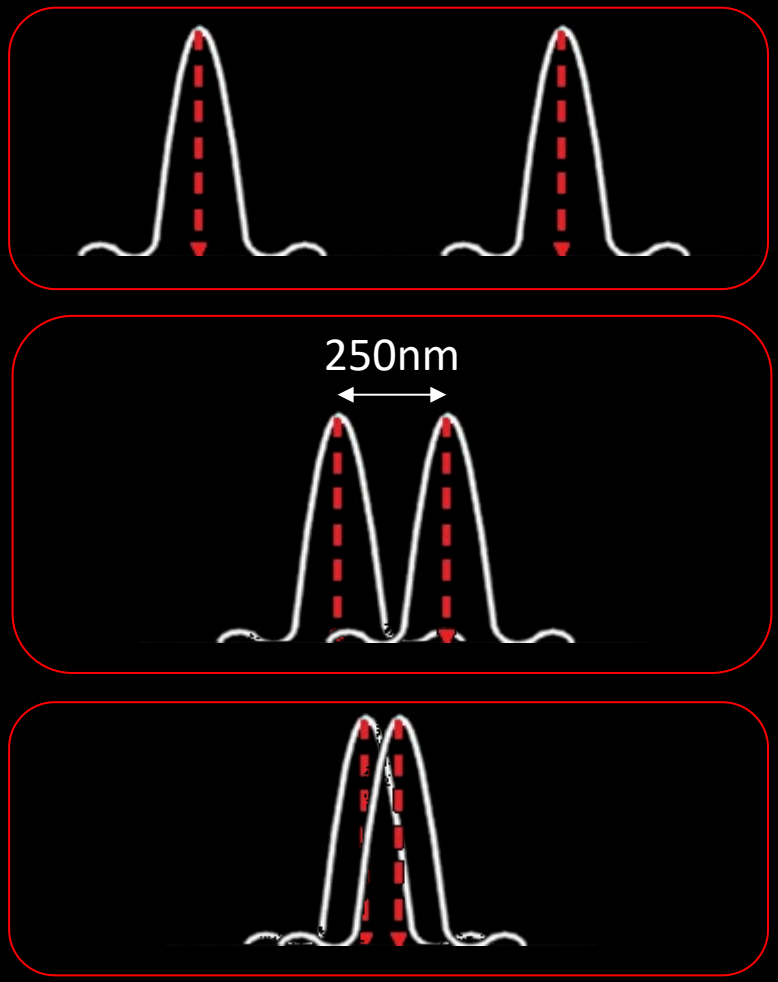
Single Molecule Localization Microscopy

Johan Slotman

12-11-2026

Erasmus
Optical
Imaging Centre

Resolution

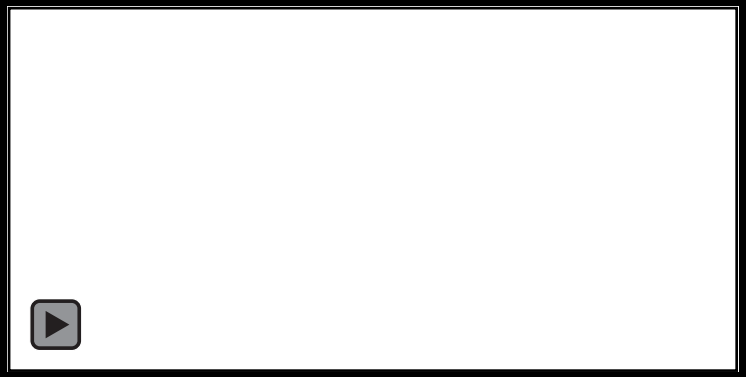


http://www.microscopyu.com/articles/superresolution/stormintro.html

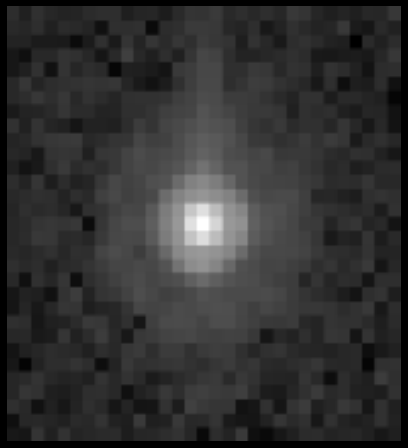
Super resolution microscopy
Single Molecule Localization Microscopy

1. Detect single molecules by a microscope
   - First observation of single fluorescent molecules in 1989 by William E. Moerner





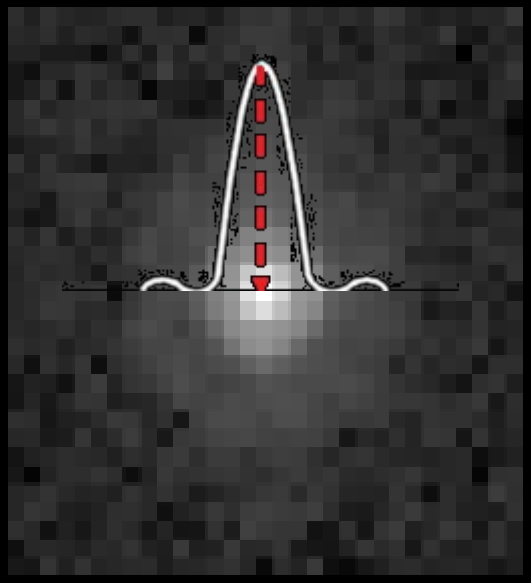
Androgen Receptor – mEos2
Hep3b
Live cell



PSF
(Point Spread Function)
of a Single Fluorophore

Single Molecule Localization Microscopy

1. Determine the precise position of a single molecule
   - Using Gauss fitting



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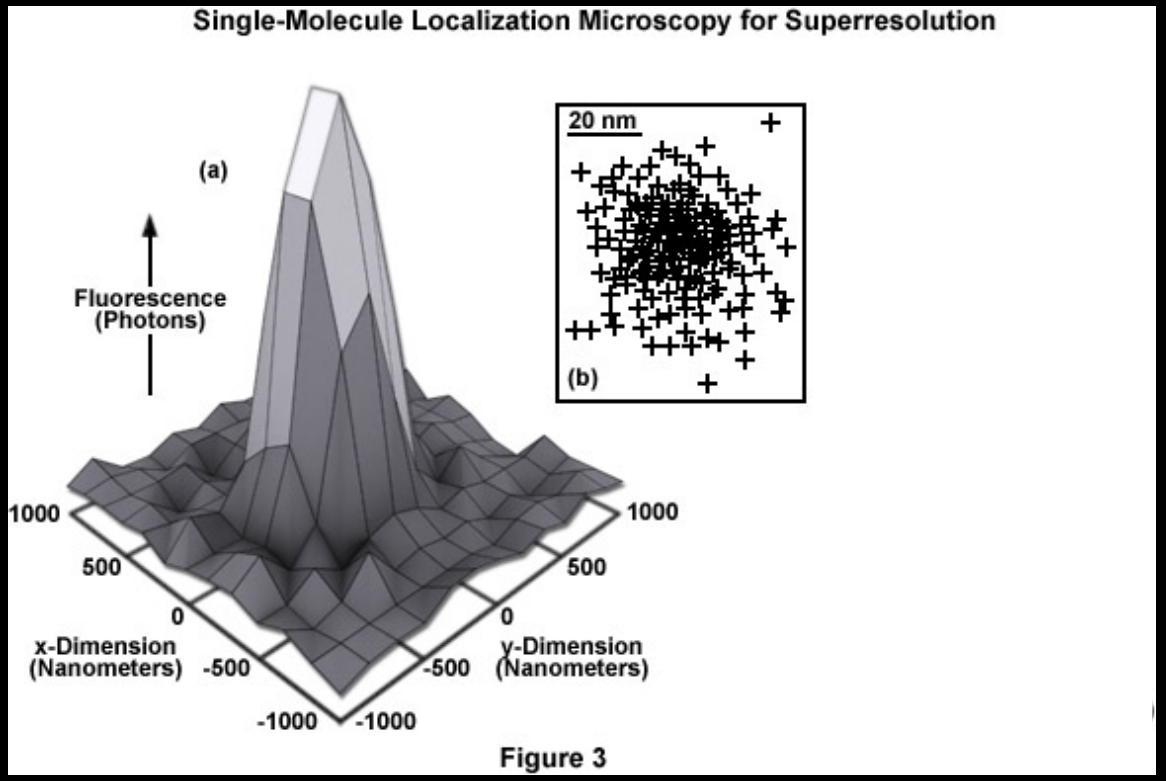
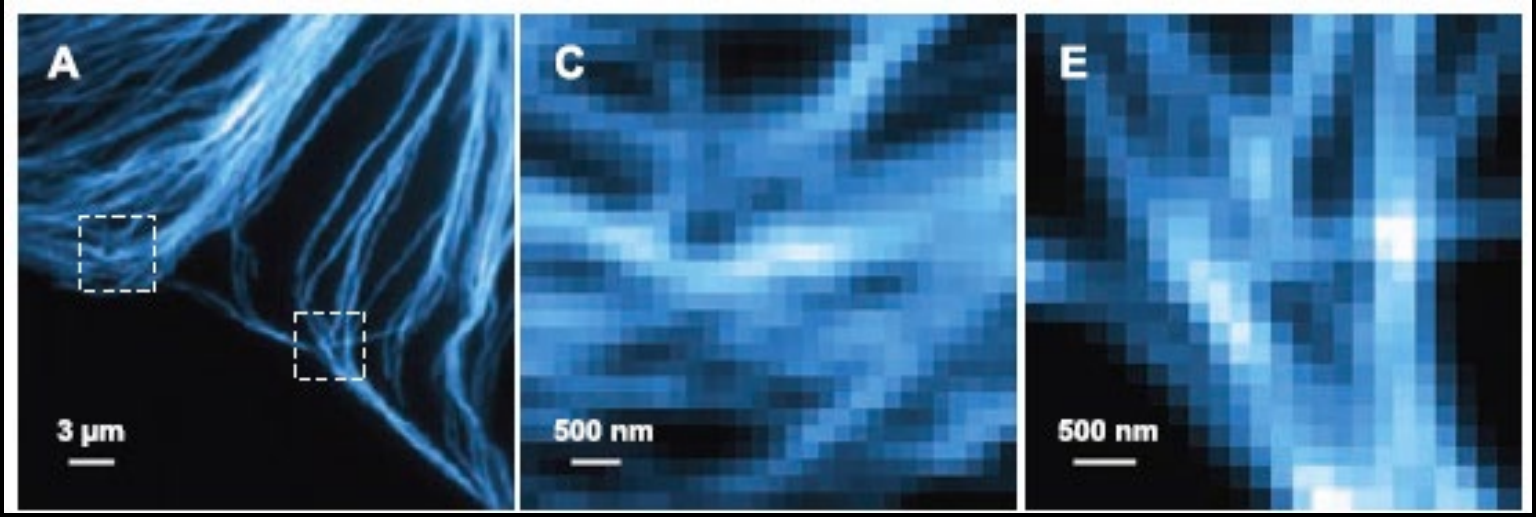


