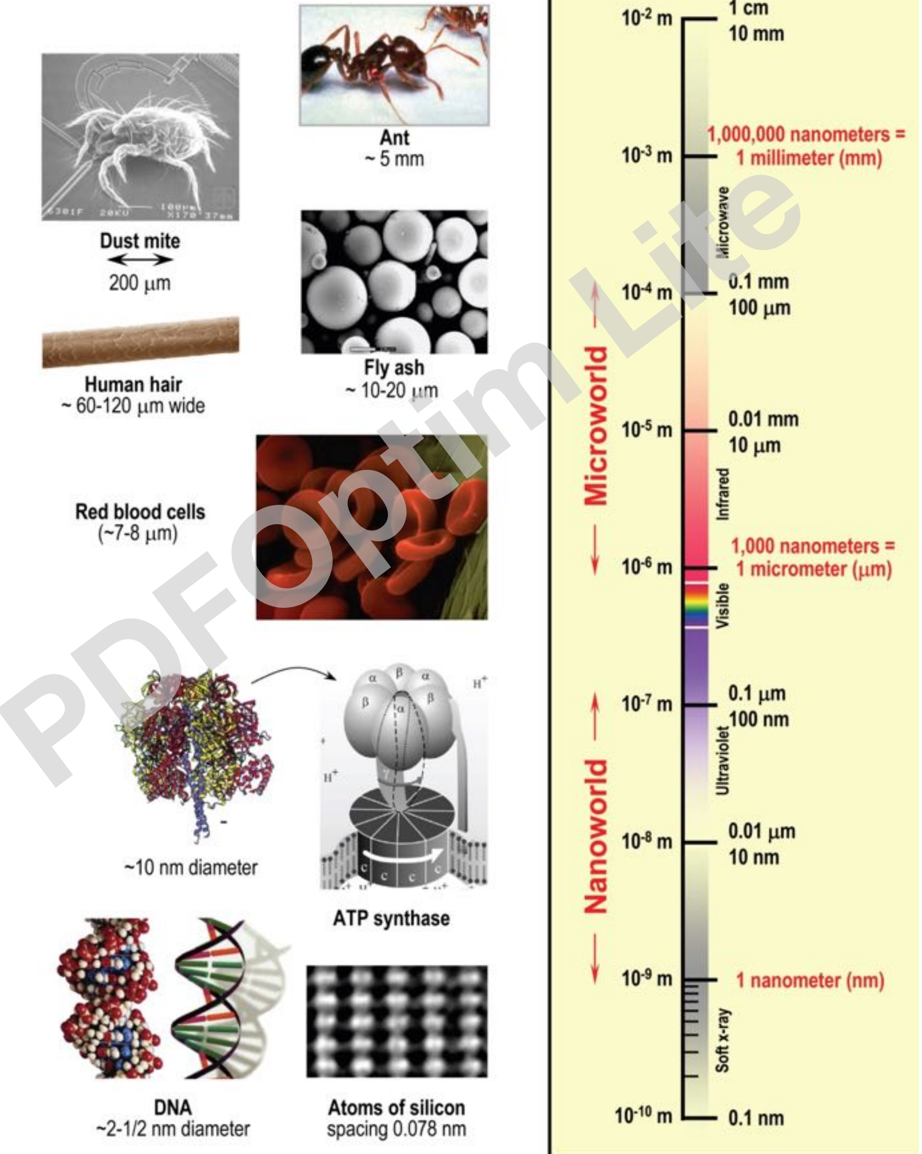
New Microscopies:

Single molecules Imaging & Superresolution microscopies

PDFOptim Lite

Brahim Lounis
Institut d'Optique Graduate School
CNRS, University of Bordeaux
France

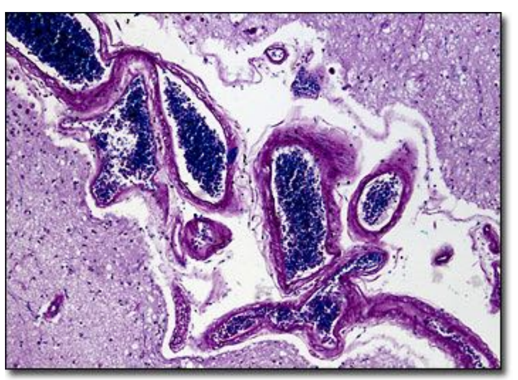
The scale of things vs radiation wavelengths



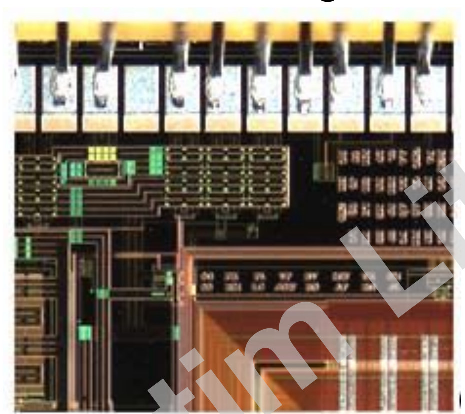
PDFOptim Lite

Common modalities of optical microscopy

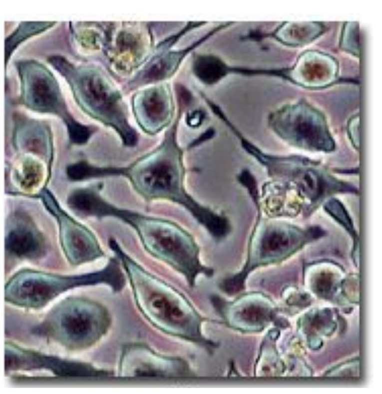
Bright field microscopy
Scattering, reflection, absorption



Dark field
Scattering

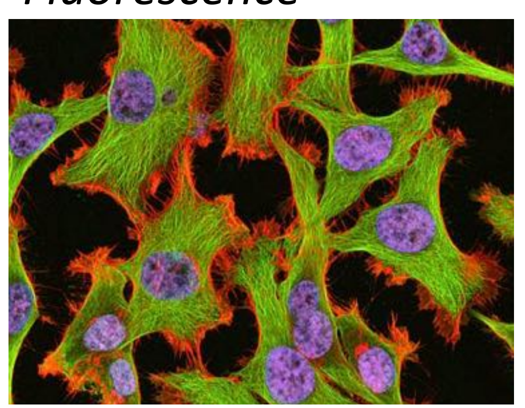


Phase contrast
Phase



Differential Interference contrast (Nomarsky) , *Phase gradients*

Fluorescence microscopy
Fluorescence



PDFOptim Lite

Single Molecule detection

Why?

No ensemble averaging (distributions)

- inhomogeneous broadening free Spectroscopy
- Isolate a single quantum system
- Sub-wavelength localization possible
- Extreme sensitivity to local environment
- Time evolution, no synchronization needed

How?

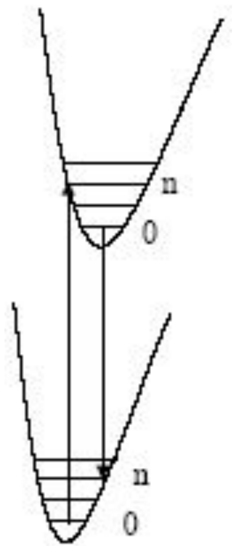
Far Field Optical Detection Techniques

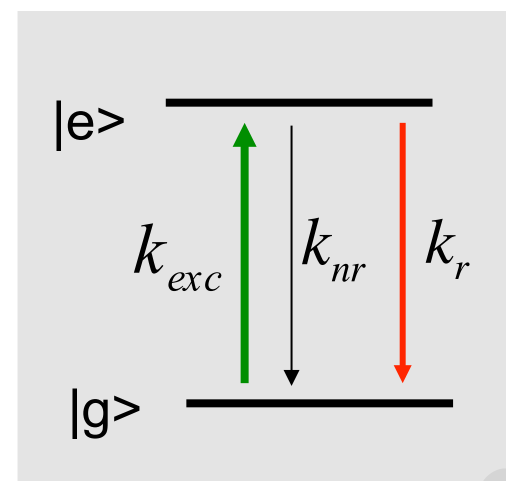
Make sure that :

- only one nanoobject interacts with the laser in the excitation volume
- the signal from the nanoobject dominate all sources of background

PDFOptim Lite

Fluorescent molecules as a two level system





k_{exc} Excitation rate (s^{-1})

$$k_{exc} = \sigma \frac{\lambda}{hc} I$$

σ: absorption cross-section

PDFOptim Lite

Desexcitation rate (s^{-1}) $$k_{des} = k_r + k_{nr}$$

non-radiative decay rate k_{nr}

Radiative desexcitation rate

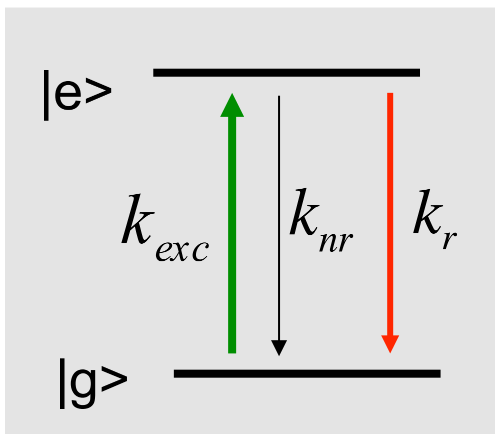
T_{fluo} radiative lifetime

$$k_r = \frac{1}{T_{fluo}}$$

Fluorescence quantum yield

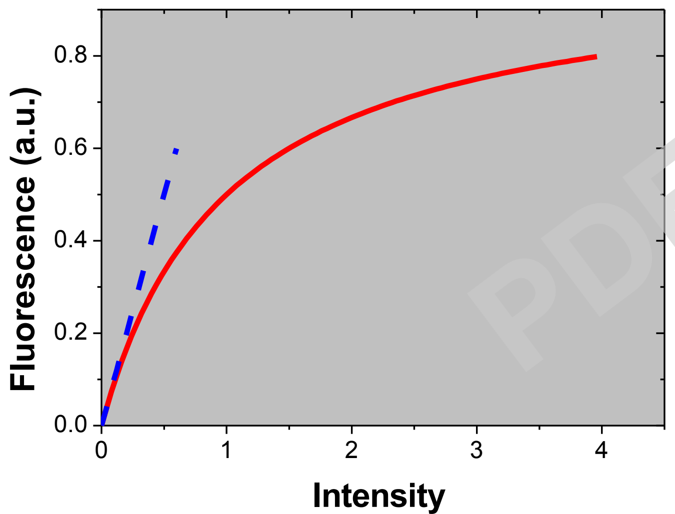
$$\eta = \frac{k_r}{k_r + k_{nr}}$$

Fluorescence Emission rate



$$R_{fluo} = k_r P_e = \frac{1}{T_{fluo}} \frac{I/I_s}{1+I/I_s} \text{ (fluorescence rate)}$$

$$I_s = (k_{nr} + k_r)\frac{hc}{\sigma\lambda} \quad \text{(saturation intensity)}$$



At low excitation intensity: linear regime

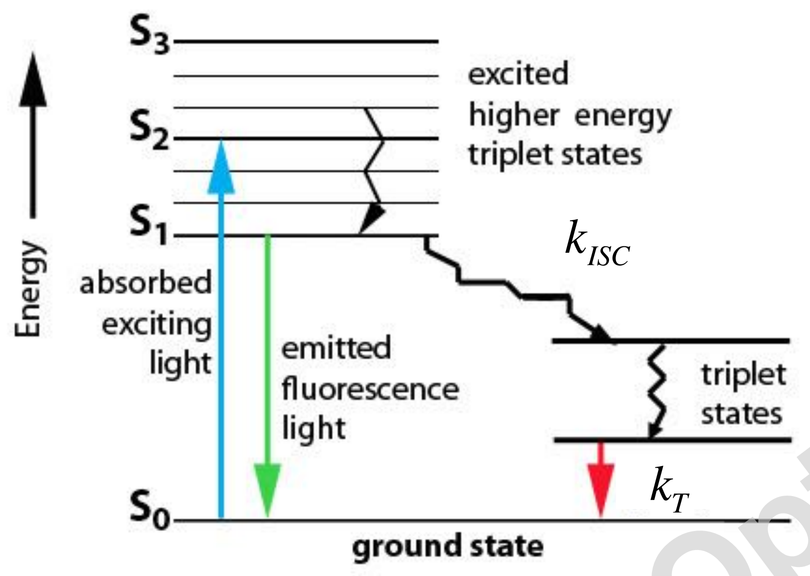
$$\text{For } I << I_s, \quad R_{fluo} \approx \eta \frac{\sigma\lambda}{hc} I$$

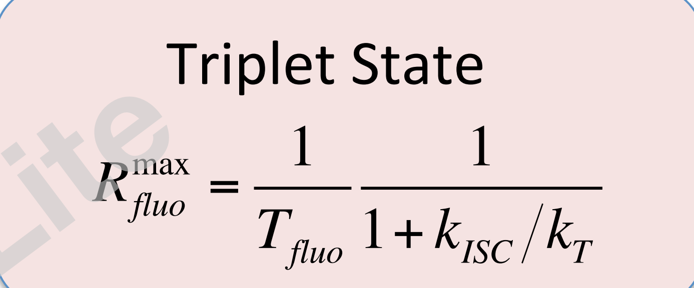
At high intensity: saturation

$$\text{For } I >> I_s, \quad R_{fluo}^{\max} = k_r = \frac{1}{T_{fluo}}$$

PDFOptim Lite

A good fluorophore for single molecule detection





- large absorption cross section,
  low excitation intensities, less background
  @ Room T: few 10^{-16} cm^2, @ Low T: ~0.1 μm^2

- large quantum yield and short excited state radiative lifetime (~few ns)
- low triplet yield, no bottleneck state
- good photostability (low photobleaching)

PDFOptim Lite

Fluorescence labeling (1)

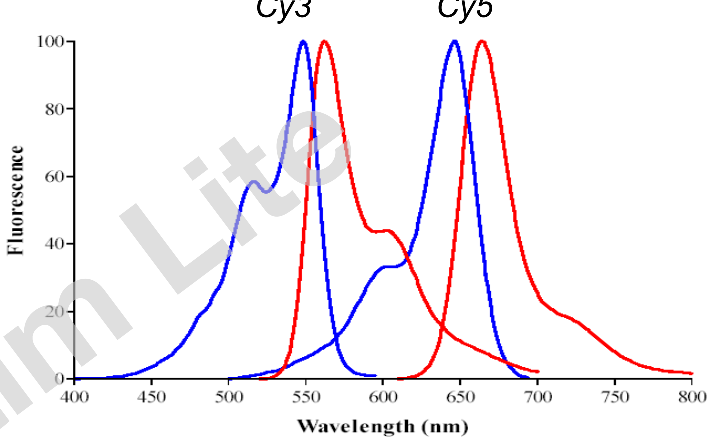
Dyes chemically attached to the proteins or to a ligand (e.g. nanobody)

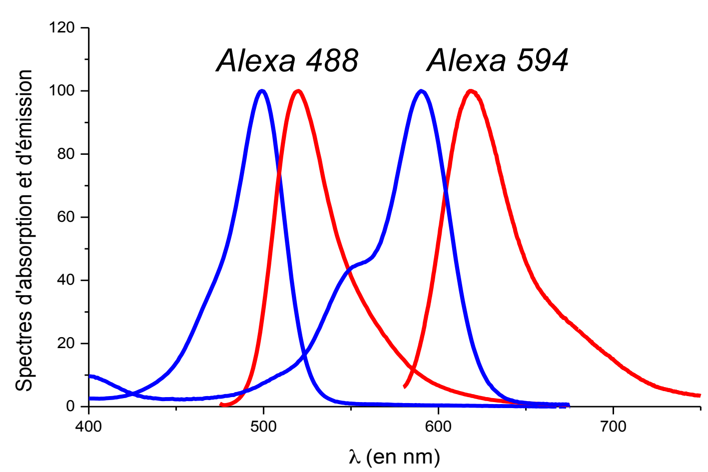
Cy3

Cy5

Alexa 488

Alexa 594





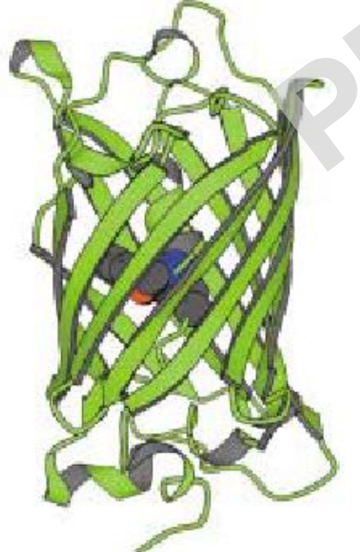
Optical spectra given by the length of the double conjugated bounds

PDFOptim Lite

Fluorescence labeling (1)

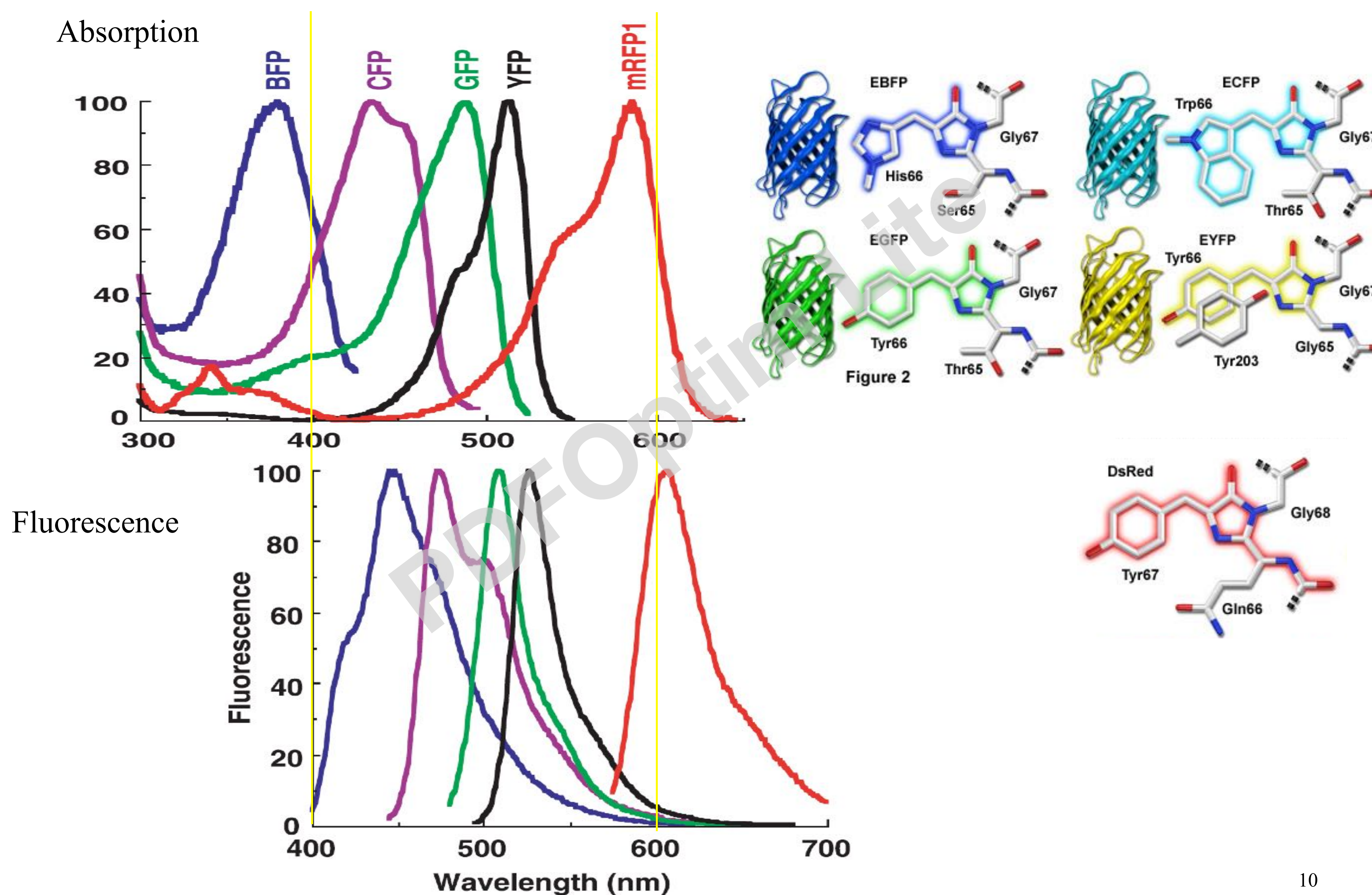
Fluorescent proteins, genetically fused to the protein of interst

The jelly fish *Aequorea Victoria*



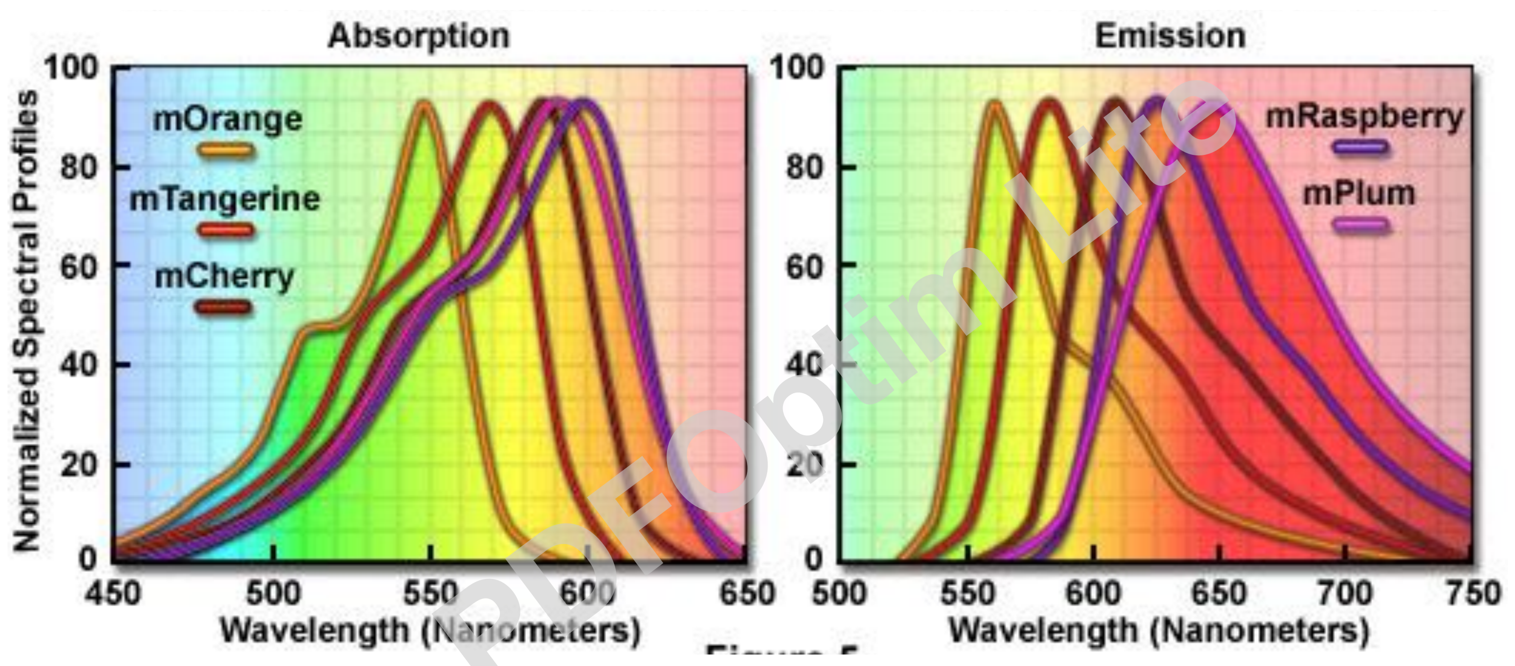
PDFOptim Lite

A few mutants of the GFP



PDFOptimLite

Red and orange mutants

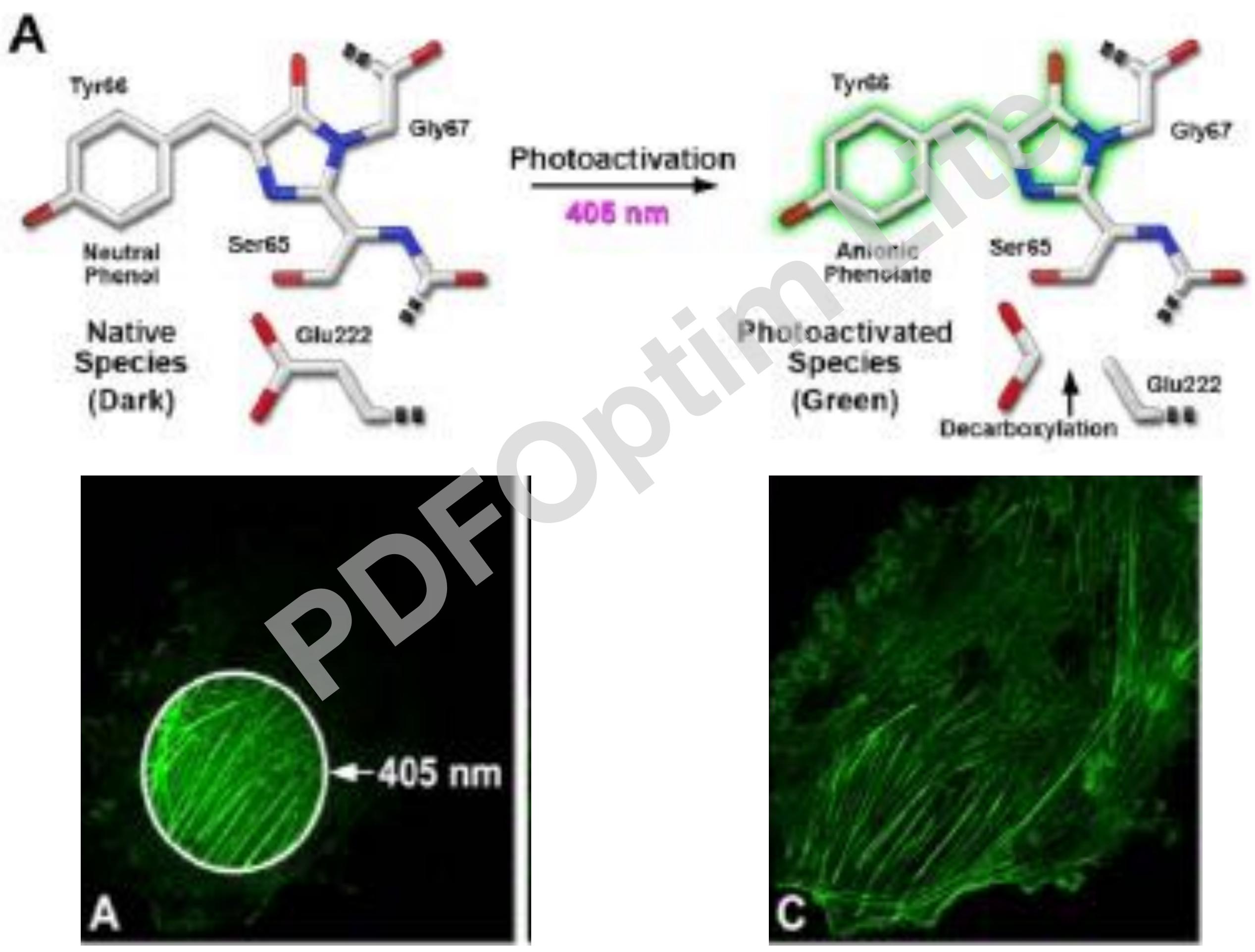


PDFOptim Lite

This rich photophysics can be used to engineer interesting nanoscale emitters in the cells…

