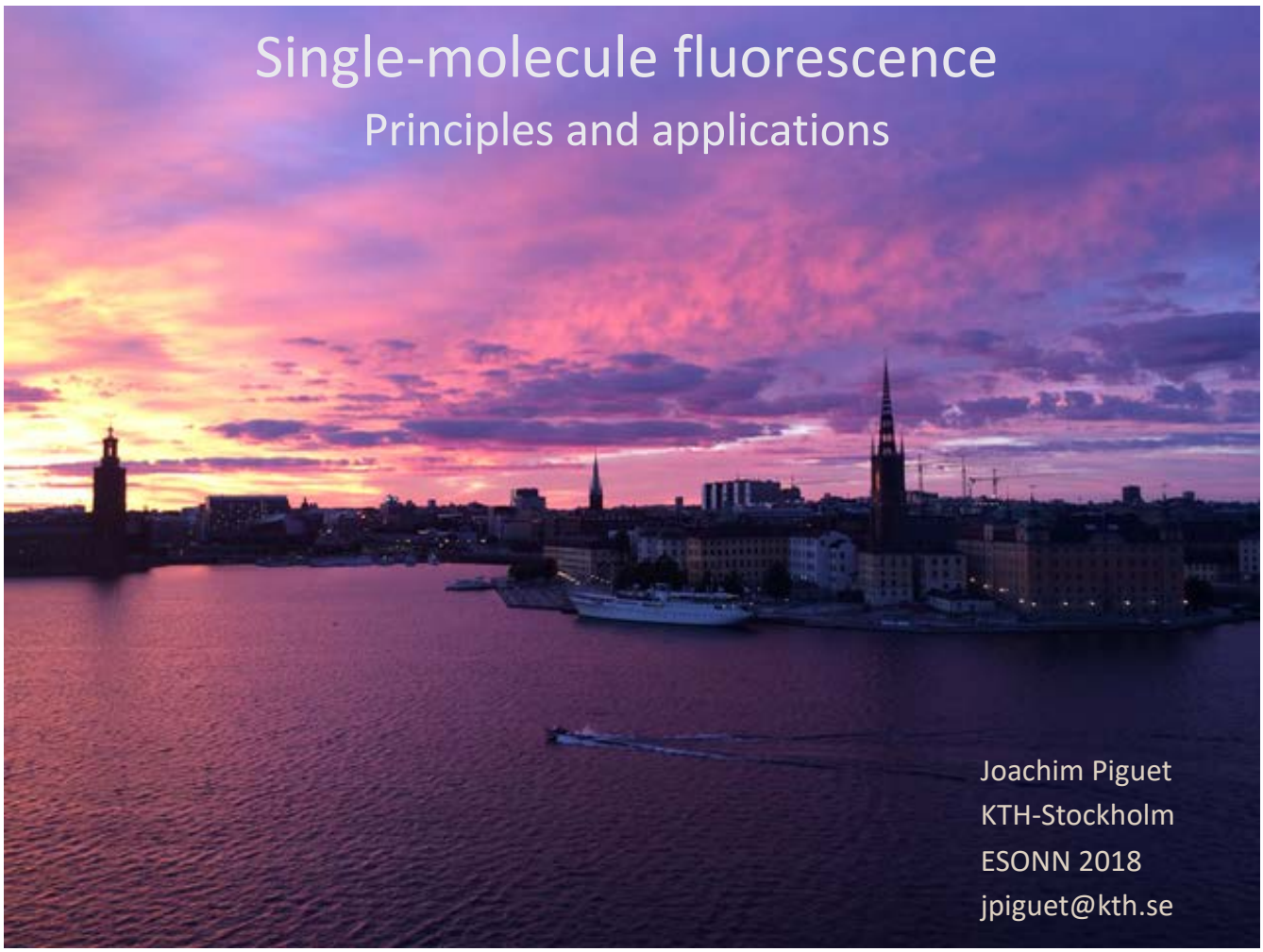


Course structure

A. Fundamentals
B. Microscopy and fluorescence imaging setup
C. Single-molecules imaging and tracking
D. Blinking
E. Fluorescence nanoscopy
F. Single-molecule detection and fluorescence signal correlation
G. Functional devices based on single-molecule
H. Useful litterature

Observing a nanomachine at work:
Single-molecule imaging or spectroscopy (SMI or SMS)

Advantages:

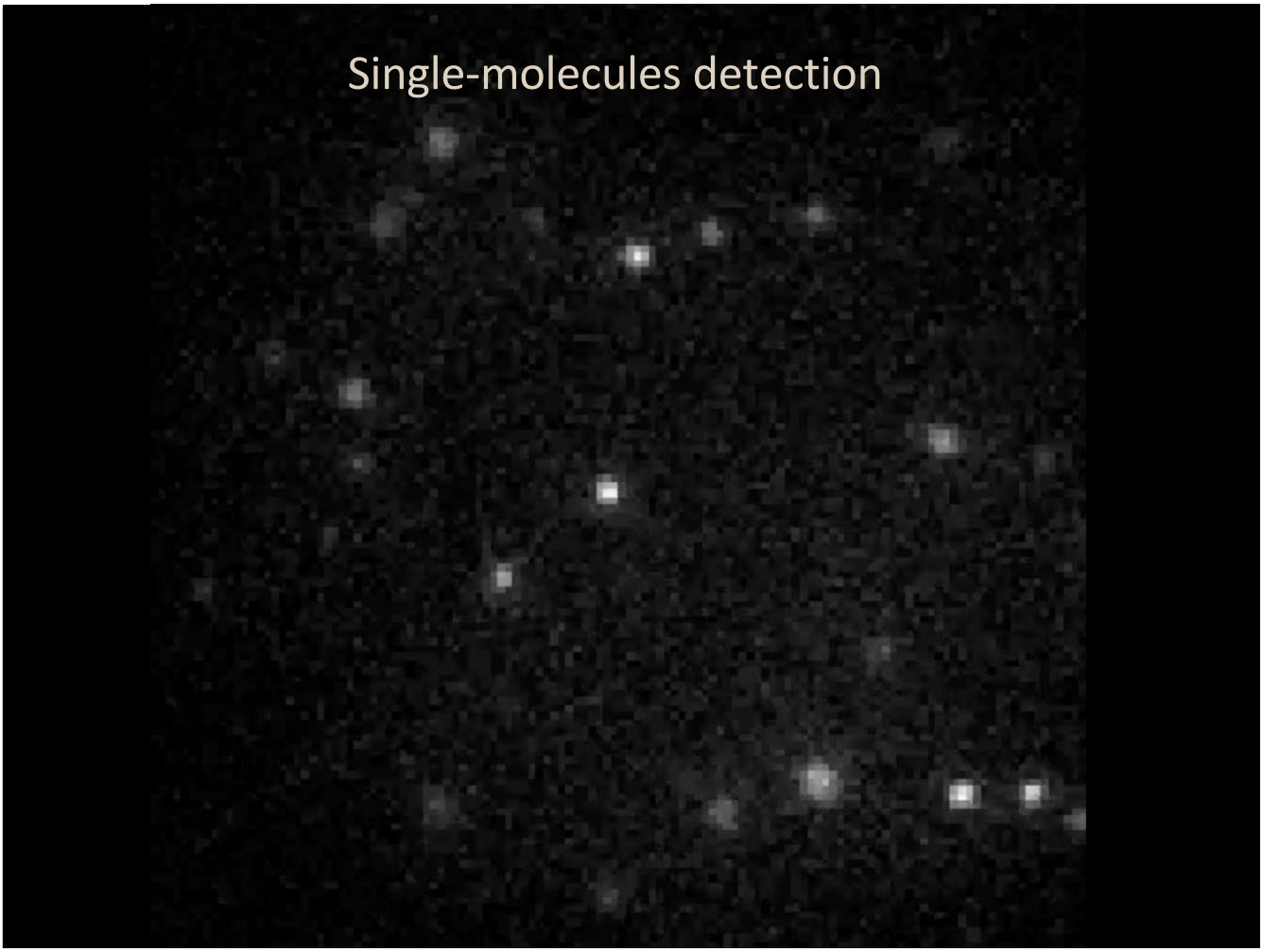
- Observation of the function and the motion of nano-objects in real-time in living systems
- Measurements on single molecule and not on ensemble of molecules
- Statistical analysis based on individual events
- Non-invasive and highly specific measurements
- Large choice of fluorophores and labels

Observing a nanomachine at work:
Single-molecule imaging or spectroscopy (SMI or SMS)

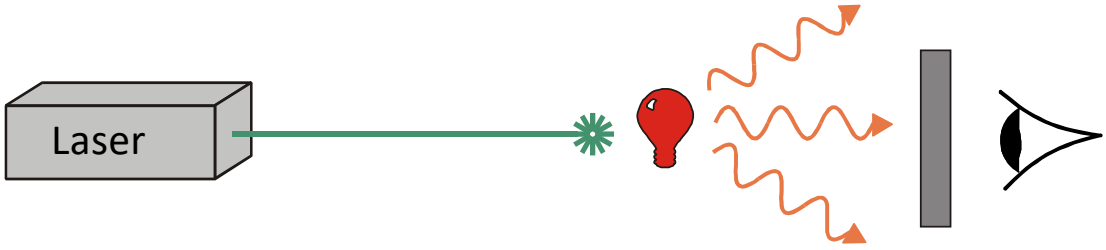
Concept:

- Control of the apparent dilution of the probe
  - Controlled labeling,
  - Photobleaching,
  - Photoactivatation,
  - Photoswitching,
  - Blinking,
  - Volume reduction,
  - Ground state depletion
  - Interference patterns

Single-molecules detection

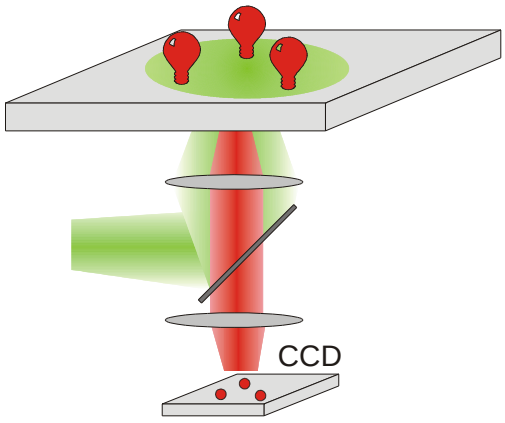


Measurement principle: Fluorescence microscopy

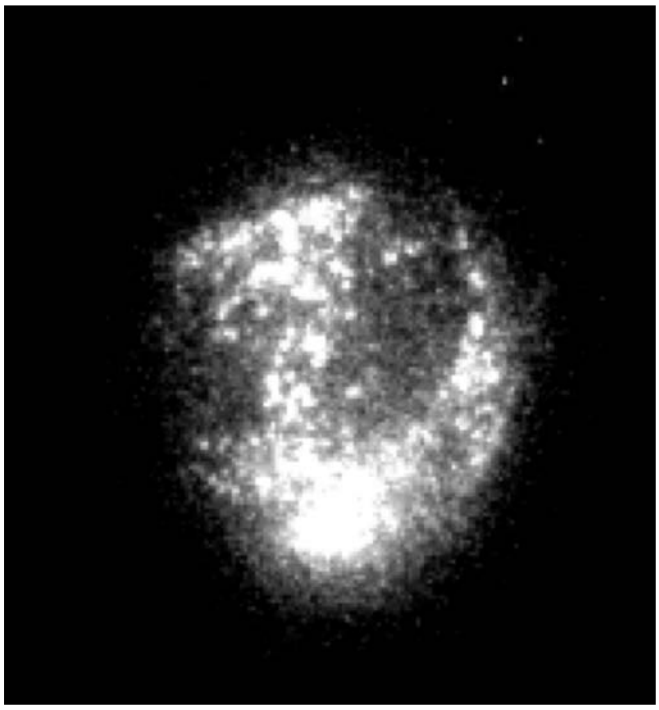


Single molecule measurements depends on the possibility to dilute the sample.

- 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

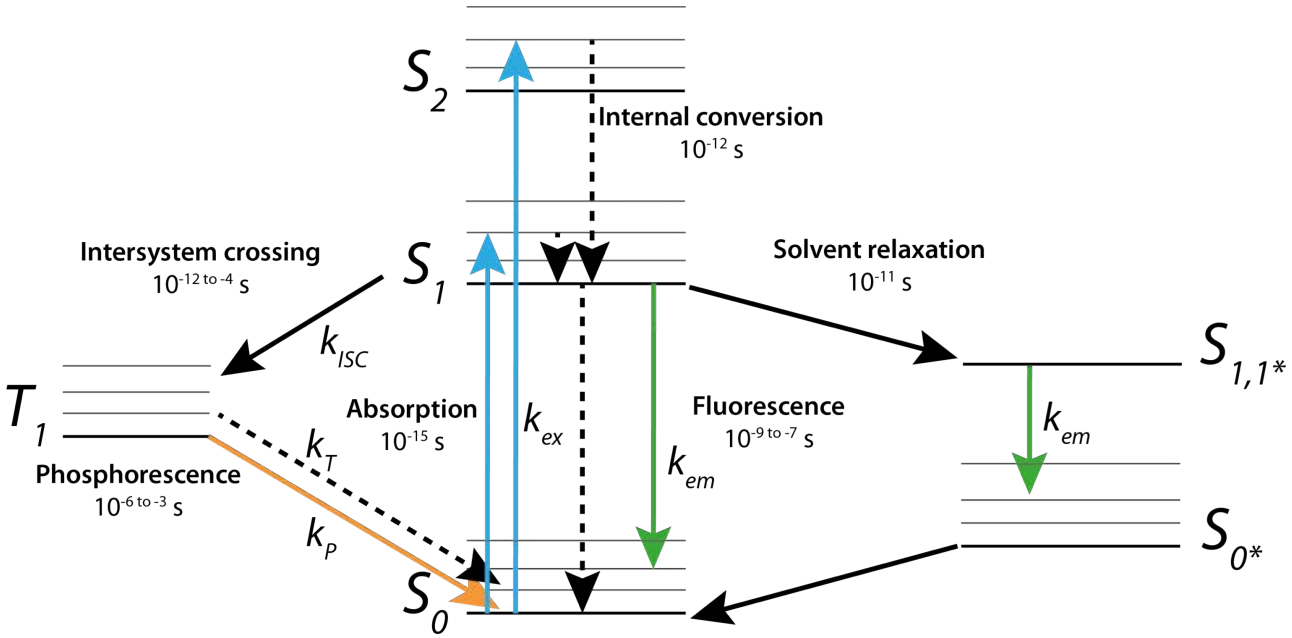


Benefits of single-molecule experiments



- No ensemble averaging
- No temporal averaging (no need to synchronize).
- Access to novel experimental parameters (in particular to a higher position accuracy).
- 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: Signal emitted by a single molecule

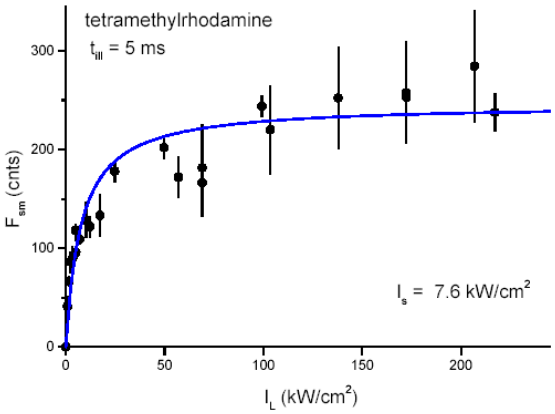
$$R(I_{ex}) = R_{max}\frac{I_{ex}/I_{sat}}{1+I_{ex}/I_{sat}}$$

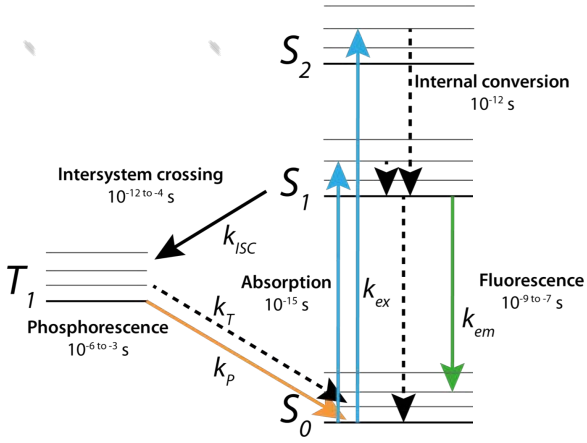
$$R_{max} = \frac{k_f q_f}{(1+k_{ISC}/k_T)}$$

$$I_{sat} = \frac{(k_f)}{\sigma\cdot(1+k_{ISC}/k_T)}$$

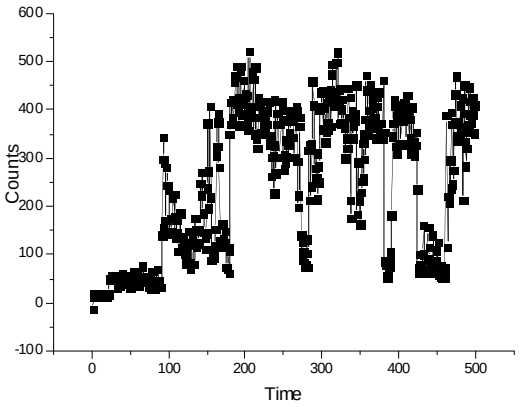
$$\sigma = 3\sigma_{av}\cos^2\theta$$

R = fluorescence emission rate,
I_{ex} = laser excitation intensity,
I_{sat} = saturation intensity,
k_T = triplet decay rate,
k_{ISC} = inter-system crossing rate,
s = absorption cross-section





Fundamentals: Fluorescence timetrace

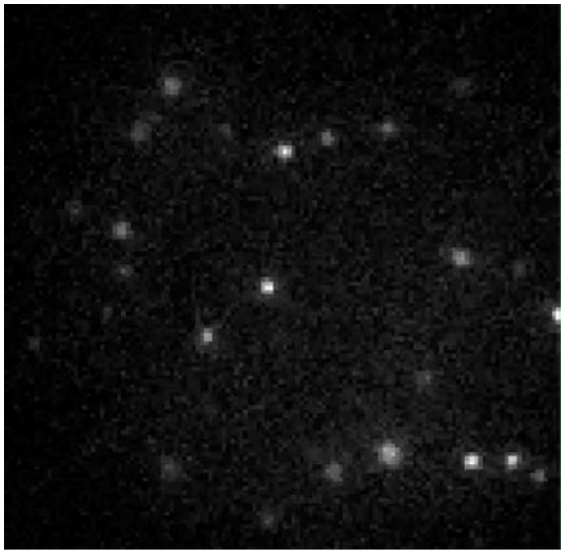


Blinking can have several origins:

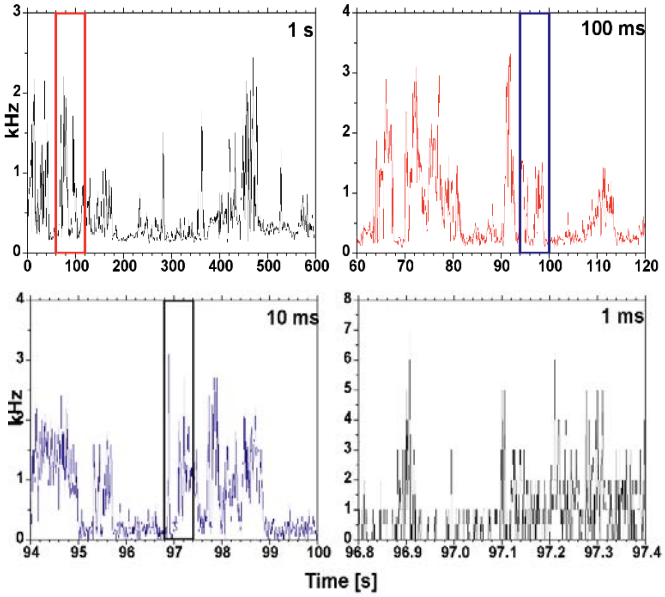
- Triplet and other dark states.
- Modifications in the environment: Changes of the spectrum or of the fluorescence quantum yield.
- Chemical reactions (e.g. protonation) or complex formations (with O_2).
- photo-ionisation.
- etc…

Fundamentals: Blinking of quantum dots

Blinking of semi-conductor nanoparticles (quantum dots) is very pronounced due to photo-ionisation



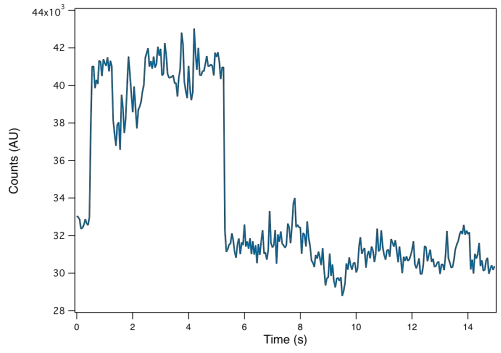
NK1 receptor (GPCR) labelled with Qdot 655 (Life technologies) on 293T cells



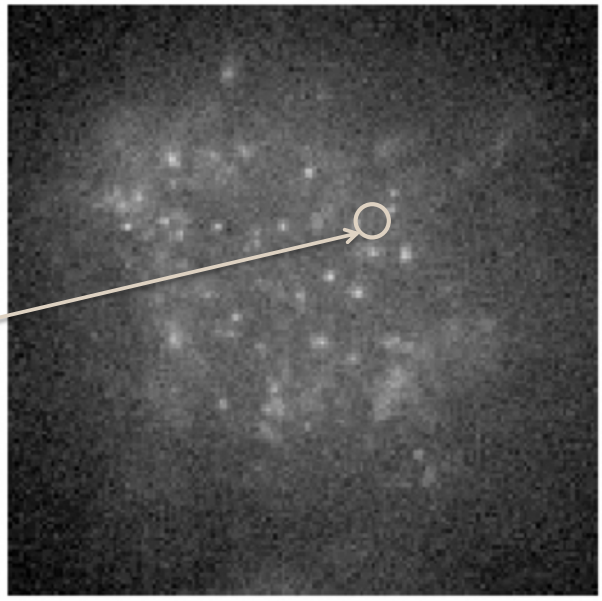
Blinking occurs at every timescale

Michalet, X, 2001 *Single Molecule, 2(4), 261–276.*

Fundamentals: Photobleaching



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



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

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

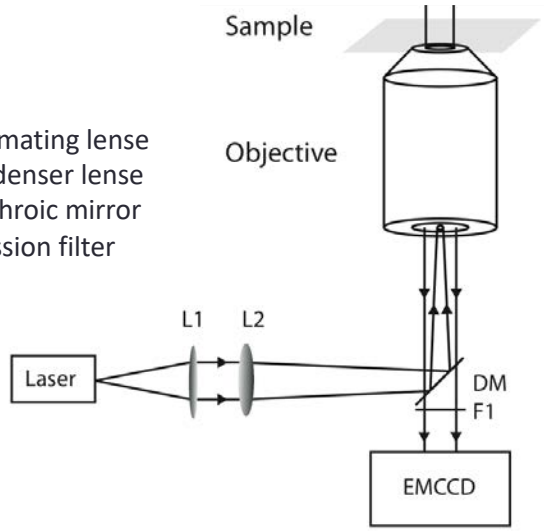
Typical fluorophores

Fluorophore	λ_{exc}	$\lambda_{em}{}^{max}$	QE	τ_f	I_s	k_∞	$\tau_{bl}{}^{\infty}$	N_{max}	ε	Brightness
	(nm)	(nm)	(in H_2O)	(ns)	(kW/cm^2)	(kcnts/ms)	(ms)	(10^6 photons)	($M^{-1}cm^{-1}$)	Rel to EGFP
fluorescein	488	520	0.71	-	-	-	-	< 0.1	68000	146
TMR	514	580	0.28	2.1	5.6±1.6	4.0±1.	11.5±2.5	0.6±0.2	95000	81
Cy3	532	568	0.14	~1	-	-	-	-	150000	64
Cy5	630	670	0.18	~1	2.2±1.0	2.3±0.3	14.8±0.3	0.67±0.11	250000	136
Atto488	501	523	0.80	4	-	-	-	-	90000	218
Atto565	563	592	0.90	4	-	-	-	-	120000	327
STAR635	634	654	0.51	2.8	-	-	-	-	63000	97
QF640R	630	670	-	-	-	-	-	-	105000	295
Atto647N	644	669	0.65	3.5	-	-	-	-	150000	295
Alexa647	650	665	0.33	1	-	-	-	-	237000	237
ECFP	439	476	0.40	-	-	-	-	-	32500	39
mTurquoise	434	474	0.84	3.7	-	-	-	-	30000	76
EGFP	488	512	0.60	3.2	13±3	2.9±0.2	2.8±0.2	0.14±0.05	55000	100
EYFP	514	527	0.61	3.7	9±2	3.1±0.3	2.6±0.1	0.14±0.05	83400	154
mCitrine	516	529	0.76		-	-	-	-	77000	177
mCherry	587	610	0.22	1.46	-	-	-	-	72000	48
mPlum	590	649	0.10	~1	-	-	-	-	41000	12
PA-GFP d	400	515	0.13	-	-	-	-	-	20700	8
PA-GFP a	504	517	0.79	-	-	-	-	-	17400	42
PA-mCherry a	564	595	0.46	-	-	-	-	-	18000	25
Dronpa (G)	503	517	0.85	-	-	-	-	-	95000	245
mEOS2 (G)	506	519	0.74	-	-	-	-	-	56000	126
mEOS2 (R)	573	584	0.66	-	-	-	-	-	46000	92

Far-field microscopy imaging setups

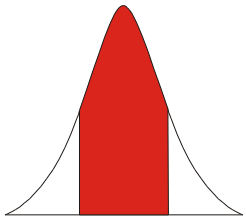
Set-up: Wide-field laser microscopy

- 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

Laser illumination:
Truncated gaussian profile



Resolution:

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

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

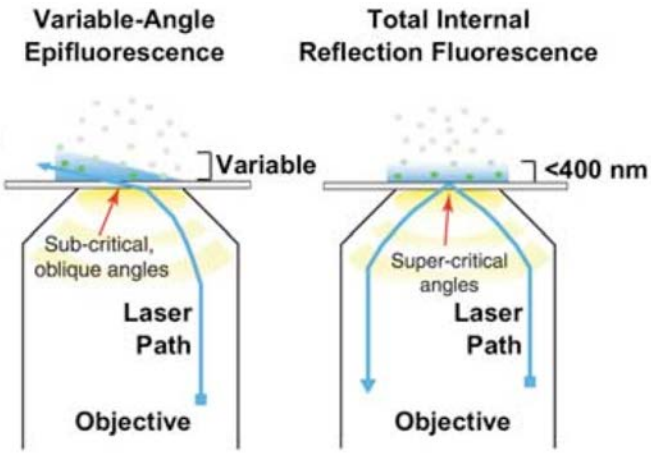
$$\varnothing_{\text{illumination}} = \frac{f_{\text{objective}}}{f_{\text{condenser}}}\varnothing_{\text{aperture}}$$

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

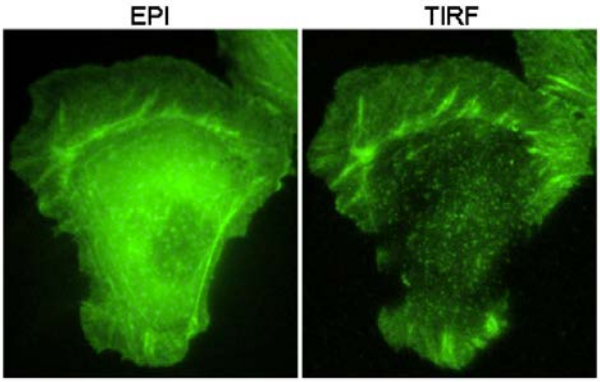
Set-up: Total-Internal Reflection Fluorescence (TIRF) Microscopy

The illumination incident light is totally reflected

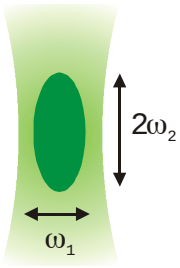
-> An evanescent wave illuminate only a few hundreds on nanometer



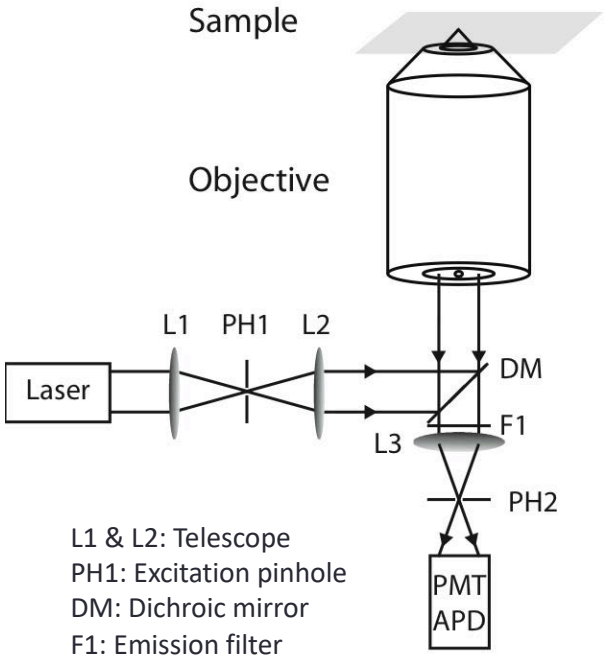
www.photonics.com



Set-up: Confocal laser scanning microscopy

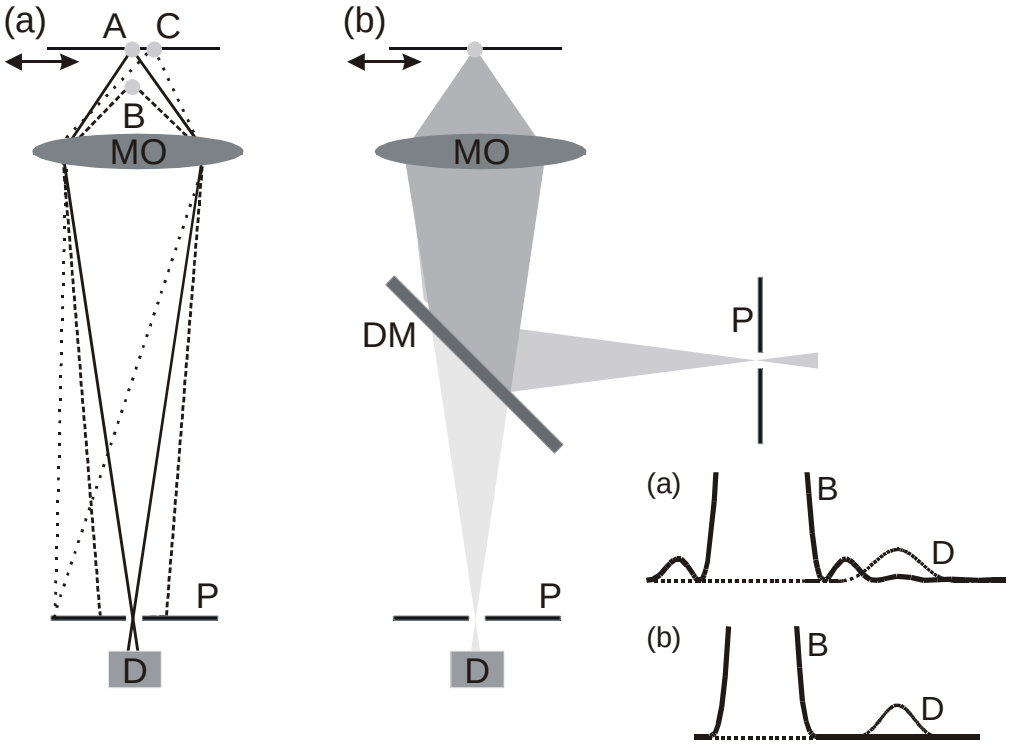


- Confocal illumination: Only a **small volume** (the confocal volume) is efficiently detected and illuminated.
- An image is obtained by **scanning** the sample and recording the fluorescence intensity as a function of the position.
- Slow measurement, high contrast
- Detection using highly-sensitive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



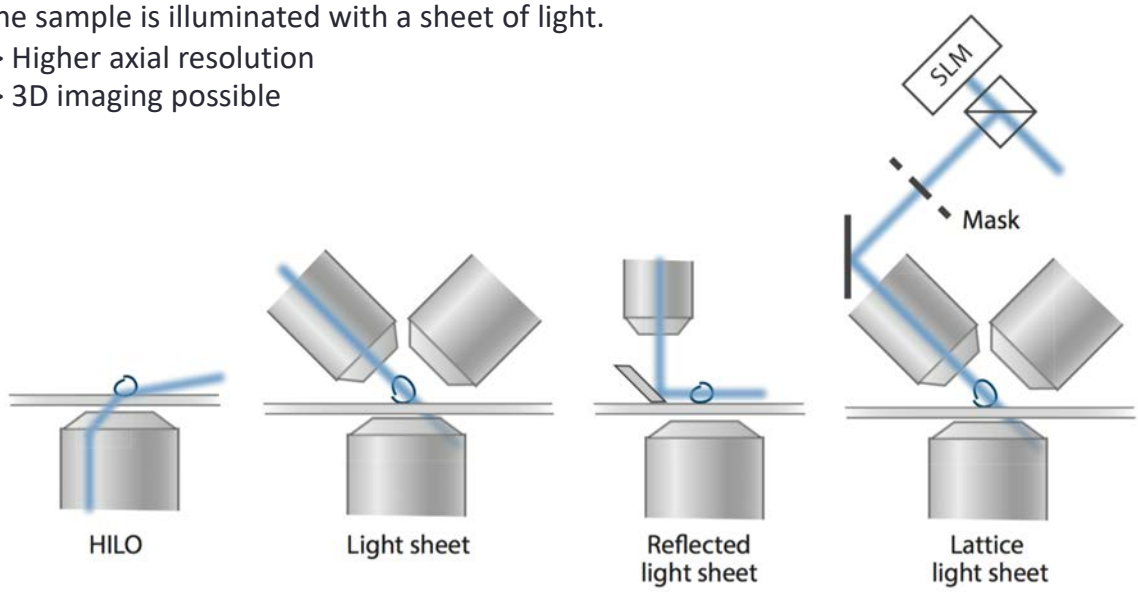
L1 & L2: Telescope
PH1: Excitation pinhole
DM: Dichroic mirror
F1: Emission filter
L3: Focusing lense
PH2: Emission pinhole

Set-up: Confocal laser scanning microscopy: principle



Set-up: Light-sheet illumination

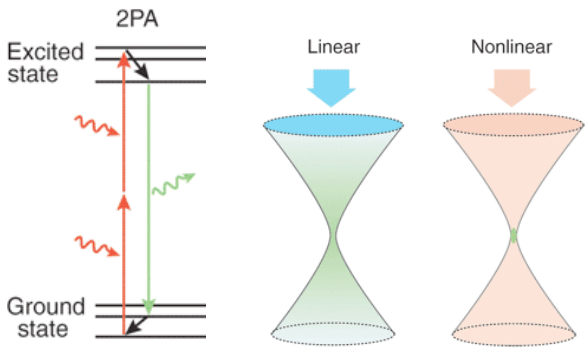
The sample is illuminated with a sheet of light.
-> Higher axial resolution
-> 3D imaging possible

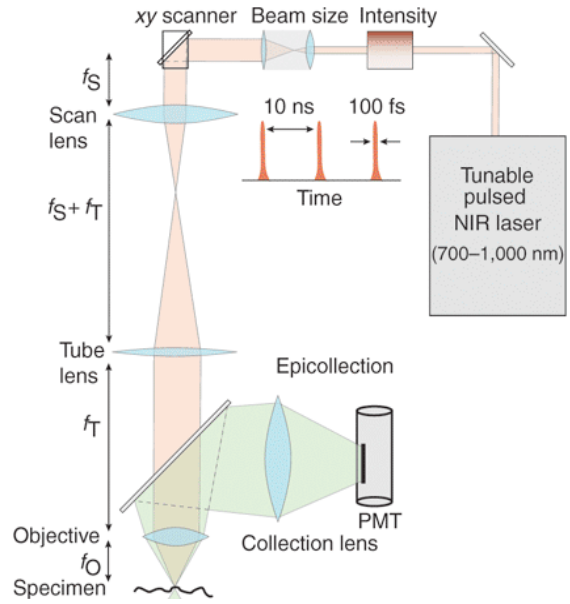


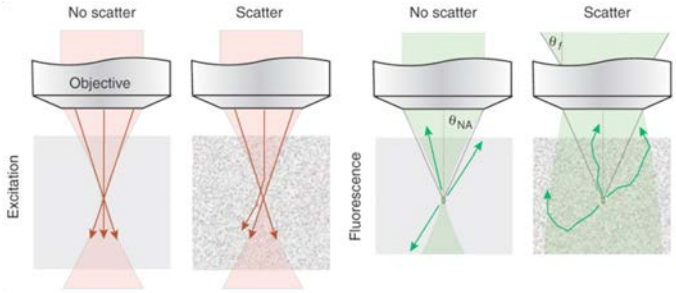
Set-up: Two-photons microscopy

The fluorophore is excited by two consecutive photons of half of the electronic transition energy

- The photon density is sufficient only close to the focus
- Confocal-like excitation volume
- The scattering of the surrounding is lower at higher wavelength. The signal to noise increases
- It is possible to measure deeper in tissues.

