Lecture 15

Single Molecule Localization Microscopy

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localization microscopy – one technique, many acronyms

sptPALM

GSDIM

dSTORM

d^{4}STORM

PALM

STORM

P-FPALM

rapidSTORM

FPALM

SPDM

BALM

PALMIRA

RPM

SOFI

DAOSTORM

SALM

CHIRON

LOBSTER

FIONA

PRILM

3B

uPAINT

single molecule localization microscopy

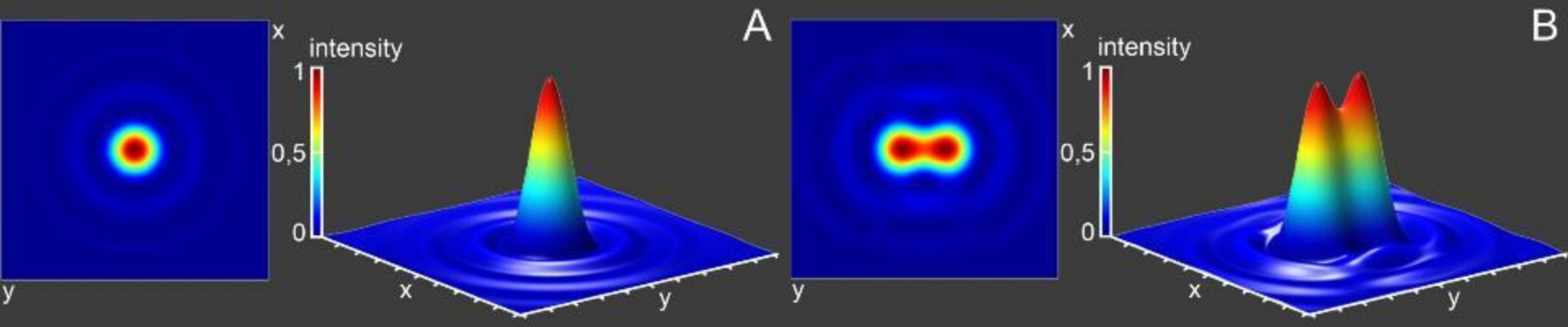
Outline:

- introduction and general idea of single molecule localization microscopy
- first approaches: “original” (F)PALM and STORM
- dSTORM, SPDM, GSDIM – using standard fluorophores
- 3D
- live-cell (4D)
- quantitativ analysis using the additional single molecule information
- alternative approaches
- conclusion

introduction to localization microscopy

problem in light microscopy: resolution limited by diffraction

→ $I_{im}(x_2, y_2) = PSF \otimes P_{fl}(x_1, y_1)$



Rayleigh criterion: $D = 0.61\frac{\lambda}{NA}$

introduction to localization microscopy

general idea:

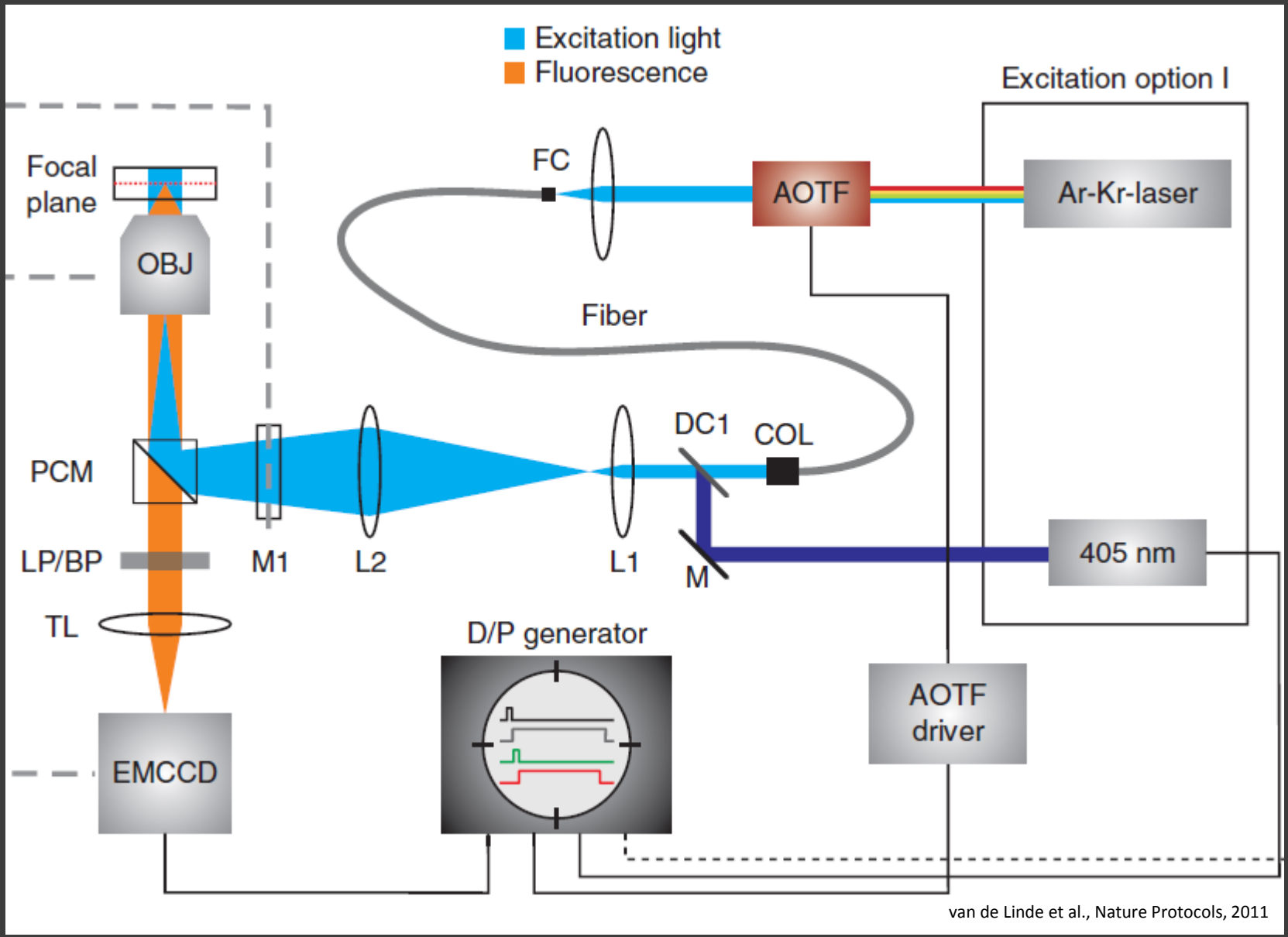
look at signals of single molecules individually instead of all fluorophores at the same time

→ this allows a very precise determination of the molecule position

→ reconstruct super-resolution image from position data of the detected molecules

introduction to localization microscopy

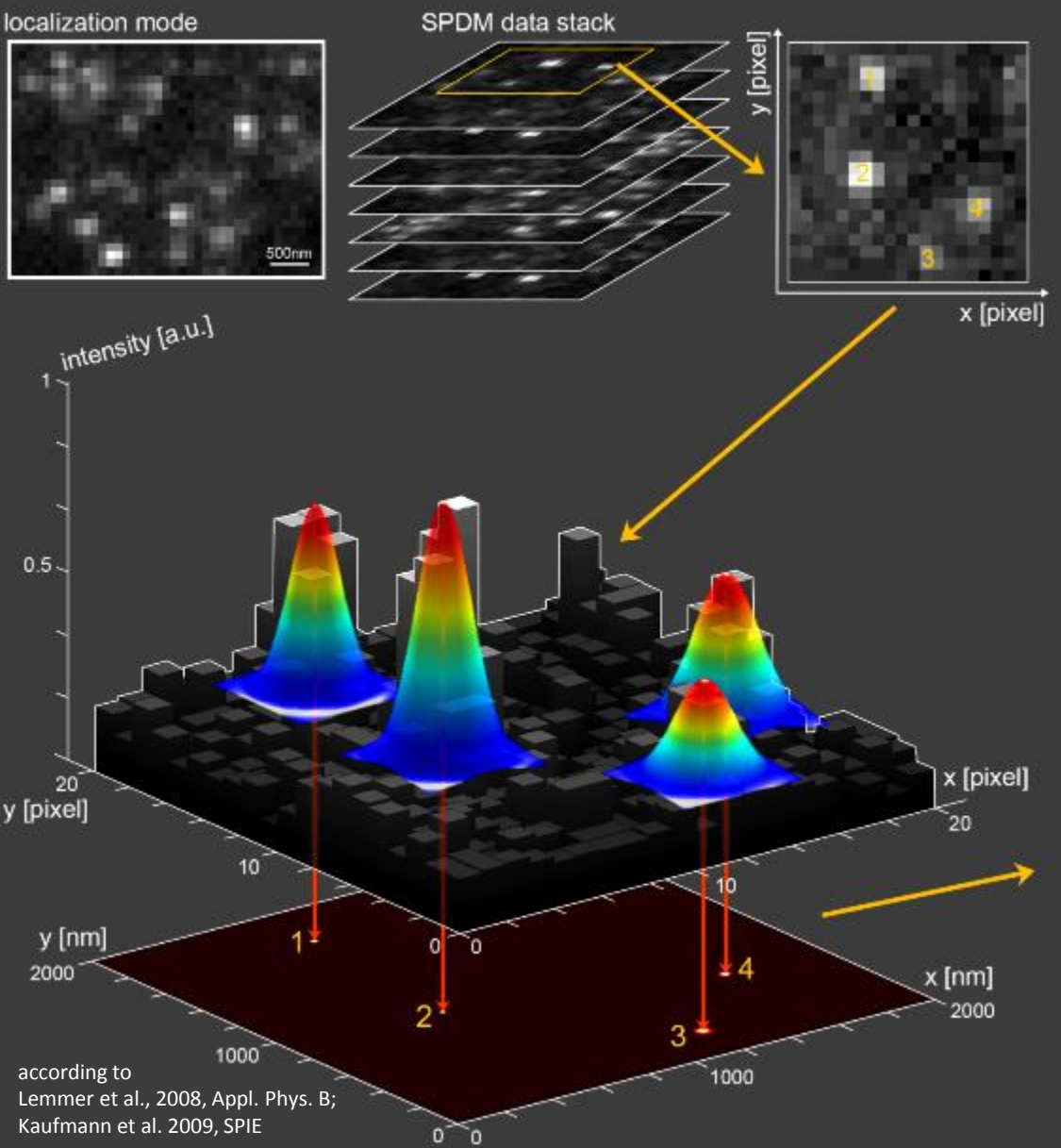
setup

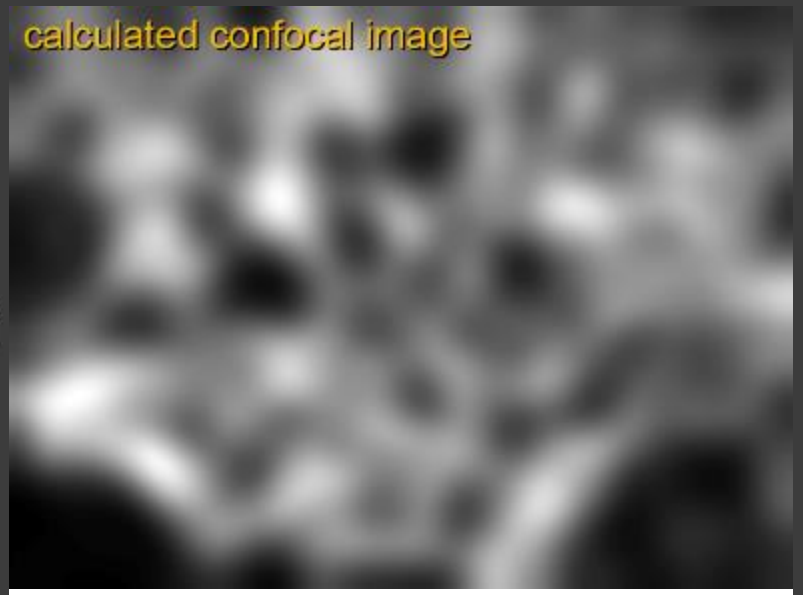


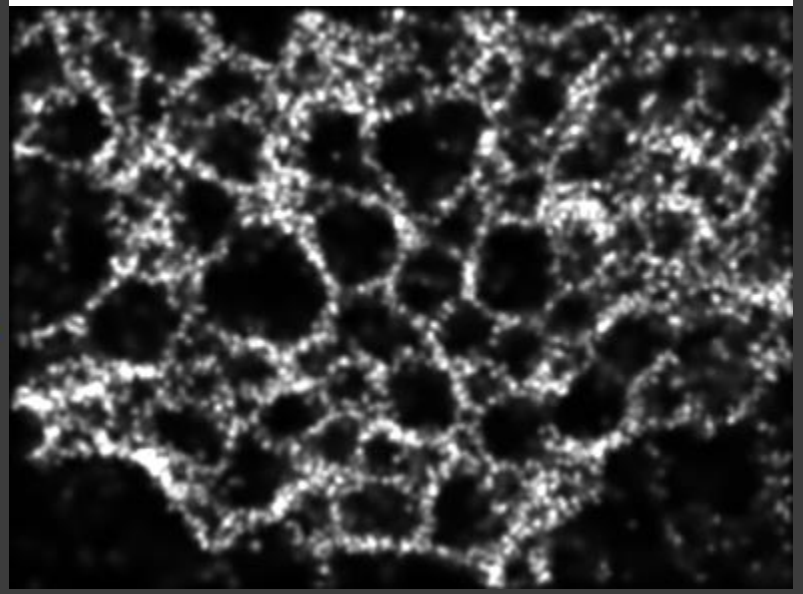
van de Linde et al., Nature Protocols, 2011

principle of localization microscopy

image reconstruction







according to
Lemmer et al., 2008, Appl. Phys. B;
Kaufmann et al. 2009, SPIE

principle of localization microscopy

position determination

localisation accuracy σ of a single molecule is depended on

- width of the PSF *s*
- number of detected photons *N*
- background intensity *b*
- size of the pixels on the camera *a*

$$\sigma^2 = \frac{s^2 + a^2/12}{N} + \frac{8\pi s^4 b^2}{a^2 N^2}$$

typical model function: 2D Gaussian + linear background

$$I(x,y) = I_0 \exp\left[-\frac{(x - x_0)^2 + (y - y_0)^2}{2s^2}\right] + b$$

principle of localization microscopy

resolution

structural resolution in localization microscopy is dependent on:

- the localization accuracy of the individual molecules
- density of detected molecules (sampling theorem – Nyquist resolution)

⇨ $$structural\ resolution = \sqrt{(2.35\ \bar{\sigma}_{xy})^2 + (2\ \bar{d}_{NN})^2}$$

$$= \sqrt{(2.35\ \bar{\sigma}_{xy})^2 + 4/\rho}$$

$\bar{\sigma}_{xy}$: mean localization accuracy

$\bar{d}_{NN}$: mean distance to next neighboring molecule(s)

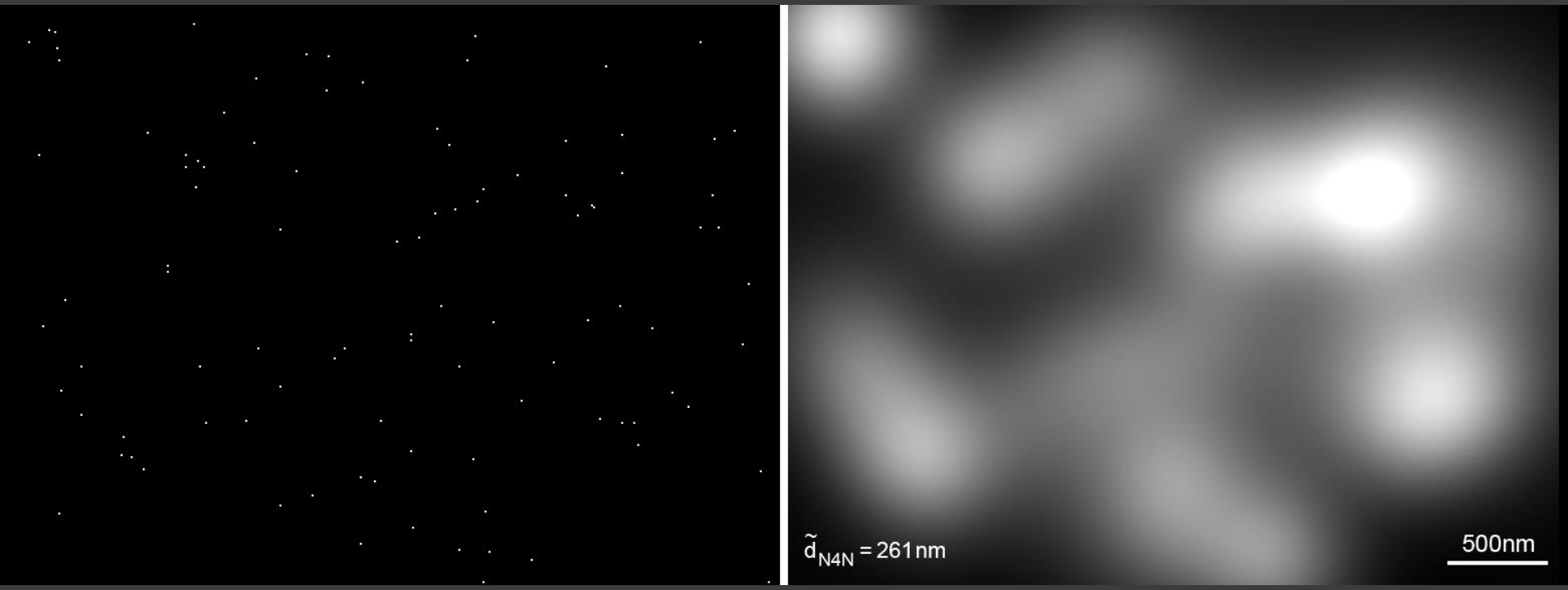
ρ: local density of detected molecules

principle of localization microscopy

resolution

structural resolution in localization microscopy is dependent on:

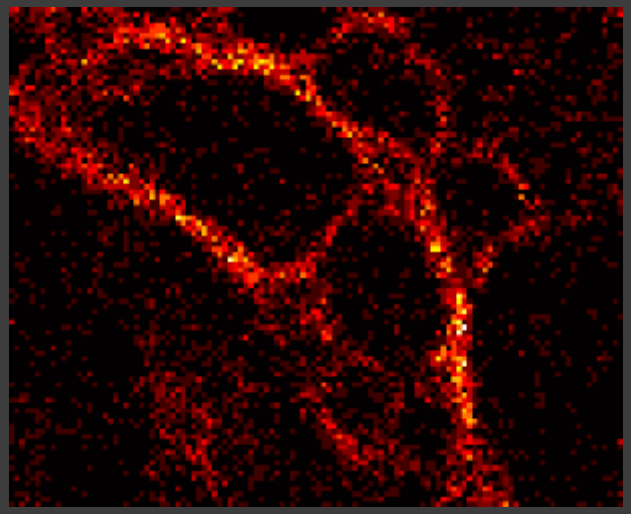
- the localization accuracy of the individual molecules
- density of detected molecules (sampling theorem – Nyquist resolution)



