_{off}}{k_{on}}$$

unit: (M)
equilibrium is dynamic!

differential equation

$$v = -\frac{d[A]}{dt} = k_{on}[A][B] - k_{off}[AB]$$

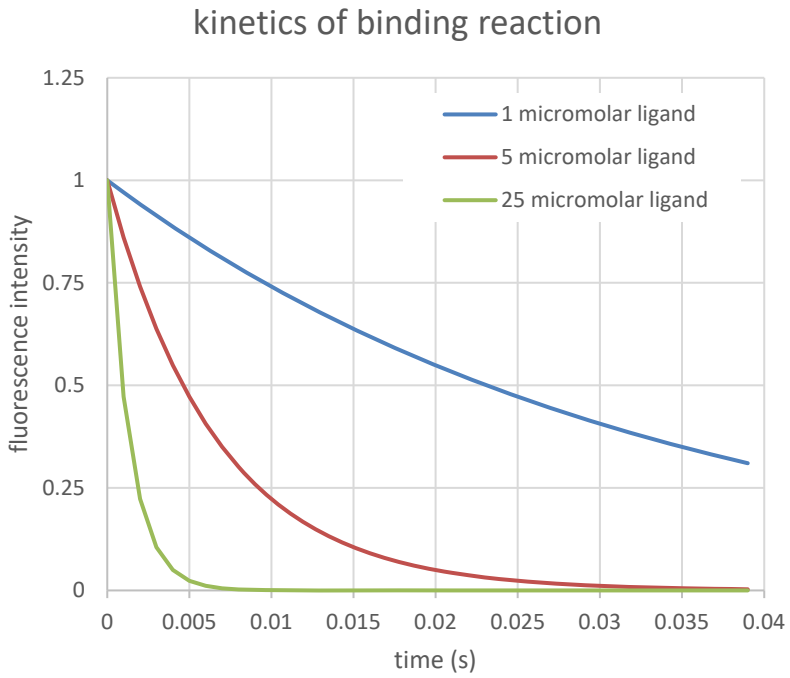
pseudo first order conditions

$$v = -\frac{d[A]}{dt} = k_{on}[A][B]_0 - k_{off}[AB] \quad = k_{on,app}[A] - k_{off}[AB]$$

$$[A](t) = A_0 e^{-(k_{on,app} + k_{off})t}$$

$$[AB](t) = A_0 (1 - e^{-(k_{on,app} + k_{off})t})$$

Quiz:



You measure the association reaction of a receptor (at 1 nM concentration) with a ligand, using fluorescence quenching.

Based from the observed kinetic curves for the association reaction with different ligand concentrations (to the right), **calculate the bimolecular association rate constant**.

On and off rates, meaning and importance

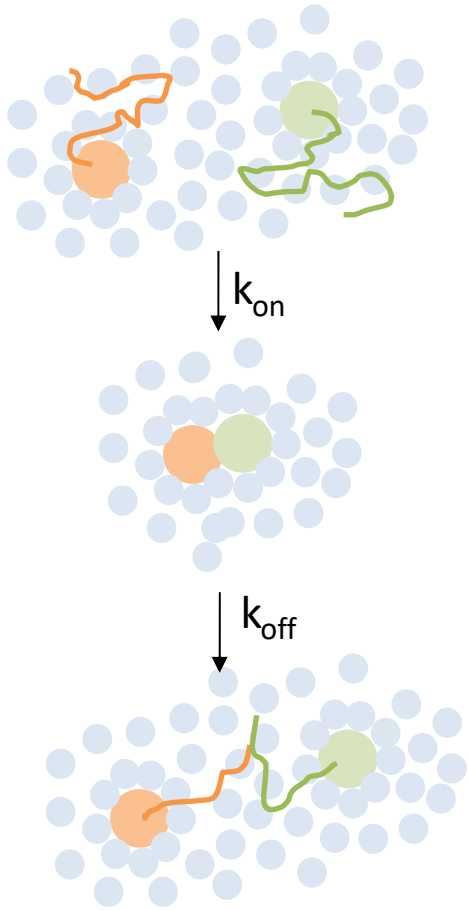
***k_{on}*: on-rate**

$1/k_{on}$: time it takes for a protein to bind its target / ligand

***k_{off}*: off-rate**

τ_R : residence time $\tau_R = 1 / k_{off}$

the time the complex remains together until dissociation



Energetics of bimolecular binding

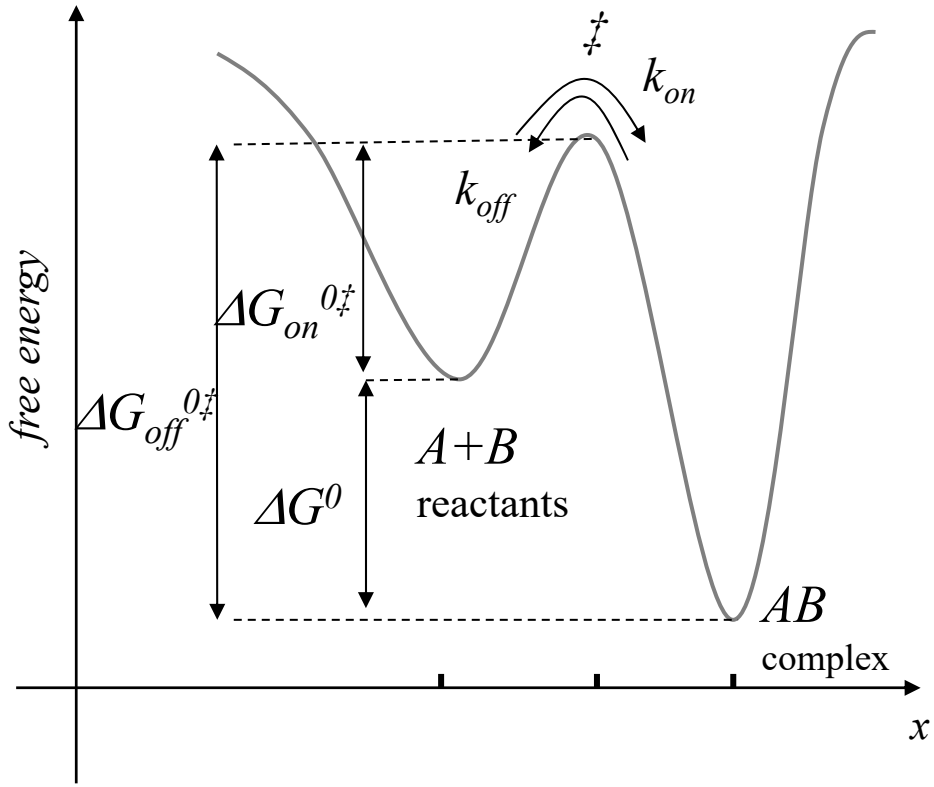
dynamic equilibrium

$$K_D = \frac{[A][B]}{[AB]} = \frac{k_{off}}{k_{on}}$$

relation of the rate constants to free energies:

$$K_D = \frac{k_{off}}{k_{on}} = \frac{A_0 e^{-\Delta G^{\ddagger}_{off}/RT}}{A_0 e^{-\Delta G^{\ddagger}_{on}/RT}}$$

$$= e^{-\left(\Delta G^{\ddagger}_{off} - \Delta G^{\ddagger}_{on}\right)/RT} = e^{-\Delta G/RT}$$



The maximal on-rate: Speed limit of reactions

most biological binding reactions involve an encounter complex

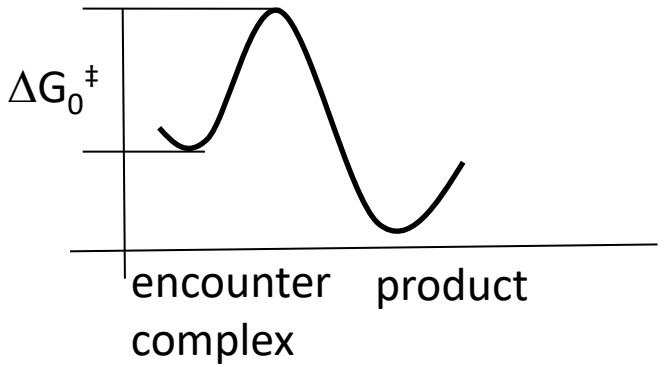
$$A + B \underset{k_d}{\overset{k_o}{\rightleftarrows}} [A...B] \xrightarrow{k_2} AB$$

The frequency of collision is given by diffusion

- if every collision results in the product

  → **diffusion control, speed limit**

- if barrier involved, reaction is slower



barrier:

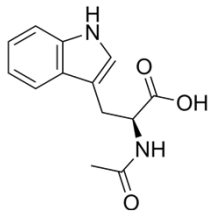
desolvation

entropy (small binding sites)

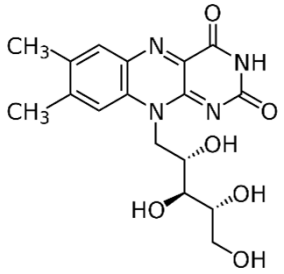
chemical reaction: energy barrier

Quenching reactions do often not exhibit a barrier

○ N-acetyl-tryptophanamide



Δ riboflavine



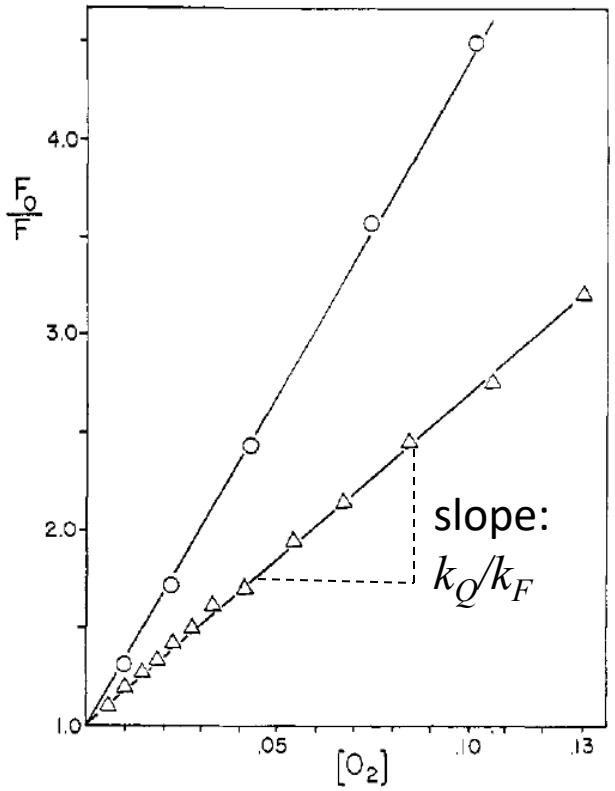


FIGURE 4: Oxygen quenching of *N*-acetyl-L-tryptophanamide (○) and riboflavine (Δ) in 0.1 M sodium phosphate, pH 7.0.

