Super resolution microscopy

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What does a microscope do?

➠ ***Why do we need microscopes?***

To observe “small objects”, microscopes main contributions:

What does a microscope do?

➠ ***Why do we need microscopes?***

To observe “small objects”, microscopes main contributions:

➠ Magnification

➠ Resolution

➠ Contrast

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➠ ***Why do we need microscopes?***

To observe “small objects”, microscopes main contributions:

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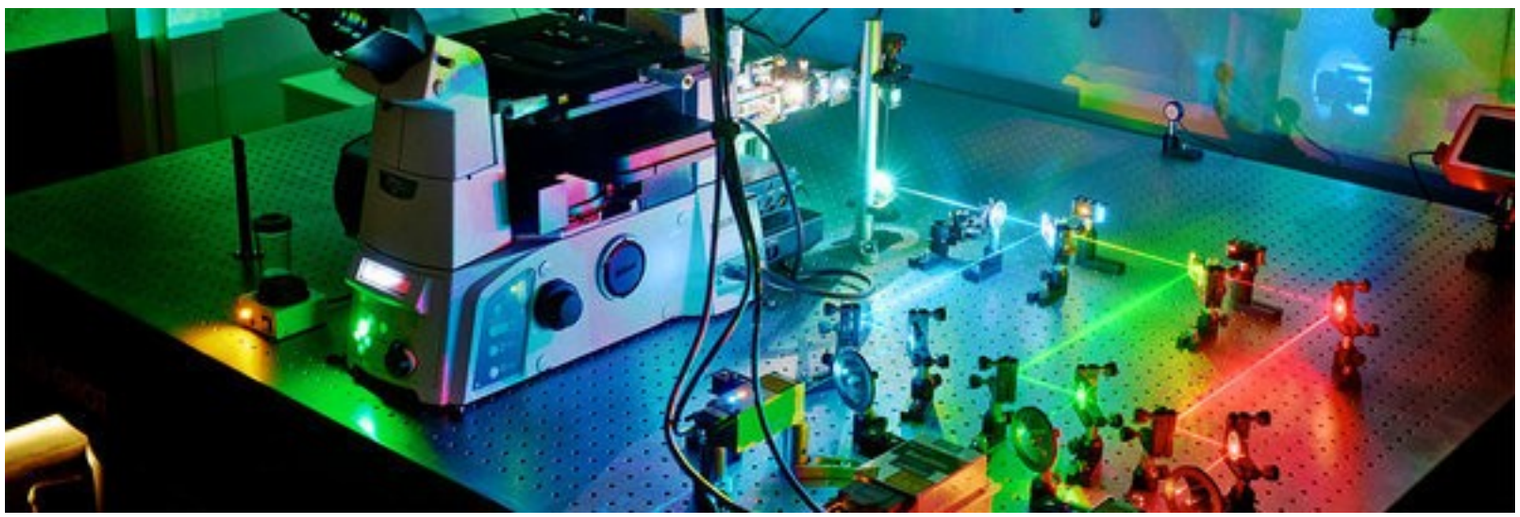
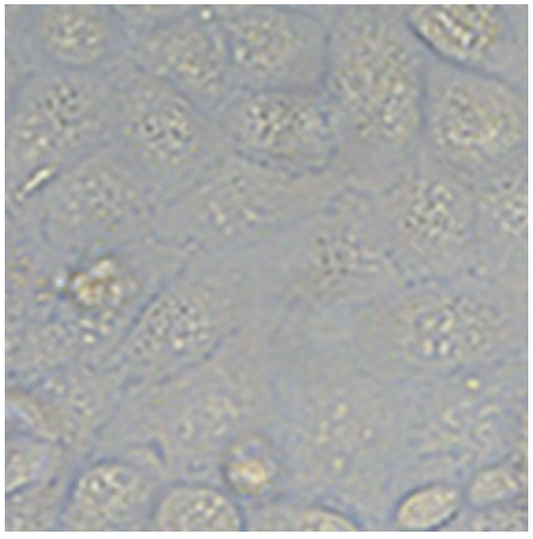
➠ Resolution

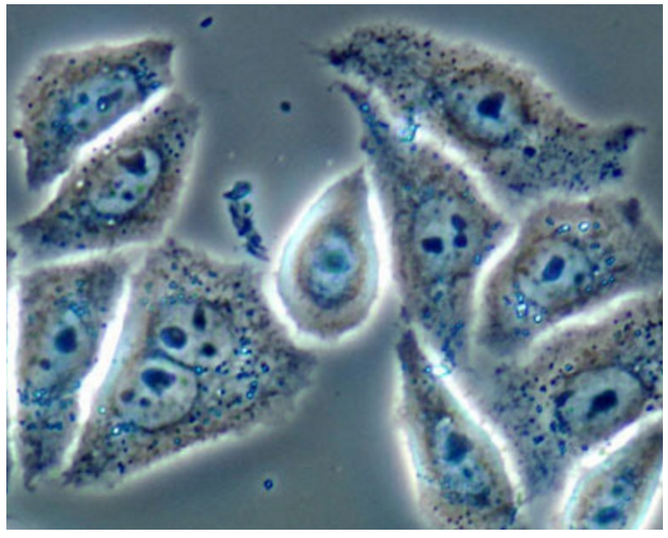
➠ Contrast



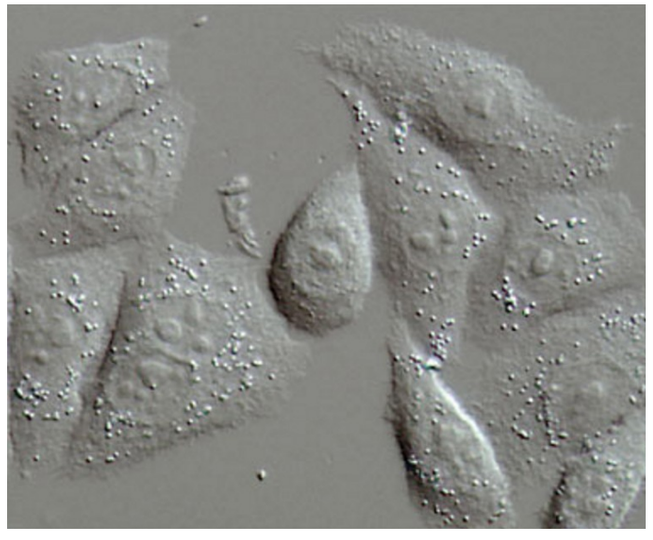
Contrasting methods



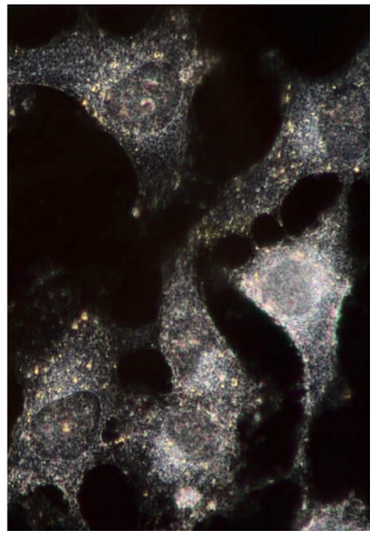
Bright field



Phase contrast



DIC*

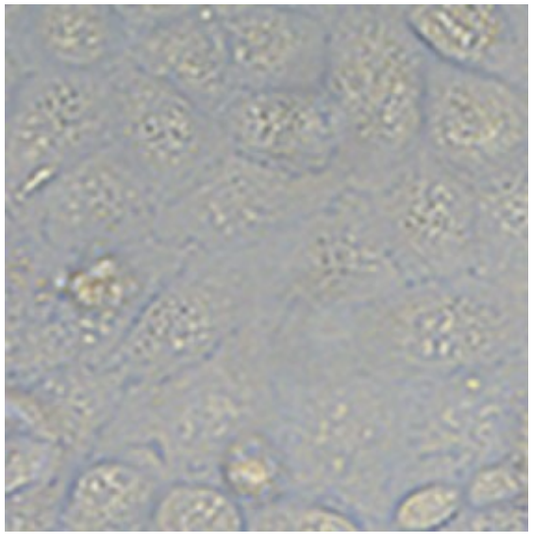


Dark field

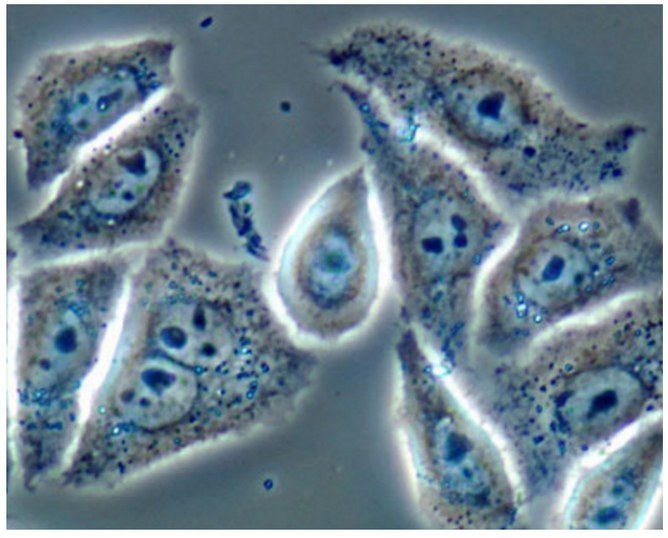
...

**Differential interference contrast*

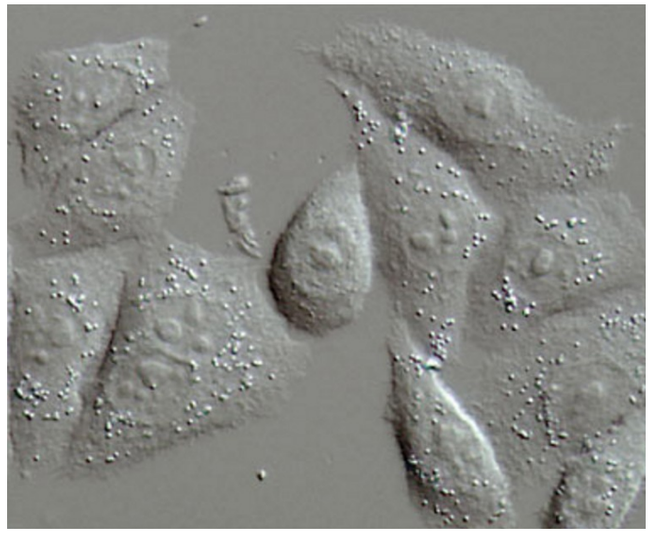
Contrasting methods



Bright field



Phase contrast



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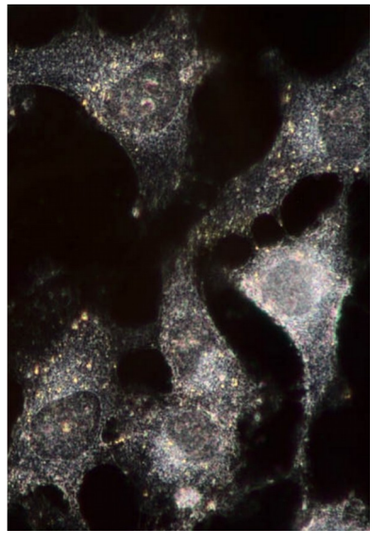
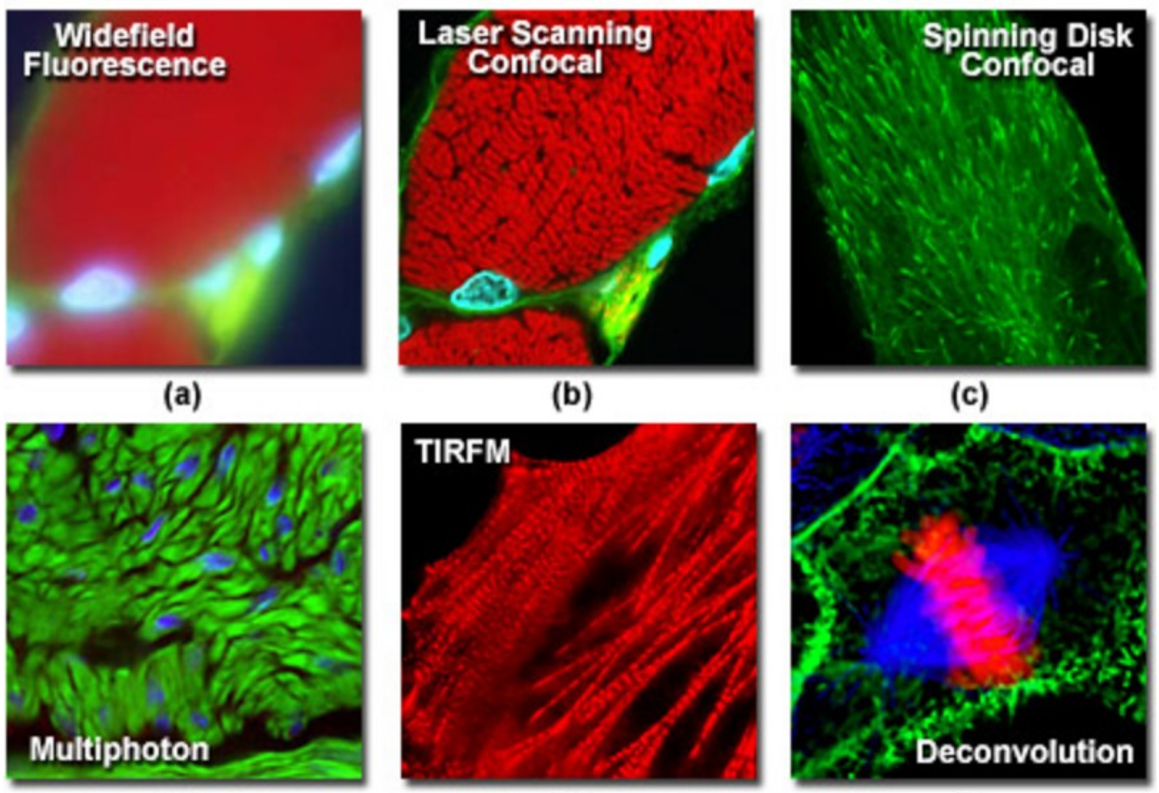


Dark field

...

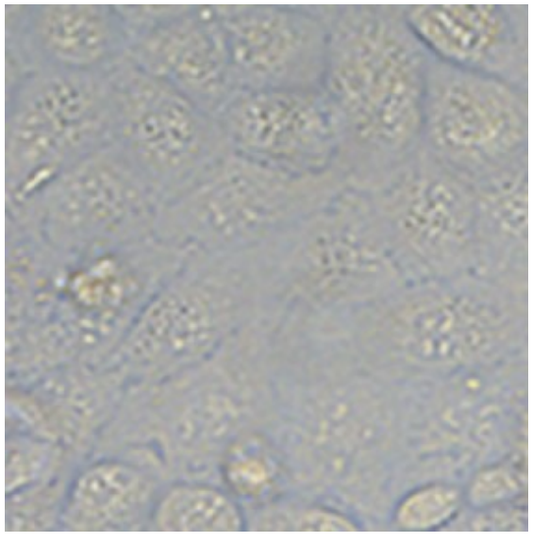
**Differential interference contrast*

Fluorescence microscopy



High contrast
Specific labelling
Multiple labelling
Live compatible
...

Contrasting methods



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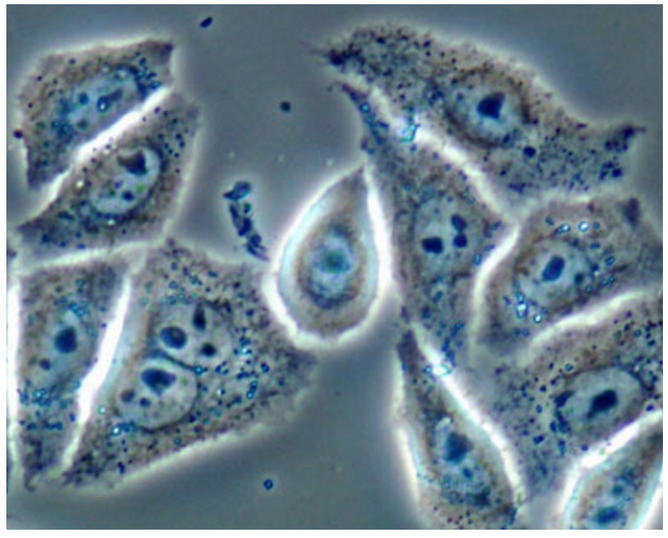
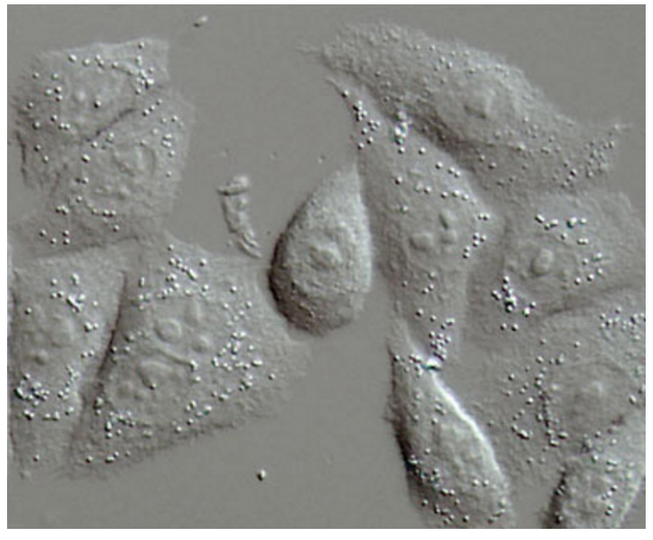


Phase contrast



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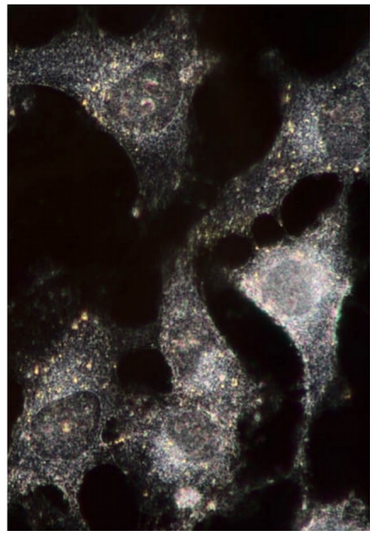

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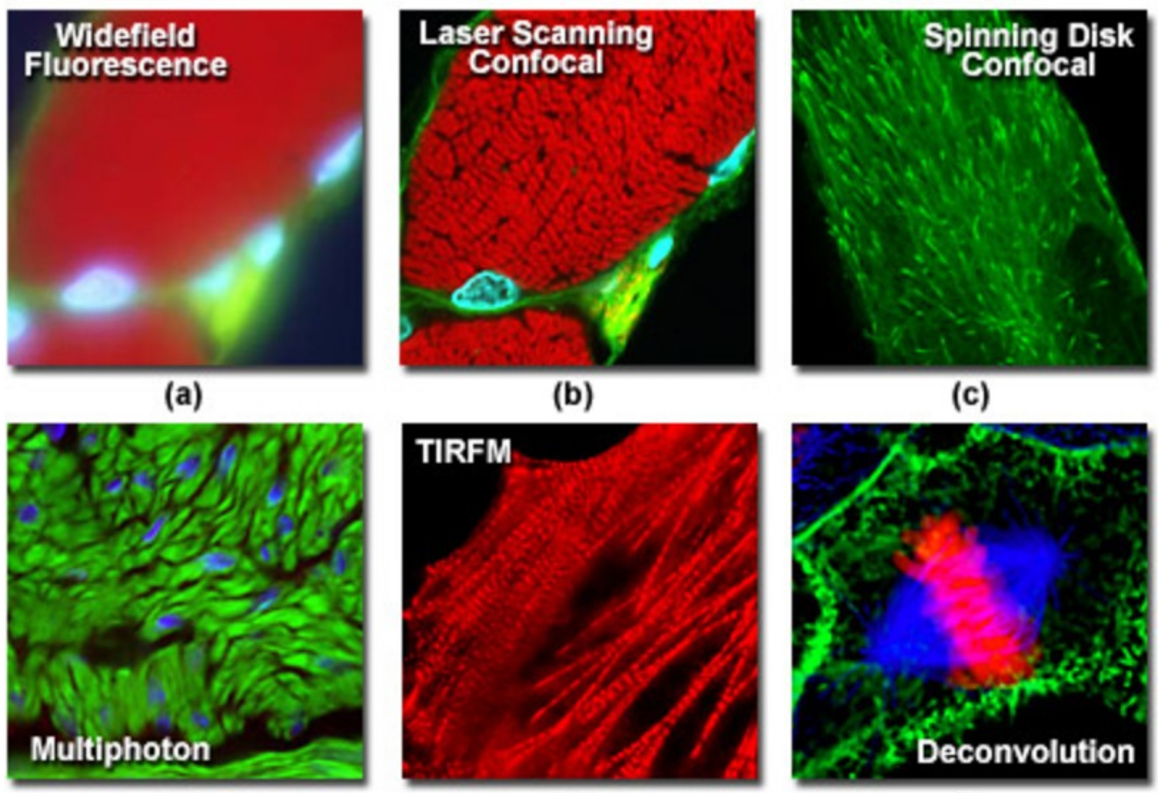
**Differential interference contrast*

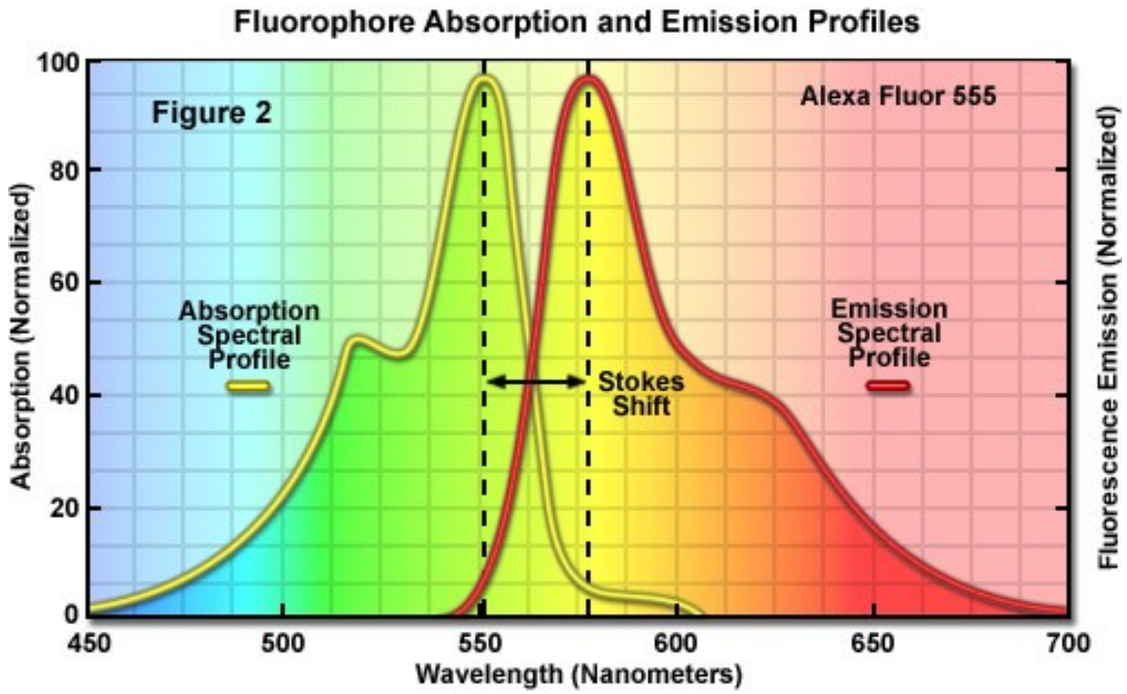
Fluorescence microscopy



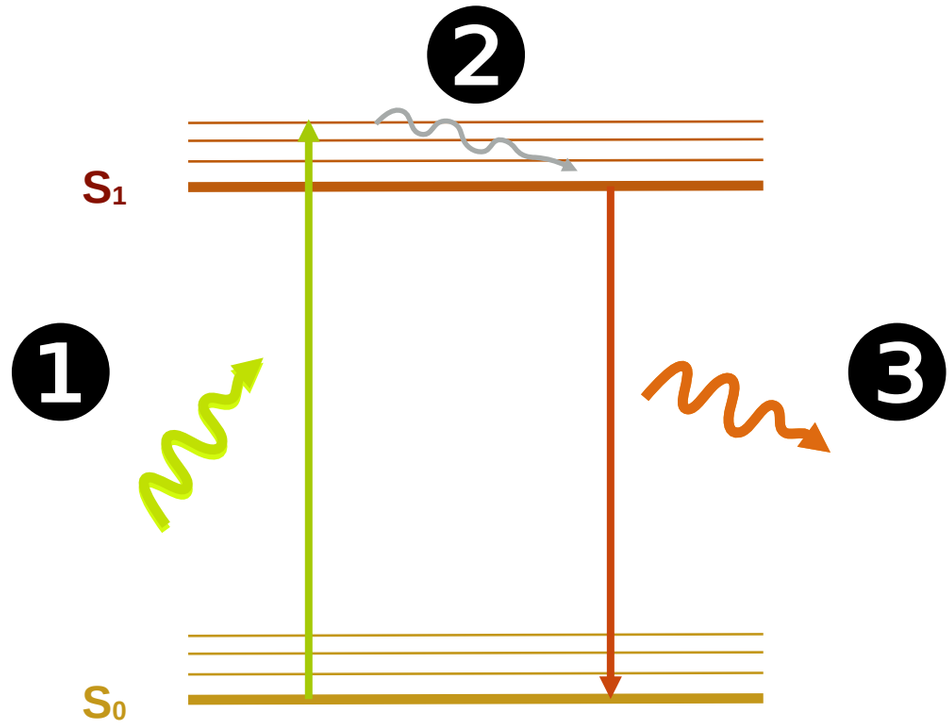
High contrast
Specific labelling
Multiple labelling
Live compatible
...

Fluorescence

Absorption / Emission spectra are shifted (stokes shift)



Jablonski diagram



Fluorescence

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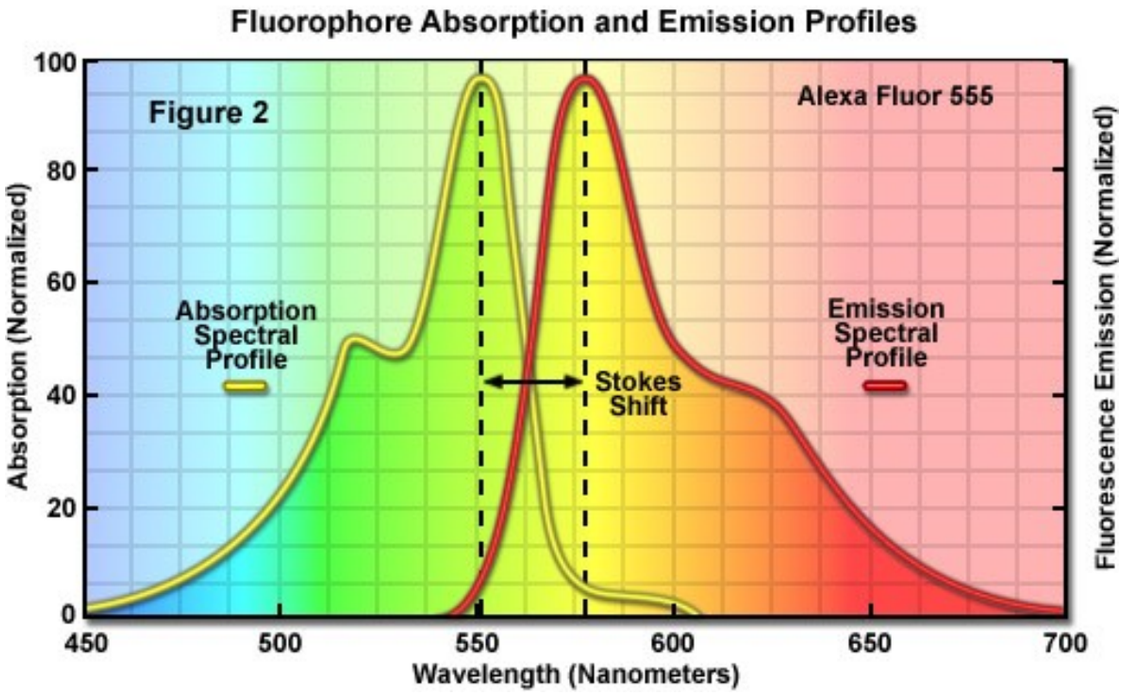

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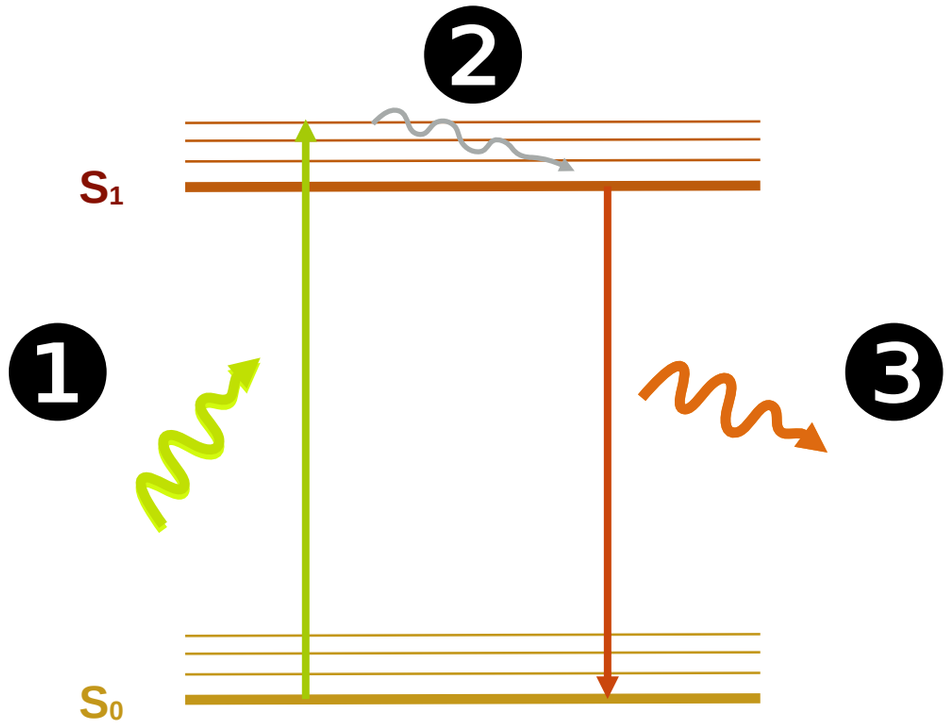
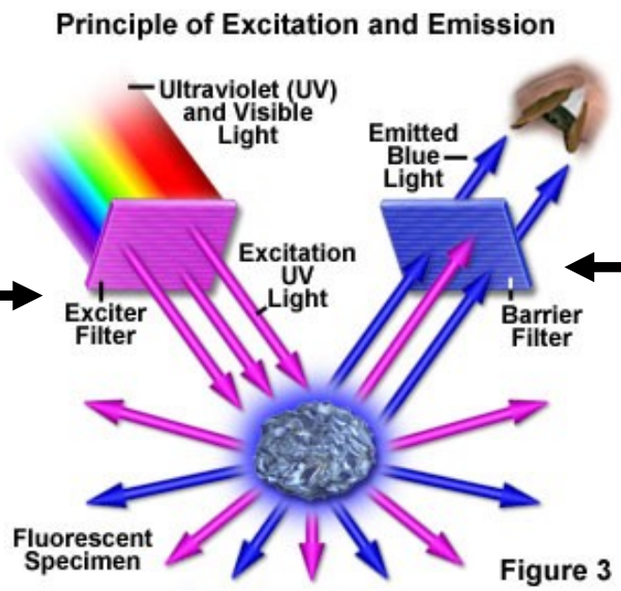


Excitation filter



Emission filter

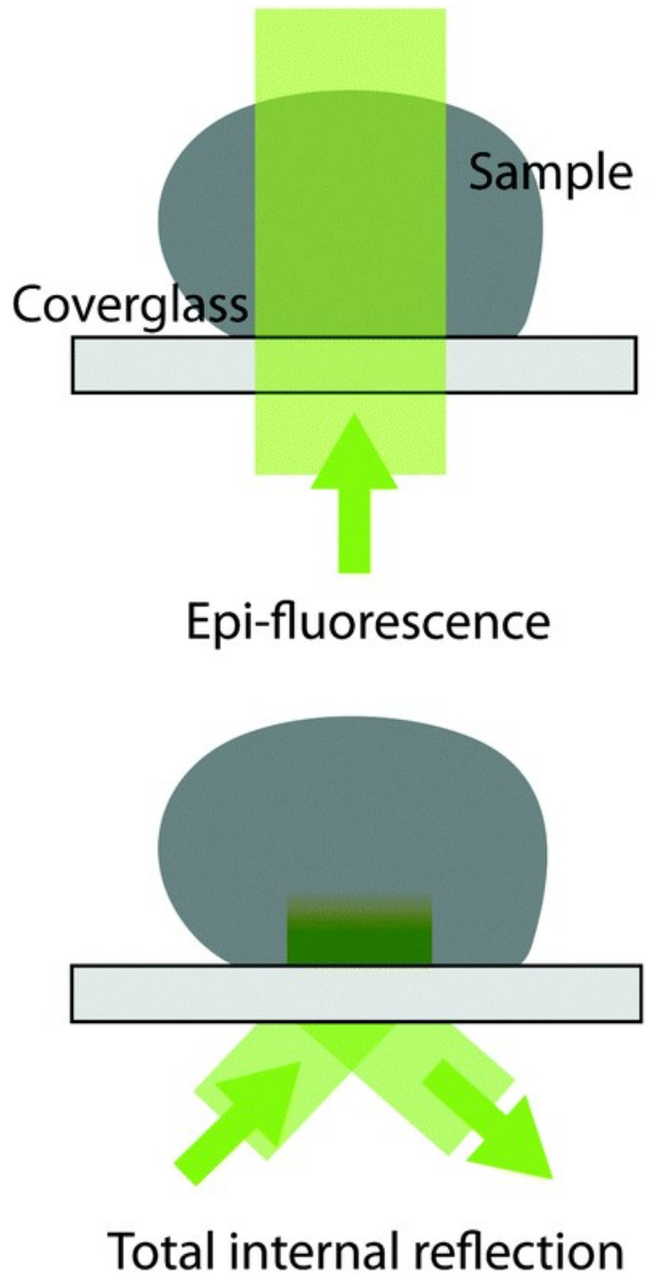
Excitation modes

Wide field imaging

Laser scanning imaging

Excitation modes

Wide field imaging



Laser scanning imaging

Excitation modes

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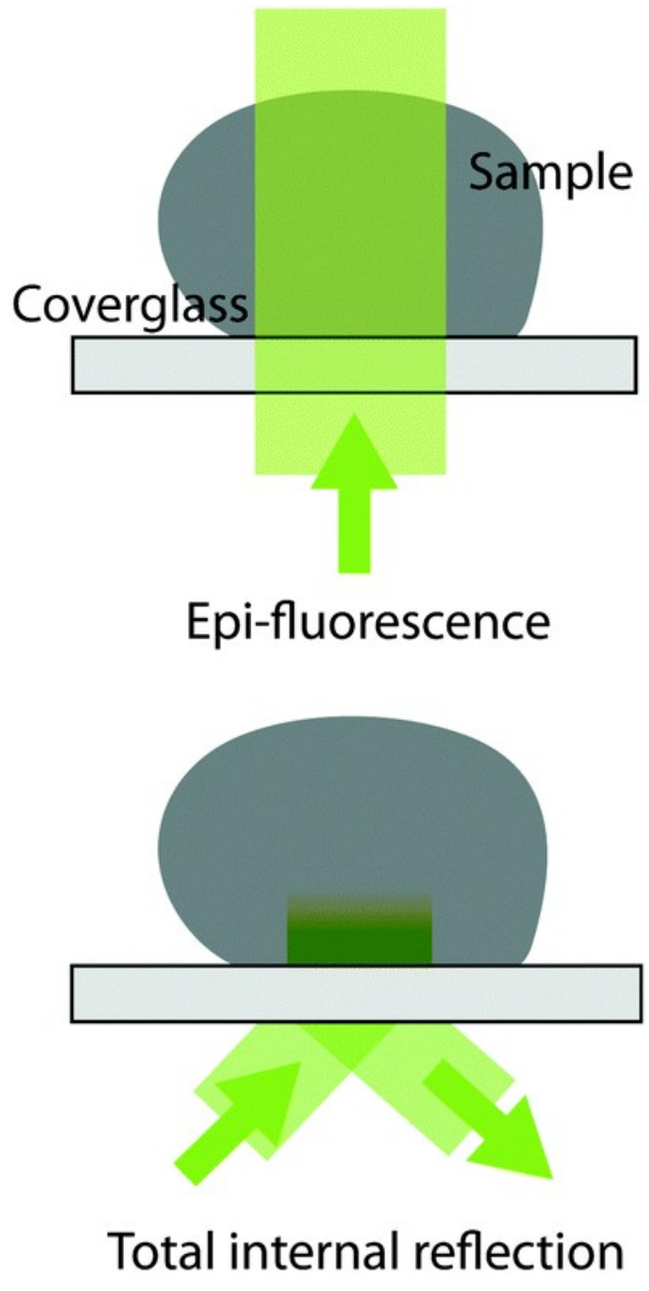
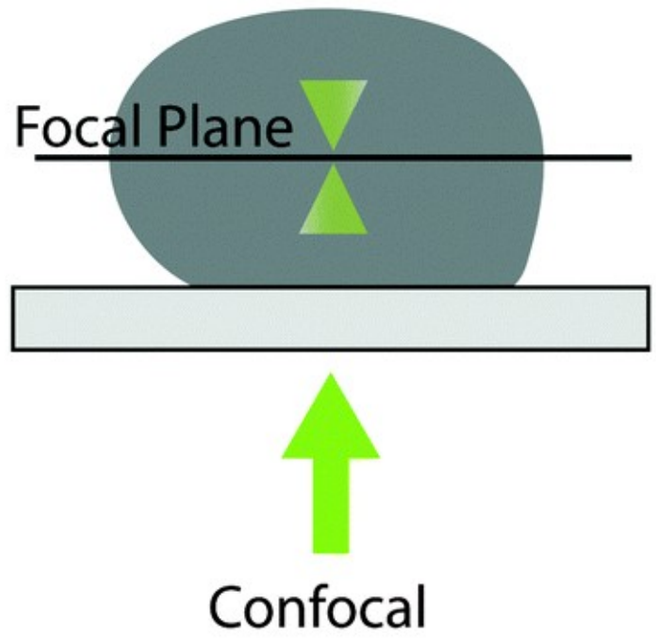
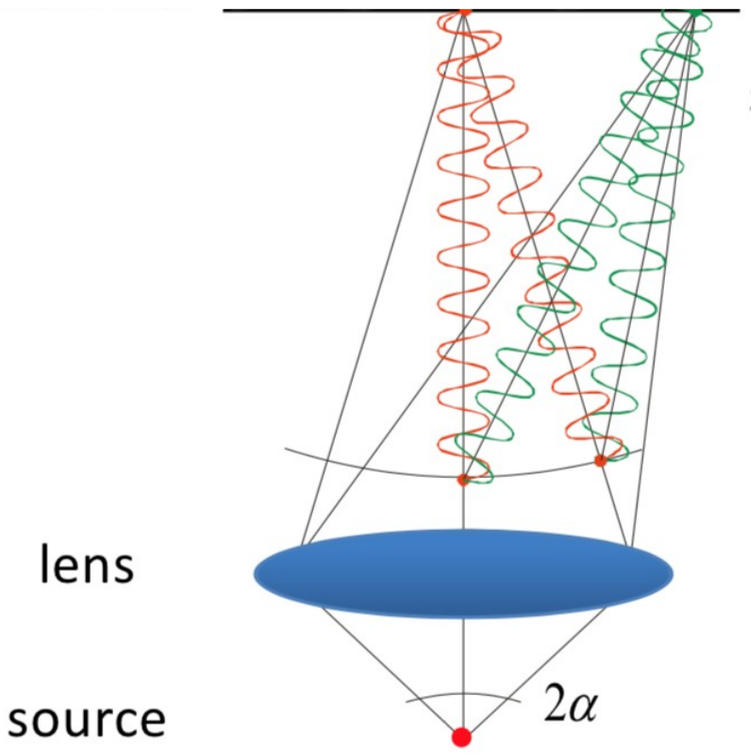


Laser scanning imaging

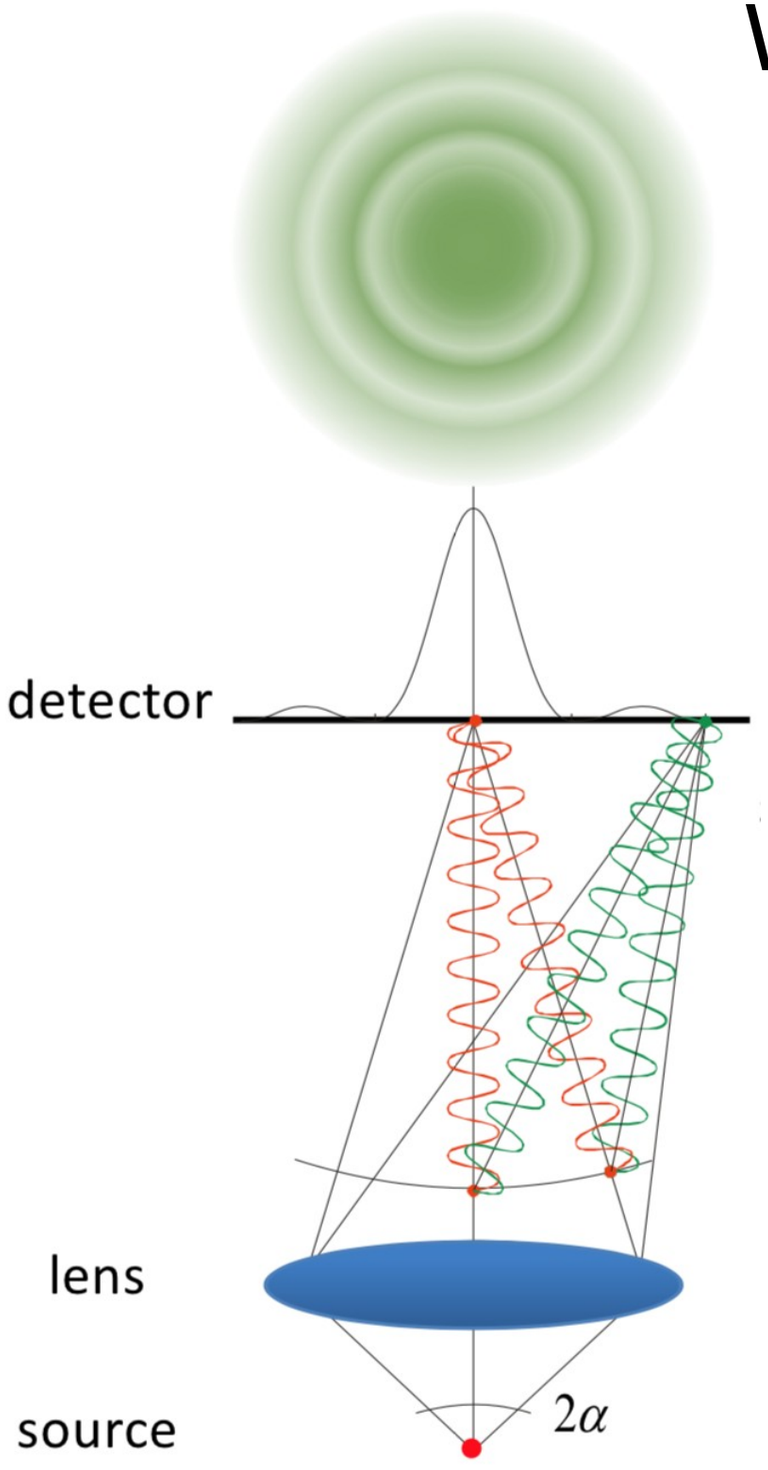


Microscope Point Spread Function



Microscope Point Spread Function

Wave + aperture —> diffraction!



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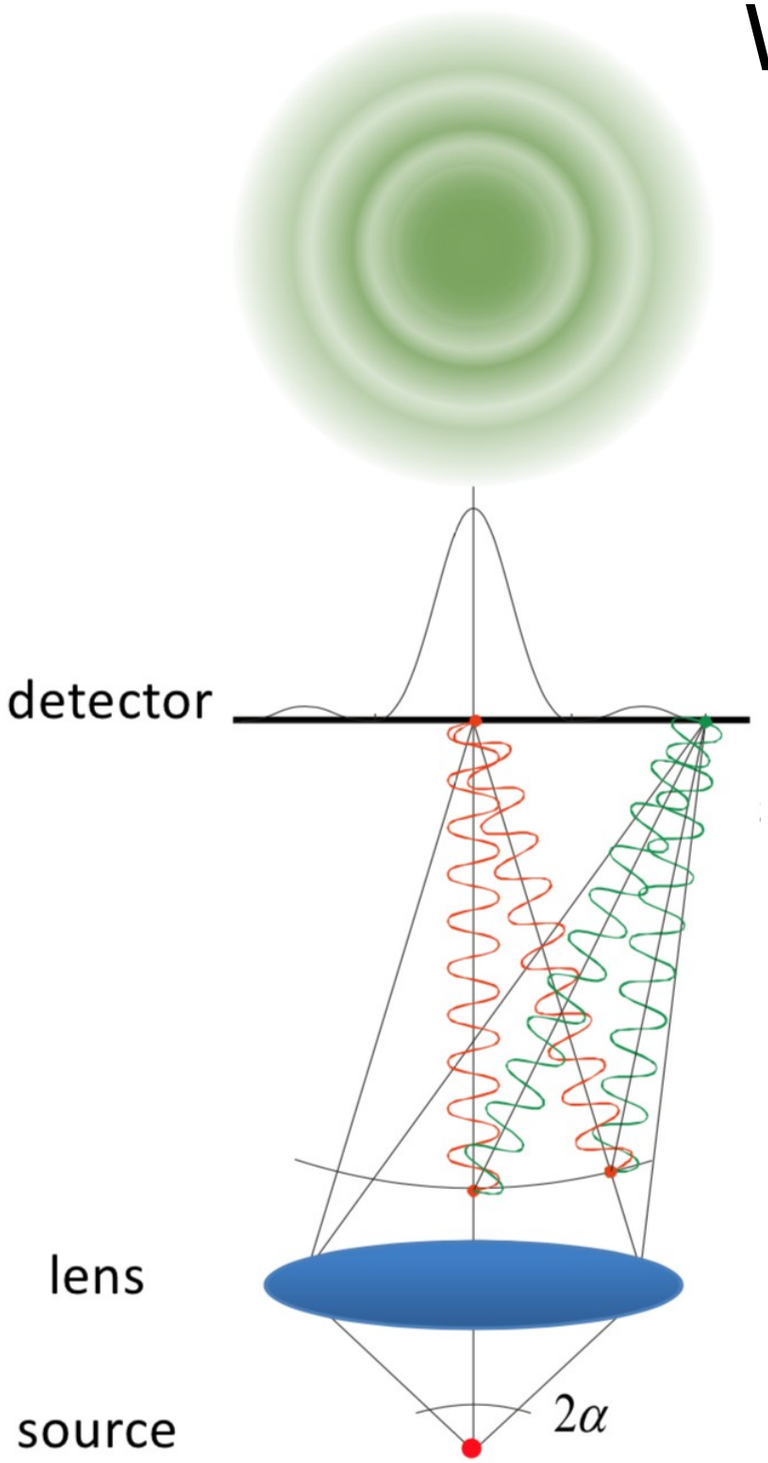
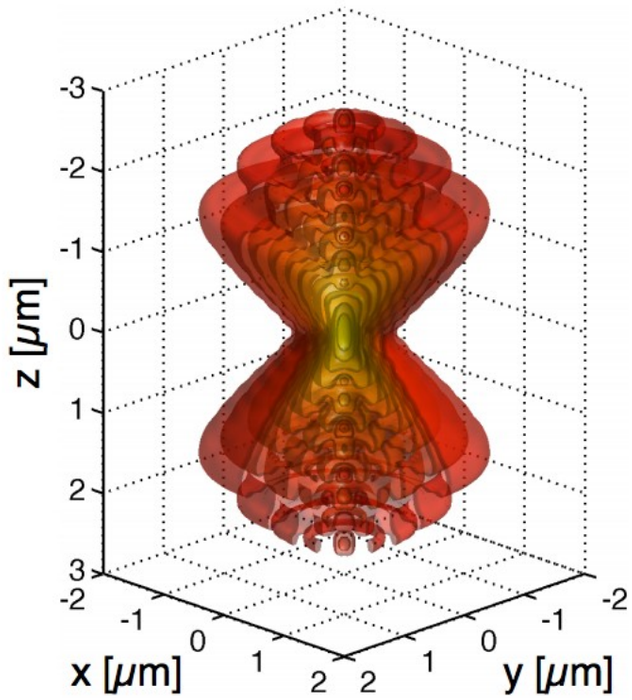
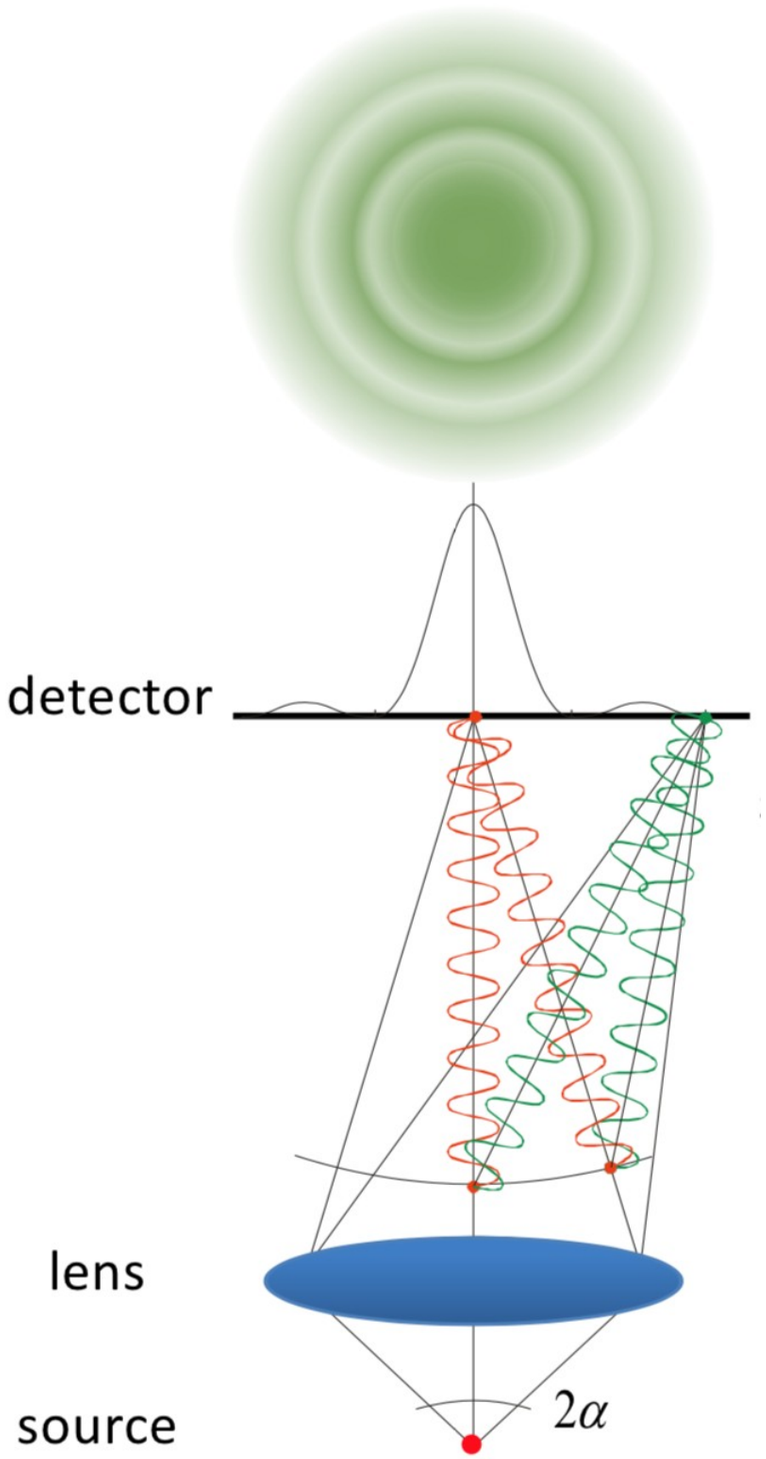


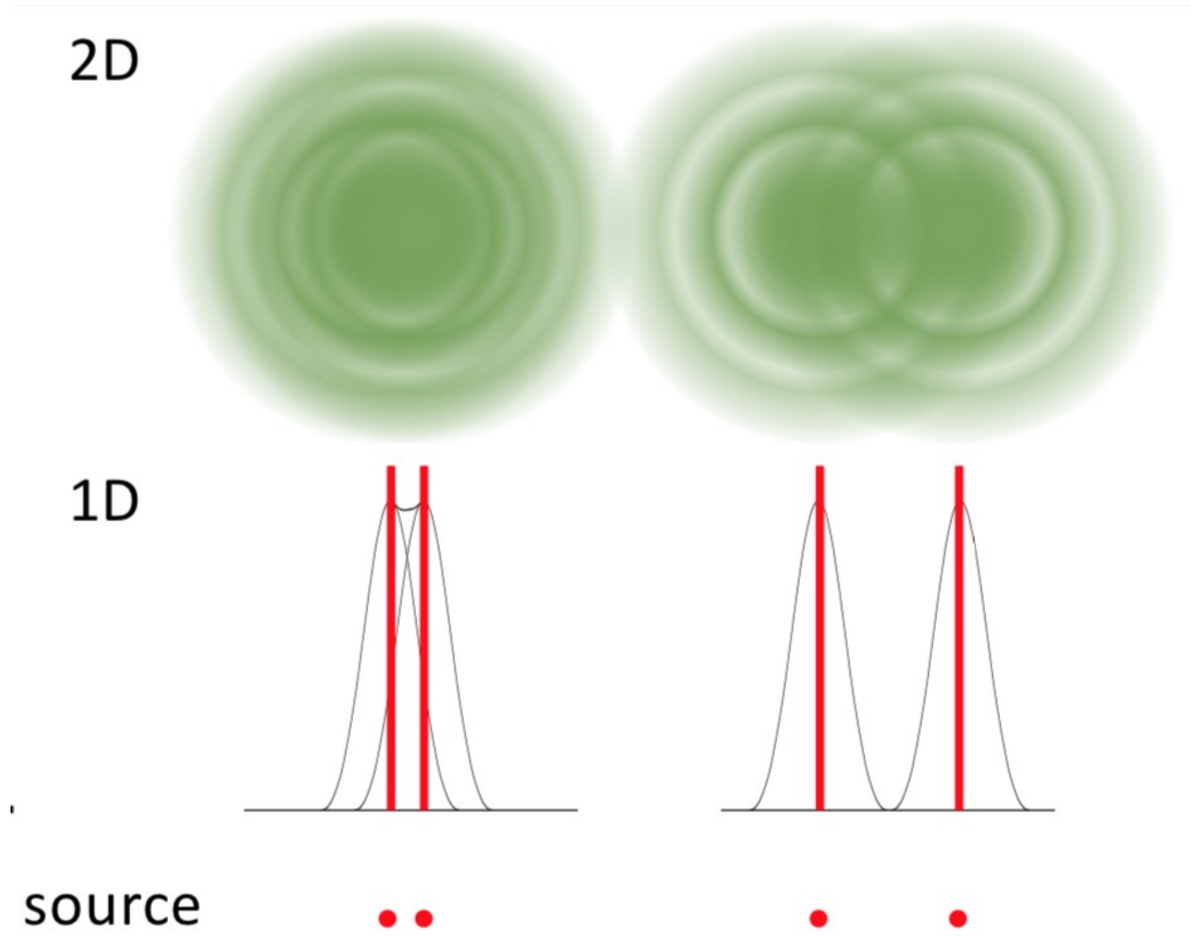


Point spread function (High Numerical Aperture objective, RI~1.5):

- **~200 nm in XY**
- **~500 nm is Z**

Resolution





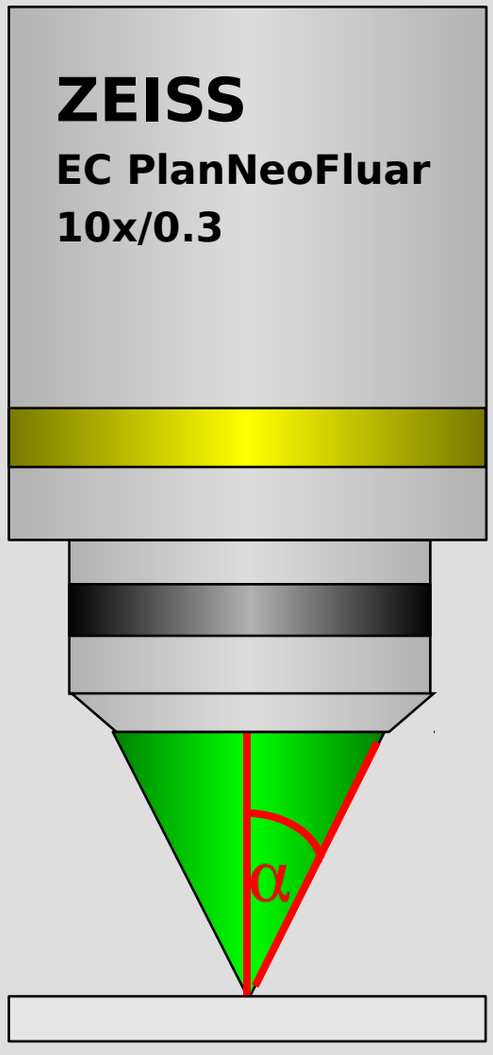
Criterion: shortest distance allowing separation of two objects imaged through the microscope

$$NA = n\sin\alpha \qquad r_{xy} = \frac{0.61\lambda}{NA} \qquad r_z = \frac{\lambda n}{NA^2}$$

$$NA = 1.4 \qquad \lambda = 550nm \qquad r_{xy} \cong 240nm \qquad r_z \cong 500nm$$

En microscopie la resolution depend de :

-> L’ouverture numerique
-> La longueur d’onde



$$d = 0{,}61\frac{\lambda}{NA}$$

$$NA = n \cdot \sin\alpha$$

d resolution

λ wavelength

NA numerical aperture

α opening angle

n refractive index

Influence de l’ouverture numerique sur la résolution

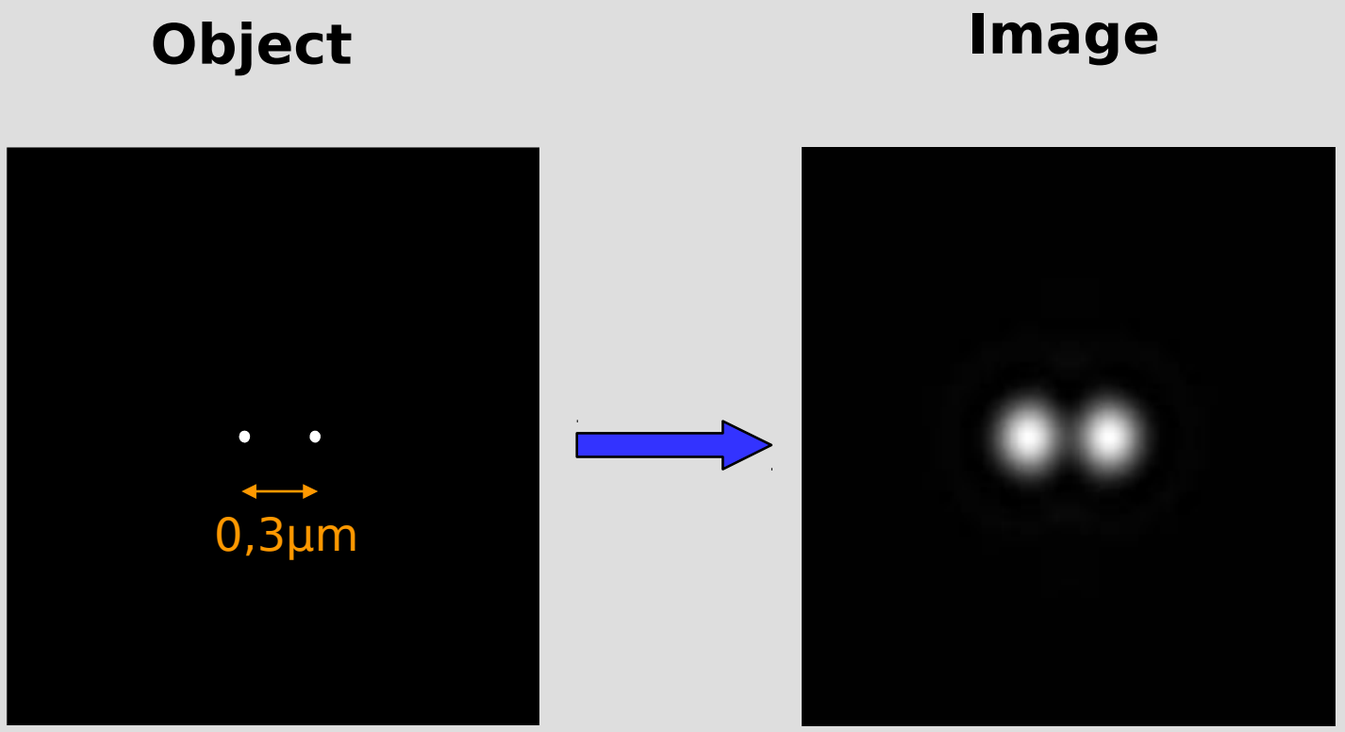
Principle effect

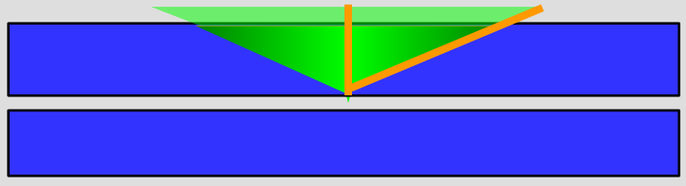
High numerical aperture objectives have a large opening angle.