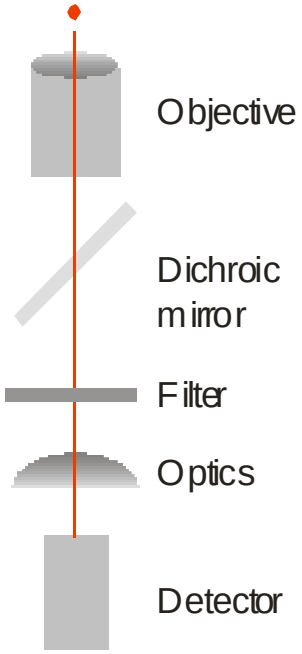






Helmchen, F., & Denk, W. (2005). *Nature methods, 2(12), 932–940*

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

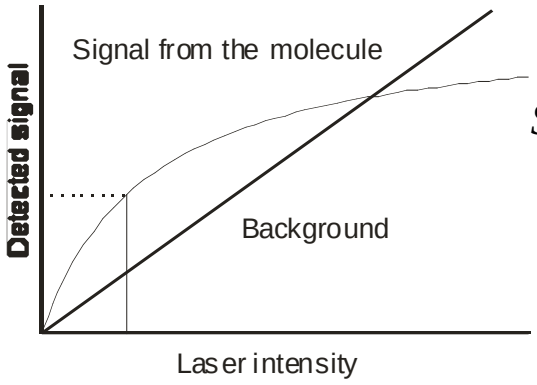
Filters and dichroics: η_{fil}=40%

Optics: η_{opt}=90%

Detector: η_{det}=50%

In total: η_{tot}=5%

Set-up: Signal/noise ratio



$$SNR=\frac{\eta_{det}RT_{int}}{\sqrt{\eta_{det}RT_{int}+C_bPT_{int}+N_dT_{int}}}$$

T_{int}=integration time

C_bP=background

N_d=noise from the detector

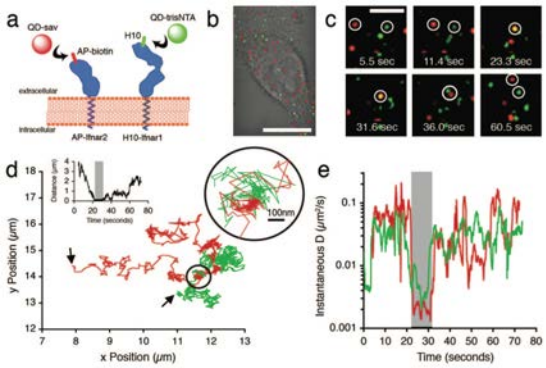
Noise sources

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

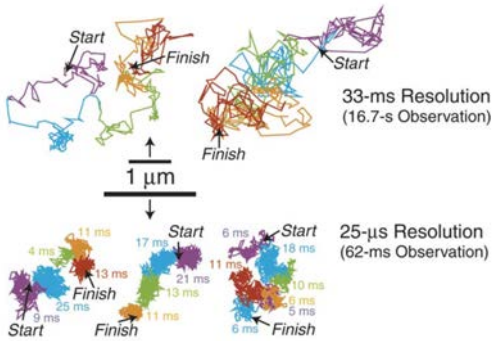
Background

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

Practically: It is best to work **slightly below I_{sat}**. Reduce as much as possible the autofluorescence. 100-200 detected counts from the molecule may be sufficient to get an SNR of 8-10 (5-10 ms measurement time). The effective number of images that can be recorded depends on the photo-bleaching.

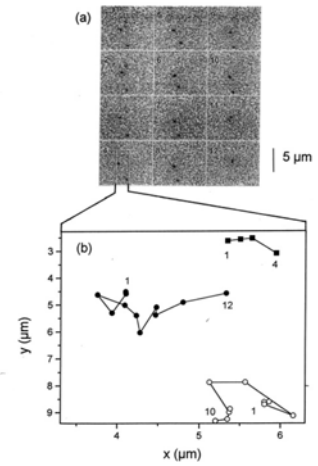


Roullier V et al. (2009) *Nano Lett*



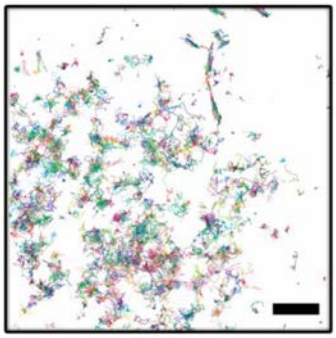
Kusumi A et al. (2005) *Semin Immunol* 17:3–21

Single-molecule imaging and tracking



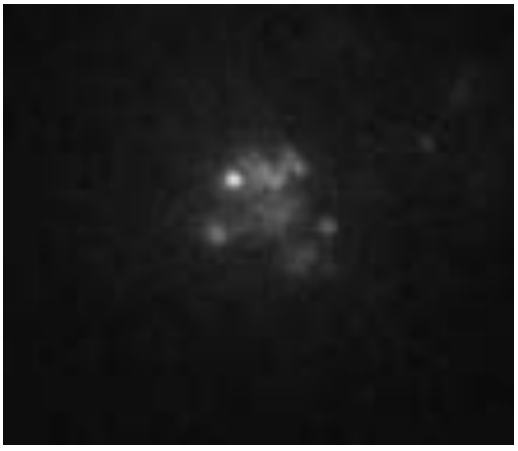
Schmidt T et al.(1995) *Journal of Physical Chemistry* 99:17662–17668

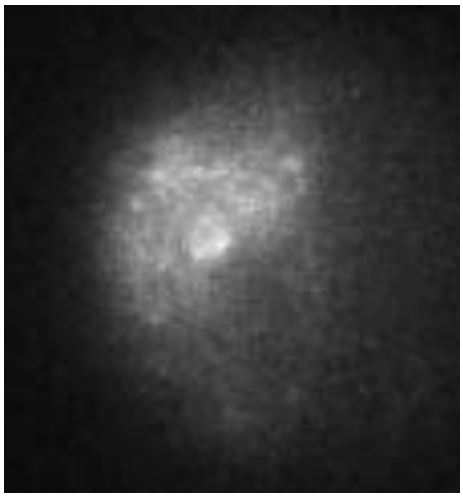
Manley S et al. (2008). *Nat Methods* 5:155–157



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

Cell autofluorescence

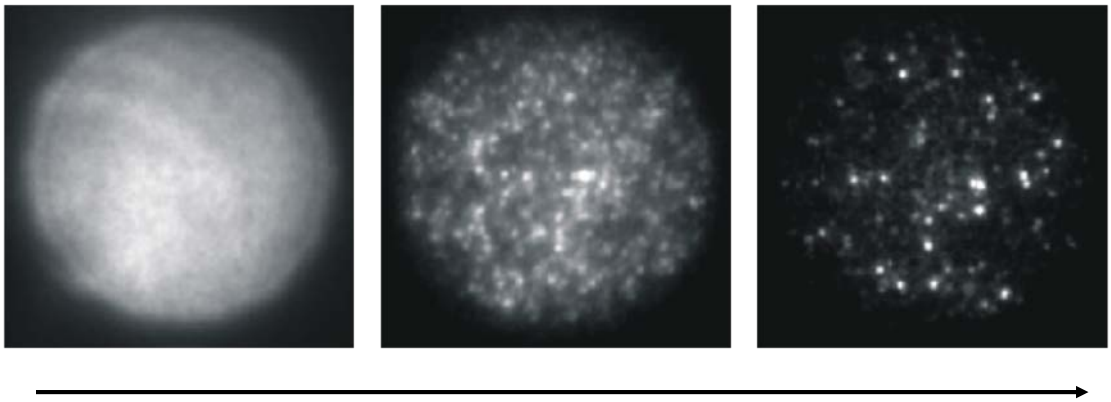




- Many biomolecules are fluorescent: flavines, NADH, FAD, chlorophyll.
- Cell autofluorescence is localized. Sometimes there is autofluorescence in the membrane. Most of all, the Golgi apparatus is usually very brillant showing small vesicles. In general stressed cells are more autofluorescent.

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

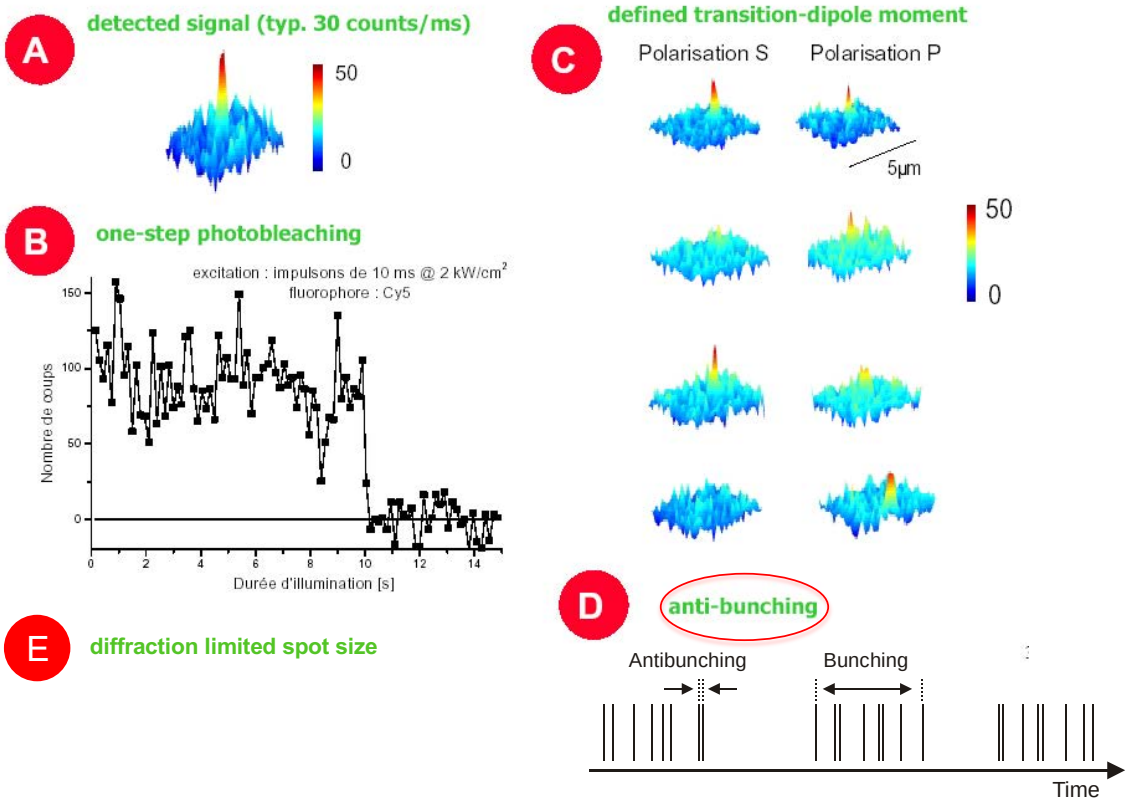
2: Careful control of the concentration



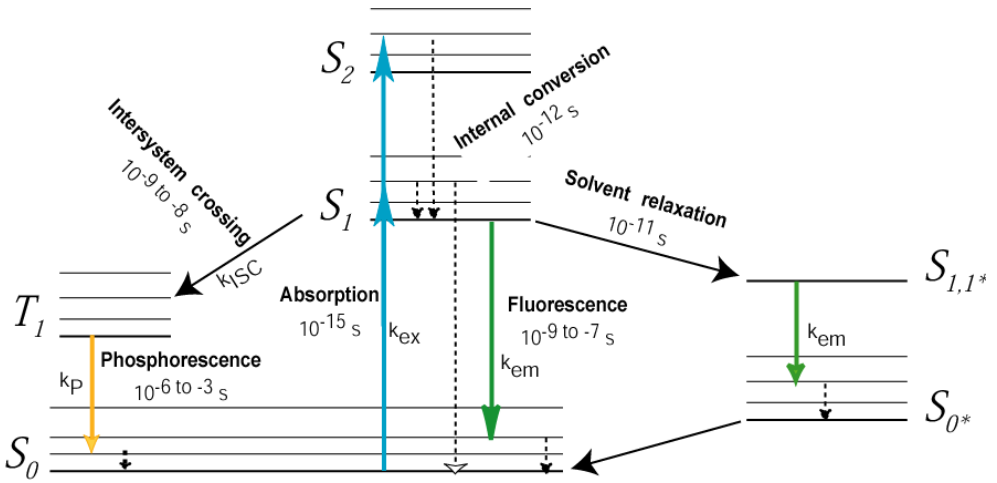
Concentration

- 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

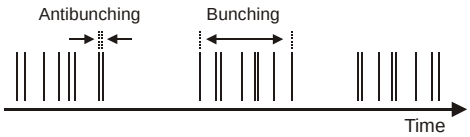


3: Detected molecules must exhibit several characteristic features of single molecules



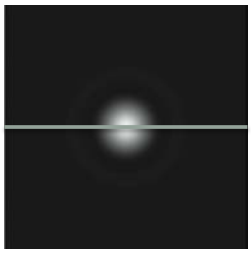
D anti-bunching

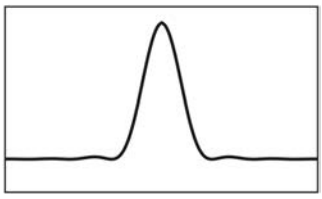
Anti-bunching is the "absolute" proof of the presence of single-molecule

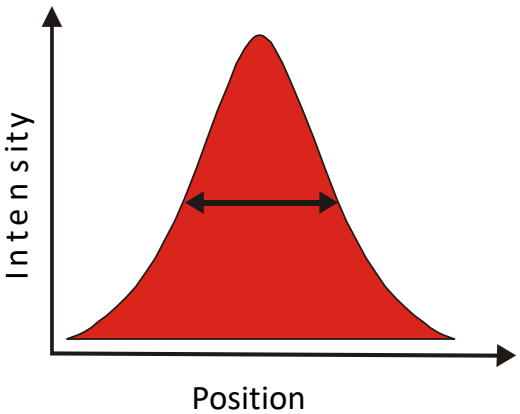


Determination of the position of a single molecule

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







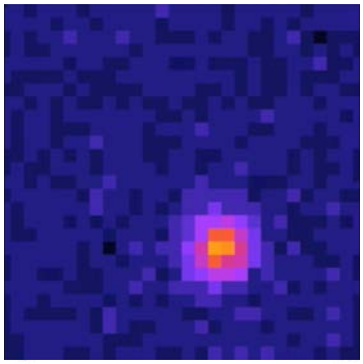
The image of a single molecule on the camera can be very well approximated by a gaussian of width ~0.5*λ.

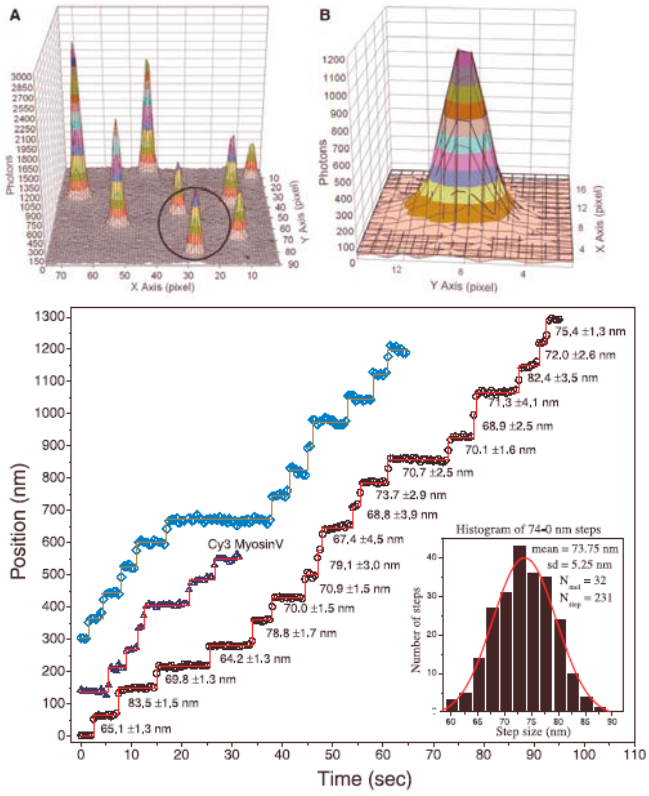
The precision of the localisation depends on the width of the PSF (s), the pixel edge length (a) the background noise (b) and the number of collected photons (N):

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

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

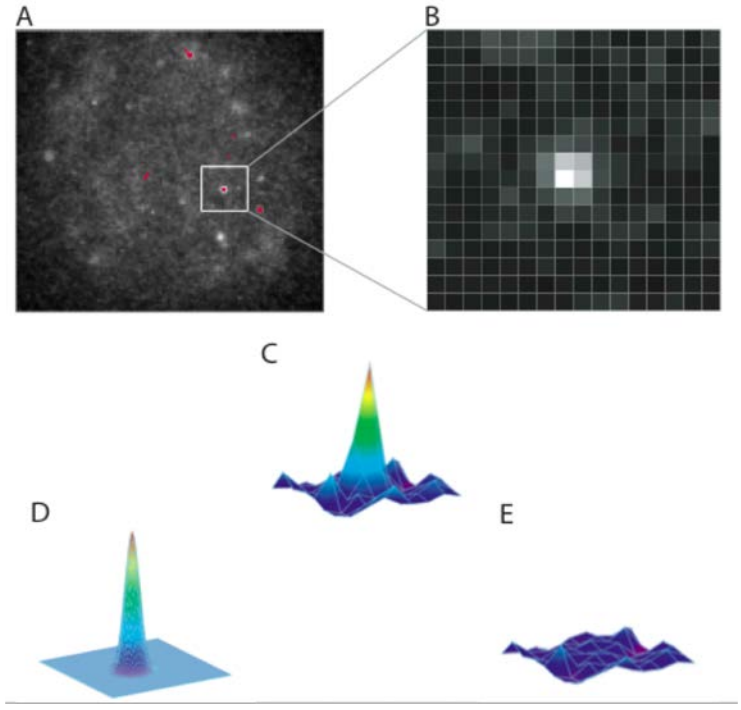




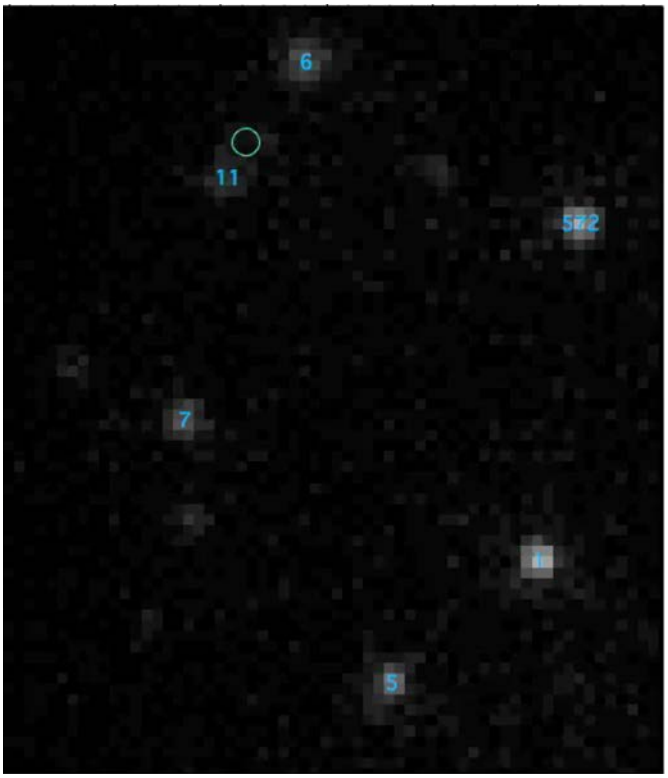
A. Yildiz, J.N. Forkey, S.A. McKinney, T. Ha, Y.E. Goldman, P.R. Selvin, *Science* **300**, 2061 (2003)

Single-molecule tracking fluorescent peaks

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



Single-molecule tracking movie

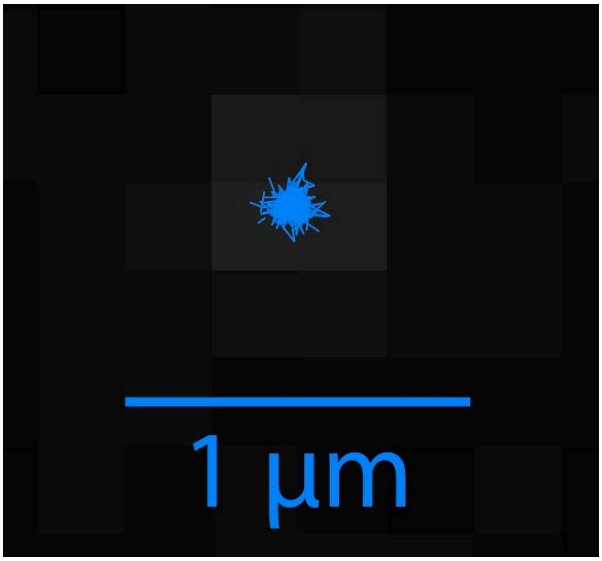


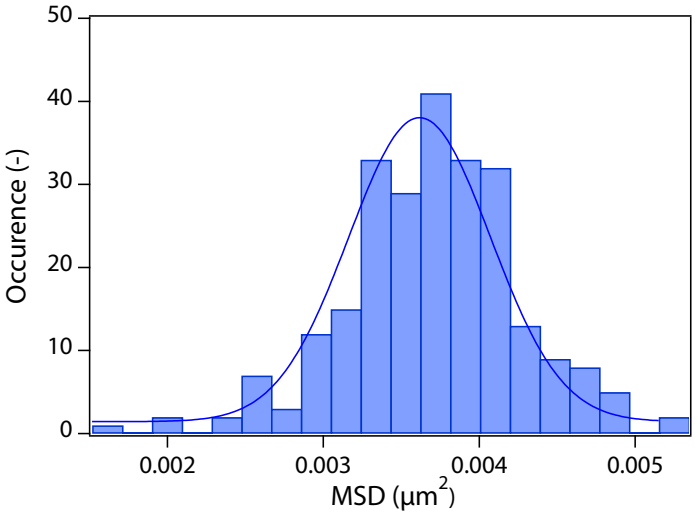
NK1R in HEK 293 cells labeled with Qdots 655

1. Localization of every single molecule in a movie
2. Test of single molecule properties
3. Tracking peaks along the movie: trajectories extraction
4. Trajectories control
5. Diffusion parameter computation

Single-molecule tracking noise

Due to noise, even an immobilized molecule will show an apparent displacement.

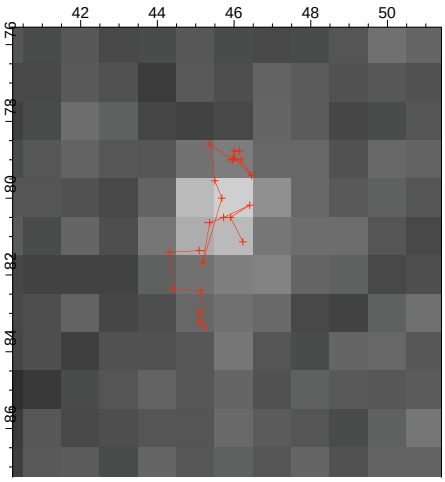


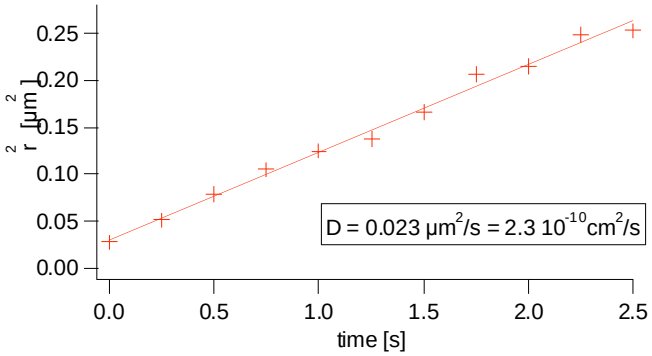


Localization accuracy **30 nm**

Single-molecule tracking

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





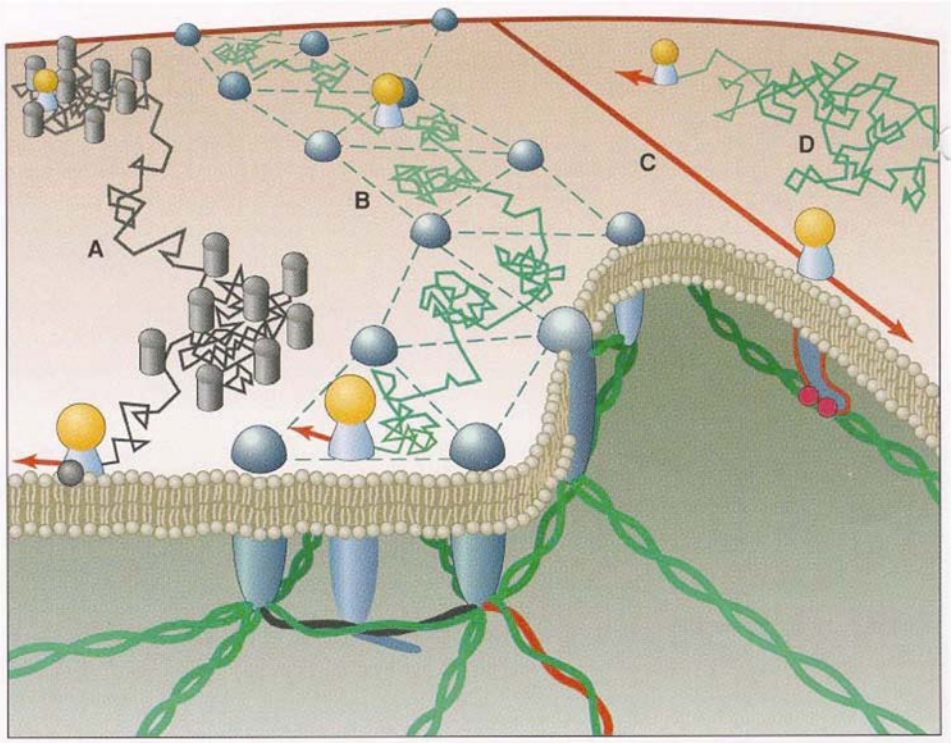
$$\mathrm{MSD}(n\delta t) = \frac{1}{N-1-n}\sum_{j=1}^{N-1-n}\left\{[x(j\delta t + n\delta t) - x(j\delta t)]^2 + [y(j\delta t + n\delta t) - y(j\delta t)]^2\right\},$$

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

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

Single-molecule tracking 4

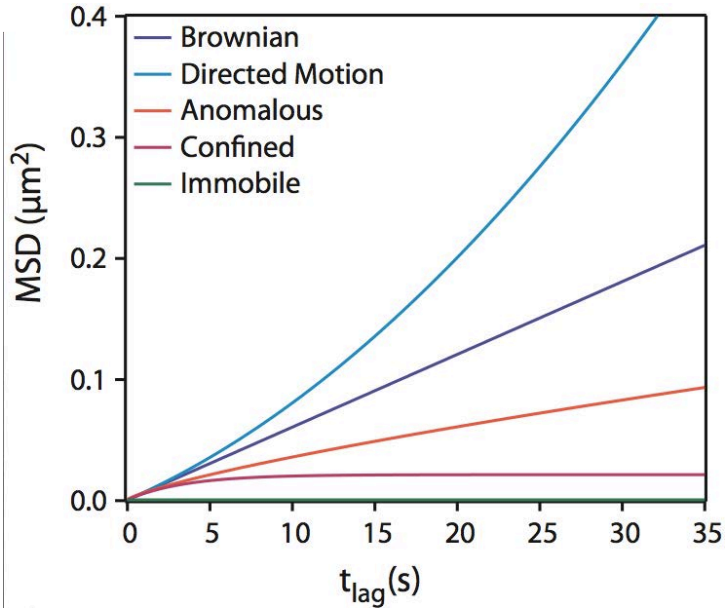
- Examples of trajectories: (A) Diffusion with obstacles, (B) Diffusion within corrals, (C) Directed motion, (D) Brownian motion



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 6

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



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

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

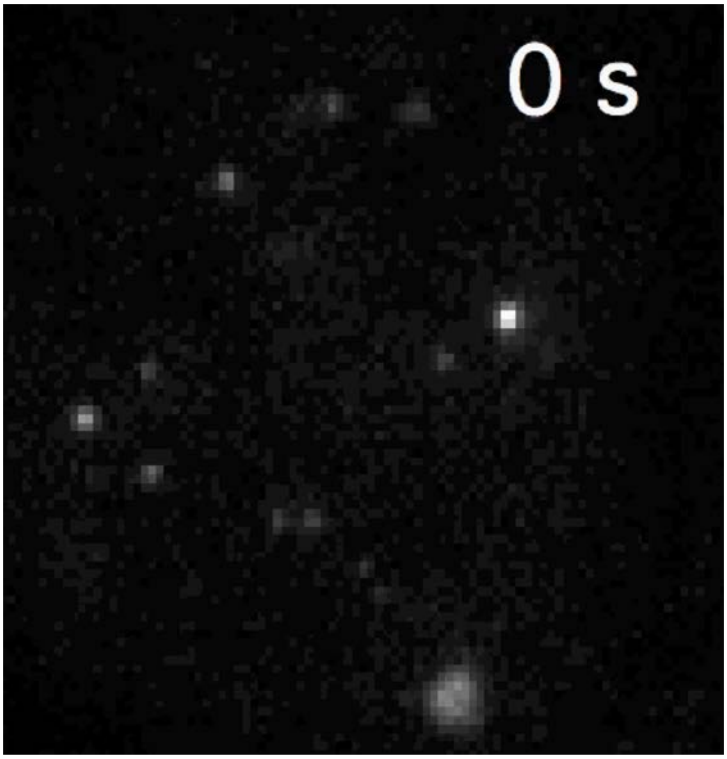
$$\mathrm{MSD}\,(t_{lag}) = 4Dt_{lag}^{\alpha}$$

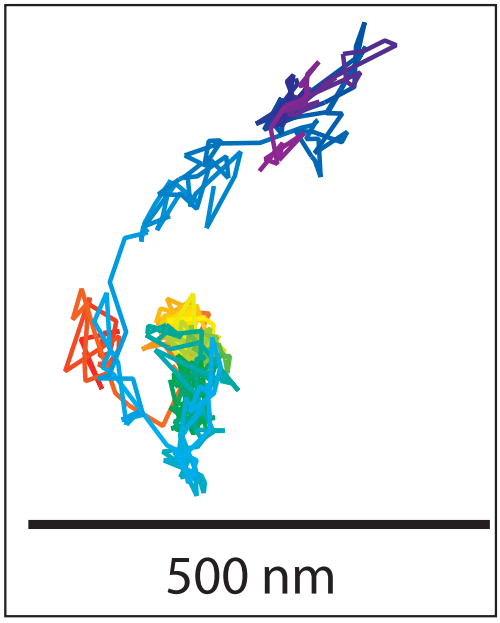
$$\mathrm{MSD}\,(t_{lag}) = \frac{L_c^2}{3} \cdot \left(1 - exp\left(\frac{-12 \cdot D_0 \cdot t_{lag}}{L_c^2}\right)\right)$$

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

Single-molecule tracking 13

Changes of the diffusion behaviour within a trajectory. Self-similarity analysis

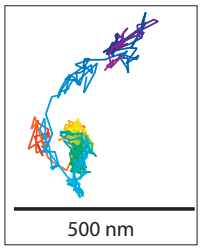




Single-molecule tracking 14

Changes of the diffusion behaviour within a trajectory. Self-similarity analysis

A



Data available:

Trajectory
MSD(t)
Instantaneous D
MSS analysis
Trajectory segment categorization

Moment scaling spectrum analysis give directly the diffusion type of a molecule or a fraction of a molecule trajectory.

