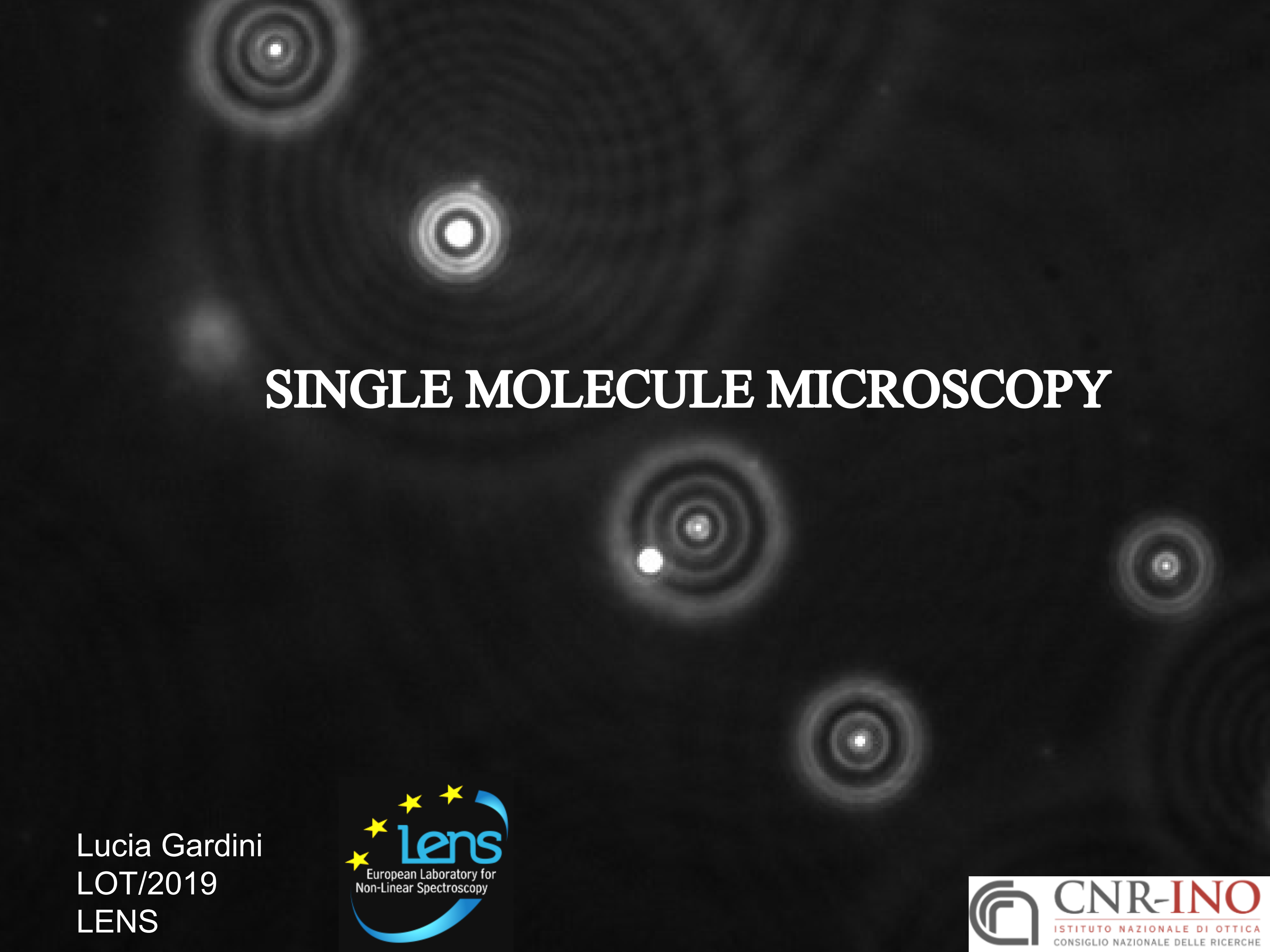
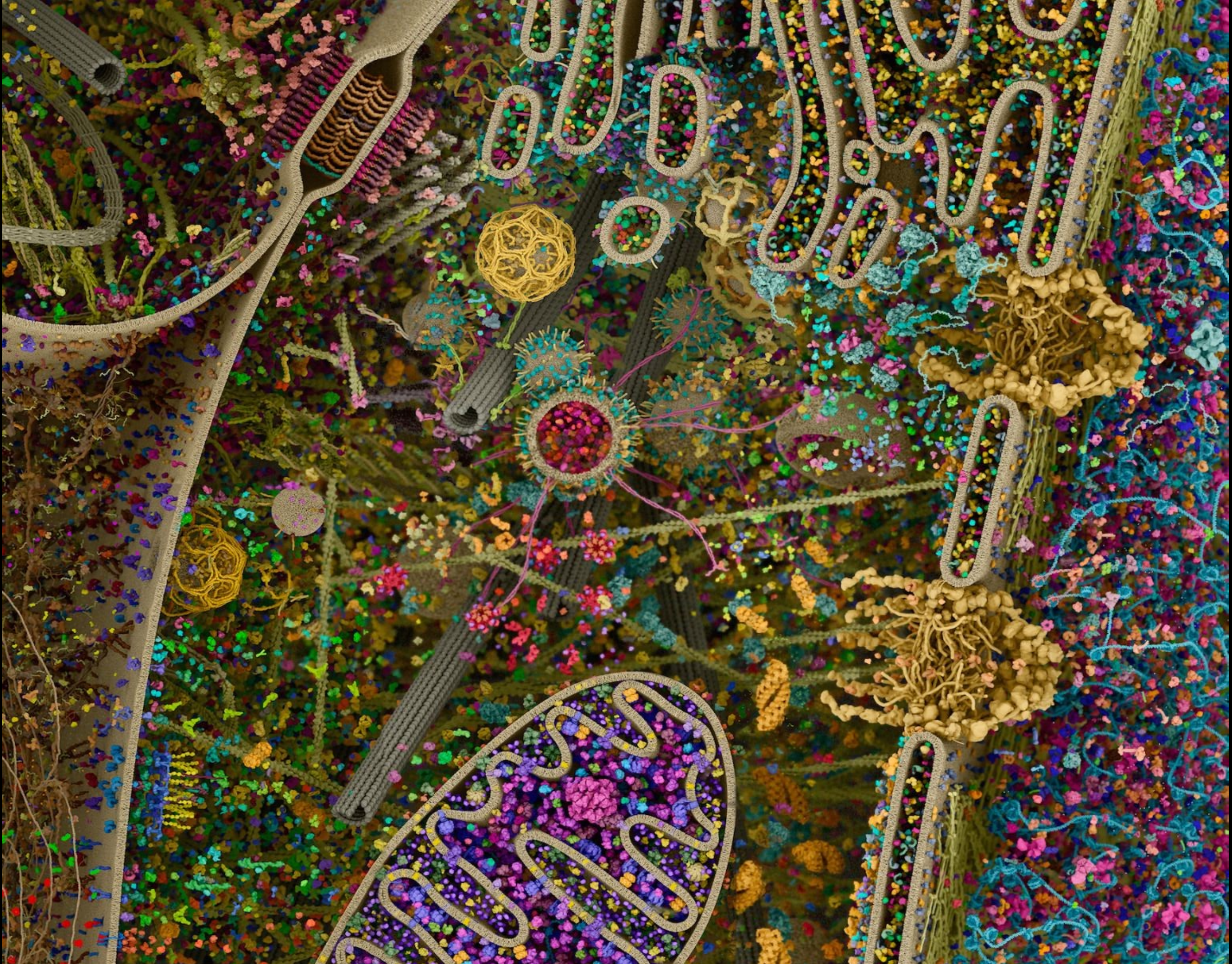


# SINGLE MOLECULE MICROSCOPY

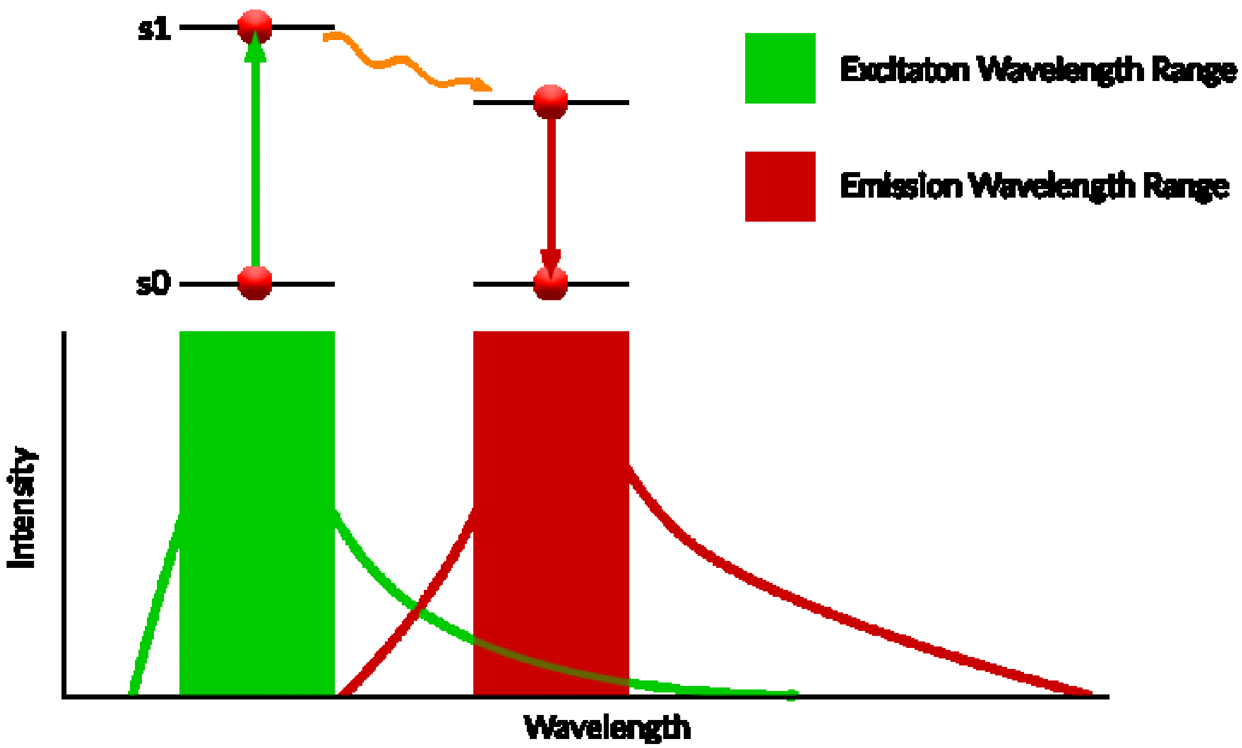
Lucia Gardini
LOT/2019
LENS

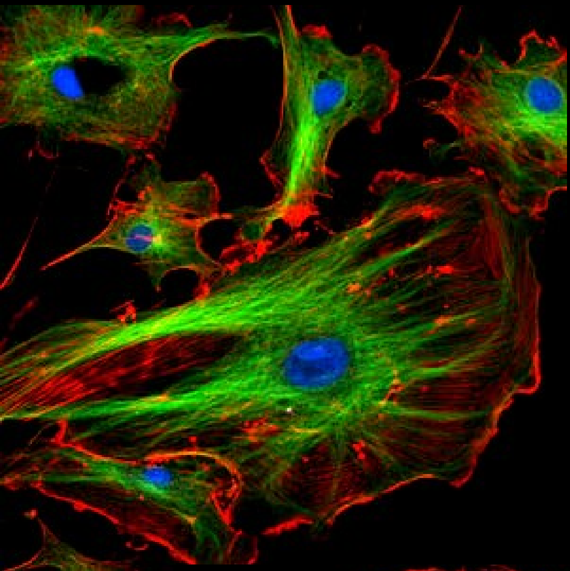
lens
European Laboratory for Non-Linear Spectroscopy

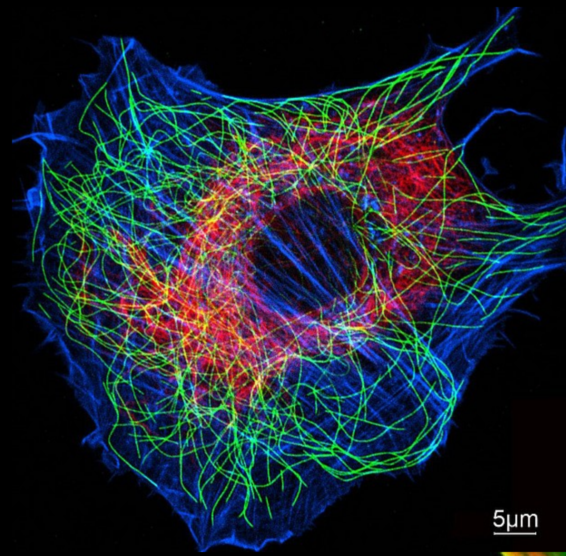
CNR-INO
ISTITUTO NAZIONALE DI OTTICA
CONSIGLIO NAZIONALE DELLE RICERCHE

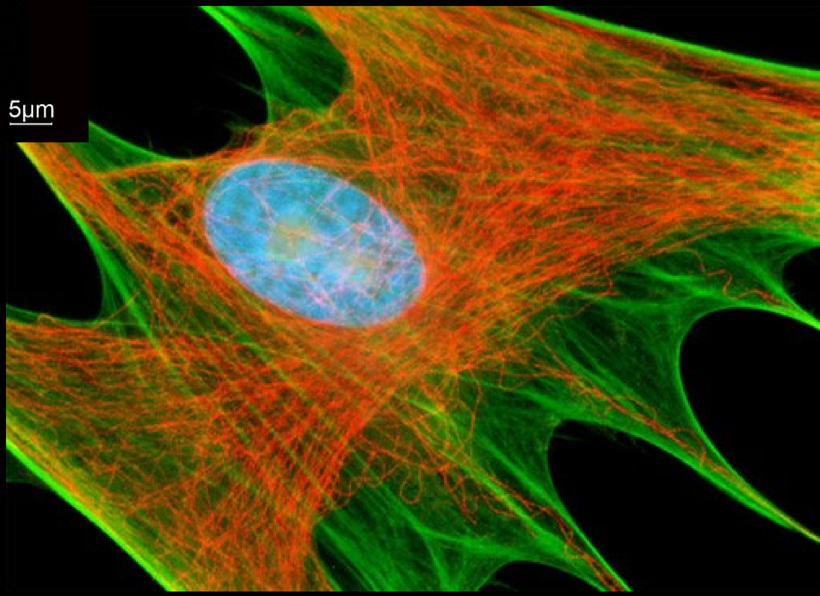


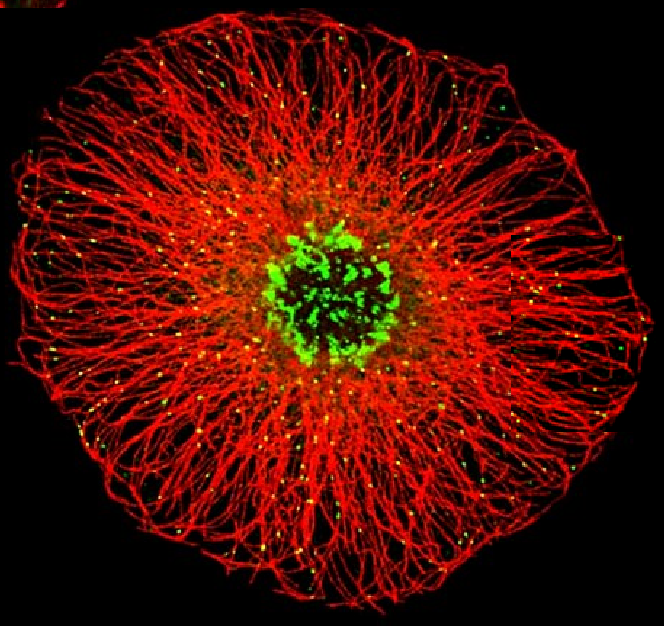
# Fluorescence microscopy






5μm





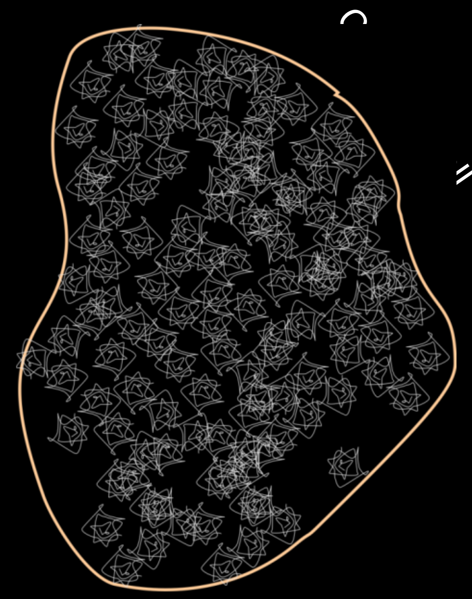
# Single-molecule fluorescence microscopy

Information on distributions and time trajectories that would otherwise be hidden

