Single-molecule fluorescence

Principles and applications

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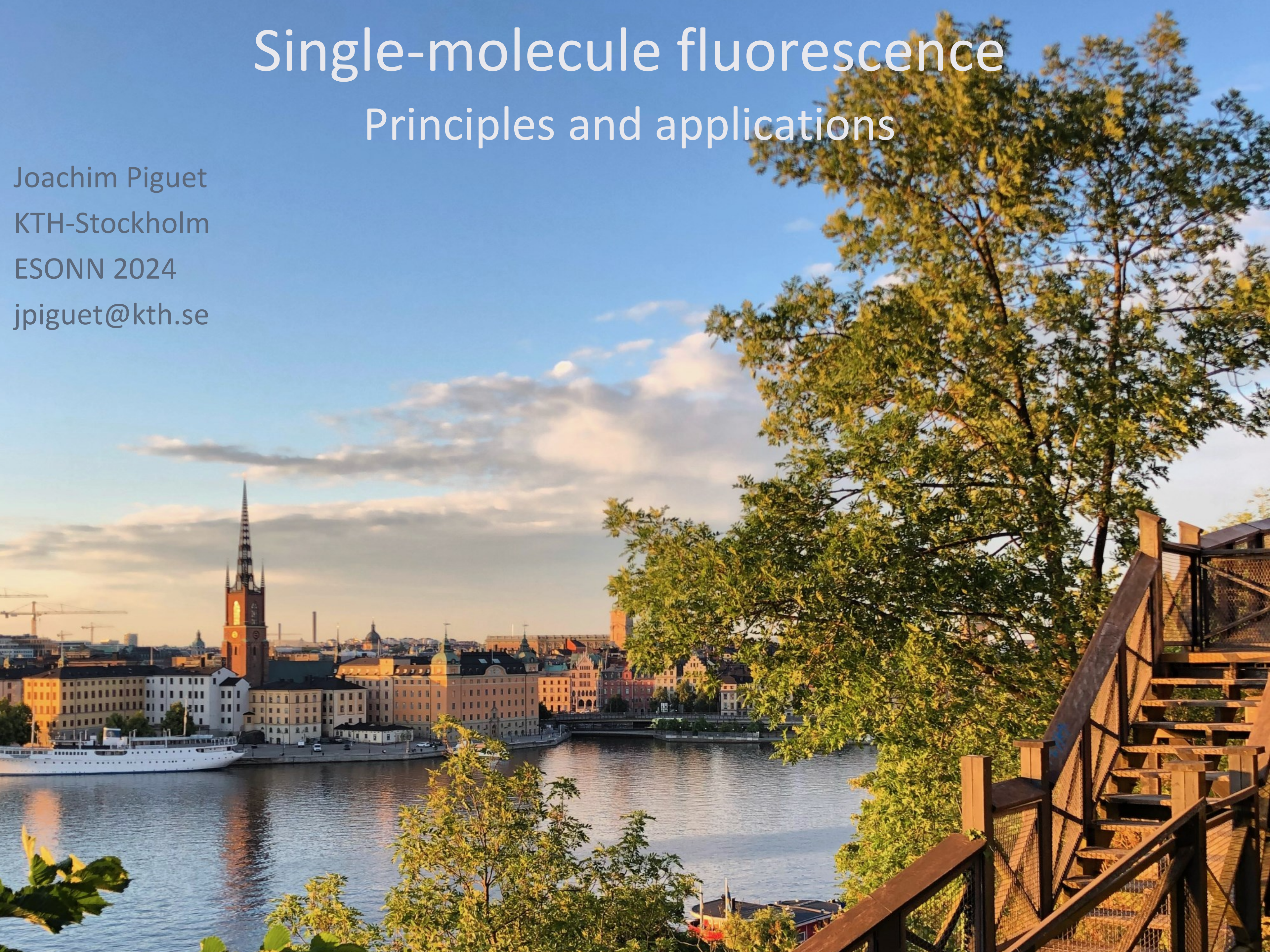


From Single-molecule localization to super resolution microscopy

A story of dilution and photon budget

1. Fundamentals
2. Microscopy and fluorescence imaging setup
3. Single-molecules imaging and tracking
4. Blinking
5. Fluorescence nanoscopy
6. Functional devices based on single-molecule
7. Single-molecule detection and fluorescence signal correlation
8. Useful literature

How to use single fluorophore properties to gain knowledge on nanoscopic objects ?

Why is it useful?:

- Observation of the function and the motion of **nano-objects** in **real-time** in **living systems**
- Measurements on **single molecule** and not on ensemble of molecules
- Statistical analysis based on **individual events**
- **Non-invasive and highly specific** measurements
- Large choice of **fluorophores and labels**

Observing nanoscopic objects at work:
Single-molecule imaging or spectroscopy (SMI or SMS)

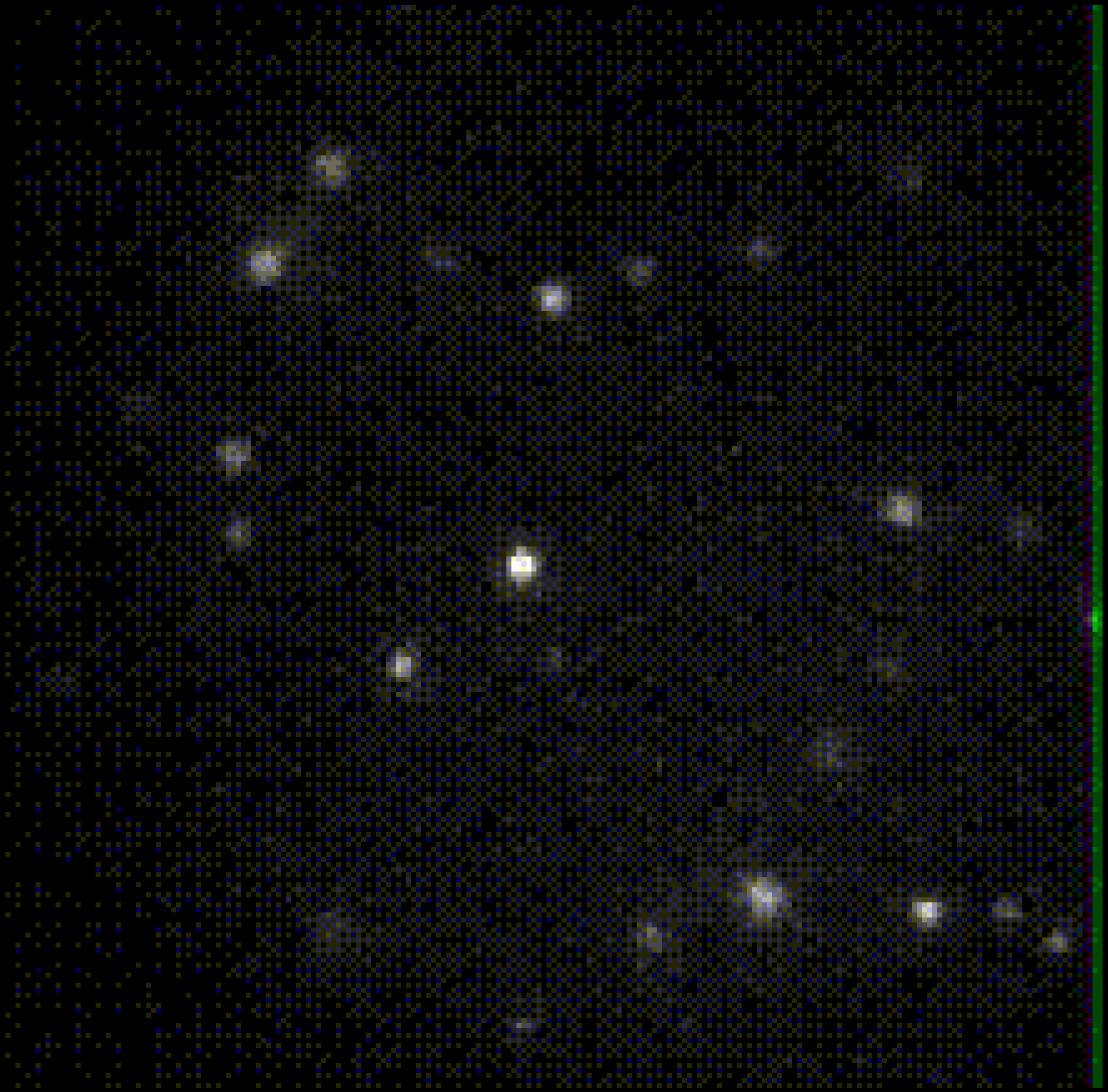
Control of the apparent dilution of the probe

- Controlled labeling
- Photobleaching
- Photoactivatation
- Photoswitching
- Blinking
- Volume reduction
- Ground state depletion
- Interference patterns

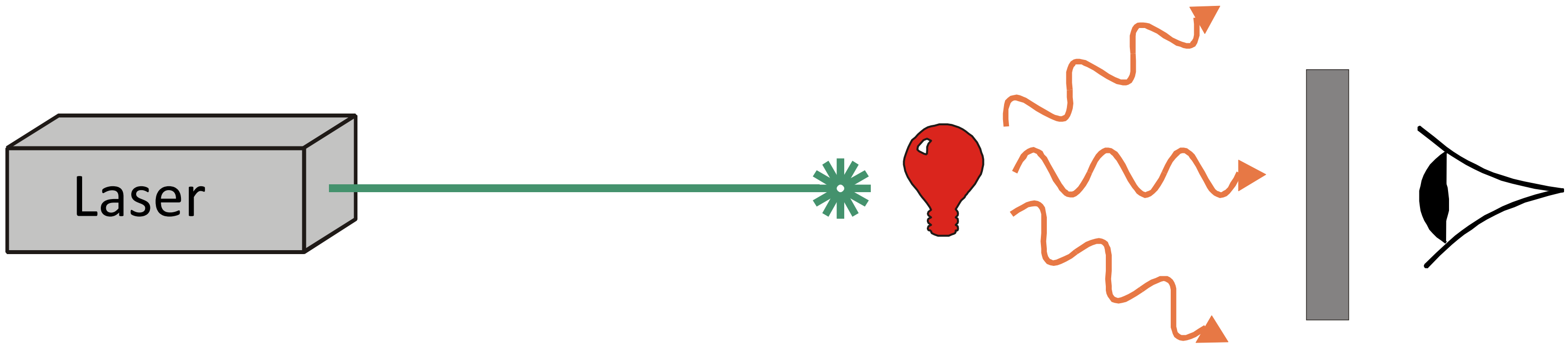
High budget of photon/localization

- Organic fluorophore
- Controlled environment
- Repetitive labelling of the same location
- Quantum-dots