PDFOptim Lite

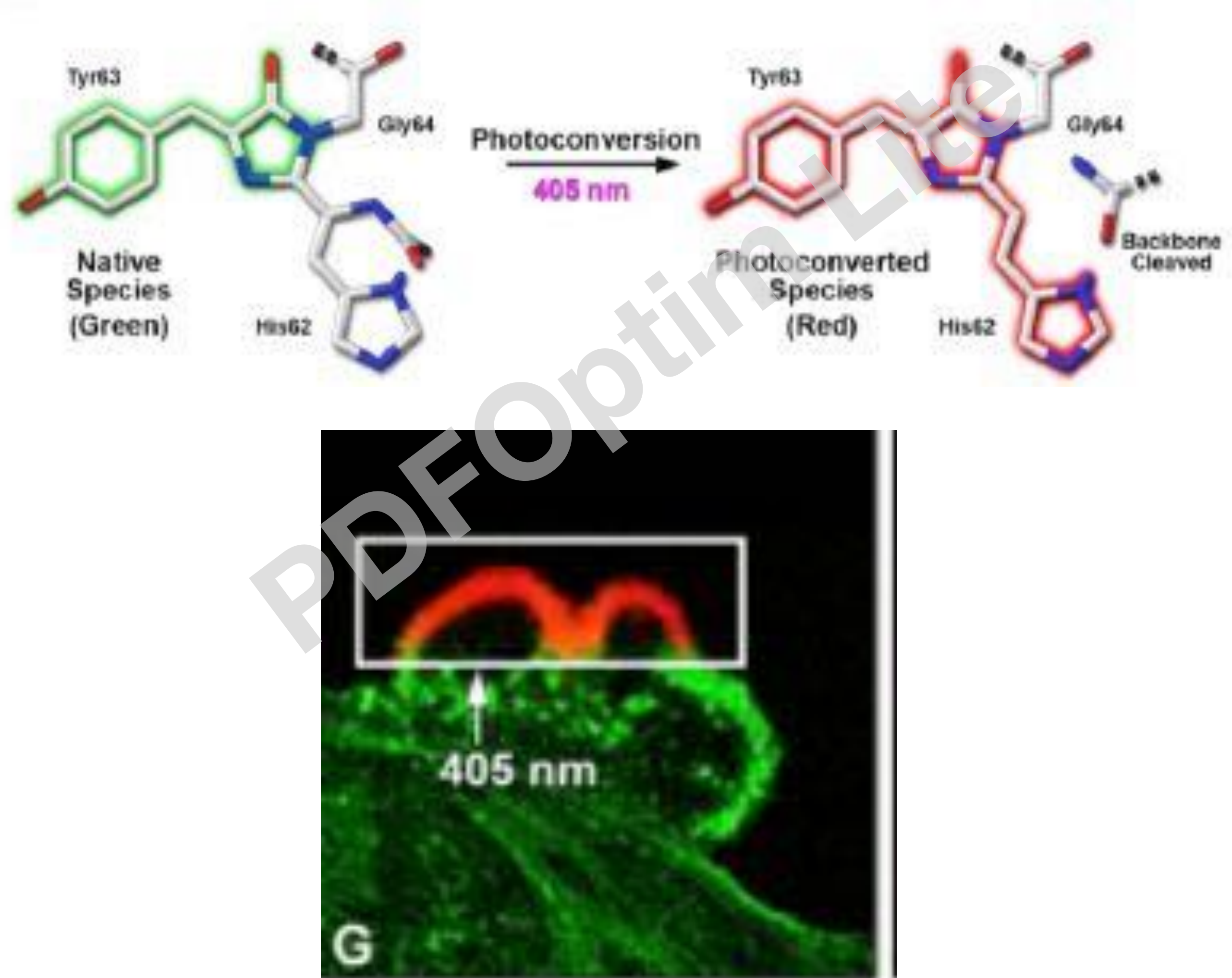
Photoactivateable FPs are dark and are irreversibly activated by irradiation. For example irradiation of PA-GFP with intense violet light results in a 100-fold increase in green fluorescence. It is presumed that the violet light causes the decarboxylation of Glu222, which aids in the formation of the anionic fluorescent form of the chromophore, see below.



PDFOptim Lite

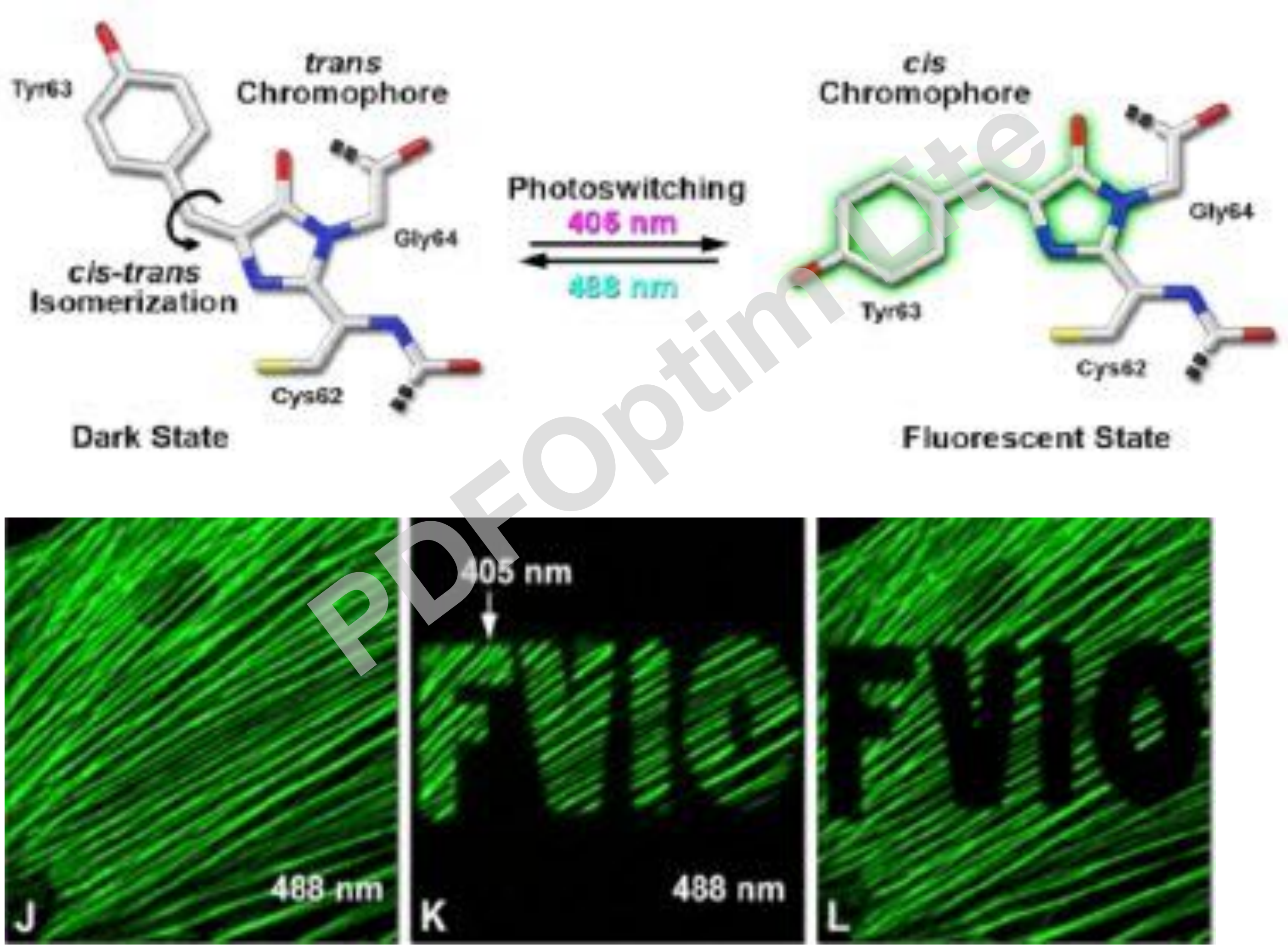
(J. Cell Science 2007,120, 4257)

Photoconvertable FPs can be irreversibly converted from a green fluorescent form to a red fluorescent form by violet or ultraviolet irradiation e.g. Kaede, KikGR, Dendra2 and Eos. The photoconversion is presumably associated with a cleavage occurring between the amide nitrogen and the alpha carbon of His62 that is followed by oxidation of the His62 sidechain.



PDFOptim Lite

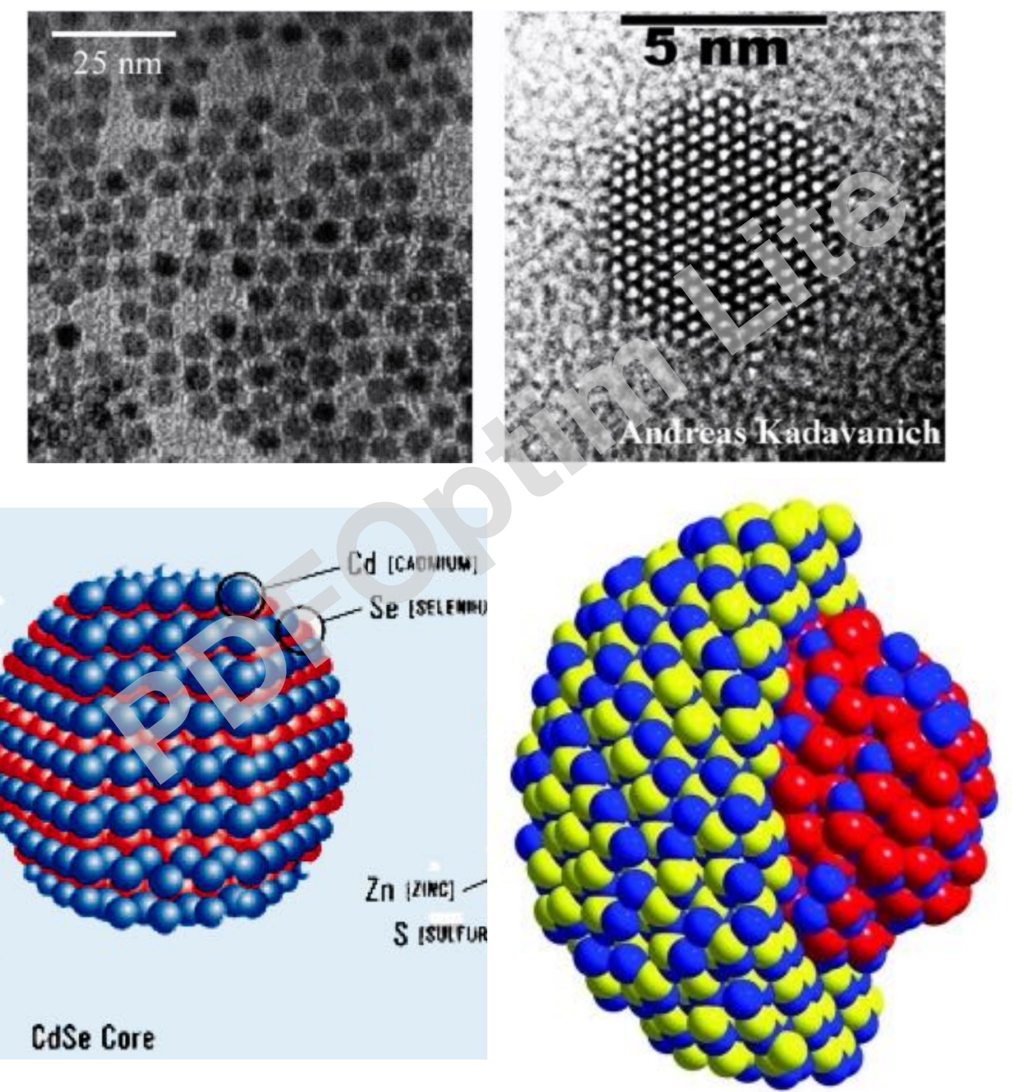
Photoswitchable FPs are dark and are reversibly activated by irradiation. Photoswithable FPs such as Dronpa, mTFP0.7 and KFP switch between the dark E (or trans) state and the fluorescent Z (or cis) state, see below.



PDFOptim Lite

Fluorescence labeling (3)

Nanocrystals of Semiconductors



PDF-Optim Lite

Emission Properties (1)

Quantum Confinement

Type-I QD

e⁻ h⁺

CdSe

ZnS

Radial distance

0

Potentials

e⁻

CdSe

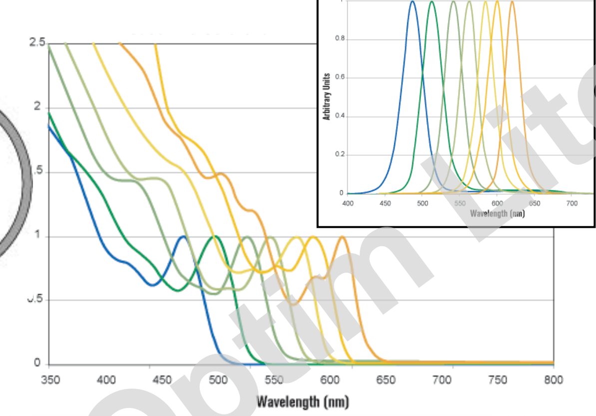
h⁺

ZnS

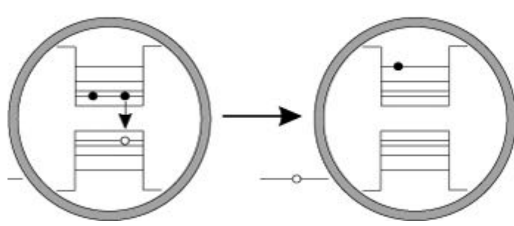
Organic Coating

-V

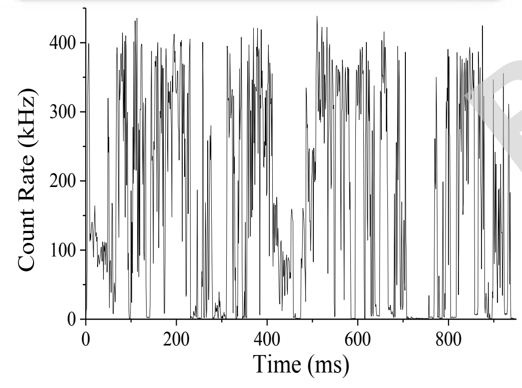
Absorption & Emission



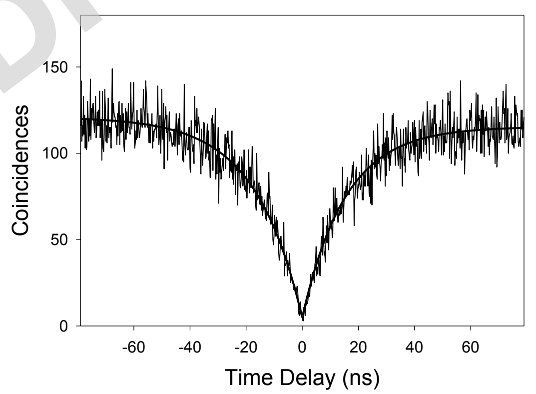
Auger Process

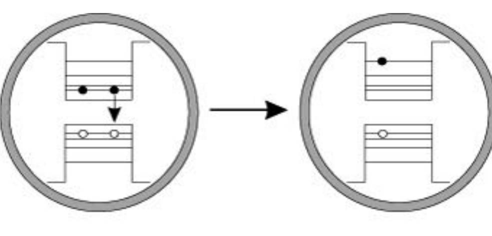


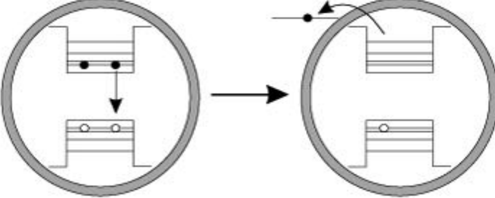
Luminescence Blinking



Photons Antibunching



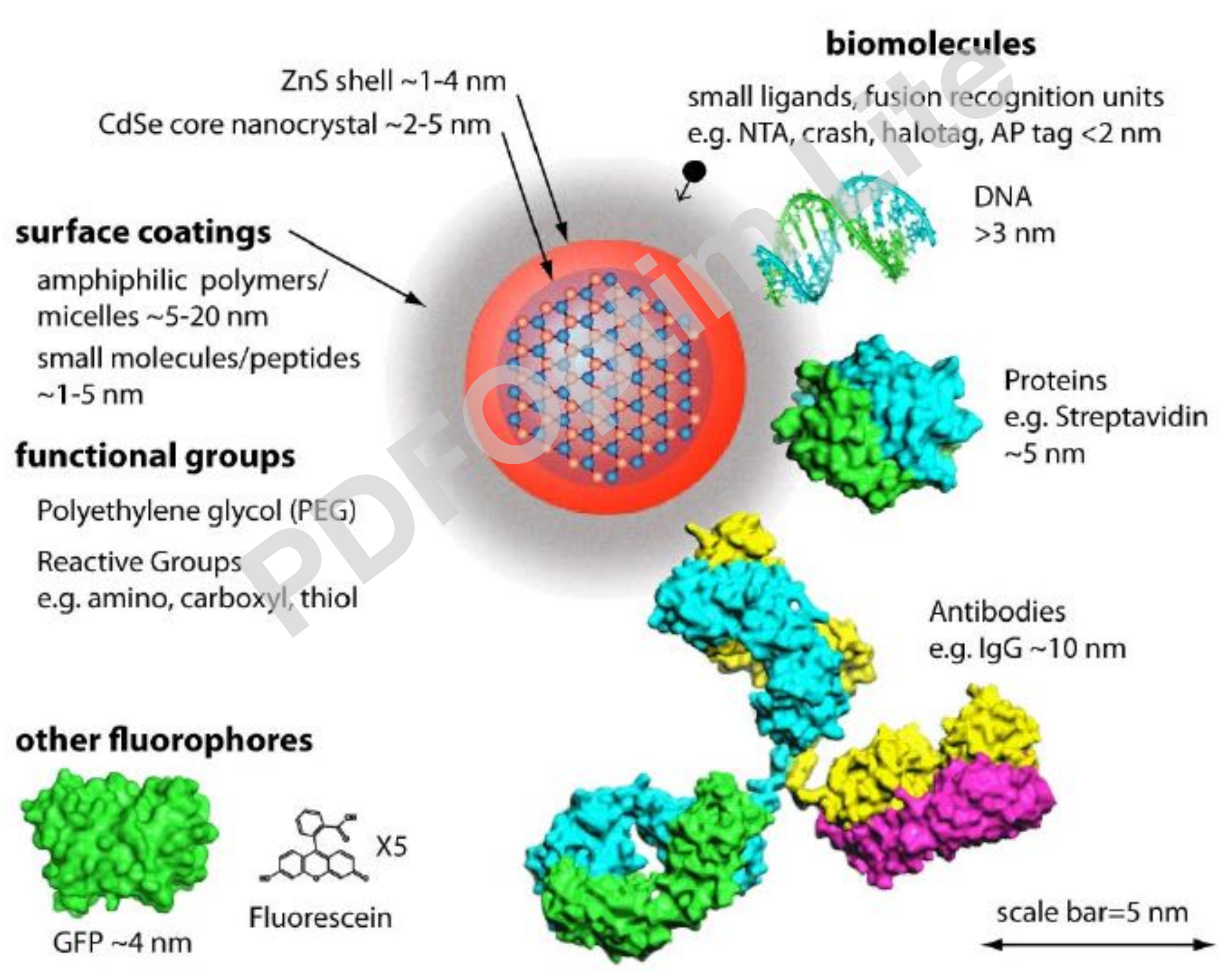




PDFOptim Lite

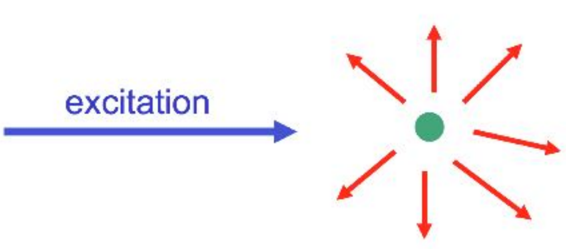
Fluorescence labeling (3)

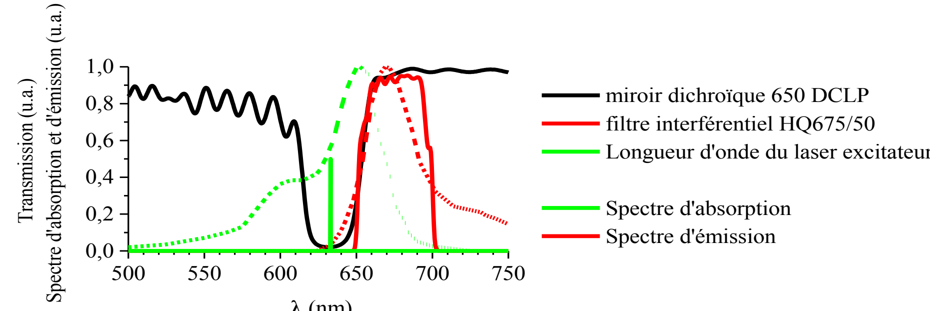
Semiconductor quantum dots bioconjugation



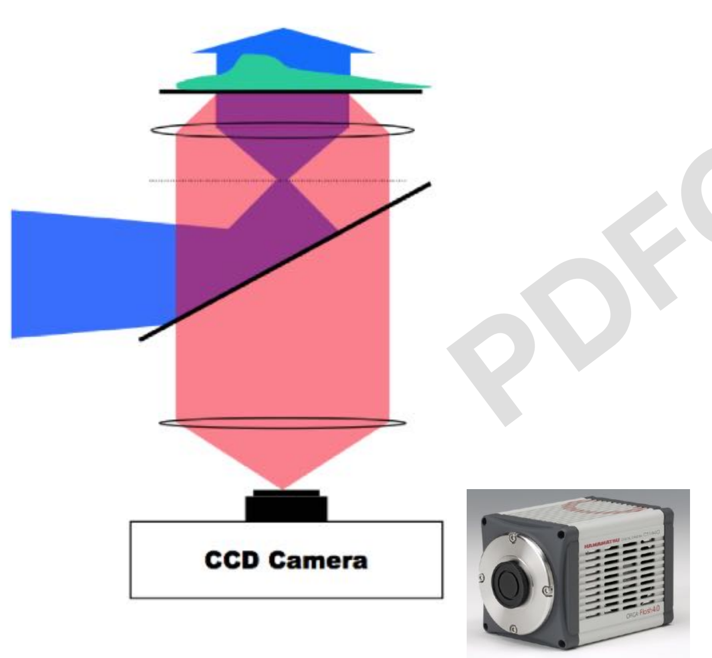
PDFOnline Lite

"Standard" fluorescence microscopy techniques





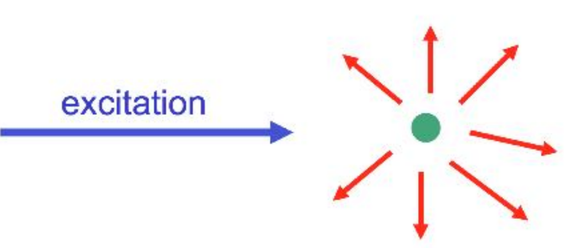
Wide-field fluorescence microscopy

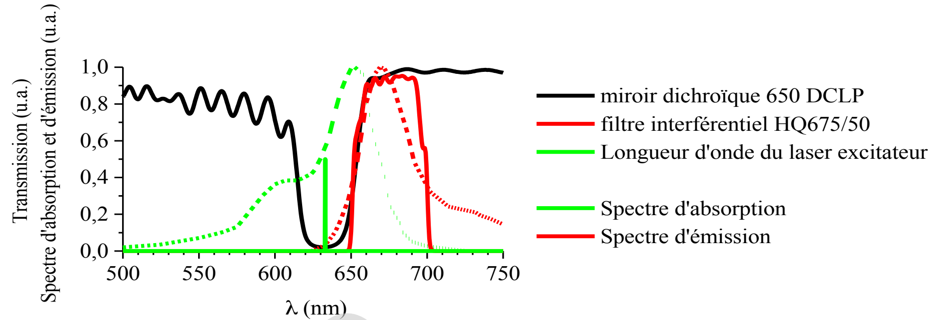


Resolution 1.22 λ / 2 NA

PDFOptim Lite

"Standard" fluorescence microscopy techniques





Wide-field fluorescence microscopy

CCD Camera

Resolution 1.22 λ / 2 NA

Confocal microscopy:

localized excitation + spatially filtered detection

pinhole

Laser Light

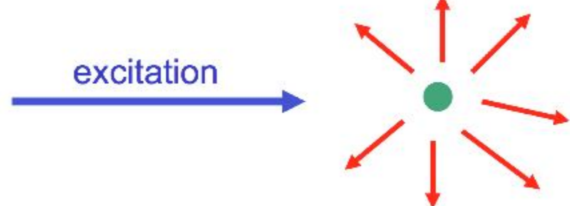
Specimen

+ laser scanning

Improved resolution 0.86 λ / 2 NA

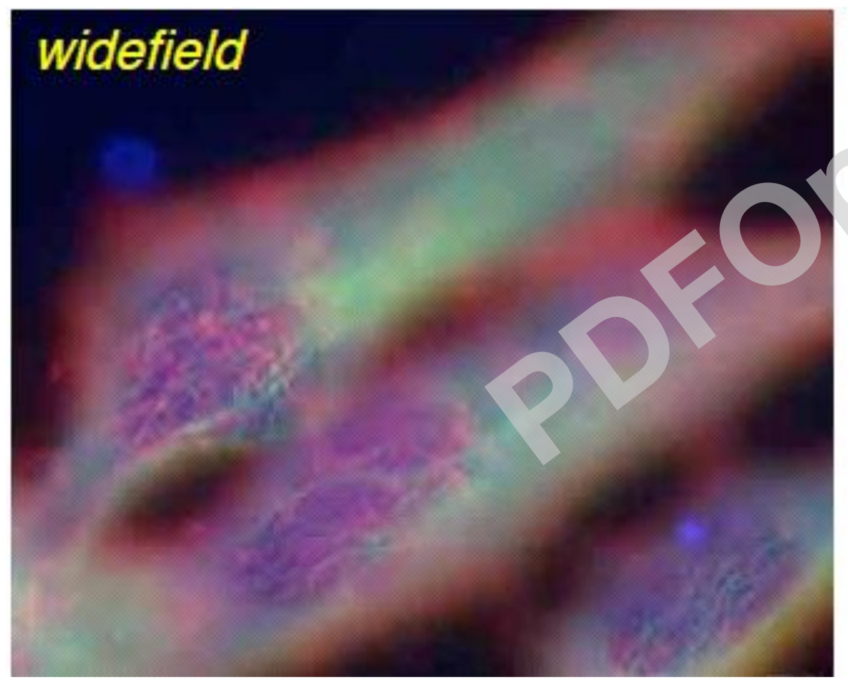
PDFOptim Lite

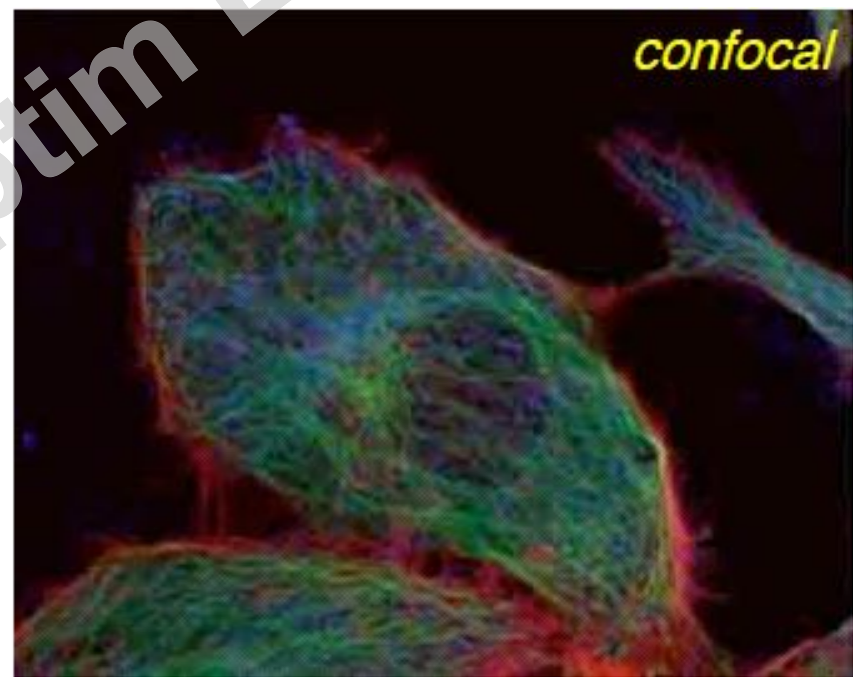
“Standard” fluorescence microscopy techniques