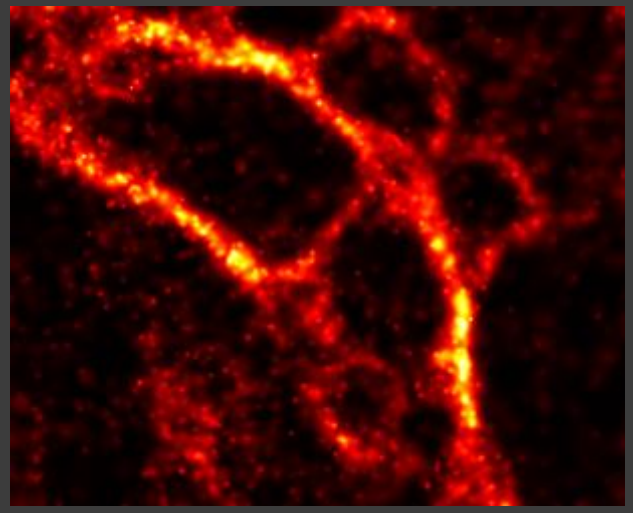
principle of localization microscopy

image reconstruction

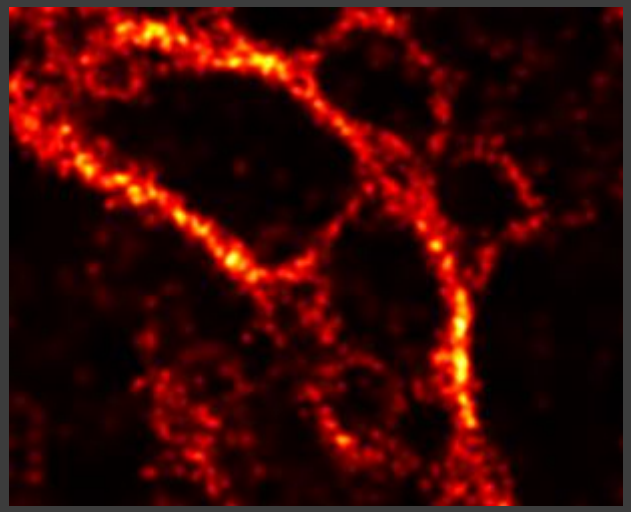
scatter plot



histogram with equal bins



visualisation of σ_{xy}



visualisation of structural resolution

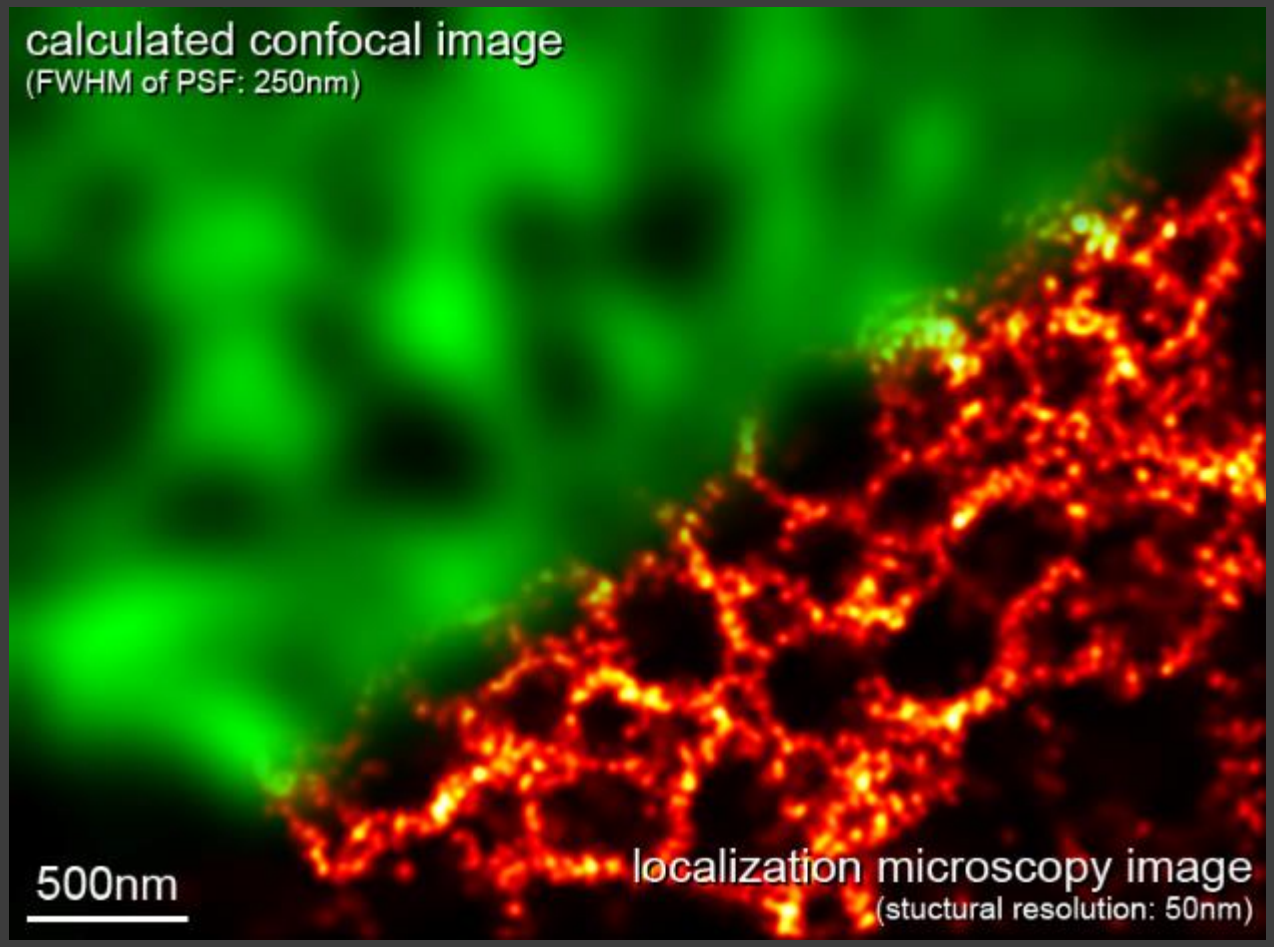
more about visualisation of localization microscopy data:

Baddeley et al., Microscopy and Microanalysis, 2010

principle of localization microscopy

summary

enhanced structural resolution down the 20 nm range



+

fluorophores are detected individually

single molecule information

- positions
- number of det. photons
- …

(F)PALM and STORM

(some) history of localization microscopy

localisation of single molecules / point-like objects

Burns et al., 1985	theoretical paper about super-resolution distance measurements using spectral characteristics
Betzig, 1995	first measurements with SNOM under cryo conditions
Bornfleth et al., 1998	CLSM measurements of 3D distances < 60 nm using fluorescent markers of different wavelengths (@ RT)
Heilemann et al., 2002	using single molecule live time instead of colours to measure distances of 40 nm

localisation of many molecules to reconstruct structural information

2006: (PALM, FPALM, STORM) – photo-switchable / photo-activatable dyes

2008: (dSTORM, SPDM, GSDIM) – using standard fluorophores

(F)PALM – (fluorescence) photo activated localization microscopy

uses photo-activatable fluorophores (e.g. PA-GFP, caged Fluorescein, …)

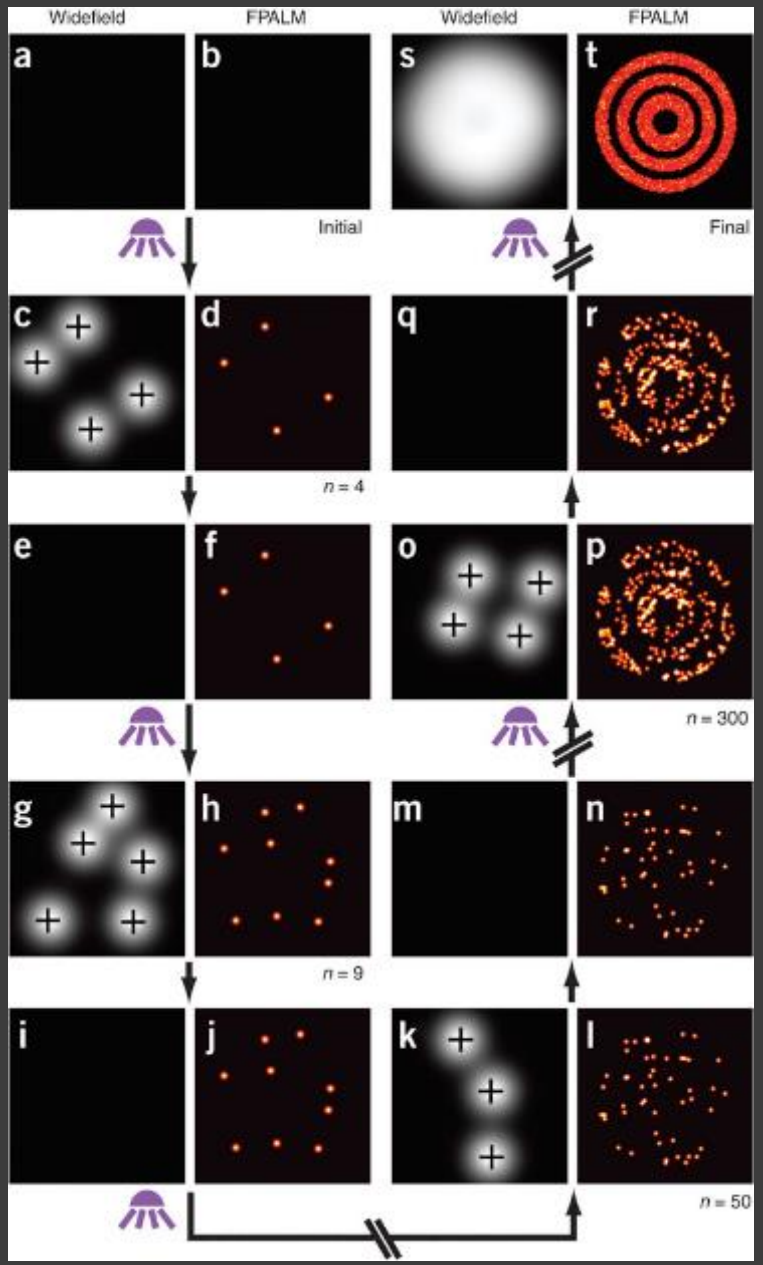
- at the beginning all fluorophores are “dark” (not fluorescent at their excitation wavelength)
- fluorophores can be “activated” to a “bright” state
- after bleaching the molecules they do not reappear

→ irreversible process

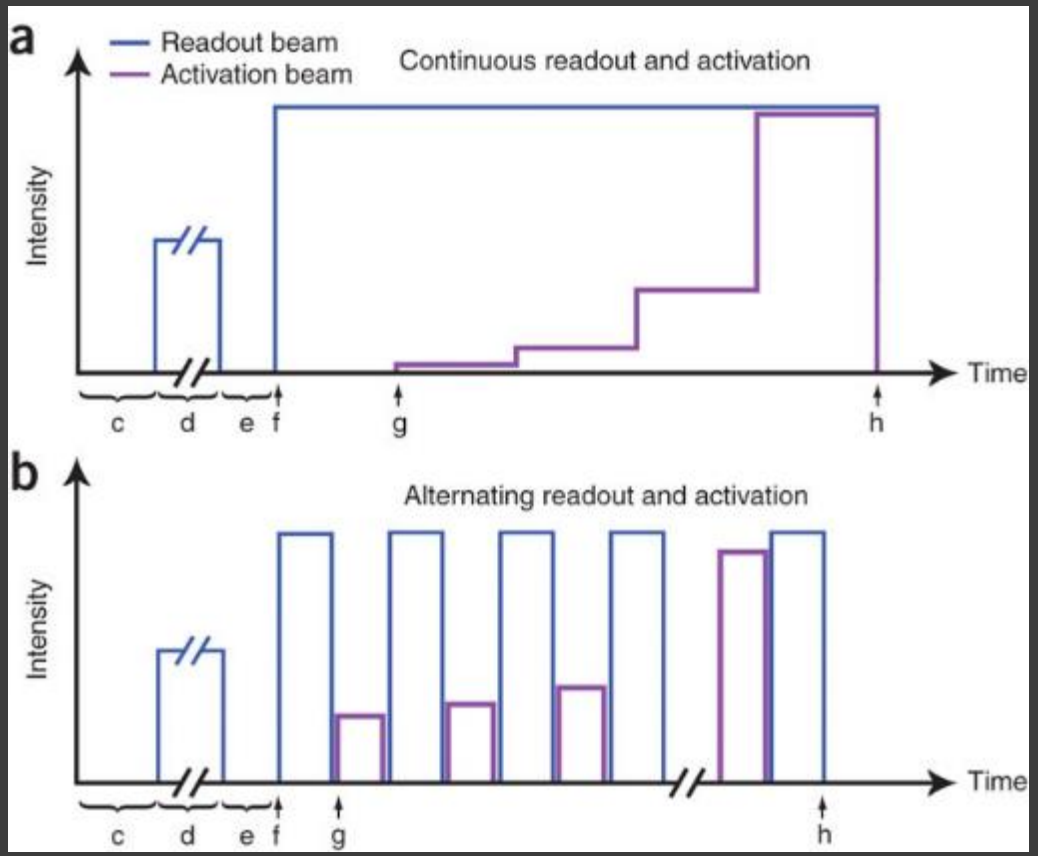
original publications:

- PALM: Betzig et al., Science, 2006
- FPALM: Hess et al., Biophysical Journal, 2006

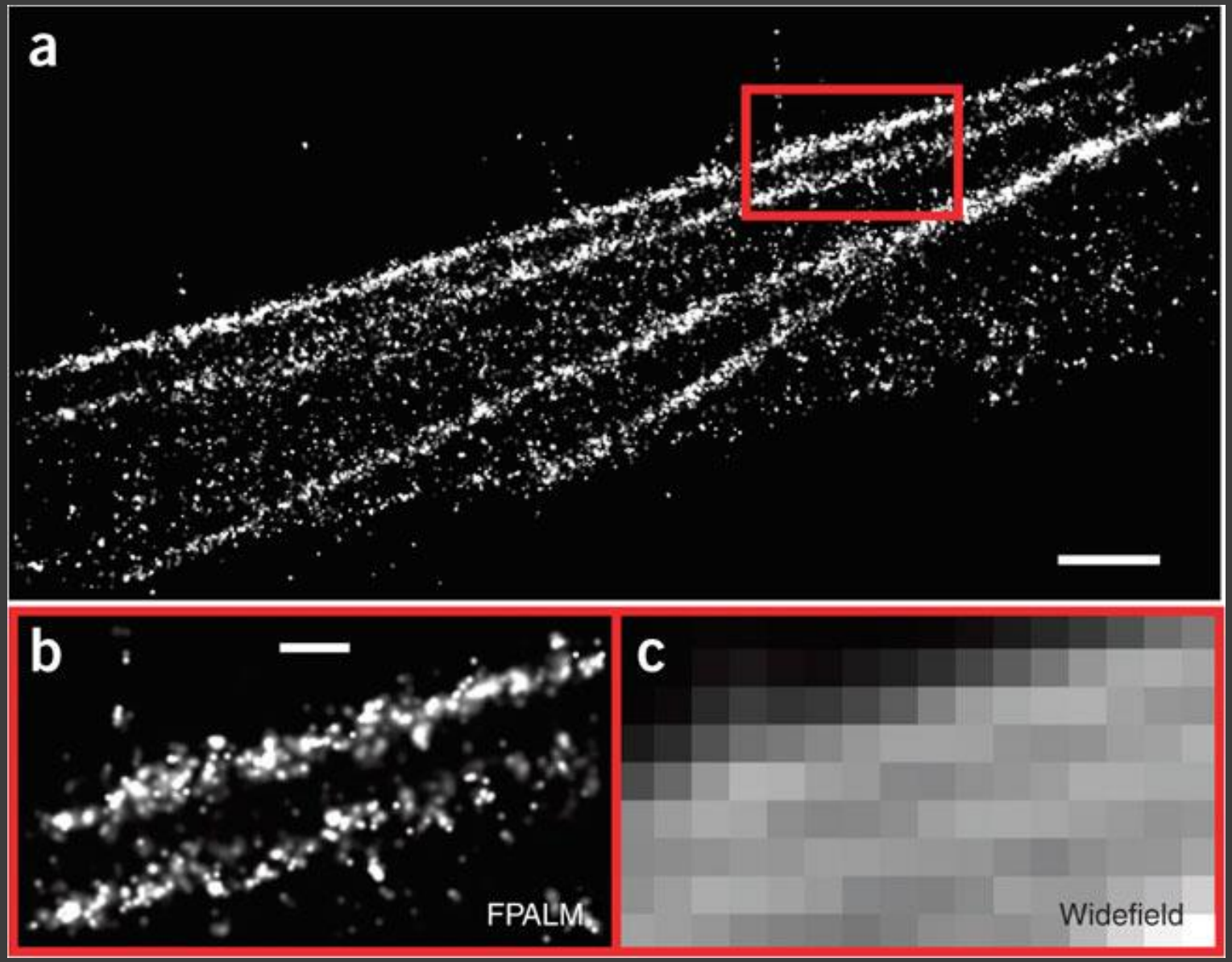
(F)PALM – (fluorescence) photo activated localization microscopy



Gould et al., Nature Protcols ,2009



(F)PALM – (fluorescence) photo activated localization microscopy



Dendra2-actin

Gould et al., Nature Protcols, 2009

STORM – stochastic optical reconstruction microscopy

uses photo-switchable fluorophores (dye pairs (e.g. Cy3-Cy5) or proteins like Dronpa)

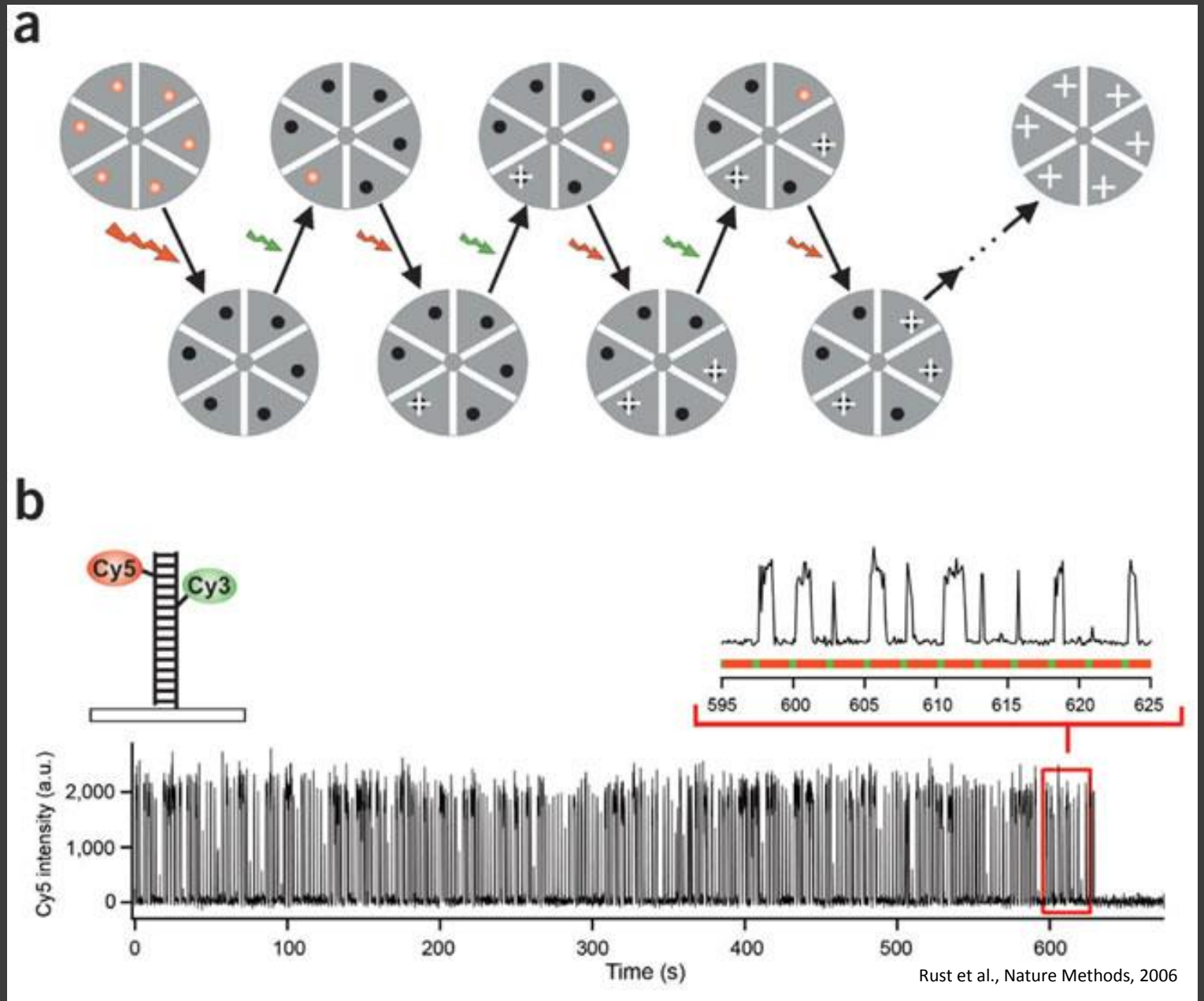
- fluorophores can be switched many times between a “bright” and a “dark” state