Single-molecules detection

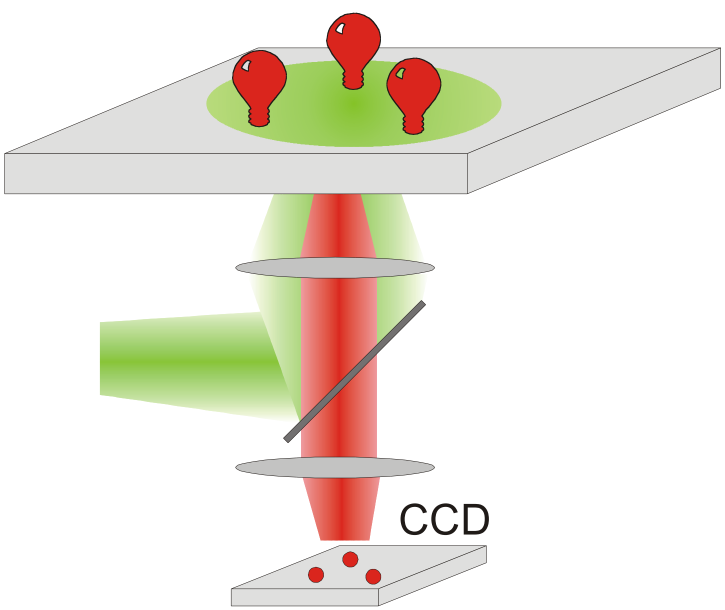


Measurement principle: Fluorescence microscopy

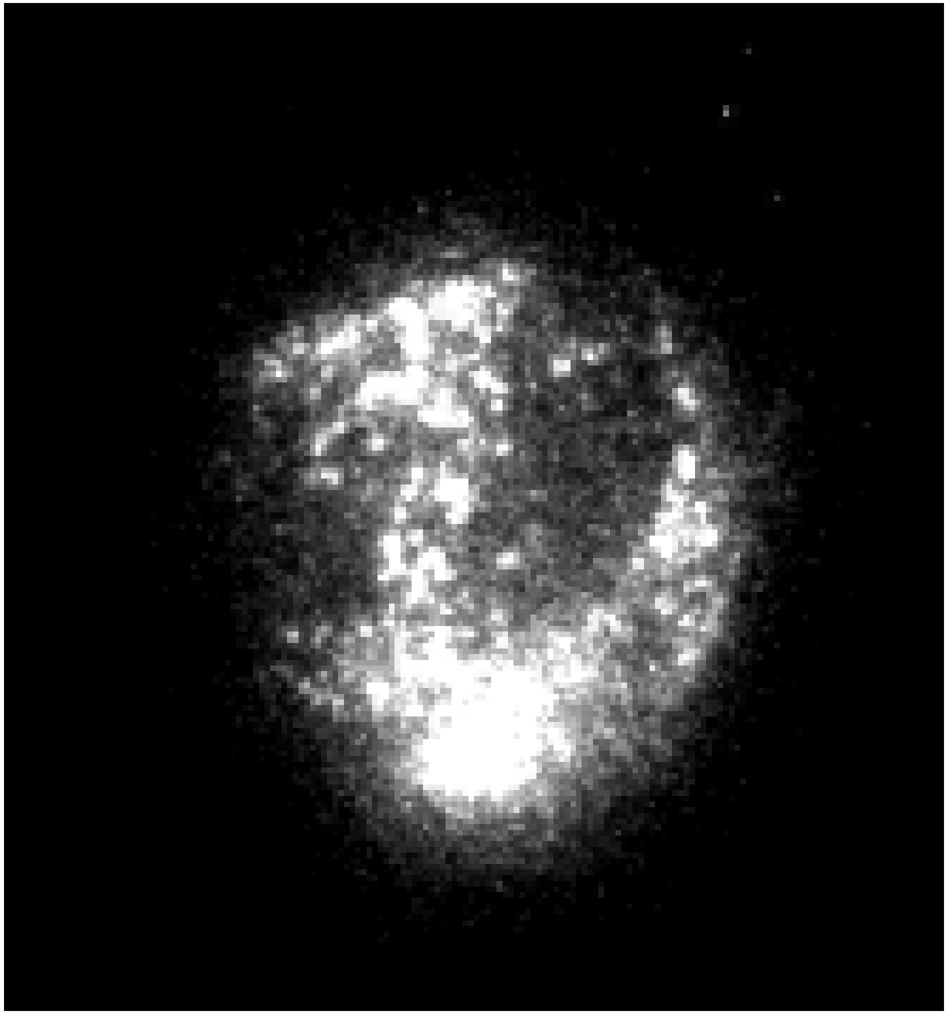


Single molecule measurements depends on the possibility to dilute the sample.

- in a cell membrane
- in an artificial membrane
- on a biomodified surface
- in a thin polymer sheet spin coated on a glass surface
- in a microfluidics device

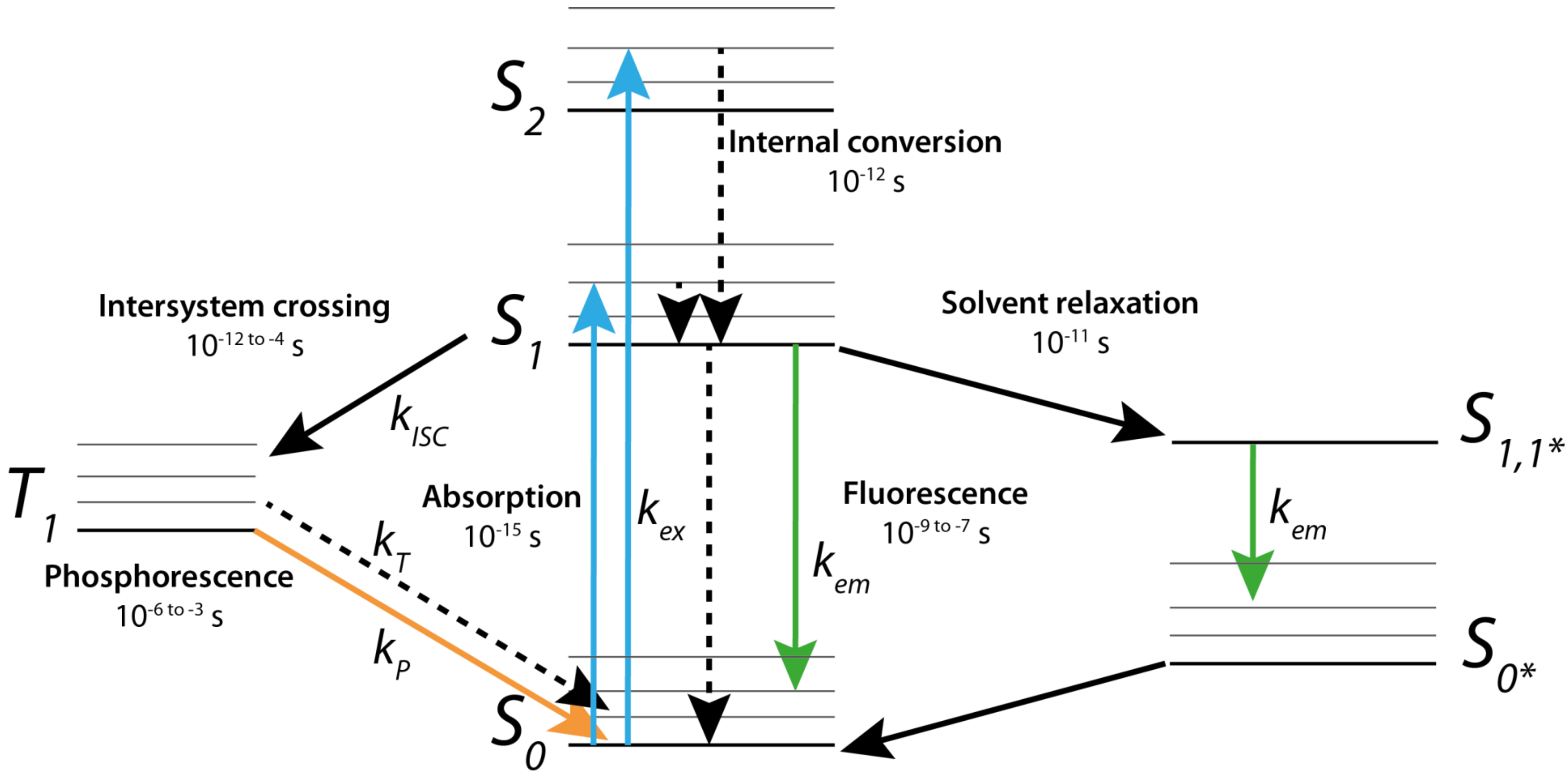


Benefits of single-molecule experiments



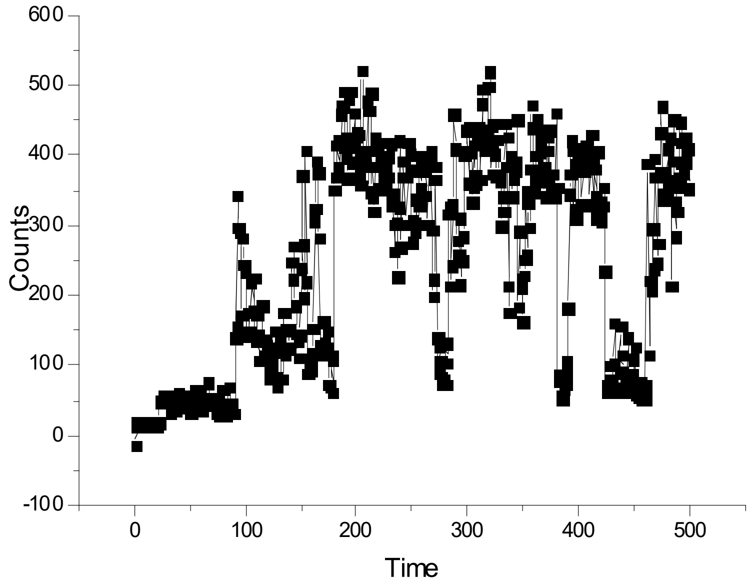
- No ensemble averaging
- No temporal averaging (no need to synchronize).
- Access to novel experimental parameters (e.g. higher position accuracy, blinking properties).
- Resolution unlimited measurement, beyond Abbe limit (superresolution microscopy)

Fundamentals: Jablonski's diagram



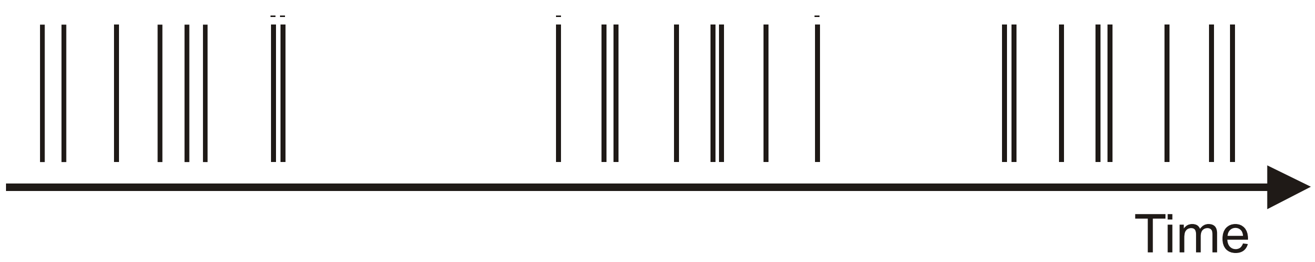
- Fluorescence lifetime: $1/\,k_f = t_f = 1/(k_{em} + \Sigma k_i)$, radiative lifetime $t_r = 1/k_{em}$
- Fluorescence quantum yield $q_f = t_f/t_r$ = # emitted photons/# absorbed photons

Fundamentals: Fluorescence timetrace



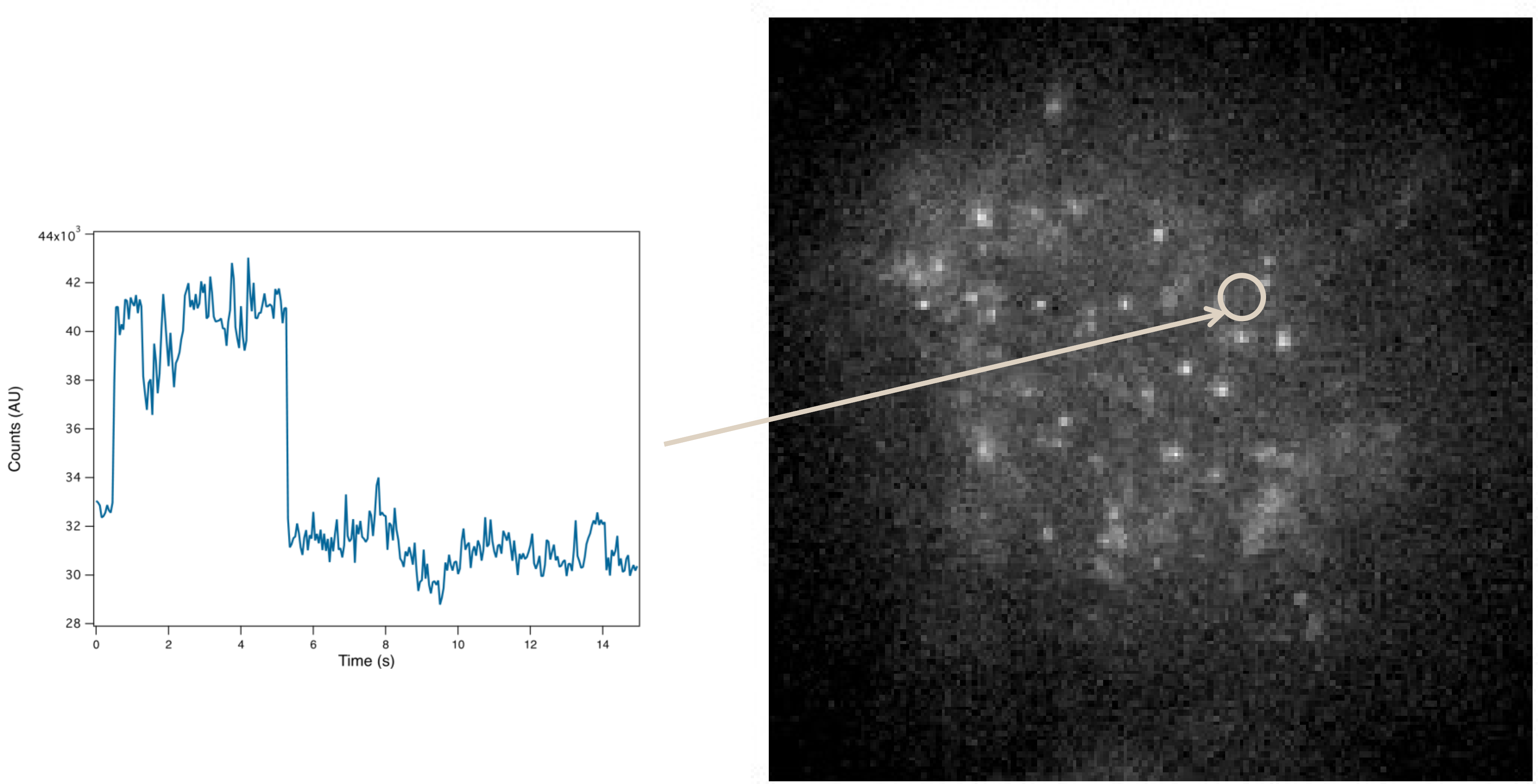
Blinking can have several origins:

- Triplet and other dark states.
- Modifications in the environment: Changes of the spectrum or of the fluorescence quantum yield.
- Chemical reactions (e.g. protonation) or complex formations (with O_2).
- photo-ionisation.
- etc…



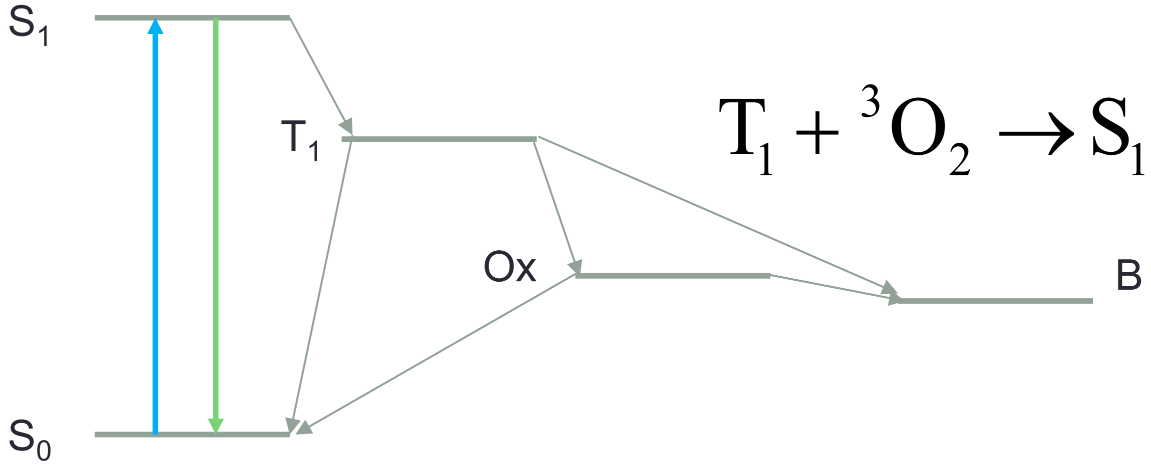
Fundamentals: Photobleaching

A considerable amount of energy is flowing through single molecules.
After a limited amount of time (~ s), they will undergo photodestruction.



Serotonin receptor labelled with a tris-NTA-Atto647N probe in 293T cells.

Fundamentals: Mechanism of Photobleaching



$$T_1 + {}^3O_2 \rightarrow S_1 + {}^1O_2$$

Most common mechanism:

The molecule goes into the triplet state (S1->T1) and then decays producing higly reactive singlet oxygen that will oxidize the fluorophore.

Photobleaching quantum yields are typically in the order of 10^{-5}-10^{-6}.

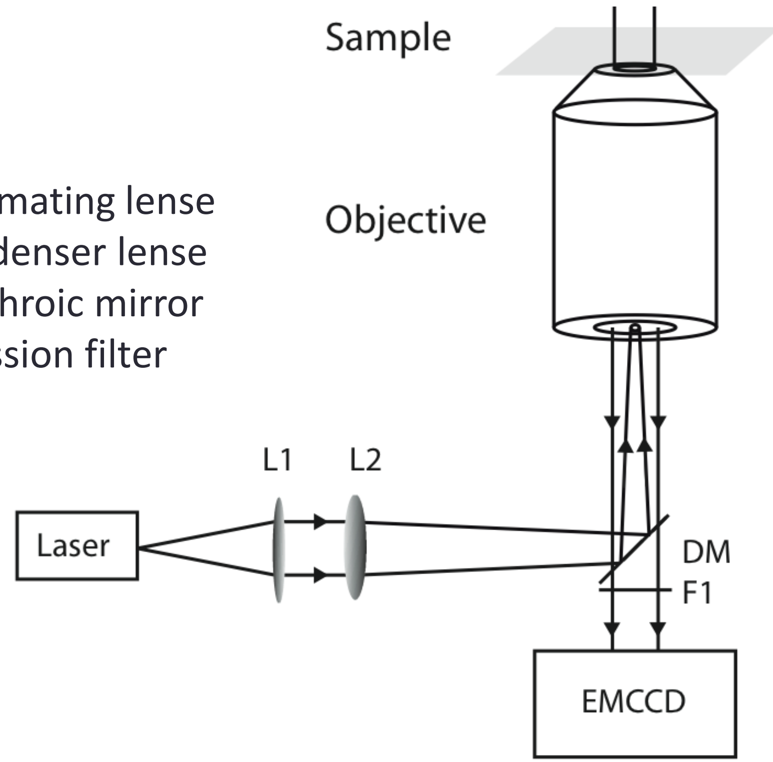
Photobleaching drastically reduces the measurement time.

Far-field microscopy imaging setups

Set-up: Wide-field laser microscopy

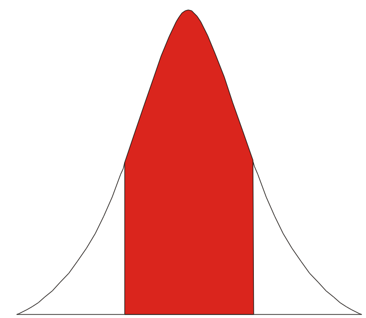
- Illumination of a wide region using a laser (clean gaussian illumination).
- The polarization, the excitation intensity and the excitation wavelength are controlled.
- Detection using highly-sensitive CCD cameras.

L1: Collimating lense
L2: Condenser lense
DM: Dichroic mirror
F1: Emission filter



illumination $f_{\text{condenser}}$ aperture

Laser illumination:
Truncated gaussian profile



Resolution:

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

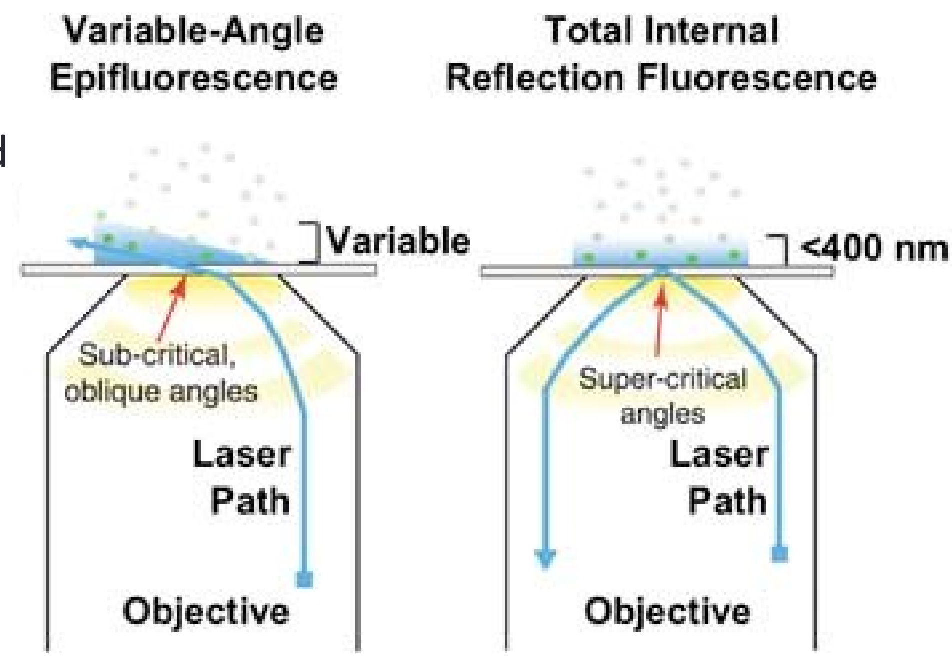
$$NA = n \cdot \sin\vartheta$$

Resolution = capability to discriminate the light emitted by two different sources

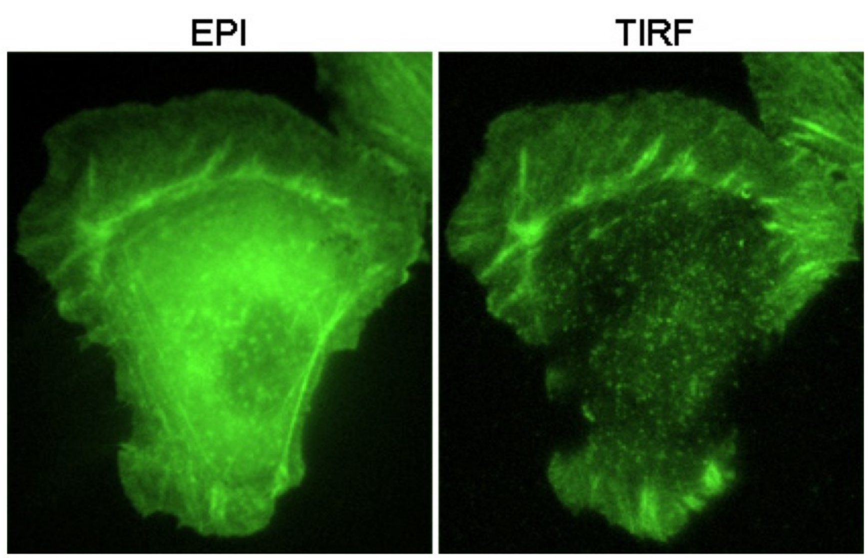
Set-up: Total-Internal Reflection Fluorescence (TIRF) Microscopy

The illumination incident light is totally reflected

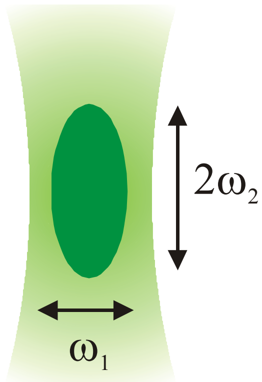
-> An evanescent wave illuminate only a few hundreds on nanometer