The higher the NA, the better the resolution of the microscope.

$$NA = 1{,}4$$

$$\alpha \approx 67°$$





Influence de l’ouverture numerique sur la résolution

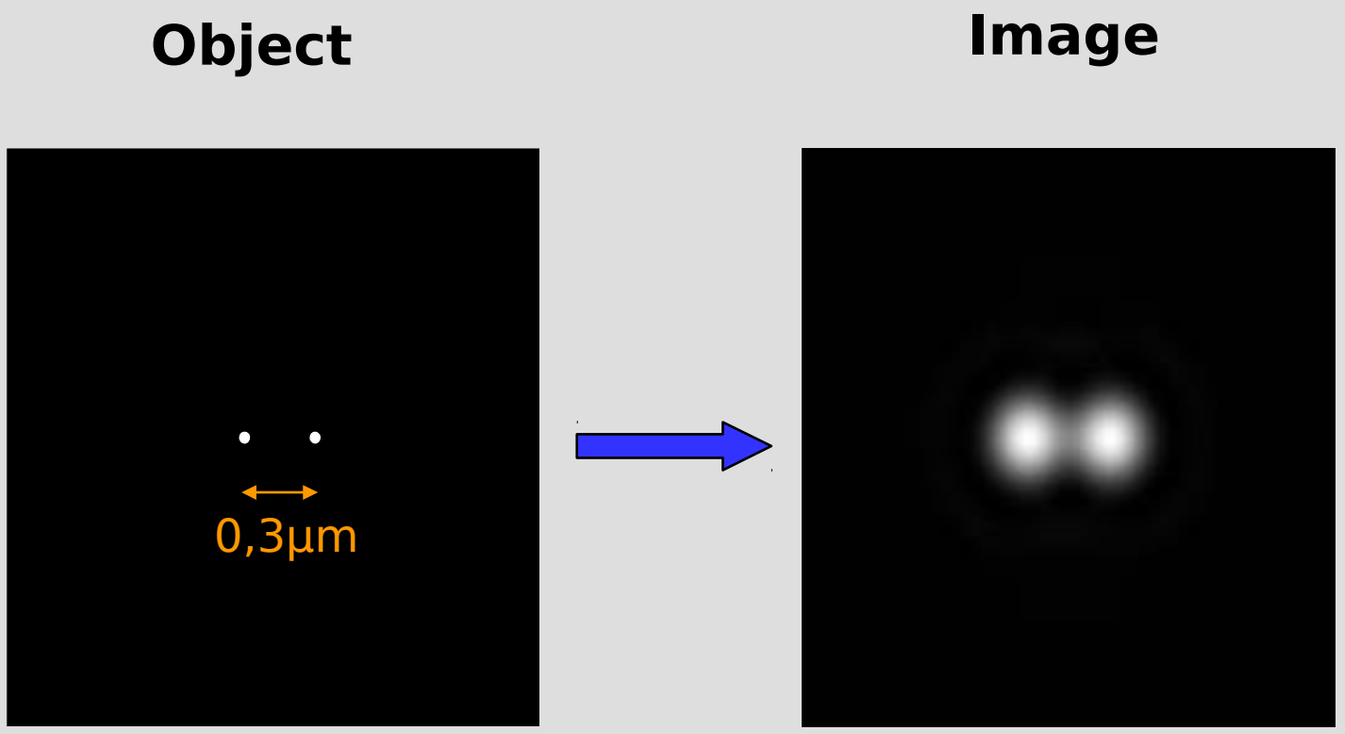
Principle effect

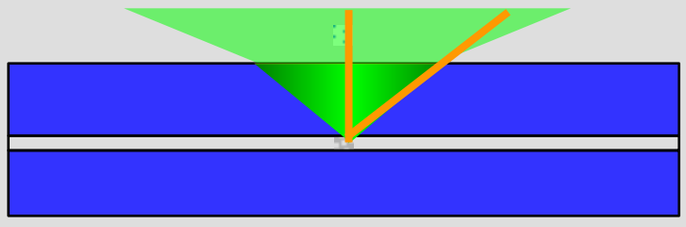
High numerical aperture objectives have a large opening angle.

The higher the NA, the better the resolution of the microscope.

$$NA = 1{,}2$$

$$\alpha \approx 52°$$





Influence de l’ouverture numerique sur la résolution

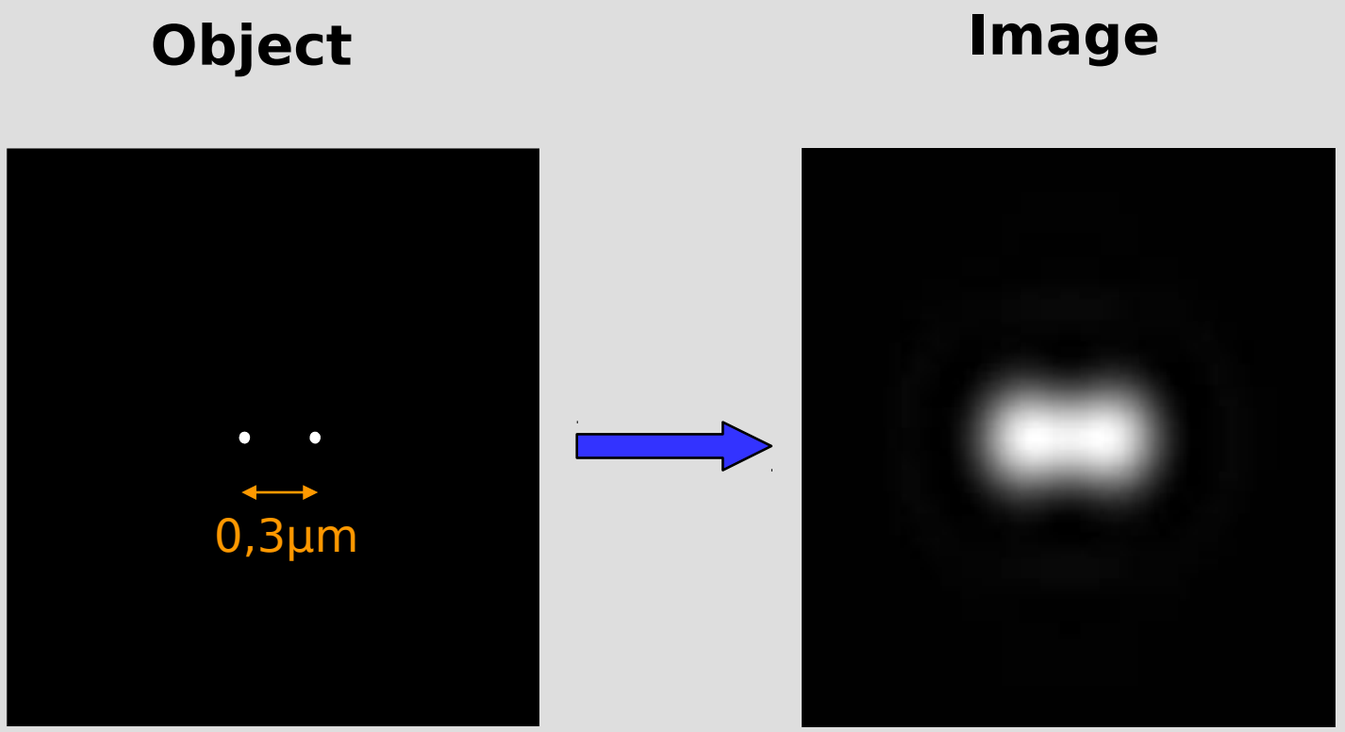
Principle effect

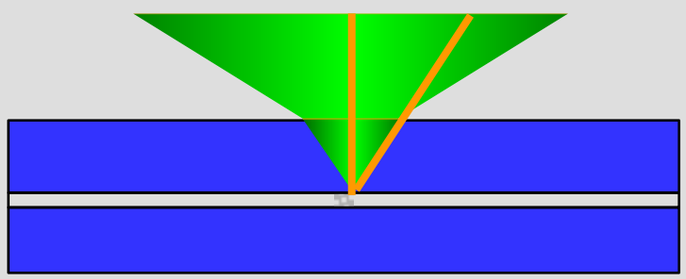
High numerical aperture objectives have a large opening angle.

The higher the NA, the better the resolution of the microscope.

$$NA = 0{,}9$$

$$\alpha \approx 36°$$





Influence de l’ouverture numerique sur la résolution

Principle effect

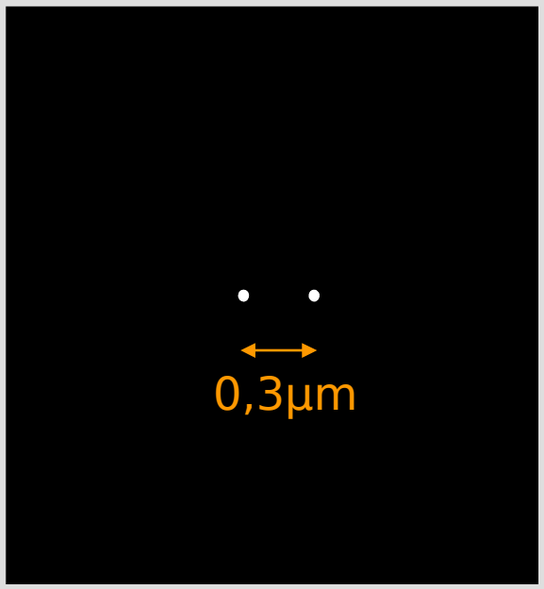
High numerical aperture objectives have a large opening angle.

The higher the NA, the better the resolution of the microscope.

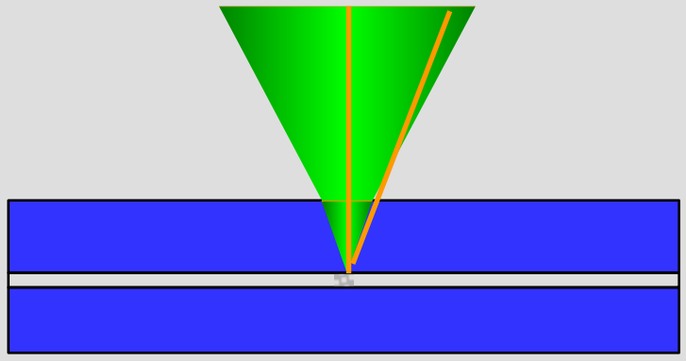
$$NA = 0{,}5$$

$$\alpha \approx 20°$$

Object



Image



Influence de l’ouverture numerique sur la résolution

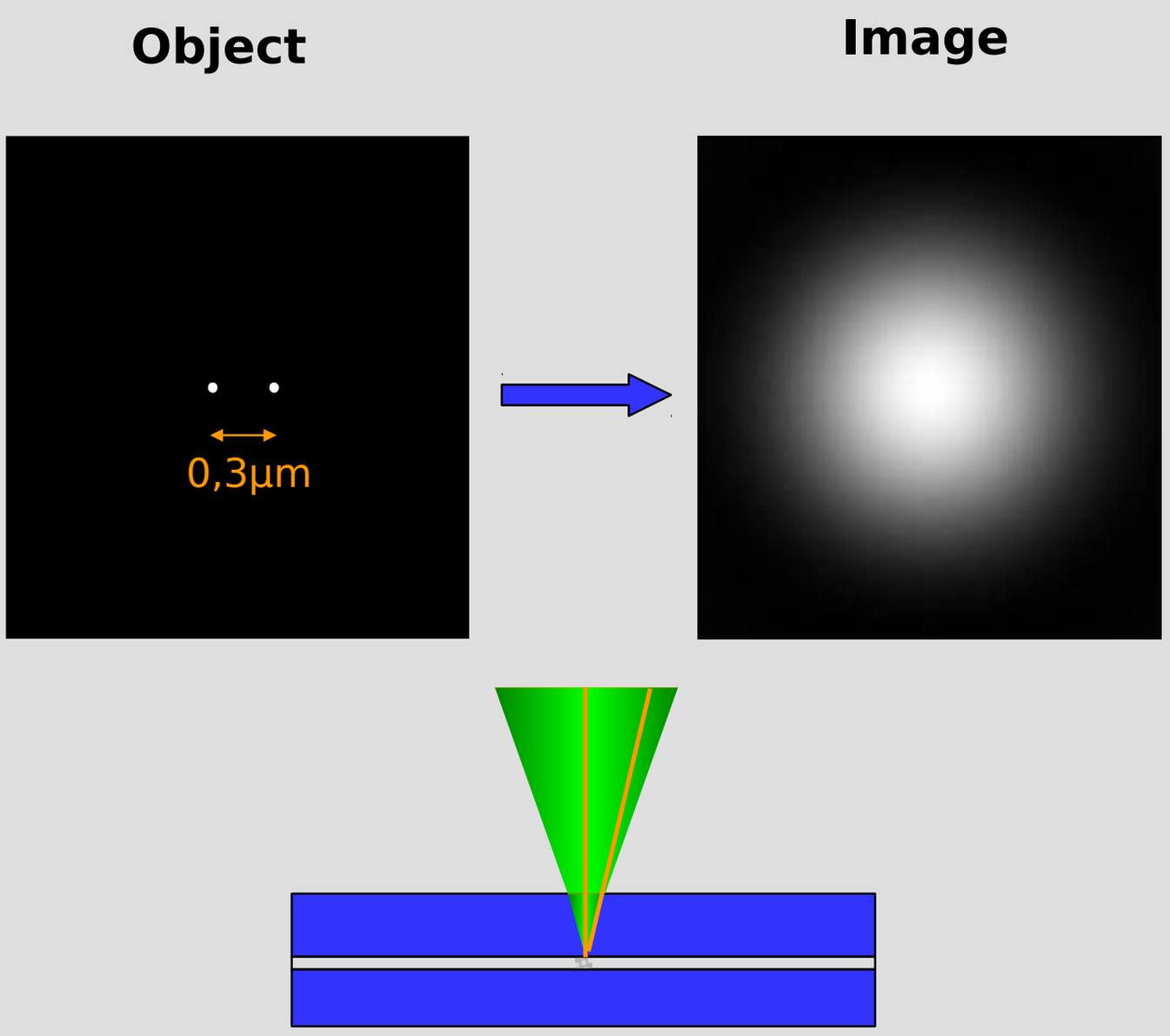
Principle effect

High numerical aperture objectives have a large opening angle.

The higher the NA, the better the resolution of the microscope.

$$NA = 0{,}3$$

$$\alpha \approx 12°$$



Influence de l’ouverture numerique sur la résolution

Principle effect

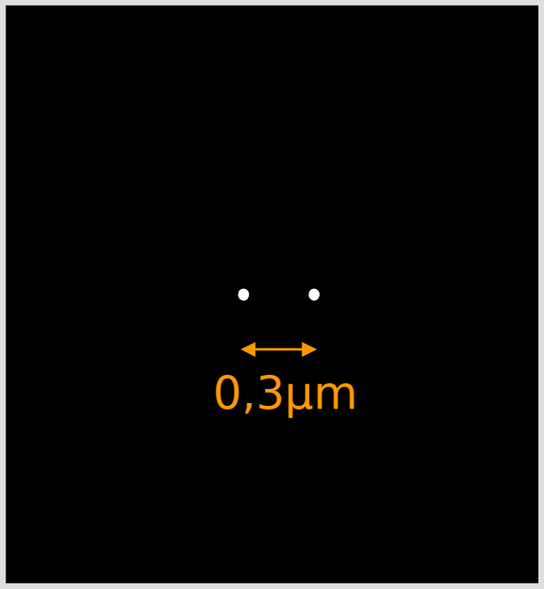
High numerical aperture objectives have a large opening angle.

The higher the NA, the better the resolution of the microscope.

$$NA = 0{,}1$$

$$\alpha \approx 6°$$

Object



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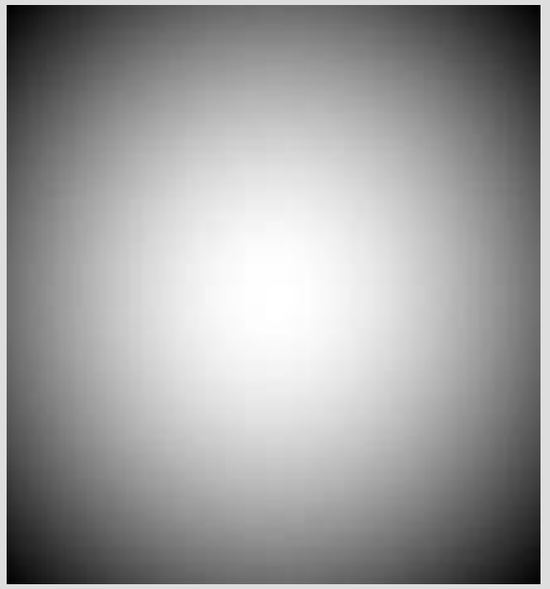
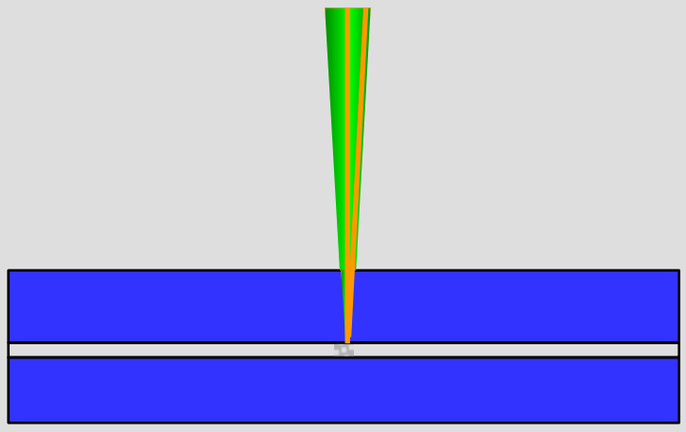




Influence de la longueur d onde sur la résolution

Principle effect

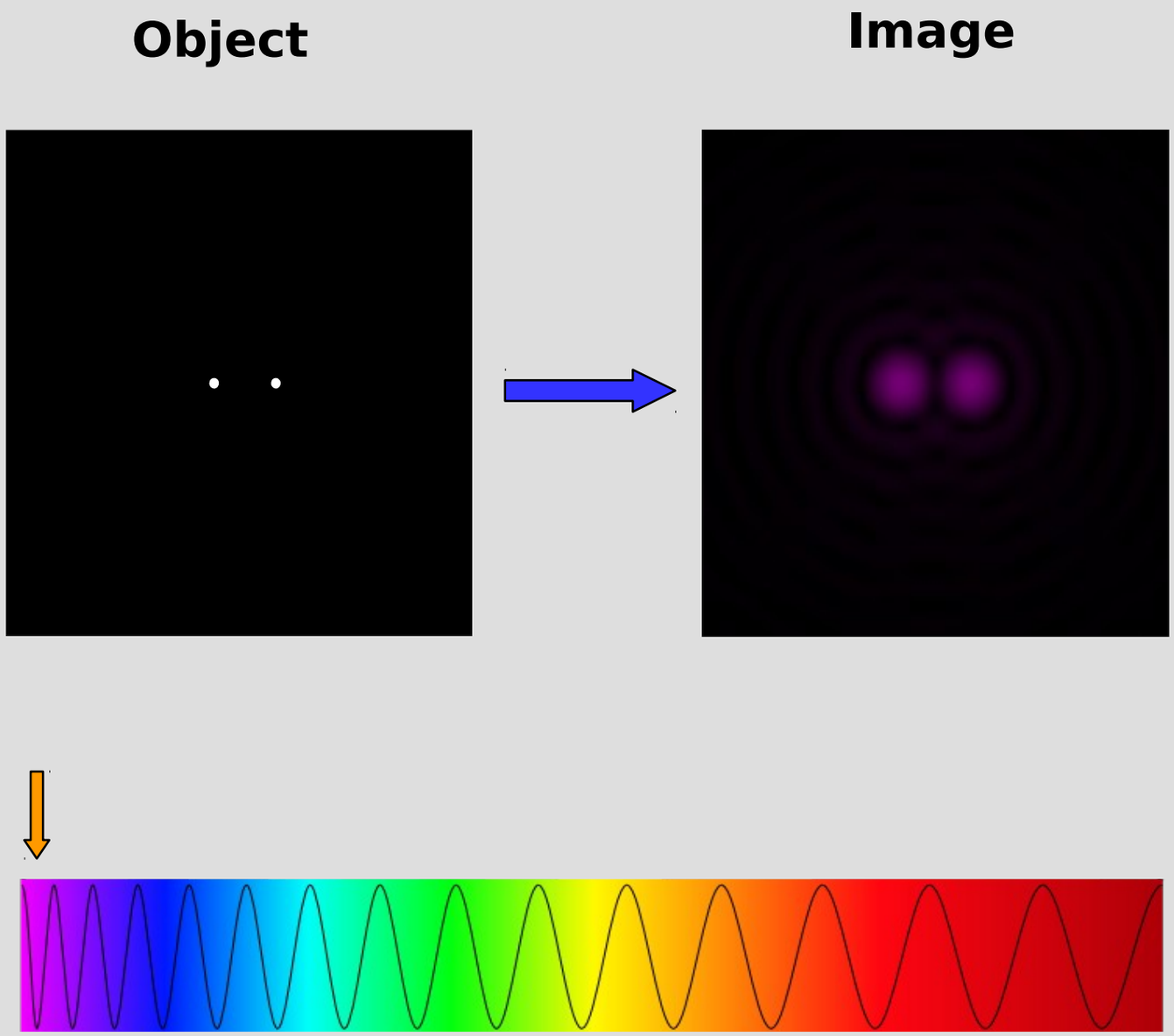
Shorter wavelengths generate smaller Point-Spread-Functions.

The shorter the wavelength, the better the resolution.

$$d = 150\text{nm}$$

$$NA = 1{,}4$$

$$\lambda = 350\text{nm}$$



Influence de la longueur d onde sur la résolution

Principle effect

Shorter wavelengths generate smaller Point-Spread-Functions.

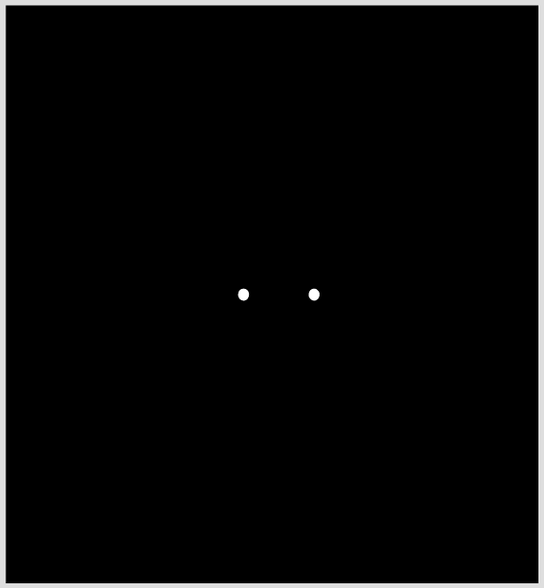
The shorter the wavelength, the better the resolution.

$$d = 210\text{nm}$$

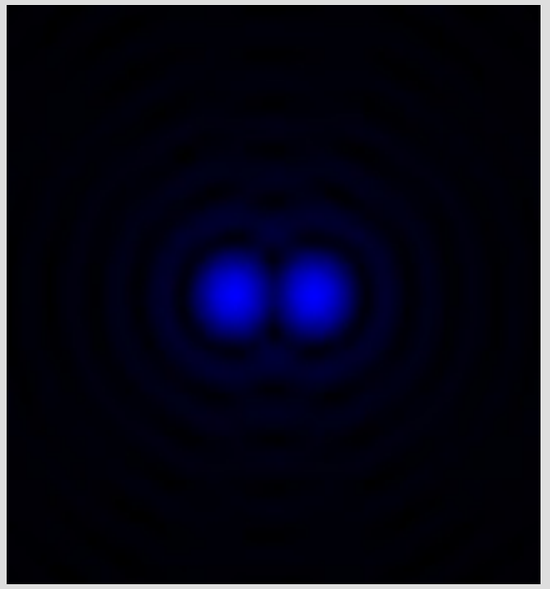
$$NA = 1{,}4$$

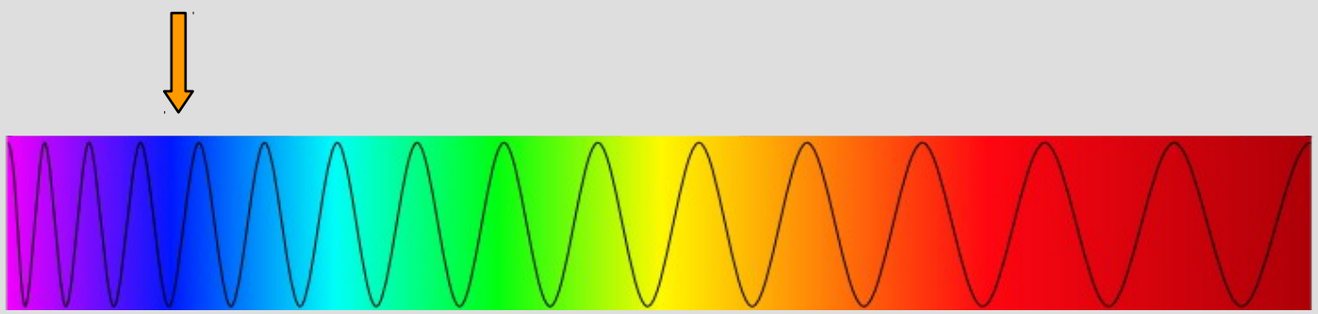
$$\lambda = 480\text{nm}$$

Object



Image





Influence de la longueur d onde sur la résolution

Principle effect

Shorter wavelengths generate smaller Point-Spread-Functions.

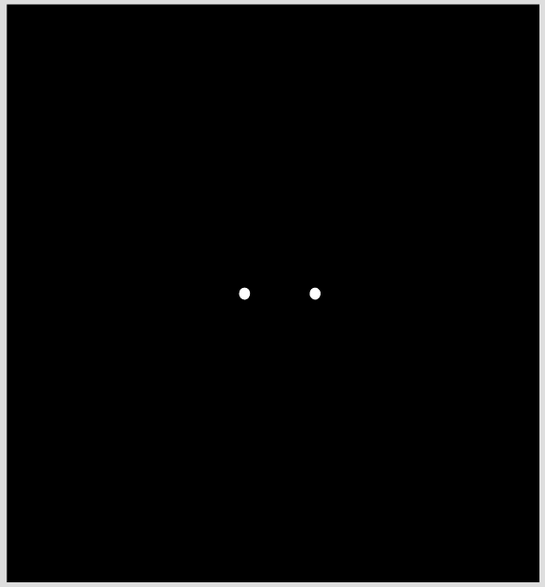
The shorter the wavelength, the better the resolution.

$$d = 230\text{nm}$$

$$NA = 1{,}4$$

$$\lambda = 520\text{nm}$$

Object



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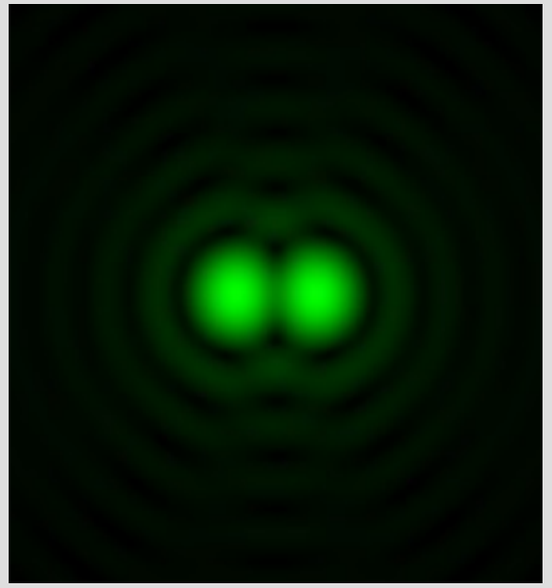
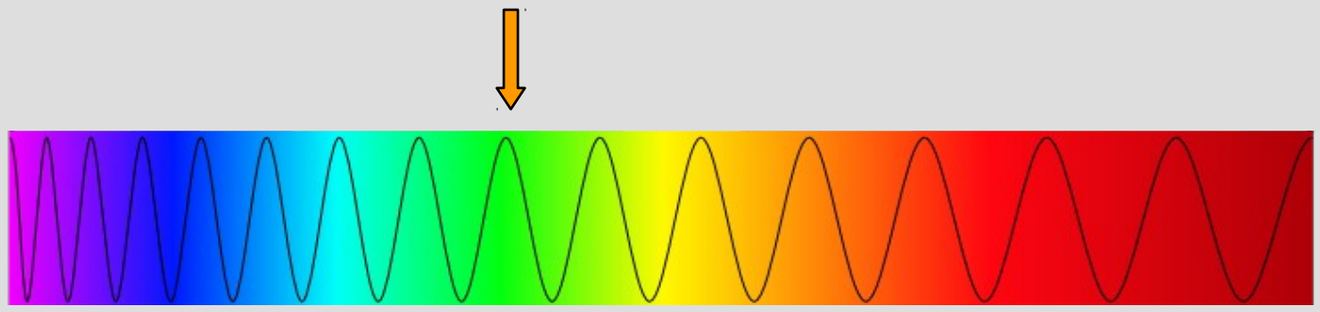




Influence de la longueur d onde sur la résolution

Principle effect

Shorter wavelengths generate smaller Point-Spread-Functions.

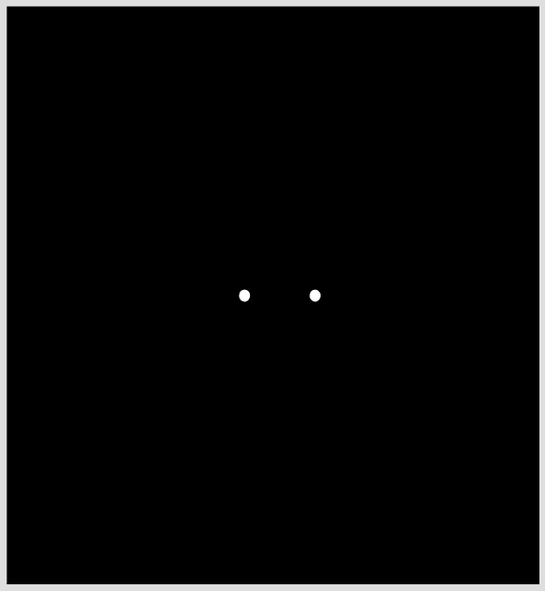
The shorter the wavelength, the better the resolution.

$$d = 250\text{nm}$$

$$NA = 1{,}4$$

$$\lambda = 570\text{nm}$$

Object



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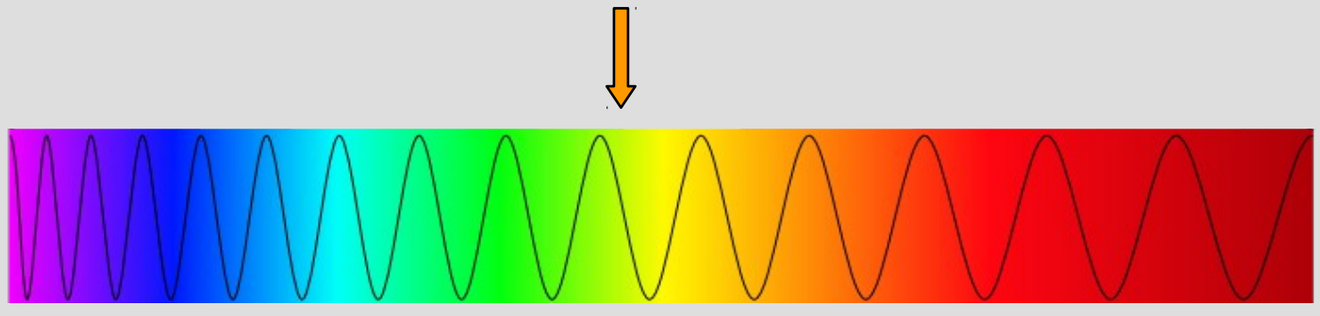


Influence de la longueur d onde sur la résolution

Principle effect

Shorter wavelengths generate smaller Point-Spread-Functions.

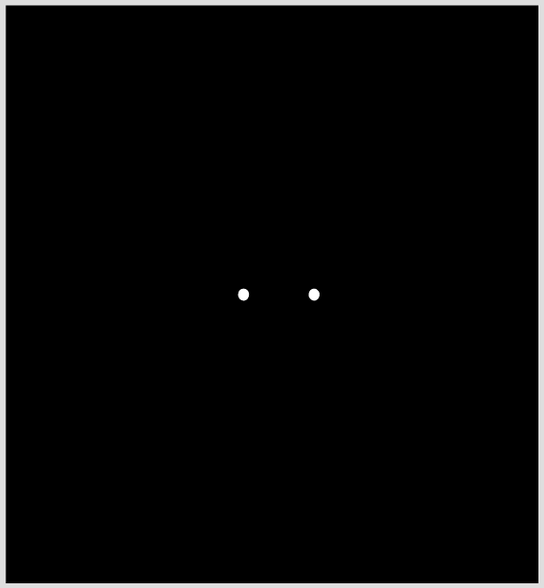
The shorter the wavelength, the better the resolution.

$$d = 265\text{nm}$$

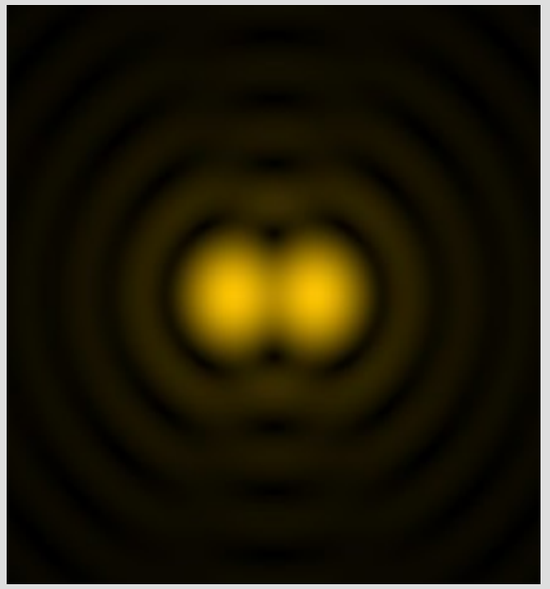
$$NA = 1{,}4$$

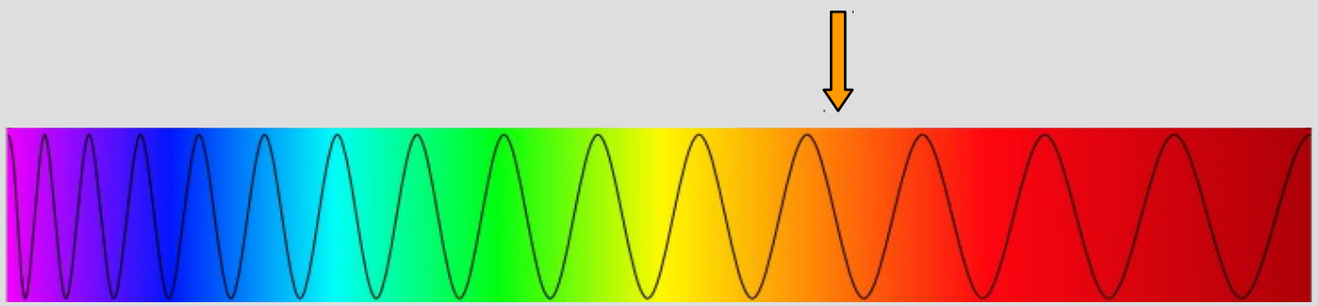
$$\lambda = 610\text{nm}$$

Object



Image





Influence de la longueur d onde sur la résolution

Principle effect

Shorter wavelengths generate smaller Point-Spread-Functions.

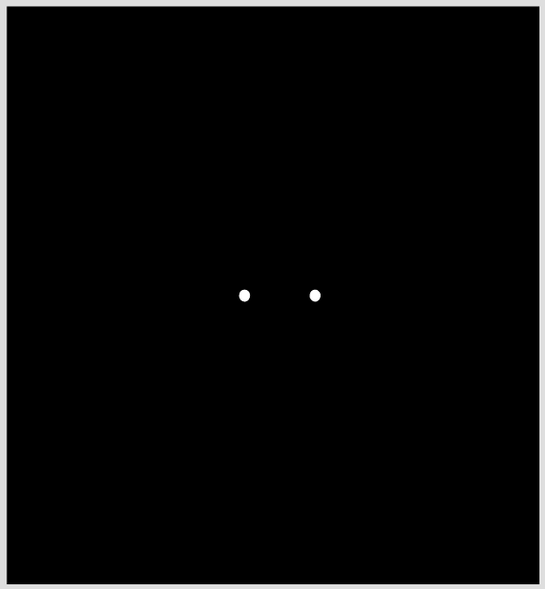
The shorter the wavelength, the better the resolution.

$$d = 280\text{nm}$$

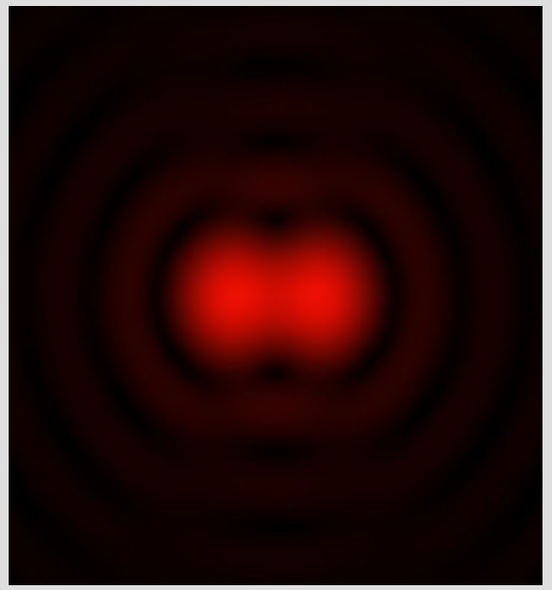
$$NA = 1{,}4$$

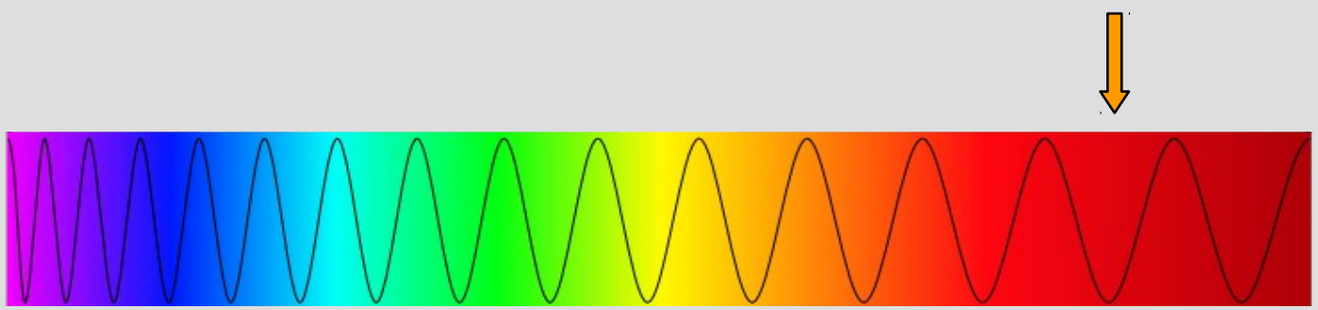
$$\lambda = 640\text{nm}$$

Object



Image





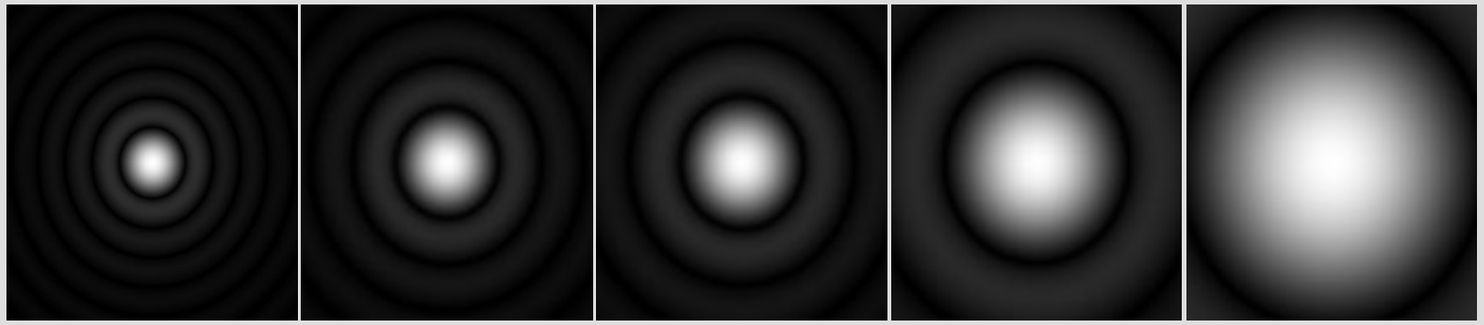
La résolution 3D en microscopie

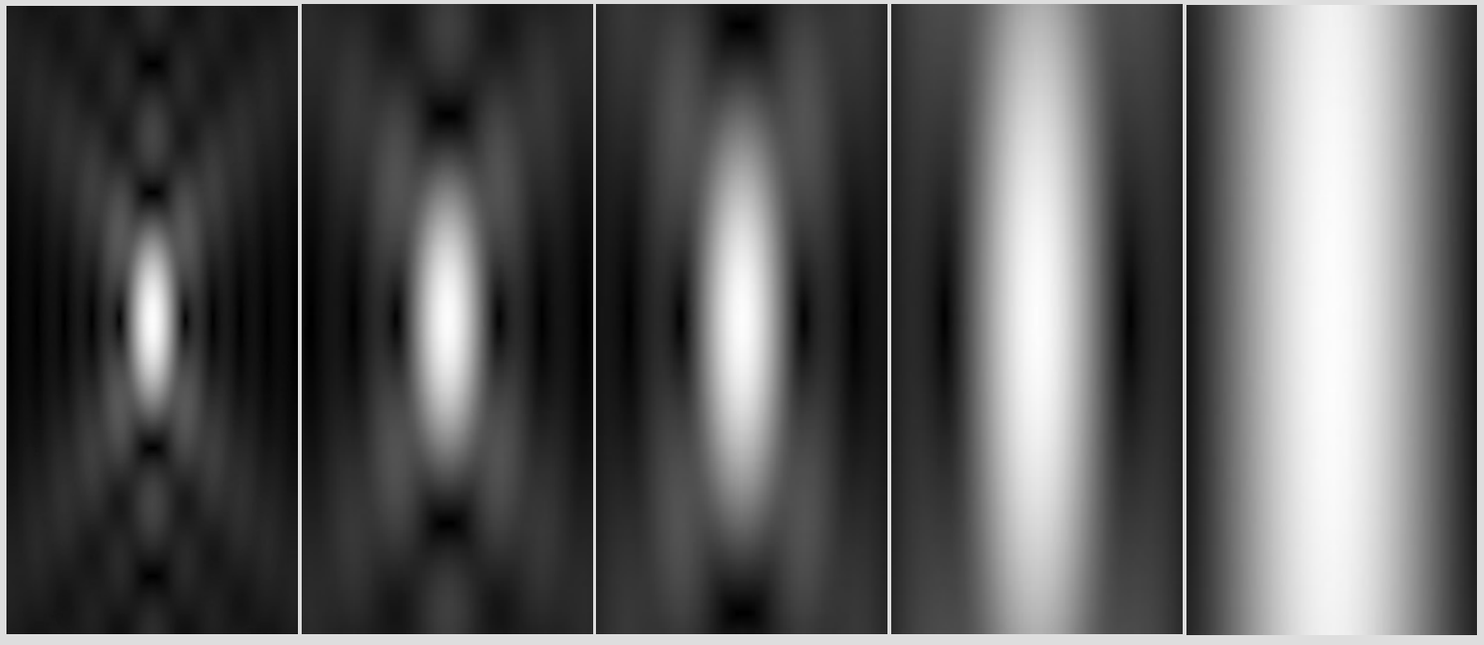
Quantitative

La resolution axiale est inférieur d‘un facteur 3-4 à la resoluiton laterale.

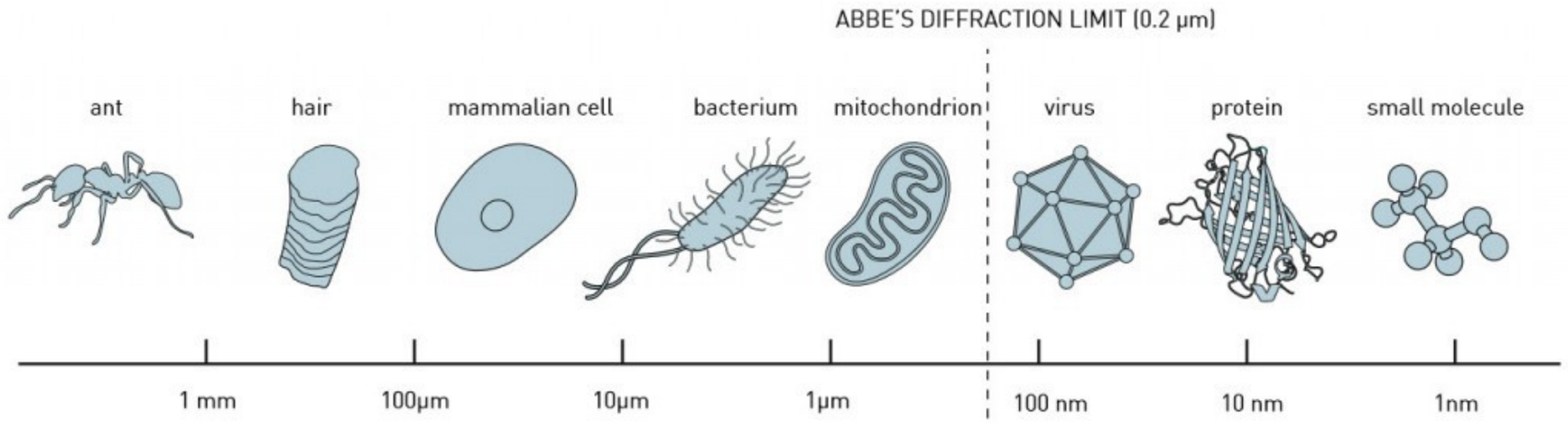
$$d_{axial} \approx 2\frac{n \cdot \lambda}{NA^2}$$

NA=1,4 NA=1,2 NA=0,9 NA=0,75 NA=0,3

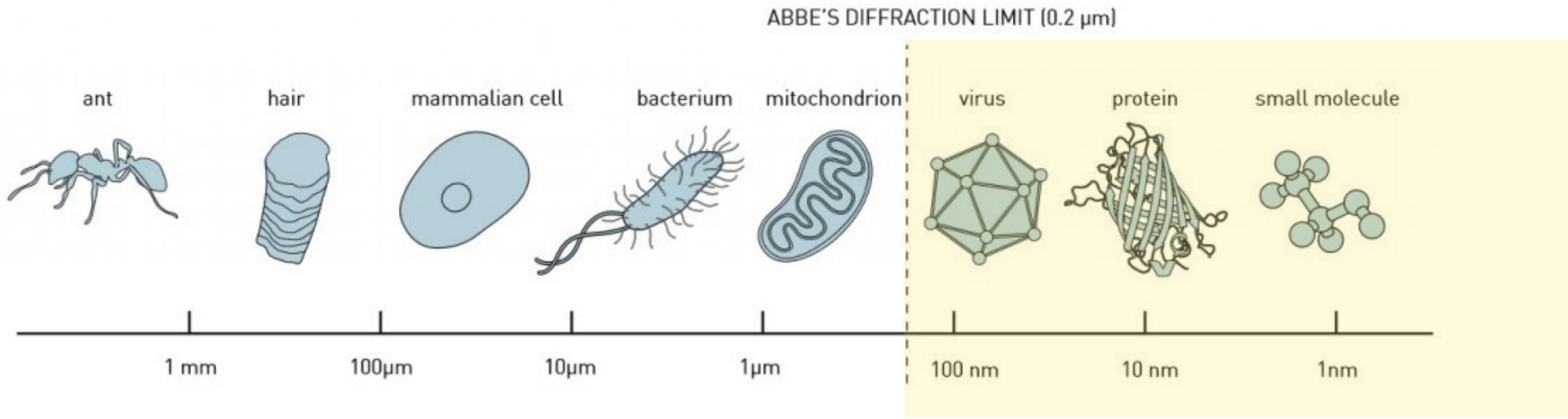




Resolution


ABBE'S DIFFRACTION LIMIT (0.2 µm)
ant
hair
mammalian cell
bacterium
mitochondrion
virus
protein
small molecule
1 mm
100µm
10µm
1µm
100 nm
10 nm
1nm

Resolution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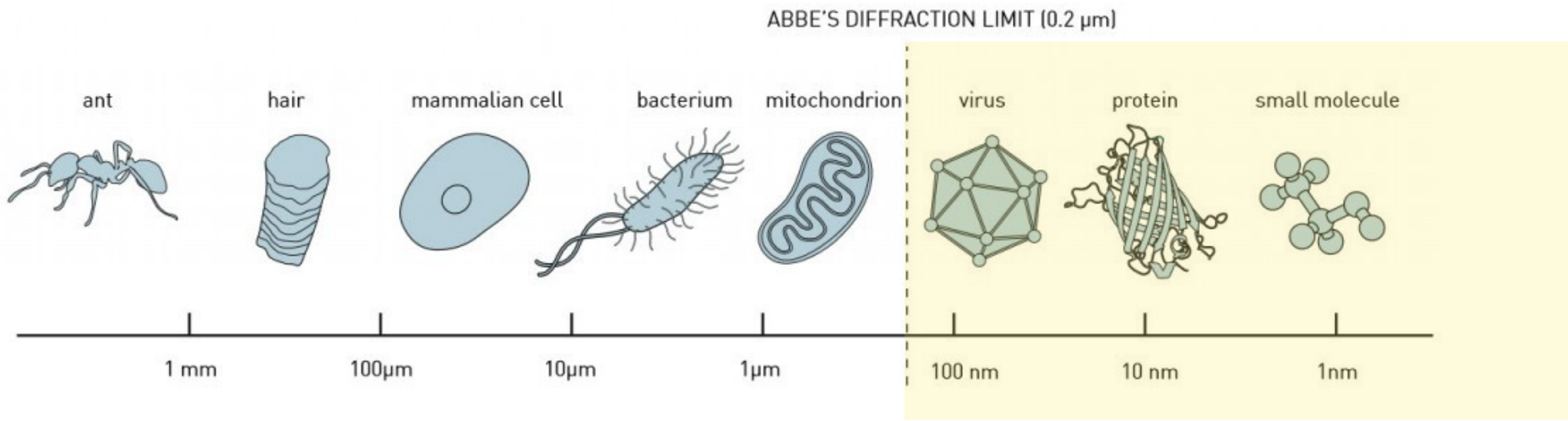
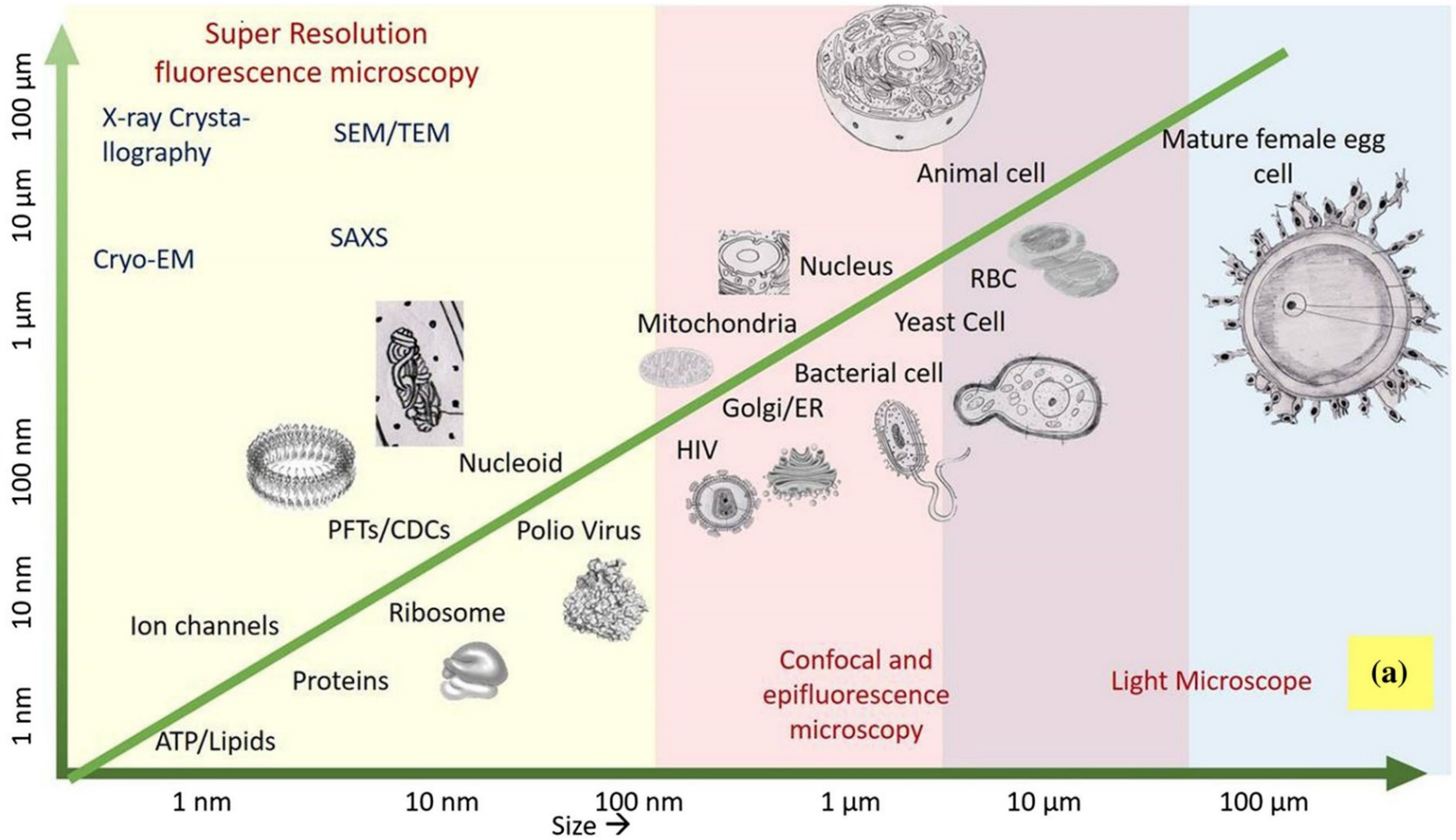


C Roobala et al.



Pushing microscopes beyond theoretical limits

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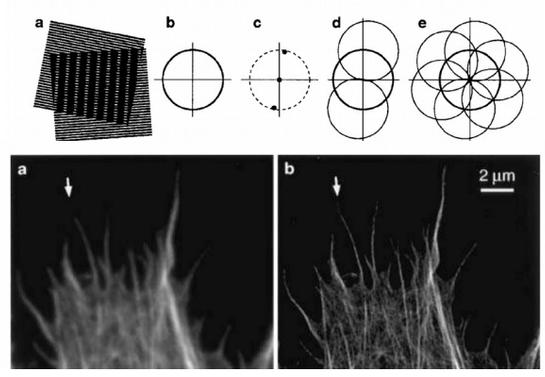
Gustafsson MGL 2000 Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy J. Microsc. 198 82–7



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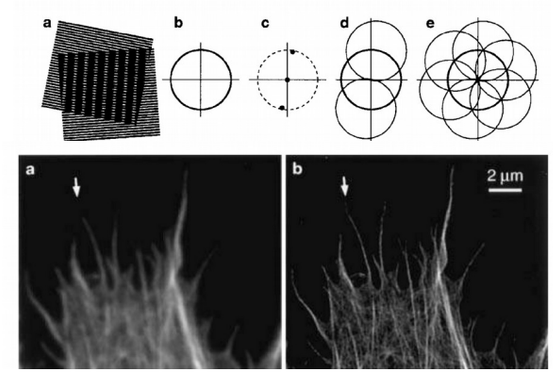

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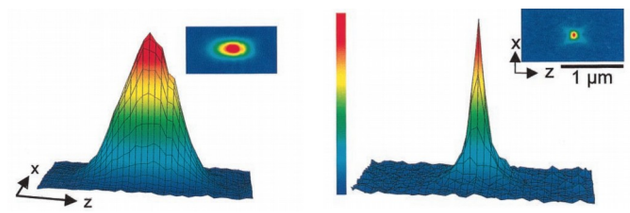

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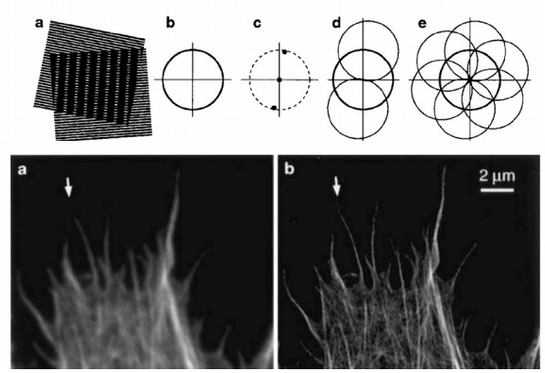

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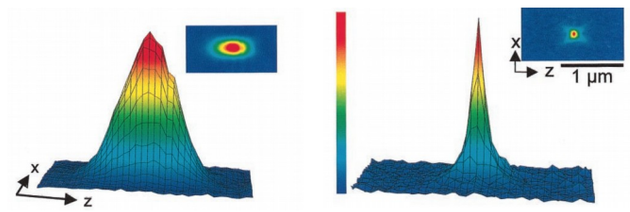

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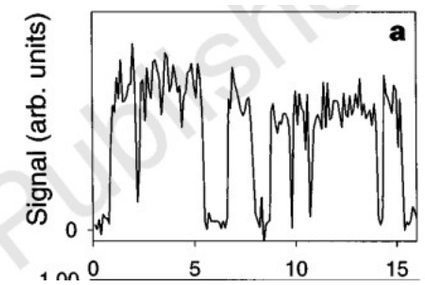

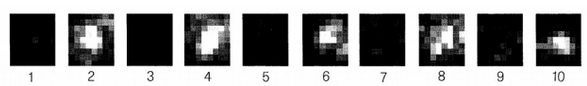

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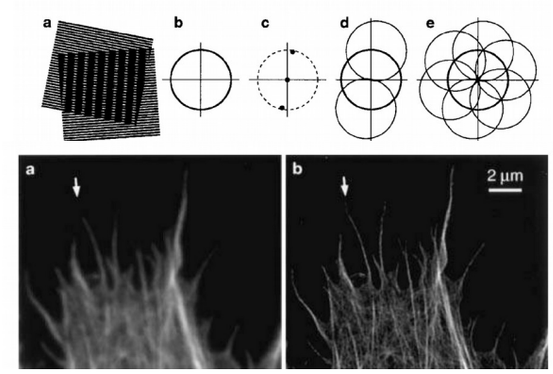

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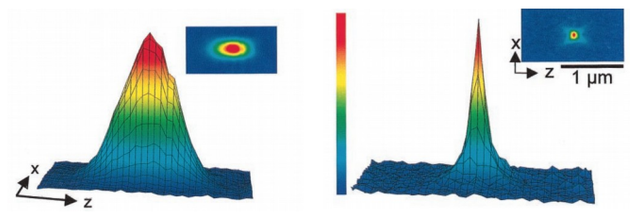

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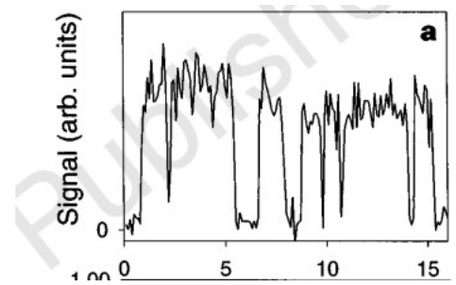
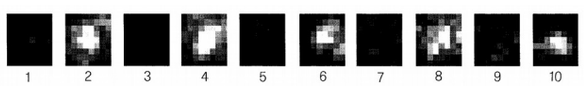
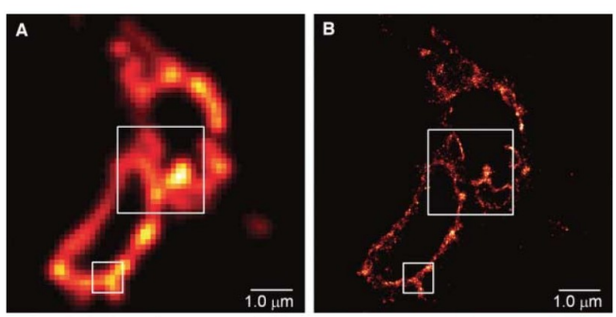




1995/2006 - E. Betzig : resolution enhancement through the localization, with sub-diffraction limit precision, of individual emitters in a sufficiently sparse population. (PALM)

Betzig E 1995 Proposed method for molecular optical imaging Opt. Lett. 20 237–237
Betzig E, PattersonGH, Sougrat R, LindwasserOW, Olenych S, Bonifacino J S, DavidsonMW, Lippincott-Schwartz J and HessHF 2006 Imaging intracellular fluorescent proteins at nanometer resolution Science 313 1642–5



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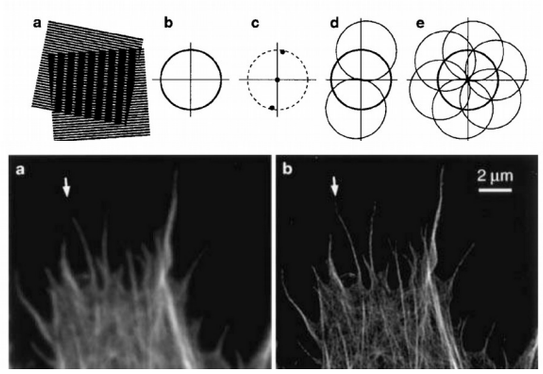

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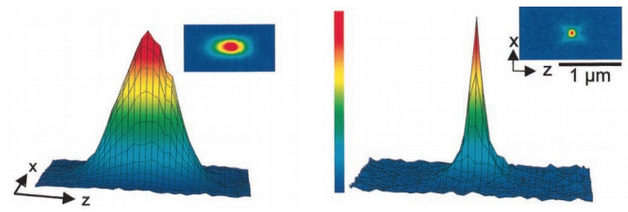

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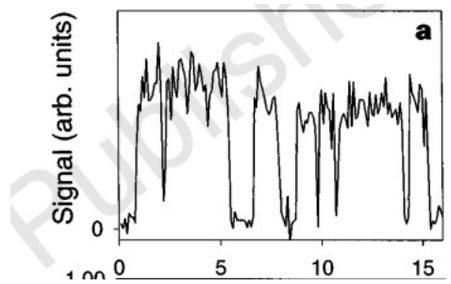

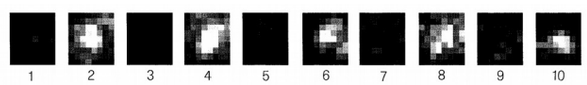

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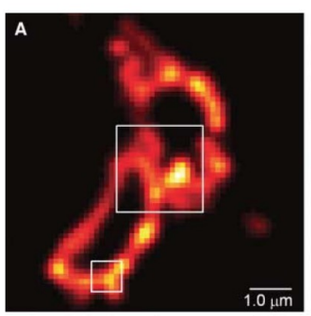

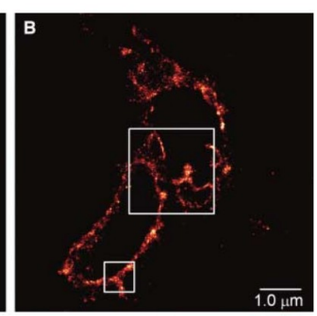

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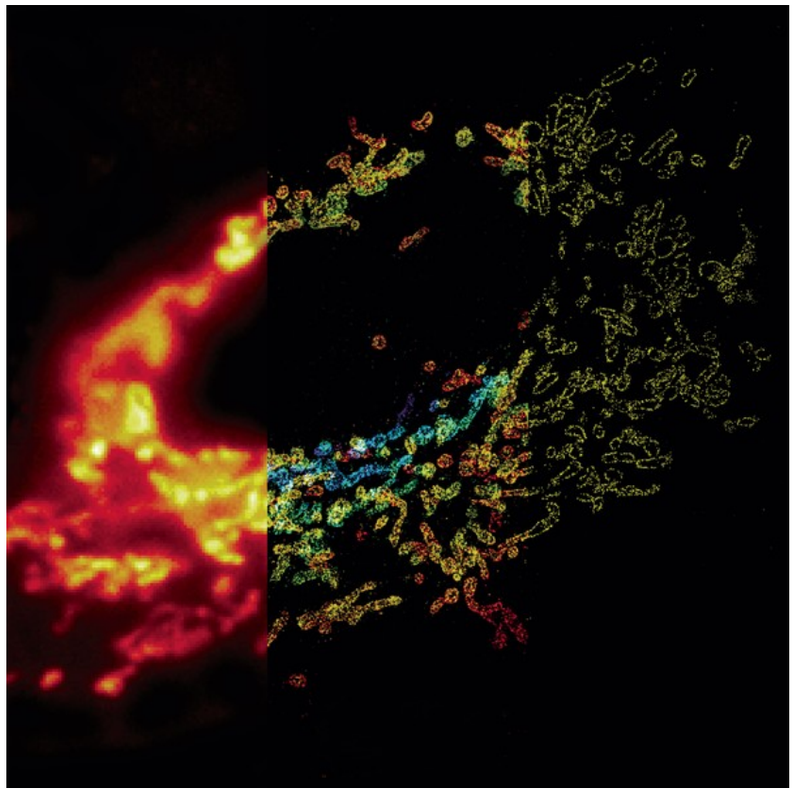

Comparison of conventional and 3D STORM image of mitochondria in a cell.