http://www.microscopyu.com/articles/superresolution/stormintro.html

Single Molecule Localization Microscopy

- Too high density of fluorescent molecules



Bates, M., Huang, B., Dempsey, G., & Zhuang, X. (2007). Multicolor super-resolution imaging with photo-switchable fluorescent probes. *Science*, *317*(5845), 1749 –1753.

Single Molecule Localization Microscopy

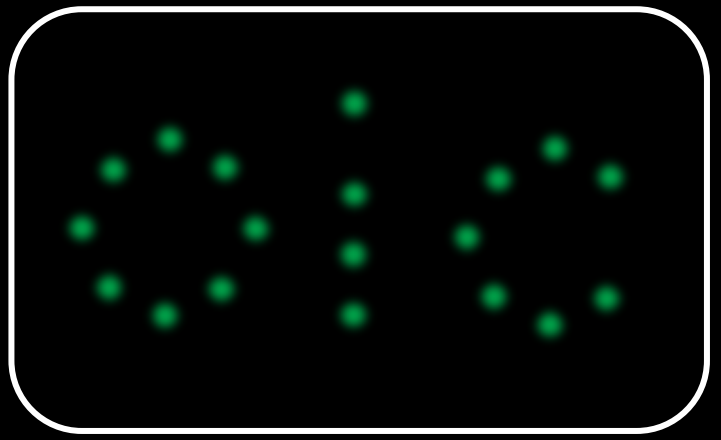


All fluorescent molecules ON

Widefield



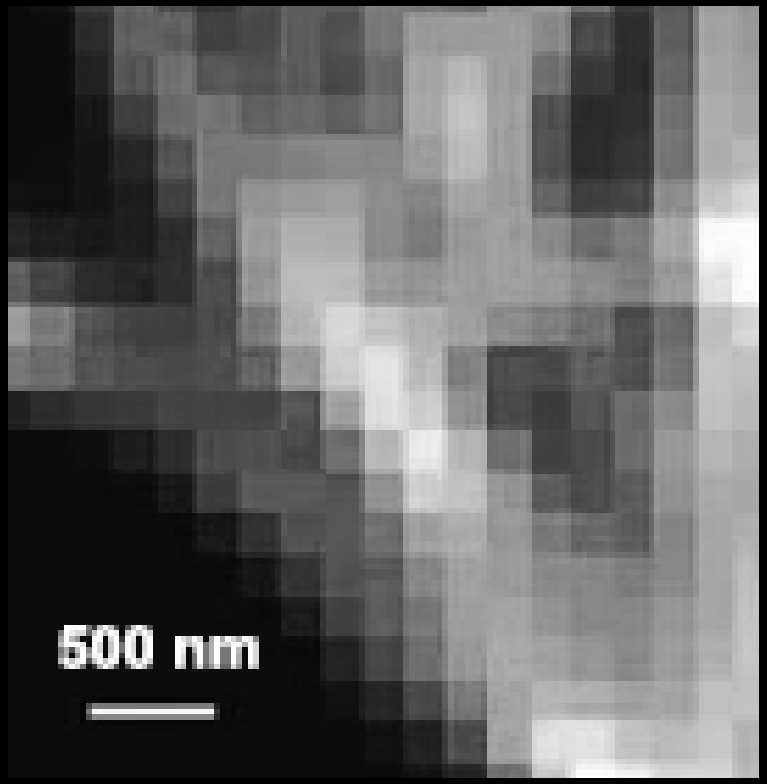
Separate fluorescent molecules in time



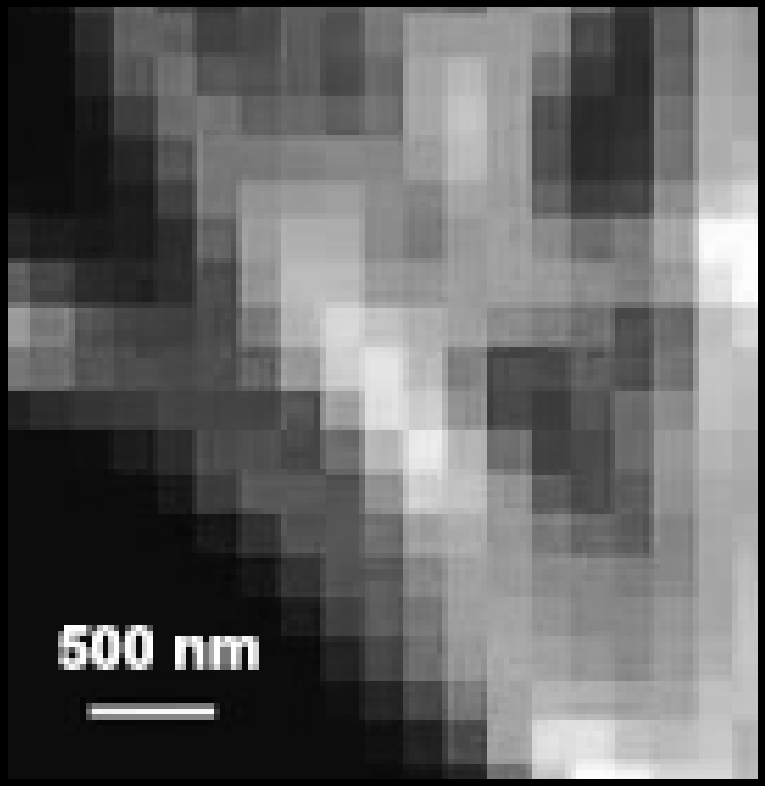
1. Localizing the molecules
2. Image Reconstruction

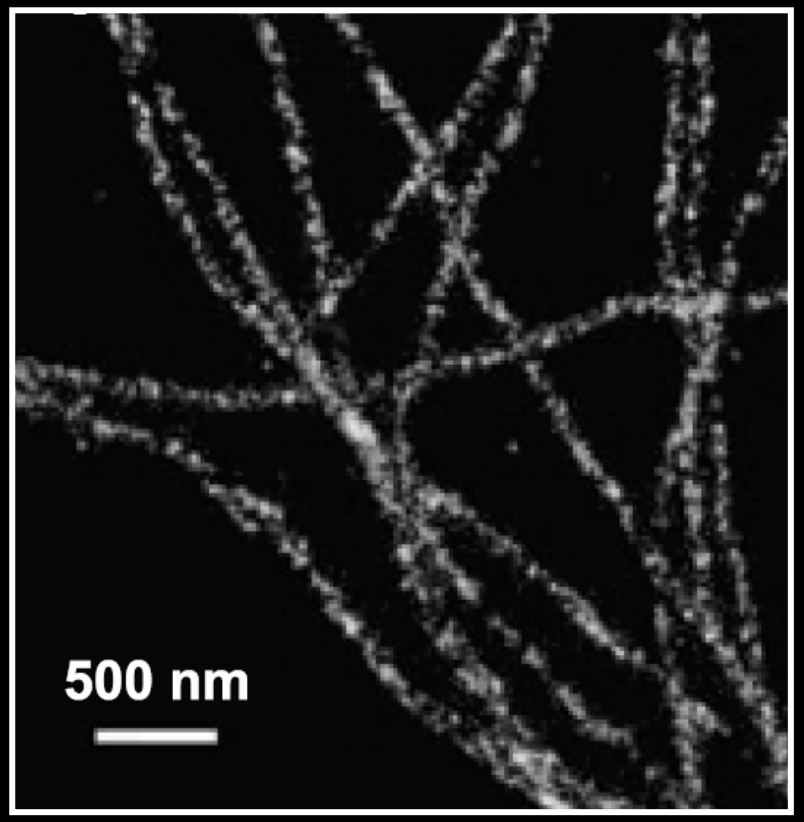
Single Molecule Localization

Single Molecule Localization Microscopy



Single Molecule Localization Microscopy





What do we need to see single fluorescent molecules in a cell

1. Microscope



2.

3.

4.

