# Quantitative Fluorescence Microscopy Techniques:

## From living cells to single molecules

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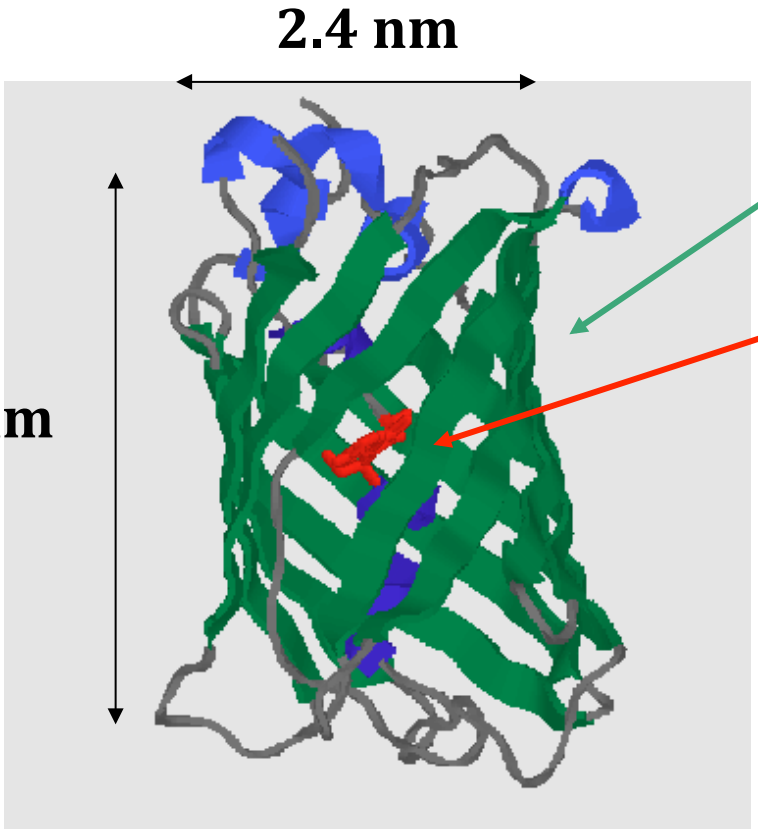
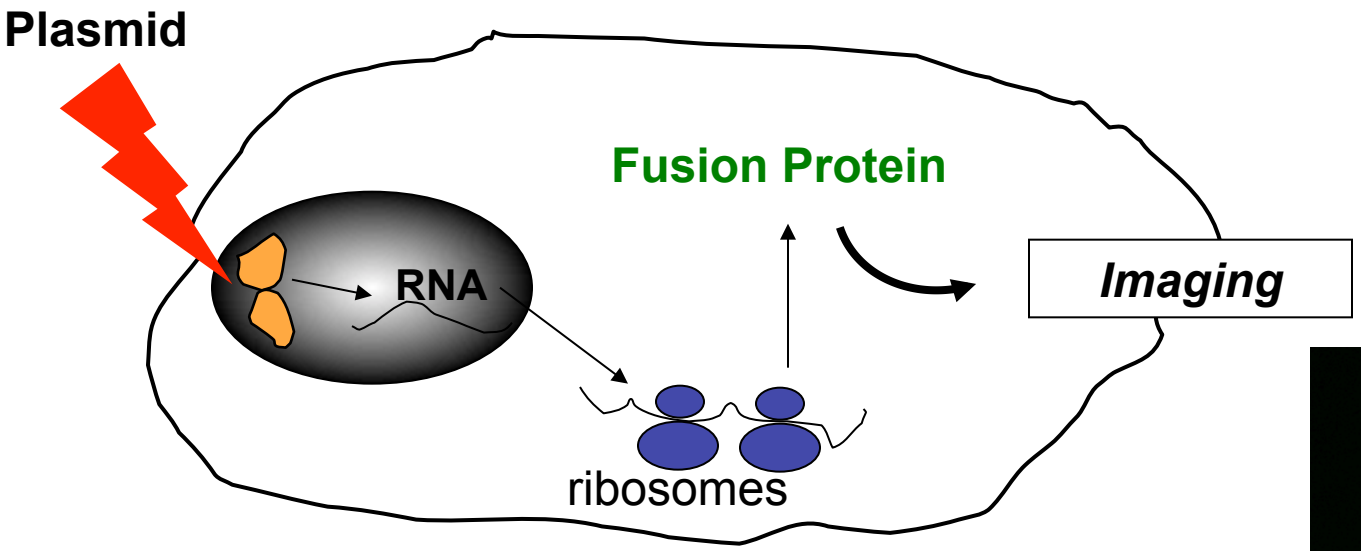
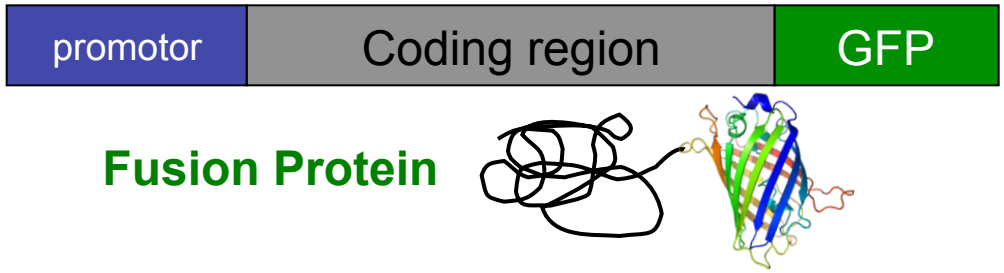
# Green Fluorescent Protein

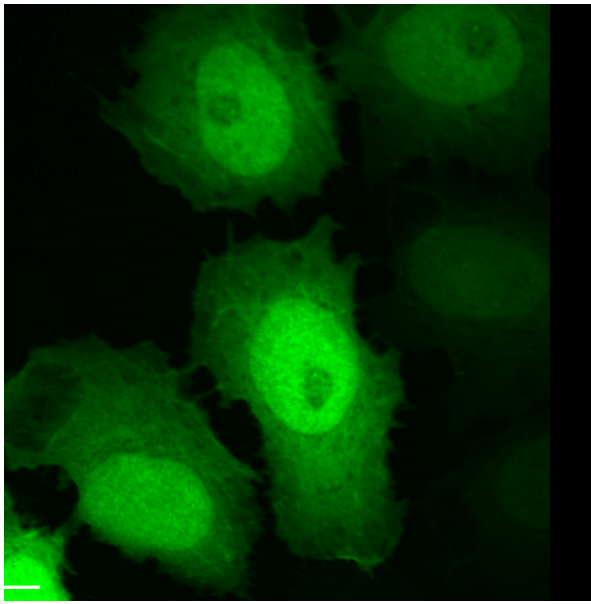



- Jellyfish Aequorea Victoria
- 238 amino-acids (1992), 27 kDa
- Expressed in a host organism (1994)

# Green Fluorescent Protein

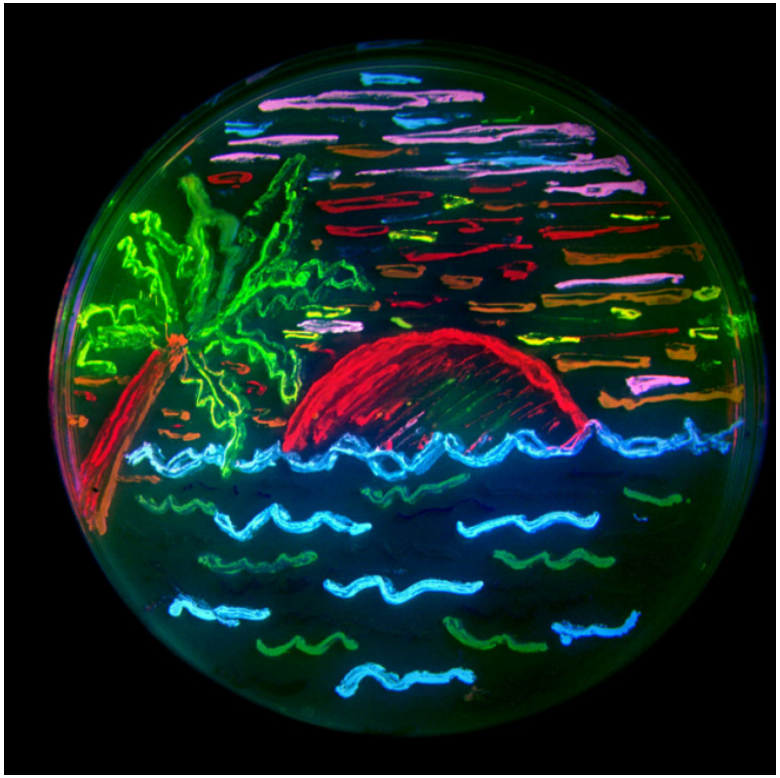


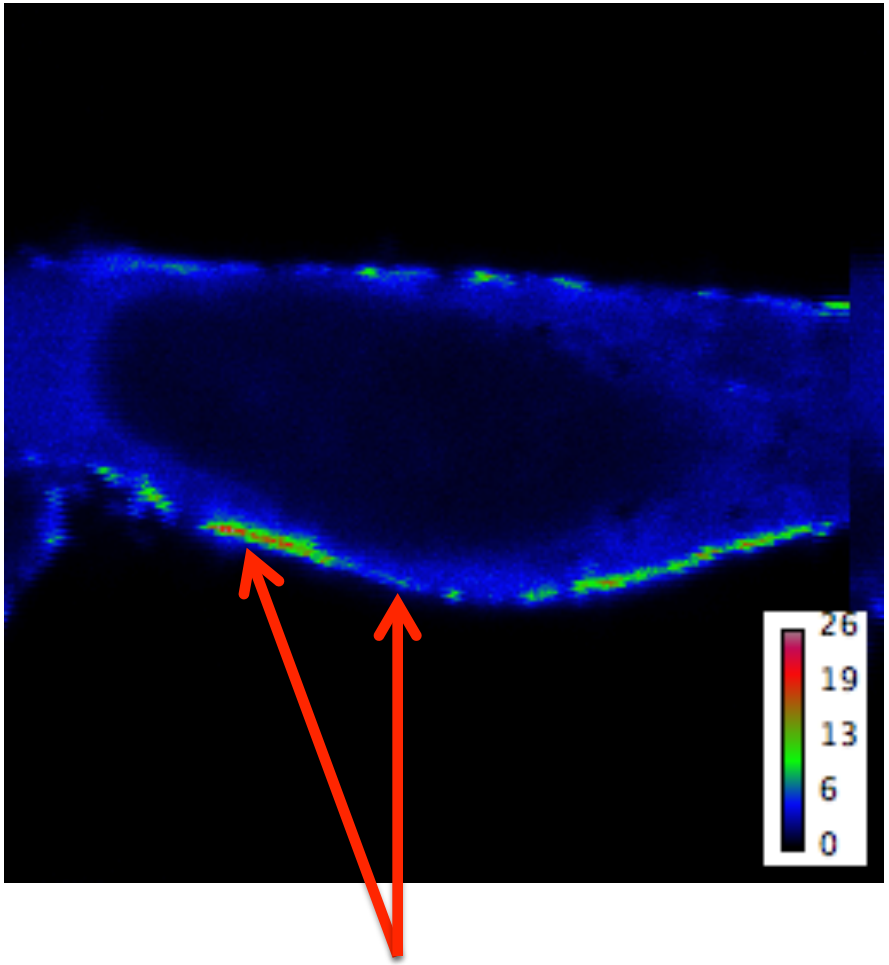



- Immunolabeling (primary and secondary antibodies)
- Chemical labeling ($NH_2$, SH, specific tags i.e. Snap Tag …)

# Limitations in fluorescence microscopy



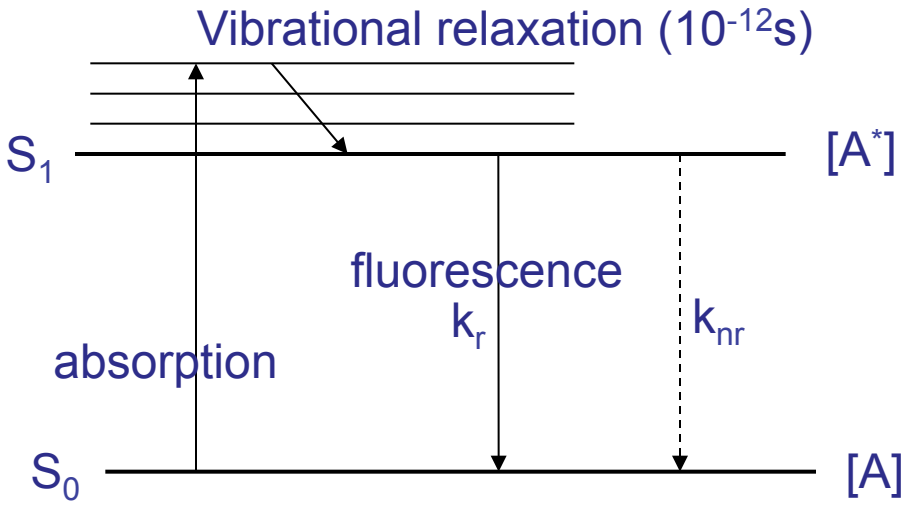
Nobel
Prize (2008)




Fluorescence intensities are relative values → depend on instrumentation and probe concentration.

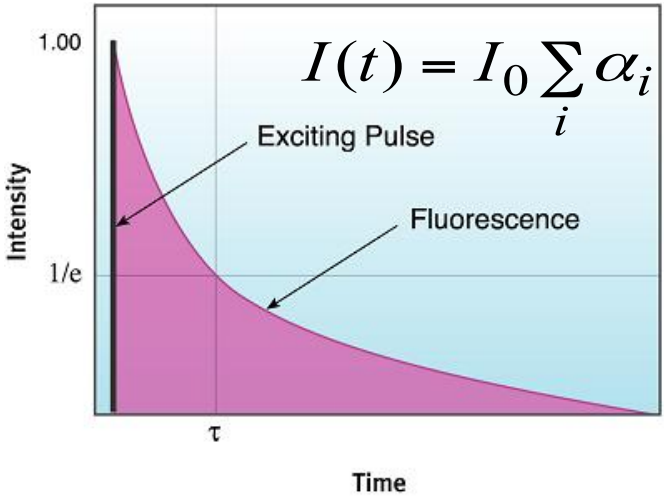
Fluorescence intensities are of limited use for quantitative imaging