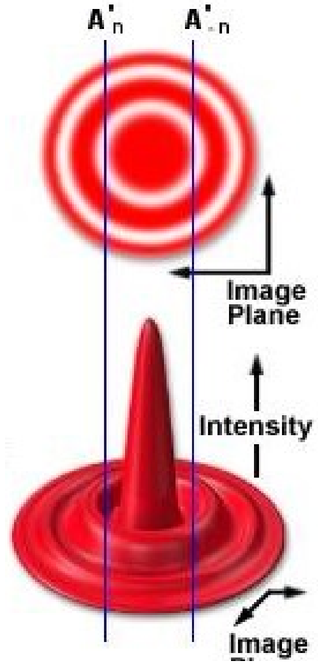
Identify and compare sub-populations

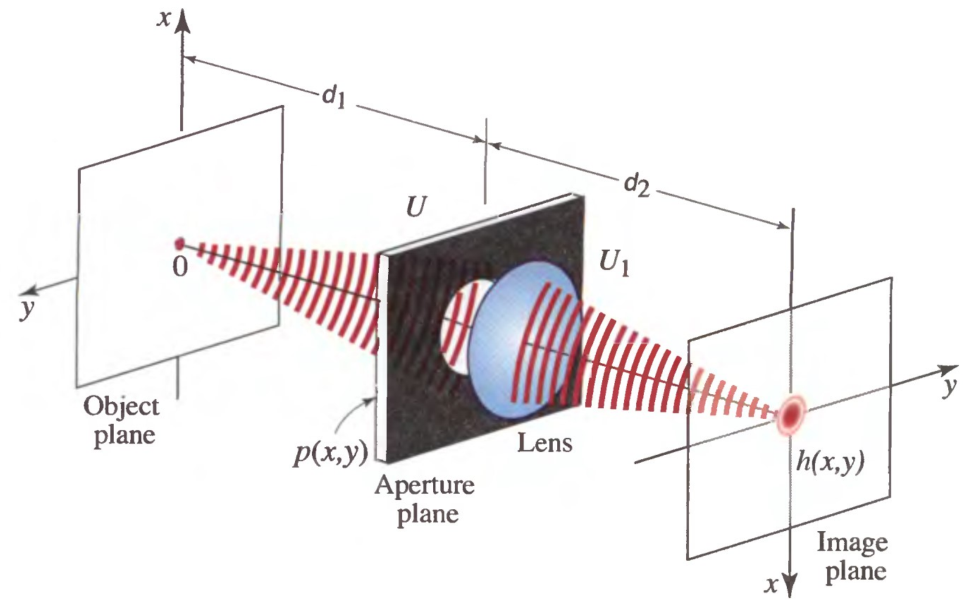
Probe biological macromolecules and provide informations on their structure and function

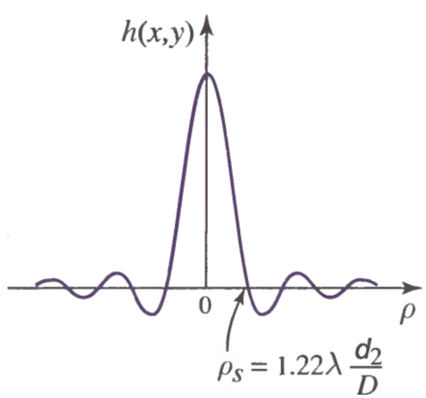
*In vitro* and *in vivo*

# PSF and image resolution




Abbe formula (x, y)

$$\rho_S = 1.22\lambda \frac{d_2}{D} \approx \frac{\lambda_0}{2n\sin\theta} = \frac{\lambda_0}{2NA}$$

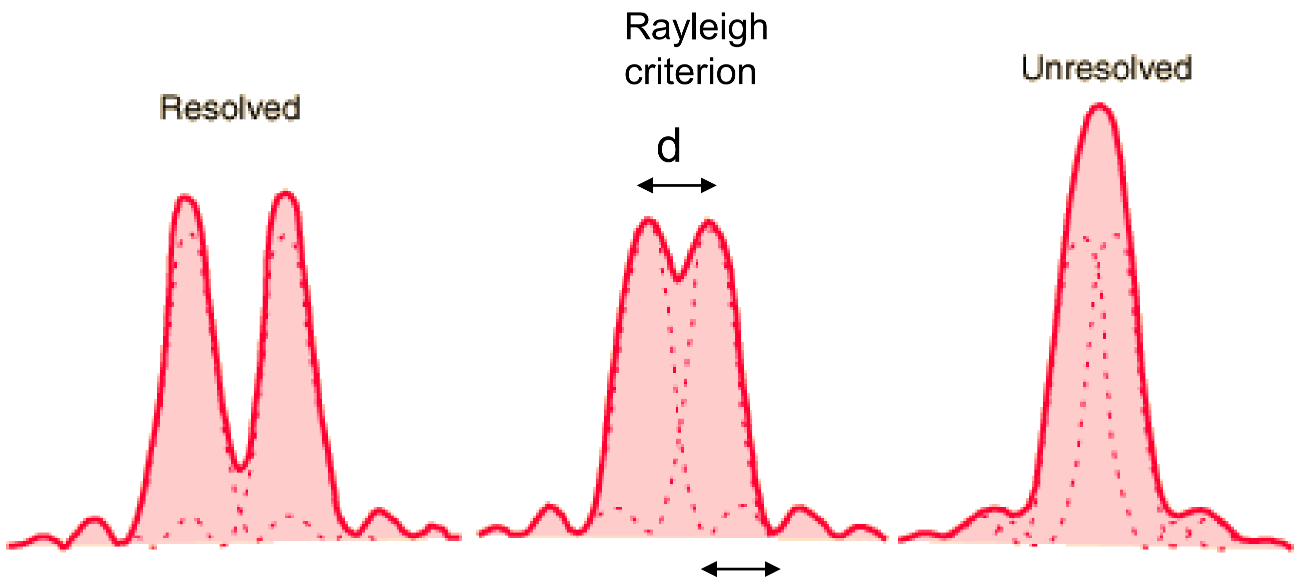
Radius of x,y PSF

Abbre forluma (z)

$2\lambda/NA^2$

Axial amplitude of the PSF

# Image resolution

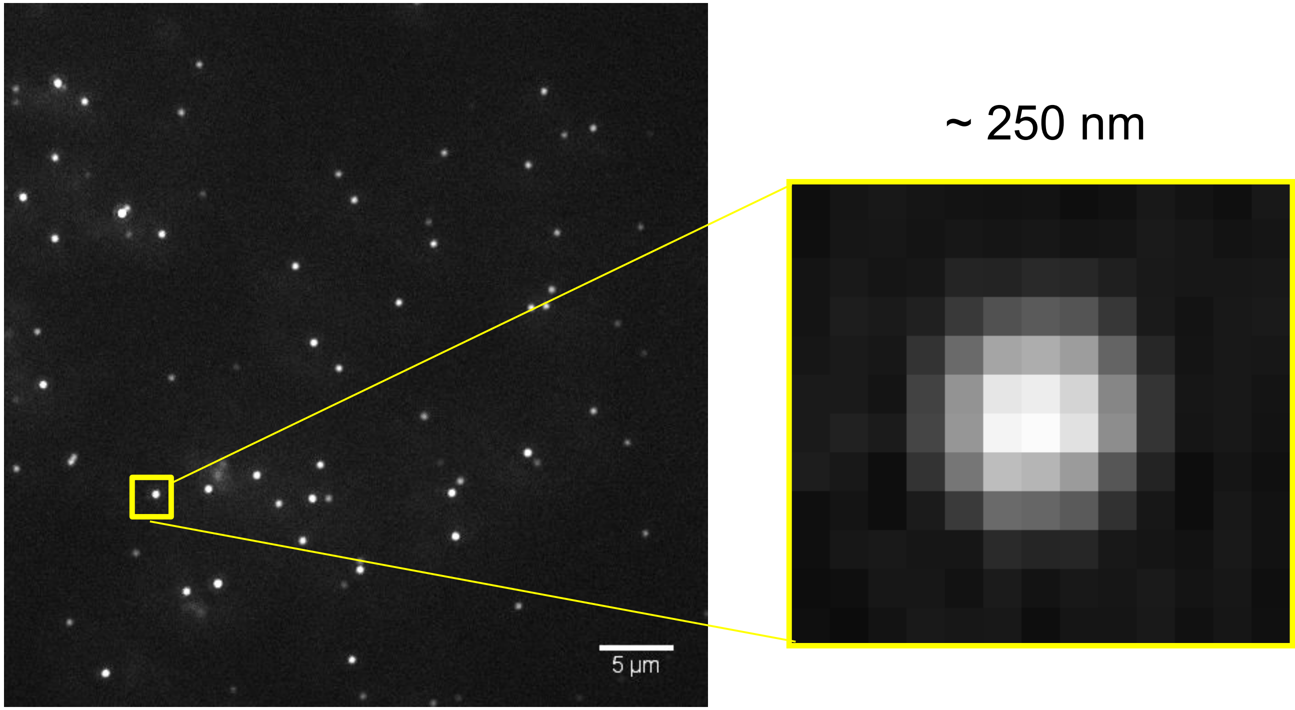



$d = \lambda/2NA$

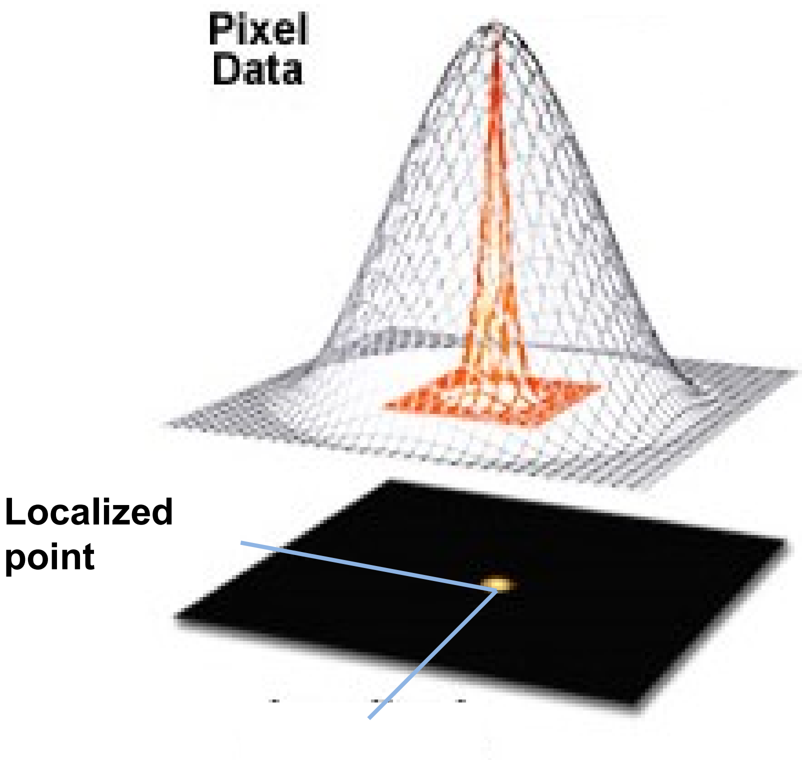
Lateral resolution ~ 250 nm

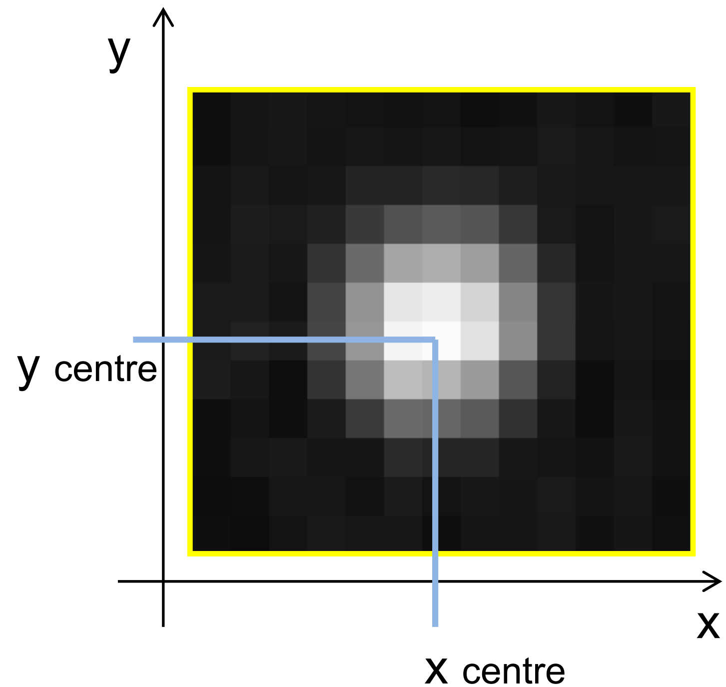
Axial resolution = $2\lambda/NA^2$ ~ 500nm

# Overcoming the diffraction limit



# Fluorescence Imaging with One Nanometer Accuracy
# FIONA




$$\sigma_{\mu} = \sqrt{\left( \frac{\sigma_{PSF}^{2}}{N} + \frac{a^{2}/12}{N} + \frac{8\pi\sigma_{PSF}^{4} b^{2}}{a^{2} N^{2}} \right)}$$

[redacted]

Thompson et al. *Biophys.J.* 2002

# Fluorescence Imaging with One Nanometer Accuracy
# FIONA

**b** ↓

**?**

**N** ↑

High quantum yield fluo probes

High NA aperture objectives (1.45)

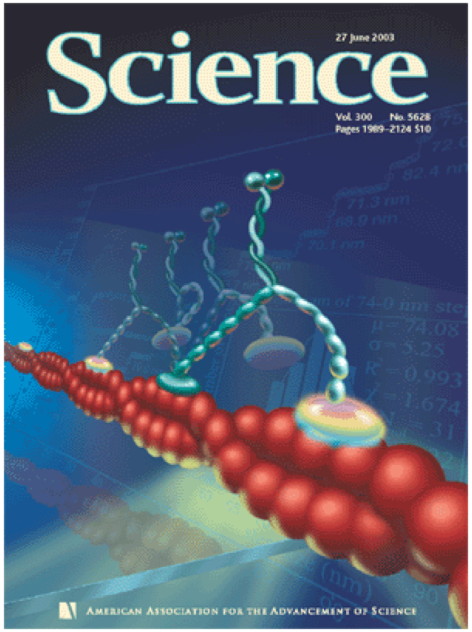
High sensitivity CCD cameras such as EMCCD

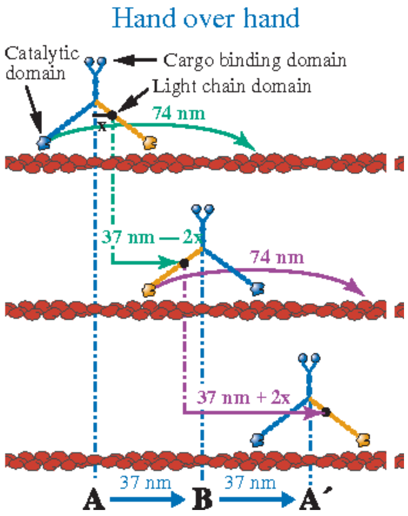
$$\sigma_{\mu} = \sqrt{\left( \frac{\sigma_{PSF}^{2}}{N} + \frac{a^{2}/12}{N} + \frac{8\pi\sigma_{PSF}^{4} b^{2}}{a^{2}N^{2}} \right)}$$

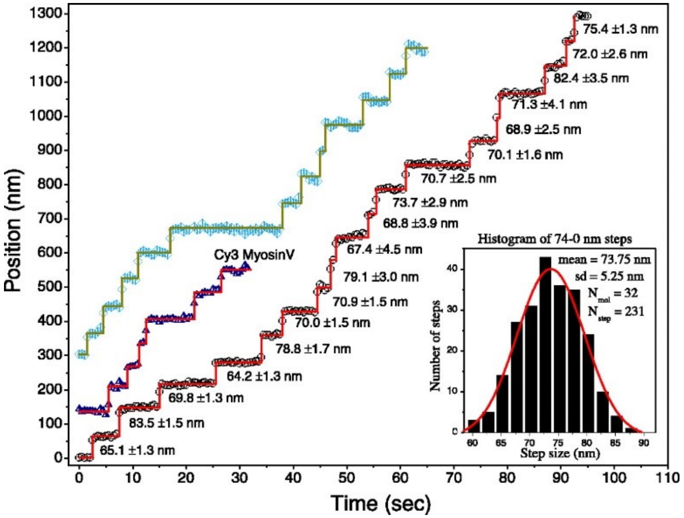
[redacted]

Thompson et al. *Biophys.J.* 2002

Yildiz et al. *Science* 2003




[redacted]

$N \approx 10^4$ photons

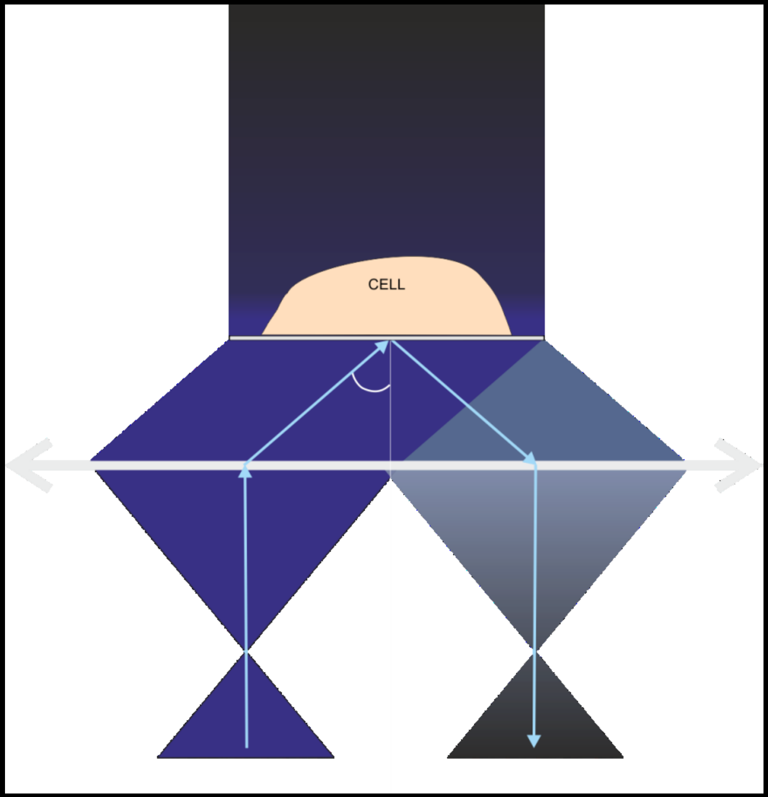
$\sigma_\mu \approx 1{,}5\,\mathrm{nm}$

High quantum yield fluo probes

High NA aperture objectives (1.45)