What do we need to see single fluorescent molecules in a cell

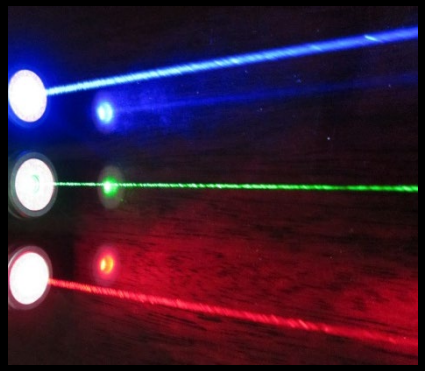
1. Microscope



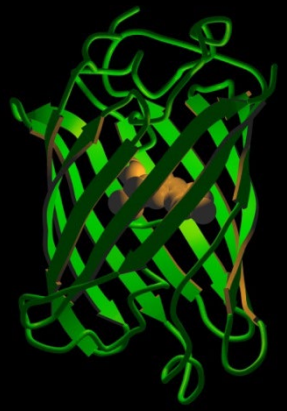
2. Sensitive camera



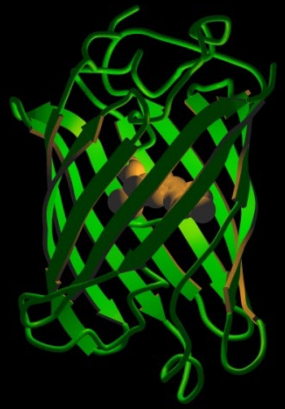
3. Powerful lasers



4. The right fluorophores



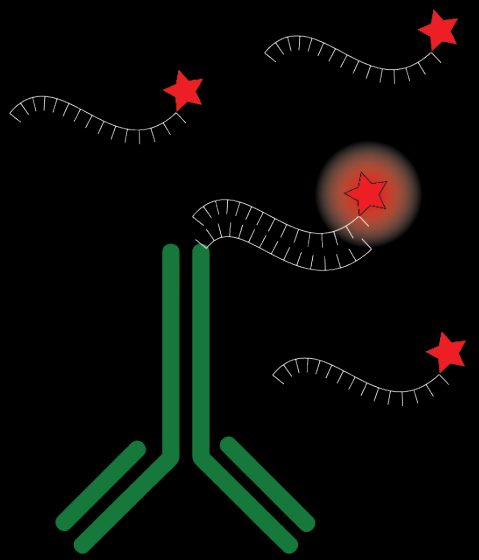
The right fluorophores



- Optical highlighters (Fluorescent Proteins)
  - Photoactivation Localization Microscopy **(PALM)**
  - Genetic encoded labeling of your proteins of interest



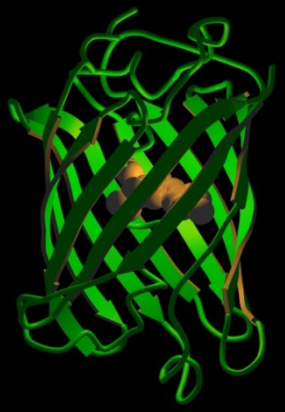
- Synthetic dyes
  - Stochastic Optical Reconstruction Microscopy **(STORM)**
  - Immunofluorescence
  - Using imaging buffer

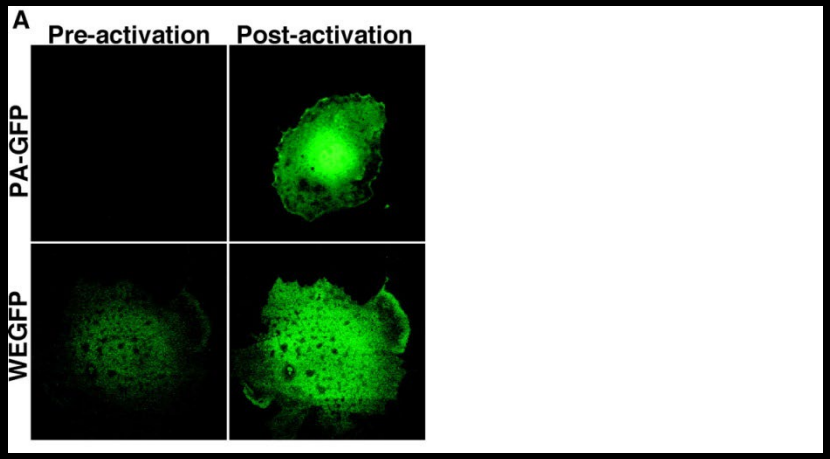


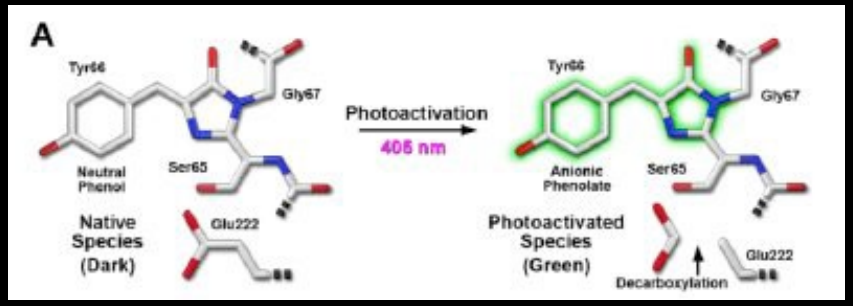
- Point Accumulation for imaging in nanoscale topology (DNA-PAINT)
- Immunostaining
- DNA docking and DNA imaging strands

Optical highlighters (Fluorescent Proteins)

- Photoactivatable GFP

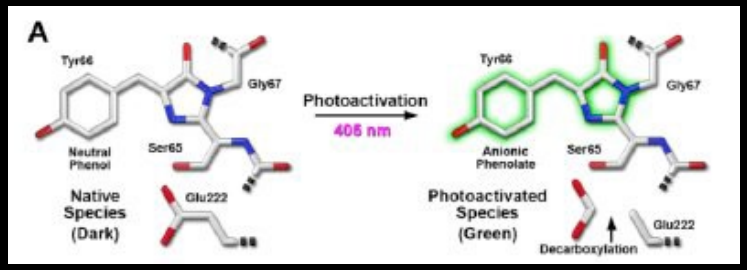


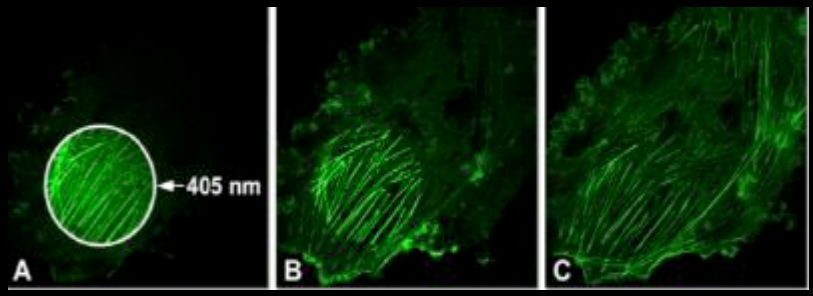




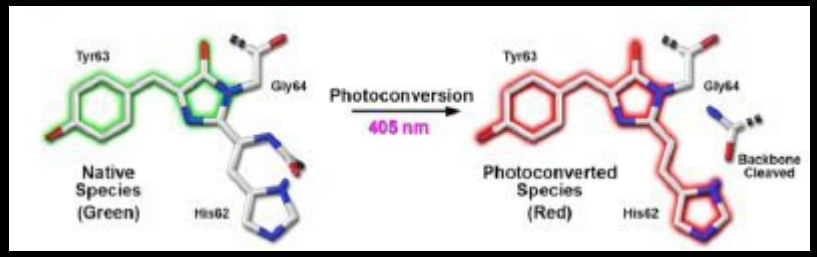
Patterson, G. H. & Lippincott-Schwartz, J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* **297,** 1873–7 (2002).