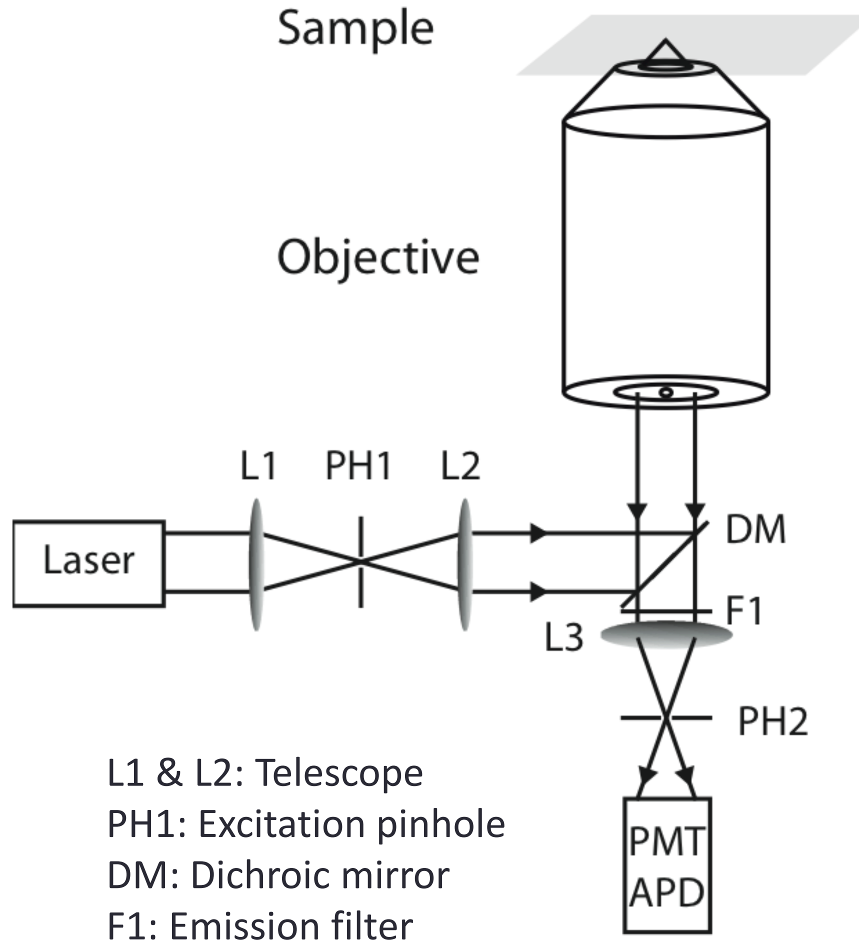
www.photonics.com



Set-up: Confocal laser scanning microscopy

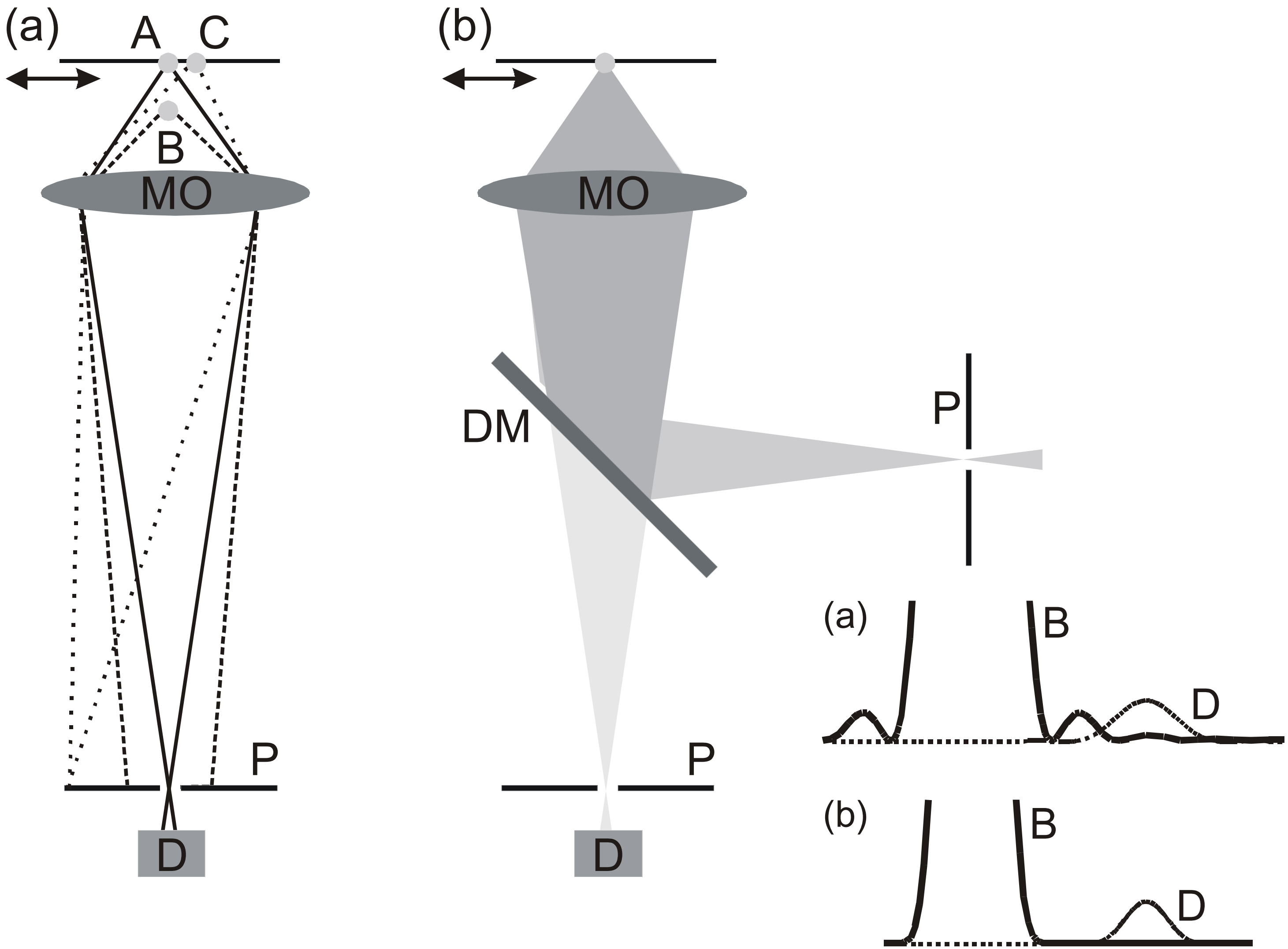


- Confocal illumination: Only a **small volume** (the confocal volume) is efficiently detected and illuminated.
- An image is obtained by **scanning** the sample and recording the fluorescence intensity as a function of the position.
- Slow measurement, high contrast
- Detection using highly-sensitive single-photon avalanche photo-diodes (APD).
- The fluorescence can be split into multiple components using cube polarizer or dichroic mirror.

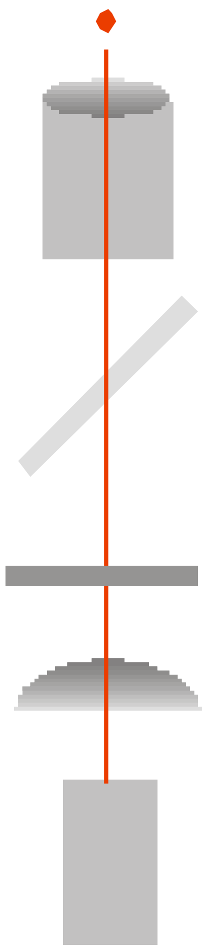


L1 & L2: Telescope
PH1: Excitation pinhole
DM: Dichroic mirror
F1: Emission filter
L3: Focusing lense
PH2: Emission pinhole

Set-up: Confocal laser scanning microscopy: principle



Set-up: Detection efficiency



$$\eta_{obj} = 0.5 \cdot [1 - \sqrt{1 - (NA/n)^2}\,]$$

For NA=1.2 and n=1.33, η_{obj}=28%

But it depends on the orientation of the fluorophore

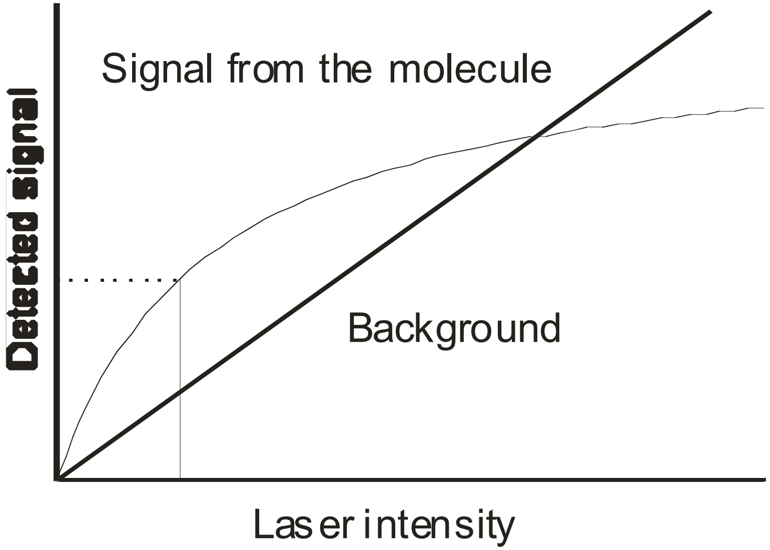
Filters and dichroics: η_{fil}=40%

Optics: η_{opt}=90%

Detector: η_{det}=50%

In total: η_{tot}=5%

Set-up: Signal/noise ratio



$$SNR = \frac{\eta_{det} R T_{int}}{\sqrt{\eta_{det} R T_{int} + C_b P T_{int} + N_d T_{int}}}$$

T_{int}=integration time

$C_b P$=background

N_d=noise from the detector

Noise sources

- Statistical noise of the signal
- Background
- Noise from the detector.

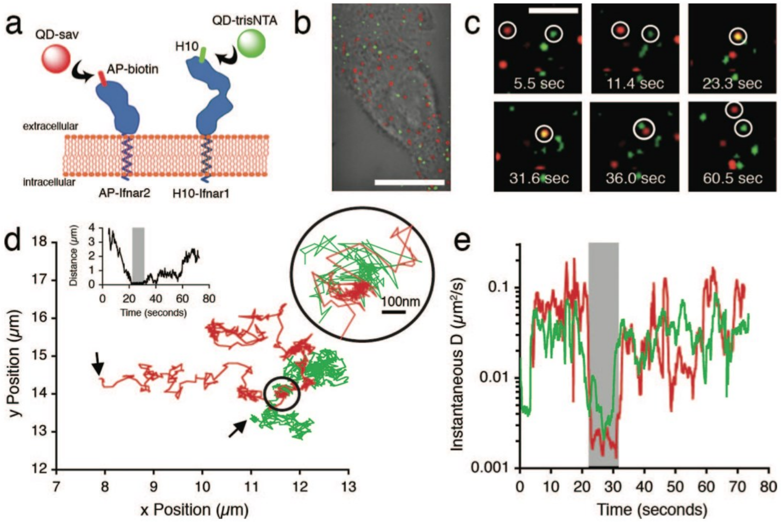
Background

- Stray photons
- Autofluorescence from the filters and optics
- Impurities in the sample (cell autofluorescence)
- Raman signal from the solvent (minimal background).

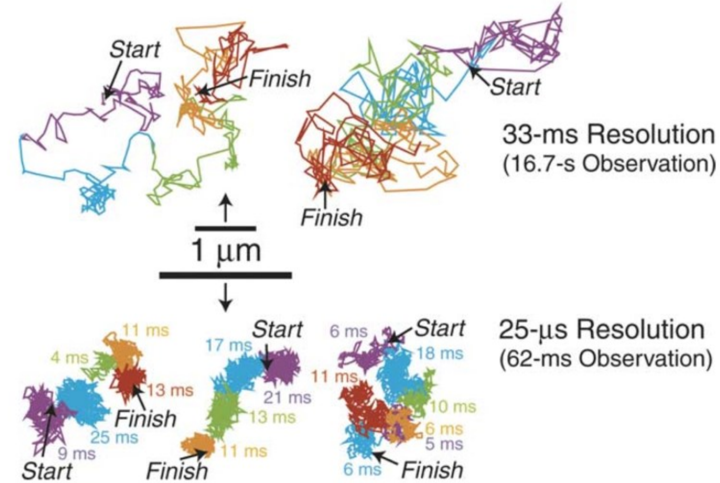
Tips

- Work slightly below I_{sat}.
- Reduce as much as possible the autofluorescence. Work as far as possible in the red part of the spectra
- 100-200 detected counts from the molecule may be sufficient to get an SNR of 8-10 (5-10 ms measurement time).
- The camera noise and efficiency have a significant impact on the localization precision.
- The effective number of images that can be recorded depends on the photo-bleaching rate.