High sensitivity CCD cameras such as EMCCD b

# TOTAL INTERNAL REFLECTION MICROSCOPY
## (proximity to the membrane)




100 nm depth in the sample

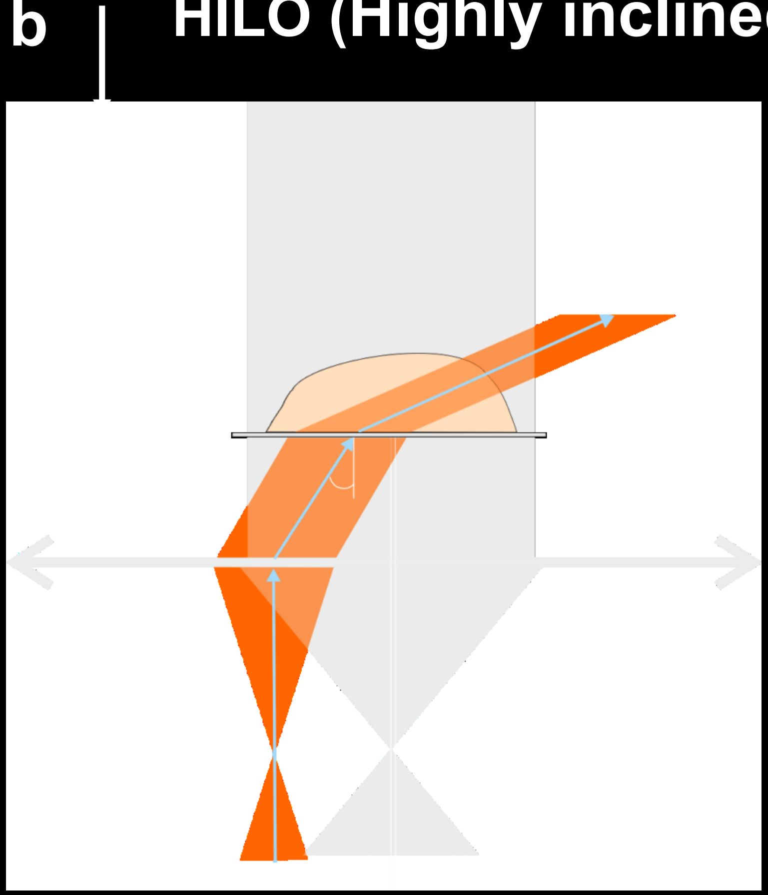
Small volume excited

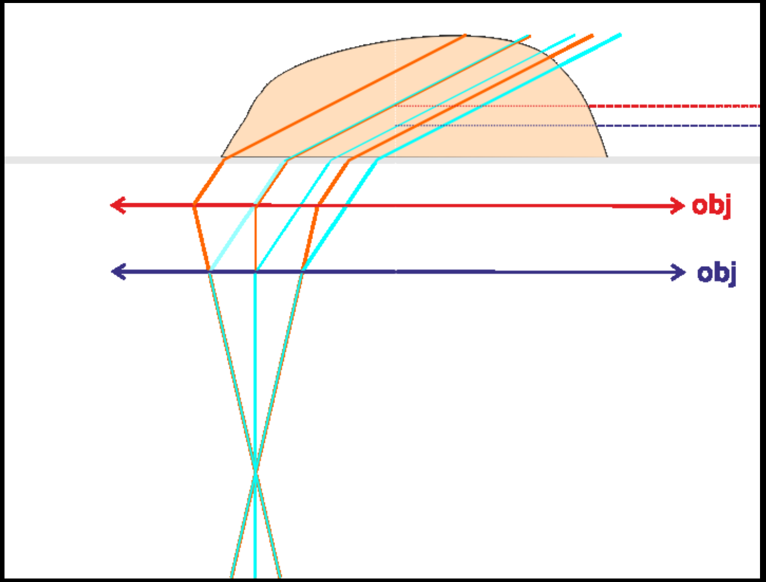
High S/N

# INCLINED ILLUMINATION

## HILO (Highly inclined and Laminated Optical sheet)

b

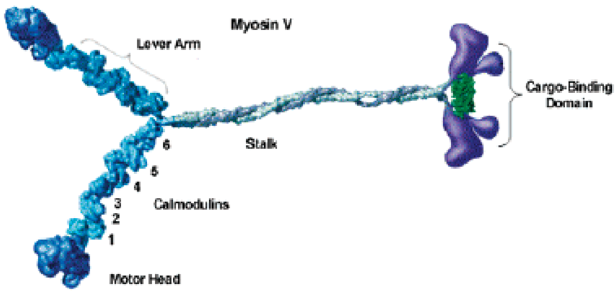




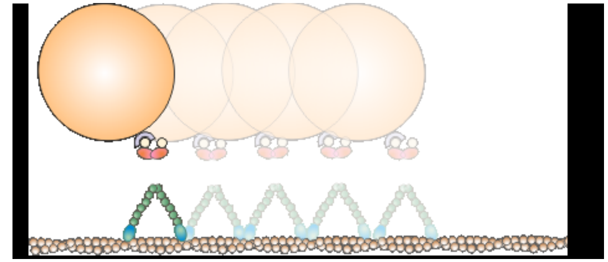

THE ILLUMINATION BEAM ALWAYS PASSES THROUGH THE CENTER OF THE SPECIMEN PLANE ALLOWING OPTICAL SECTIONING

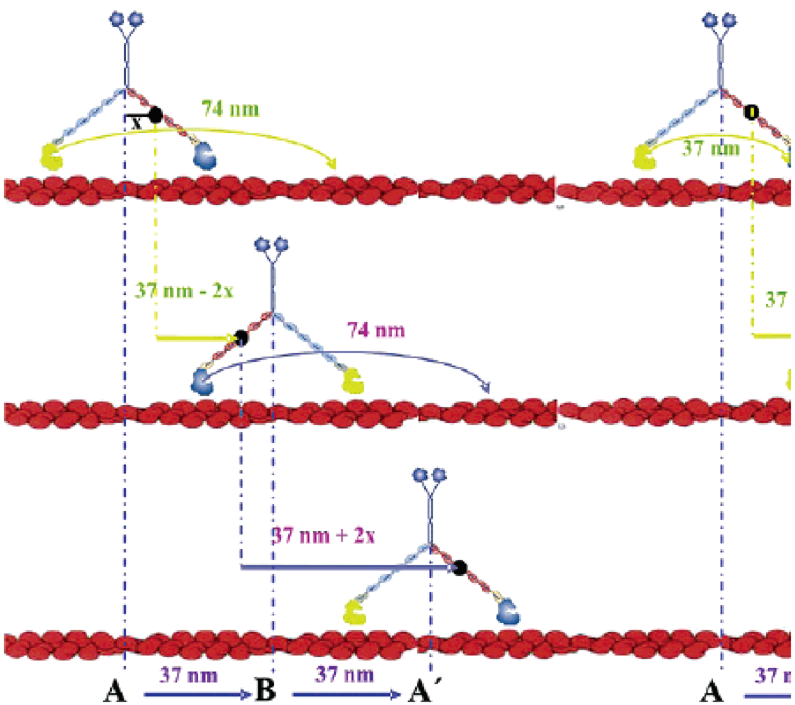
8 FOLD HIGHER SIGNAL/BACKGROUND COMPARED TO TRADITIONAL WIDEFIELD MICROSOCPY

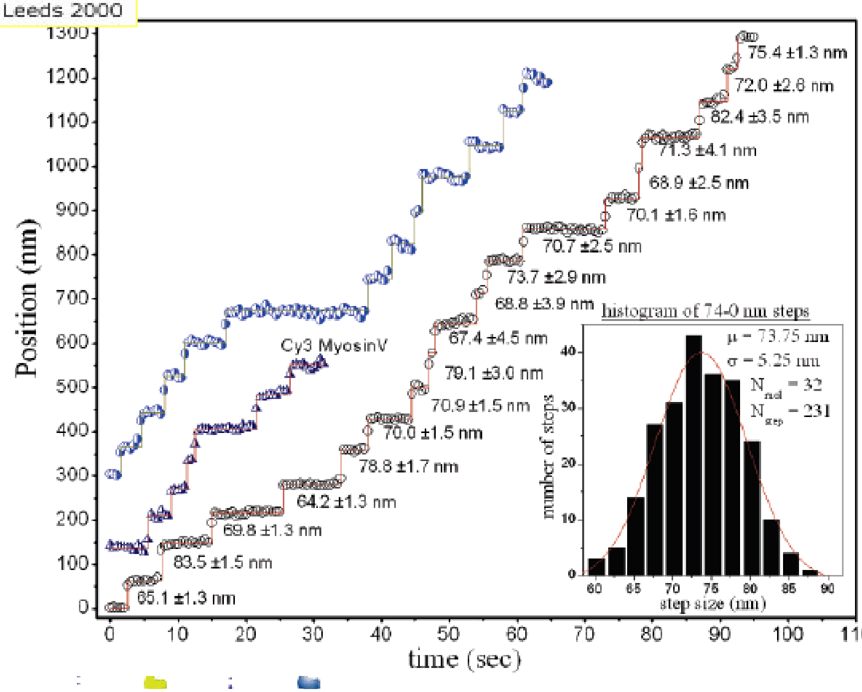
# Application in vitro: myosin V walks hand over hand







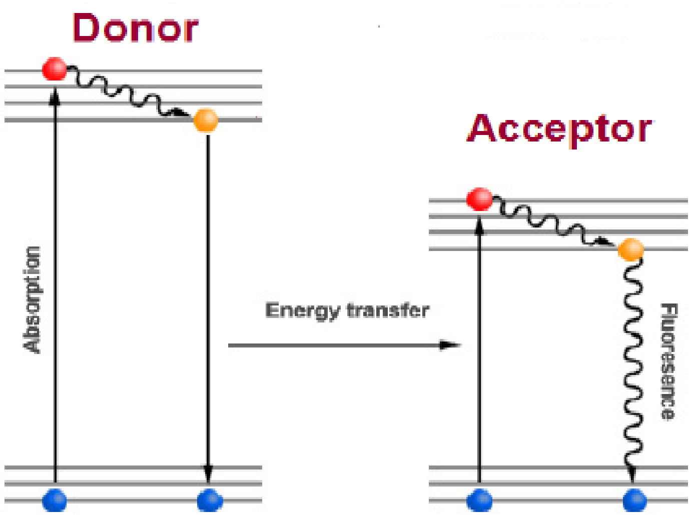


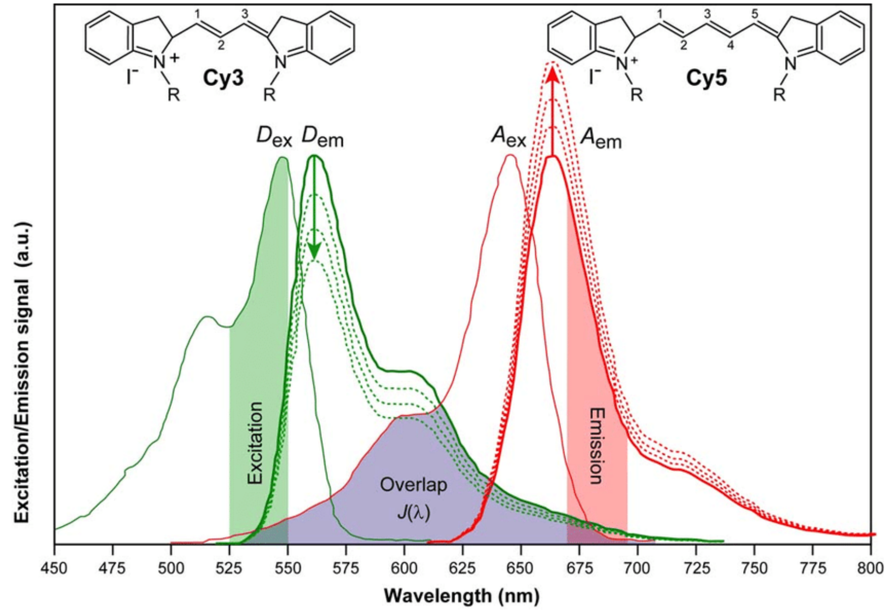




Accuracy: 1.5 nm. Time resolution: 0.5 s
10^4 photons collected

Yildiz et al. Science 300:2061, 2003

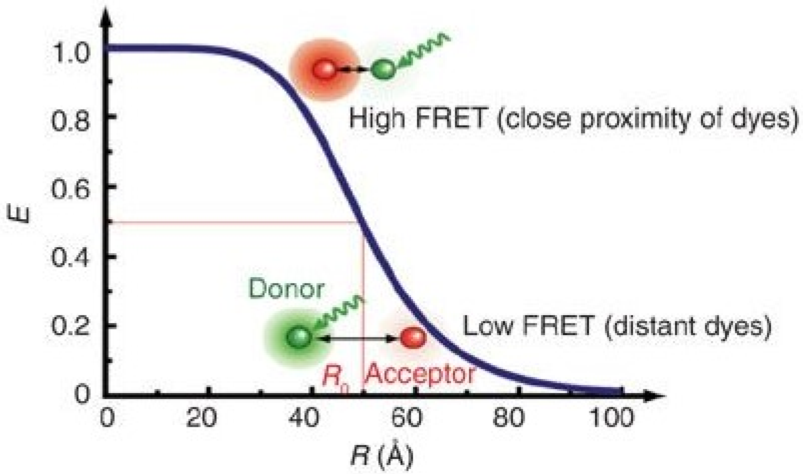
# Singel Molecule FRET (Forster Resonance Energy Transfer)



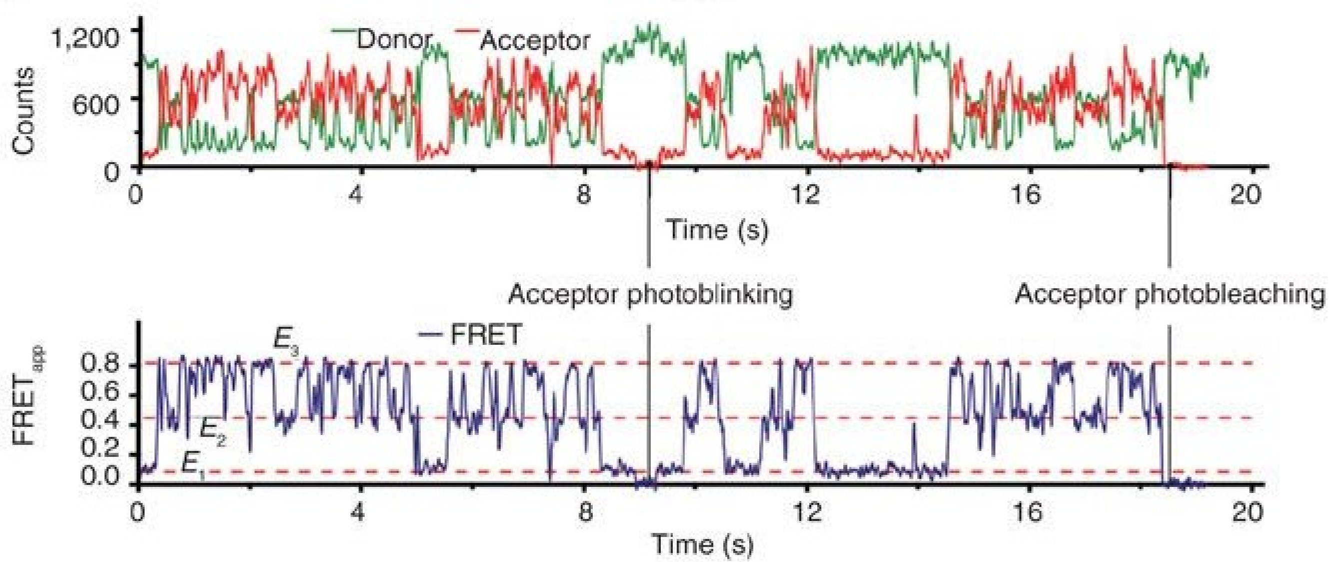

Distanze tipiche tra 30 e 80 Å

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

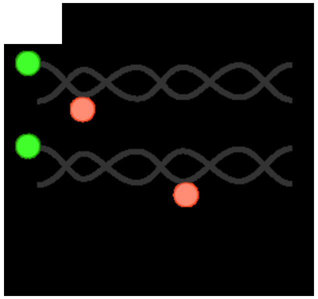


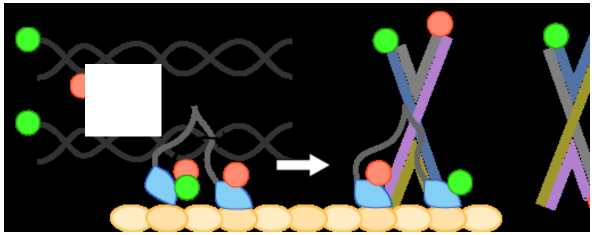

R = dist.donor/acc
$R_0$ = dist.caratteristica (50% di en. trasferita)

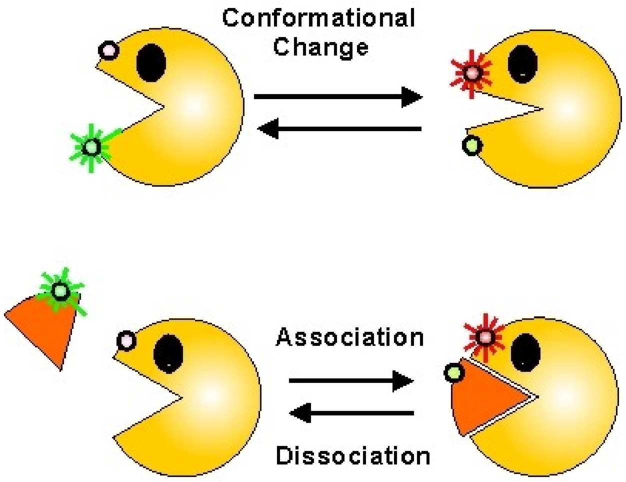
Es.Cy3/Cy5 $R_0$= 60 Å (6 nm)