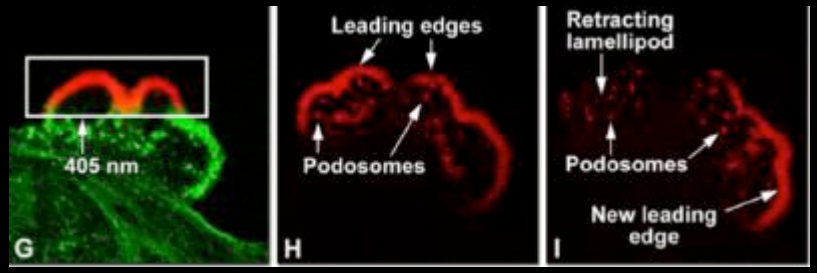
- Photoactivatable FPs



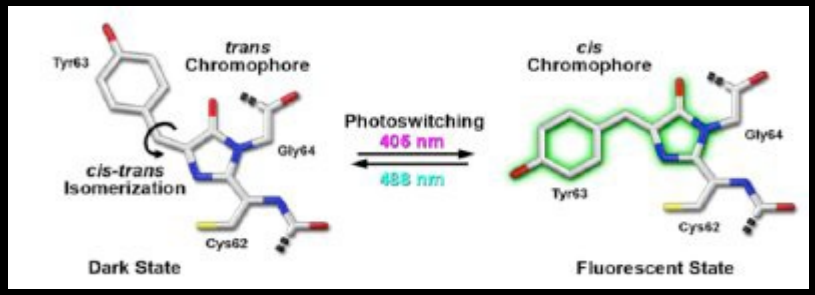


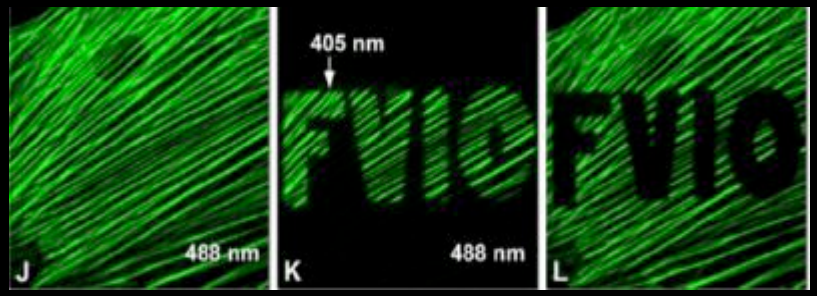
- Photoconvertable FPs





Reversible Photoswitchable FPs





http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP-Highlighters.html

Photoconvertible Fluorescent Proteins



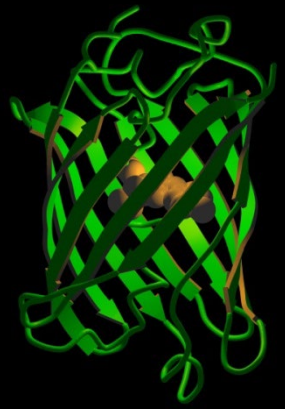
RAD54-mEos2

Photoswitched

405 nm light

Image reconstruction

The right fluorophores



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- Synthetic dyes
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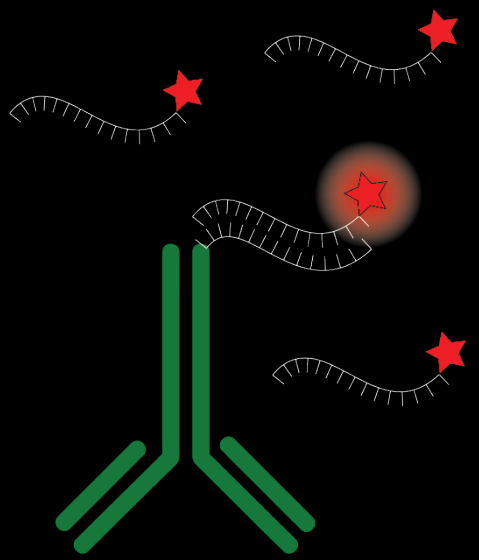
  - Immunofluorescence
  - Using imaging buffer



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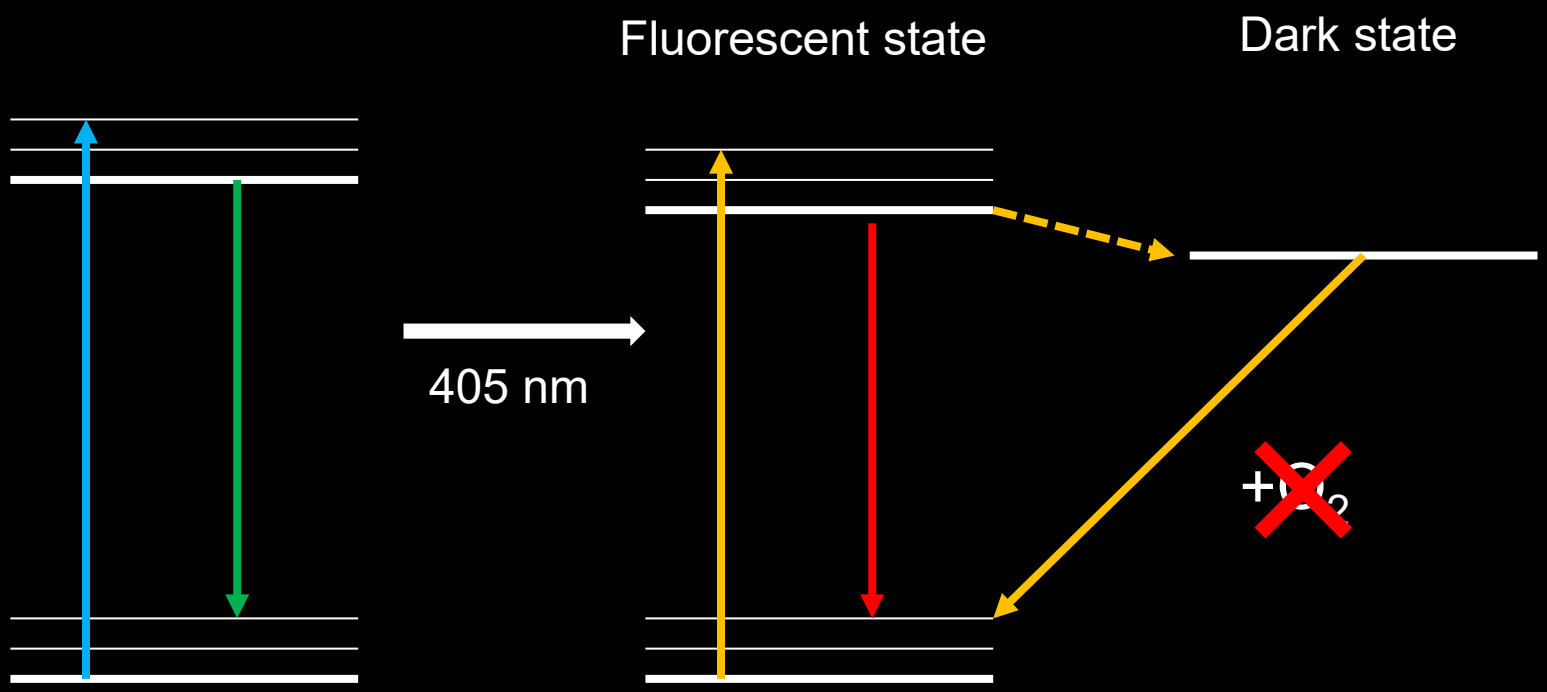
Mechanism of photoswitching



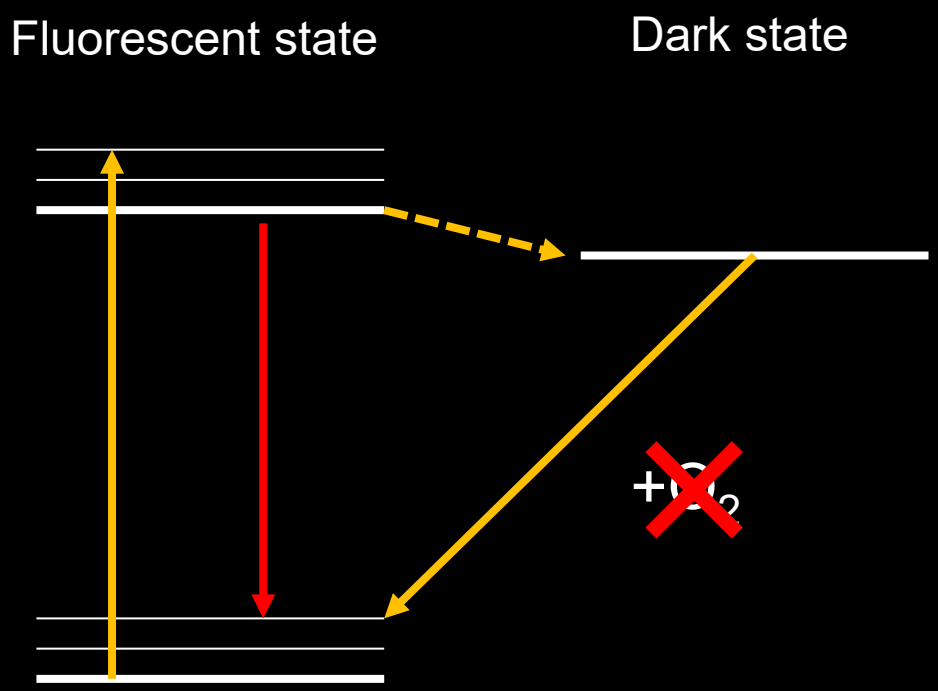
PALM

dSTORM

Mechanism of photoswitching

Stabilization of dark-state

- Imaging buffer
  - Oxygen scavenger
    - Glucose oxidase + Catalase
    - Glucose



Van de Linde, S., et. al (2011). Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nat. Protocols*, *6*(7), 991–1009.

dSTORM

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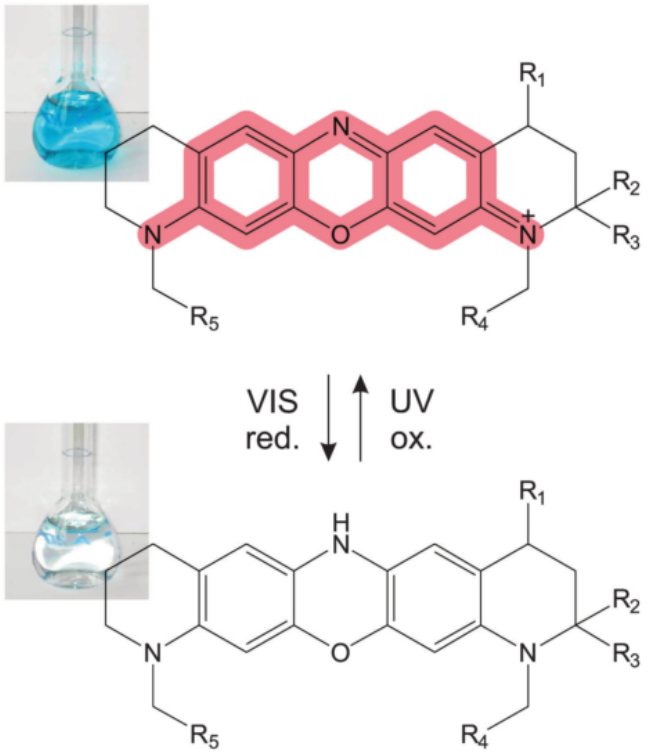
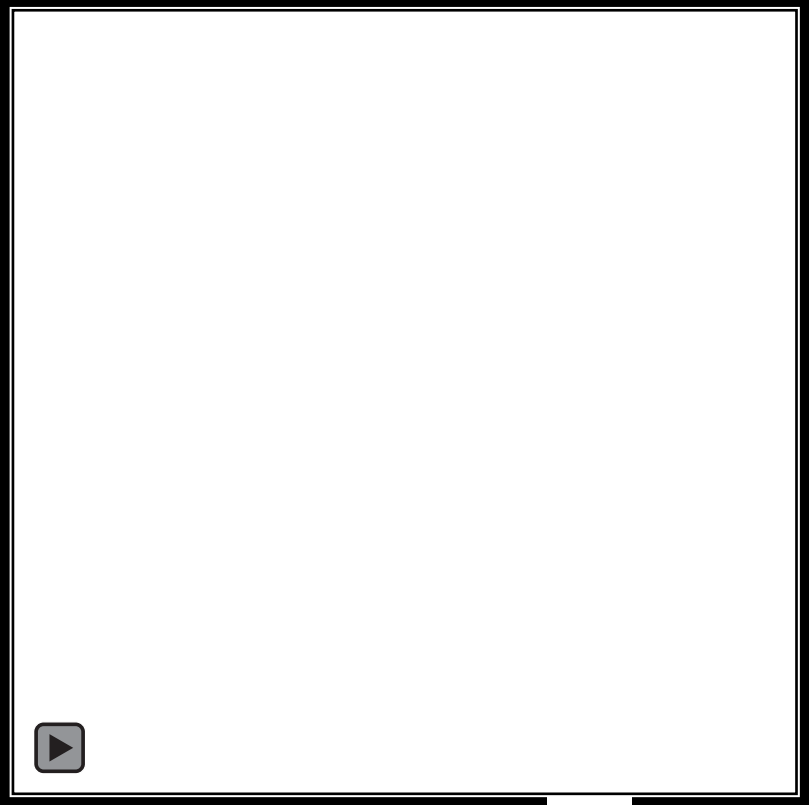