Stern – Vollmer equation

$$\frac{I_0}{I_F} = 1 + \frac{k_Q}{k_F}[Q]$$

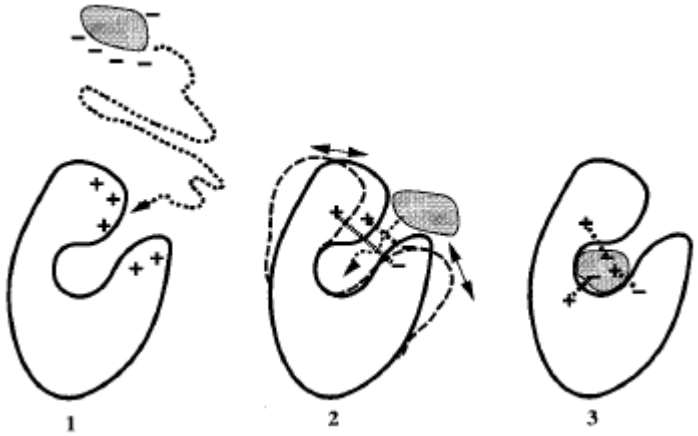
From a plot of $1/I_F$ vs. $[Q]$, the value of k_Q/k_F can be determined.

k_Q is often close to the rate constant of a diffusion controlled reaction with $k_Q = 10^{10}\,\mathrm{M^{-1}s^{-1}}$

→ diffusion control (BB)

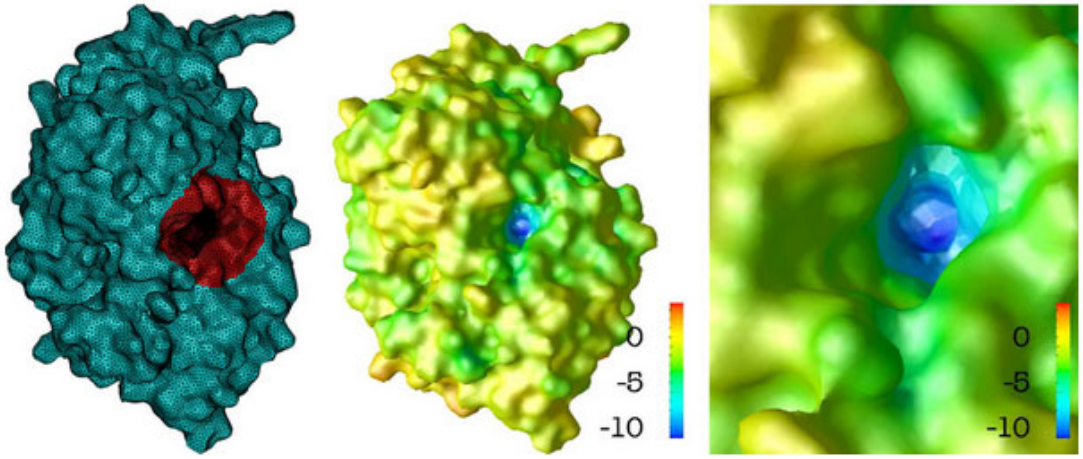
J.R. Lakowicz, G. Weber, Biochemistry 12, 1973

Considerations in proteins



electrostatic guidance
orientation of the
enzyme, substrate

→ can be very fast
→ sensitive to salt



acetylcholinesterase

http://www.math.colostate.edu/~yzhou/research/research.html

Transcription factors are highly specifc DNA binding proteins

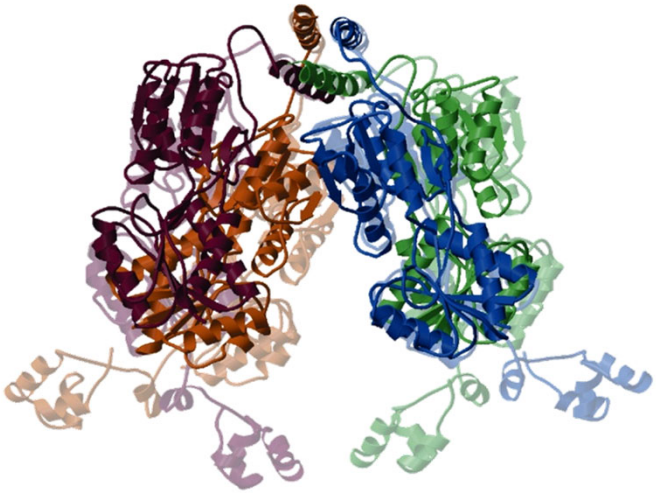


Figure 28-7d
Lehninger Principles of Biochemistry, Fifth Edition
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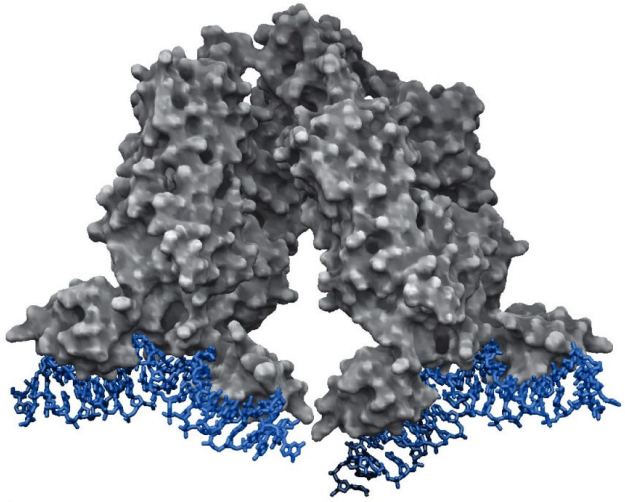

Figure 28-7c
Lehninger Principles of Biochemistry, Fifth Edition
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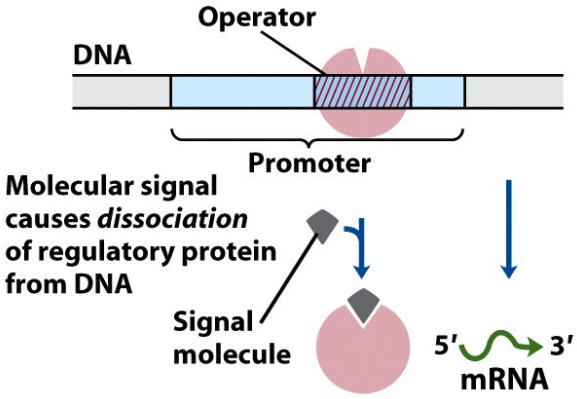
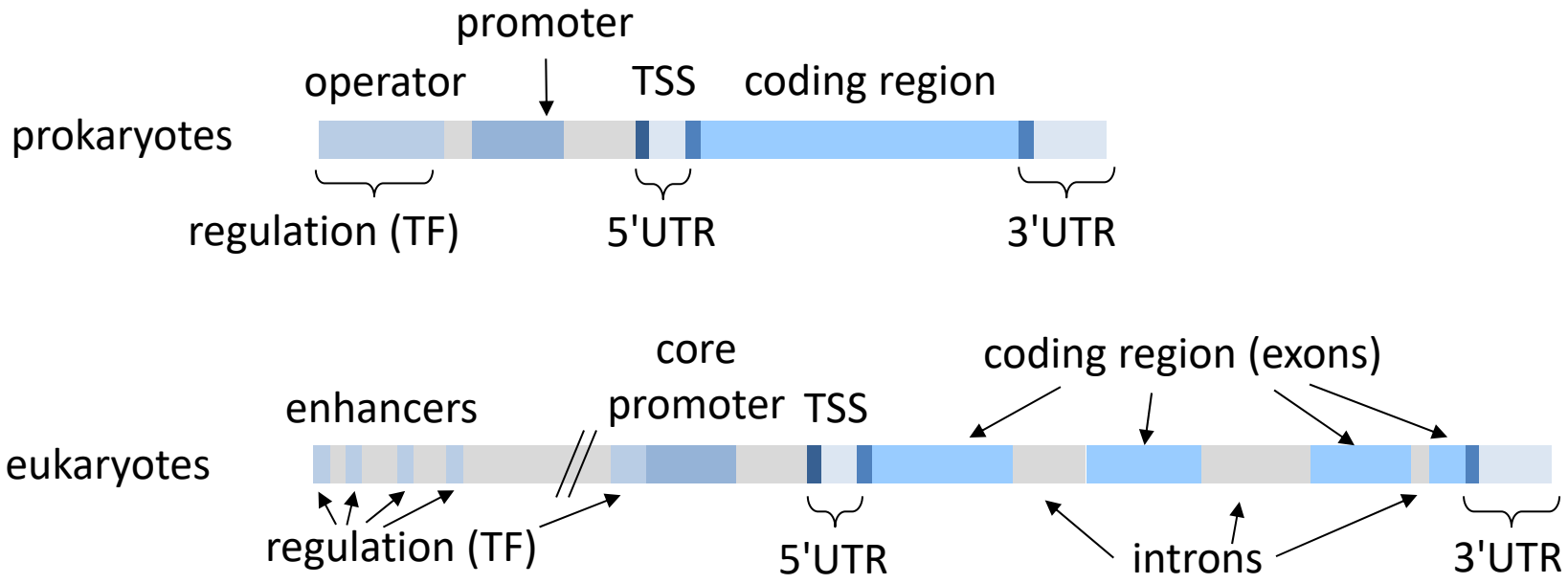
Example: Lac repressor

- binds a specific site in the Lac operon
- represses genes required for digestion of lactose
- in the presence of lactose, it dissociates and genes are expressed



