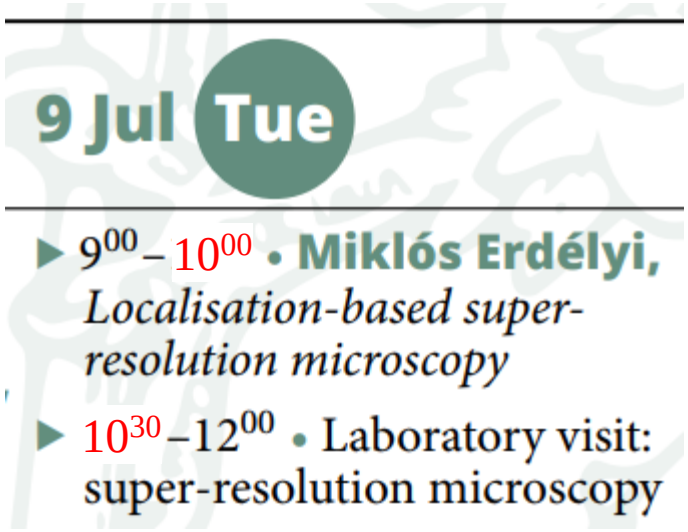
Morning section on single molecule localization based super-resolution microscopy (SMLM)

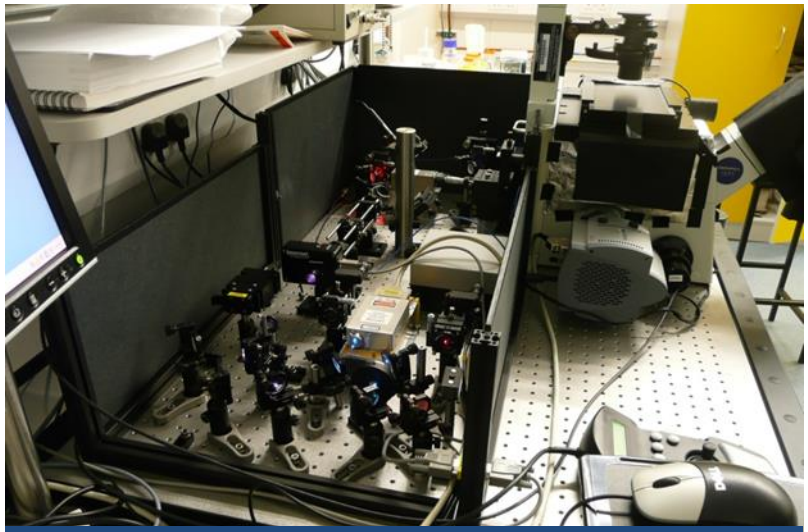
Prezentation: general intro to SMLM (9:00-10:00)
Lab visit on hardware, software and applications (2×45 minutes 10:30-12:00)



Lasers in Medicine and Life Sciences Summer School, Szeged
9th July 2019

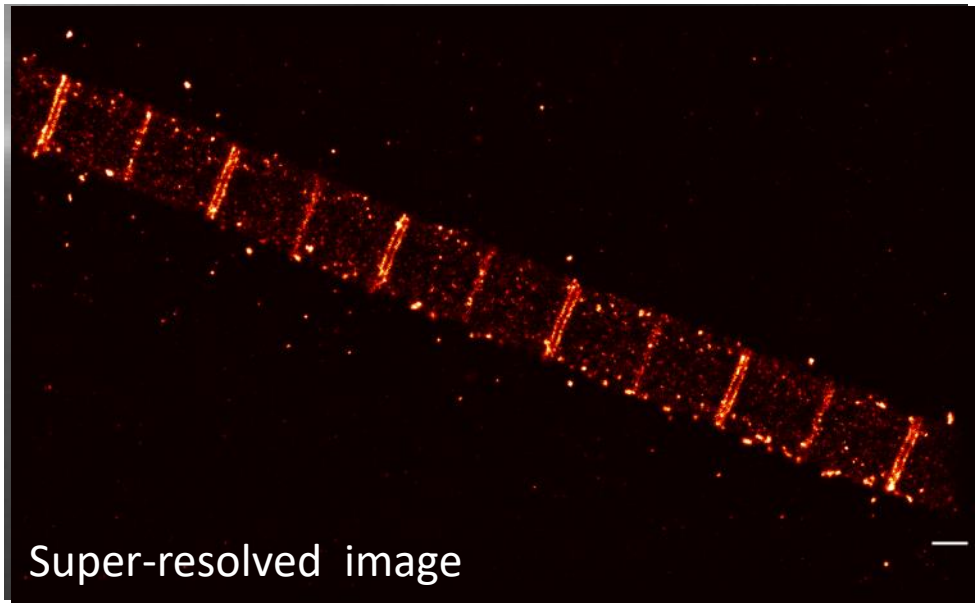
Principles of localisation based super-resolution microscopy

Miklós Erdélyi
University of Szeged



„Traditional" fluorescence microscope

??



Super-resolved image

Layout of the introductory presentation

Diffraction limited imaging

- The point spread function
- Rayleigh resolution limit
- Image formation of a wide-field microscope

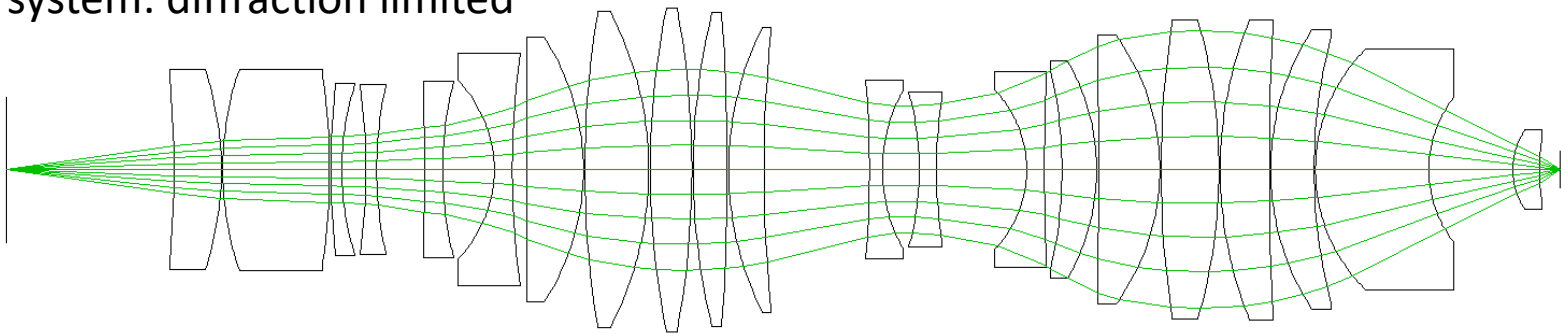
From diffraction limited imaging to localization based super-resolution microscopy

- Localisation based super-resolution microscopy
- Examples
- Issues and artefacts

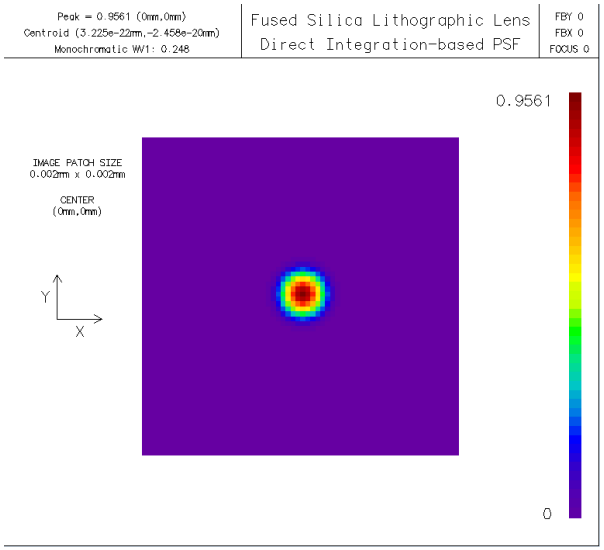
Diffraction limited imaging – Point Spread Function

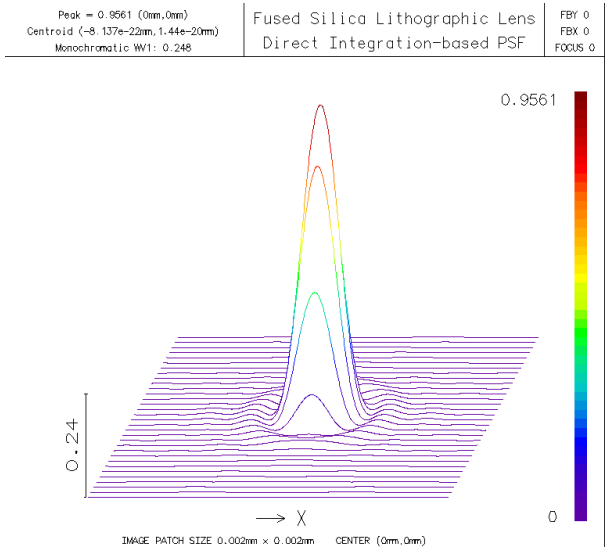
Object: point-like source

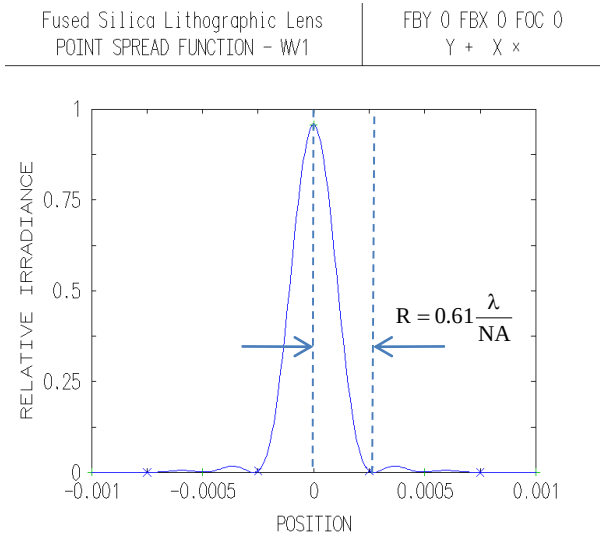
Imaging system: diffraction limited



Diffraction limited point spread function = Airy pattern







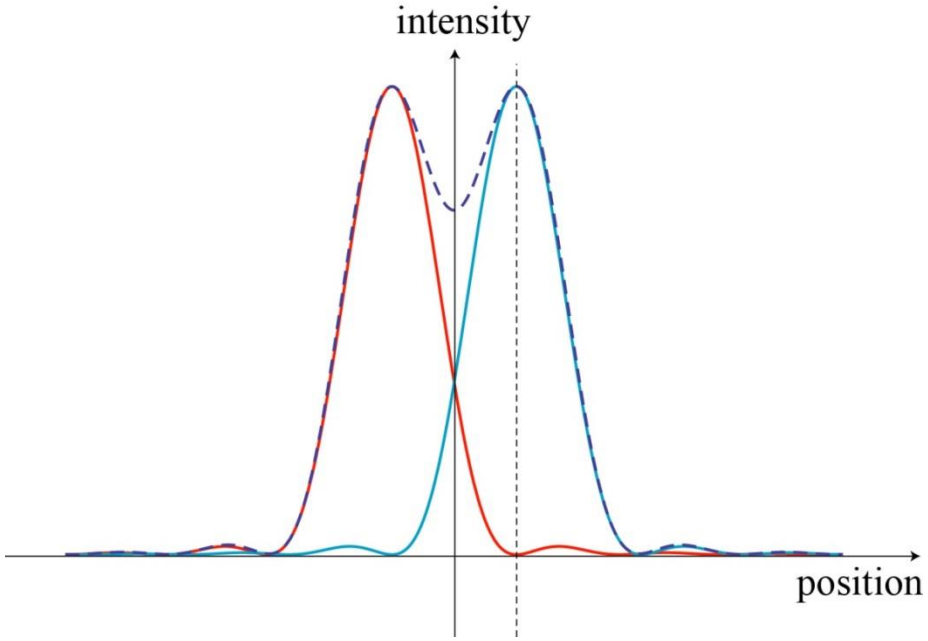
Cylindrical symmetry

Ring system

FWHM(λ, NA)

Spatial resolution – the Rayleigh criterion

Shape of PSF → resolution limit

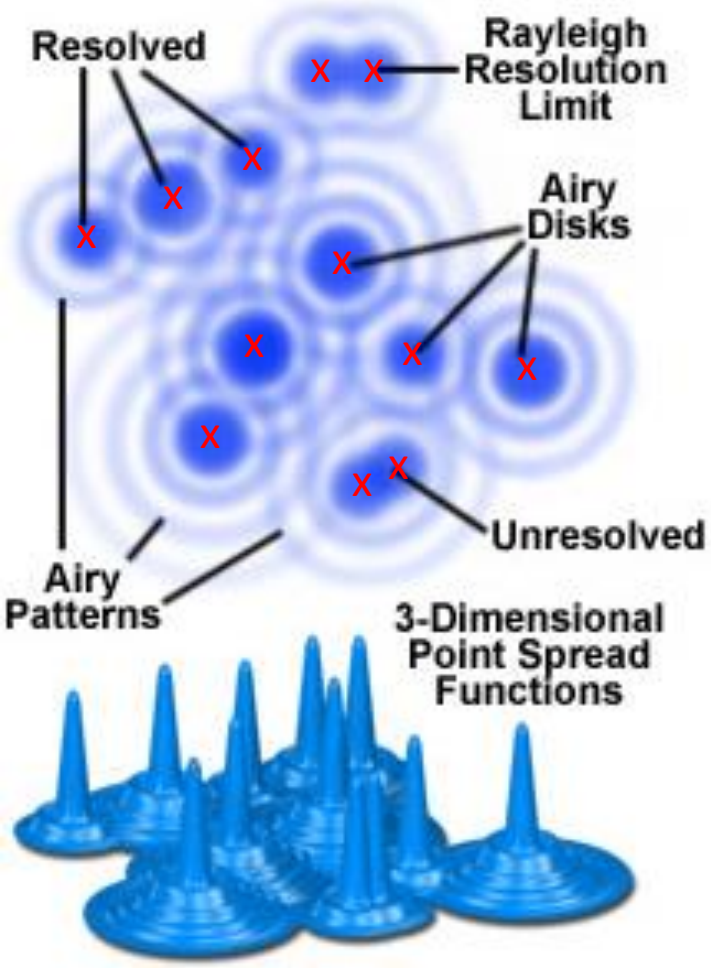


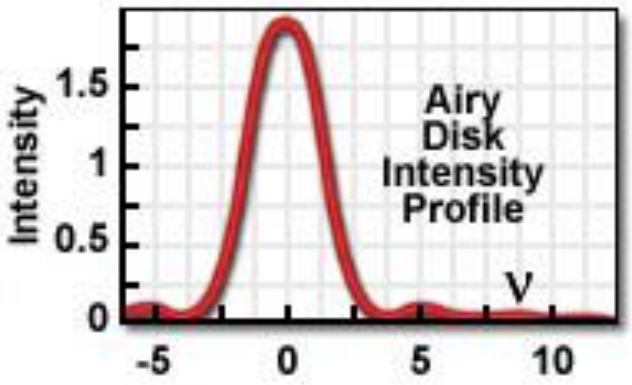
Two diffraction spots can be resolved, if their separation is larger than the distance between the main peak and the first minimum.

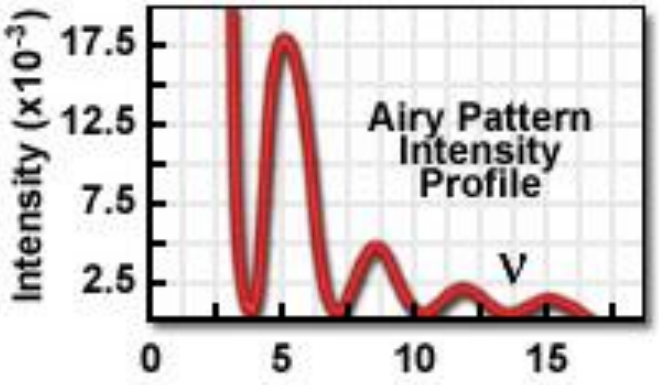
In the limiting case the minimum between the two peaks is approximately 80% of the main peaks.

Measured Image = distribution of dye molecules ⊗ PSF

Measured Image = x ⊗ 



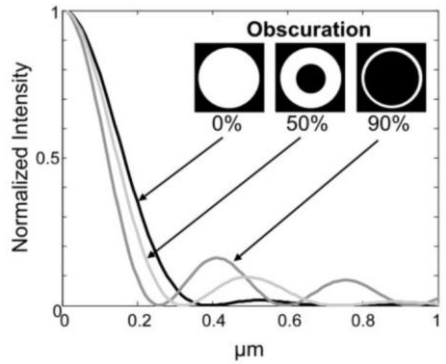




$$R = k_1 \cdot \frac{\lambda}{NA}$$

How can spatial resolution be enhanced?

- Wavelength reduction (autofluorescence, optics)
- Using high NA lenses (immersion objectives)
- Manipulation of the k_1 factor



But PSF engineering typically introduces significant loss of light!