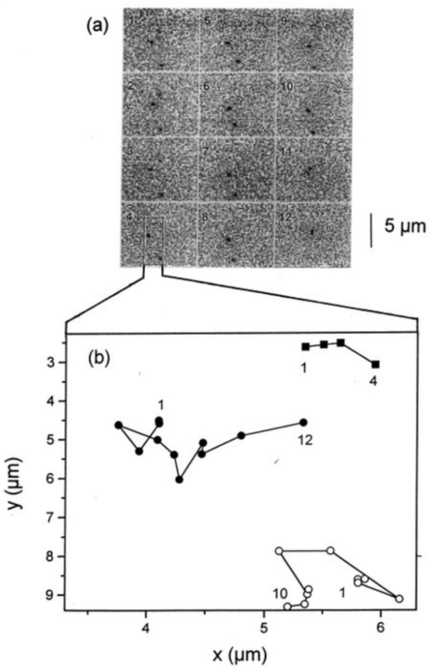
Single-molecule imaging and tracking



Roullier V et al. (2009) *Nano Lett*

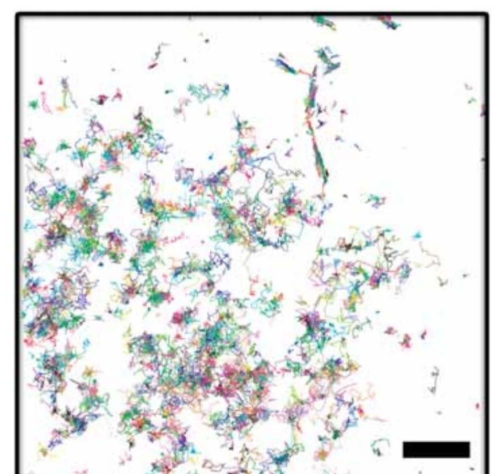


Kusumi A et al. (2005) *Semin Immunol* 17:3–21



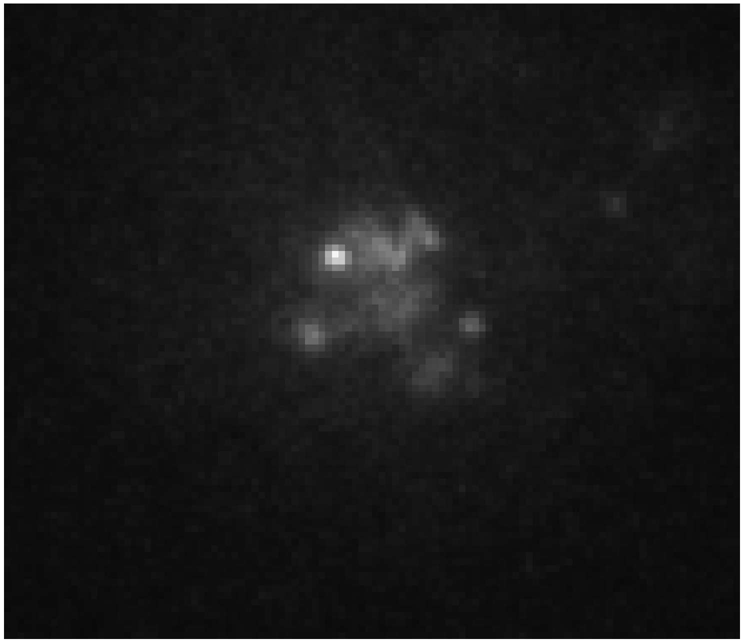
Schmidt T et al.(1995) *Journal of Physical Chemistry* 99:17662–17668

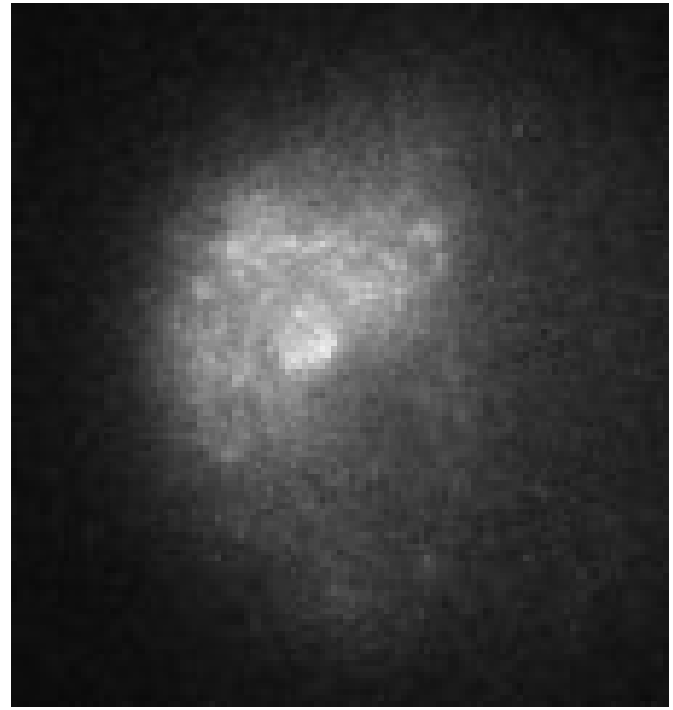
Manley S et al. (2008). *Nat Methods* 5:155–157



How to be sure that these are single molecules?
1: Well characterize the system without single molecules

Cell autofluorescence

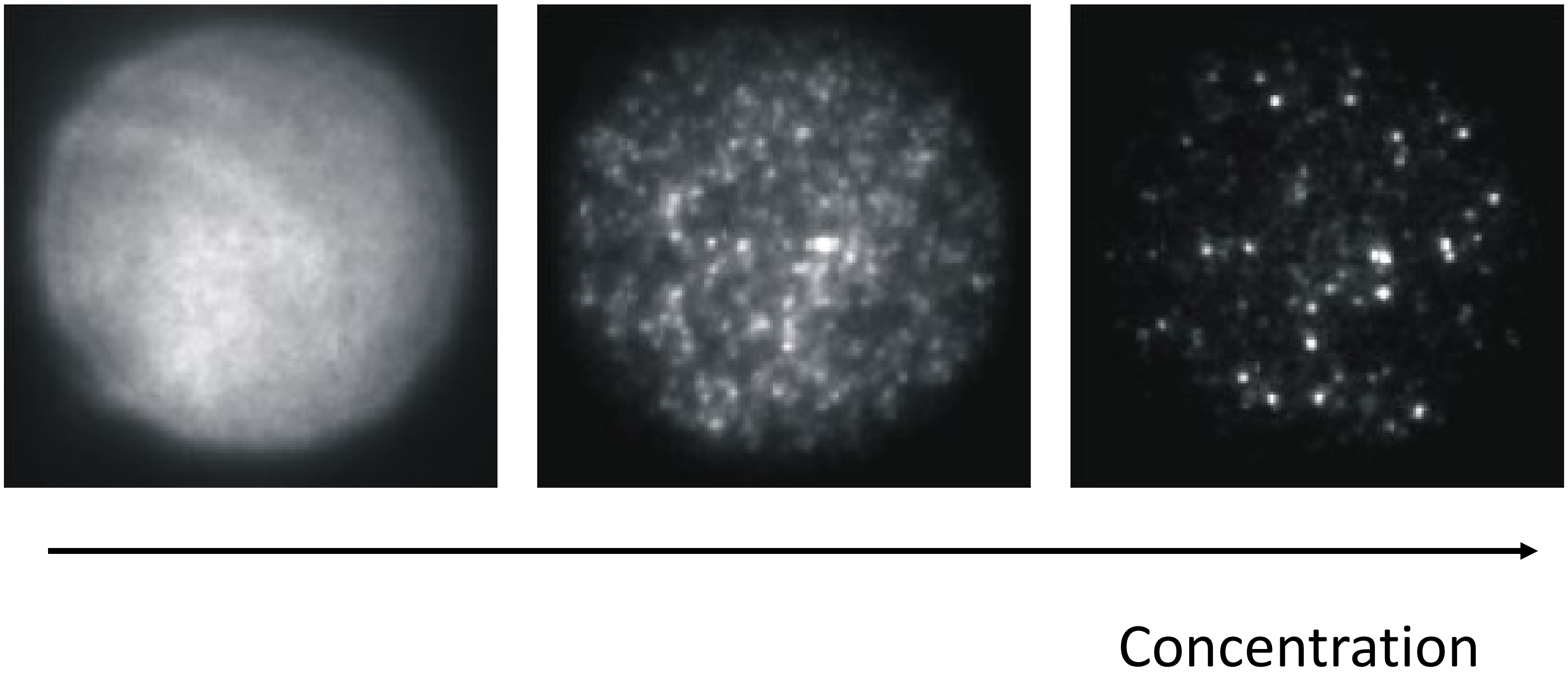




- Many biomolecules are fluorescent: flavines, NADH, FAD, chlorophyll.
- Cell autofluorescence is localized. Sometimes there is autofluorescence in the membrane. Most of all, the Golgi apparatus is usually very brillant showing small vesicles. In general stressed cells are more autofluorescent.

=> **Only a detailed analysis of the autofluorescence allows unambiguous statements about single-molecule measurements.**

2: Careful control of the concentration

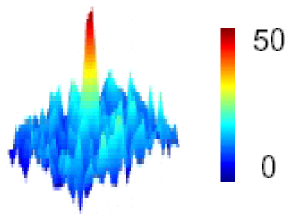


- Molecules of a dye (Rhodamine 6G) diluted in a polymer (PVA).

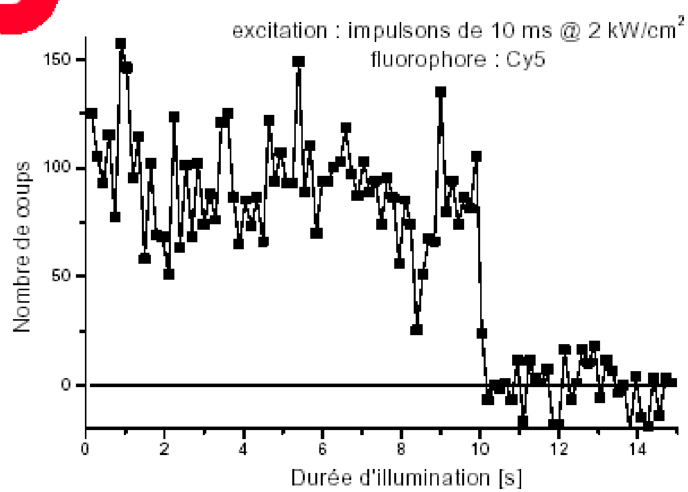
- It is important to have full control on the concentration of the fluophores.