Fig. 6 Reversible photoswitching of oxazine dyes like ATTO 655 in presence of thiol-containing reducing agents such as MEA and upon irradiation at 640 nm and 405 nm, respectively. During the reaction oxygen is consumed and a stable colorless dark state is formed.[60,61]

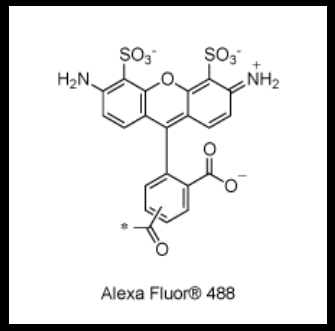
van de Linde, S. & Sauer, M. How to switch a fluorophore: from undesired blinking to controlled photoswitching. *Chem. Soc. Rev.* (2013). doi:10.1039/c3cs60195a

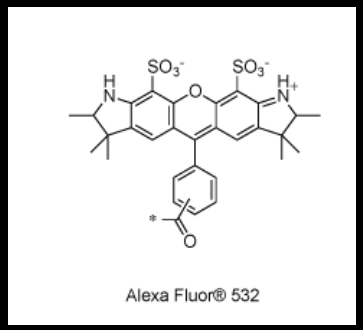
dSTORM

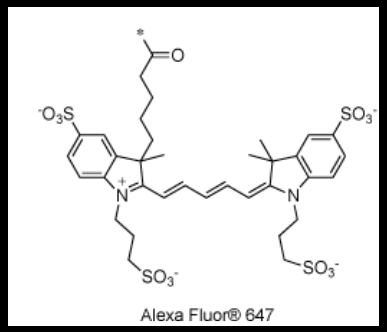


Alexa647

2 µm
20 frames/s

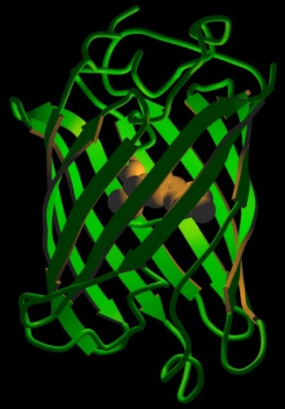






2 µm

The right fluorophores



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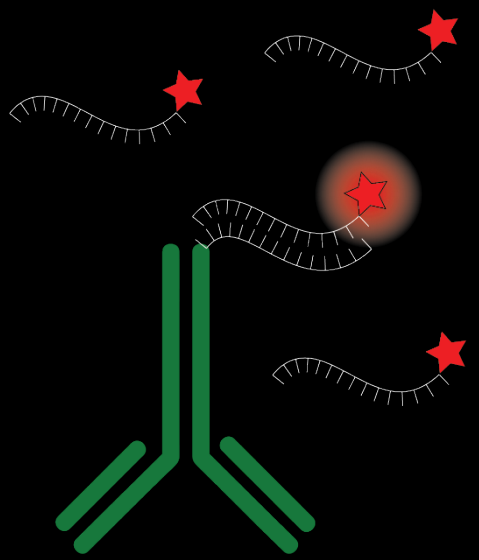
  - Using imaging buffer



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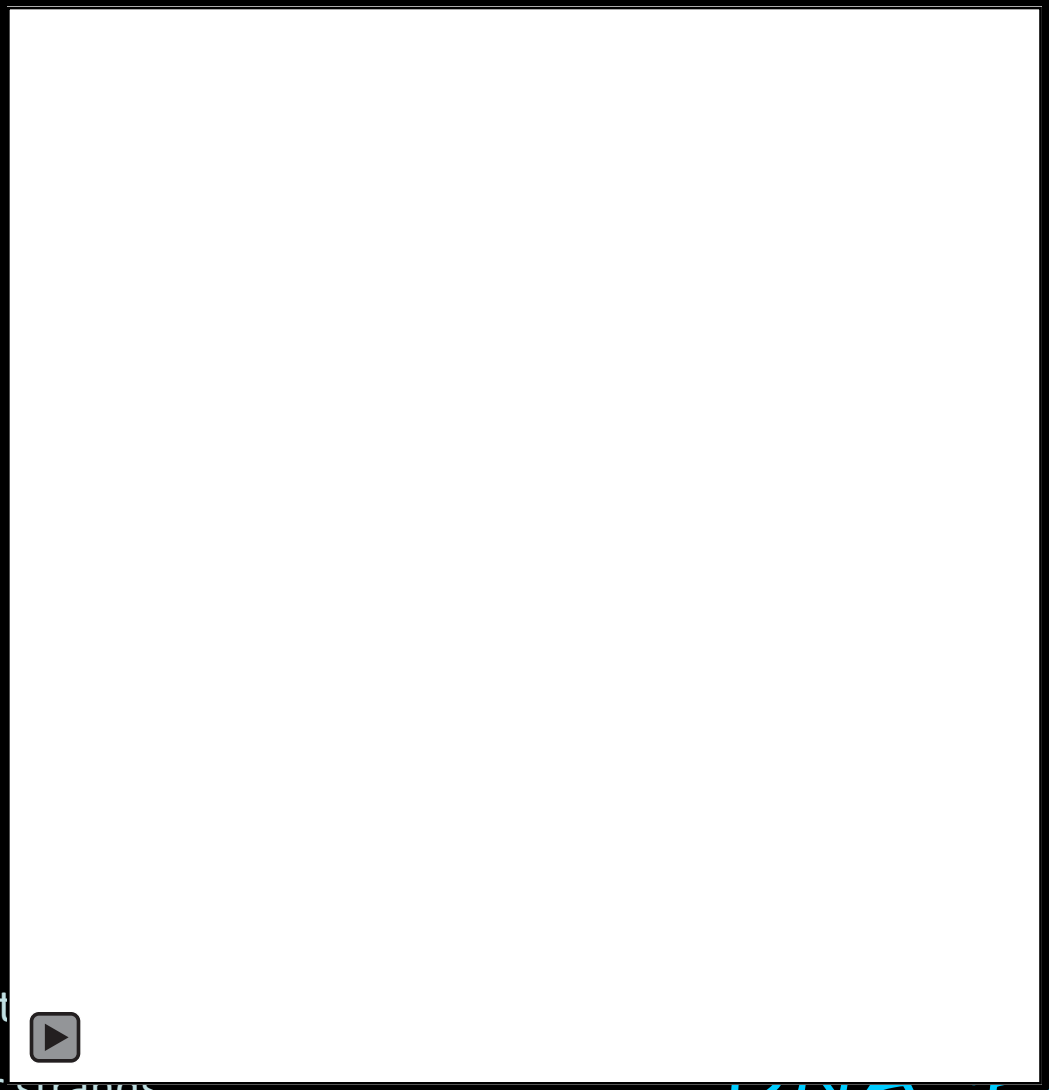
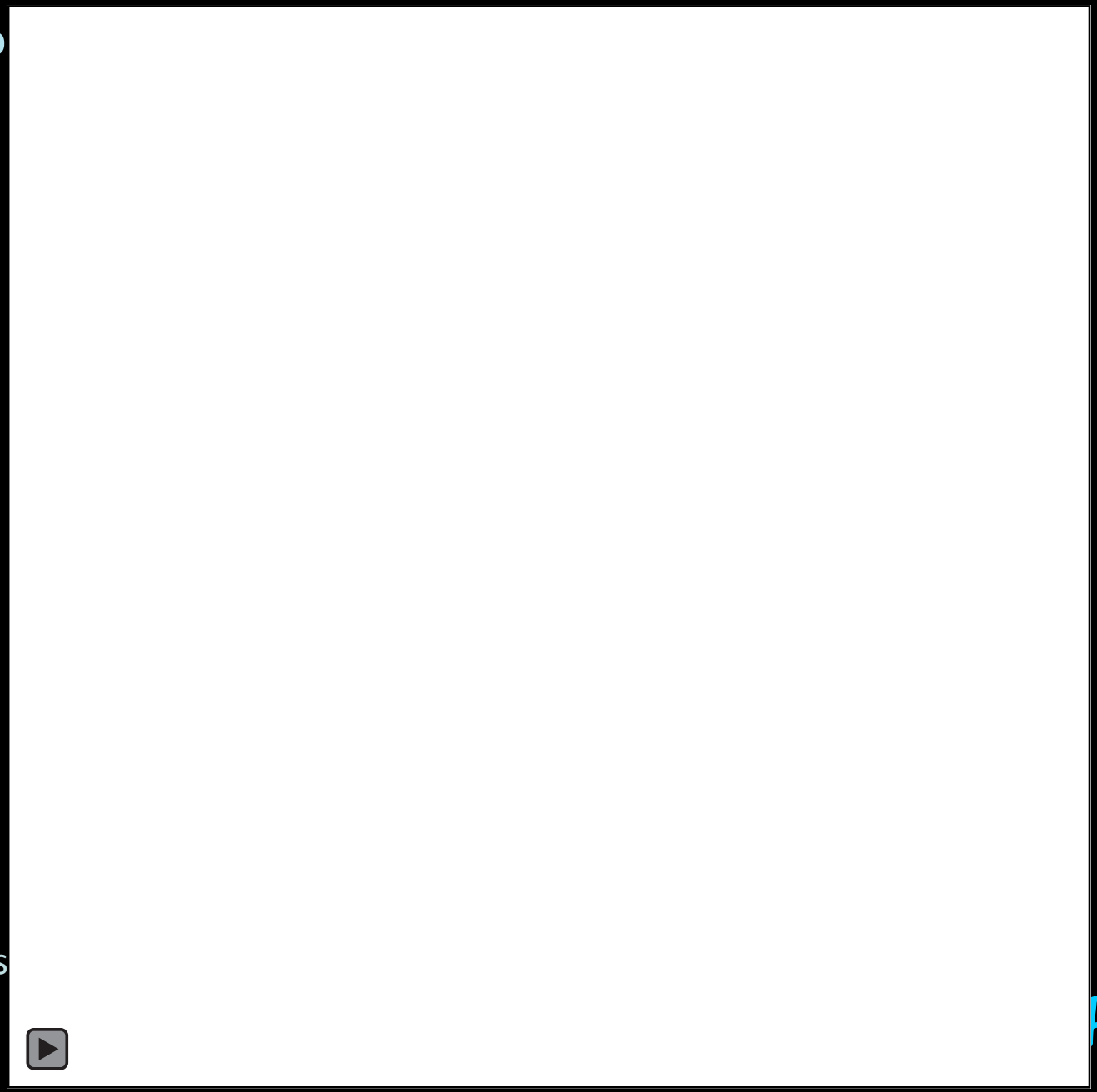
Localisation by immobilisation