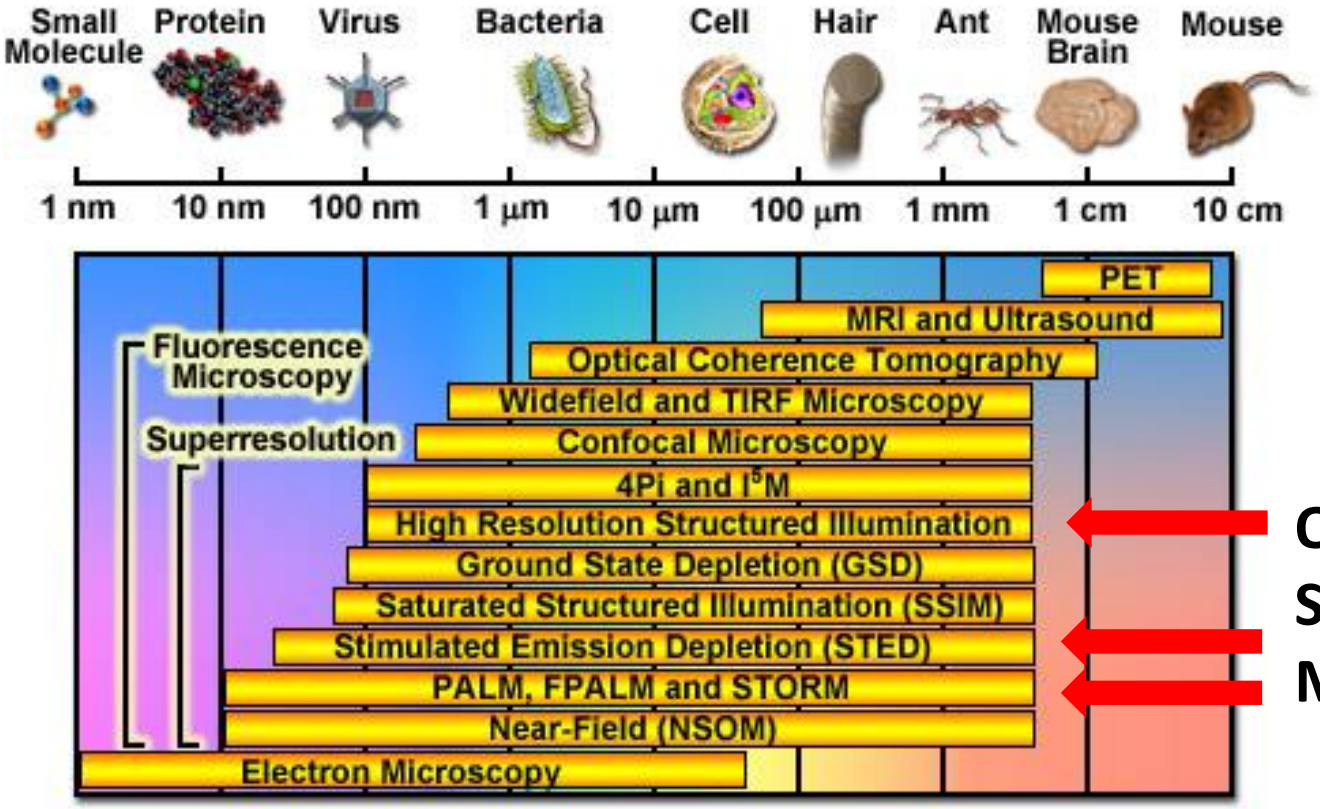
IN THE VISIBLE REGION: R≈250-300 nm

Microscopy methods

Scanning/wide field
Transmission/reflection
Bright field/dark field
Optical/electron
Fluorescence/non fluorescence
Intensity/polarization
Diffraction limited/super-resolution
……

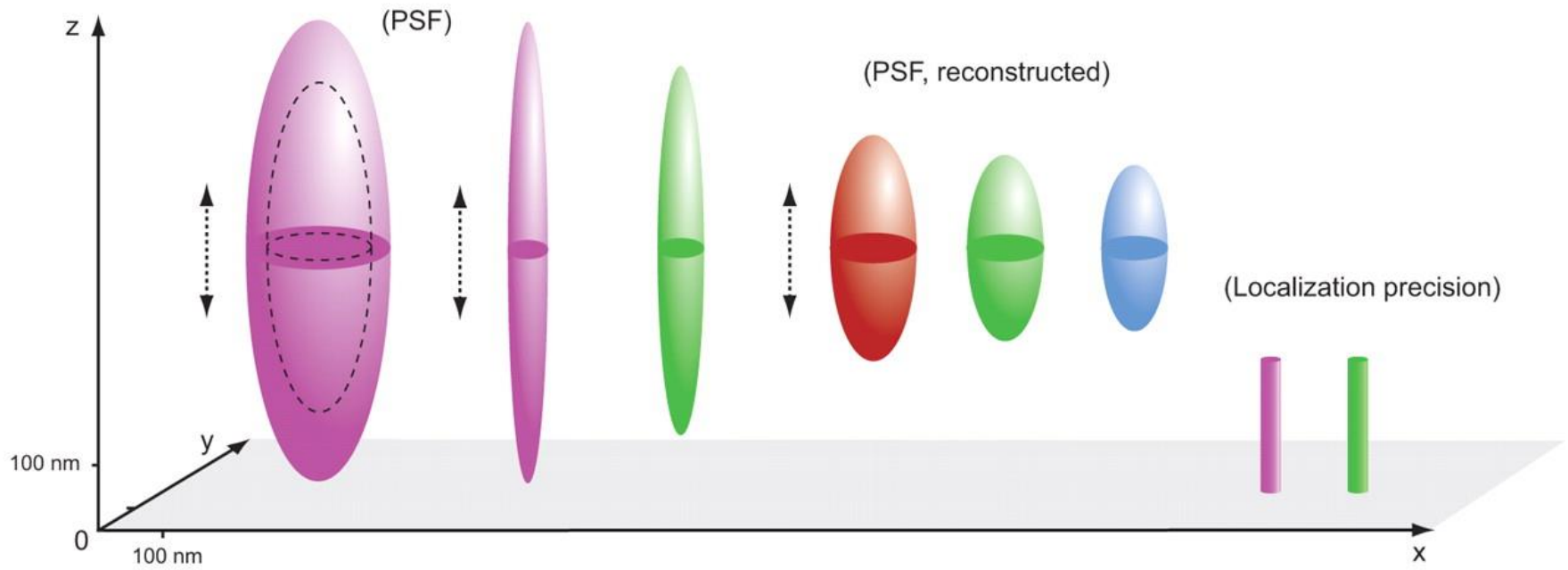
Localization microscopy: Optical, super-resolution, wide-field and fluorescence method

Super-resolution methods in microscopy



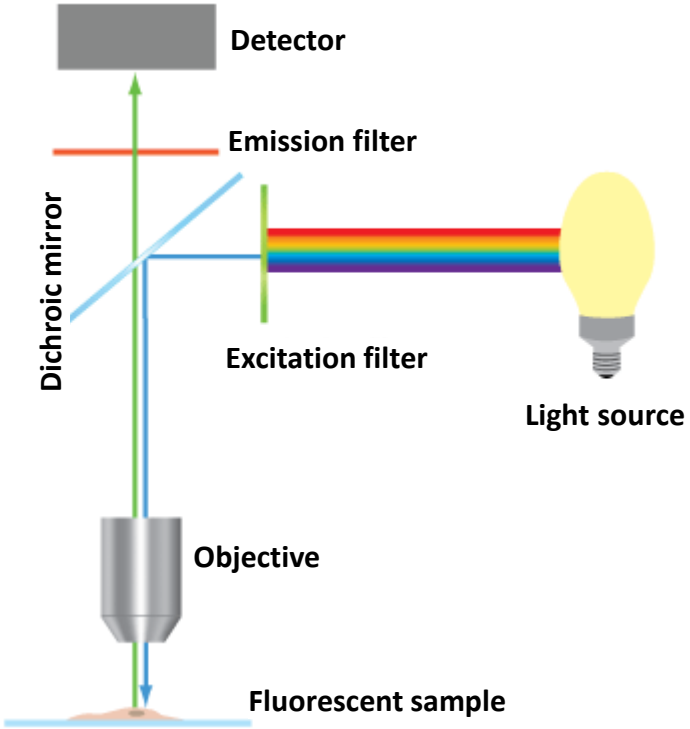
PSFs in different microscopic methods

	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520

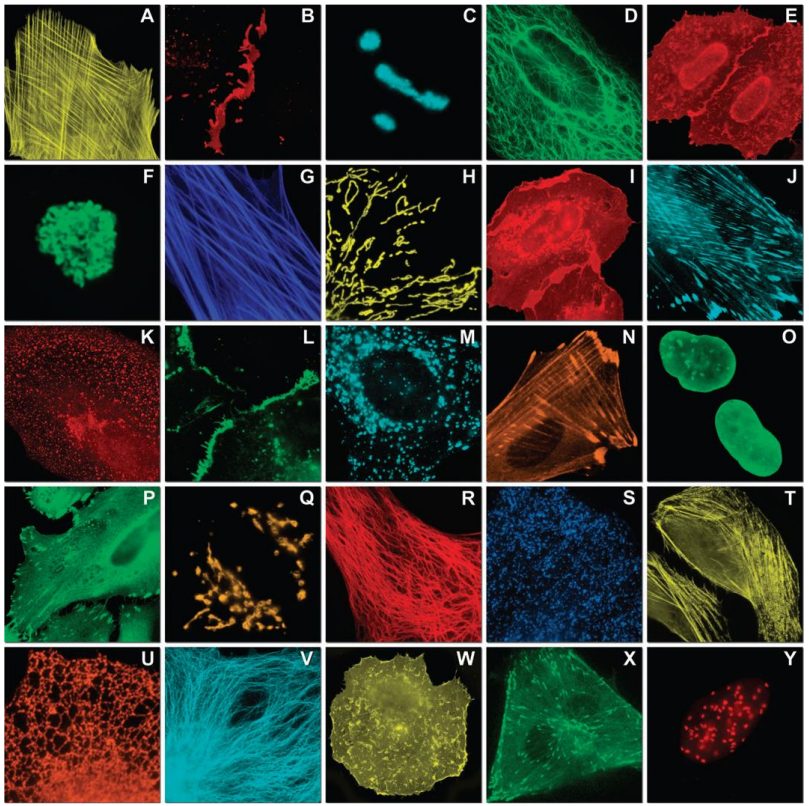


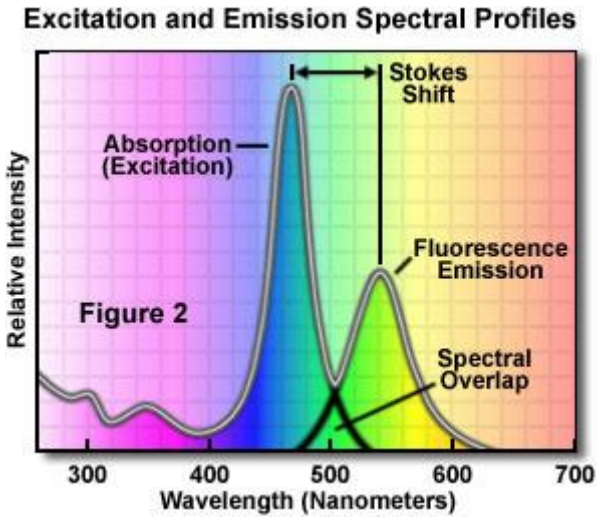
~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Fluorescence microscopy



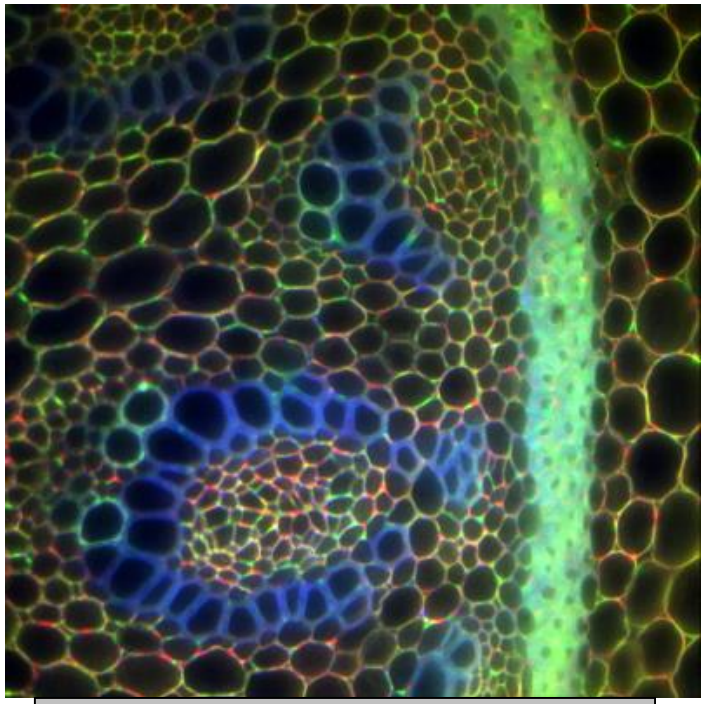
Point-like source= fluorescent dye molecule





High specificity!

Optical Fluorescence Microscopy – Multicolour -

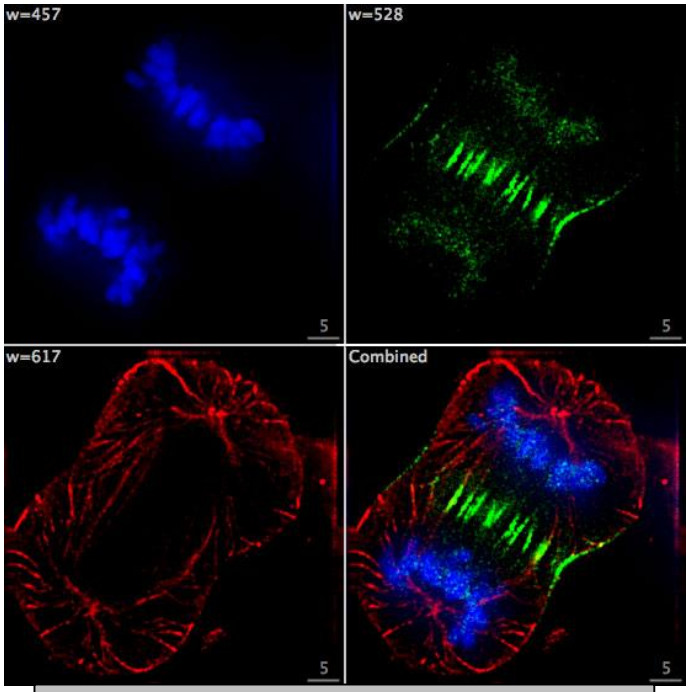


Convallaria cross section.

3 channel autofluorescence.

Supercontinuum (“white laser”) light illumination.

[Laser Analytics group 2010]



A human cancer cell dividing.

3 Fluorescent labels:

DAPI (stains DNA) – blue;

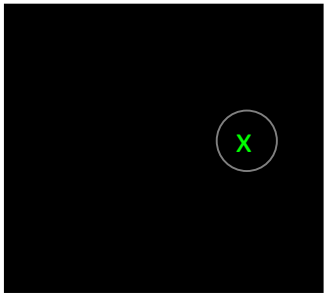
INCENP (a protein) – green;

Tubulin (cell skeleton) – red.

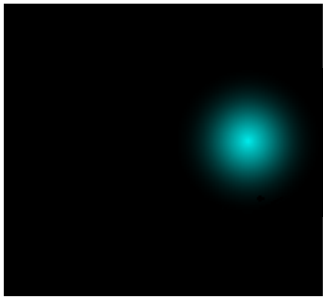
[F. Lamiot, 2010]

Principles of localization method – single isolated molecule –

Object → Image → Fitting → Reconstruction

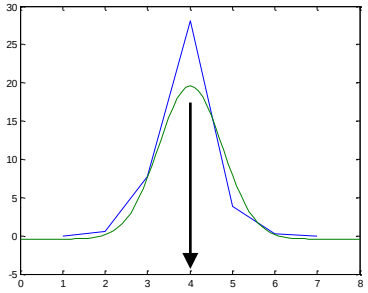


A single fluorescent molecule is a point-like light source.



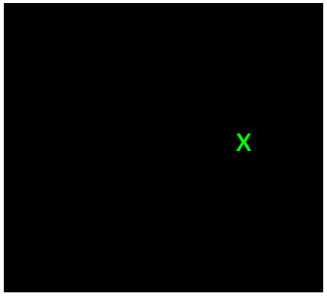
Its image is blurred.

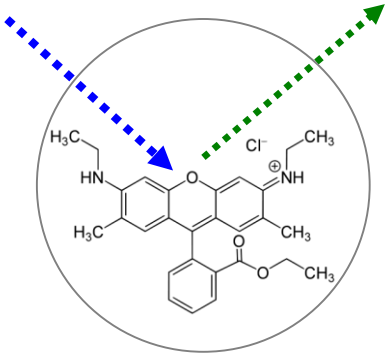
(And pixelated.)

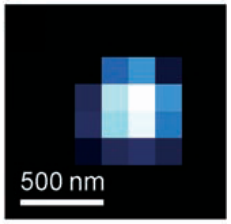


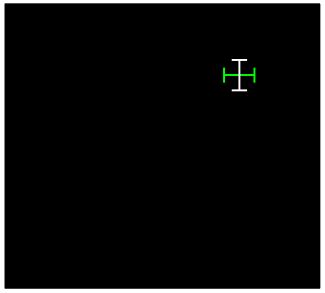
The centre of the fitted curve corresponds to the position of the molecule.

(Accuracy depends on the signal to noise ratio.)