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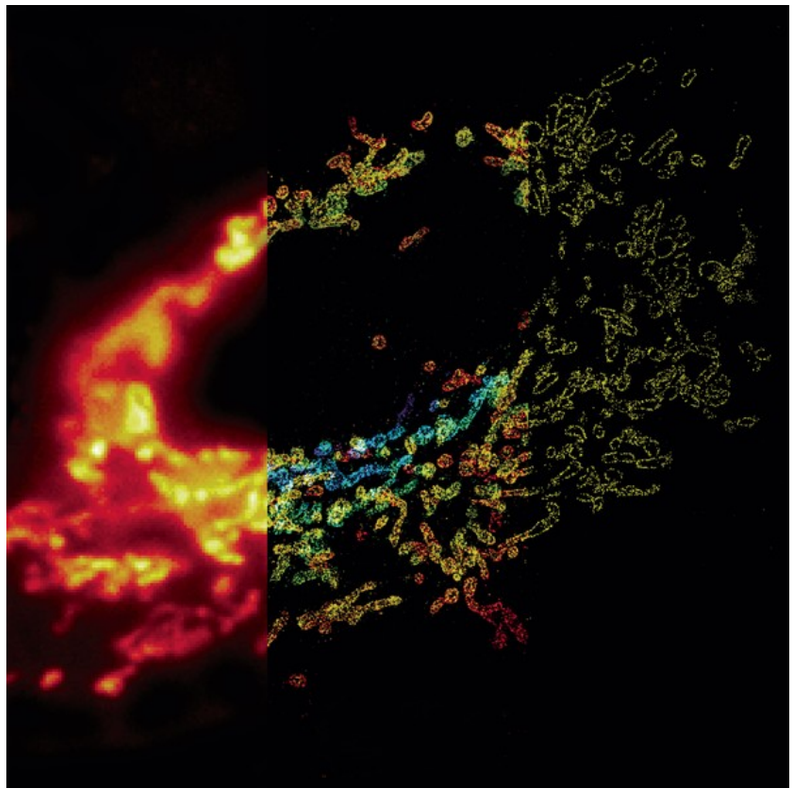

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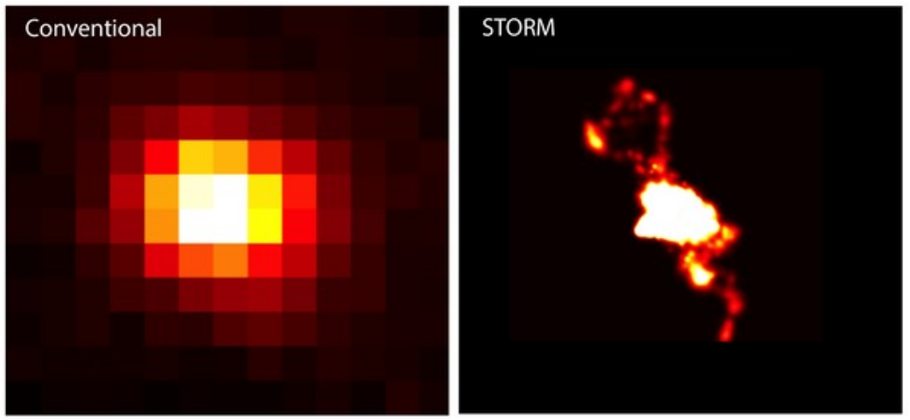
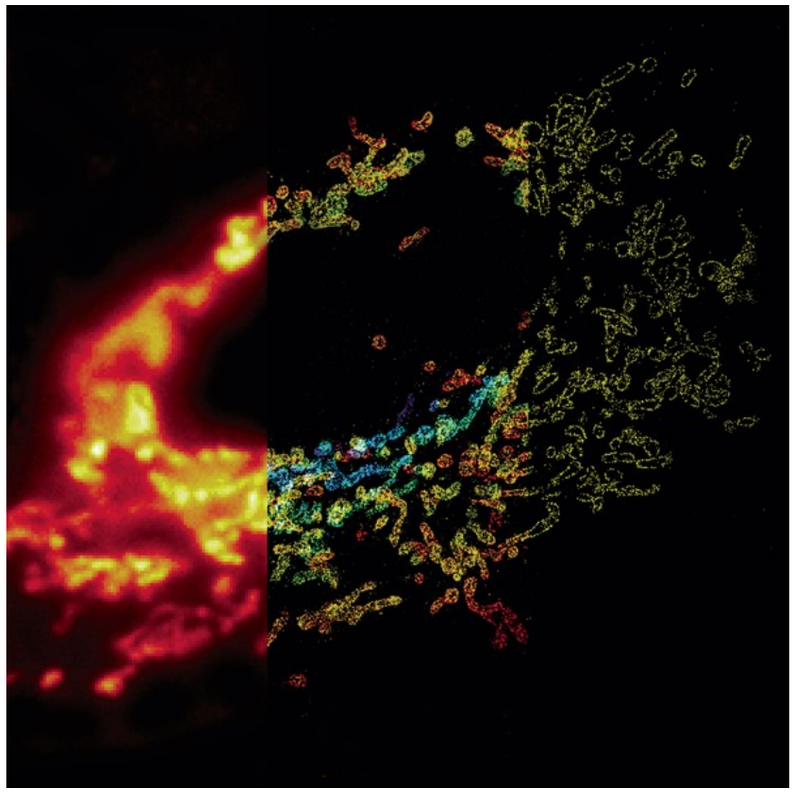


Comparison of conventional (upper) and STORM (bottom) images of chromatin in the nucleus. A specific locus of the chromatin is labeled by fluorescence in situ hybridization with photoswitchable dyes.

Comparisons



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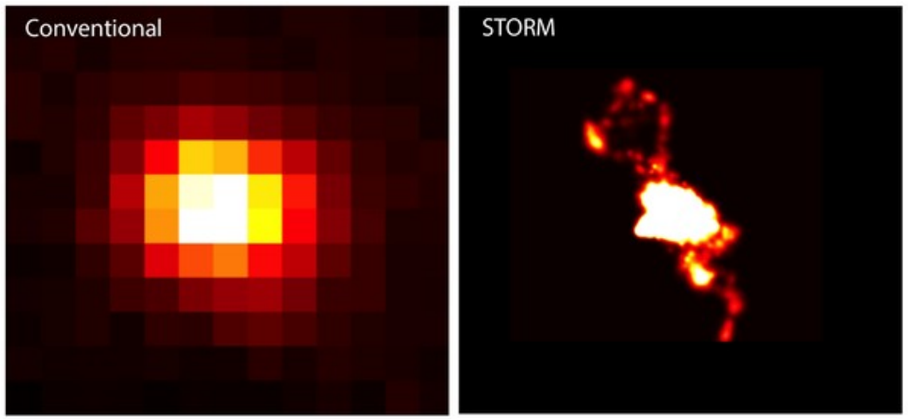

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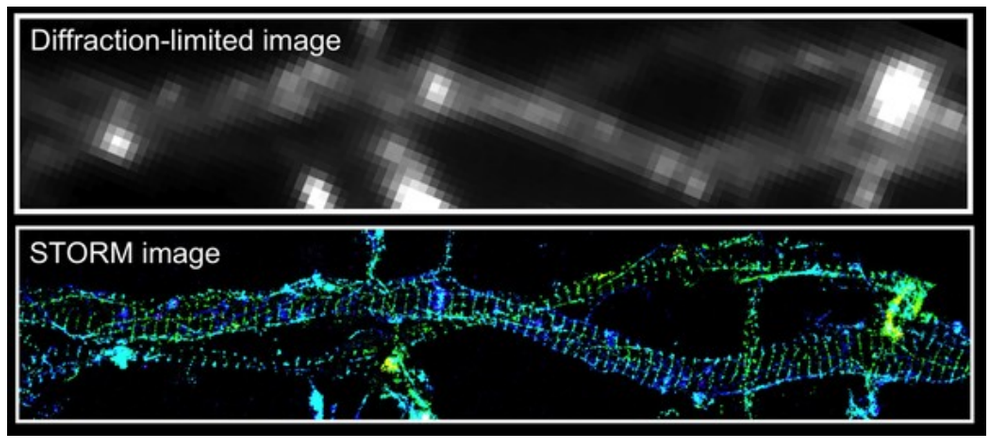

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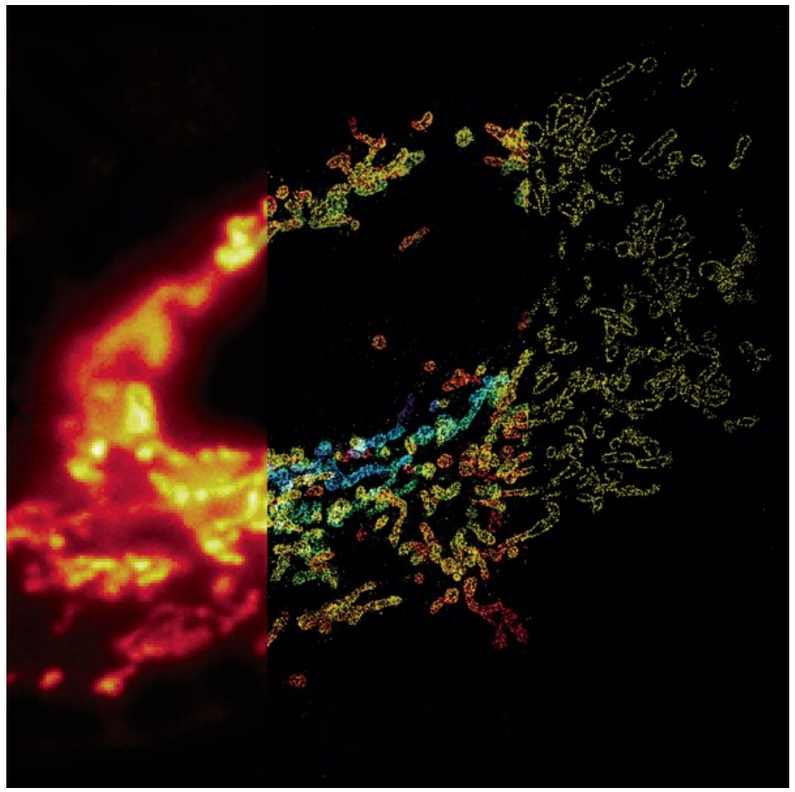

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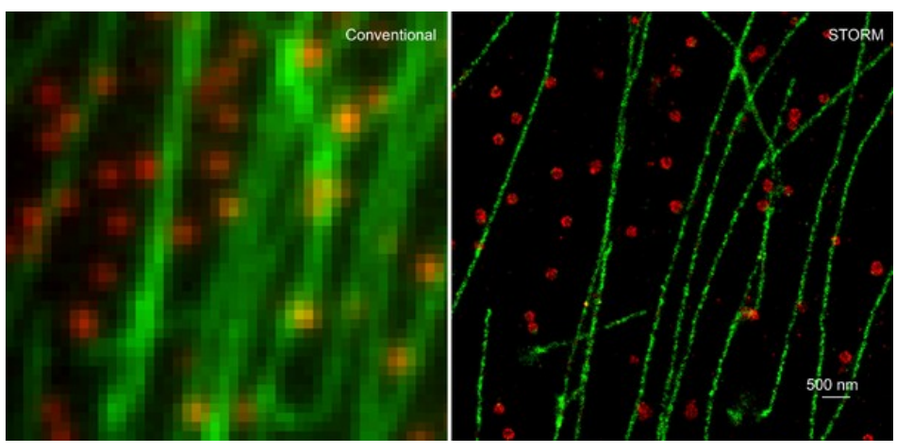

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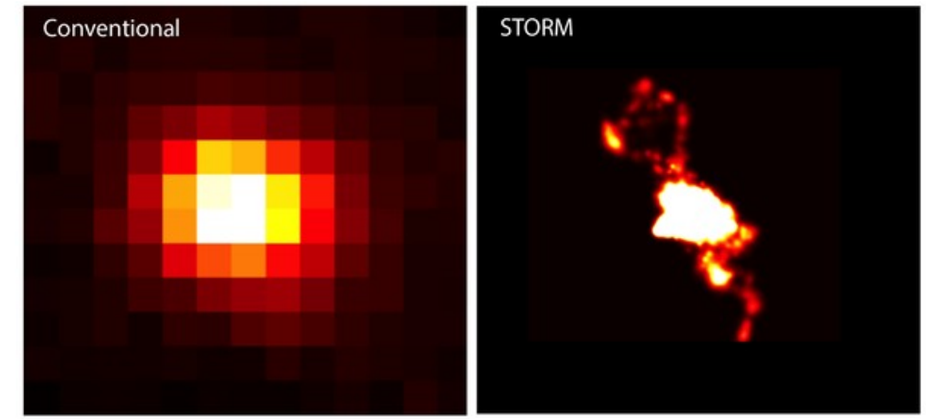

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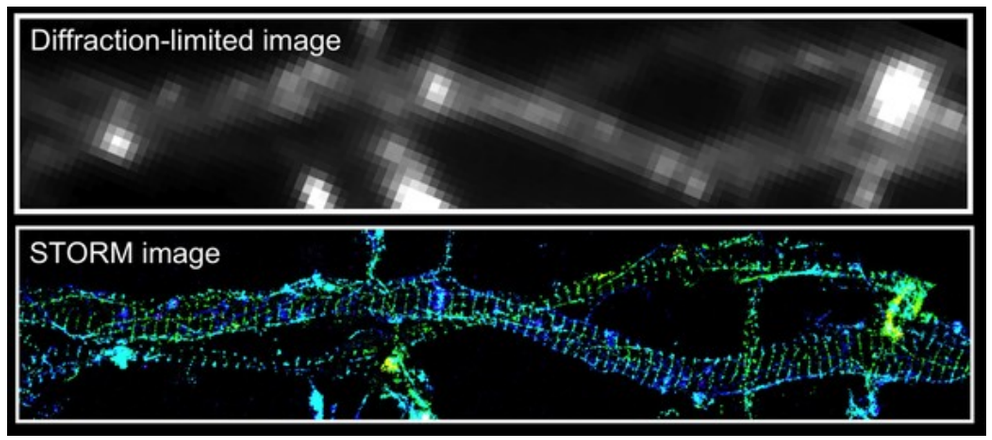

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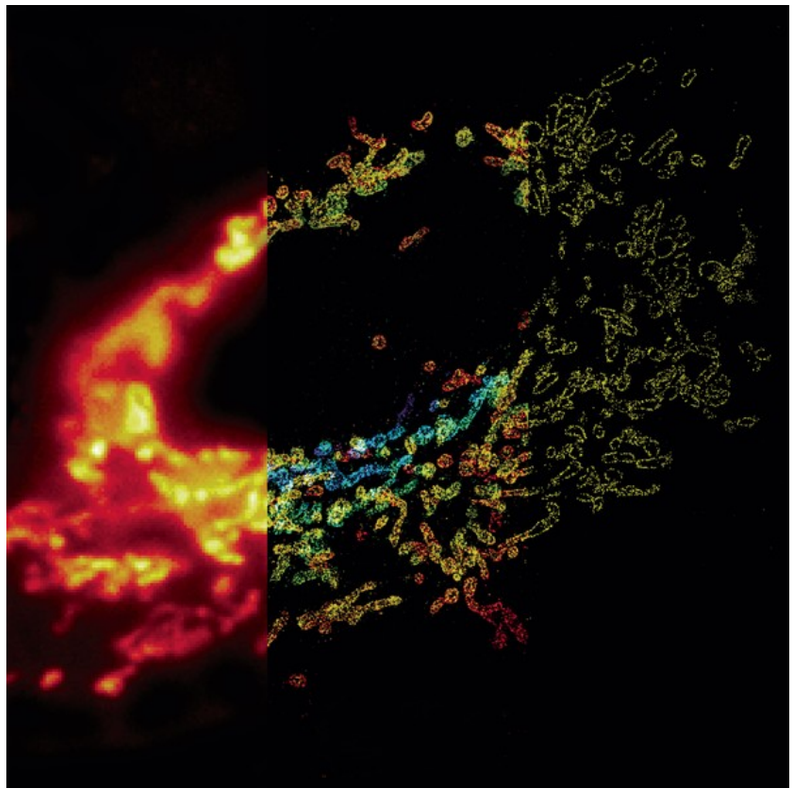

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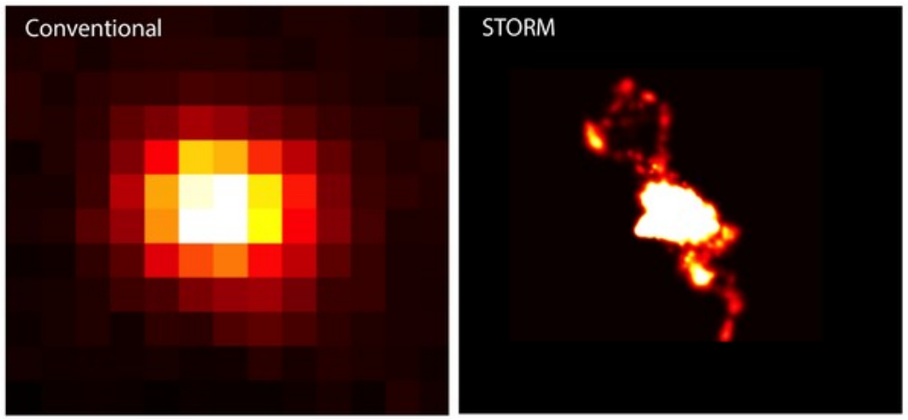

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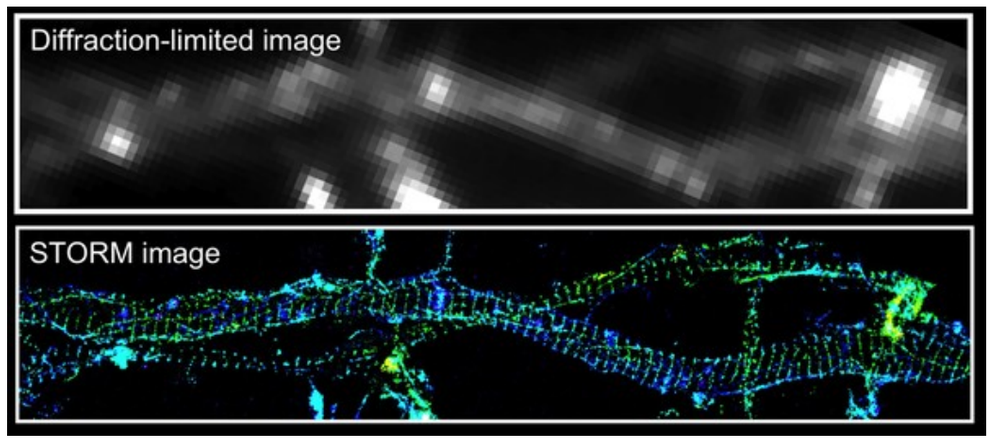

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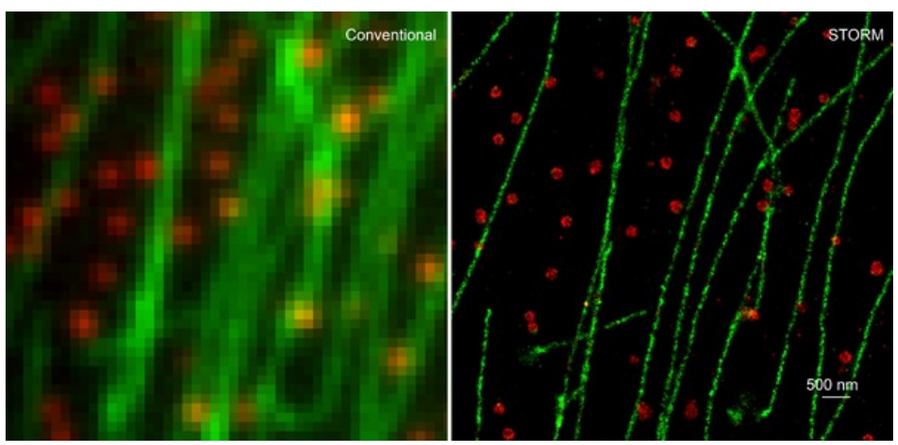

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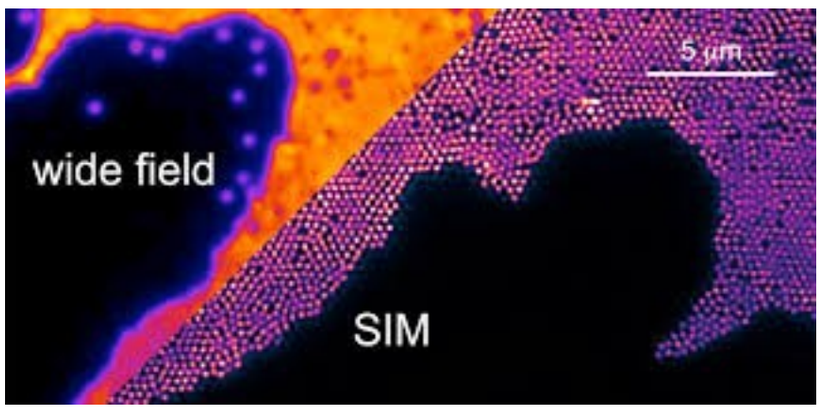
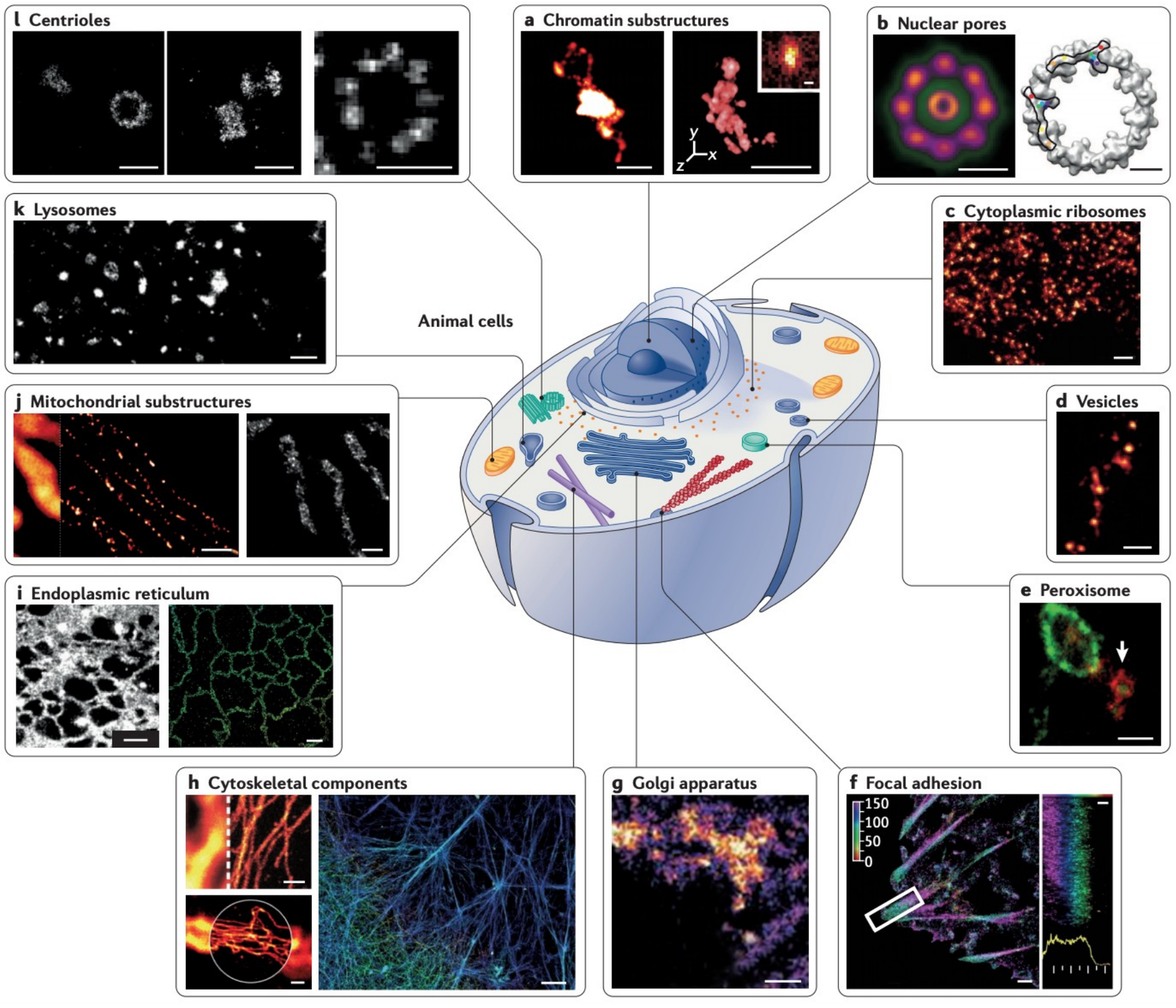


Structured Illumination Microscopy image of fluorescent beads

What is beyond the diffraction limit

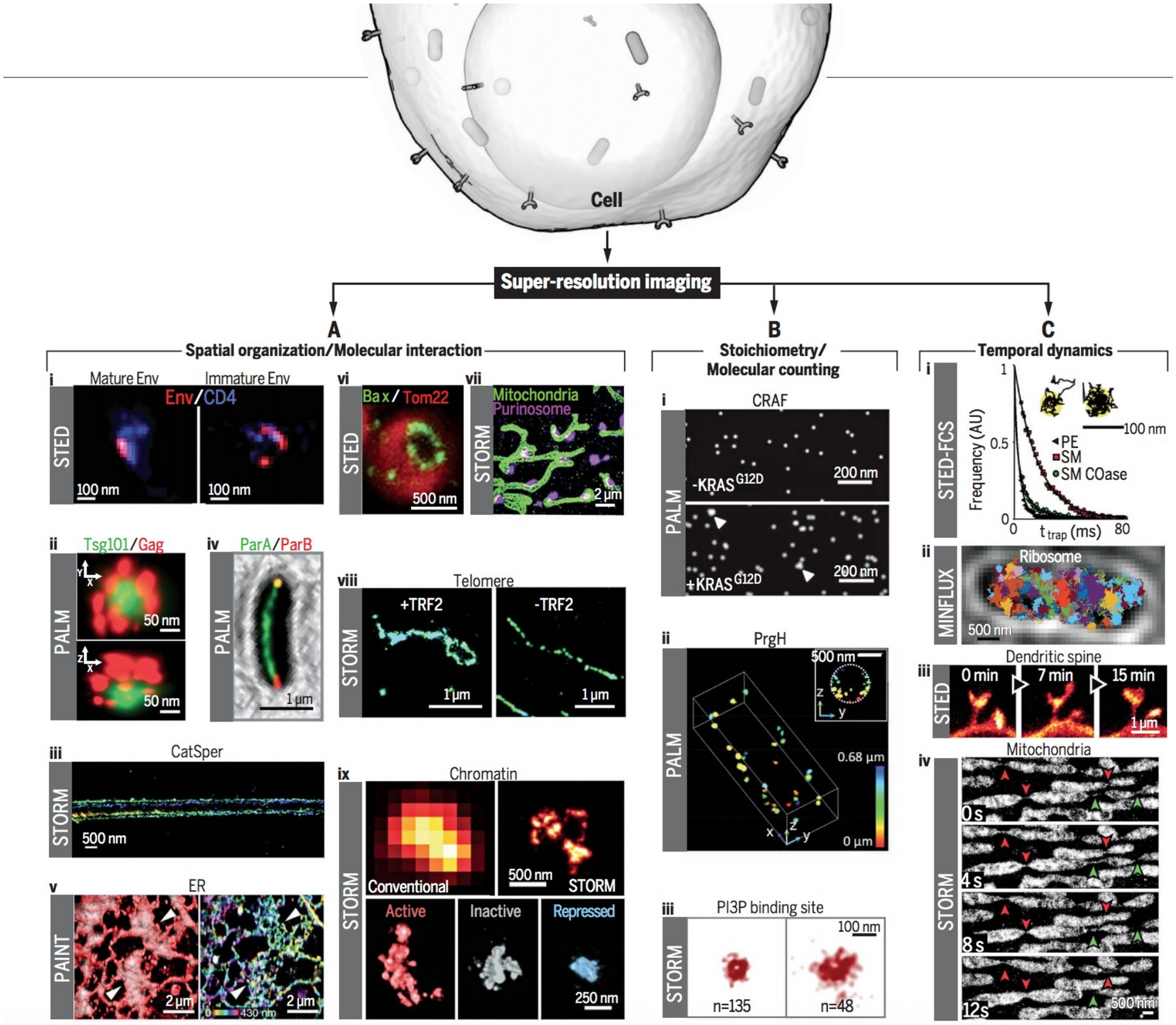


Christophe Leterrier

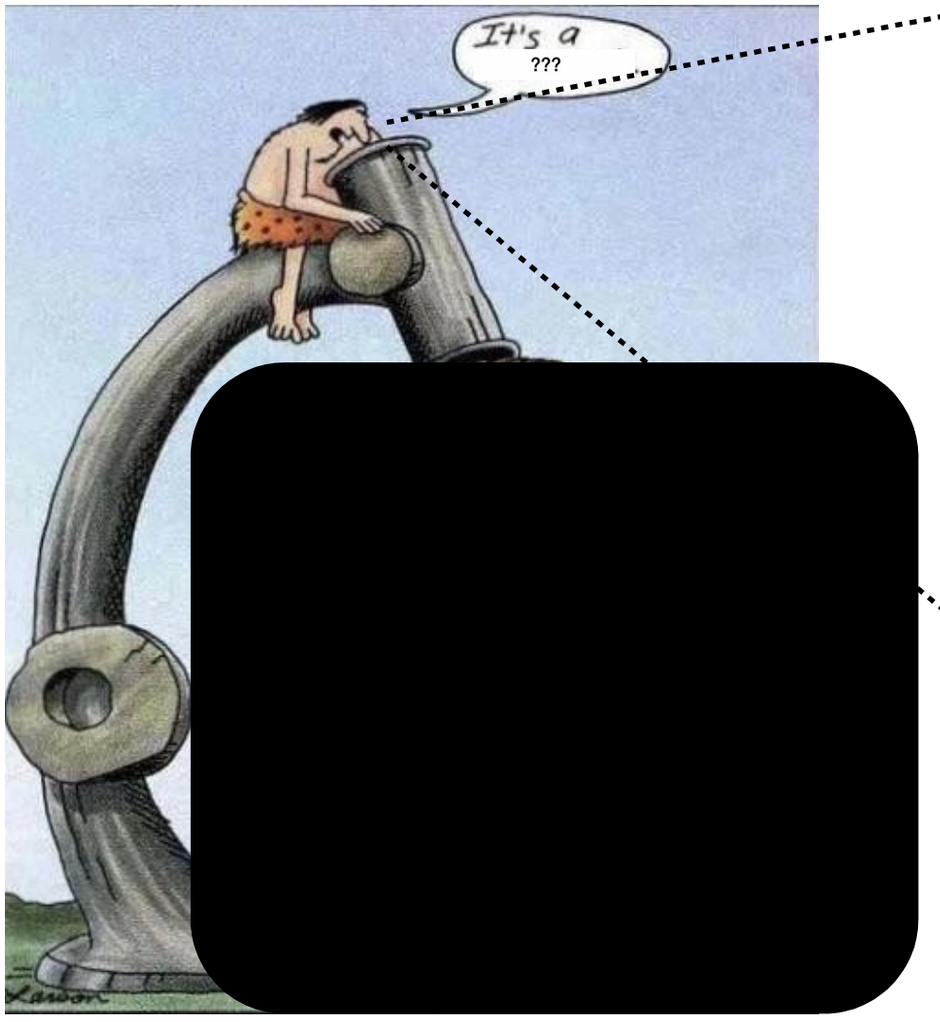
https://www.nikonsmallworld.com/techniques/structured-illumination-microsco

https://www.nikonsmallworld.com/techniques/super-resolution-microscopy

Beyond pretty images



Not only resolution matters, ...



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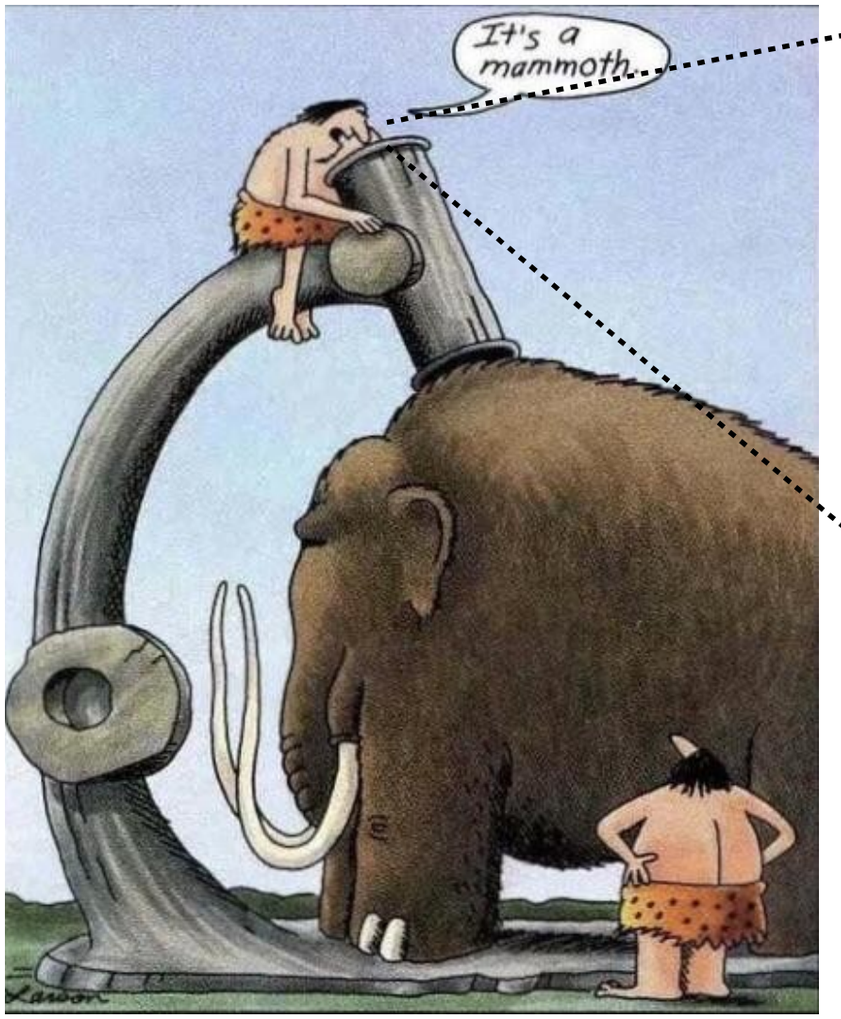

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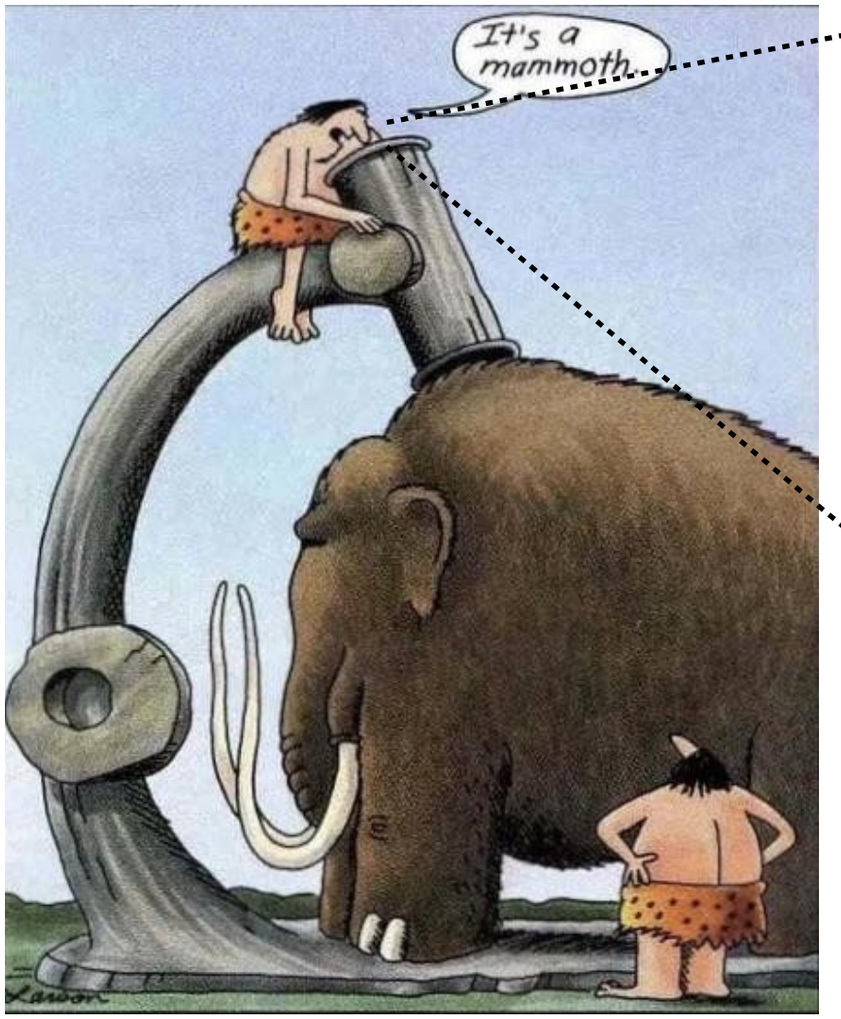
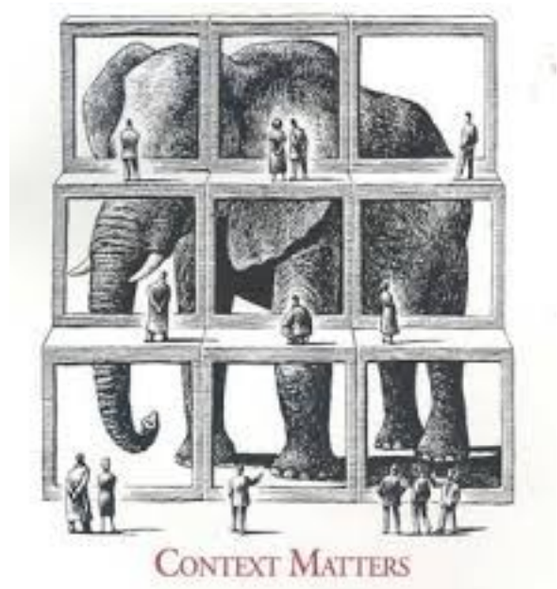


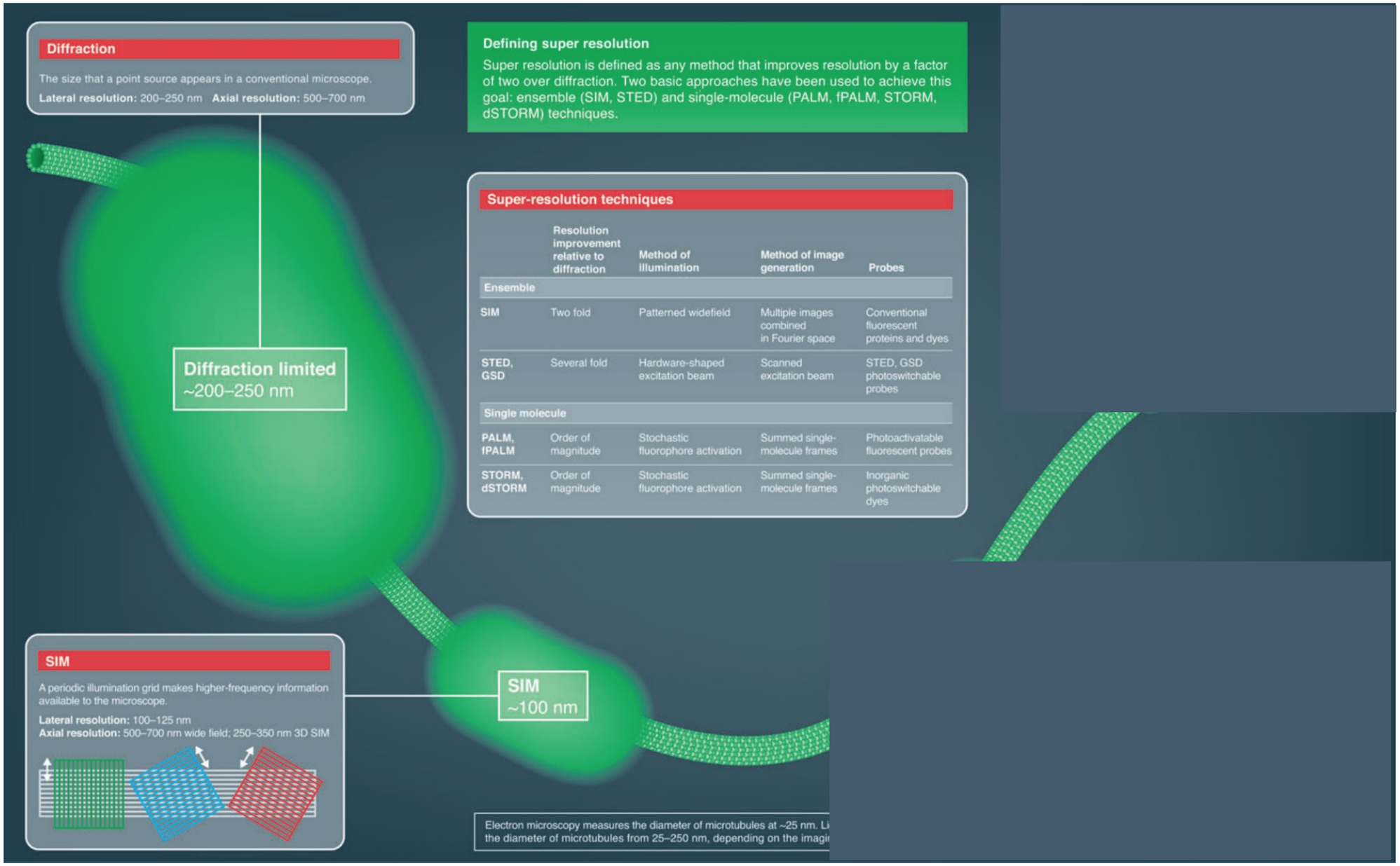
Context :

- FOV size
- 3D (depth)
- t (dynamics)
- λ (colors)
- ...



Super resolution microscopies

Super resolution microscopies



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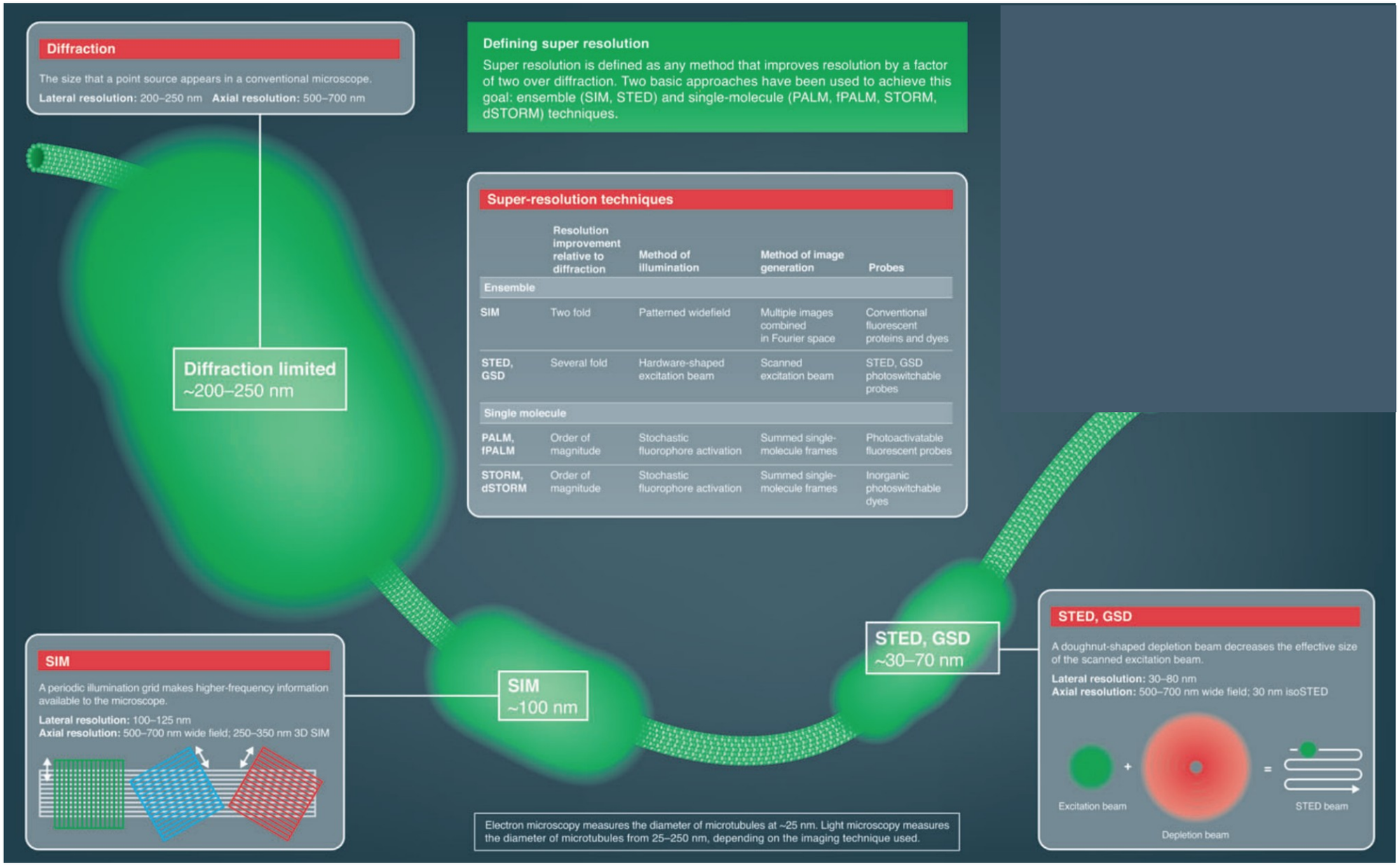

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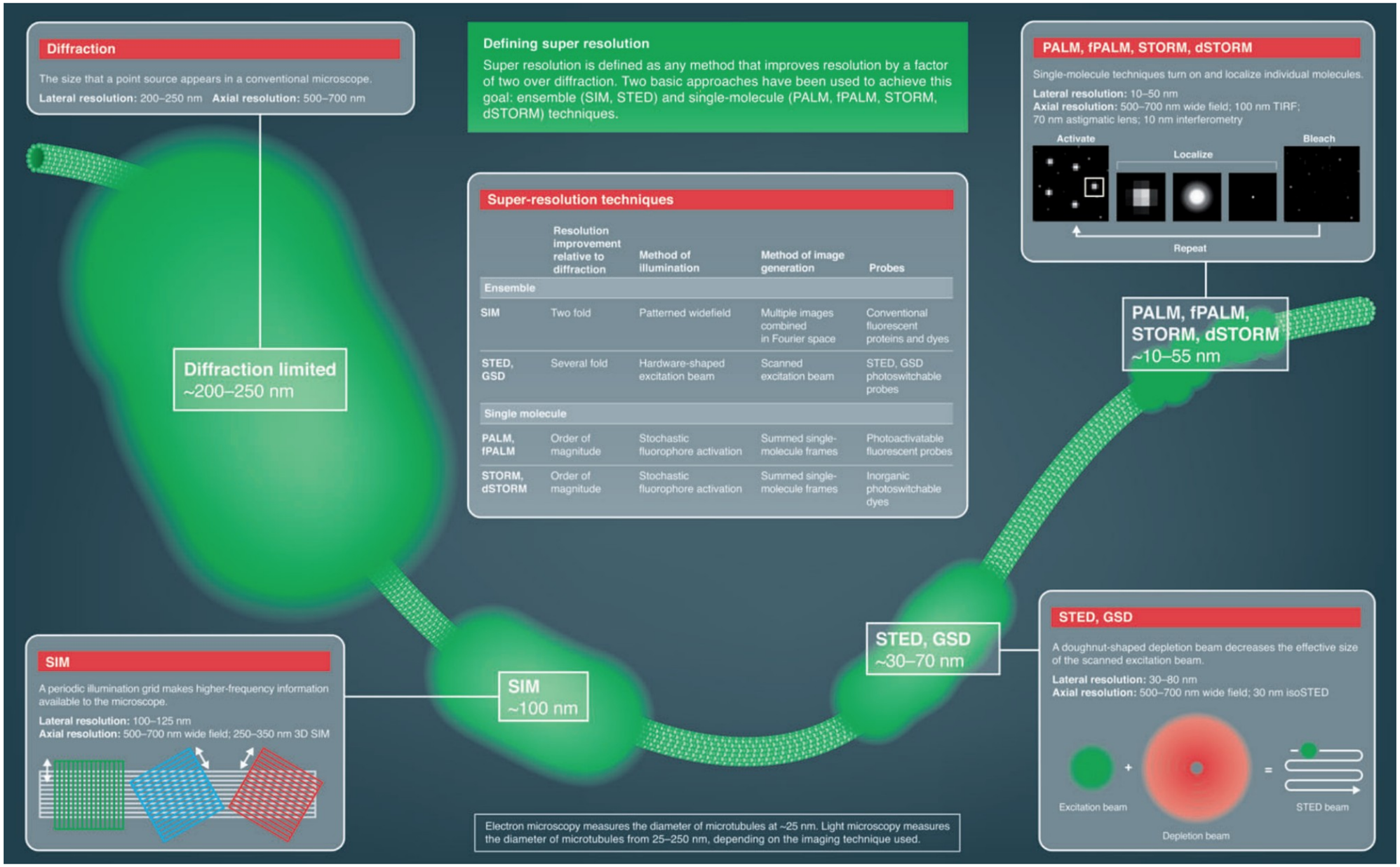


Outline

1. Introduction

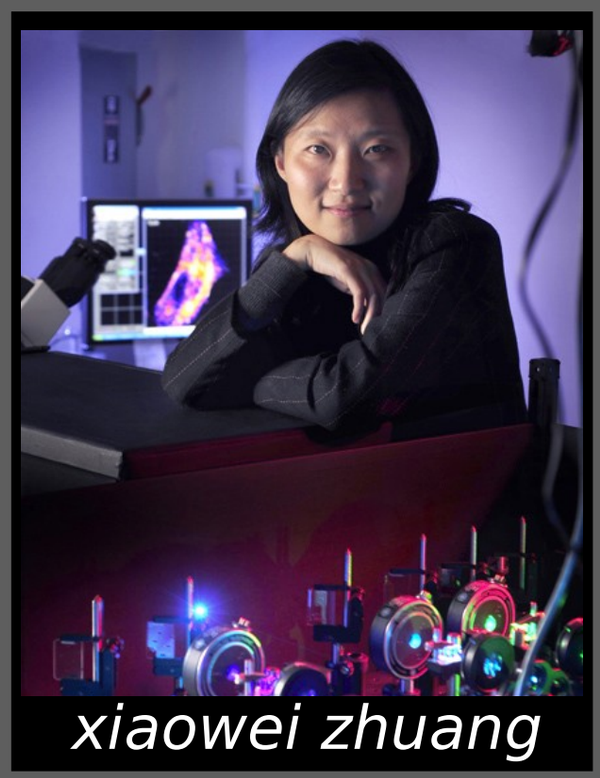
2. Single molecule super resolution microscopy

3. Structured illumination super resolution microscopy

4. Case study

Single molecule Localization microscopy (SMLM)

Eric Betzig



xiaowei zhuang

Single molecule Localization microscopy (SMLM)

Concept

Techniques (PALM, STORM, Paint, etc…) / Fluorescent probes

How to do SMLM

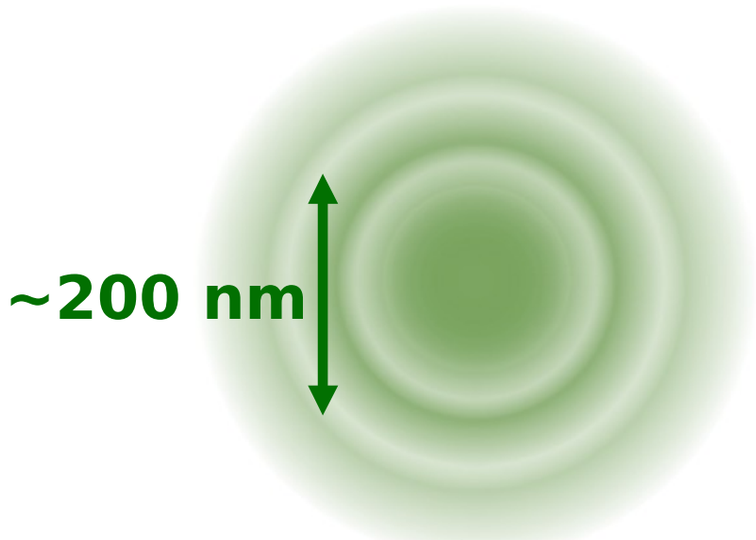
3D

Multicolor (Im registration)

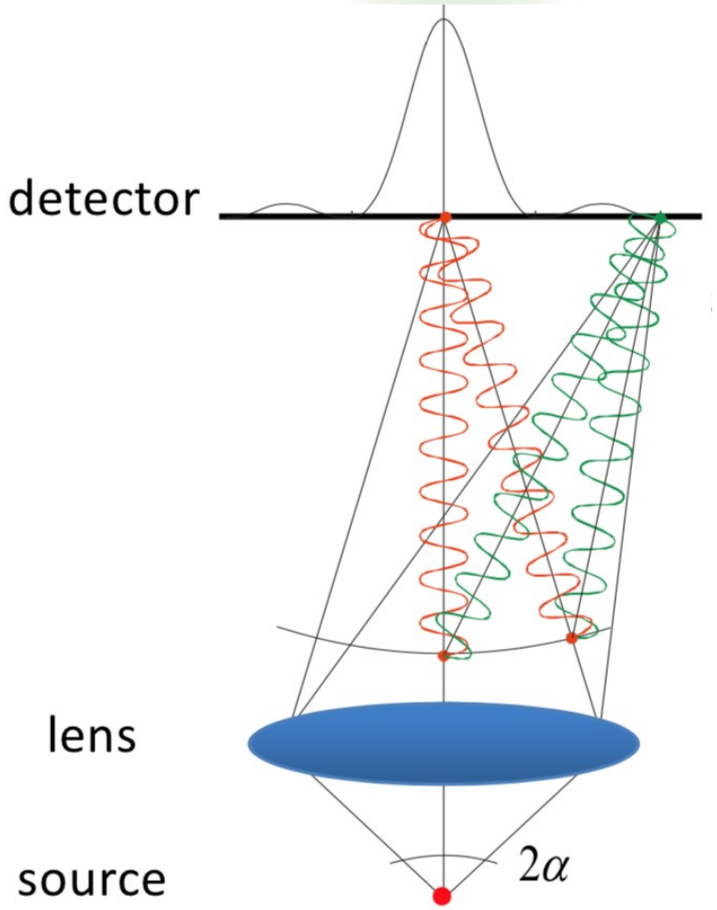
Analysis (drift, quantification, coloc, clustering, etc…)

Concept

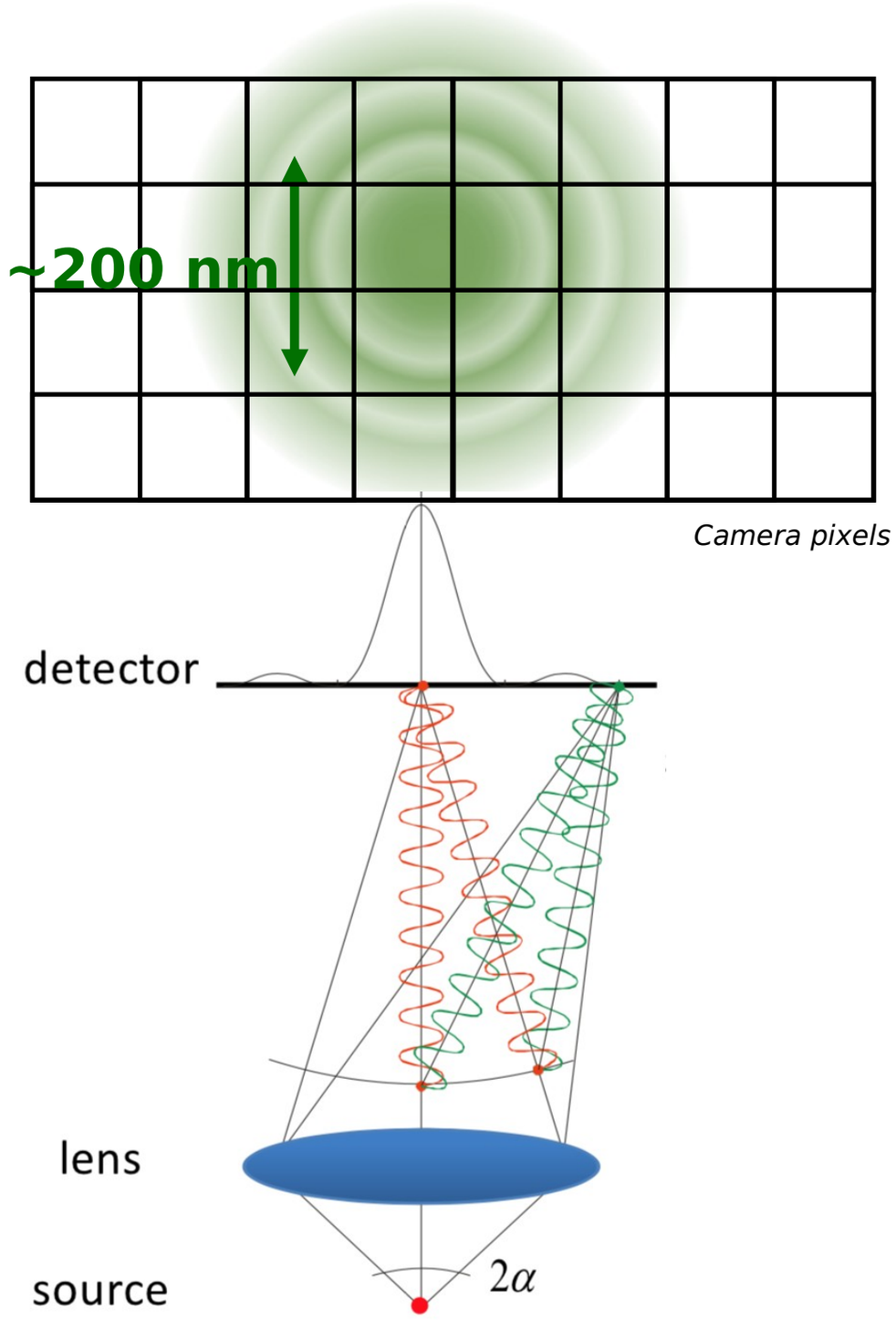
SMLM - Concept



Camera pixels



SMLM - Concept



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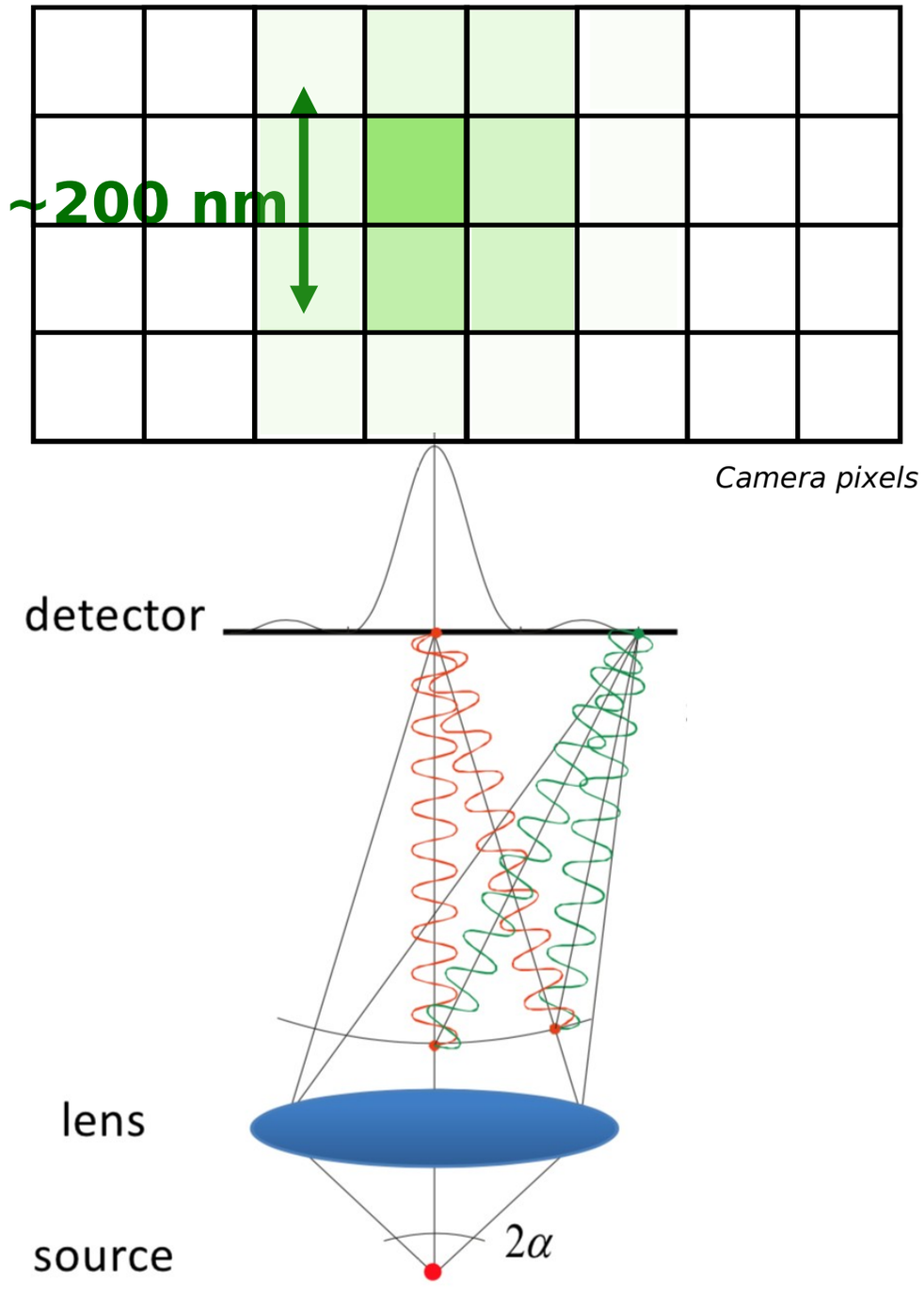