- Off-state : Free imager st
- On-state : Bound imager strands

DNA PAINT

Multip



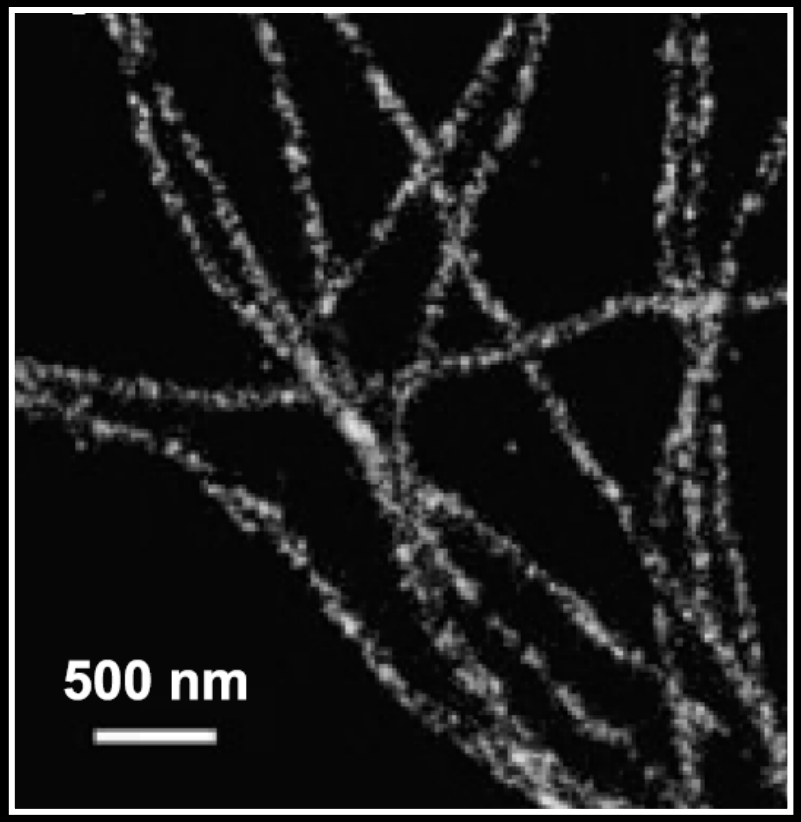
- Imager s

PAINT

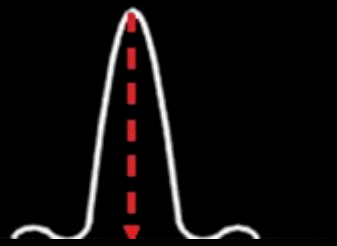
Comparing SMLM techniques

	PALM	dSTORM	DNA-PAINT
labeling	Genetic	Antibodies	Antibodies
localization	once	multiple	Multiple
resolution	+	++	+++
brightness	+	+++	++
speed	++	++	+
multi color	+	++	+++
live cell	++ (smt) halotag	-	-

Image reconstruction



- Detecting frame by frame signals above background



- Fit these signals to a 2D gaussian distribution

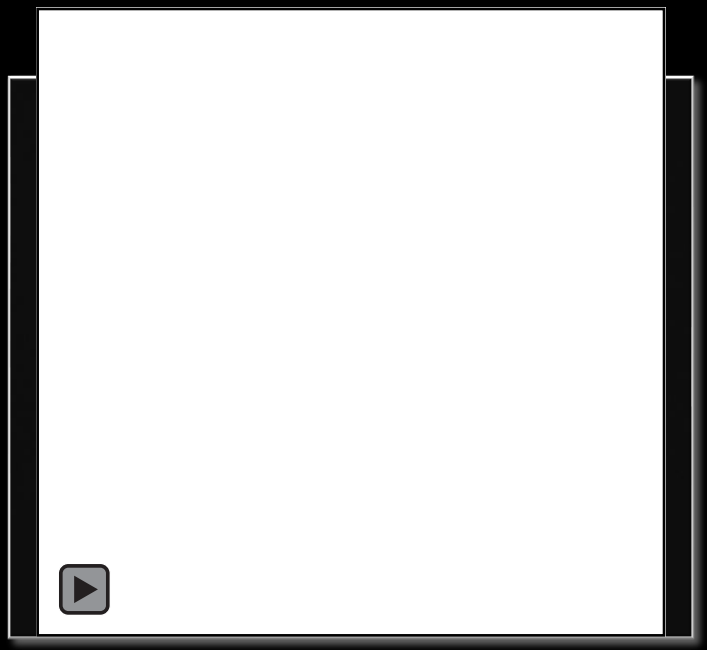
Results

File Edit Font Results

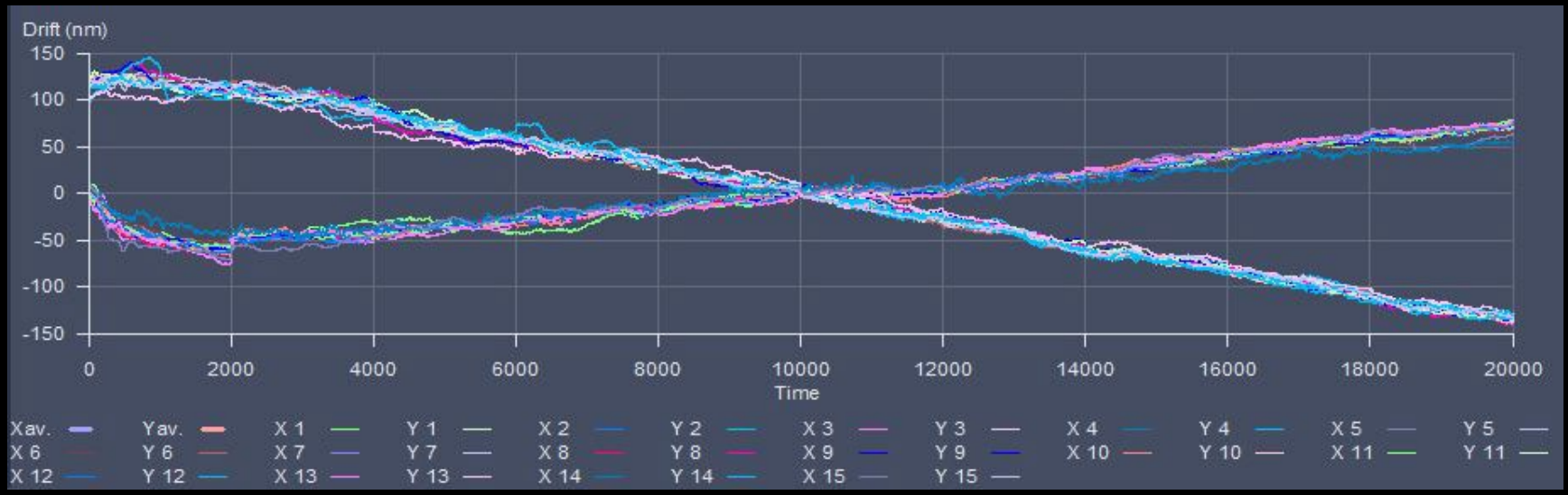
	Index	X	Y	Precision	PSF	First_Frame	N_detections	Photons
1	4040	38020.900	35409.200	19	143.100	494	1	247
2	6419	38046.500	35402.600	18.400	159.500	782	1	295
3	9923	38020.600	35404.500	26.500	142.700	1234	1	179
4	10290	38023.200	35389.700	17.100	151	1289	1	396
5	11411	38287.200	35579.400	7.900	137.300	1461	1	869
6	12270	38015.600	35378.500	12.200	164.200	1597	1	593
7	12820	37974.800	35389.400	14.700	168	1681	1	629
8	15282	38013.300	35440.500	13.600	136.100	2094	1	420
9	15943	38010.900	35377.700	28.400	158.500	2206	1	226
10	16088	38272.400	35532.800	21.900	153.100	2230	1	215
11	18520	38011.100	35375.800	18.800	146.600	2646	1	272
12	20401	38013.700	35373.100	17.400	159.400	2977	1	341
13	22402	37832.500	35641.900	14.800	152.300	3353	1	438
14	22830	38027.800	35372.100	31.500	153.400	3439	1	143
15	24481	37995.100	35382.400	32.100	145.800	3770	1	158