Localization procedure

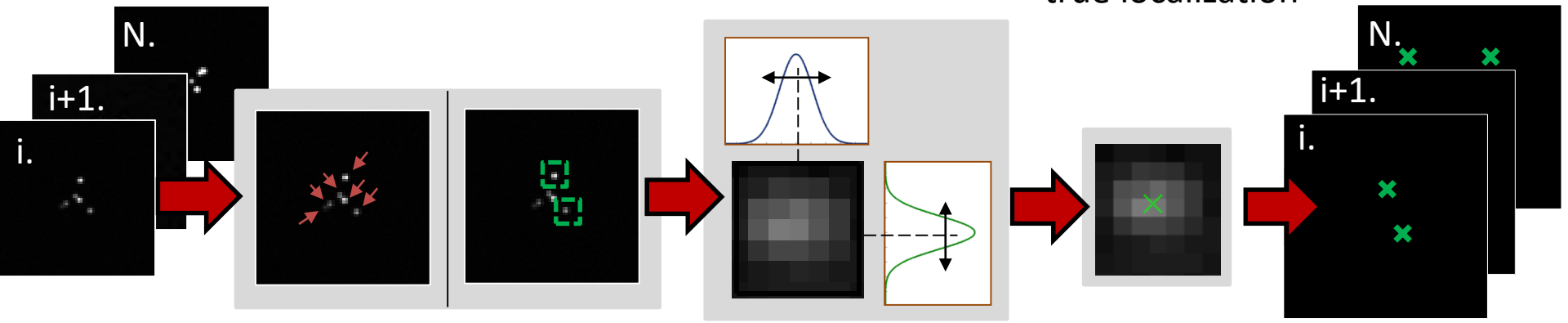
Collection of image stacks

Segmentation

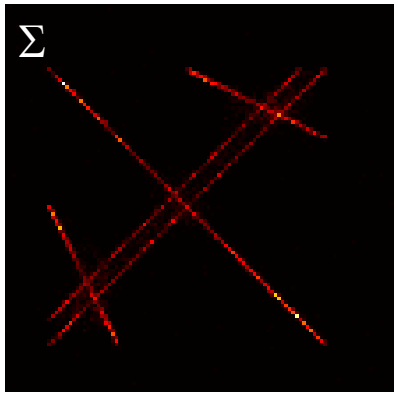
Fitting

Judging, selection of true localization

Storage, Registration



Visualization



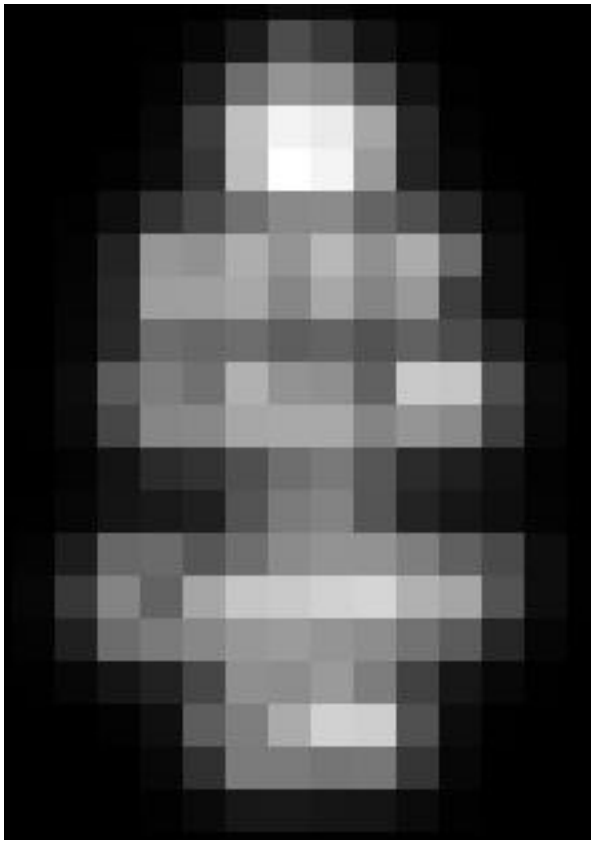
Principles of the localization method - densely labelled sample -

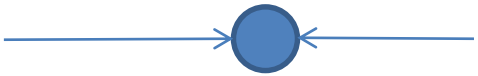
Object

2 μm

Aerial Image

Pixelated Image

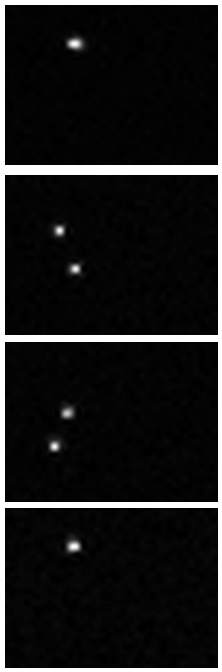




$FWHM_{PSF}$=220nm

Pixel size=160nm

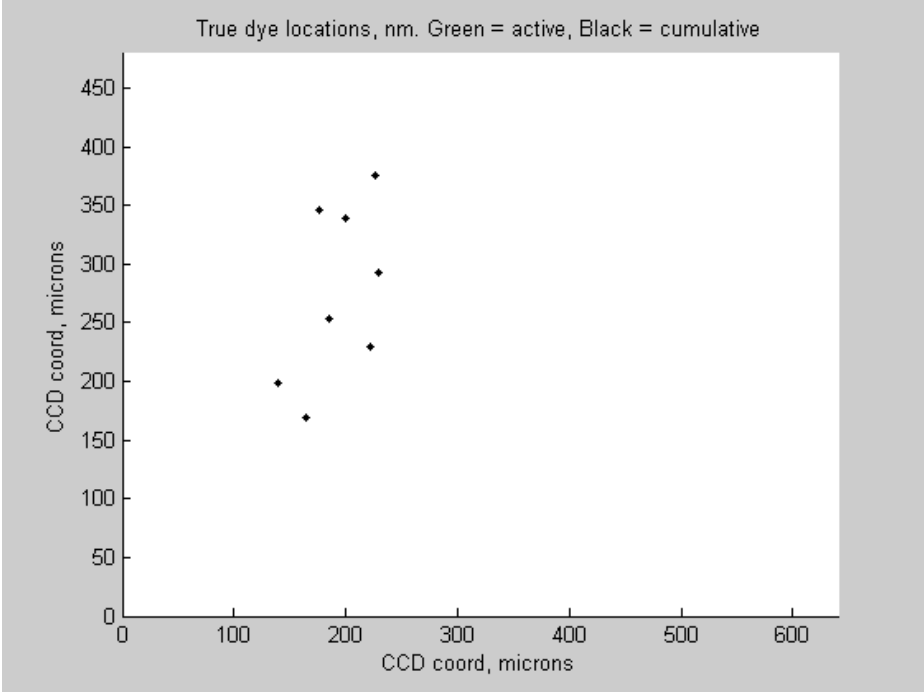
However, if a sparse subset of molecules can be switched on …



…
2000 simulated frames

Simulated Images

… then exact positions of many molecules can be found!



Accuracy of the Gaussian PSF model

Gaussian fitting is applied because:

- It is simple and fast (coordinates can be separated)
- Only the peak position is important ($a \approx s$ => substructure cannot be resolved)
- It is very close to the exact PSF (vector diffraction at high NA)

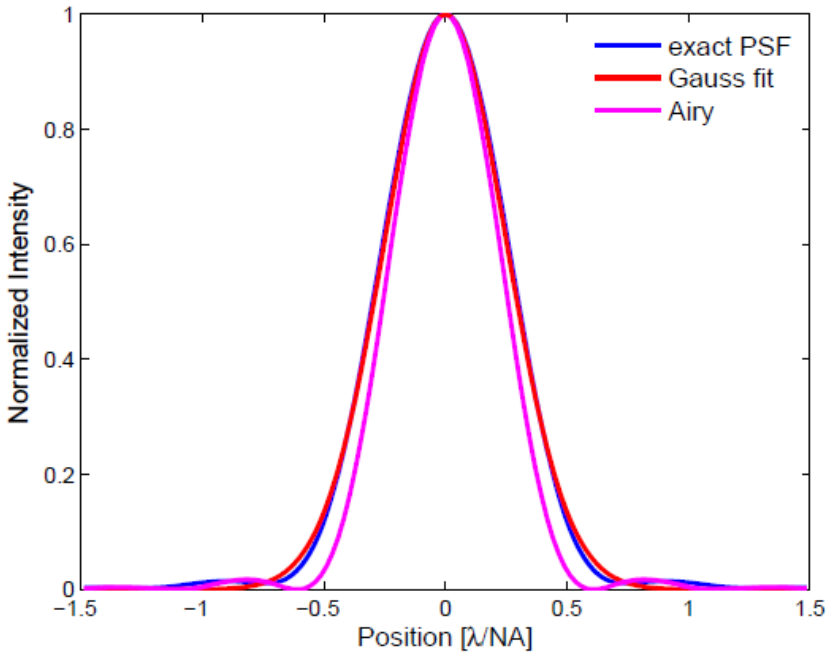
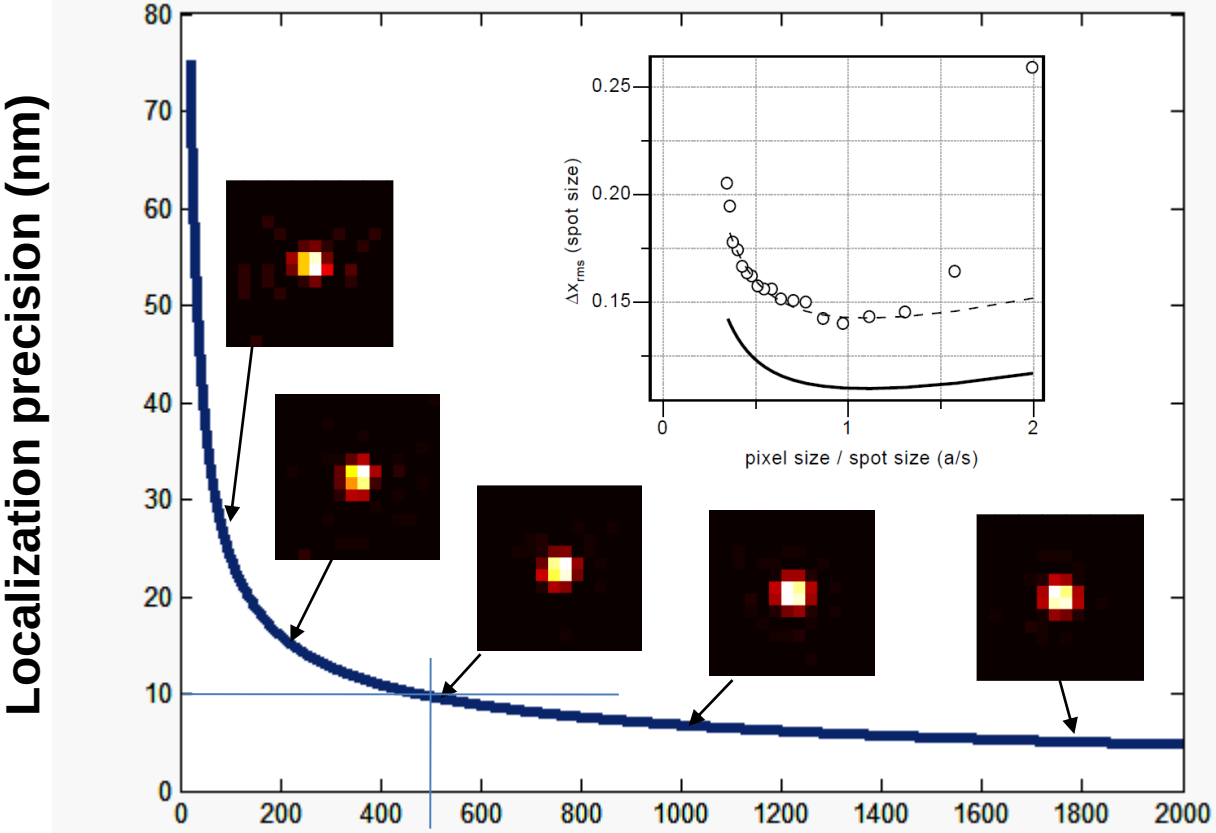


Fig. 1. Dipole orientation averaged PSF for a 100×/1.25 water immersion objective, an Airy-distribution for the same NA, and a Gaussian with standard deviation $\sigma = 0.25\lambda/\mathrm{NA}$.

Localization precision (photon number)

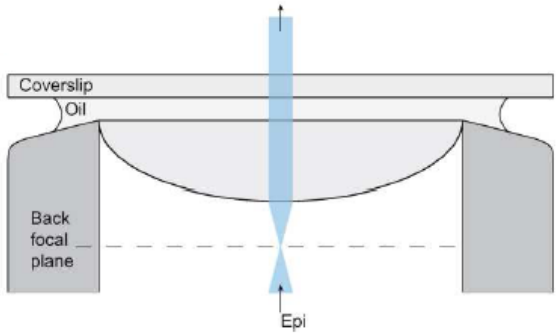
$$\langle (\Delta x)^2 \rangle = \frac{s^2 + a^2/12}{N} + \frac{8\pi s^4 b^2}{a^2 N^2}$$

N: photons
s: PSF size
a: pixel size
b: background noise

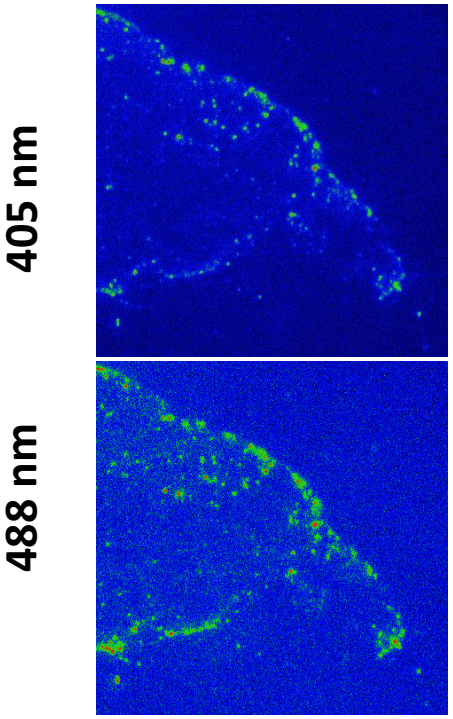


Flurescent background reduction: EPI versus TIRF illumination

EPI

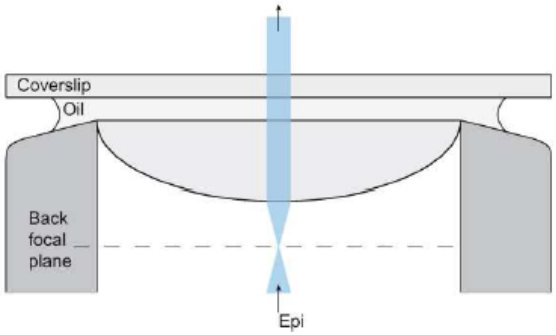


- Excitation beam is focused into the centre of the back focal plane
- The whole volume of the sample is excitated
- Sectioning is limited by DOF ($\propto \lambda/NA^2$)
- High fluorescence background