MSS = 0.5 -> Free diffusion
MSS > 0.5 -> Directed diffusion
MSS < 0.5 -> Restricted motion
MSS = 0 -> Immobile

Single-molecule tracking 15: multiparameter analysis

Using trajectory shape, size, velocity, symmetry to increase information content of single molecules

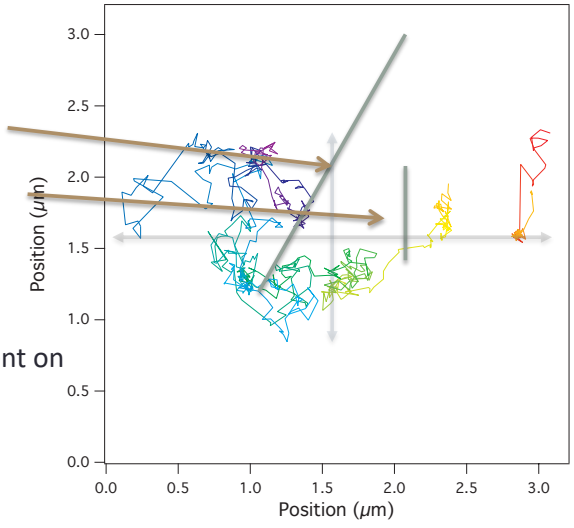
Basic information

1. Net displacement

- Complete trajectory

- Segment of trajectory

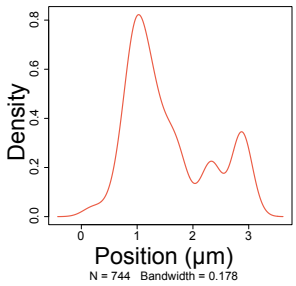
2. Efficiency:
- Ratio of net displacement on sum of all single steps



Shape related information

3. Asymetry
- Aspect ratio of the trajectory
- 0.254 -> Brownian motion

Distribution along main axis

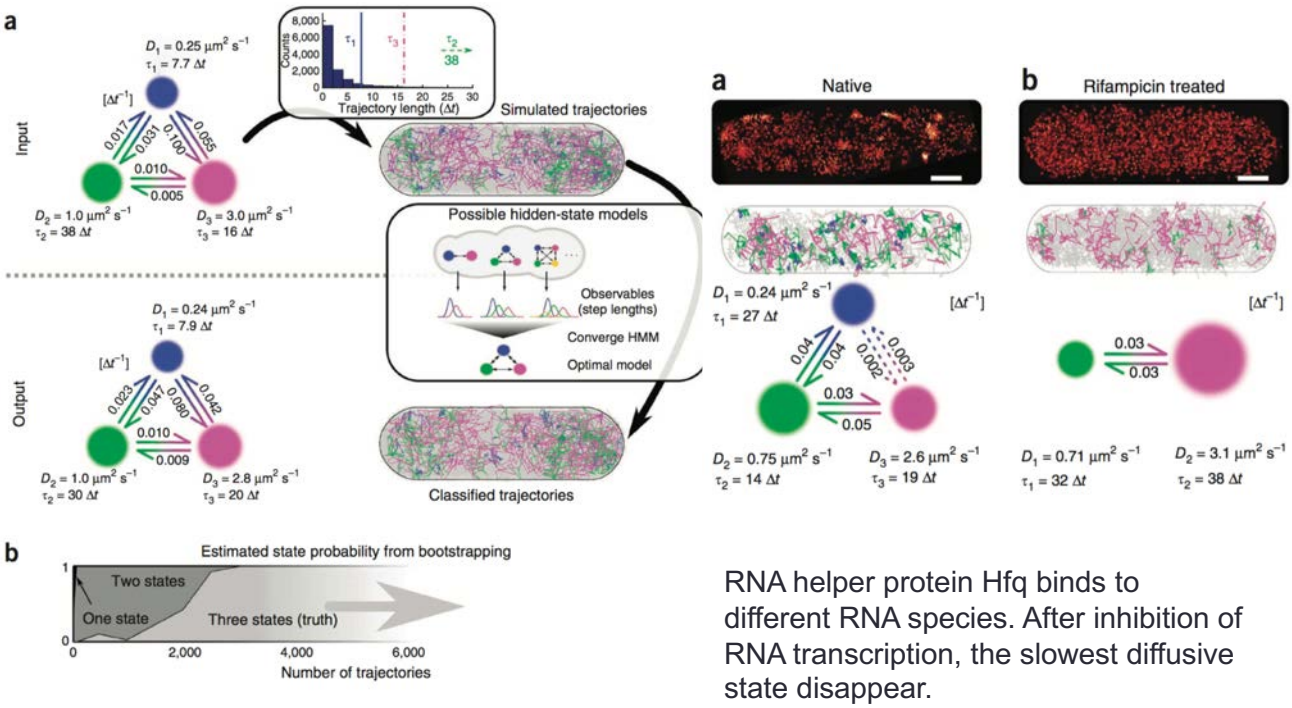


4. Skewness
5. Kurtosis
6. Length of displacement

Based on Helmuth, J Struct Biol, 2007

Single-molecule tracking 16: Subdiffusive states

Variational Bayes SPT (vbSPT) allows to extract multiple unknown subdiffusive states

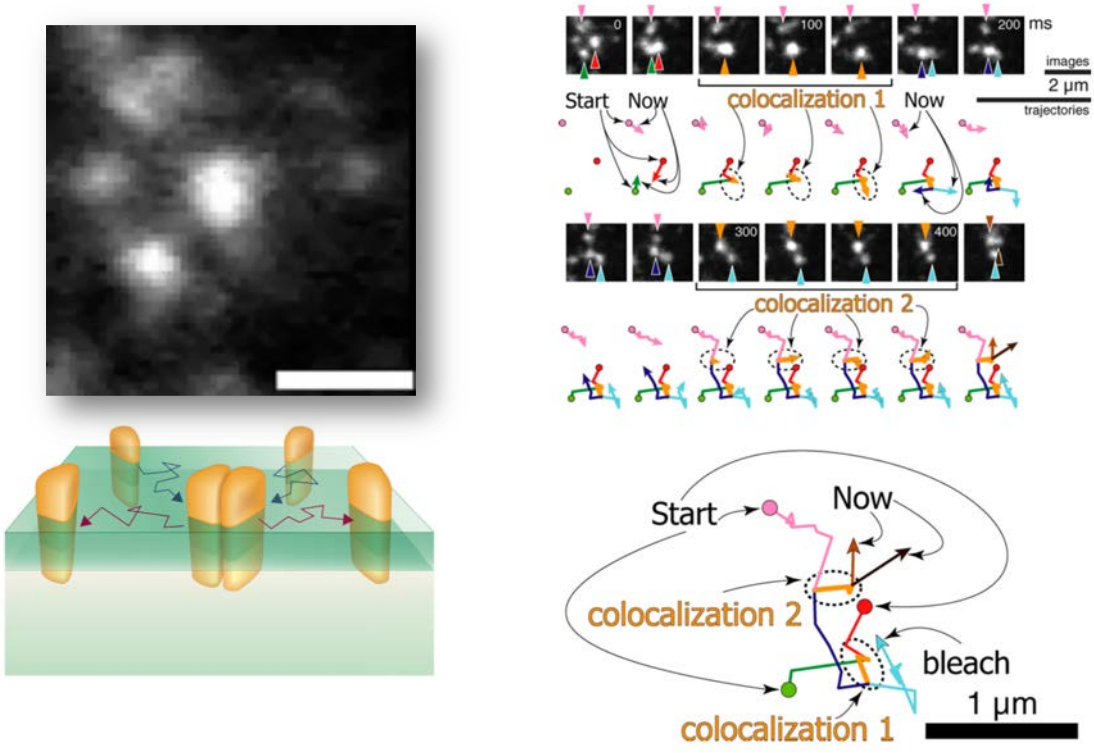


RNA helper protein Hfq binds to different RNA species. After inhibition of RNA transcription, the slowest diffusive state disappear.

Persson, F., *Nat Methods* **10,** 265–269 (2013).

Single-molecule tracking 17: Interactions

Diffusion of N-formyl peptide receptor in CHO cells

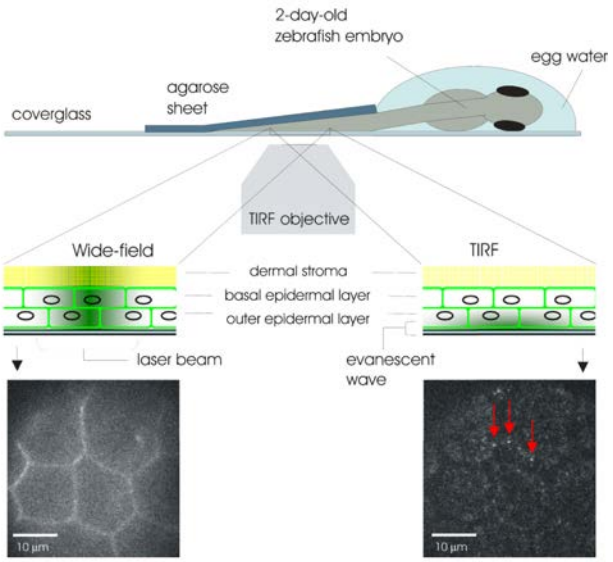


Kasai, R. S., et al. (2011) *The Journal of Cell Biology*, *192*(3), 463–480. http://doi.org/10.1083/jcb.201009128

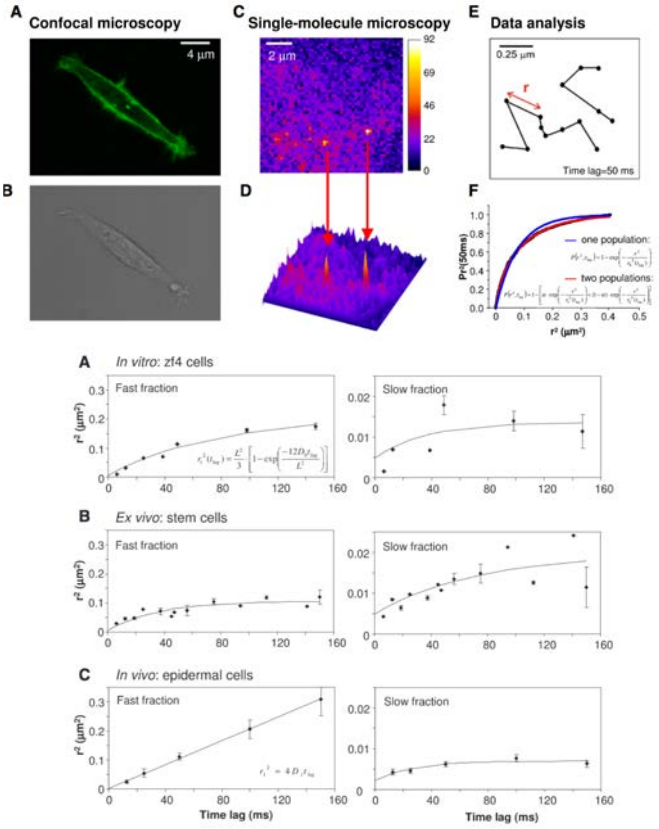
Kasai, R. S., & Kusumi, A. (2014). *Current Opinion in Cell Biology*, *27*, 78–86. http://doi.org/10.1016/j.ceb.2013.11.008

Single-molecule tracking 18: SMT in living organisms

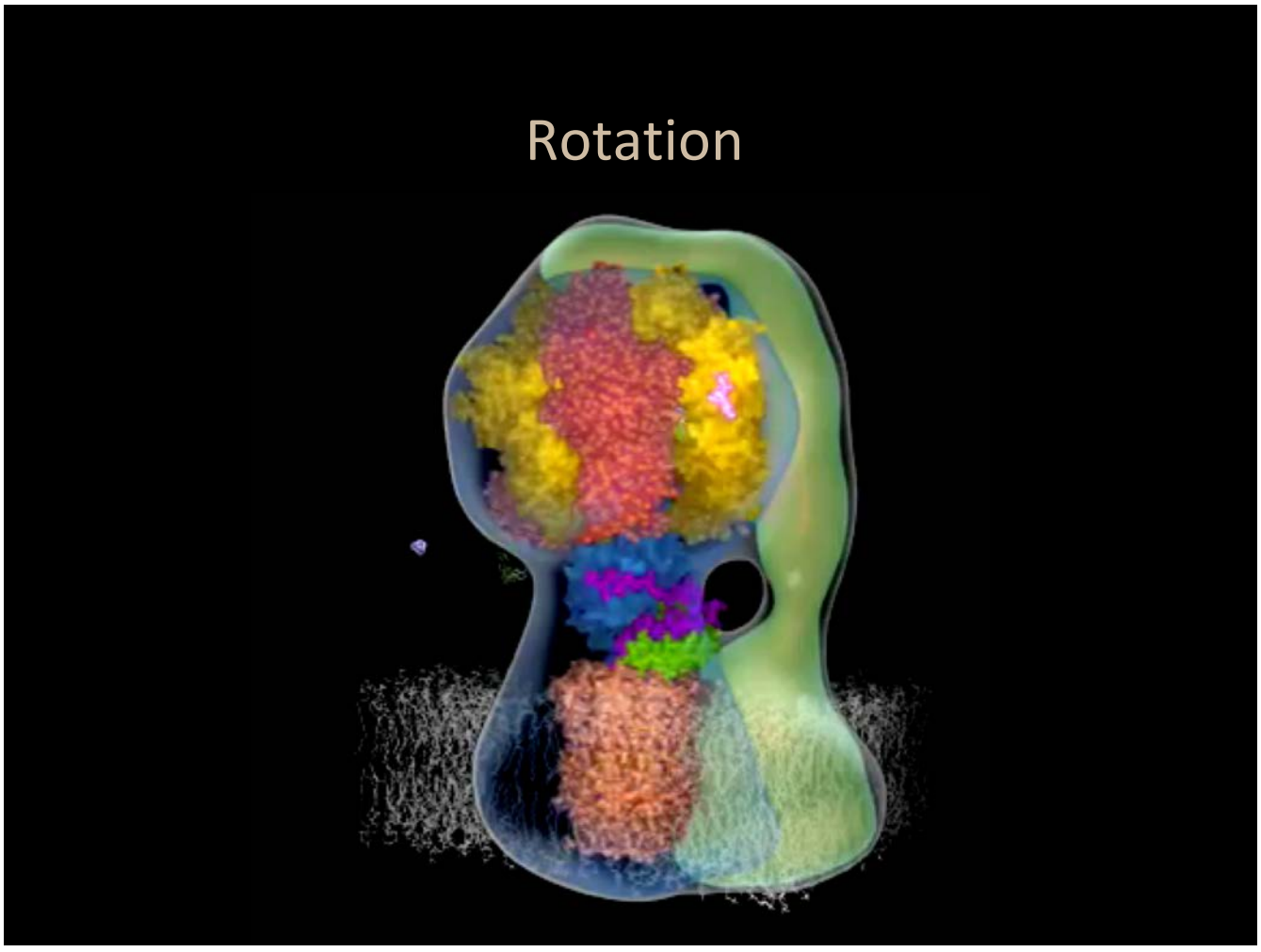
Are the *ex vivo* or *in* vitro single-molecule tracking experiments relevant?



Schaaf, M. J. M. *et al. Biophys J* **97,** 1206–1214 (2009).

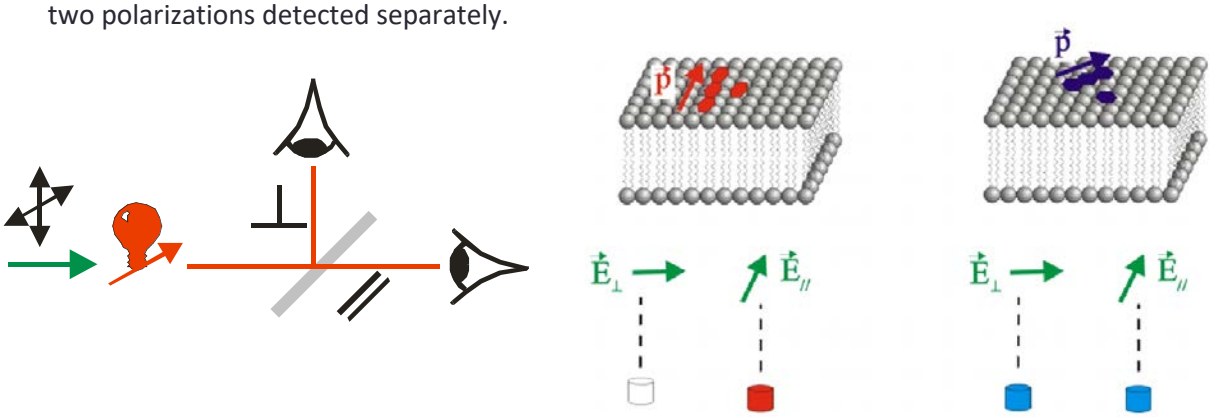


Domains exist in epidermal cells in embryo

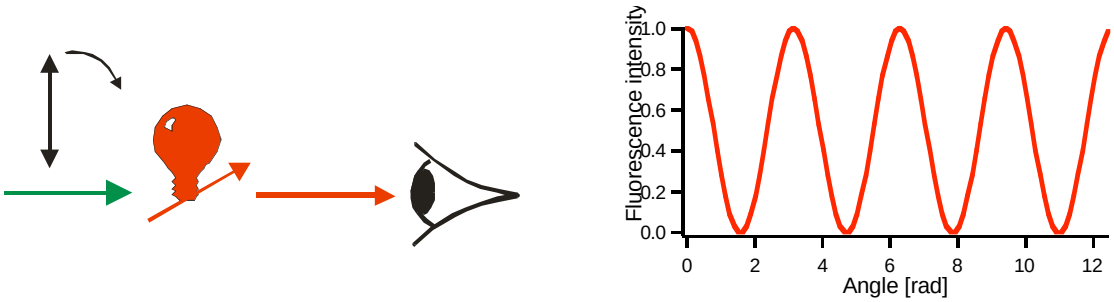


Polarization allows monitoring of rotation. Two methods.

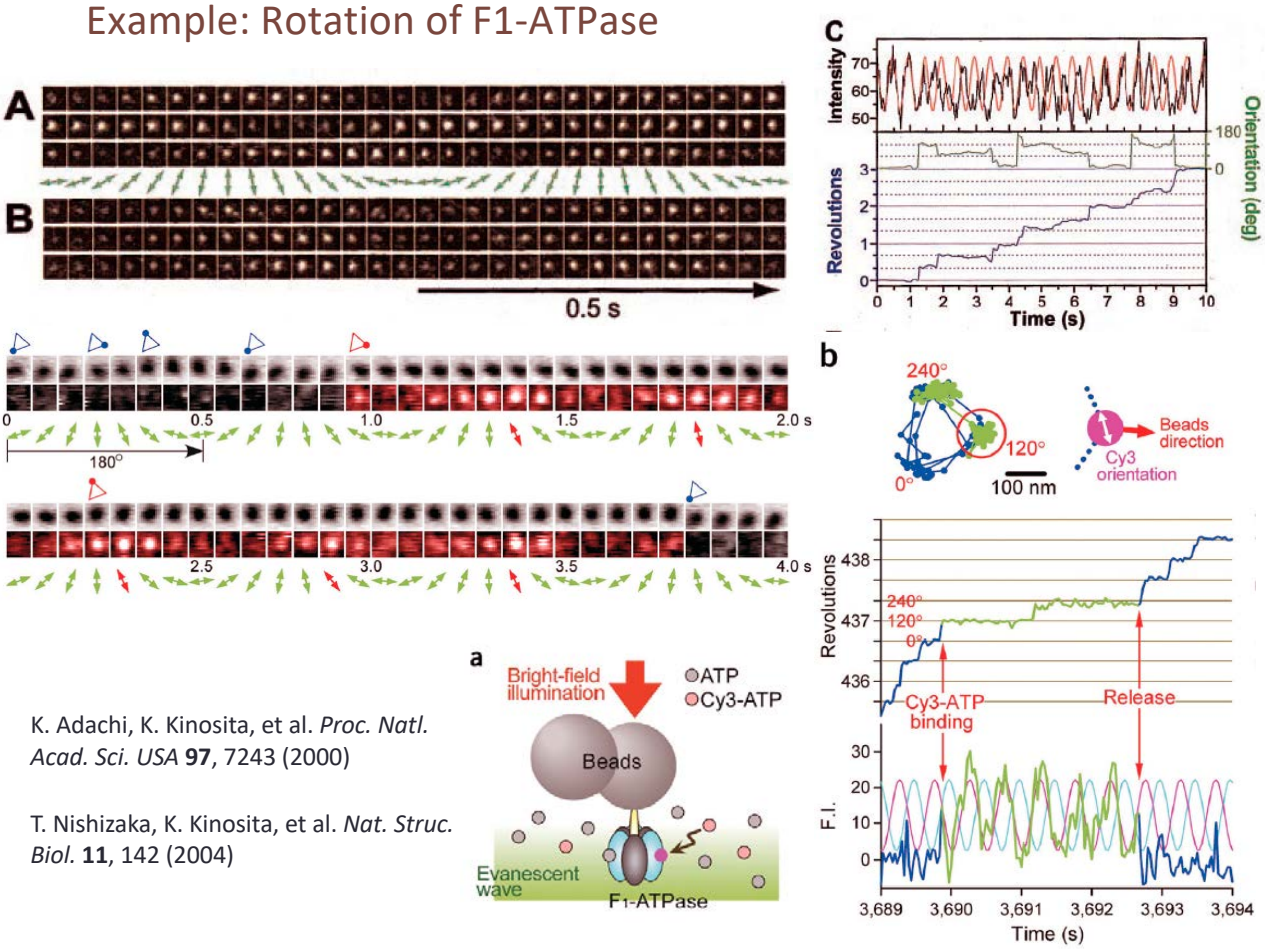
The single molecule is excited using circularly polarized light. The fluorescence is split into two polarizations detected separately.



The single molecule is excited using polarized light. The polarization is continuously rotated yielding a fluorescence intensity which varies with $\cos^2$(angle).



Example: Rotation of F1-ATPase

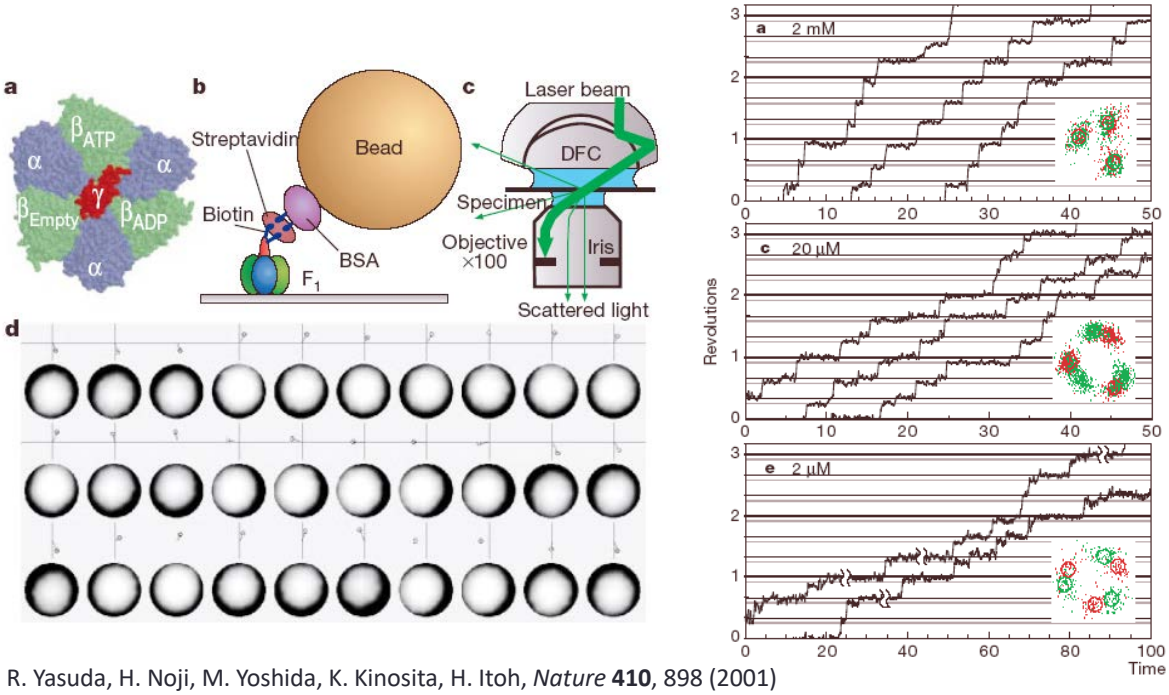


K. Adachi, K. Kinosita, et al. *Proc. Natl. Acad. Sci. USA* **97**, 7243 (2000)

T. Nishizaka, K. Kinosita, et al. *Nat. Struc. Biol.* **11**, 142 (2004)

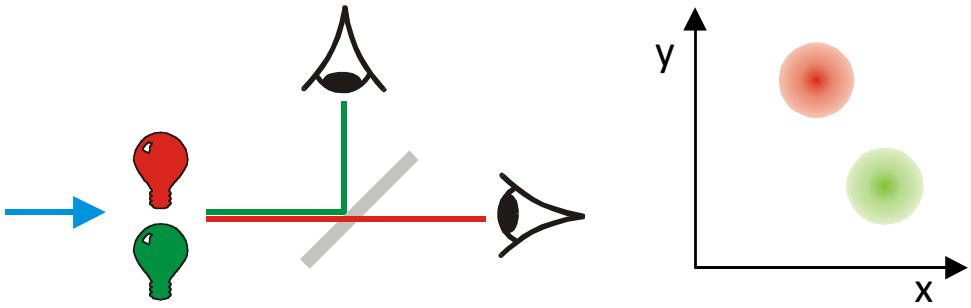
Single-Particle Tracking (example: Rotation of F1-ATPase)

Instead of labelling the biomolecule with a fluorophore, a golden bead is used. Photobleaching is not anymore an issue and so extensively long traces can be recorded. Moreover, the time resolution is much better (submillisecond). The drawback is the size of the bead (ca. 40 nm) that may influence the properties of the biomolecule. Detection of the bead is usually performed using DIC microscopy.



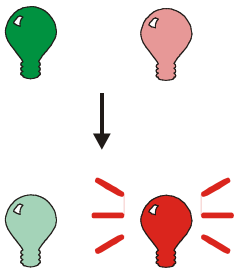
R. Yasuda, H. Noji, M. Yoshida, K. Kinosita, H. Itoh, *Nature* **410**, 898 (2001)

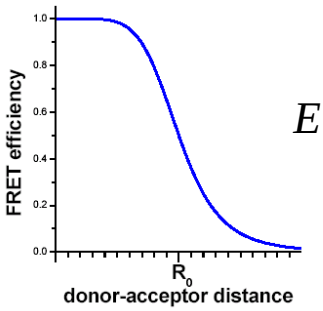
Two colours: 2 labelled biomolecules



- Owing to the precision in the position measurements, the interaction between 2 biomolecules can be observed with a precision in the order of 30 nm.

Fluorescence resonance energy transfer (FRET)

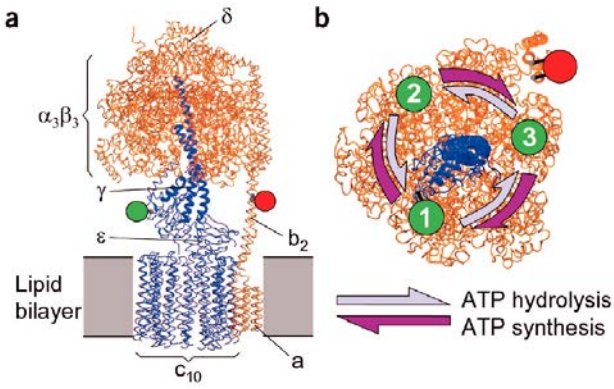


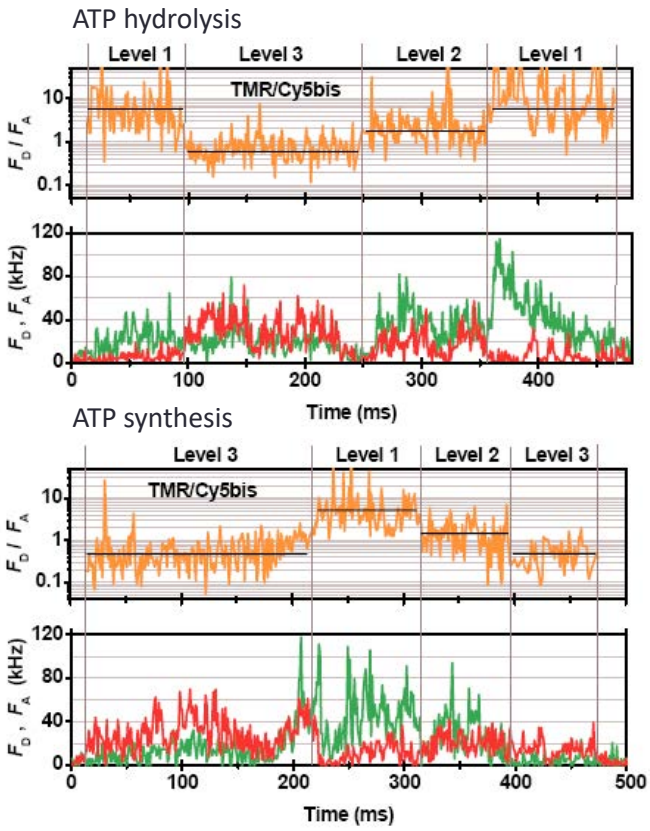


$$E = 1 - \frac{F_{DA}}{F_D} = \frac{R_0^6}{R_0^6 + R^6}$$

- In the formula, E is the FRET efficiency; F_{DA} is the fluorescence of the donor with acceptor present; F_D is the fluorescence of the donor with acceptor absent; R_0 is Förster's radius; R is the distance.
- Allow the observation of the interaction between two biomolecules with nanometer precision.
- Allow the observation of conformational changes.
- R_0 depends on the donor-acceptor pair. It is possible to vary R_0 by carefully choosing the pair.