3: Detected molecules must exhibit several characteristic features of single molecules

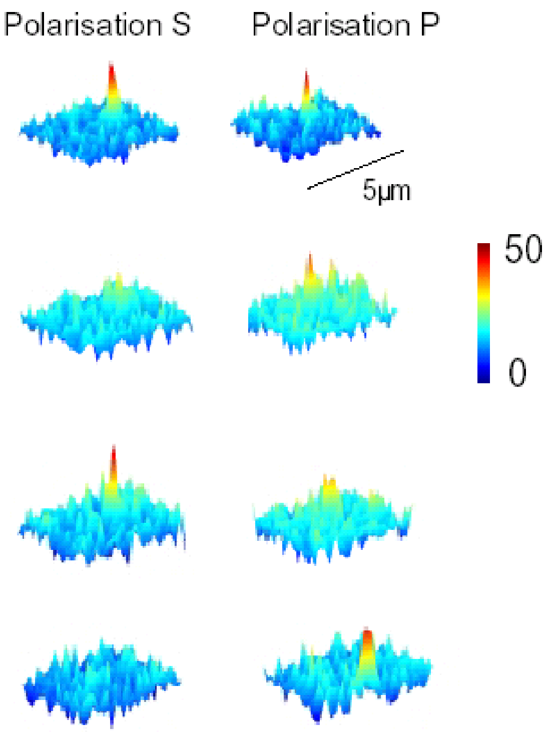
A **detected signal (typ. 30 counts/ms)**



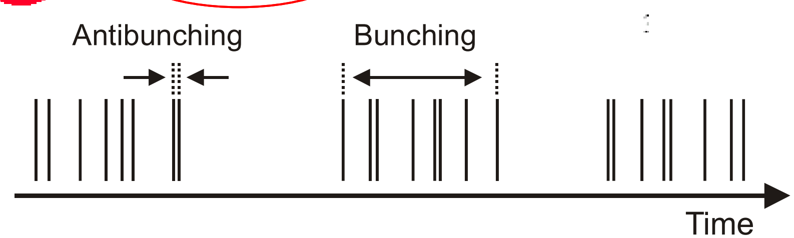
B **one-step photobleaching**



C **defined transition-dipole moment**

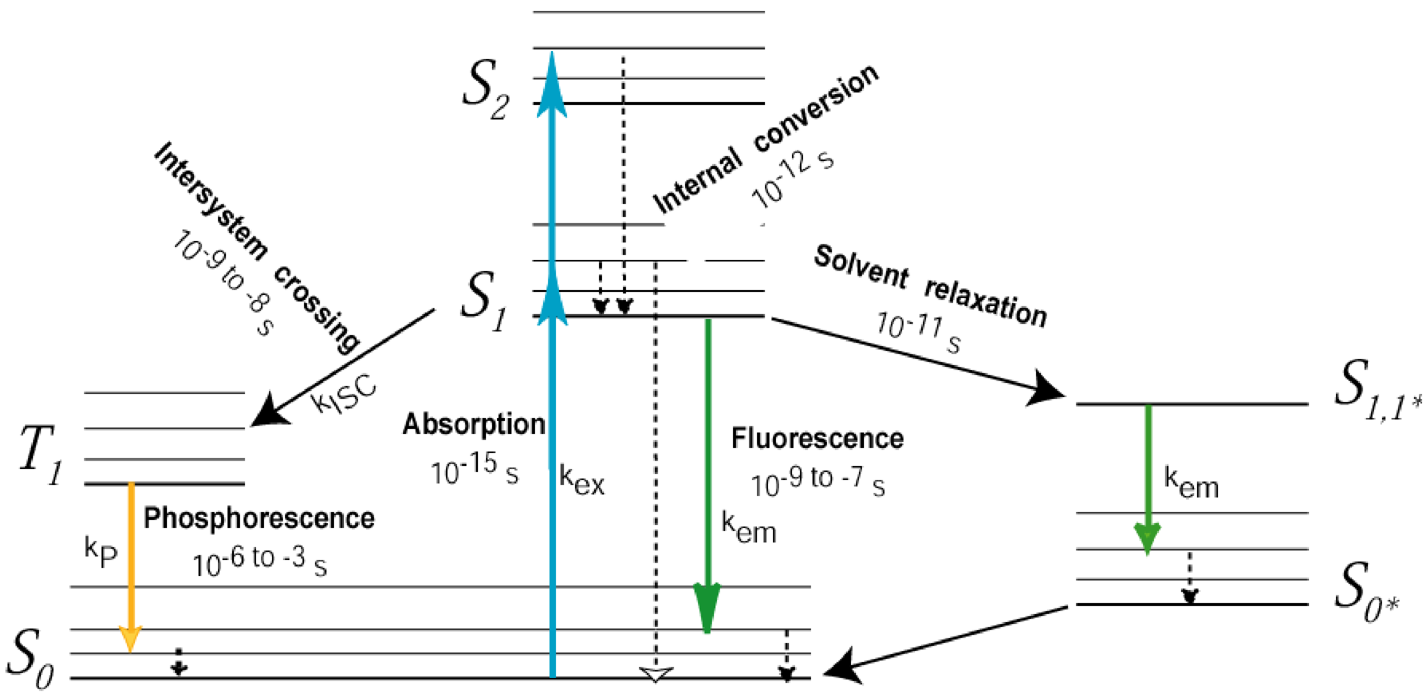


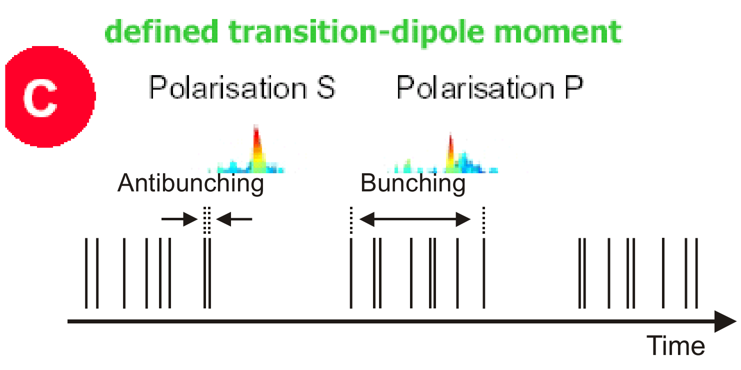
D **anti-bunching**



E **diffraction limited spot size**

3: Detected molecules must exhibit several characteristic features of single molecules

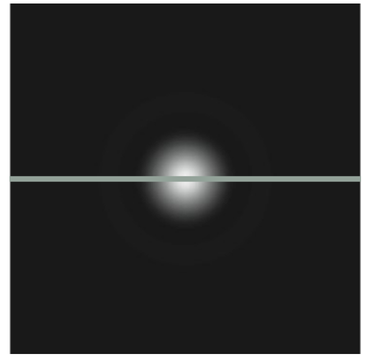


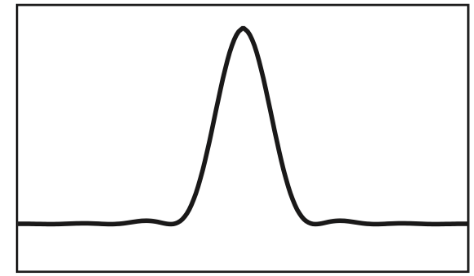


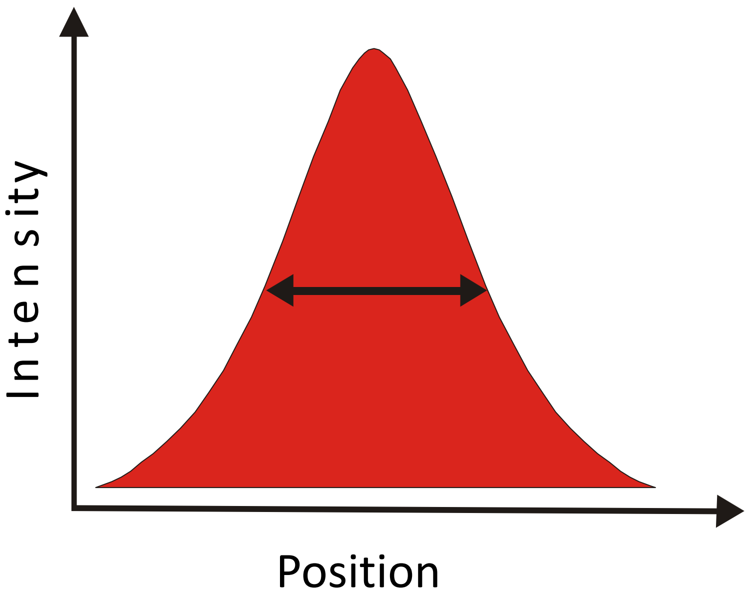
Anti-bunching is the "absolute" proof of the presence of single-molecule

Determination of the position of a single molecule

The size of the image of a single point photon emitter is limited by the diffraction







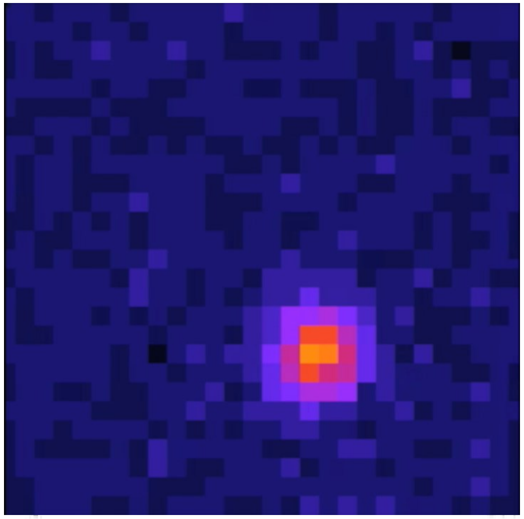
The image of a single molecule on the camera can be very well approximated by a gaussian of width ~0.5*λ.

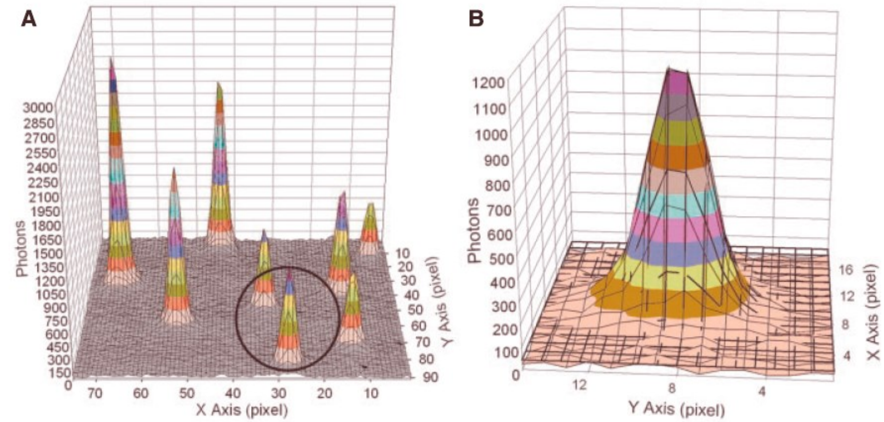
The precision of the localisation depends on the width of the PSF (s), the pixel edge length (a) the background noise (b) and the number of collected photons (N):

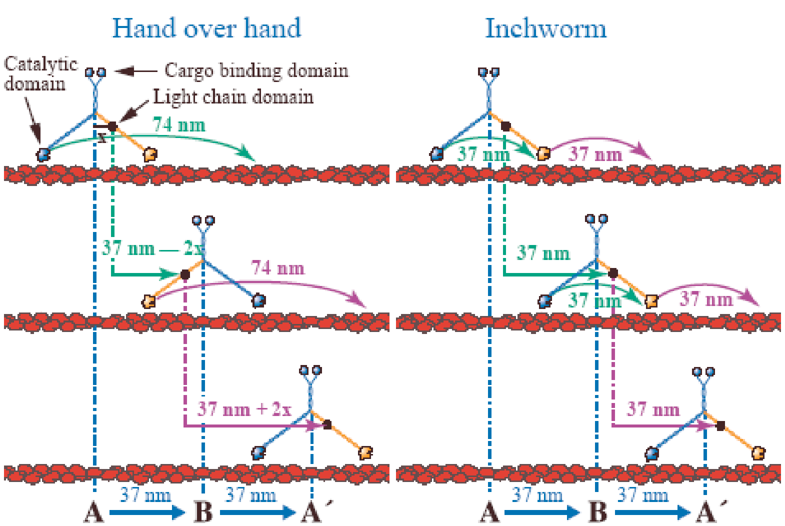
$$\sigma \approx \sqrt{\frac{s^2 + (a^2/12)}{N} + \frac{4\sqrt{\pi s^3 b^2}}{aN^2}},$$

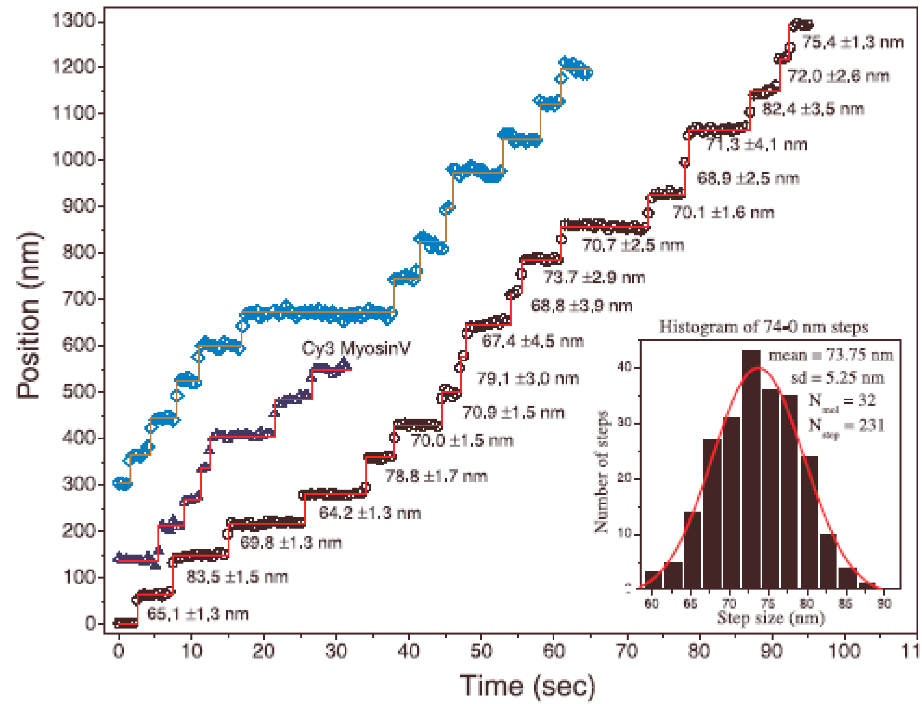
The experimental precision is a few tens of nanometer :
50 nm (fluorophore), 30 nm (organic dyes) and 5 nm (quantum dots)