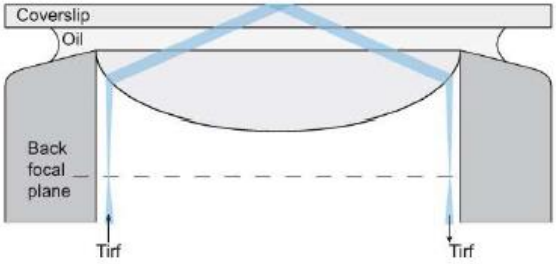


Flurescent background reduction: EPI versus TIRF illumination

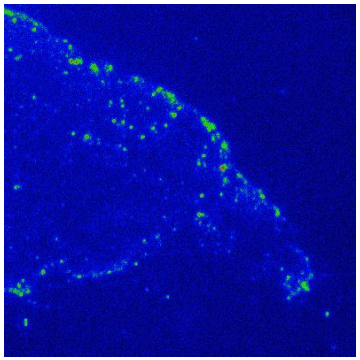
EPI

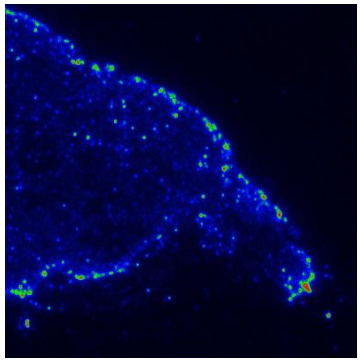


TIRF

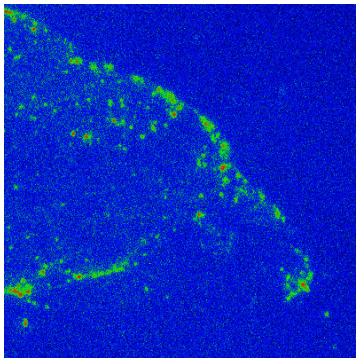


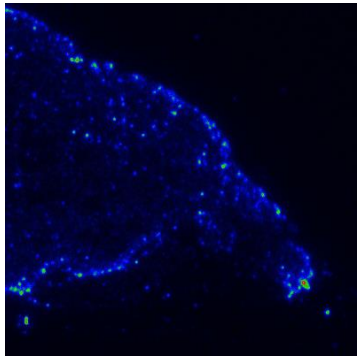
405 nm





488 nm

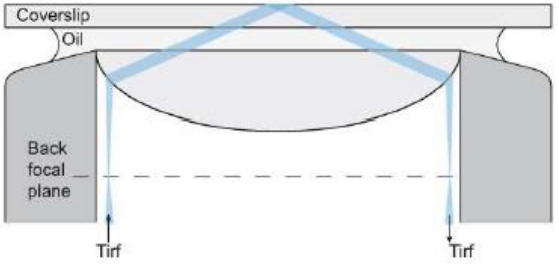


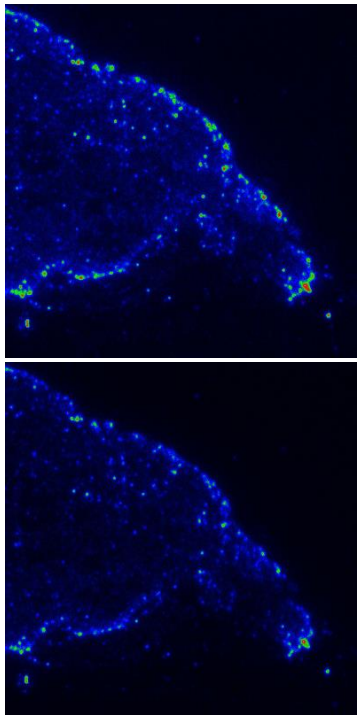


Flurescent background reduction: EPI versus TIRF illumination

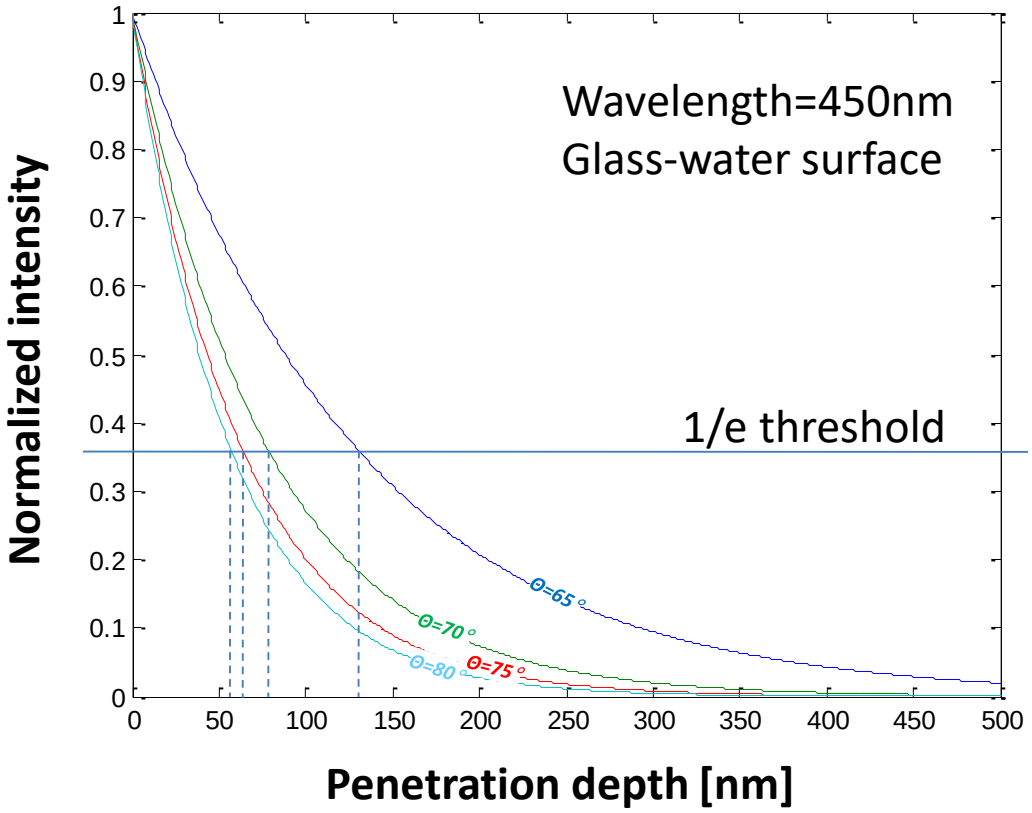
- Excitation beam is focused on the edge of the back focal plane
- Very thin section of the sample is excited
- Sectioning is limited by the penetration depth of the evanescent field (<150nm)
- Reduced fluorescence background

TIRF

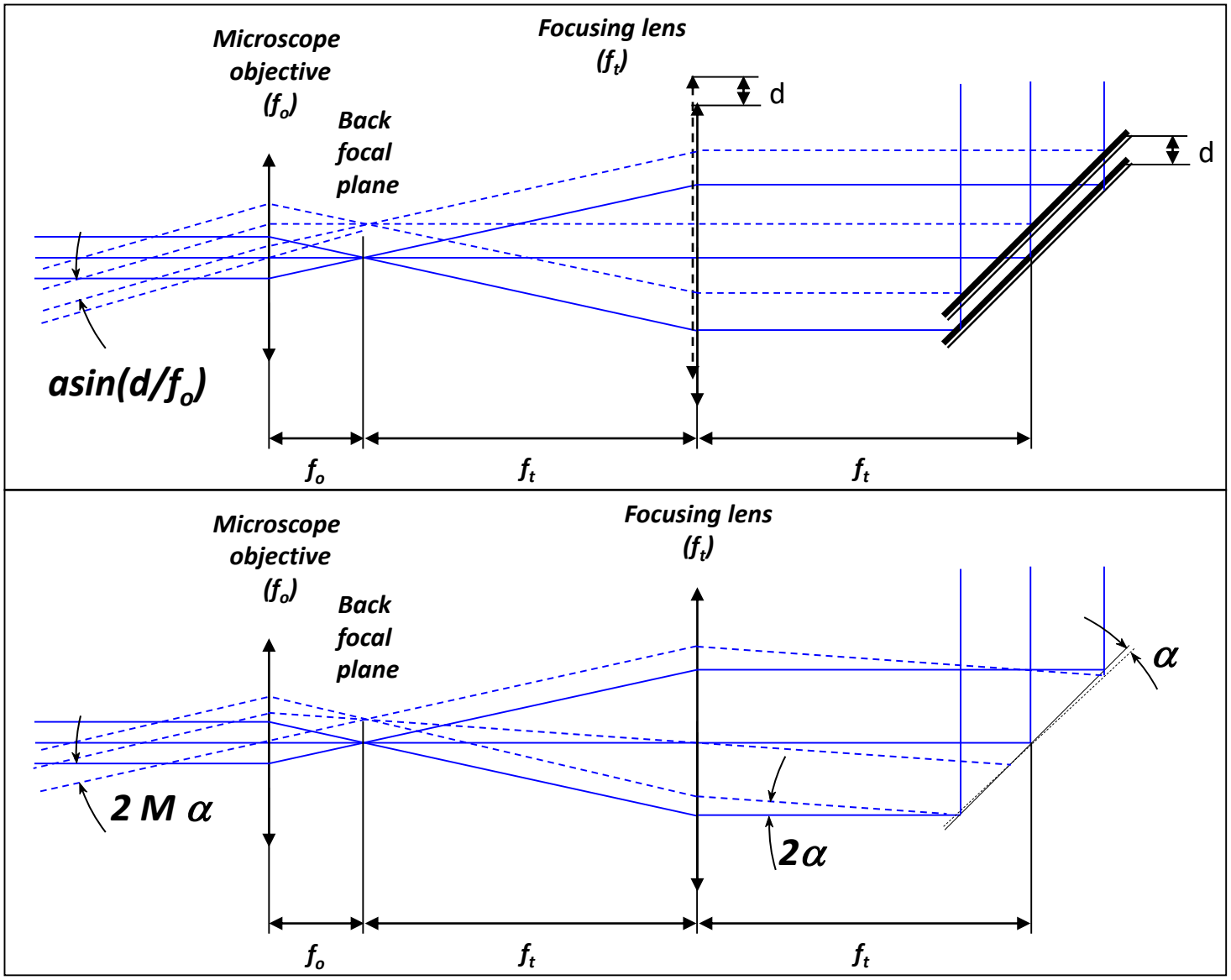




Penetration depth inTIRF mode



Laser illumination (excitation) of the sample: EPI versus TIRF



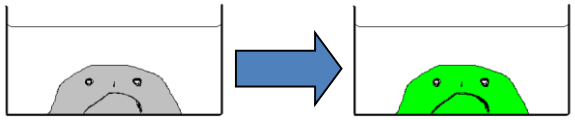
See more during the lab visit!!

Localization microscopy: a five-step experiment

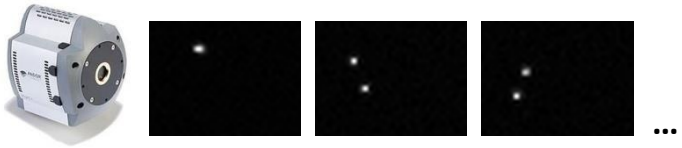
Find a switchable fluorophore

PALM, STORM, dSTORM, FPALM, FIONA, GSDIM …

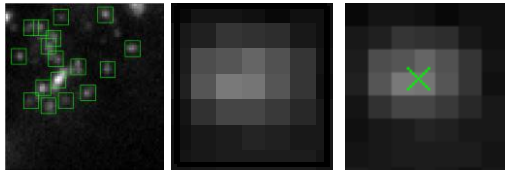
Label some living cells



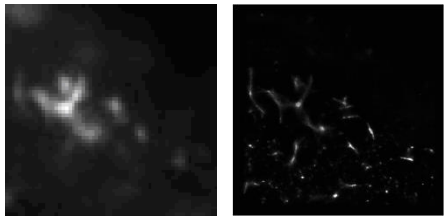
Capture ~10,000 images



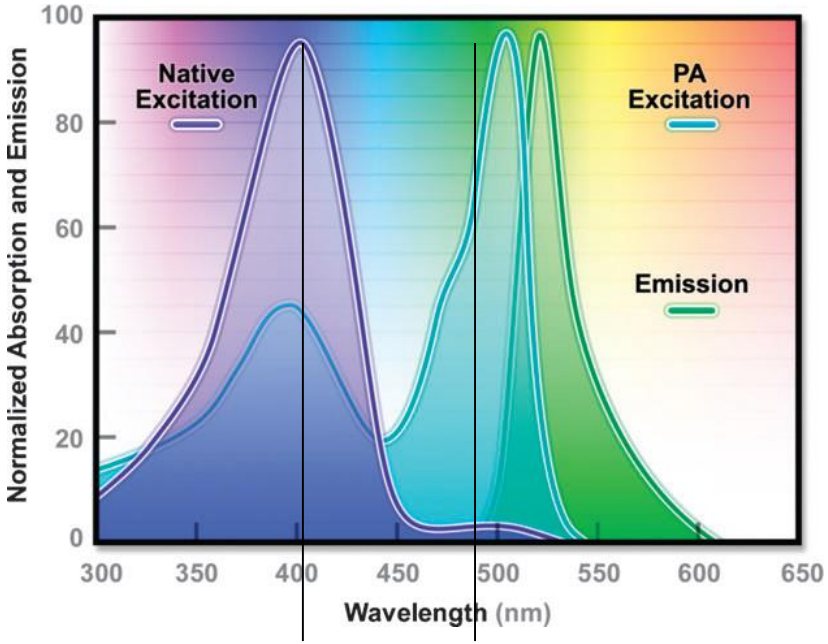
Localize fluorophores



Reconstruct Hi-res image



Photoactivated localization microscopy (PALM)



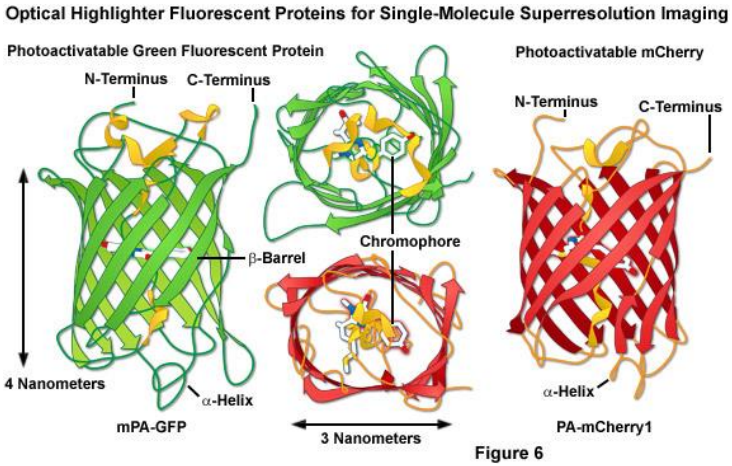
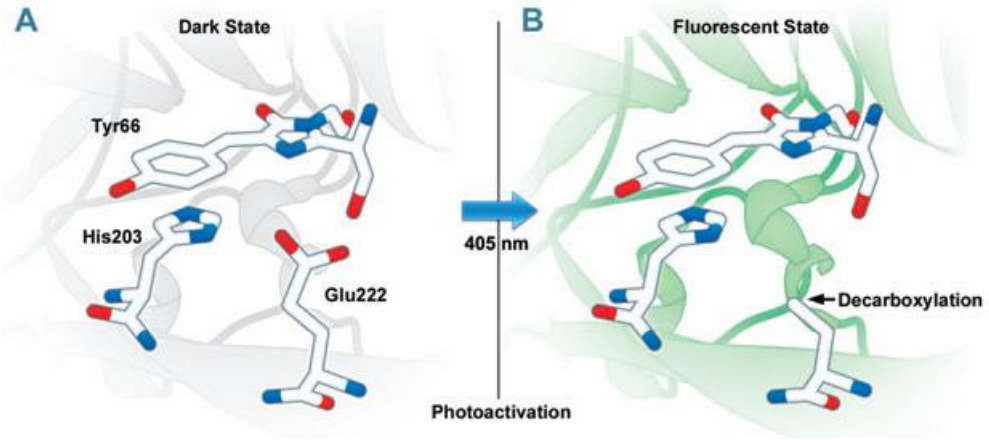
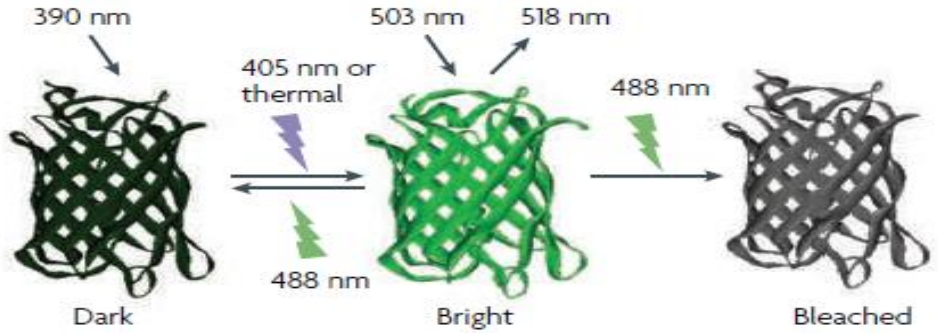


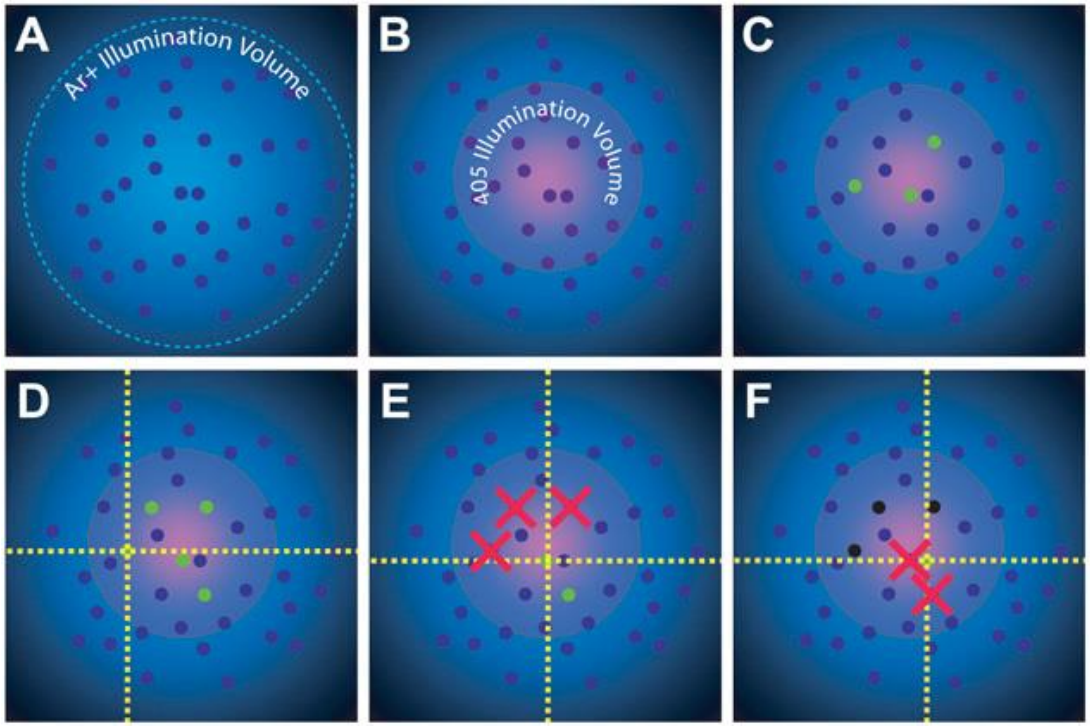
Figure 6



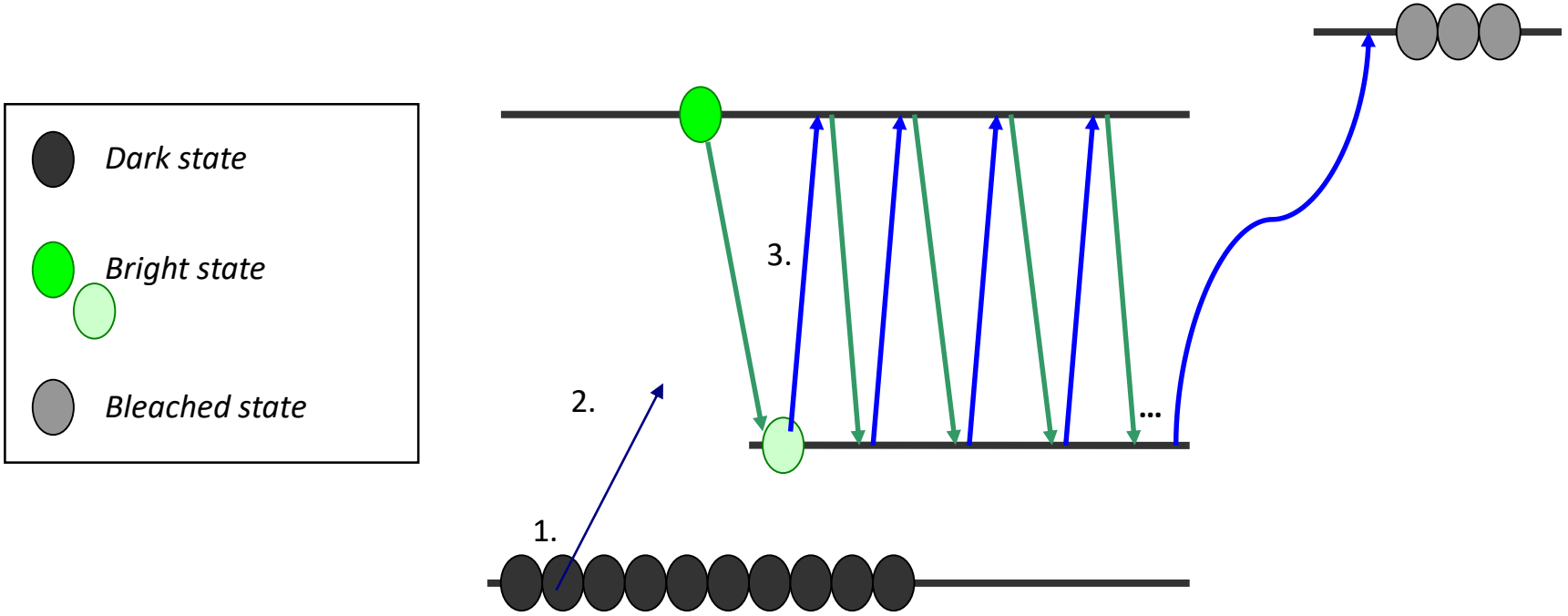
Photoactivated localization microscopy (PALM)