# Fluorescence lifetimes are absolute values




Lifetime $\tau$ = average time spent in the excited state = $1/(k_r+k_{nr})$

Fluorescence lifetimes are absolute values, independent of the instrumentation and probe concentration




$$I(t) = I_0 \sum_i \alpha_i \exp(-t/\tau_i)$$

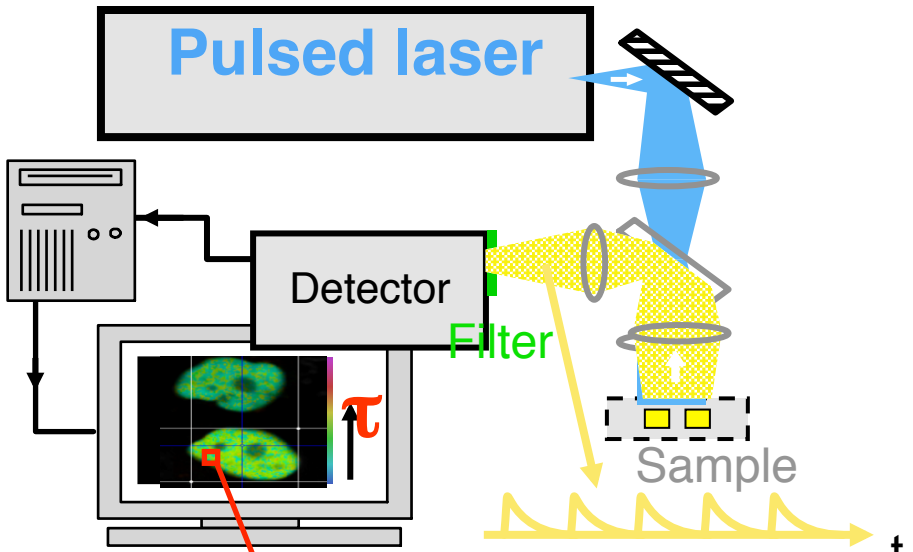
Mean lifetime

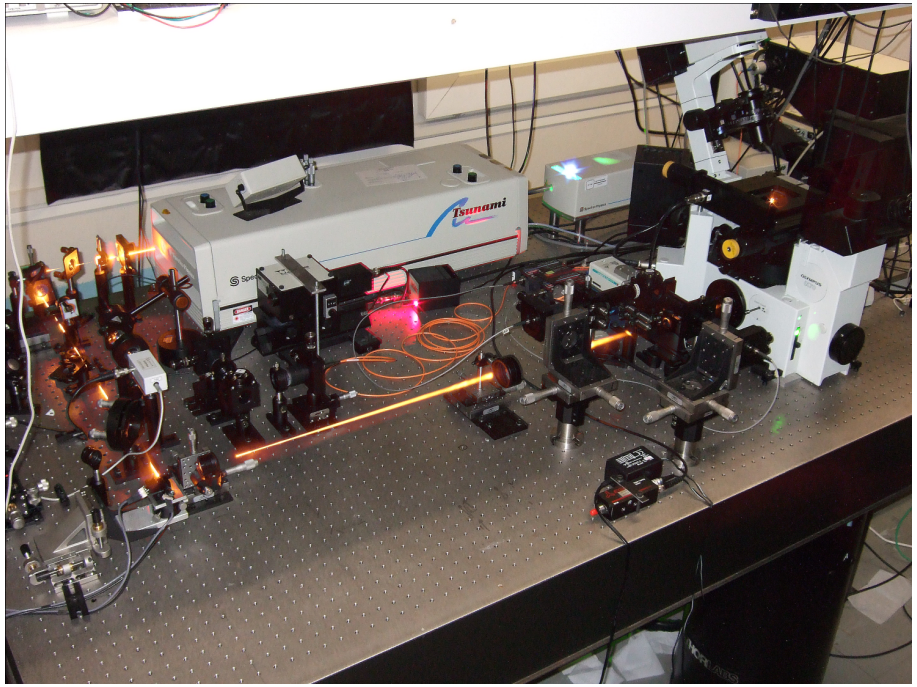
$$<\tau> = \sum_i \alpha_i \tau_i$$

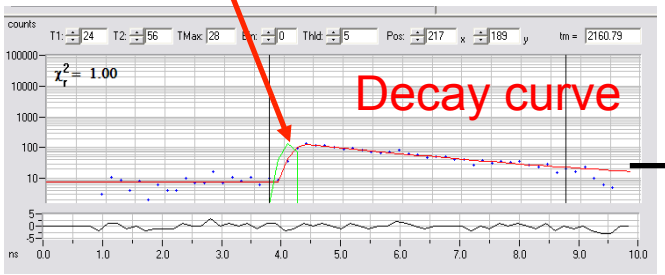
10 ps < $\tau$ < 100 ns. Usually: 1-5 ns.

# Fluorescence lifetime imaging Microscopy (FLIM)

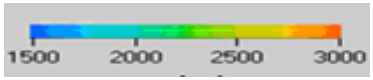
Lifetimes are measured for each pixel of the microscope image.



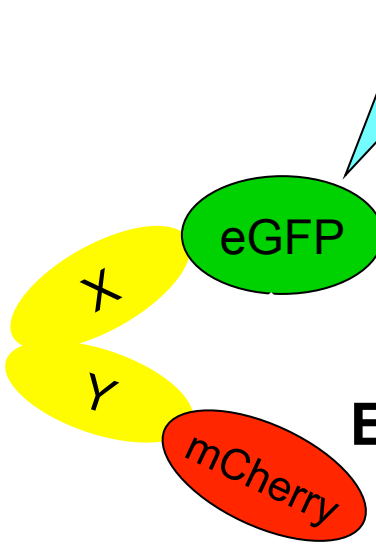




Lifetime




Confocal microscopy or two photon microscopy. Low number of photons ($10^3$-$10^4$ photons): usually one or two lifetimes → mean lifetime.

# FRET-FLIM: an ideal tool for monitoring molecular interactions in cells




**Emission : $I_1$ et $\tau_D$**

**Emission : $I_2$ et $\tau_{DA}$ : $\tau_D > \tau_{DA}$**

**Energy Transfer**

**$E = 1 - \tau_{DA}/\tau_D$**

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

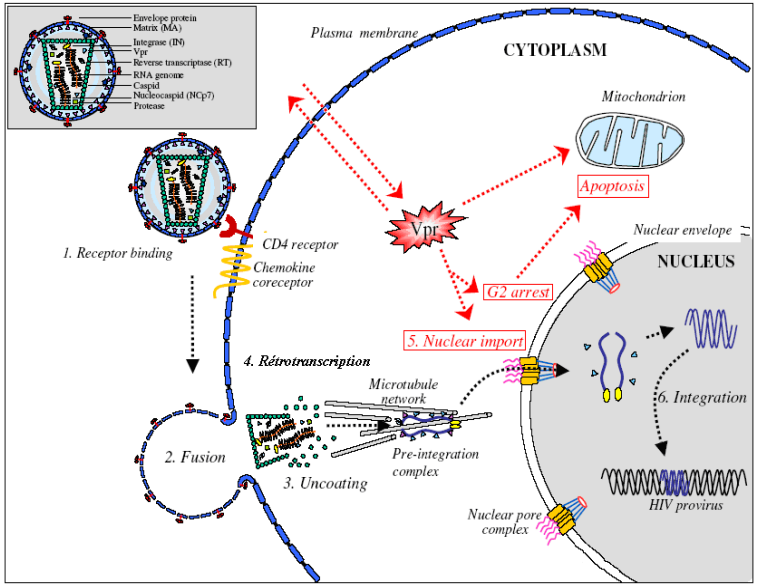
**$\tau_{X\text{-}GFP}$ ↘**

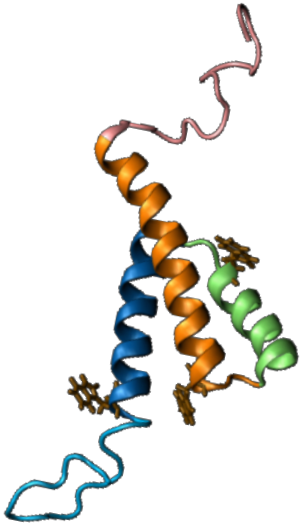
**X-Y Interaction**

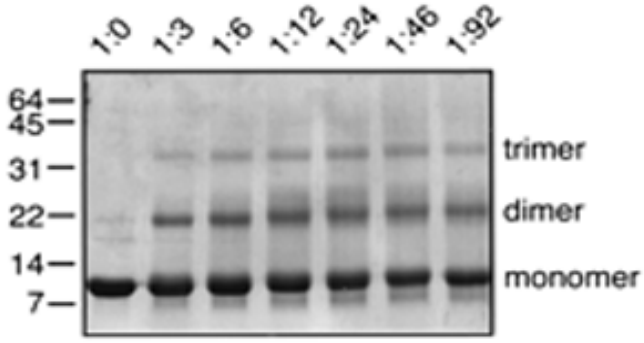
**$\tau_{X\text{-}GFP} = \tau_{GFP}$**

Fluorescence lifetime decreases when FRET → proof of molecular interaction.

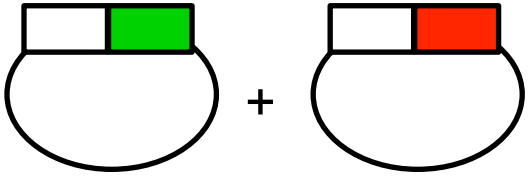
# Oligomerization of HIV-1 Vpr

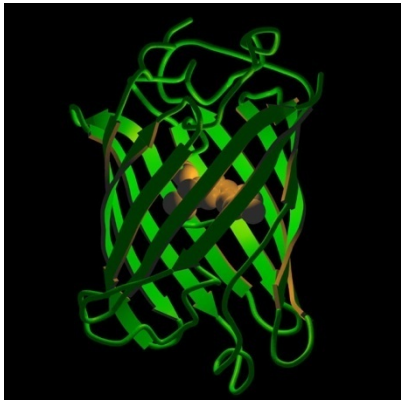


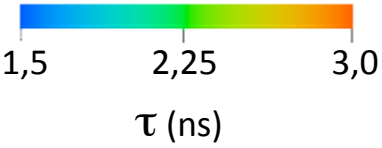



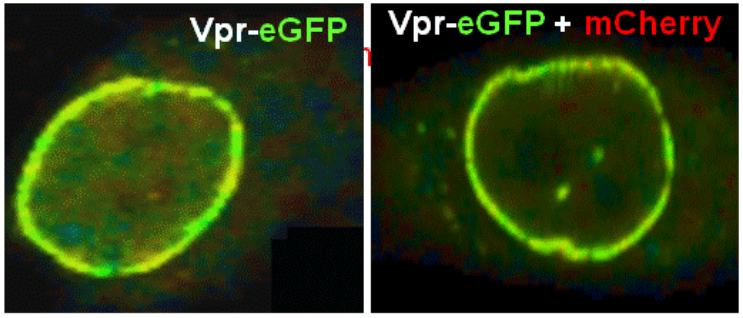



Vpr eGFP + Vpr mCherry





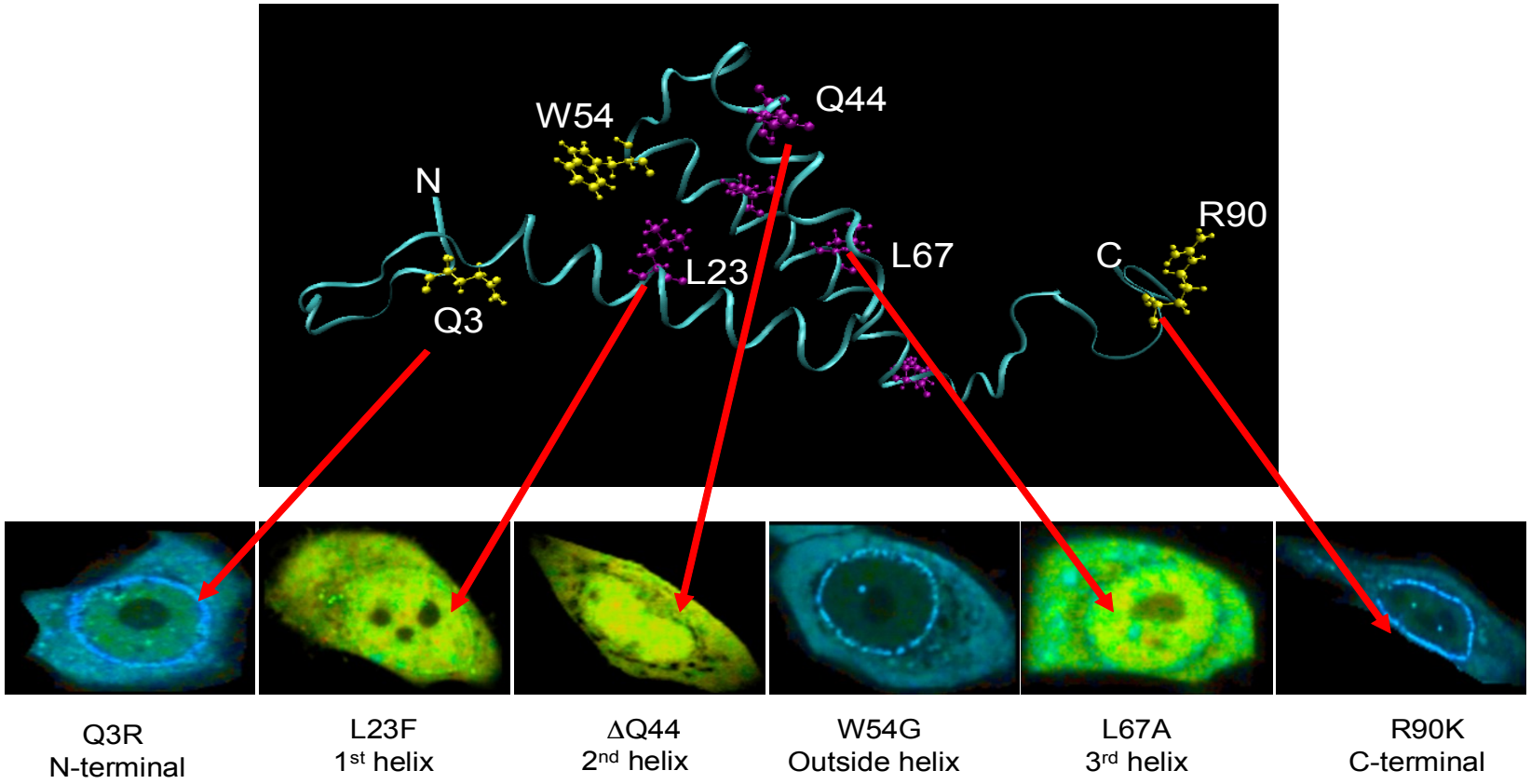



E=15% E=23%

Vpr oligomerizes in the whole cell

# Determinants of Vpr oligomerization

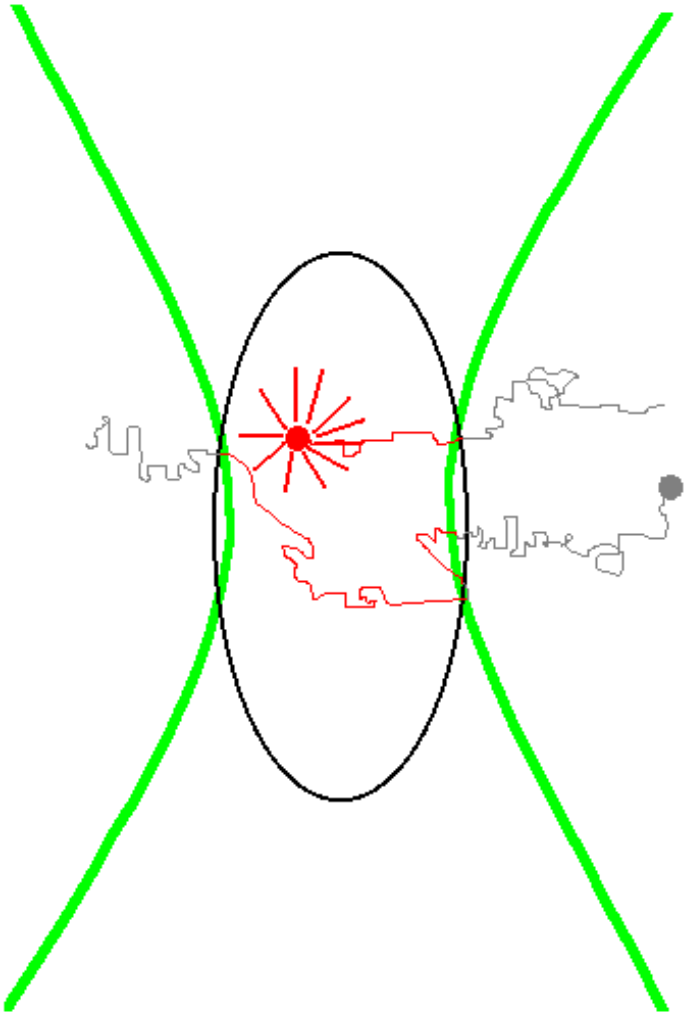


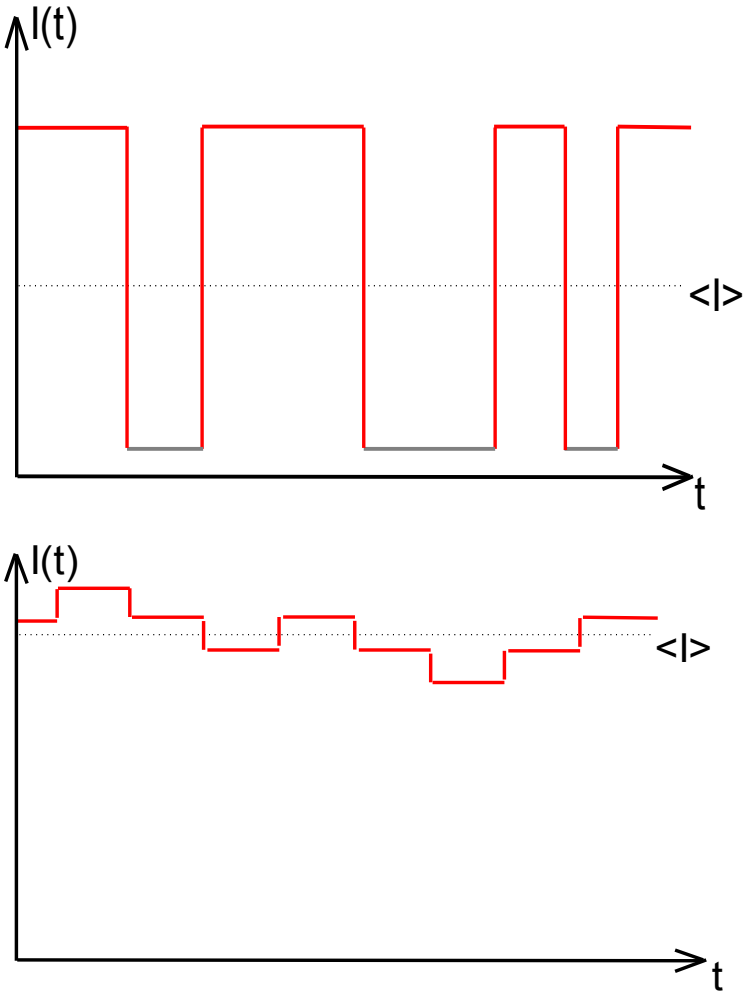

- Vpr oligomerization and binding to the membrane are correlated

-Vpr oligomerization is critically dependent on residues in the $\alpha$-helices

- What's about the stoichiometry?