Architecture of a gene



RNA polymerase binds at the **promoter**

Gene expression is regulated at the **operator** and **enhancer regions**

Gene regulation by transcription factors - Repression

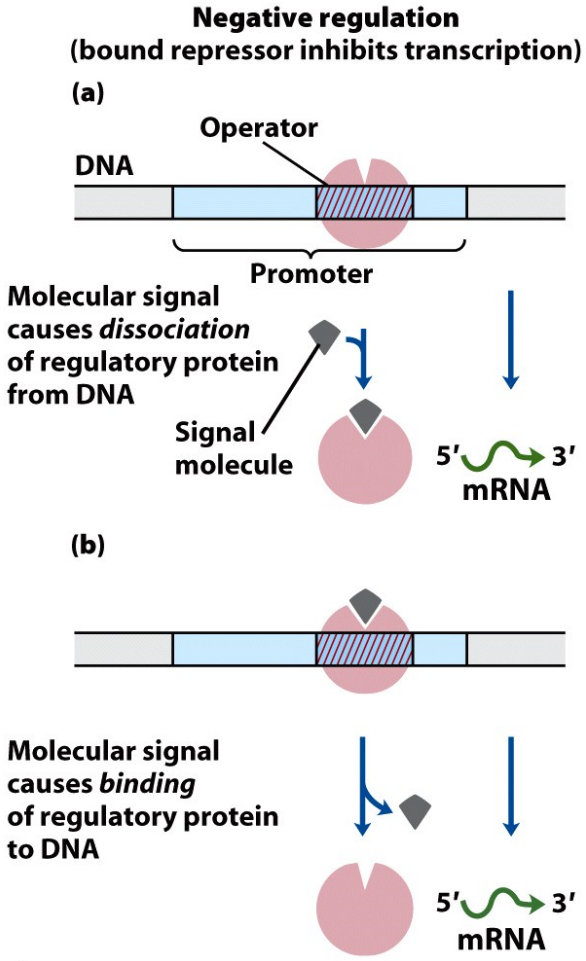
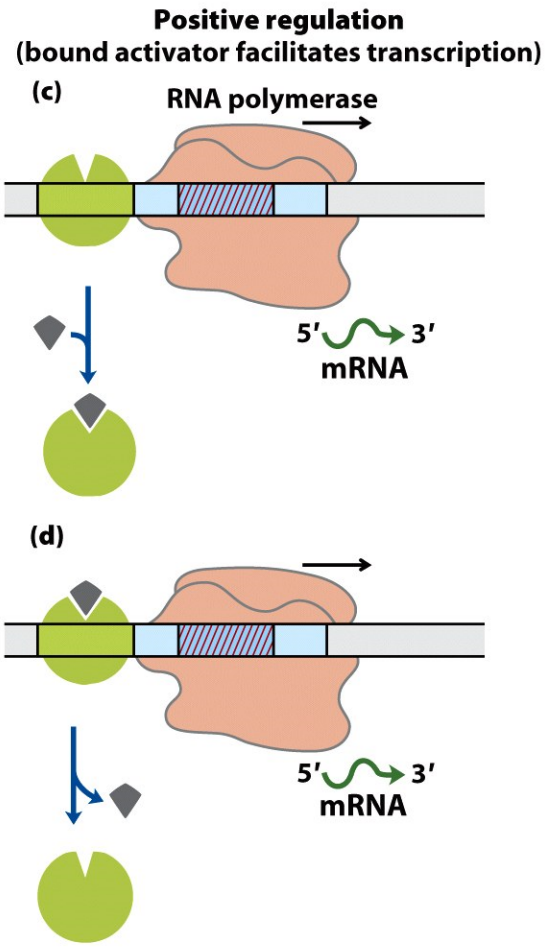


Figure 28-4
Lehninger Principles of Biochemistry, Fifth Edition

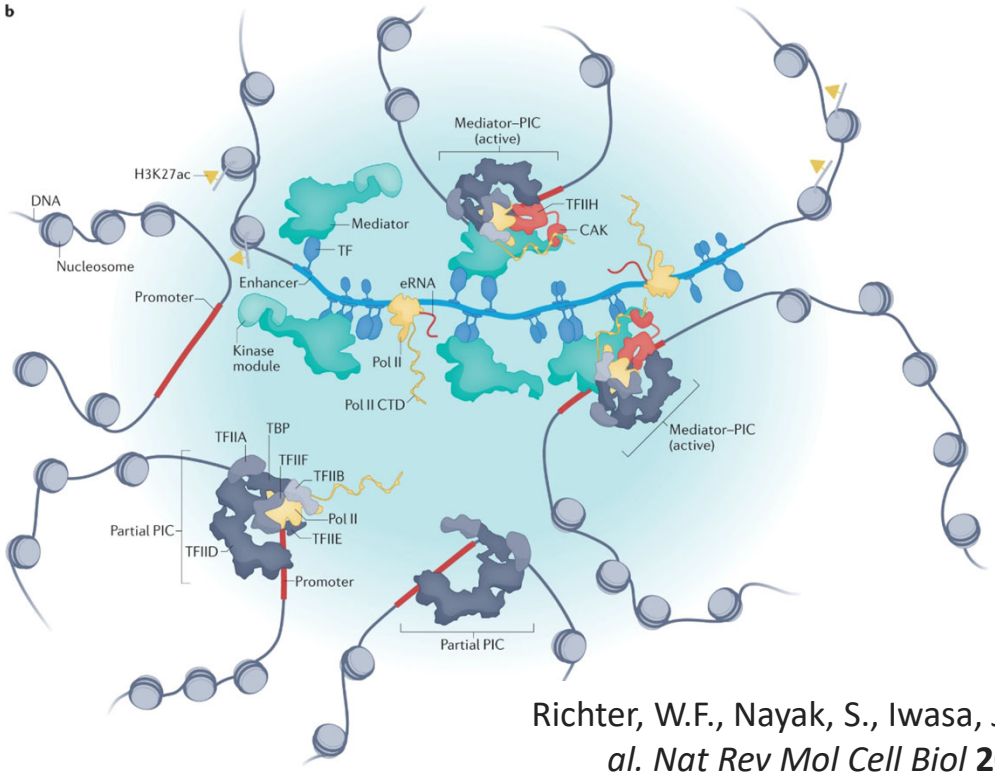
- Repressor binds to the operator
- prevents RNA Pol binding / transcription initiation
- external signal → dissociation
- transcription initiation

- Repressor binds in the presence of the signal
- Repressor dissociates and transcription ensues when the signal is removed

Gene regulation by transcription factors - Activation



- Activator binds to the operator controlled by signal
- Recruits RNA Pol, and/or stabilizes its binding
- → Direct or indirect interactions between transcription factor and transcription machinery
- Initiation of transcription



Richter, W.F., Nayak, S., Iwasa, J. *et al. Nat Rev Mol Cell Biol* **2022**

Example: Binding of transcription factors to DNA

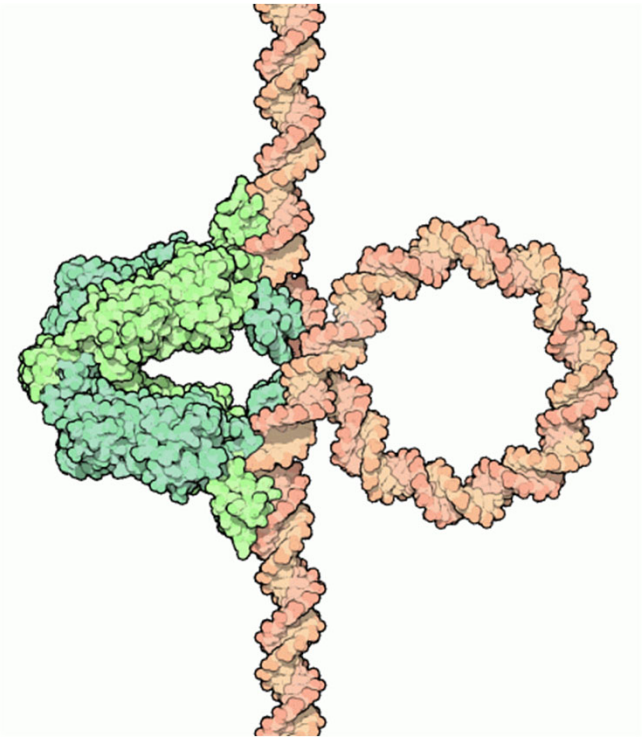
In 1970, Riggs et al. measured the association rate of LacI repressor and its operator on DNA

$$[R]+[DNA_{site}] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [DNA_{site}:R]$$

$$\frac{d[DNA_{site}:R]}{dt} = k_{on}[R][DNA_{site}] - k_{off}[DNA_{site}:R]$$

Estimated k_{on} using the Smoluchovski equation, with $D \sim 10^{-7}$ cm^{2}s^{-1}

$$k_{DS} = 4\pi D_{3D} b \sim 10^8 \, \mathrm{M}^{-1} \, \mathrm{s}^{-1}$$

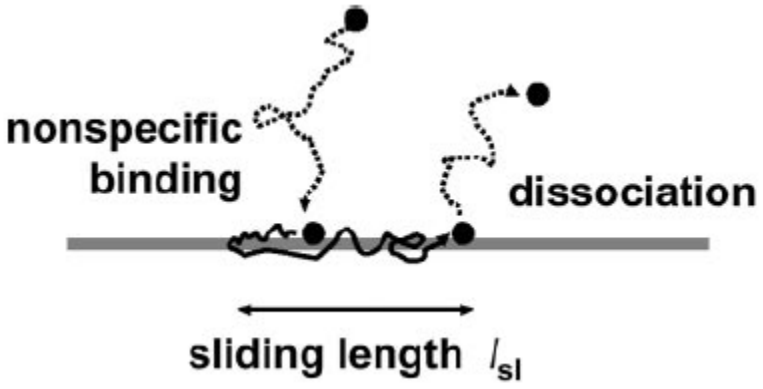


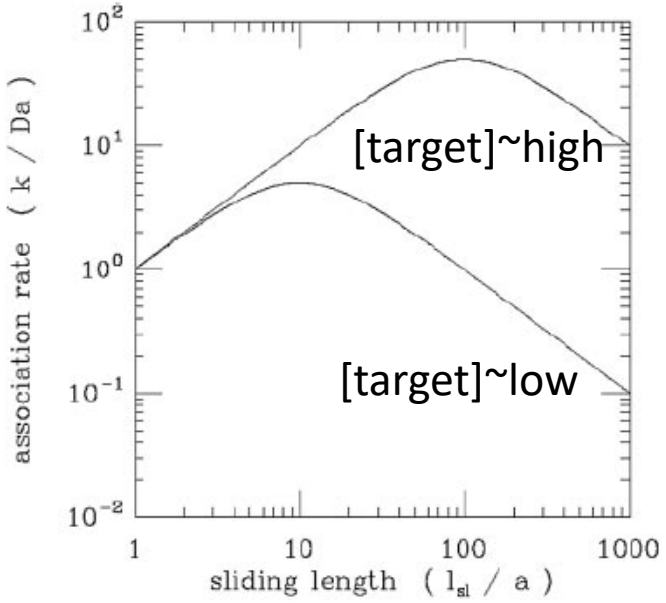
measured value: $k_{on} \sim 10^{10}$ s^{-1} !