Example: Conformational changes in F_0F_1-ATP synthase



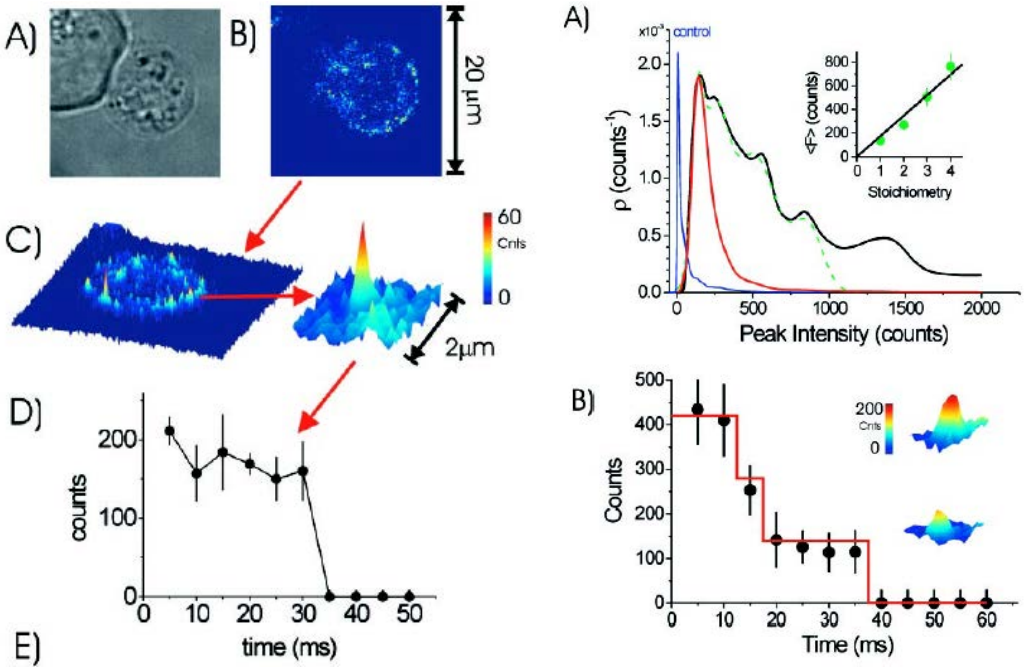


M. Diez, P. Gräber et al.,

Nat. Struc. Biol. **11**, 135 (2004)

Signal intensity/photobleaching: Information about the presence of multiple fluorophores

- Example: Oligomerization of the L-type Ca^{2+} channel

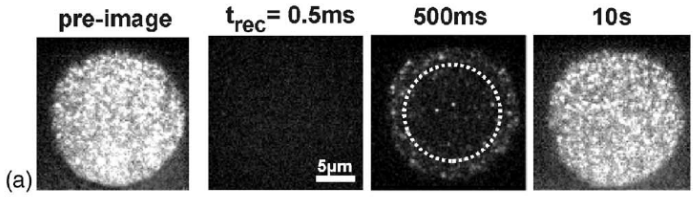


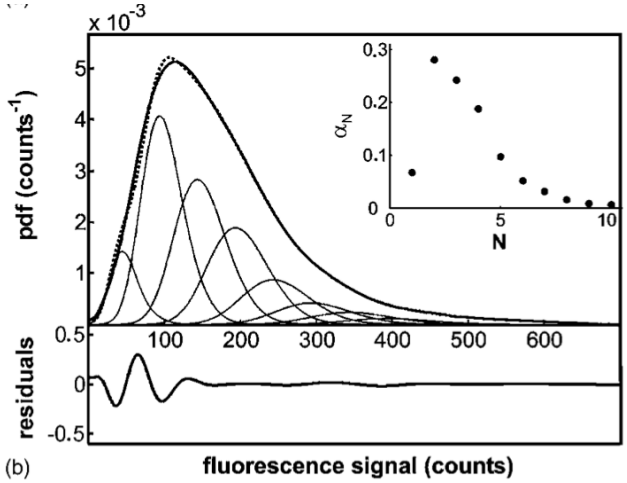
G. Harms, Th. Schmidt et al.,*Biophys. J.* **81**, 2639 (2001)

Signal intensity/photobleaching: Information about the presence of multiple fluorophores

Thinning out clusters while conserving stoichiometry of labeling (TOCCSL)

FRAP followed by trackin of single-molecule diffusing in the photobleached region





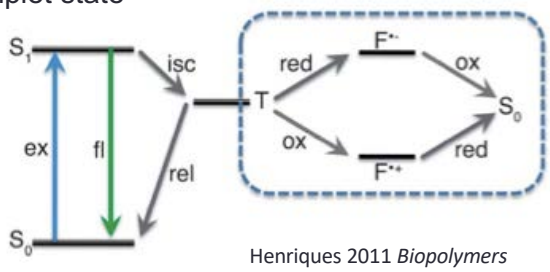
The probabilty density function of the cluster is fitted with a multiple normal distribution centered at multiple of single-molecule fluorescence

Moertelmaier, et al (2005). *Applied Physics Letters, 87(26), 263903*

Blinking 1: Controlled intermittent emission of single molecule

Organic fluorophore

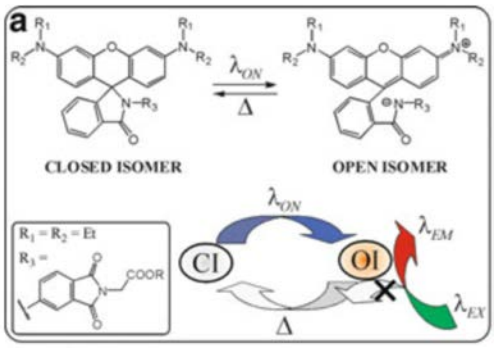
Triplet state



Henriques 2011 *Biopolymers*

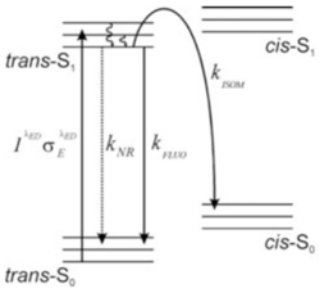
Triplet state can be stabilized using reductant and oxidant solutions leading to a controlled blinking of the dye.

Isomerization



P. Tinnefeld, *Far-Field Optical Nanoscopy*, Springer, 2015.

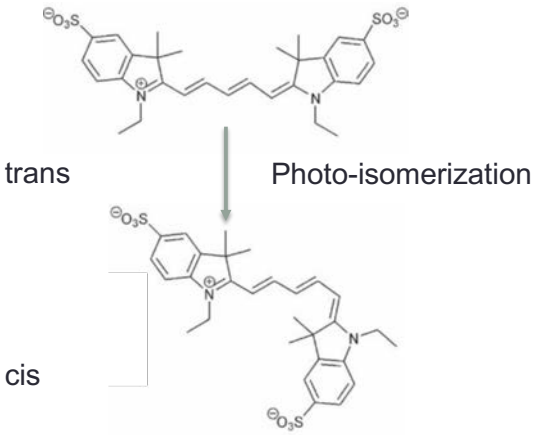
Cy5 cis-trans isomerization



$$\Phi_{FLUO} = \frac{k_{FLUO}}{k_{FLUO} + k_{ISOM} + k_{NR}}$$

$$\Phi_{ISOM} = \frac{k_{ISOM}}{k_{FLUO} + k_{ISOM} + k_{NR}}$$

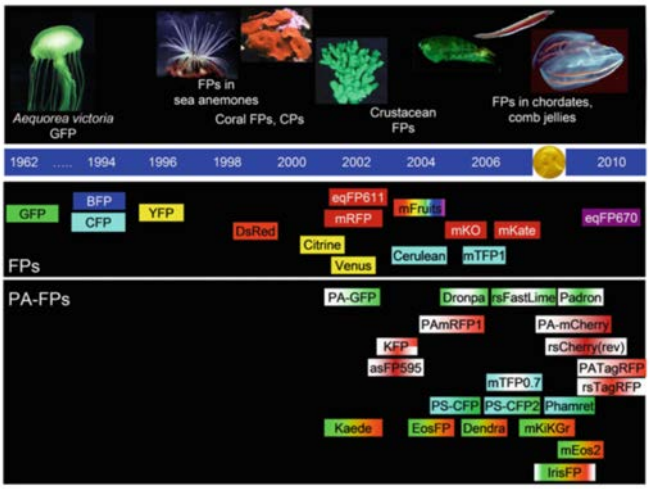
P. J. Walla, *Modern Biophysical Chemistry*. John Wiley & Sons, 2015.

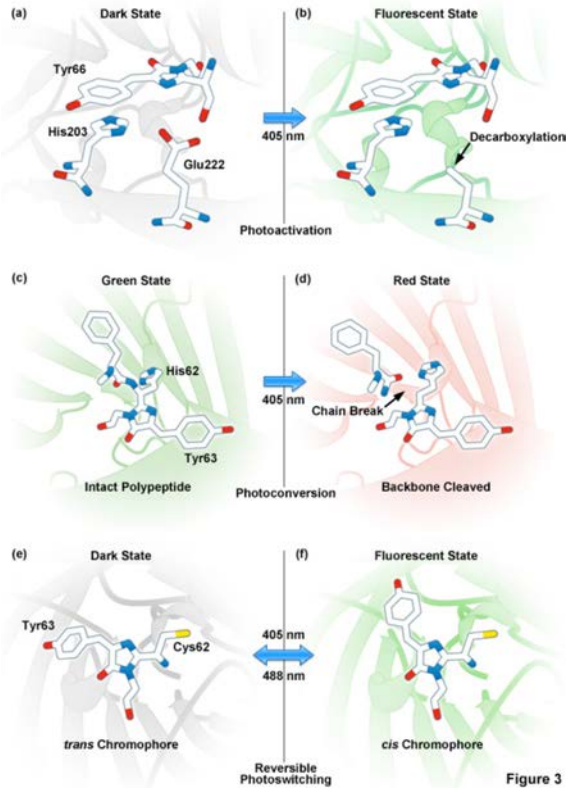


E. M. S. Stennett, et al, *Chem Soc Rev*, vol. 43, no. 4, pp. 1057–1075, 2014.

Blinking 2: Controlled intermittent emission of single molecule

Fluorescent proteins

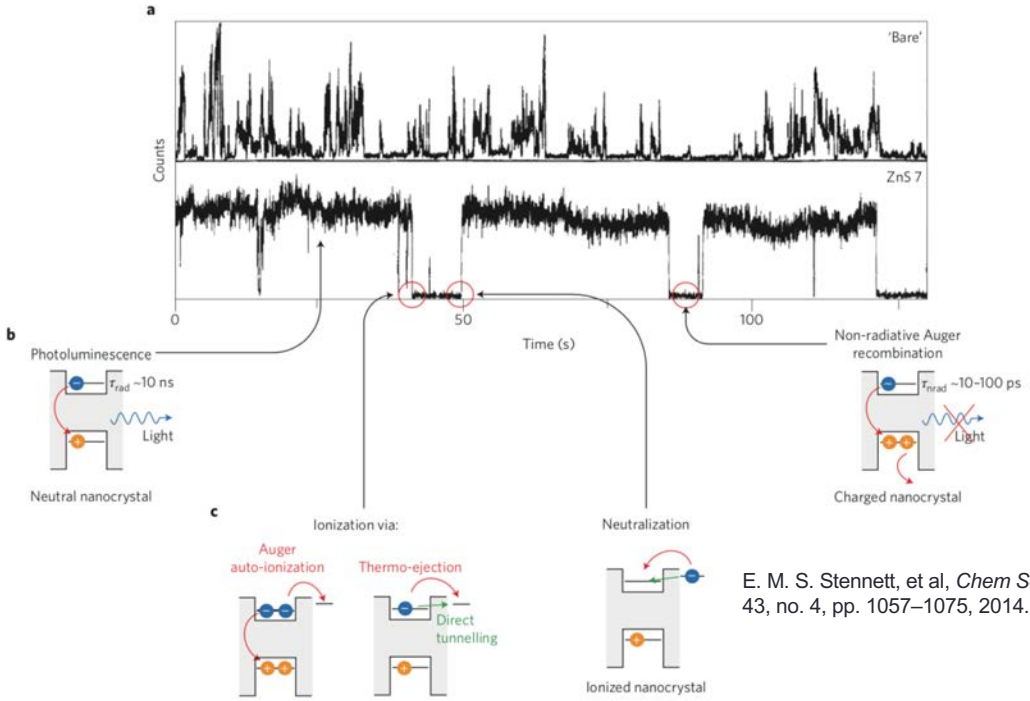




http://zeiss-campus.magnet.fsu.edu/articles/probes/highlighterfps.html

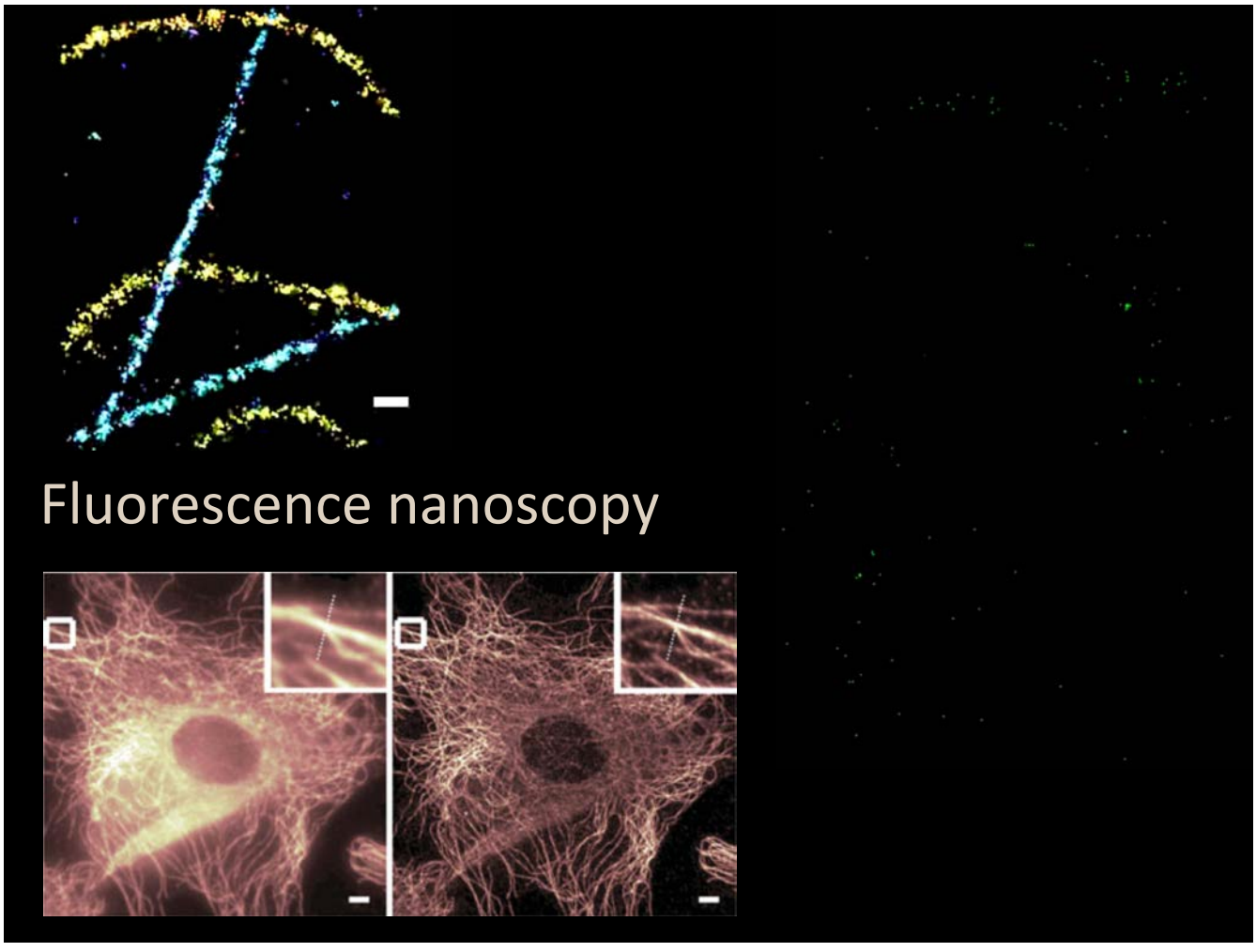
Blinking 3: Controlled intermittent emission of single molecule

Nanocrystals of semi conductors (quantum dots)



E. M. S. Stennett, et al, *Chem Soc Rev*, vol. 43, no. 4, pp. 1057–1075, 2014.

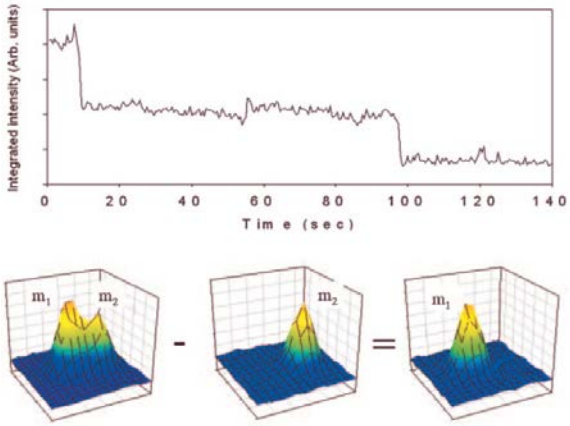
Quantum dots are naturally blinking molecules mainly due to photo-ionization at the surface of the particle. There is no easy method to control QD blinking.



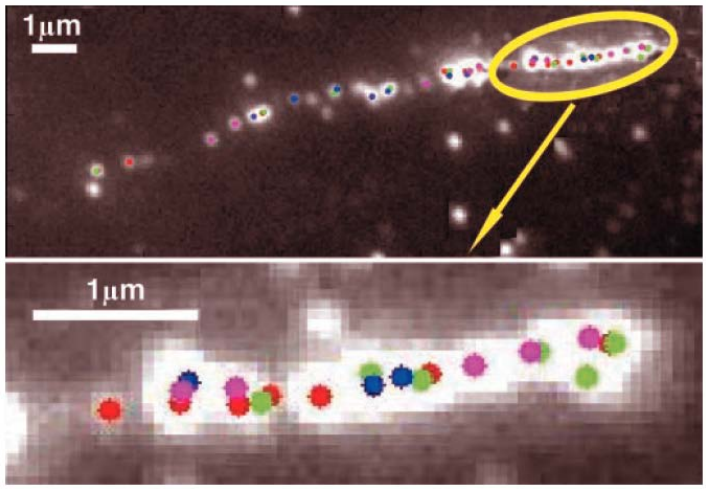
Photobleaching: Distance measurements in the nanometer range

Distance measurements have the same accuracy as position measurements (10-30 nm). Principle: the last molecule to photobleach is fitted using a gaussian. This gaussian is then used to fit the two-molecule emission profile, and so on.

Example in a two-molecule case



DNA mapping



X. Qu, D. Wu, L. Mets, N.F. Scherer, *Proc. Natl. Acad. Sci. USA* **101**, 11298 (2004)

M.P. Gordon, T. Ha, P.R. Selvin, *Proc. Natl. Acad. Sci. USA* **101**, 6462 (2004)

Dyes for superresolution microscopy (nanoscopy)

TABLE 1. Spectral properties of photoswitchable fluorescent proteins and organic dyes

		Before activation		After activation		
Fluorophore	Activation (nm)	Absorption max (nm)	Emission max (nm)	Absorption max (nm)	Emission max (nm)	Reference(s)
Fluorescent proteins						
PA-GFP	405	400	515	504	517	Patterson and Lippincott-Schwartz (2002)
PS-CFP2	405	400	468	490	511	Chudakov et al. (2004)
Dendra-2	405	490	507	553	573	Gurskaya et al. (2006)
Kaede	405	508	518	572	582	Ando et al. (2002)
EosFP, mEos2	405	506	516	571	581	Wiedenmann et al. (2004), McKinney et al. (2009)
mKikGR	405	507	517	583	593	Tsutsui et al. (2005), Habuchi et al. (2008)
Dronpa	405	—	—	503	518	Habuchi et al. (2005)
Dronpa-2	405	—	—	486	513	Ando et al. (2007)
bs-Dronpa	405	—	—	460	504	Andresen et al. (2008)
rsFastLime	405	—	—	496	518	Stiel et al. (2007)
eYFP	405	—	—	514	529	Dickson et al. (1997), Biteen et al. (2008)
PA-mCherry	405	—	—	570	596	Subach et al. (2009)
Synthetic dyes						
Cy5	350–570[a]	—	—	649	670	Bates et al. (2005, 2007), Heilemann et al. (2005), Dempsey et al. (2009a)
Cy5.5	350–570[a]	—	—	675	694	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Cy7	350–570[a]	—	—	747	776	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Alexa Fluor 647	350–570[a]	—	—	650	665	Bates et al. (2005, 2007), Dempsey et al. (2009a)
Photochromic rhodamine B	375	—	—	565	580	Folling et al. (2007)
Rhodamine spiroamides	375			537–591[b]	555–620[b]	Belov et al. (2009)
Azido-DCDHF		—	—	570	613	Lord et al. (2009)

PA, photoactivatable; GFP, green fluorescent protein; PS, photoswitchable; CFP, cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; azido-DCDHF, azido-2-dicyanomethylene-3-cyano-2,5-dihydrofuran.

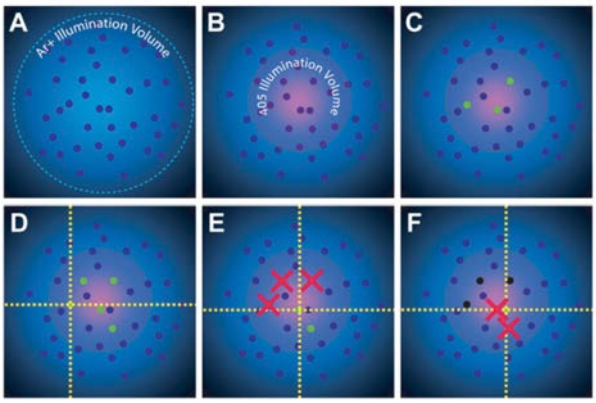
[a]Dependent on the activator dye, if present.

[b]Dependent on the specific compound.

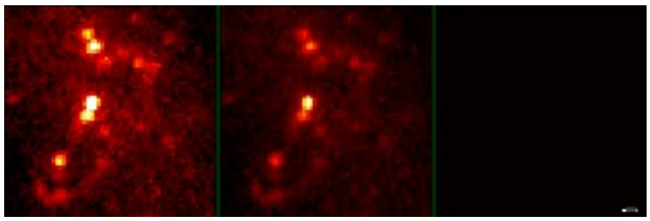
Bates M, Jones SA, Zhuang X (2013) *Cold Spring Harbor Protocols* 2013

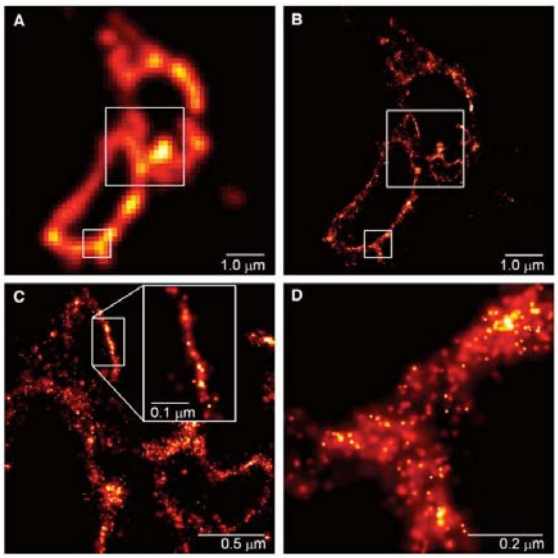
Super-resolution 1: PALM

Photoactivatated localization microscopy (PALM)



Hess, *Biophys J*, 2006





Betzig, *Science,* 2006

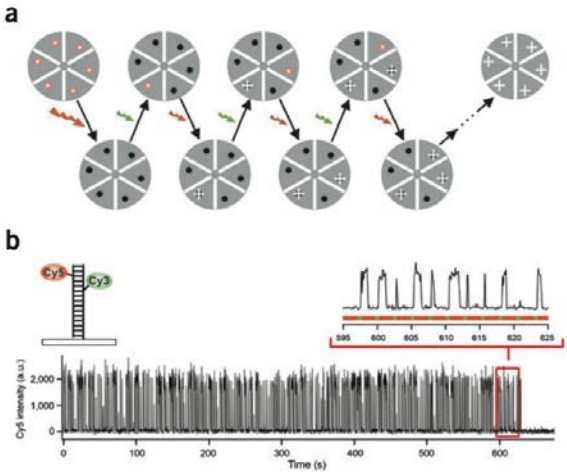
Fluorescent protein is activated or switched at low rate: molecules appear as single fluorescent spots.

Requirements:
-Photoactivatable or photoconvertible fluorescent protein (eg. mEOS2, Dronpa PA-GFP, PA-mCherry, EYFP)
-Relatively low protein expression

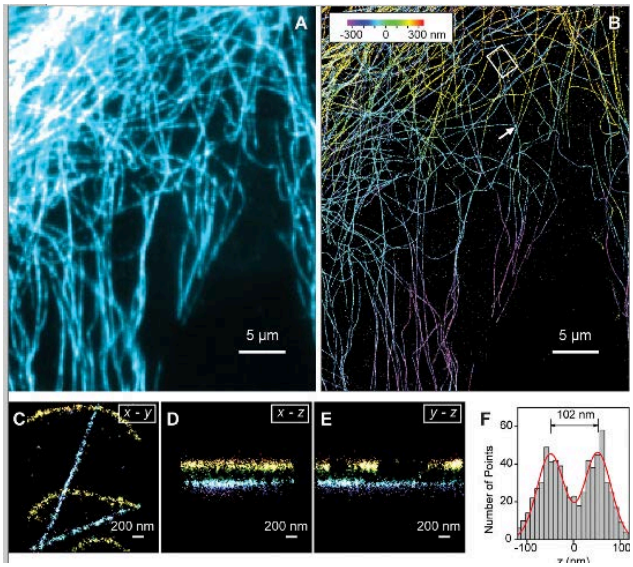
Super-resolution 2: STORM

Stochastic optical reconstruction microscopy (STORM)

Principle



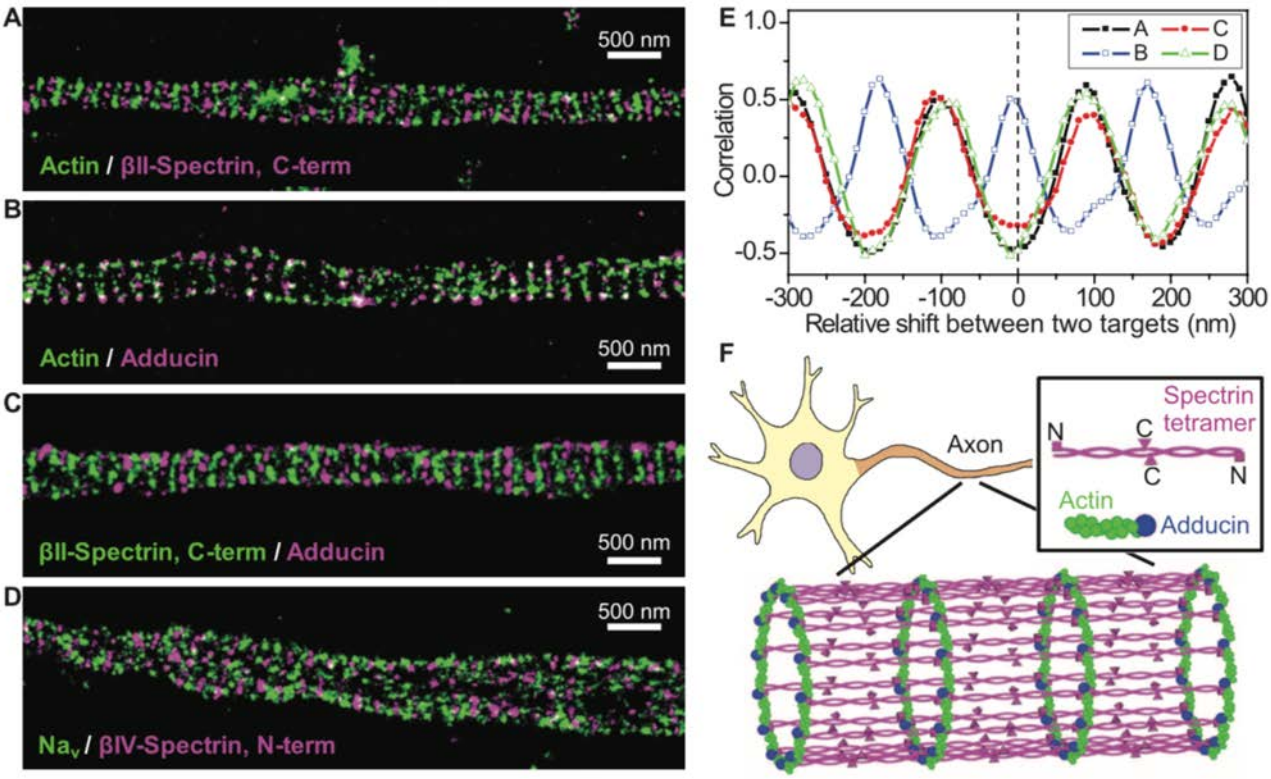
Rust, *Nat Meth*, 2006



Huang, *Science,* 2008

A dye is maintained in a dark state using strong excitation light. A fraction of the dye is reactivated using another wavelength. This can be mediated using another dye (STORM) or without (dSTORM)
Requirements:
-A pair of dye (eg Cy5 & Cy3) in close proximity (nanometer range) or a dye with stable triplet state
-An adapted buffer environnement.
-Post-translational labelling (generally outside cells or *in vitro*)

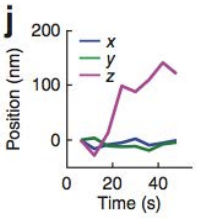
Super-resolution 3: STORM in neurons

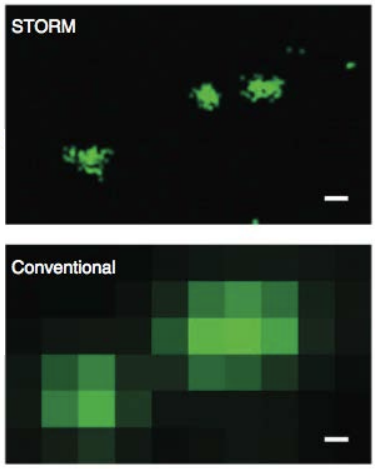


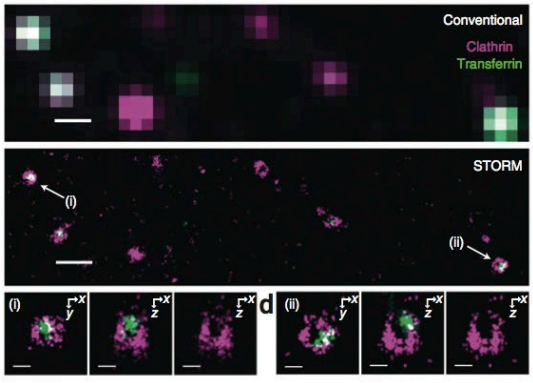
K. Xu, G. Zhong, and X. Zhuang, *Science*, vol. 339, no. 6118, pp. 452–456, Jan. 2013.

Super-resolution 4: Dynamic STORM in living cell

Dynamic STORM image of tranferrin in live cell





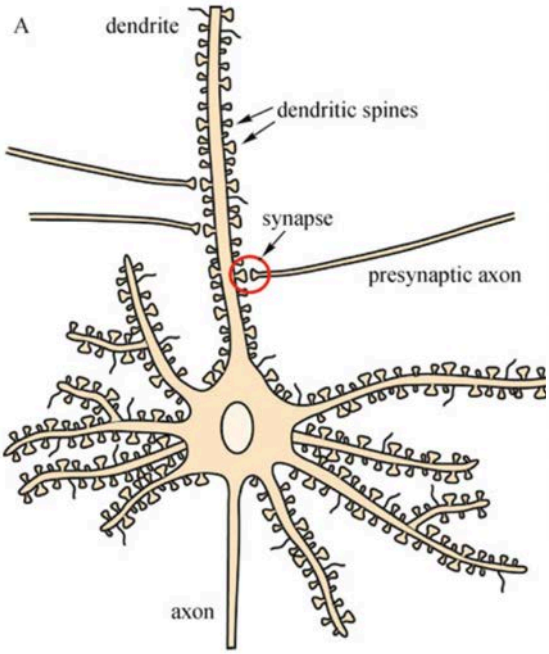


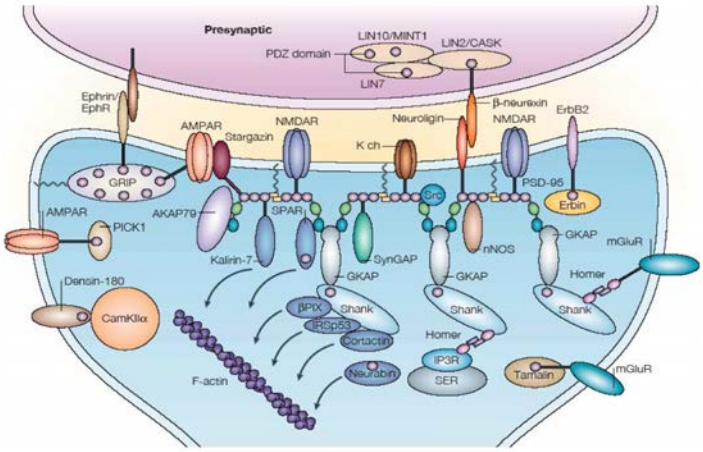
Dual color superresolution image of transferrin in clathrin-coated pits.

Jones, S. A., Shim, S.-H., He, J., & Zhuang, X. (2011) *Nature methods, 8(6), 499–505*

Super-resolution 5: Dynamic STORM in neuron

How do receptors diffuse in post-synaptic membrane ?

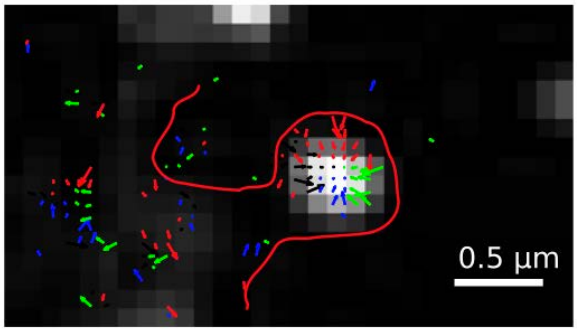




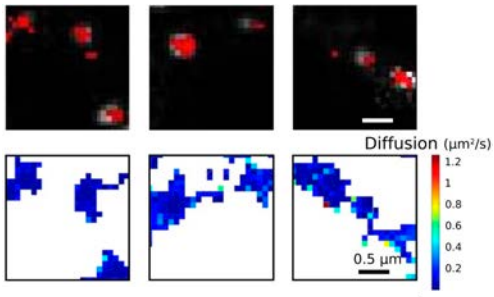
Smrt RD, Zhao X (2010) Frontiers in Biology 5:304–323.