Wide-field fluorescence microscopy

Confocal microscopy:
localized excitation + spatially filtered detection



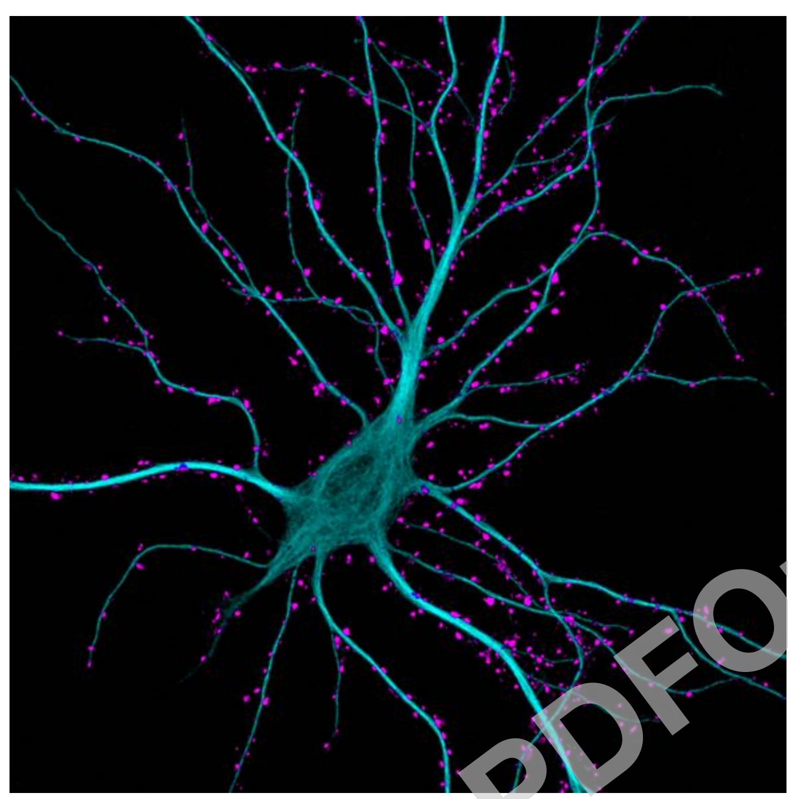


Resolution 1.22 λ / 2 NA

Improved resolution 0.86 λ / 2 NA
+ Axial resolution

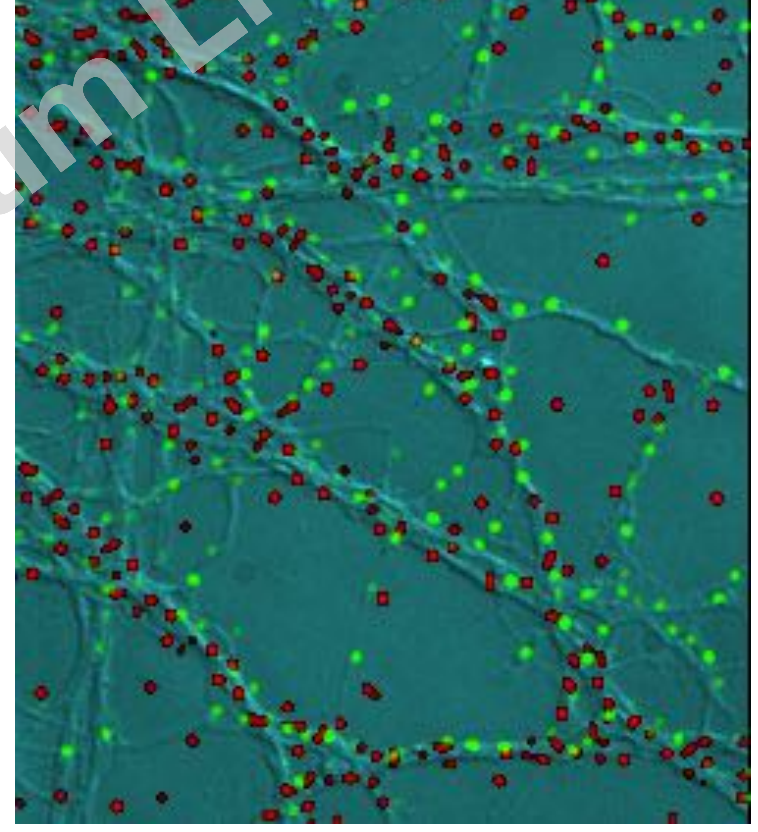
PDFOptim Lite

Fluorescence microscopy



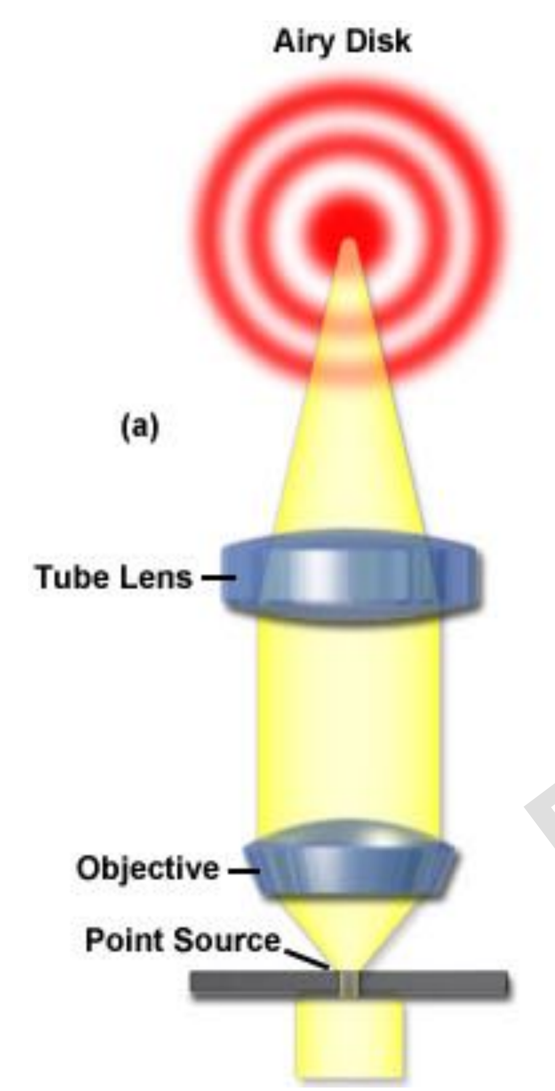
Fluorescence image of neuron

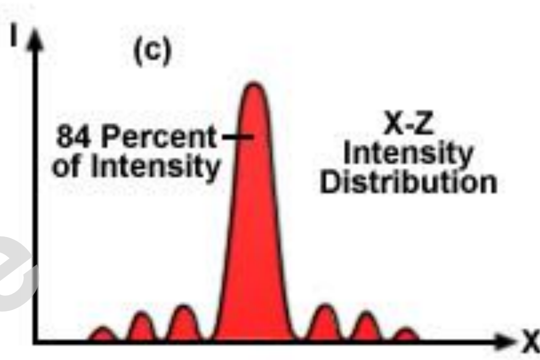
Live cell imaging



PDFOptim Lite

Diffraction limit

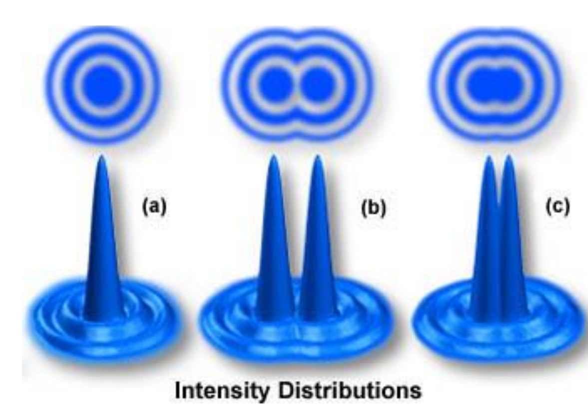




diffraction spot width $$\Delta x = \frac{1.22\lambda}{2NA}$$

High N.A. = 1.49, oil immersion
Δx = 214 nm @ λ= 500 nm,

Resolution :
Rayleigh criterium

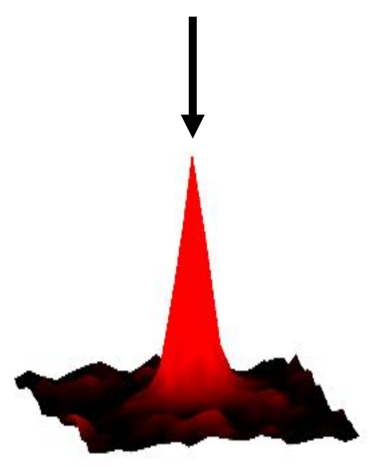


PDFOptim Lite

E. Abbe (1873), *Arch. Mikroskop. Anat.* 9, 413.

Pointing accuracy

The pointing accuracy i.e. precision of the localization of the fluorophore is only limited by the signal to noise ratio



$$\left\langle (\Delta x)^2 \right\rangle = \frac{s^2 + p^2/12}{N} + \frac{8\pi s^4 B^2}{p^2 N^2}$$

N: number of the collected photons, s: PSF width, p: Pixel size B: Background signal

Typically , for a SNR of ~ 30

The pointing accuracy is ~ 40nm

In the limit of a SNR limited by the photon number noise:

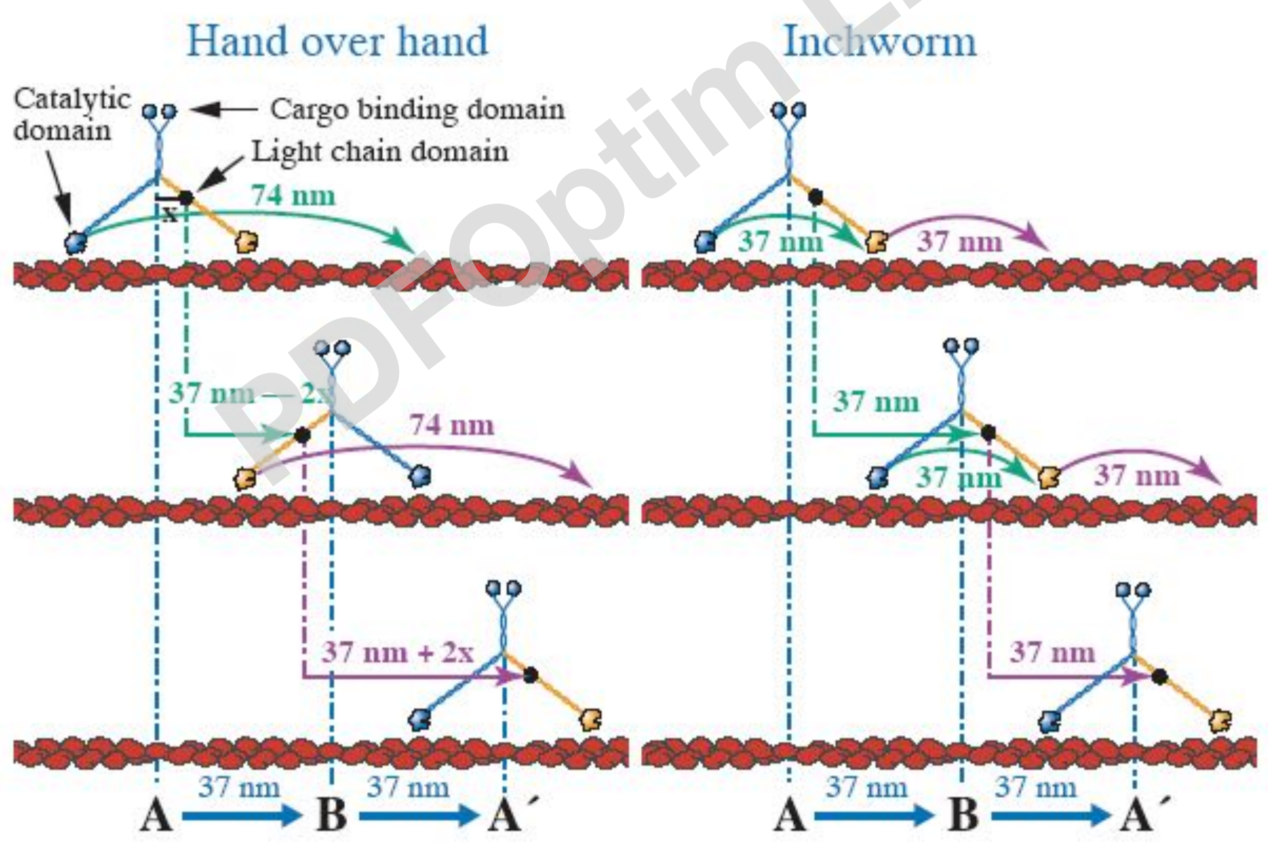
$$\left\langle (\Delta x)^2 \right\rangle \approx \frac{\lambda/2}{\sqrt{N}}$$

N. Bobroff, Rev. Sci. Inst. 57 (1986) 1152

PDFOptim Lite

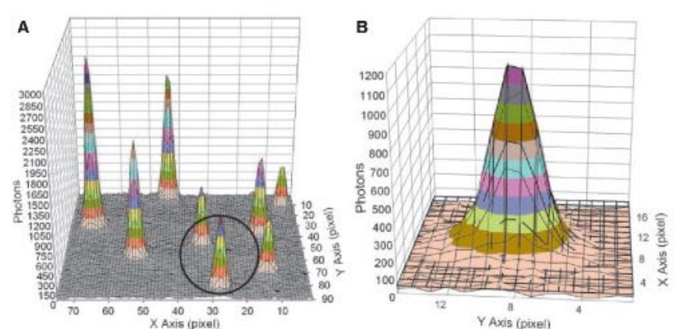
Myosin V Motility

- Cargo carrying processive molecular motor
- 37 nm center of mass steps along the actin filament at each ATP hydrolysis
- two heads held together by coiled-coil stalk



PDFOptim Lite

Myosin V Walks Hand-Over-Hand:



Single molecules detected with high S/N ratio
Pointing accuracy < 5nm

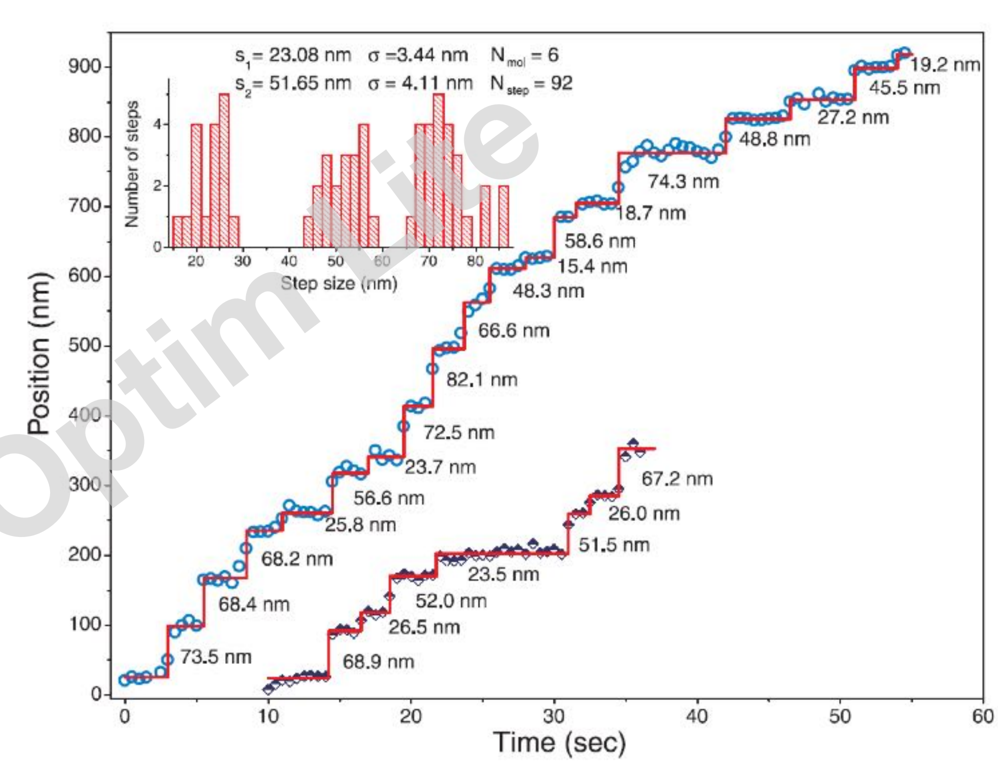


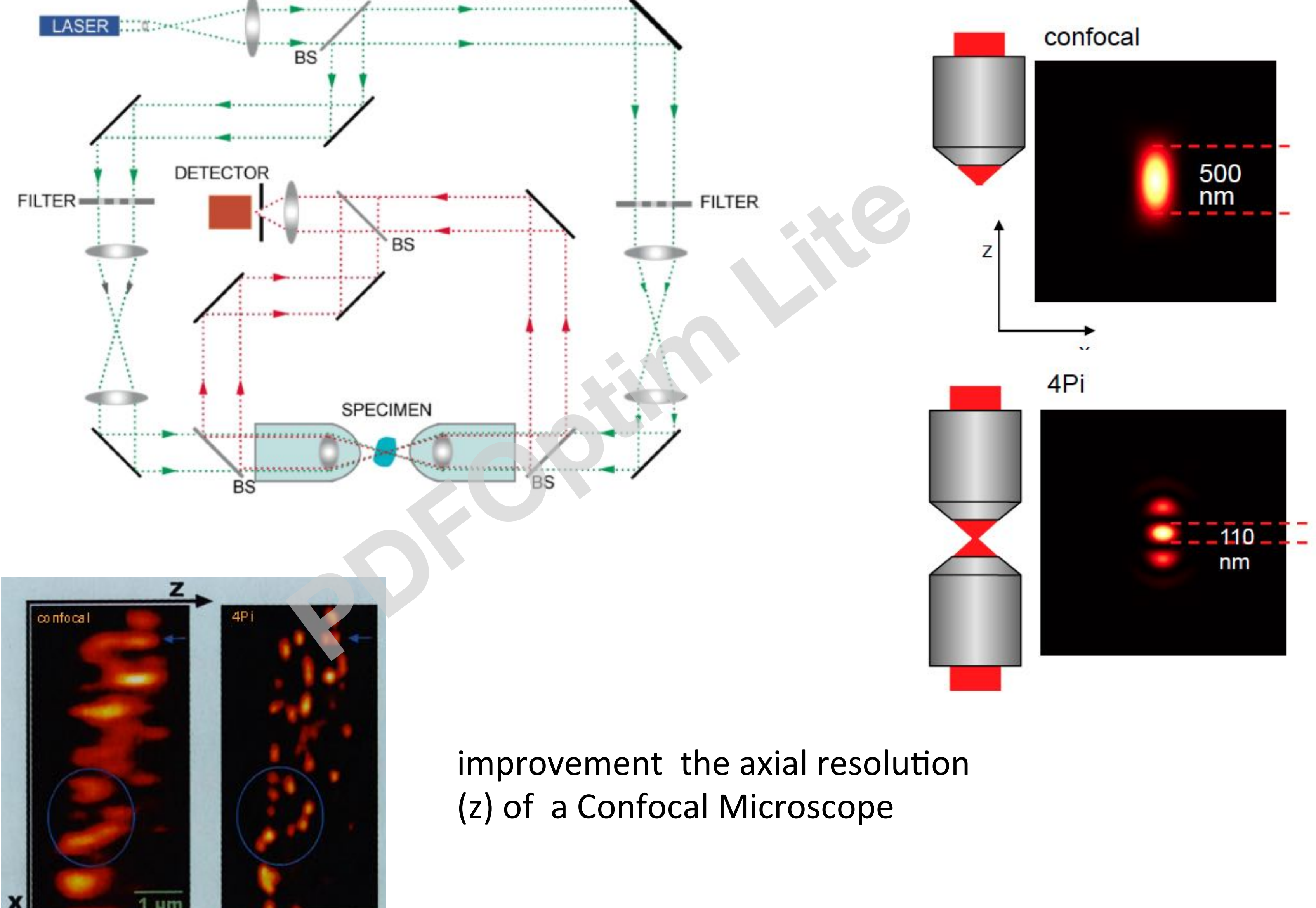
Fig. 4. Stepping traces of two different BR-labeled myosin V molecules displaying alternating 52-23 steps, and histogram of a total of six myosin V's taking 92 steps. Due to the 0.5-s time resolution of measurements, some steps are missed and yield 74-nm apparent steps, the sum of two steps. On the basis of the alternating step size, we infer that the dye is 7 nm from the center of mass along the direction of motion.

PDFOptimLite

Ahmet Yildiz,[1] Joseph N. Forkey,[3] Sean A. McKinney,[1,2]
Taekjip Ha,[1,2] Yale E. Goldman,[3] Paul R. Selvin[1,2]*

SCIENCE VOL 300 27 JUNE 2003

4 PI confocal microscopy



PDFOptim Lite

improvement the axial resolution (z) of a Confocal Microscope

Super-resolution microscopies

i.e. which provide images with resolution below the diffraction limit

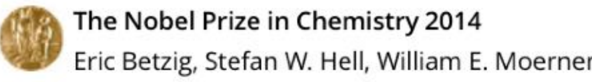
- revolutionized imaging capabilities offered by far-field optical microscopy in complex environments
- development mainly driven by the need for improved resolutions to study biological processes in live samples.
- optical resolutions down to the scale of individual biomolecules in order to give access to nanoscale molecular organizations

based on:
controlling fluorescence emission volumes to highly localized regions, using structured illumination schemes or stochastic detection of single emitters

PDFOptim Lite

Super-resolution microscopies

i.e. which provide images with resolution below the diffraction limit



Share this: 1.2K

The Nobel Prize in Chemistry 2014

Photo: Matt Staley/HHMI

Eric Betzig

Prize share: 1/3

© Bernd Schuller, Max-Planck-Institut

Stefan W. Hell

Prize share: 1/3

Photo: K. Lowder via Wikimedia Commons, CC-BY-SA-3.0

William E. Moerner

Prize share: 1/3

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

PDFOptim Lite

Super-resolution microscopies

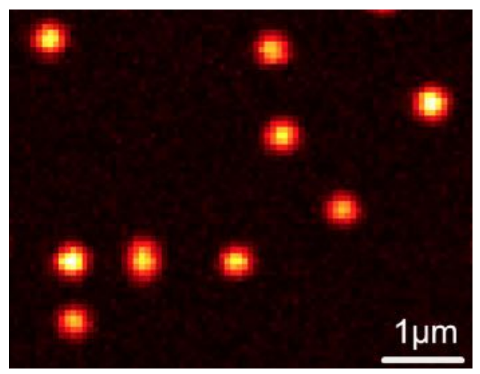
i.e. which provide images with resolution below the diffraction limit

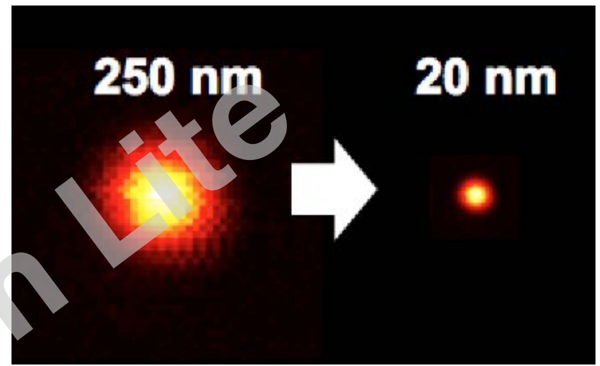
- **based on single molecule detection: PALM, STORM, uPAINT etc…**

- based on controlled fluorescence emission volumes (STED, RESOLFT…)

PDFOptim Lite

Single molecule detection allows localizing isolated emitters with nanometer resolutions





Every single molecule detection corresponds to a diffraction limited spot

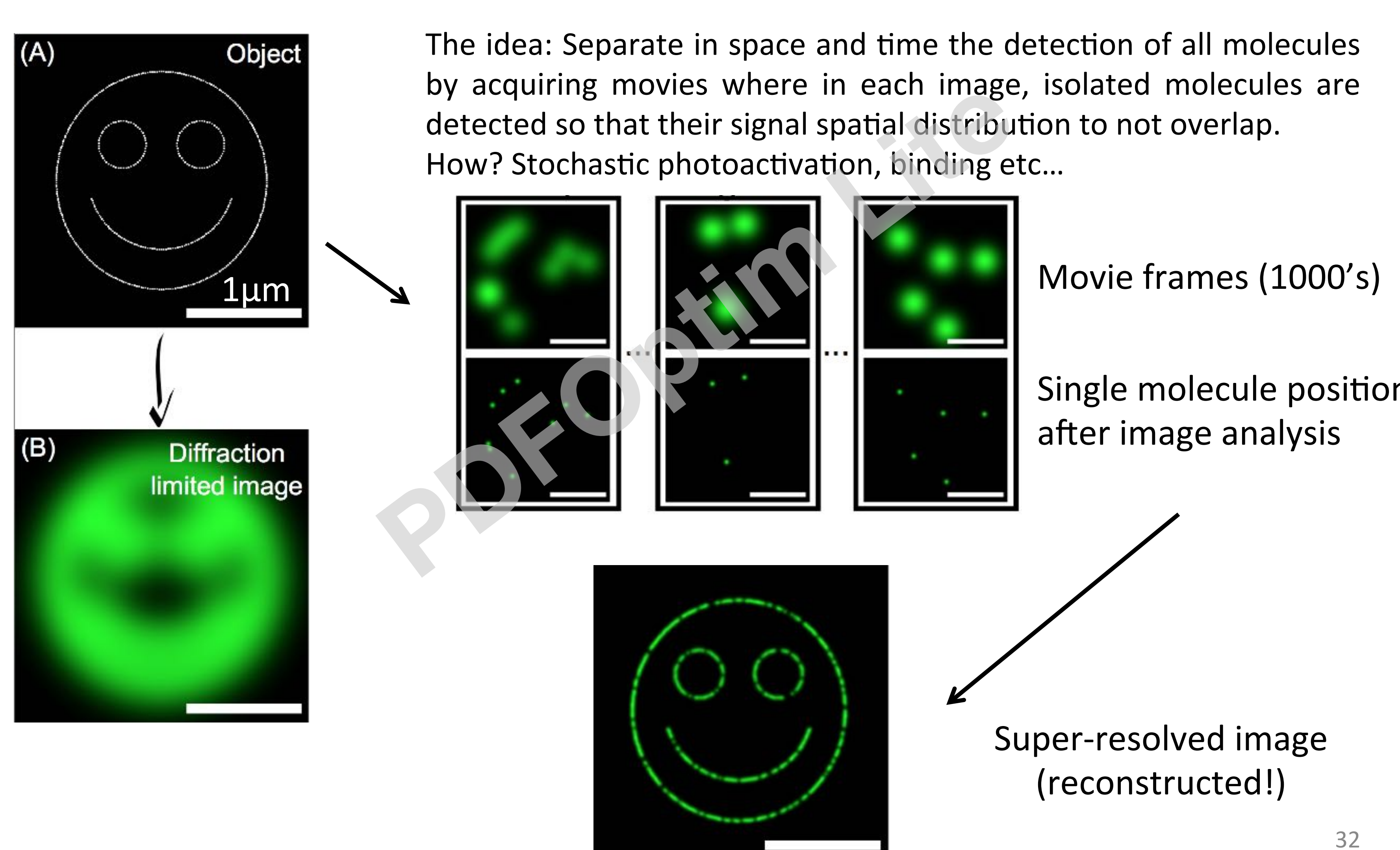
With the pre-knowledge that each spot corresponds to a single molecule, determination of the center of each spot by 2D curve fitting provides the position of the emitting molecule with nanometer resolution (limited by signal to noise ratio).