1970, Riggs et al.

This is 1-2 orders of magnitude faster than the theoretically allowed maximal value!

DNA binding kinetics: Sliding model





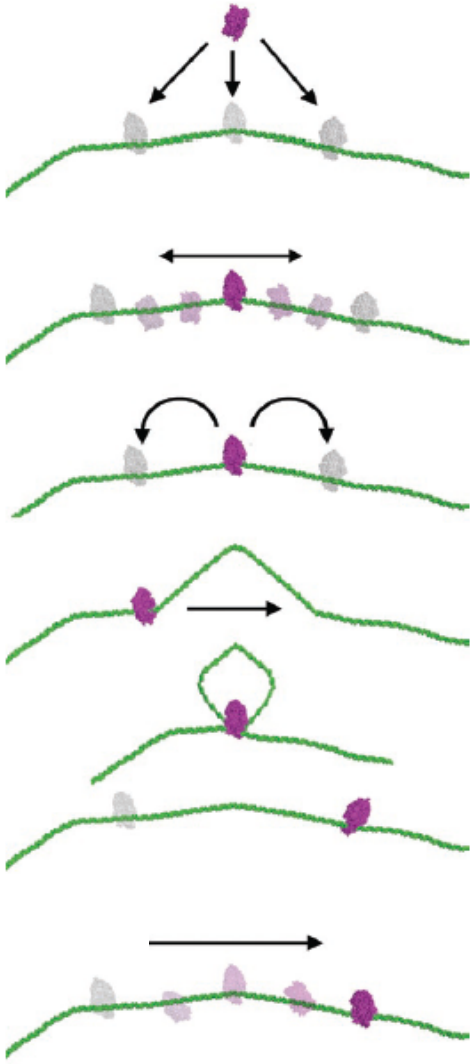
- nonspecific sequences increase binding rate (longer DNA → faster binding)
- two nonpalidromic restriction enzyme sites are more efficiently cleaved when separated by less than 50 bp → sliding length
- barriers on the order of kT

→ rotation and 1D/3D diffusion combination.

$$\tau_{fac} = \frac{1}{4\pi D_{3D} l_{sl} [\text{site}]} + \frac{L\, l_{sl}}{D_{1D}}$$

Halford & Marko
NAR 2004

DNA transcription factor binding



Random collision

Sliding

Hopping

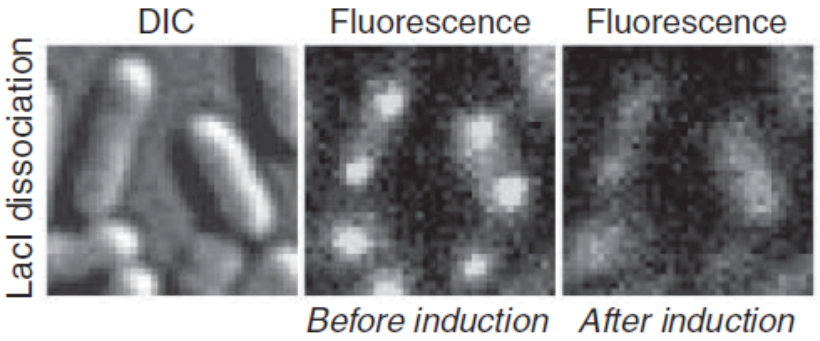
Intersegmental transfer

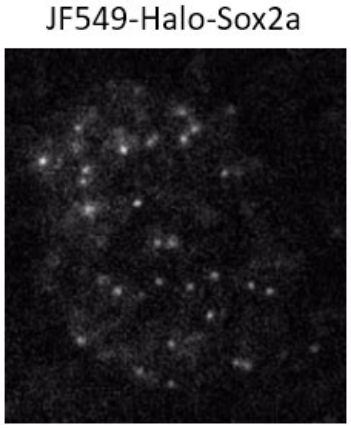
limited by DNA
persistence length

Active translocation

Gorman & Greene,
NSMB 2008

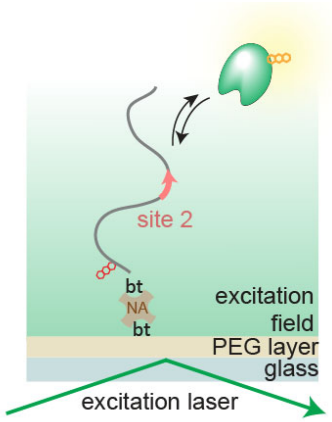
Observing 4-Protein and DNA interactions

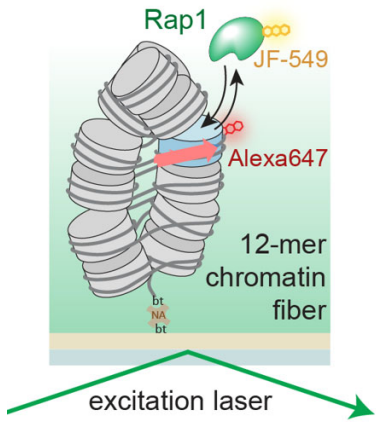




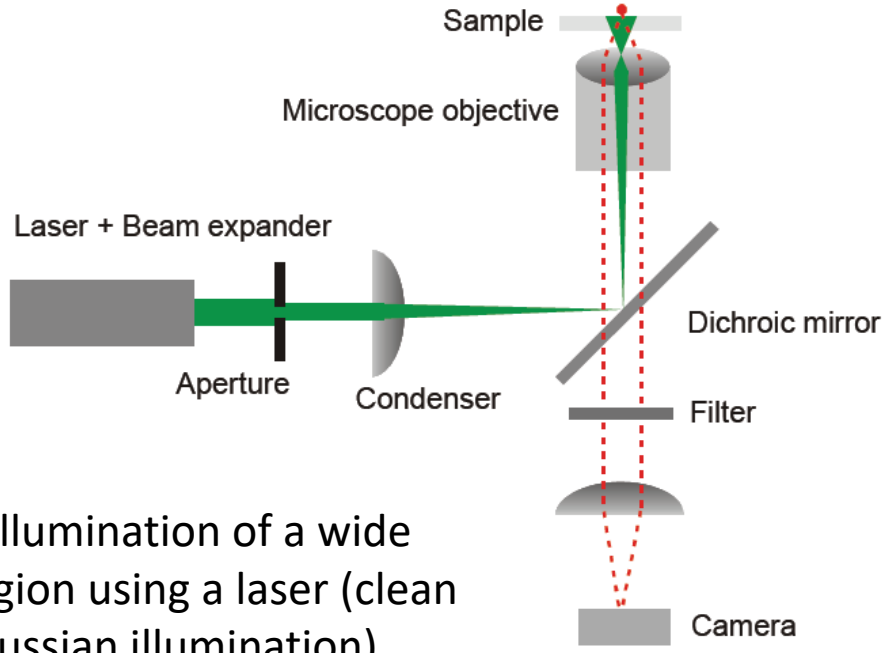
Observing single-molecules in cells

Studies in vitro using defined DNA/chromatin constructs





Observing dynamics in cells: Wide-field microscopy



• Illumination of a wide region using a laser (clean gaussian illumination).

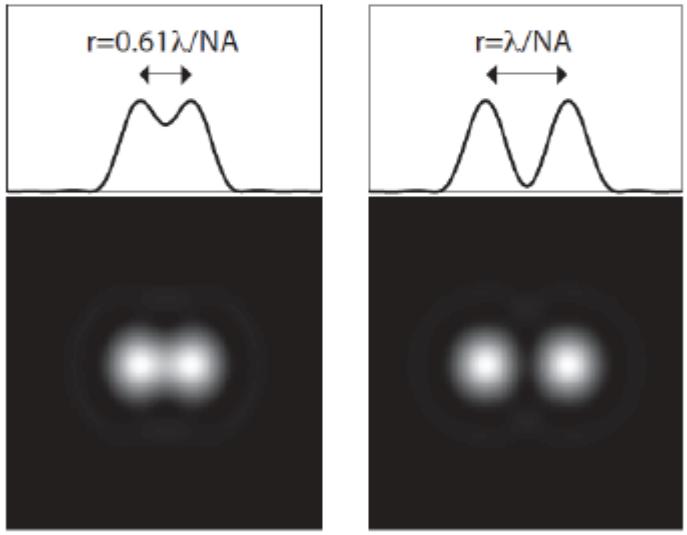
• The polarization, the excitation intensity and the excitation wavelength are controlled.

• Detection using highly-sensitive CCD cameras

Optical resolution - The Raleigh limit:

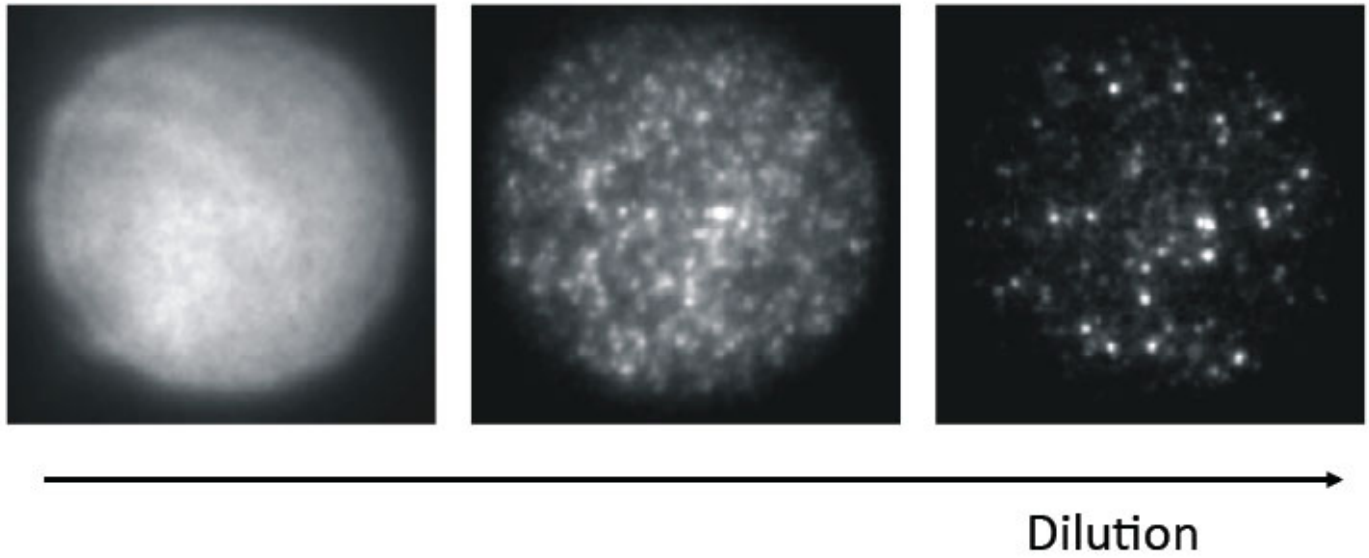
$$r = 0.61\frac{\lambda}{NA}$$

$$NA = n \cdot \sin\vartheta$$



PSF: point spread function (diffraction limited)

Careful control of the fluorophore concentration



- Molecules of a dye (Rhodamine 6G) diluted in a polymer (PVA).
- It is important to have full control on the concentration of the fluophores.