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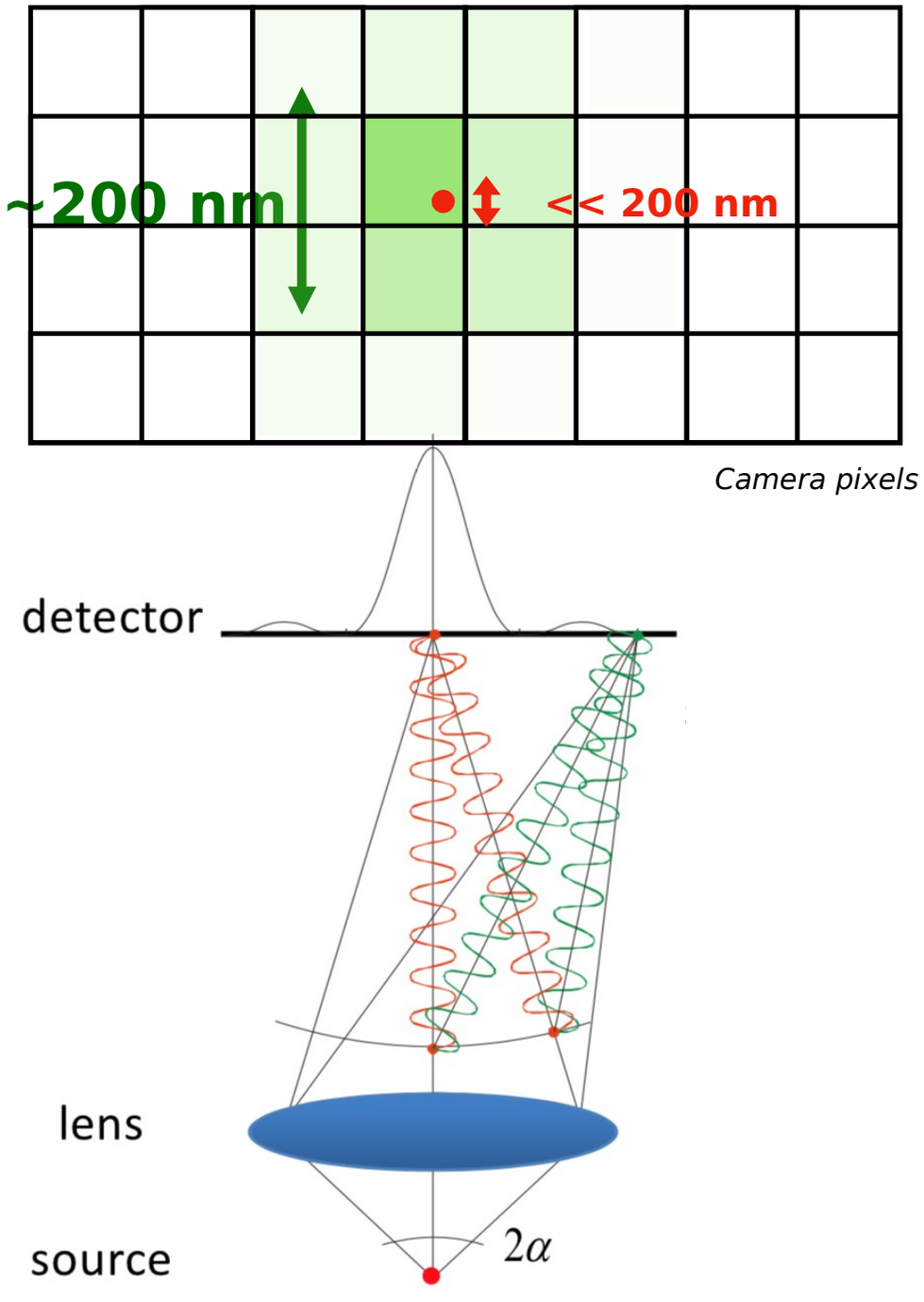
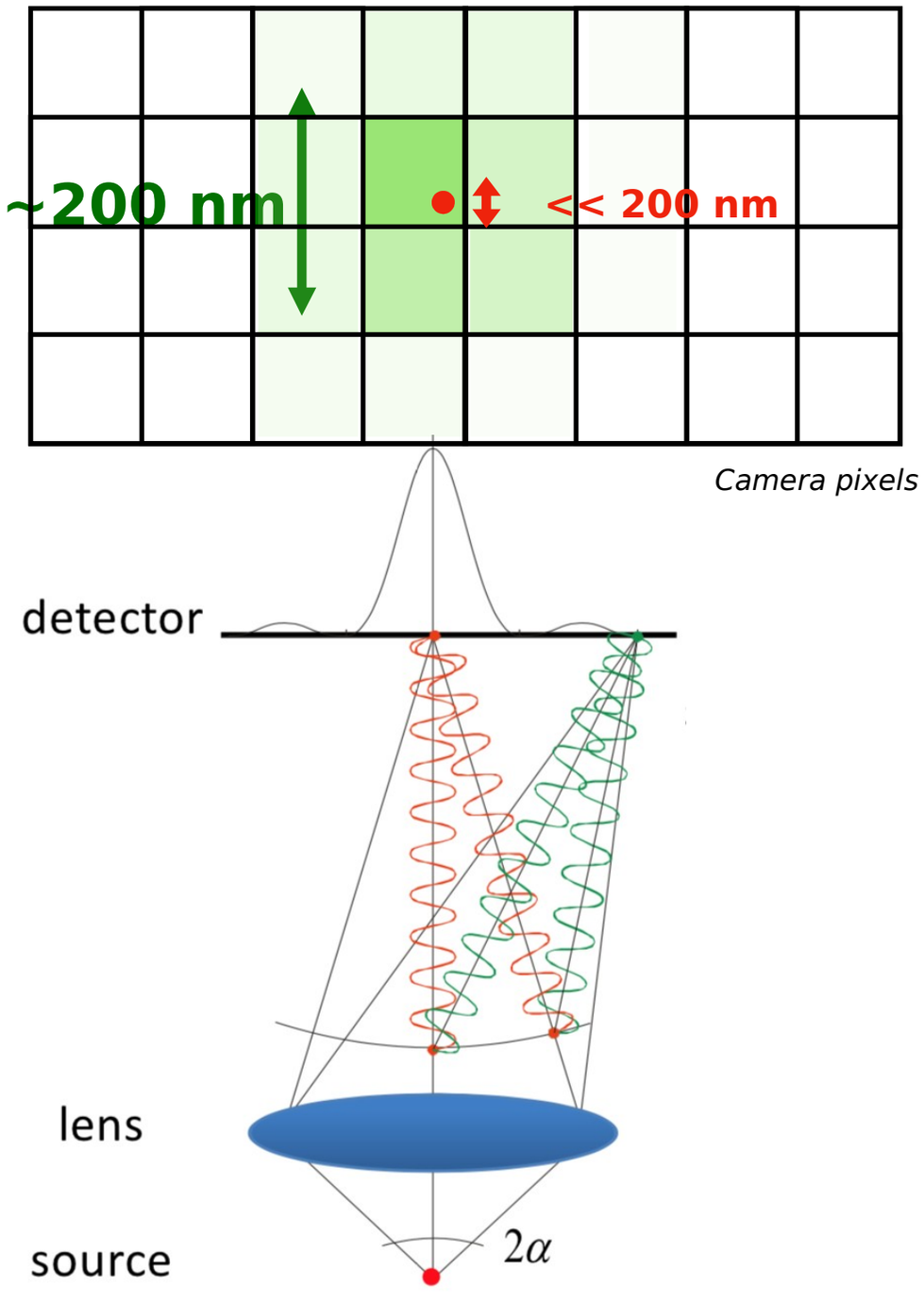


If you know there's **only one molecule** behind that PSF, then you can tell **precisely where that molecule is**

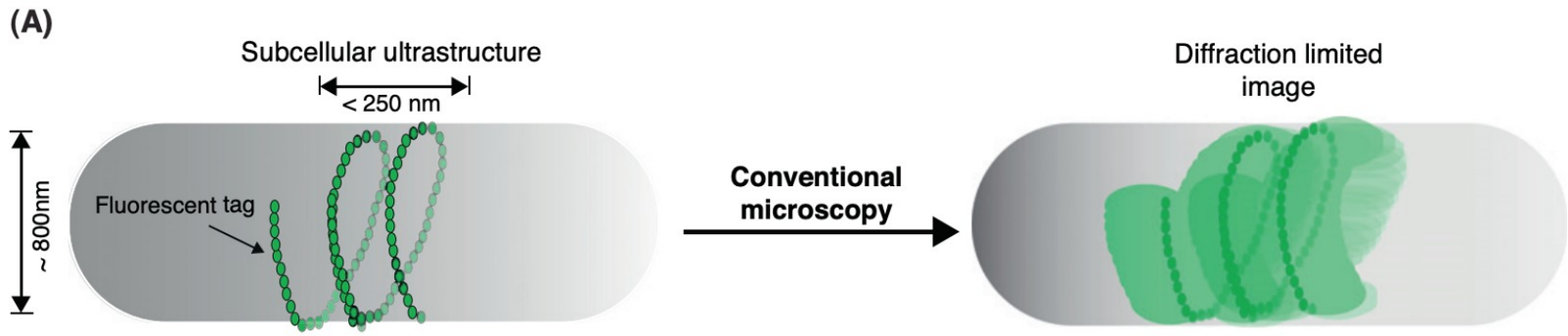
SMLM - Concept



If you know there's **only one molecule** behind that PSF, then you can tell **precisely where that molecule is**

➠ Go to single molecule conditions to localize all fluorescent molecules one by one

SMLM - Concept



Cattoni et al, Current Opinion in Microbiology, 15(6):758-63, Dec 2012

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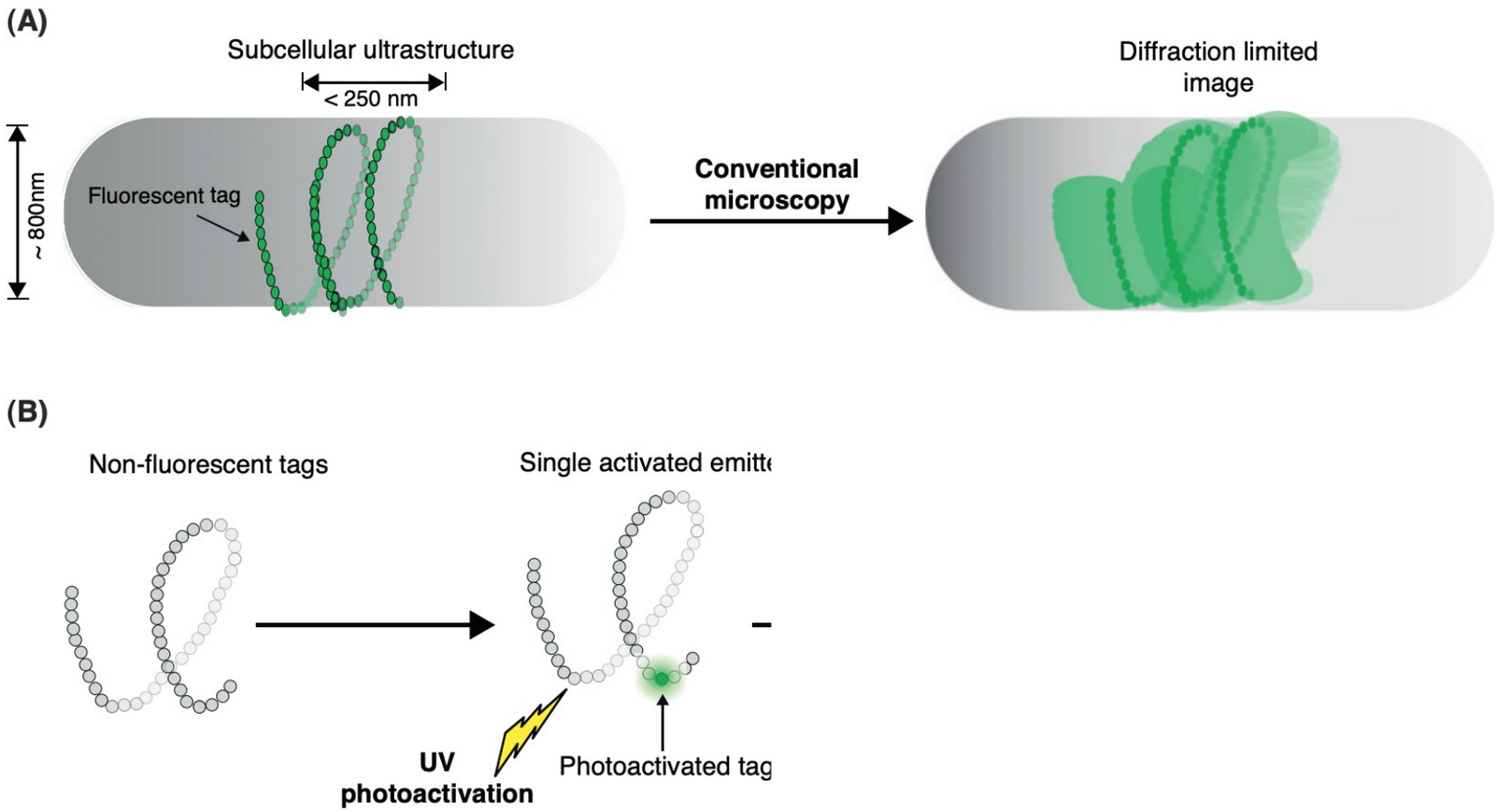

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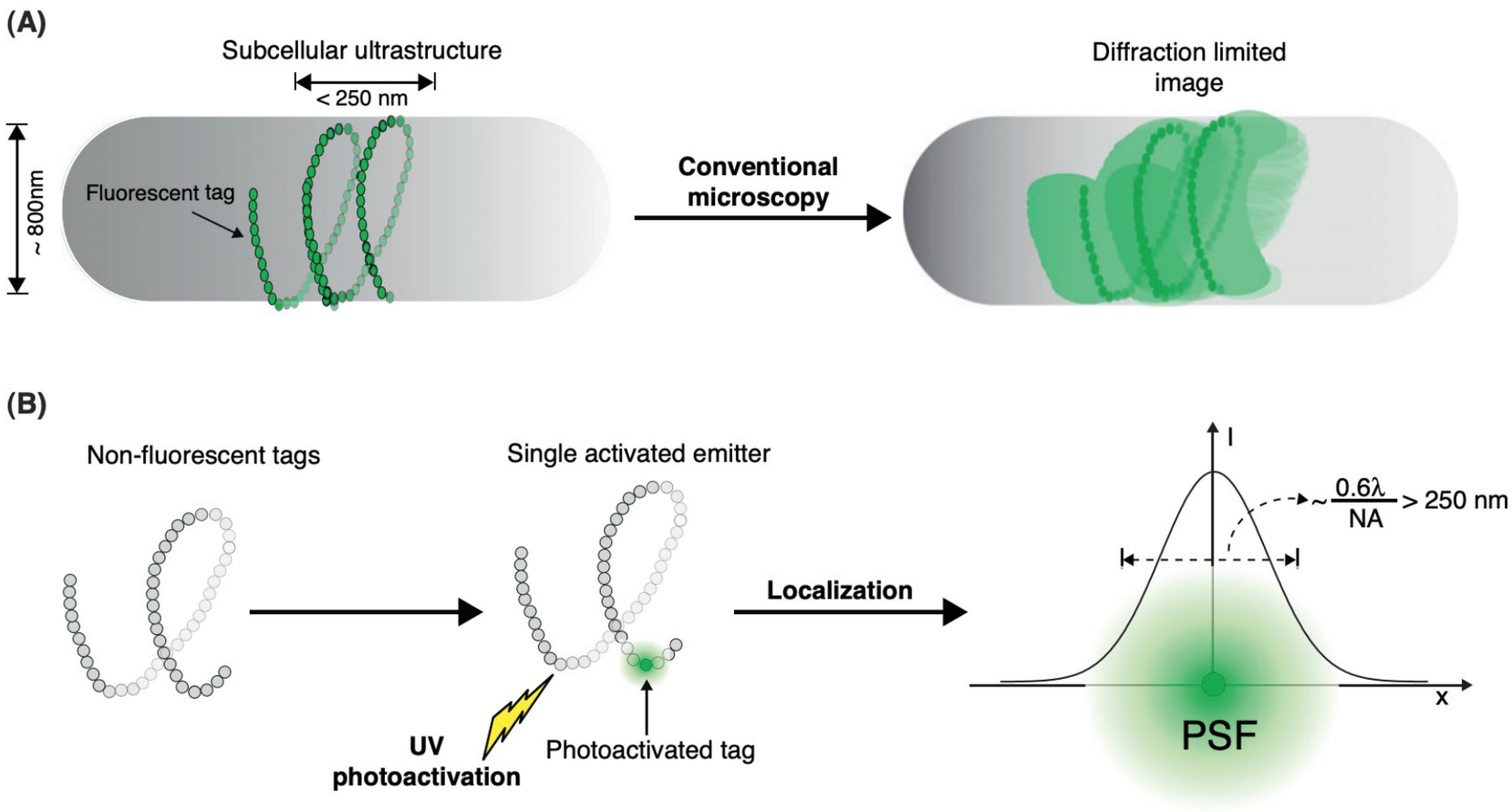

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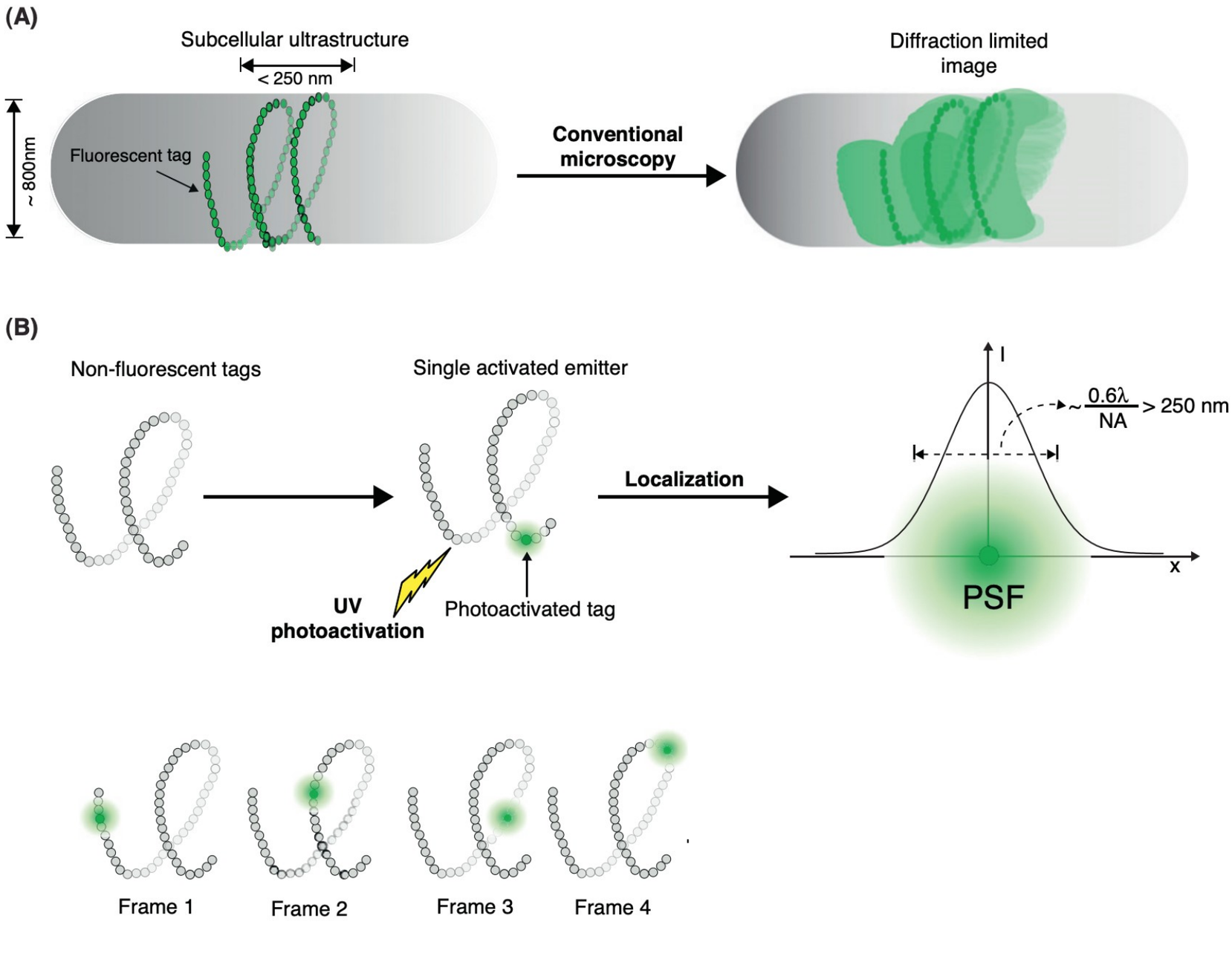

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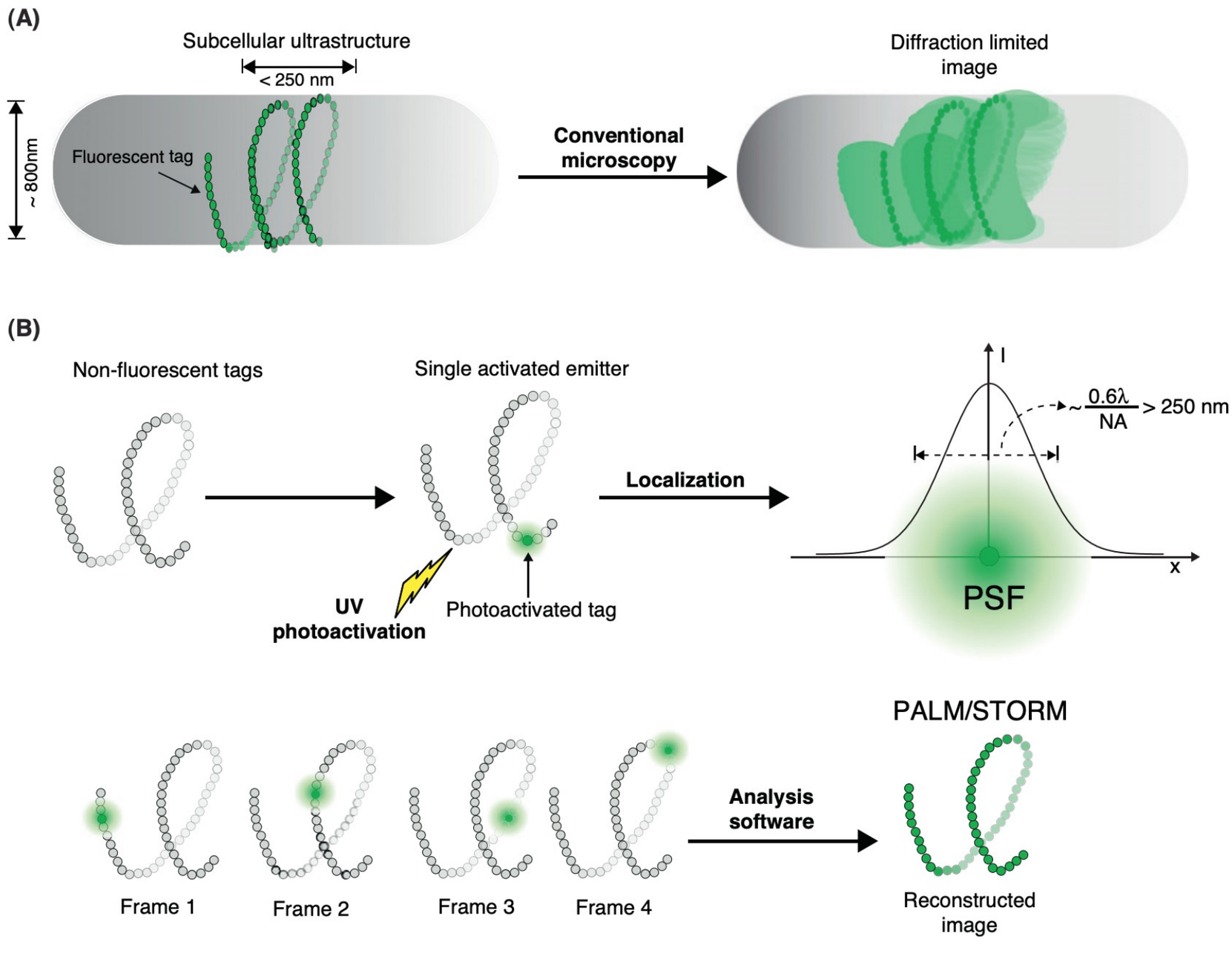

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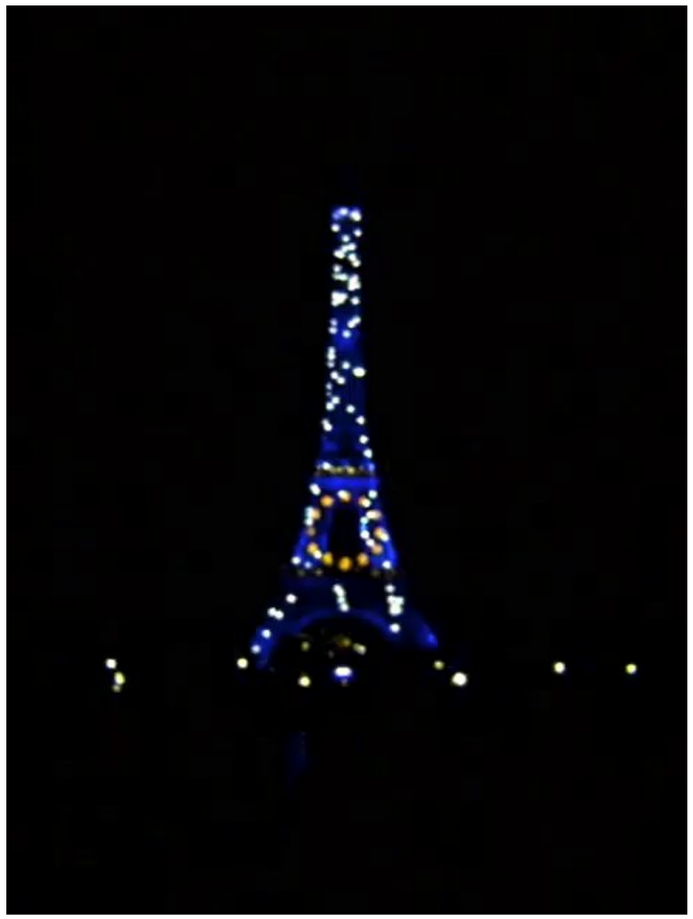

Ricardo Henriques | LMCB

SMLM - Concept

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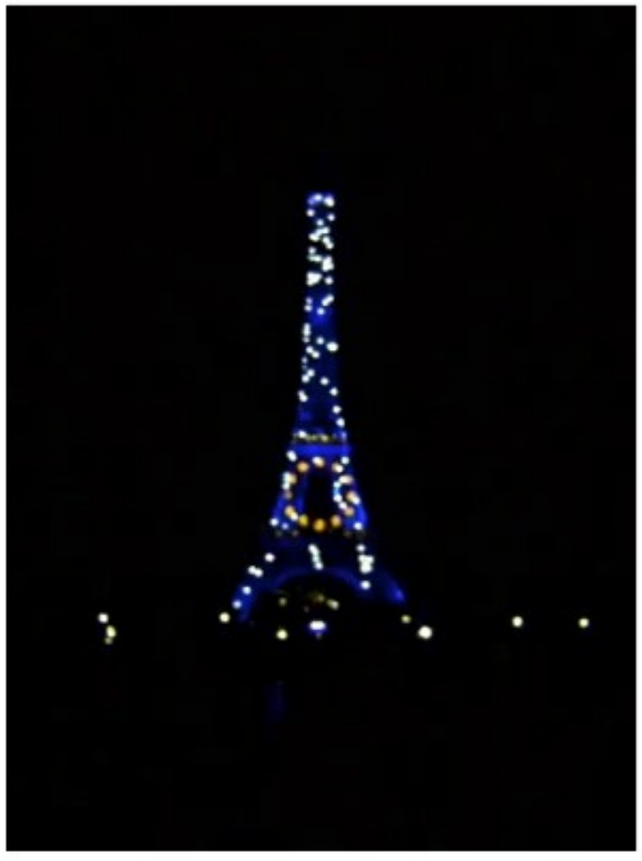

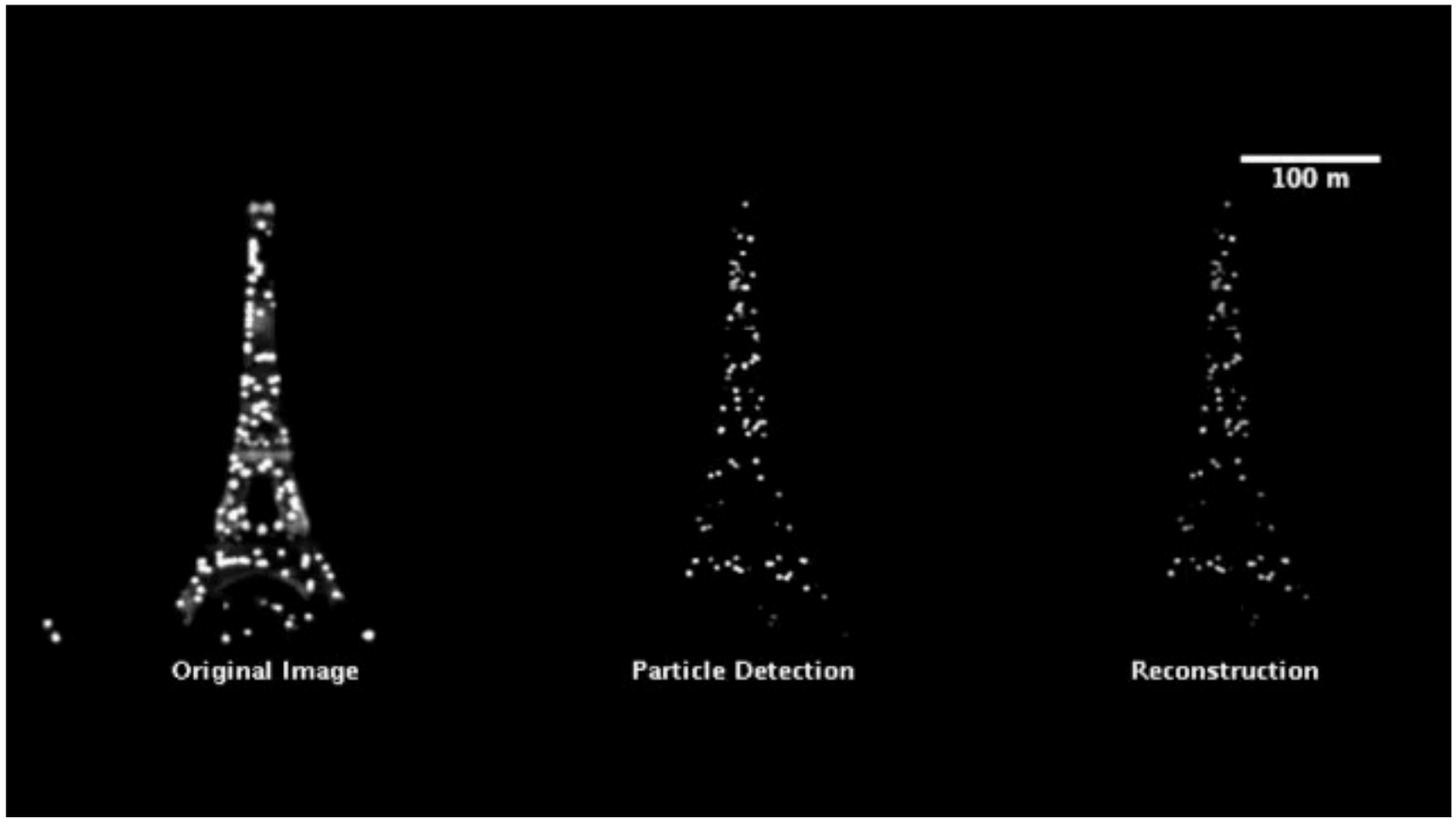

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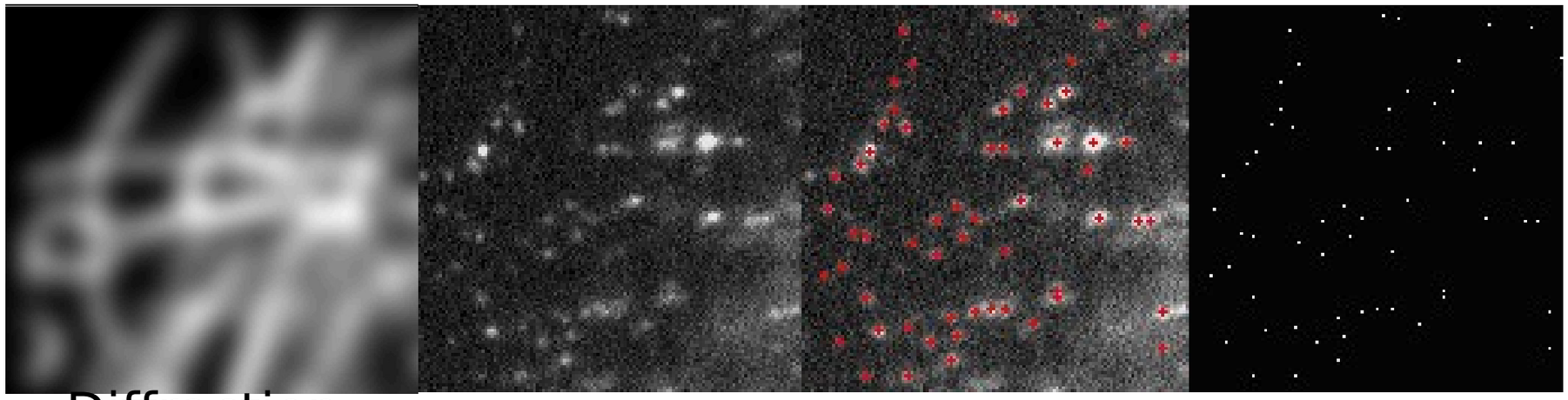

Diffraction limited image

Single molecules condition

Detect and localise single fluorescent molecules

Retrieve and cumulate localisations only

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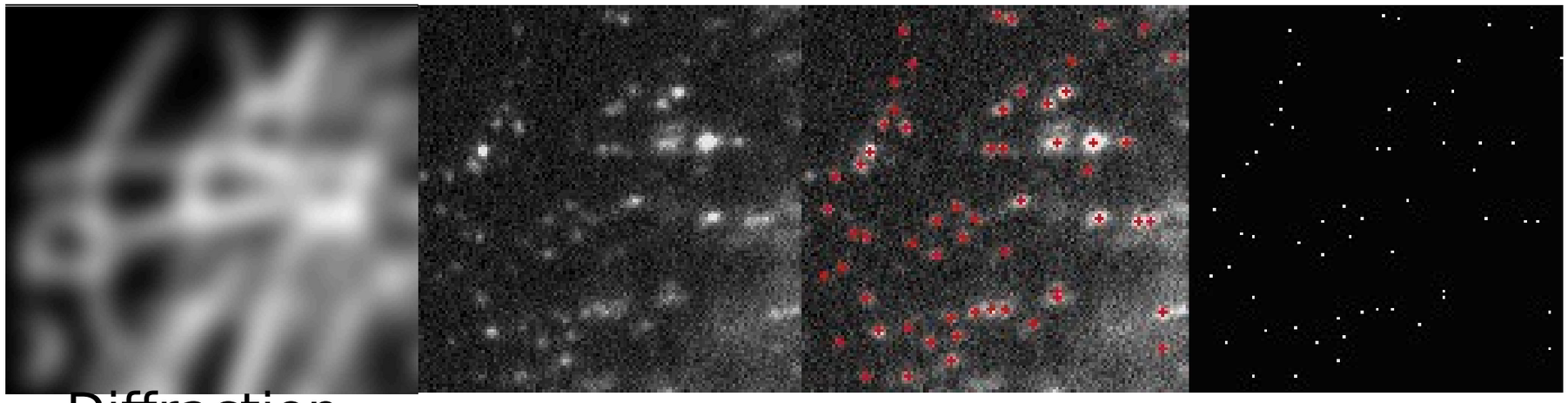

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Repeat many times (**N** frames)

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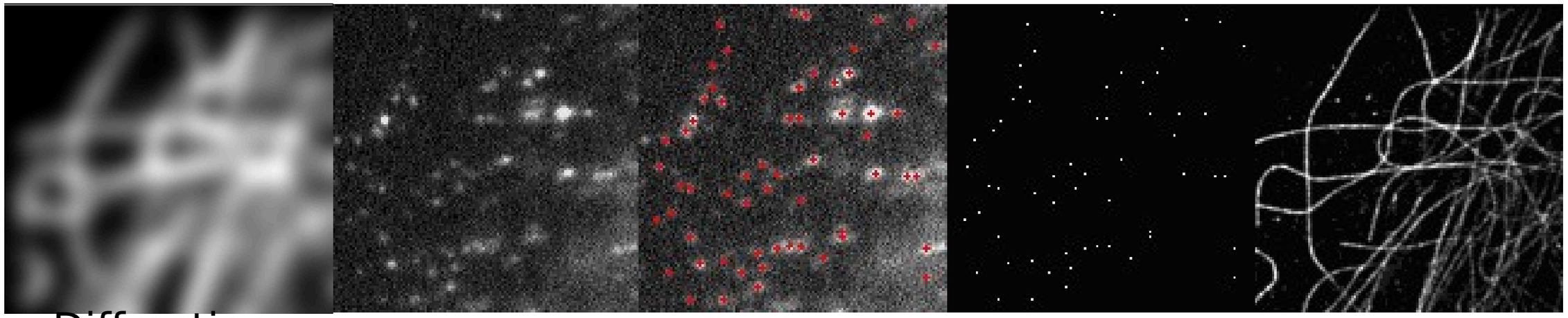

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Super resolved image
Cumulation of **X** molecules localisations through **N** frames

Repeat many times (**N** frames)

Typically:
- **N** ~10^4 frames
- **X** ~$10^{5\text{-}6}$ molecules

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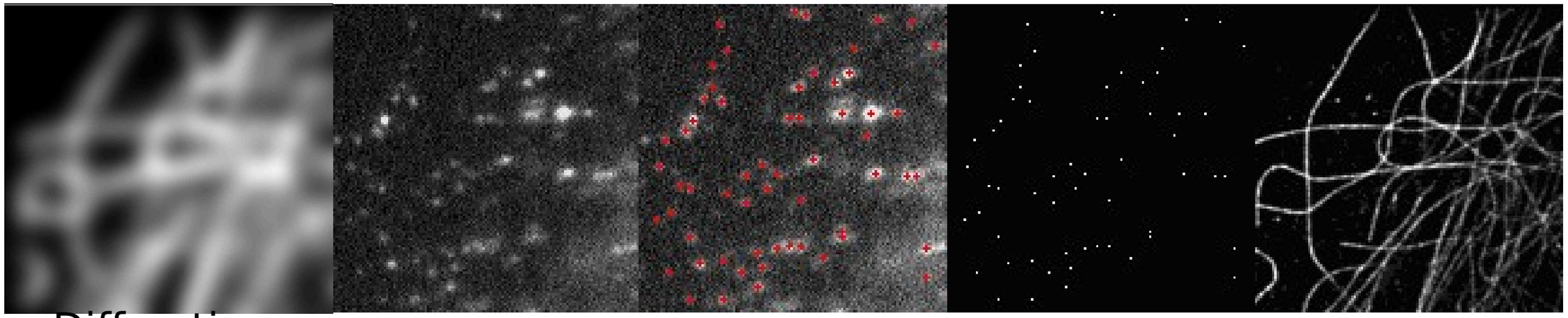

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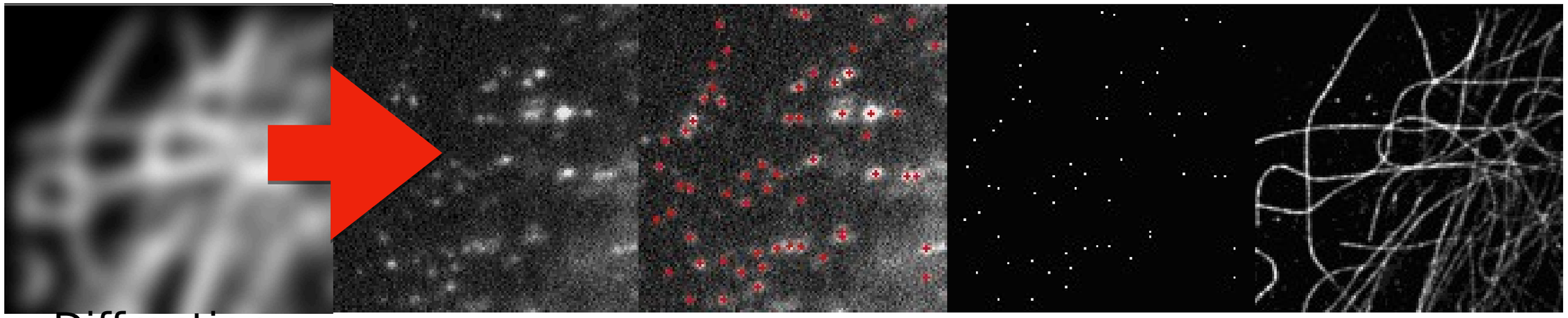

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How to make them blink?

SMLM techniques:

STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY (STORM) Organic Fluorophores (Cy5, Alexa647...)

Rust et al 2006

PHOTOACTIVATION LOCALIZATION MICROSCOPY (PALM) Genetically Encoded Fluorescent Proteins (PAGFP, EOS...)

Betzig et al 2006

POINT ACCUMULATION IN NANOSCALE TOPOGRAPHY (PAINT, uPAINT, Organic Fluorophores (ATTO dyes) **DNA-PAINT)**

Sharonov et al 2006, Giannone et al 2010, Jungmann et al 2014

(F)PALM, (d)STORM, GSDIM, PALMIRA, (u)PAINT, BALM, SMACM, SPDM, etc...

Conceptually the same :

stochastically establish the on state at the single-molecule level, so that only a single fluorophore within a distance larger than the diffraction limit is able to emit. Each fluorophore can then be separately localized.

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It’s all about making fluorophores blink!

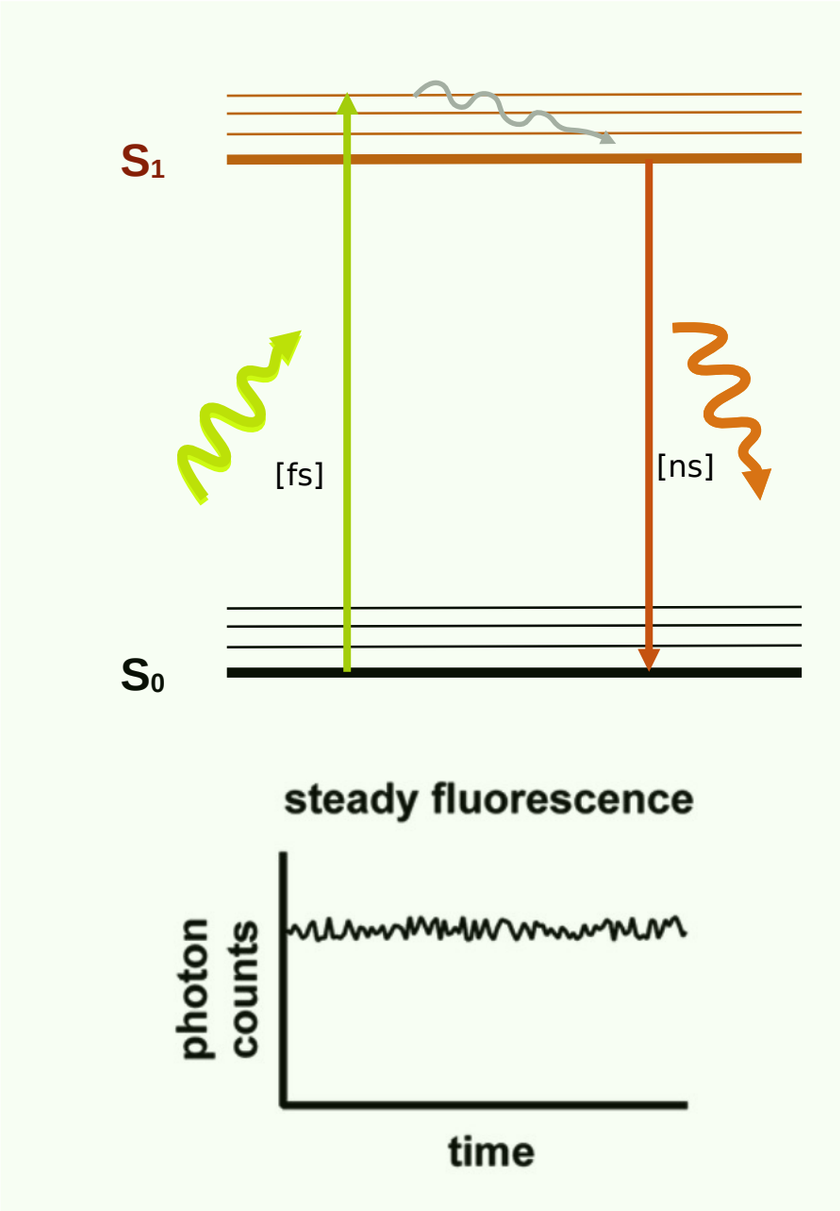
Techniques / Fluoroscent probes

STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY (STORM)

Organic Fluorophores

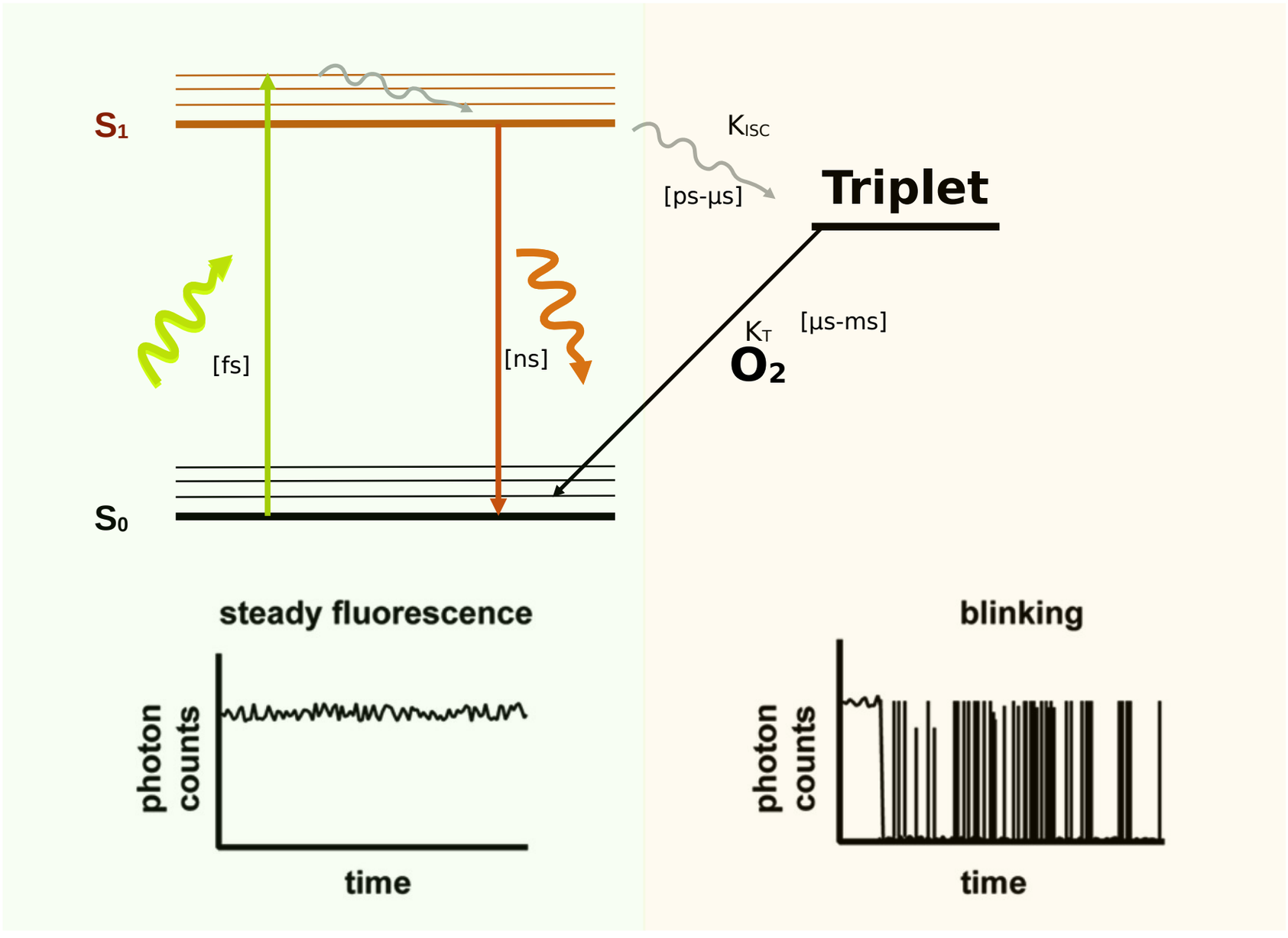
STORM / Organic dyes

Jablonski diagram



STORM / Organic dyes

Jablonski diagram



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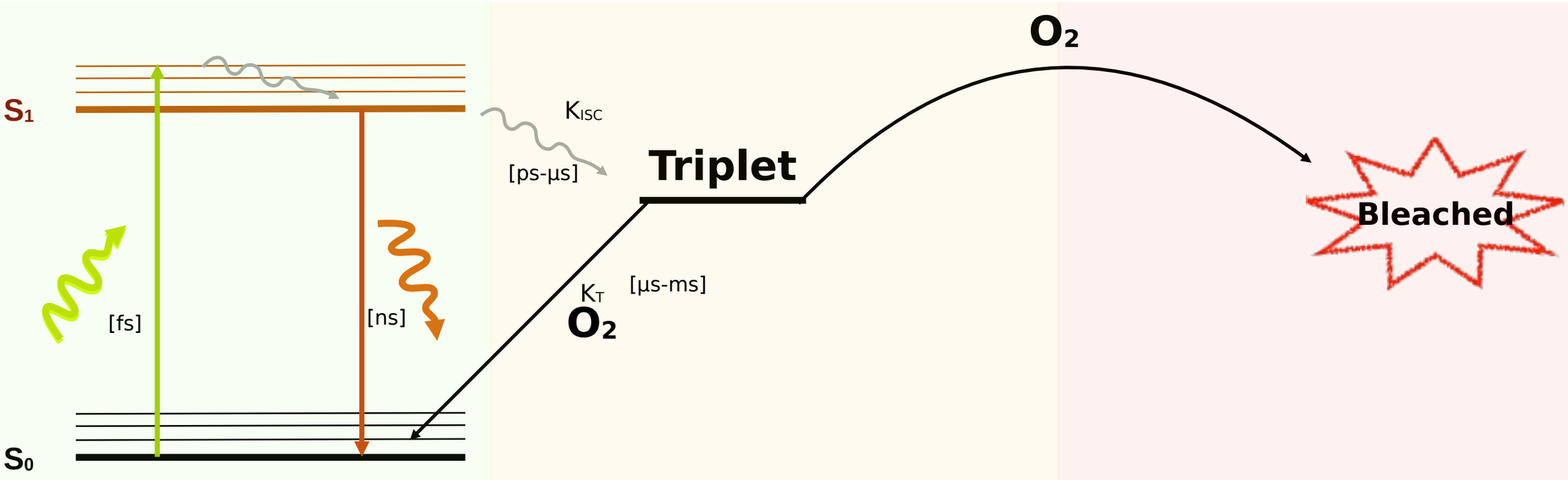
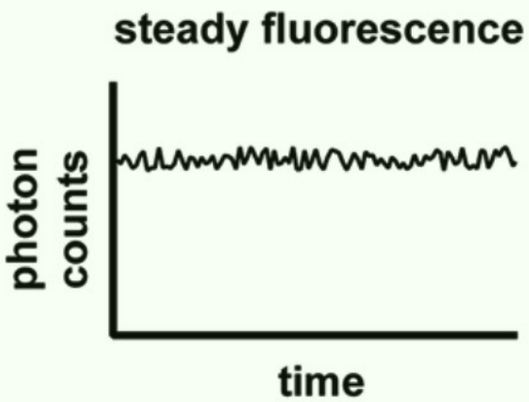
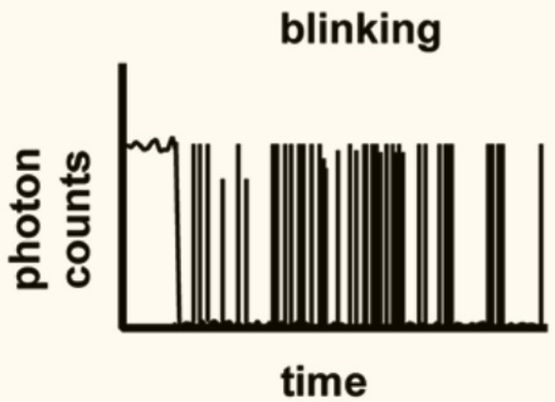
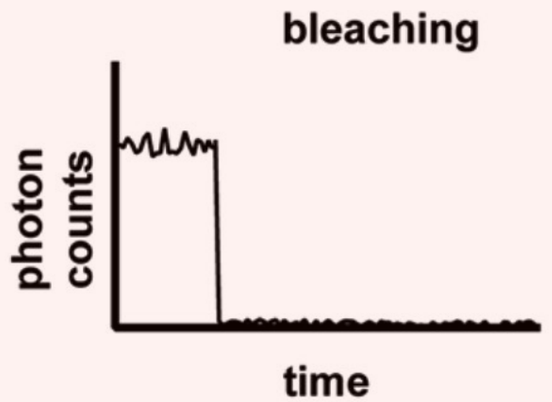




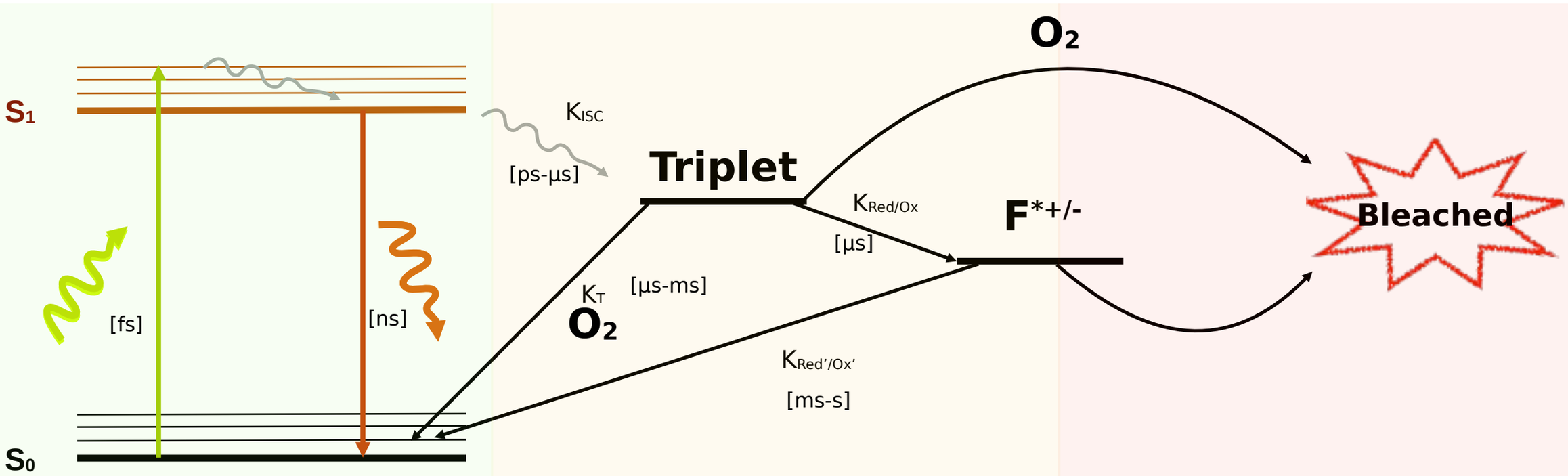


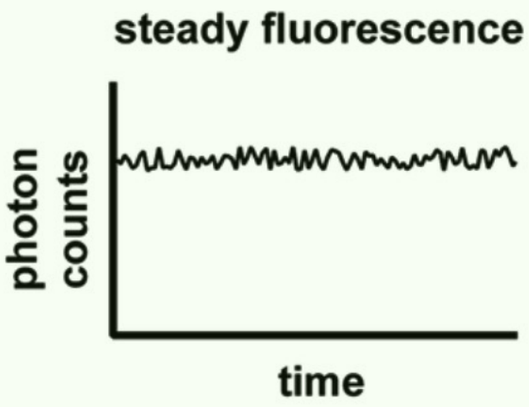


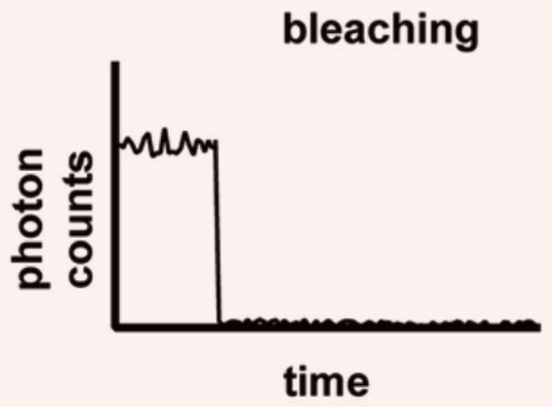
STORM / Organic dyes

Jablonski diagram

Oxygen scavenger + Oxidizing/Reducing agents = **Control of blinking properties**







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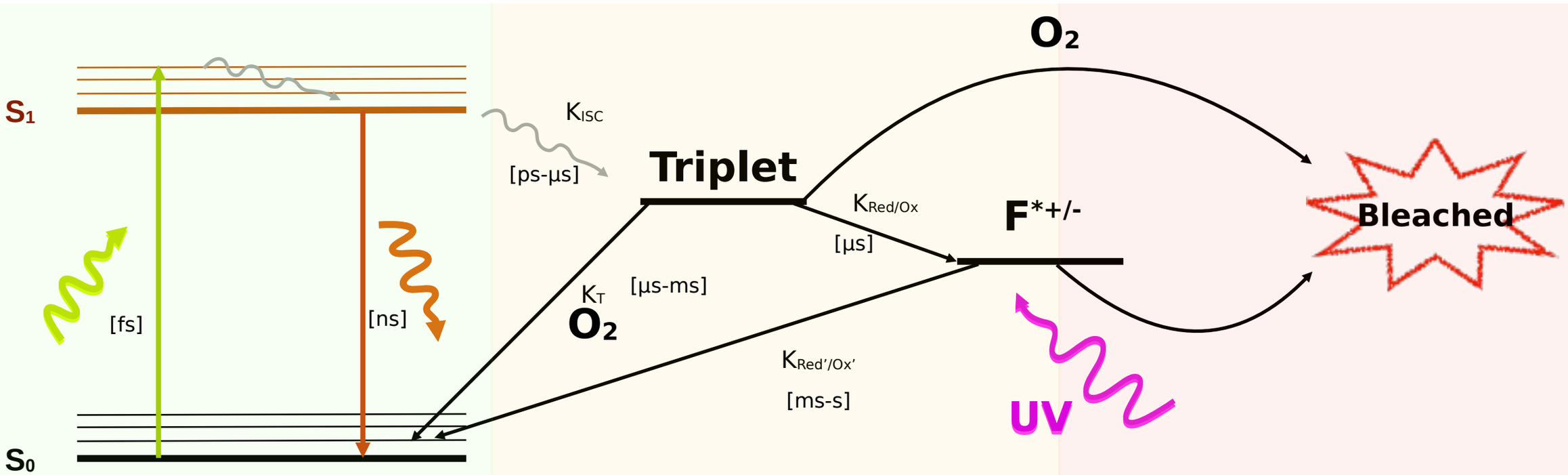
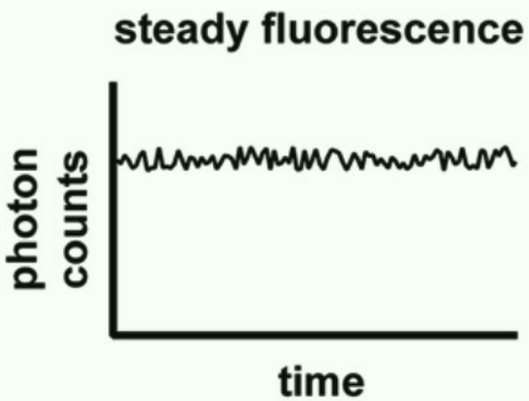
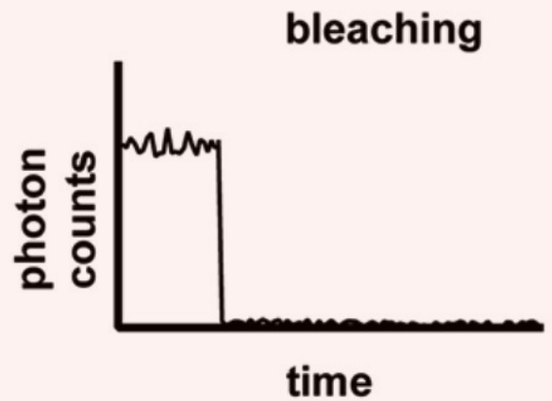






PHOTOACTIVATION LOCALIZATION MICROSCOPY (PALM)