→ reversible process

original publication:

- Rust et al., Nature Methods, 2006

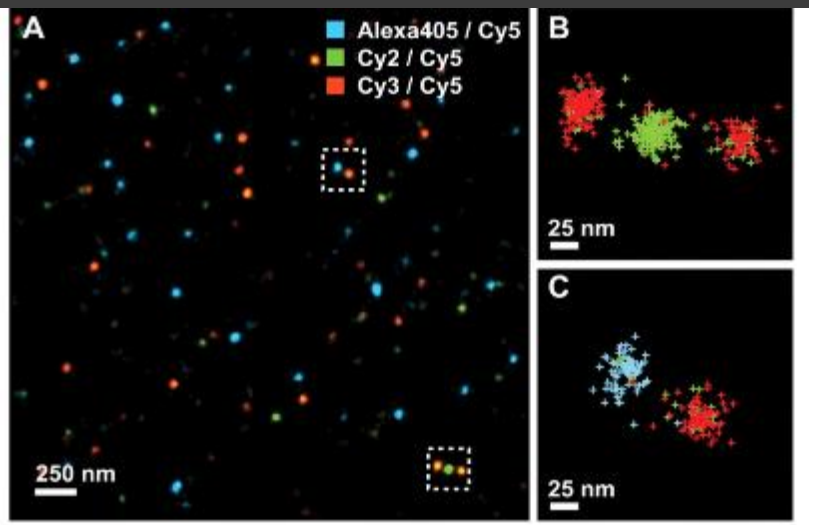
STORM – stochastic optical reconstruction microscopy

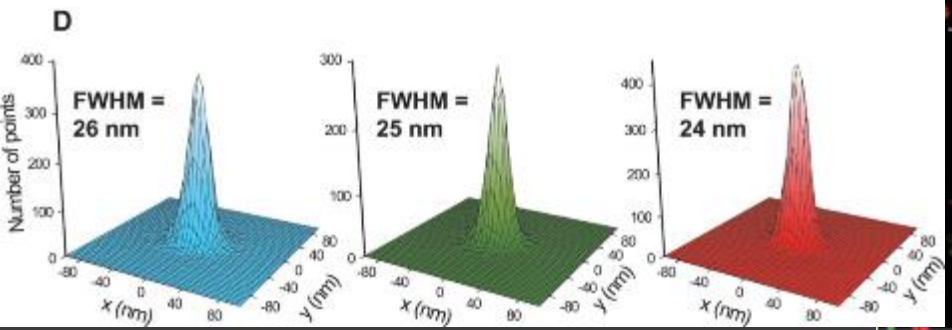


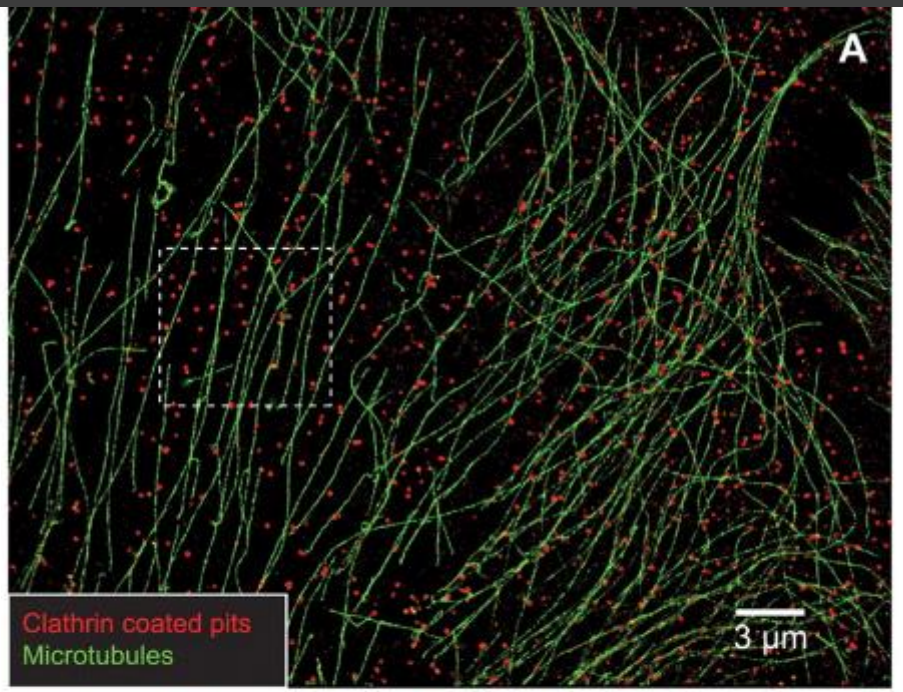
Rust et al., Nature Methods, 2006

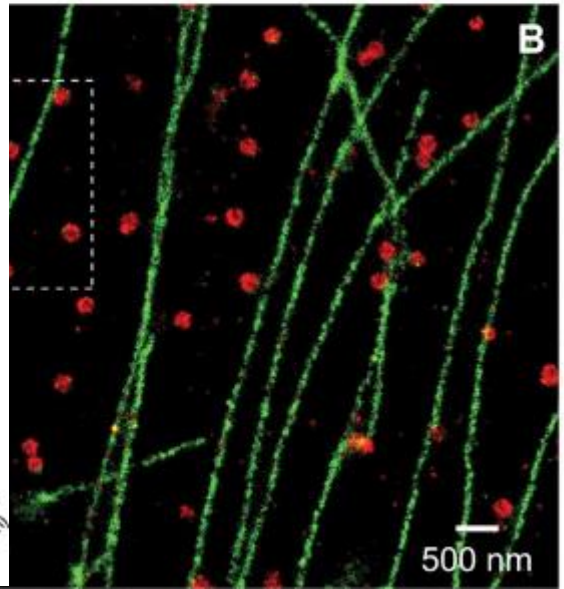
STORM – stochastic optical reconstruction microscopy

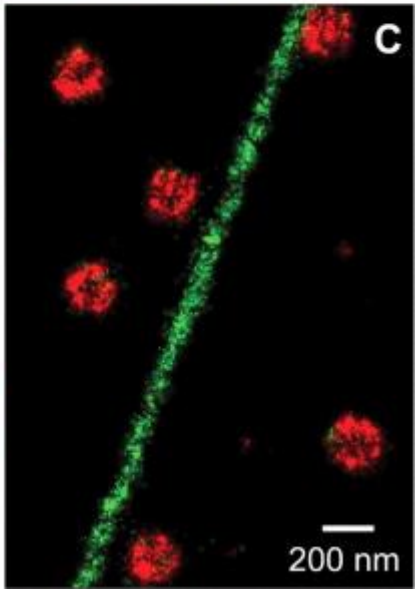
Bates et al., Science, 2007











dSTORM, SPDM, GSDIM, …

dSTORM, SPDM, GSDIM, …

direct STROM

spectral position determination microscopy

ground state depletion microscopy followed by individual molecule return

uses standard fluorophores (e.g. Alexa and Atto dyes, GFP, YFP, RFP, …)

- switching mechanism based on a light induced long-lived “dark” state
- stochastic recovery to “bright” (fluorescent) state is used for optical isolation of the single molecule signals

original publication:

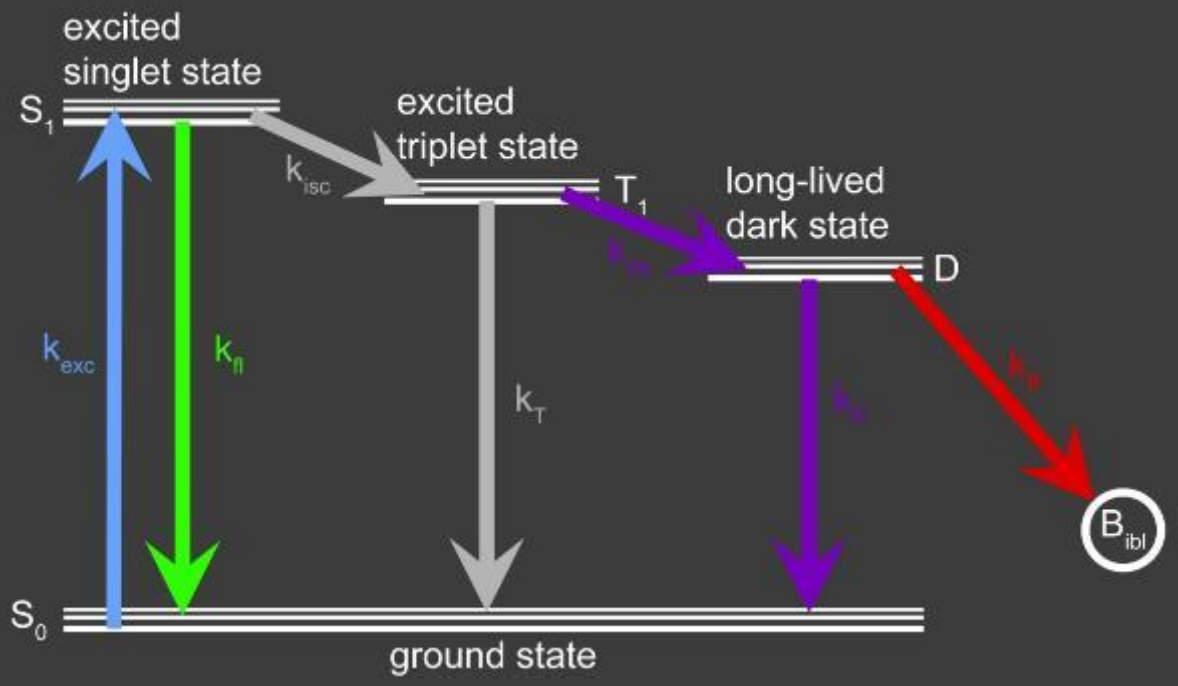
- dSTORM: Heilemann et. al., Angewandte Chemie International Edition, 2008
- SPDM: Lemmer et al., Applied Physics B, 2008
- GSDIM: Fölling et al., Nature Methods, 2008

dSTORM, SPDM, GSDIM, …

light induced long-lived (ms – 100 s) dark state

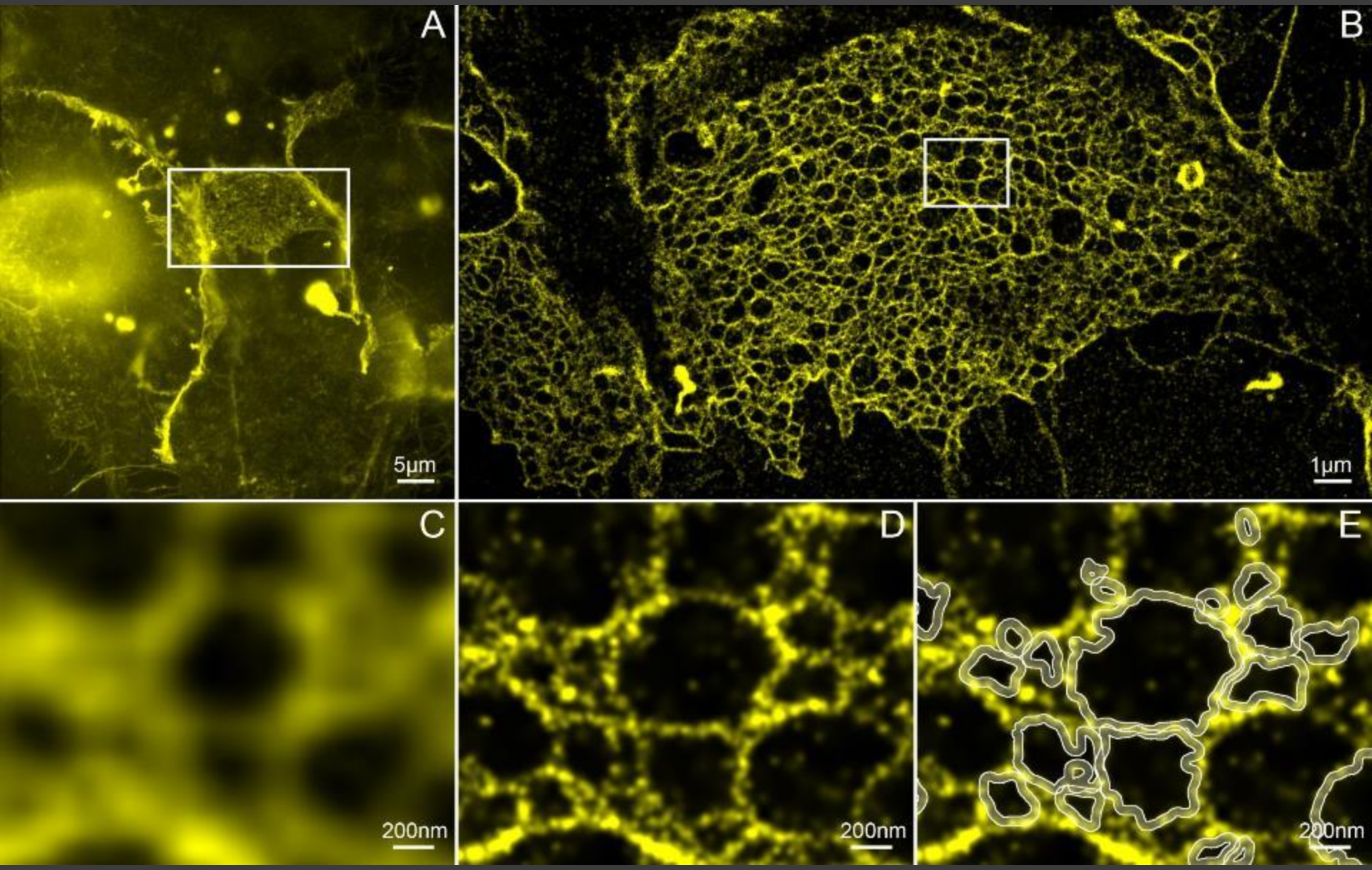
critical parameters for driving fluorophores into the long-lived dark state:

- illumination intensity
- wavelength
- embedding medium



statistical recovery of fluorophores from the light induced long-lived dark state can be used for optical isolation of single molecules