Reducing the background: Total Internal Reflection Fluorescence Microscopy

Restriction of the sample volume at an interface:

Total internal reflection
at a boundary of changing refractive index, light arriving at a critical angle will be reflected (swimming pool!)

Critical (TIR) angle: (complete reflection)

$$\Theta_C = sin^{-1}\left(\frac{n_1}{n_2}\right)$$

However, an **evanescent field** penetrates into solution (approx 200 nm):

$$I(z) = I(0)\exp(-z/d)$$

$$d = \frac{\lambda_0}{4\pi}(n_2^2 sin\Theta_2 - n_1^2)^{-1/2}$$

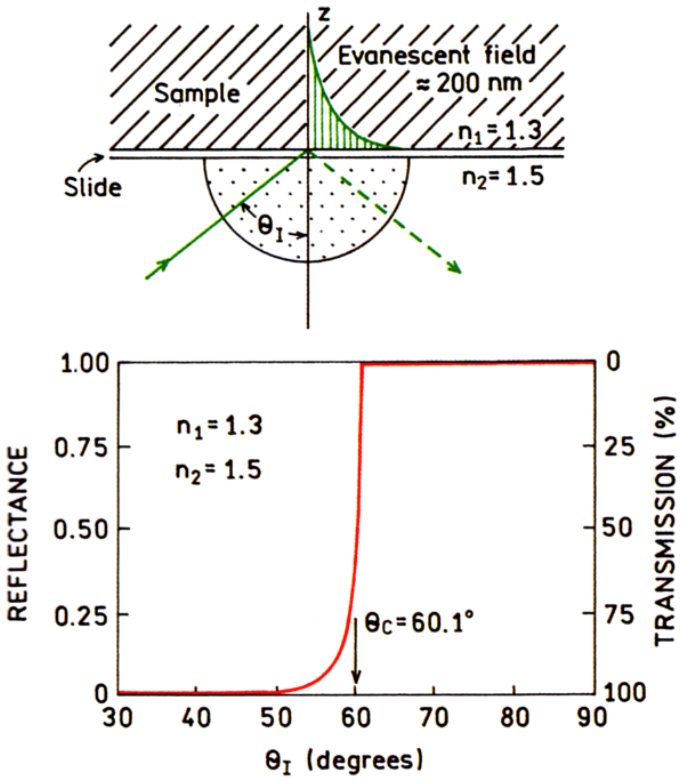
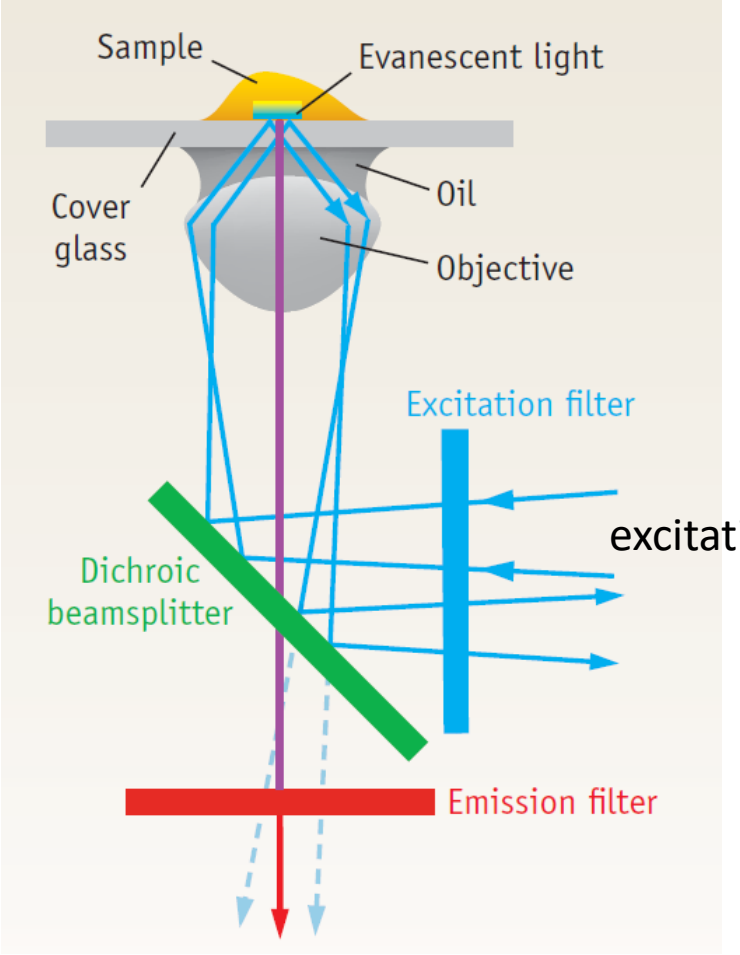


Figure 23.5. **Top**: Optical geometry for total internal reflection (TIR). **Bottom**: Calculated reflectance and transmittance for n_2 = 1.5 and n_1 = 1.3.

Setting up a TIRF microscop



Normal microscope setup (widefield -> imaging of a large area)

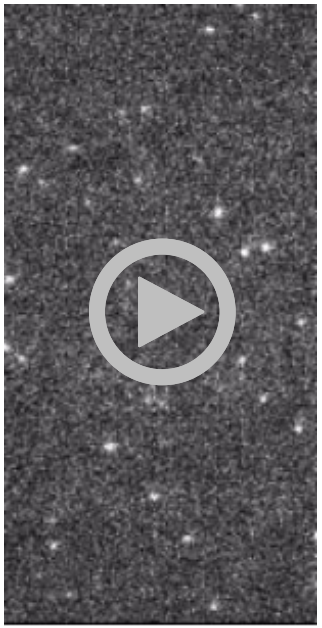
Objective with very **high numberical aperture** required (NA > 1.38, ideally NA = 1.45)

Excitation with **lasers** for even illumination, high intensity

Detection using **EMCCD cameras** -> this allows the imaging of many single molecules at the same time, however at lower time resolution compared to confocal microscopy (ms)

Why is TIRF microscopy required?

50 nM of a labeled protein



moving in and out of the TIRF angle

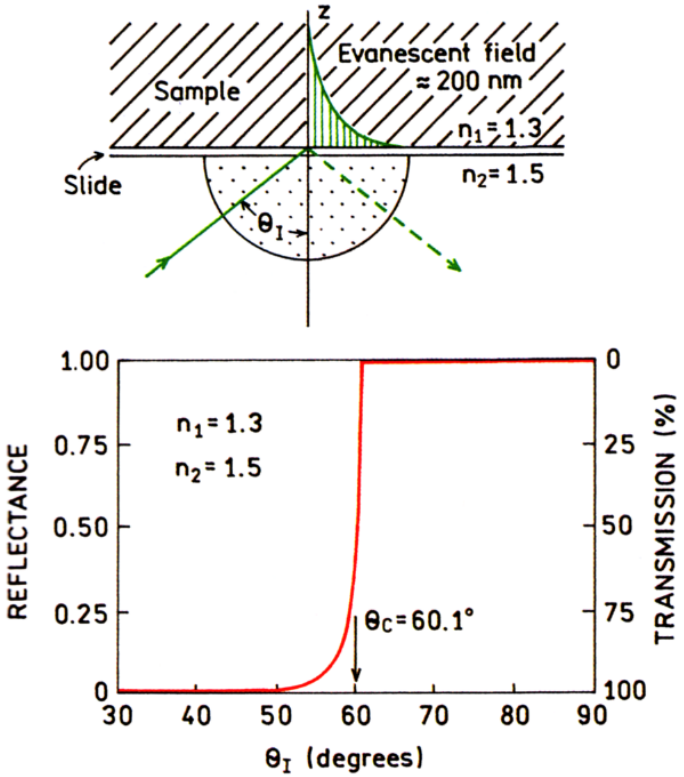
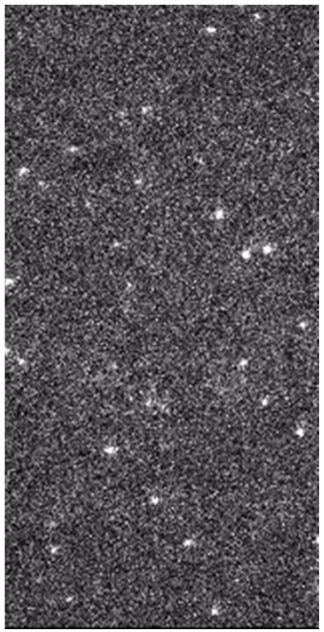


Figure 23.5. **Top**: Optical geometry for total internal reflection (TIR). **Bottom**: Calculated reflectance and transmittance for n_2 = 1.5 and n_1 = 1.3.

movement in and out of the TIRF angle: Under non-TIRF conditions, the background fluorescence is overwhelming the signal completely.

Why is TIRF microscopy required?