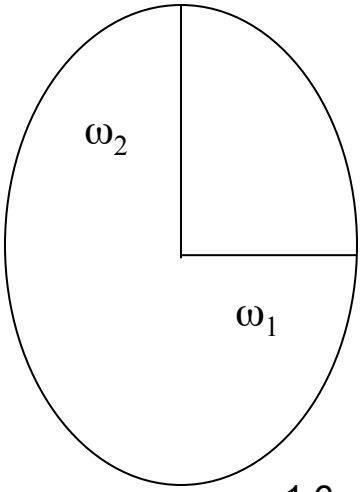
# Fluorescence correlation spectroscopy






Direct measurements of the average number of molecules in the focal volume, diffusion constant and **the brightness of the dye**.

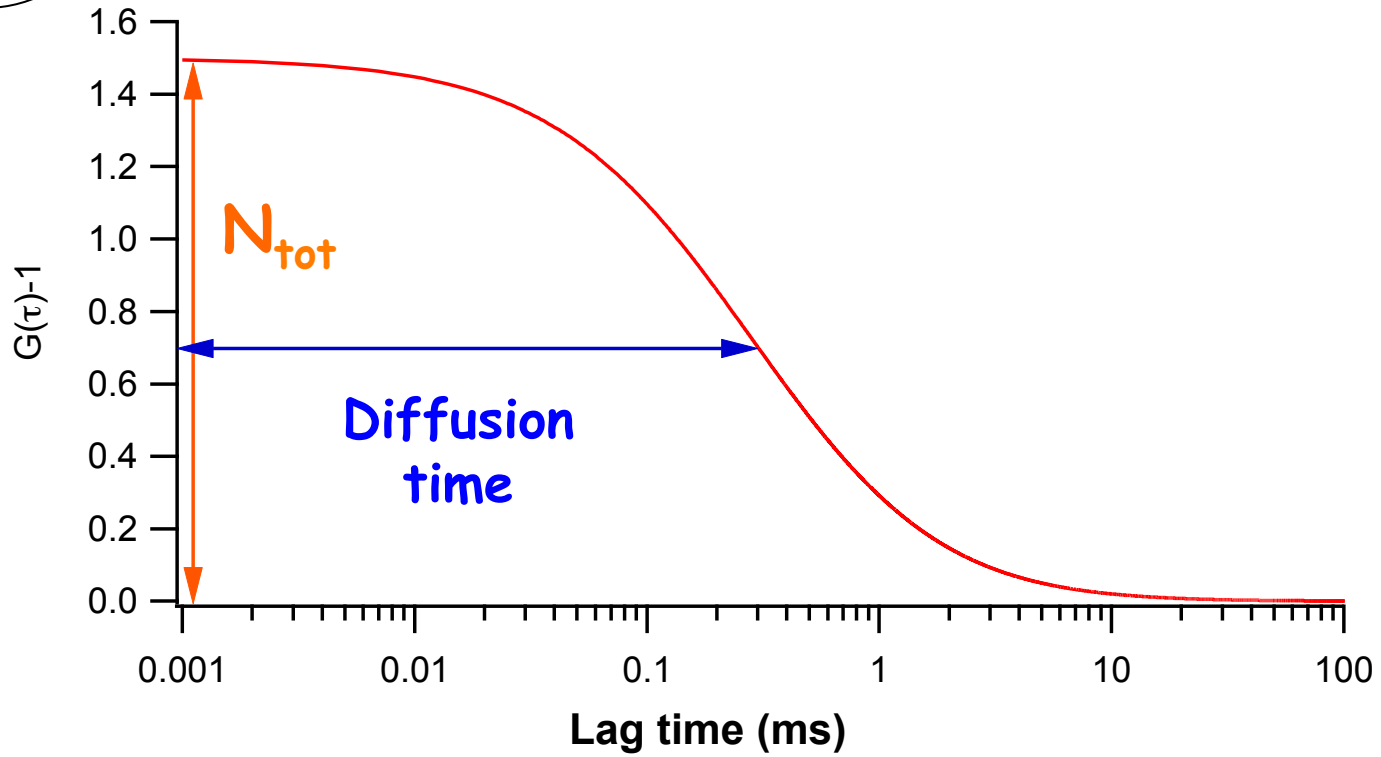
# Fluorescence correlation spectroscopy



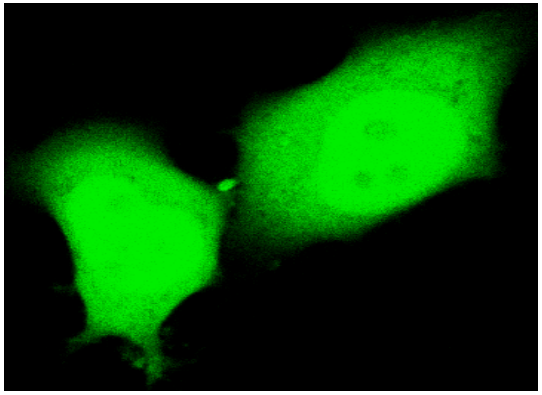

$$G(\tau) = 1 + \frac{< \delta I(t)\delta I(t+\tau) >}{< I >^2}$$

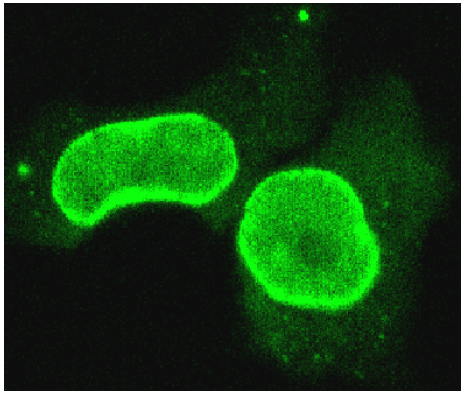
Free diffusion (Brownian) in 3D

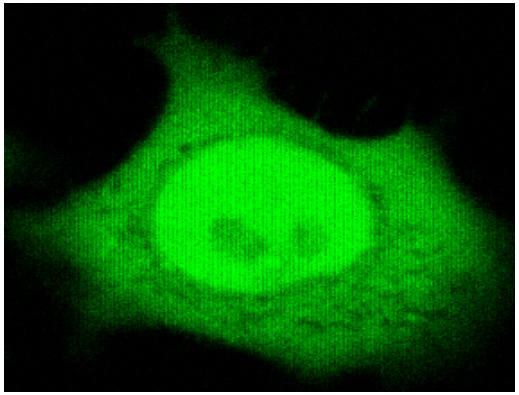
$$G(\tau) = \frac{1}{N}\left(\frac{1}{1+4D\tau/\omega_1^2}\right)\left(\frac{1}{1+4D\tau/\omega_2^2}\right)^{1/2}$$

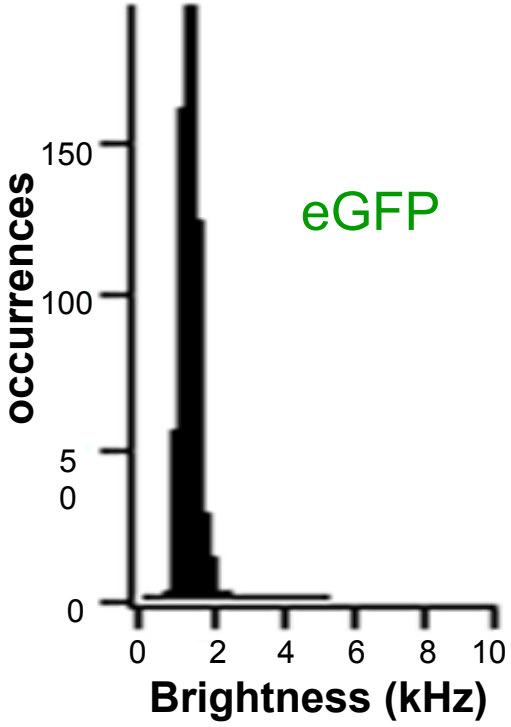


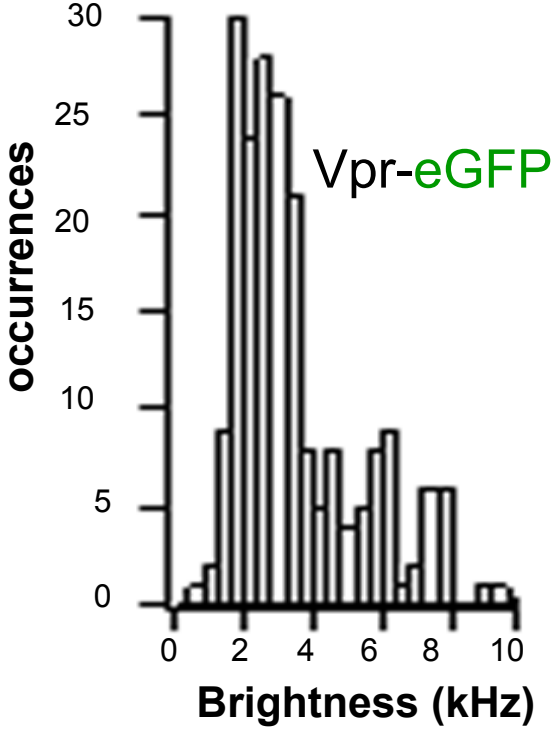
# Stoichiometry of Vpr oligomers: FCS

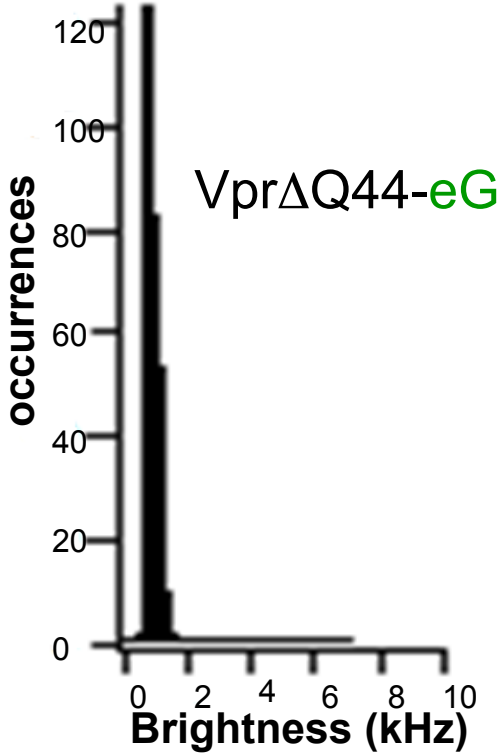








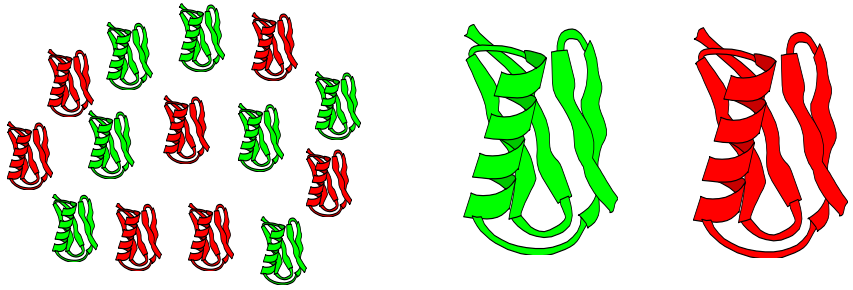




Vpr forms mainly dimers and trimers in the cytoplasm. The Vpr mutant is monomeric.

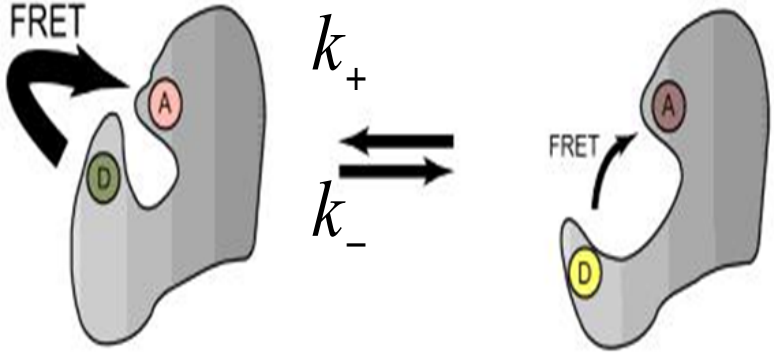
# Why Single molecule experiments ?

To overcome average effect of ensemble measurements



Individual approach: same protein molecules may not be identical (!)

Analysis of stochastic processes



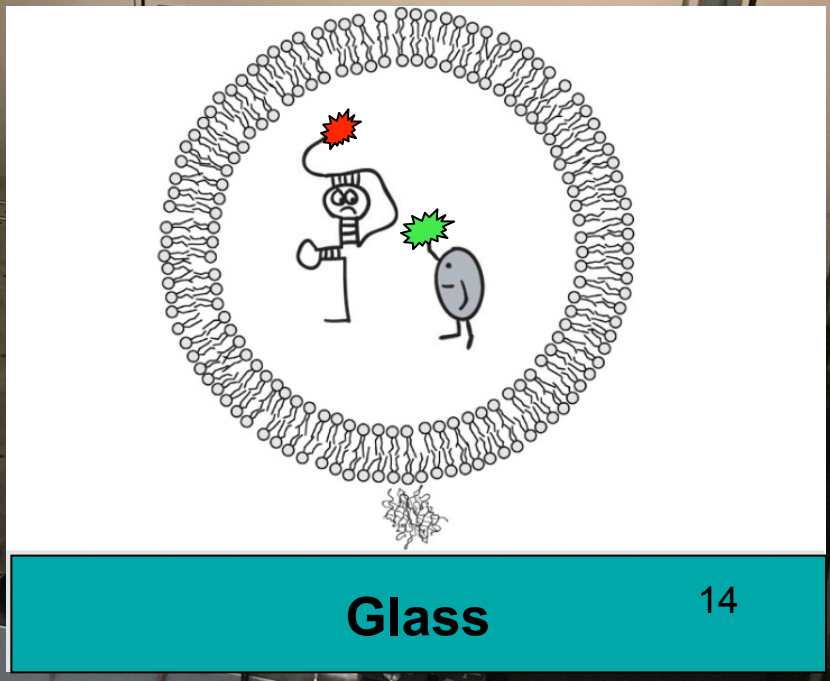

Kinetic constant can be measured even at equilibrium (!)