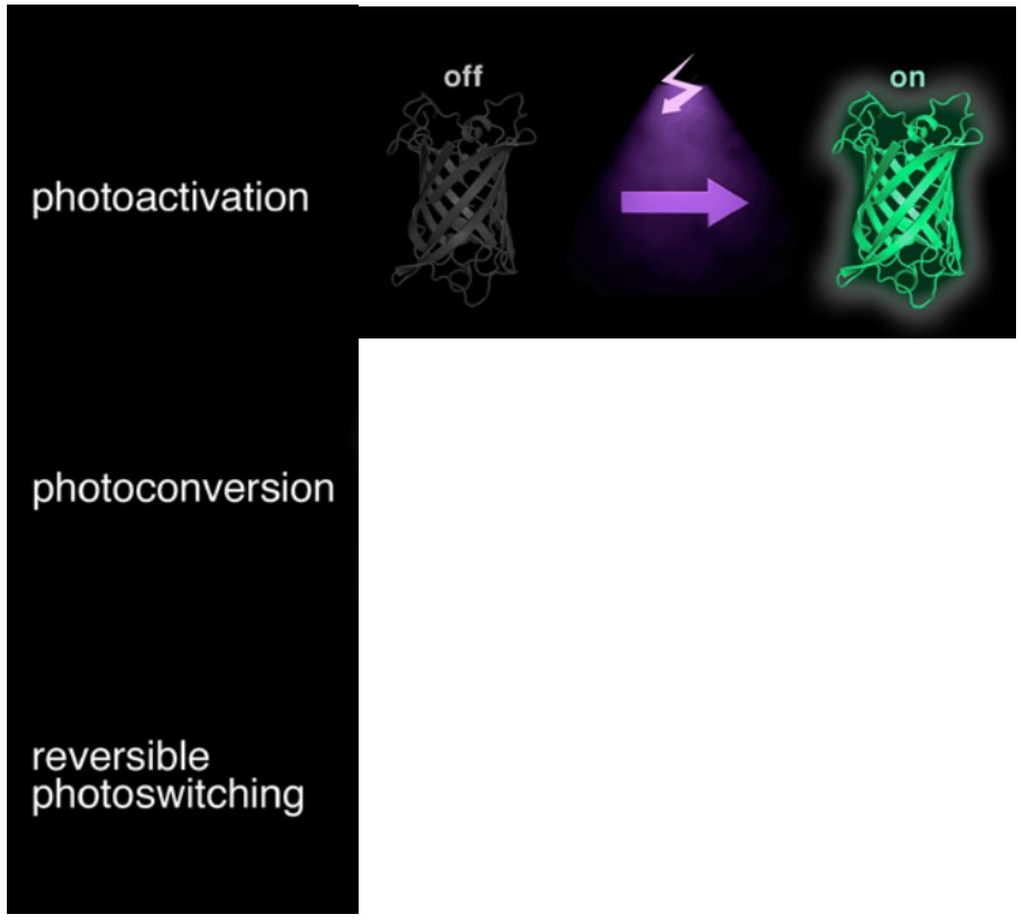
Genetically Encoded Fluorescent Proteins

PALM / Fluorescent proteins

Light of a shorter wavelength ➟ Control of photo switching events

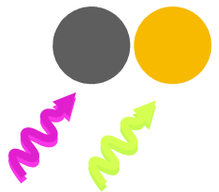
PALM / Fluorescent proteins

Light of a shorter wavelength ➟ Control of photo switching events



PA-GFP

PA-mCherry



PALM / Fluorescent proteins

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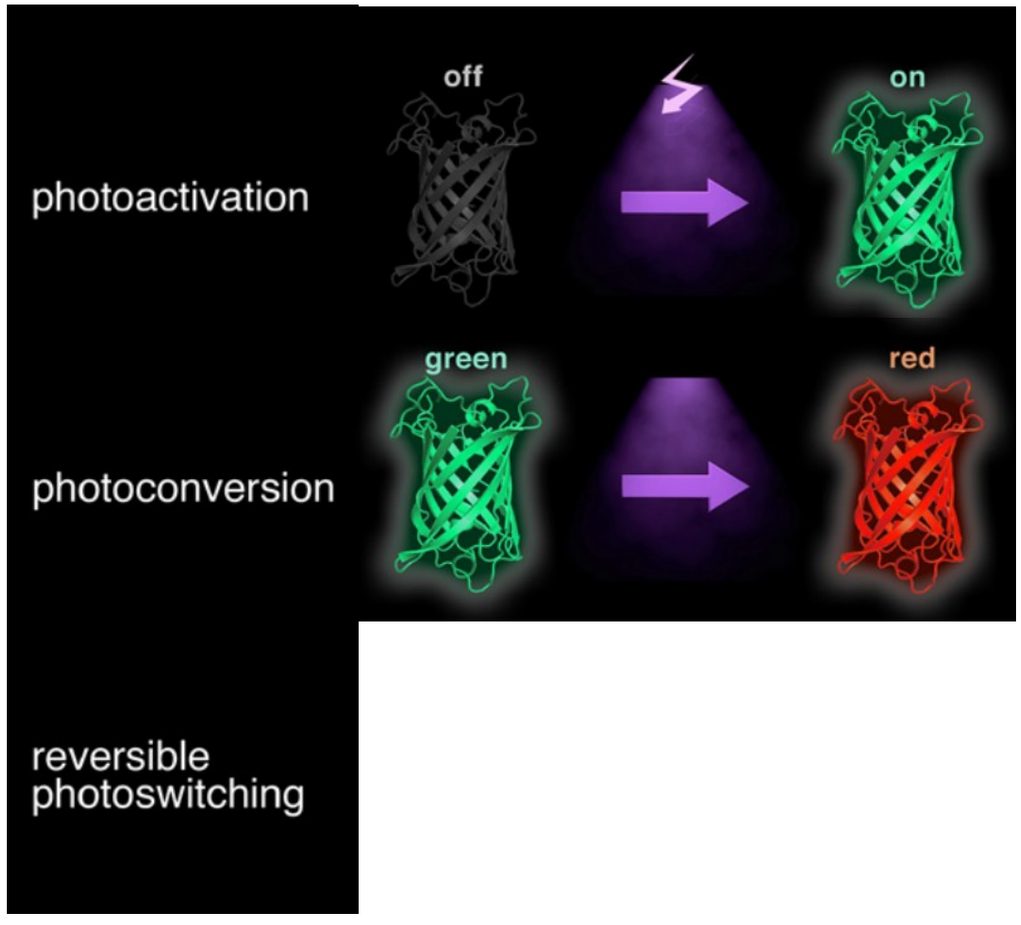

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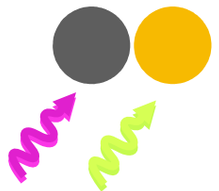
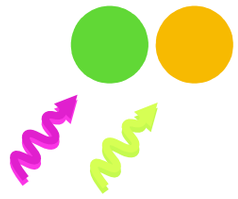
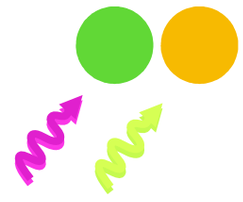


mEos3.2



mMaple 2 or 3



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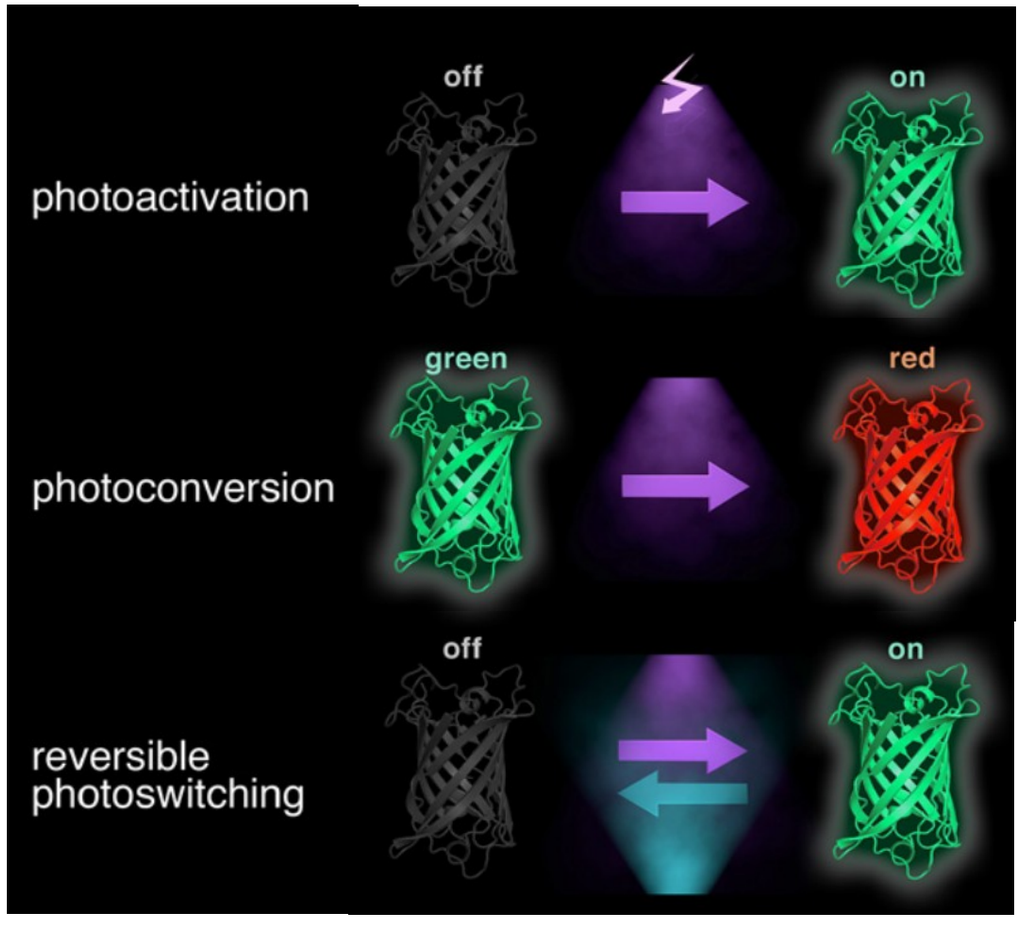

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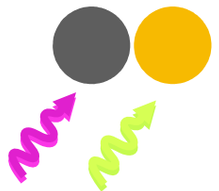

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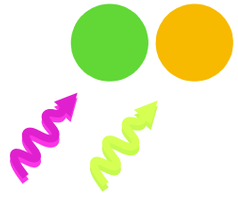
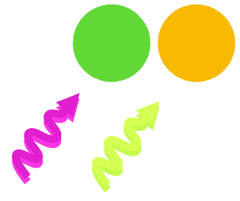


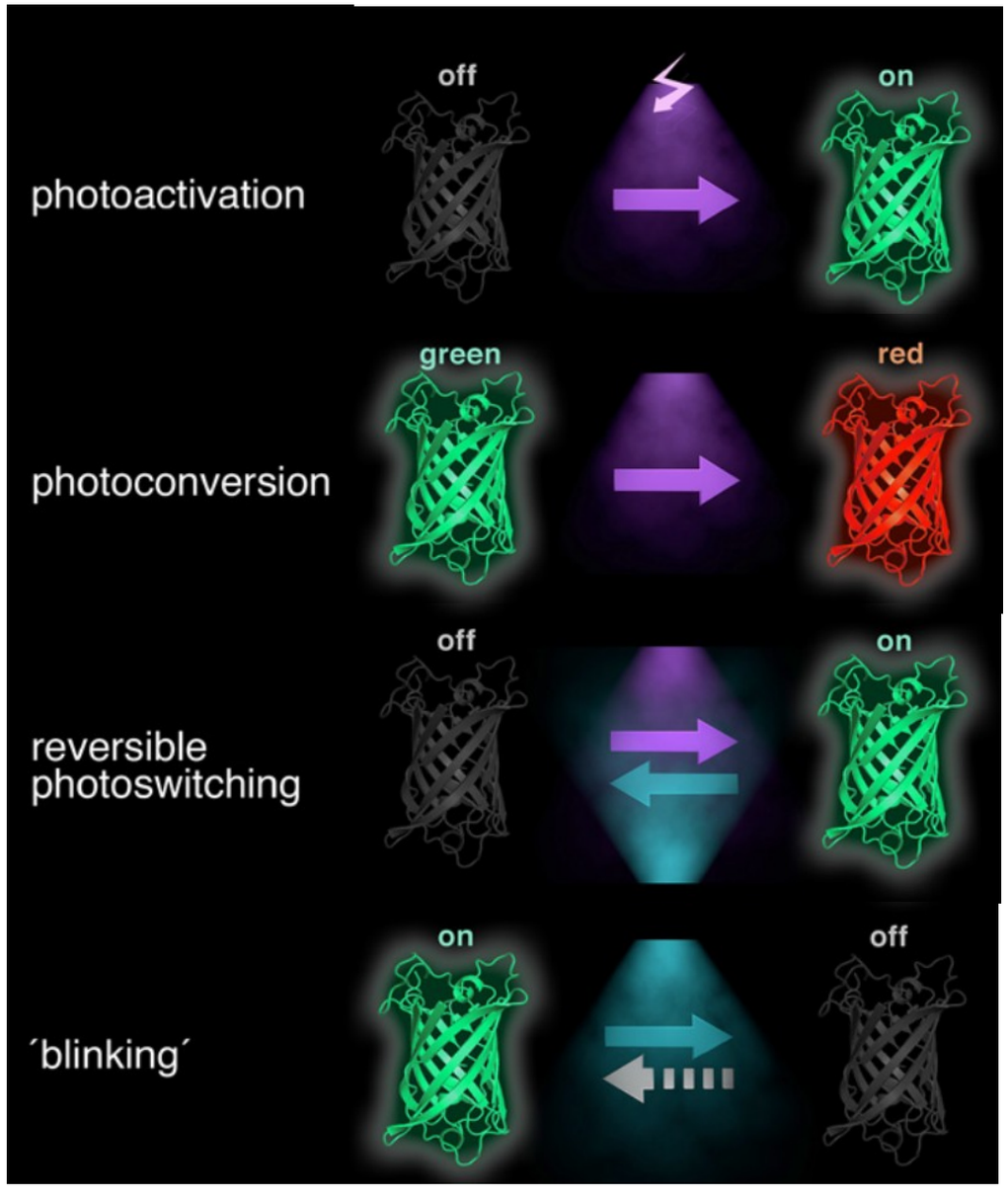
mMaple 2 or 3



Dronpa

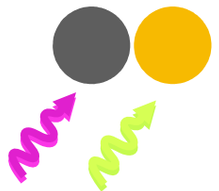
PALM / Fluorescent proteins

Light of a shorter wavelength ➟ Control of photo switching events

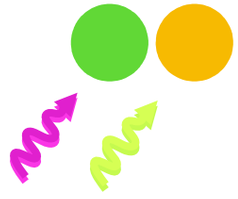


PA-GFP

PA-mCherry



mEos3.2



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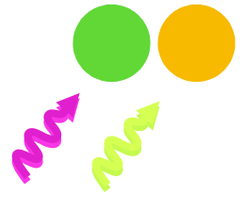
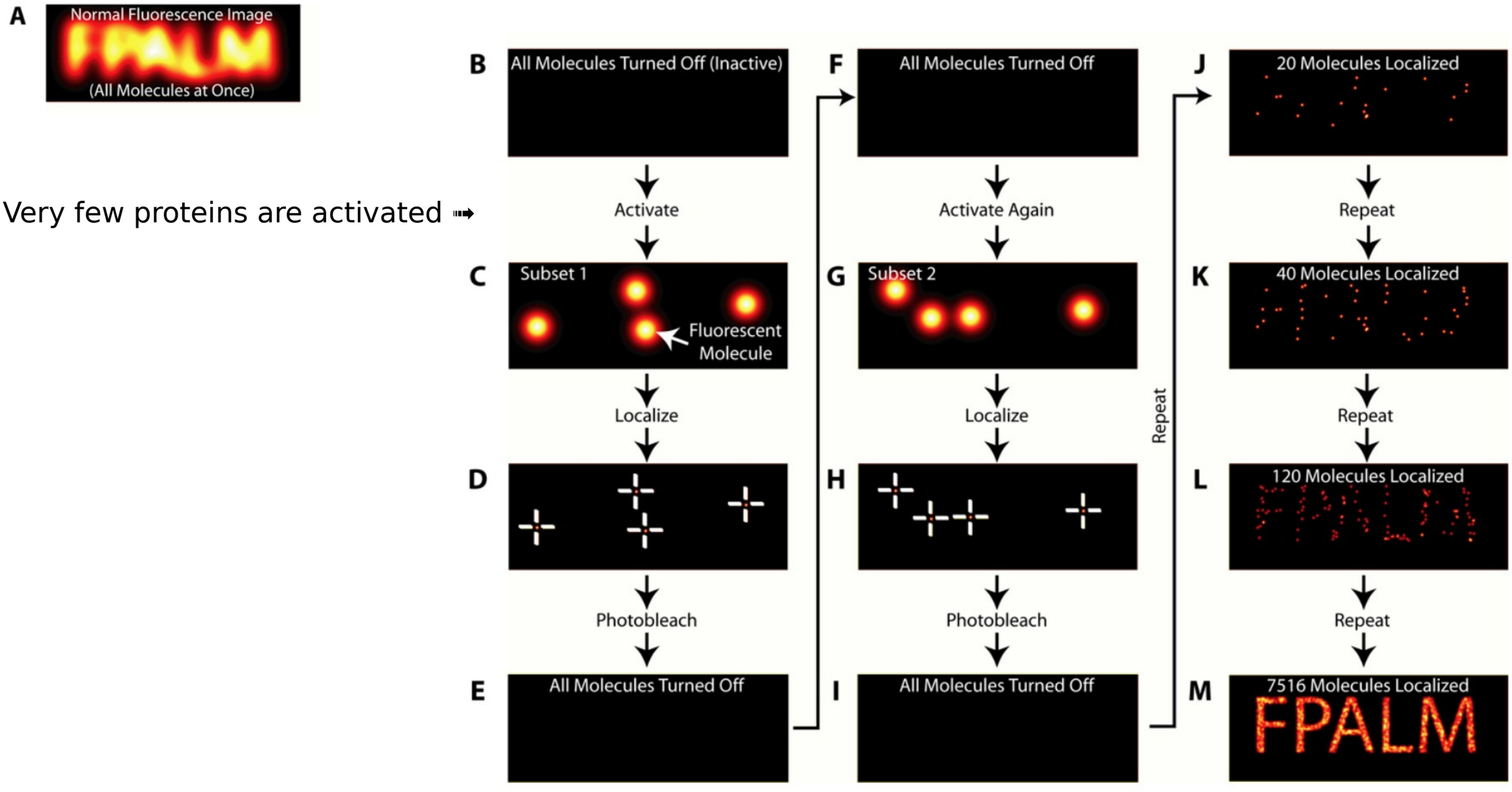


Dronpa

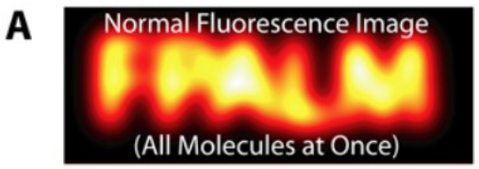
PALM

Very few proteins are activated ➟



Activation/bleaching

PALM



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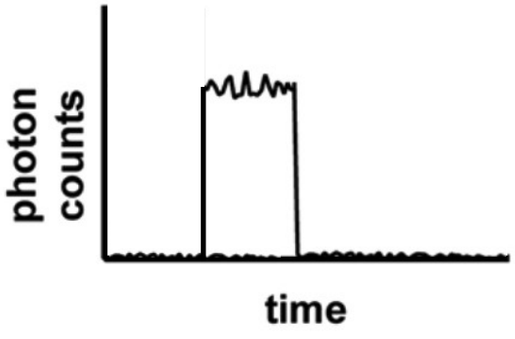
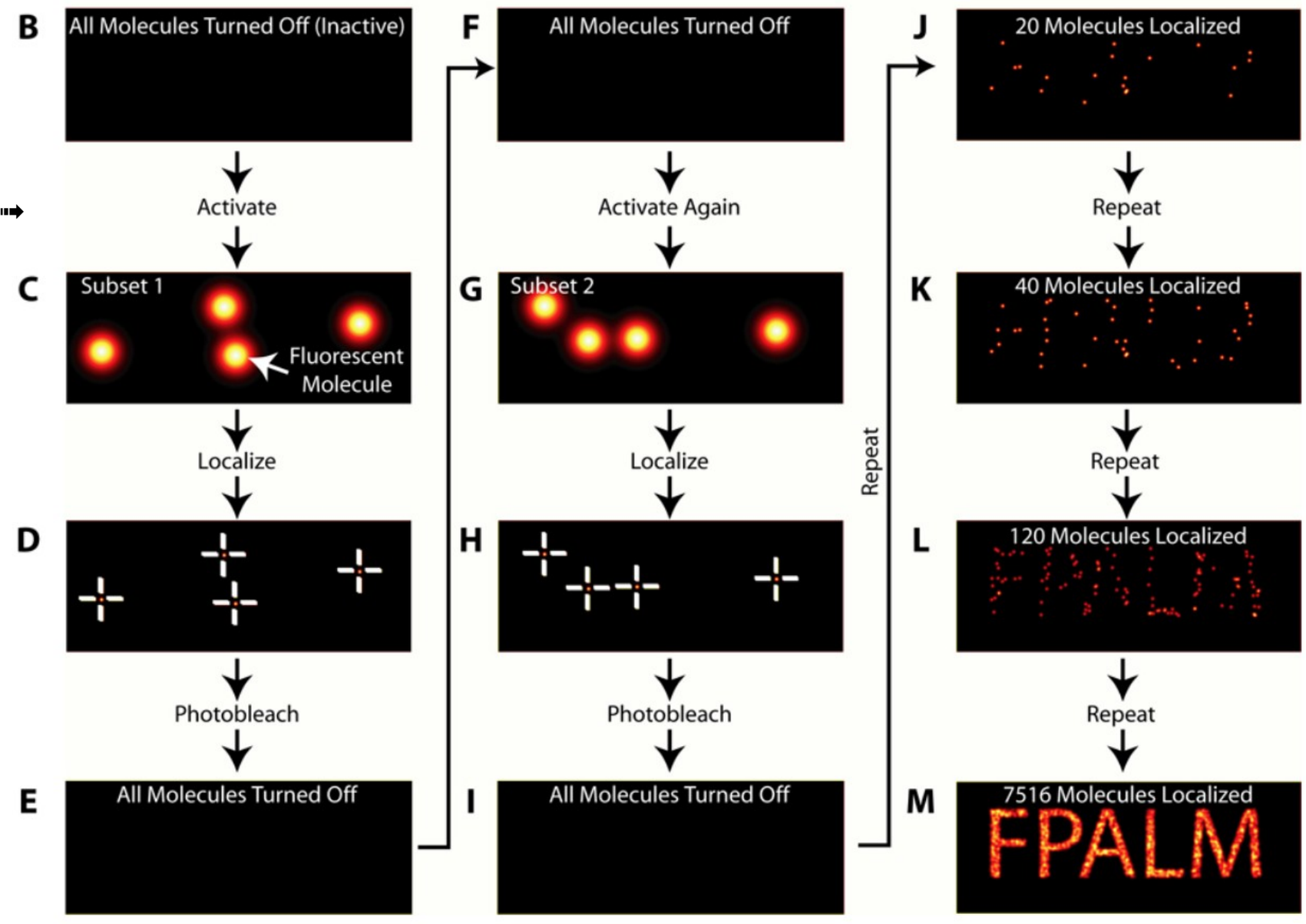
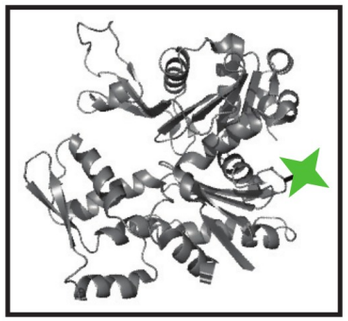




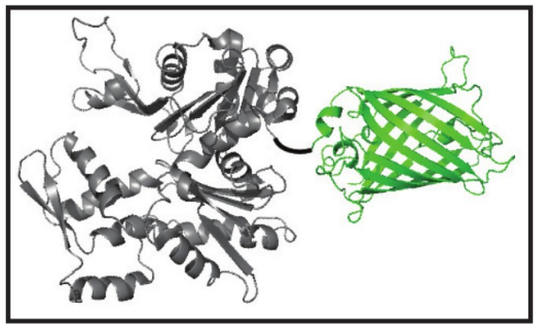
Activation/bleaching

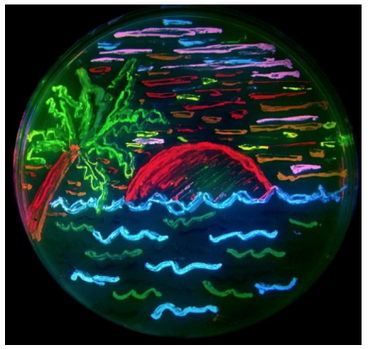
Fluorescent proteins vs Organic dyes



Organic Dyes

➠ **Control of blinking properties**

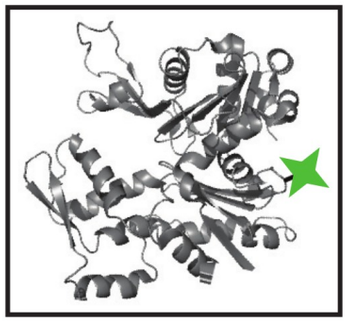




Fluorescent Protein

➠ **Control of photo activation/switching events**

Fluorescent proteins vs Organic dyes



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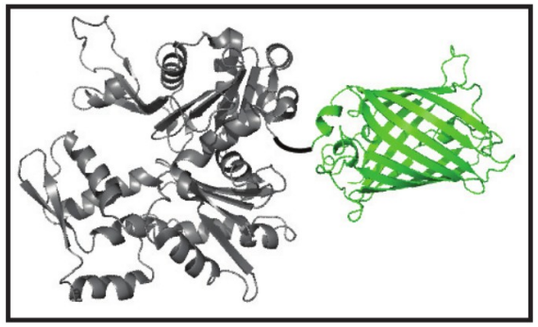

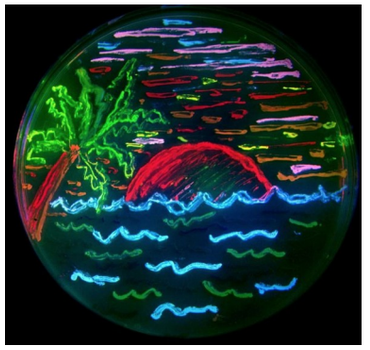

Fluorescent Protein

➠ **Control of photo activation/switching events**

Labeling endogenous proteins
High S/N
Photostable
Small label size
Many commercially available dyes
More versatile for multicolor imaging

Fluorescent proteins vs Organic dyes

Organic Dyes

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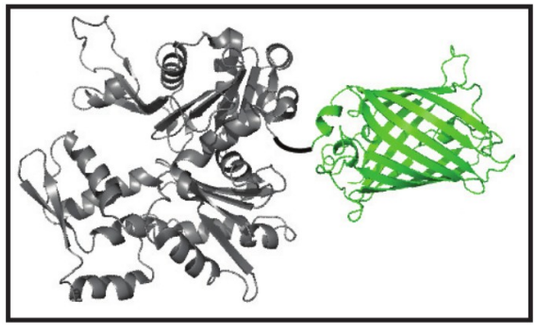

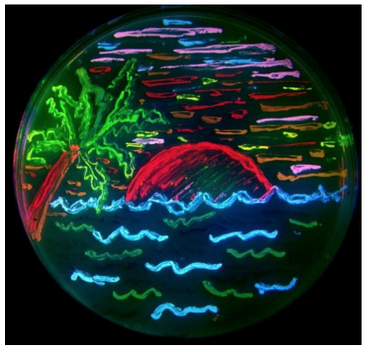

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Introduction of the dye into POI
Washing
Specificity of labelling / Potential non-specific labelling
Lower labelling efficiency

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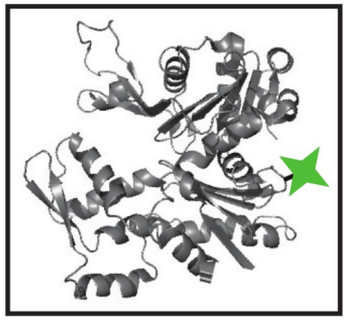

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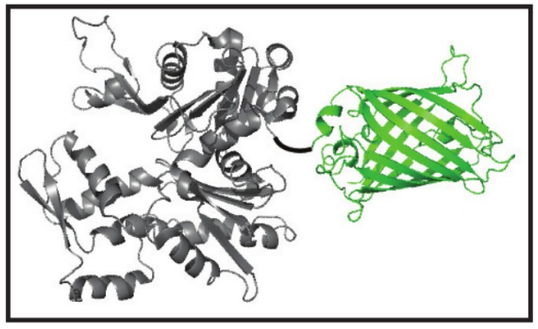
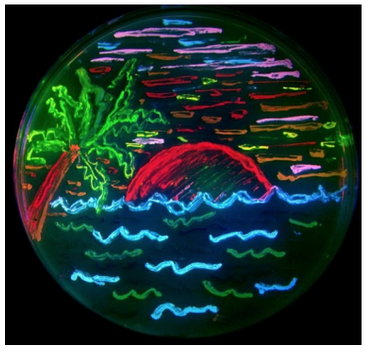


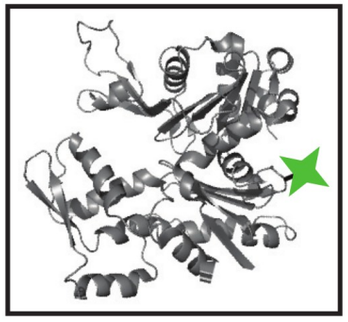


Fluorescent Protein

➠ **Control of photo activation/switching events**

Live sample labelling
Genetically encoded
High labelling efficiency
High specificity
No permeabilization, staining
High labelling density
Single molecule counting (1:1 labelling)
Single molecule tracking

Fluorescent proteins vs Organic dyes



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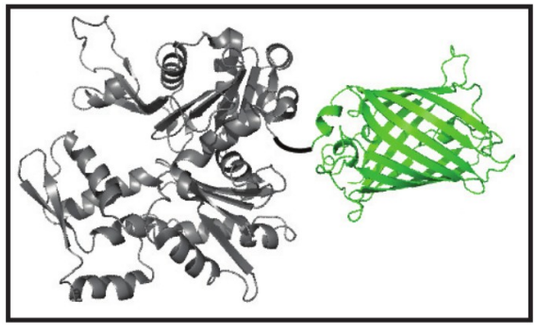

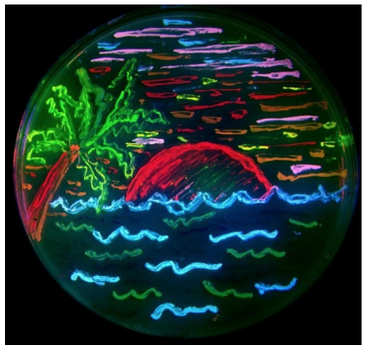

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High labelling efficiency
High specificity
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High labelling density
Single molecule counting (1:1 labelling)
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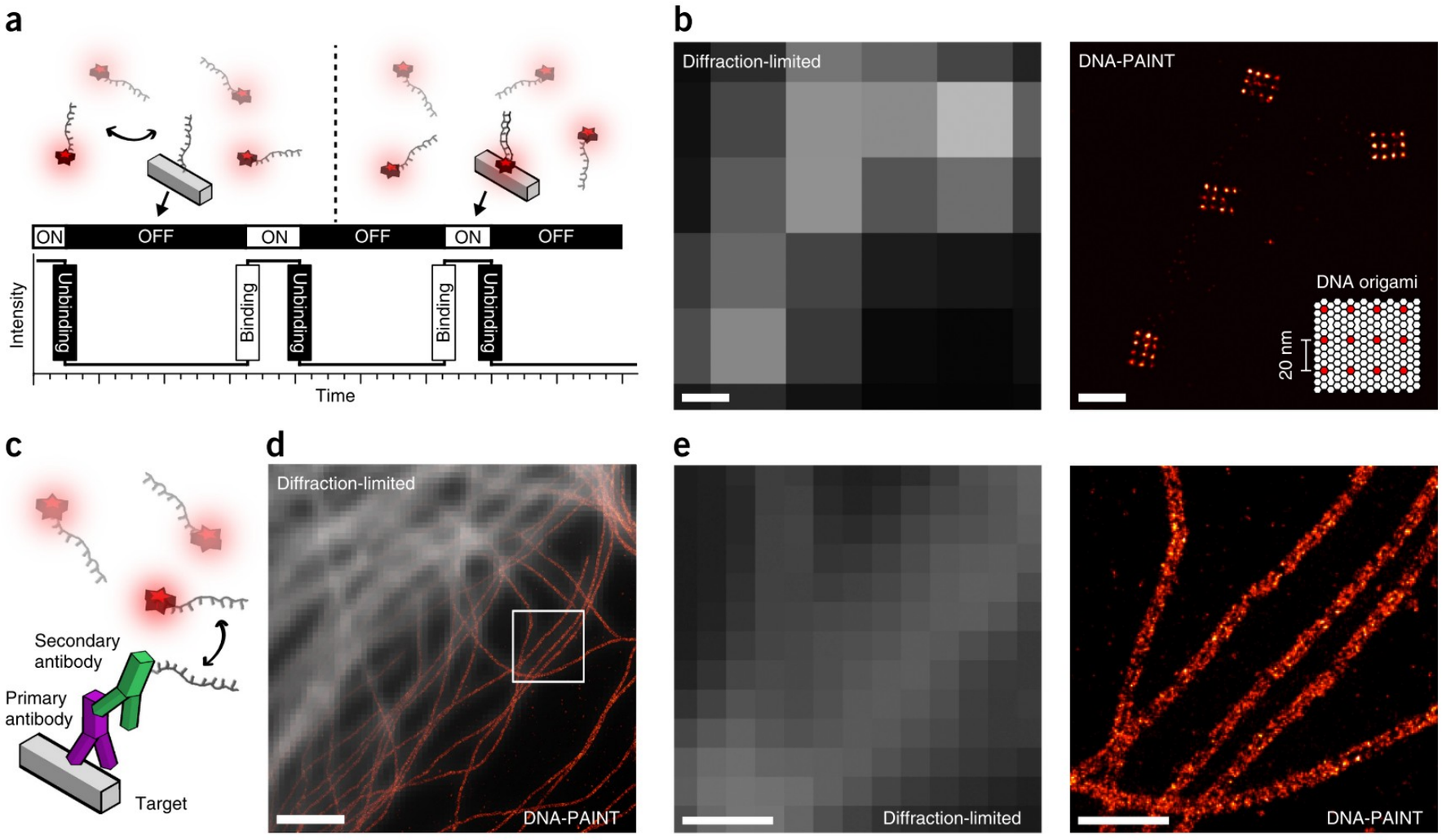
Lower S/N (Quantum yield)
Maturation rate/efficiency
Oligomerization state
Photo-toxicity
Protein fusion toxicity/ functionality
Multicolor imaging challenging

POINT ACCUMULATION IN NANOSCALE TOPOGRAPHY (PAINT, uPAINT, DNA-PAINT)

DNA-PAINT

Slow affinity ➟ High binding/unbinding rates



All the advantages of STORM +
- Infinite labelling
- Sequential imaging (pseudo-multicolor)

How to do SMLM

Critical features of SMLM

Critical features of SMLM

Single molecule conditions

Critical features of SMLM

Single molecule conditions

Probe size

Critical features of SMLM

Single molecule conditions

Probe size

Labelling density

Critical features of SMLM

Single molecule conditions

Probe size

Labelling density

Localization precision

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Localization precision

Sample stability

Critical features of SMLM

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Probe size

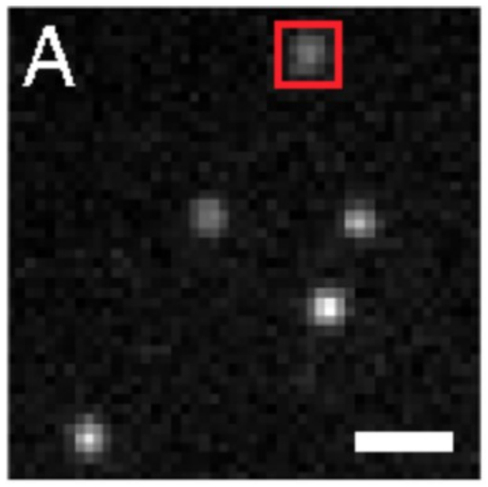
Labelling density

Localization precision

Sample stability

Contaminants

How to know I’m in single molecules conditions?



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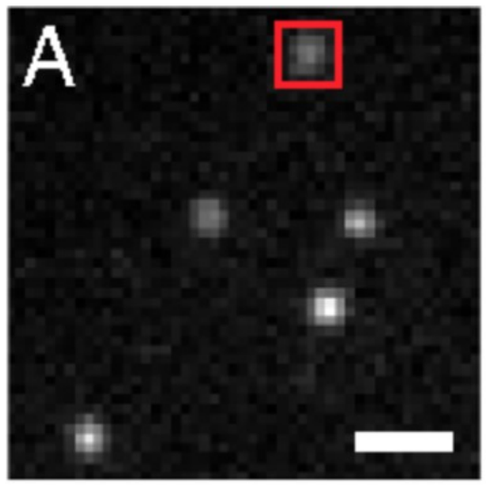

- ✔ **Spatially isolated spot**

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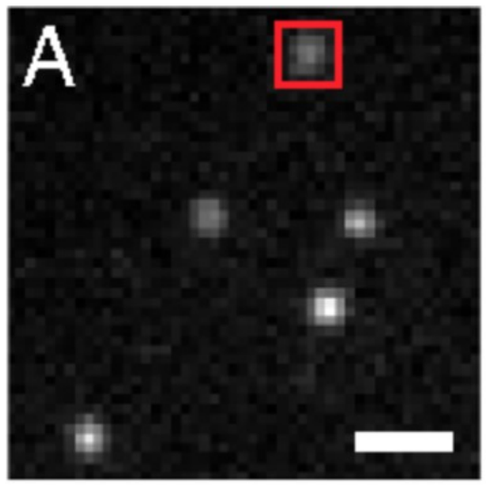

- ✔ **Spatially isolated spot**
- ✔ **Diffraction limited spot (=PSF)**

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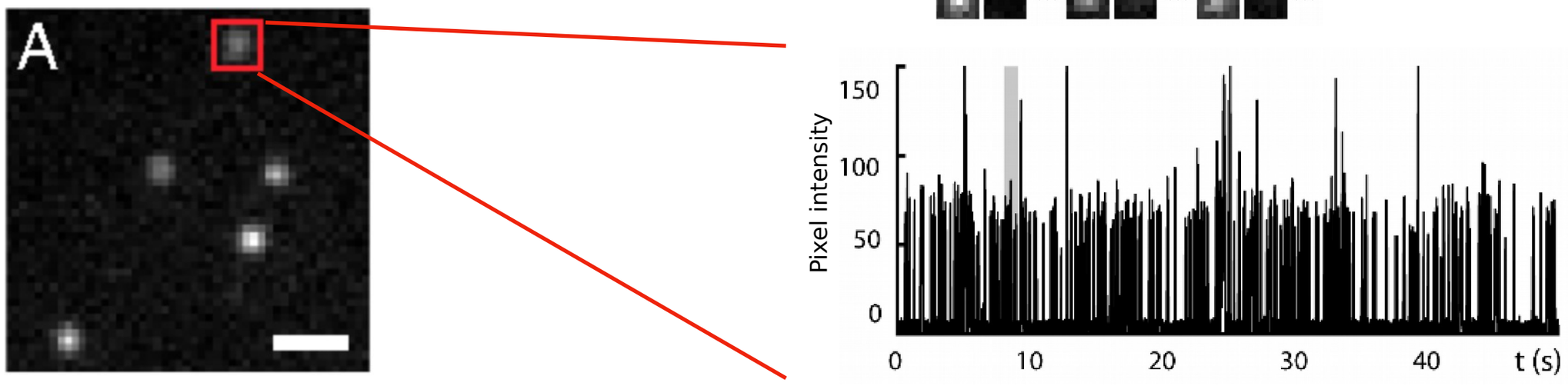

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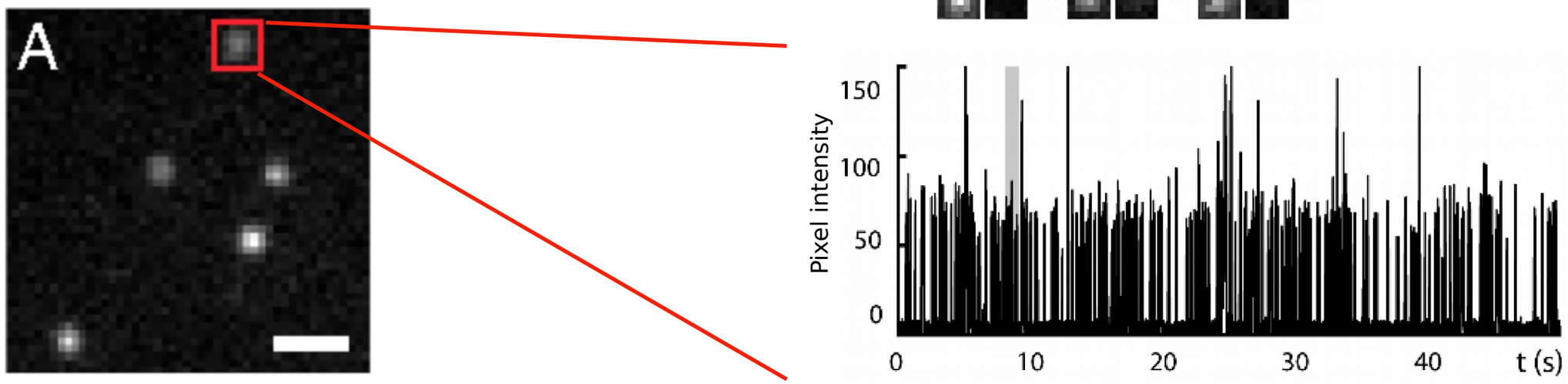

- ✔ **Spatially isolated spot**
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- ✔ **ON states of ~the same intensity**

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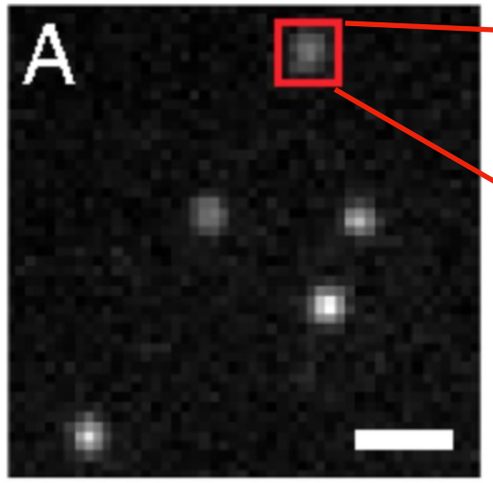

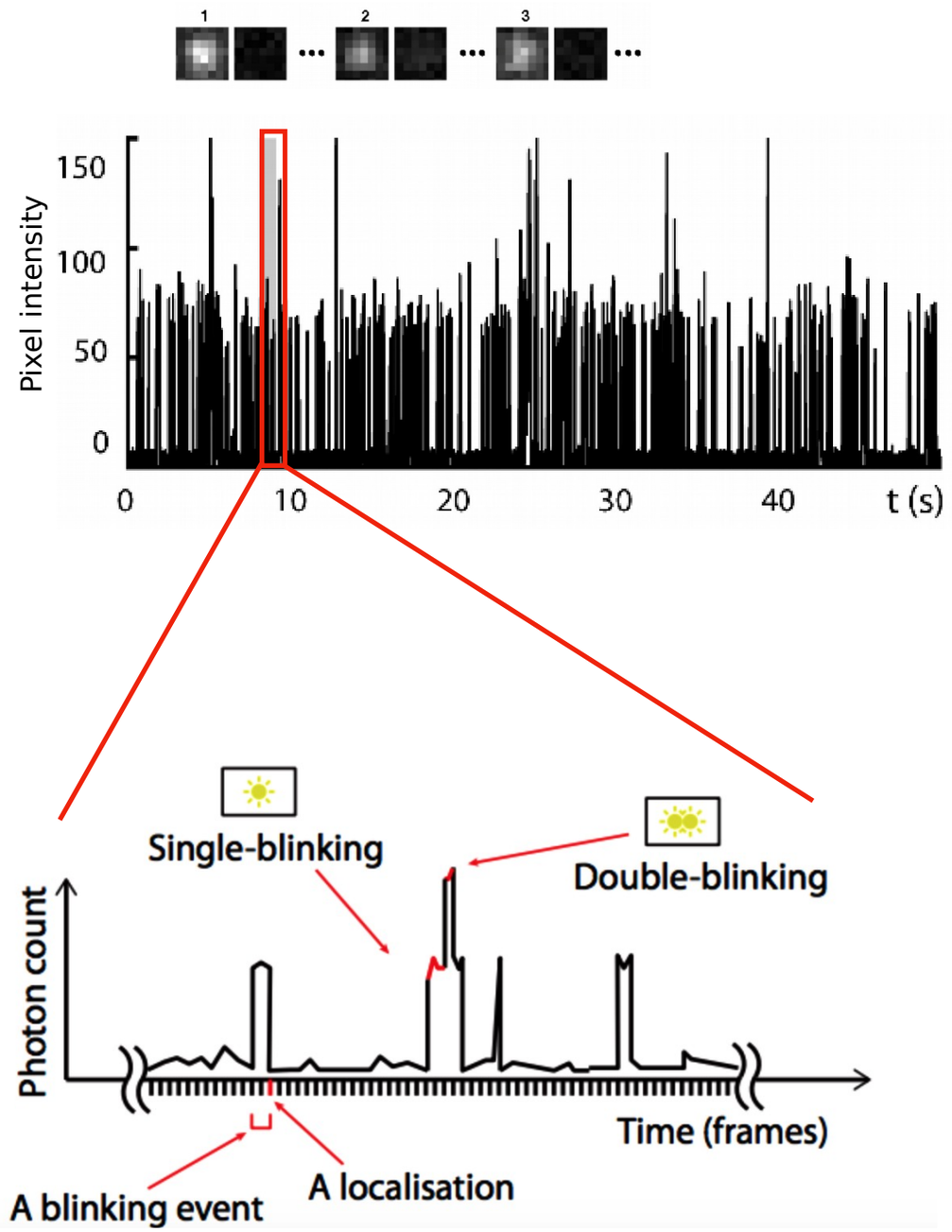

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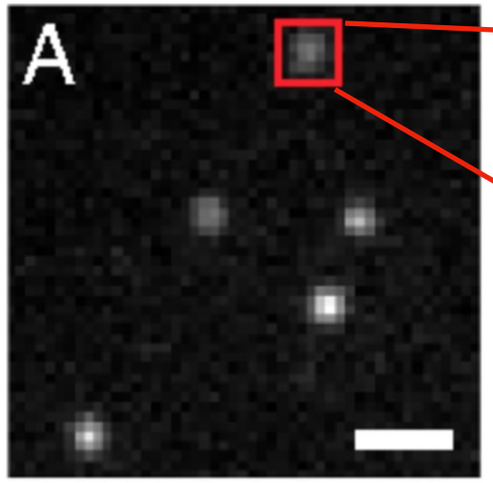

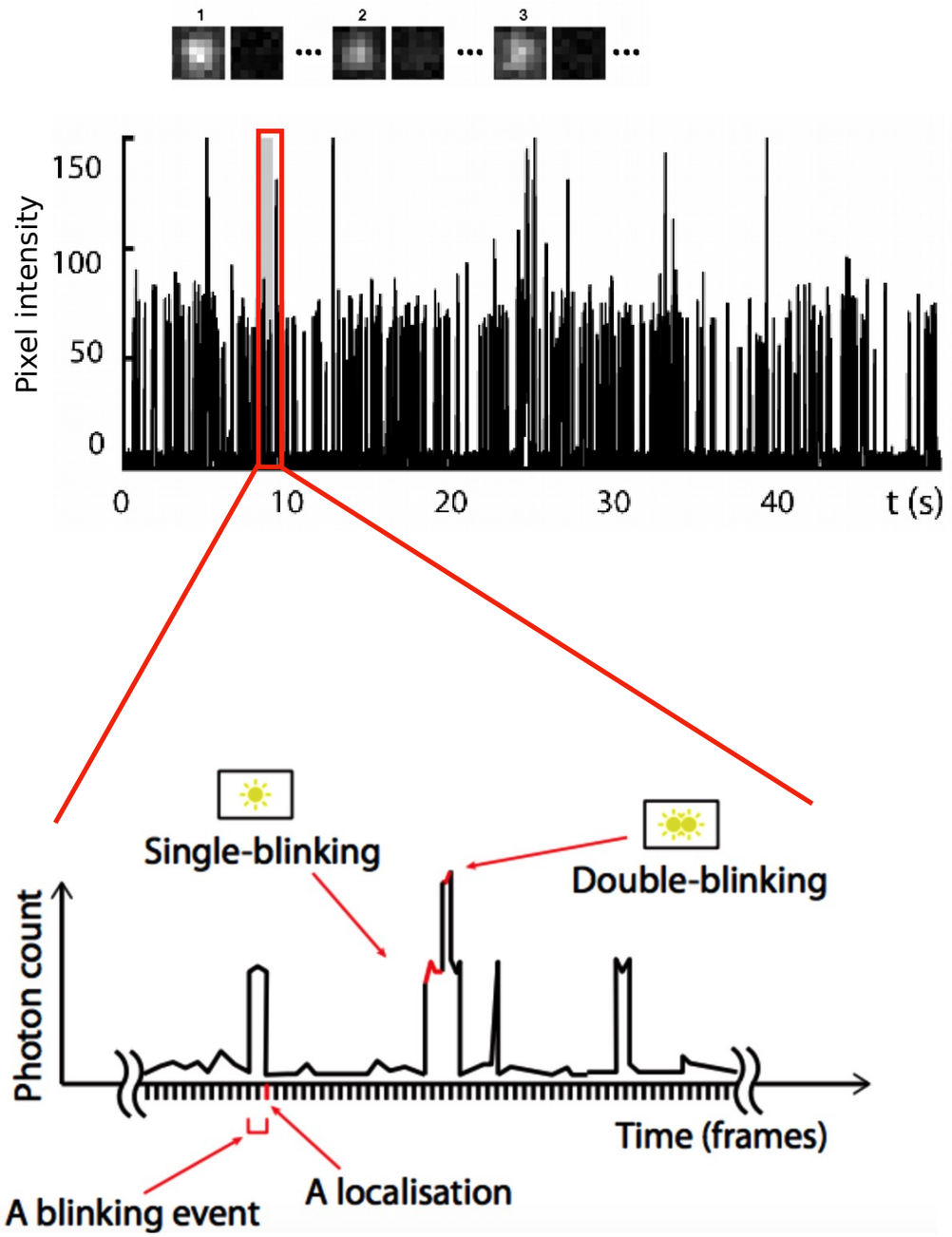

- ✔ **Spatially isolated spot**
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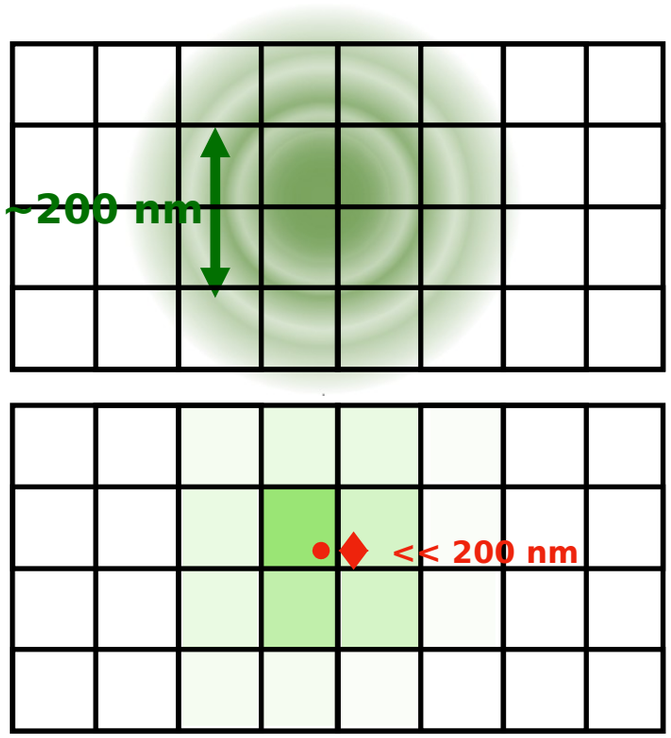
- ✔ **Single step photobleaching/blinking**

PSF sampling



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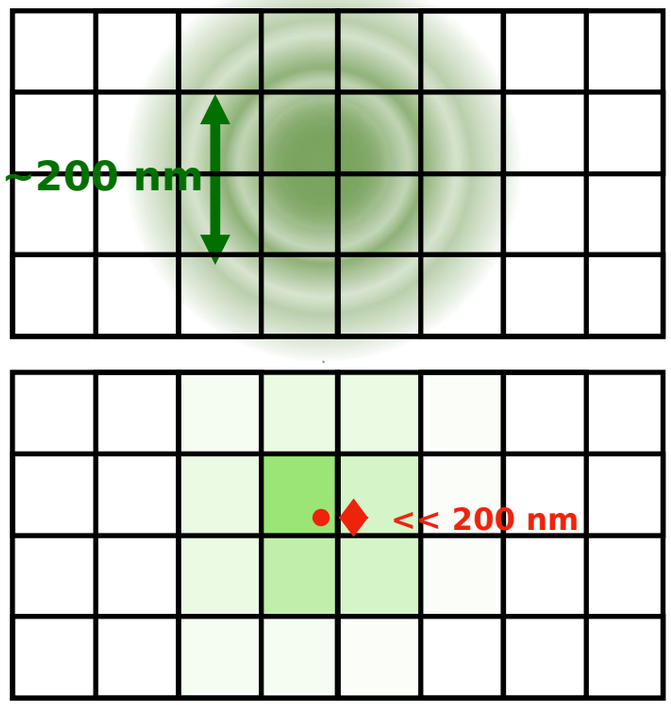

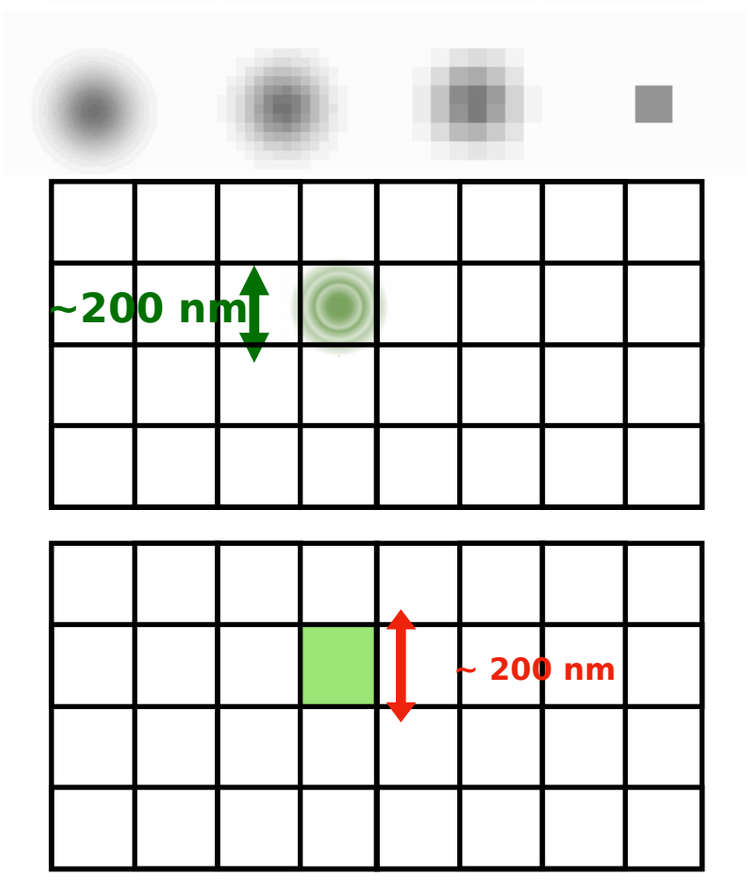

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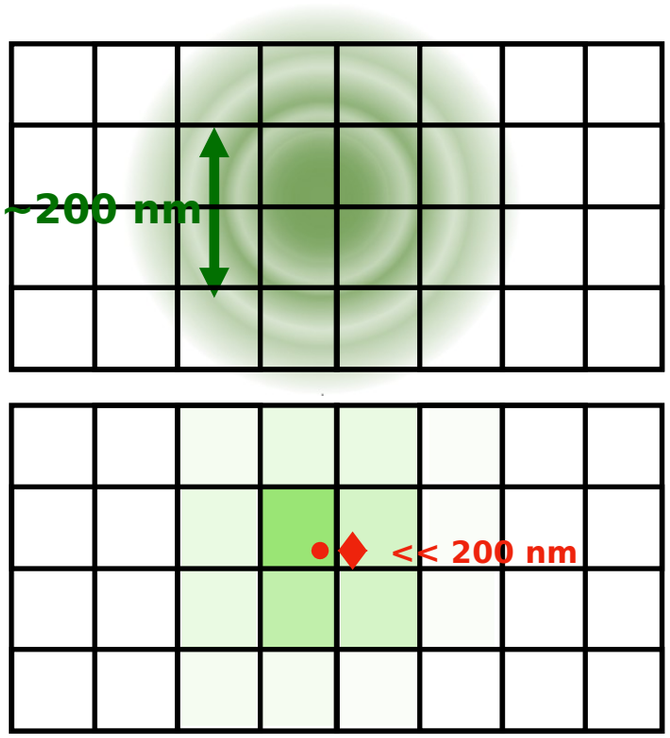
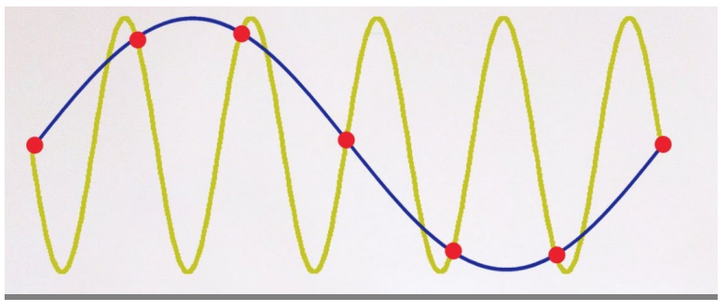
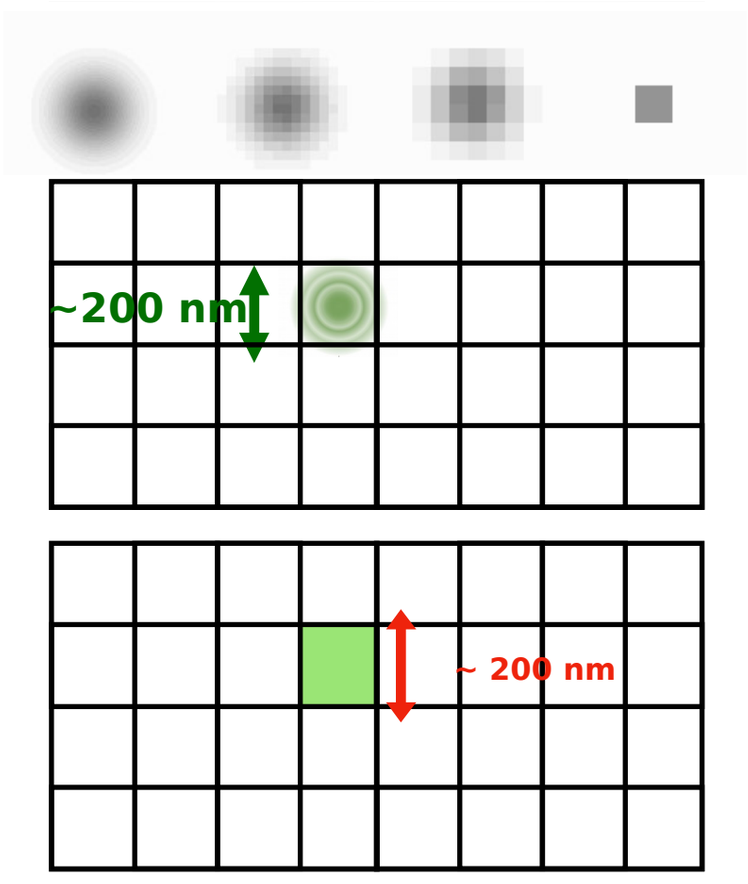


Nyquist theorem

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency





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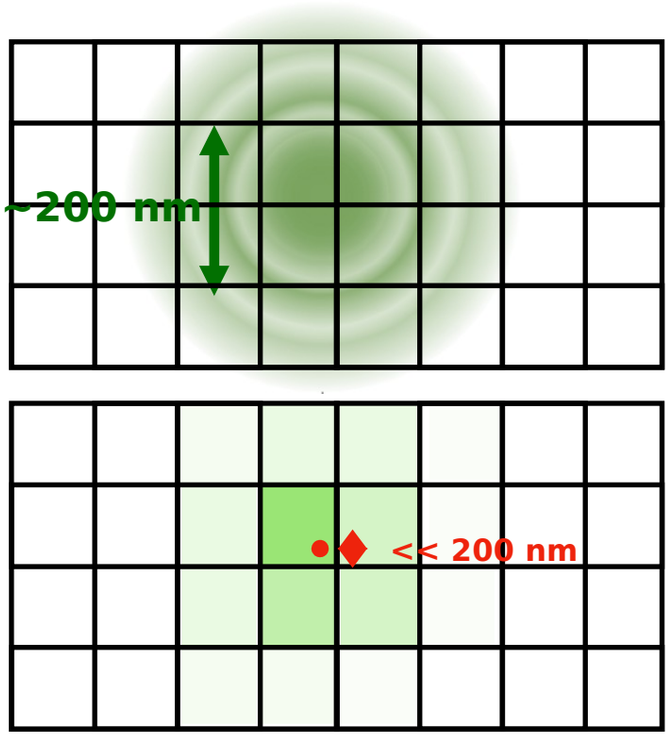

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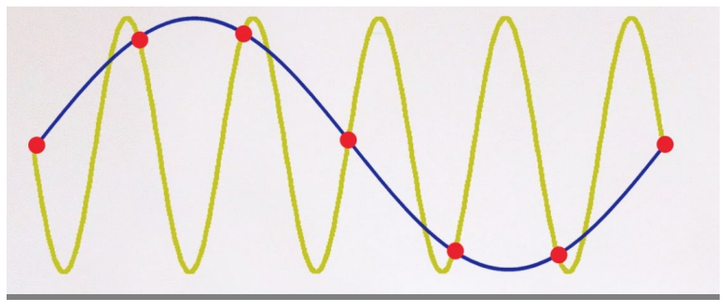
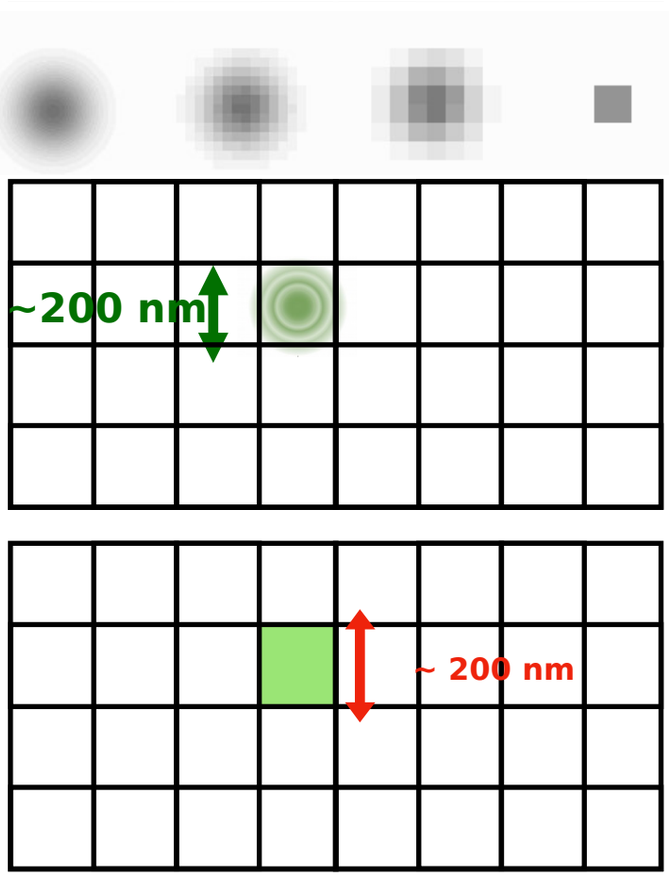
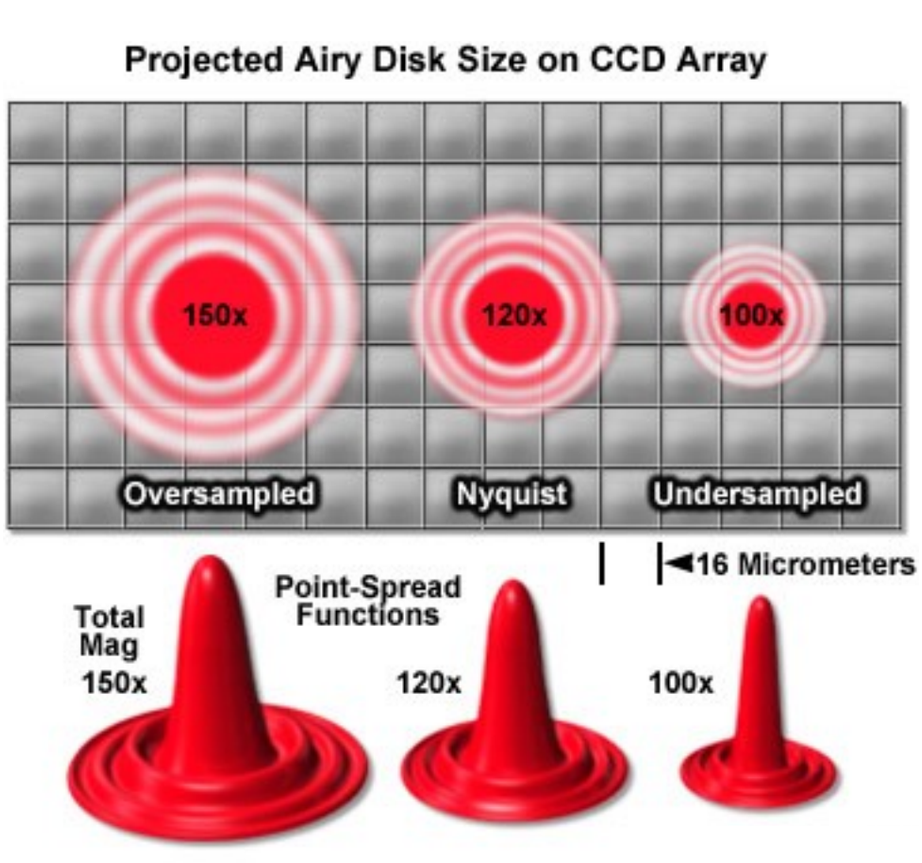


Ex : for a NA 1.4, 100X objective imaging in the green (509 nm), the lateral resolution is 222 nm hence represented as 22.2 µm on the camera plane. The camera pixel size should therefore be no more than 11 µm.