Example: The motion of the molecular motor myosin V





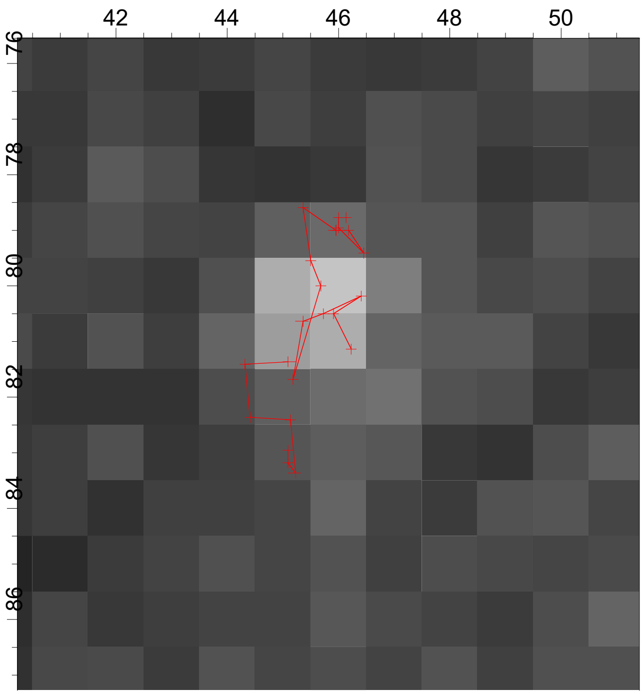


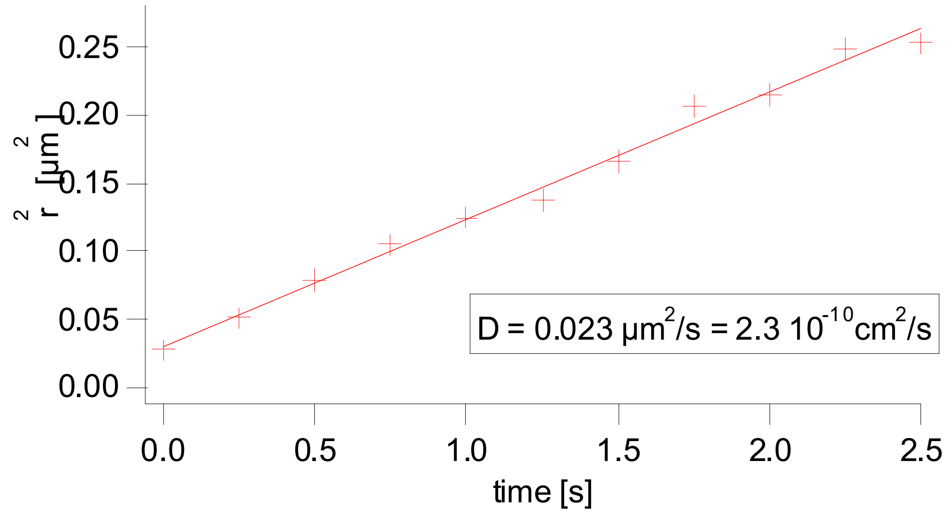


A. Yildiz, J.N. Forkey, S.A. McKinney, T. Ha, Y.E. Goldman, P.R. Selvin, *Science* **300**, 2061 (2003)

Single-molecule tracking

The mean square displacement (*MSD*) is calculated from the extacted trajectories





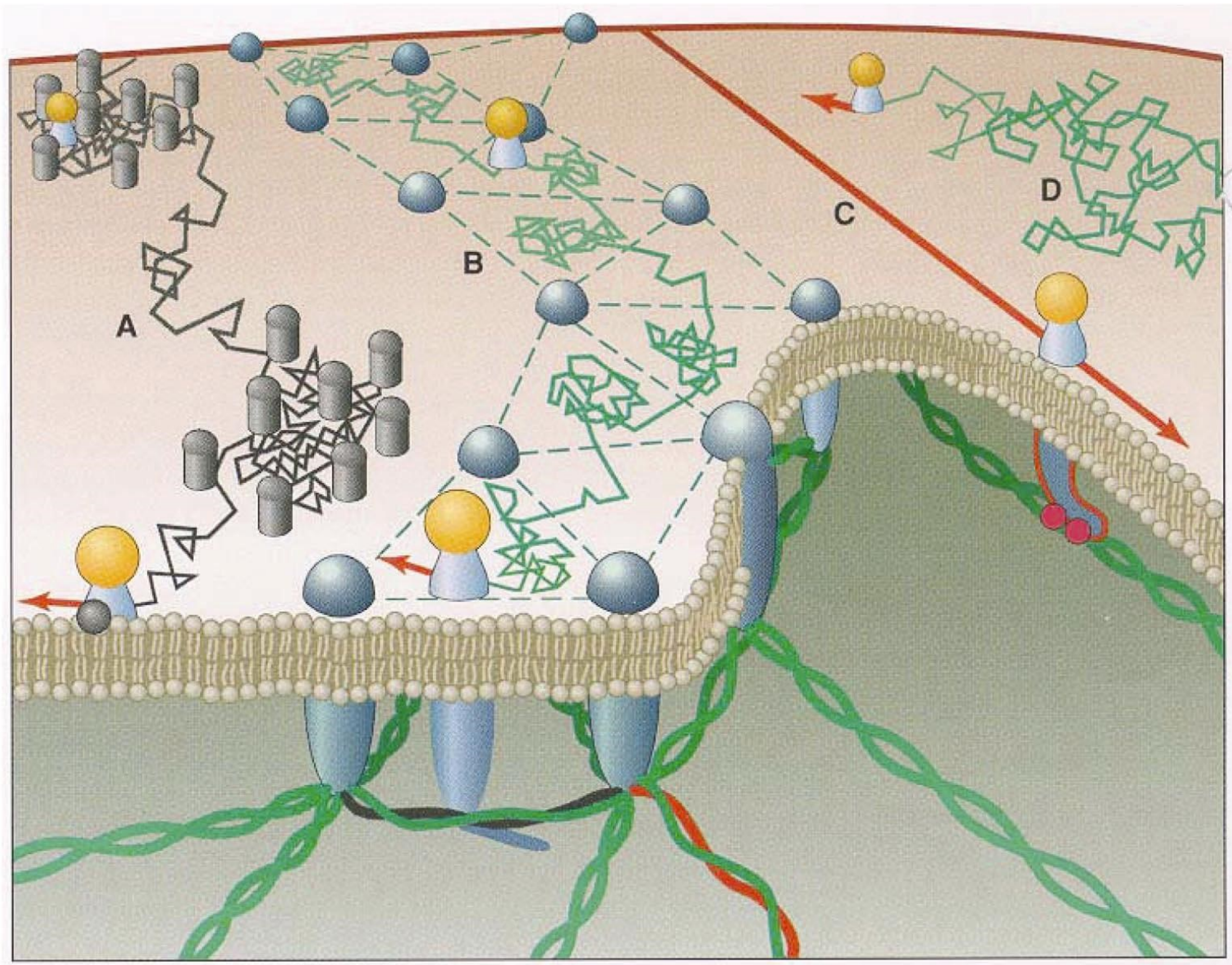
$$\mathrm{MSD}(n\delta t) = \frac{1}{N-1-n} \sum_{j=1}^{N-1-n} \{[x(j\delta t + n\delta t) - x(j\delta t)]^2 + [y(j\delta t + n\delta t) - y(j\delta t)]^2\},$$

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

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

Single-molecule tracking 2

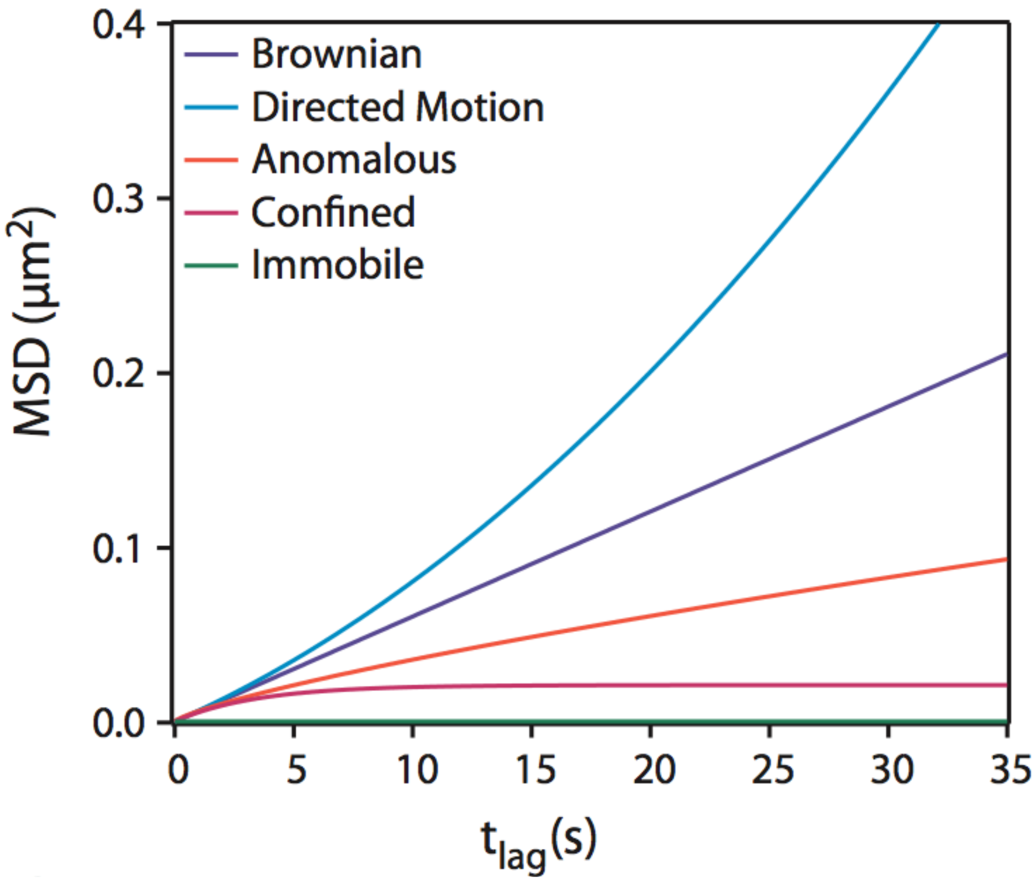
- Examples of trajectories: (A) Diffusion with obstacles, (B) Diffusion within corrals, (C) Directed motion, (D) Brownian motion



Jacobson, K., Sheets, E. D., & Simson, R. (1995). Revisiting the fluid mosaic model of membranes. *Science (New York, NY)*, *268*(5216), 1441–1442.