Readout laser (488 nm)			
Activation laser (405 nm)			



Sparse fluorescence by photoactivation



1. All fluorophores are initially “switched off”
2. Activate sparsely with UV
3. Image sparse dyes until they bleach
4. Repeat steps 2 and 3

Photoactivatable fluorescent proteins

List of PAFPs [edit]

PAFP Properties[3]									
PAFP	Absorbance$_1$ (nm)	Emission$_1$ (nm)	Absorbance$_2$ (nm)	Emission$_2$ (nm)	Photoconversion wavelength	Reversibility	Brightness$_1$*	Brightness$_2$*	Reference
Kaede (protein)	508	518	572	580	ultraviolet	none	2.64X	0.60X	[4]
Eos (protein)	506	516	571	581	ultraviolet	none	1.30X	0.70X	[5]
IrisFP	488	516	551	580	ultraviolet	none	0.66X	0.49X	[6]
IrisFP	488	516	390	?	490 nm	reversible, 390 nm	?	?	idem
IrisFP	551	580	440	?	550 nm	reversible, 440 nm	?	?	idem
KikGR/Kikume	507	517	583	593	ultraviolet	none	0.60X	0.64X	[7]
Dronpa	503	518	390	?	490 nm	reversible, 390 nm	?	?	[8]
PAGFP	400	?	504	517	ultraviolet	none	0.08X	0.42X	[9]
PS-CFP	402	468	490	511	ultraviolet	none	0.17X	0.16X	[10]
KFP1	?	?	590	600	green	variable	0.004X	0.13X	
*Brightness values are relative to EGFP.									

Advantages:

High labelling specificity
Life-cell imaging

Limitations:

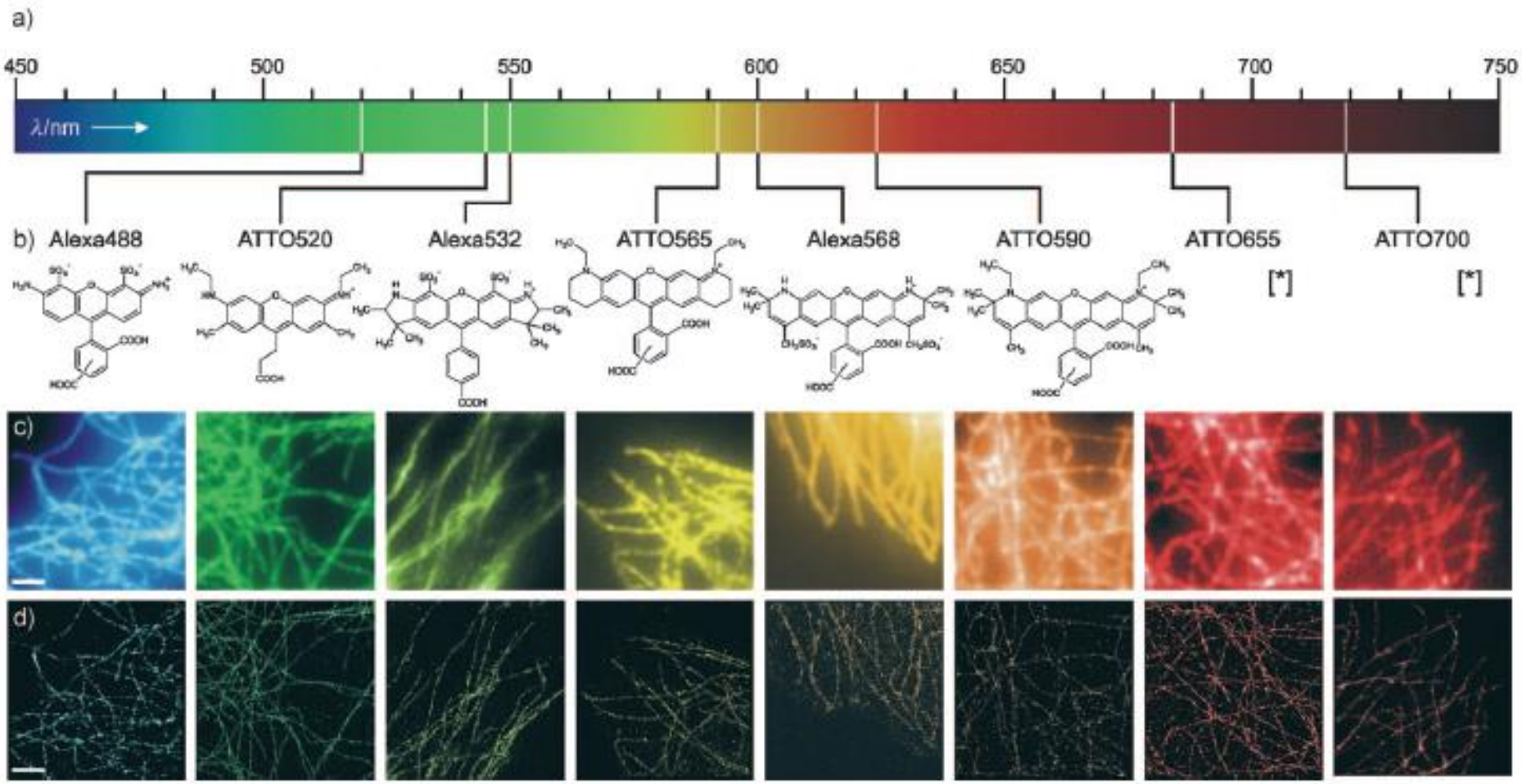
low quantum efficiency
Complicated labelling procedure

William E. Moerner

Eric Betzig

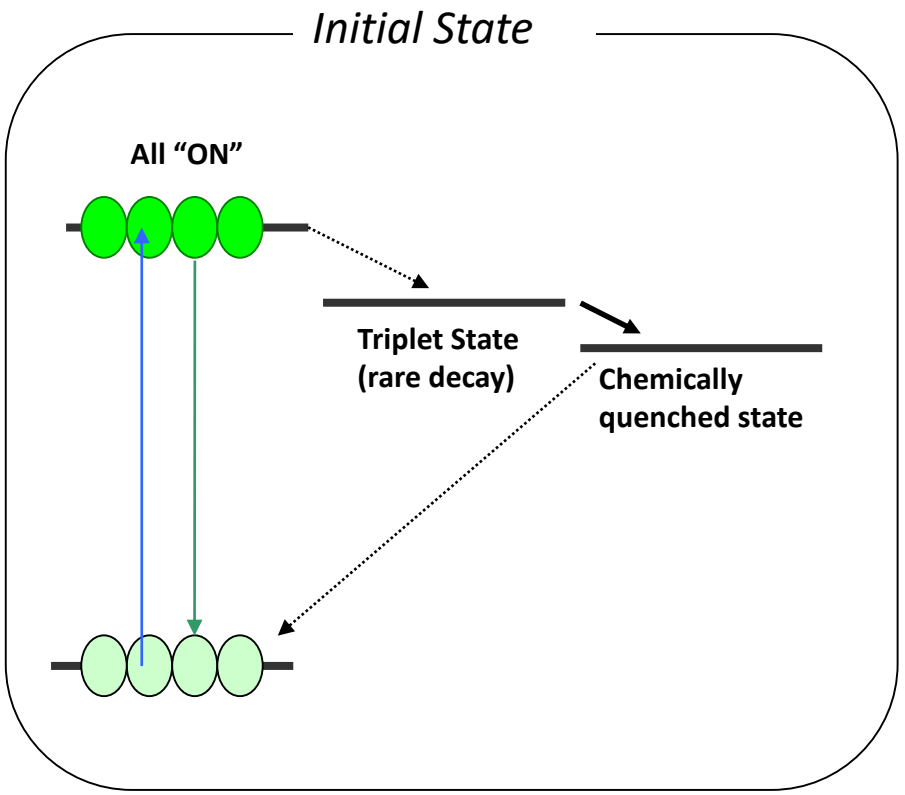
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, William E. Moerner and Stefan W. Hell *"for the development of super-resolved fluorescence microscopy"*.

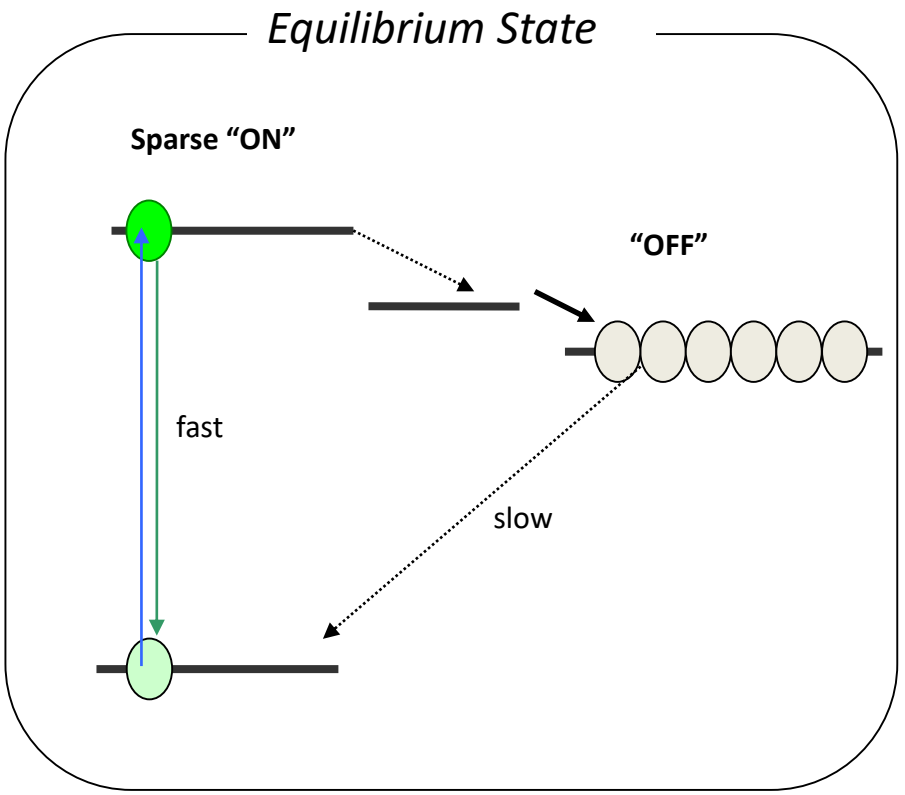
Direct stochastic optical reconstruction microscopy (dSTORM)



M. Heilemann, ... M. Sauer, *Angew. Chem. Int.*, 48, 6903 (2009)

Sparse fluorescence by quenching





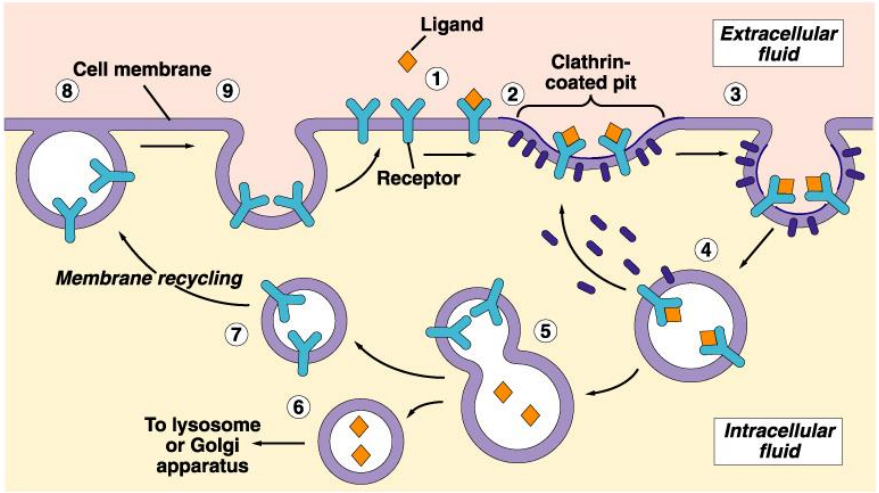
1. All fluorophores are initially "switched on"
2. Triplet quenching
3. Slow reactivation
4. … Sparse "on" population