# Single molecule FRET

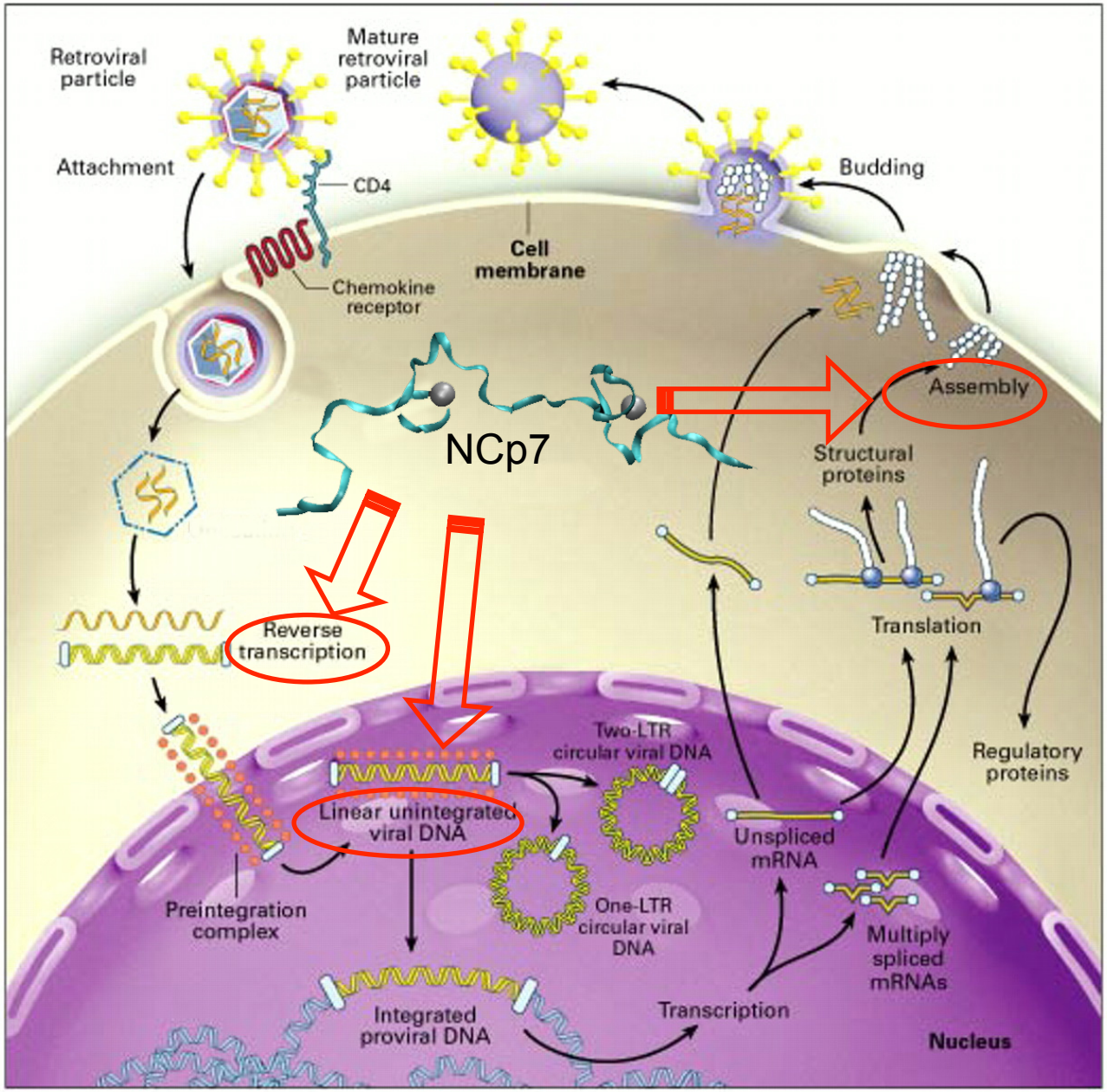
Wide-field TIRF cw laser excitation (405, 488, 532, 561, 632 nm)
Oil objective 100x NA=1.49
2 EMCCDs

Biotinylated Large unilamellar vesicle (100 nm)
(local concentration ~3.1 μM)

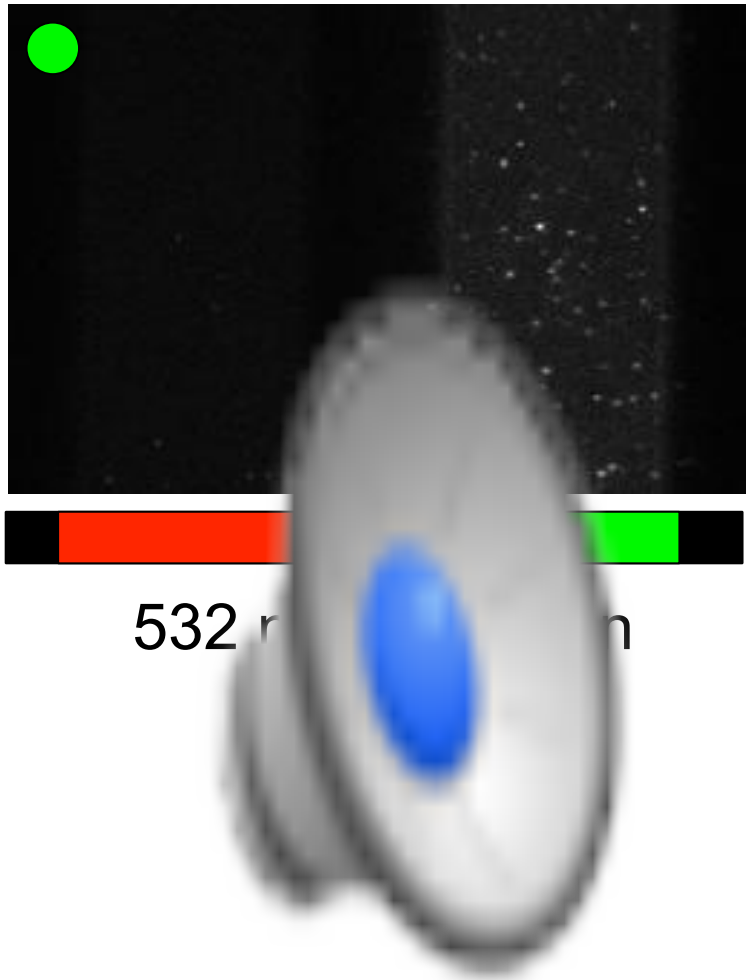
Quartz slide with biotinylated PEG/streptavidin

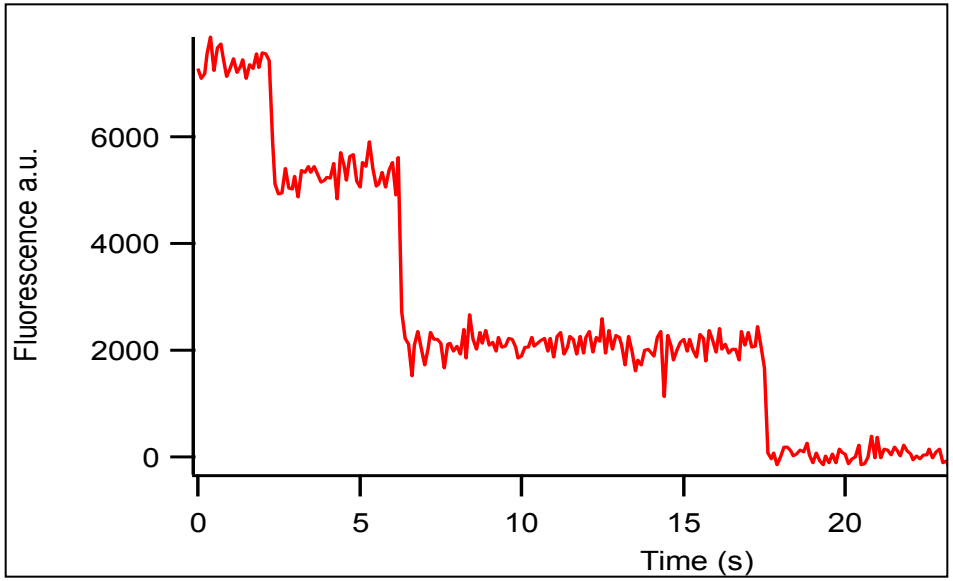


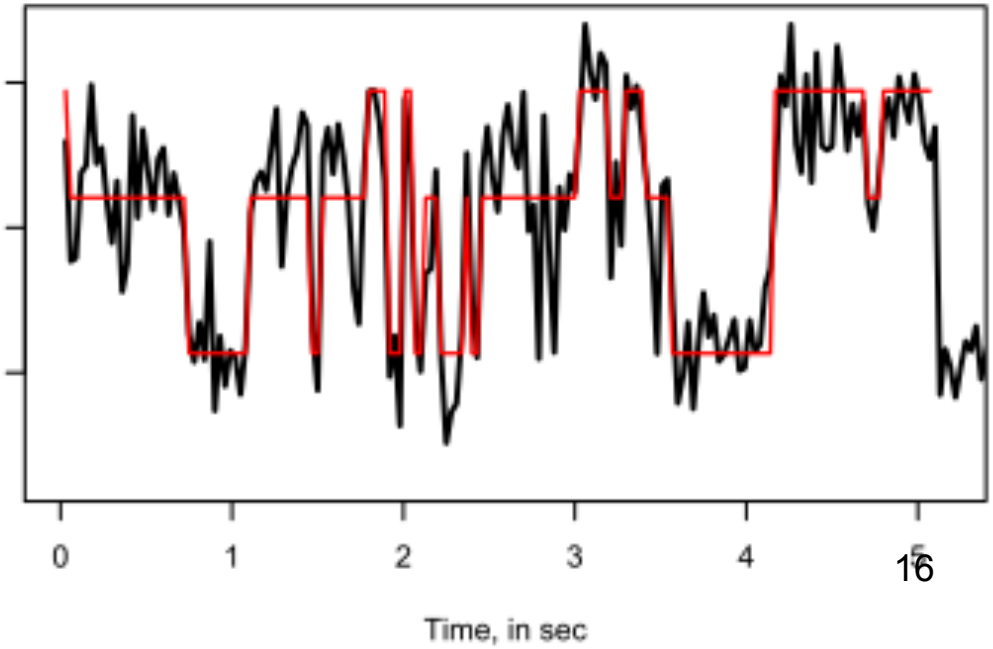
# The HIV-1 nucleocapsid protein (Ncp7)



# Single molecule FRET

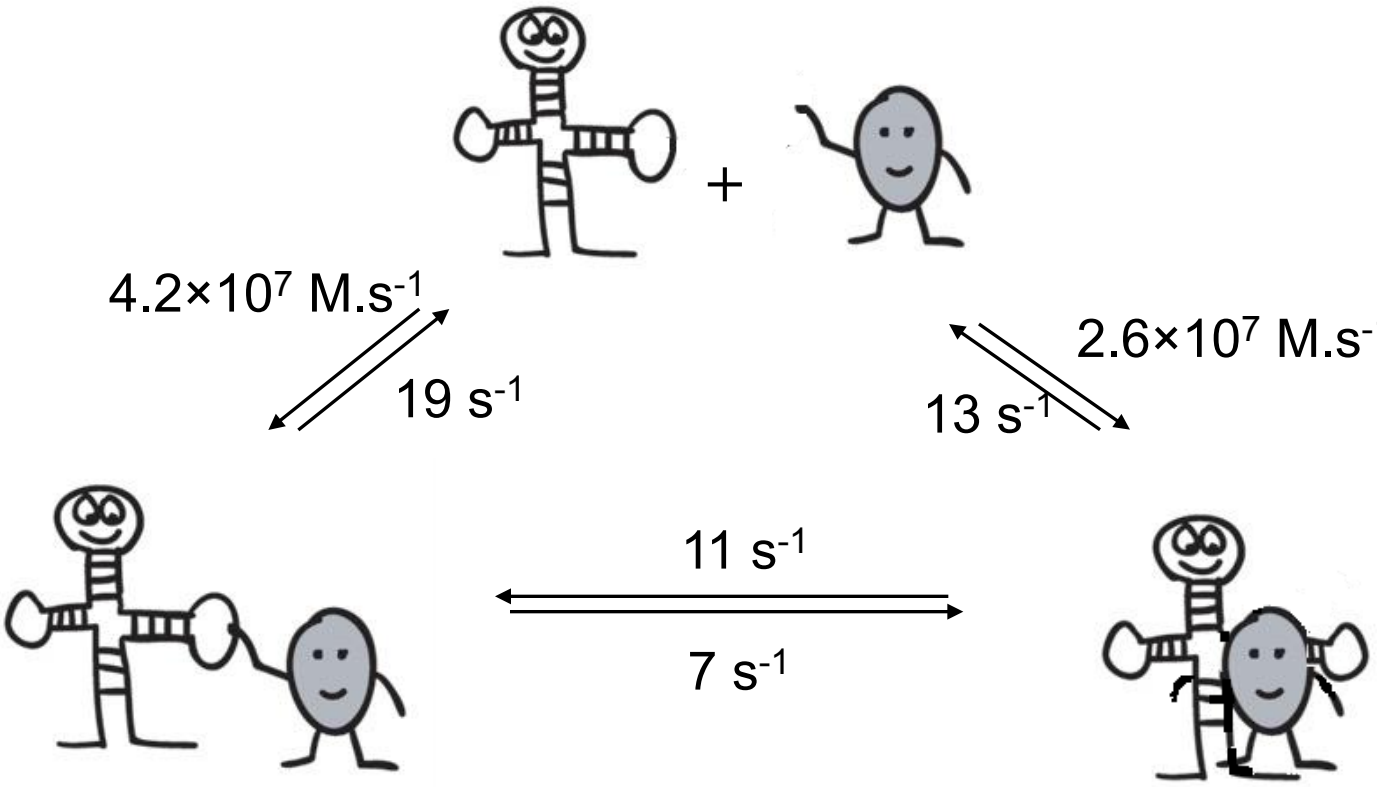


# A highly dynamic binding

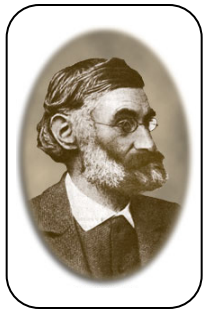
Binding constants for TMR-NCp7 with Cy5-NAs.

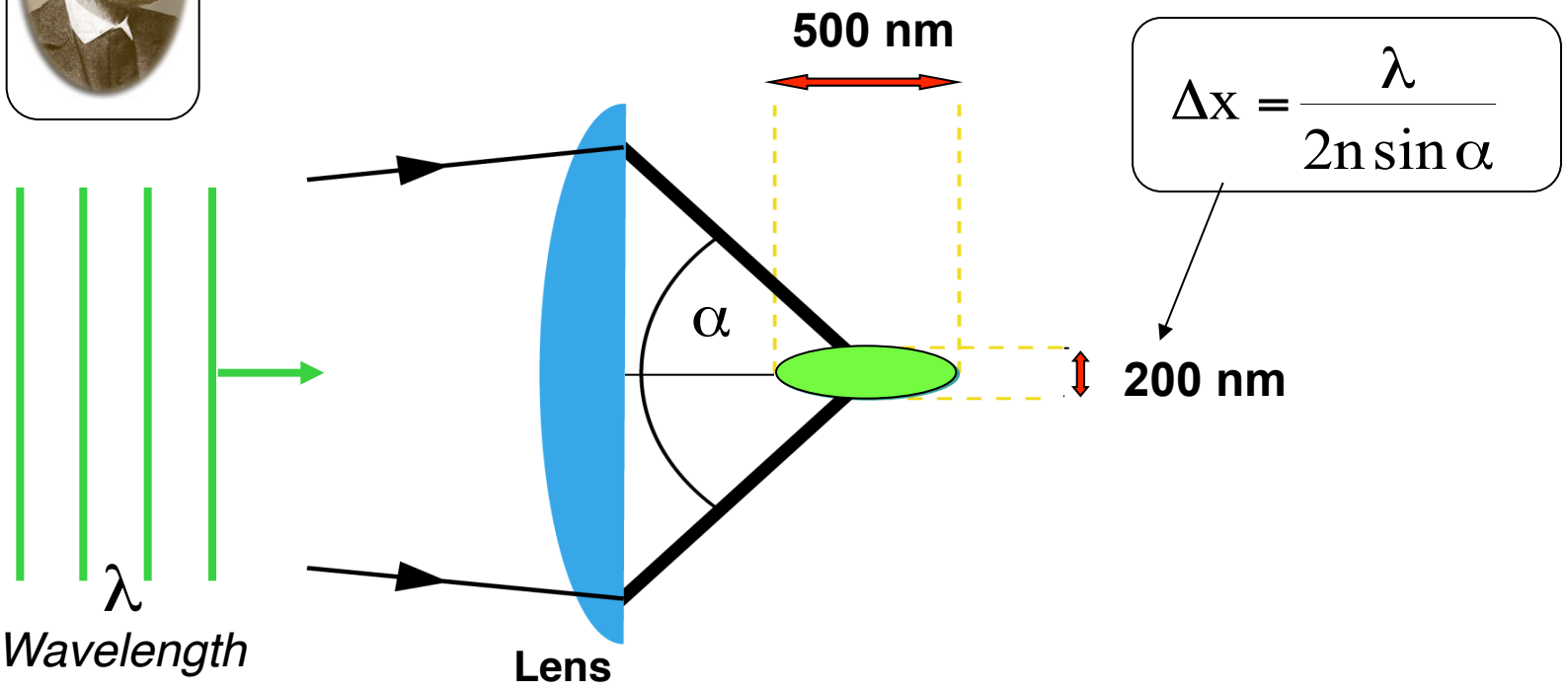



Two modes of binding mainly driven by (NMR data):

- hydrophobic interactions through the zinc-fingers,
- electrostatic interactions via the $N_{term}$ part.