Requirement : molecules are isolated so that their signal spatial distribution do not overlap

PDFOptim Lite

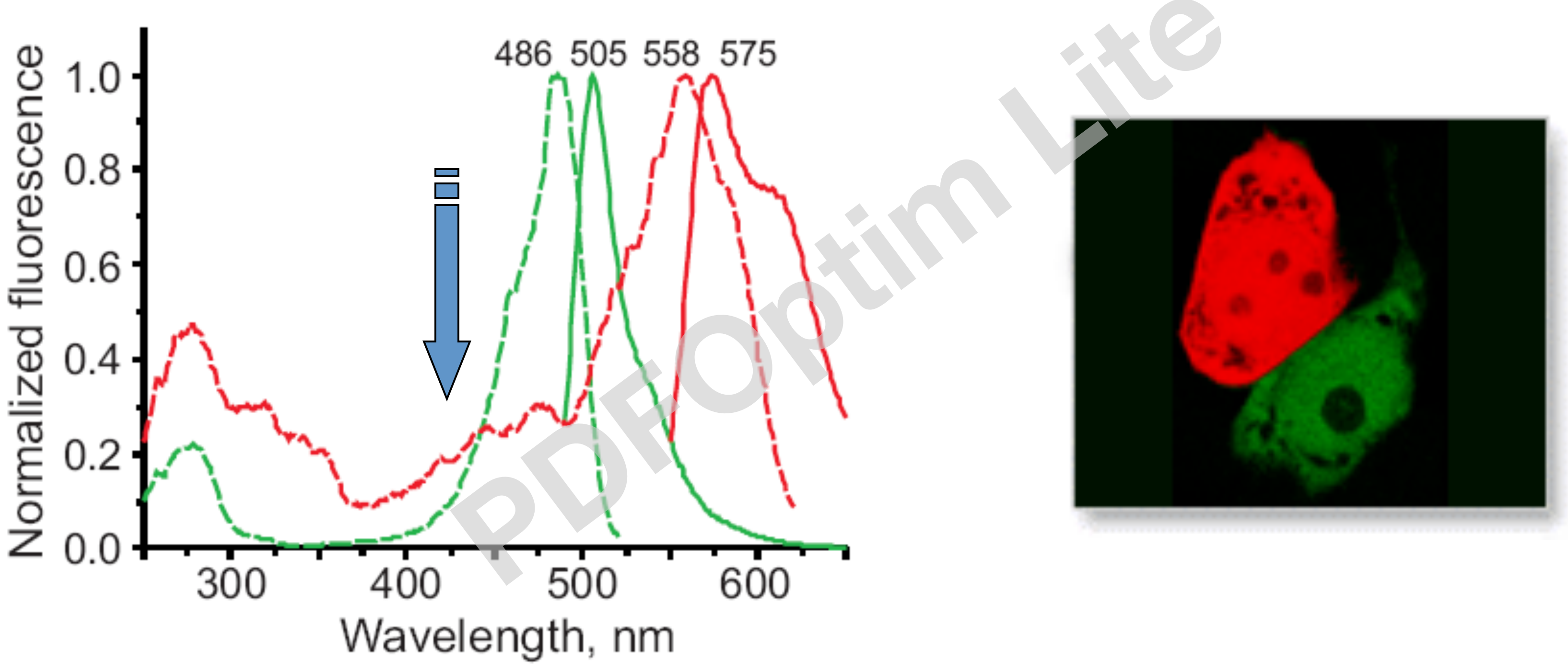
Super-resolution imaging methods based on single molecule detection (PALM, STORM, uPAINT …)

The idea: Separate in space and time the detection of all molecules by acquiring movies where in each image, isolated molecules are detected so that their signal spatial distribution to not overlap.
How? Stochastic photoactivation, binding etc…



PDFOptim Lite

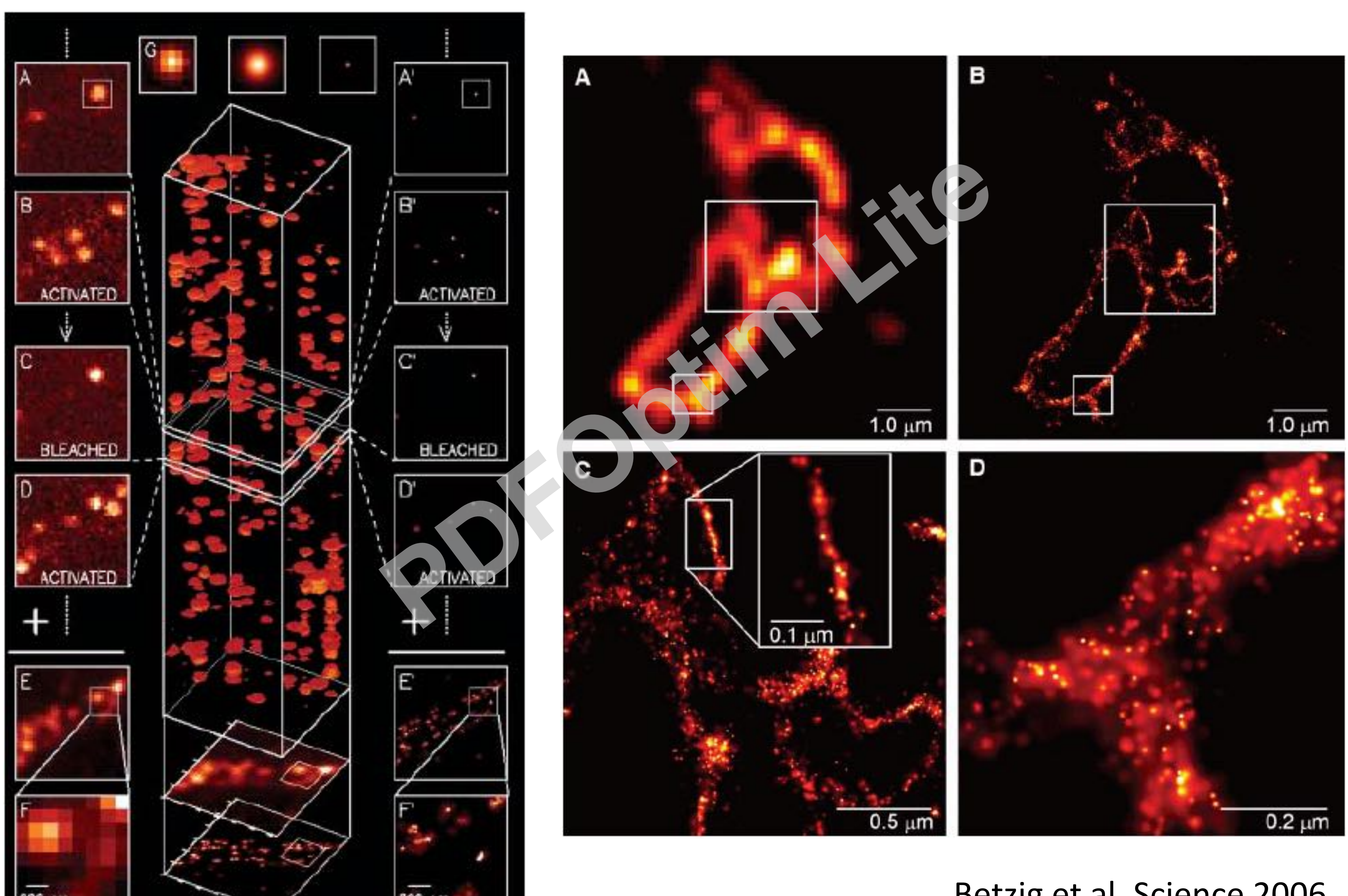
Photo-switchable Fluorescent Protein



PDFOptim Lite

Gurskaya NG et al. 2006 *Nat. Biotechnol.*

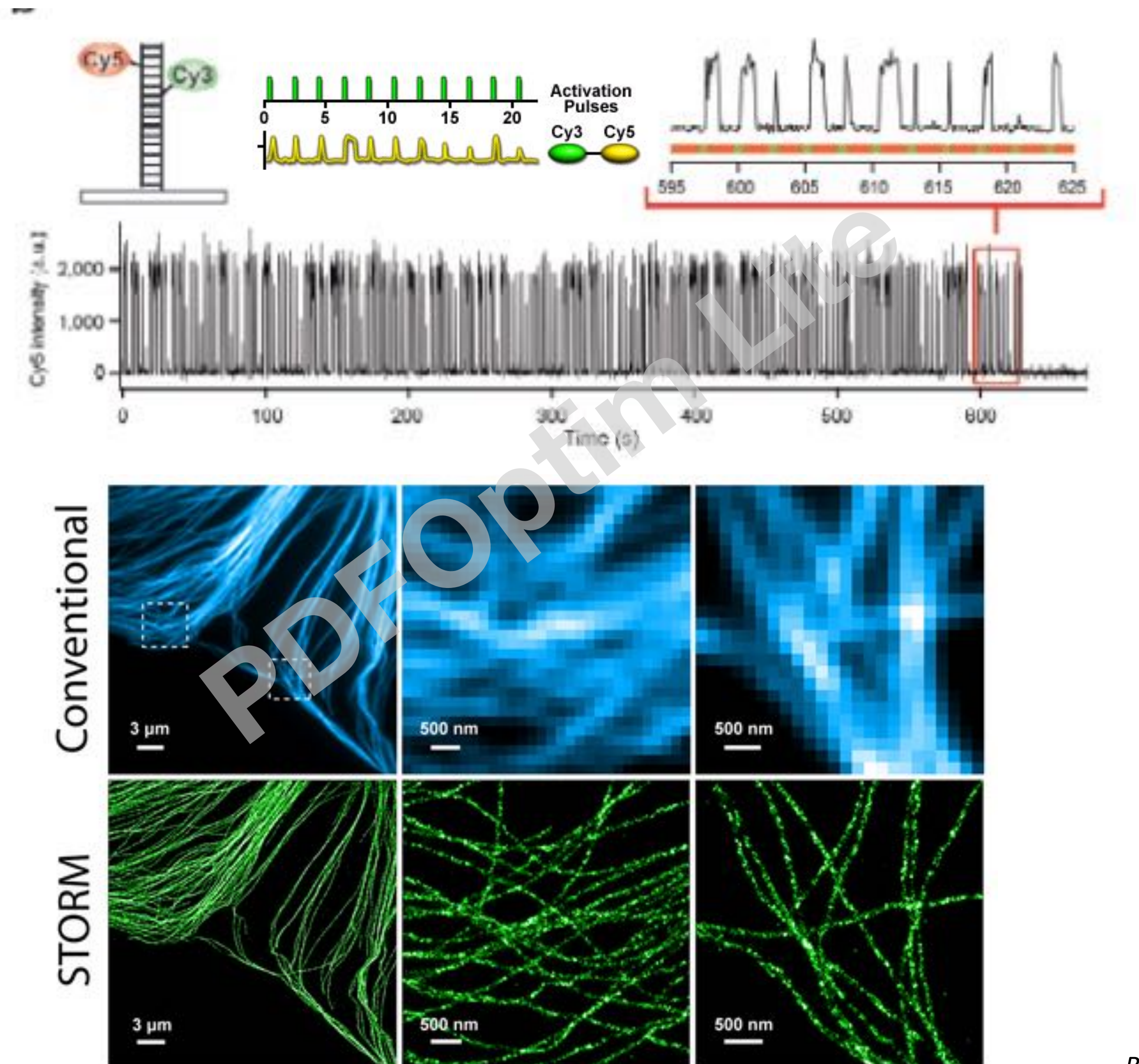
PhotoActivable Light Microscopy (PALM)



PDFOptim Lite

Betzig et al, Science 2006

Stochastic optical reconstruction microscopy (STORM)



Rust 2006 Nat Meth

PDFOptim Lite

Table 1 Photoswitchable fluorophores used in super-resolution fluorescence microscopy

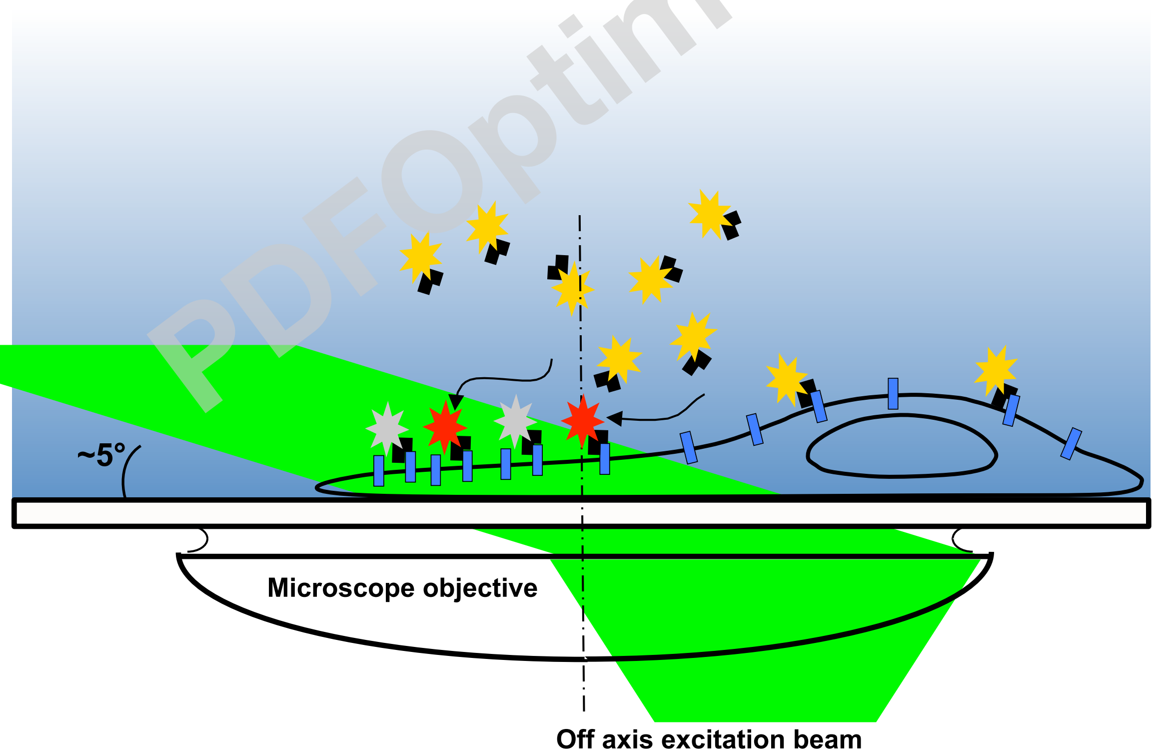
Fluorophore		Activation wavelength (nm)	Before activation		After activation		Reversible
			Ex[a] (nm)	Em (nm)	Ex (nm)	Em (nm)	
Cyan/dark-to-green FP	PA-GFP	405	400	517	504	517	No
	PS-CFP2		400	468	490	511	
Green-to-red FP	Kaede	405	508	518	572	582	No
	EosFP	405	505	516	569	581	
	Dendra2	405–488	490	507	553	573	
Dark-to-red FP	PAmCherry	405	NF		564	595	No
Reversible FP	Dronpa	405	NF		503	518	Yes
	Dronpa2				486	513	
	Dronpa3				487	514	
	rsFastLime				496	518	
	bsDronpa				460	504	
	EYFP	405	NF		513	527	
Caged dyes	Caged fluorescein	<405	NF		497	516	No
	Caged Q-rhodamine[d]				545	575	
Cyanine dyes	Cy5 & Alexa 647	350–570[c]	NF		647	665	Yes
	Cy5.5				674	692	
	Cy7				746	773	
Photochromic rhodamine	SRA545	375	NF		Green	545	Yes[f]
	SRA552					552	
	SRA577					577	
	SRA617					617	

PDFOptim Lite

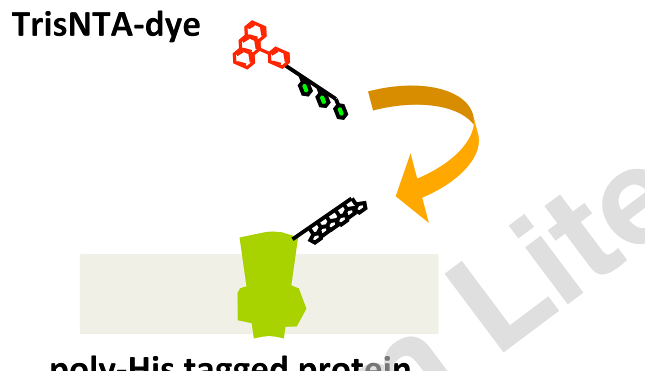
universal Point Accumulation Imaging in the Nanoscale Topography (uPAINT)

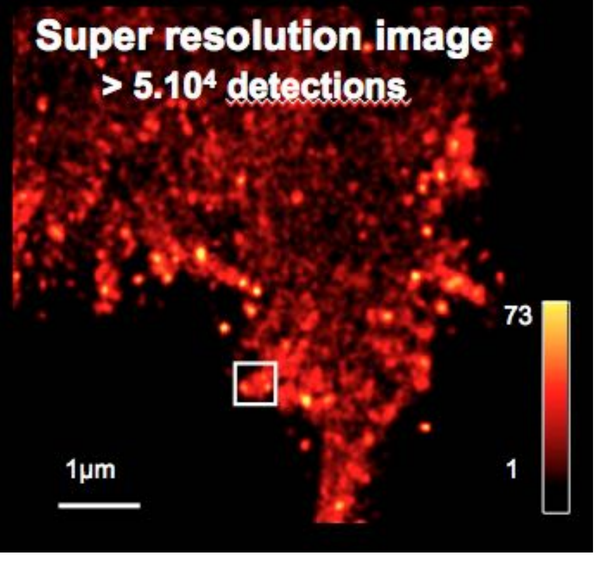
- do not need stochastic photoswitching of emitters
- captures real-time molecular interactions to control the density of fluorescent emitters
- can use any binding entity conjugated to fluorophores having high specificity toward a target molecule (*e.g.* ligand, antibody)
- allows to image and track endogenous receptors at high
- can combining single molecule FRET and dual-color uPAINT allow the specific super-resolution imaging and tracking of interacting receptors activated by their cognate ligand in live cells

PDFOptim Lite



uPAINT Example : Unprocessed real time uPAINT images
Model transmembrane protein on fibroblasts (Atto dyes)

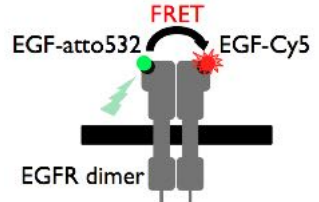




Unprocessed real time uPAINT images

PDFOptim Lite

uPAINT : Super-resolution imaging of endogenous EGFR dimers in live cells by smFRET



Donor

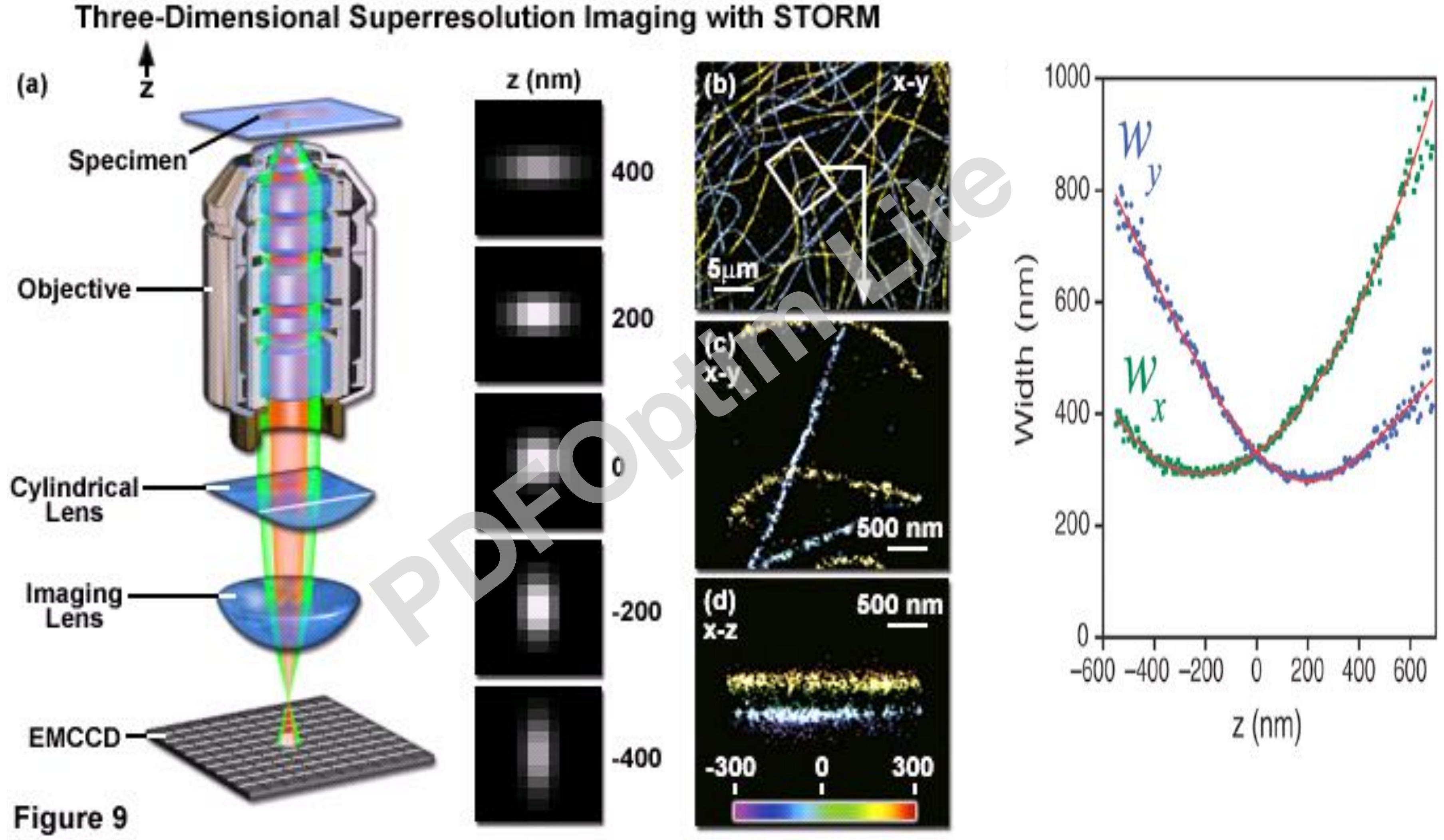
500
0
5 µm
1 µm
1 µm

Acceptor (EGFR dimers)

500
0
5 µm
1 µm
1 µm

PDFOptim Lite

Super-resolution en 3D (PALM / STORM / uPAINT etc…)