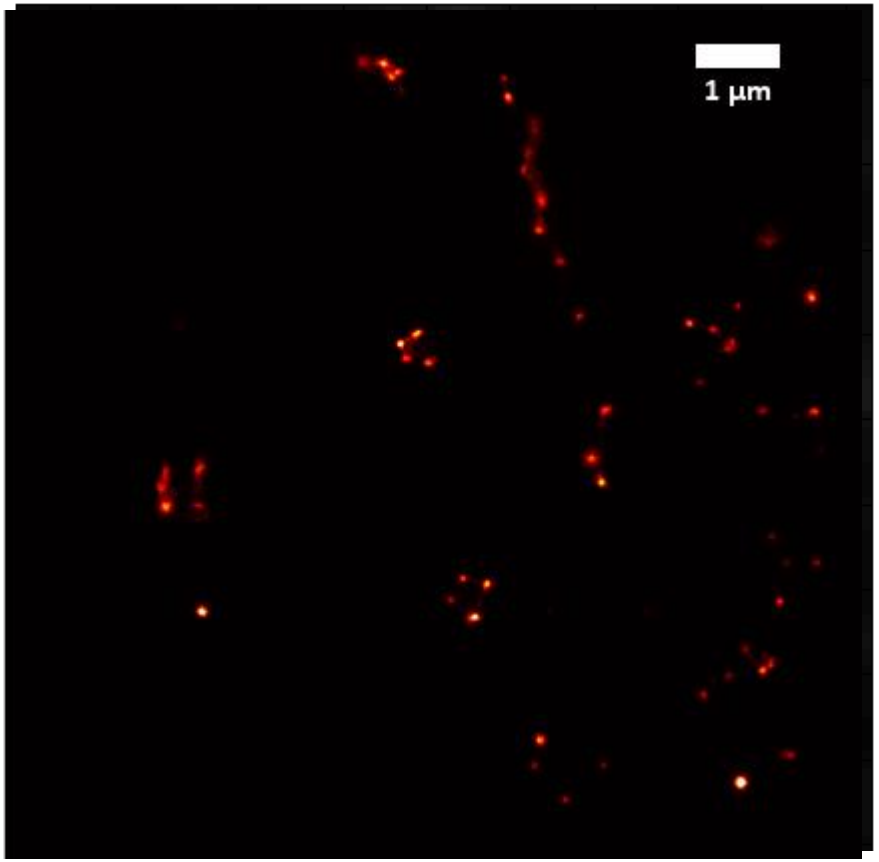
1-5 kW/cm^2

STORM dyes

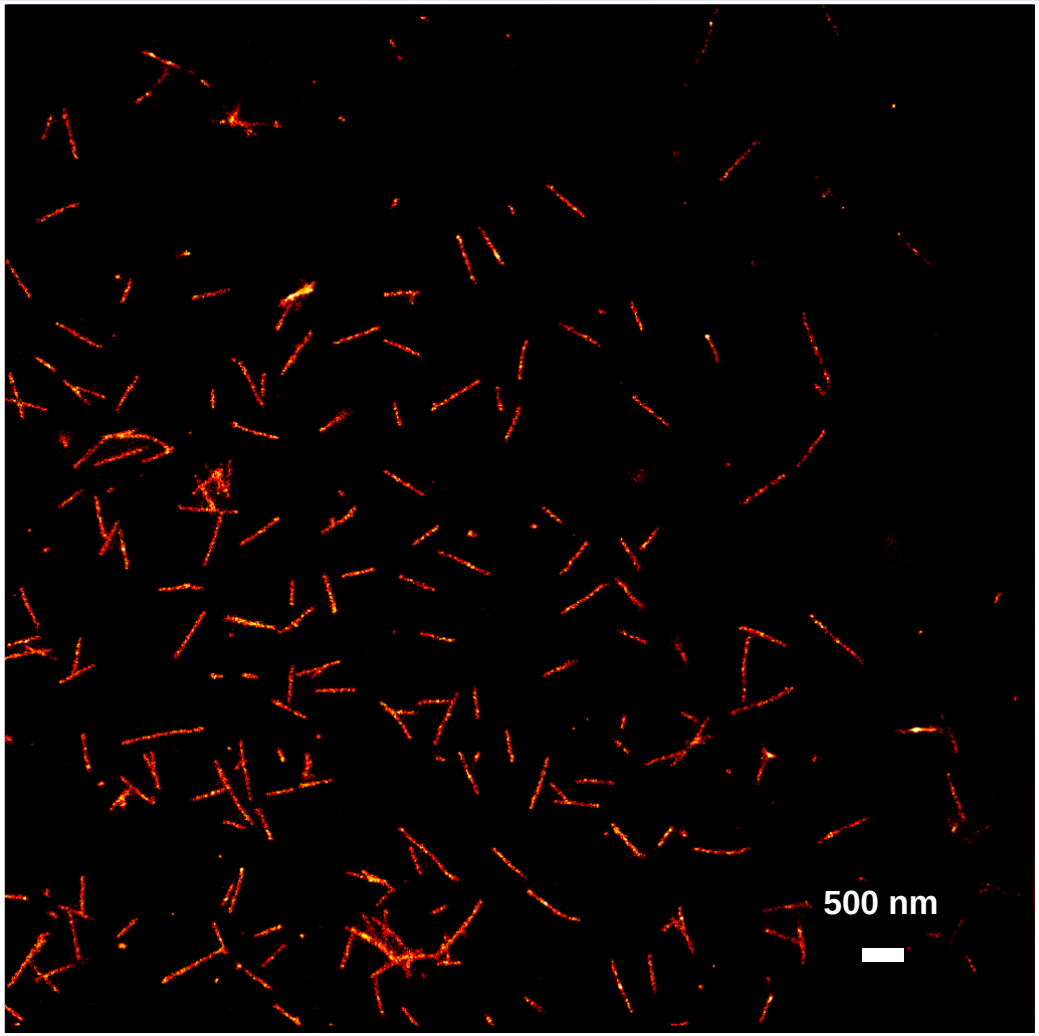
Dye		Excitation Maximum (nm)	Emission Maximum (nm)	Extinction (M^{-1} cm^{-1})	Quantum Yield	Detected Photons Per Cycle		Equilibrium Duty Cycle		Survival Fraction (400 s)		# Switching Cycles (Mean)	
						MEA	βME	MEA	βME	MEA	βME	MEA	βME
Blue-absorbing	Atto 488	501	523	90,000	0.8	1,341	1,110	0.00065	0.0022	0.98	0.99	11	49
	Alexa 488	495	519	71,000	0.92	1,193	427	0.00055	0.0017	0.94	1	16	139
	Atto 520	516	538	110,000	0.9	1,231	868	0.0015	0.00061	0.92	0.86	9	17
	Fluorescein	494	518	70,000	0.79	1,493	776	0.00032	0.00034	0.51	0.83	4	15
	FITC	494	518	70,000	0.8	639	1,086	0.00041	0.00031	0.75	0.9	17	16
	Cy2	489	506	150,000	0.12	6,241	4,583	0.00012	0.00045	0.12	0.19	0.4	0.7
Yellow-absorbing	Cy3B	559	570	130,000	0.67	1,365	2,057	0.0003	0.0004	1	0.89	8	5
	Alexa 568	578	603	91,300	0.69	2,826	1,686	0.00058	0.0027	0.58	0.99	7	52
	TAMRA	546	575	90,430	0.2	4,884	2,025	0.0017	0.0049	0.85	0.99	10	59
	Cy3	550	570	150,000	0.15	11,022	8,158	0.0001	0.0003	0.17	0.55	0.5	1.6
	Cy3.5	581	596	150,000	0.15	4,968	8,028	0.0017	0.0005	0.89	0.61	5.7	3.3
	Atto 565	563	592	120,000	0.9	19,714	13,294	0.00058	0.00037	0.17	0.26	4	5
Red-absorbing	Alexa 647	650	665	239,000	0.33	3,823	5,202	0.0005	0.0012	0.83	0.73	14	26
	Cy5	649	670	250,000	0.28	4,254	5,873	0.0004	0.0007	0.75	0.83	10	17
	Atto 647	645	669	120,000	0.2	1,526	944	0.0021	0.0016	0.46	0.84	10	24
	Atto 647N	644	669	150,000	0.65	3,254	4,433	0.0012	0.0035	0.24	0.65	9	39
	Dyomics 654	654	675	220,000	–	3,653	3,014	0.0011	0.0018	0.79	0.64	20	19
	Atto 655	663	684	125,000	0.3	1,105	657	0.0006	0.0011	0.65	0.78	17	22
	Atto 680	680	700	125,000	0.3	1,656	987	0.0019	0.0024	0.65	0.91	8	27

Clathrin mediated endocitosis (EGF labelled by Alexa 647)





$A\beta_{42}$ fibrils in vitro (labelled by Alexa 647)



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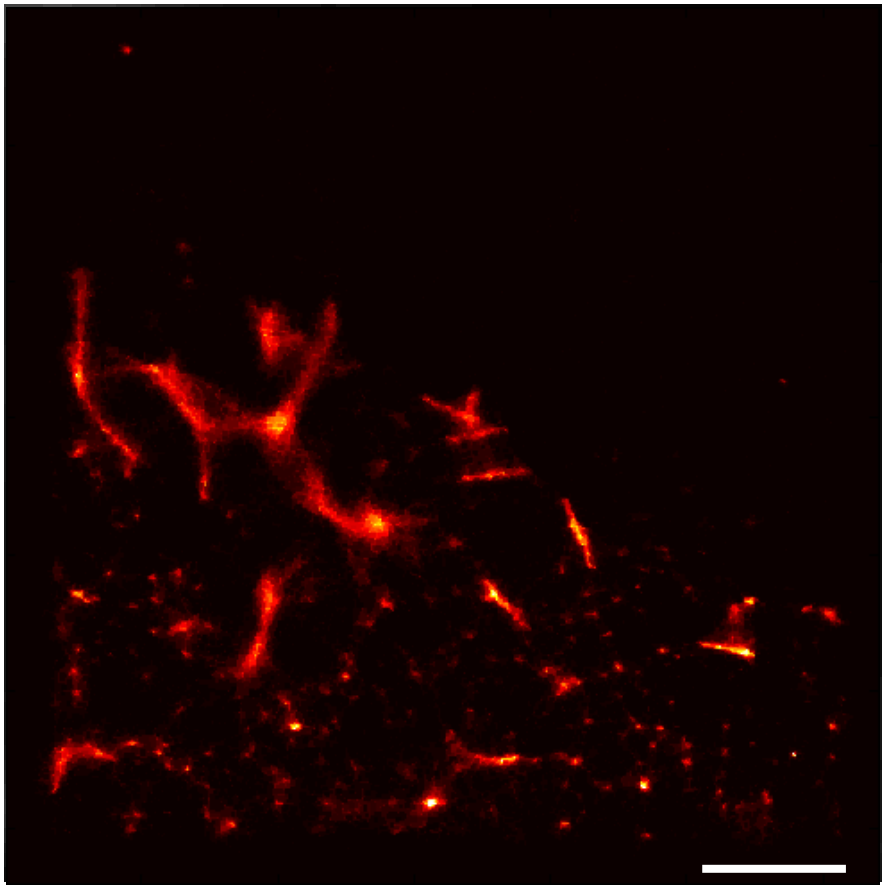
In Situ Measurements of the Formation and Morphology of Intracellular β-Amyloid Fibrils by Super-Resolution Fluorescence Imaging

Gabriele S. Kaminski Schierle,[†] Sebastian van de Linde,[‡] Miklos Erdelyi,[†,∥] Elin K. Esbjörner,[§] Teresa Klein,[‡] Eric Rees,[†] Carlos W. Bertoncini,[⊥] Christopher M. Dobson,[§] Markus Sauer,[*,‡] and Clemens F. Kaminski[*,†,¶]

$A\beta_{42}$ fibrils in cell (labelled by Alexa 647)



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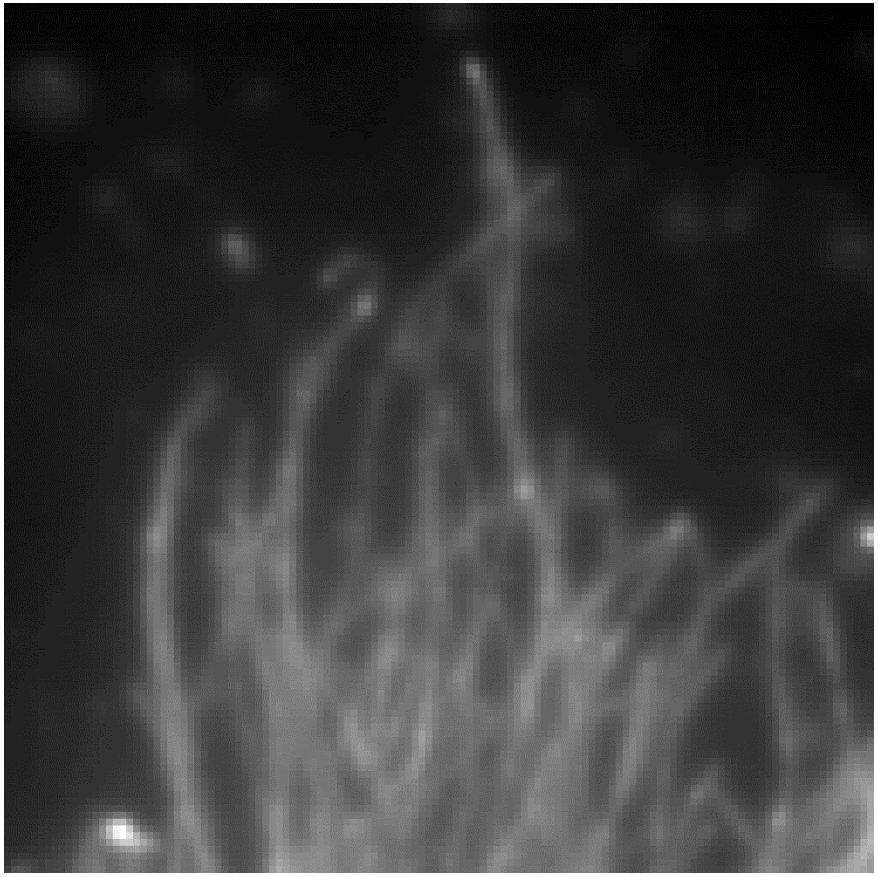
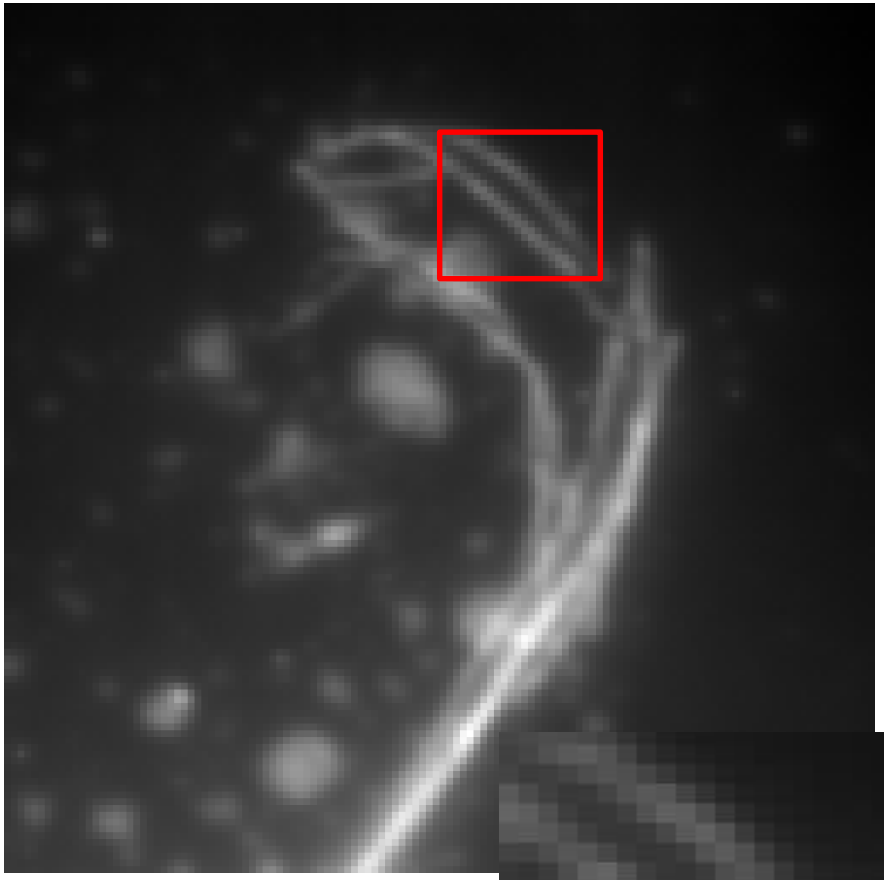
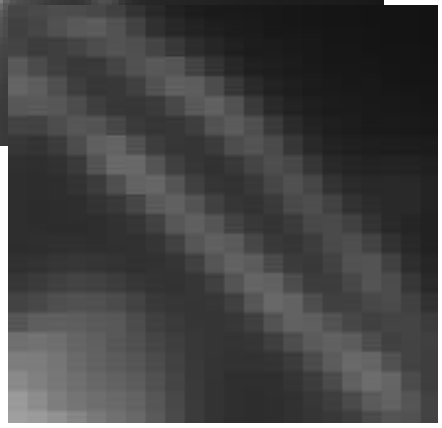
Microtubules in cells







Issues – labelling -

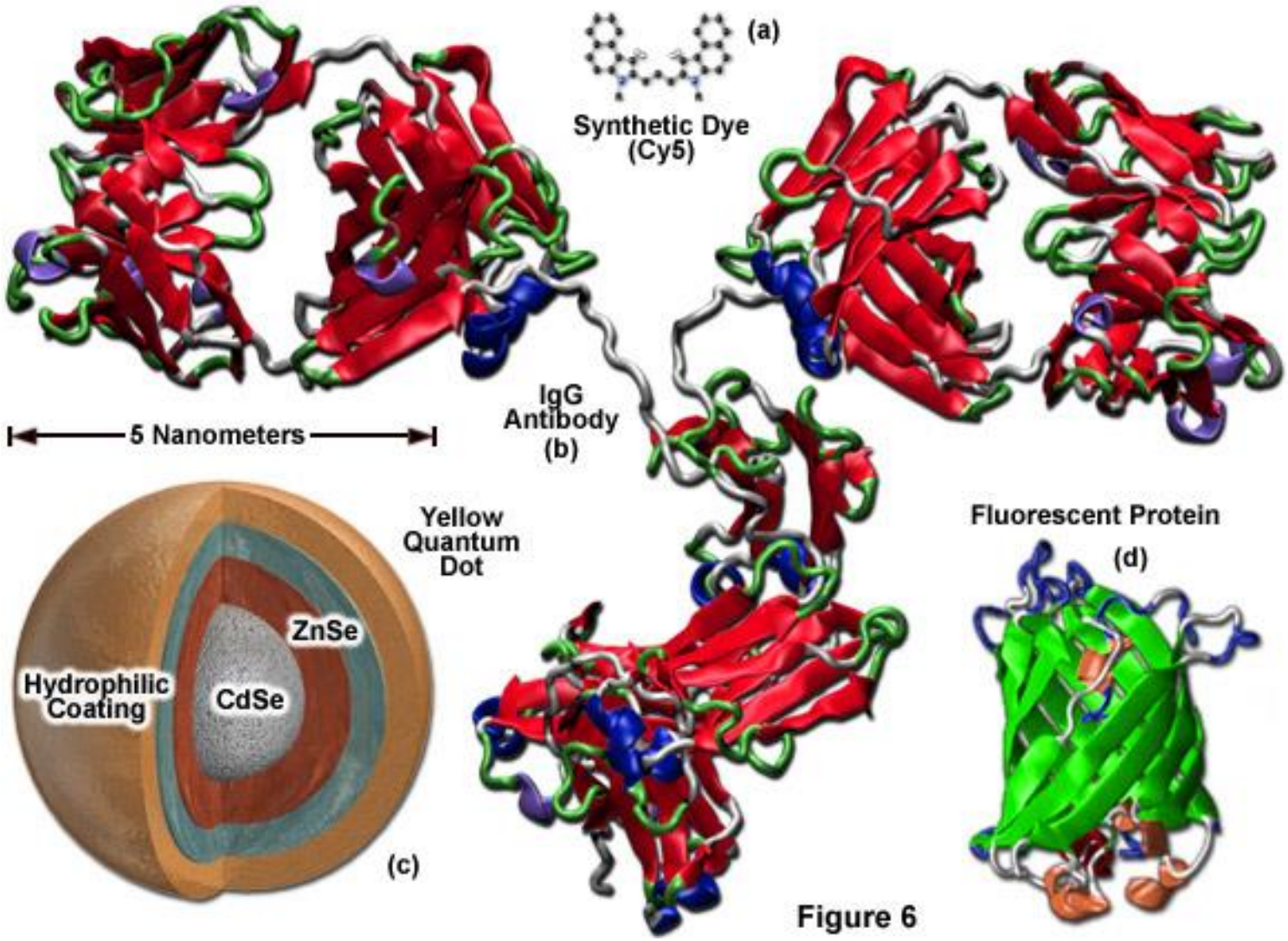
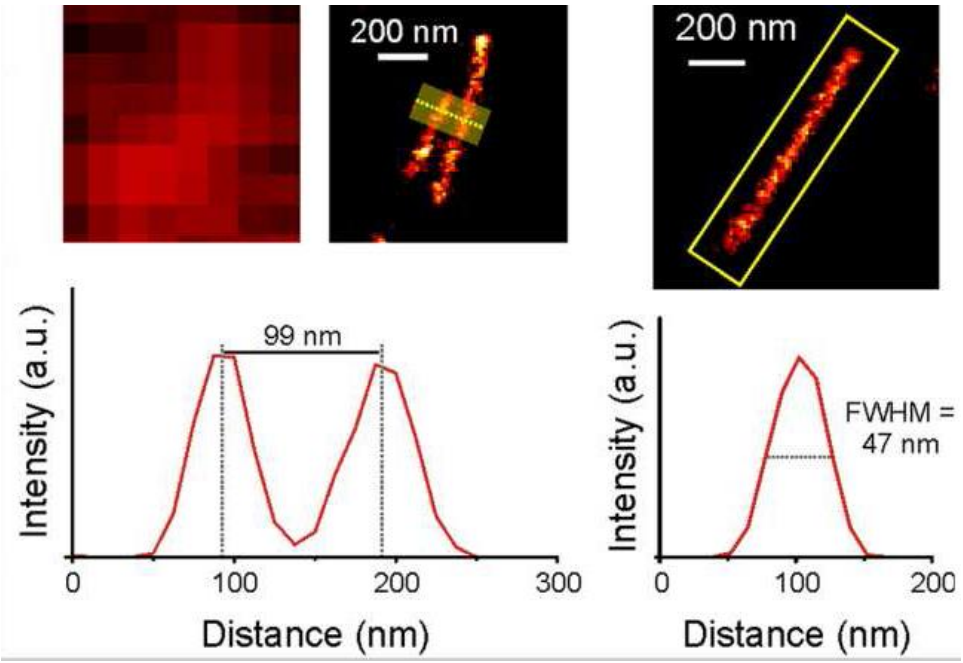