# Why Single molecule imaging ?

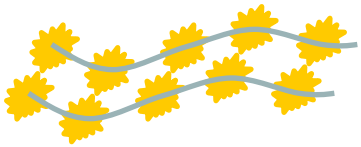




# To perform Superlocalization !

## Widefield microscopy

resolution ≥ **200 nm**



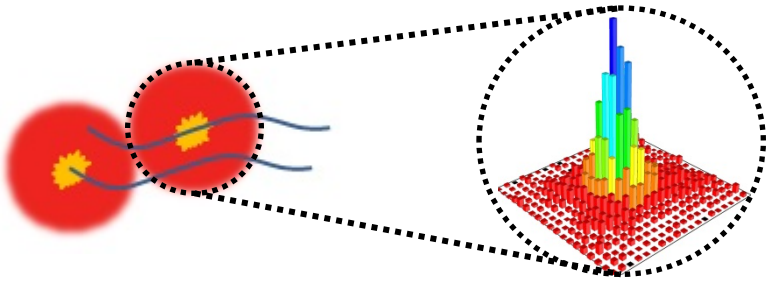
Sample

Overlapping PSF

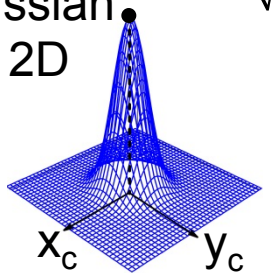
Image

## Super-localization microscopy

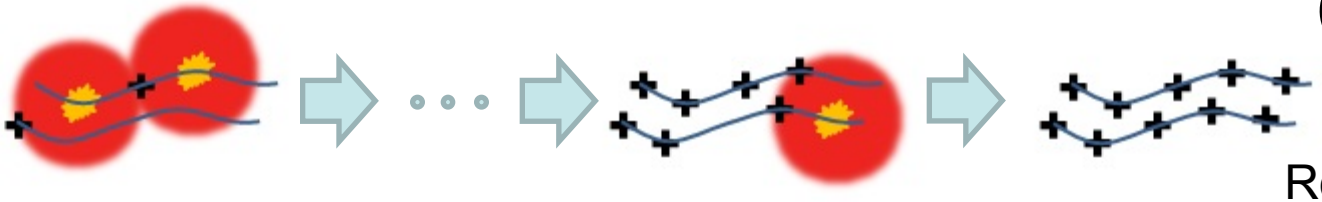
$$\Delta r \approx \frac{\lambda/2}{\sqrt{N_{ph}}}$$



Gaussian fit 2D




Single emitter

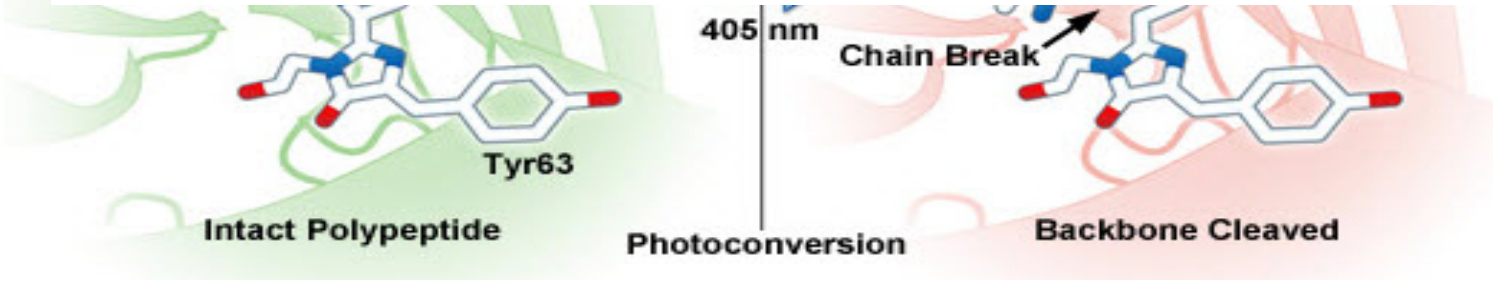


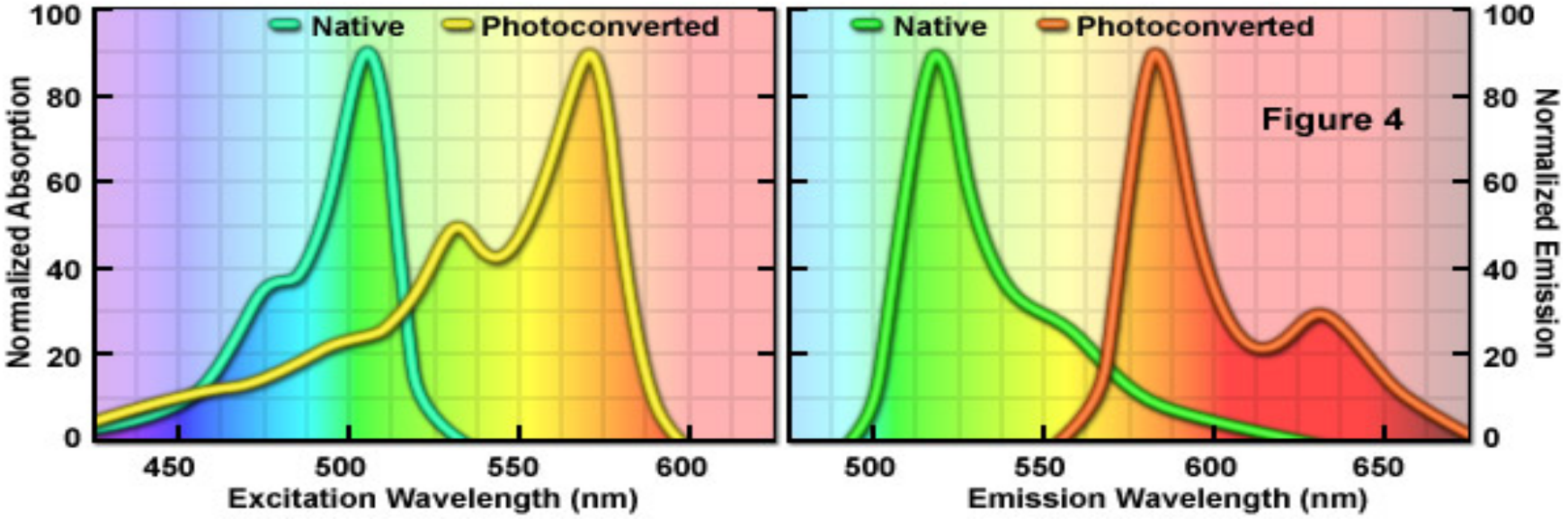
**Reconstructed image**

Resolution ≈ **20 nm**

# Photo-switchable Fluorescent Protein

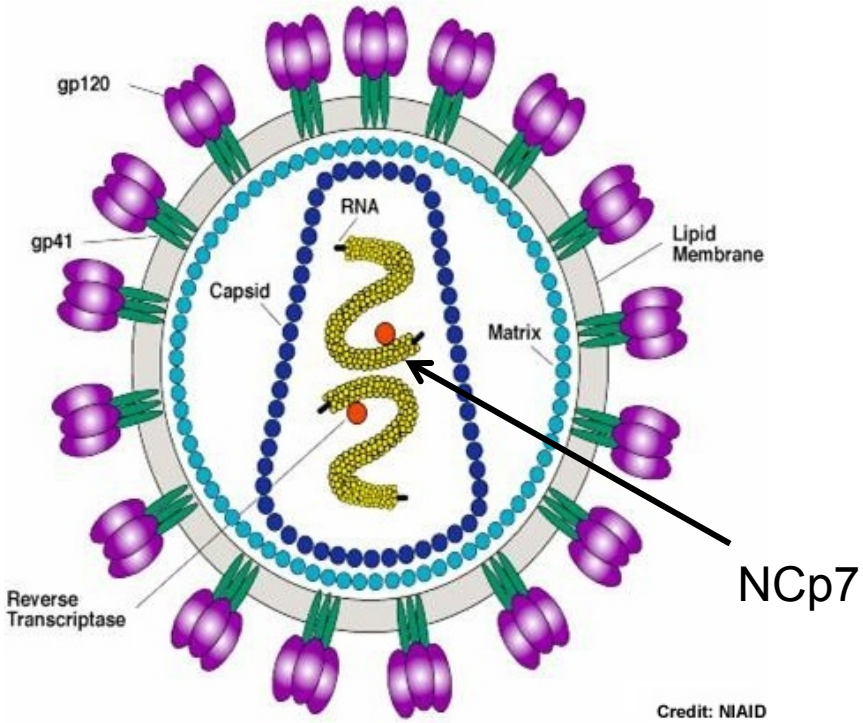
## PhotoActivation Localisation Microscopy PALM



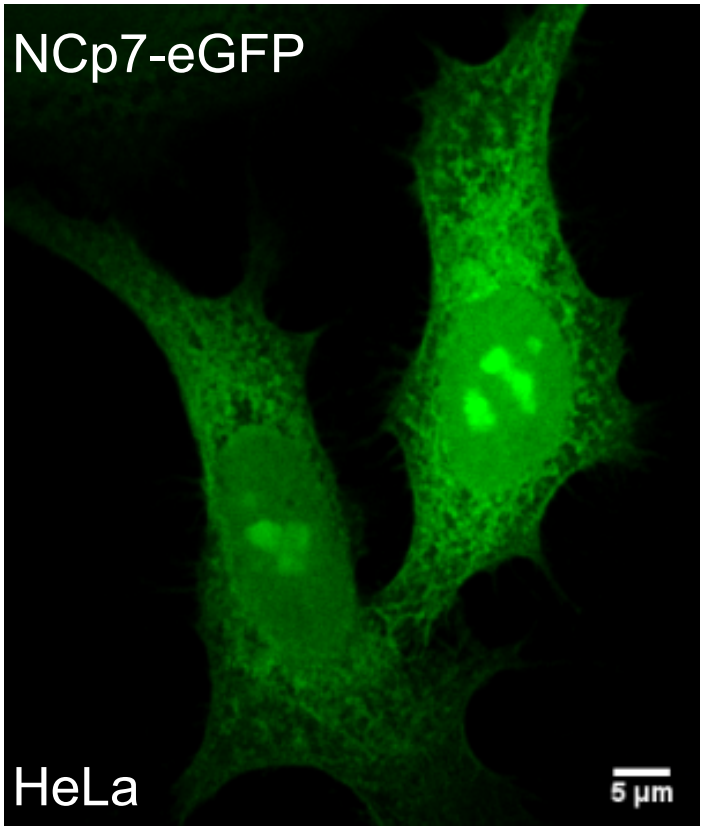




UV illumination leads to a photoinduced chemical modification of the optically active part of mEOS2

# Nucleocapsid protein NCp7



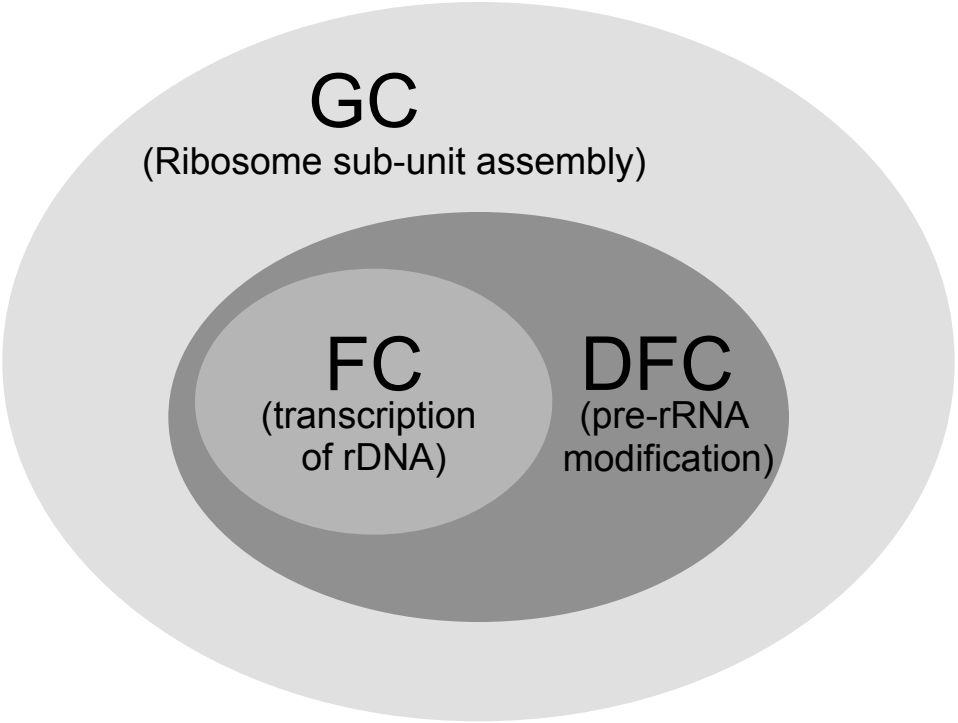

HIV-1 virion structure




Confocal microscopy study

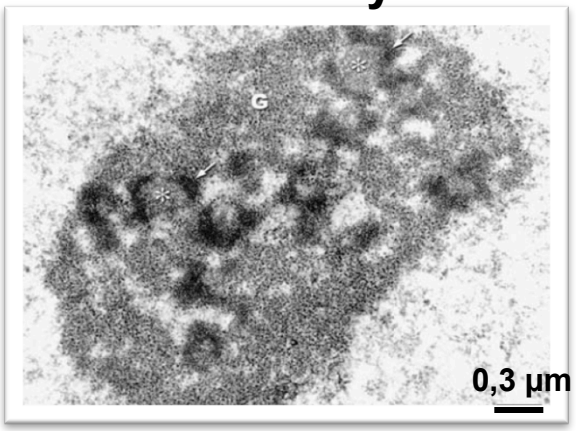
Localization of NCp7 in the nucleolus is common among retroviruses (HIV, RSV, MMTV); observed in different cell types (HeLa, QT6)

# Nucleolus




FC – **F**ibrillar **C**enter
DFC – **D**ense **F**ibrillar **C**omponent Fibrillarin
GC – **G**ranular **C**omponent Nucleophosmin

**EM study**

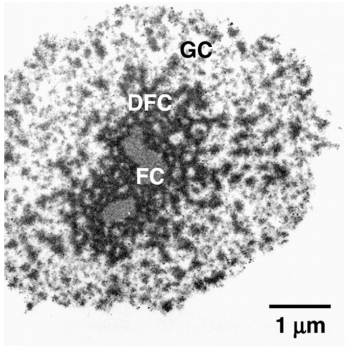



Different compartments

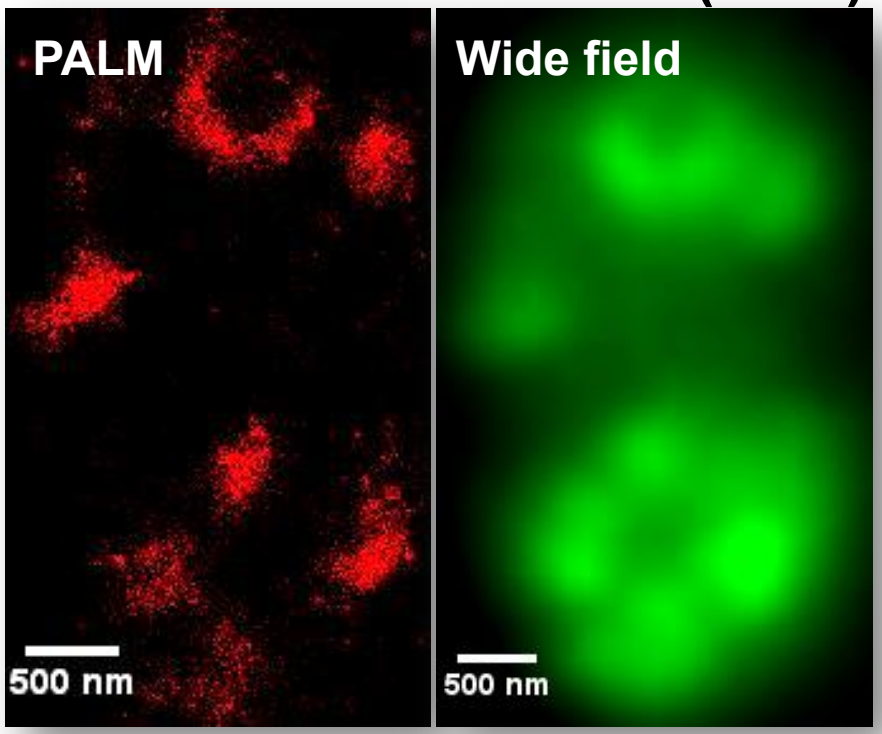
Different stages of ribosome biogenesis

Boisvert et al., *Nat Rev Mol Cell Biol*, 8(2007)