Super-resolution microscopies

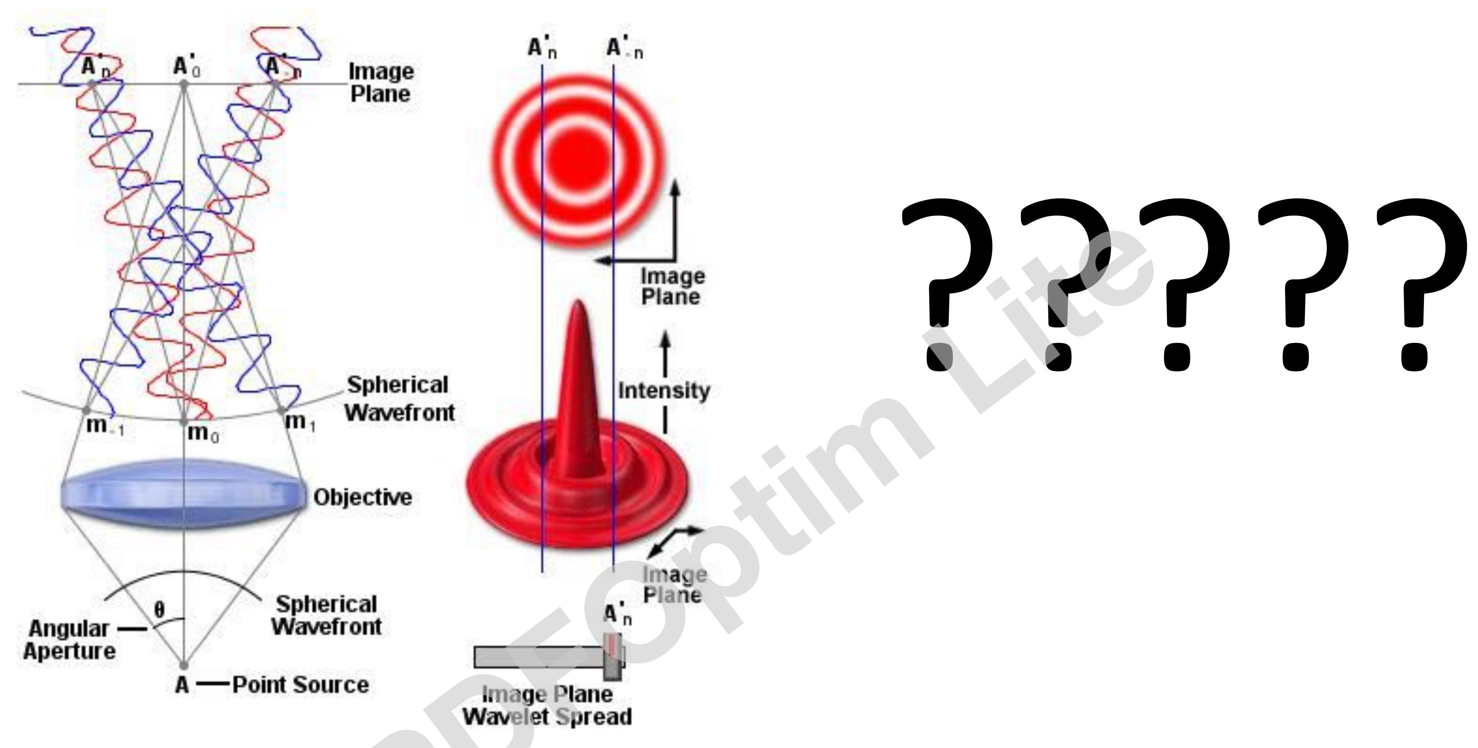
i.e. which provide images with resolution below the diffraction limit

- based on single molecule detection: PALM, STORM, uPAINT etc…

- **based on controlled fluorescence emission volumes (STED, RESOLFT…)**

PDFOptim Lite

”confocal microscopy” beyond diffraction limit



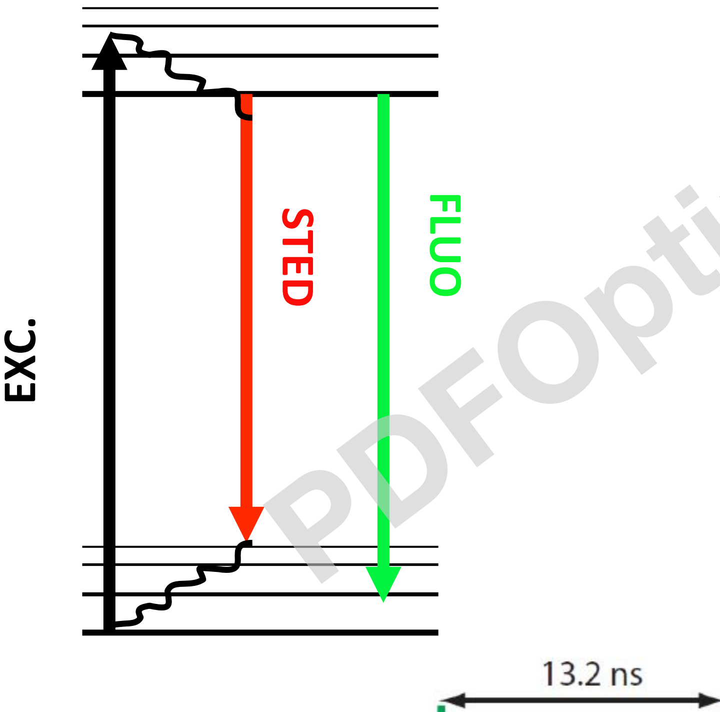
PDFOptim Lite

Diffraction limit – distribution of light intensity

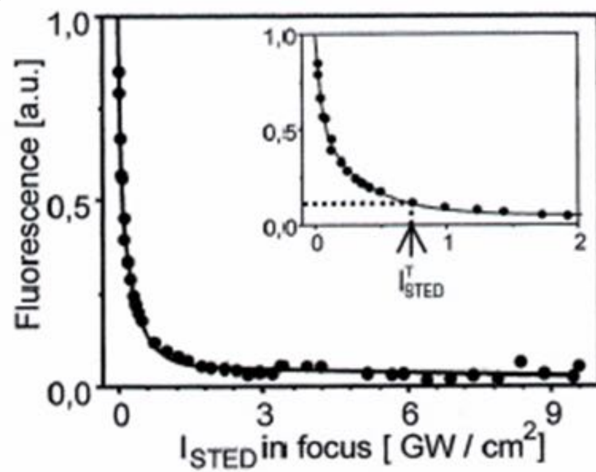
However, if the process is nonlinear function of intensity, then the localization <u>is not limited by the wavelength</u>

STimulated Emission Depletion microscopy (STED)

Stimulated Emission

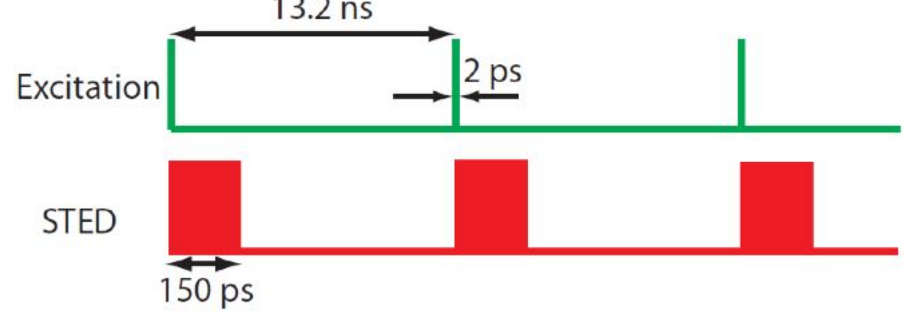


Experimental proof of the non-linear dependence of the excited state population with depletion beam intensity



Exc: 561 nm, 2 ps

Dep: 760 nm, 150 ps



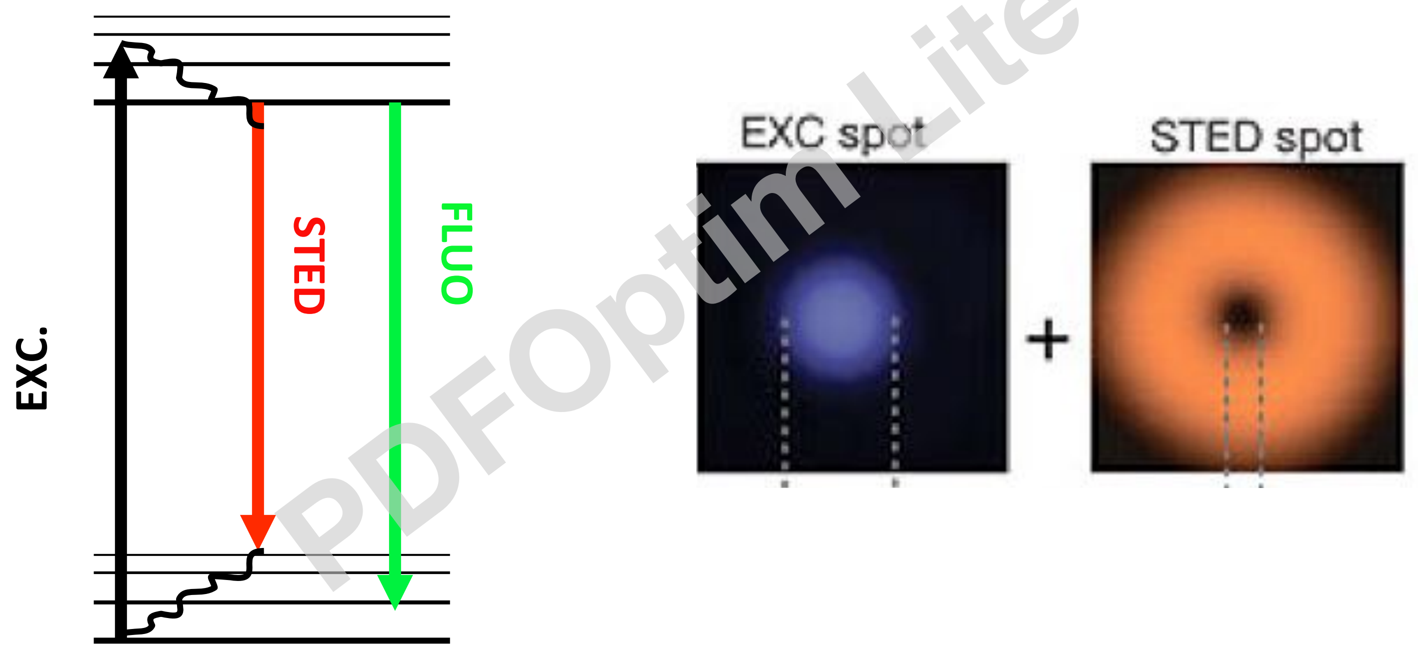
Hell, 1994

PDFOptim Lite

STimulated Emission Depletion microscopy (STED)

Stimulated Emission

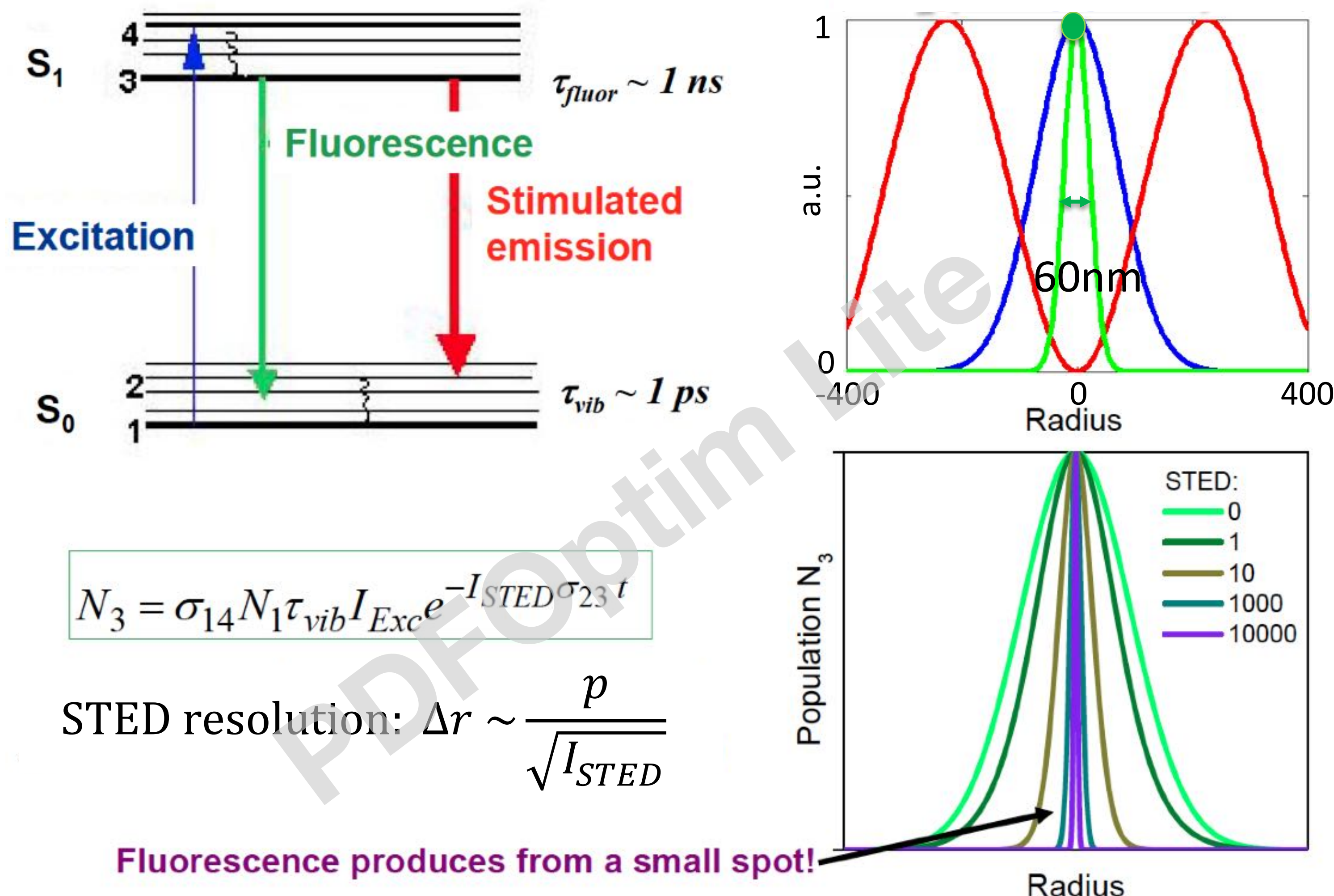
Structured illumination



PDFOptim Lite

Hell, 1994

Reduction of the fluorescence emission volume

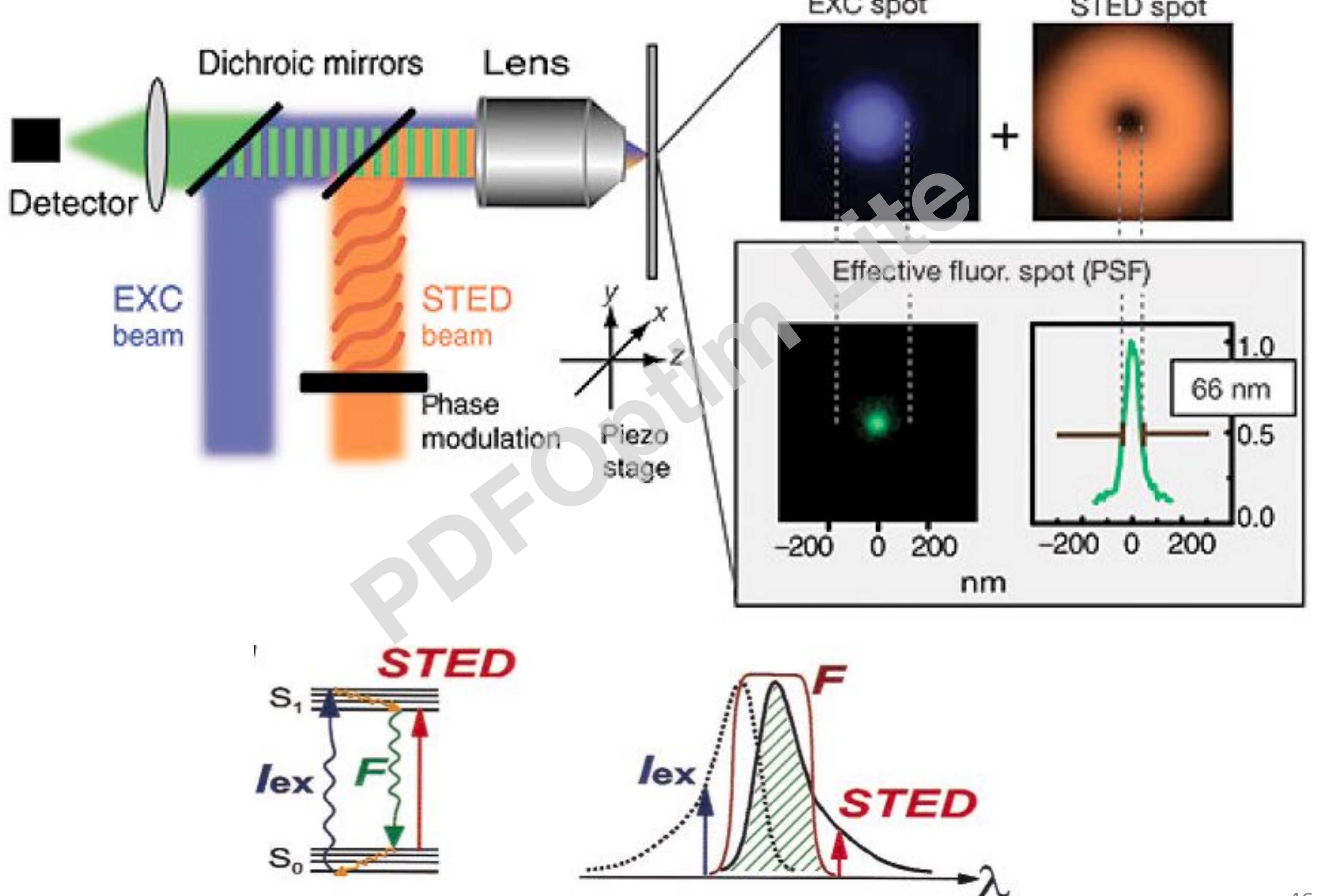


$$N_3 = \sigma_{14} N_1 \tau_{vib} I_{Exc} e^{-I_{STED}\sigma_{23} t}$$

STED resolution: $\Delta r \sim \dfrac{p}{\sqrt{I_{STED}}}$

In practice , one generally uses a depletion beam with a doughnut shape to deplete molecules excited around the center of the excitation beam

Experimental setup

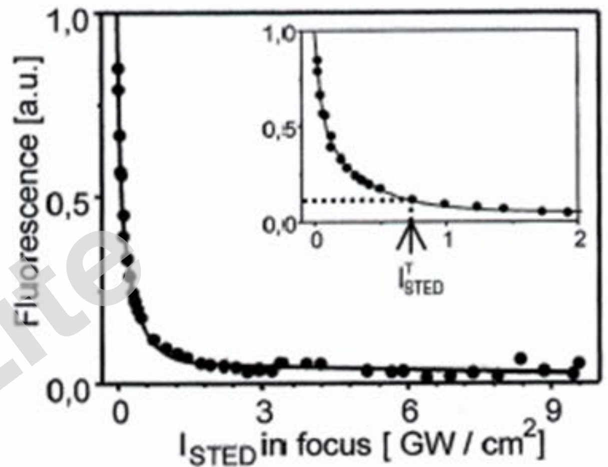


PDFOptim Lite

Illustrations

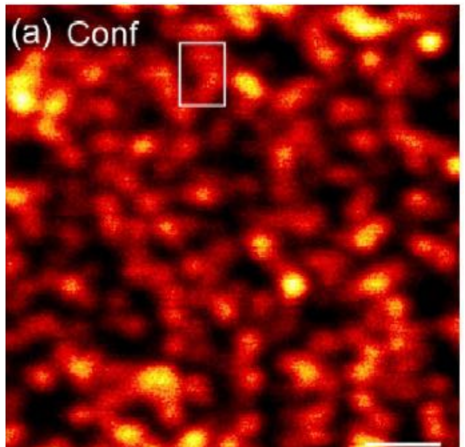
Experimental proof of the non-linear dependence of the excited state population with depletion beam intensity

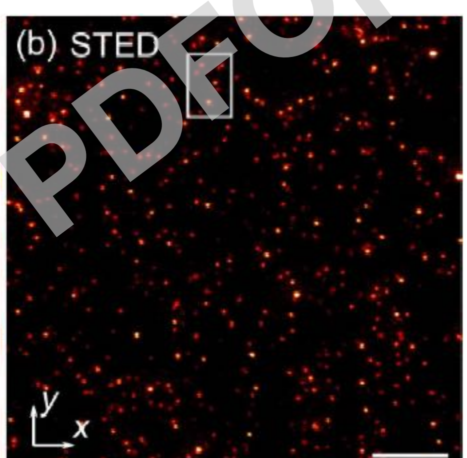
$$N_3 = \sigma_{14} N_1 \tau_{vib} I_{Exc} e^{-I_{STED}\sigma_{23}\, t}$$

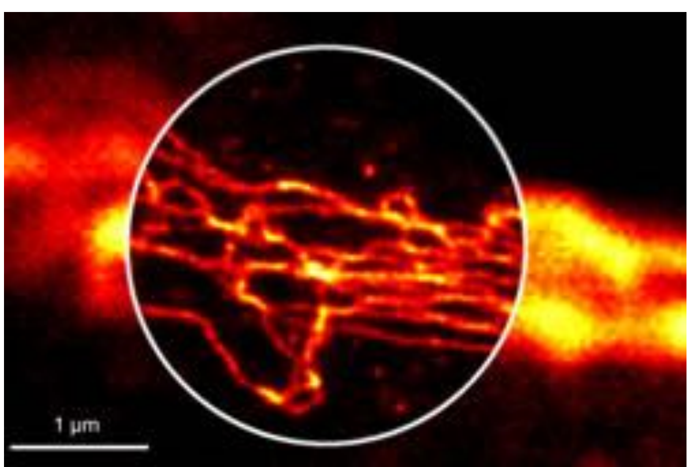


The quality of the "zero" in the depletion beam is crucial

PDFOptim Lite

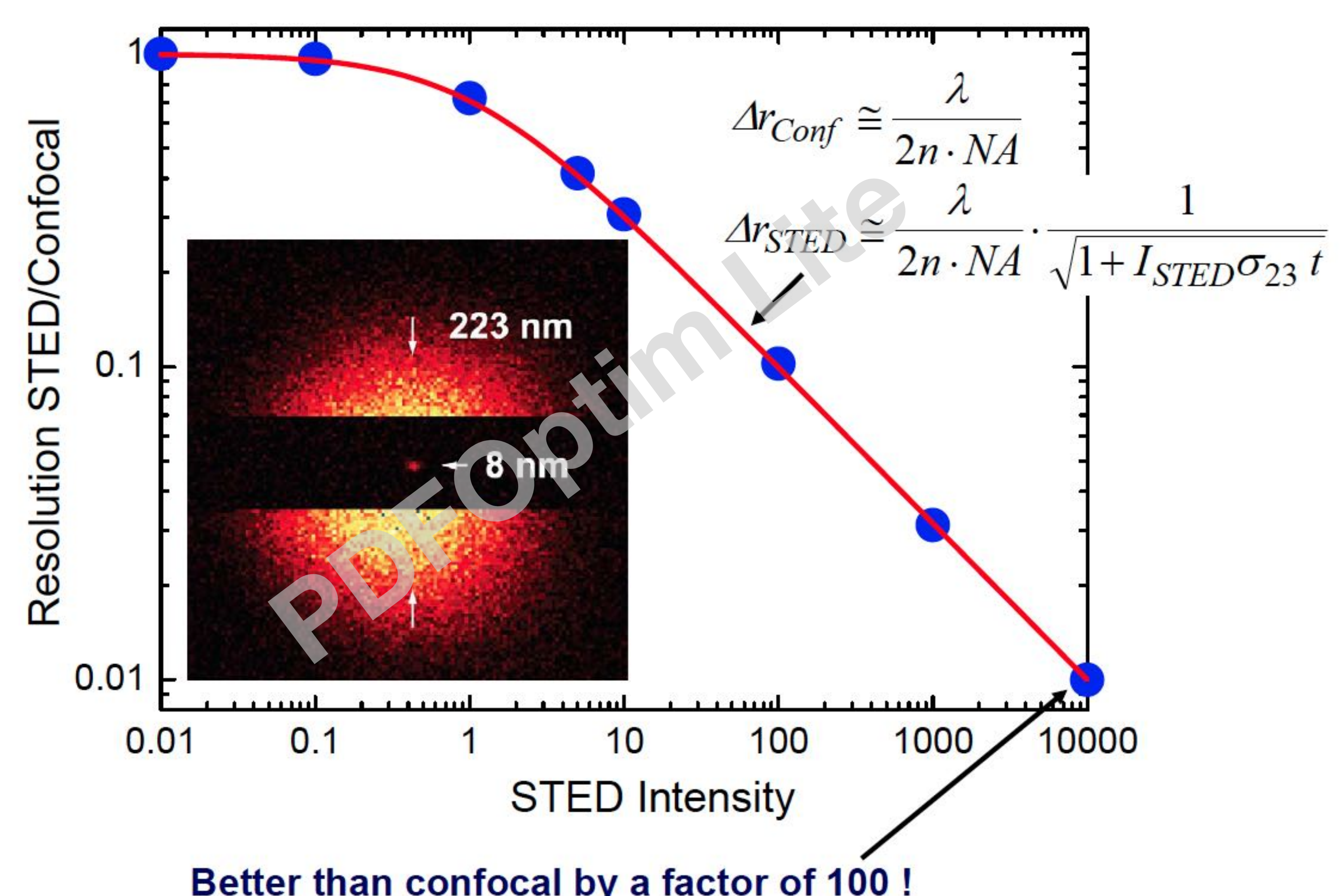






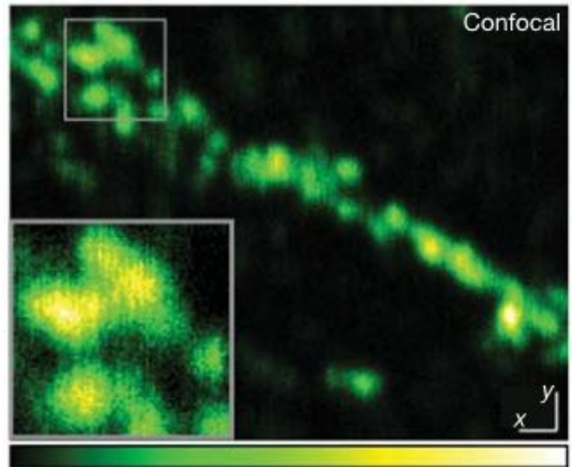
Images of 24nm fluorescent beads and of neuron form S. Hell Group

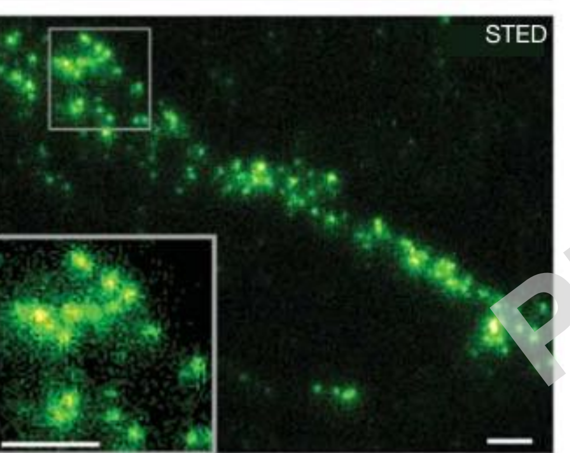
Résolution du STED



PDFOptim Lite

STED Nanoscopy



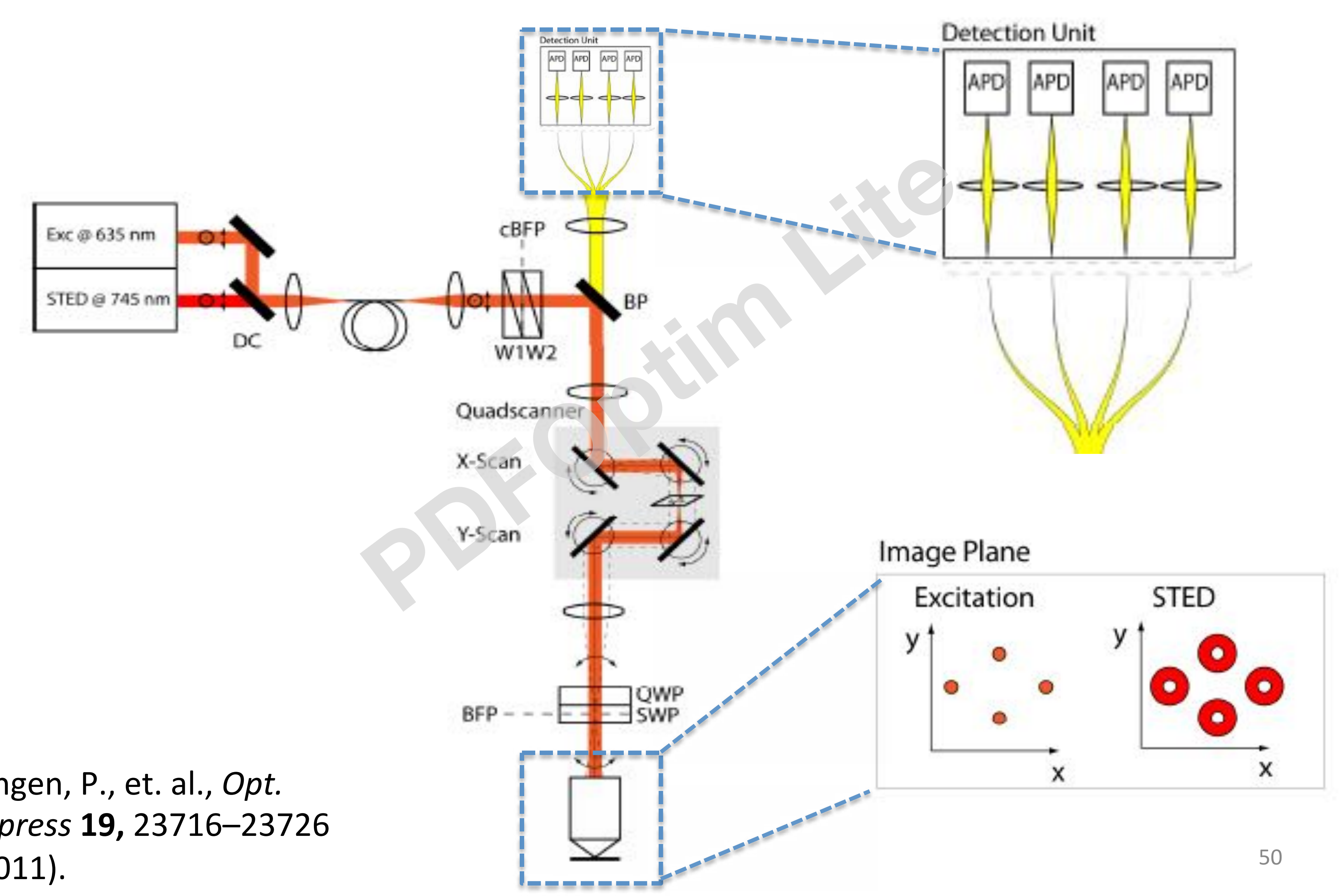


- STED nanoscopy techniques are based on single point-scanning
- Gain in spatial resolution requires dense pixelation and hence long recording times
- This constitutes a drawback for fast wide field imaging
- need parallelization

PDFOptim Lite

Willig, K. I., et al. *Nature* **440,** 935–939 (2006).