50 nM of a labeled protein



moving in and out of the TIRF angle

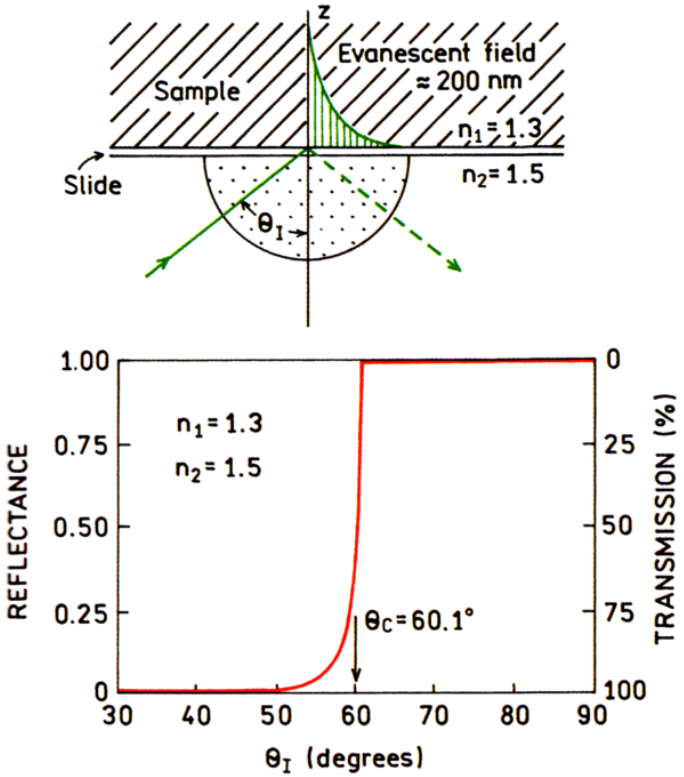
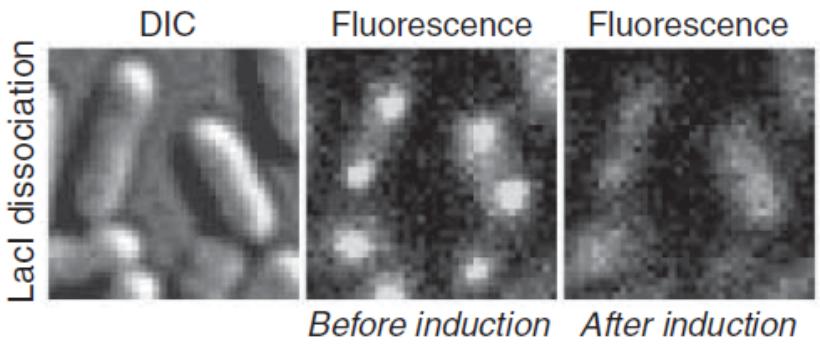


Figure 23.5. **Top**: Optical geometry for total internal reflection (TIR). **Bottom**: Calculated reflectance and transmittance for n_2 = 1.5 and n_1 = 1.3.

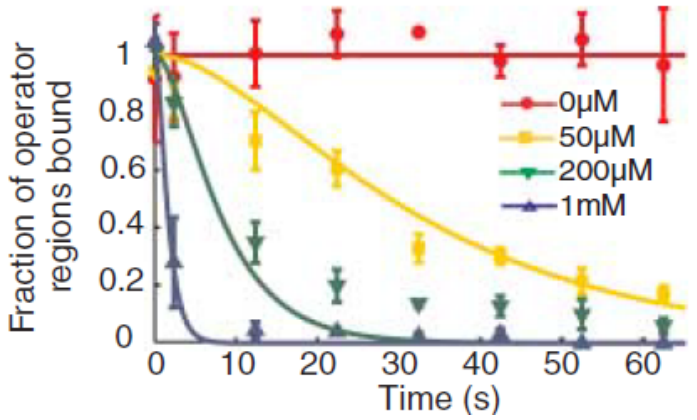
movement in and out of the TIRF angle: Under non-TIRF conditions, the background fluorescence is overwhelming the signal completely.

Lac repressor dynamics in living cells

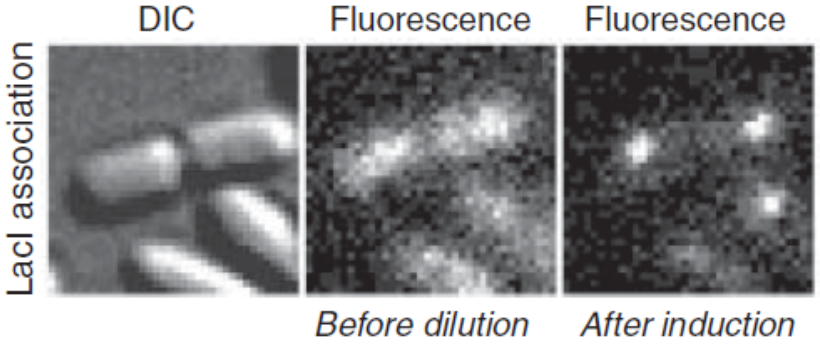
Ligand addition → TF dissocation



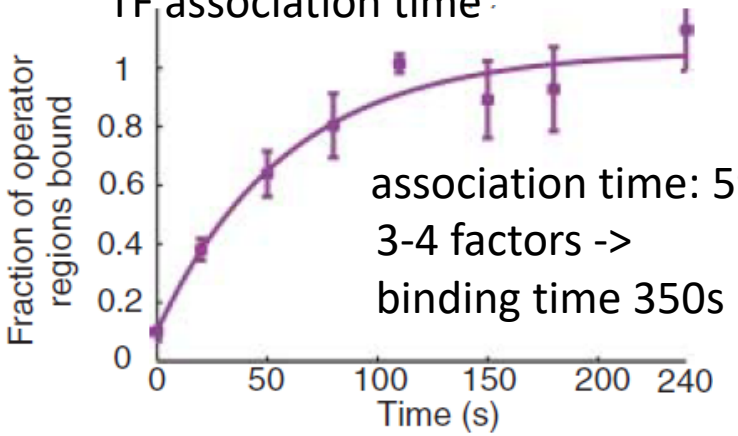
TF dissociation time



Ligand removal → TF association



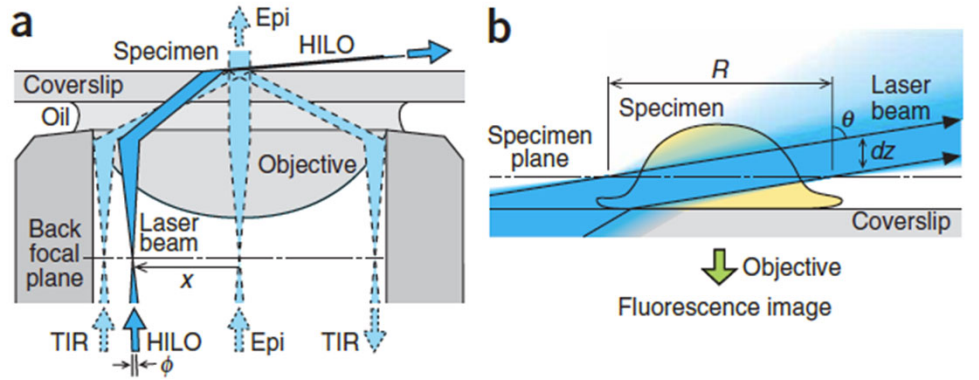
TF association time

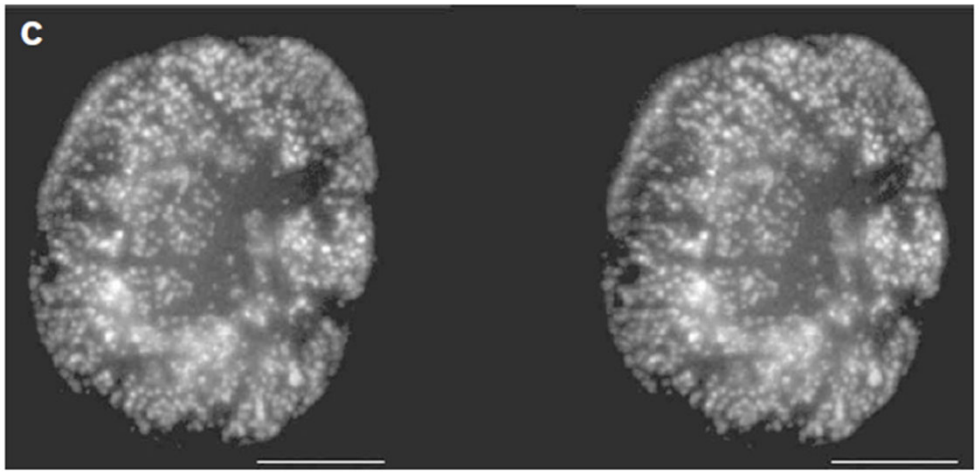


association time: 59 s
3-4 factors ->
binding time 350s

Elf, Li, Xie, Science 2007

Detecting TF dynamics in Mammalian Cells





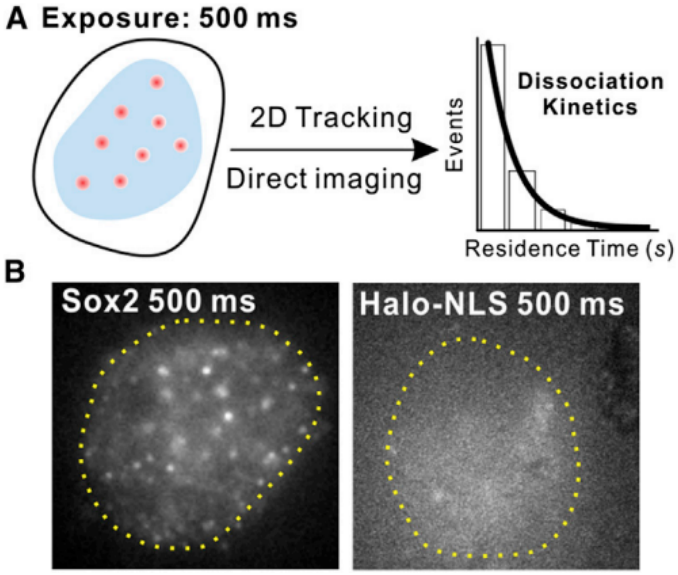
HILO: Highly inclined thin illumination

light sheet sectioning cells

greatly increases signal-to-noise (~ fold) compared to epifluorescence

Tokunaga et al. Nature Methods 2008

Detecting transcription factor residence times



Chen et al., Cell 2014

Measuring time until a single-molecule disappears:

→ dissociation process

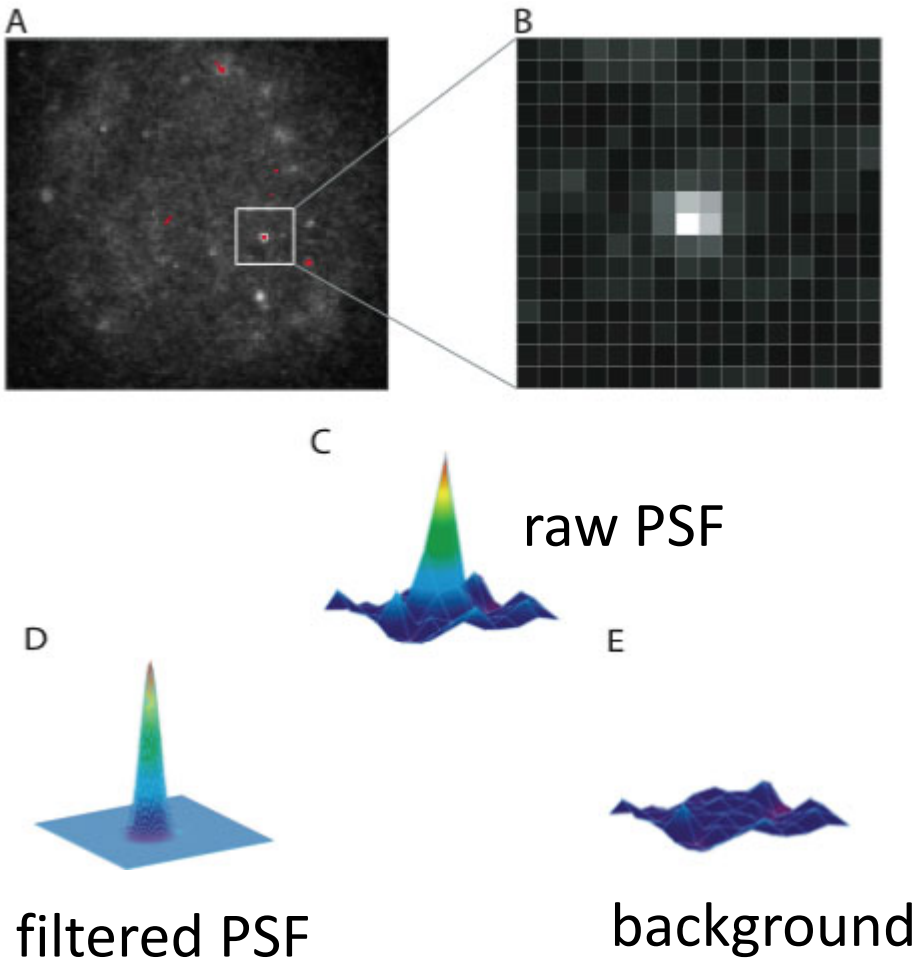
Generation of lifetime histograms:

→ residence time distribution at a given site

analysis with multiexponential distribution:

→ specific sites
→ non-specific sites
→ different DNA sequence and chromatin context
→ activity

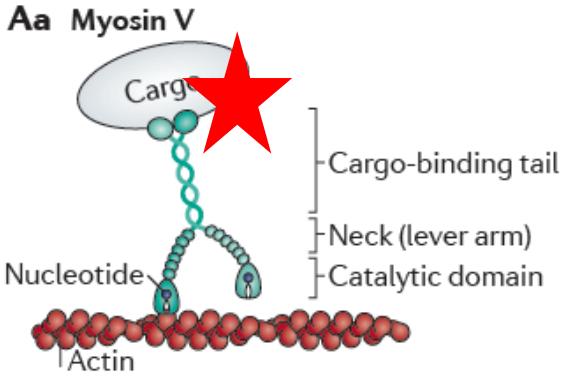
Single-molecule tracking



In a second step, single-molecule images are fitted using a two-dimensional gaussian.

The precision of the Gaussian peak position << resolution of 0.61λ/NA !!

Fluoresence Imaging with One-Nanometer Accuracy (FIONA) – works well for individual molecules!



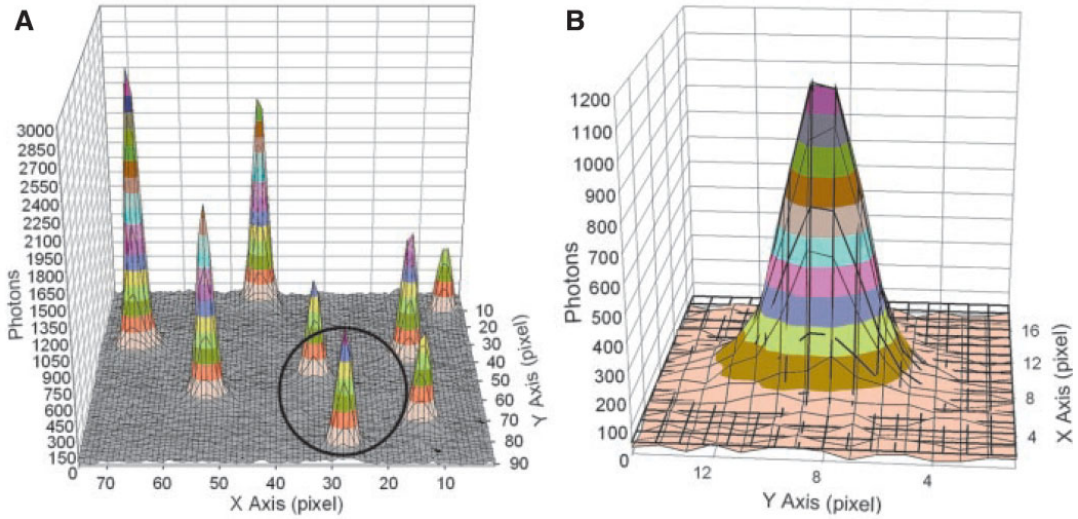
Fit of fluorescence emission (>1000 photons) with 2d – gaussian

Exact position determination with 1.5 nm accuracy

Dense samples → Overlap of peaks!

Single molecule imaging

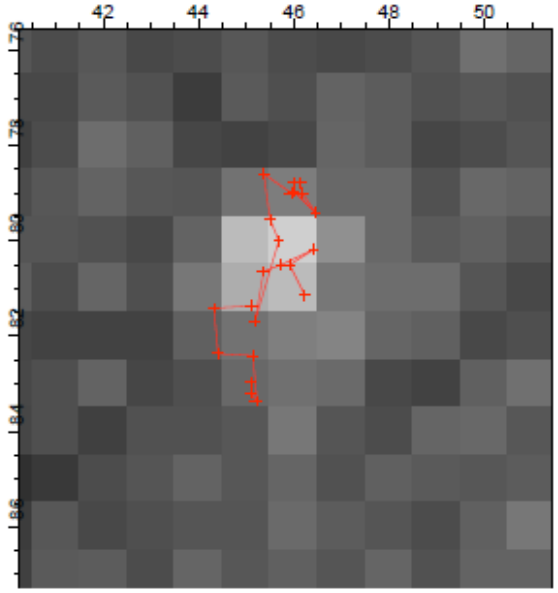
Yildiz et al., Science 2003

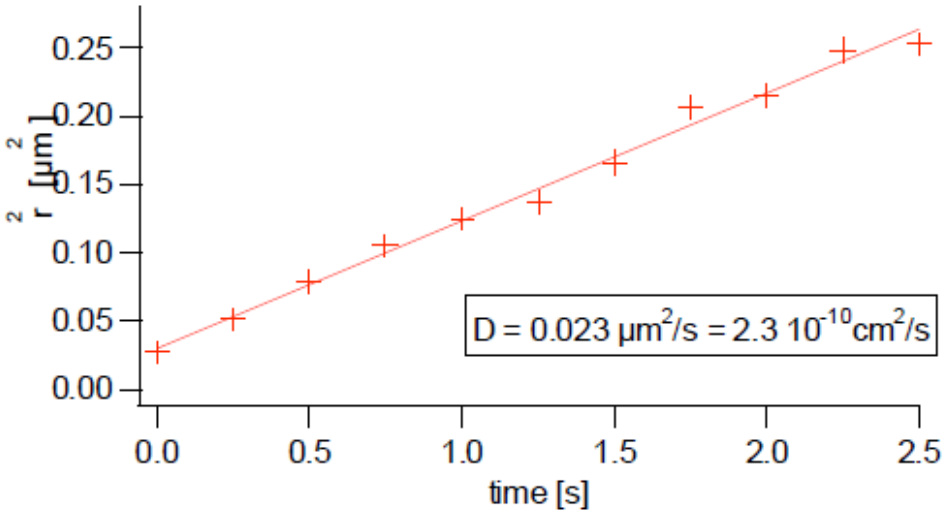


If single fluorophores can be observed, extremely high accuracy can be obtained!

How can a dense sample be imaged, one fluorophore **at a time?**

Single-molecule tracking





$$MSD(n\delta t) = \frac{1}{N-1-n}\sum_{j=1}^{N-1-n}\left\{[x(j\delta t + n\delta t) - x(j\delta t)]^2 + [y(j\delta t + n\delta t) - y(j\delta t)]^2\right\},$$

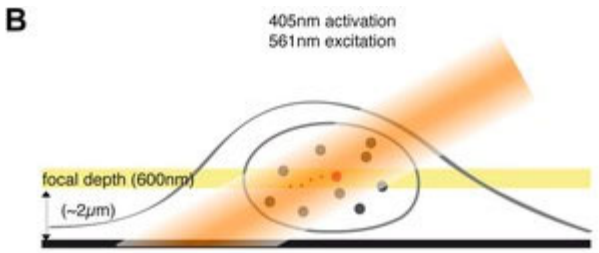
The mean square displacement (*MSD*) is calculated

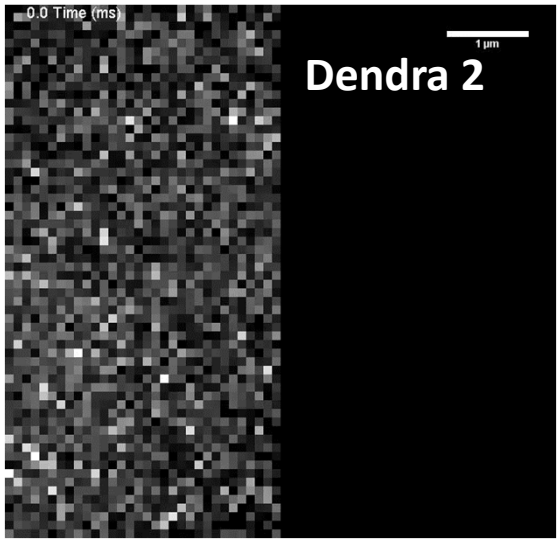
$$MSD(\tau) = 4D\tau$$

With *MSD*: mean square displacement, *D* diffusion coefficient , *t=ndt* time, *N* total # of measurements