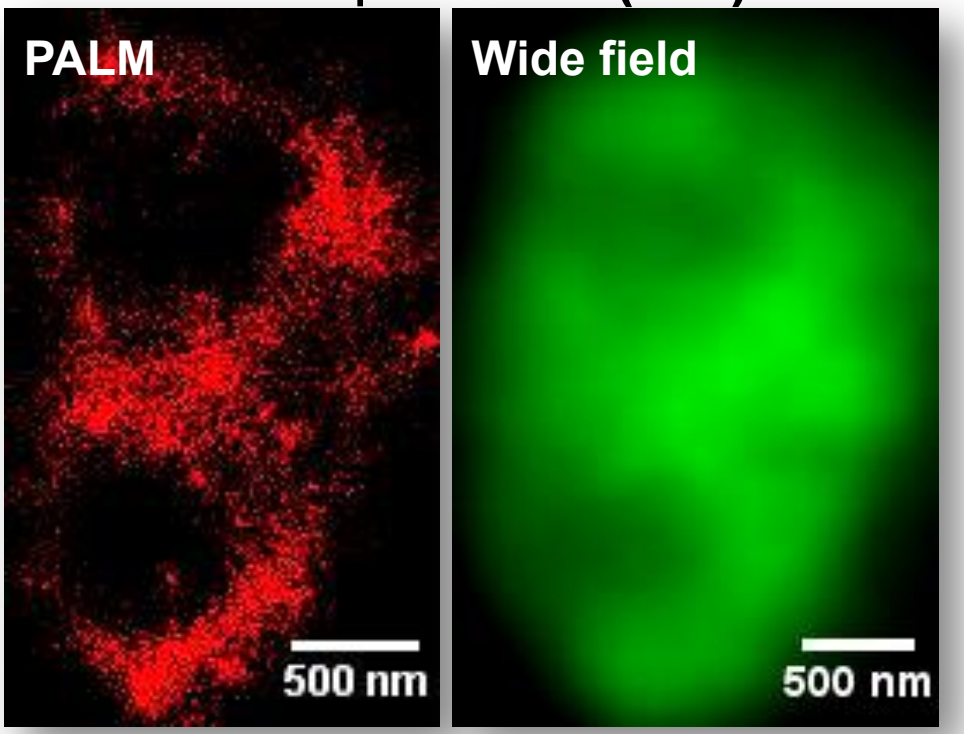
# Nucleolar proteins (PALM)




Fibrillarin **(DFC)**

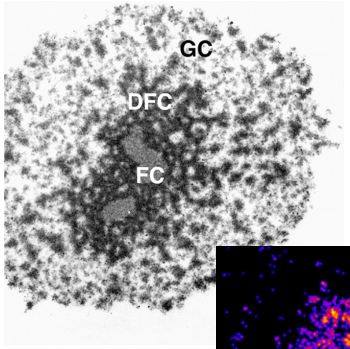


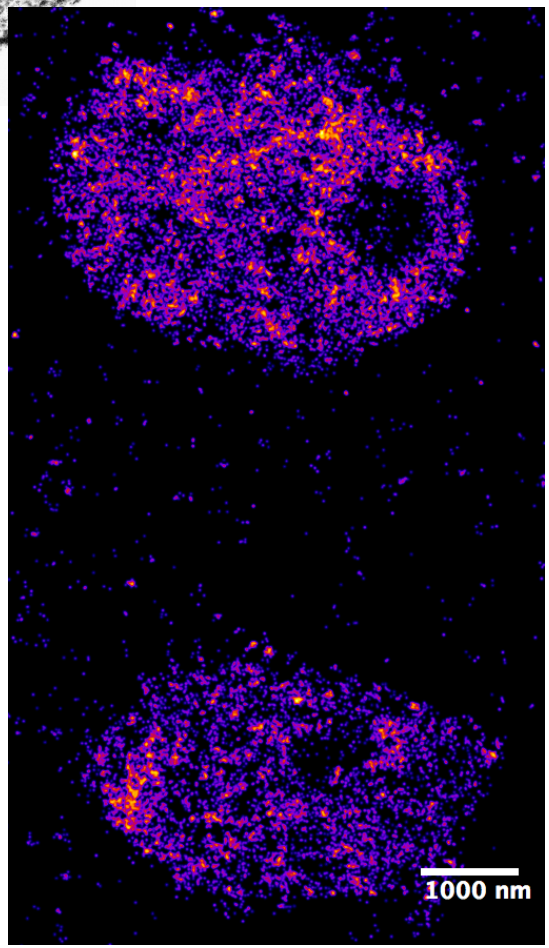

Nucleophosmin **(GC)**



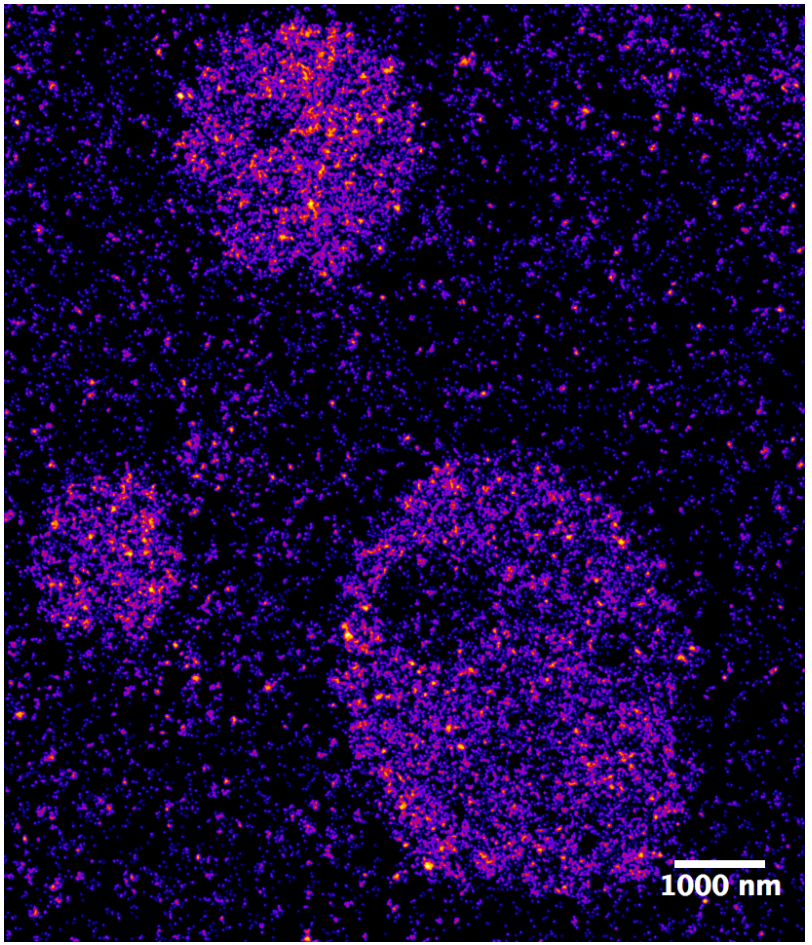

PALM is an alternative method to electron microscopy for biological samples

# NCp7 (PALM)



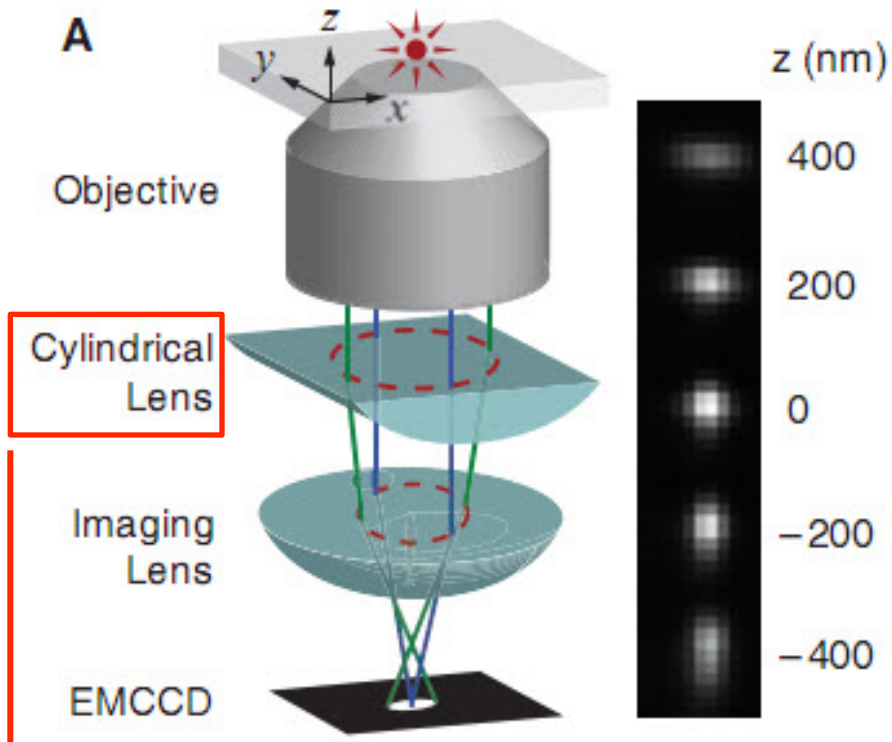

mEos2-NPM



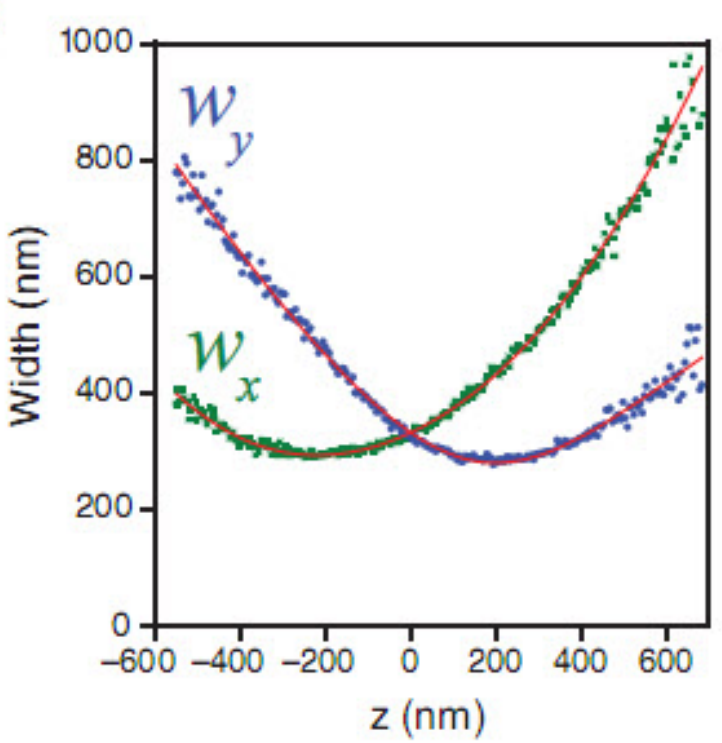

mEos2-NCp7

**Localization in the granular component of the nucleolus**

# 3D Superlocalization

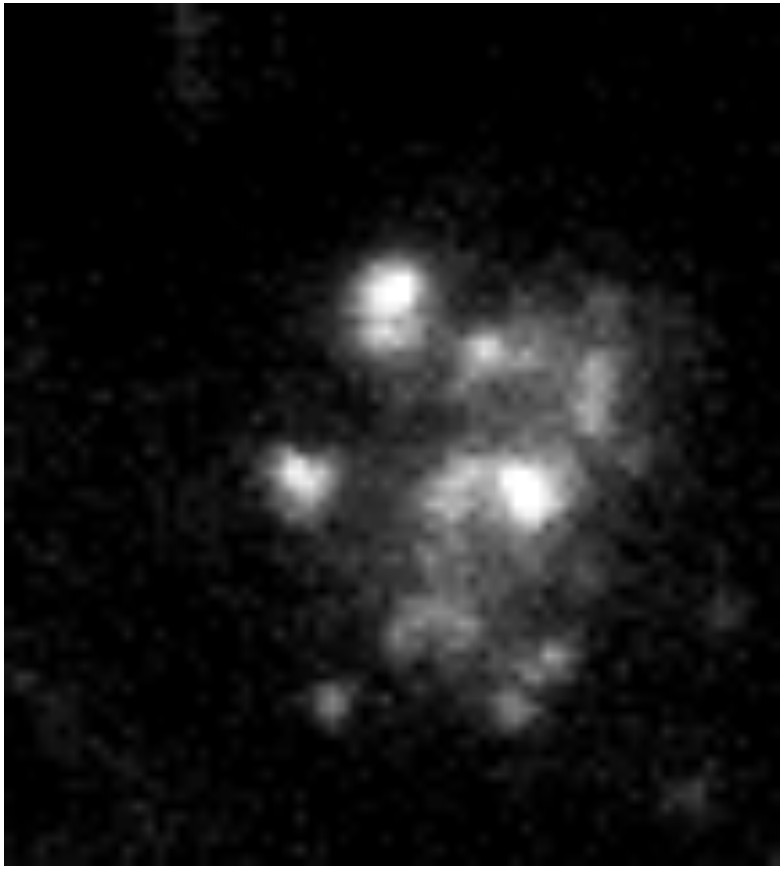



Astigmatism controled with adaptive optics

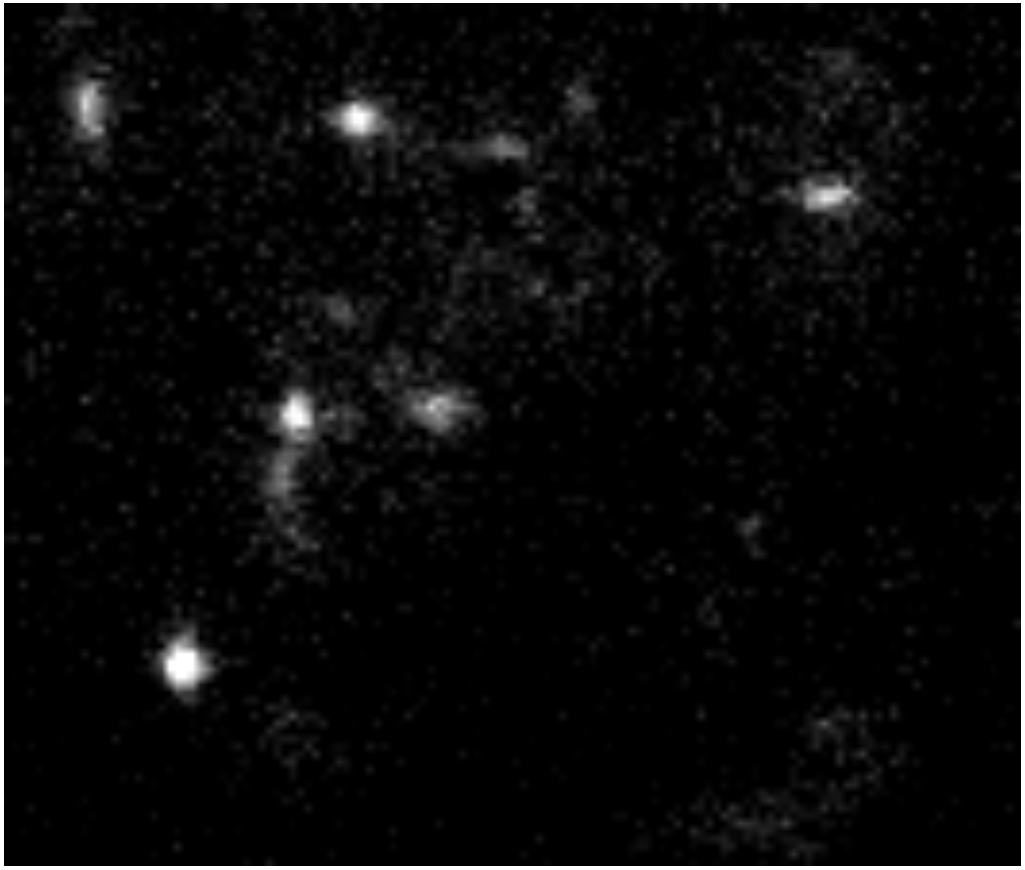



Calibration curve of image width $w_x$ and $w_y$ as a function of z obtained with fluorescent beads