Data analysis

Drift correction



- Using fiducial markers
  - Fluorescent beads
  - Gold nanoparticles (50-100nm)
- Correction for drift during image acquisition
- Also useful for channel alignment

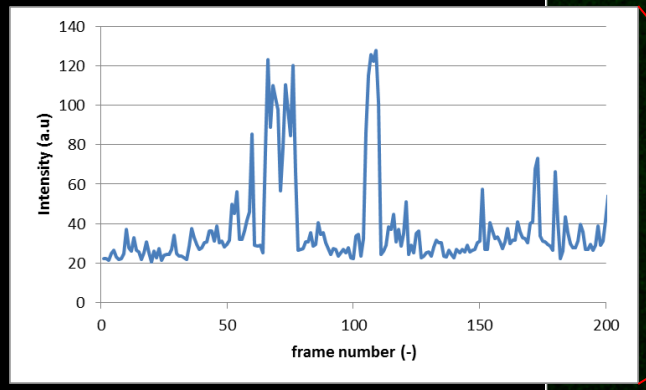


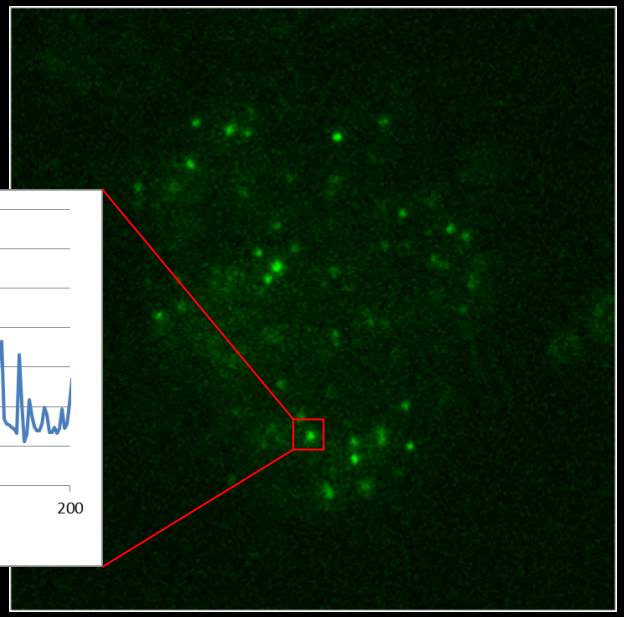
Data analysis

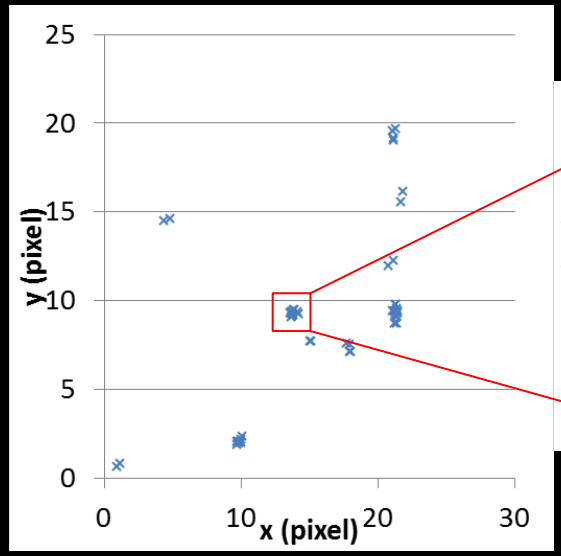
Grouping of single detections in to molecules

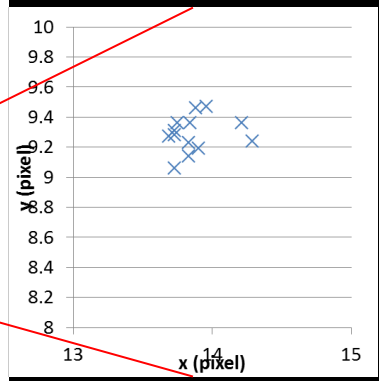
Erasmus Optical Imaging Centre

- Determine the average position of multiple detections of the same fluorescent molecule
- Criteria
  - Time
  - Distance
- Difficult at higher label density
- Measuring behaviour of fluorophores in standardized sample





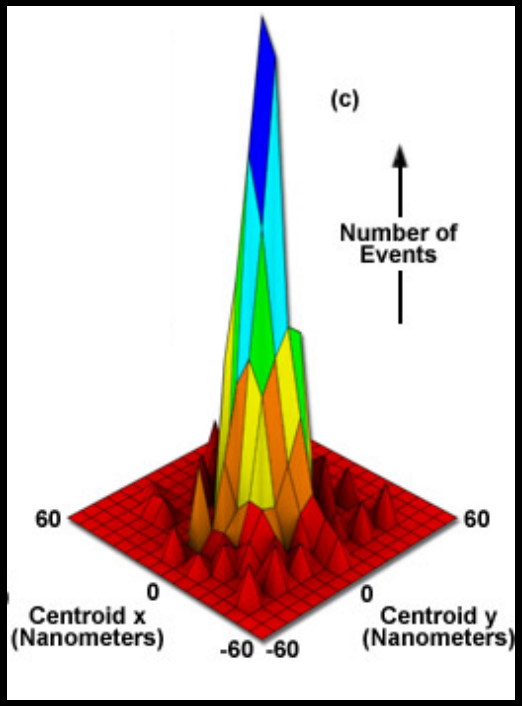




Resolution in Single Molecule Microscopy

- x-y resolution depends on the precision that a molecule can be localized

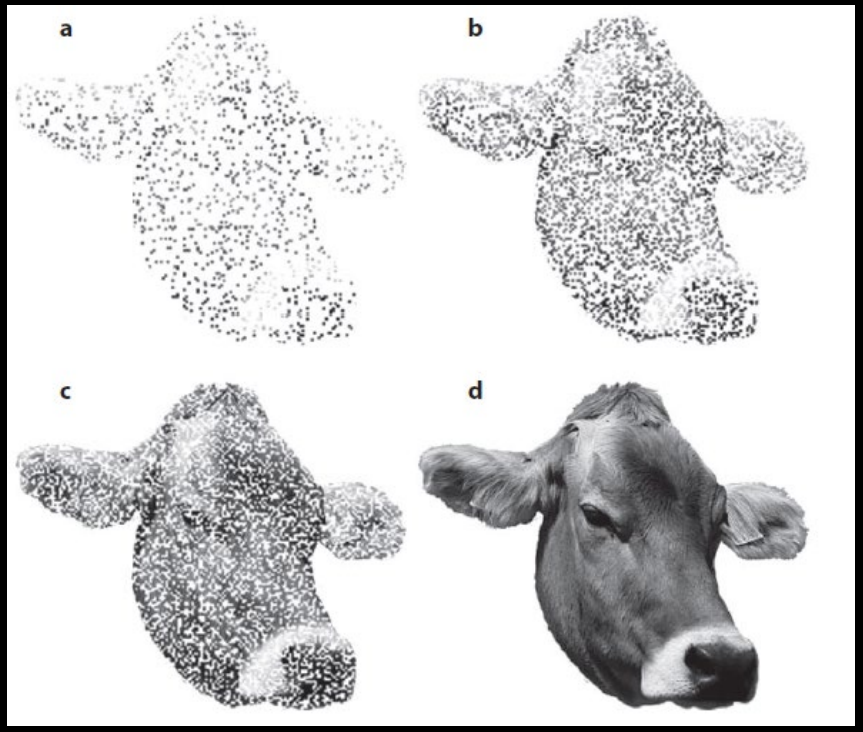
- $\sigma^2_{x,y} \approx \frac{s^2}{N}$



Patterson, G., & Davidson, M. (2010). Superresolution imaging using single-molecule localization. *Annual review of …*, *61*, 345–367.

Resolution in Single Molecule Microscopy

- Resolution depends on the labeling density
  - Nyquist theory

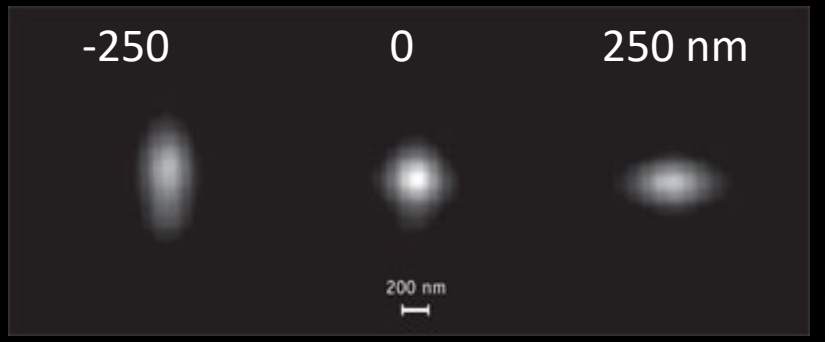


Patterson, G., & Davidson, M. (2010). Superresolution imaging using single-molecule localization. *Annual review of …*, *61*, 345–367.