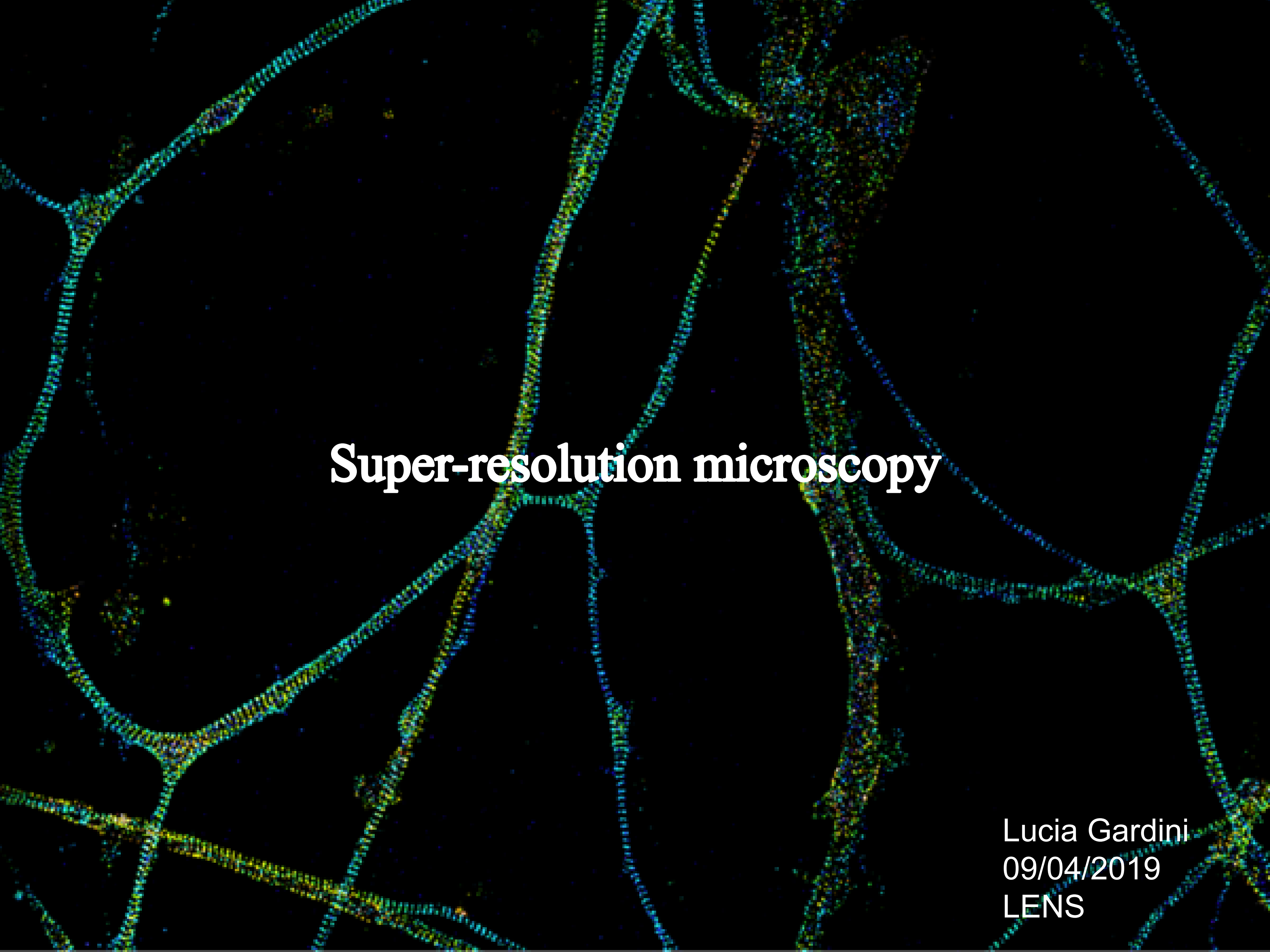


# Probing conformational changes and displacements









# Super-resolution microscopy

Lucia Gardini
09/04/2019
LENS

# Single molecule localization microscopy: SUPER-RESOLUTION MICROSCOPY

## NOBEL PRIZE IN CHEMISTRY 2014

Photo: Matt Staley/HHMI
**Eric Betzig**
Prize share: 1/3

Photo: Wikimedia Commons, CC-BY-SA-3.0
**Stefan W. Hell**
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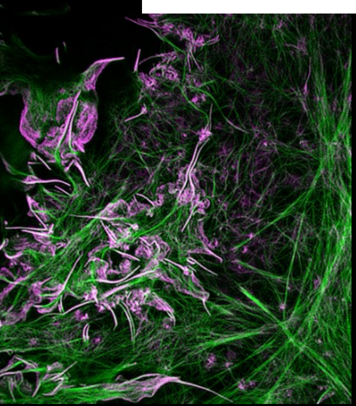
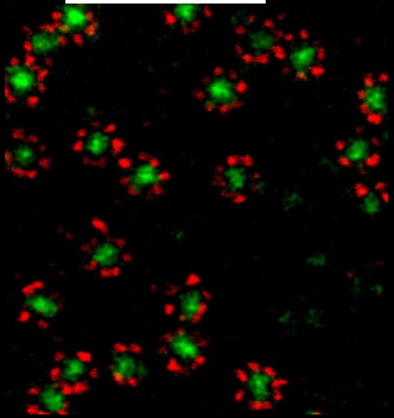
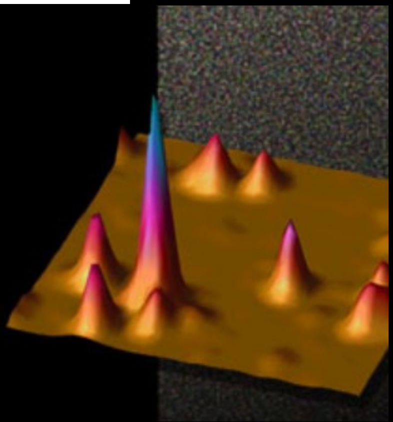
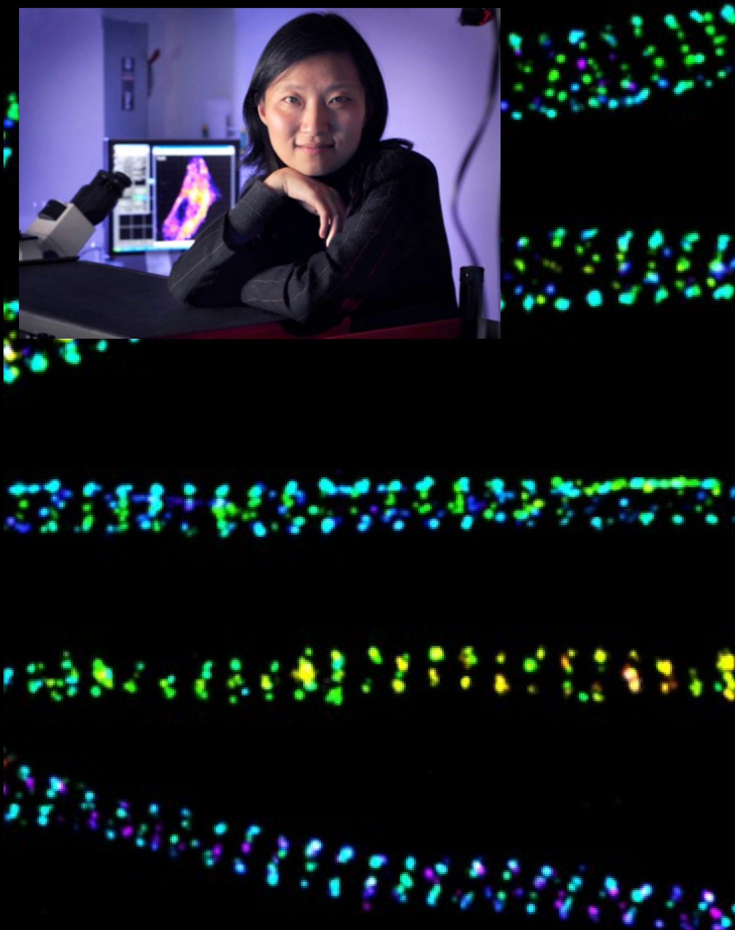
Photo: K. Lowder via Wikimedia Commons, CC-BY-SA-3.0
**William E. Moerner**
Prize share: 1/3



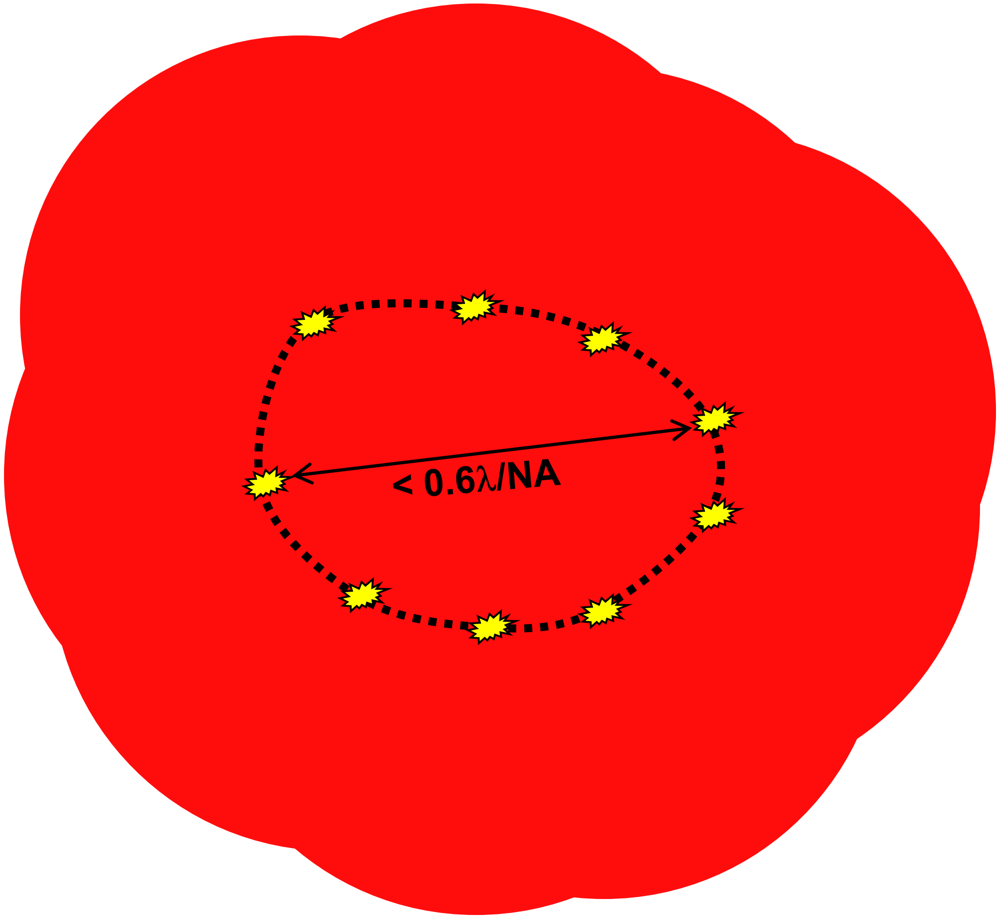




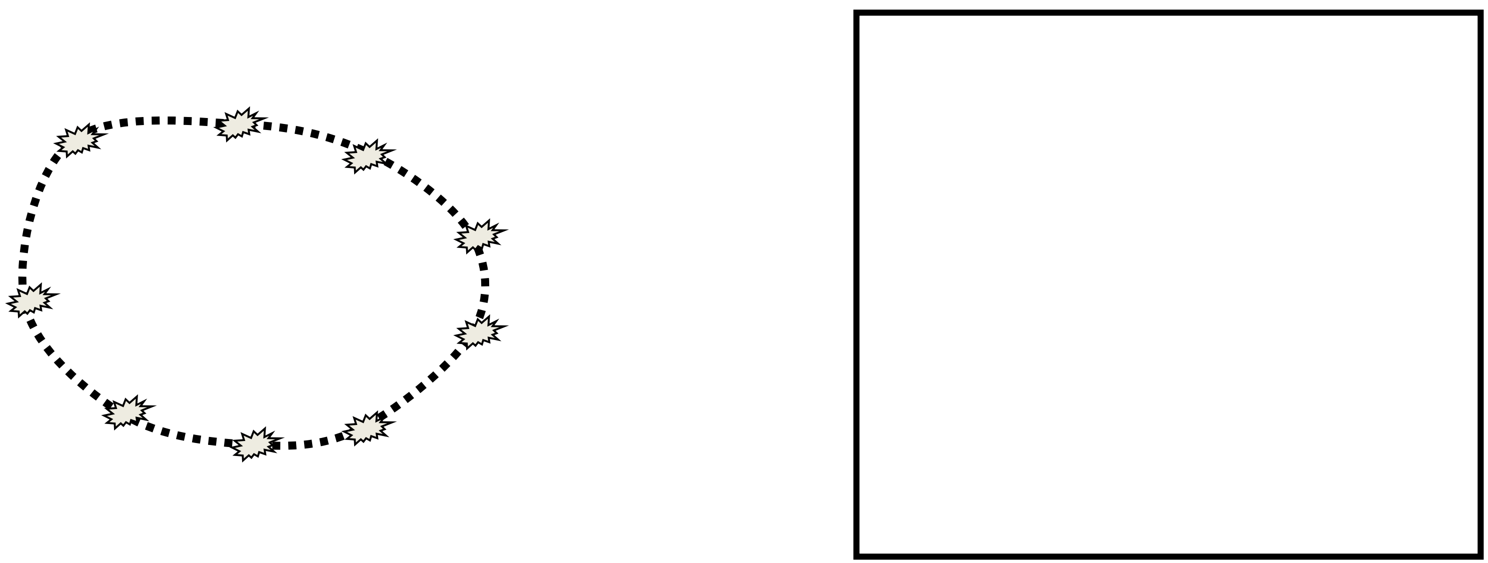
## Xiaowei Zhuang
## BREACKTHROUGH PRIZE 2019



# *The principle of PALM and STORM*



# *The principle of PALM and STORM*



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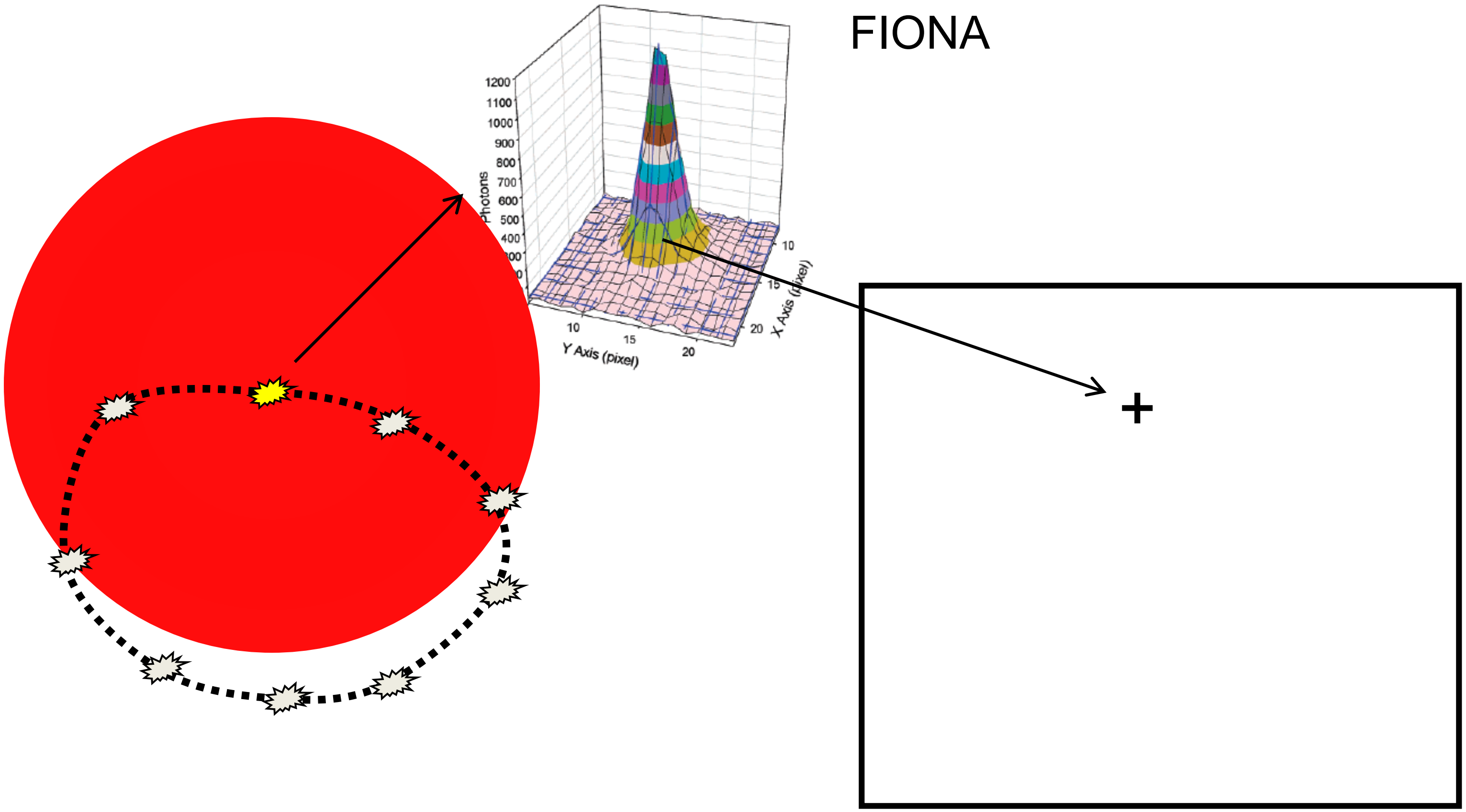