Single-molecule tracking 3

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



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

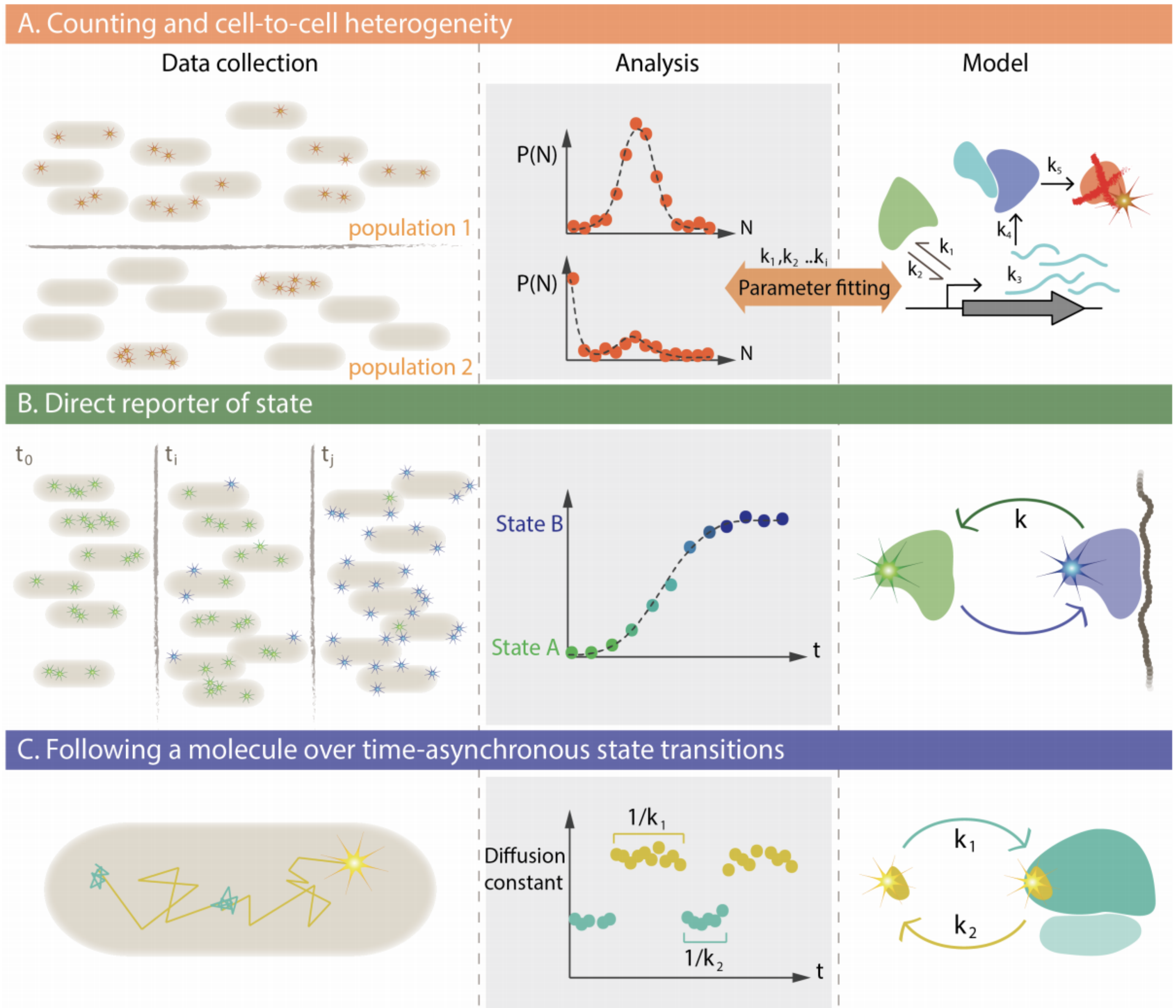
$$\mathrm{MSD}\left(t_{lag}\right) = 4Dt_{lag} + \left(\mathrm{v}t_{lag}\right)^2$$

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

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

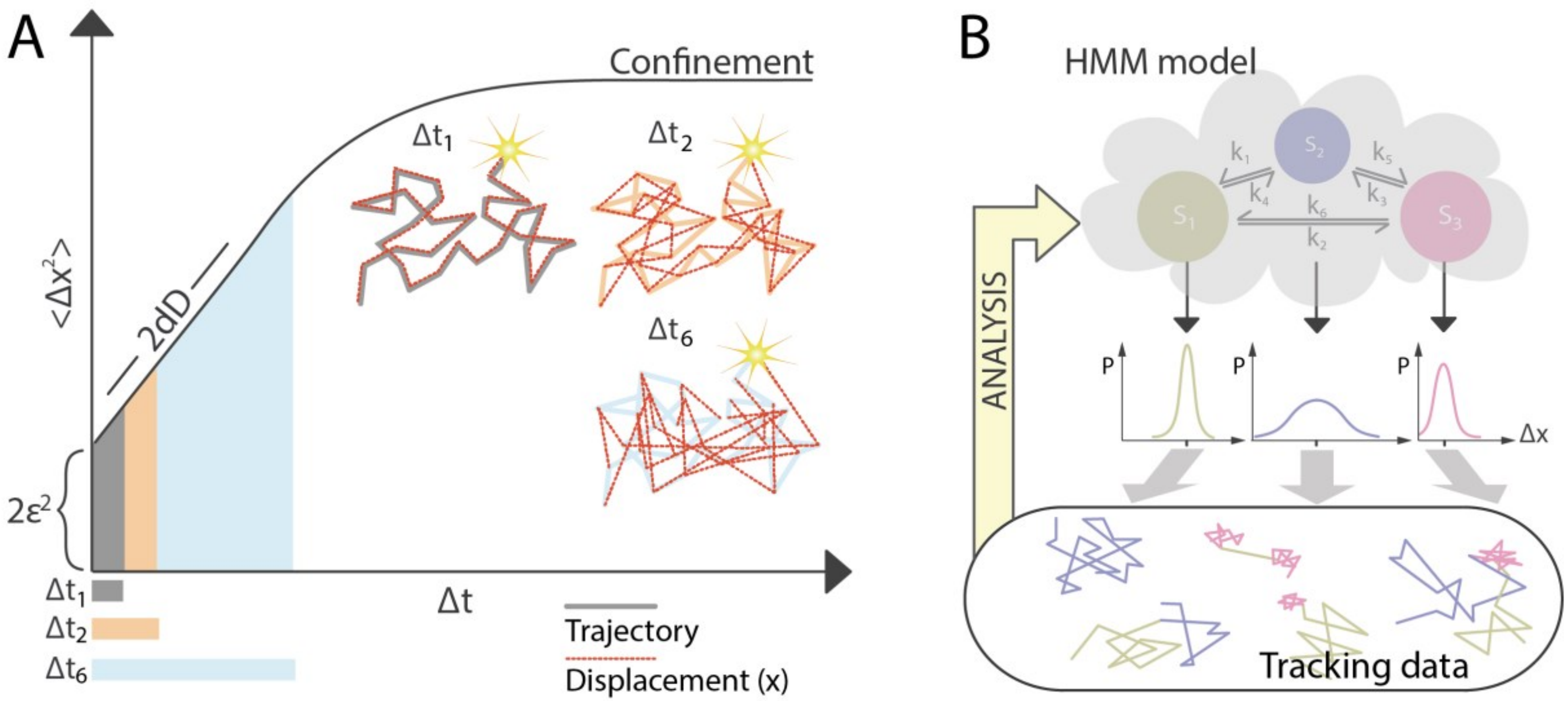
$$\text{noise} = 4Dt_{aq} + 2\sigma^2$$

Single-molecule tracking: Heterogeneity of states



J. Elf and I. Barkefors Single-molecule kinetics in living cell https://arxiv.org/pdf/1809.03265.pdf

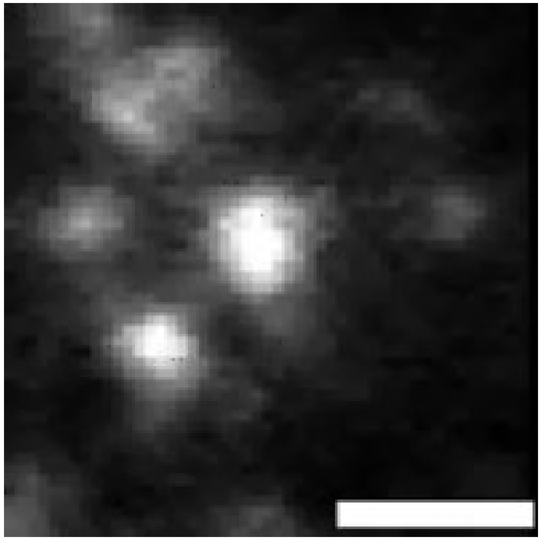
Single-molecule tracking: Heterogeneity of states

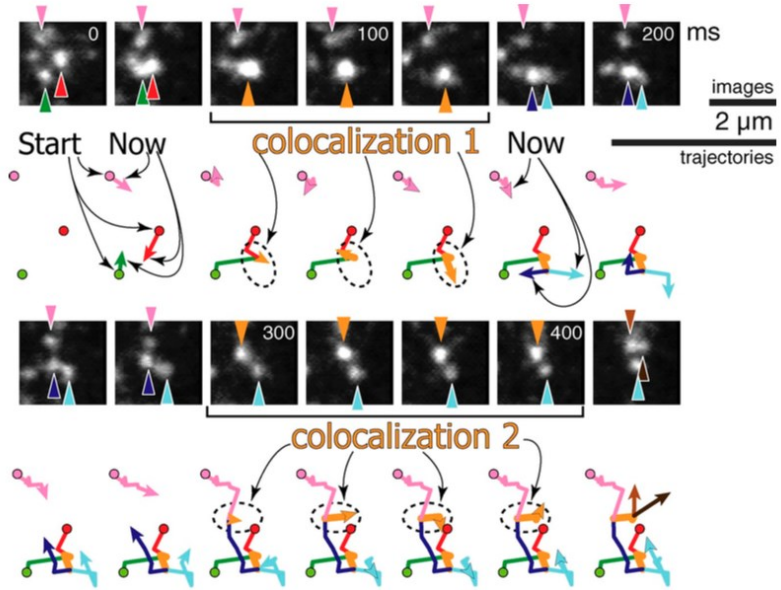


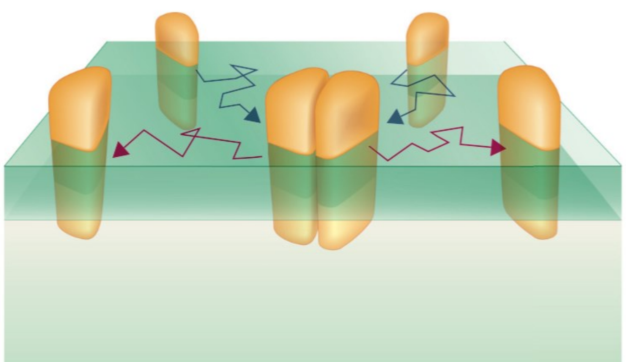
J. Elf and I. Barkefors Single-molecule kinetics in living cell https://arxiv.org/pdf/1809.03265.pdf

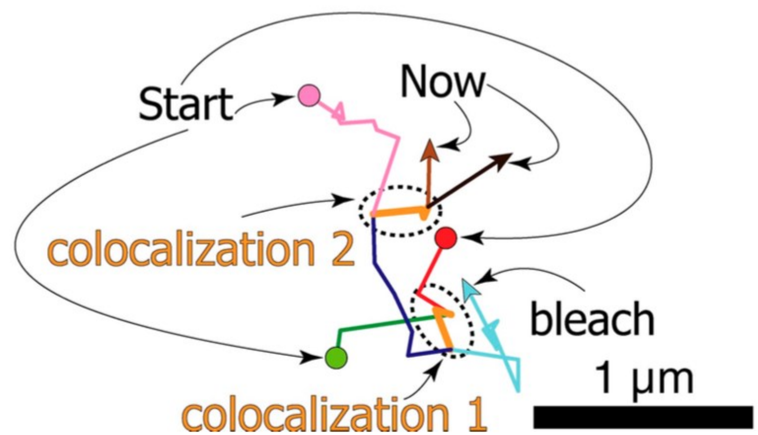
Single-molecule tracking: Interactions

Diffusion of N-formyl peptide receptor in CHO cells









Kasai, R. S., et al. (2011) *The Journal of Cell Biology*, *192*(3), 463–480. http://doi.org/10.1083/jcb.201009128

Kasai, R. S., & Kusumi, A. (2014). *Current Opinion in Cell Biology*, *27*, 78–86. http://doi.org/10.1016/j.ceb.2013.11.008