Localization precision

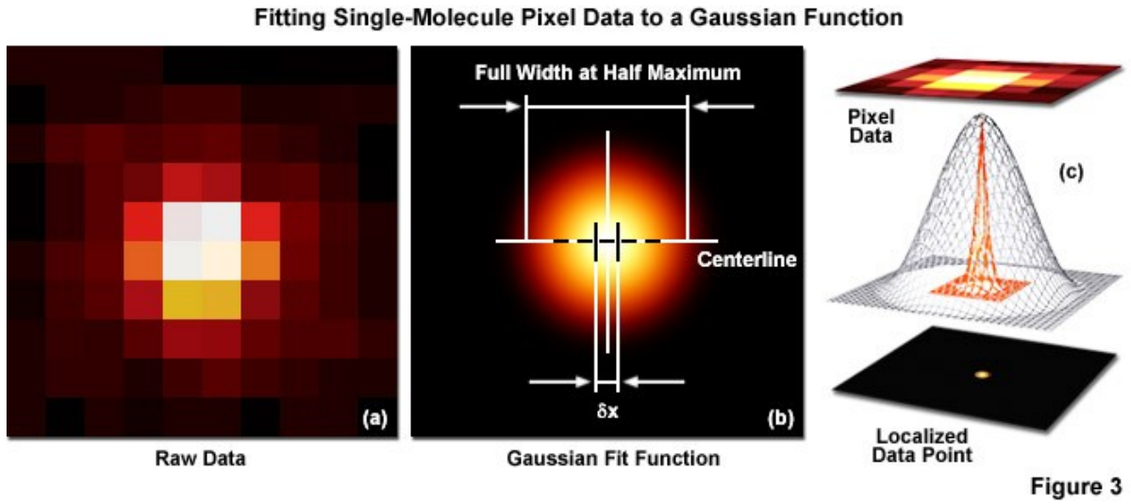


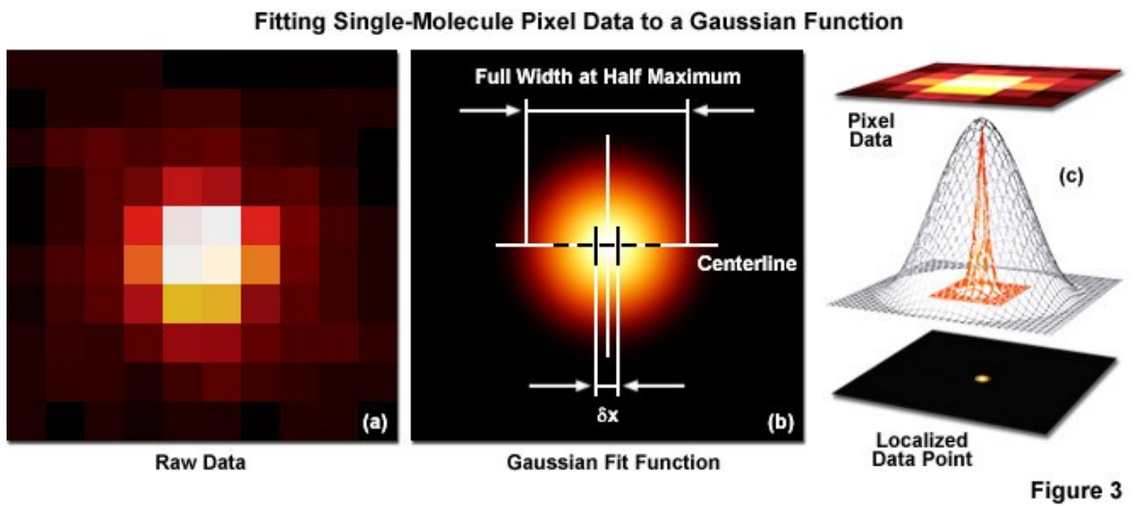
Figure 3

Localization precision

Localization precision is determined by the equation:

$$\sigma = \sqrt{\frac{s^2}{N} + \frac{\left(\frac{a^2}{12}\right)}{N} + \frac{4\sqrt{\pi}s^3b^2}{aN^2}}$$

Thompson et al. (2002)



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Where:

σ is localization precision,

s is the std of the Gaussian function,

N is the number of photons captured,

a is the pixel size of the detector and

b is the std of the background fluorescence.

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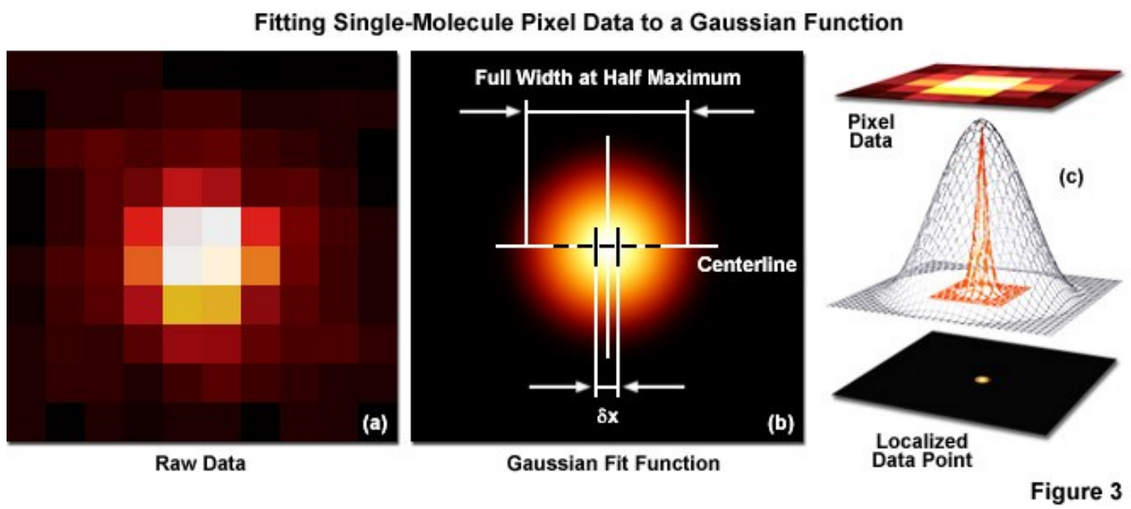
photon shot noise **pixelation noise** **background noise from the sample**



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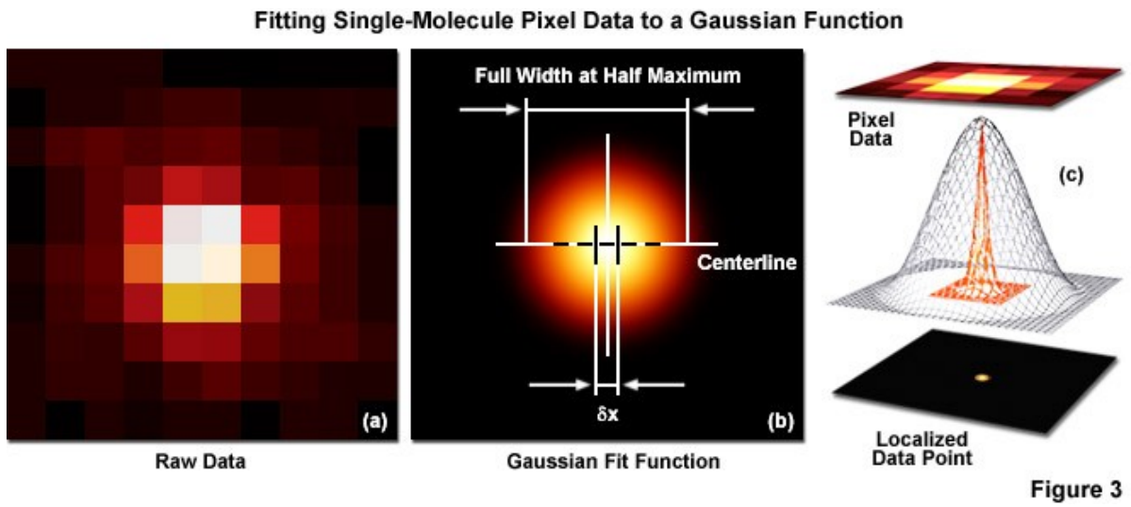


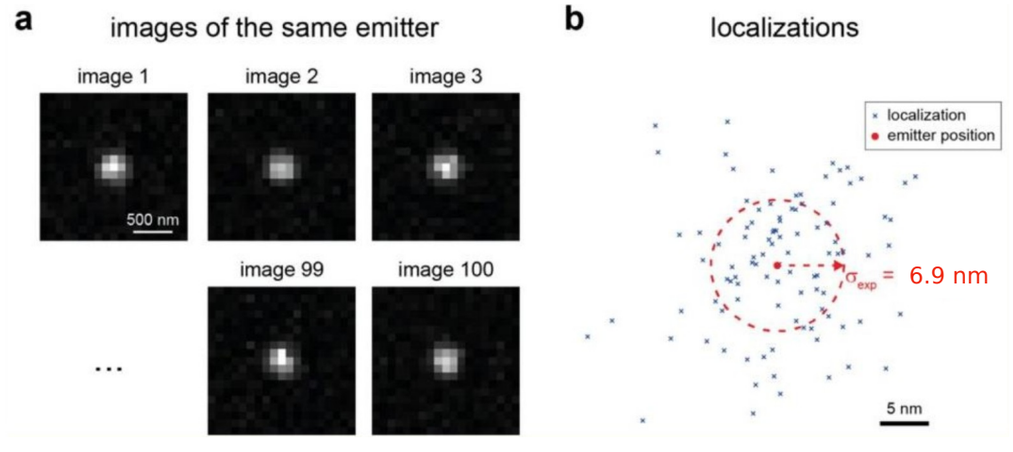
Figure 3

Alexa Fluor 647

σ = 150 nm ($FWHM \cong \lambda/2 \cong$ 350 nm)

$\boldsymbol{N}$ = **1000 photons** ; a = 100 nm ; b = 2 photons

$\boldsymbol{\Delta x_{loc} = 6.9\ nm}$



Localization precision

Localization precision is determined by the equation:

$$\sigma = \sqrt{\frac{s^2}{N} + \frac{\left(\frac{a^2}{12}\right)}{N} + \frac{4\sqrt{\pi}s^3b^2}{aN^2}}$$

Thompson et al. (2002)

photon shot noise **pixelation noise** **background noise from the sample**

Where:

σ is localization precision,

s is the std of the Gaussian function,

N is the number of photons captured,

a is the pixel size of the detector and

b is the std of the background fluorescence.

With small effective pixel sizes and low background noise, the equation is often simplified to:

$$\sigma \approx \frac{s}{\sqrt{N}}$$

➠ The more photons you collect, the more precise you get

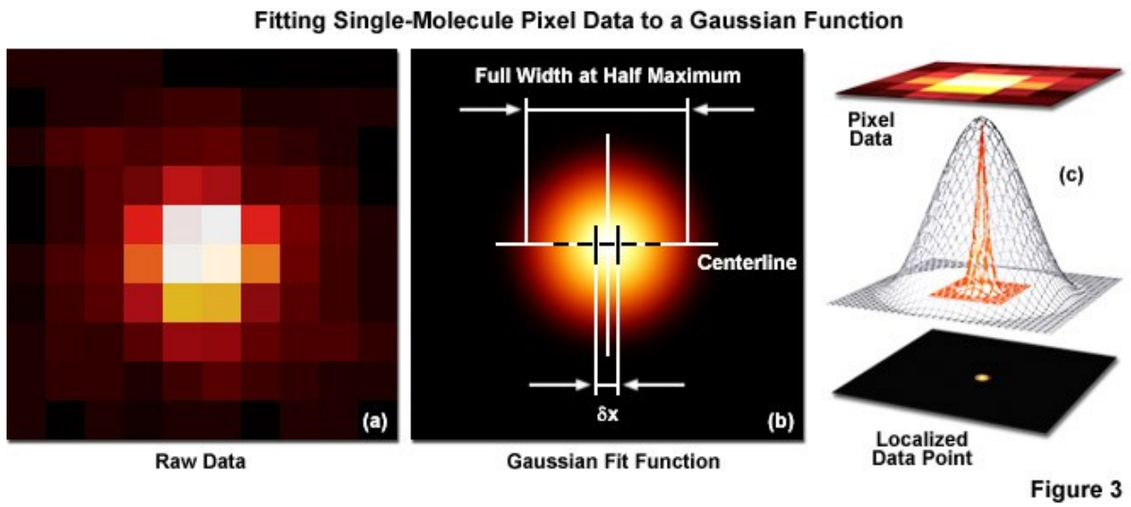


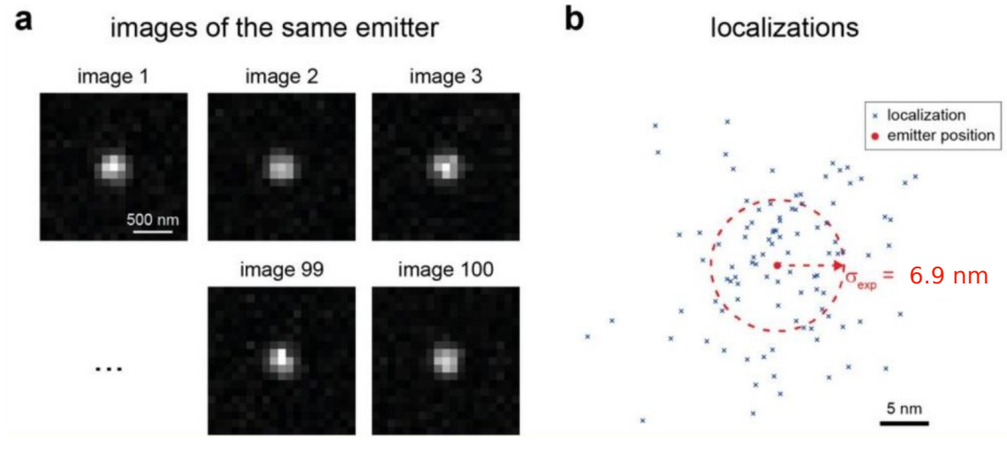
Figure 3

Alexa Fluor 647

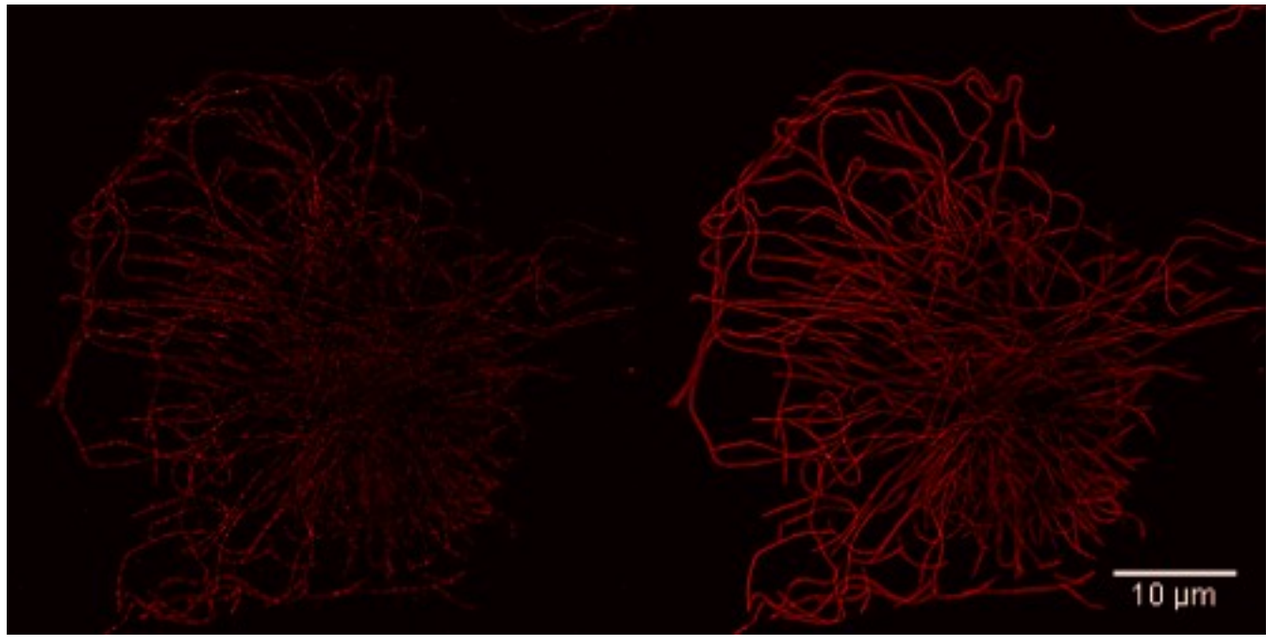
$\sigma = 150$ nm $(FWHM \cong \lambda/2 \cong 350$ nm$)$

$\boldsymbol{N = 1000}$ **photons** ; $a = 100$ nm ; $b = 2$ photons

$\Delta x_{loc} = \mathbf{6.9\ nm}$



Molecular density



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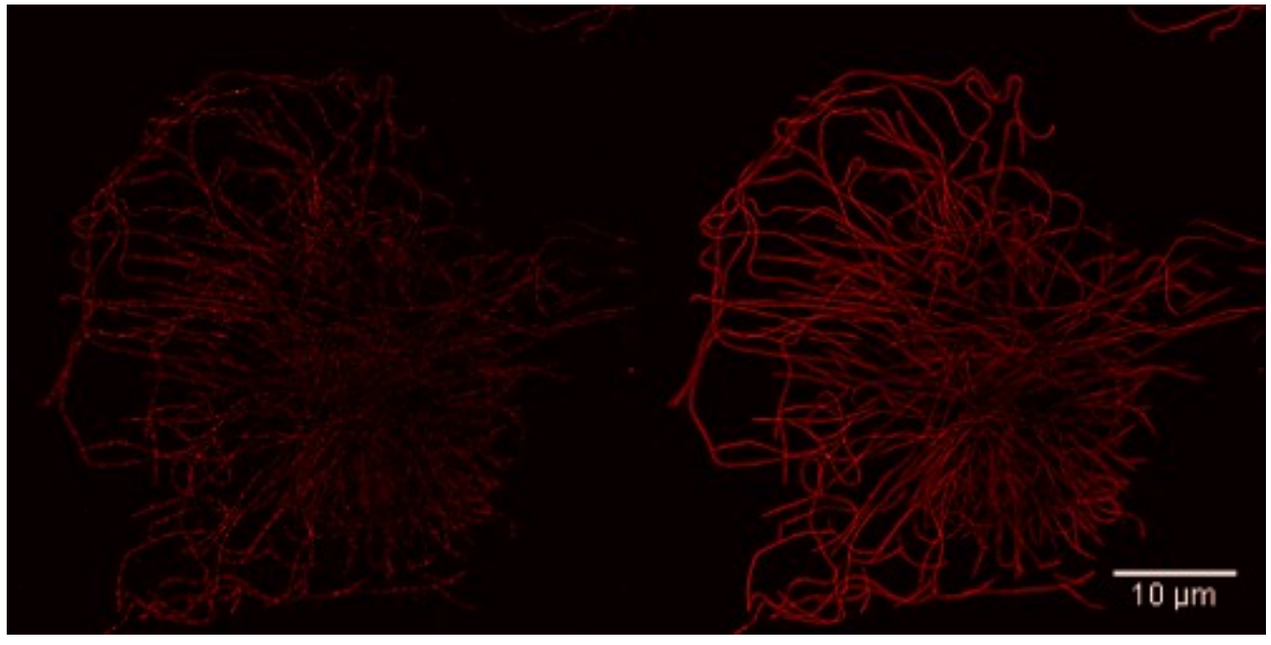

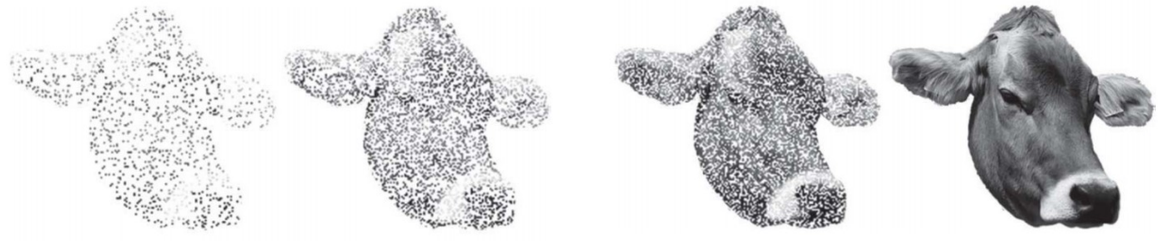

➠ high localization precision means nothing if there aren’t enough fluorophores present to resolve a structure

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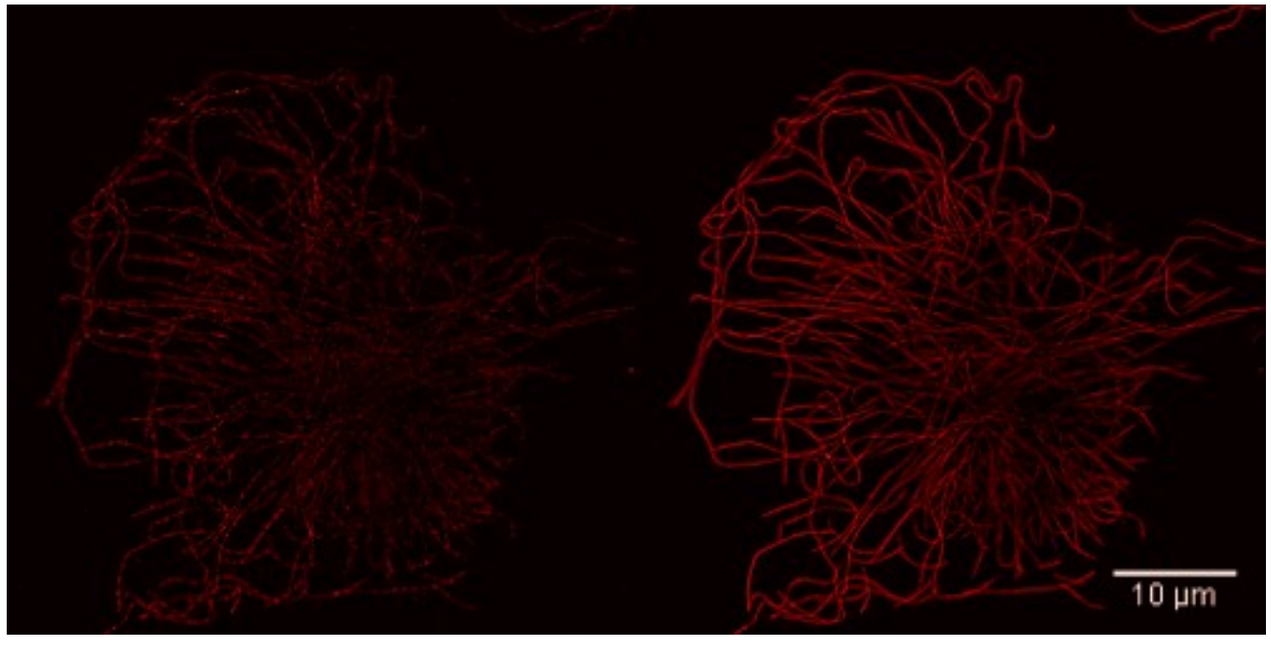

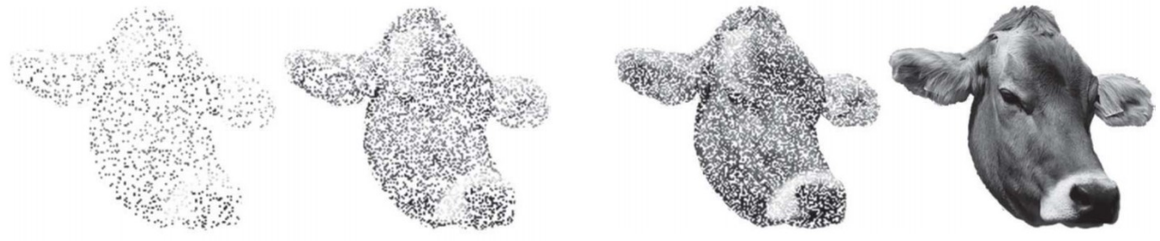

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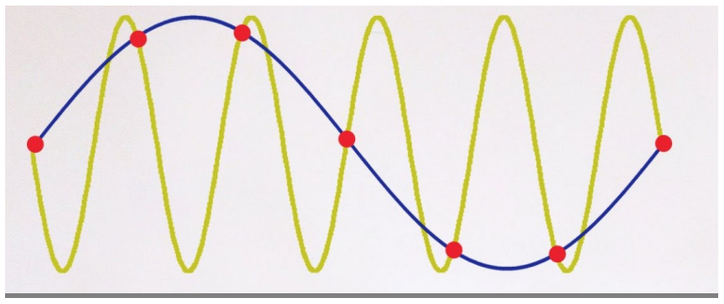
Nyquist theorem

To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency



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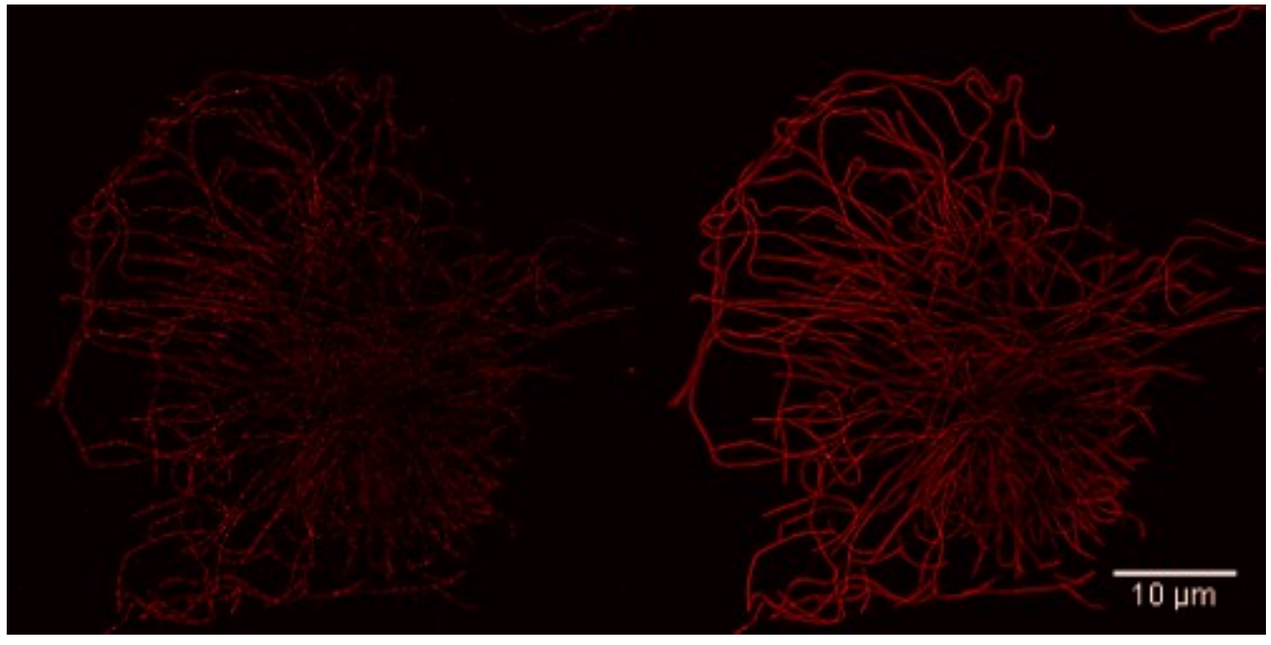

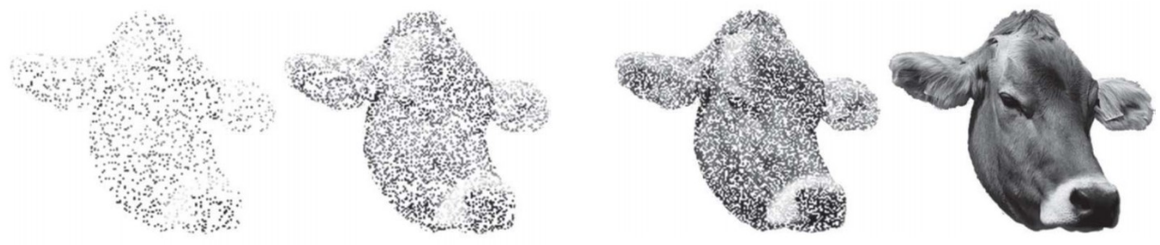

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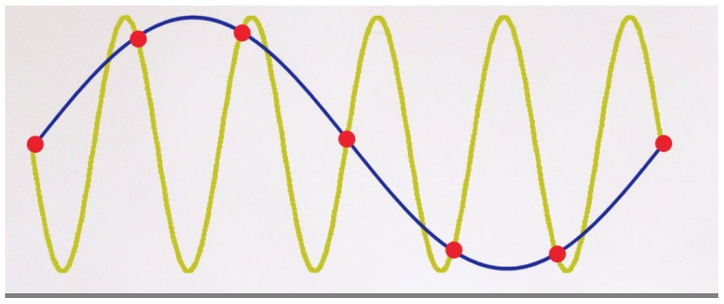
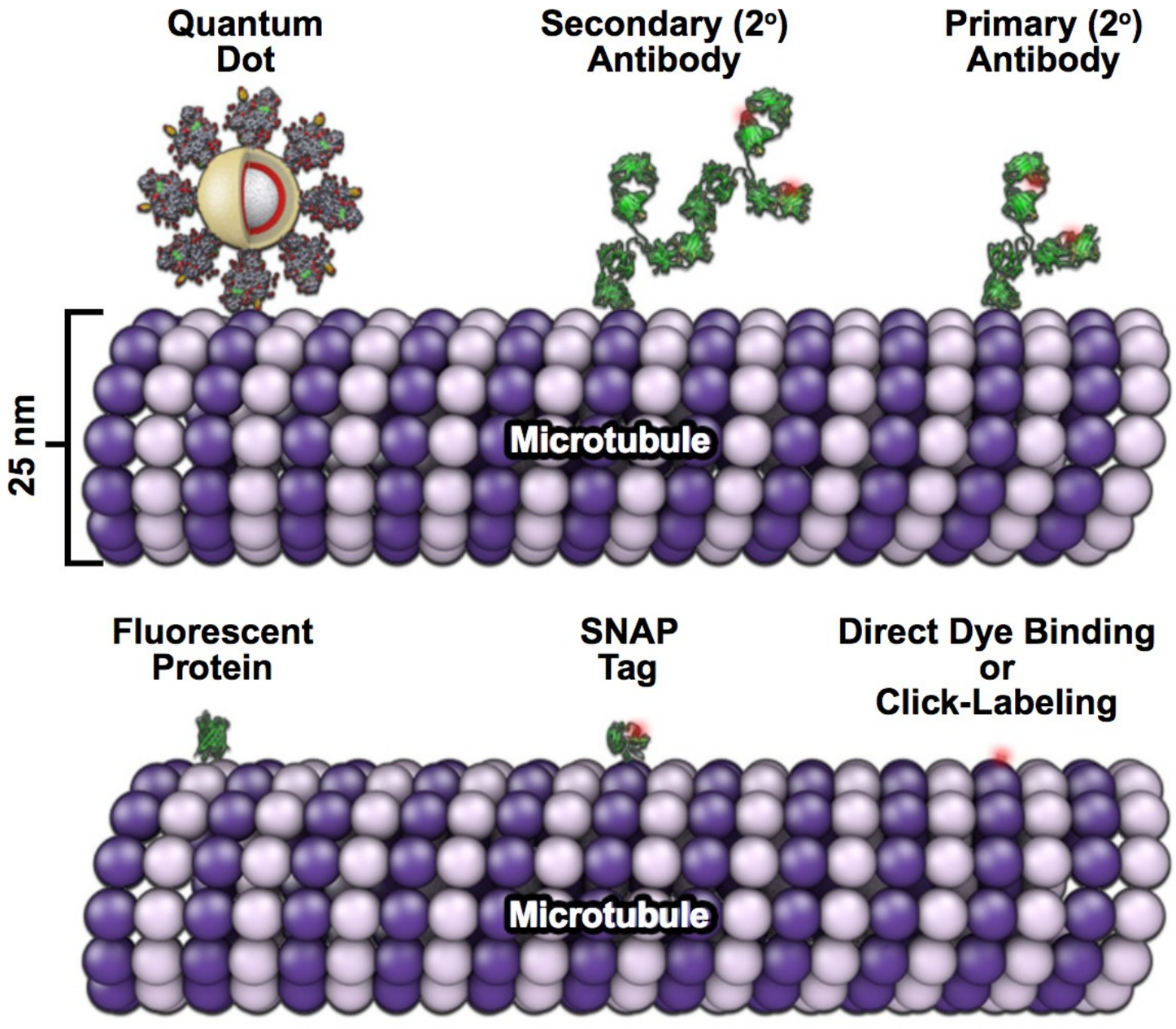


➠ **distance between two neighbouring molecules should be at least twice as fine as the desired resolution**

if the localization accuracy is 20 nm, there should be a fluorophore every 10 nm (at least) for correct sampling.

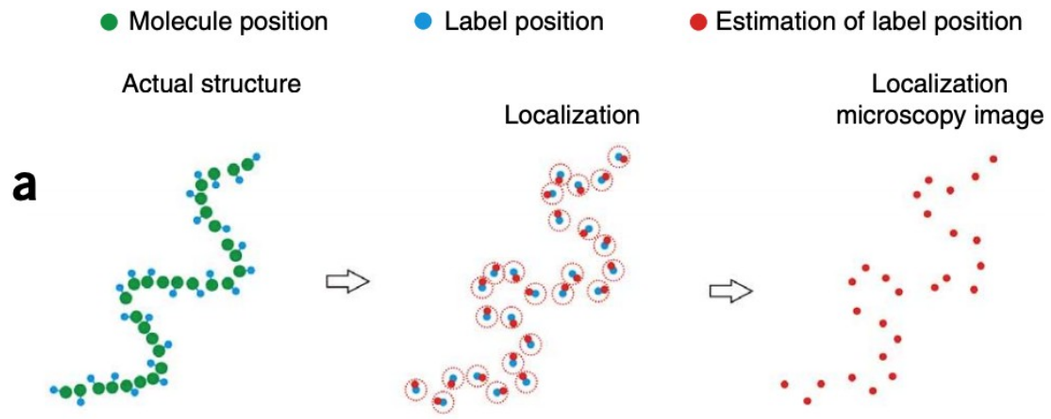
Probe size



Comparison of the relative sizes of common labeling systems for STORM. *Each label is illustrated to scale and attached to a single location on a microtubule (microtubules illustrated to scale with a characteristic diameter of ~25 nm) for reference. For labels utilizing synthetic dyes, the dye is highlighted by a red glow. (a) A quantum dot, including biological targeting moieties decorating the exterior. Note that quantum dots are relatively large, resulting in a significant distance between the fluorescent core and the molecule of interest. This illustration assumes non-antibody conjugate QDot labeling. (b) Indirect immunofluorescence: a primary antibody is directed against the target antigen (tubulin) and a fluorescently labeled secondary antibody is directed against the primary antibody. Antibody molecules are about 10-15 nanometers wide, meaning that indirect labeling can result in a displacement of 20+ nm of the fluorophore from the molecule of interest. (c) Direct immunofluorescence: a fluorescently labeled primary antibody recognizes the antigen, resulting in less (but still significant) separation compared to indirect labeling. (d) Fluorescent protein labeling – FPs are about 2x2x4 nm and are expressed attached directly to the molecule of interest via a short linker sequence, resulting in a separation of only a few nanometers from its fusion partner. (e) SNAP-tag (and other similar chemical labels) are roughly the size of FPs, but rely on an exogenously introduced synthetic dye. (f) A small molecule dye specific for tubulin: such chemically specific dyes bind the molecule of interest most closely.*

Probe/ligand = displacement of the fluorophore from the molecule of interest !!

Probe size / localisation precision / density



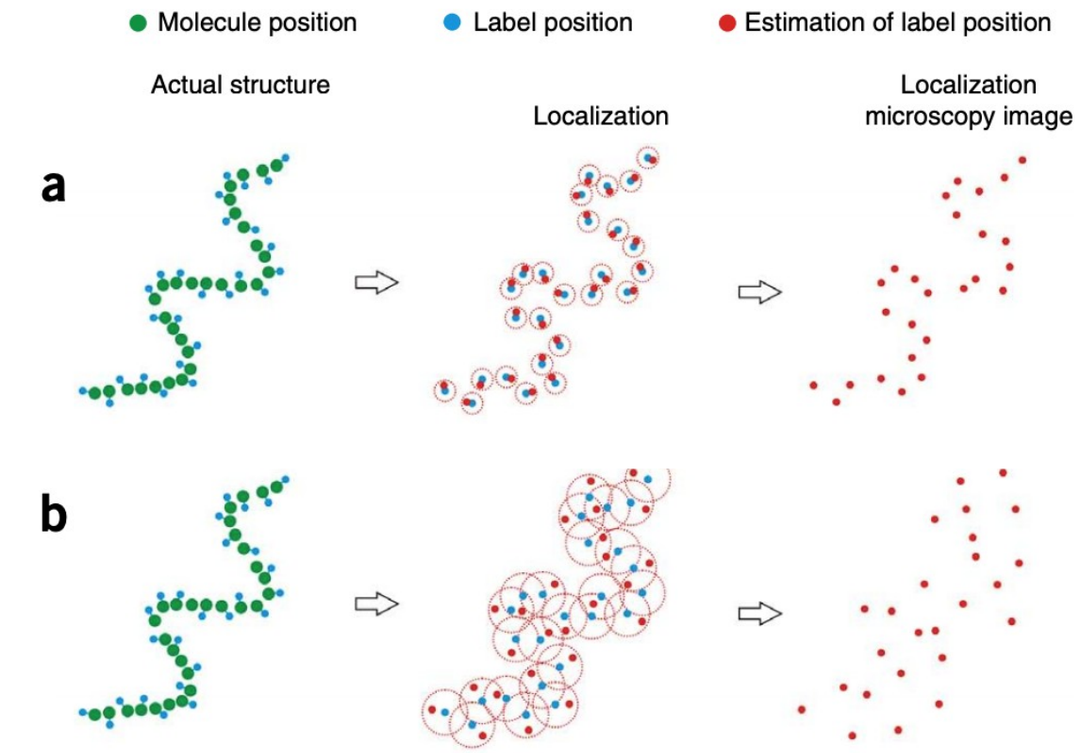
Small label (close to target)

High photon (good localization precision)

=> good reconstruction

Deschout et al., Nature Methods 2014

Probe size / localisation precision / density



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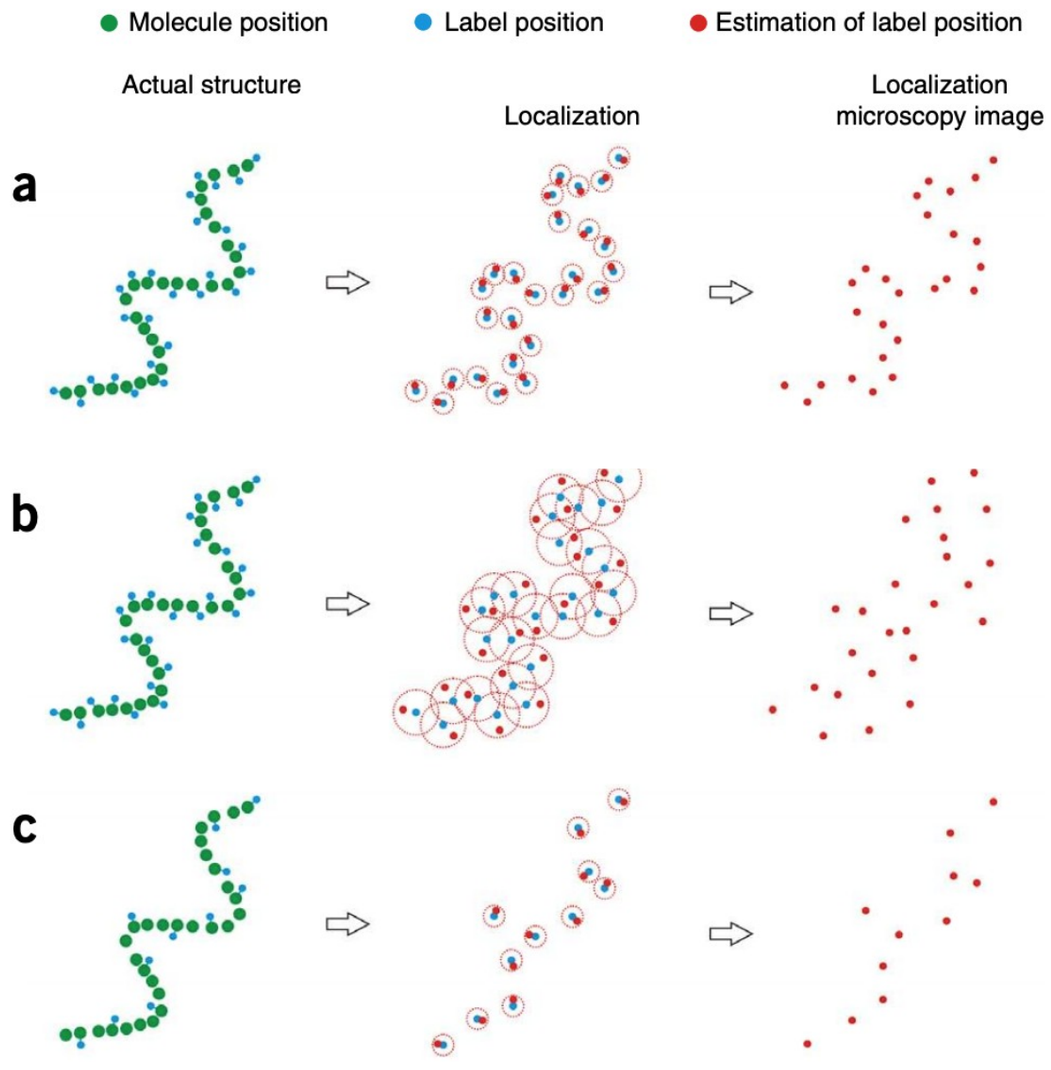
Small label (close to target)

low photon (bad localization precision)

=> bad reconstruction

Deschout et al., Nature Methods 2014

Probe size / localisation precision / density



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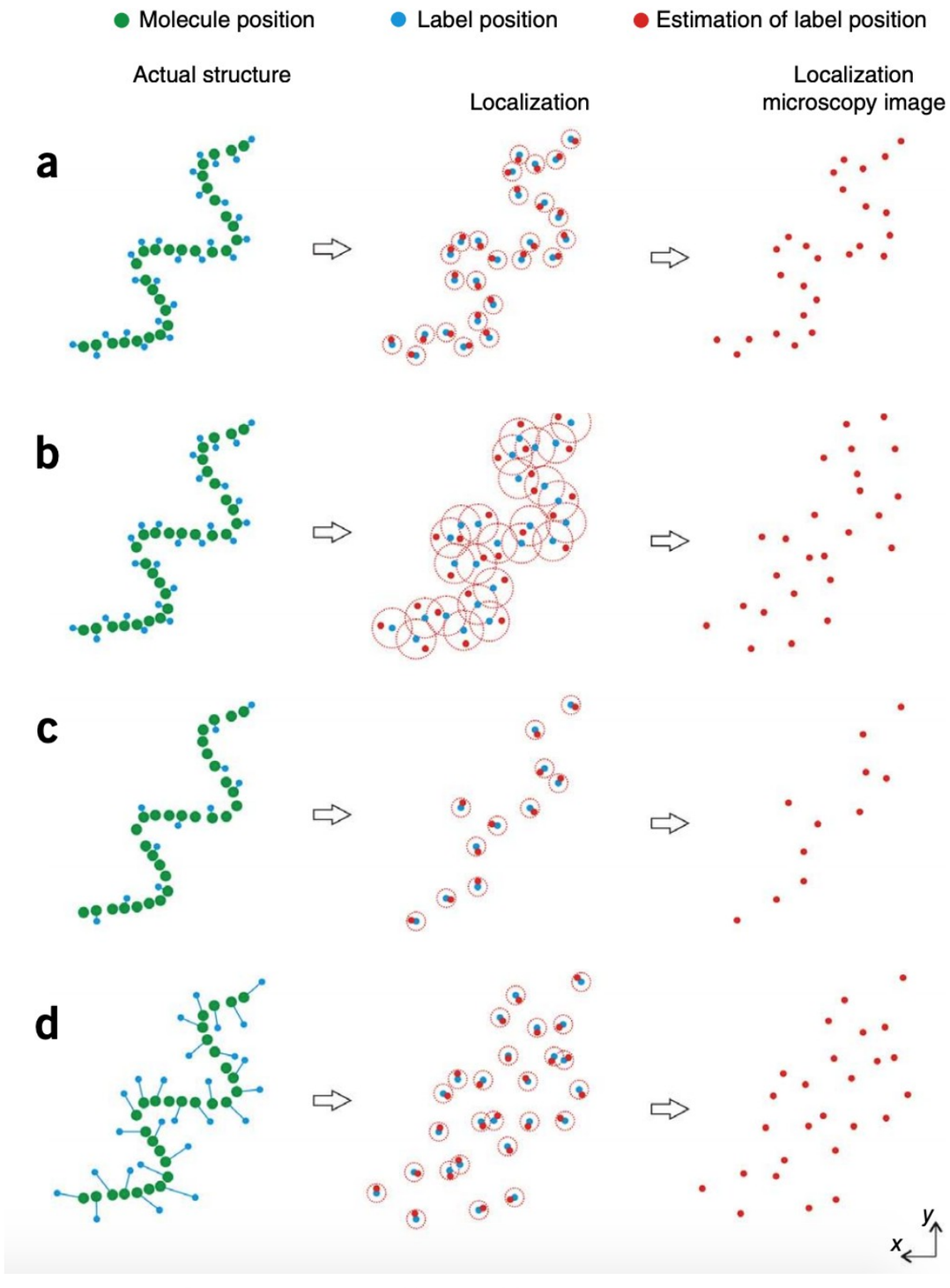

Deschout et al., Nature Methods 2014

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low photon (bad localization precision)

=> bad reconstruction

Small label (close to target)
high photon (good localization precision)

low density

=> bad reconstruction

large label (Far from target)

high photon (good localization precision)

=> bad reconstruction

Sample drift

Thermal Drift (AC, illumination, electronics, etc…)
Vibration (camera fan, doors, electromechanical motion of the setup, etc…)
Mechanical Instability (gravitational strain)
Adding Reagents
Immersion media evaporation
Etc…

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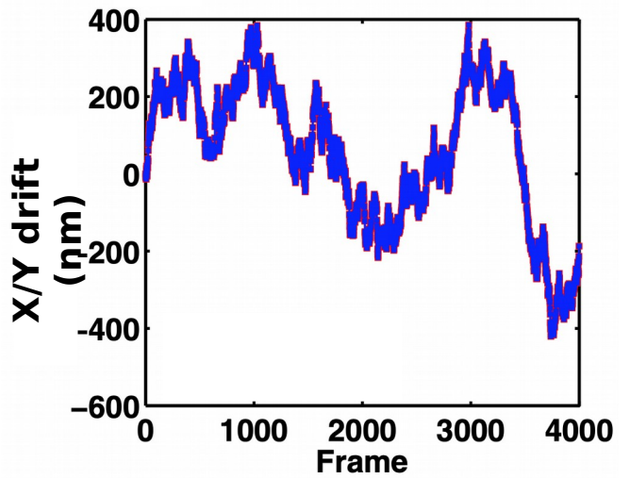
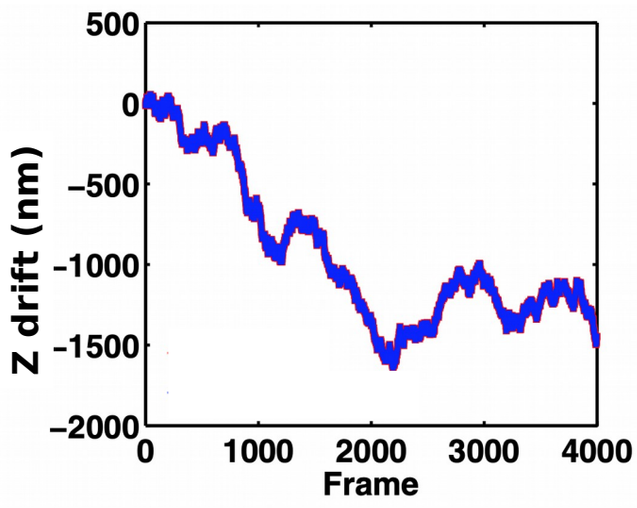
➠ Typical drifts can range from few hundred nanometers to a few microns in three directions.





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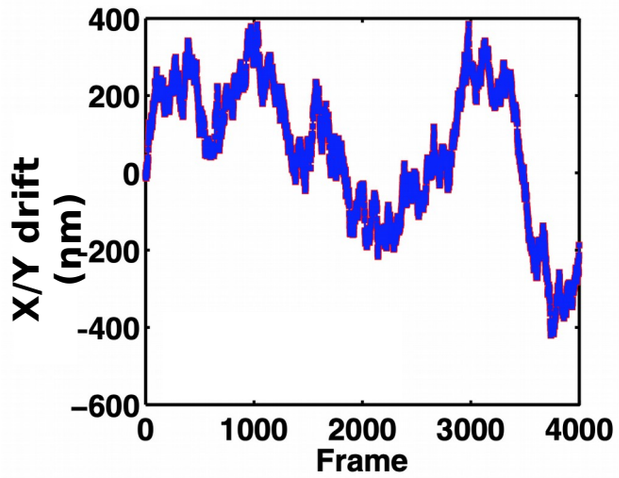

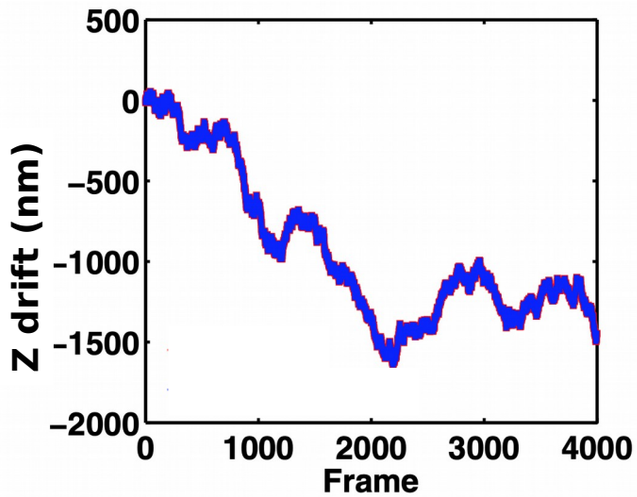

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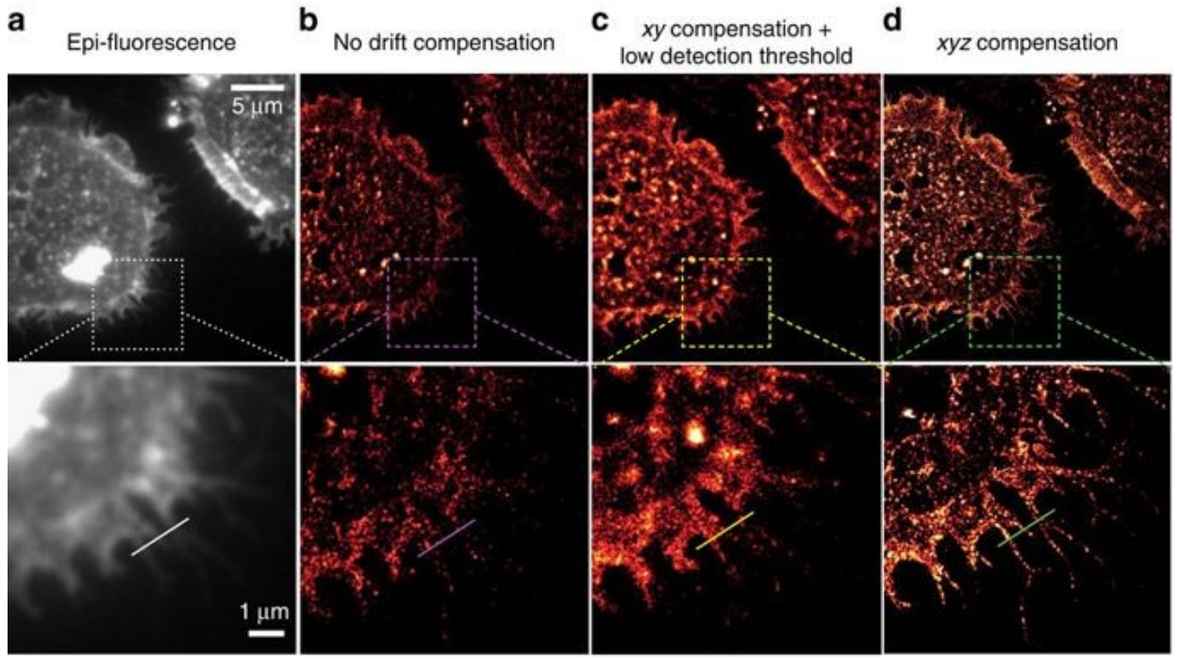

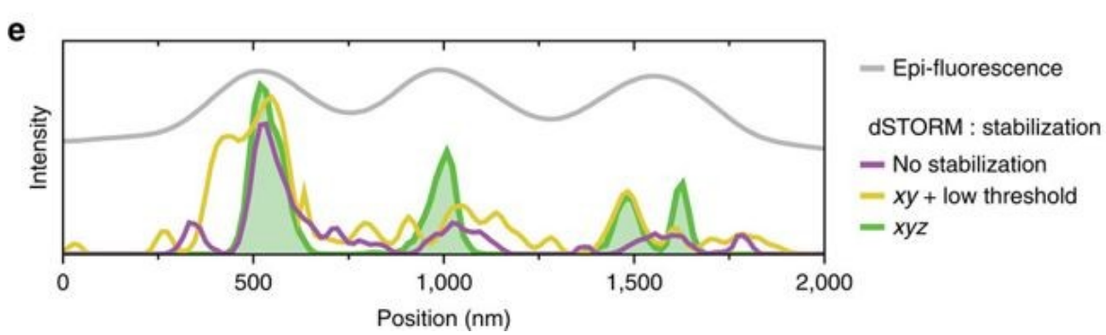

➠ **reduce resolution**

➠ **cause the sample to move out of focus**

➠ **possibly lead to misinterpretation of the results.**

XY sample drift correction

Small fluorescent beads stuck to the coverslip (tetraspeck, gold nanoparticles, nanodiamands, etc...)