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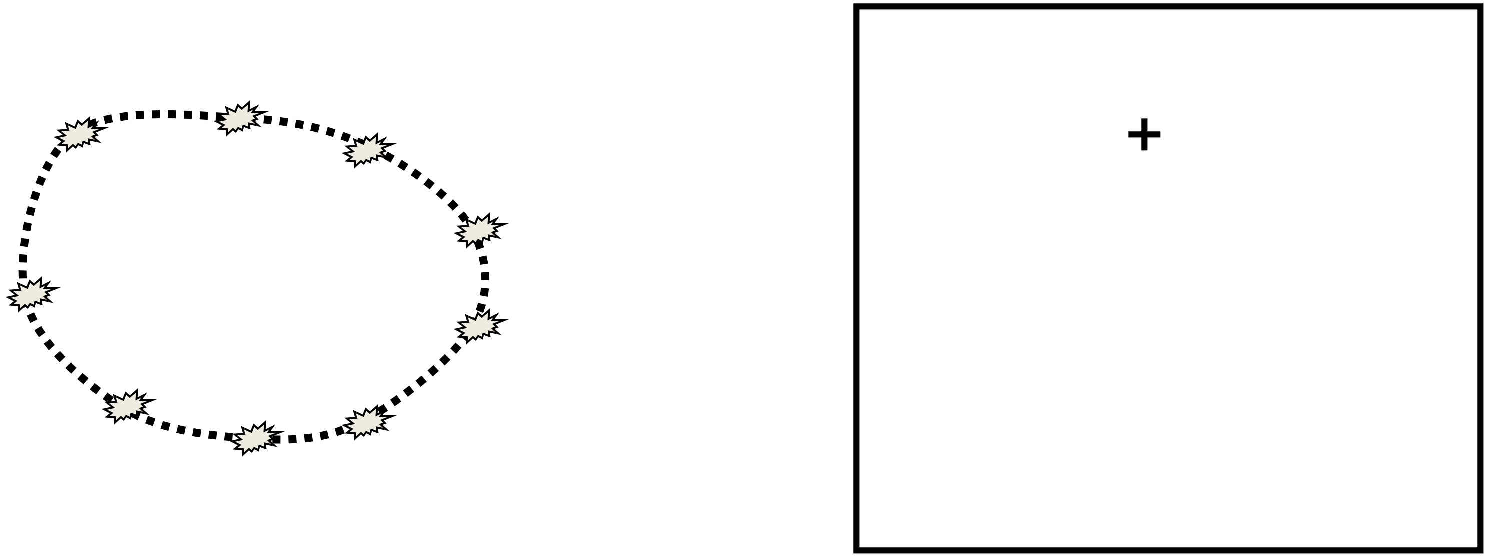

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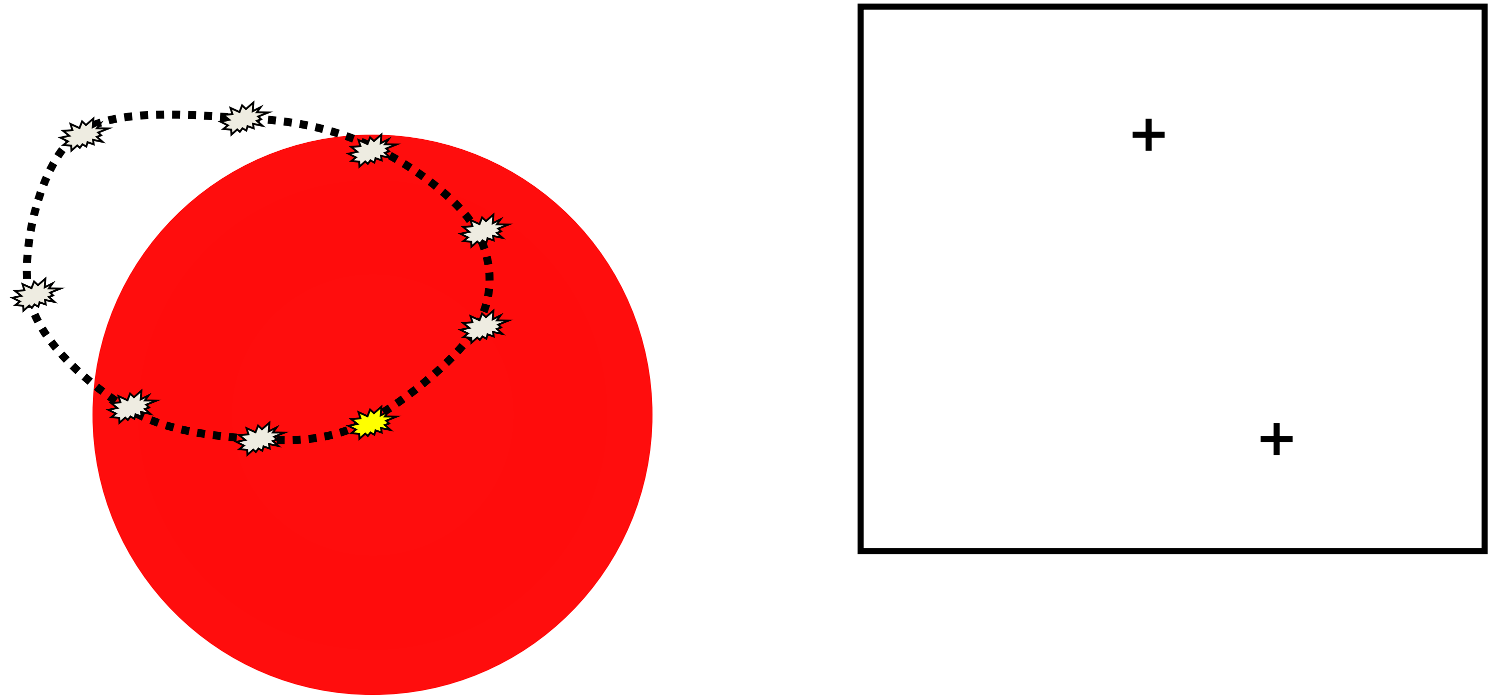

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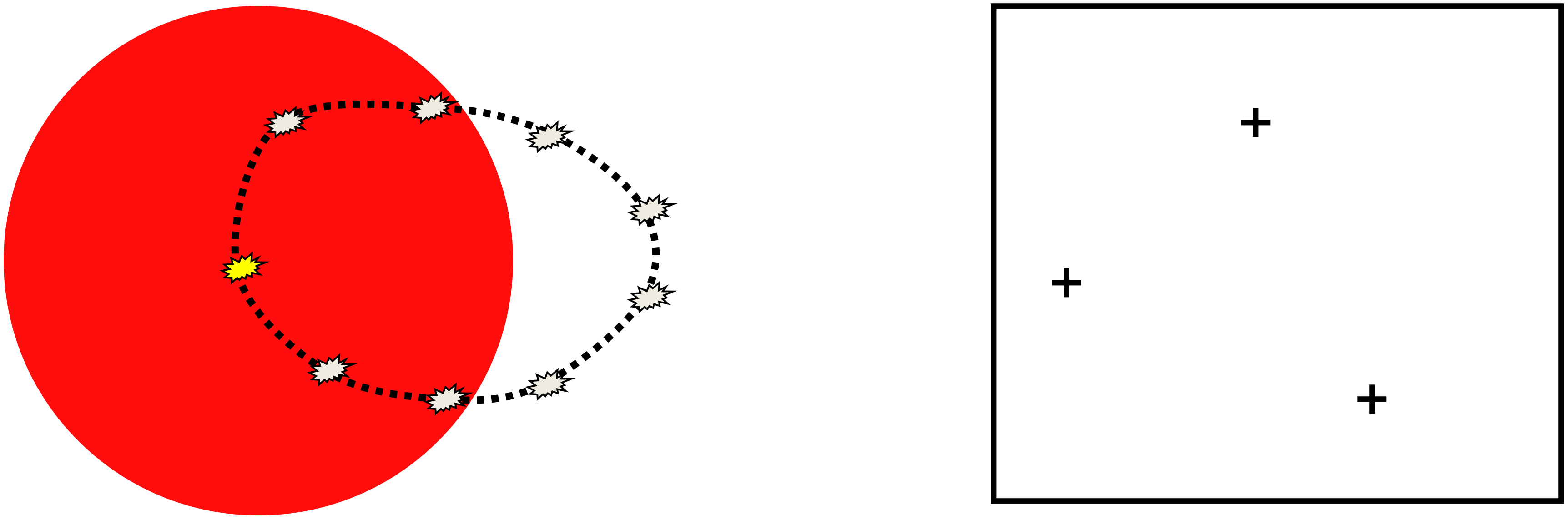

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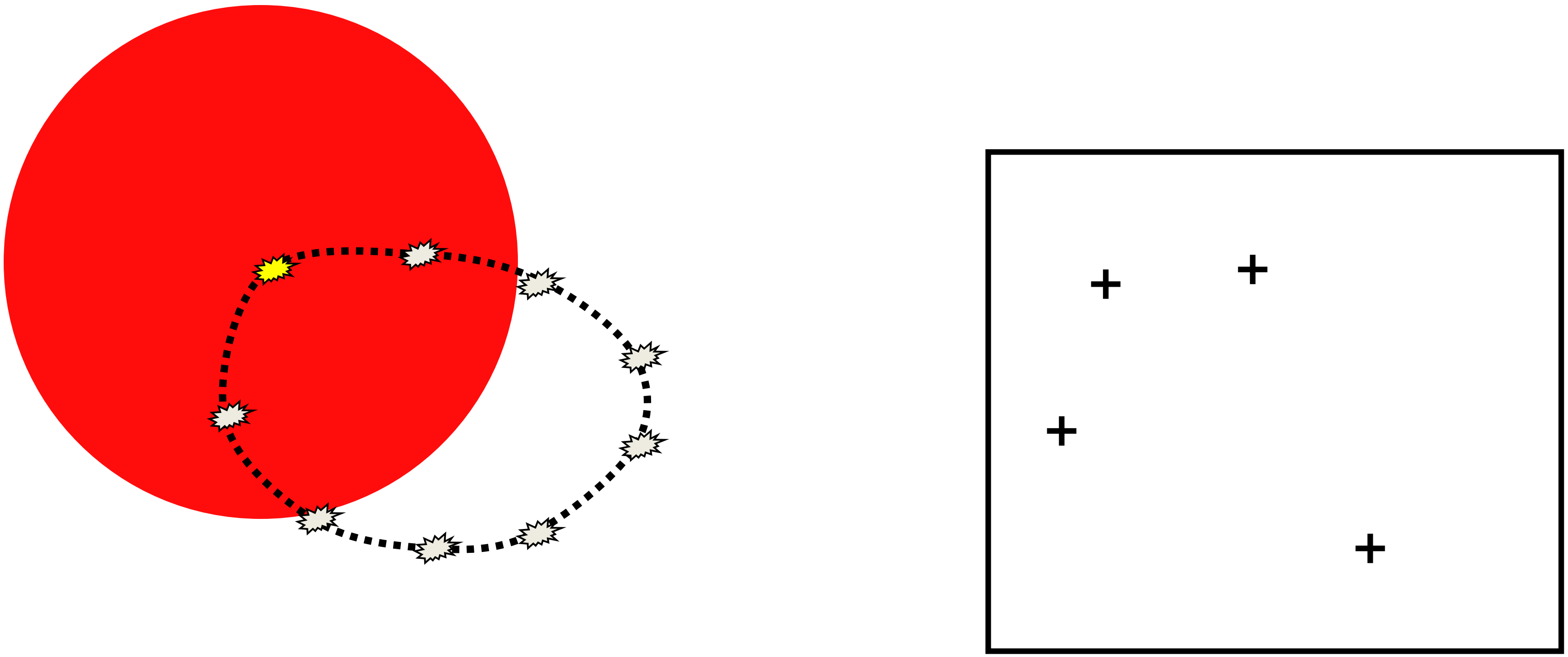
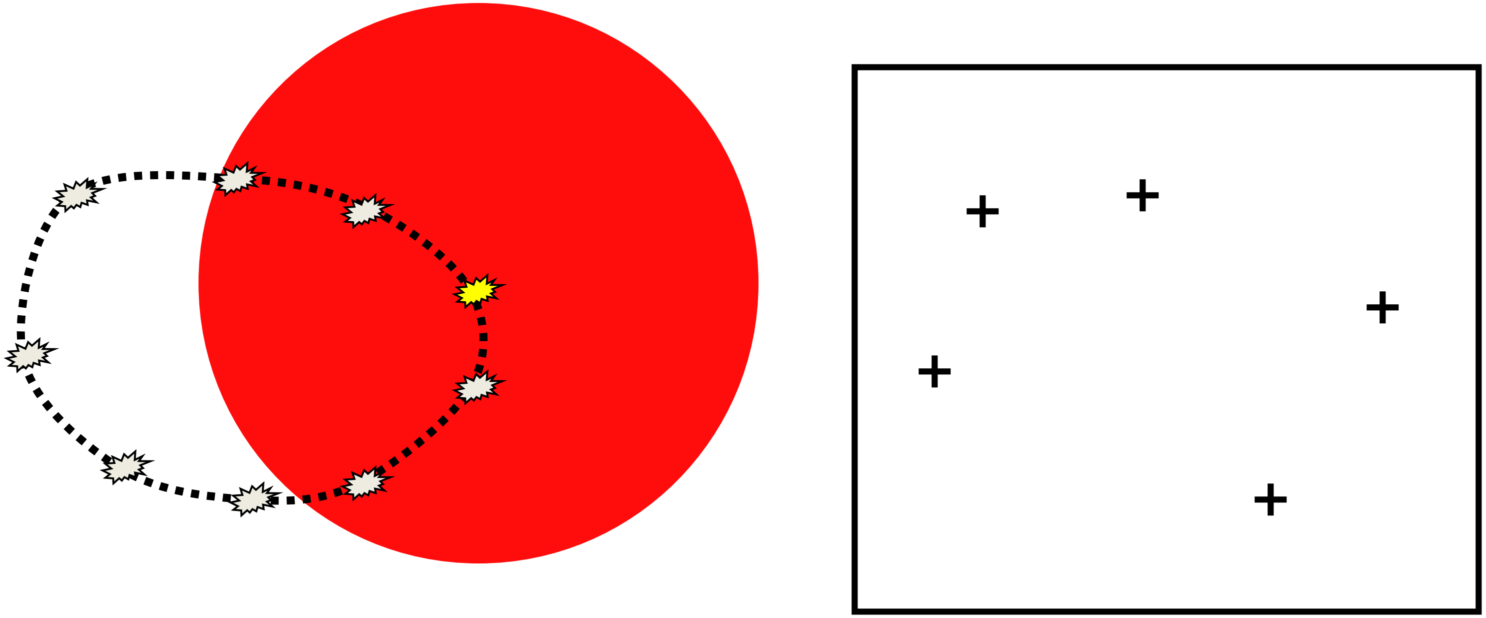


# *The principle of PALM and STORM*



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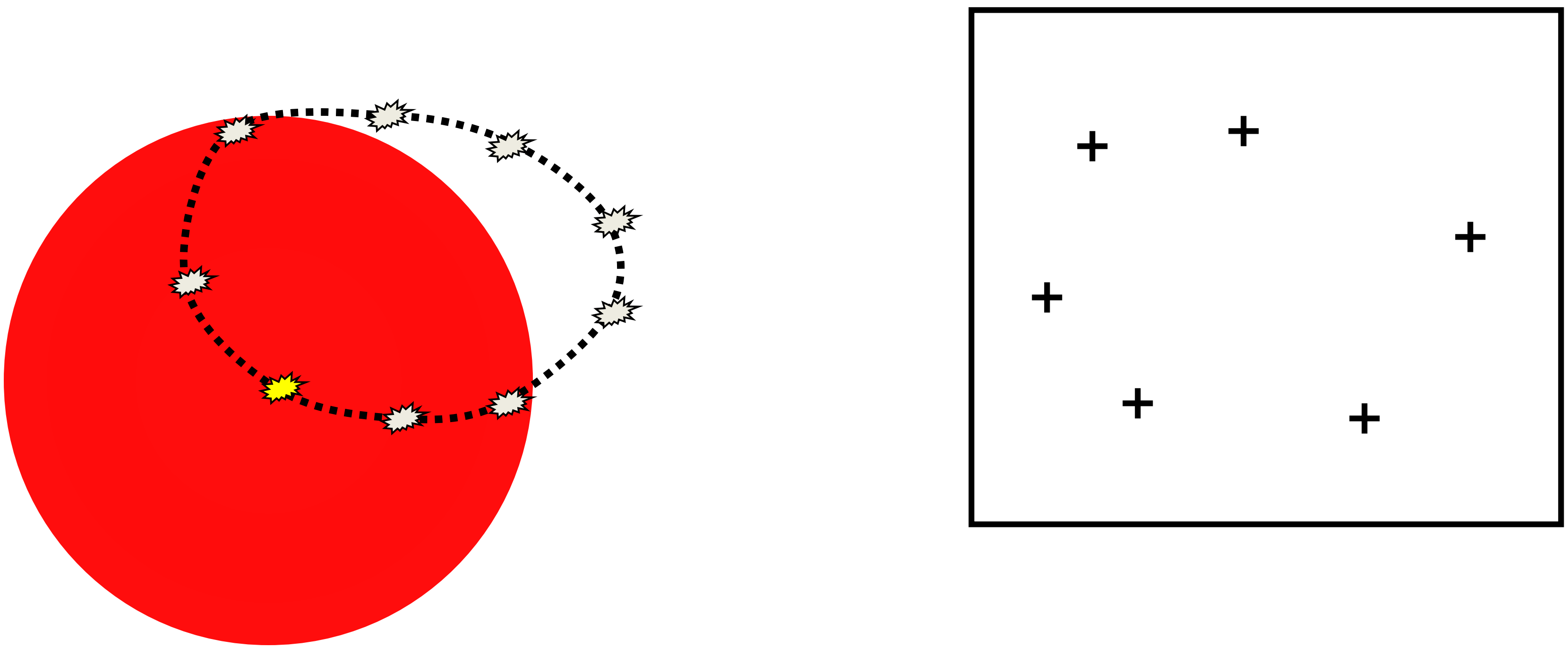