Multi-doughnut STED microscopy



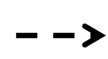
PDFOptim Lite

Bingen, P., et. al., *Opt. Express* **19,** 23716–23726 (2011).

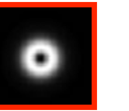
STED Parallelization

Multi doughnuts configuration:

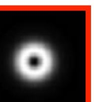
- **Depletion power limit**
- **Experimentally Complicated**



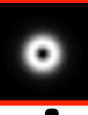
APD



APD



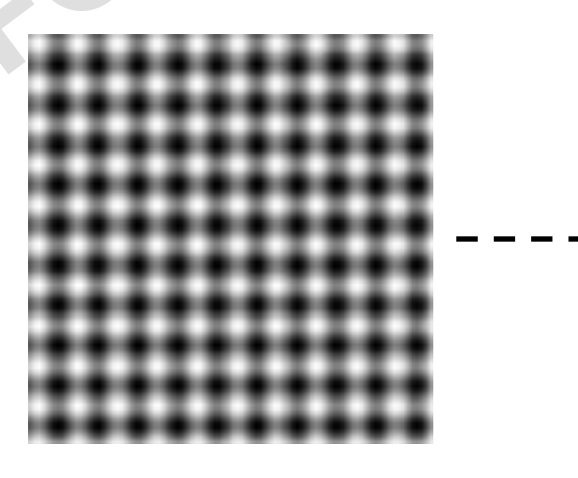
APD



APD

Large STED parallelization:

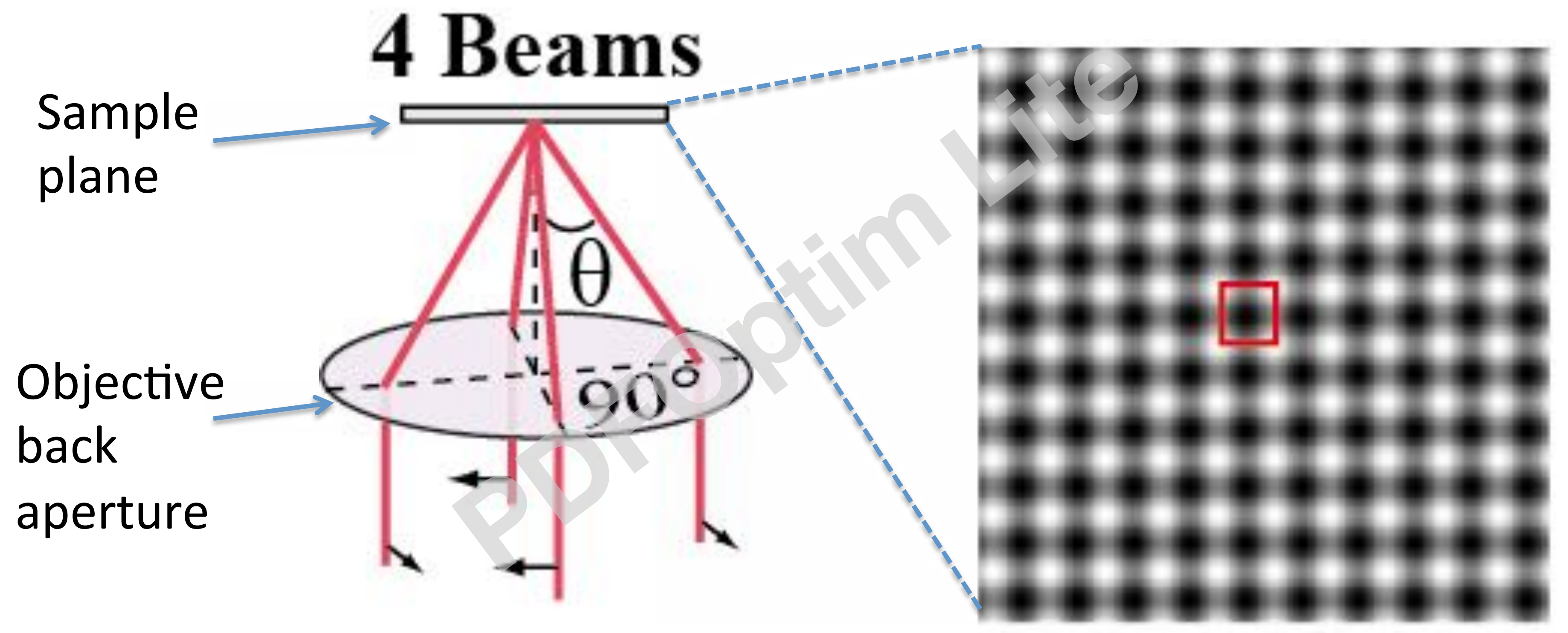
- **wide-field excitation**
- **optical lattices for depletion**
- **A fast camera for detection**



2D detector

PDFOptim Lite

Lattice-STED microscopy

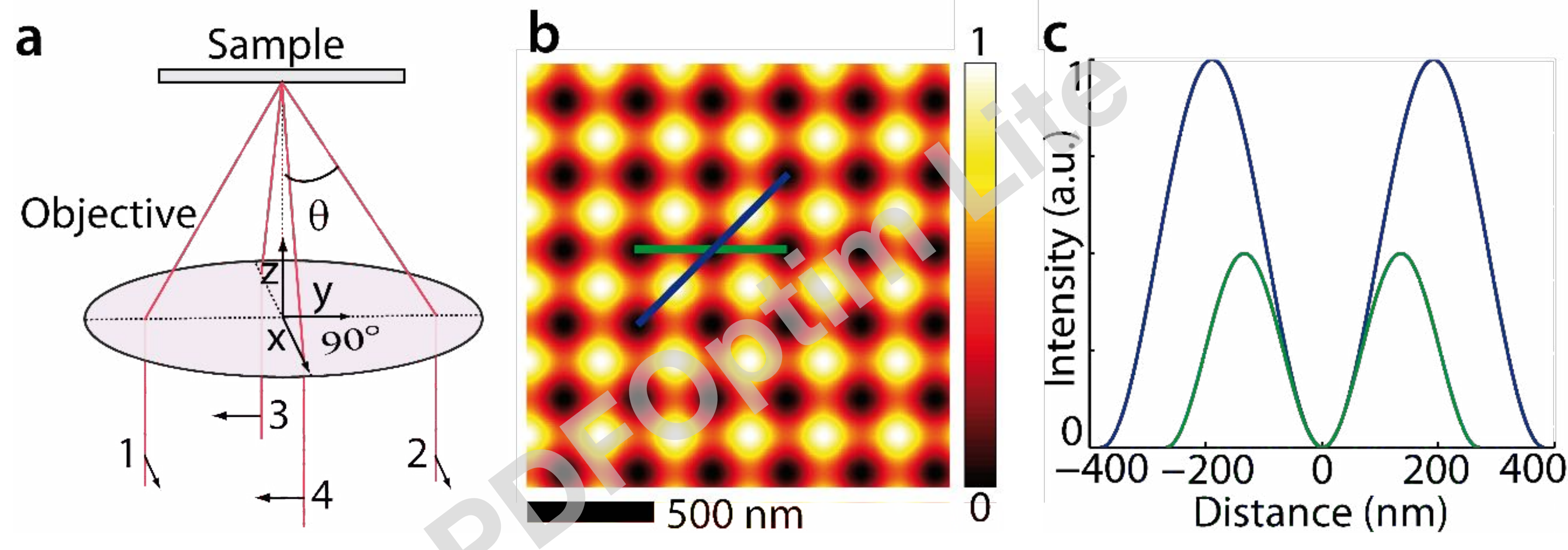


PDFOptim Lite

Optical lattices

Period: $p = \frac{\lambda}{2n\,sin\theta}$

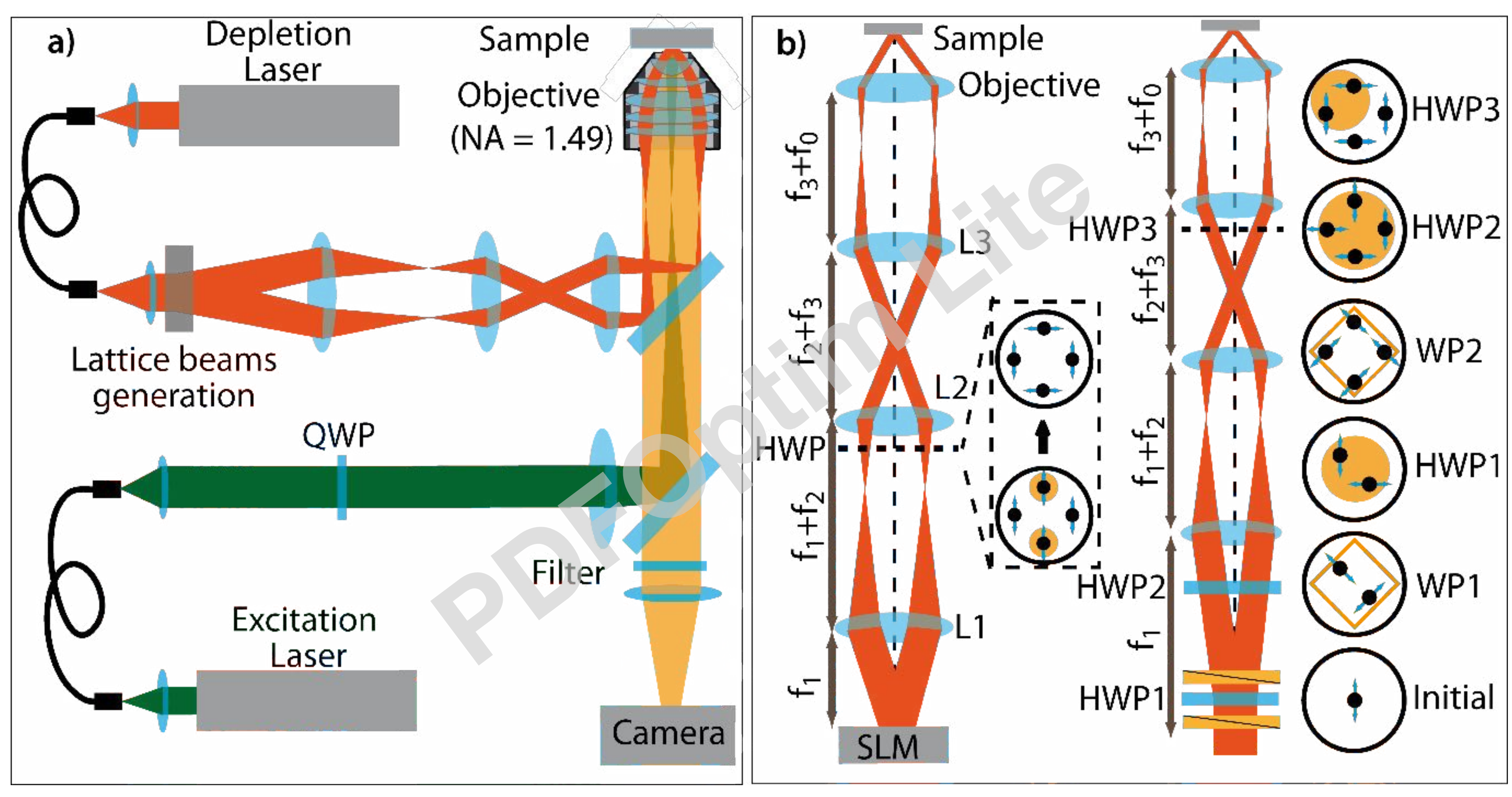
Lattice-STED microscopy



PDFOptim Lite

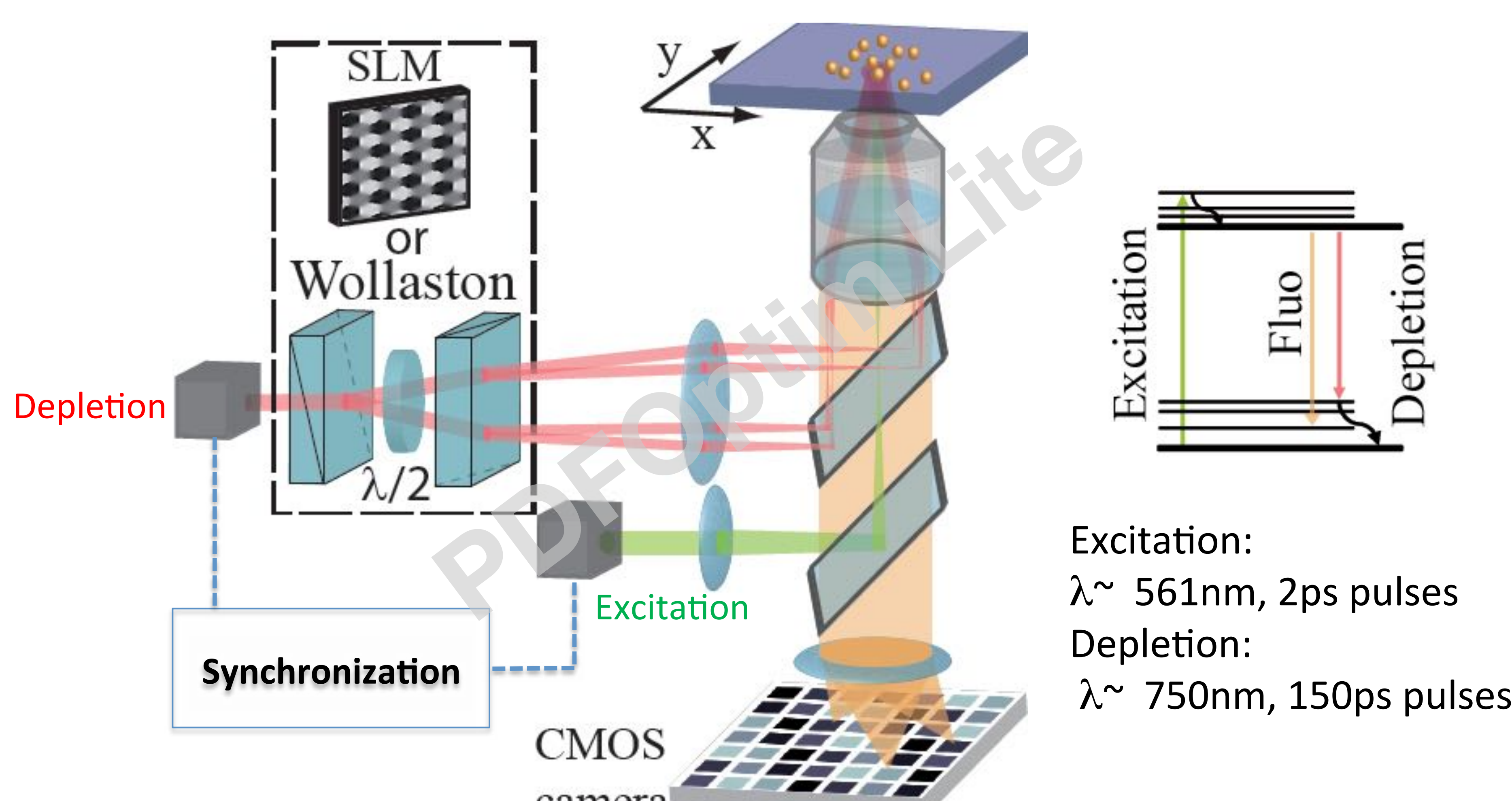
Period: $p = \frac{\lambda}{2n\, sin\theta}$

Lattice-STED nanoscopy Setup



Excitation: λ~ 561nm, 2ps pulses
Depletion: λ~ 750nm, 150ps pulses

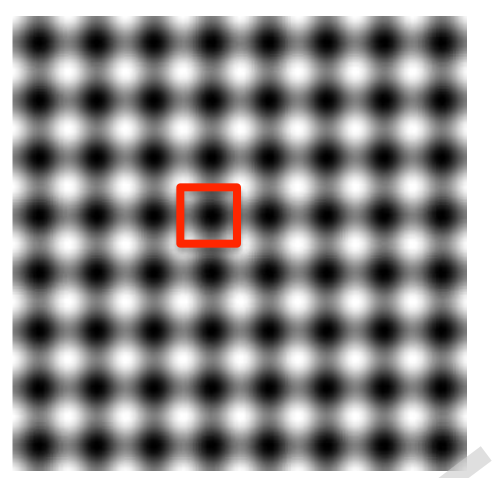
In-STED nanoscopy Setup

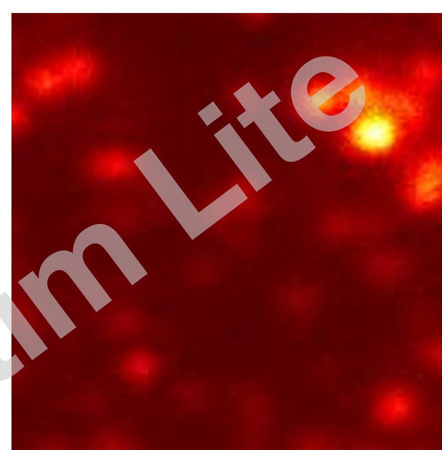


PDFOptim Lite

Lattice-STED Images acquisition

Depletion pattern

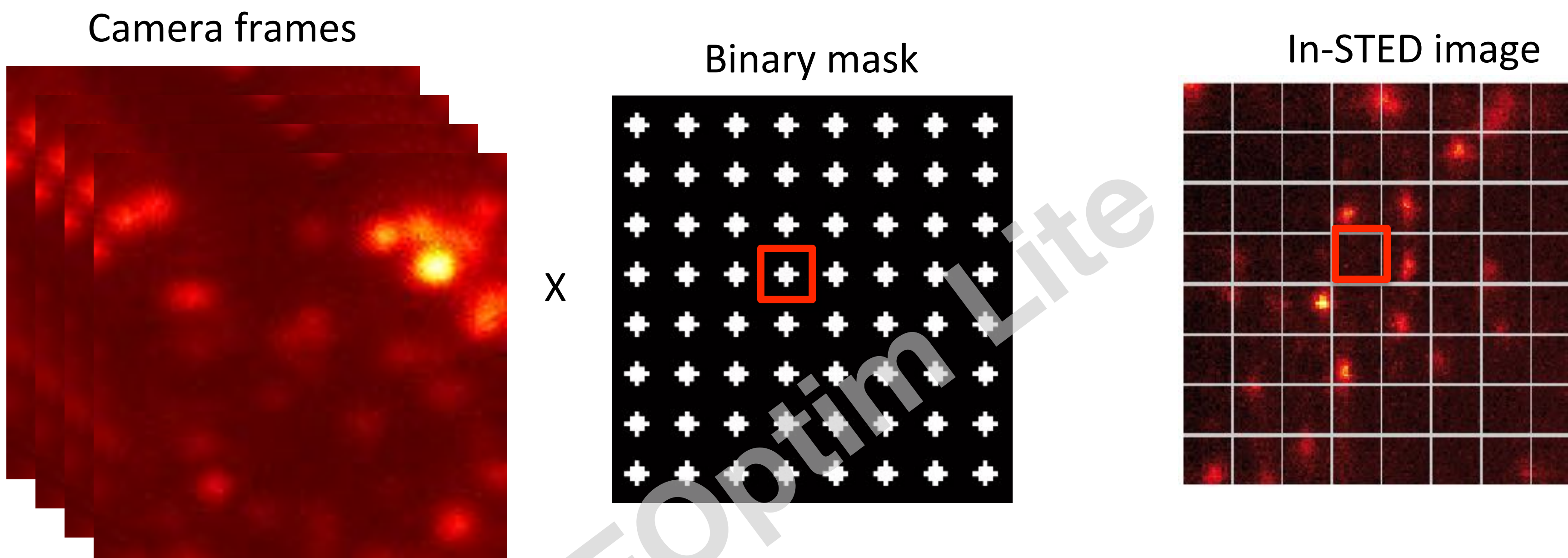




PDFOptim Lite

- **scan the sample over a unit cell** in the presence of the wide field excitation and the depletion pattern, while acquiring a fluorescence image for each scanning step
- A stack of camera frames is produced

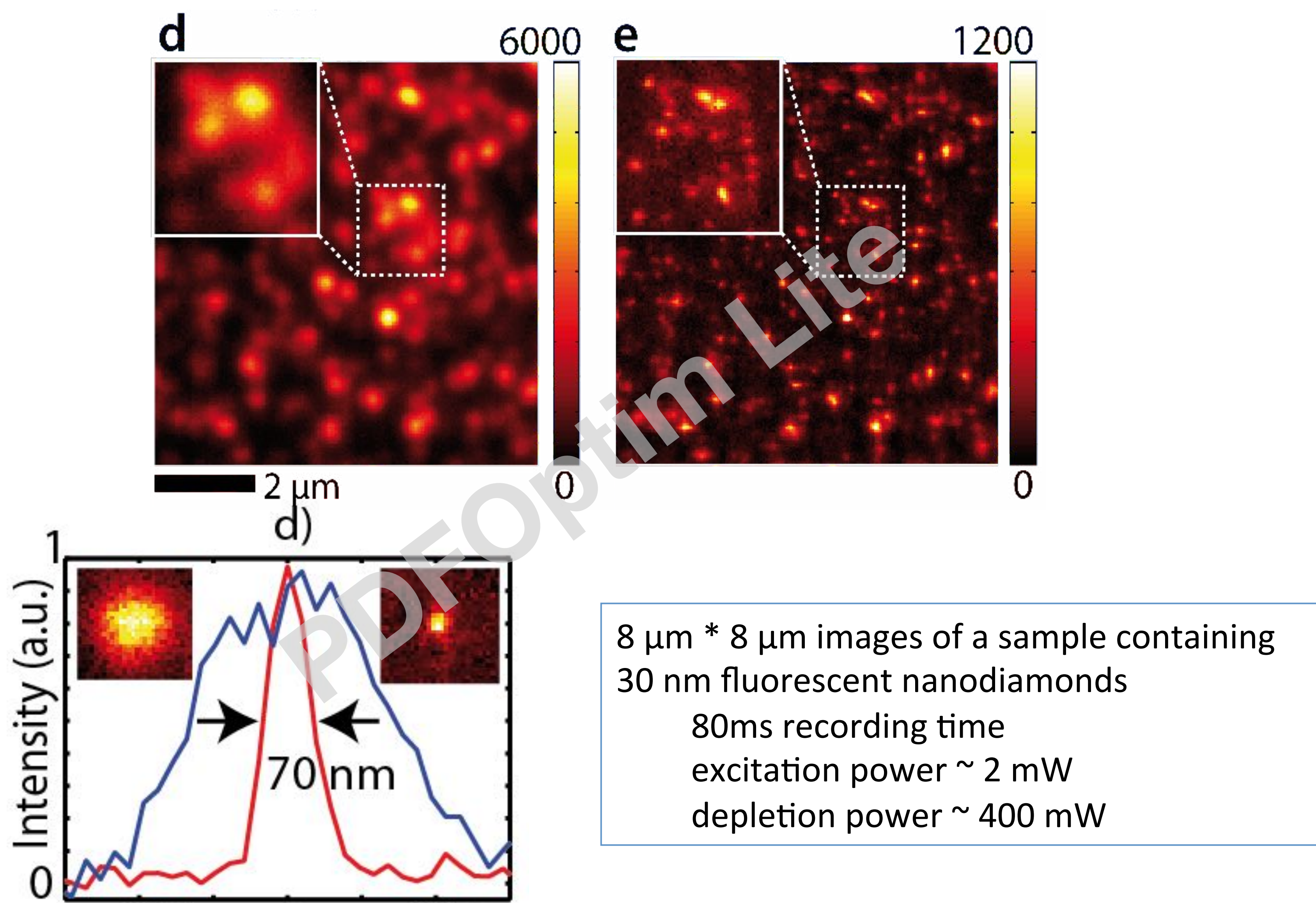
Lattice-STED Images acquisition



PDFOptim Lite

- Overlay a binary mask on the images, the transparent parts corresponding to the minima positions of the depletion pattern.
- CMOS camera + digital mask → Array of parallelized "point detectors" recording an image of the size of the lattice unit cell.
- The In-STED image obtained by assembling all the unit cell images together.