- **diffraction limited**
- **bright (good localisation precision)**
- **photostable**

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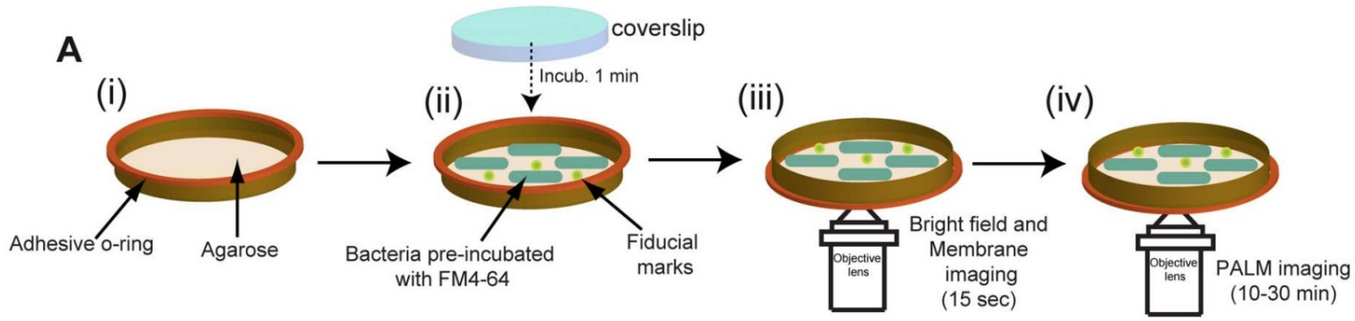

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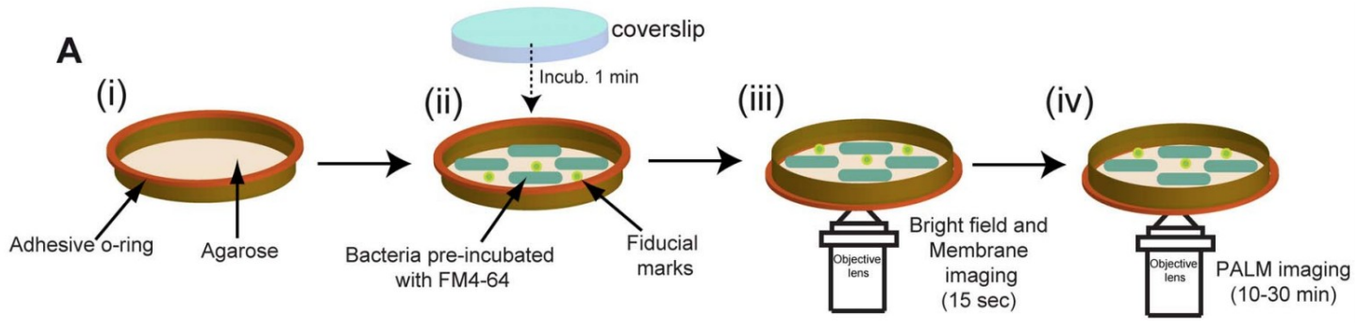
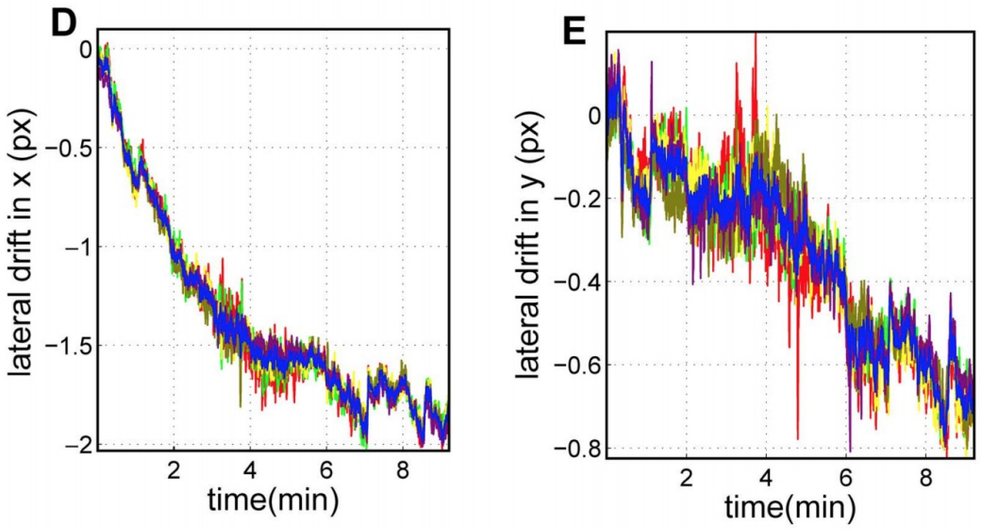


Track fiducial marks position vs time



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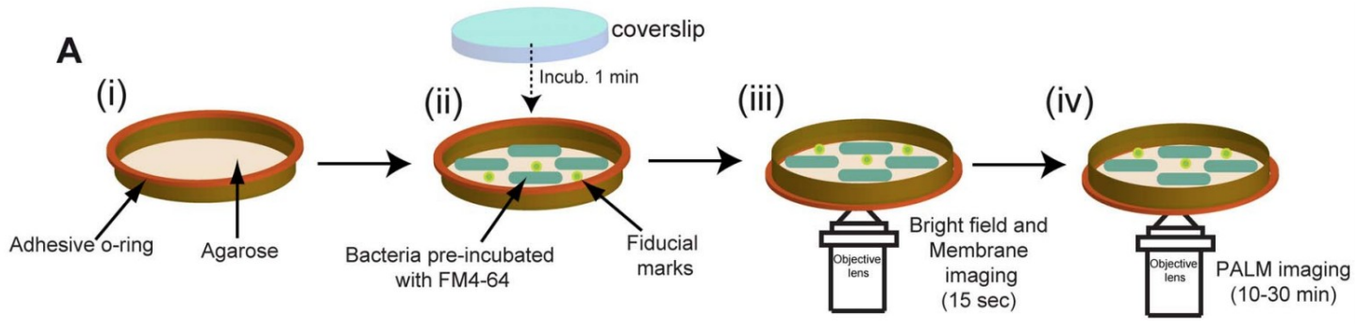

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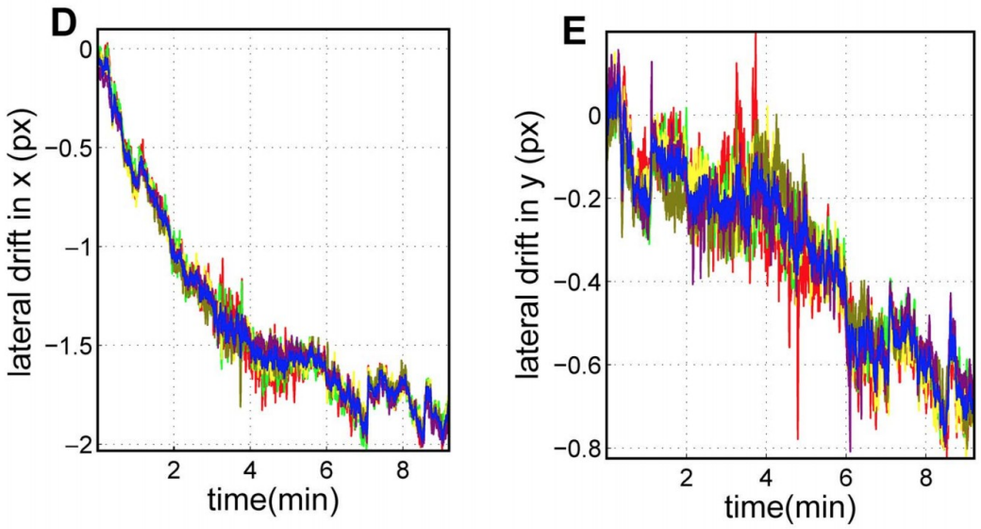
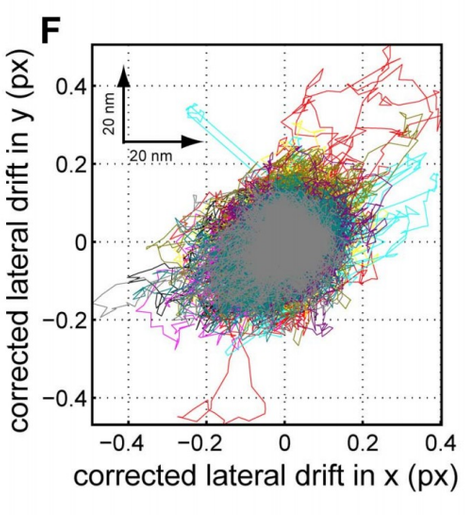


Lateral displacement after drift correction



Residual noise:

dx = 5.4 nm and dy = 6.1 nm

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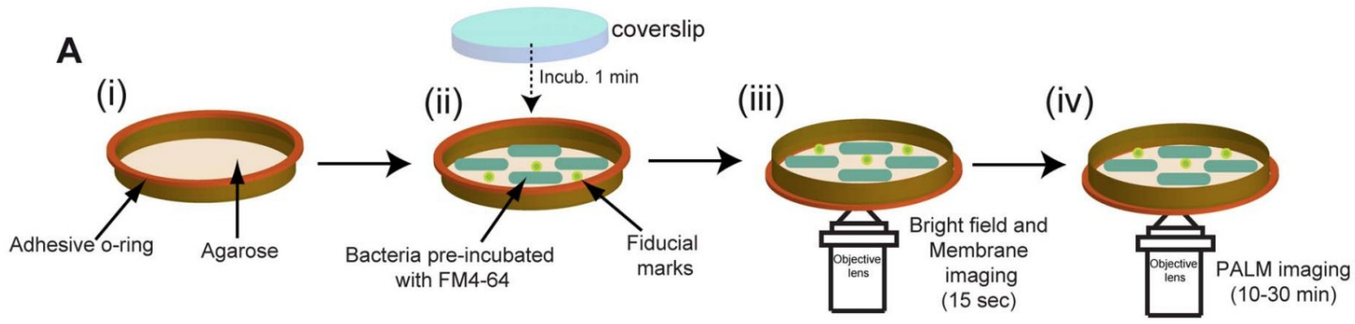

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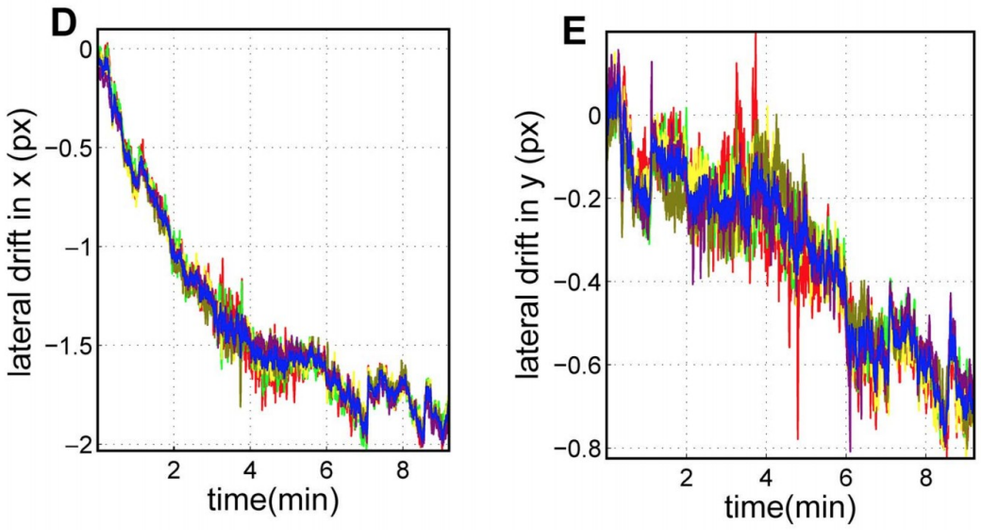
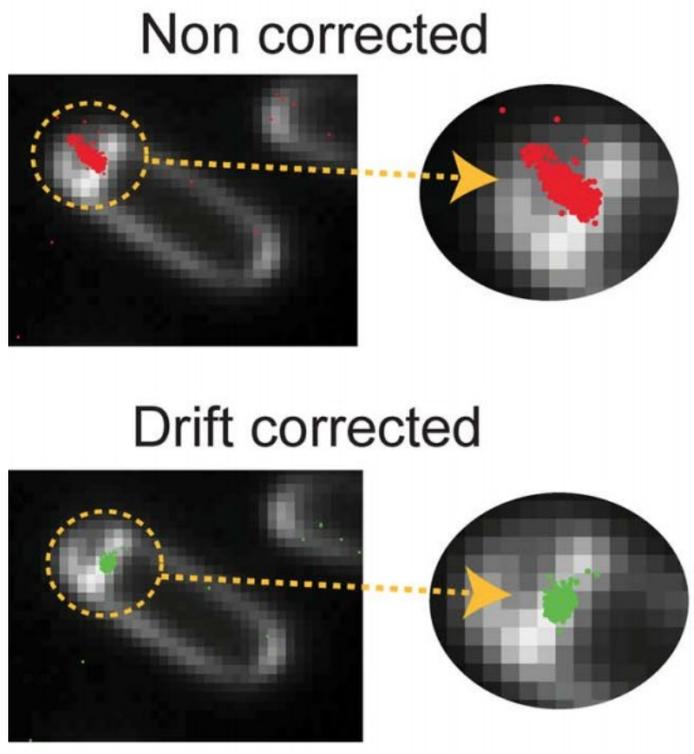


Subtract fiducial marks mean position to PALM localisations



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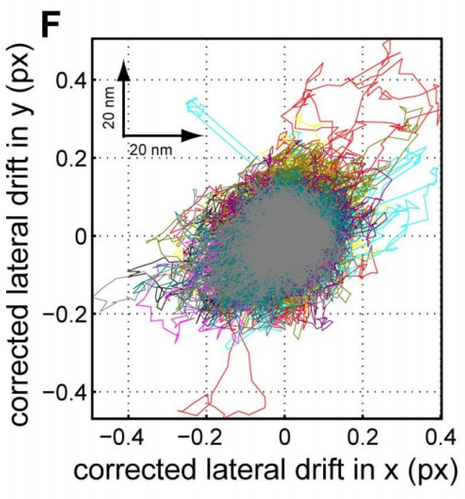

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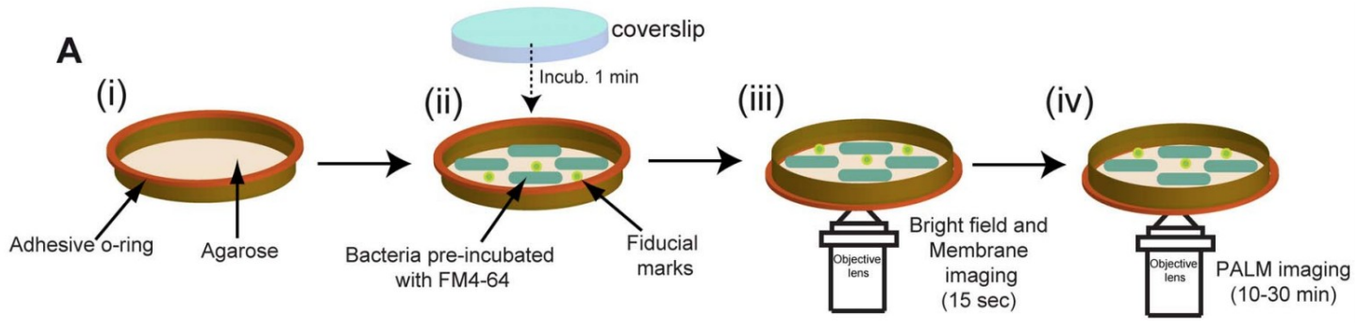

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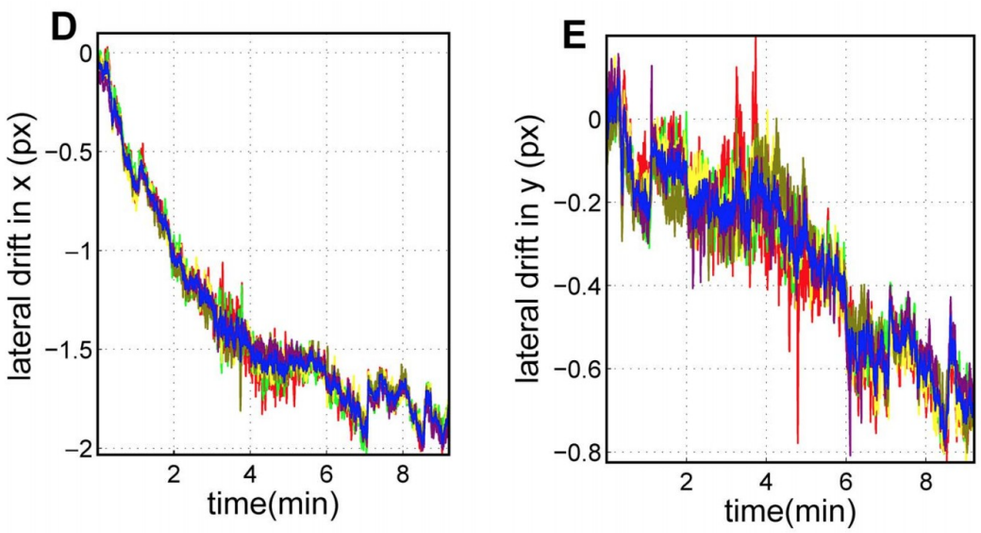

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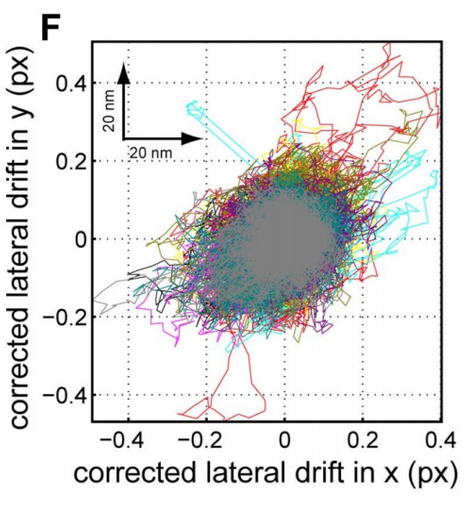

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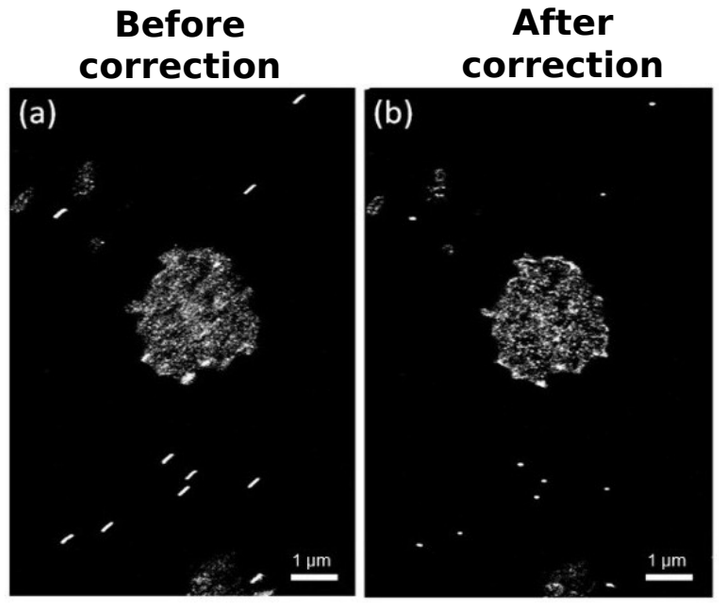
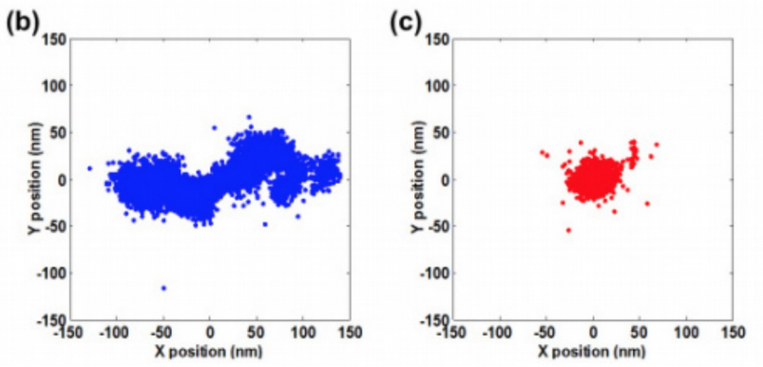
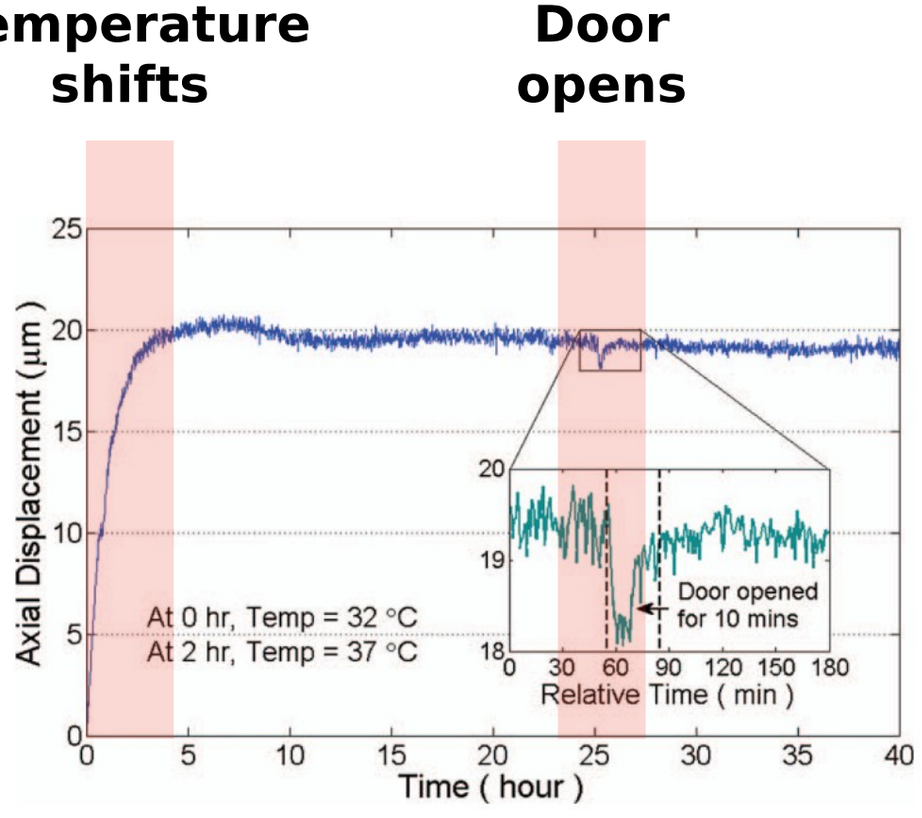




Z sample drift correction



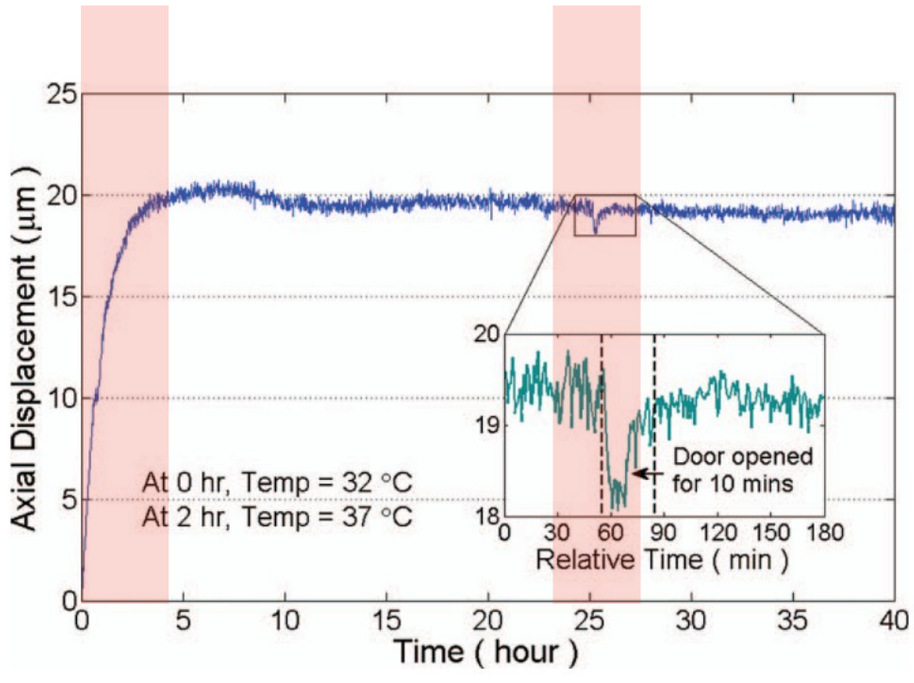
Z sample drift correction

Different methods:
- Image correlation
- Reflected Laser beam
- Total internal reflection beam

TRADE-OFFS :
Simplicity
Range
Speed
Resolution

Temperature shifts

Door opens



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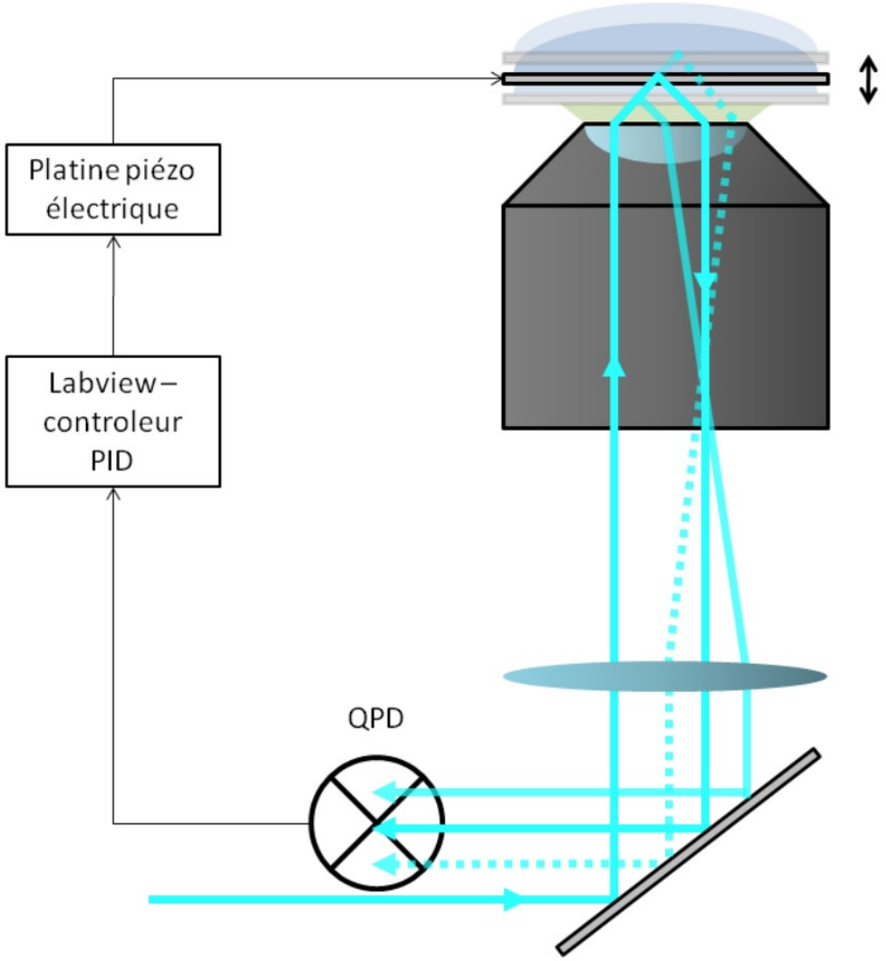

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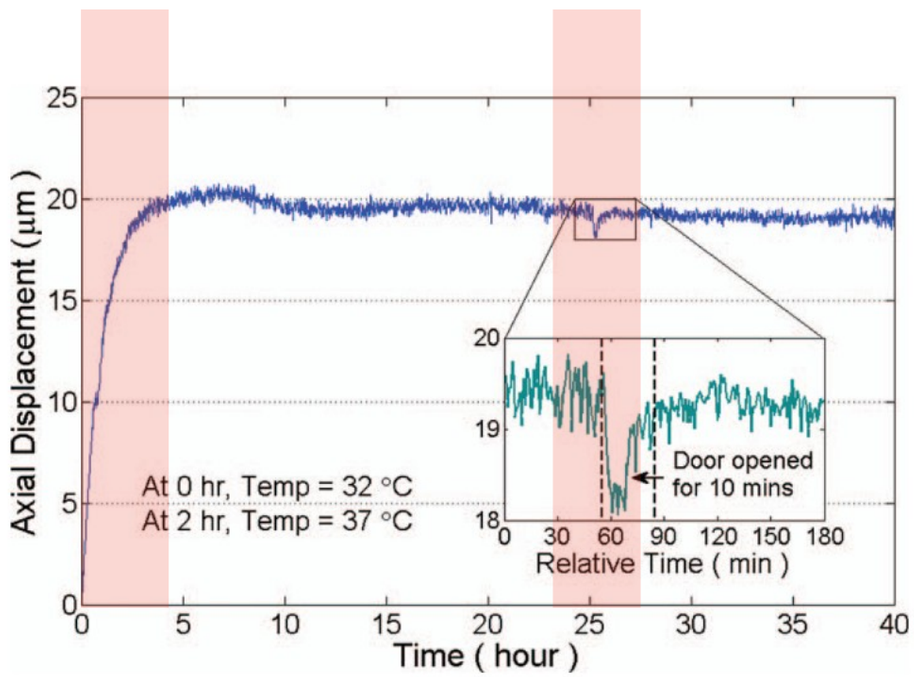
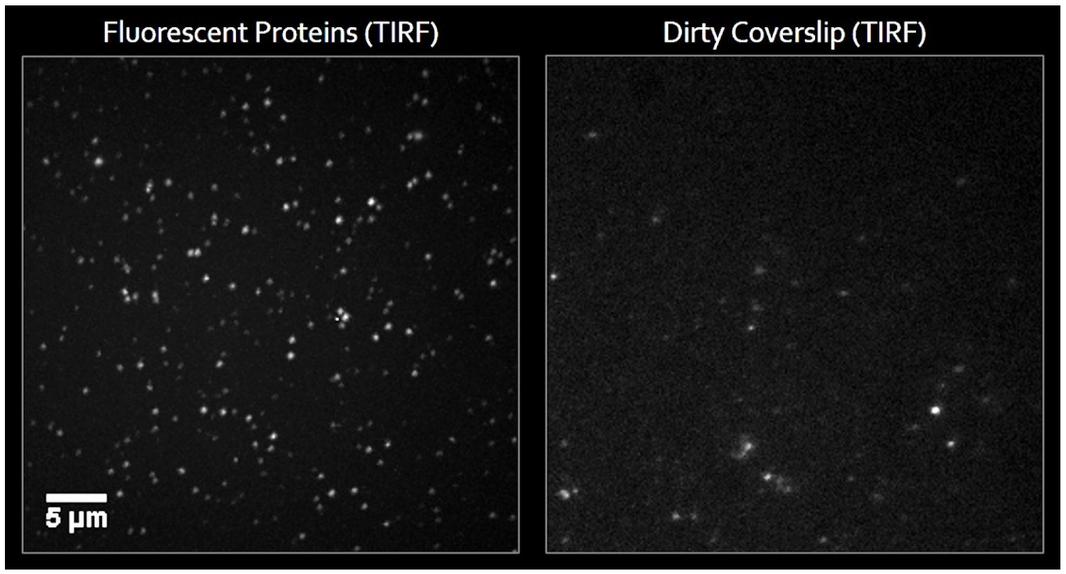


Contaminants

At the single molecule level, even single molecules of dirt may be detected!

Contaminants:

- coverslips are dirty
- reagents (filter them)
- cellular debris
- tubings
- ...



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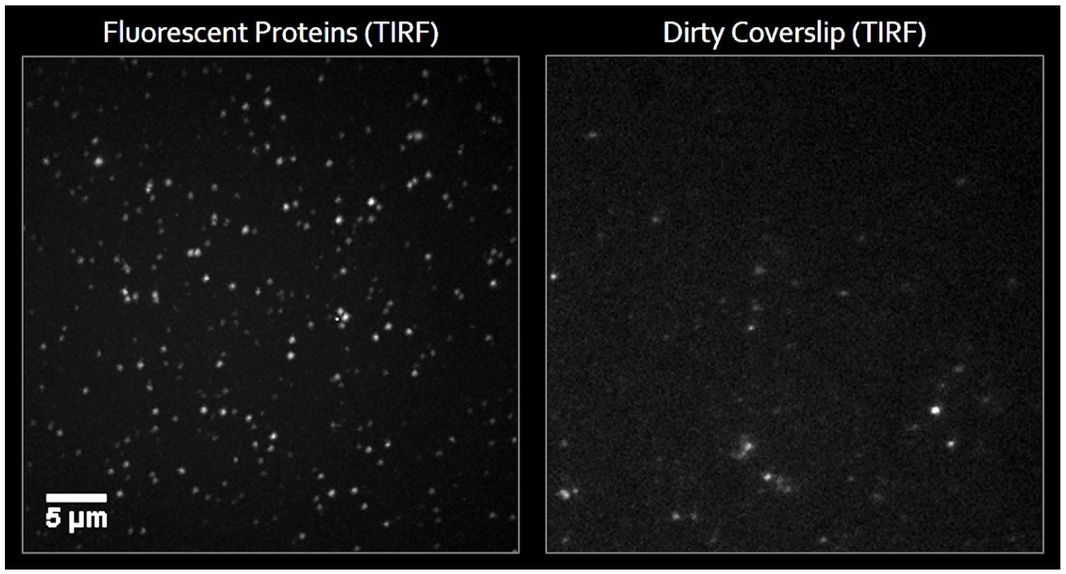


Cleaning coverslips methods :

- strong base baths (1M KOH),
- strong acids,
- acid-ethanol,
- acid piranha solution (H2SO4 + H2O2 in a ratio of 3:1)
- plasma cleaning

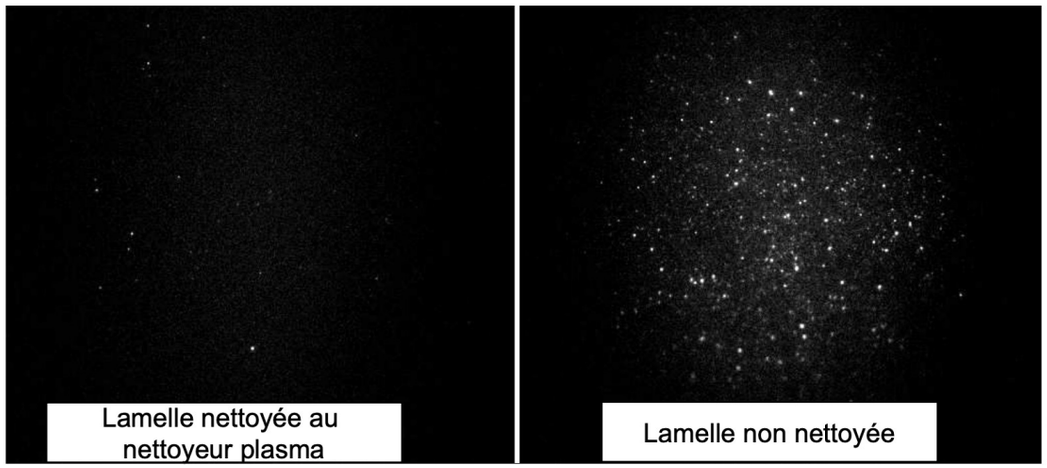


Image reconstruction

How to go from a list of coordinates to a picture?

Post-processing analysis

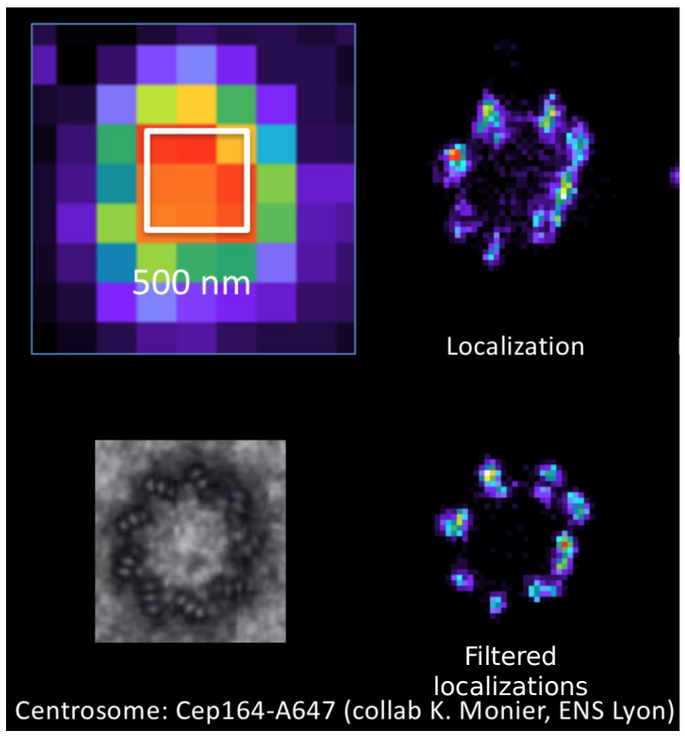
- Removing molecules with poor localization (intensity, size, circularity, etc…)
- Merging of reappearing molecules (stitching)
- Lateral drift correction

Image reconstruction

How to go from a list of coordinates to a picture?

Post-processing analysis

- Removing molecules with poor localization (intensity, size, circularity, etc…)
- Merging of reappearing molecules (stitching)
- Lateral drift correction



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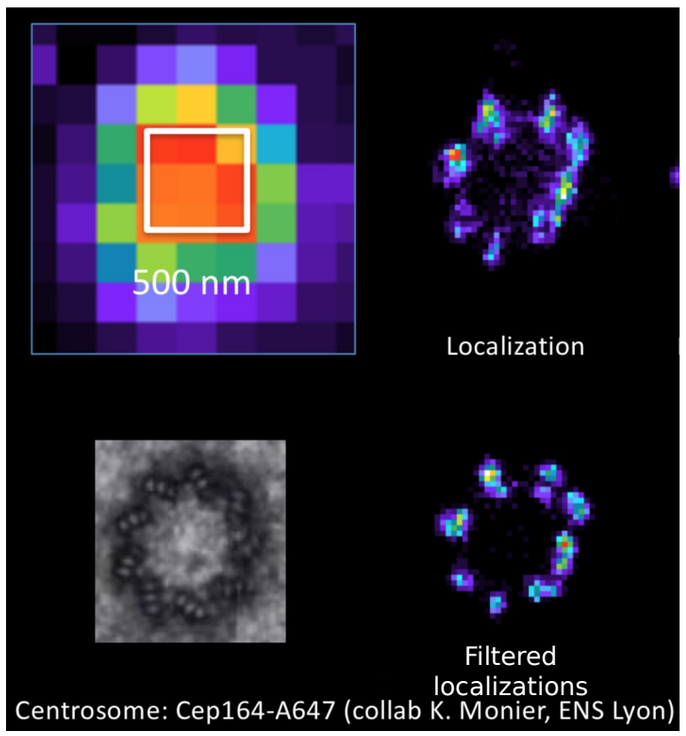
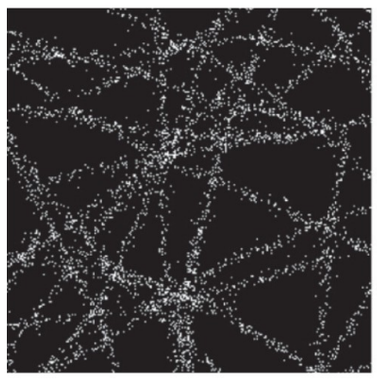
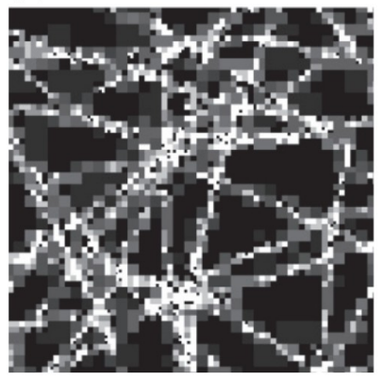
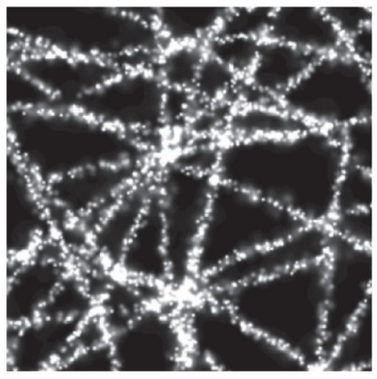
Visualization :

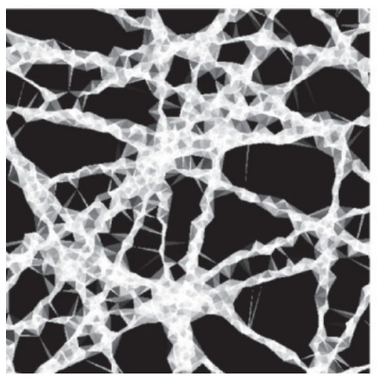
- Scatter plot
- Histogram (binning)
- Gaussian rendering (blur the points with the localization precision)
- Delaunay triangulation
- etc…



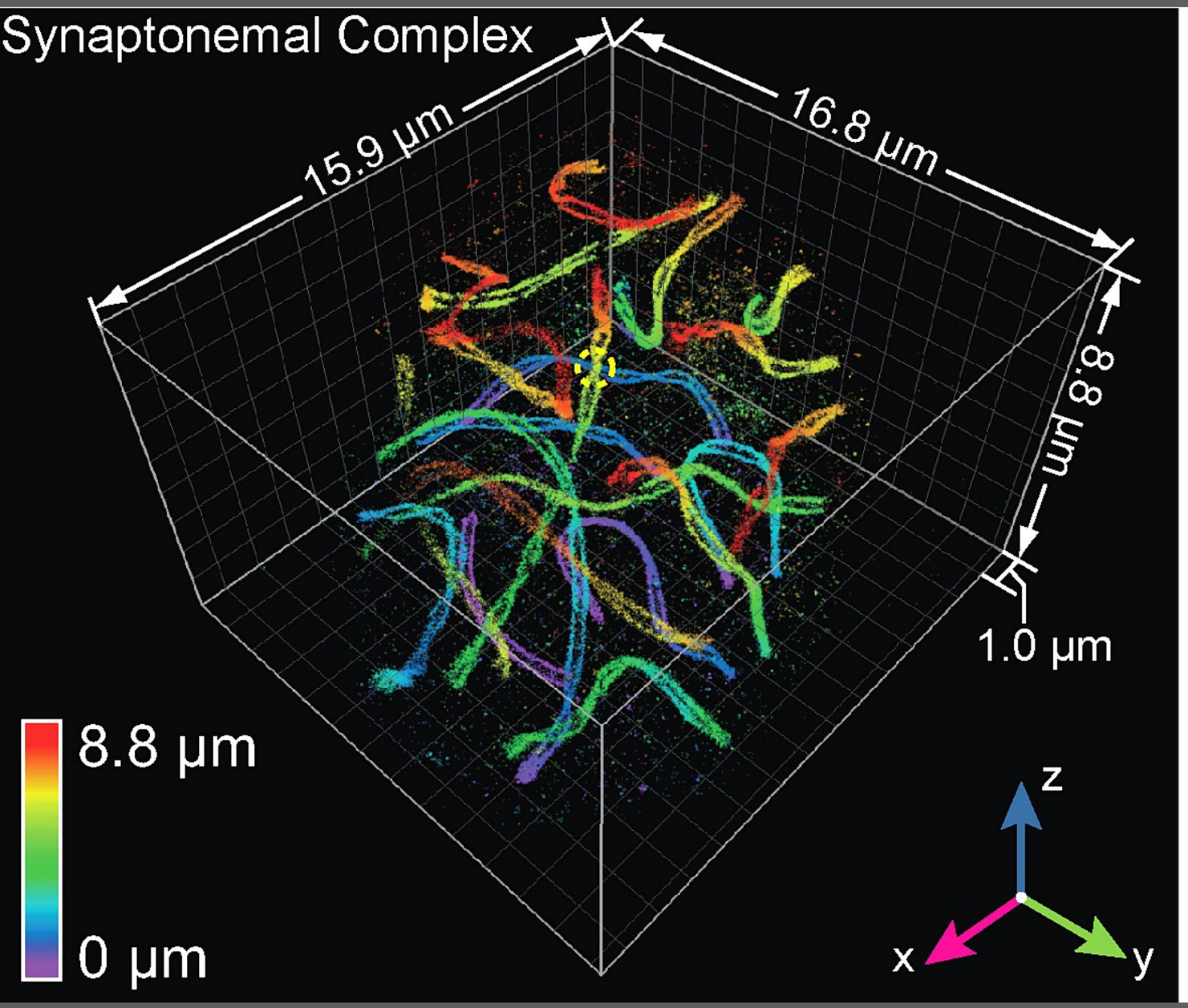


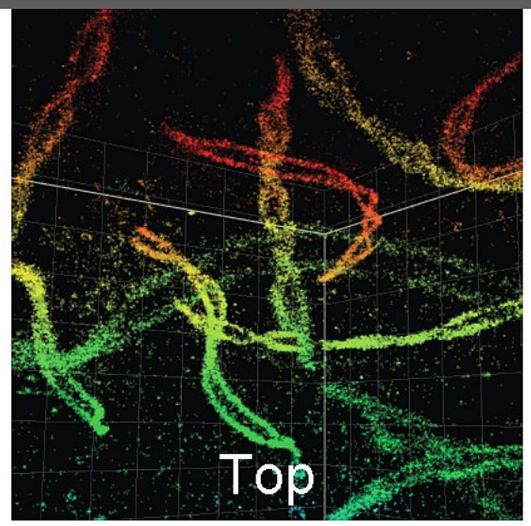


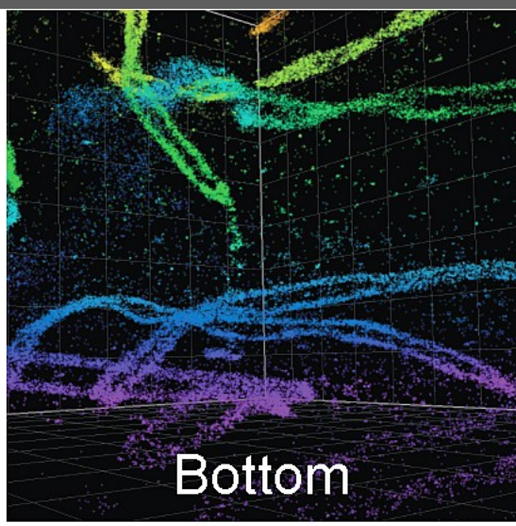




3D


Synaptonemal Complex
15.9 µm
16.8 µm
8.8 µm
1.0 µm
8.8 µm
0 µm
z
x
y


Top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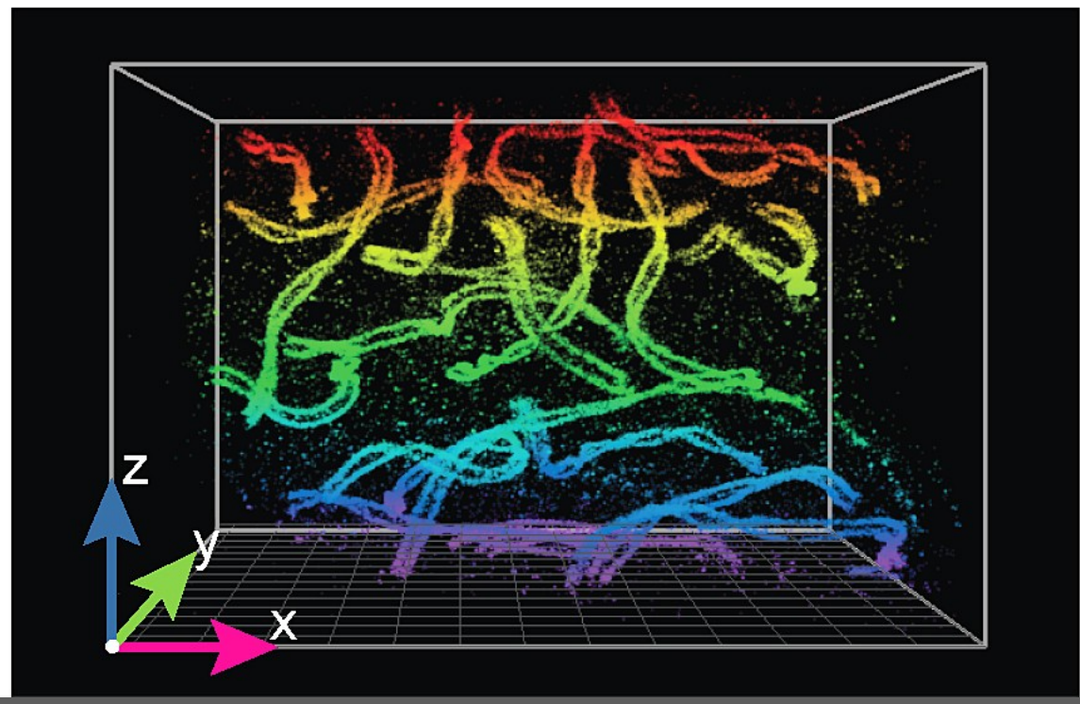
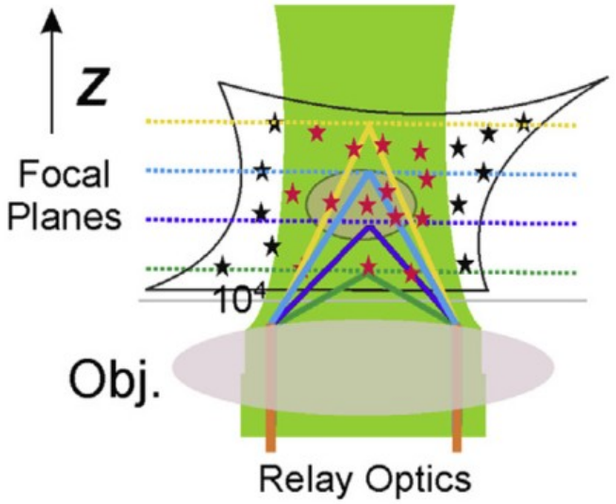
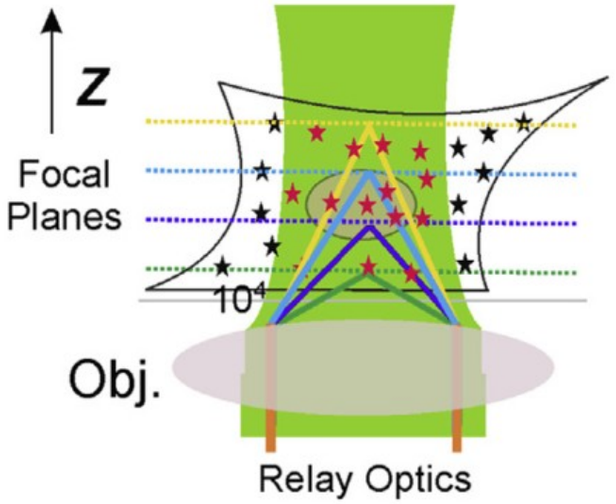

z
y
x

Z scanning



<u>Principle</u> : scanning over different z depths and recording the corresponding images

Z scanning



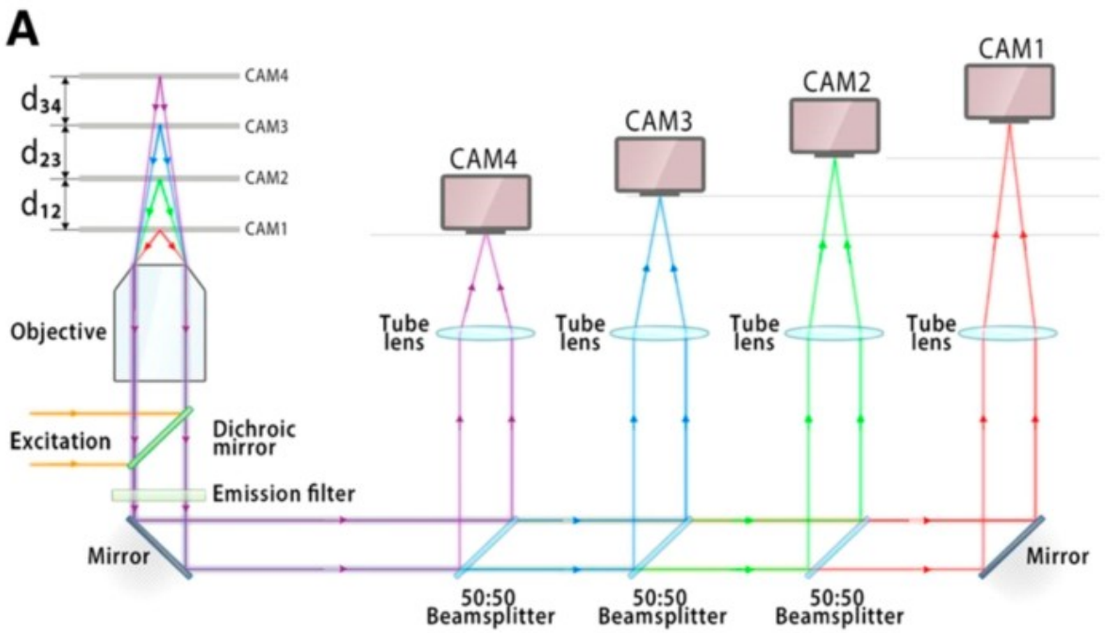
<u>Principle</u> : scanning over different z depths and recording the corresponding images

<u>Pros</u> :
- Accessibility

<u>Cons</u> :
- Photobleaching
- Slow

restricted to extremely slow dynamic processes

Bi & Multi-Plane Microscopy



<u>Principle</u> : record simultaneously different focal planes on several detectors

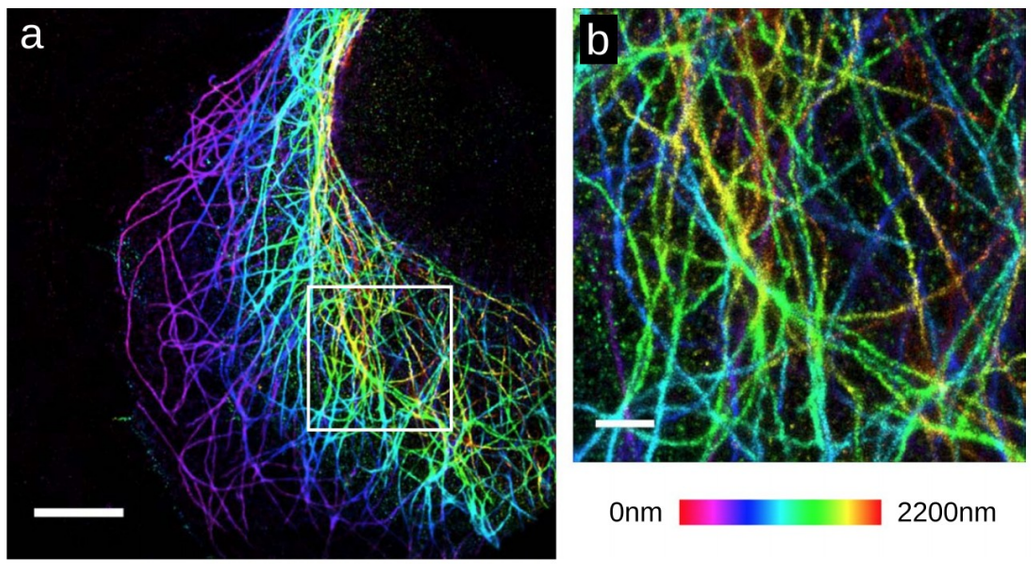
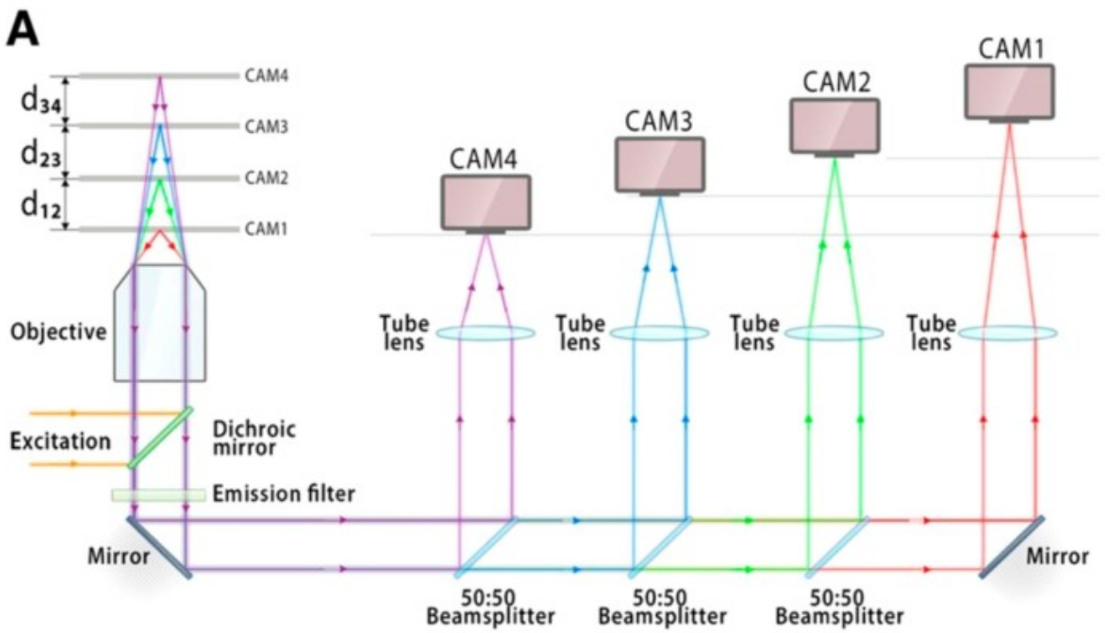


Figure 5. 3D SMLM image of Alexa-647 labeled microtubules in U2OS cells taken using four GS3-U3-51S5M-C cameras in a quadplane configuration. (**a**) 3D SMLM image with Z color scale as shown in the color bar. (**b**) Zoom in of the area white boxed area in (**a**) with the same Z color scale. Scale bars are 5 μm in (**a**), 1 μm in (**b**).

Babcock, Scientific reports, 2018

Bi & Multi-Plane Microscopy



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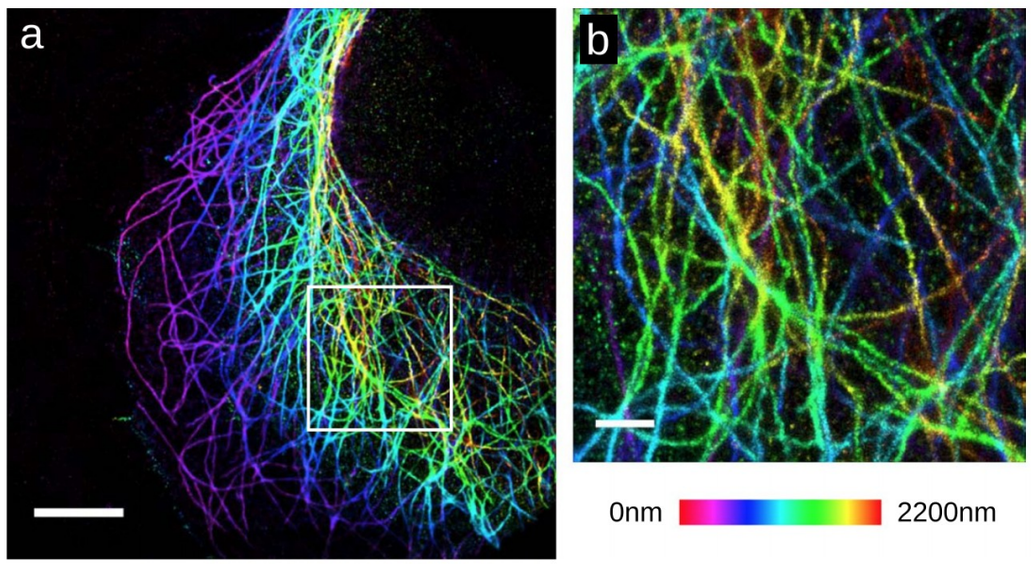

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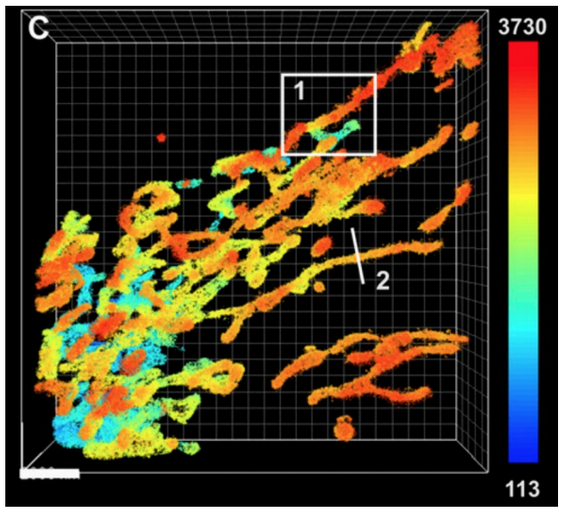
<u>Pros</u> :
- simultaneous images at different focal planes
- better temporal resolution
- large depth of field

<u>Cons</u>:
- Split photon flux
- Expensive

Babcock, Scientific reports, 2018

Multi Focus Microscopy

<u>Principle</u> : record simultaneously different focal planes on a single detector with diffractive optics



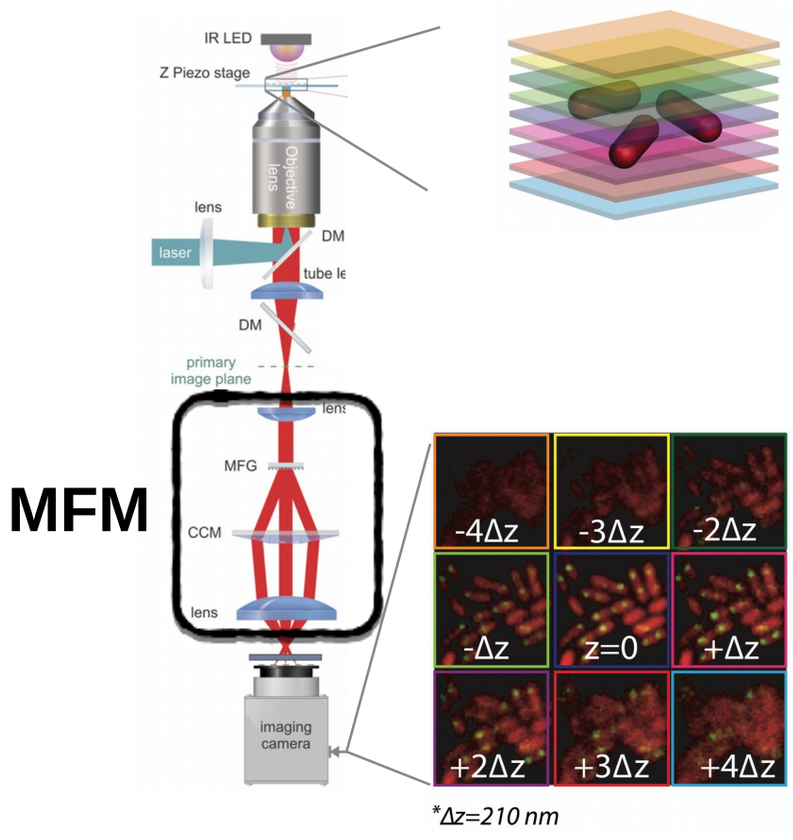
Le Gall et al, Nat Com, 2016

Hajj et al, PNAS, 2017

Oudjedi et al, Opt Exp, 2017

Multi Focus Microscopy

Multi-Focus Microscope:



MFM

Abrahamsson et al, Nat. Meth. 2012

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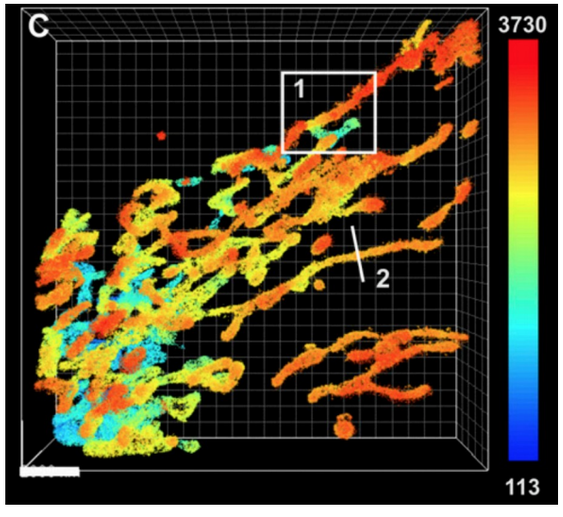

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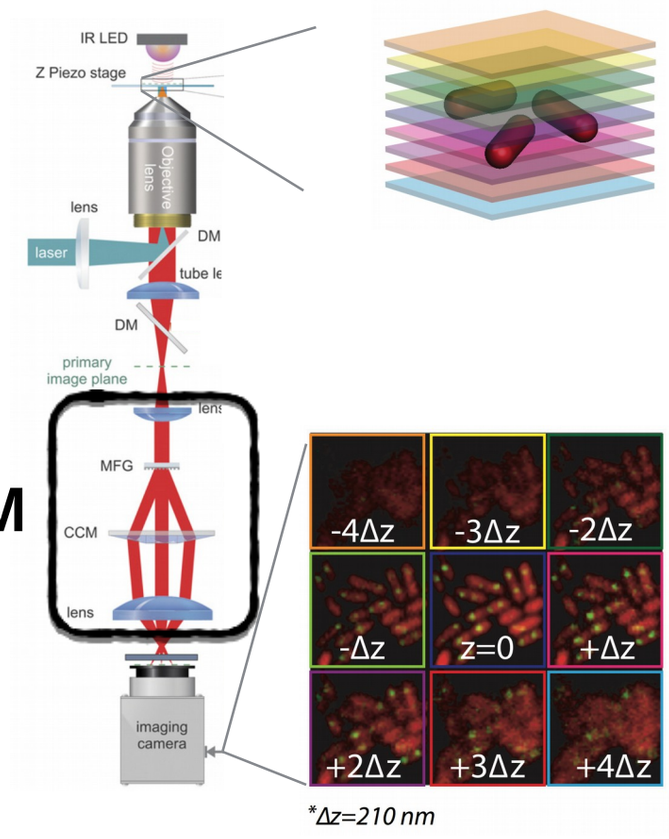

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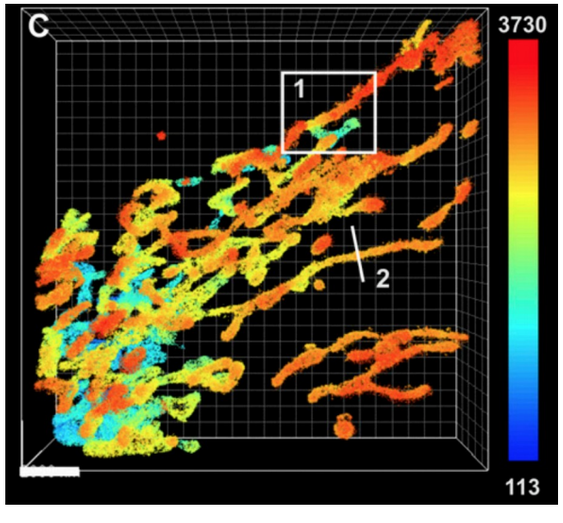


Le Gall et al, Nat Com, 2016

Hajj et al, PNAS, 2017

Oudjedi et al, Opt Exp, 2017

Principle : record simultaneously different focal planes on a single detector with diffractive optics

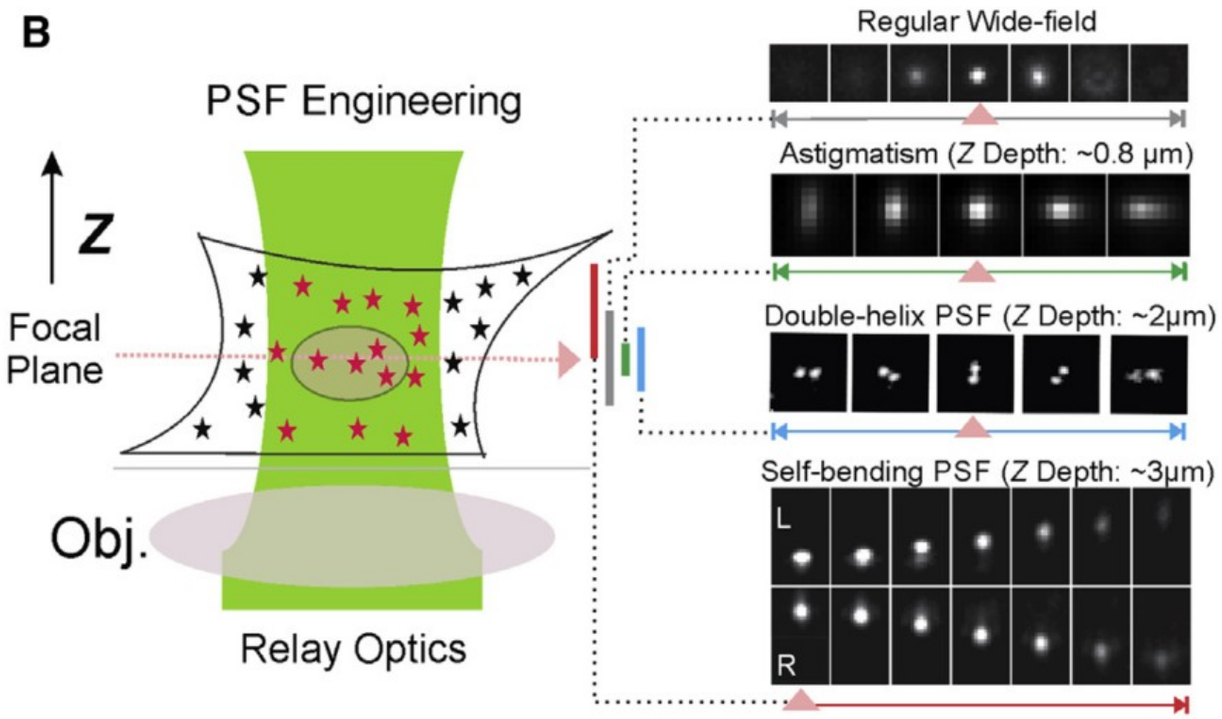
Pros:

- simultaneous images at different focal planes (>9)
- great temporal resolution
- very large depth of field (>4µm)
- Photons split based on focal plane

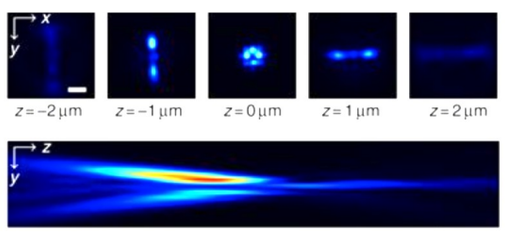
Cons:

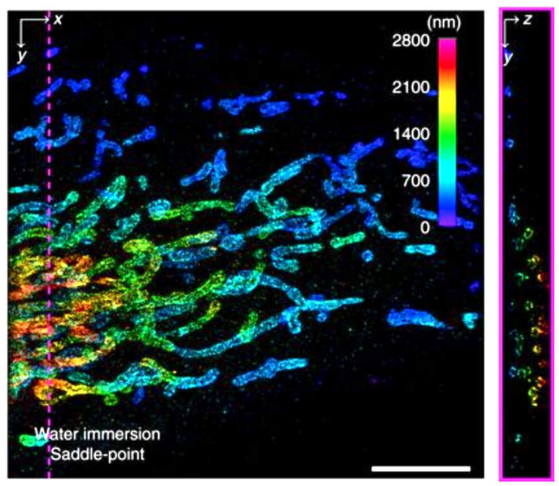
- Split field of view

PSF engineering



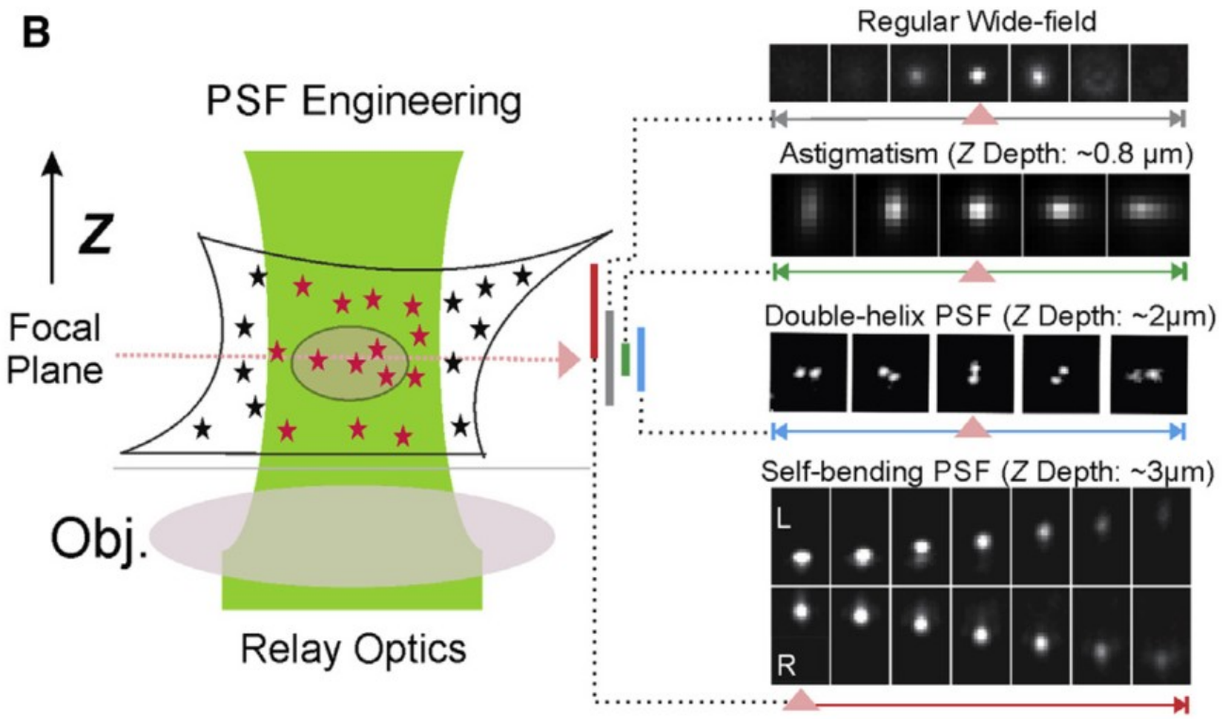
<u>Principle</u>: takes advantage of the size and shape of the intensity spot (the PSF of the microscope) when is de-focused to infer on the z-position of the particle (figure





Aristov et al, Nat Com, 2018

PSF engineering



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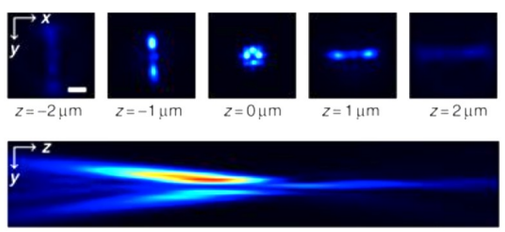
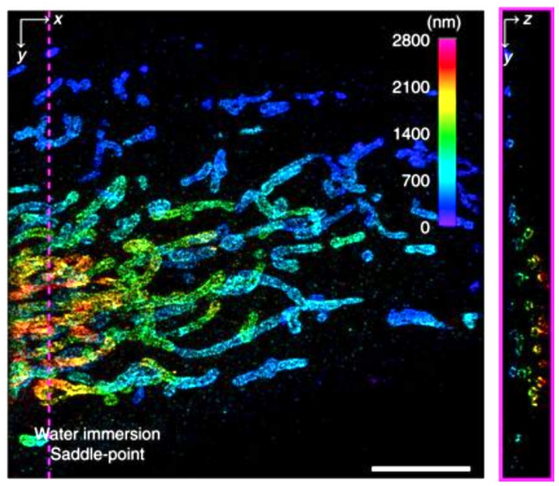
<u>Pros</u>:



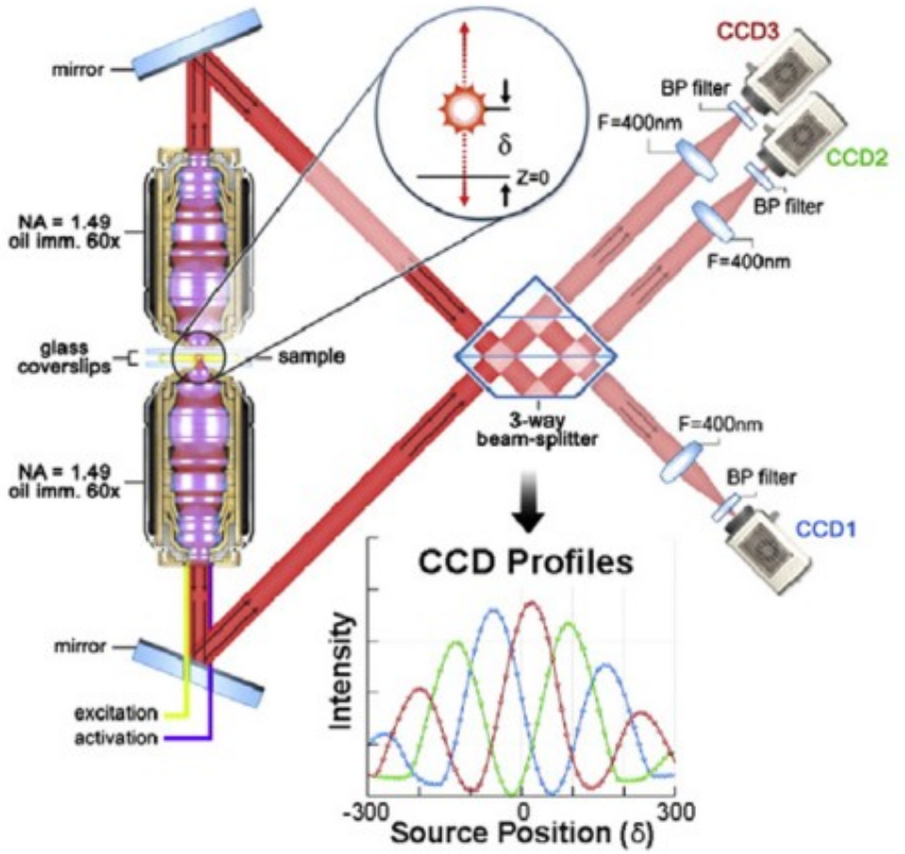


<u>Cons</u>:

- Specific localisation algorithms
- lowered emitter density per image (overlapping of PSFs from different depths)
- Phase masks are color-specific

Aristov et al, Nat Com, 2018

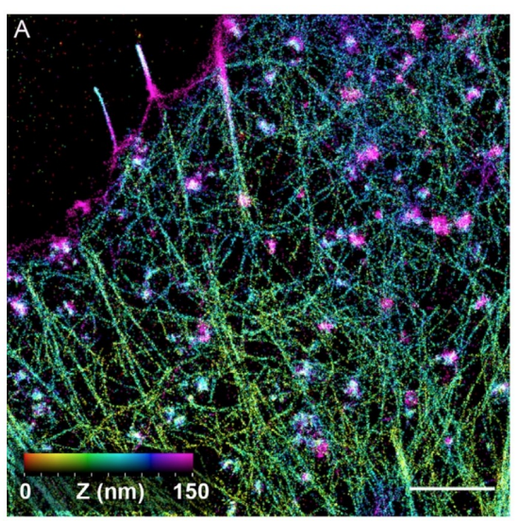
Interferometric PALM



<u>Principle</u>: self-interference of an emitted wave emanating from a single molecule near the common focus of two collection objectives

<u>Pros</u>:

- Near 3D isotropic resolution
- Photon collection efficiency

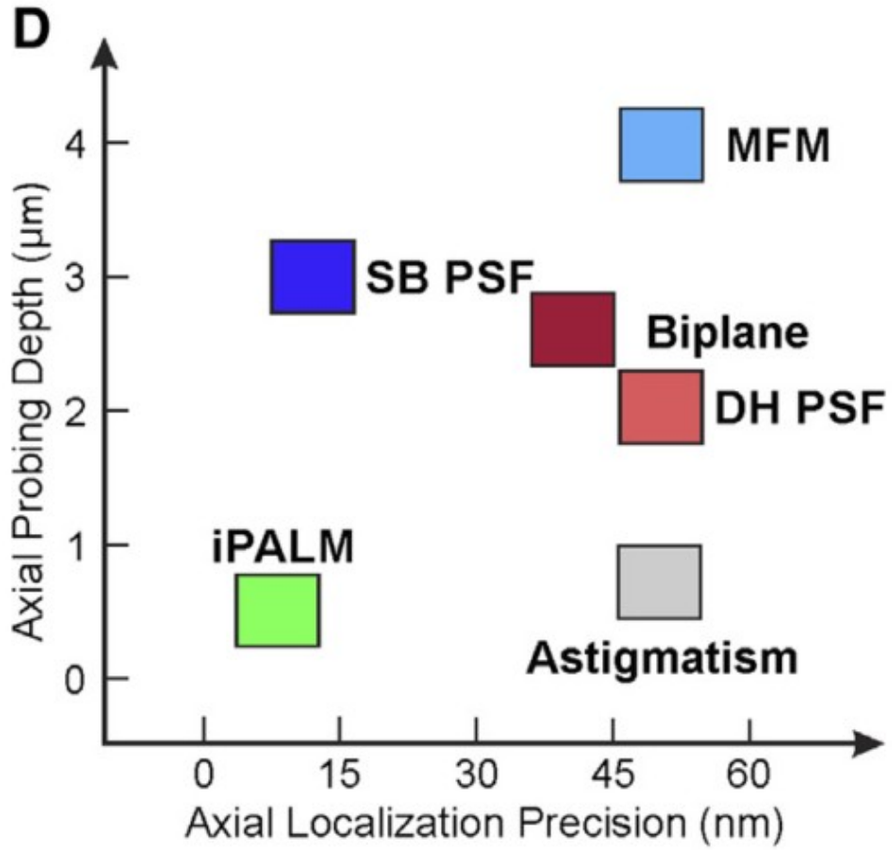


<u>Cons</u>:

- Complexity
- Limited depth of field

Methods in Cell Biology, Volume 123, ISSN 0091-679X

3D techniques DOP and Z precision



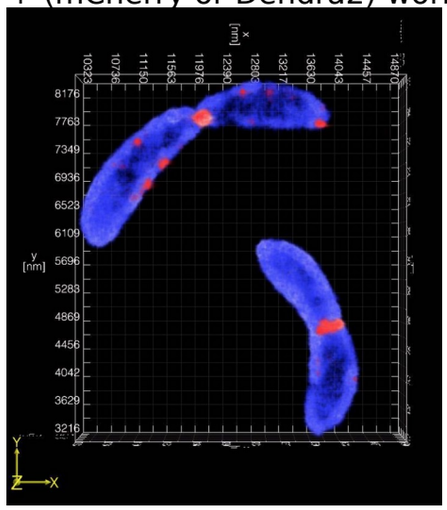
Multicolor

PALM

➠ Complicated

Green switchable FPs suck!

YFP + (mCherry or Dendra2) works ok



C.crescentus membrane proteins
Holden lab

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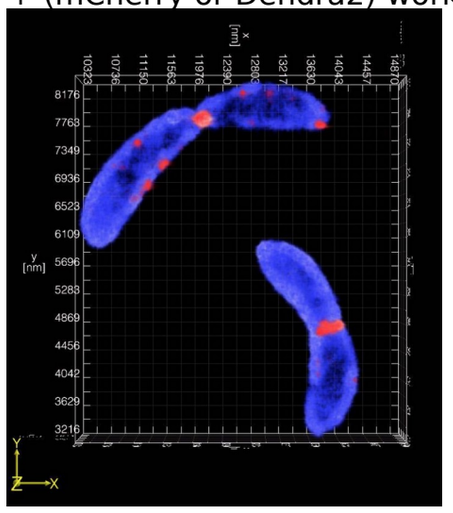
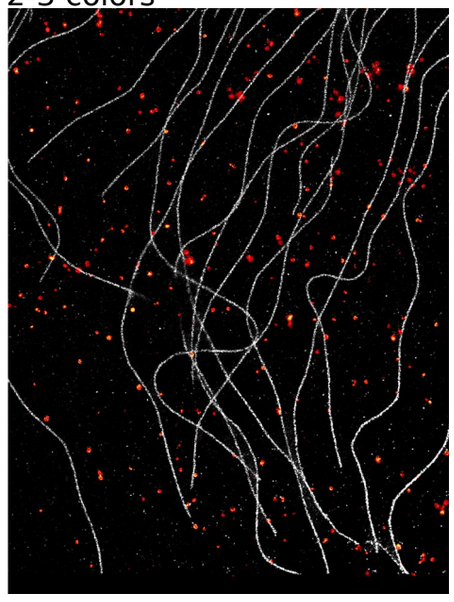


C.crescentus membrane proteins
Holden lab

STORM

➟ larger panel of dyes

2-3 colors



Dual-color Tubulin/Clathrin of epithelial cell
Abbelight

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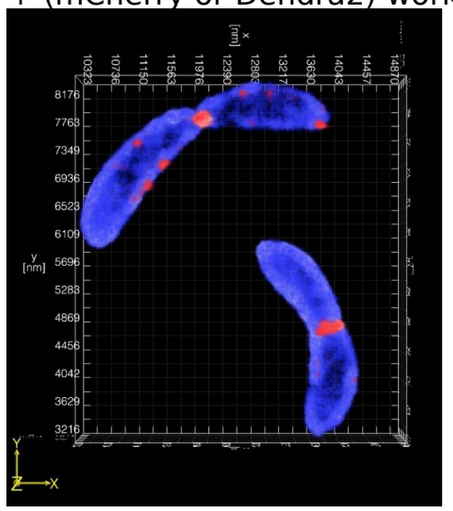

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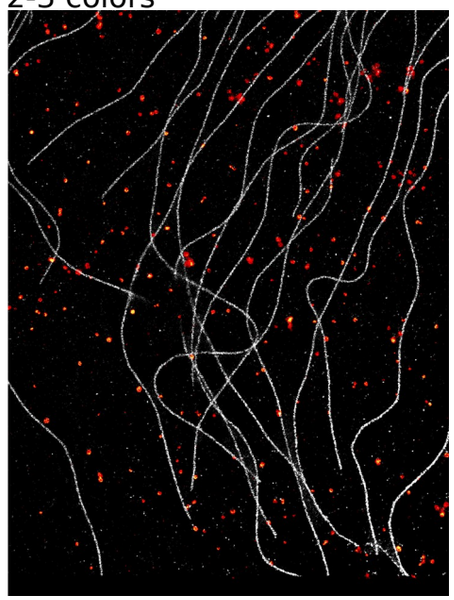
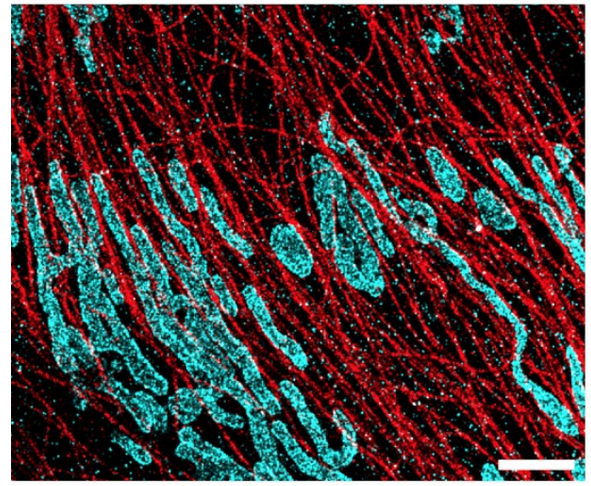


Dual-color Tubulin/Clathrin of epithelial cell
Abbelight

DNA-PAINT

➟ sequential imaging!

Many!



a-tubulin (red) and Tom20 (cyan)

Schnitzbauer et al, Nat.Meth., 2017

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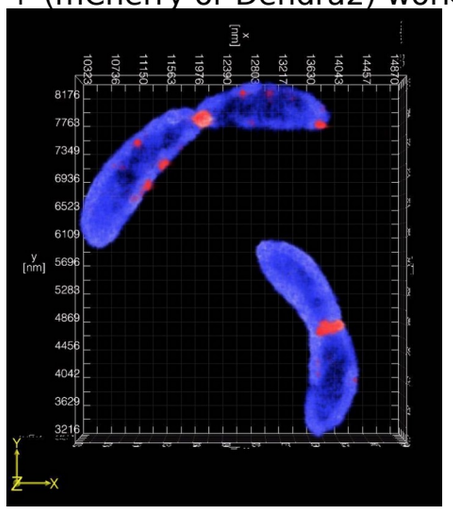

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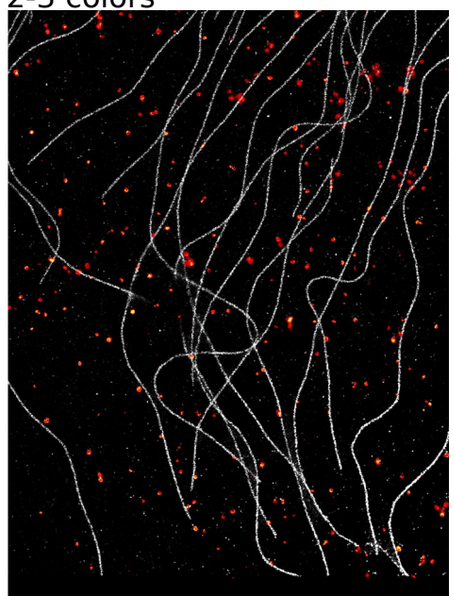

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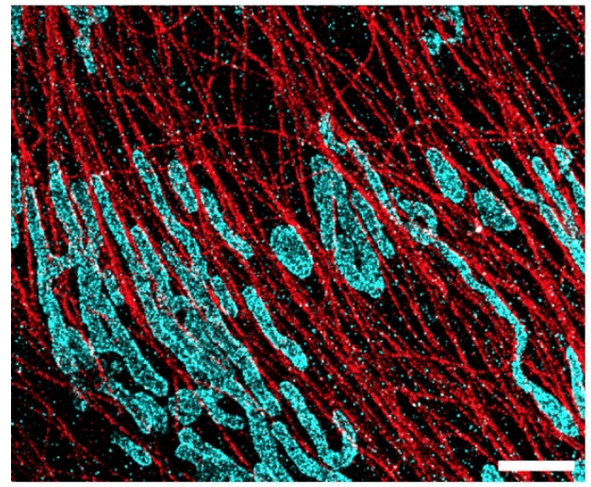

a-tubulin (red) and Tom20 (cyan)

Schnitzbauer et al, Nat.Meth., 2017

Chromatic aberrations!

Multicolor

PALM

➟ Complicated

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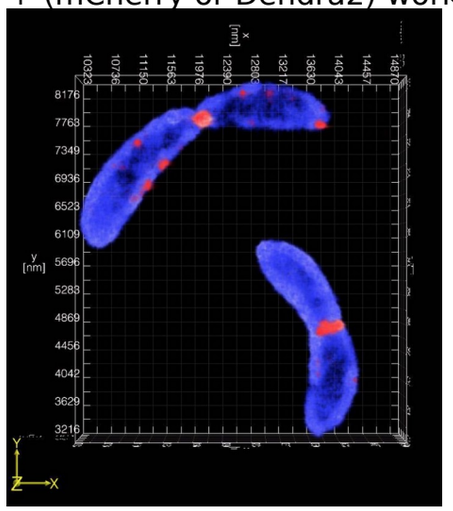

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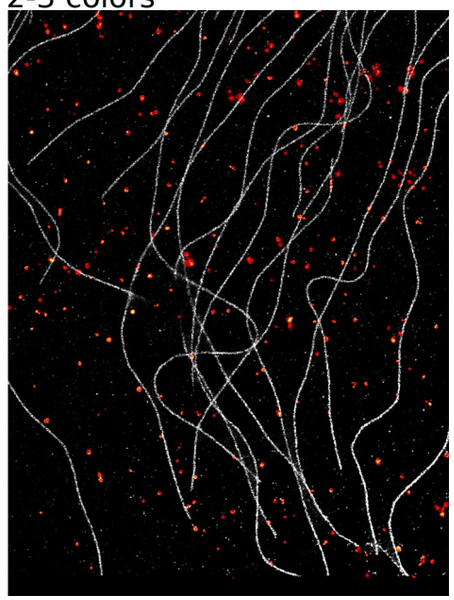

Dual-color Tubulin/Clathrin of epithelial cell
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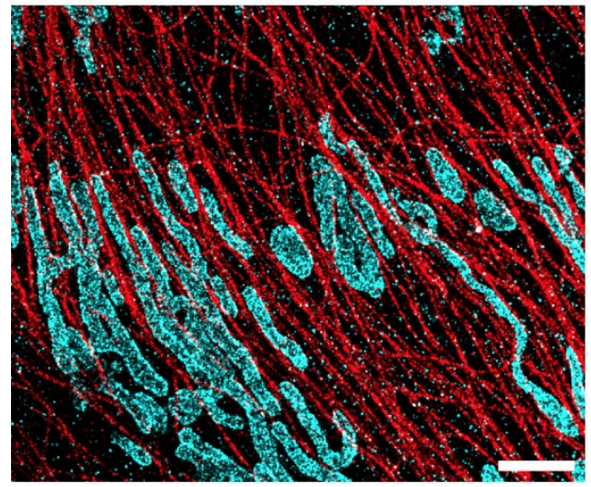


a-tubulin (red) and Tom20 (cyan)

Schnitzbauer et al, Nat.Meth., 2017

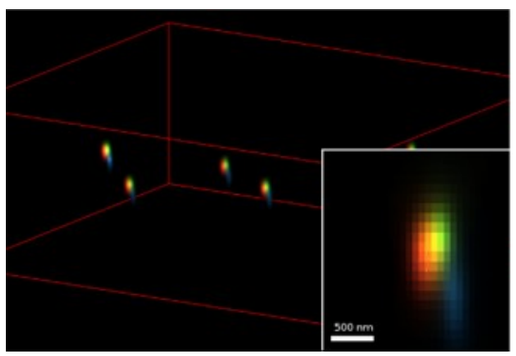
Chromatic aberrations!

➟ refractive indices for all optical glasses vary with wavelength

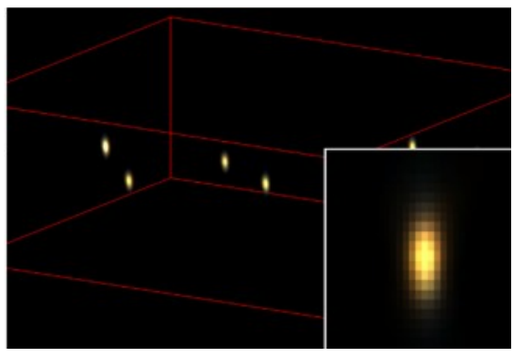
- Shifts between wavelengths

  BOTH **Lateral** AND **Axial**

With chromatic aberrations

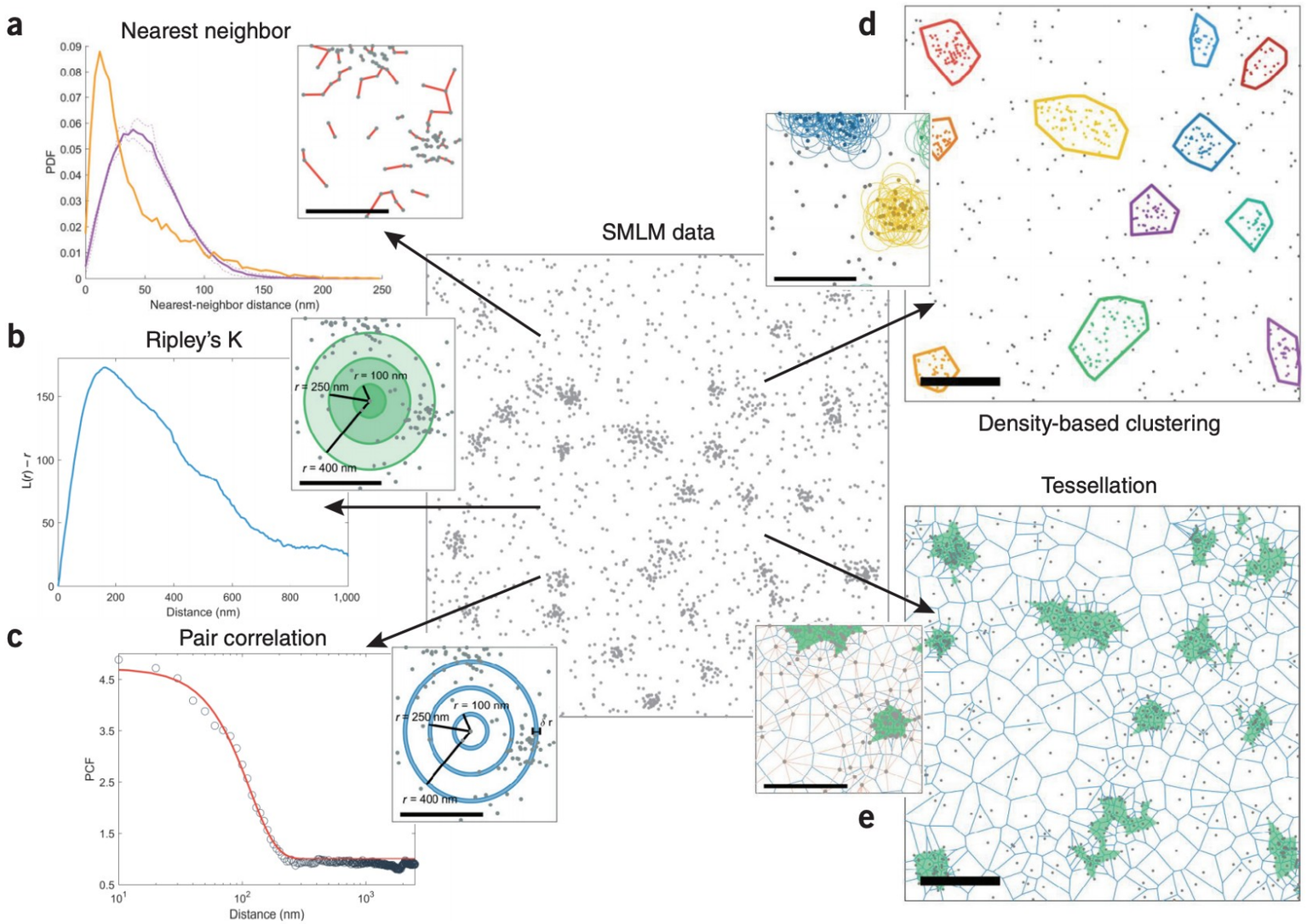


With chromatic aberrations coercetion



Analysis

Analysis

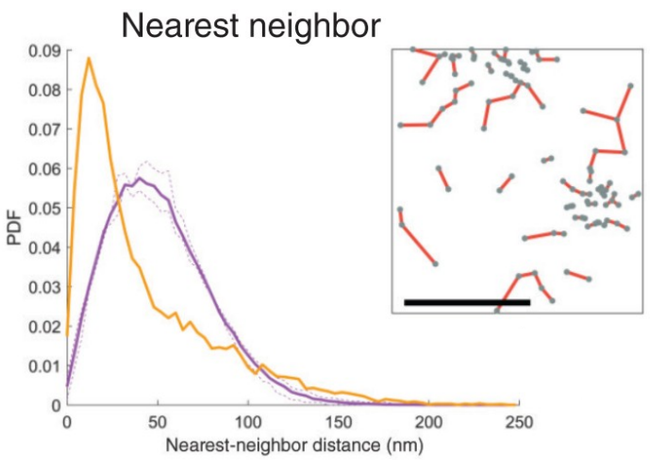


Under these conditions, are target molecules clustered or distributed randomly? Do they colocalize? Do they form particular structures? …

Clustering, Colocalization, Quantification, Single particle averaging, etc…)

Revealing order within a sea of points

Distance methods



The resulting histogram shows shorter nearest-neighbor distances in the measured data (orange) versus a random distribution of points at an identical density (purple curves)

Nearest-neighbor

Nearest-neighbor analysis measures pairwise distances (inset, red) between the closest neighboring points

➟ Sensitive self-clustering caused by multiple detection events

Revealing order within a sea of points

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The resulting histogram shows shorter nearest-neighbor distances in the measured data (orange) versus a random distribution of points at an identical density (purple curves)

Nearest-neighbor

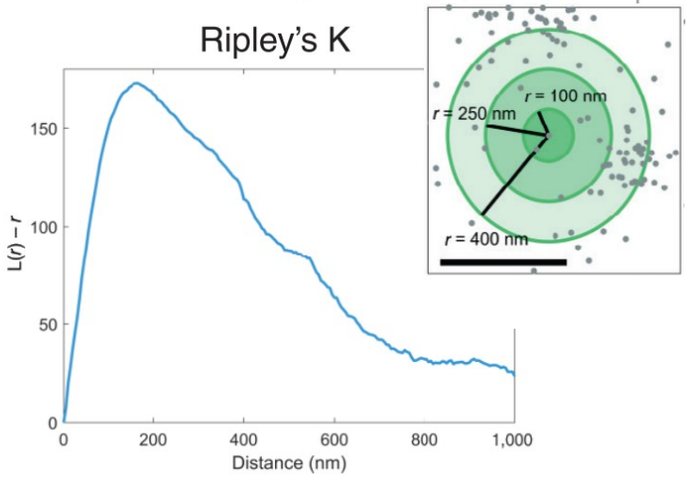
Nearest-neighbor analysis measures pairwise distances (inset, red) between the closest neighboring points

➟ Sensitive self-clustering caused by multiple detection events

Ripley's function

Ripley's K analysis measures point density as a function of circle radius around each point in the data set.

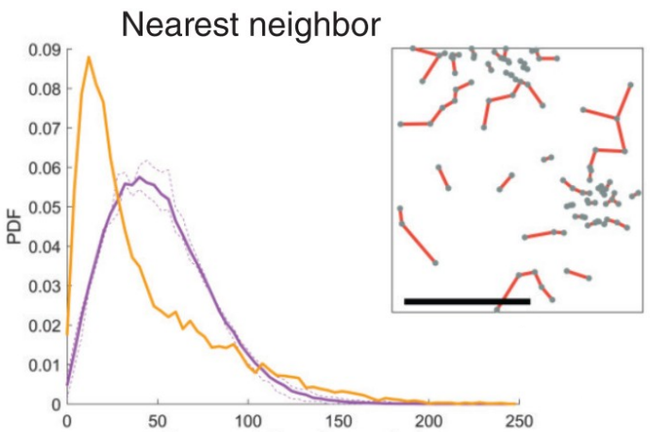
➟ possible to differentiate between potential cluster shapes



The resulting L(r) – r curve shows a peak indicative of clustering at a radius of 170 nm, corresponding to a full width at half maximum (FWHM) of a Gaussian with σ = 72 nm

Revealing order within a sea of points

Distance methods



The resulting histogram shows shorter nearest-neighbor distances in the measured data (orange) versus a random distribution of points at an identical density (purple curves)

Nearest-neighbor

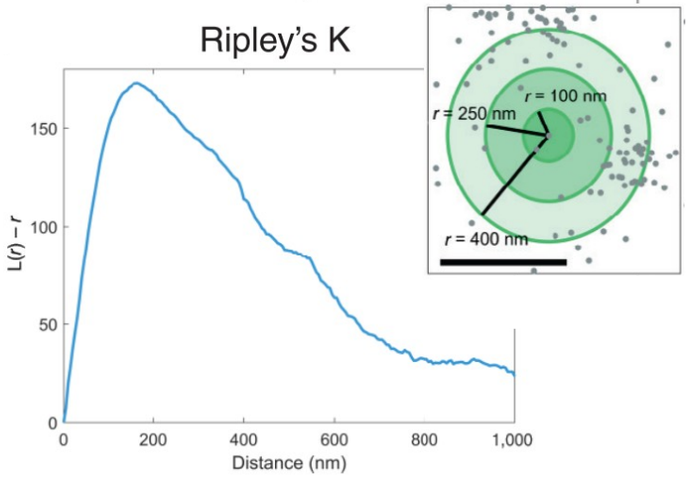
Nearest-neighbor analysis measures pairwise distances (inset, red) between the closest neighboring points

➟ Sensitive self-clustering caused by multiple detection events

Ripley's function

Ripley's K analysis measures point density as a function of circle radius around each point in the data set.

➟ possible to differentiate between potential cluster shapes



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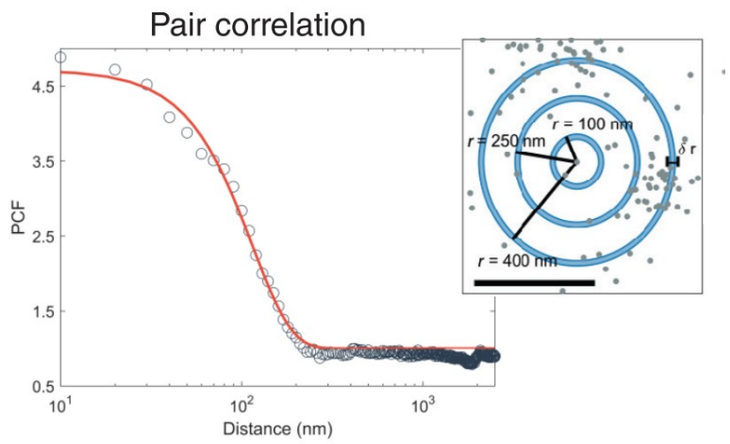


The pair-correlation curve is characteristic of Gaussian-shaped clusters with σ = 60 nm.

Pair correlation

similar to the Ripley's K calculation but replaces circles with concentric tori of width δr

➟ discriminates better between self-clustering caused by multiple detection events and multitarget clustering

Revealing order within a sea of points

Spatial clustering methods

Tesselation

– Space-subdividing technique
– One localizationàone polygon
– Statistics computed on the polygons (area, density…)

Revealing order within a sea of points

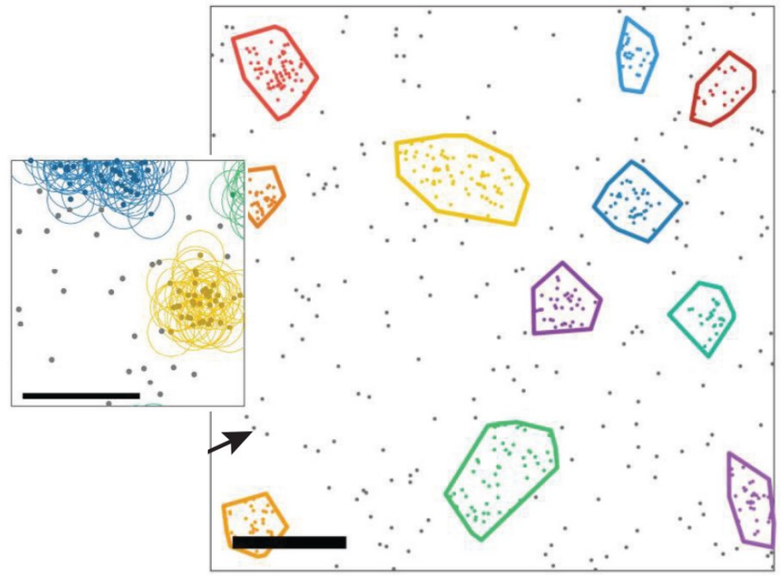
Spatial clustering methods

Density-Based Spatial Clustering Analysis with Noise

- Organizes the localizations wrt density in 3 classes – Core, density-reachable or outlier points
- Two parameters

– Radius *r*àneighborhood size
– *MinPts*àmin nb of points in *r* to be a core point with *MinPts* ≠ min number of points of a cluster

DBSCAN can cope with clusters of varying size and shape and of unknown number



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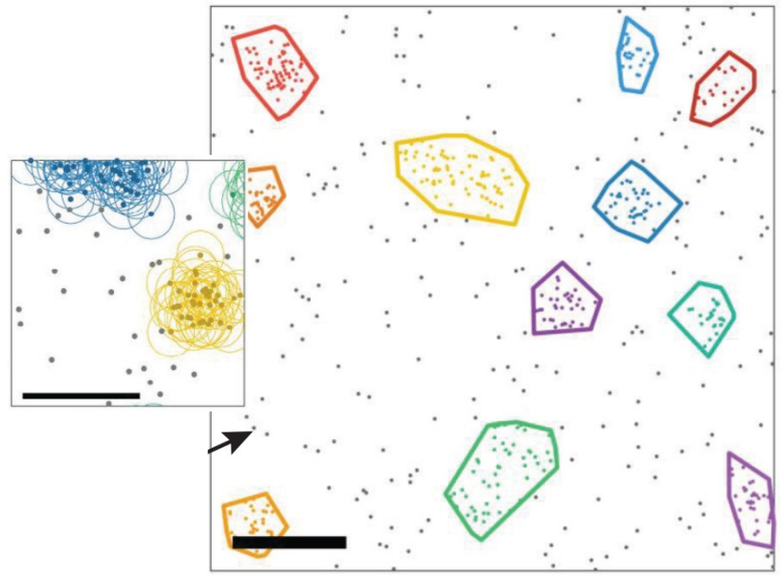

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Fails if clusters vary largely by density

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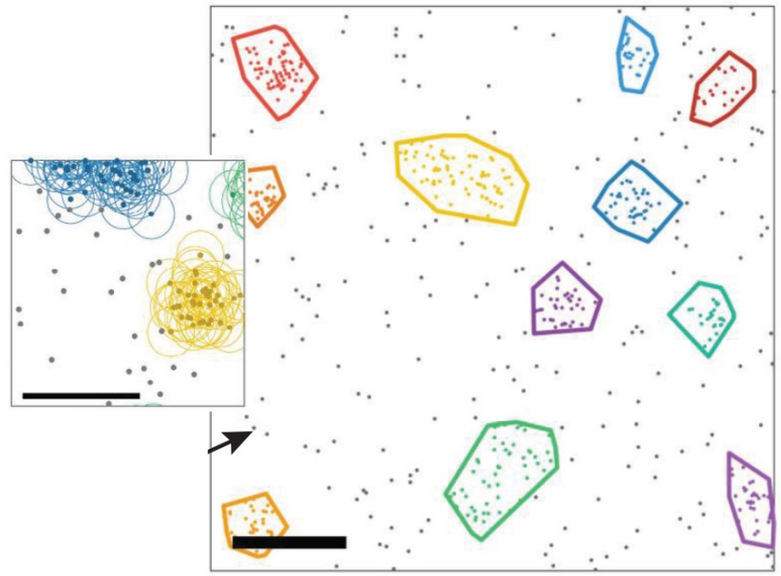

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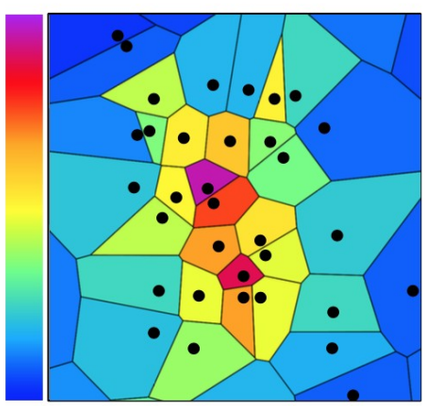
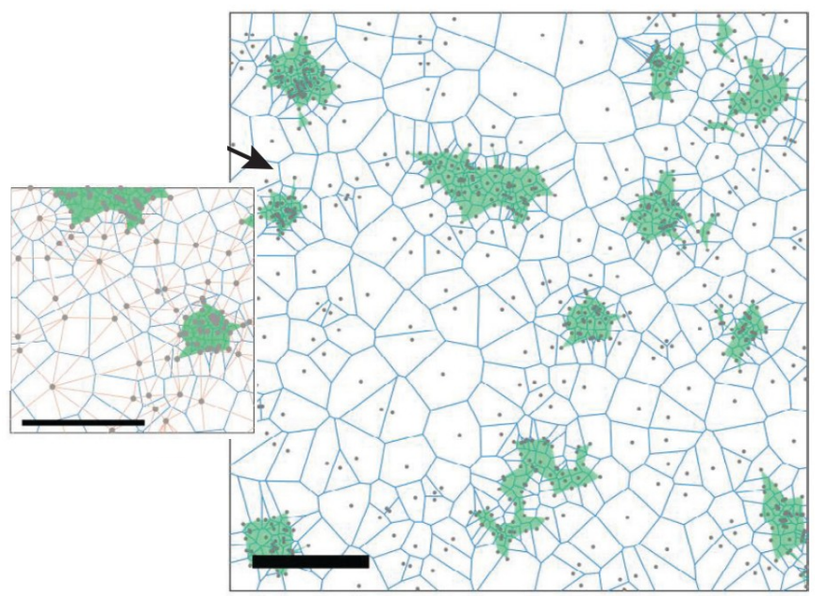
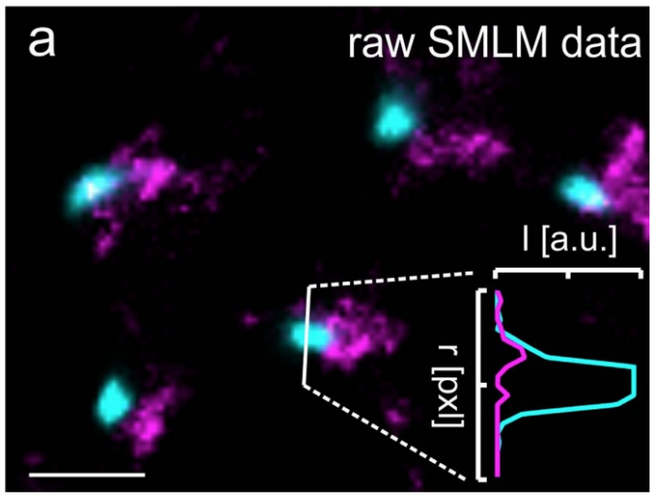
Polygon P
# of neighbors n
Polygon area A
Local density δ

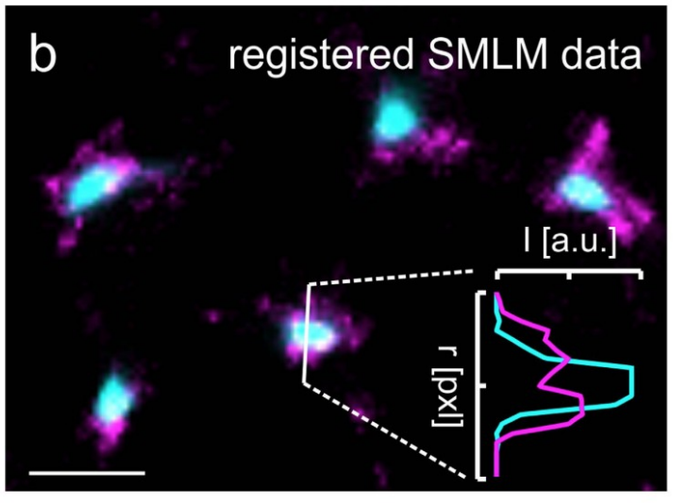


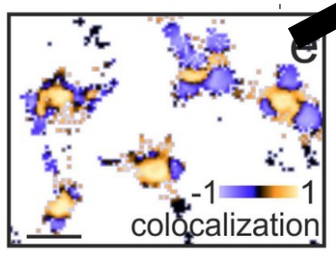
Tessellation

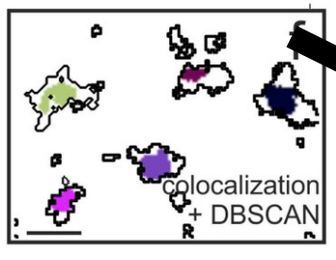


Colocalization

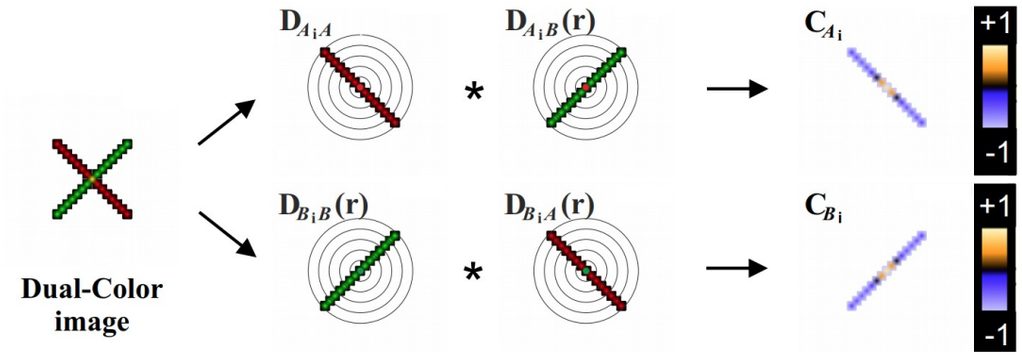








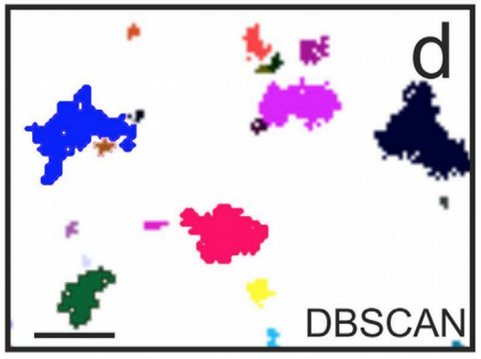
Coordinates Based Colocalization



Malkusch et al, Histochem Cell Bio, 2012

For each localization : distribution function of the neighboring localizations of the same species.
From the individual distribution functions, a correlation coefficient is calculated and weighted by the distance distribution.
As a result, each single- molecule localization of each species is attributed an individual colocalization value, which reports on the local environment of a particular red or green molecule, separately

Cluster based analysis



Clusterize localisations in one channel
Statistics in and out of clusters in second channel (density, distribution, etc…)

Molecular quantification

➟ **Each extracted coordinates correspond to an individual emitter.**

- Counting molecules using fusion protein

- Counting molecules using antibodies

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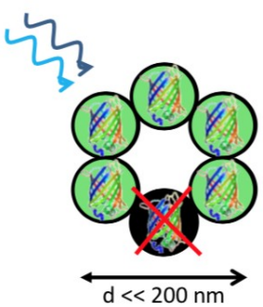

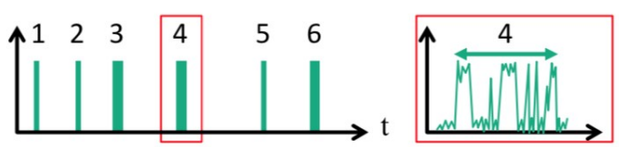

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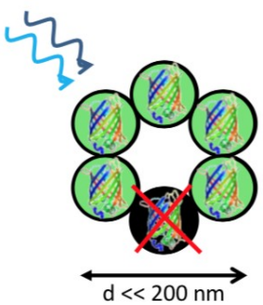

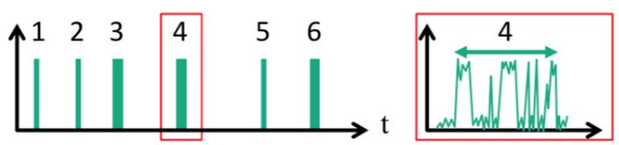

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Annibale et al (2010) ; Puchner et al (2013) ; Coltharp et al (2014) ; Durisic et al. (2014)

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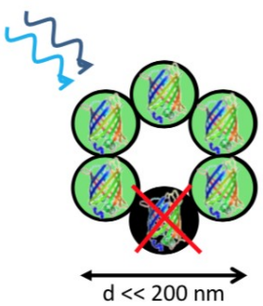

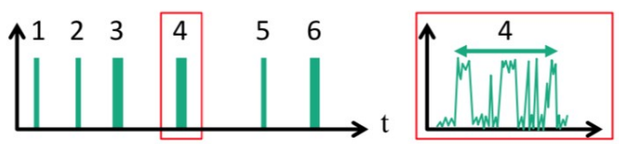

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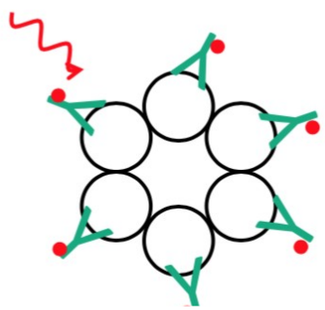

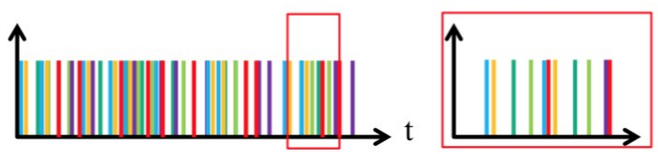

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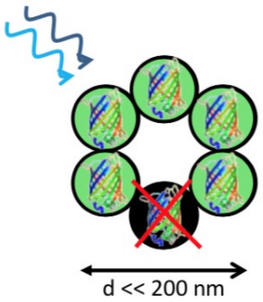

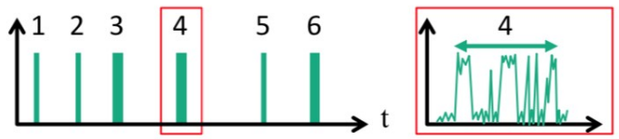

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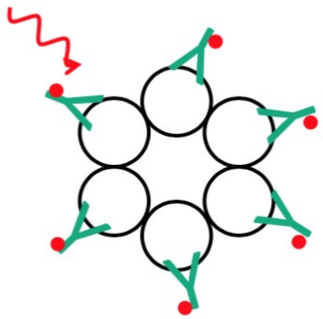

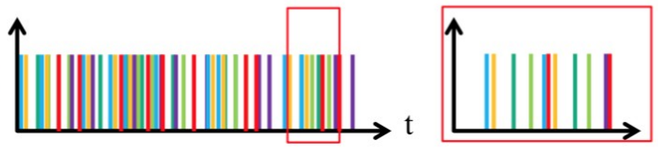

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- **Problem**: **Downcounting** due to labelling efficiency. This is a structure-dependent problem, and the control is not obvious.
- **Solutions**: Use **high efficiency labelling**, with **short linkers** for better penetration.
- **Problems**: **Labelling density** and **Over/Downcounting**.
- **Solutions**: **Direct labelling** with **short linkers** (Nanobodies, Fab, mStrav …) and tune the blinking medium to the labelling density.

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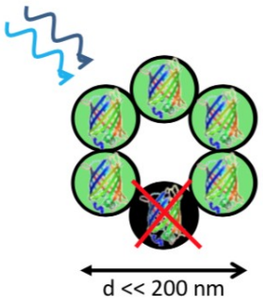

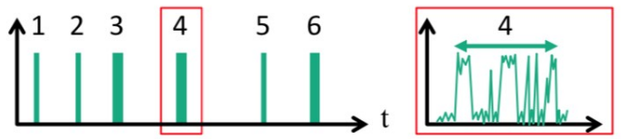

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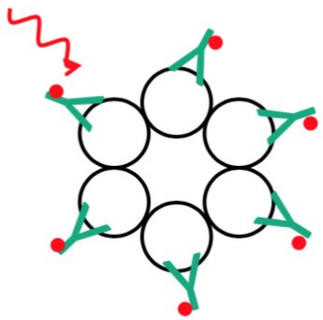

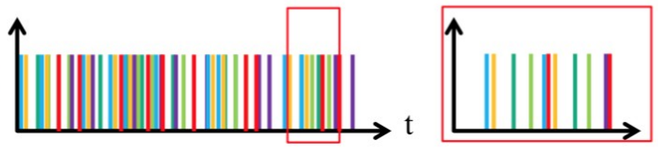

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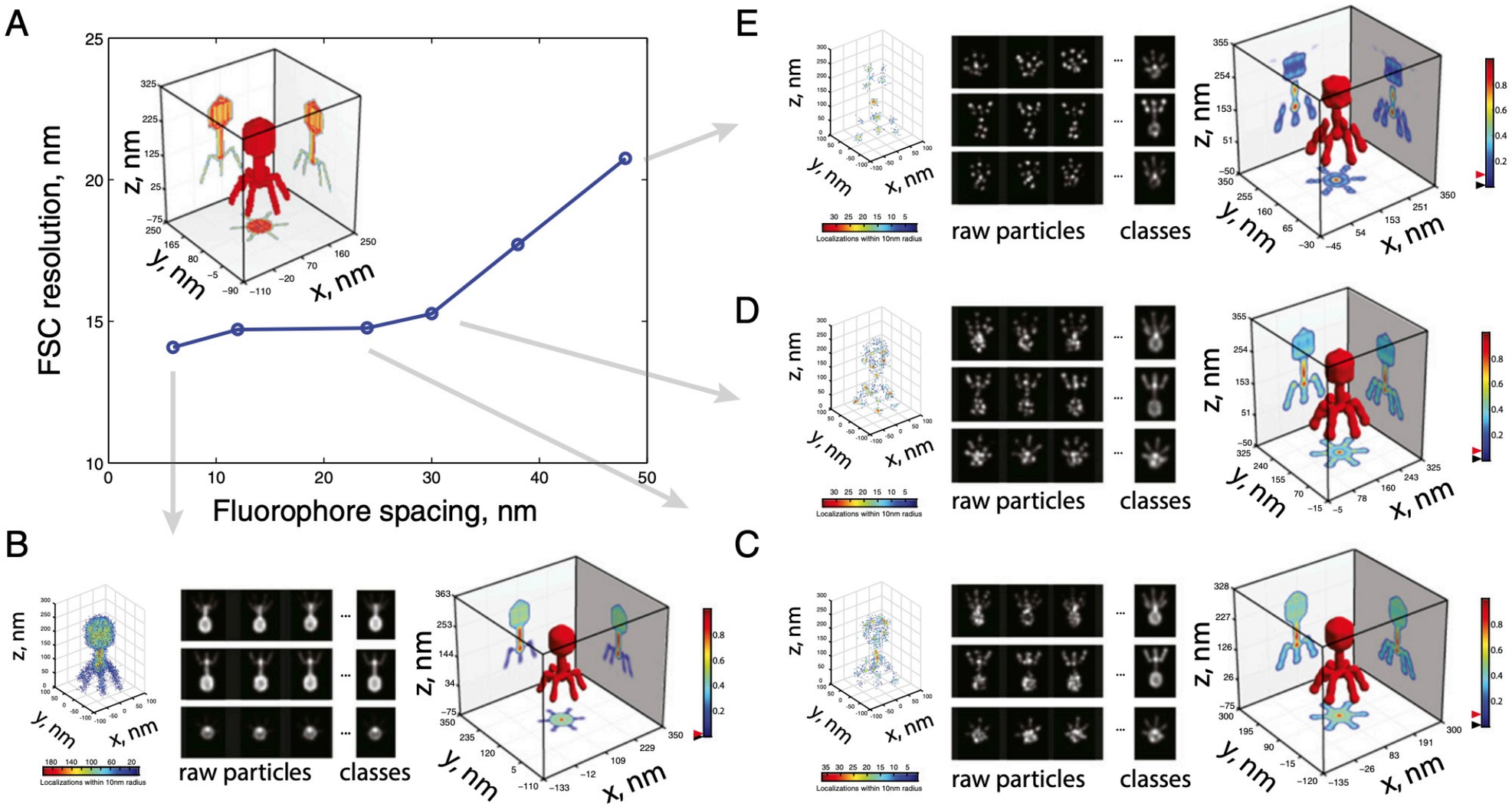
Challenging! :

- imperfect labeling
- overlapping emitters
- overcounting multiple blinking events from the same molecule
- etc…

Particle averaging



Circumvent issues with labelling density

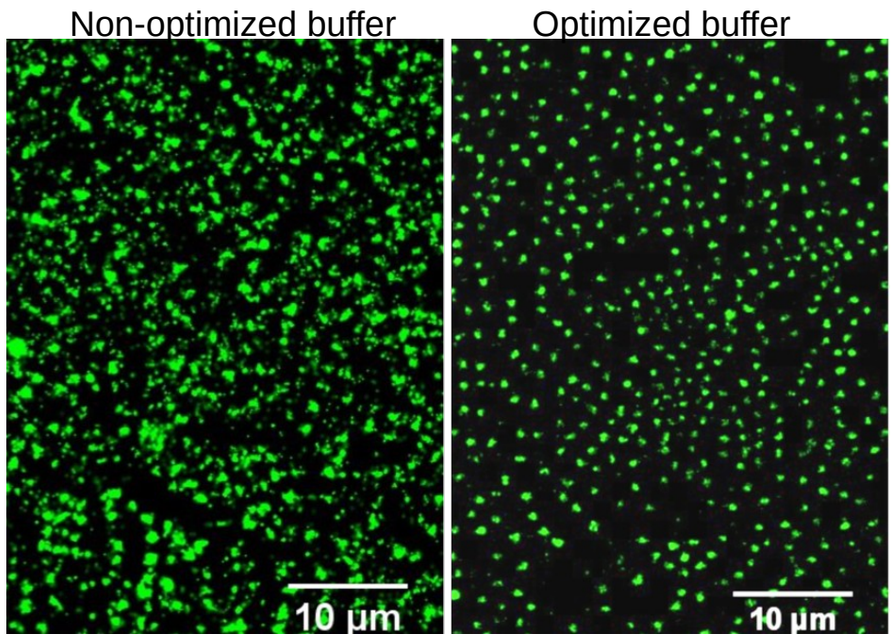
Increase resolution

Sort heterogeneity

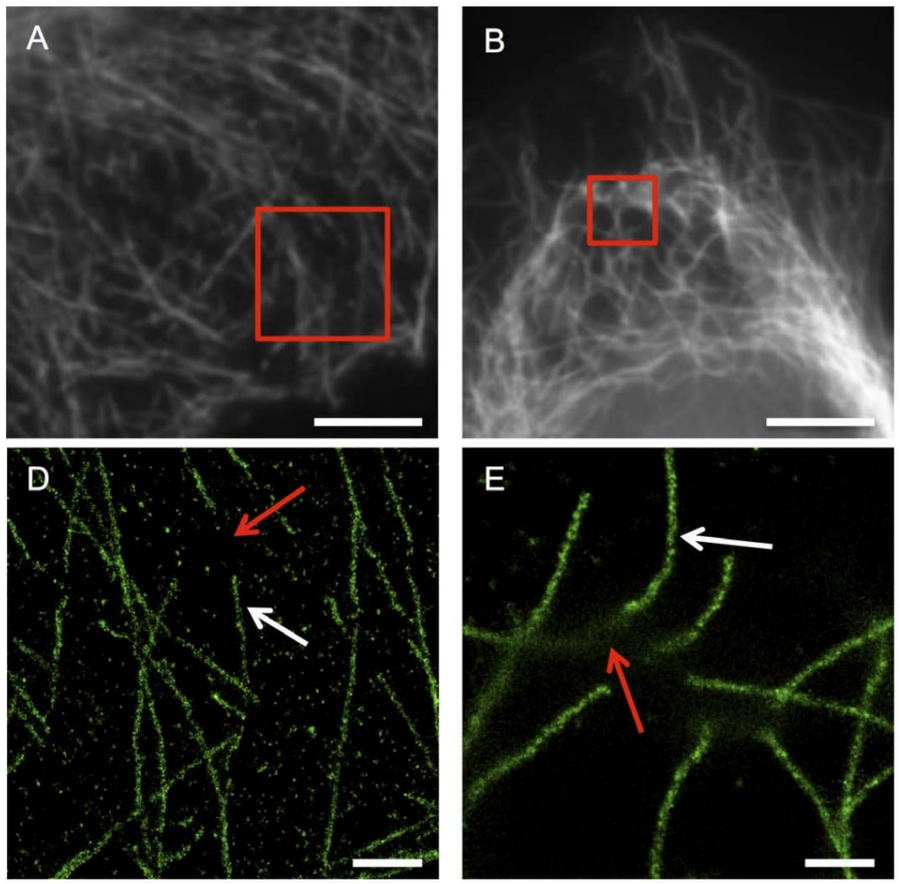
Reconstruct isotropic 3D structures from 2D images

Artefacts

Fixation
Buffer optimisation
Overlapping fluorophores
Drift
Image distortion
Color registration
Calibration (Z, pixel size, etc…)
etc…



Fixation artefacts



Whelan et al, scientific reports, 2015

Conclusions

SMLM is very powerful & popular Super resolution technique

➠ Localize, count & track bio-molecules with nanometric resolution

Maximize the number of photons during the acquisition

➠ Best fluorophores

➠ Optimizing buffer solutions

➠ Optimizing the acquisition parameters

Use the appropriate labeling strategy

➠ PAINT, Fab, Nanobodies, etc...

Data processing

➠ Drift correction

➠ Check number of photons

➠ Check PSF shape

➠ Labeling density

➠ Image registration

Ressources

Vangindertael et al, An introduction to optical super-resolution microscopy of the adventurous biologist, Methods and Applications in Fluorescence, 2018