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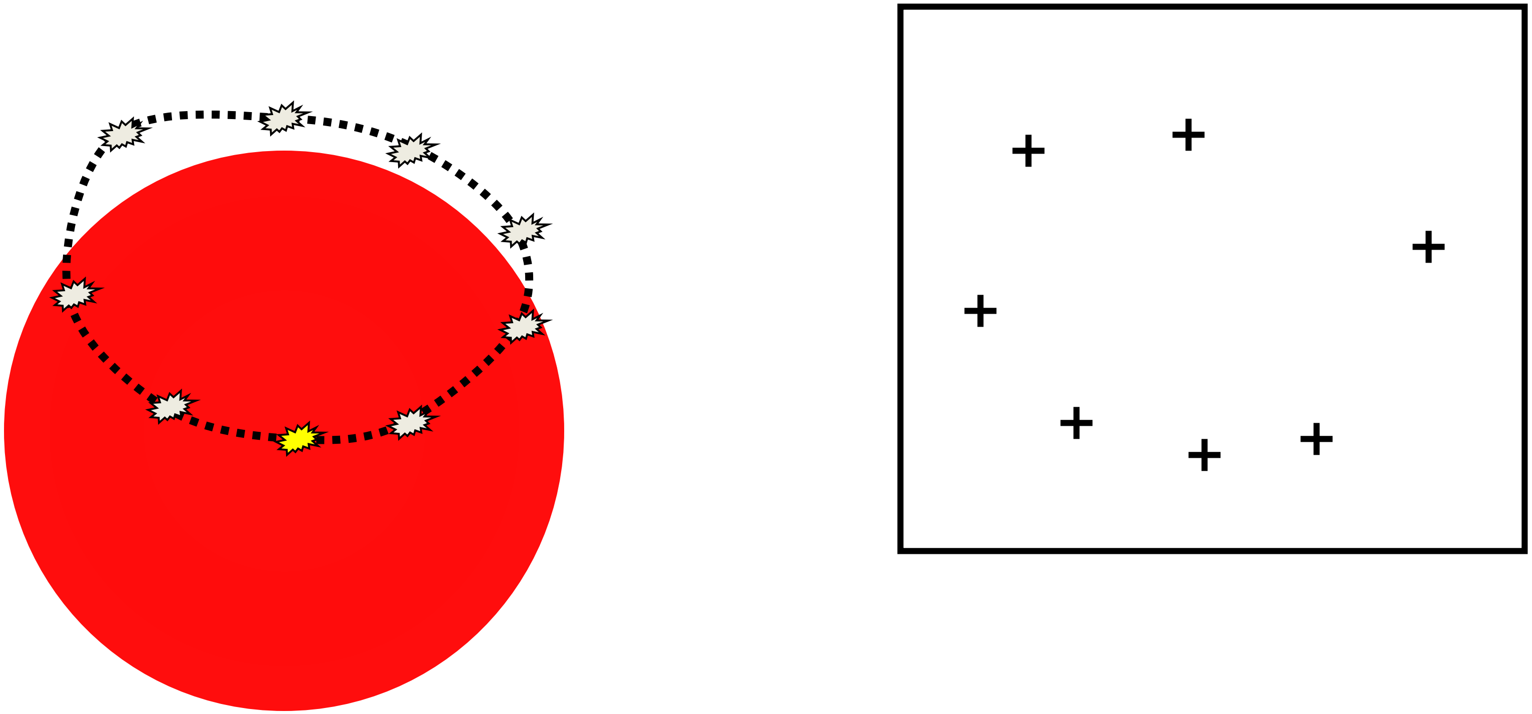

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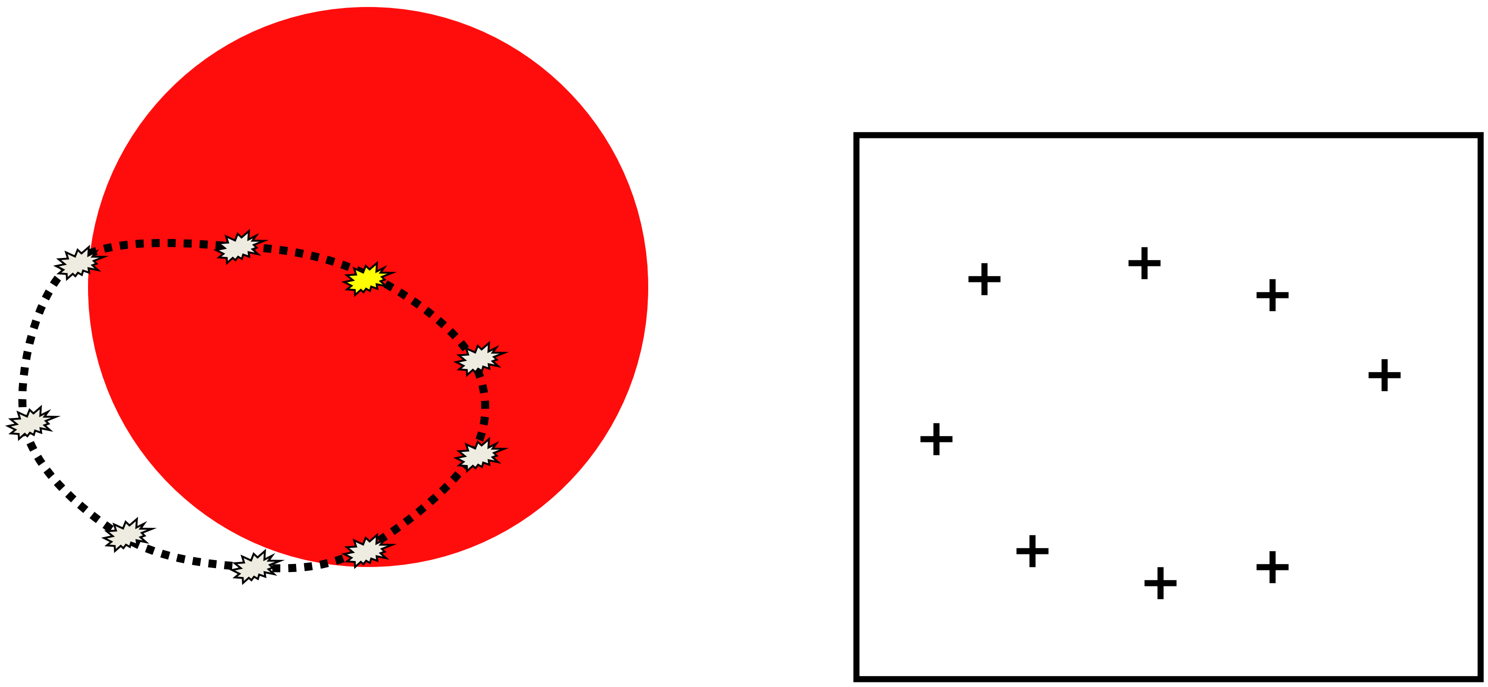
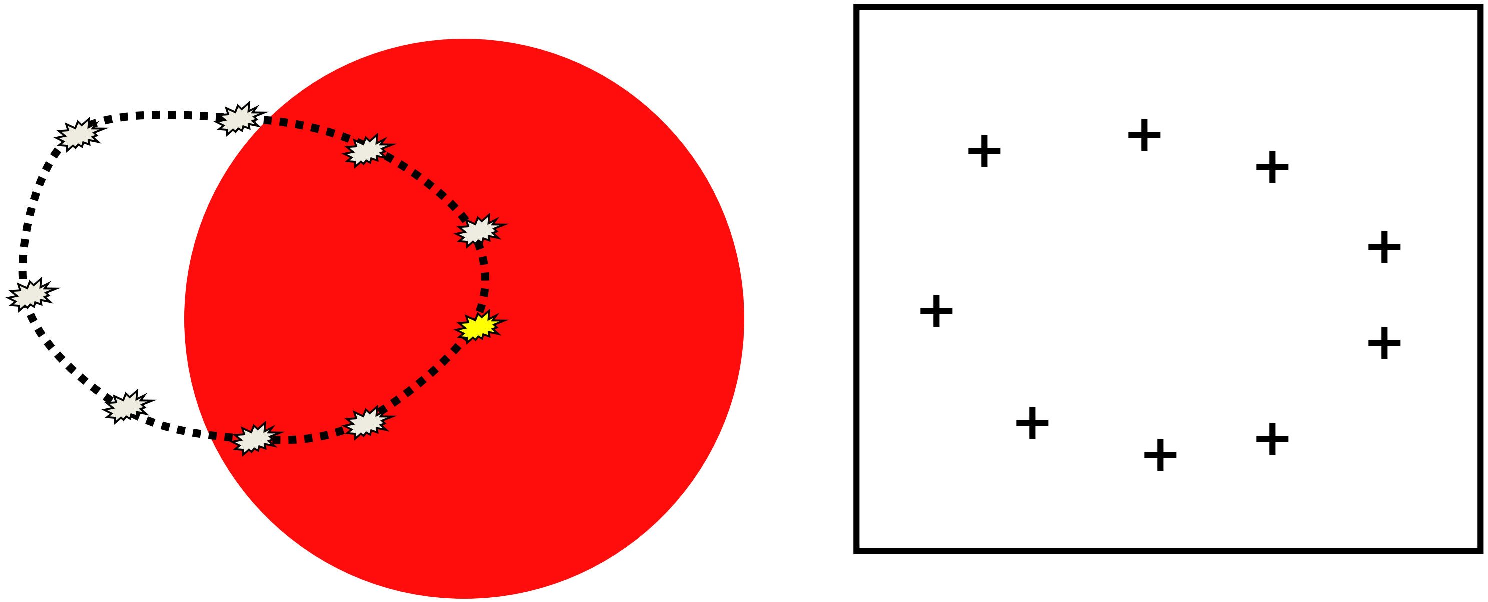
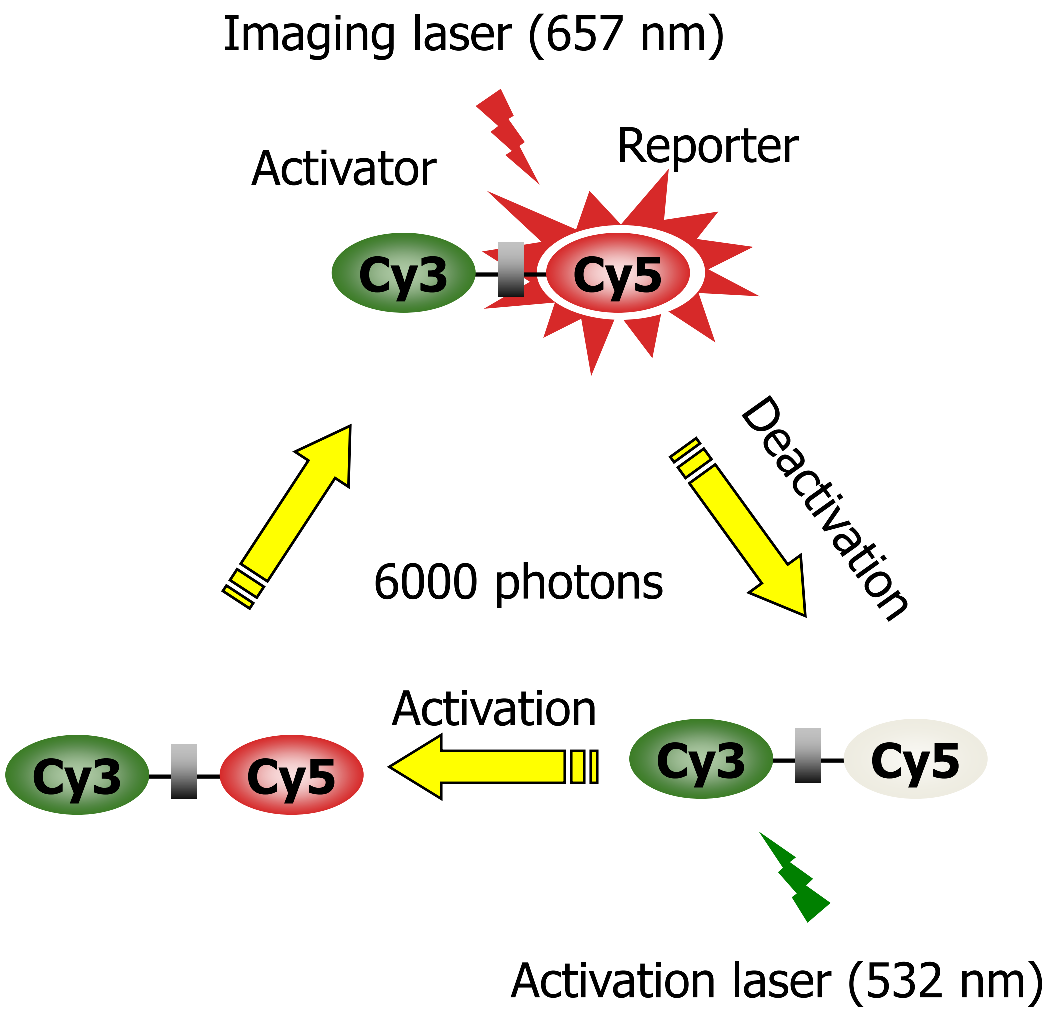
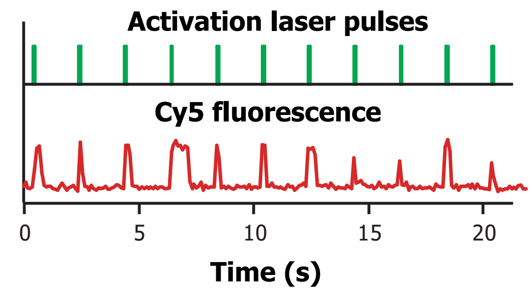