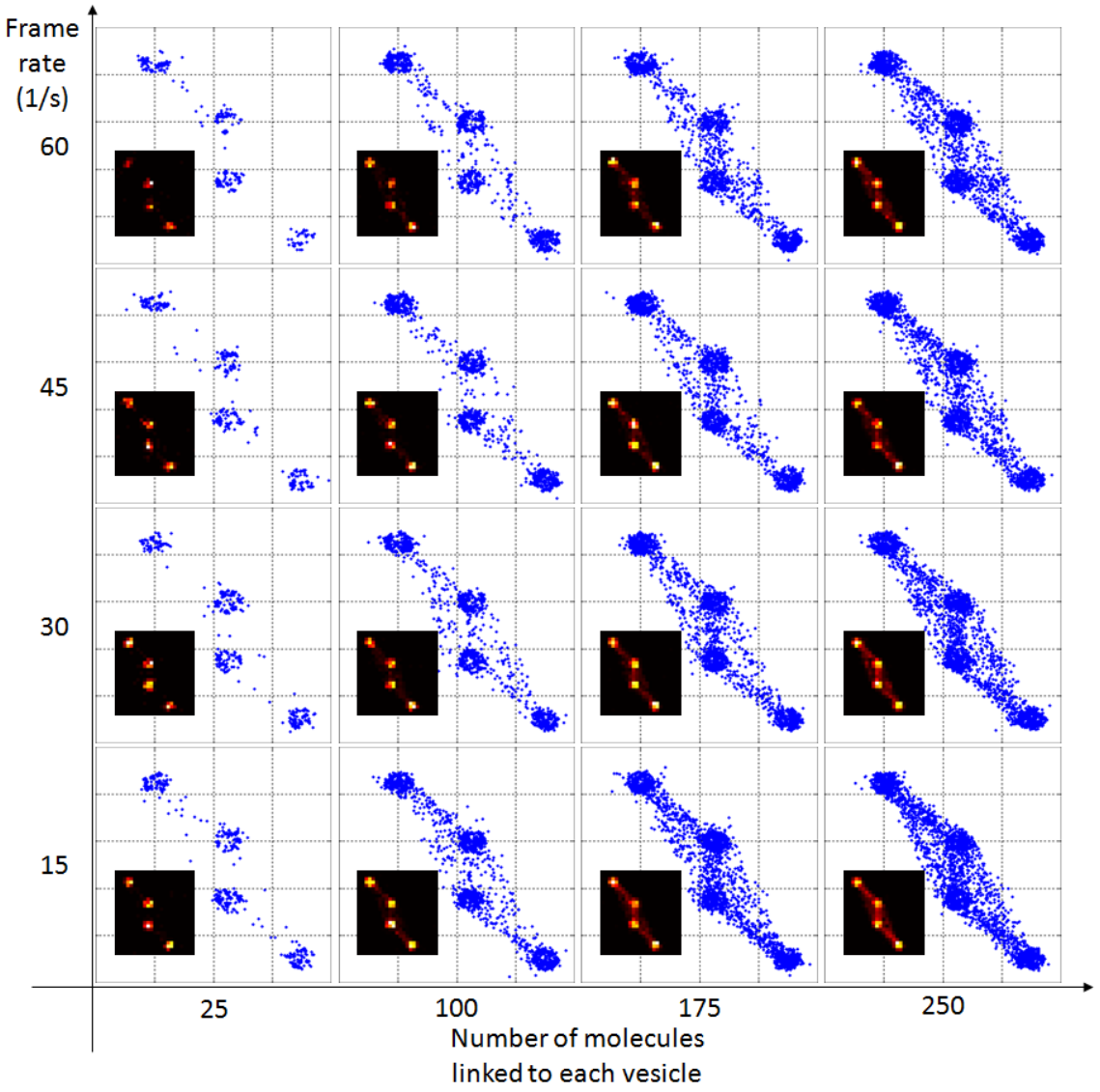
Figure 6

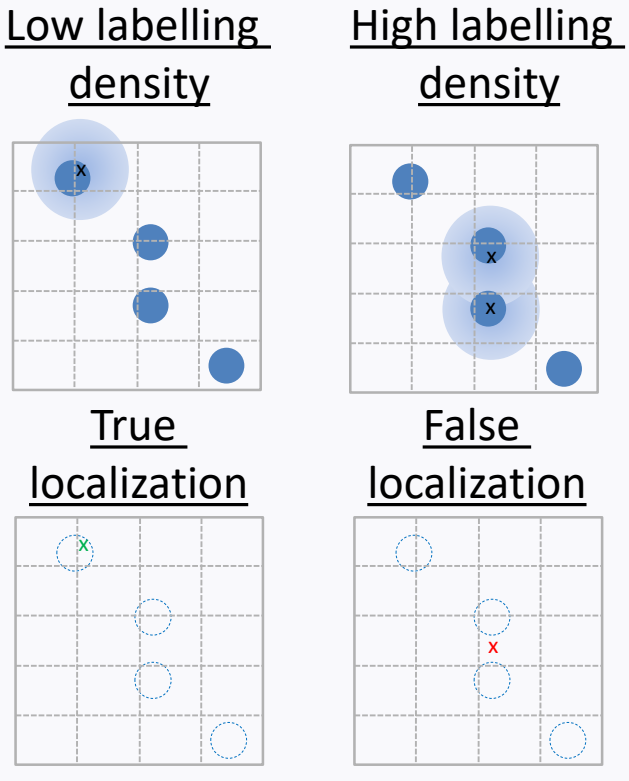
Issues – labelling -

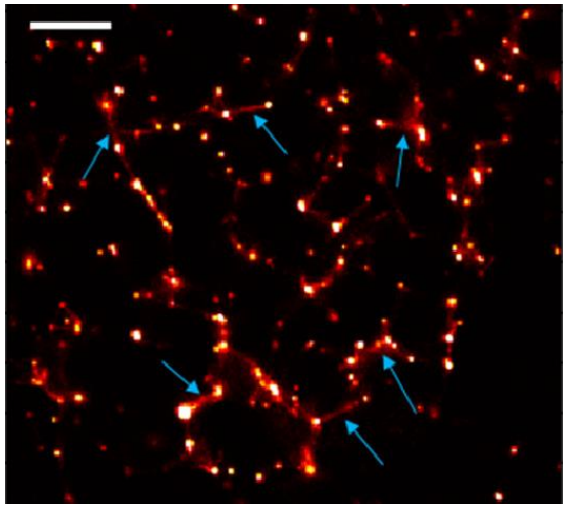


Short linkers and small dye molecules are required!

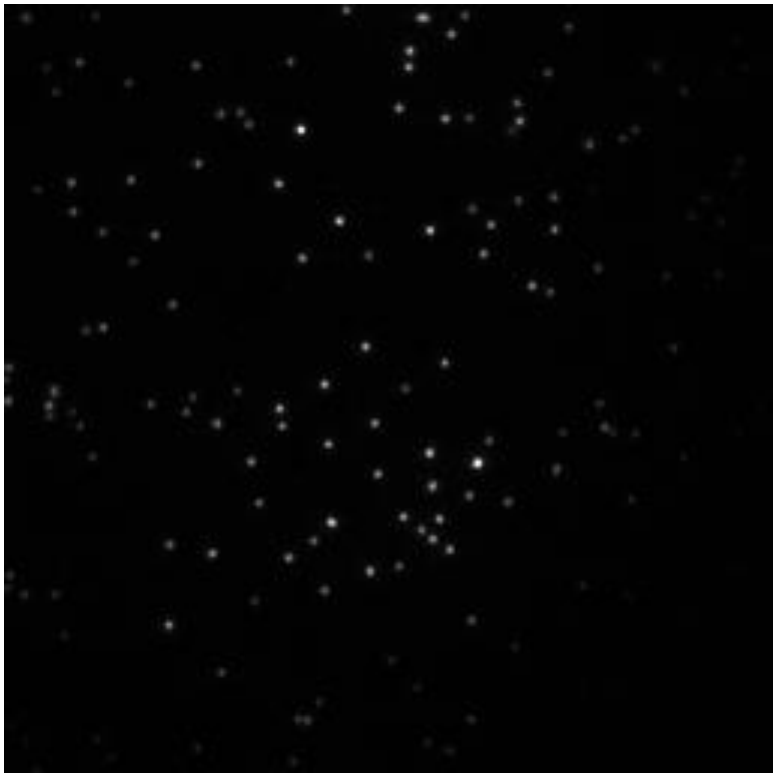
Issues – labelling density –

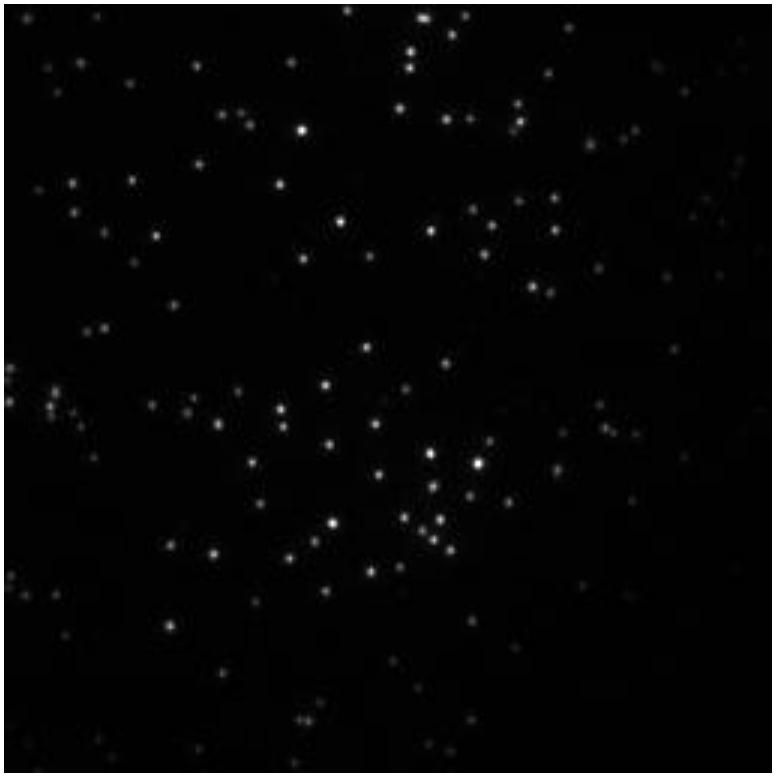






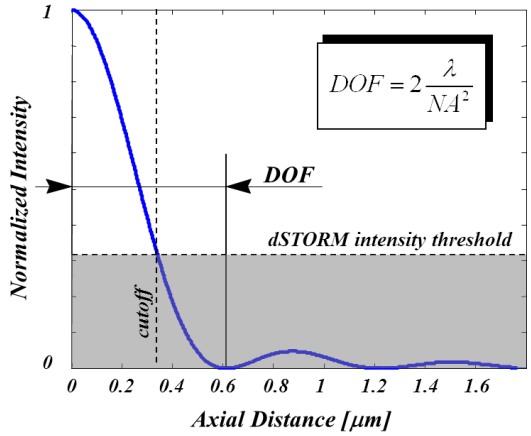
Issues – mechanical drift -



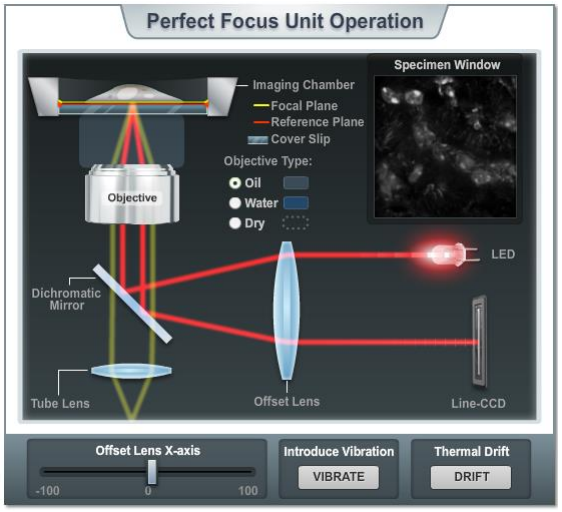


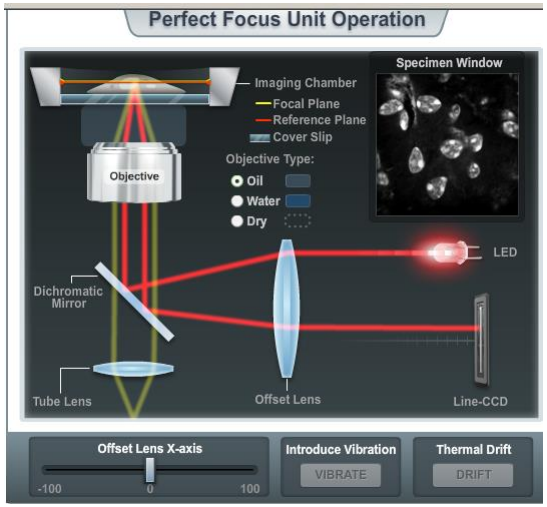
260nm correction

Sample: fluorescent beads
Sampling frequency: 1 frame /s
Duration: 20min



Autofocus system using a separated illumination port (Nikon PFS)





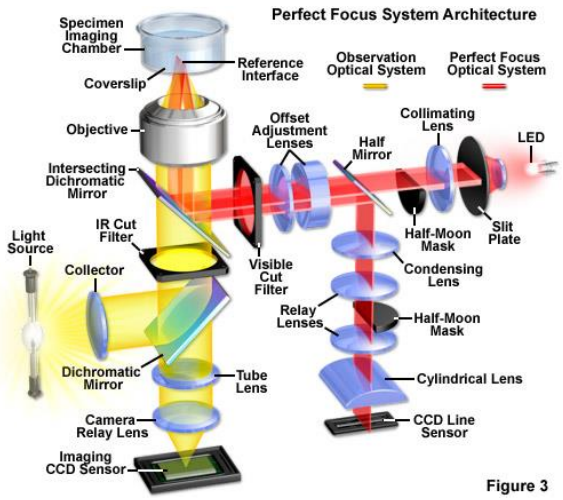
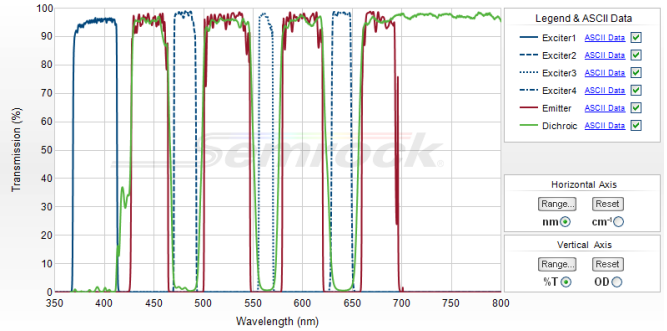