# 3D PALM with Eos labeled nucleolar protein

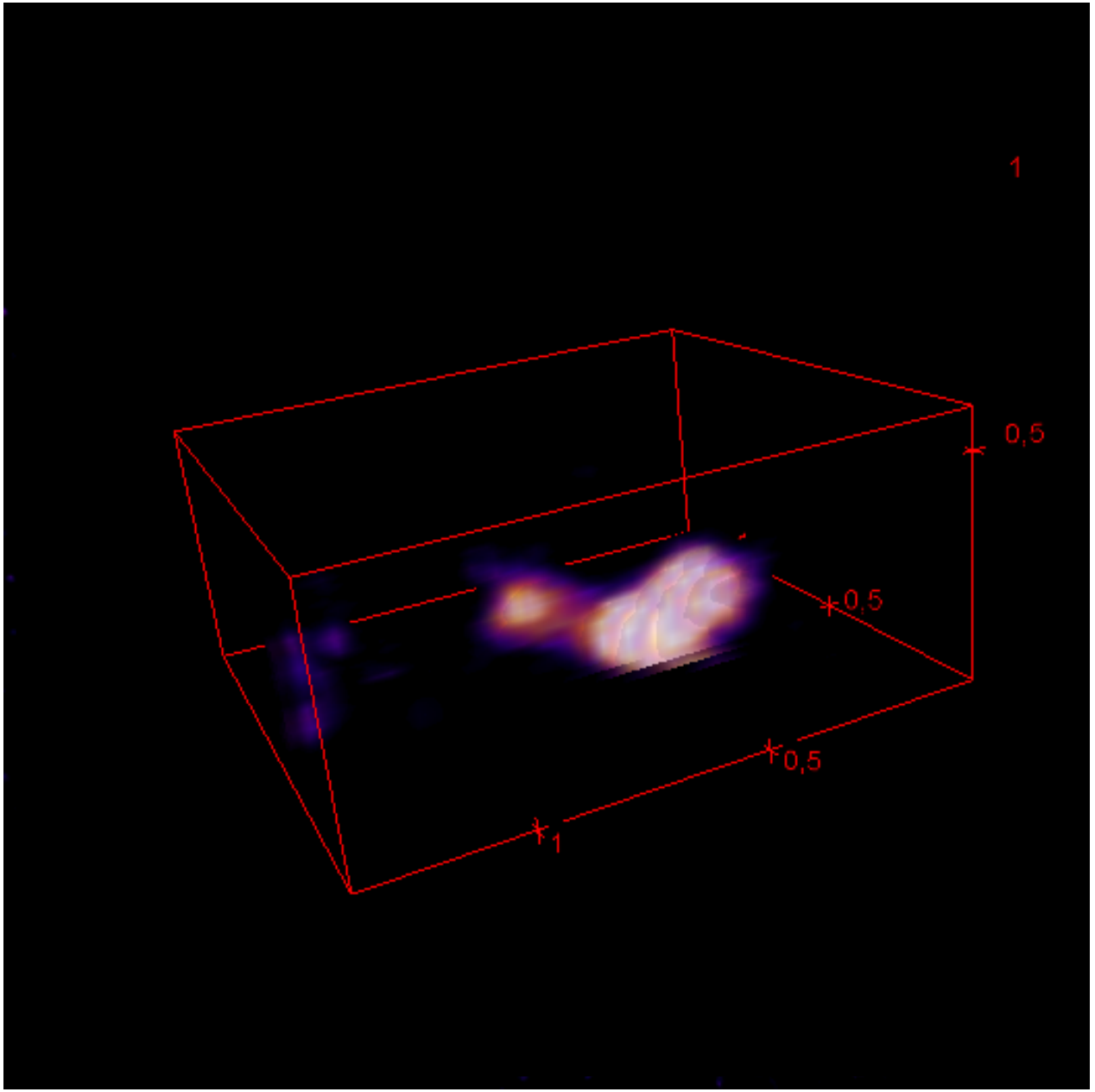


Without astigmatism

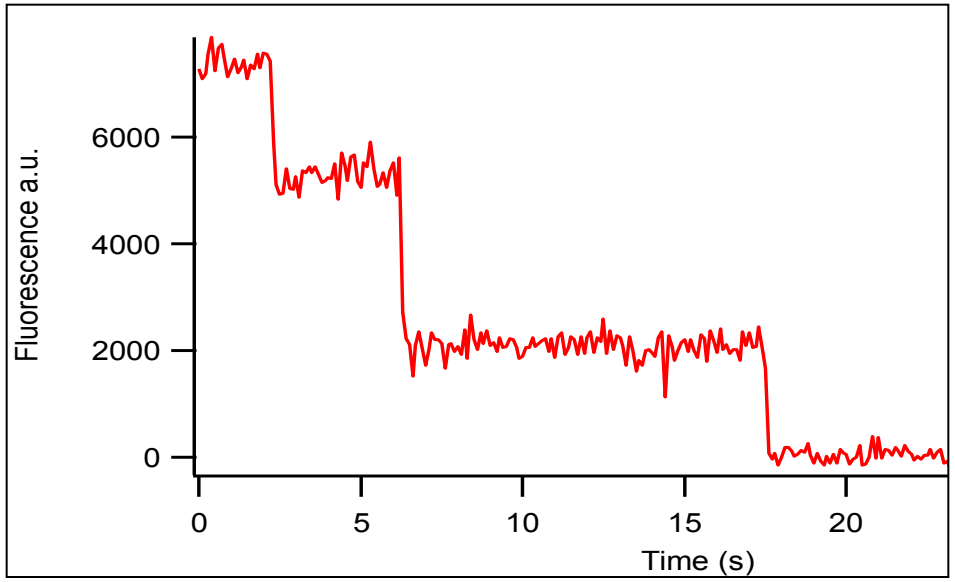


With astigmatism

# 3D PALM with Eos labeled nucleolar protein



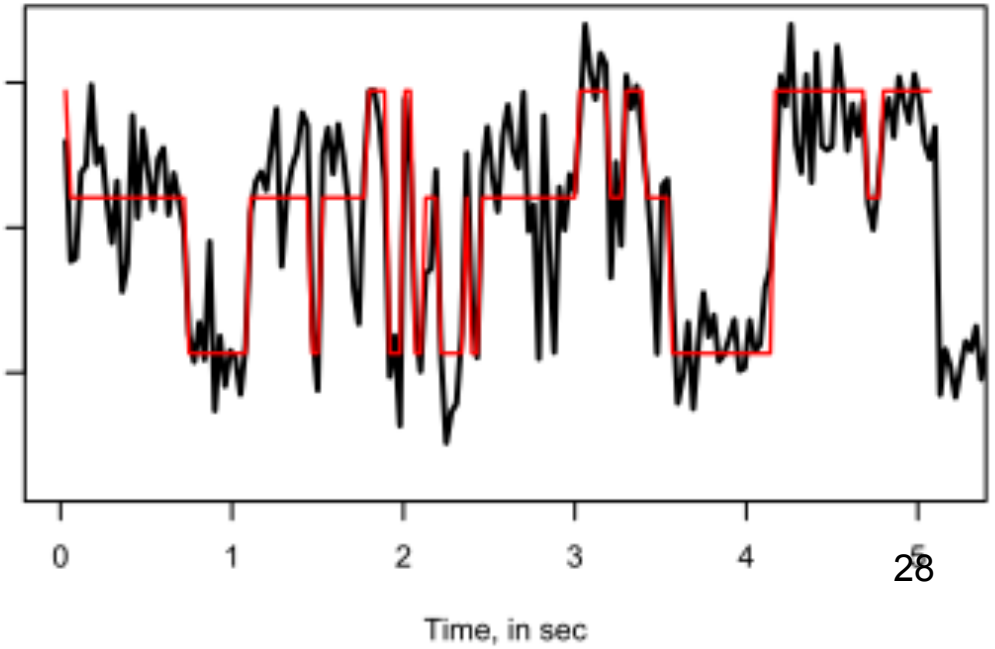
# Limitations in single molecule experiments



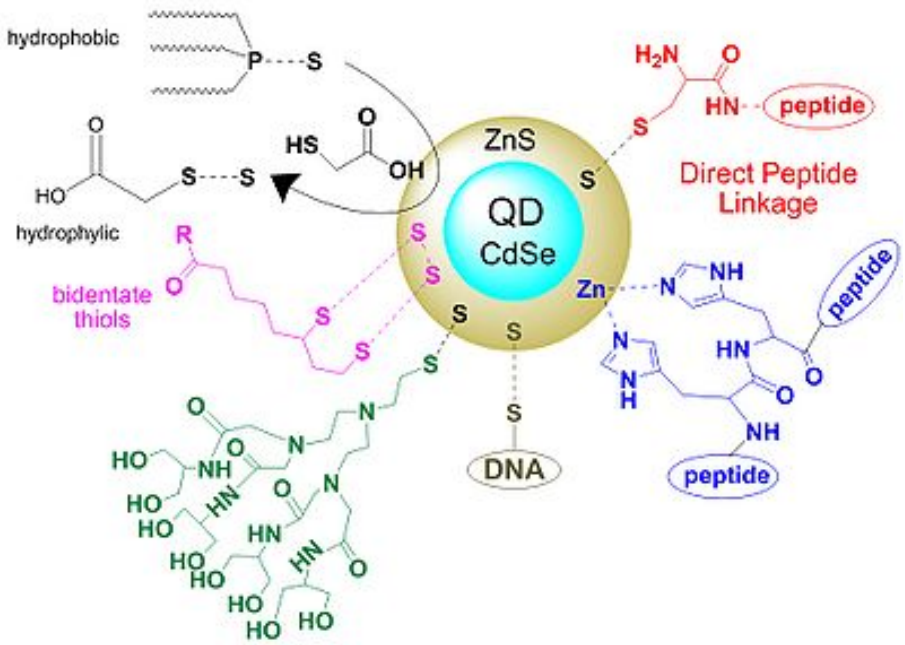

Observation time limited to few tens of seconds.

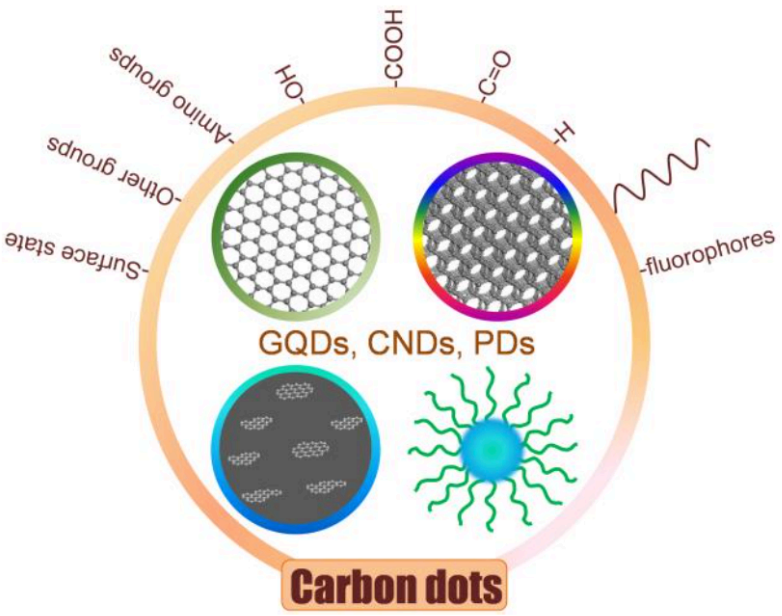
Temporal resolution is also limited by the brigthness of the dye.

In addition, very low S/N ratio.



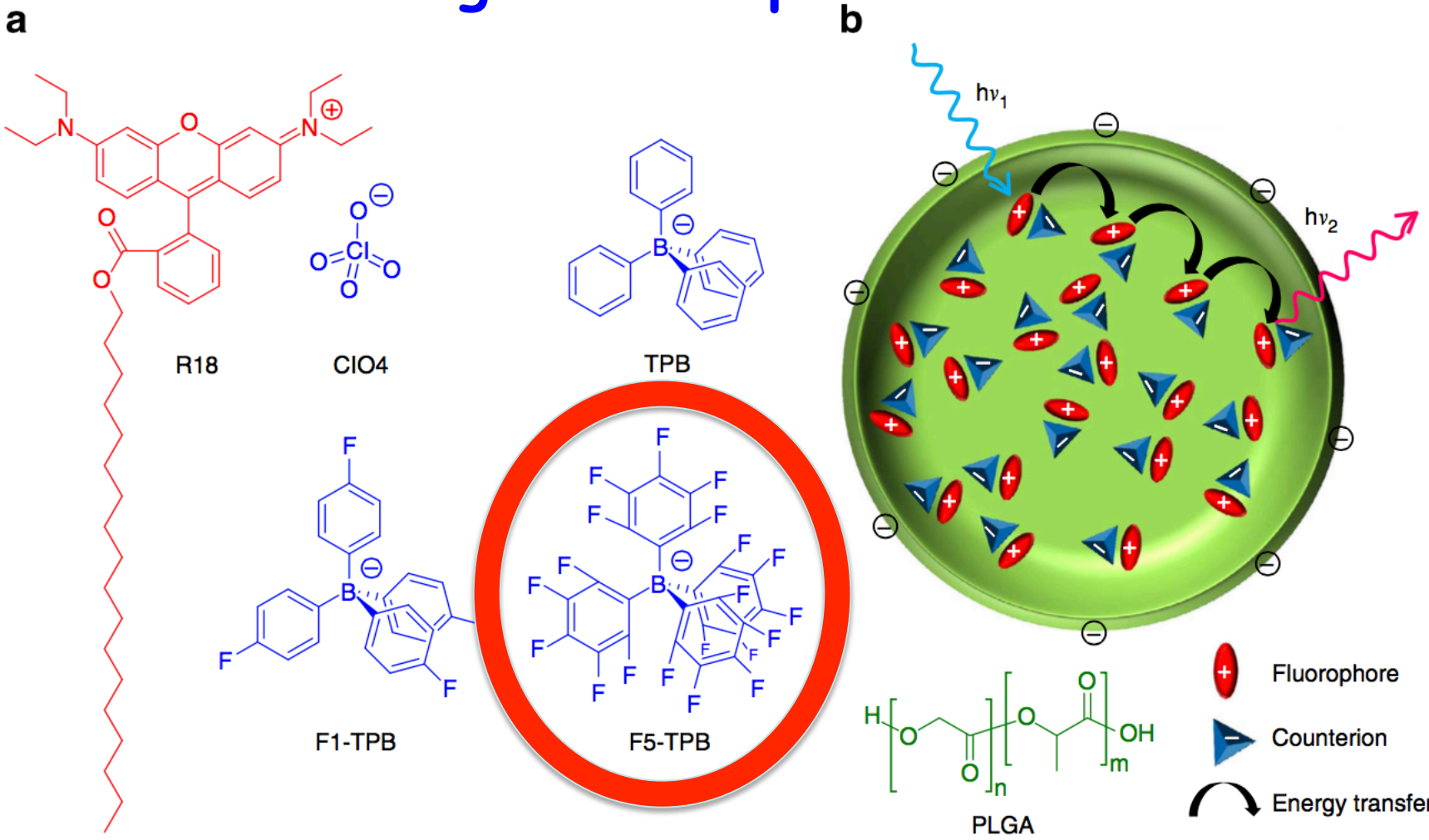
# Development of ultrabright nanoparticles