dSTORM, SPDM, GSDIM, …



EYFP-Cld3

Kaufmann et al., PLoS ONE, 2012

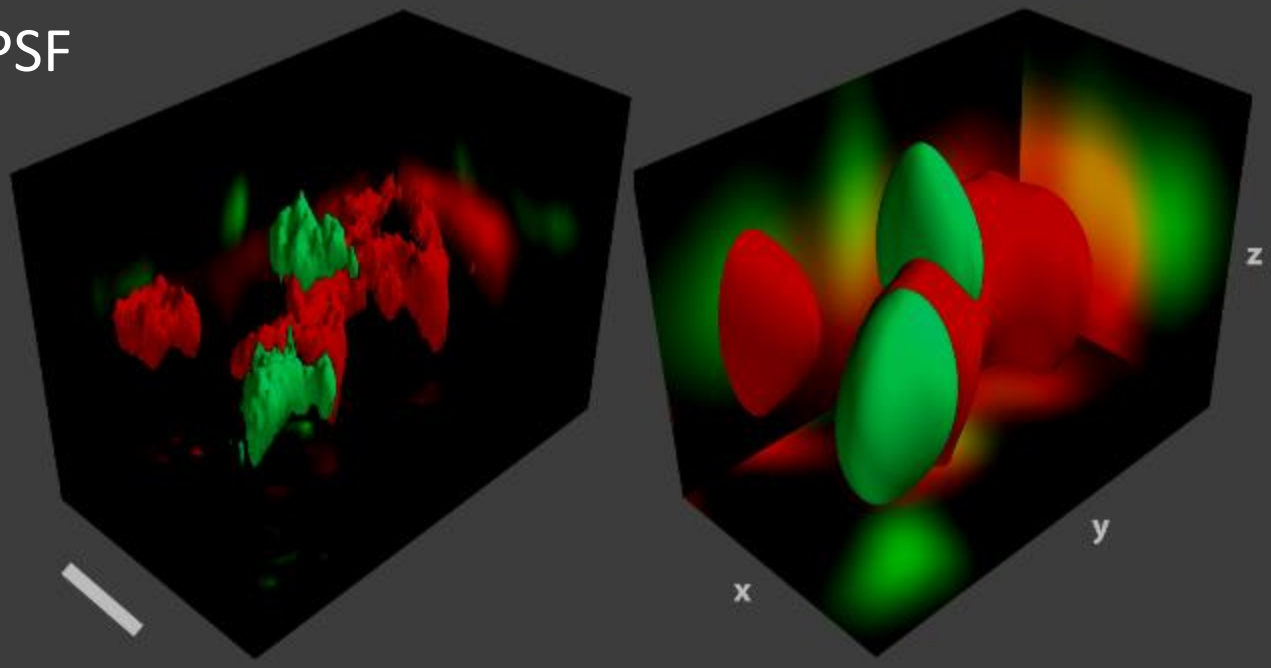
3D

astigmatic (elliptical) PSF

biplane

double helical PSF

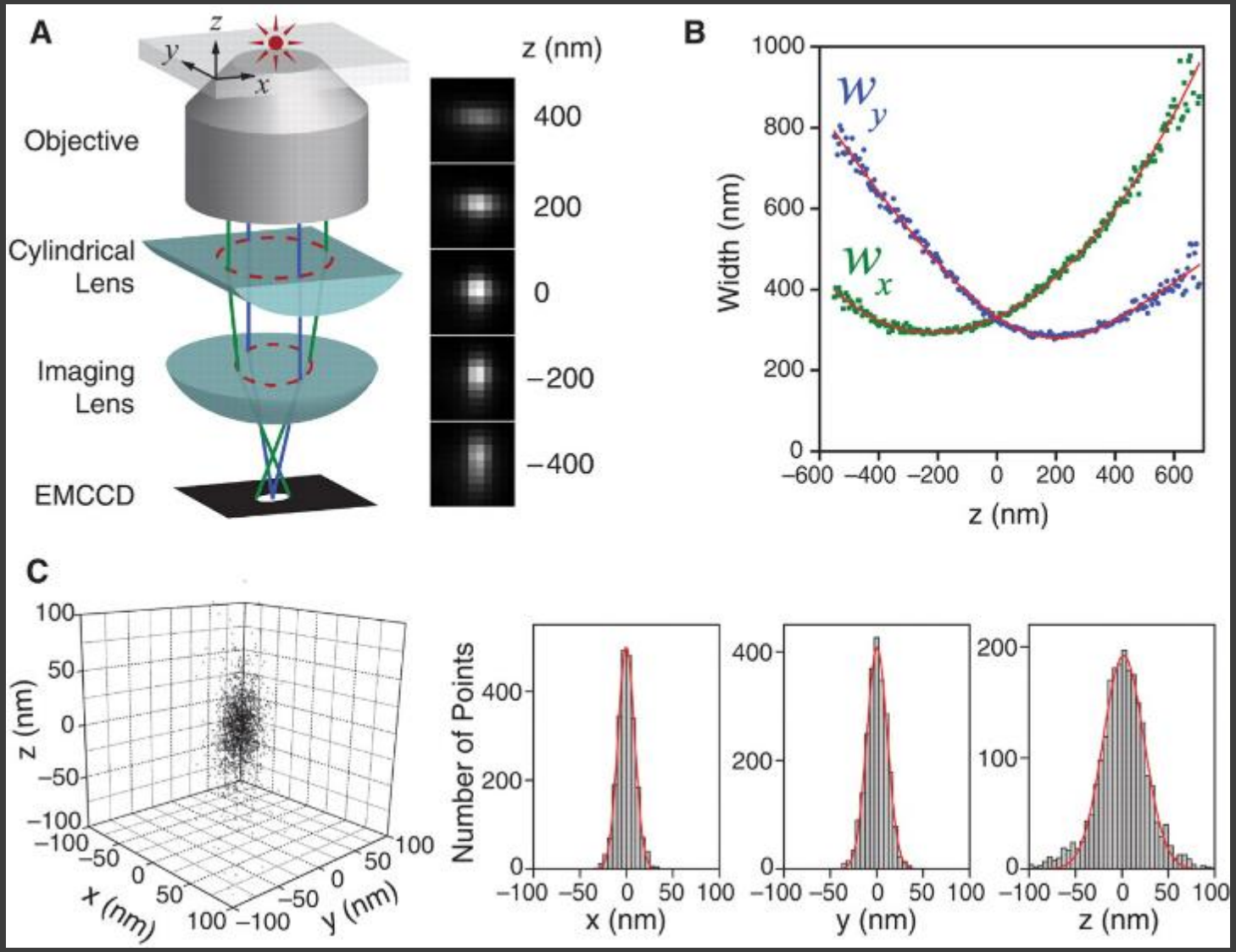
iPALM



Baddeley et al., PLoS ONE, 2011

3D

astigmatic imaging system



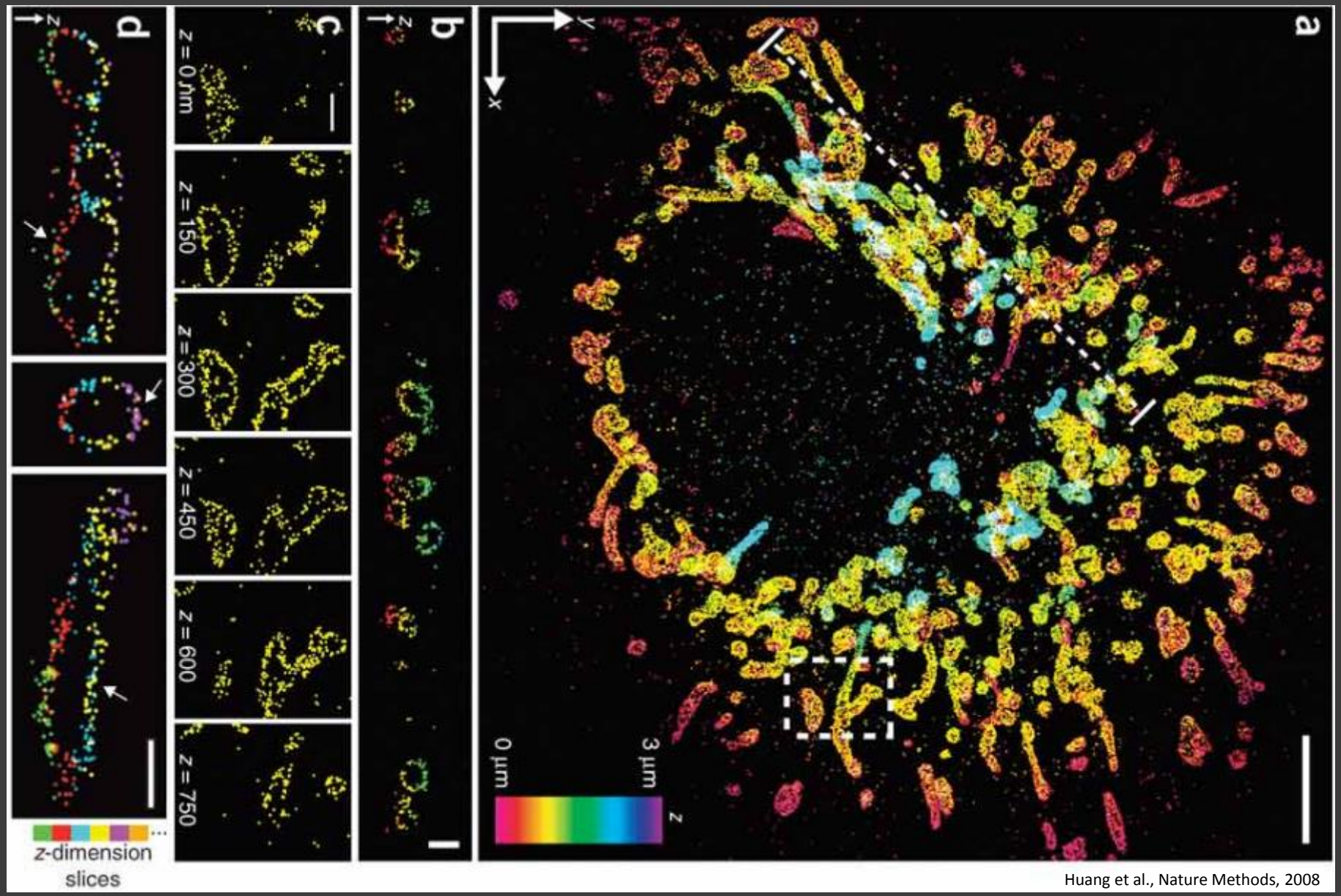
resolution
lateral: 30 nm
axial: 50 nm

Huang et al., Science, 2008

3D

Alexa405-Cy5-mitochondria

astigmatic imaging system



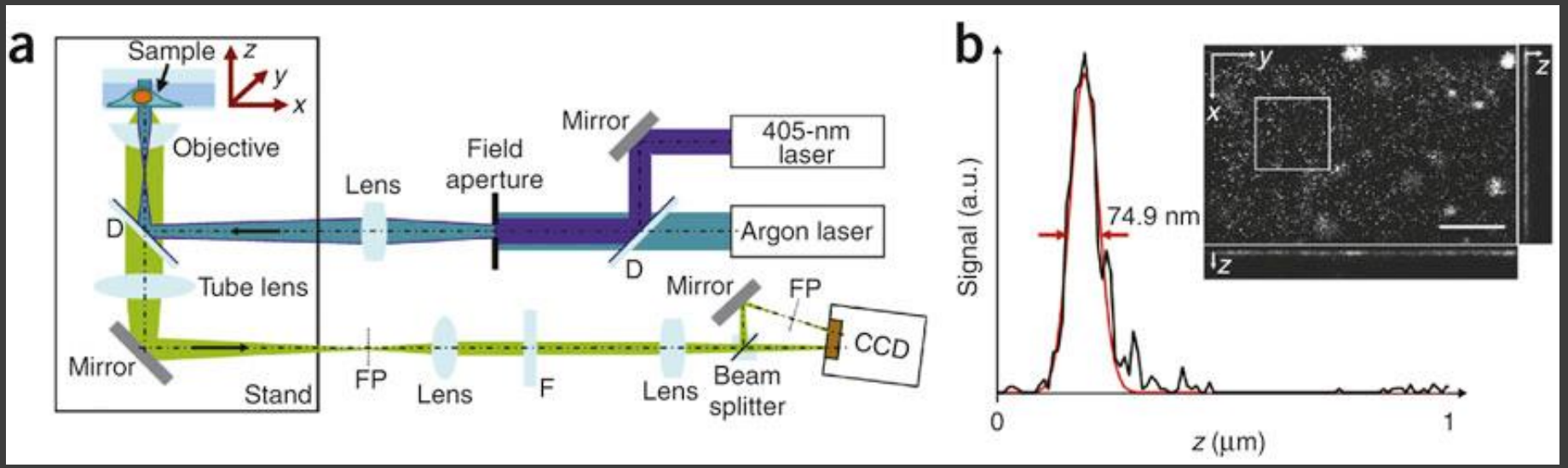
Huang et al., Nature Methods, 2008

3D

biplane imaging

imaging of two different axial plane simultaneously

→ fitting of 3D-PSF yields 3D position of the fluorophore



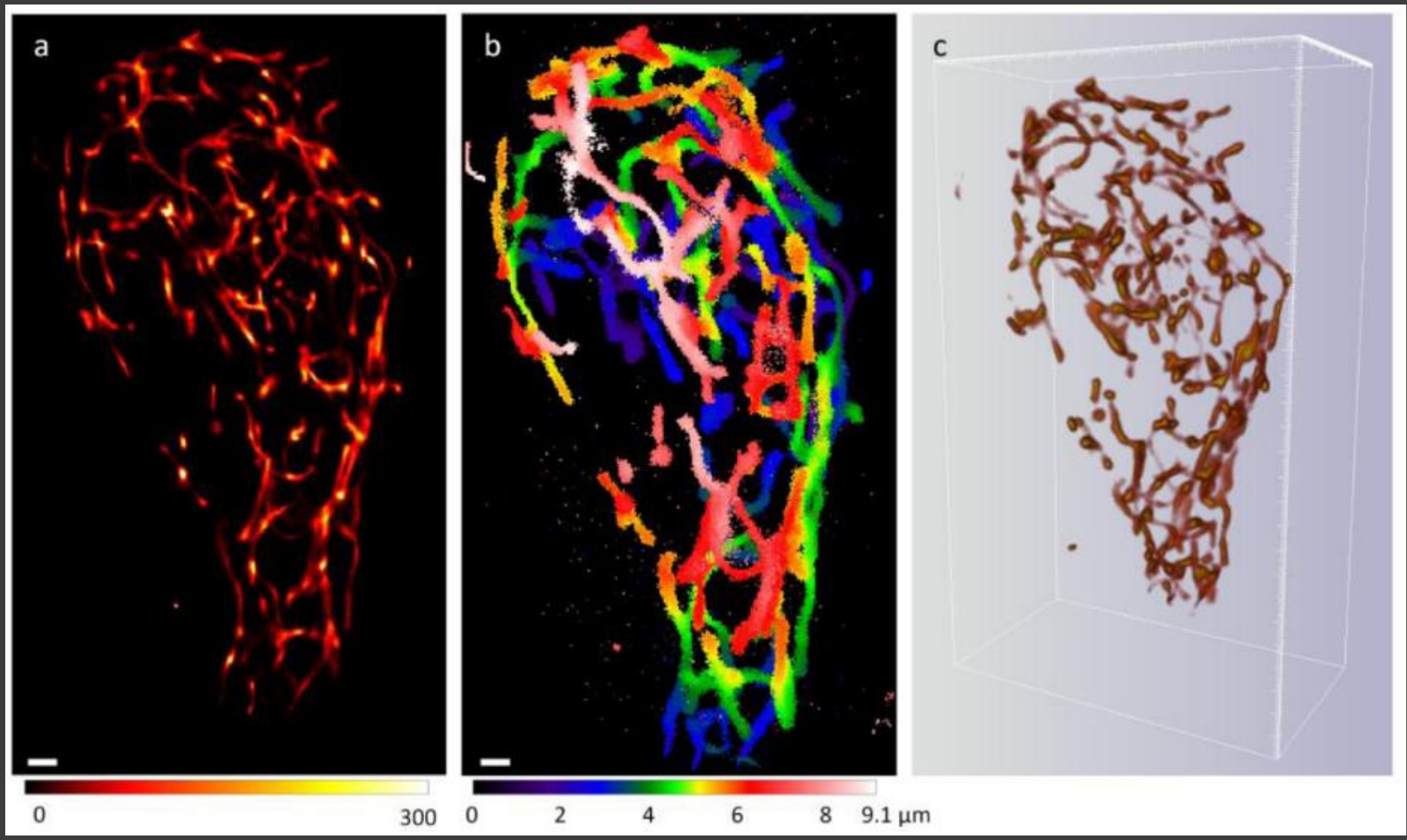
Juette et al., Nature Methods, 2008

resolution
lateral: 30 nm
axial: 60 nm

3D

biplane imaging

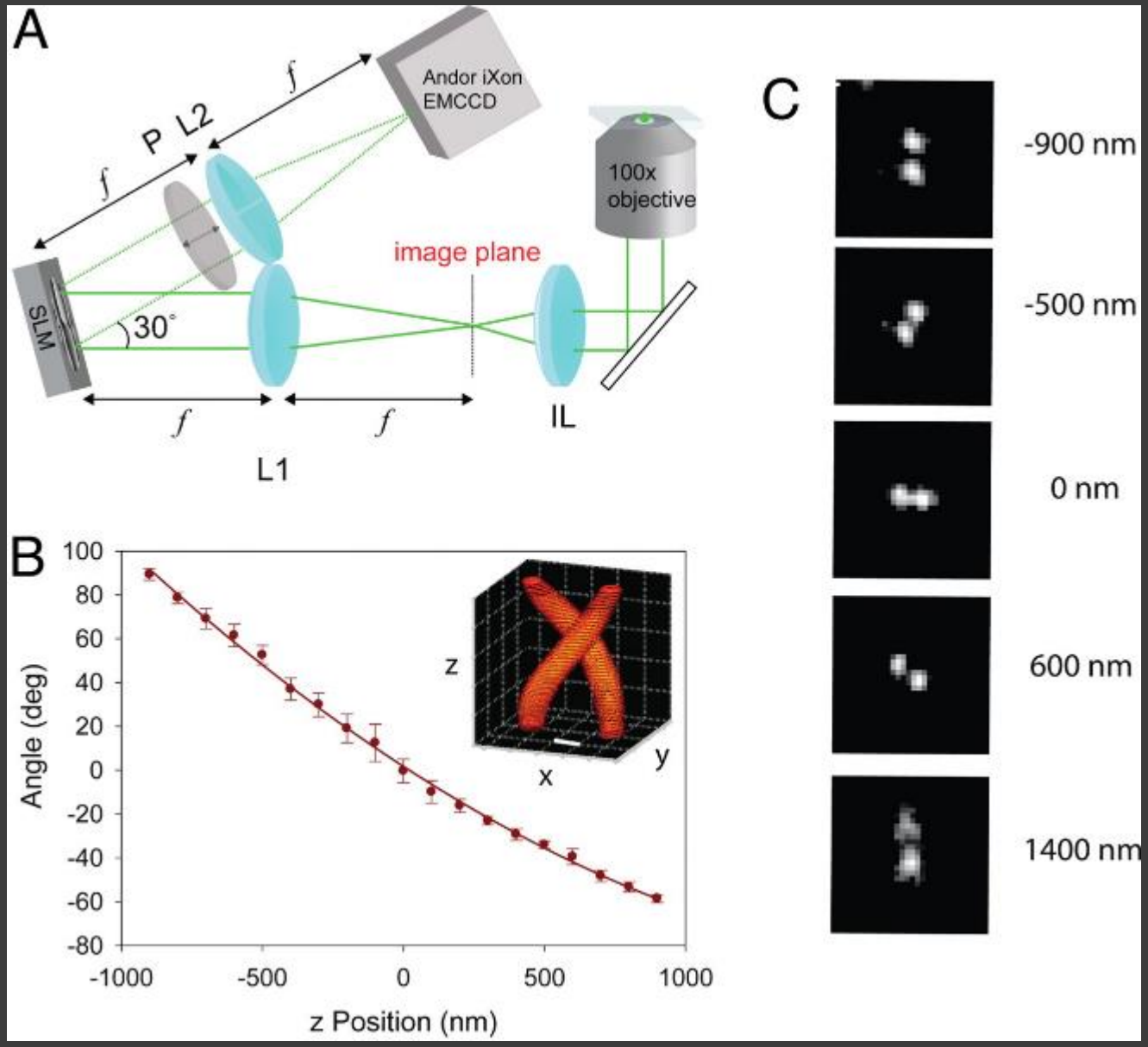
mtEos2-mitochondria



Mlodzianoski et al., Optics Express, 2011

3D

double helical PSF



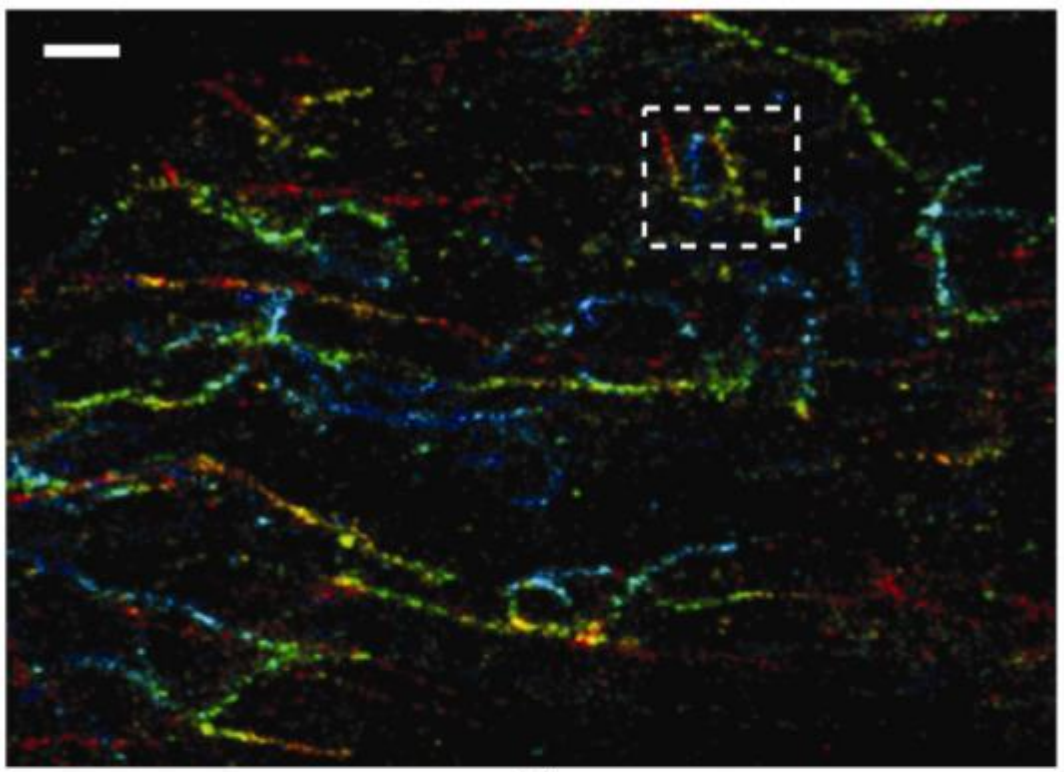
fitting of two 2D Gaussians

→ 3D position of the molecule

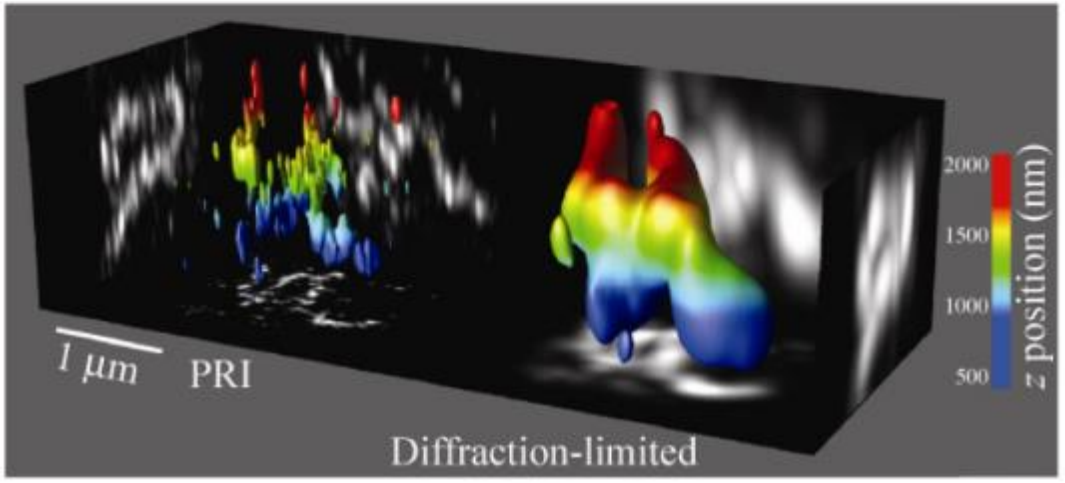
Pavani et al., PNAS, 2009

3D

double helical PSF

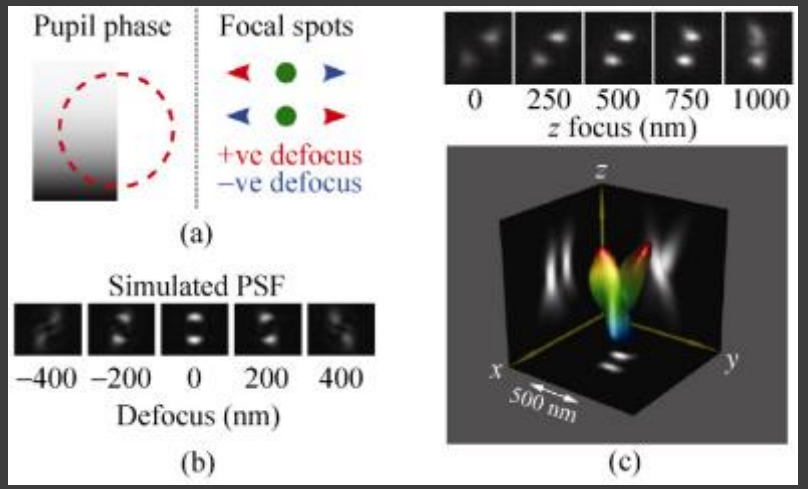


(a)