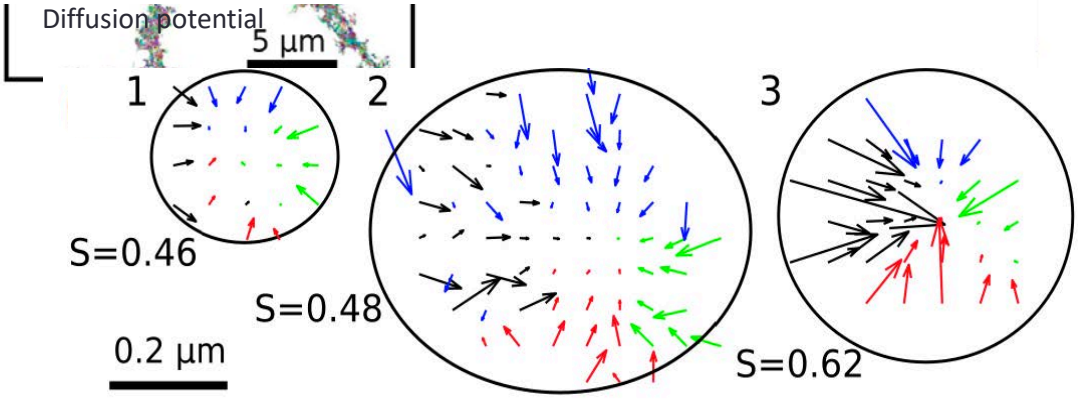
Super-resolution 6: Dynamic STORM in neuron

How do receptors diffuse in post-synaptic membrane ?



Diffusion mapping





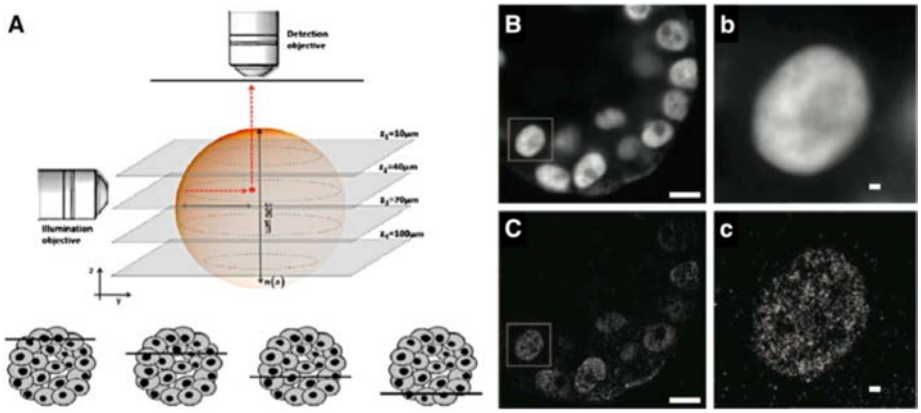
Hoze N et al. (2012) Proc Natl Acad Sci USA 109:17052-17057

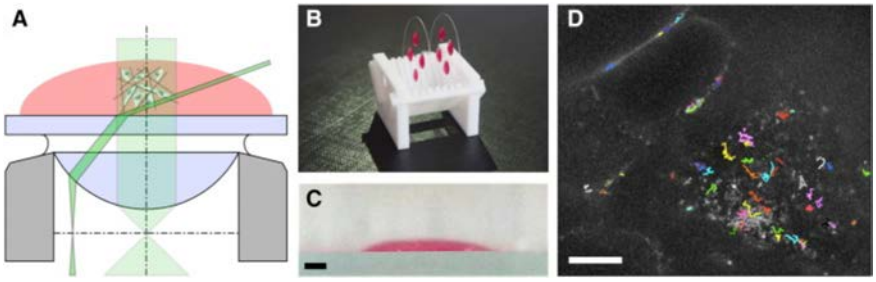
Single-molecule tracking/superresolution: Light-sheet

Single-molecule localization in 3D is achieved by using a light-sheet to illuminate the sample

Light-sheet microscopy on spheroid.

A second objective is used for illumination



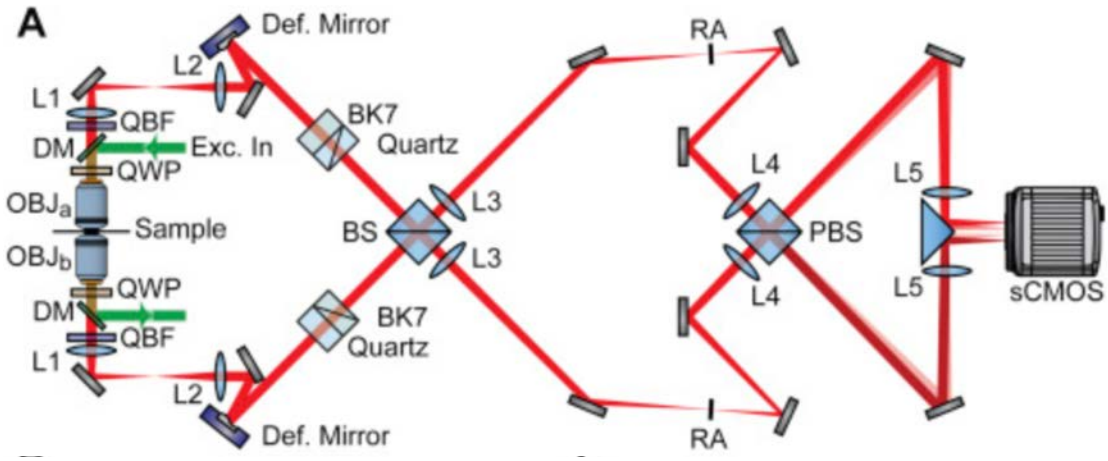


Highly Inclined Laminated Optical Sheet (HILO) of a 3D cell culture in collagene

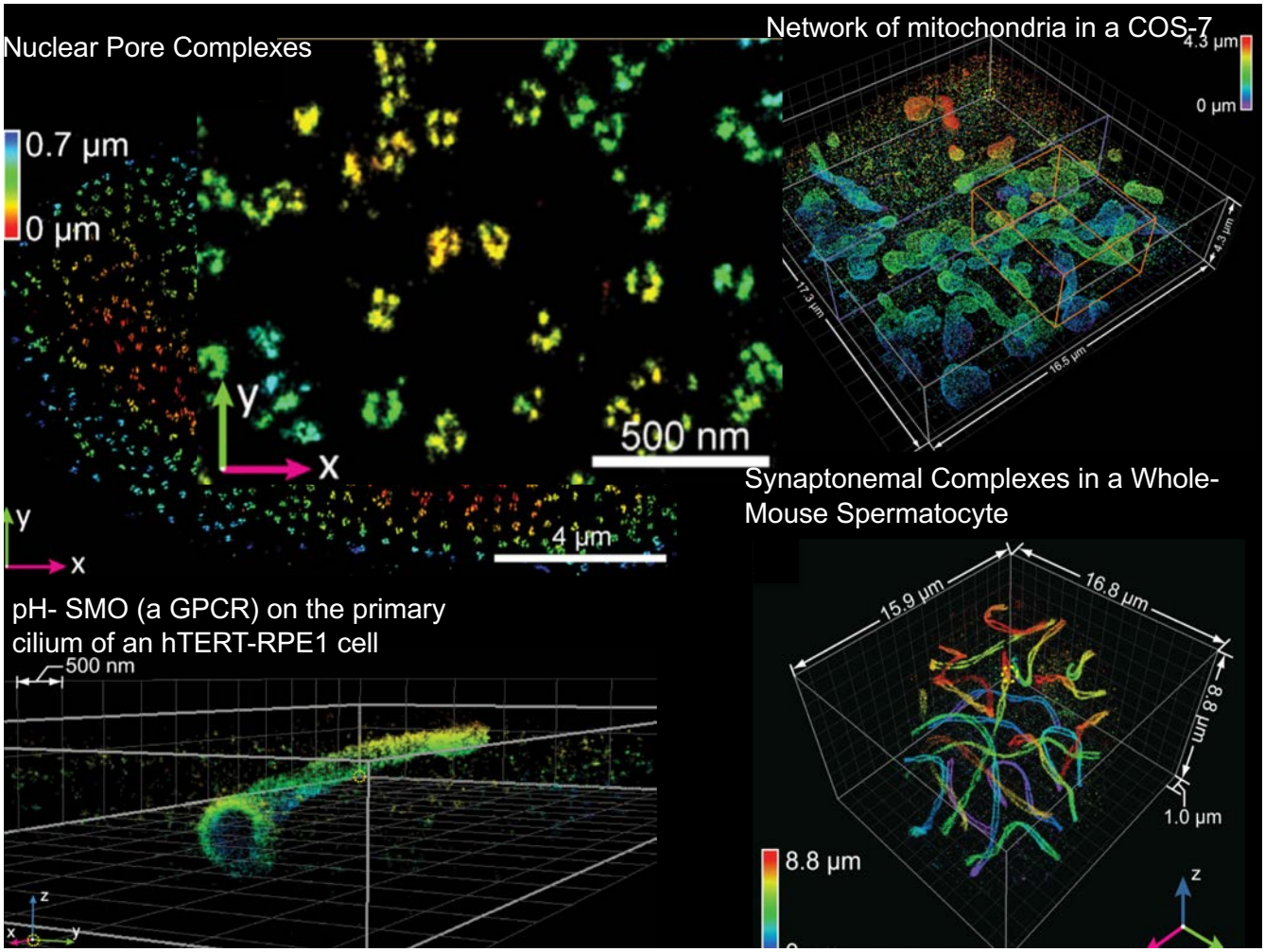
Lauer, F. M., *Adv. Drug Deliv. Rev.* **79-80,** 79–94 (2014).

Super-resolution 6: 3D image of whole cells

Whole-cell 4Pi single-molecule switching nanoscopy (W-4PiSMSN): Combination of PALM, 4Pi-microscopy and iPALM

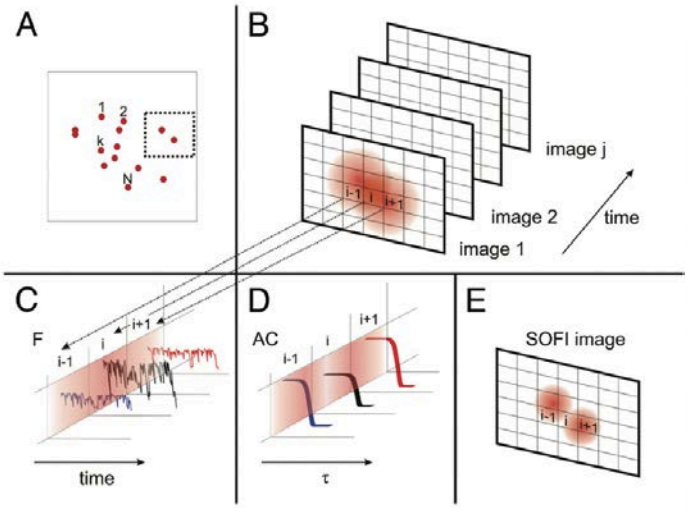


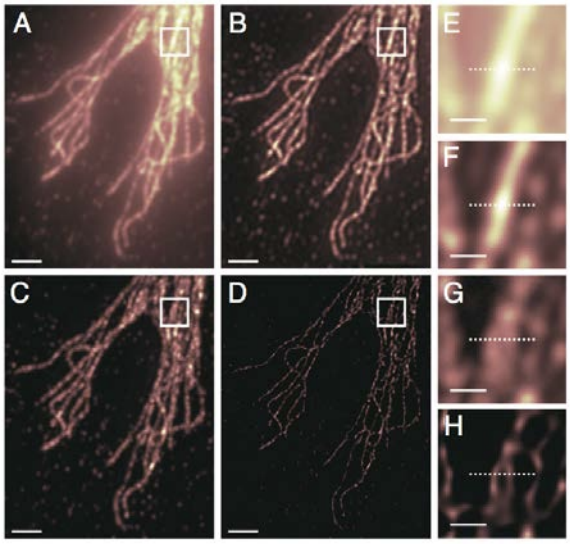
- The lateral resolution is increased by a factor 1.7 thanks to the 2 objectives
- The 4Pi configuration of the objective allows to reach a 10 nm axial resolution



Super-resolution 7: SOFI

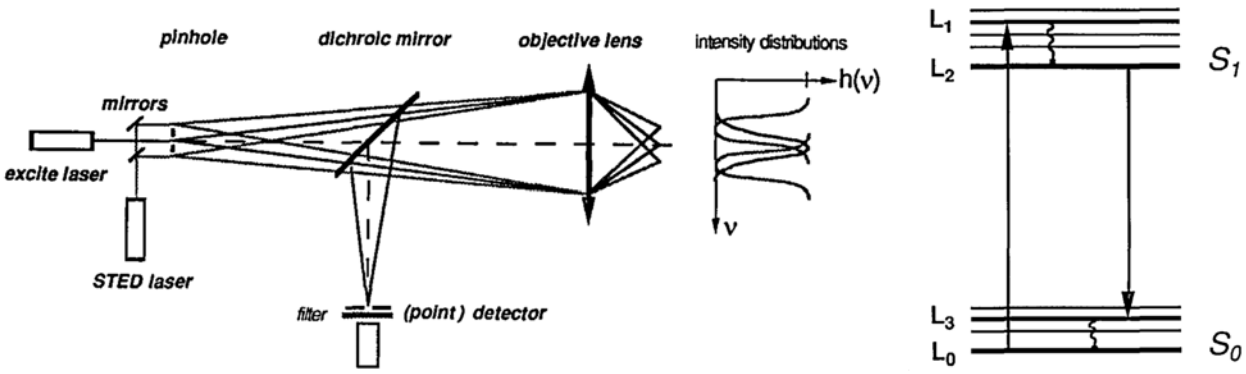
Blinking of fluorescent probe is used to increase image resolution !



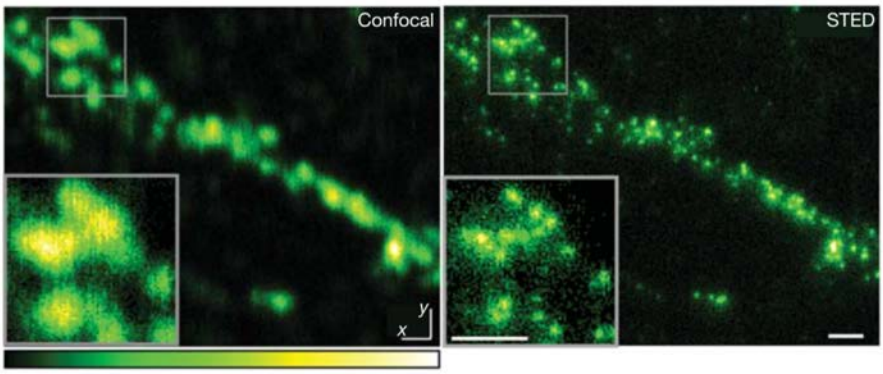


Dertinger, T., Colyer, R., Iyer, G., Weiss, S., & Enderlein, J. (2009) *PNAS, 106(52), 22287–22292*

Super-resolution 8 : Confocal STED microscopy

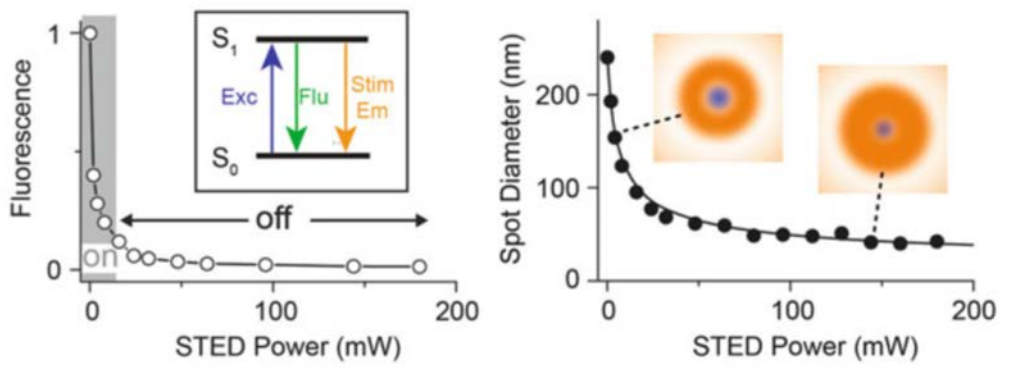


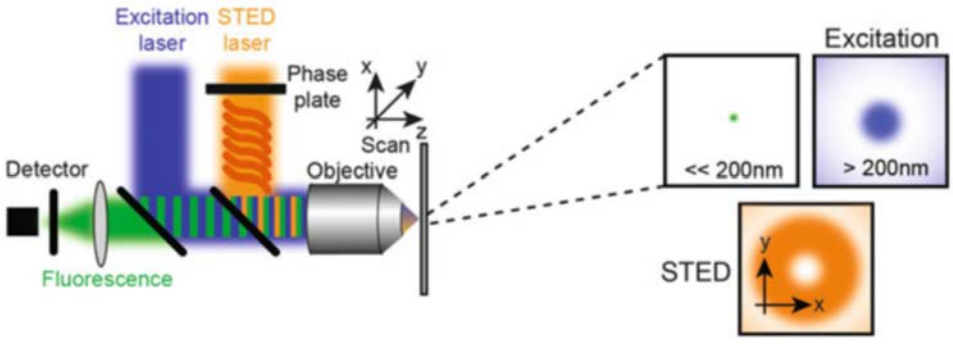
Hell, S. W., & Wichmann, J. (1994). *Optics Letters*, *19*(11), 780–782.



Willig, K. I., Rizzoli, S. O., Westphal, V., Jahn, R., & Hell, S. W. (2006). *Nature Cell Biology*, *440*(7086), 935–939. http://doi.org/10.1038/nature04592

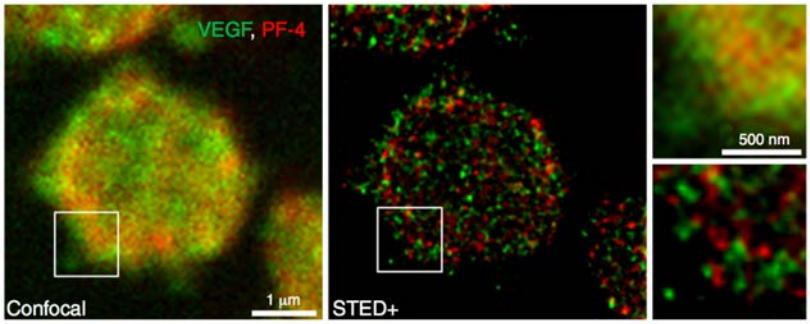
Super-resolution 9: Confocal STED microscopy

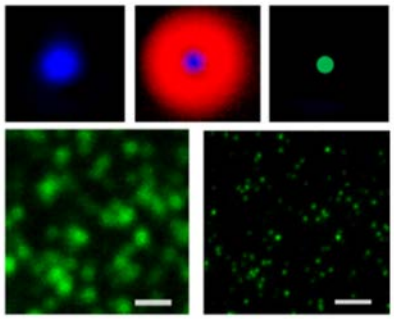




Blom, H., & Widengren, J. (2014). *Current Opinion in Chemical Biology*, *20*, 127–133. http://doi.org/10.1016/j.cbpa.2014.06.004

Super-resolution 9: Confocal STED microscopy

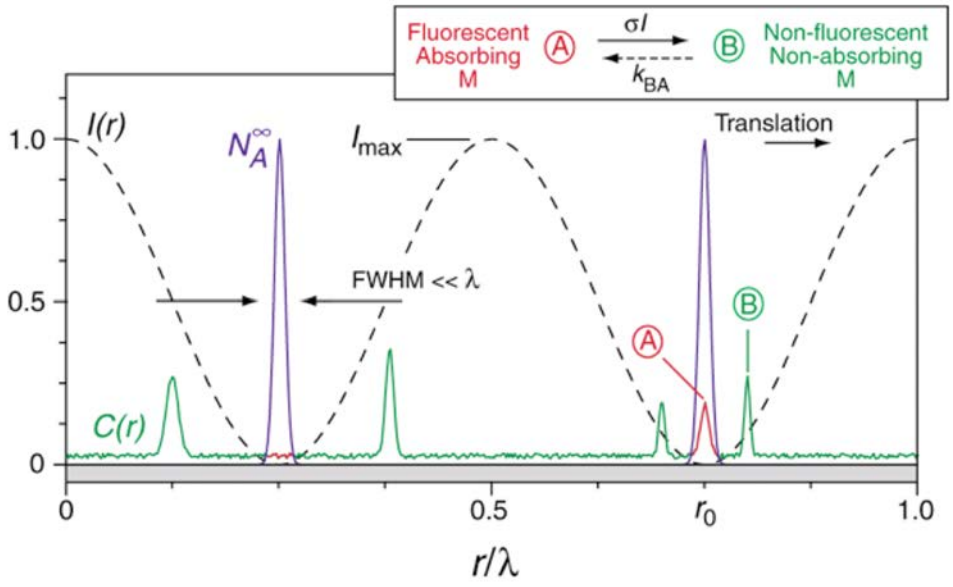




P. Tinnefeld, *Far-Field Optical Nanoscopy*, Springer, 2015.

Super-resolution 10: RESOLFT

Use of another "dark" molecular state to break the diffraction limit.
Molecules outside the intensity minimum are switched off



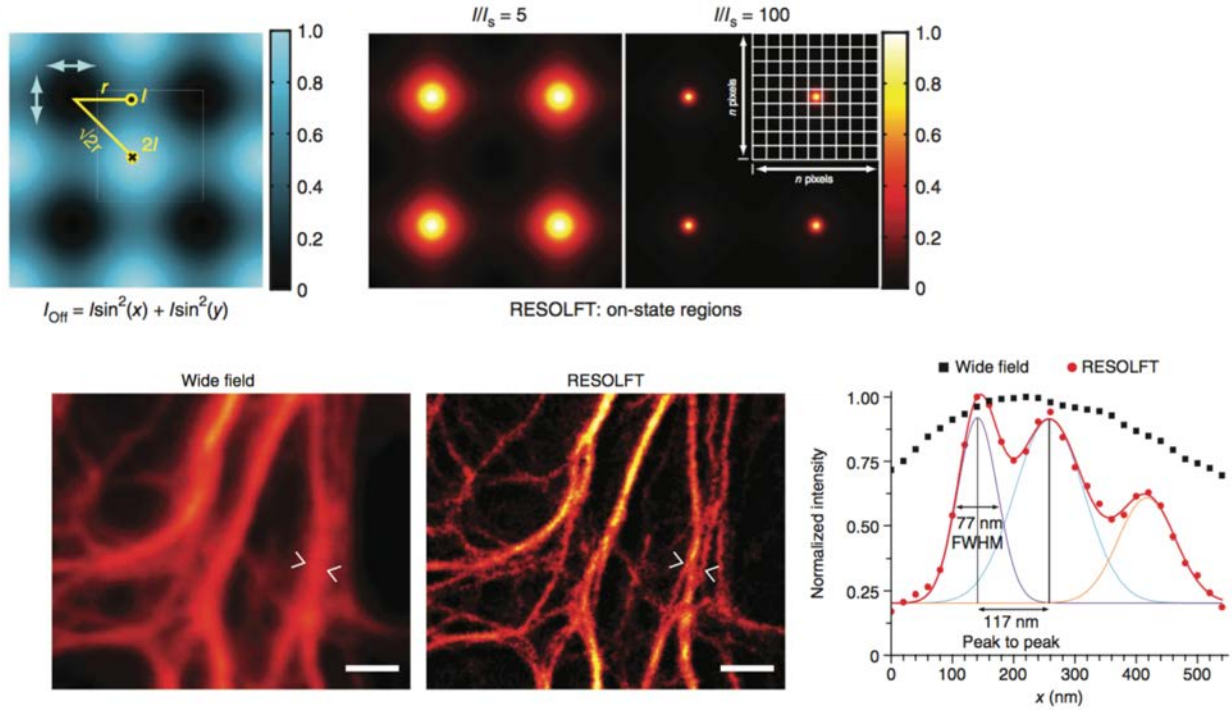
The energy required to switch off the molecule are low compare to STED:

- Less damage to the sample
- Possibility to use pattern for illumination

Hell, S. W. Toward fluorescence nanoscopy. *Nat Biotechnol* **21,** 1347–1355 (2003).

Super-resolution 11: RESOLFT

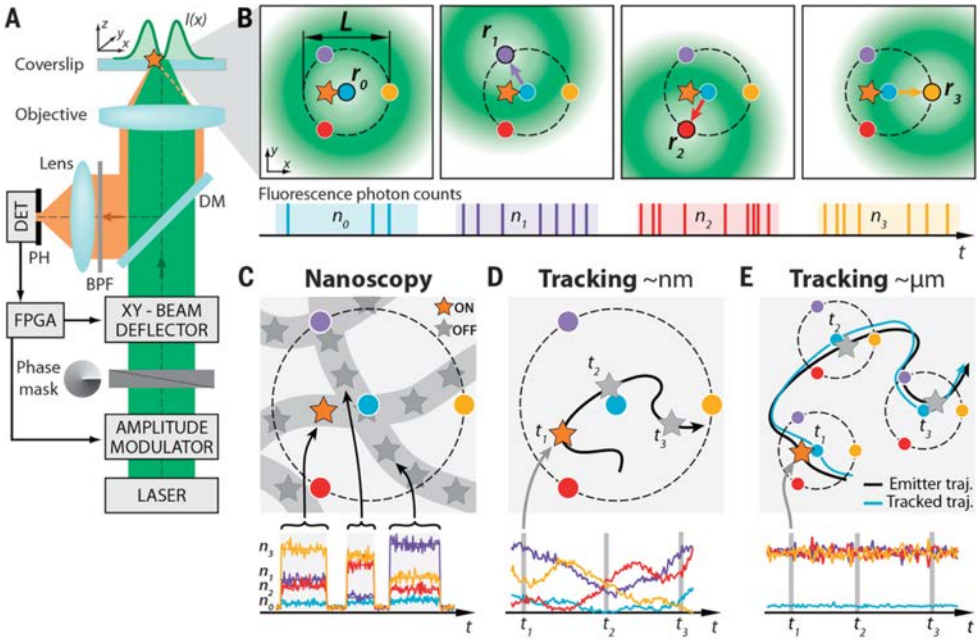
The low energy required to populate the dark state allow to use a network of foci



Chmyrov, A. *et al.* Nanoscopy with more than 100,000 'doughnuts'. *Nat Methods* **10,** 737–740 (2013).

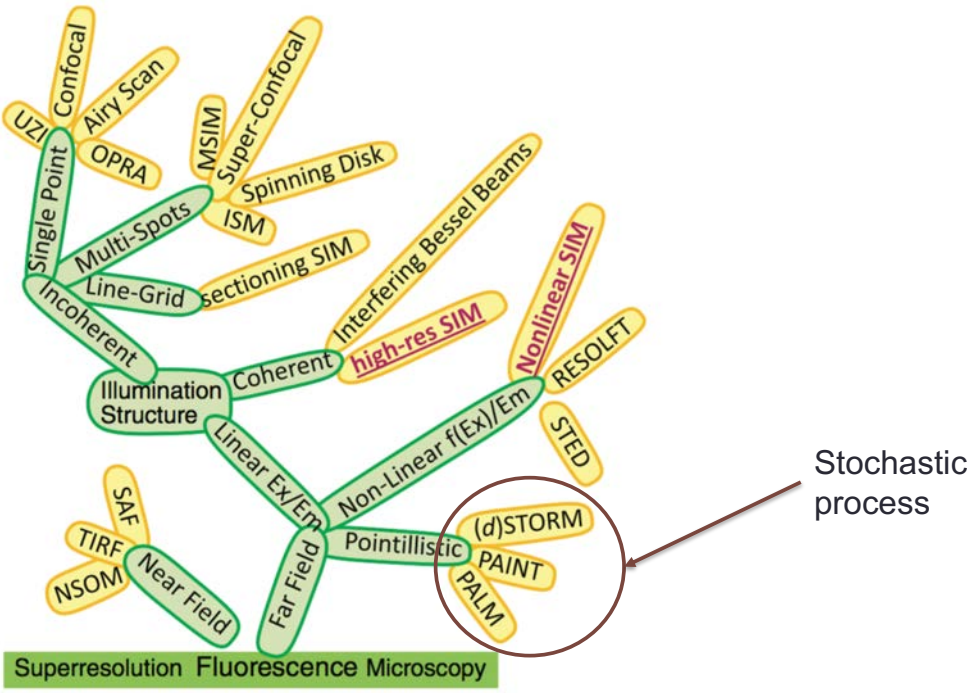
Super-resolution 12: Miniflux

Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes



Balzarotti, F. *et al. Science* **355,** 606–612 (2017).

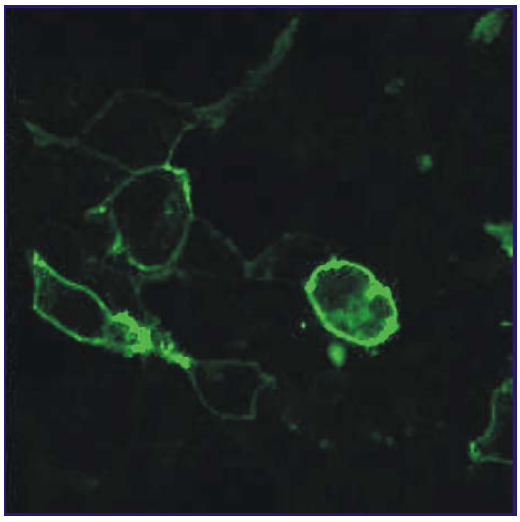
Super-resolution



Hell, S. W. *et al.* The 2015 super-resolution microscopy roadmap. *Journal of Physics D: Applied Physics* 1–35 (2015). doi:10.1088/0022-3727/48/44/443001

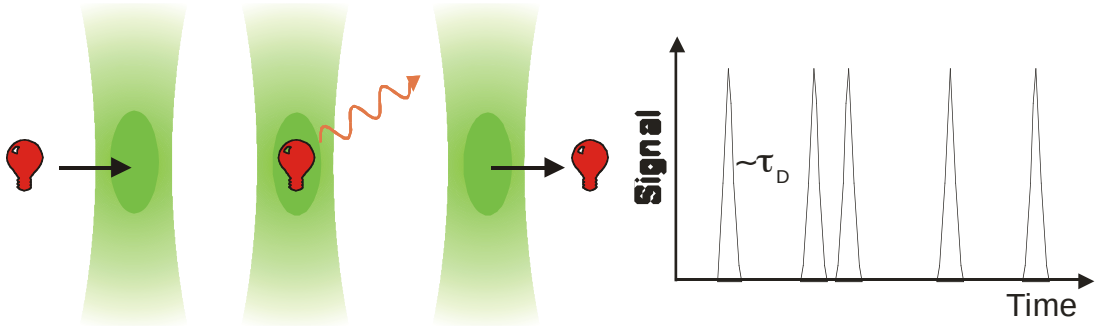
Single-molecule detection and fluorescence signal correlation

Rapidly diffusing molecules: Single-molecule detection (SMD) and fluorescence correlation spectroscopy (FCS)



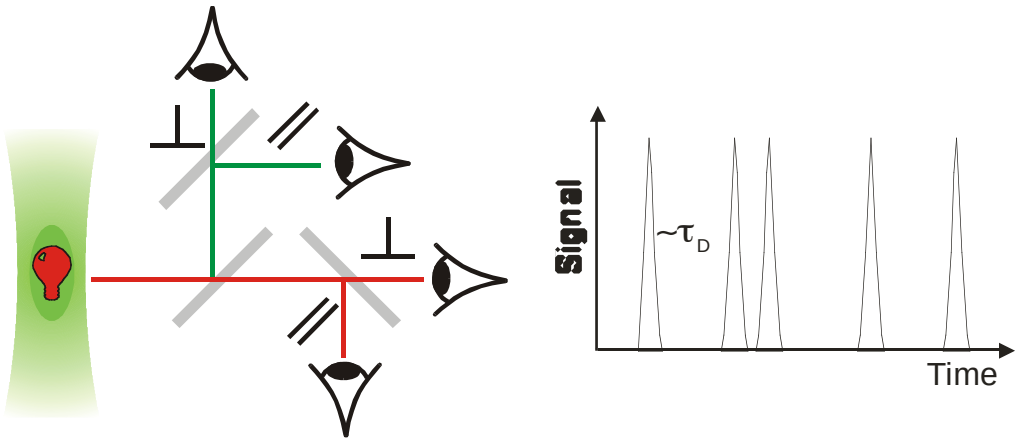
- Diffusion is too fast to be measured by single-molecule tracking
- High concentration of label
- Single-molecule measurement in solution

Confocal volume fixed at a particular location



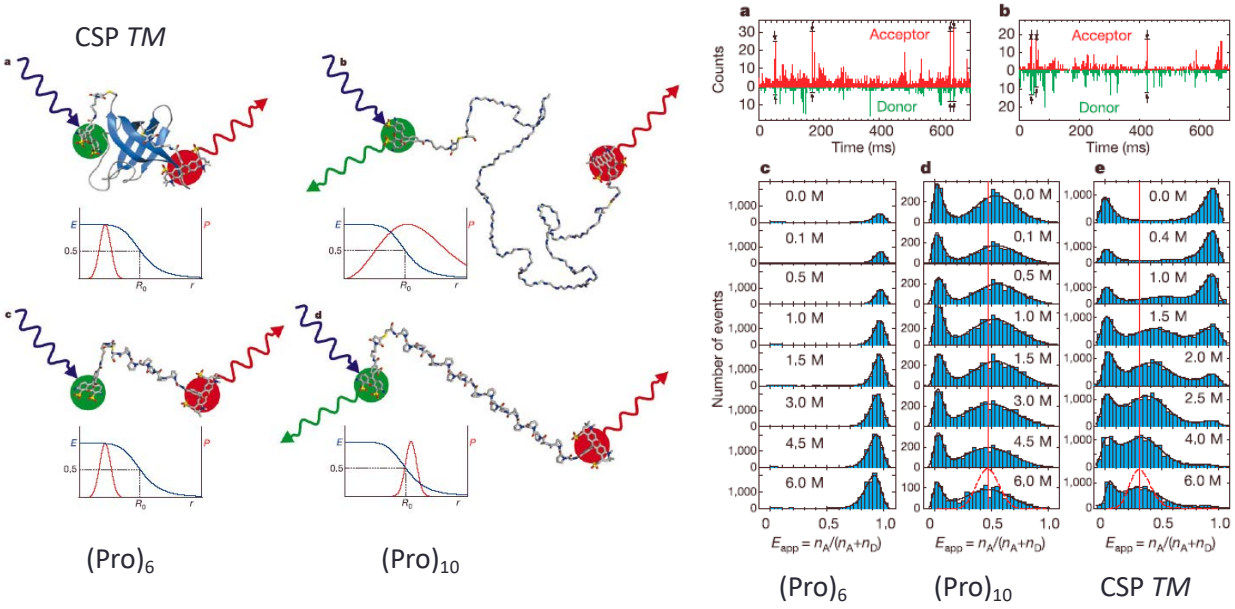
- Remark: The signal emitted by a single molecule when it is diffusing through the confocal volume is extremely small. It is necessary to average over several single molecules.