Figure 3

http://www.microscopyu.com/tutorials/flash/focusdrift/perfectfocus/index.html

LF405/488/561/635-4X-A-000



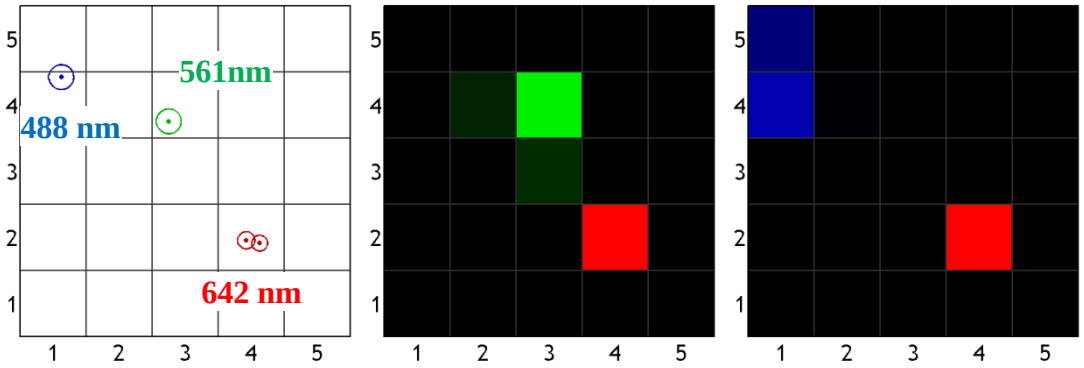
- In Nikon this is a separated channel from the excitation path (cannot be directly implemented into IX71 frame)
- Requires a dichroic mirror that reflect at the applied wavelength (around 870nm)

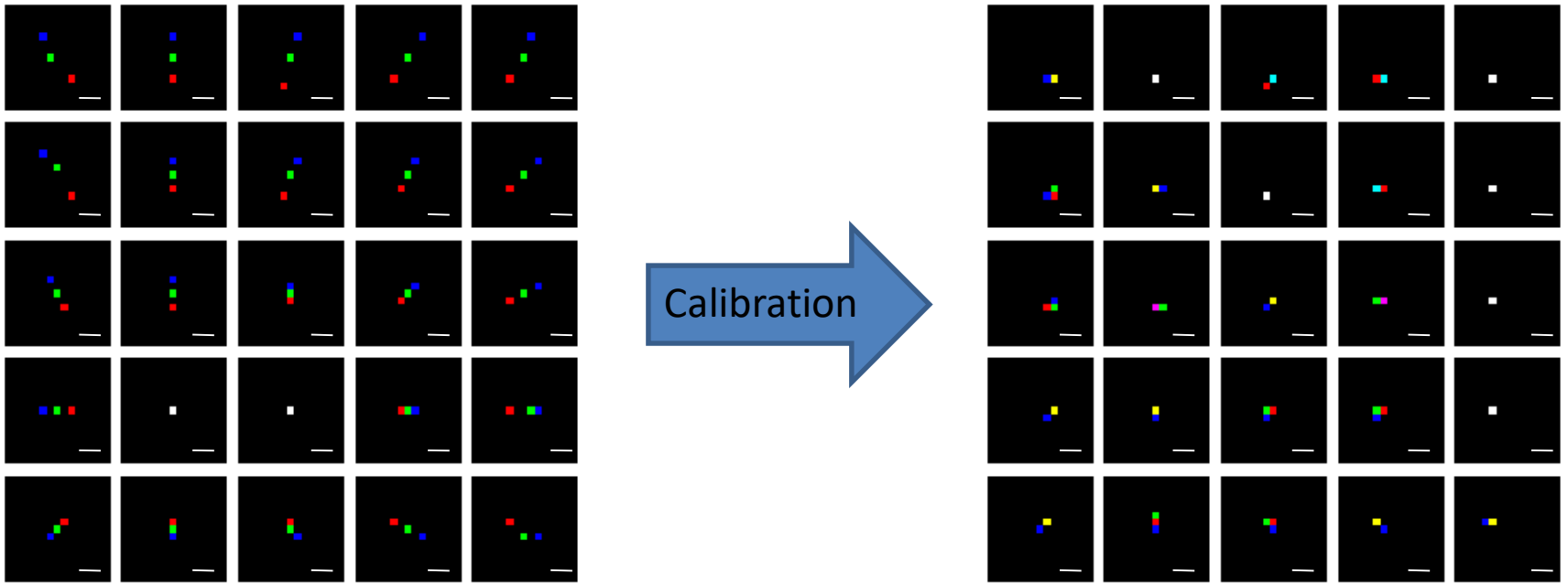
Under ideal circumstances, the focusing precision of the PFS is usually less than one third of the objective focal depth. (http://www.microscopyu.com/articles/livecellimaging/perfectfocus.html)

See more during the lab visit!!

Issues - chromatic optical offset -

(CCD pixel)





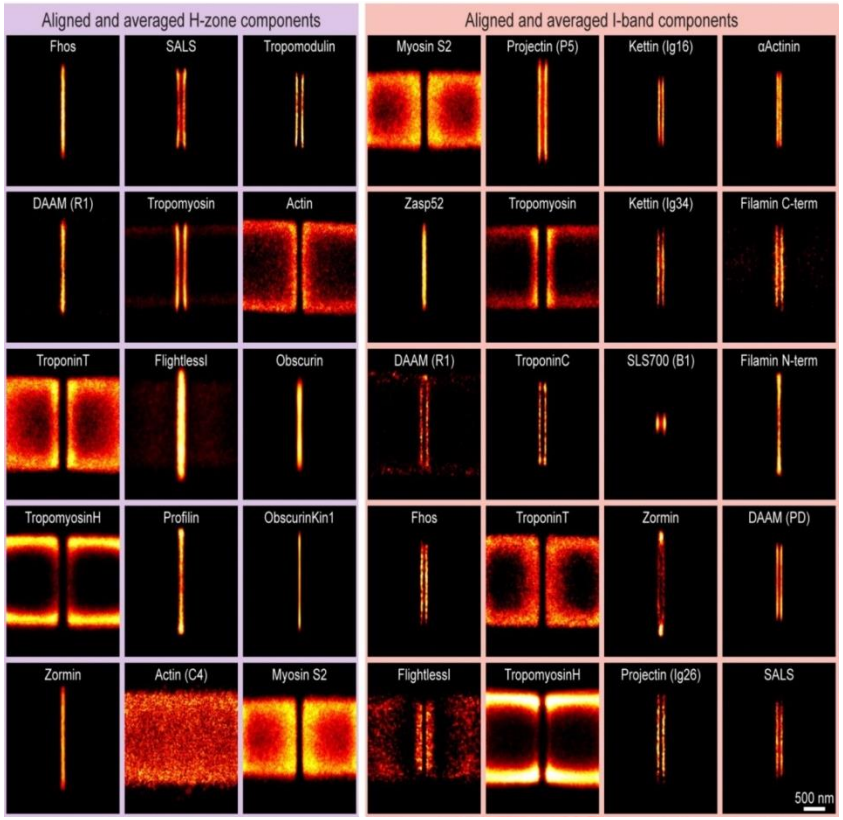
Super-resolution microscopy roadmap

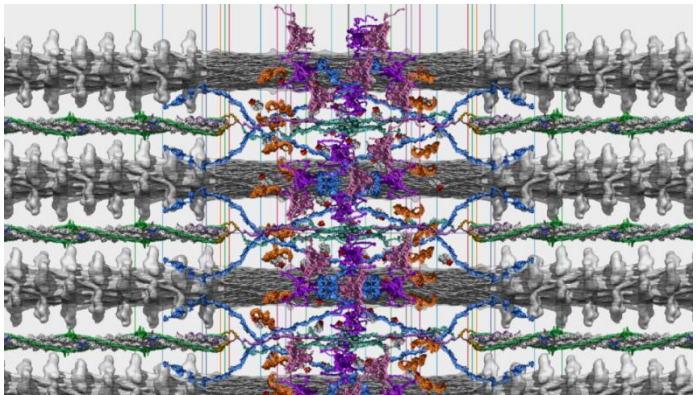
Stefan W Hell[2,3], Steffen J Sahl[2], Mark Bates[2], Xiaowei Zhuang[4], Rainer Heintzmann[5,6,7], Martin J Booth[8,9], Joerg Bewersdorf[10,11], Gleb Shtengel[12], Harald Hess[12], Philip Tinnefeld[13], Alf Honigmann[14], Stefan Jakobs[2,16], Ilaria Testa[15,17], Laurent Cognet[18], Brahim Lounis[18], Helge Ewers[19], Simon J Davis[1], Christian Eggeling[1], David Klenerman[20], Katrin I Willig[21], Giuseppe Vicidomini[22], Marco Castello[22], Alberto Diaspro[22] and Thorben Cordes[23]

Contents

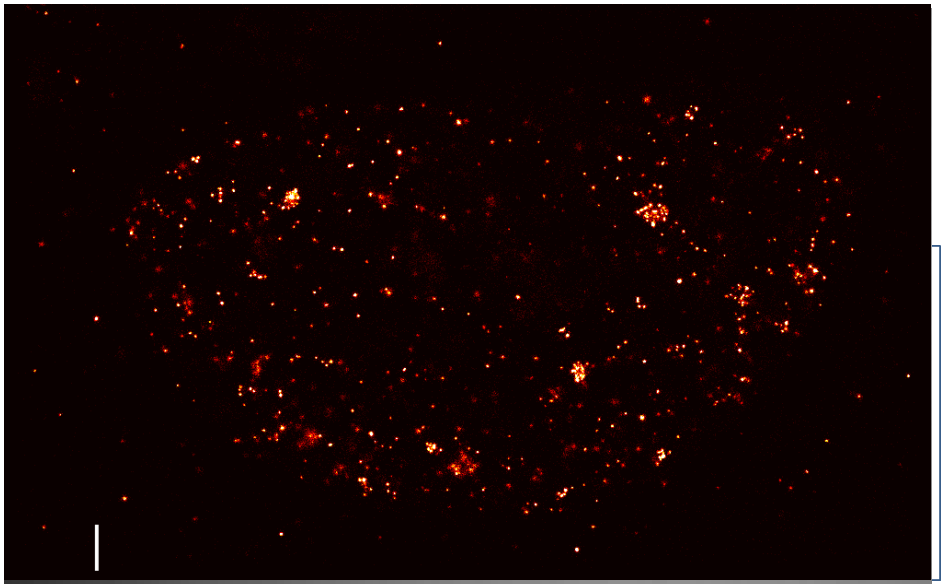
Quantitative STORM

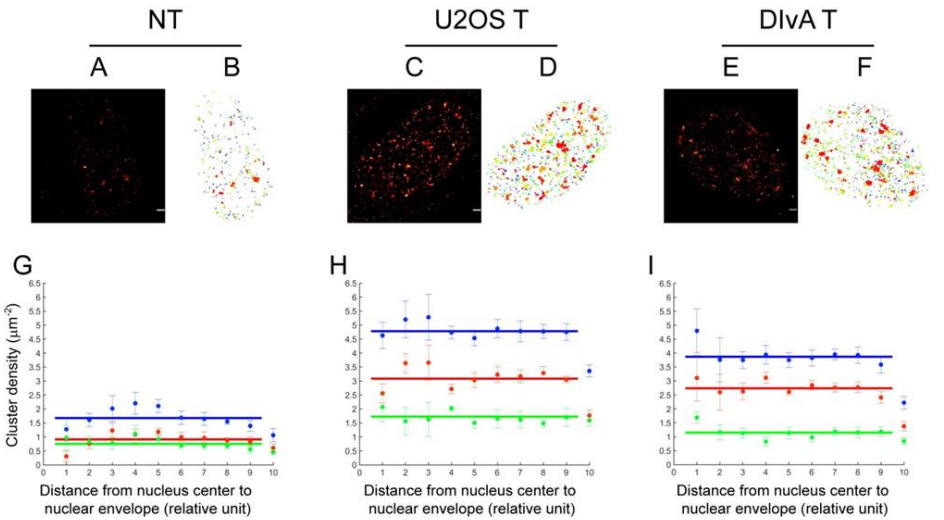
- Geometrical parameters





- Number of target molecules





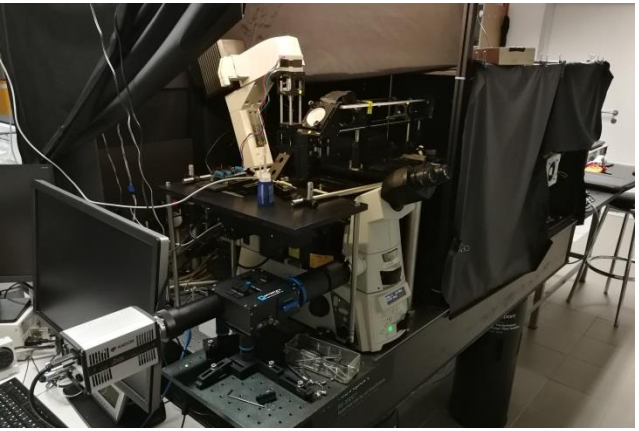
Conclusions

- The image of a point-like source (PSF) has spatial extent because of diffraction
- Spatial resolution is limited by the wavelength and NA
- Sub-diffraction imaging requires special super-resolution methods
- Super-resolution can be achieved via localization method
- Switchable fluorescent molecules are required for STORM
- Special localization algorithm is required
- Imaging parameters are critical
- Implementation of super-resolved images is critical

For more details: Lab visit

HARDWARE

optical system



sample preparation

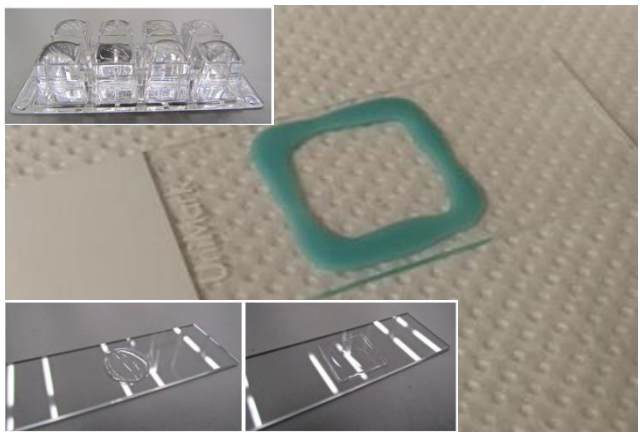
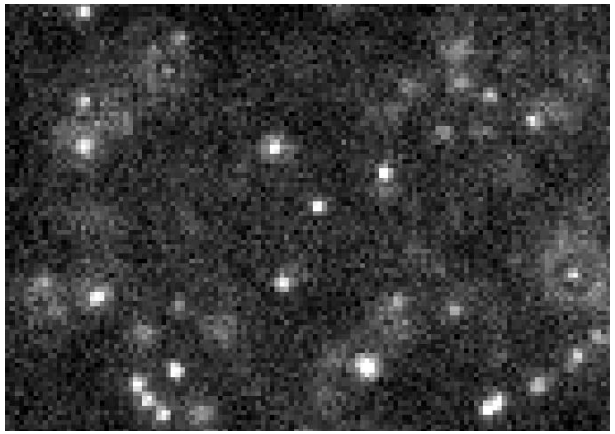
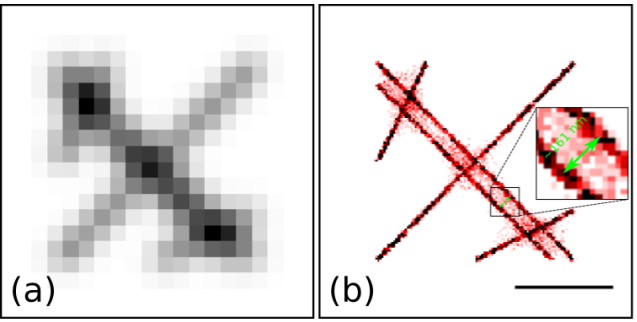


image acquisition

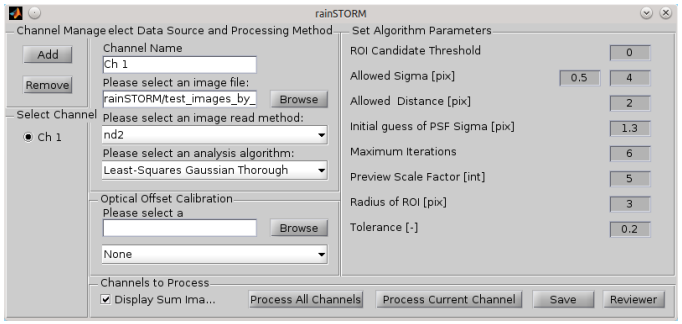


SOFTWARE

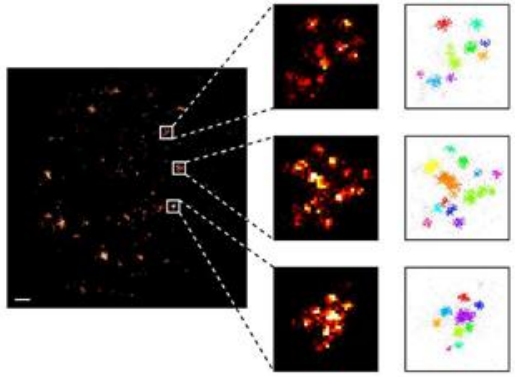
test sample generator



localization code



quantitative evaluation



APPLICATIONS

Thank you and see you in the lab!