Alexa680-β-tubulin

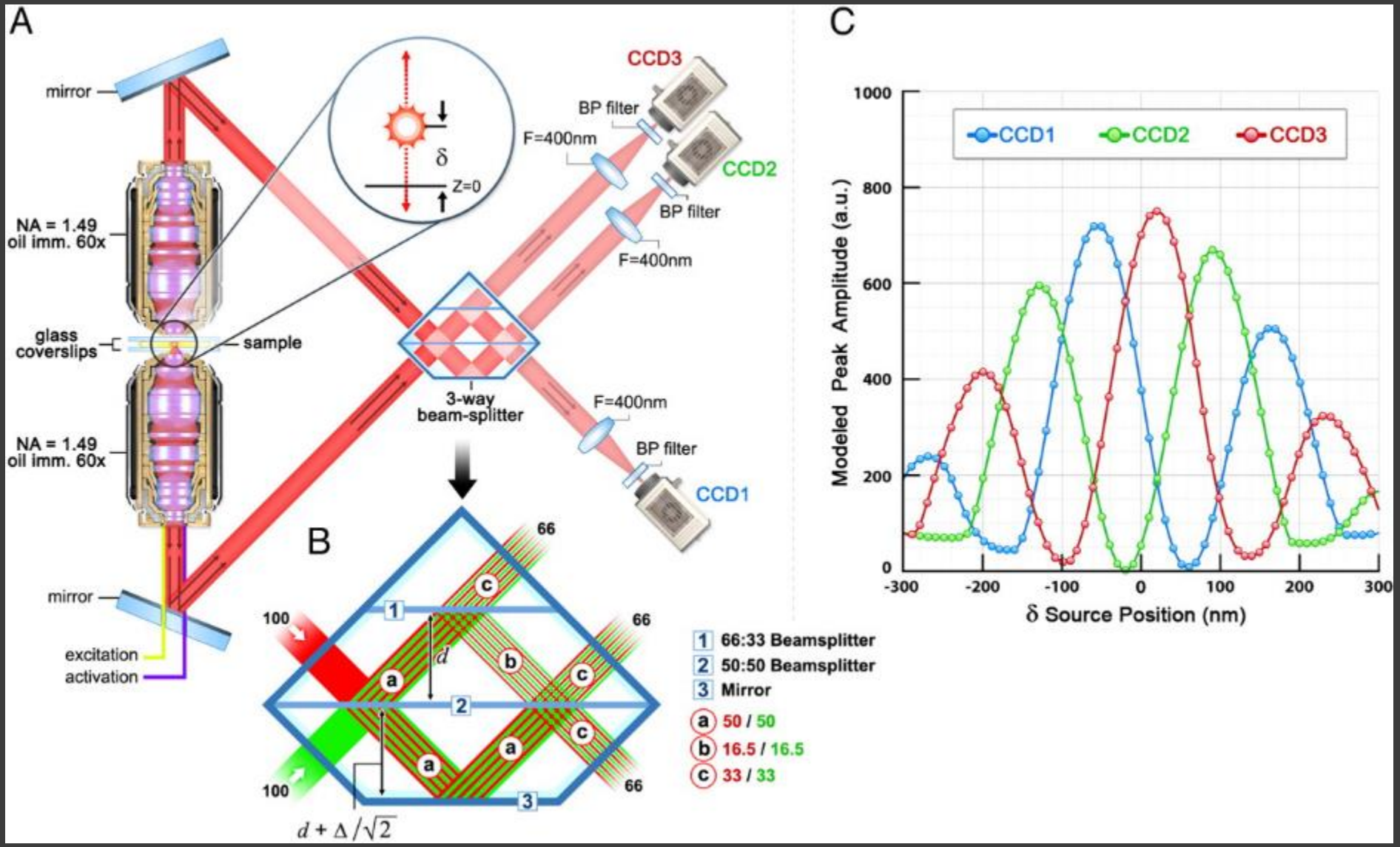
resolution
xy: 30 nm
z: < 100 nm



Baddeley et al., Nano Research, 2011

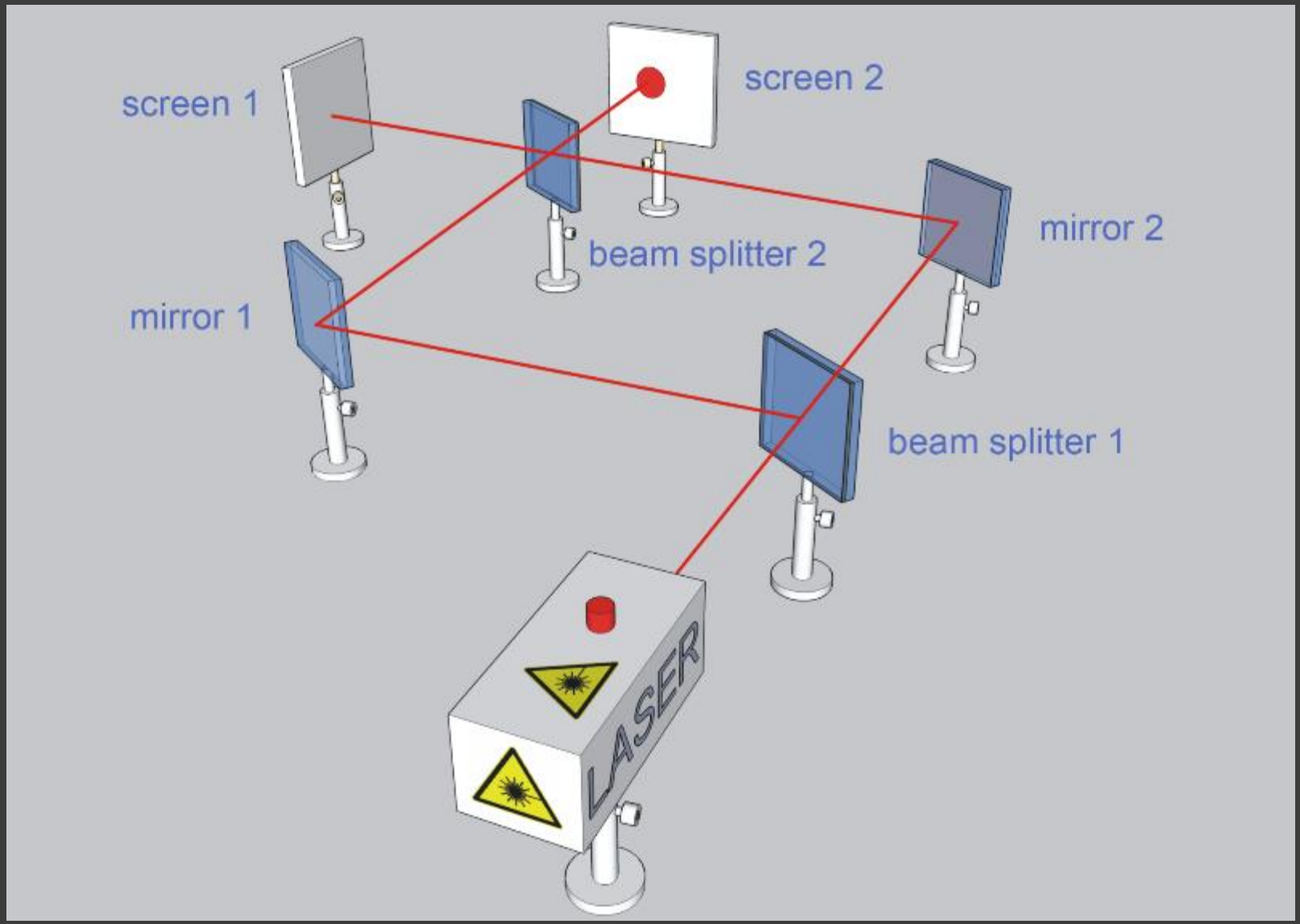
3D

iPALM



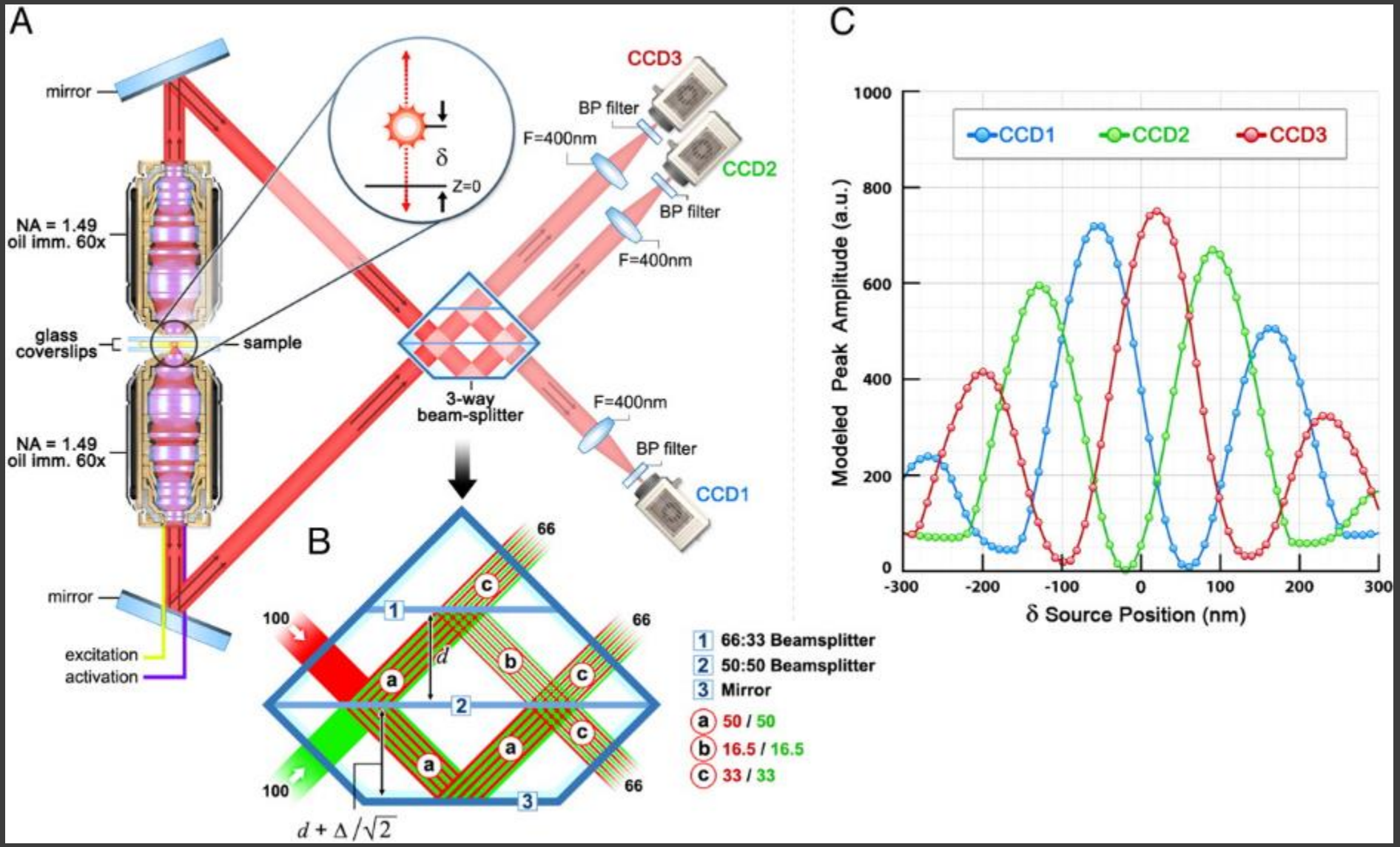
Shtengel et al., PNAS, 2009

Mach-Zehnder-Interferometer



3D

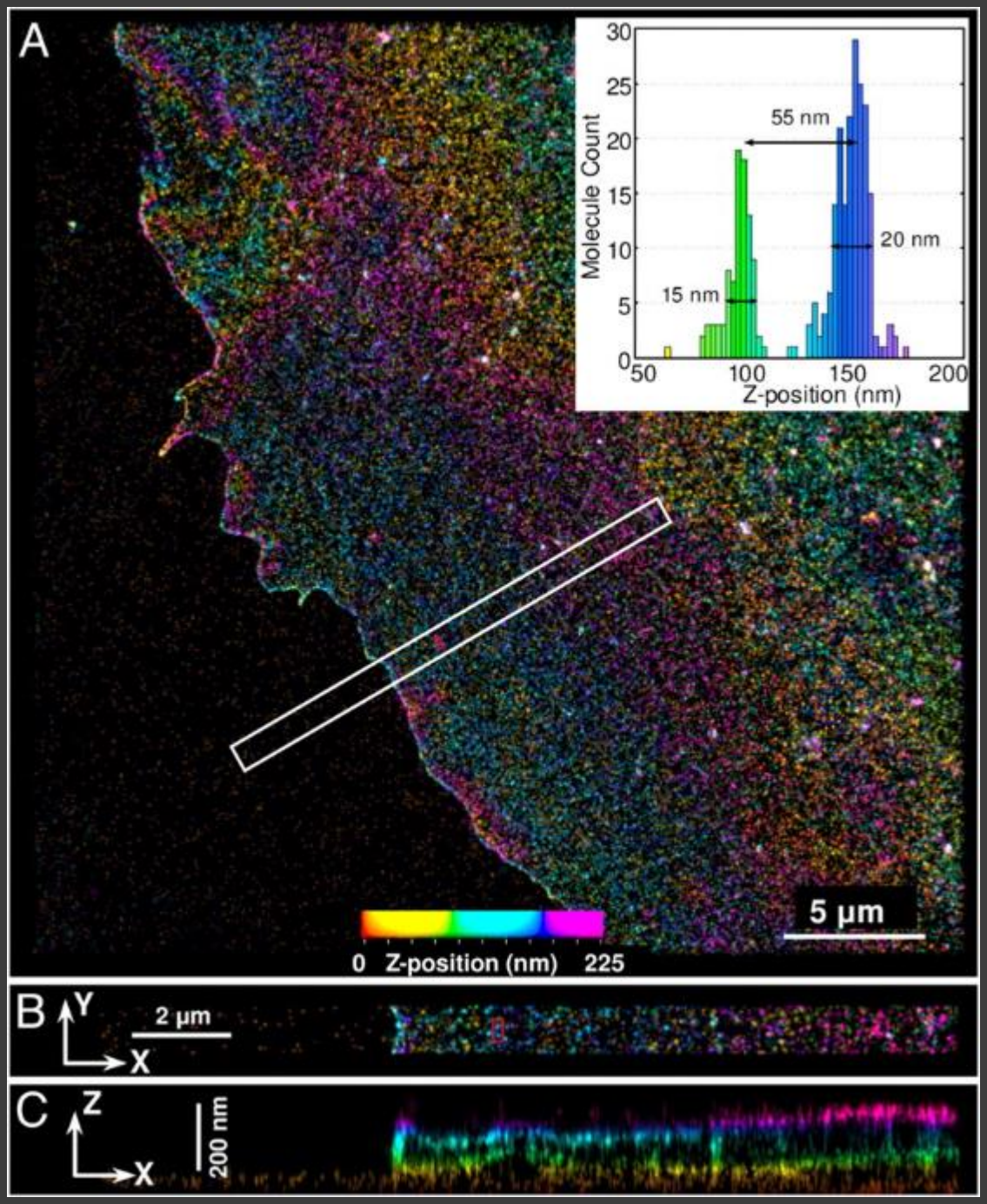
iPALM



Shtengel et al., PNAS, 2009

3D

iPALM

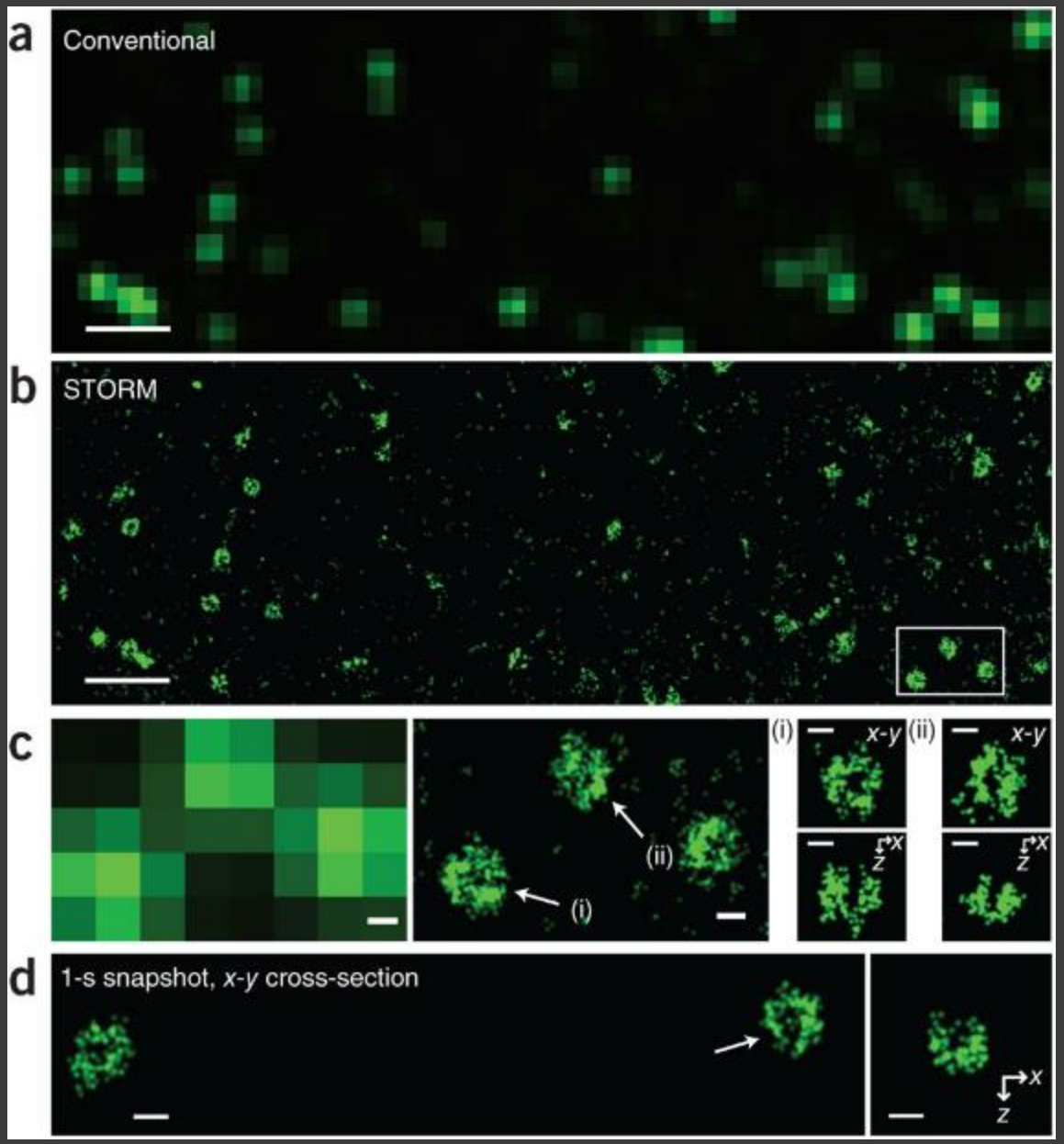


resolution:
50 nm in all 3 directions

Shtengel et al., PNAS, 2009

two examples for “live-cell” applications

live-cell STORM (dSTORM)