Multiparameter Fluorescence Detection (MFD) or Single-Molecule Detection (SMD)



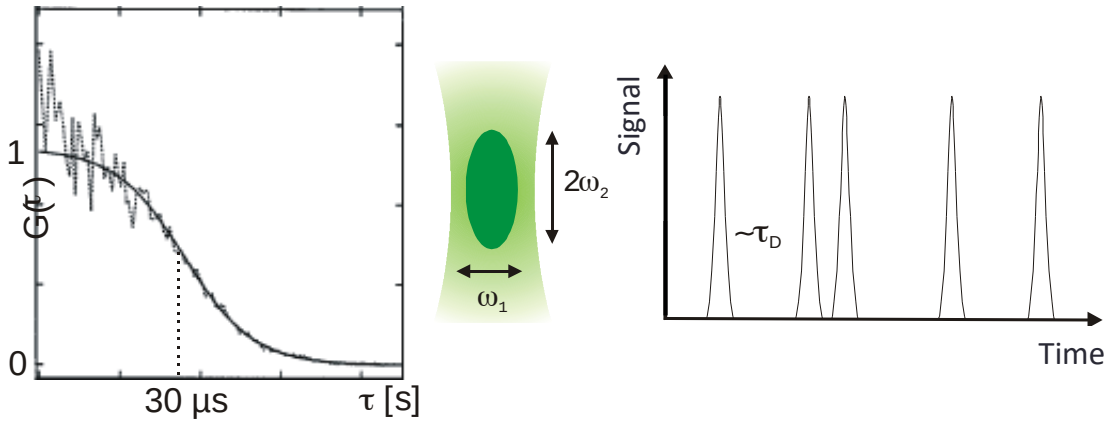
- Many parameters are simultaneously measured: colour, FRET efficiency, polarization (anisotropy), fluorescence lifetime, arrival time (absolute time), intensity, diffusion.
- By correlating the parameters, novel observations can be made.

Example: protein folding



B. Schuler, E.A. Lipman, W.A. Eaton, *Nature* **419**, 743 (2002)

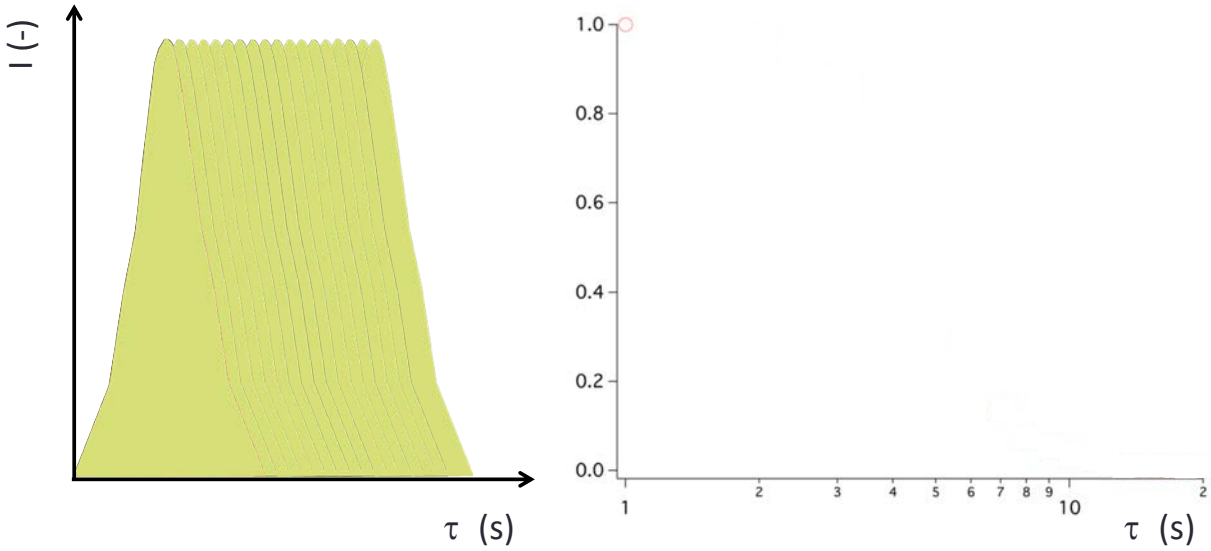
Fluorescence autocorrelation function



$$G'(\tau) = \langle I(t) \cdot I(t+\tau) \rangle = \lim_{T \to \infty} \frac{1}{2T} \int_{-T}^{T} \langle I(t) \cdot I(t+\tau) \rangle dt$$

- *G'* denotes the non-normalized autocorrelation function, *t* the time difference, *I* the intensity and *T* the measurement time

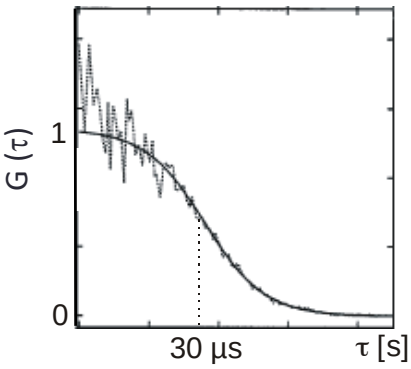
Fluorescence autocorrelation function



$$G'(\tau) = \langle I(t) \cdot I(t+\tau) \rangle = \lim_{T \to \infty} \frac{1}{2T} \int_{-T}^{T} \langle I(t) \cdot I(t+\tau) \rangle dt$$

- *G'* denotes the non-normalized autocorrelation function, *t* the time difference, *I* the intensity and *T* the measurement time

Influence of diffusion

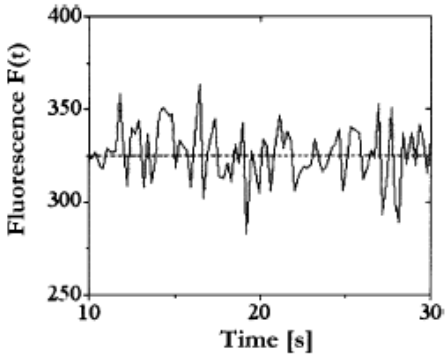


$$\tau_D = \frac{\omega_1^2}{4D} \quad D = \frac{k_B T}{6\pi\eta r} \quad r = \sqrt[3]{\frac{3M / N_A}{4\pi\rho}}$$

$$\frac{\tau_{D,ref}}{\tau_{D,\,unknown}} = \frac{D_{unknown}}{D_{ref}} = \sqrt[3]{\frac{M_{ref}}{M_{unknown}}}$$

- In the formulas: t_D: diffusion time, w_1: diameter of the focus, D: diffusion constant, M: molecular weight, N_A: Avogadro number, r: density, r: hydrodynamic radius, h: viscosity. This model is only correct for spherical molecules.
- For proteins r=1.2 g/cm^3; For nucleic acids r=1.8 g/cm^3; For lipids r=0.9-1.1 g/cm^3.
- When the molecular weight changes by a factor of two, the diffusion constant only changes by a factor of 1.26 (detection limit).
- With w_1=230 nm, t_D=37 ms for a usual dye (M=550 D, D=360 mm^2/s) and 235 ms for an antibody (M=140 kD).

Extension to multiple molecules: fluorescence correlation spectroscopy (FCS)



$$G'(\tau) = \langle I(t) \cdot I(t+\tau) \rangle$$

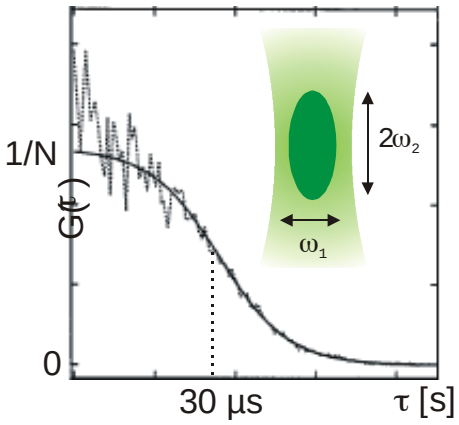
$$I(t) = I + \langle \delta I(t) \rangle$$

$$G'(\tau) = \langle I \rangle^2 + \langle \delta I(t) \delta I(t+\tau) \rangle$$

$$G(\tau) = \frac{G'(\tau)}{\langle I \rangle^2} - 1 = \frac{\langle \delta I(t) \delta I(t+\tau) \rangle}{\langle I \rangle^2} \quad \text{ou} \quad G(\tau) = \frac{G'(\tau)}{\langle I \rangle^2}$$

- In the formulas: G: normalized autocorrelation function. dI: intensity fluctuation from the averaged intensity.

Fluorescence autocorrelation function for multiple molecules



The number of molecules in the confocal volume fluctuates with poissonian statistics

$$\lim_{x\to 0}\langle \delta I(t)\delta I(t+\tau)\rangle / \langle I^2\rangle = \frac{1}{N}$$

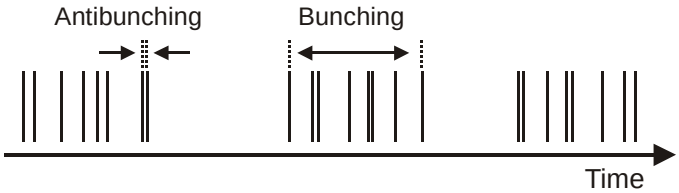
For a gaussian profile in the confocal volume:

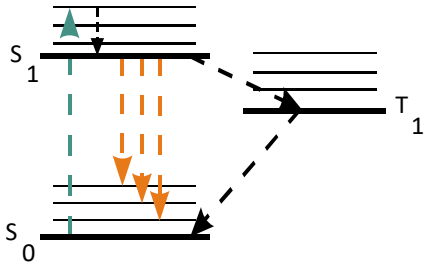
$$G(\tau) = \frac{1}{N}\cdot\frac{1}{1+4D\tau/\omega_1^2}\left[\frac{1}{1+4D\tau/\omega_2^2}\right]^{1/2}$$

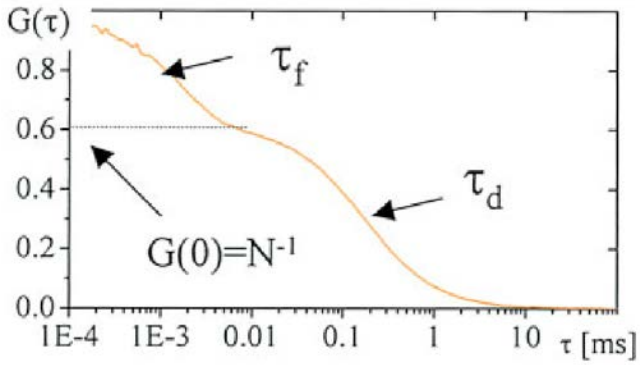
$$\omega_2 = S\cdot\omega_1 \qquad V = \omega_1^2\cdot\pi\cdot 2\cdot\omega_2 = 2\cdot\pi\cdot S\cdot\omega_1^3 \qquad c = \frac{N}{N_A\cdot V}$$

- In the formulas: *N*: averaged number of molecules in the confocal volume, *S*: structure parameter, *V*: volume, *c*: concentration.
- *S* typically is 5; w_1 is diffraction-limited, i.e. ~0.5l=250 nm for a laser at 500 nm; *S* and w_1 are determined using a standard.

Influence of the triplet state





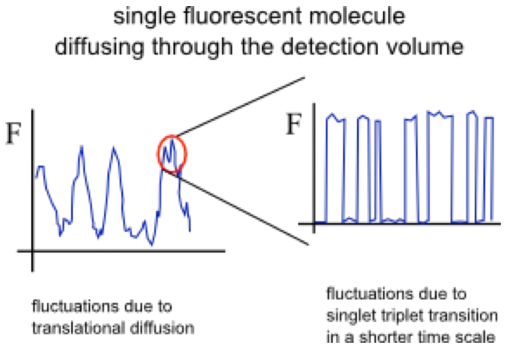


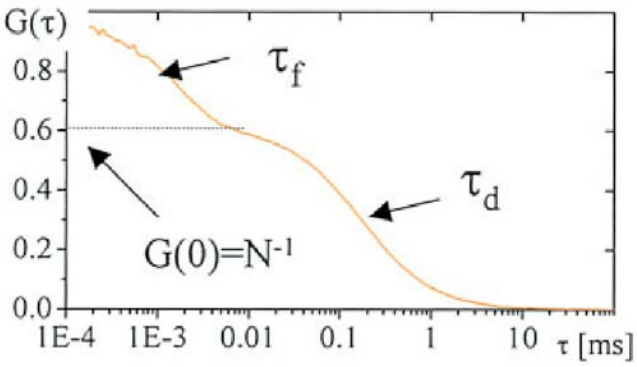
The presence of the triplet state adds a term to the autocorrelation function:

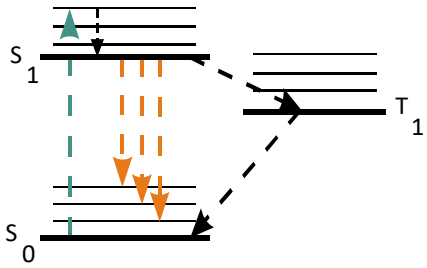
$$\frac{1-F+F\exp(-\tau/\tau_T)}{1-F}$$

With *F*: fraction of molecules in the triplet; t_T: triplet lifetime

Influence of the triplet state





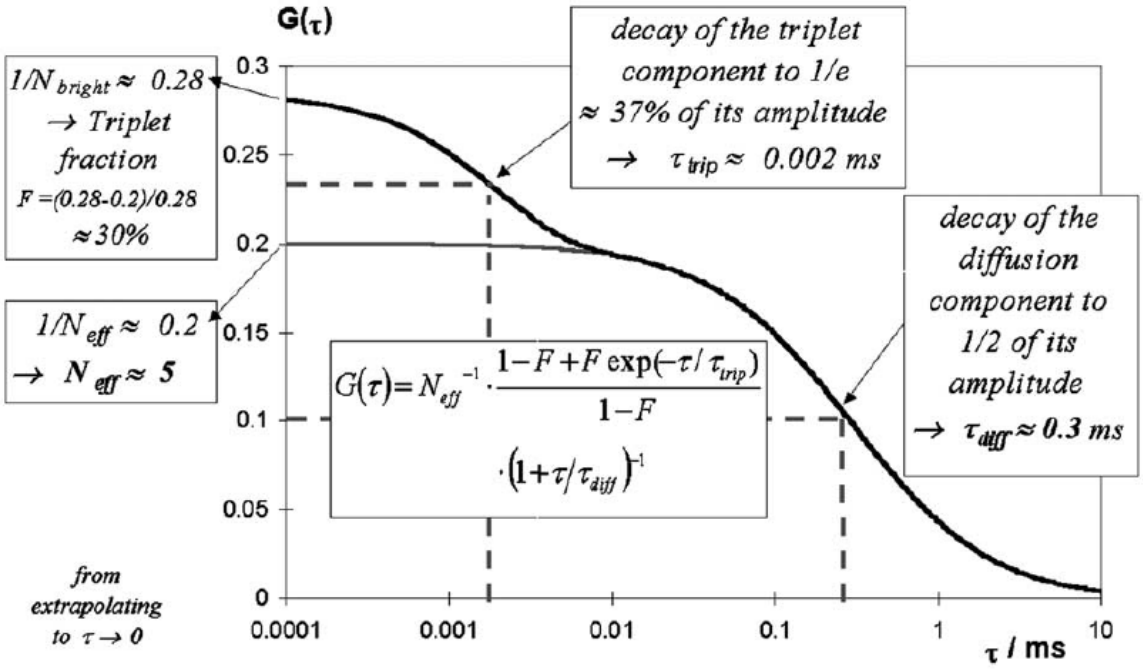


The presence of the triplet state adds a term to the autocorrelation function:

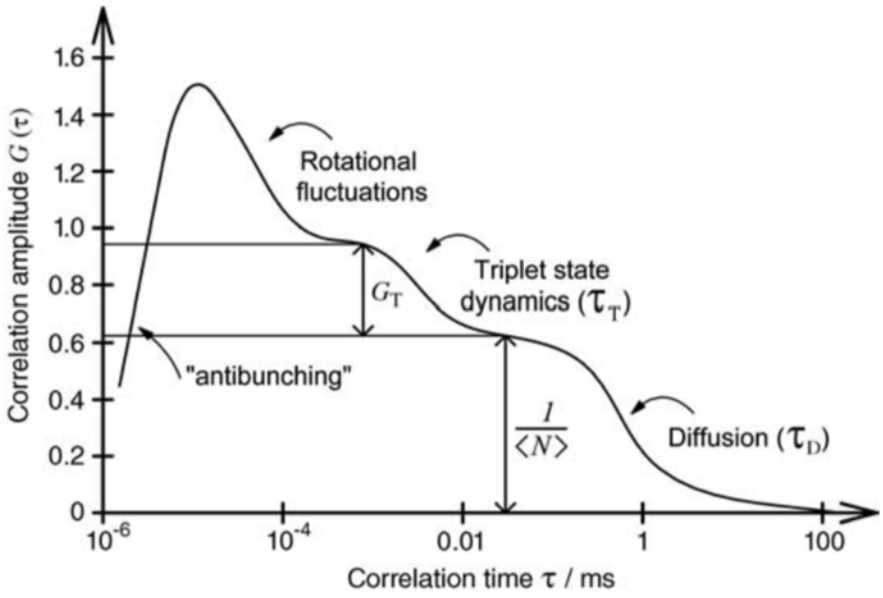
$$\frac{1-F+F\exp(-\tau/\tau_T)}{1-F}$$

With F: fraction of molecules in the triplet; t_T: triplet lifetime

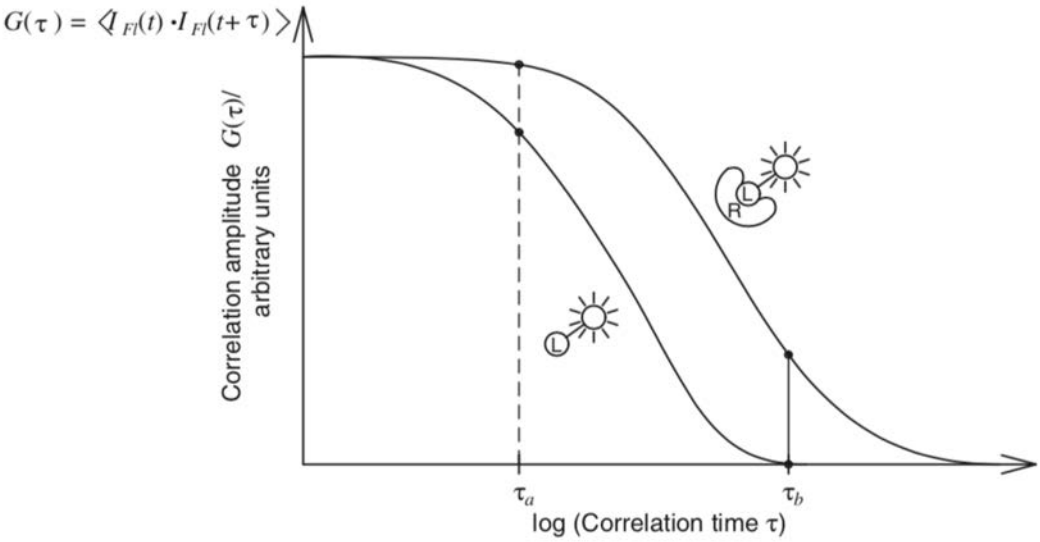
Fluorescence autocorrelation function: summary



Fluorescence autocorrelation function: summary



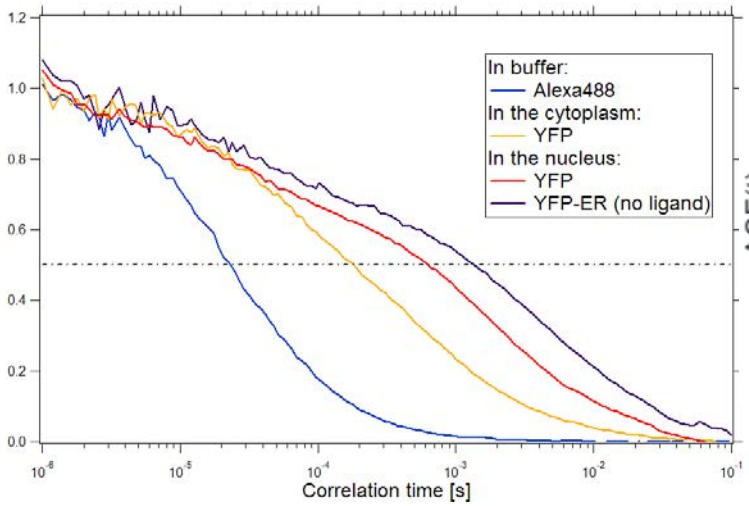
Example: Measurements of binding constants

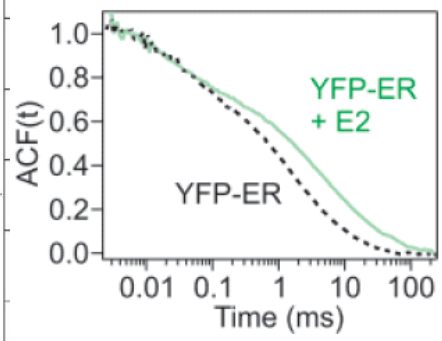


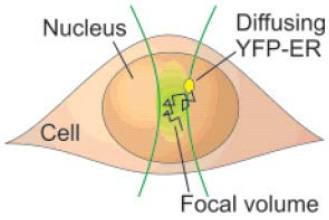
- Principle: The molecular weight of the labelled molecule drastically changes upon binding or reaction. Typical case: The binding of small ligands on receptors.
- Since the fluorescence from the various species is not correlated, the autocorrelation function is the sum of the autocorrelation functions of the different species.

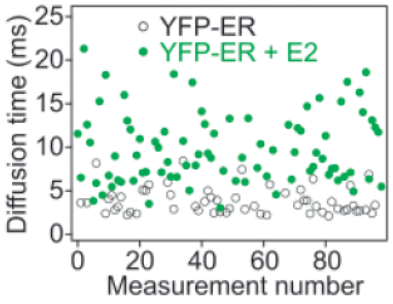
P. J. Walla, *Modern Biophysical Chemistry*. John Wiley & Sons, 2015.

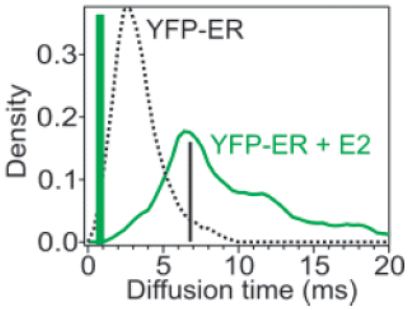
Example: The estrogen receptor



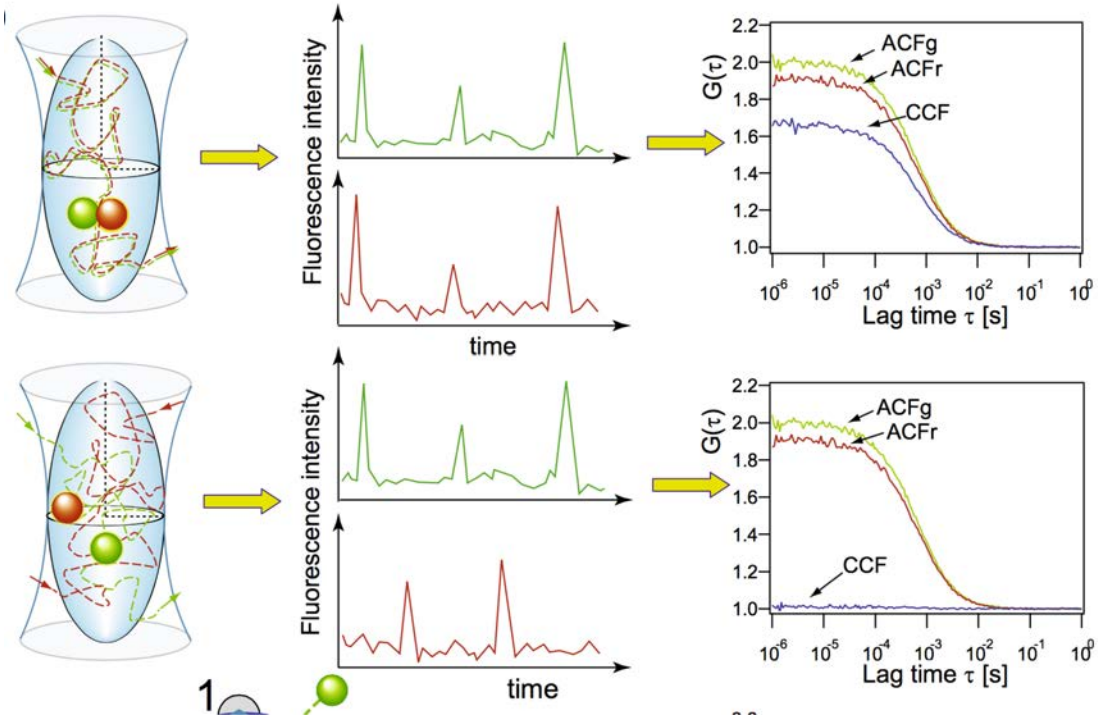






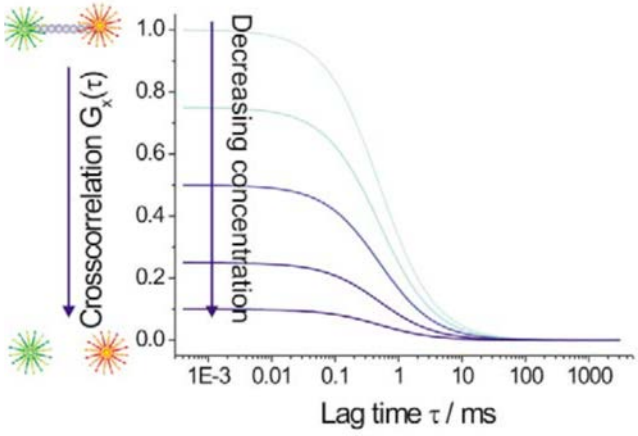


Cross-correlation

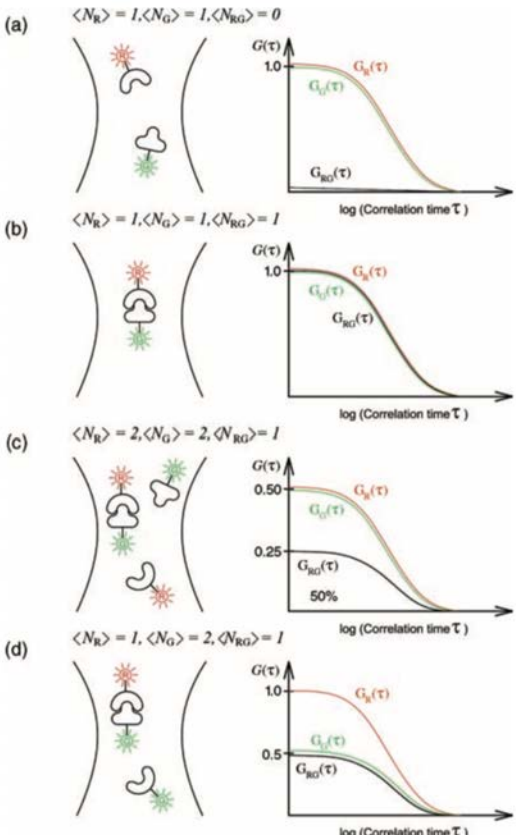


Cross-correlation

Concentration of dimers in solution



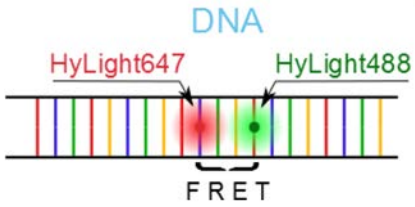
Various concentration of species

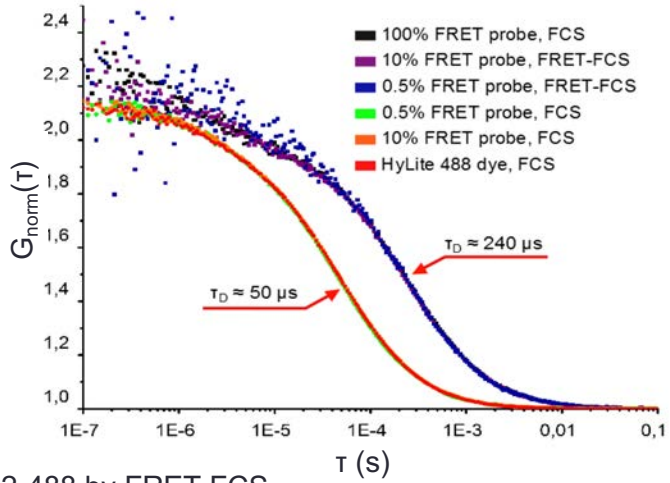


P. J. Walla, *Modern Biophysical Chemistry*. John Wiley & Sons, 2015.

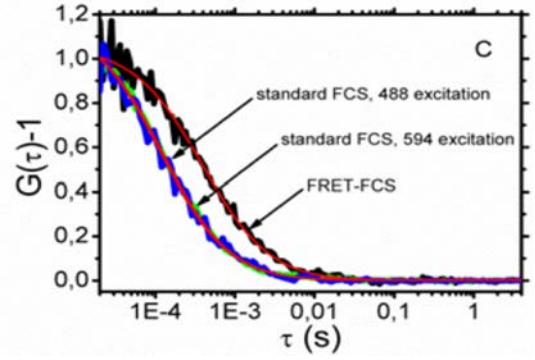
FRET-FCS

Detection of weakly interacting molecules in solution



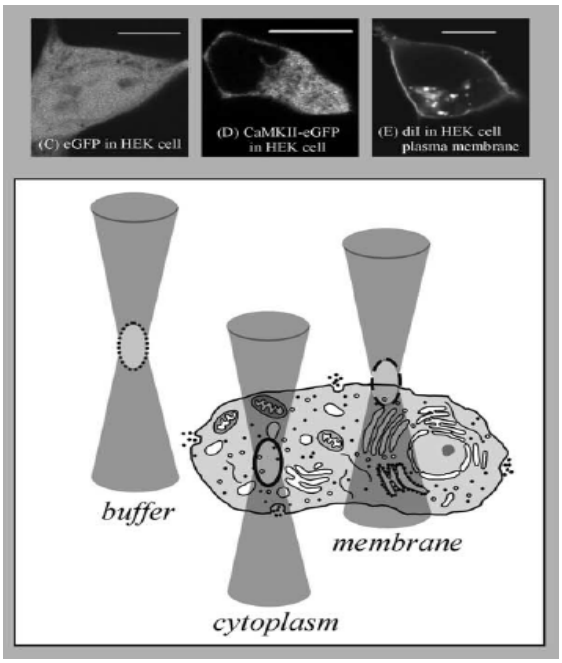


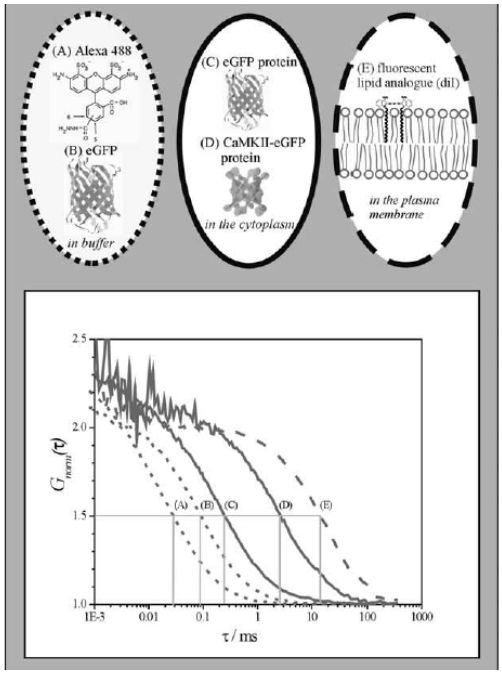
Detection of oligomers of Aβ42-647and Aβ42-488 by FRET-FCS



Amyloids β aggregation is very difficult to detect in solution:
The contrast of FRET-FCS is sufficient to detect very low aggregation