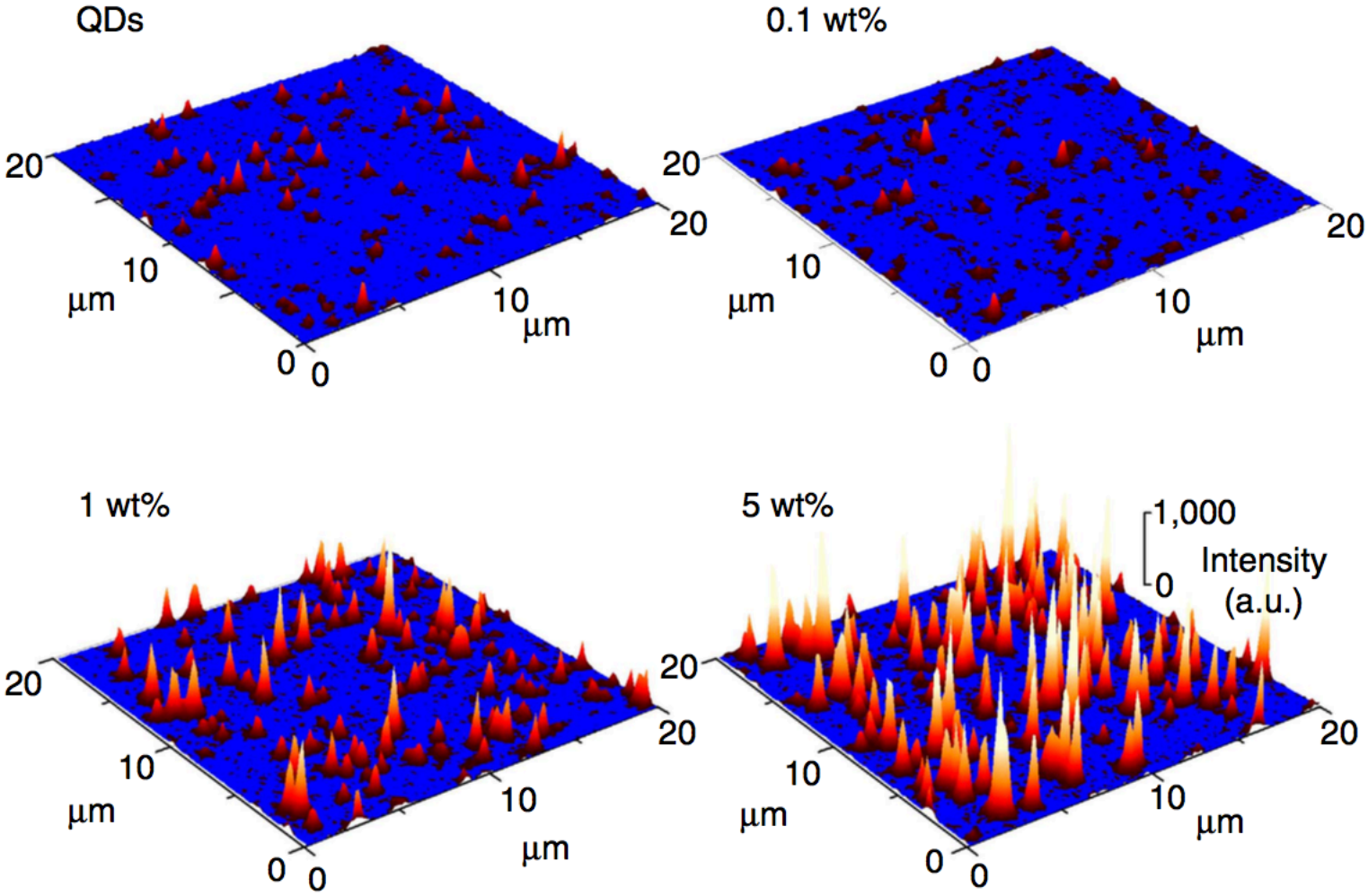

Quantum and carbon dots are made of inorganic materials that may display long term toxicity in living organisms.

# Development of ultrabright and biocompatible organic nanoparticles



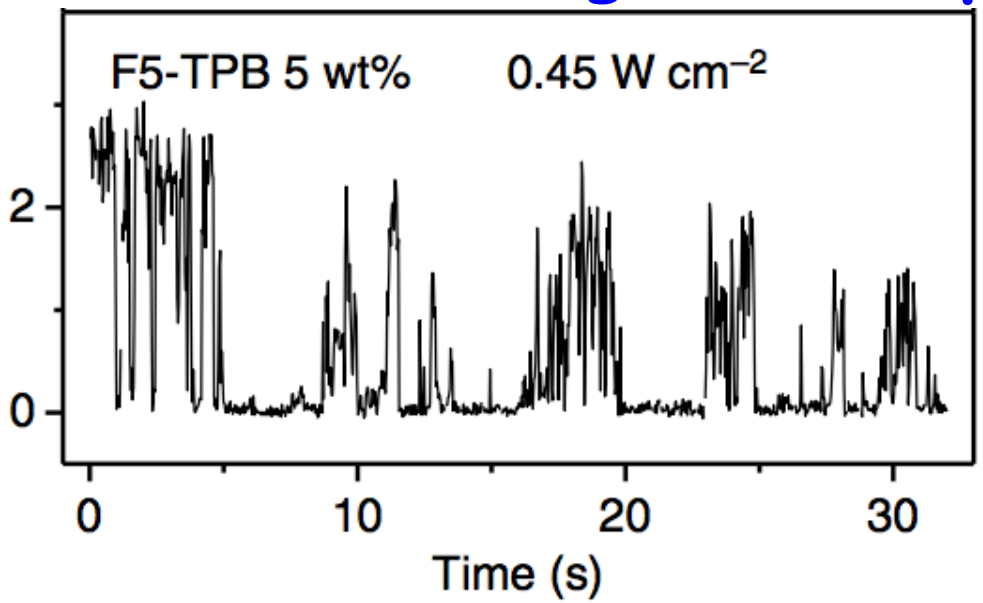

NPs based on biodegradable polymers, such as poly(D,L-lactide-co-glycolide) (PLGA), are known for many years as drug carriers

# Development of ultrabright and biocompatible organic nanoparticles



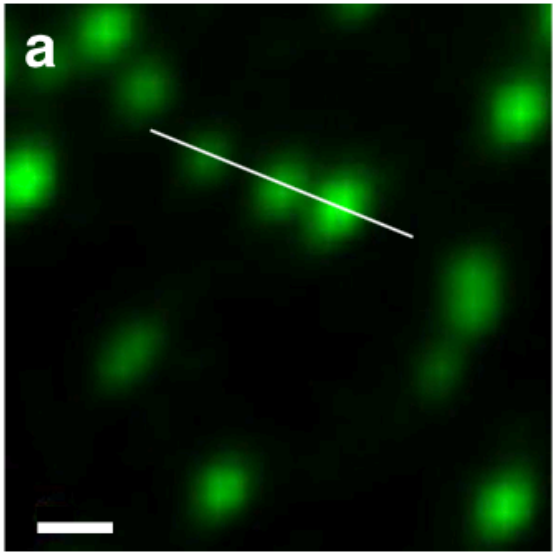

Comparison between ONPs (30 nm) and QDs under the same excitation conditions (<1 W.cm$^{-2}$).

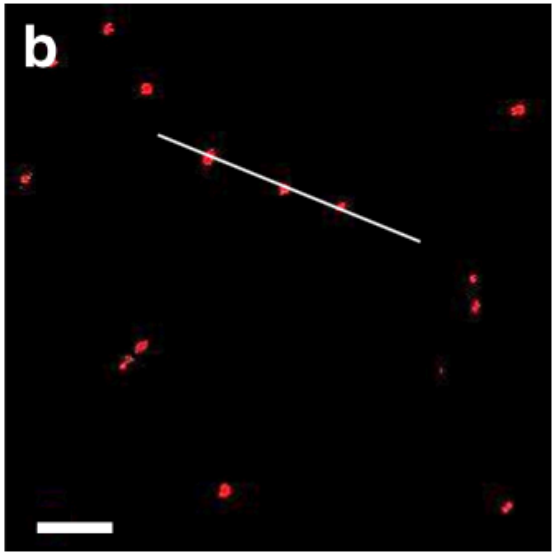
# Development of ultrabright and biocompatible organic nanoparticles



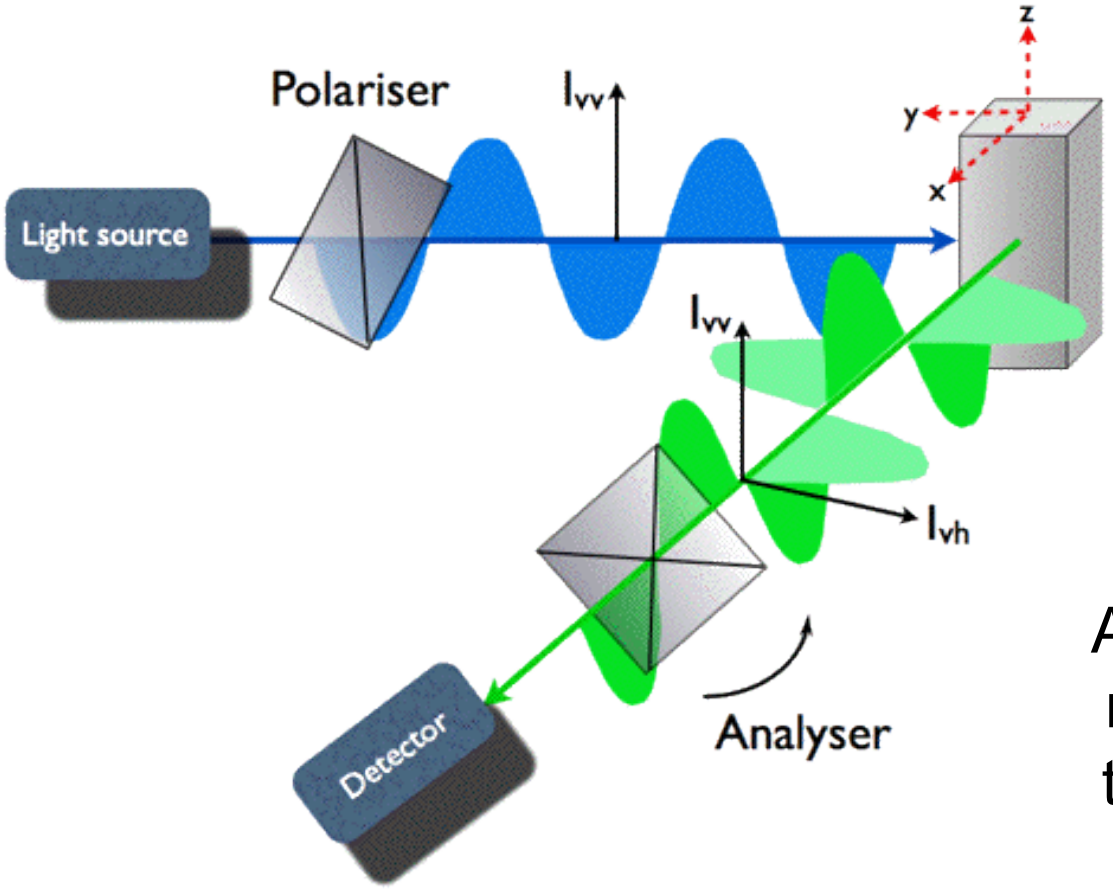

Dye doped ONPs behave like a single « super » dye.

ONPs can be used for super-resolution microscopy (scale bar 200 nm).



# Development of ultrabright and biocompatible organic nanoparticles

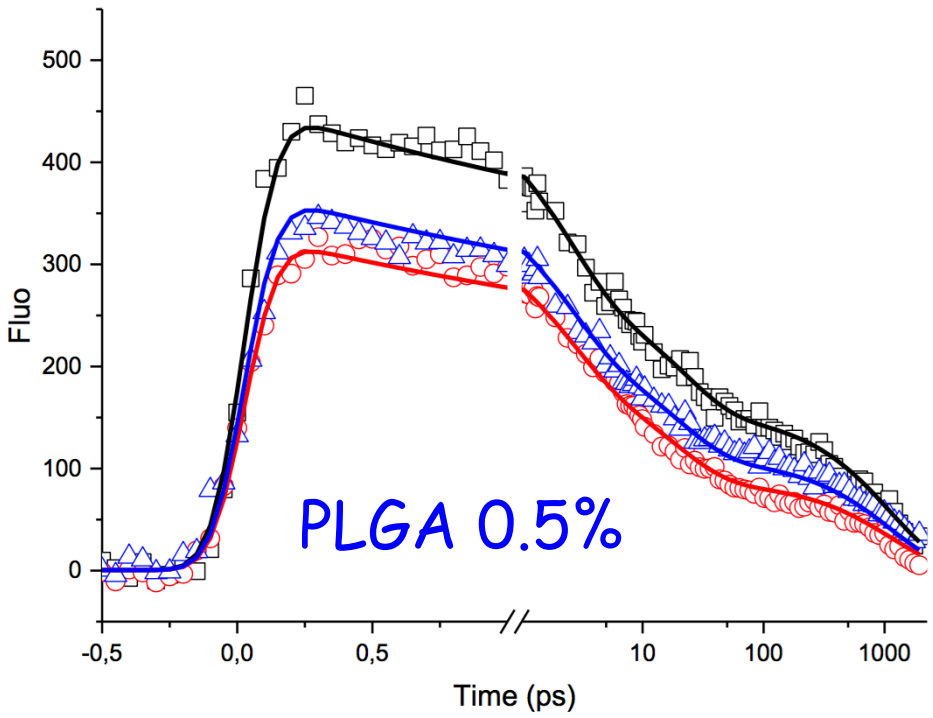


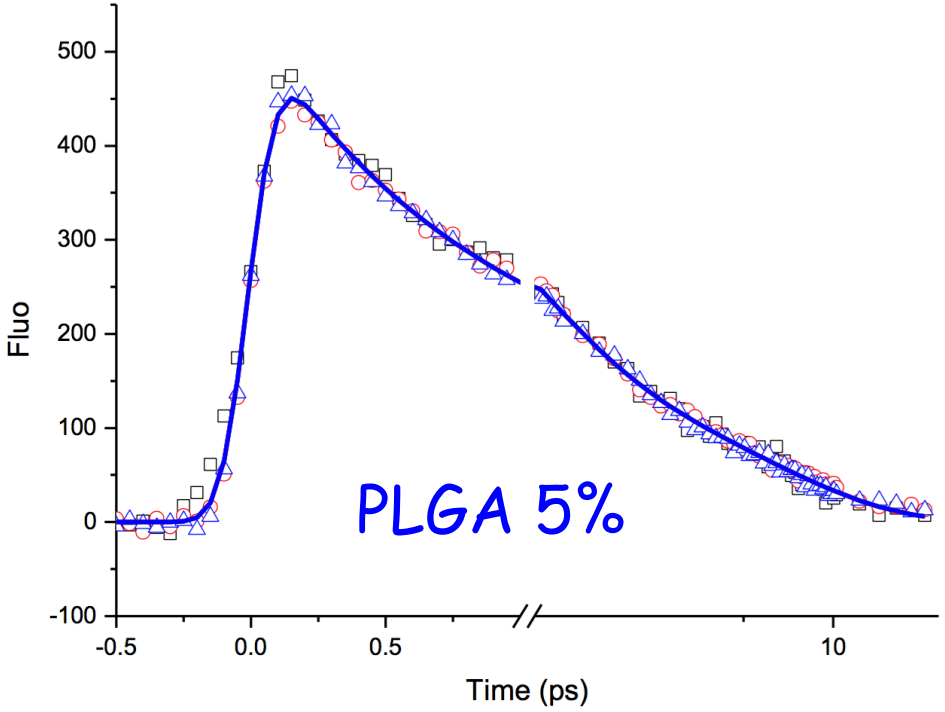

Fluorescence Anisotropy

$$r = \frac{I_{vv} - I_{vh}}{I_{vv} + 2I_{vh}}$$

Anisotropy can be time-resolved giving access to the fluorophore size.

# Development of ultrabright and biocompatible organic nanoparticles




Time-resolved anisotropy evidences ultrafast energy transfer within the particules

- time scale of the energy transfer < 200 fs.
- Coherent coupling between the dye.

# Conclusions

- FRET/FLIM is an ideal tool for monitoring protein oligomerization, protein/protein and protein/ligand interactions, but needs both partners to be labelled.

- FCS is highly suited to determine the binding stoichiometry, local concentrations of labelled proteins, the size of the diffusing species.

-Single molecule experiments can be used to determine kinetic constants of biochemical reaction and to perform super-resolution fluorescence imaging microscopy.

-ONPs represents an excellent alternative to inorganic nanoparticles.