Alexa647-CCP

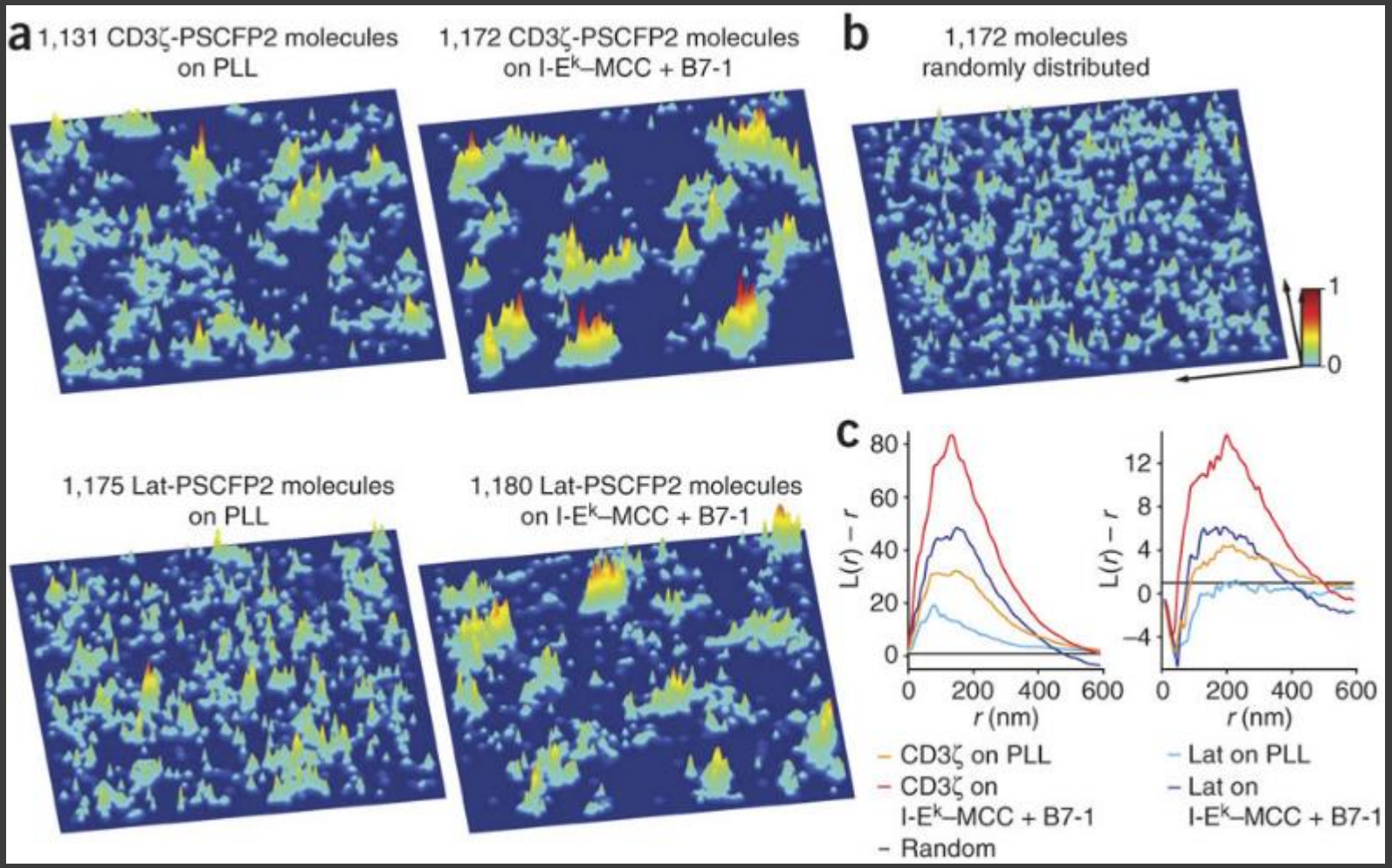
resolution

2D
spatial: 25 nm
temporal: 500 ms

3D
spatial: xy: 30 nm, z: 50 nm
temporal: 1-2 s

Jones et al., Nature Methods, 2011

hsPALM



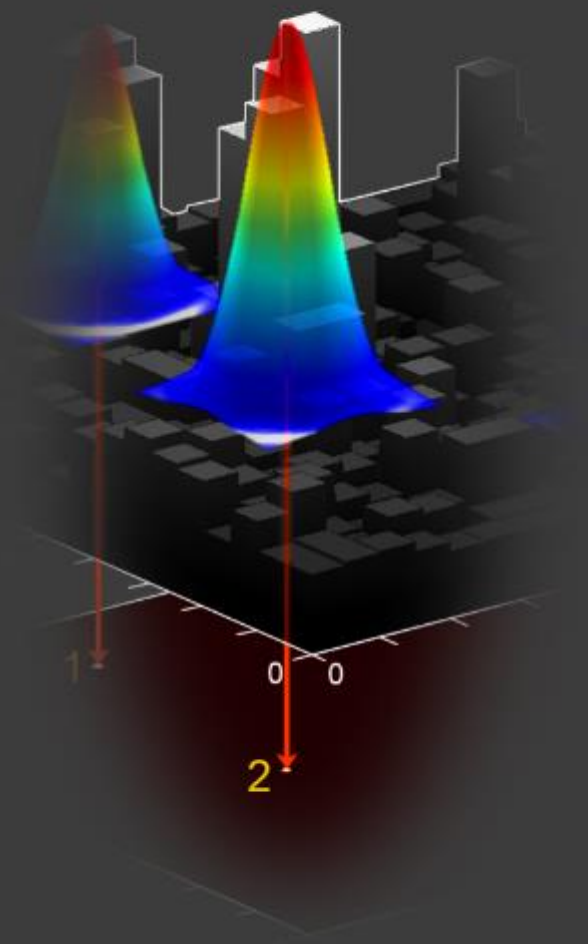
Lillemeier et al., Nature Immunology, 2009

2D, spatial resolution: 60 nm, temporal resolution: 4-10 s

how to get a lot more information from the data

the additional single molecule information

remember?



all the molecules in the image have been detected one by one

→ position of each molecule

→ number of detected photons

→ shape of the PSF

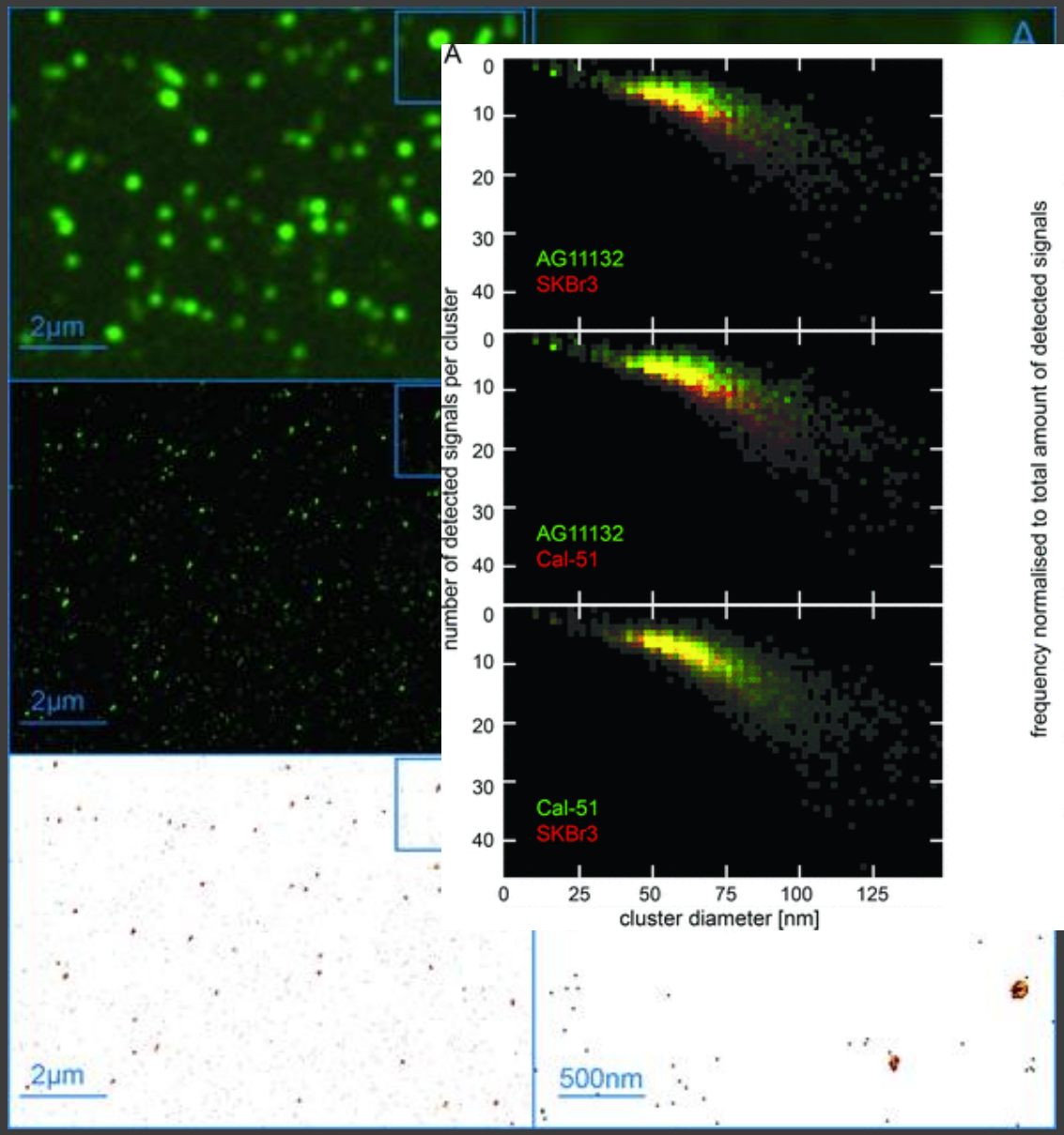
→ polarisation

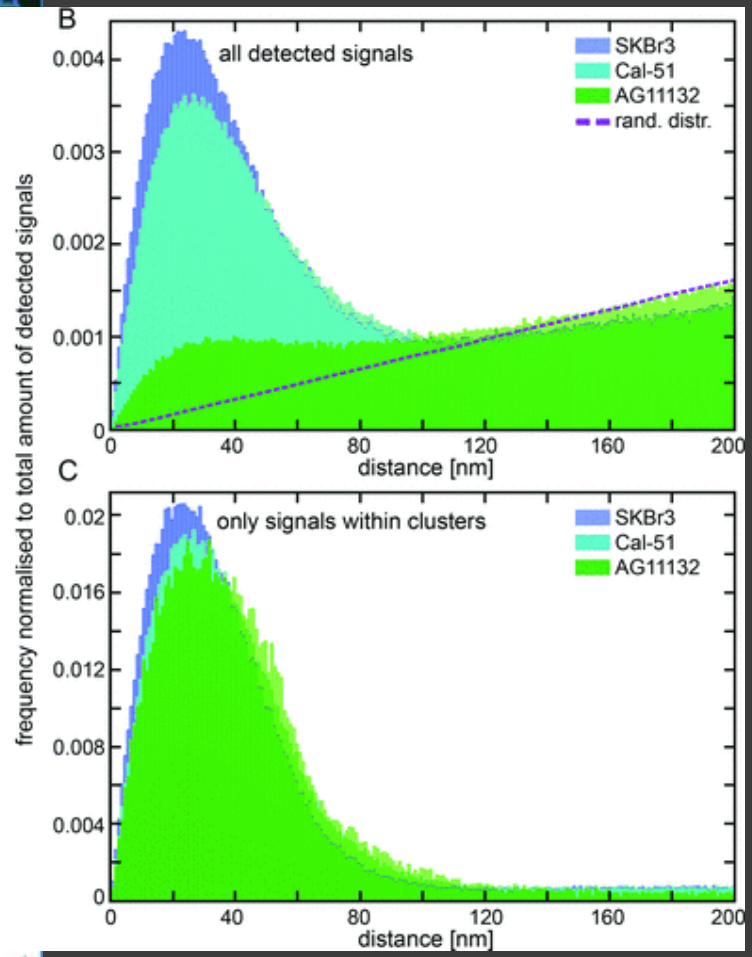
→ wavelength

→ dynamics (in living cells)

→ …

statistical analysis of small protein clusters

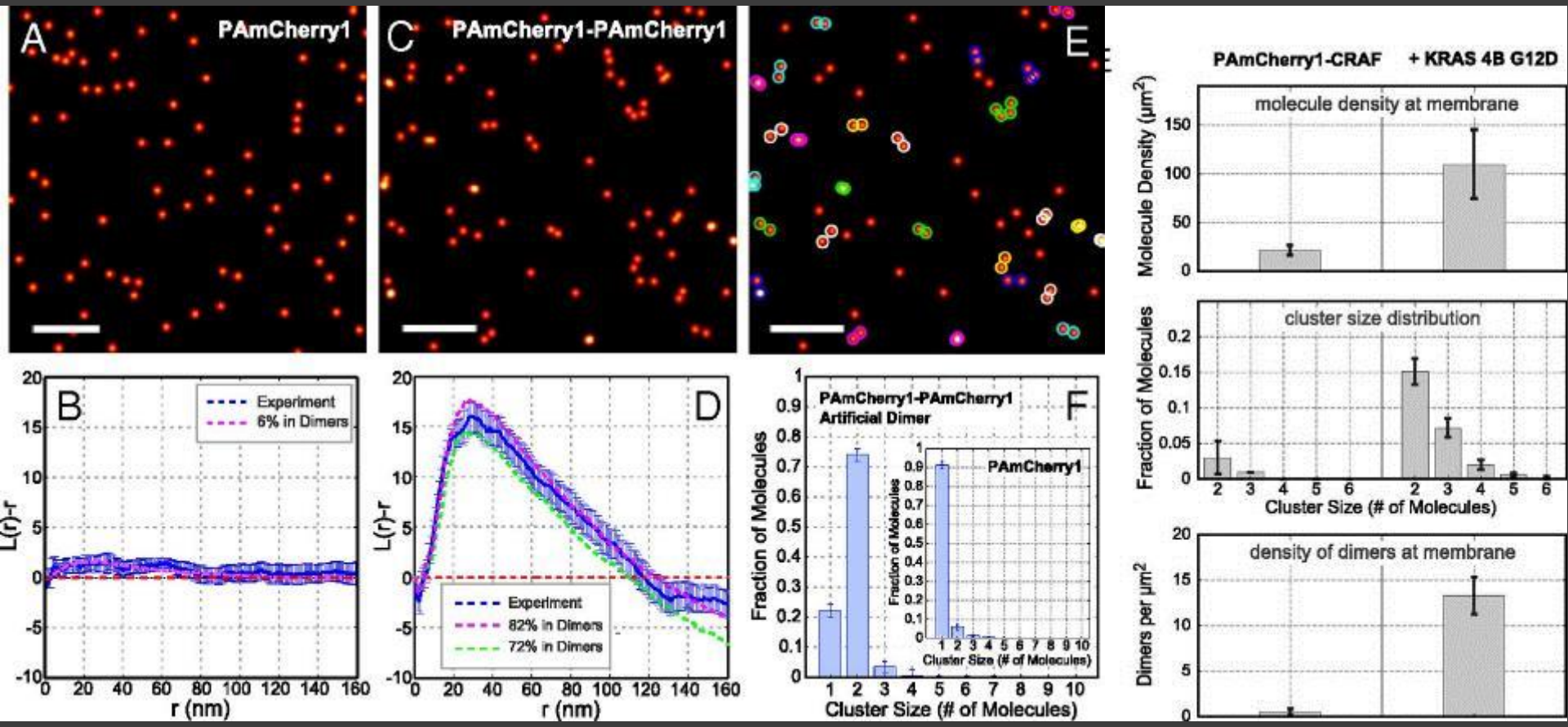




Alexa488-Her2/neu

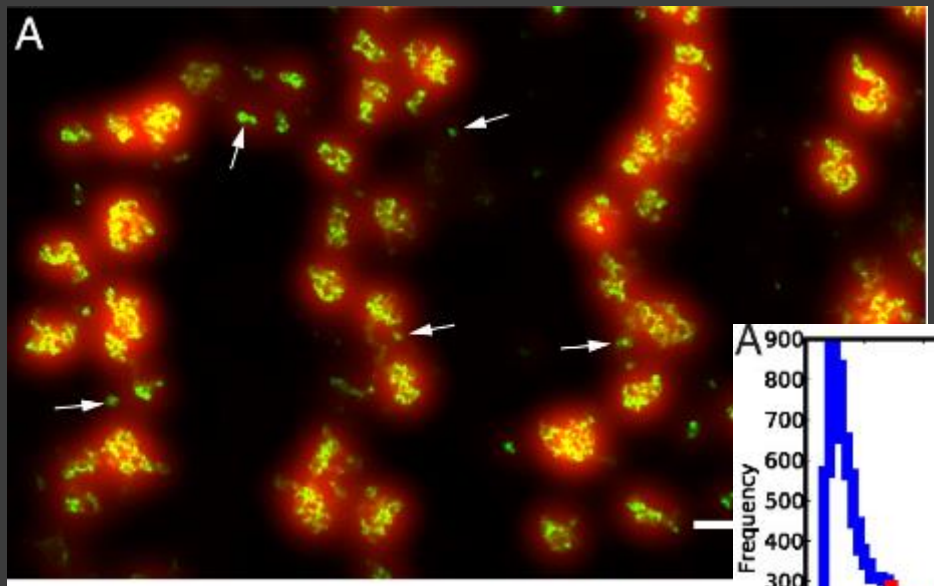
Kaufmann et al., Journal of Microscopy, 2010

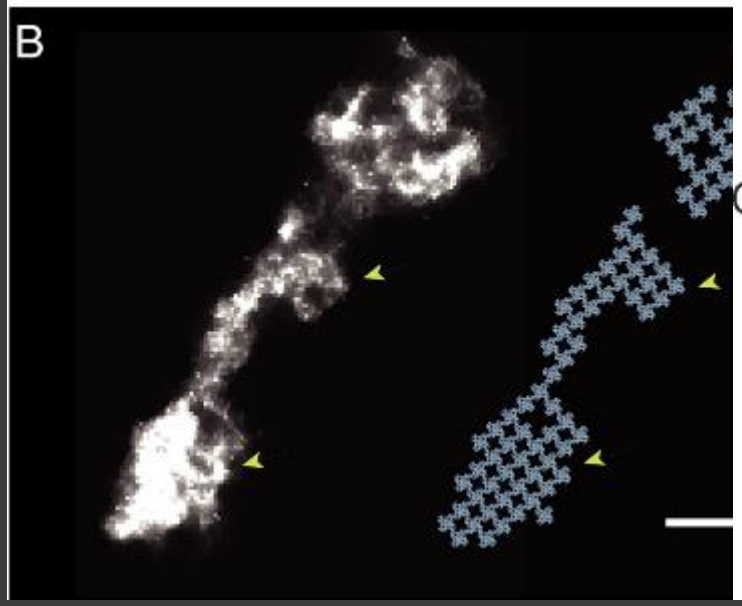
analysis of protein clusters and molecule counting