Measurements on membranes



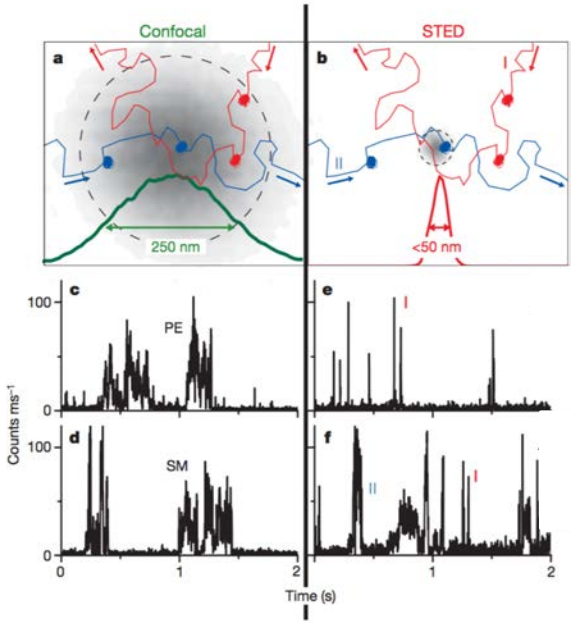


$$G(\tau) = \frac{1}{N} \cdot \frac{1}{1 + 4D\tau / \omega_1^2}$$

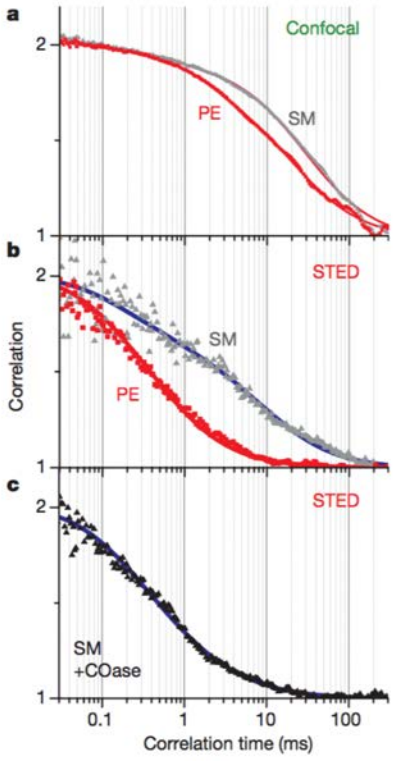
2D diffusion of membrane proteins is slow for conventional FCS

STED FCS (2D diffusion)

Illumination volume is reduced
- Access to smaller diffusion coefficient
- Access to mesoscale structures



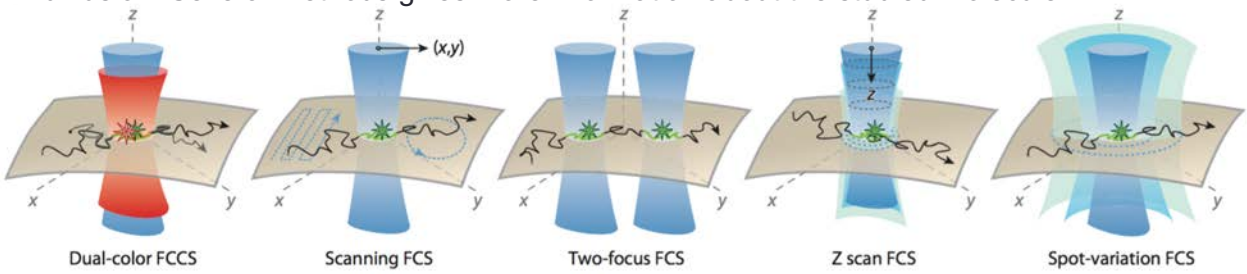
Diffusion of sphingomyelin and phospatidylethanolamine lipids in the plasmae membrane



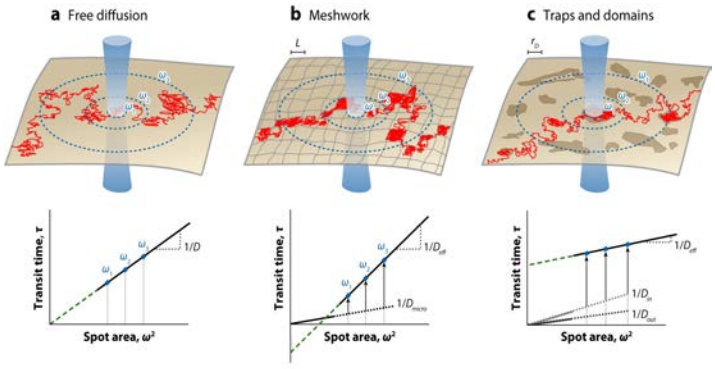
Eggeling, C., et al. (2009). *Nature*, *457*(7233), 1159–1162. http://doi.org/10.1038/nature07596

FCS-Toolbox

Standard FCS is not ideal for the detection of immobile molecule, domains or anomalous diffusion. Several methods gives more information about the studied molecule

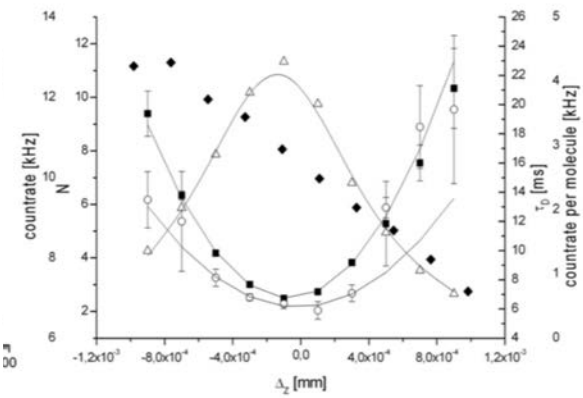


Spot variation FCS



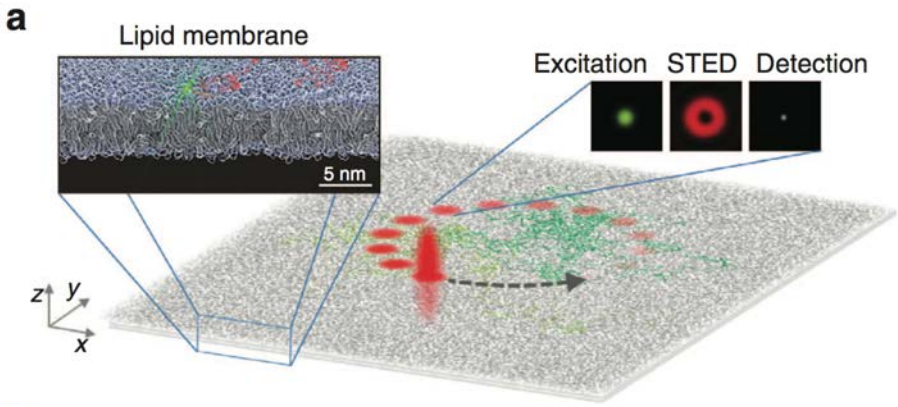
He H-T, Marguet D. 2011.
Annu. Rev. Phys. Chem. 62:417–36

Z-Scan FCS



Macháň, R. & Hof, M *BBA* **1798,** 1377–1391 (2010).

Scanning STED FCS (2D diffusion)



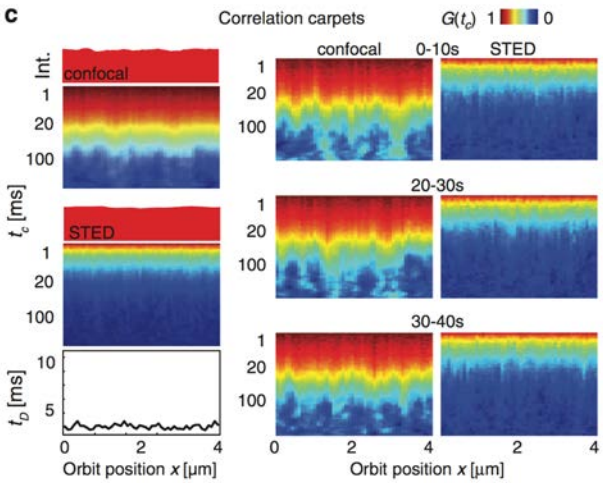
The STED beam is circularly moved in the plane of the cell membrane:

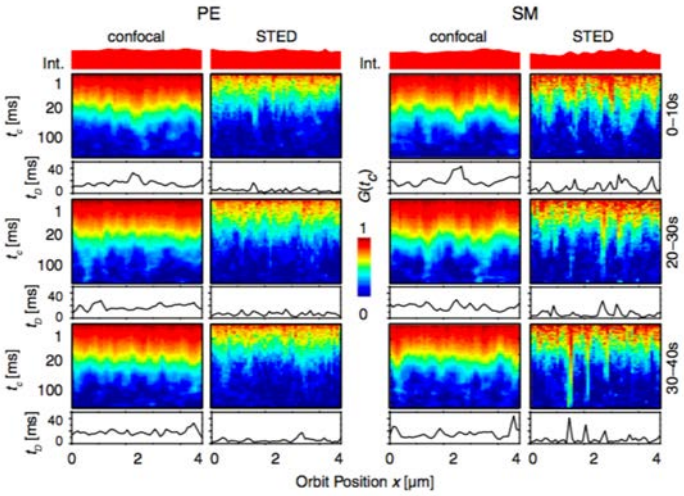
- 1-4 kHz rotating frequency
- Radius in the µm range
- 60 nm resolution (width of the STED volume)
- 1 pixel every 15 nm

Mueller V et al. (2014) Nat Commun 5:1–12.

Scanning STED FCS (2D diffusion)

Correlation carpet of simulated brownian diffusing particles





Correlation carpet of PE and SM in the plasma membrane of living cells

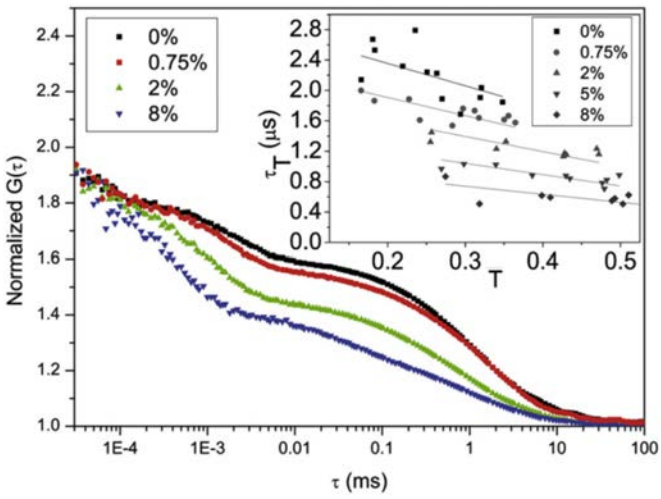
Mueller V et al. (2014) Nat Commun 5:1–12.

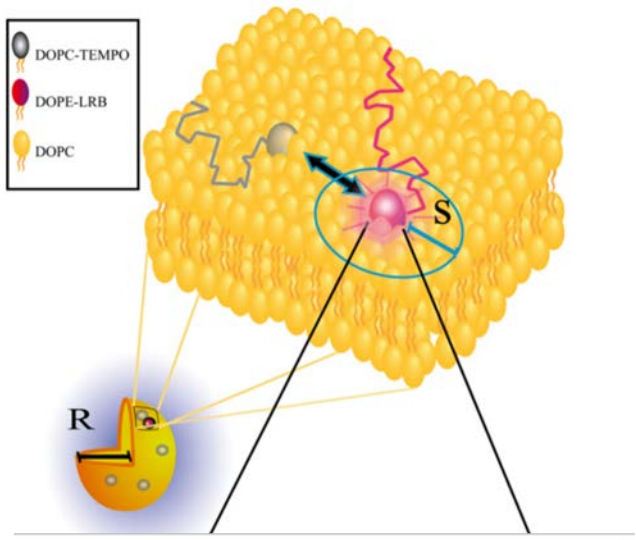
- ✓ Direct access to the heterogeneity of the cell membrane
- ✓ Possibilty to extract informations about lipids domain
- ✓ Spatial correlation

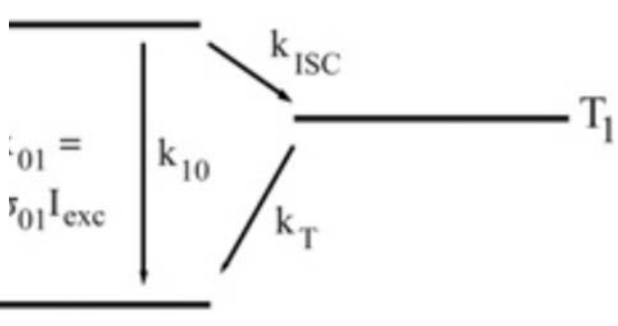
Quenching of Triplet State

FRET timescale is too short to measure interactions between diffusing particles

Triplet lifetime (up to µs) is long enough for the diffusing molecule to interact



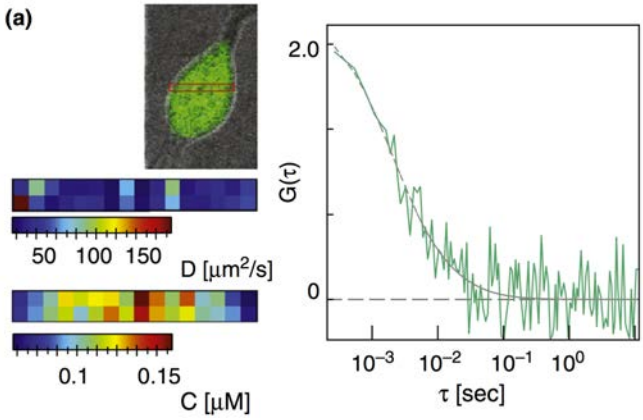


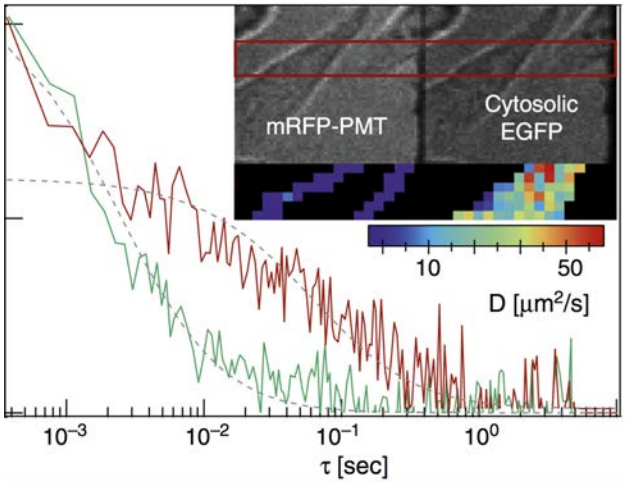


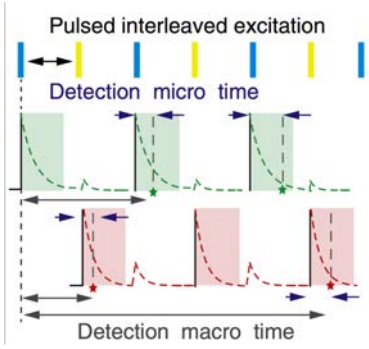
Strömqvist, J., et al.(2010). *Biophysical Journal*, *99*(11), http://doi.org/10.1016/j.bpj.2010.09.059

Imaging FCS

Two observables:
- Diffusion coefficient
- Number of molecules

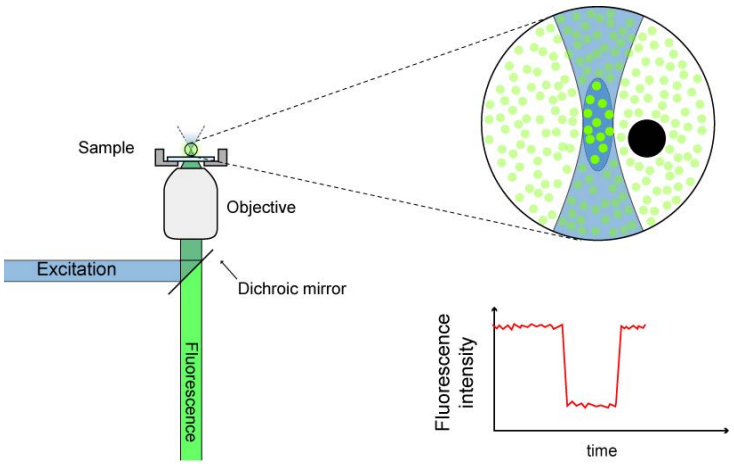


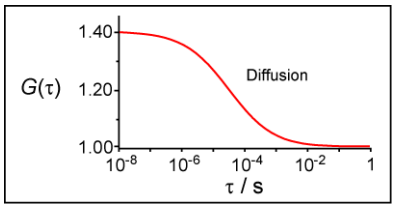




Singh, A. P., & Wohland, T. (2014). *Current Opinion in Chemical Biology*, *20*, 29–35. http://doi.org/10.1016/j.cbpa.2014.04.006

Single-molecule detection without labeling: Inverse FCS





$$G(0) = \frac{N}{\left(\frac{1}{V_q} - N\right)^2} \qquad V_q = \frac{V_{particle}}{V_{dectection}}$$

- Photon noise
- Molecular noise

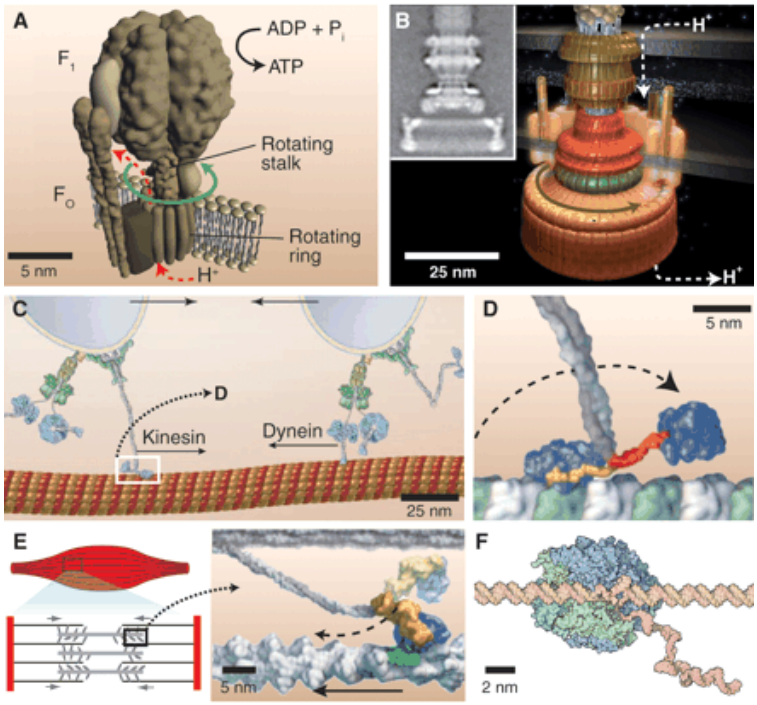
Wennmalm S. et al., *Anal. Chem.*, 2009

Functional devices based on single-molecule

Outlook: How can one integrate nano-machines into functional devices?

- Molecular motors
- Nano-bioanalytics, nano-containers: Reducing the sample volumes
- Nano-sensors: Combination with electrophysiology
- Nano-sequencer: DNA, RNA and peptide sequencing

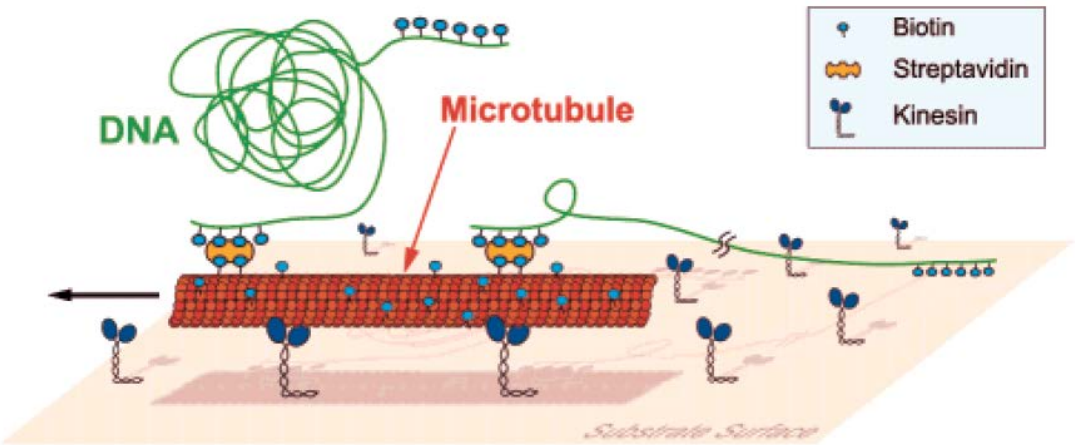
Molecular Motors



M. G. L. van den Heuvel, C. Dekker, *Science* **317,** 5836, (2007) 333 – 336

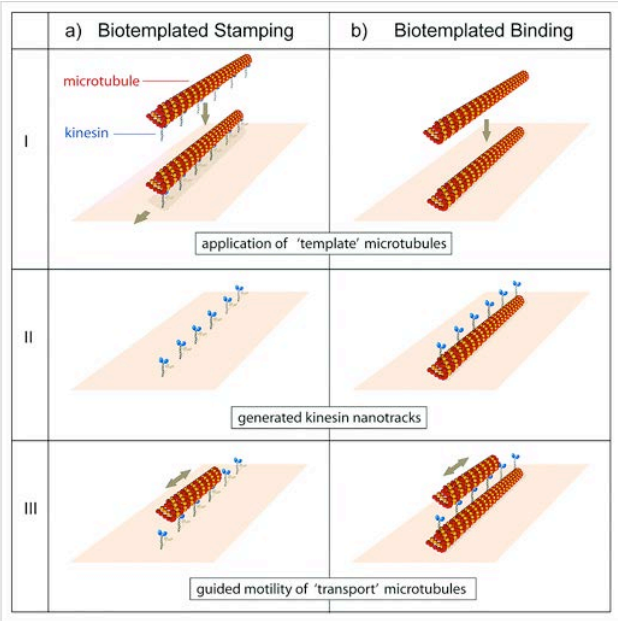
Processing motors: Moving cargos

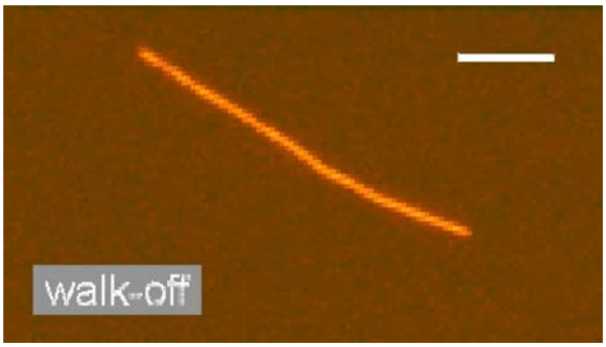
Motor: kinesin. Cargos: DNA attached to microtubules

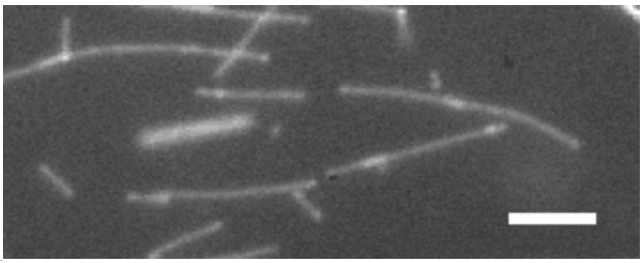


S. Diez, C. Reuther, C. Dinu, R. Seidel, M. Mertig, W. Pompe, J. Howard, *Nano Lett.* **3**, 1251 (2003)

Kinesin nanotracks



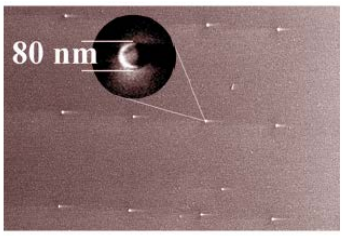


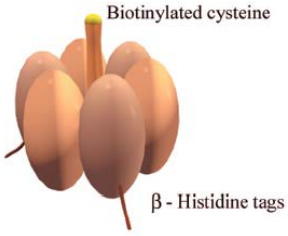


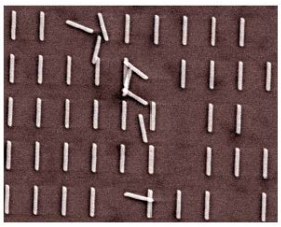
C. Reuther,[†] L. Hajdo,[‡] R. Tucker,[§] A. A. Kasprzak,[‡] S. Diez*[†] *Nano Lett.*, 2006, 6 (10), 2177–2183

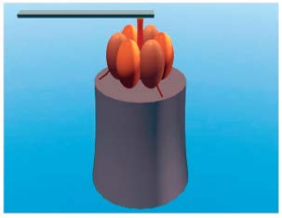
Rotary motors: F1 ATPase

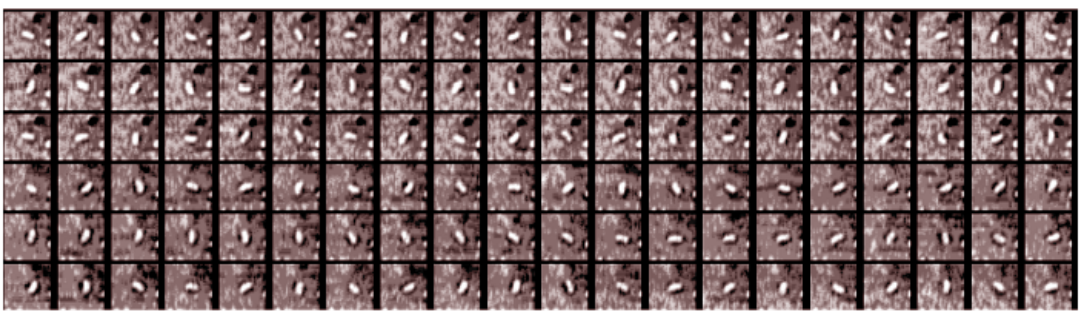
F1 ATPase attached to Ni posts by His Tags is used to rotate Ni arms





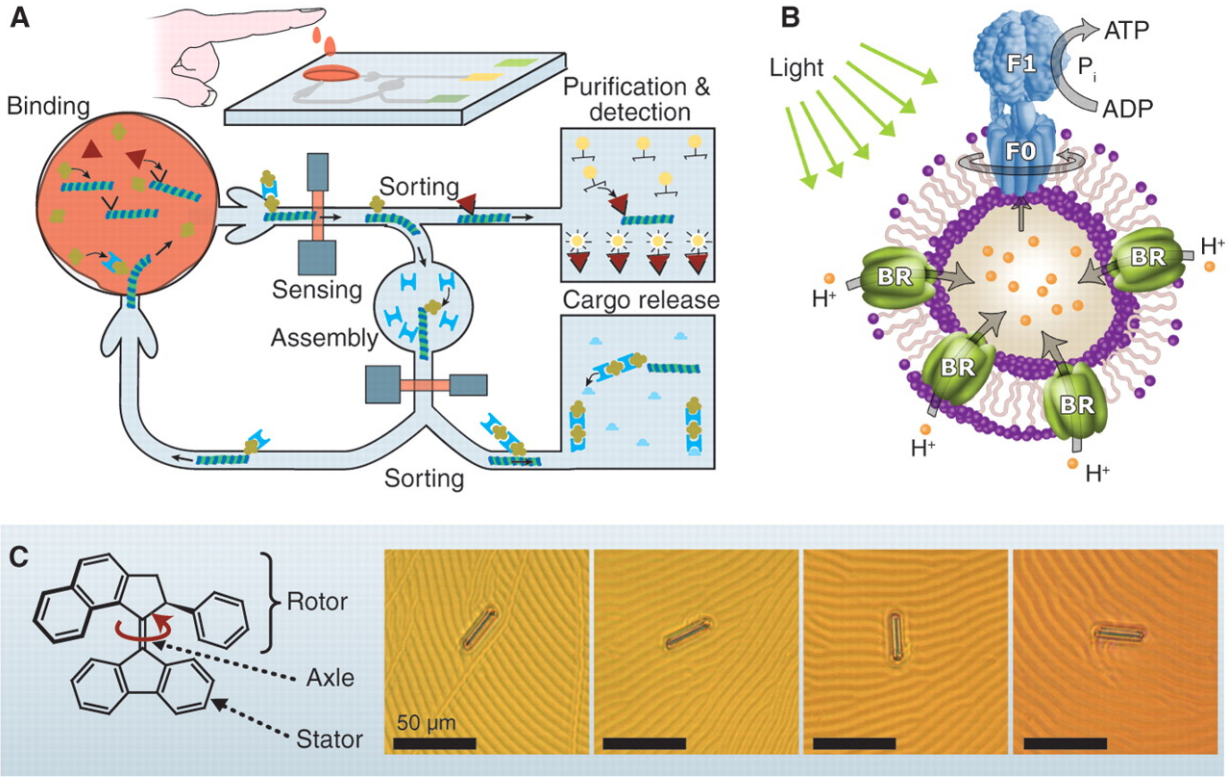






R.K. Soong, G.D. Bachand, H.P. Neves, A.G. Olkhovets, H.G. Craighead, C.D. Montemagno, *Science* **290**, 1555 (2000)

New directions with nanomotors



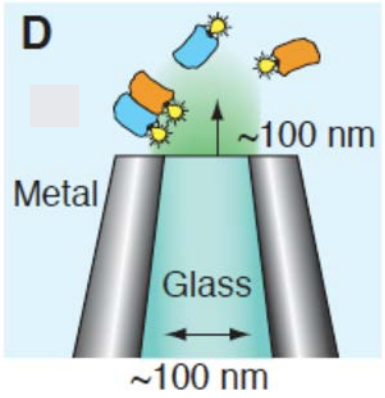
M. G. L. van den Heuvel, C. Dekker, *Science* **317,** 5836, (2007) 333 – 336

High concentration or slow diffusion: Confining the volume

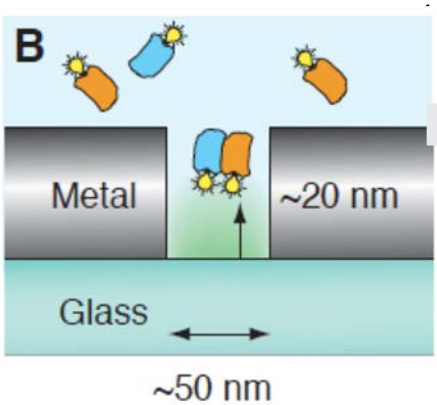
Measurement on living systems often involve high concentration on molecule or very slow diffusion coefficient, which are not compatible with conventional FCS