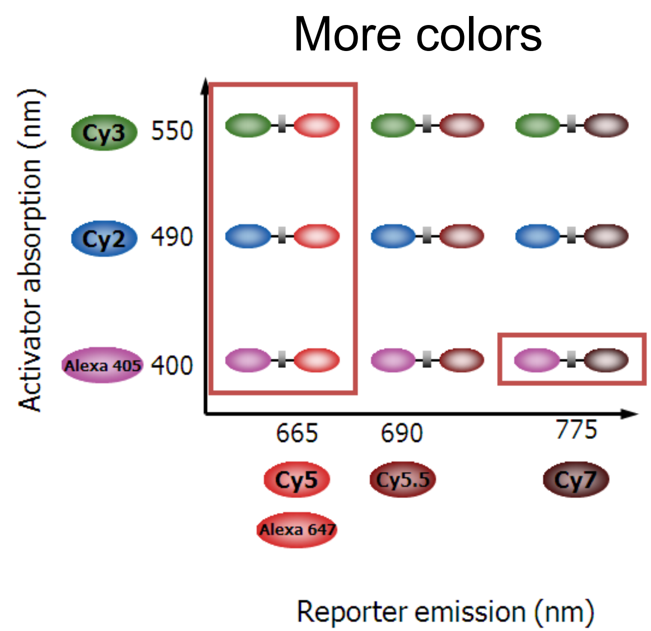
# *The principle of PALM and STORM*



# *STORM - Photo-switchable Probes*

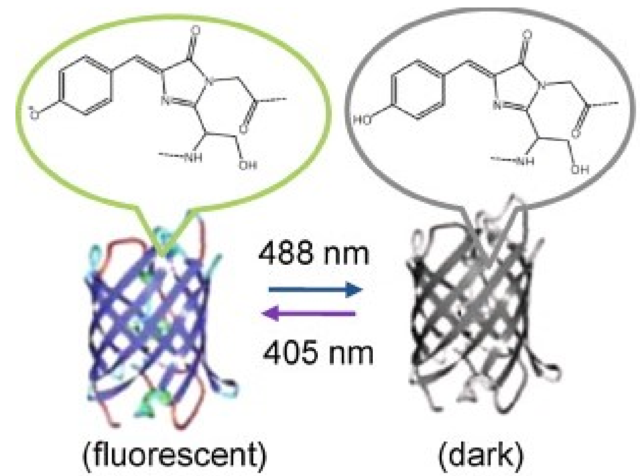


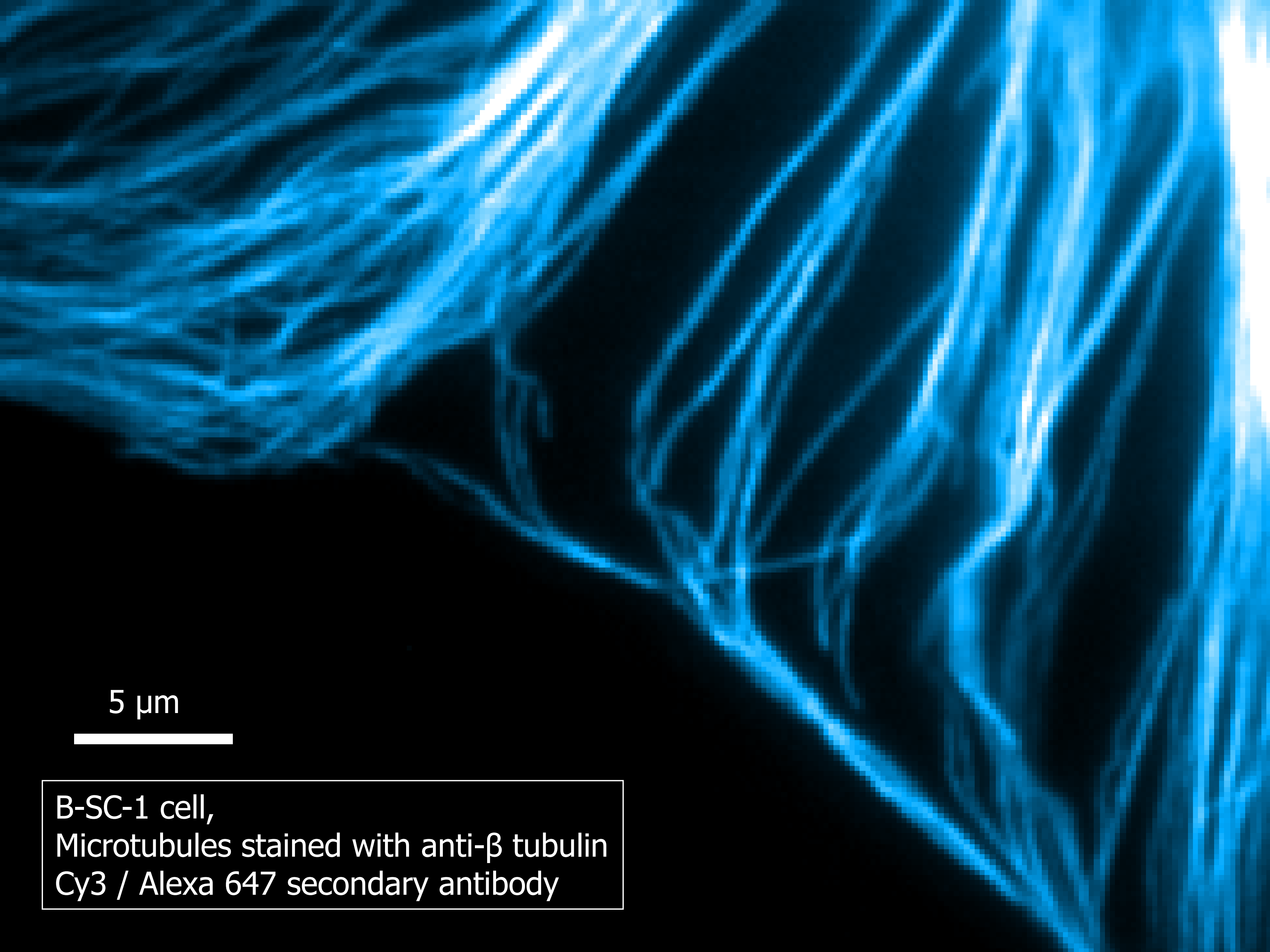



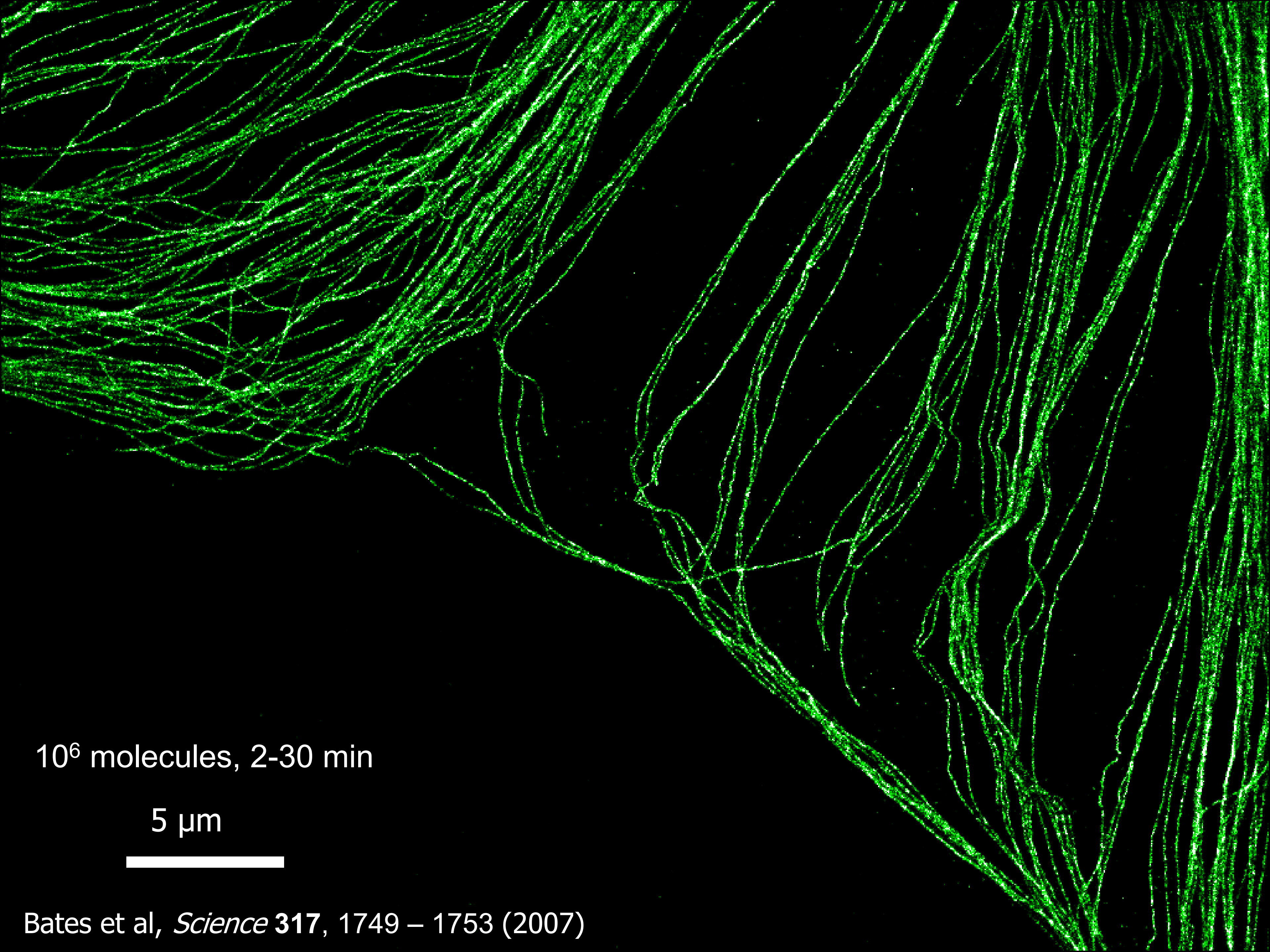
# Direct STORM

| Dye | | Sensitivity[a] |
|---|---|---|
| Blue-absorbing | Atto 488 | + |
| | Alexa Fluor 488 | + |
| | Atto 520 | + |
| | Fluorescein | – |
| | FITC | – |
| | Cy2 | – |
| Yellow-absorbing | Cy3B | + |
| | Alexa Fluor 568 | + |
| | TAMRA | – |
| | Cy3 | – |
| | Cy3.5 | + |
| | Atto 565 | + |
| Red-absorbing | Alexa Fluor 647 | ++ |
| | Cy5 | ++ |
| | Atto 647 | + |
| | Atto 647N | + |
| | Dyomics 654 | ++ |
| | Atto 655 | + |
| | Atto 680 | + |
| | Cy5.5 | ++ |
| NIR-absorbing | Dylight 750 | ++ |
| | Cy7 | ++ |
| | Alexa Fluor 750 | ++ |
| | Atto 740 | + |
| | Alexa Fluor 790 | ++ |
| | IRDye 800CW | ++ |

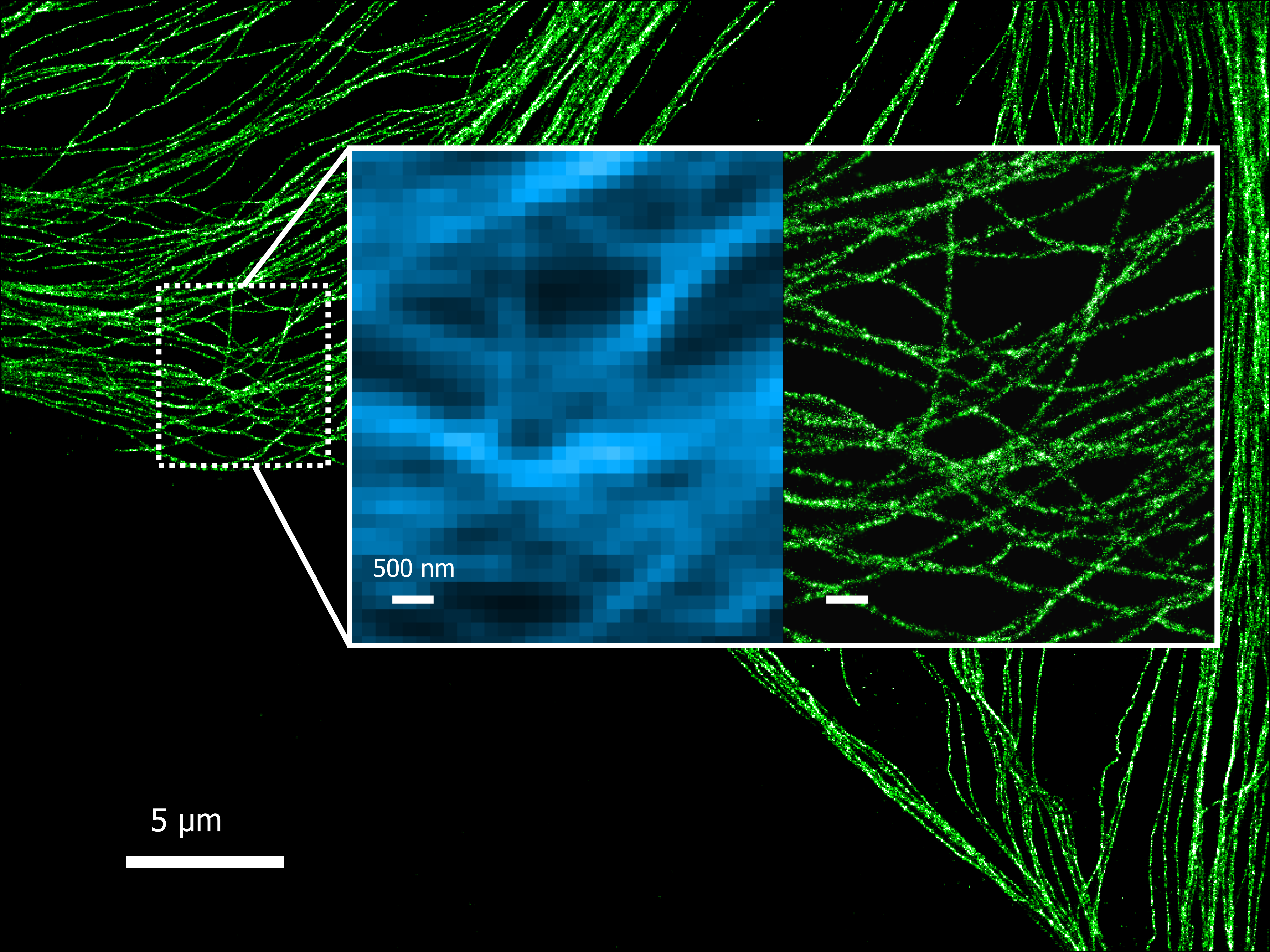



Dempsey G.T. et al Nat. Methods 2011

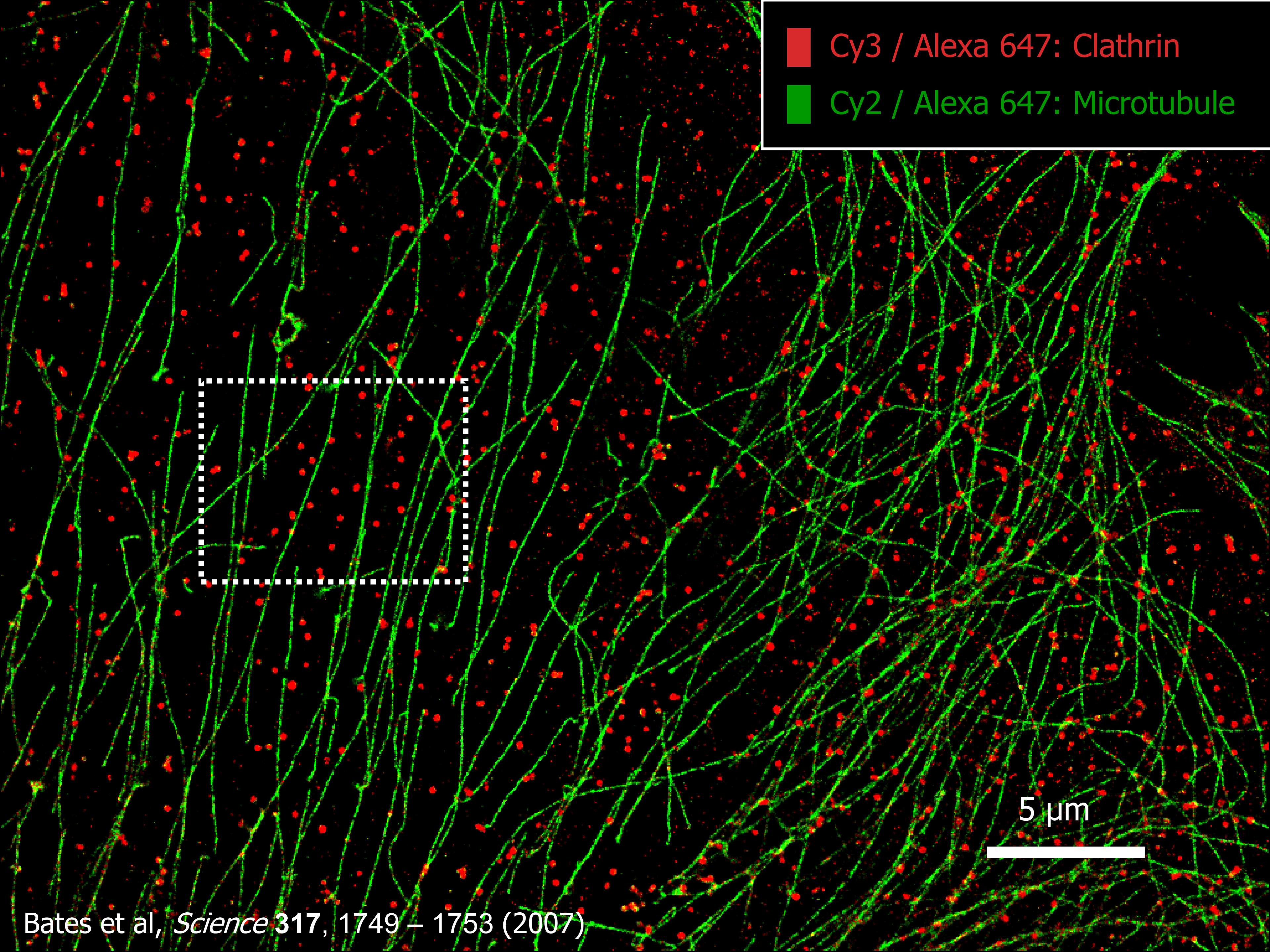

5 µm
B-SC-1 cell,
Microtubules stained with anti-β tubulin
Cy3 / Alexa 647 secondary antibody



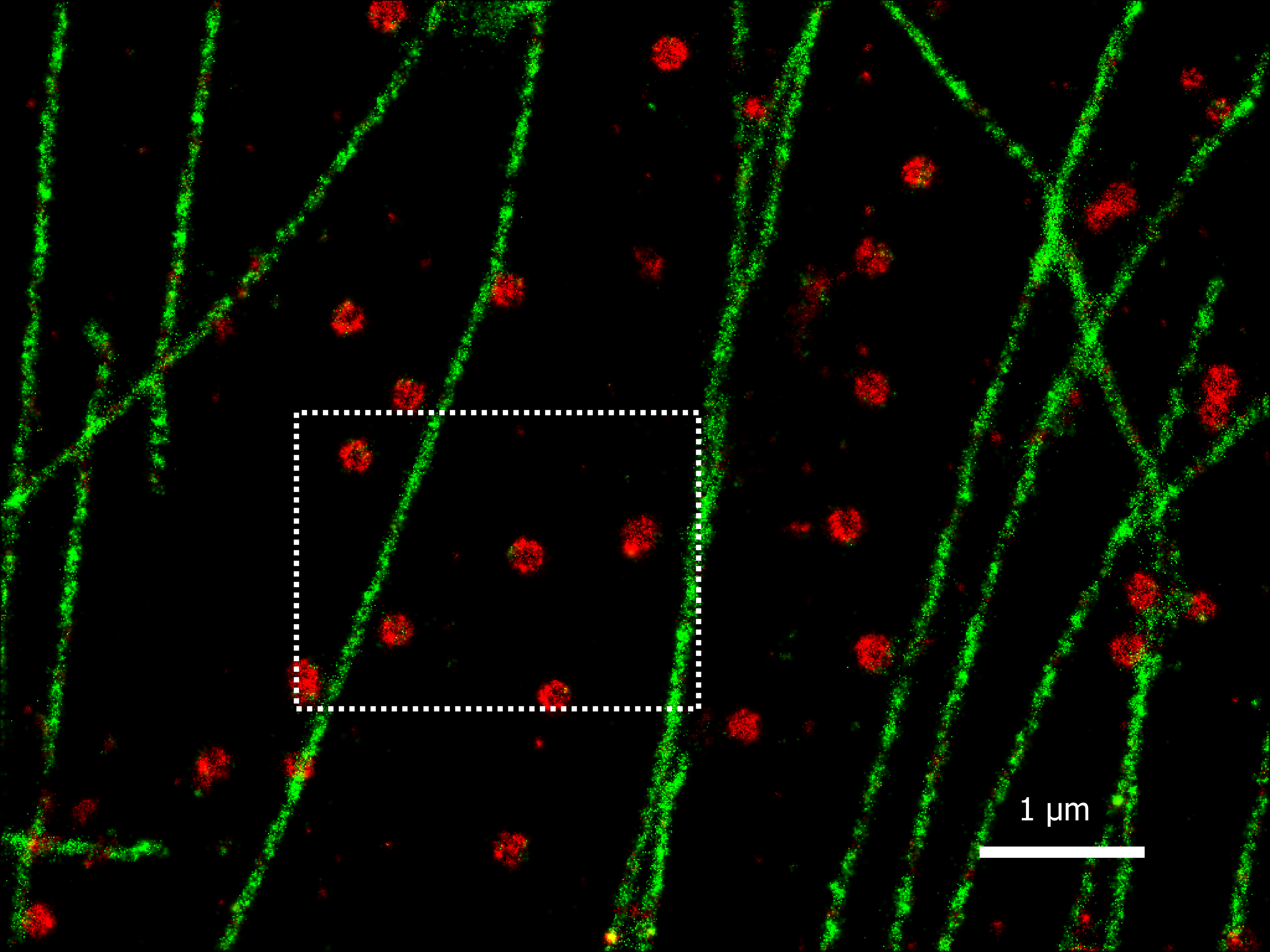

Bates et al, *Science* **317**, 1749 – 1753 (2007)