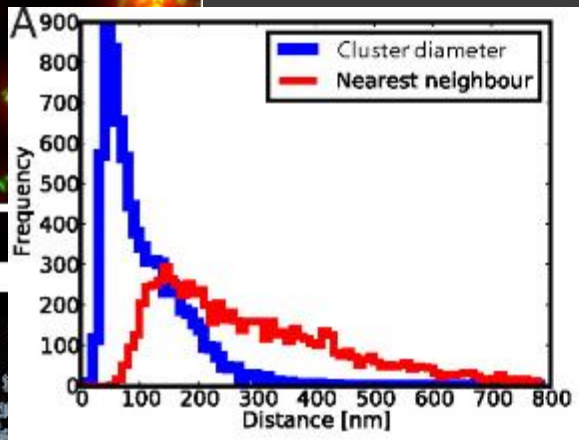


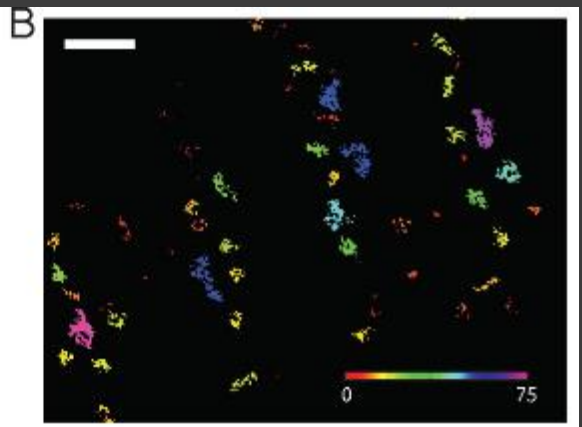
Xiaolin et al., PNAS, 2013

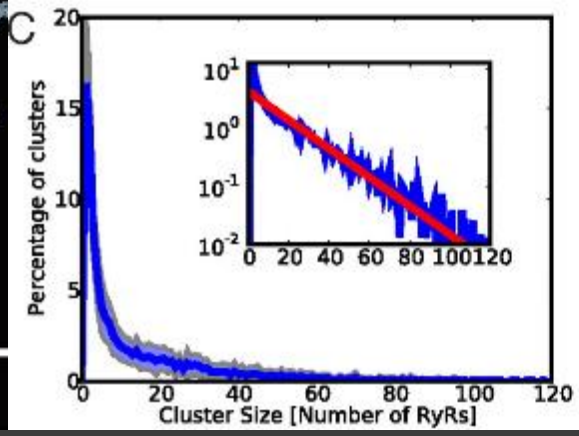
statistical analysis of large protein clusters

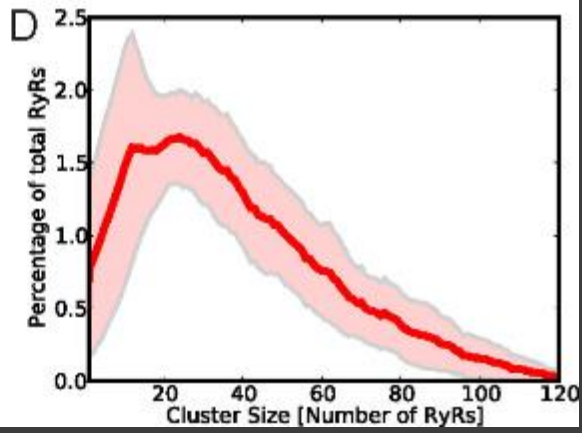






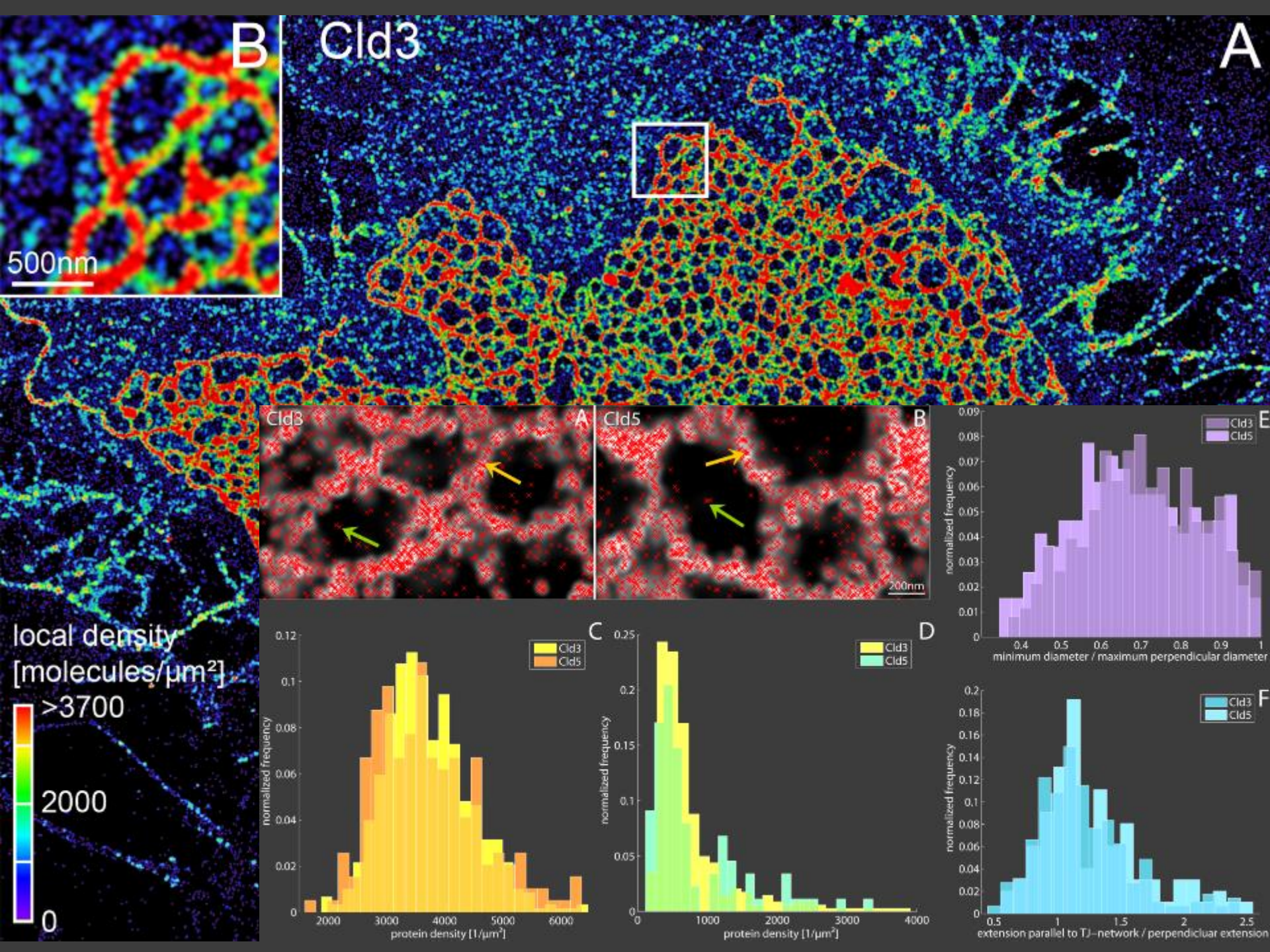




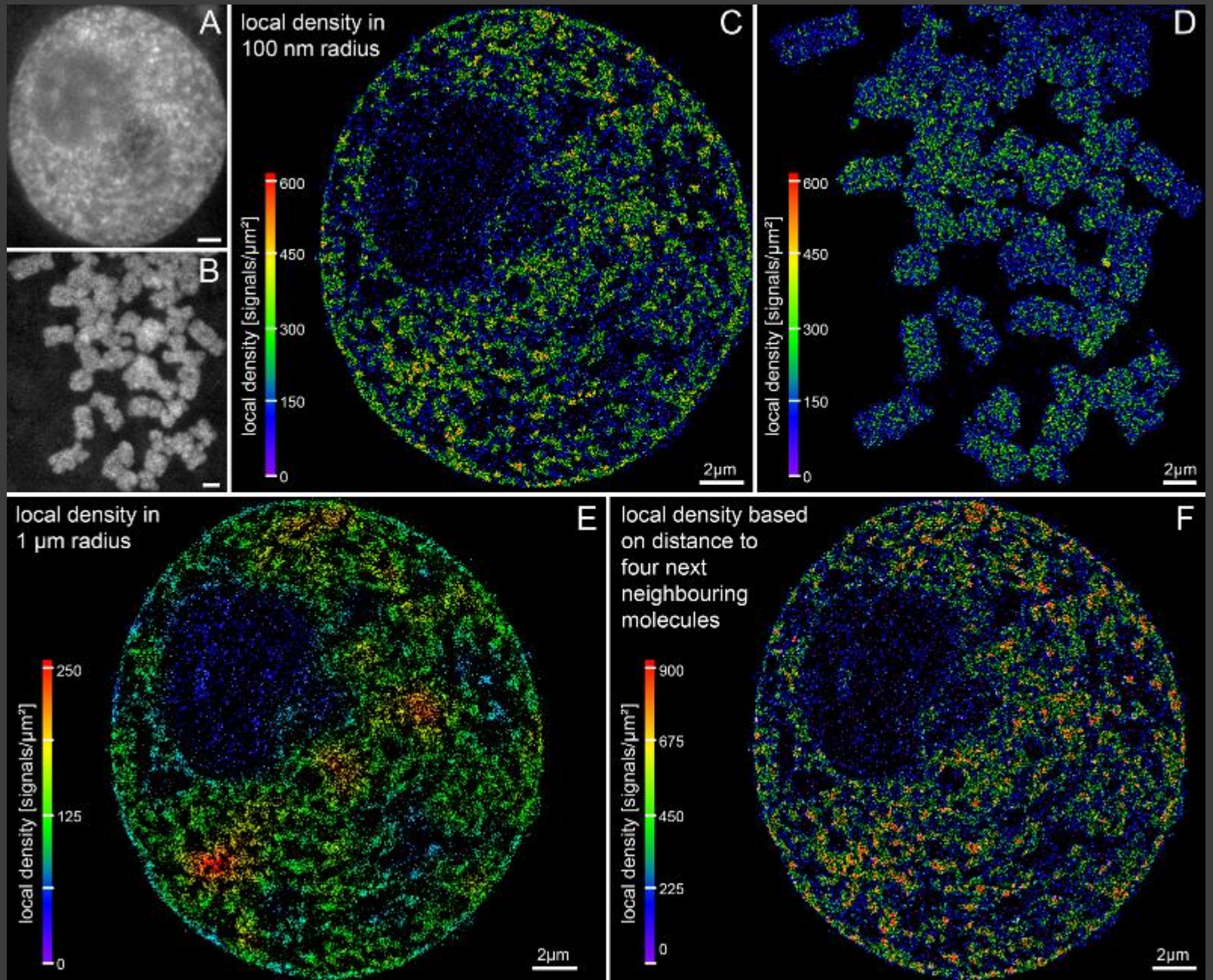


Alexa647-RyR

Baddeley et al., PNAS, 2009

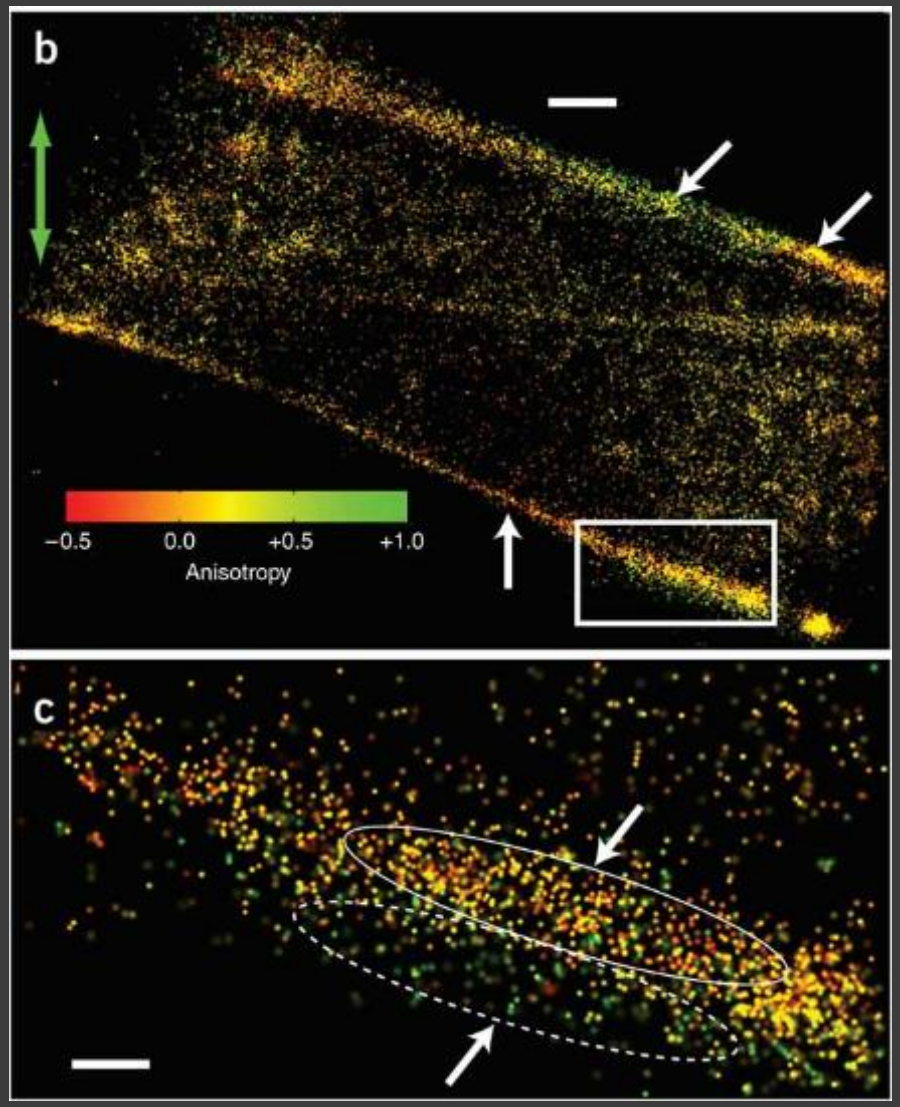

B
Cld3
A
500nm
Cld3
A
Cld5
B
200nm
local density
[molecules/µm²]
>3700
2000
0
C
Cld3
Cld5
normalized frequency
protein density [1/µm²]
D
Cld3
Cld5
normalized frequency
protein density [1/µm²]
E
Cld3
Cld5
normalized frequency
minimum diameter / maximum perpendicular diameter
F
Cld3
Cld5
normalized frequency
extension parallel to TJ-network / perpendicluar extension