Working with small volume can overcome these drawbacks

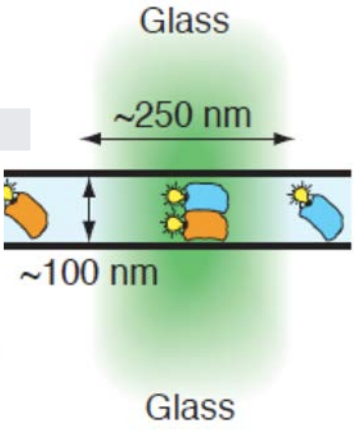
Metallic Tapered Fiber tip



Subwavelength metal nanopores

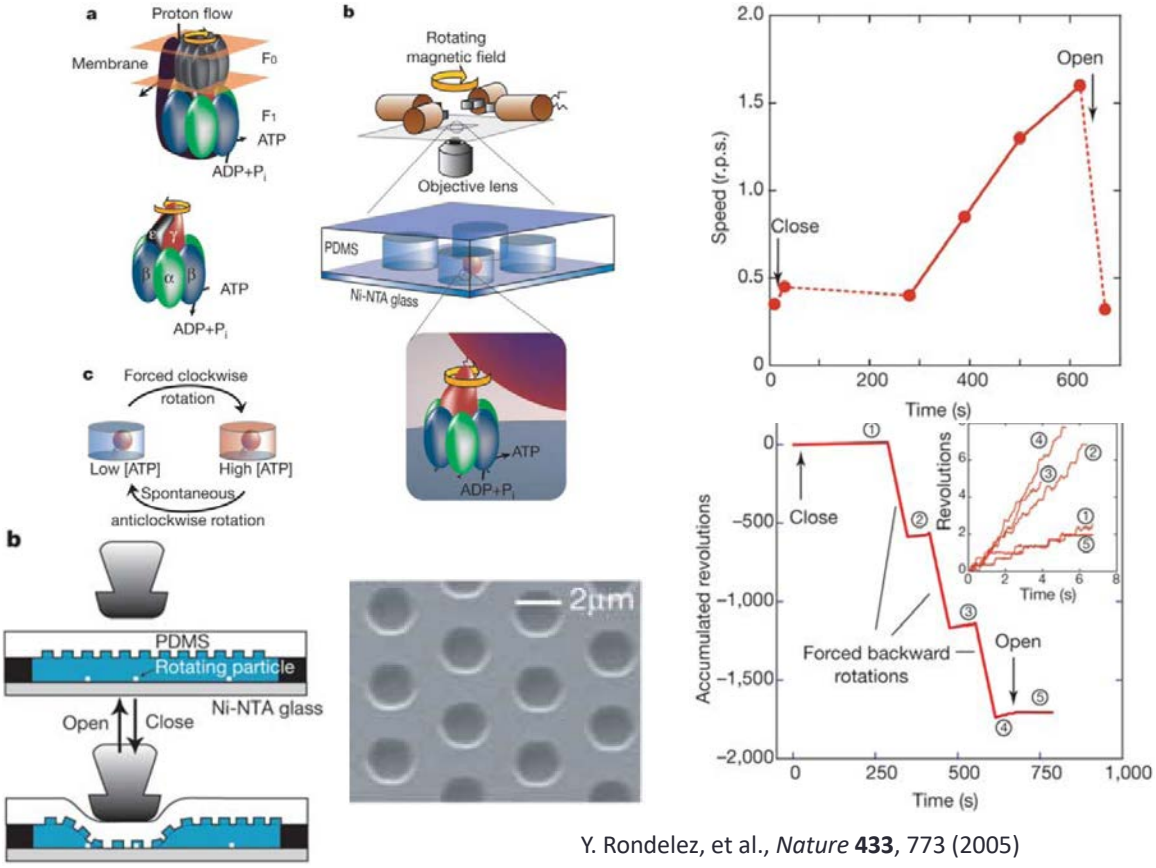


Nanofluidic



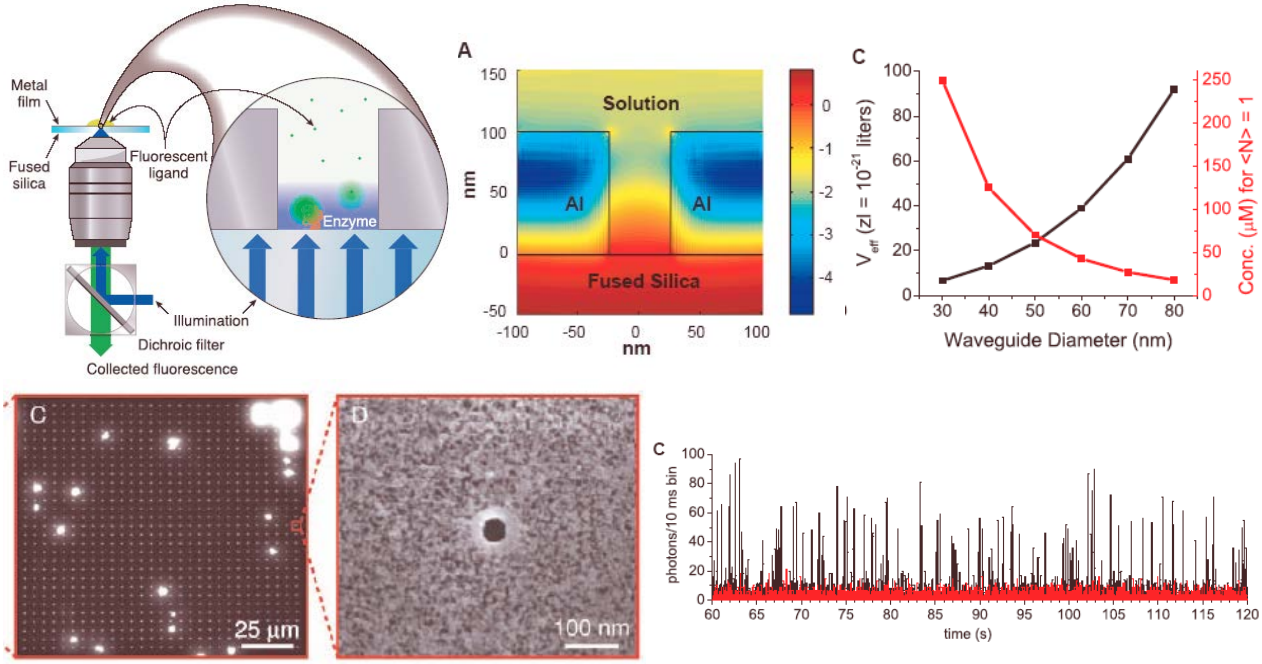
Laurence, T. A., Nature, 2003

Reducing the volumes: Femtoliter chambers



Y. Rondelez, et al., *Nature* **433**, 773 (2005)

Reducing the volumes for nano-bioanalytics: Nanowells



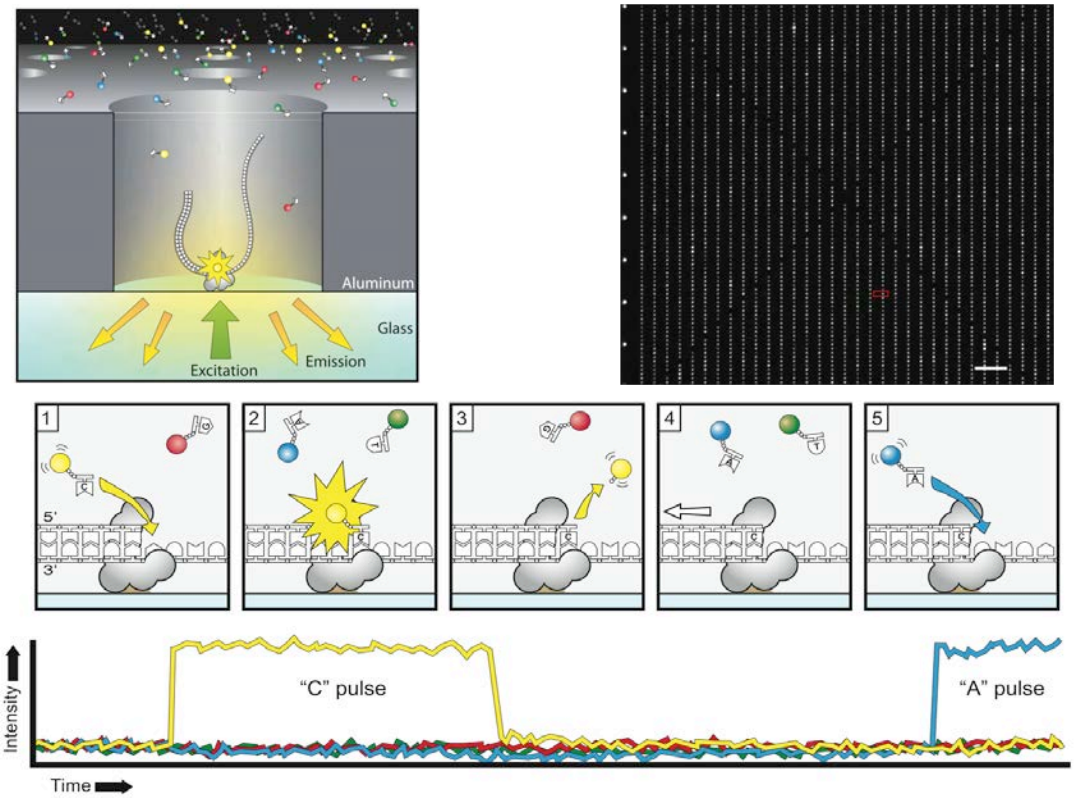
$$I(z) = \exp(-z/\Lambda)$$

$$\frac{1}{\Lambda} = \sqrt{2\frac{1}{\lambda_c^{\,2}} - \frac{1}{\lambda_m^{\,2}}}$$

Principle: Small wells in Al with a diameter smaller than the wavelength of light. The light penetrates only up to a small depth in z.

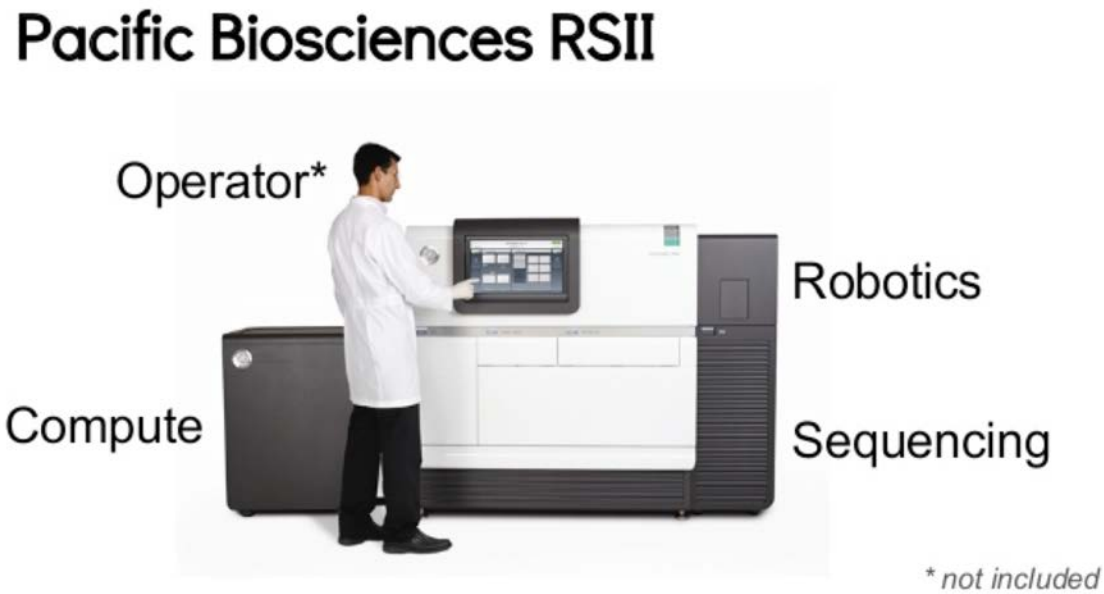
M.J. Levene, J. Korlach, S.W. Turner, M. Foquet, H.G. Craighead, W.W. Webb, *Science* **299**, 682 (2003)

Nanowells: DNA sequencing

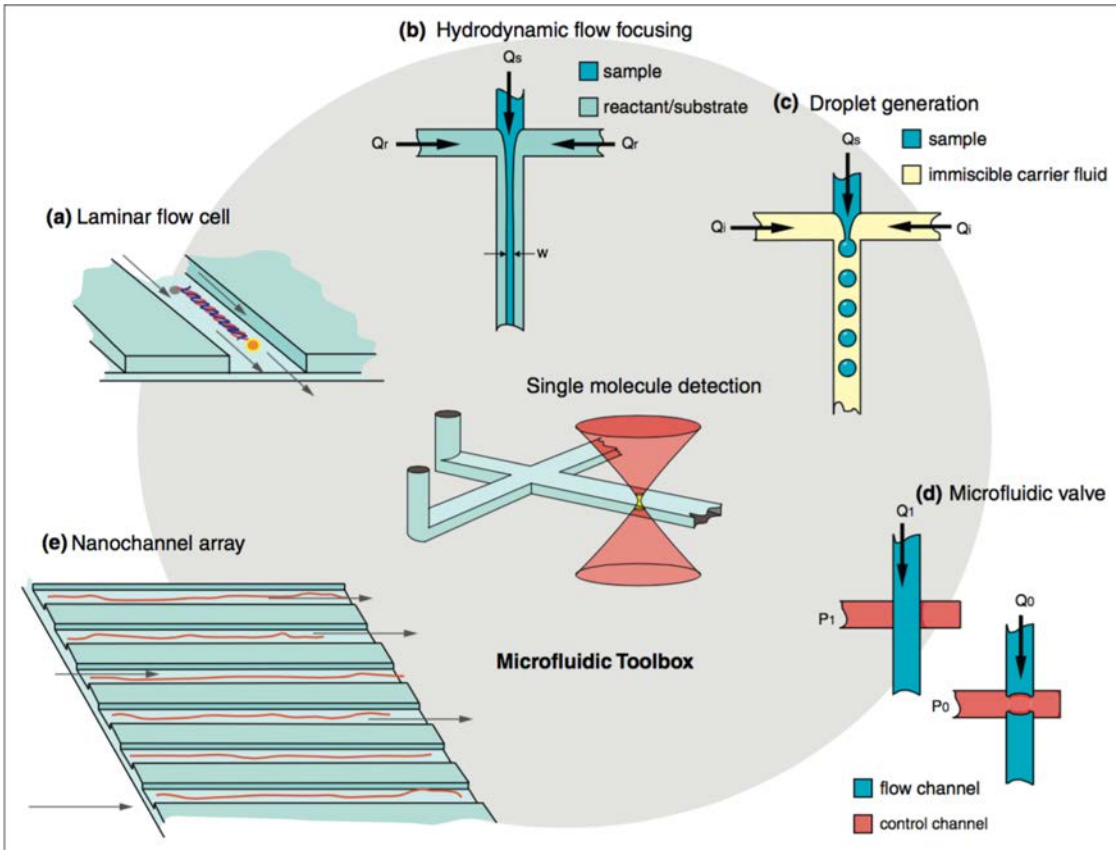


J.Eid, et al., *Science* **323** (2009) 133-138

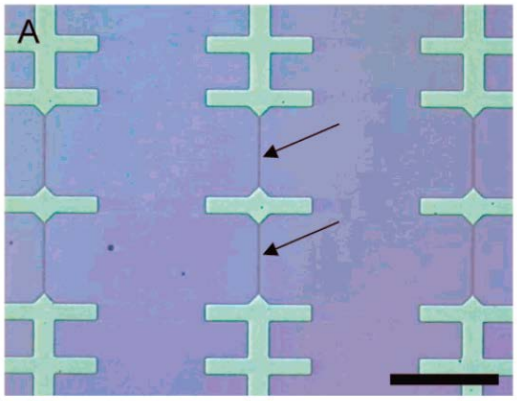
Nanowells: DNA sequencing

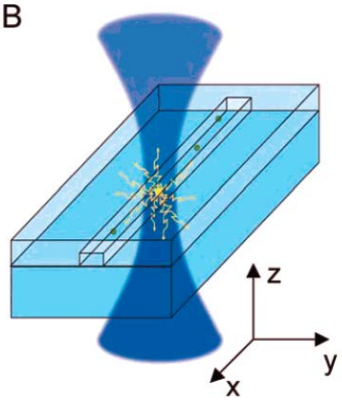


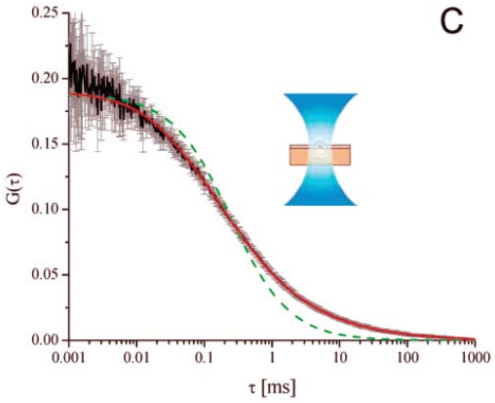
Reducing the volumes for nano-bioanalytics: Nanofluidics



Reducing the volumes for nano-bioanalytics: Nanofluidics



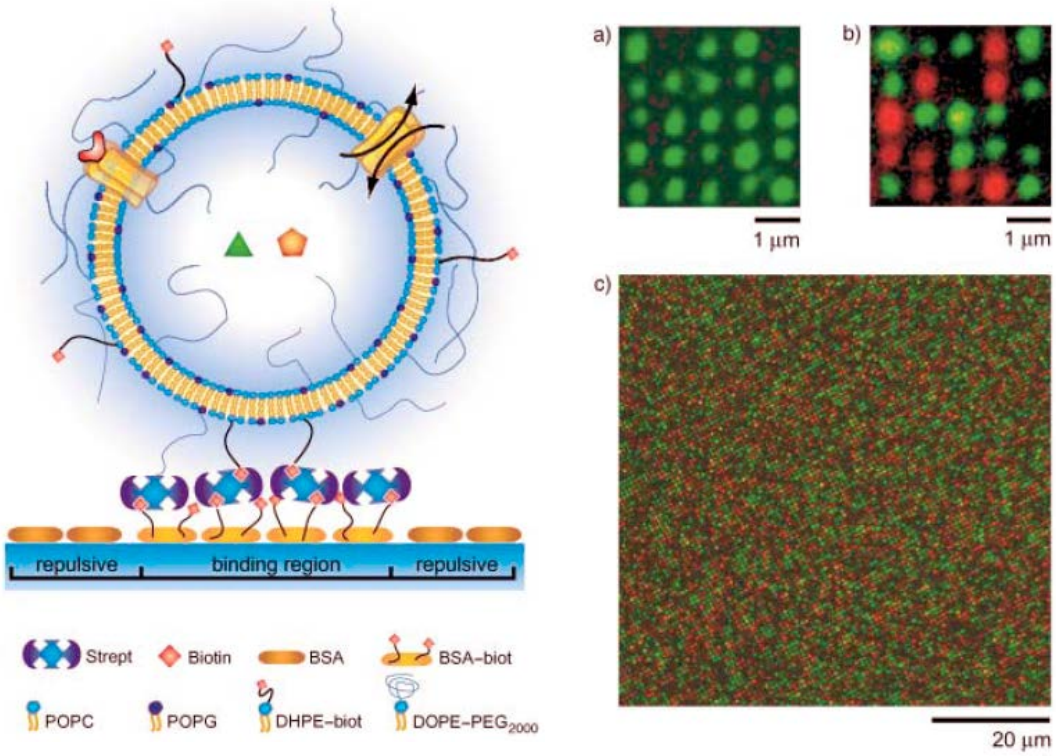




Channel height: ~250 nm
Wide channel diameter: 10 mm
Narrow channel diameter: ~350 nm
V_{eff}=~1 fL usually
V_{eff}=0.033 fL for the wide channel
V_{eff}=0.011 fL for the narrow channel

M. Foquet, J. Korlach, W.R. Zipfel, W.W. Webb, H.G. Craighead
Anal. Chem. **76**, 1618 (2004)

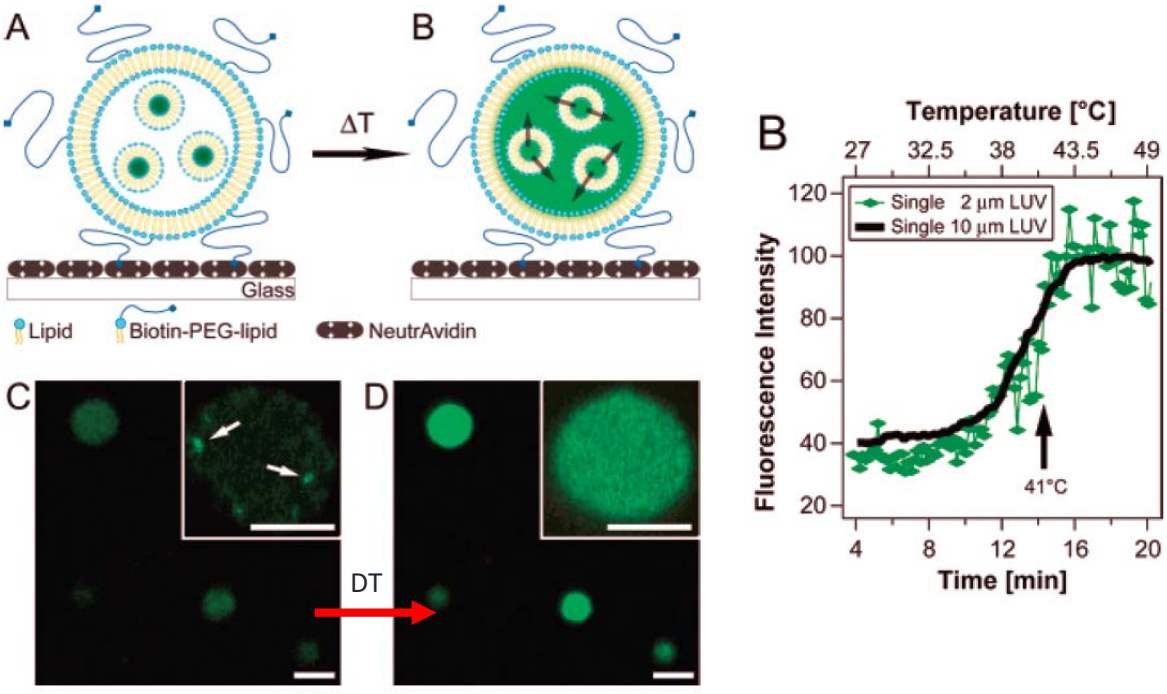
Reducing the volumes for nano-bioanalytics: Nanocontainers made out of vesicles ($V_{vesicle}$ = 1 aL)



D. Stamou, C. Duschl, E. Delamarche, H. Vogel, *Angew. Chem. Int. Ed.* **42**, 5580 (2003)

Nano-reactor: Nanochemistry in a vesicle

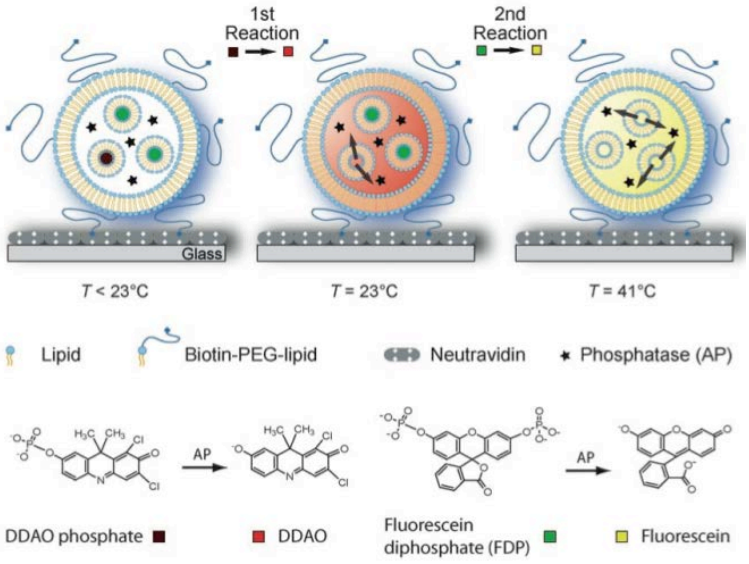
Proof of principle using fluorescein. Fluorescein is quenched in the small vesicles. After release from the small vesicles, its intensity increases.

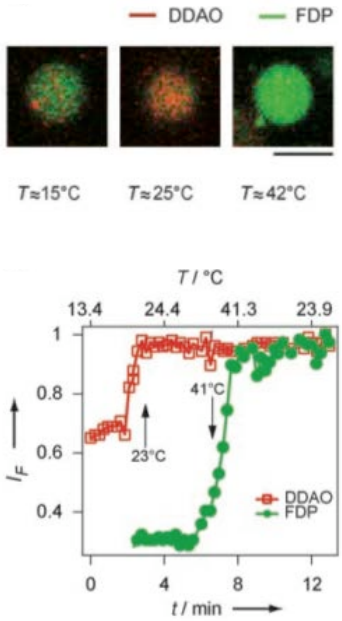


P.-Y. Bolinger, D. Stamou, H. Vogel, *J. Am. Chem. Soc.* **126**, 8594 (2004)

Nano-reactor: Nanochemistry in a vesicle

Consecutive enzymatic reactions in a single nanoreactor.

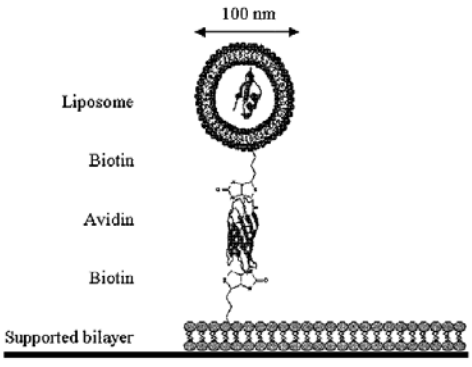


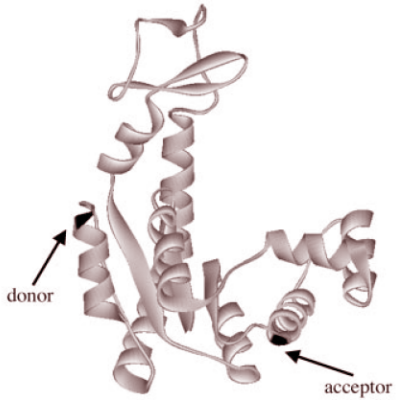


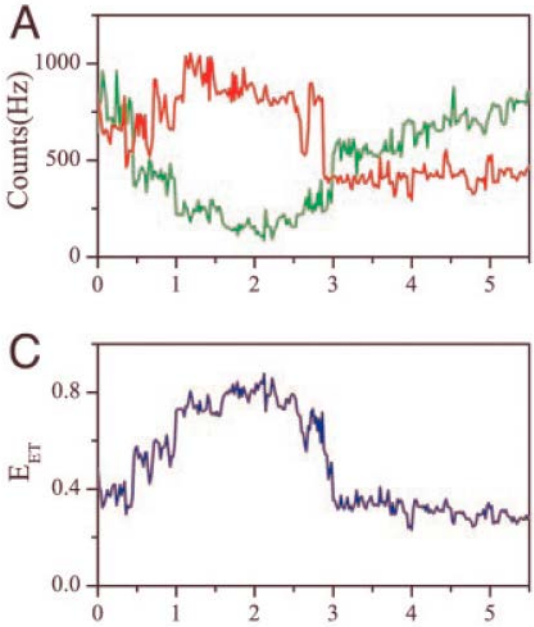
P.-Y. Bolinger, D. Stamou, H. Vogel, *Angew. Chem. Int. Ed*. **47**, 5544 (2008)

Single-molecule investigations in nano-containers

Protein folding of Adenylate Kinase

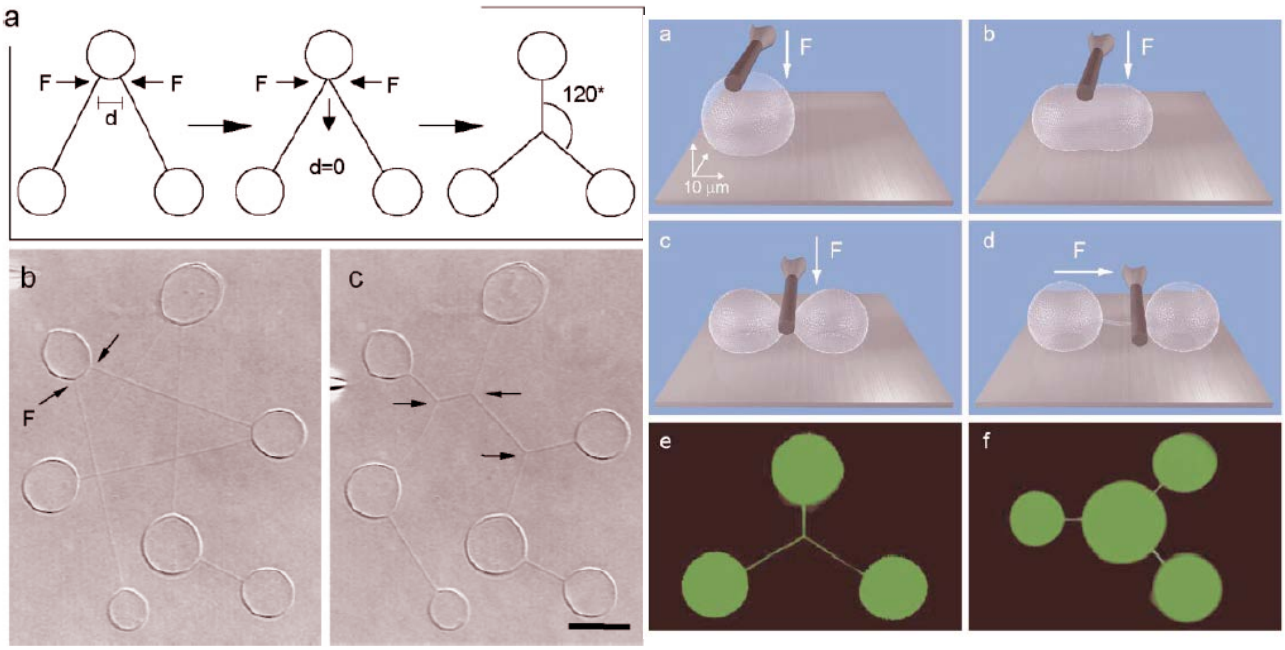






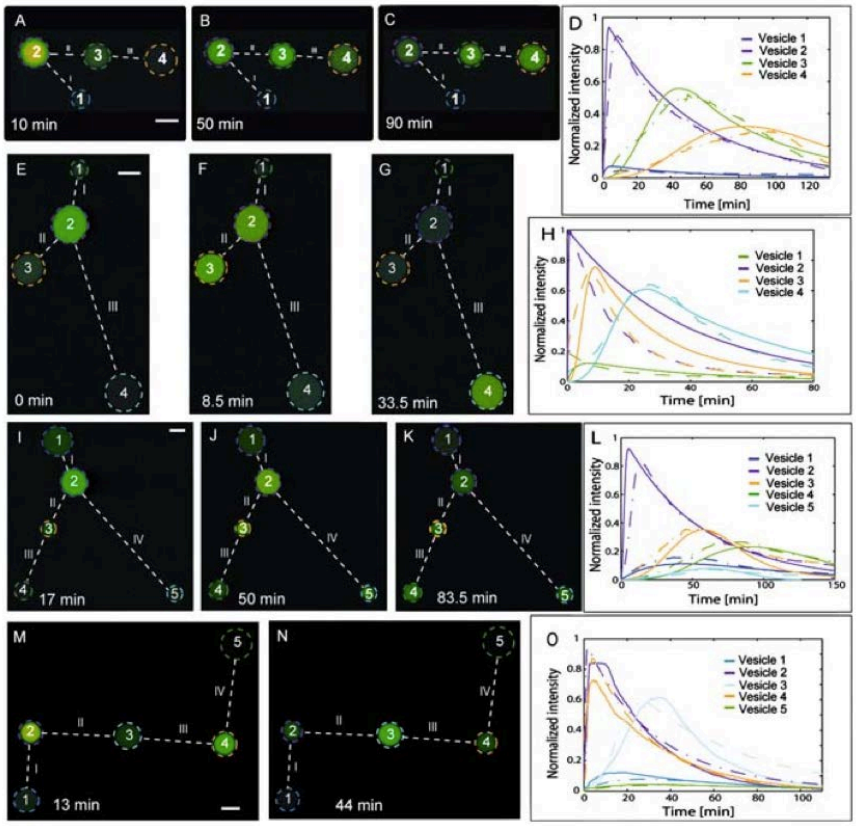
E. Rhoades, E. Gussakovsky, G. Haran, *Proc. Natl. Acad. Sci. USA* **100**, 3197 (2003)

Connecting nano-containers



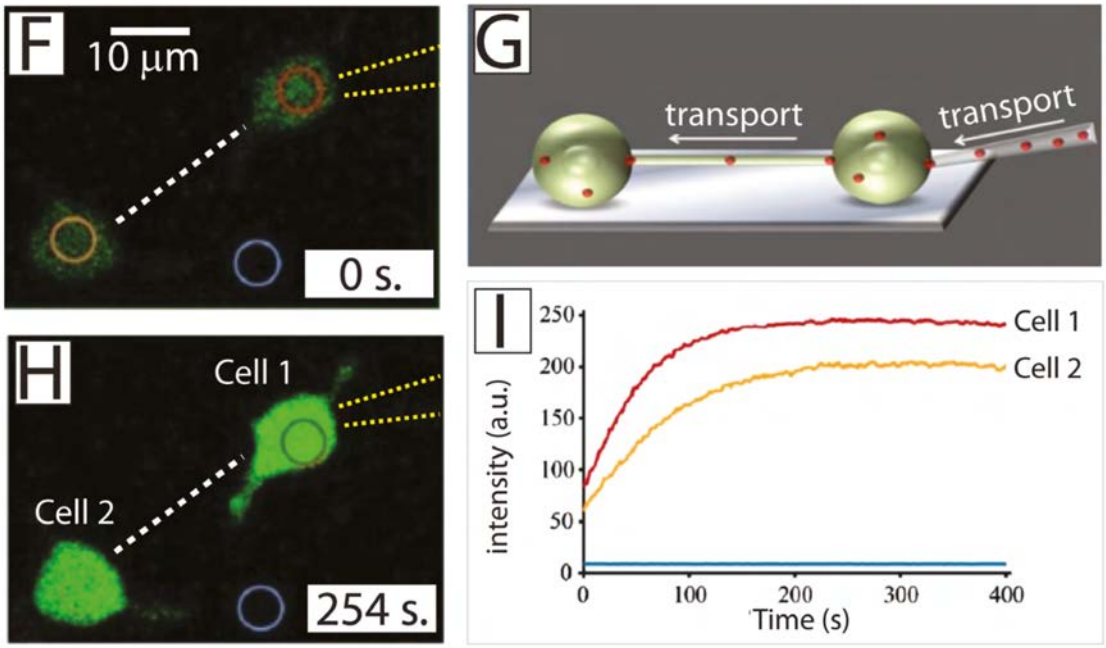
M. Karlsson, O. Orwar, et al., *Annu. Rev. Phys. Chem.* **55**, 613 (2004)

Chemistry in network of nano-containers



L. Lizana, Z. Konkoli, B. Bauer, A. Jesorka, O. Orwar 2009 *Annual Review of Physical Chemistry*, Vol. 60: 449-468

Semi-natural network of cells



Gözen, I., & Jesorka, A. (2015). Lipid nanotube networks: Biomimetic Cell-to-Cell Communication and Soft-Matter Technology. *Nanofabrication*. http://doi.org/10.1515/nanofab-2015-0003

Literature

Fundamentals

J. R. Lakowicz, *Principles of fluorescence spectroscopy* (Plenum, New-York, 1999)

R. Loudon, *The Quantum Theory of Light Sec. Ed.*(Oxford University Press, 1983)

P. J. Walla, *Modern Biophysical Chemistry*. John Wiley & Sons, 2015

Nicholas J. Turro, V. Ramamurthy, J.C. Scaiano, *Principles of molecular photochemistry : an introduction,* (University Science Books, 2009)

Single-Molecule

S. Weiss, "Fluorescence spectroscopy of single biomolecules", Science **283**, 1676 (1999)

P. Tinnefeld and M. Sauer, “Branching Out of Single-Molecule Fluorescence Spectroscopy: Challenges for Chemistry and Influence on Biology”, Angew. Chem. Int. Ed. **44,** 2642 (2005)

X. Michalet, Sh. Weiss, and M. Jäger, “Single-Molecule Fluorescence Studies of Protein Folding and Conformational Dynamics”, Chem. Rev. **106**, 1785 (2006)

C. Bräuchle, D. C. Lamb, and J. Michaelis, *Single Particle Tracking and Single Molecule Energy Transfer*. John Wiley & Sons, 2009.

Literature

Super-resolution

U. J. Birk, *Super-Resolution Microscopy*. John Wiley & Sons, 2017.

P. Tinnefeld, C. Eggeling & S. W. Hell, *Far-Field Optical Nanoscopy*. (Springer, 2015)

http://www.microscopyu.com/references/single-molecule-localization

FCS

Sergey Y. Tetin, *Fluorescence Fluctuation Spectroscopy (FFS),* Methods in Enzymology 2013

A. Gräslund, R. Rigler, and J. Widengren *Single Molecule Spectroscopy in Chemistry, Physics and Biology Nobel Symposium* (Springer, 2010)

Y. Chen et al, *Fluorescence fluctuation spectroscopy*, Methods **19**, 234 (1999)