Lattice-STED images



PDFOptim Lite

8 μm * 8 μm images of a sample containing 30 nm fluorescent nanodiamonds

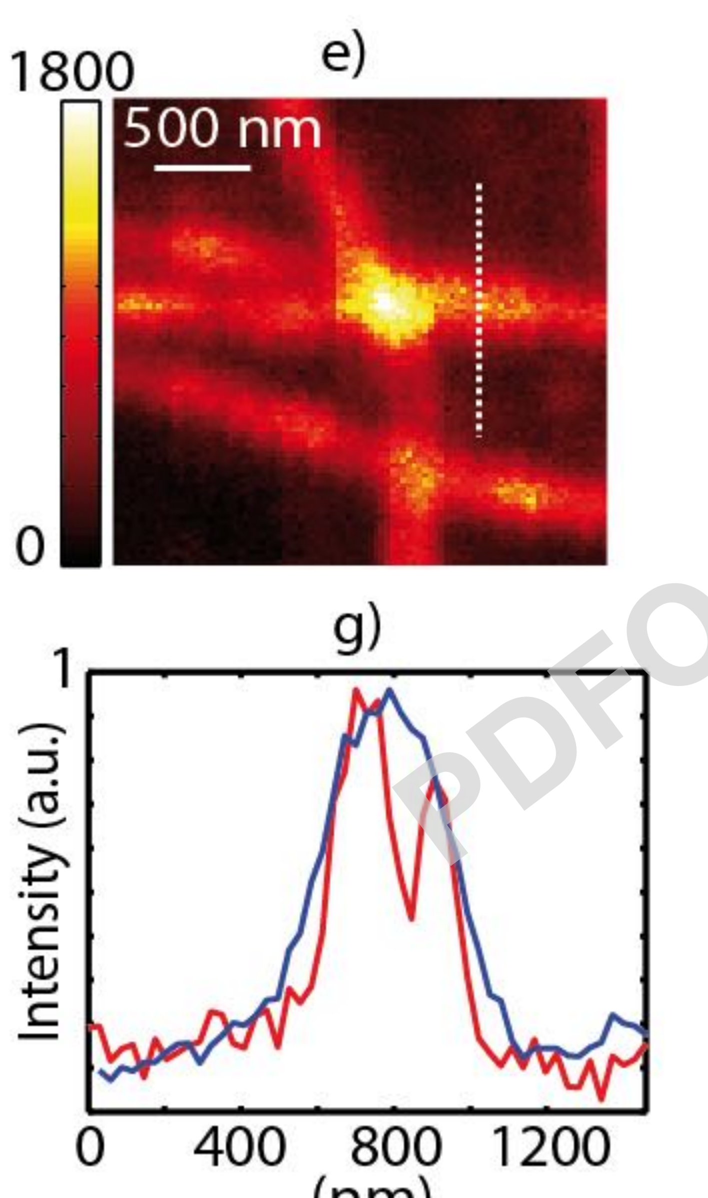
80ms recording time

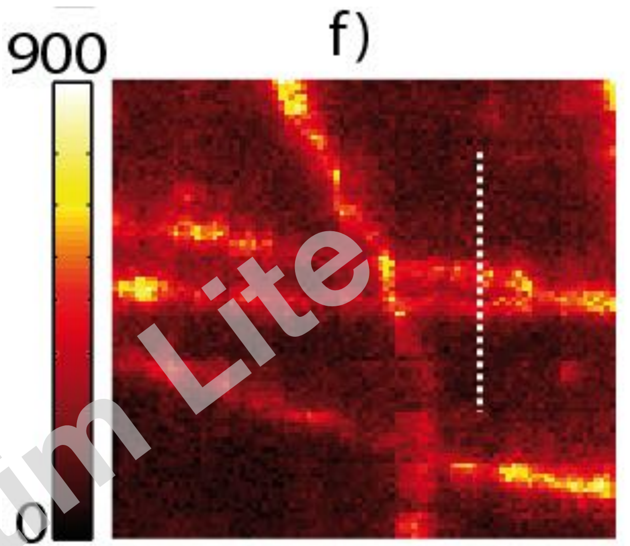
excitation power ~ 2 mW

depletion power ~ 400 mW

Yang et al. **Optics Express** (2014)

Lattice-STED images





PDFOptim Lite

Imaging of microtubules in a fixed cell

- 3 µm * 3 µm field of view
- 80ms recording time
- excitation power ~ 2 mW
- depletion power ~ 400 mW

Cryogenic super-resolution microscopy

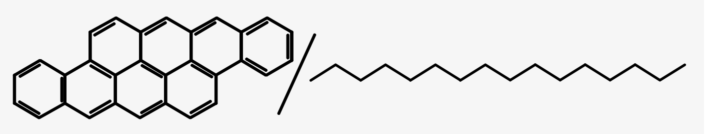
by Excited State Saturation (ESSat)

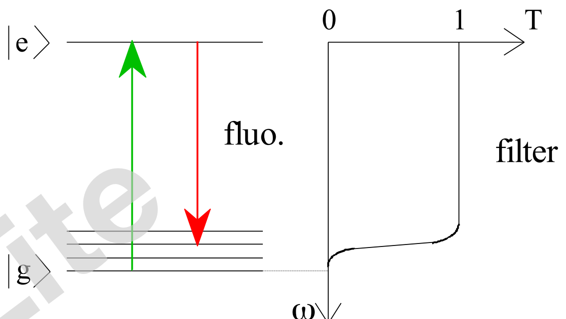
Yang et al. (2015)

PDFOptim Lite

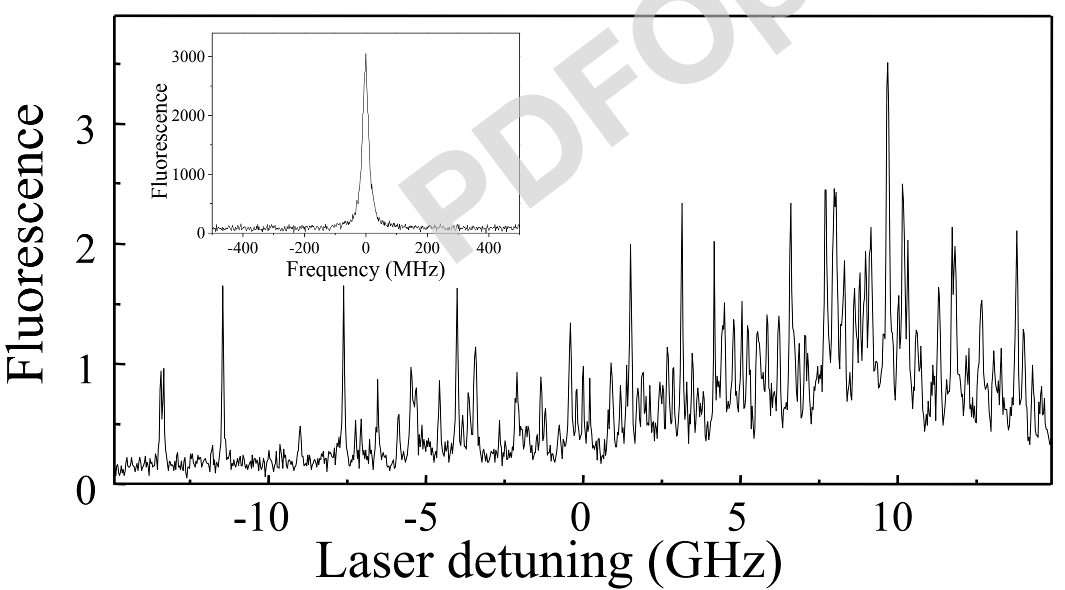
Single Molecule Spectroscopy @ Low Temp.

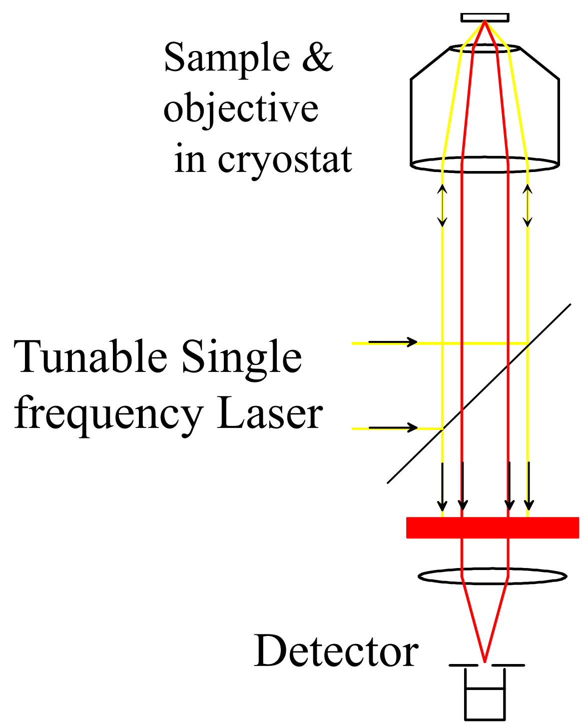
PAH molecules in Molecular Crystals





Resonant Fluorescence Excitation spectra :
- Sharp Zero Phonon Lines (T_1 limited)





(Orrit & Bernard, PRL 1990)

PDFOptim Lite

Single Molecule Spectroscopy @ Low Temp.

Intensity Saturation

γ_{hom}= 20 MHz

I ~ 250I_s

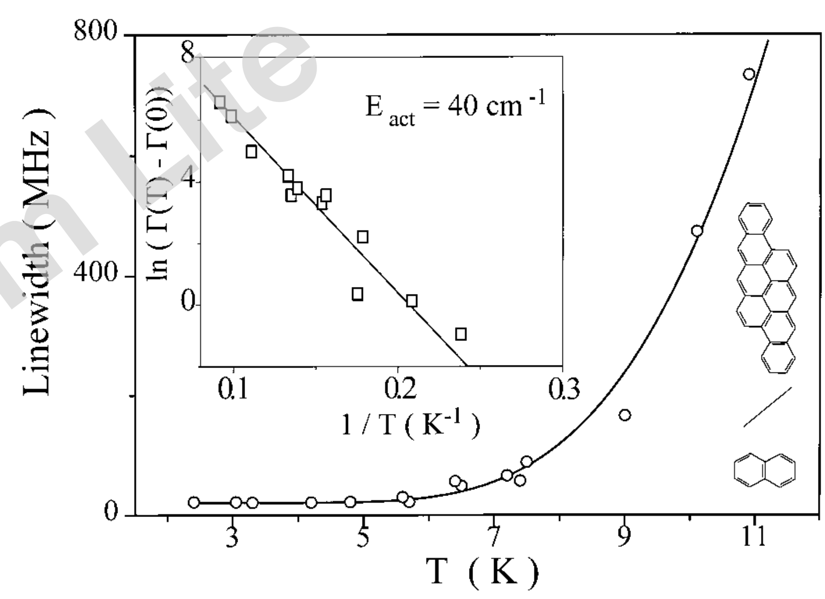
Fluorescence

0 20 40 60 80

-400 -200 0 200 400

Laser detuning (MHz)

Temperature dependence



At low temperature and weak laser excitations

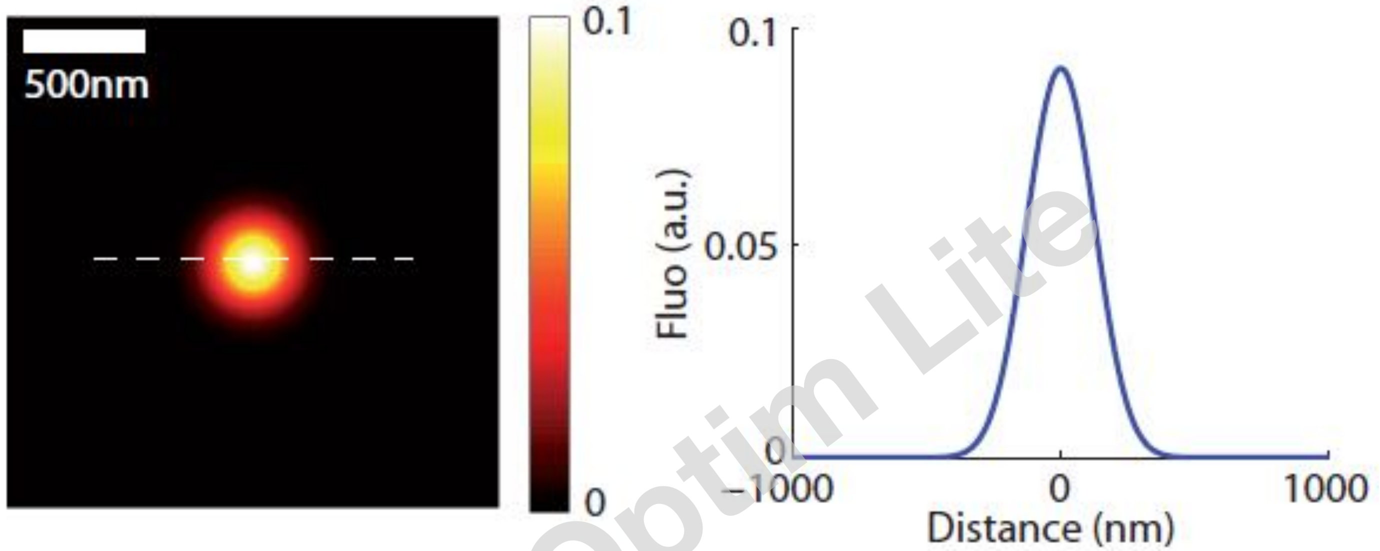
Optical resonances with high quality factors (up to 10^7-10^8)