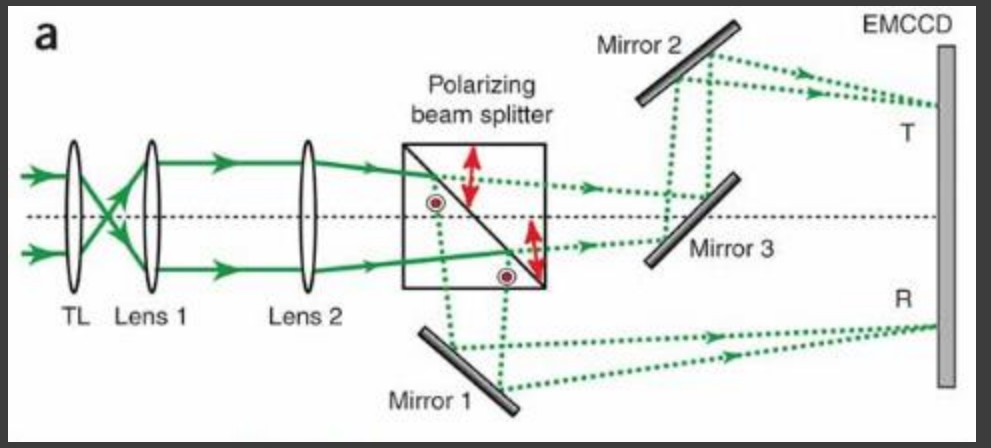
visualisation of protein densities



polarisation of the detected fluorophores

Dendra2-actin

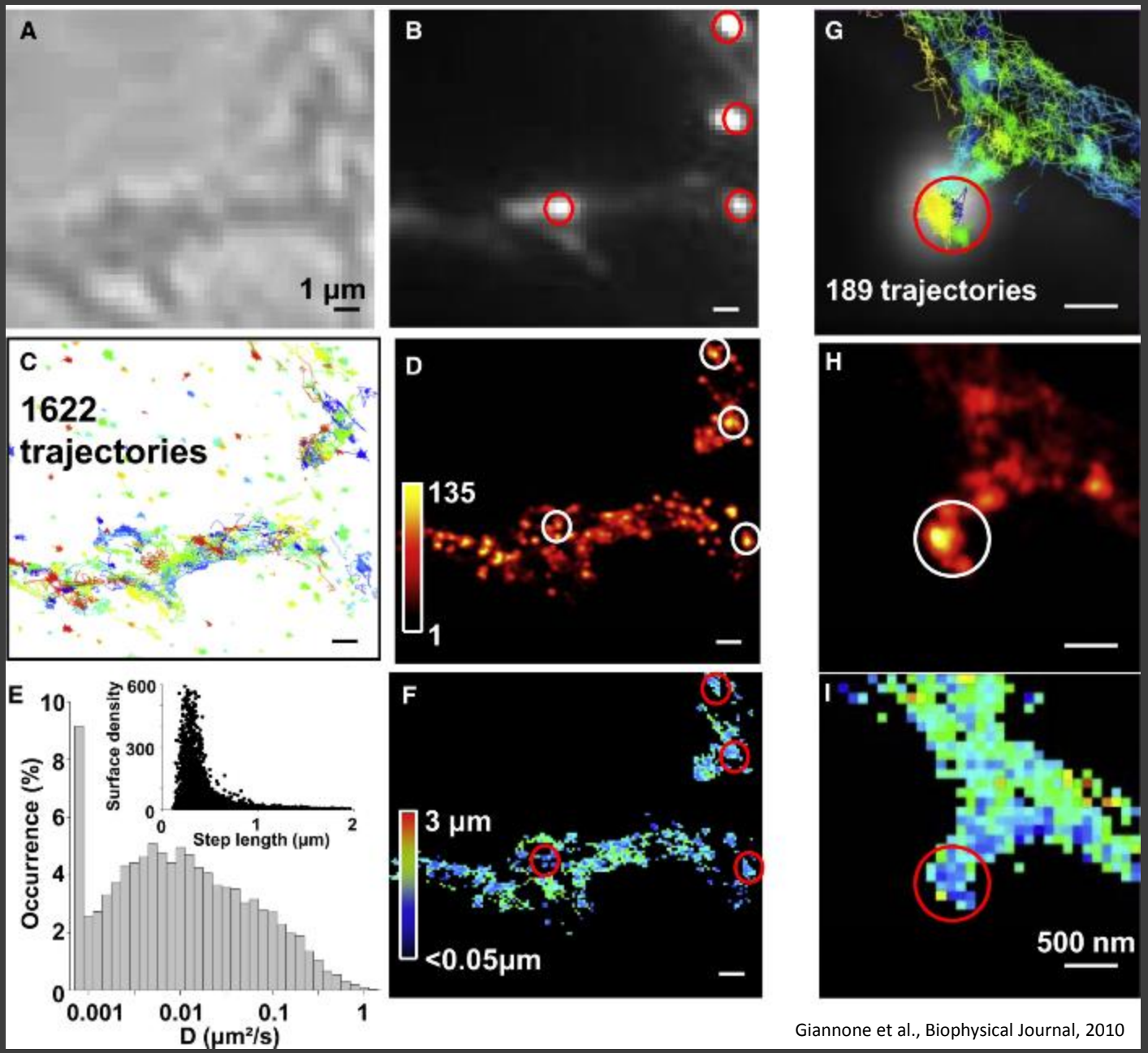




Gould et al., Nature Methods, 2008

high density particle tracking in living cells

sptPALM or uPAINT



Giannone et al., Biophysical Journal, 2010

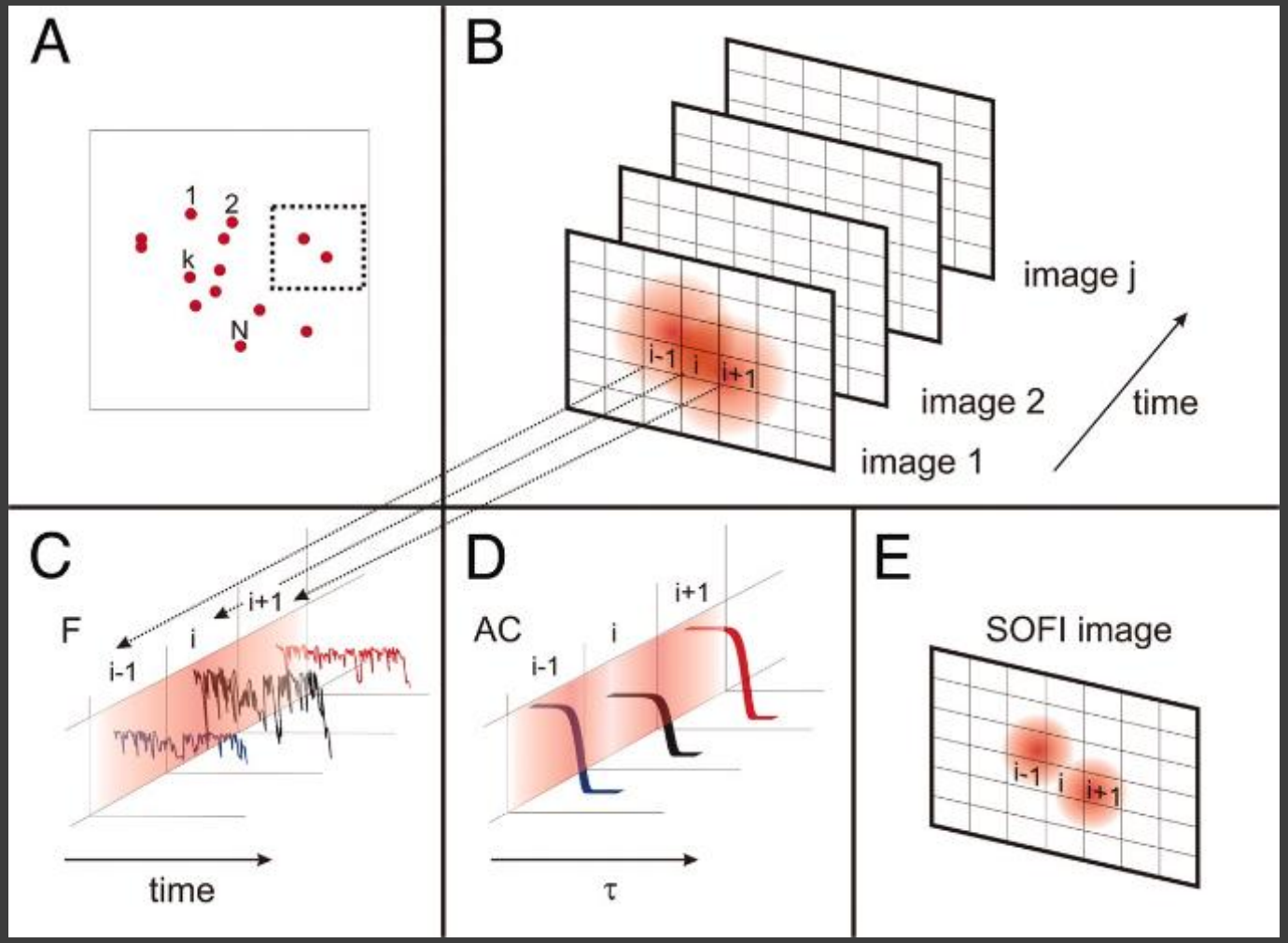
sptPALM:
Manely et al., Nature Methods, 2008

antiGluR2-AT647N-AMPAR

alternative approaches

SOFI - making the setup even more simpler

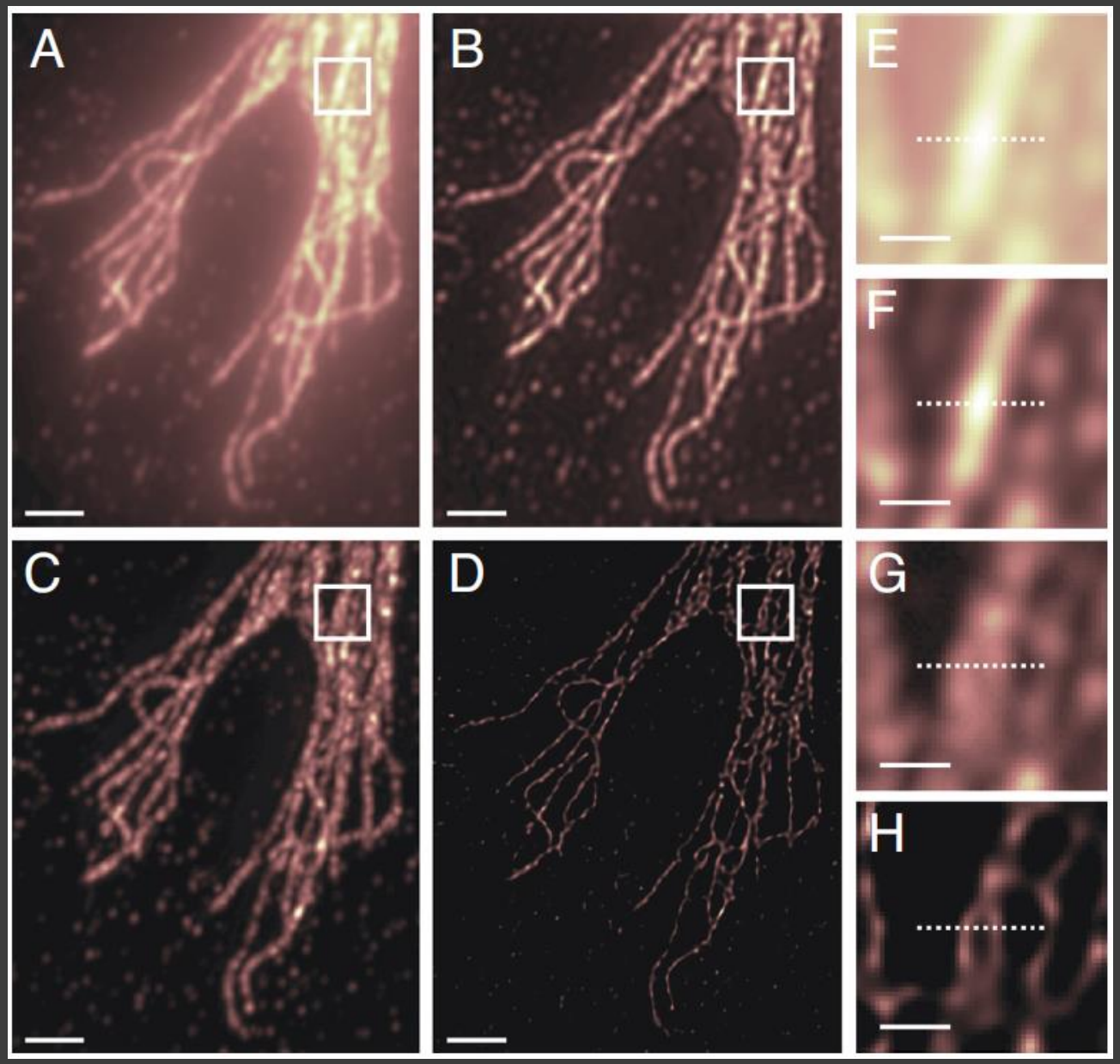
localization microscopy using a lamp!



Dertinger et al., PNAS, 2009

$$\sum_{k=1}^{N} U(\mathbf{r}-\mathbf{r}_k)\cdot\varepsilon_k\cdot s_k(t) \qquad G_2(\mathbf{r},\tau)=\sum_k U^2(\mathbf{r}-\mathbf{r}_k)\cdot\varepsilon_k^2\cdot\langle\delta s_k(t+\tau)s_k(t)\rangle$$

SOFI - making the setup even more simpler



QD625-α-tubulin

Dertinger et al., PNAS, 2009

lateral resolution:
70-100 nm

BUT!

no single molecule information

only resolution enhancement

3B analysis localization microscopy

similar approach as SOFI but some differences:

+ also based on very high molecule densities florescent in one frame

→ very fast: only several hundred frames needed for reconstruction of an image with a resolution of 50 nm → time resolution: 4 s

+ single molecule information is still accessible

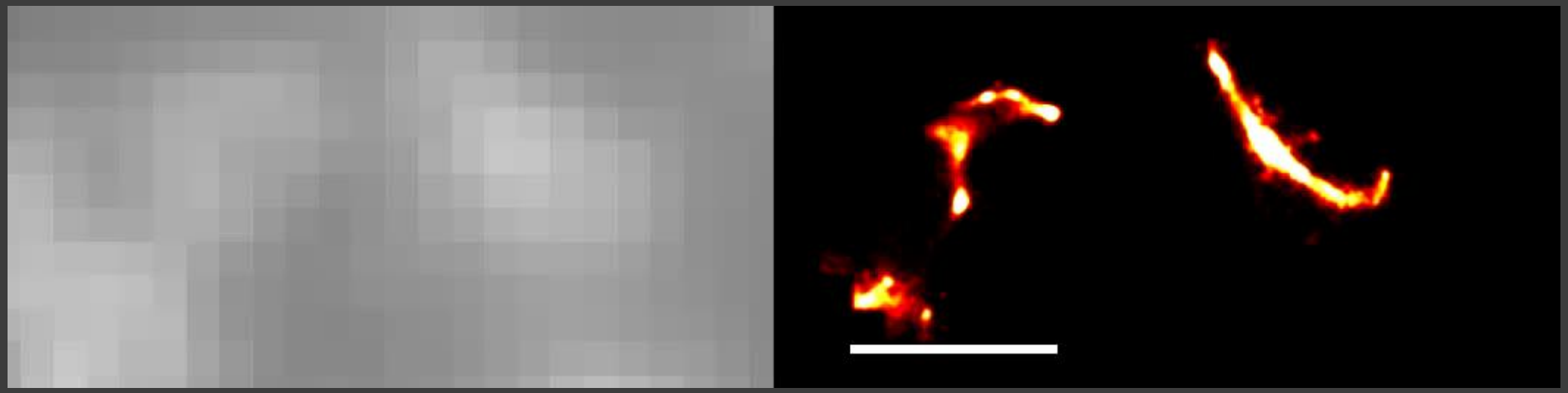
- extremely extensive computation effort

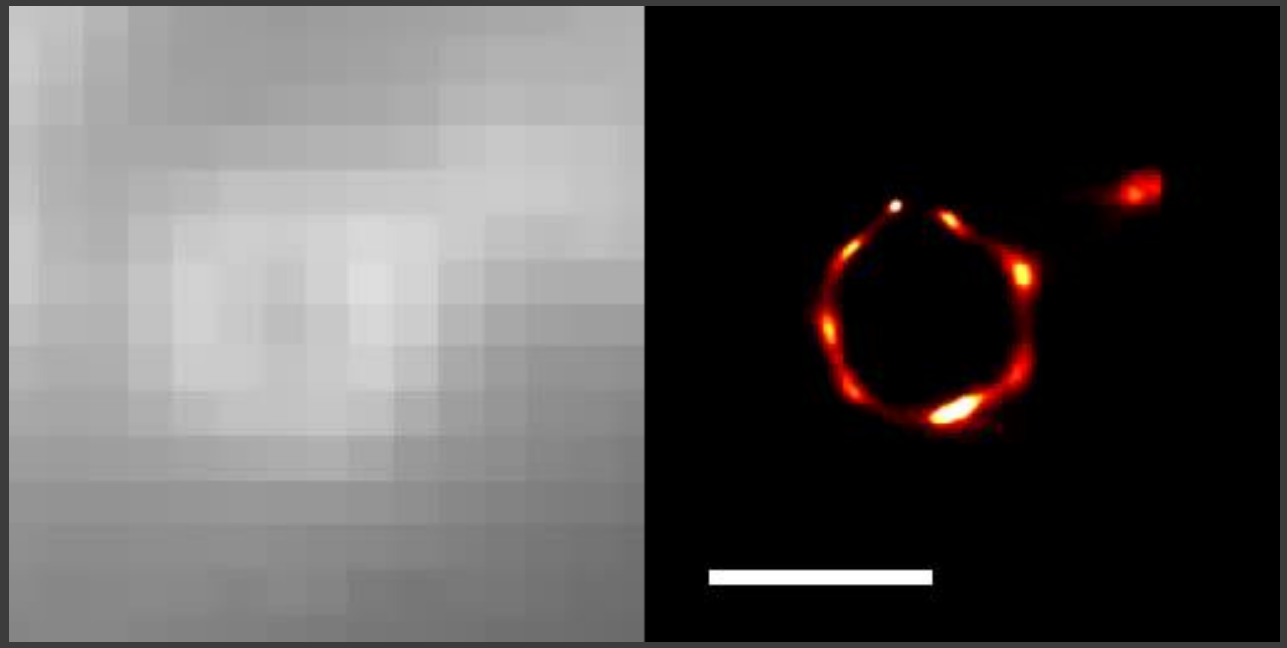
→ regions larger than 2 x 2 µm would need to be processed for days on a conventional (core i7) CPU

3B analysis localization microscopy

wide-field

reconstruction (resolution: 50 nm)





Cox et al., Nature Methods, 2011

Alexa488-podosomes

conclusion

conclusion

PALM: irreversible photo-activation

→ quantitative analyses, particle tracking, counting

→ needs (in most cases) TIRF!

STORM, dSTORM, GSDIM, SPDM: reversible photo-switching

→ resolution, fast

→ also works without TIRF → imaging deeper inside cells

SPDM and GSDIM with FPs: (ir)reversible photo-switching

→ quantitative analyses using conventional FPs

→ also works without TIRF → imaging deeper inside cells

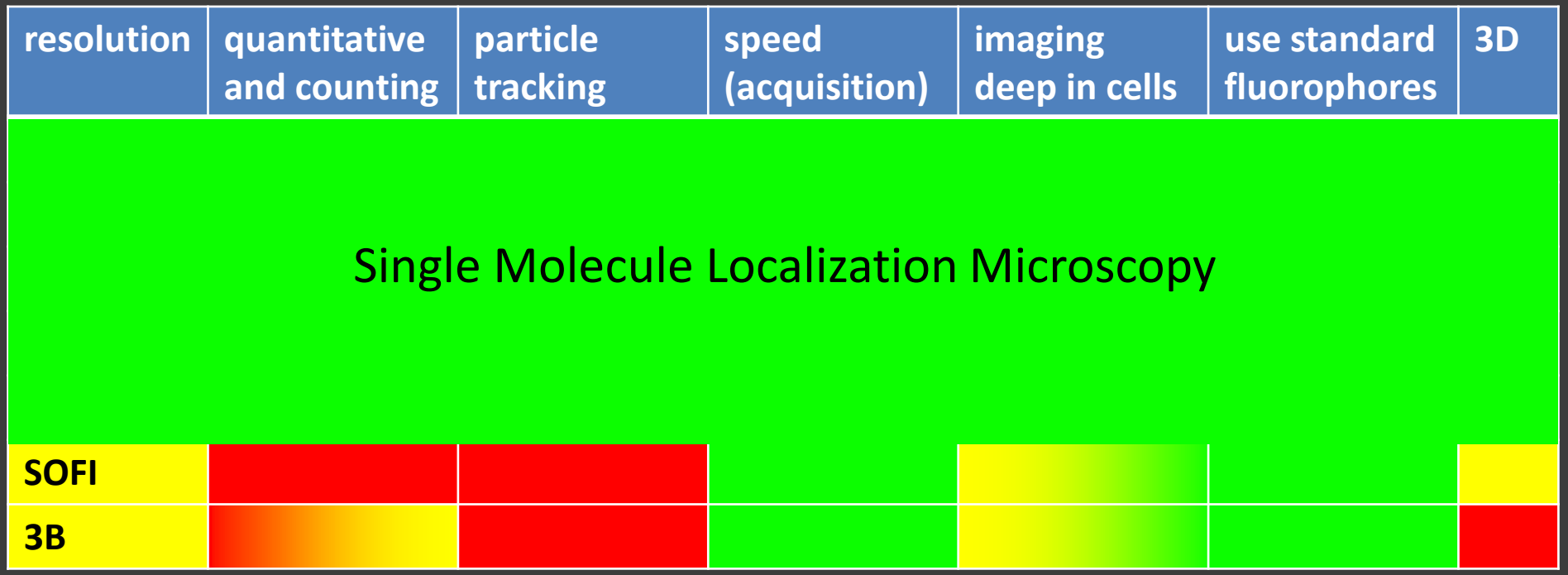
conclusion

resolution	quantitative and counting	particle tracking	speed (acquisition)	imaging deep in cells	use standard fluorophores	3D
(F)PALM						
STORM						
dSTORM						
SPDM						
GSDIM						
SOFI						
3B						

referring to the original ideas of the methods

If you have a wide-field microscope with a laser for excitation of the fluorophores and one for switching/activating you can do ALL of these methods!

conclusion



referring to the original ideas of the methods

If you have a wide-field microscope with a laser for excitation of the fluorophores and one for switching/activating you can do ALL of these methods!

links

original (F)PALM and STORM:
http://www.sciencemag.org/content/313/5793/1642.short
http://www.nature.com/nmeth/journal/v3/n10/full/nmeth929.html
http://www.sciencedirect.com/science/article/pii/S0006349506721403

dSTORM, SPDM and GSDIM (with standard fluorophores):
http://onlinelibrary.wiley.com/doi/10.1002/anie.200802376/full
http://www.springerlink.com/content/vx05p35kr3424228/
http://www.nature.com/nmeth/journal/v5/n11/full/nmeth.1257.html

3D:
http://apl.aip.org/resource/1/applab/v97/i16/p161103_s1?view=fulltext
http://www.pnas.org/content/106/9/3125.short

live-cell applications:
http://www.nature.com/nmeth/journal/v8/n6/abs/nmeth.1605.html
http://www.nature.com/ni/journal/v11/n1/full/ni.1832.html

statistical data analysis:
http://www.pnas.org/content/106/52/22275.short
http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2818.2010.03436.x/full
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0031128

links

high density particle tracking:
http://www.nature.com/nmeth/journal/v5/n2/full/nmeth.1176.html
http://www.sciencedirect.com/science/article/pii/S0006349510007137

Nat. Protoc.:
http://www.nature.com/nprot/journal/v4/n3/abs/nprot.2008.246.html
http://www.nature.com/nprot/journal/v6/n7/abs/nprot.2011.336.html

commercial systems:
http://zeiss-campus.magnet.fsu.edu/articles/superresolution/palm/introduction.html
http://www.nikoninstruments.com/en_GB/Products/Microscope-Systems/Inverted-Microscopes/Biological/N-STORM-Super-Resolution
http://www.leica-microsystems.com/products/light-microscopes/life-science-research/fluorescence-microscopes/details/product/leica-sr-gsd/

algorithms:
http://www.super-resolution.biozentrum.uni-wuerzburg.de/home/rapidstorm/
http://code.google.com/p/quickpalm/

summary and links:
http://www2.bioch.ox.ac.uk/microngroup/research/localization-microscopy.shtml