* 2D displacement

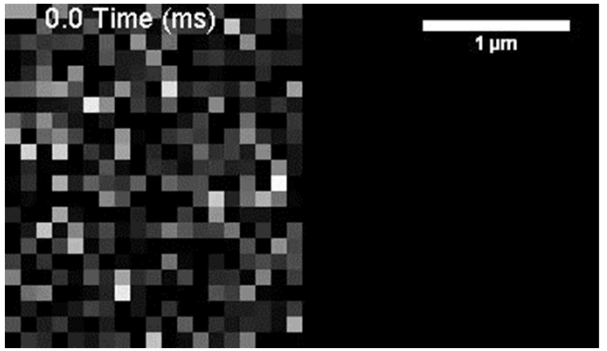
Observing molecules directly



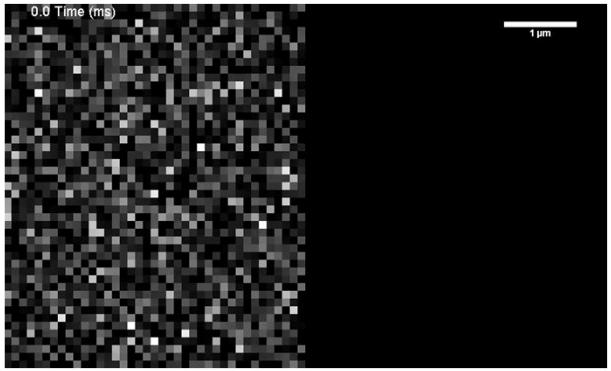


Izzedin et al. eLife 2014

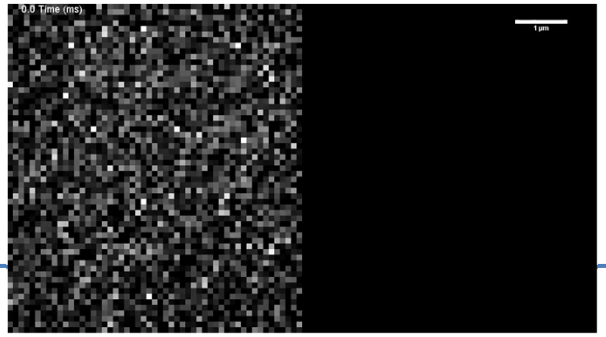
https://elifesciences.org/articles/02230



H2B
attached to chromatin
immobile

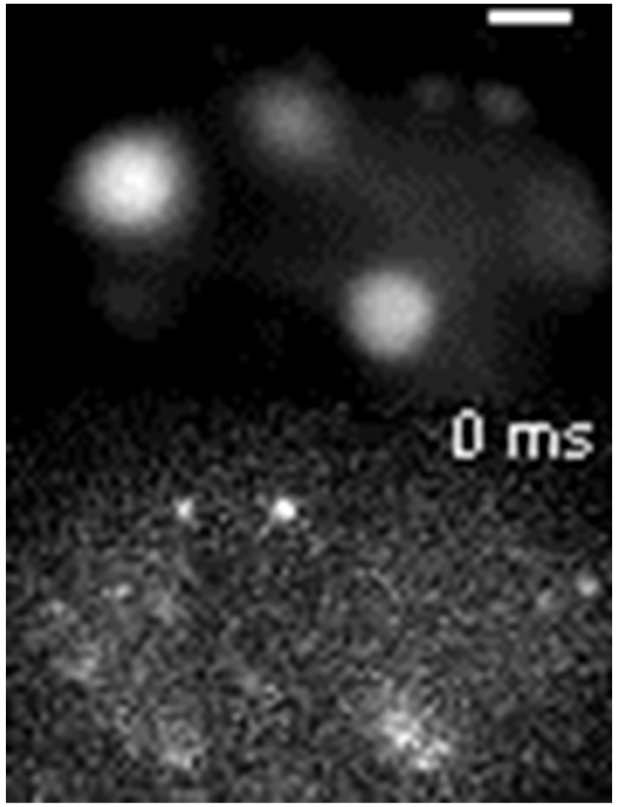


cMyc
Transcription factor



p-TEFb
Transcription elongation factor

Single molecule tracking of Sox2 in enhancer clusters



two color imaging

bright areas: compact heterochromatin

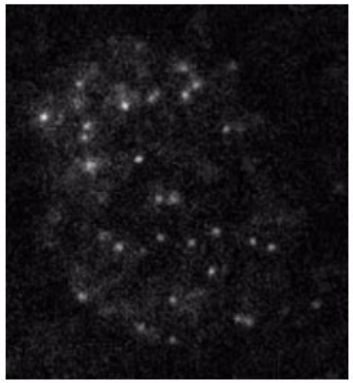
3D imaging of Sox2 motion in different chromatin states

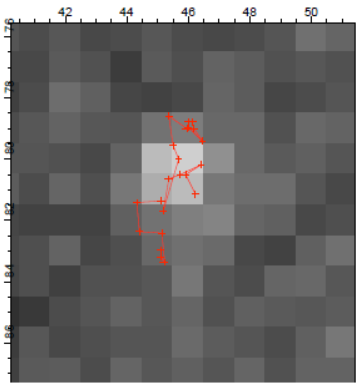
Liu et al. eLife 2014

https://elifesciences.org/articles/04236/figures

Analysis of single TF molecules reveals their dynamic behavior in cells

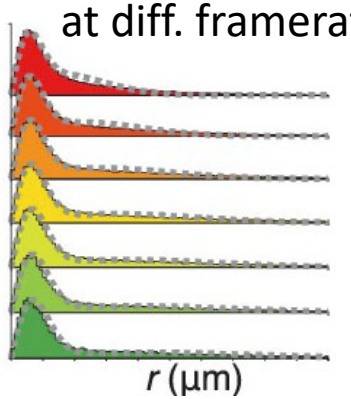
JF549-Halo-Sox2a



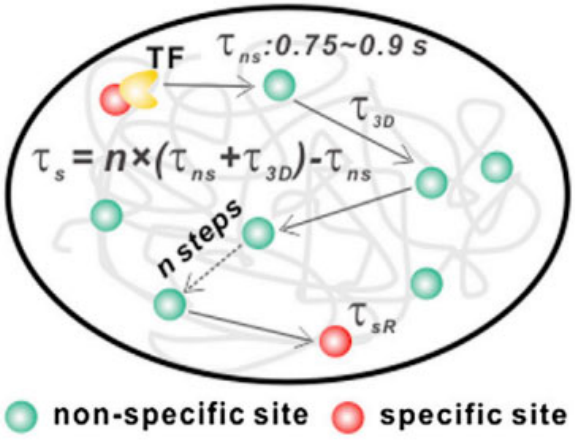


tracks

displacement histograms at diff. framerates



Hansen et al., eLife 2018

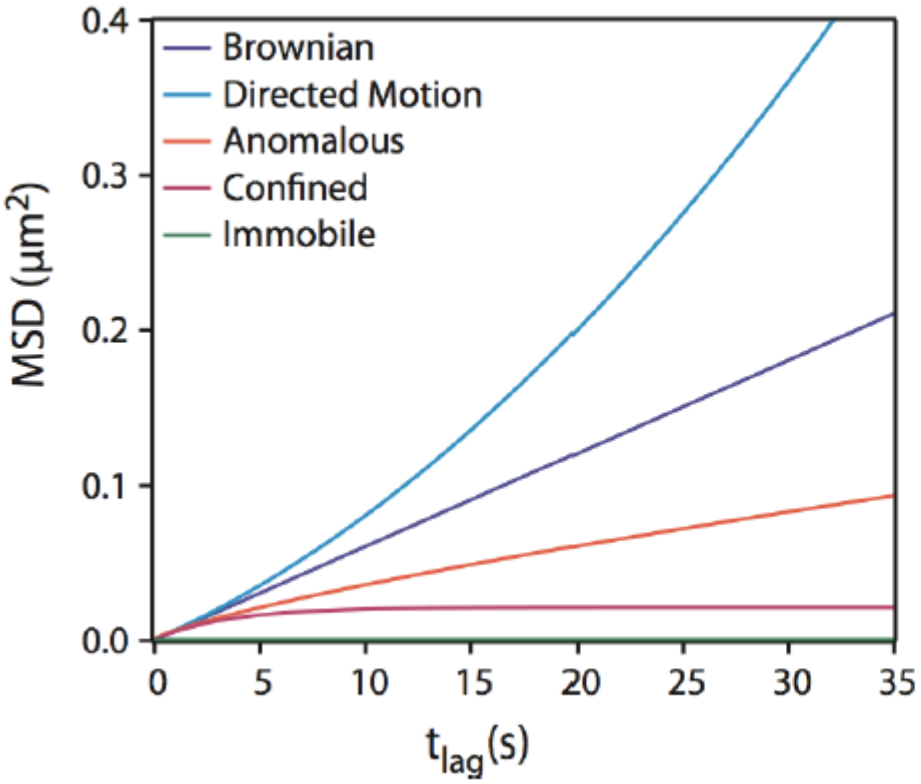


Chen et al., Cell 2014

diffusion modes:

- bound at specific sites
- bound at non-specific sites
- freely diffusing

Single-molecule tracking can differentiate between different diffusional motions



$$\mathrm{MSD}\,(t_{lag}) = 4Dt_{lag} + (\mathrm{v}t_{lag})^2$$

$$\mathrm{MSD}\,(t_{lag}) = 4 \cdot D_0 \cdot t_{lag}$$

$$\mathrm{MSD}\,(t_{lag}) = 4Dt_{lag}^{\alpha}$$

$$\mathrm{MSD}\,(t_{lag}) = \frac{L_c^2}{3} \cdot \left(1 - exp\left(\frac{-12 \cdot D_0 \cdot t_{lag}}{L_c^2}\right)\right)$$

- In case of non-Brownian diffusion, *MSD* deviates from a linear relationship.
- MSD of a free-diffusing molecule (Brownian motion) is linear with time