Extremely stable lines: no spectral diffusion, no blinking

PDFOptim Lite

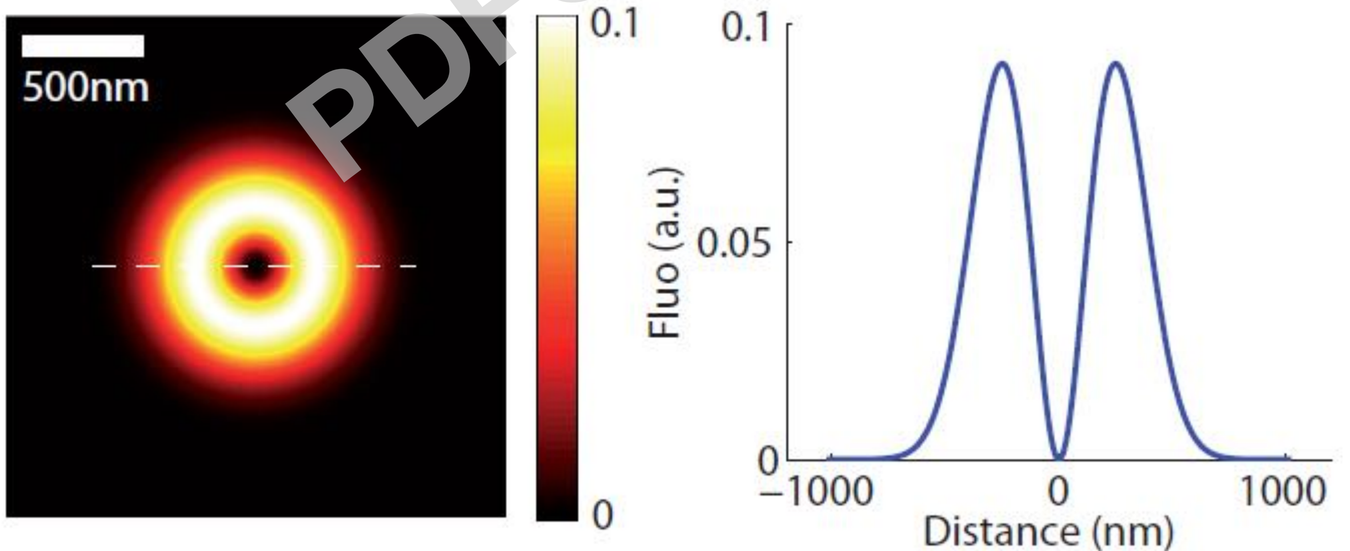
Molecule excitation at linear regime

Image of a molecule excited with a Gaussian beam

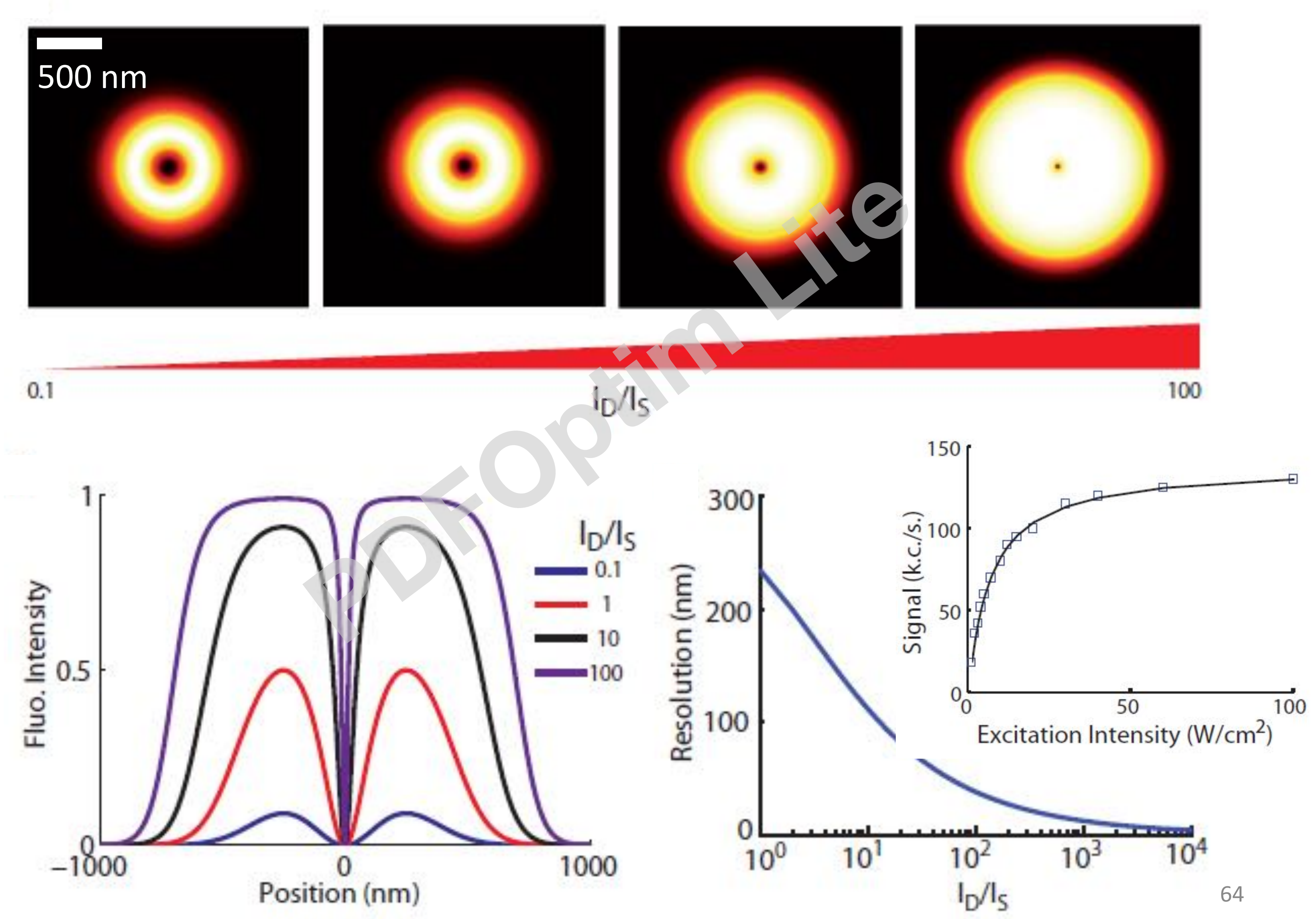


PDFOptim Lite

Image of a molecule excited with a doughnut beam

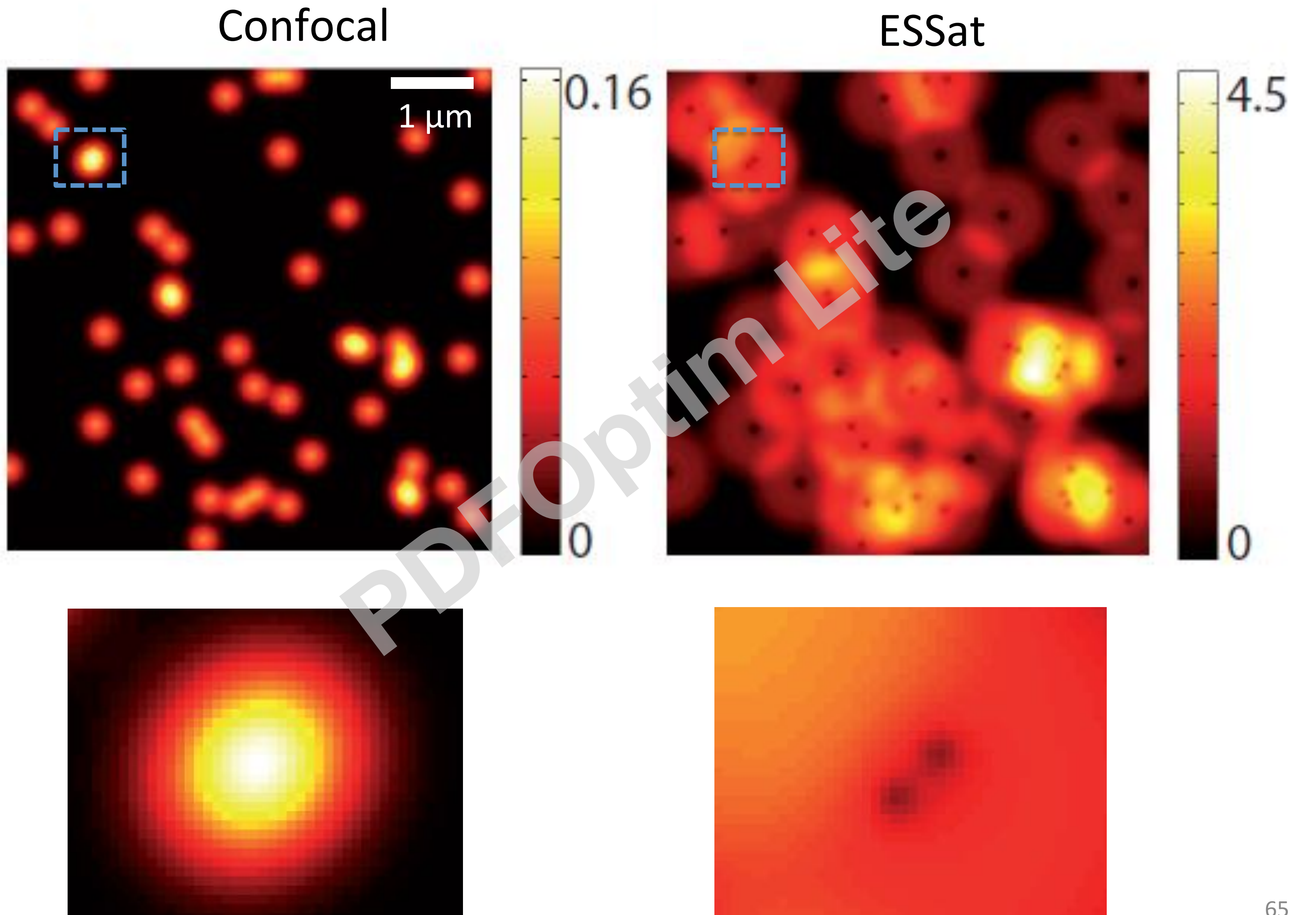


Principle of ESSat microscopy



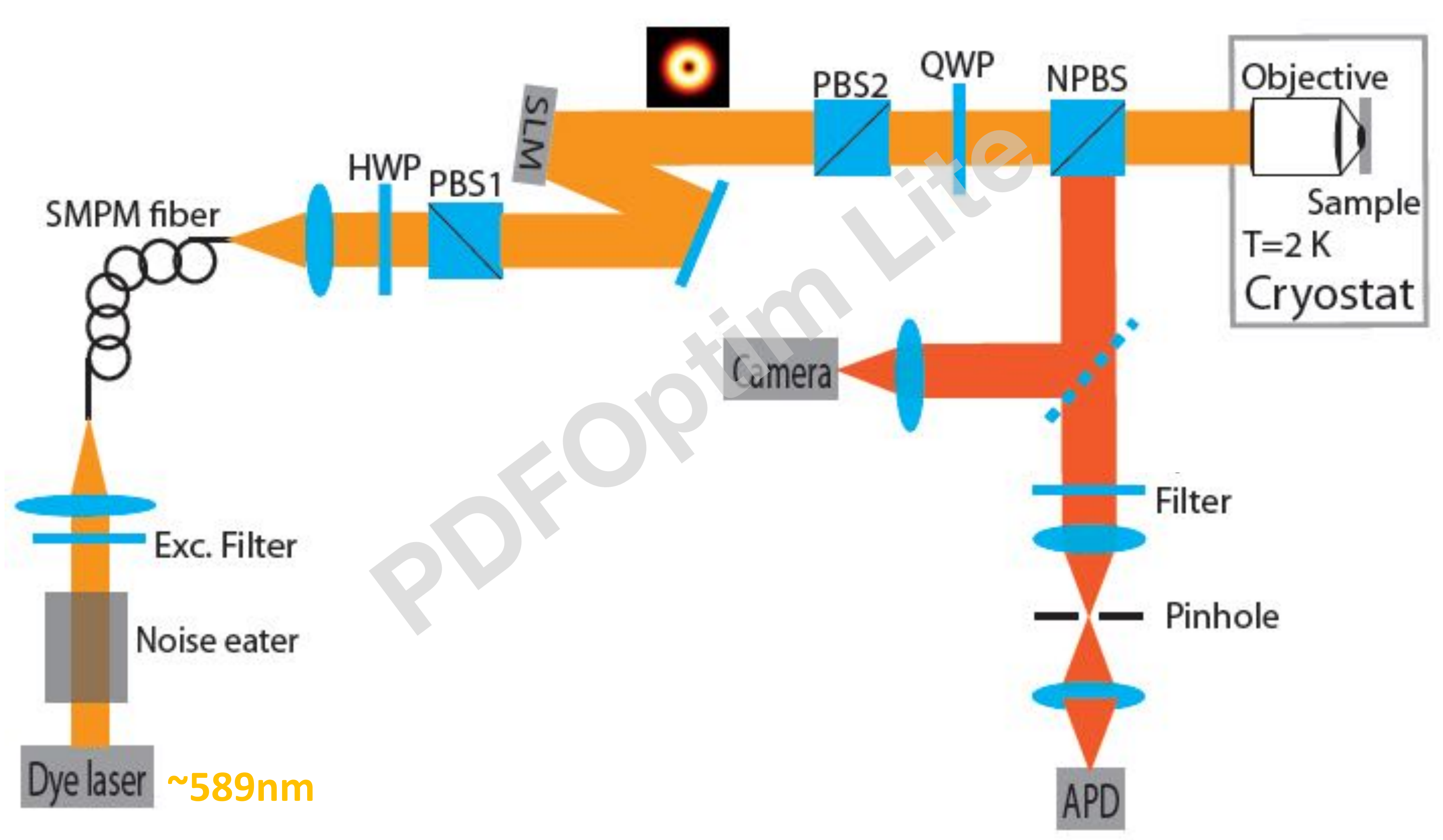
PDFOptim Lite

Simulated confocal and ESSat images



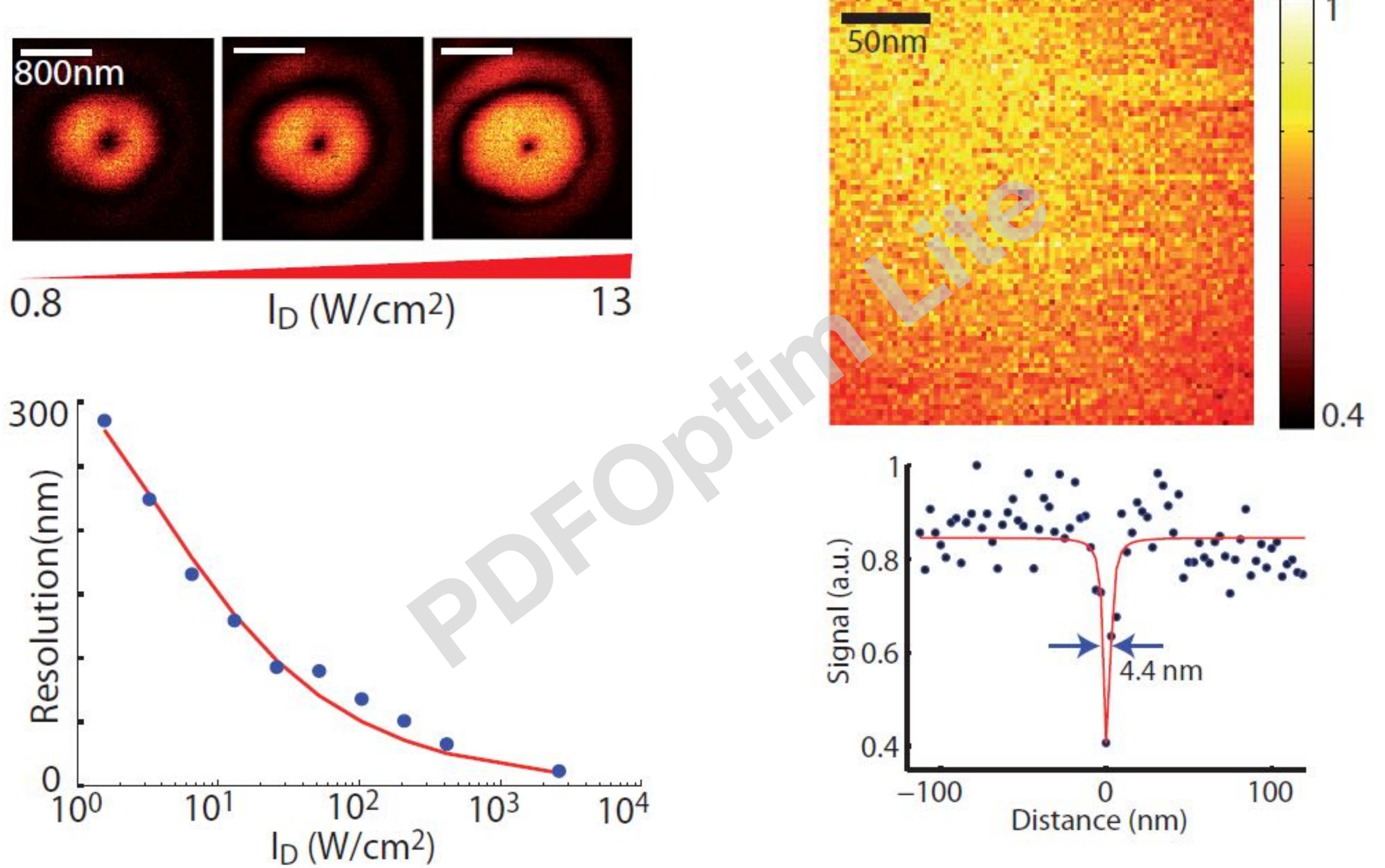
PDFOptim Lite

Experimental setup of the ESSat microscope



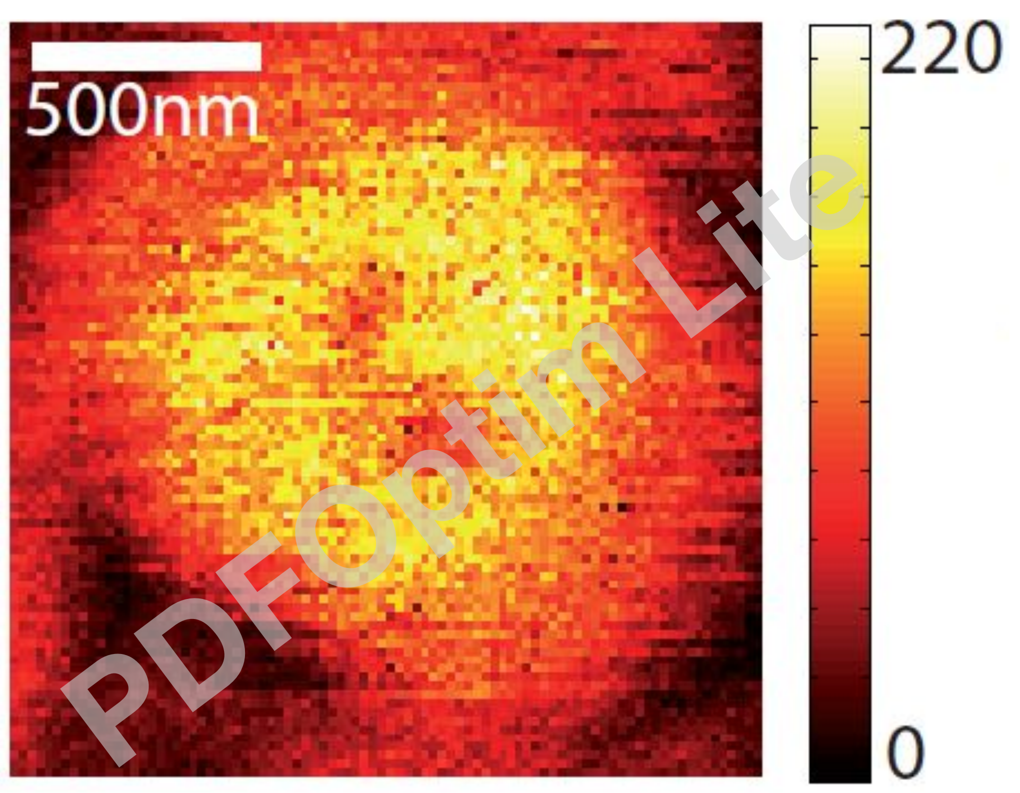
PDFOptim Lite

Resolution of ESSat microscopy



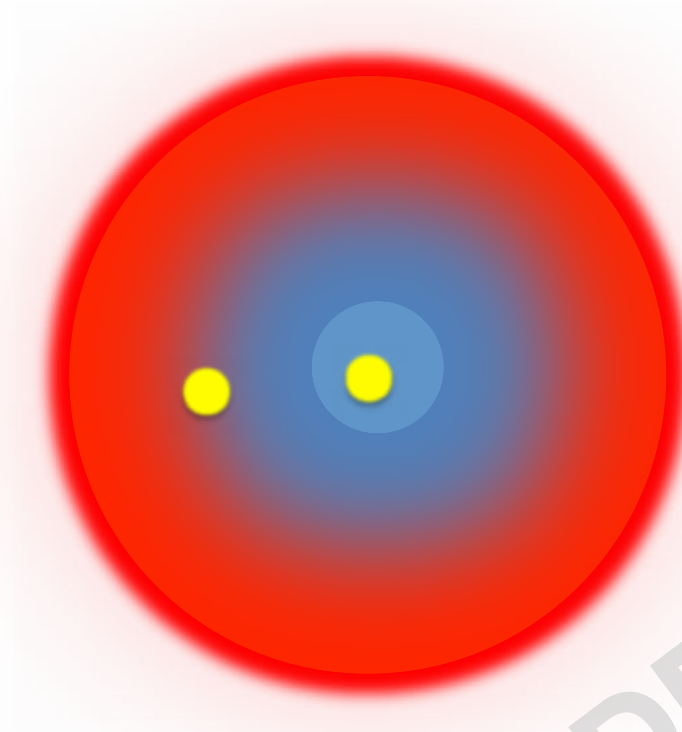
PDFOptim Lite

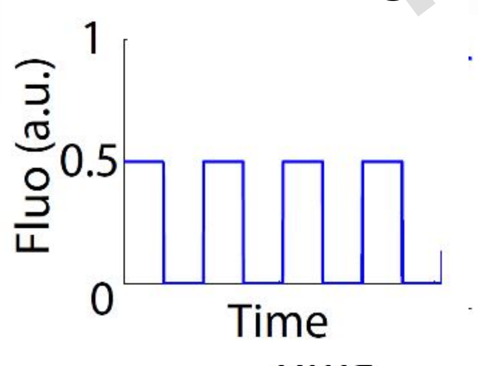
Two super-resolved molecules

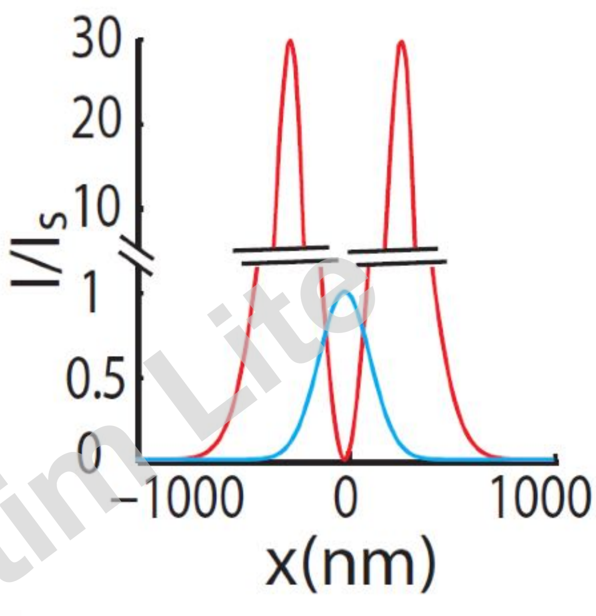


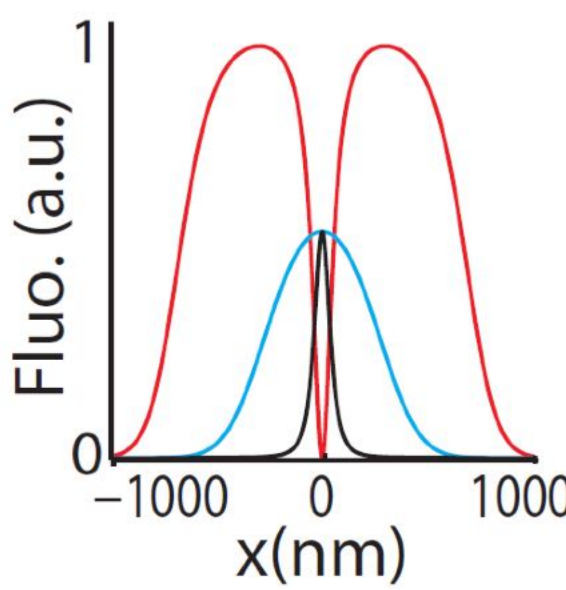
PDFOptim Lite

Modulated-ESSat microscopy



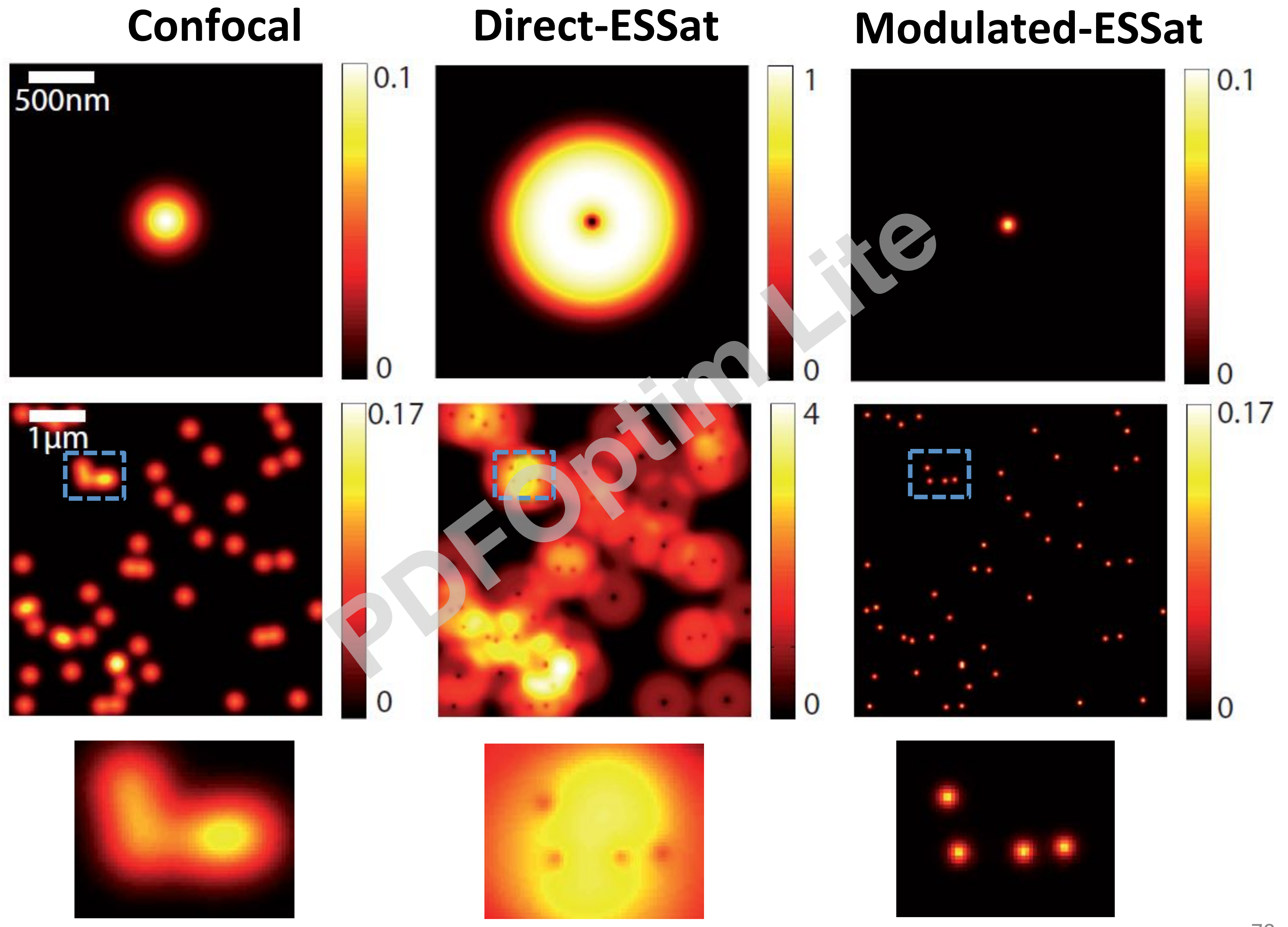






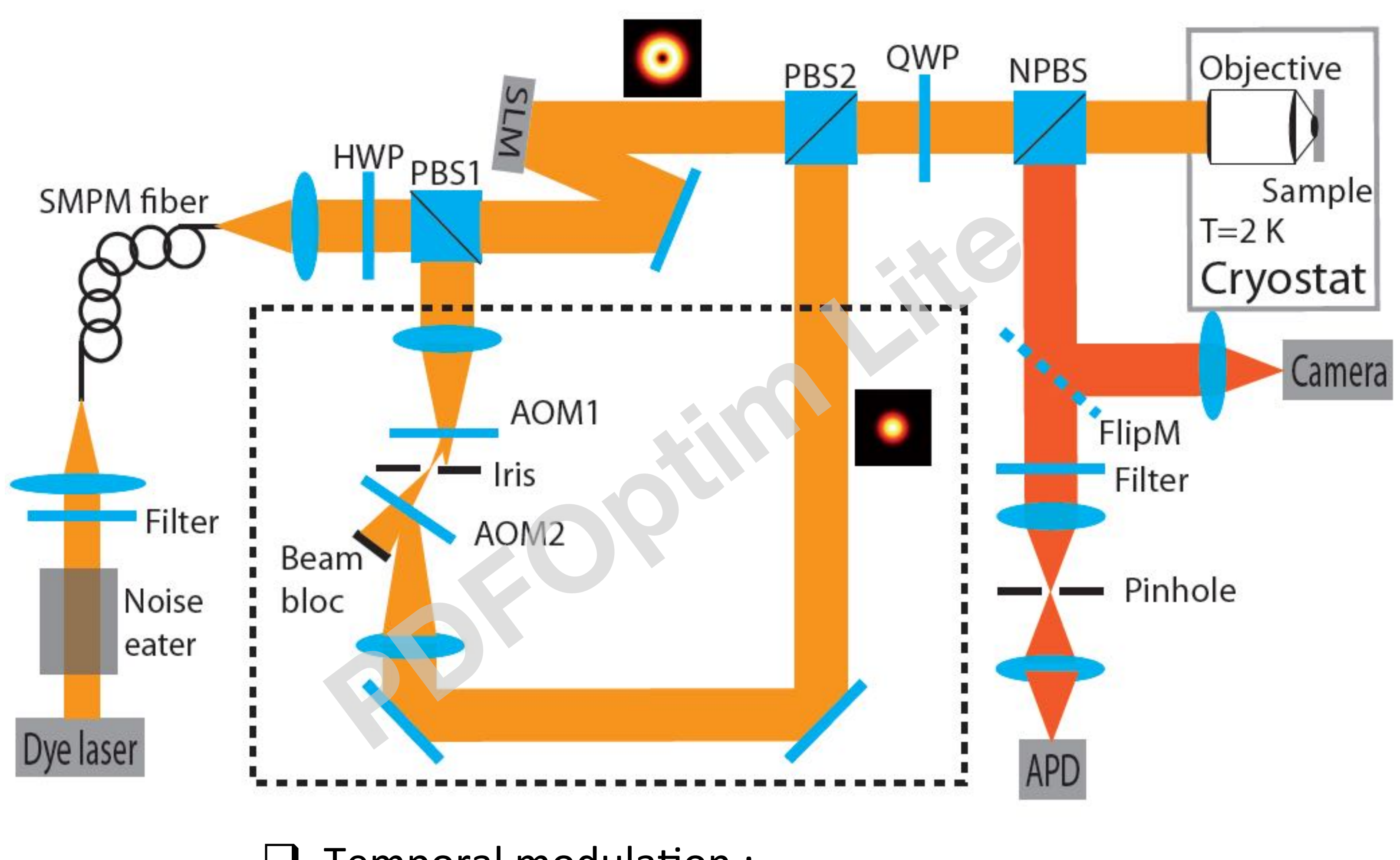
PDFOptim Lite

Simulated images of single molecules



PDFOptim Lite

Experimental setup of modulated-ESSat



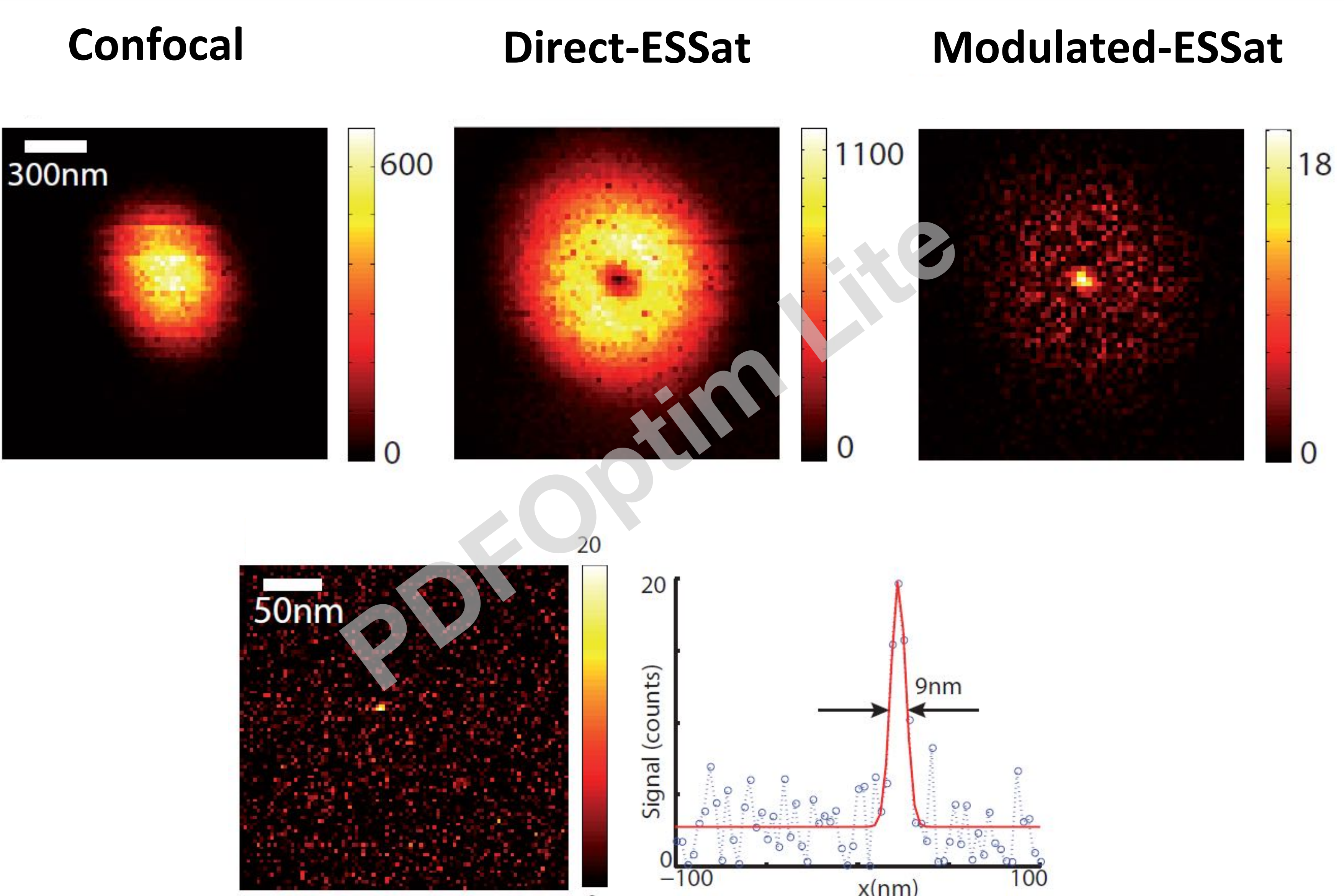
PDFOptim Lite

- ❑ Temporal modulation :

1 kHz, 500 µs "on" and 500 µs "off";

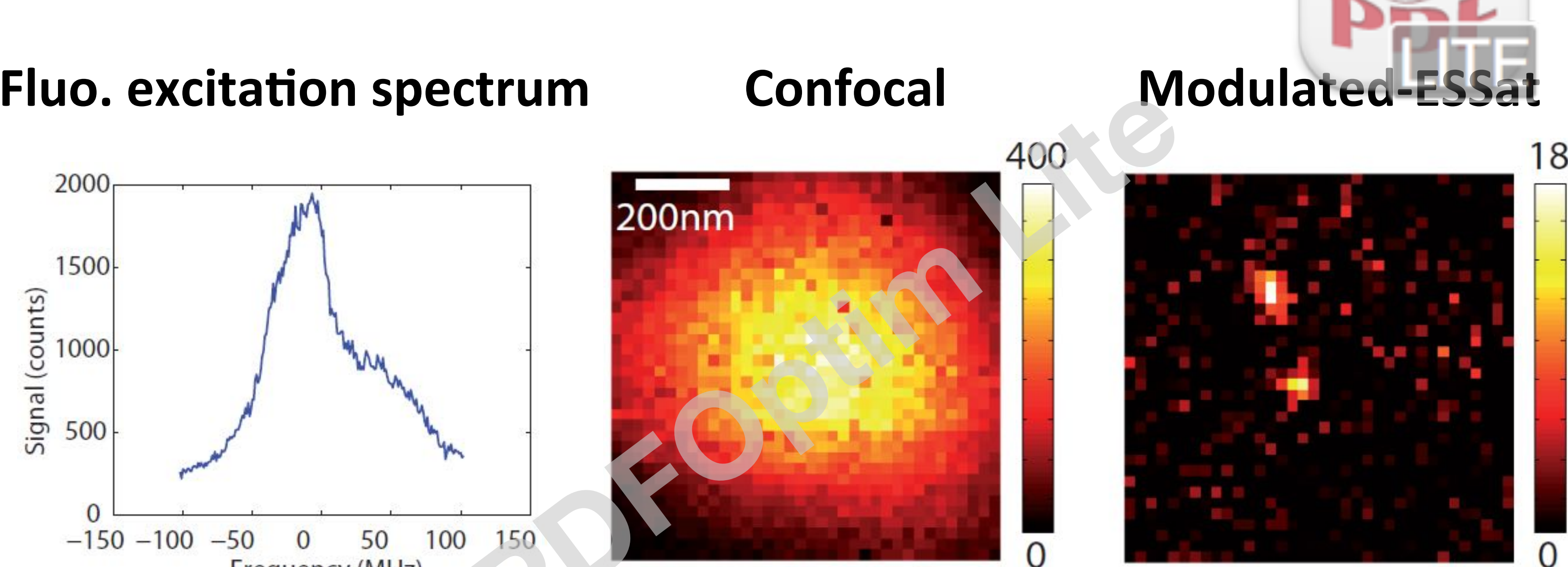
- ❑ Laser frequency shift: ~1 MHz

Resolution of modulated-ESSat microscopy



PDFOptim Lite

Two molecules with overlapping resonances



PDFOptim Lite