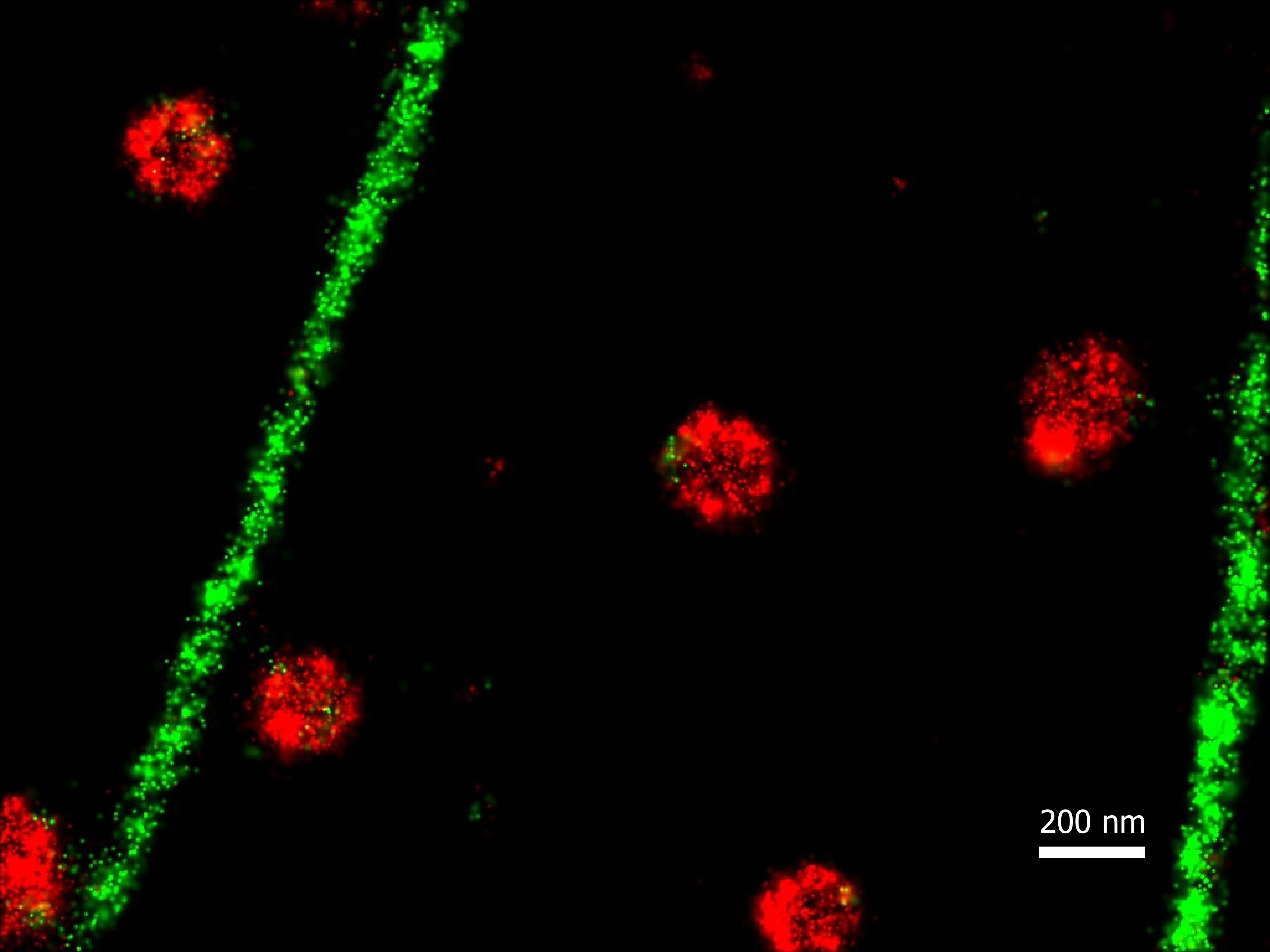


500 nm
5 µm

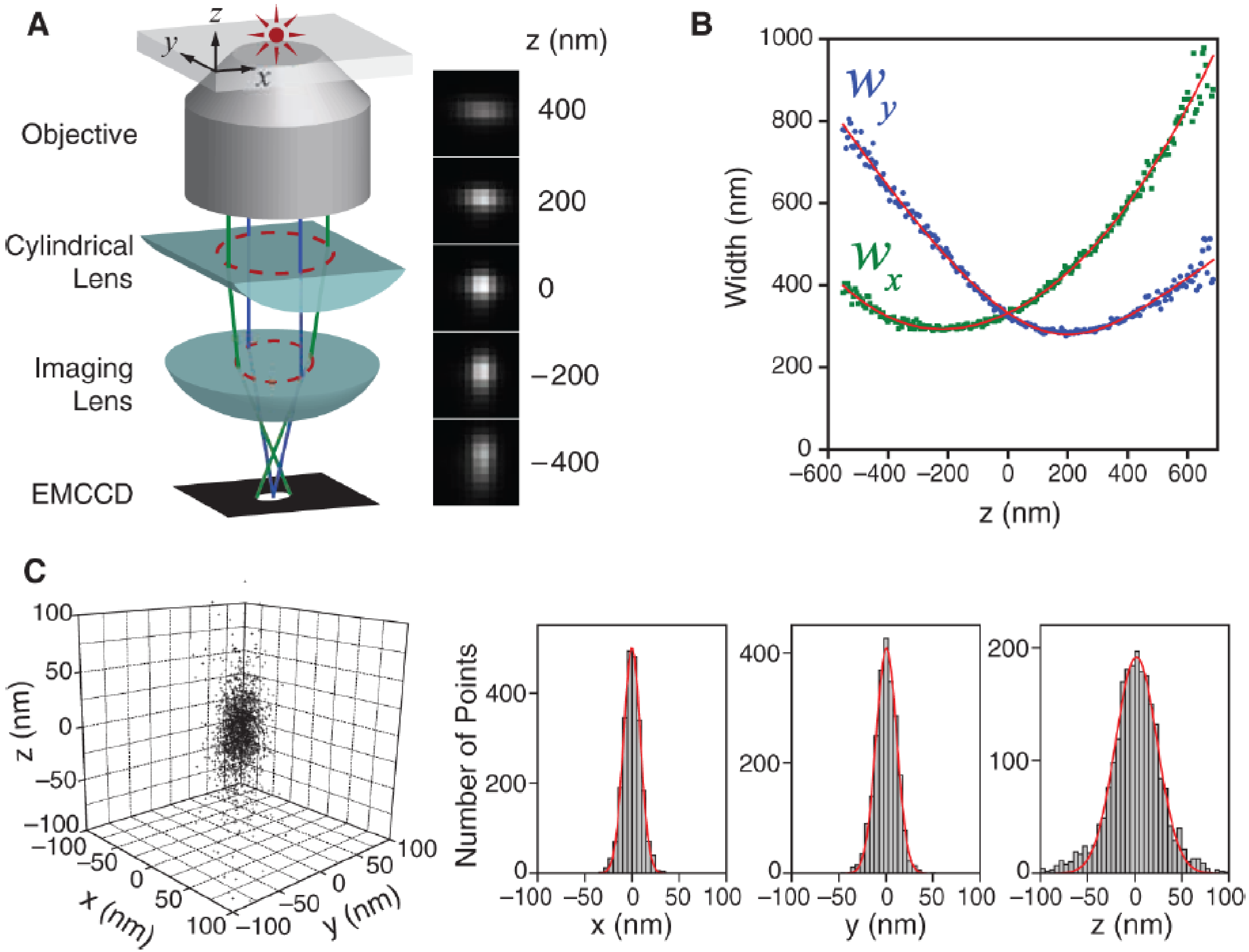



Bates et al, *Science* **317**, 1749 – 1753 (2007)


1 µm


200 nm

# *3D via astigmatic detection*



nature methods

Brief Communication | Published: 08 January 2012

# Dual-objective STORM reveals three-dimensional filament organization in the actin cytoskeleton

Ke Xu, Hazen P Babcock & Xiaowei Zhuang

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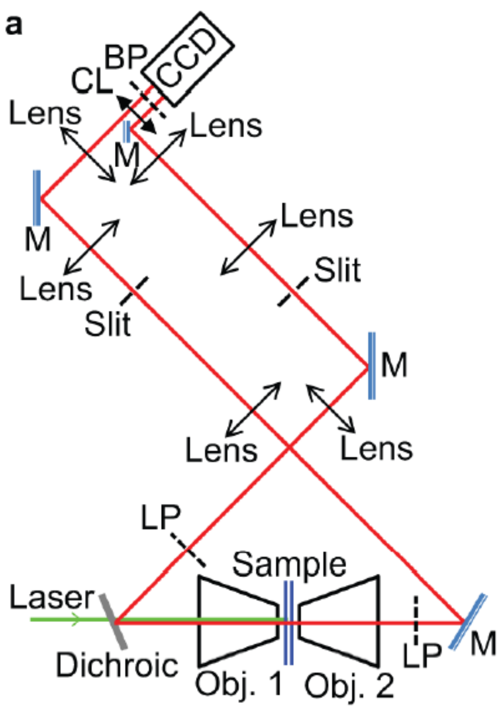
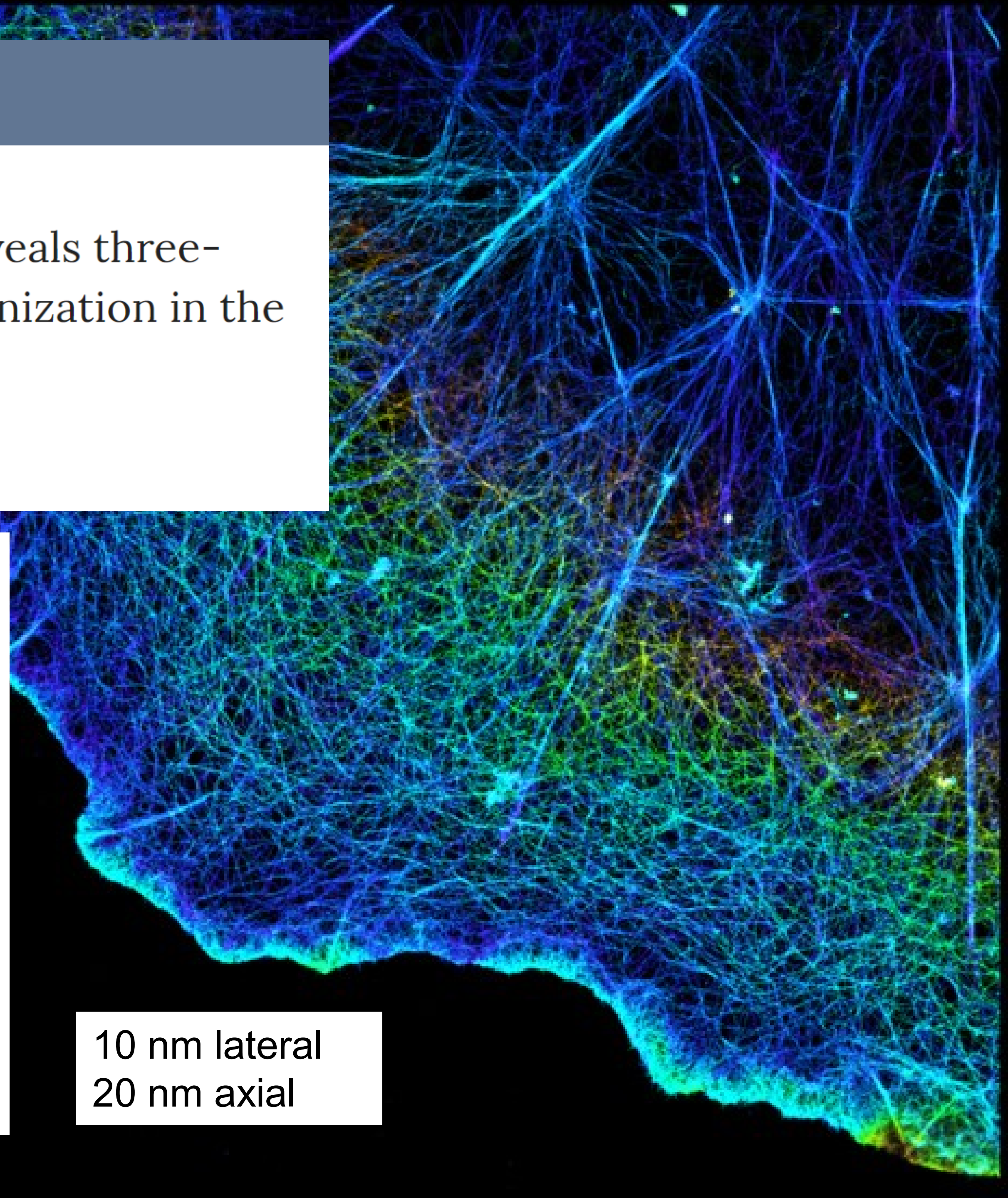
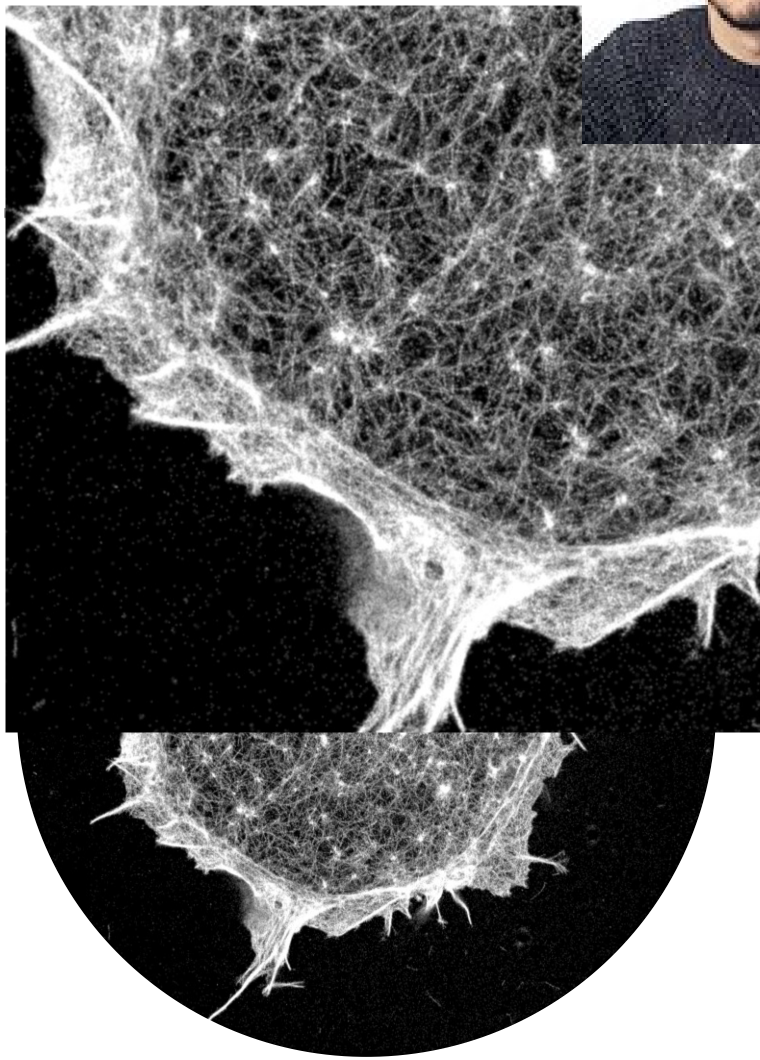
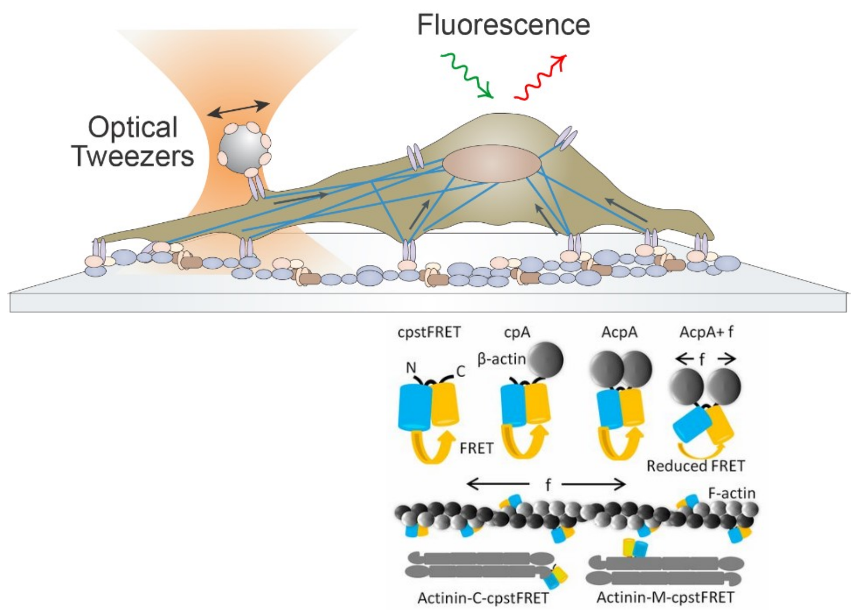


10 nm lateral
20 nm axial




Fluorescence
Optical Tweezers
cpstFRET
cpA
AcpA
AcpA+ f
N
C
β-actin
f
FRET
Reduced FRET
f
F-actin
Actinin-C-cpstFRET
Actinin-M-cpstFRET