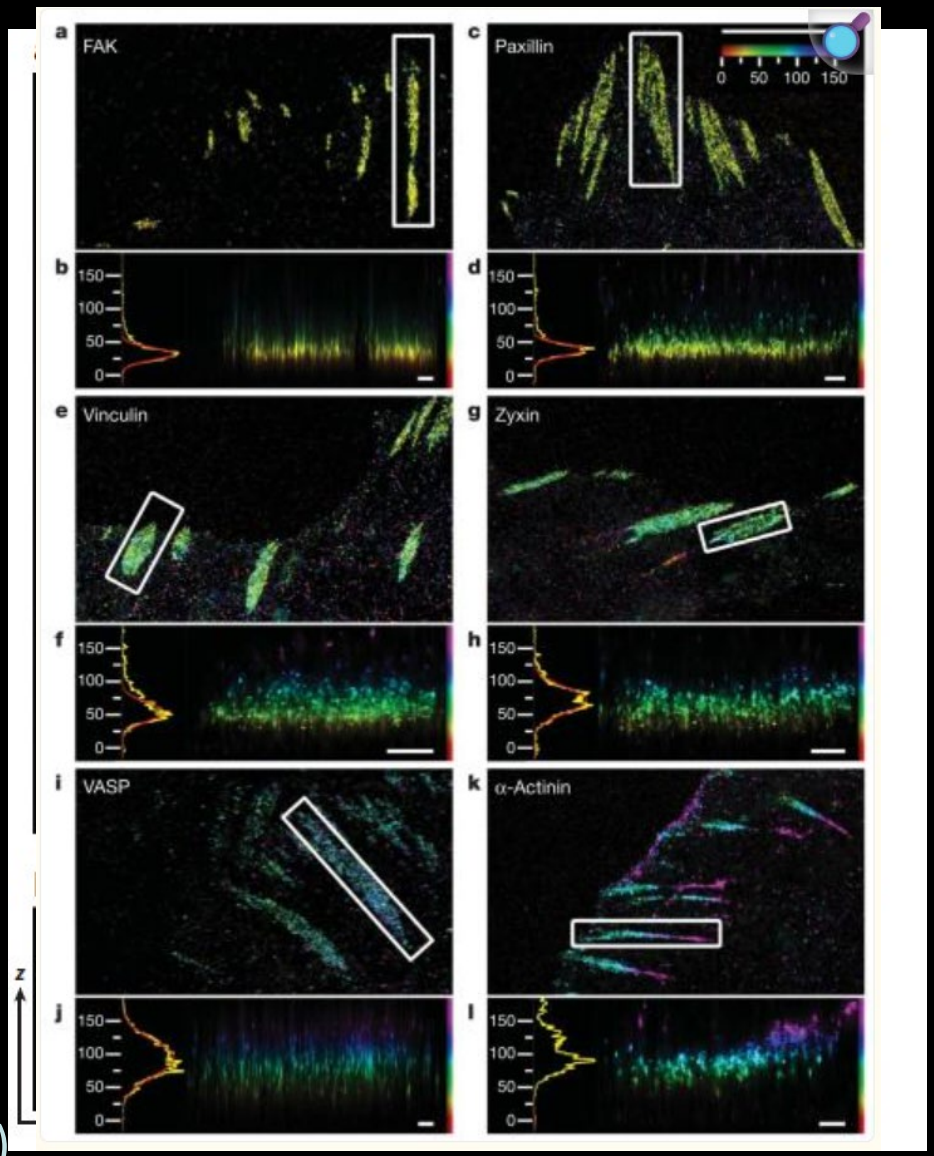
Z Resolution in Single Molecule Microscopy

- PALM/STORM in principle 2D technique

- Deformation of PSF
  - Cylindrical lens
  - 50 nm z-resolution

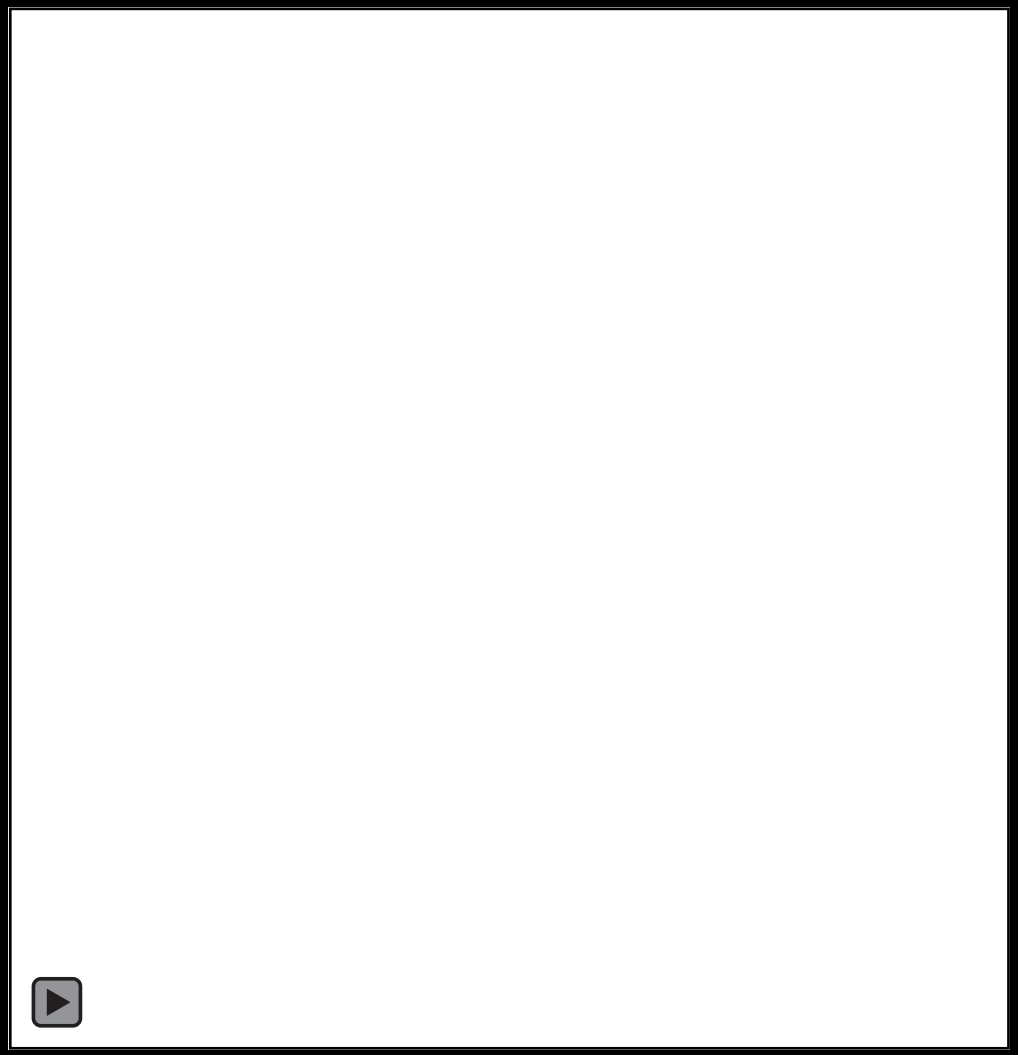


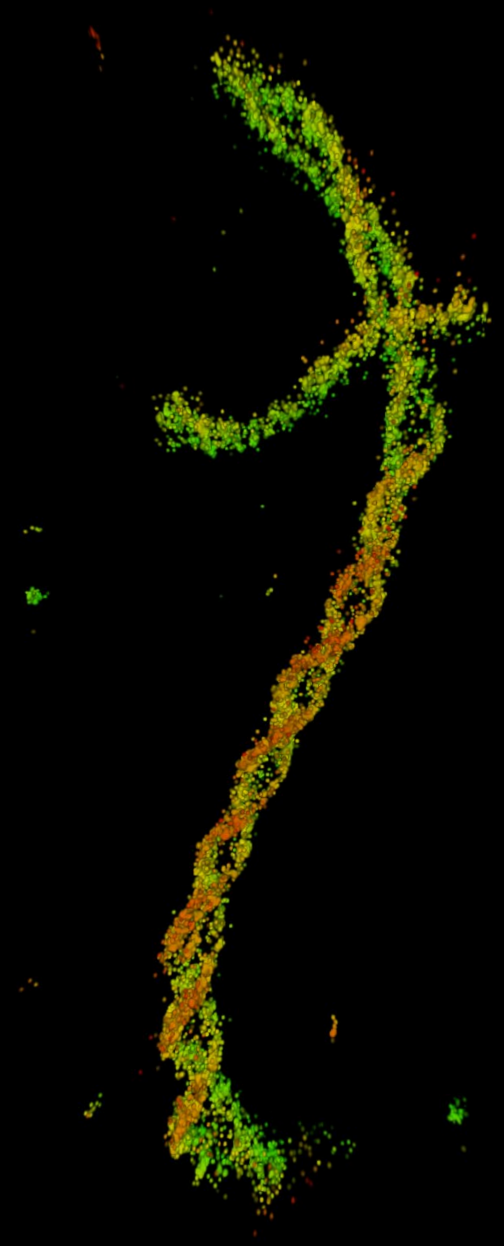
- Many other options
  - Differents PSF shapes
  - 4Pi / interferometry (iPALM)



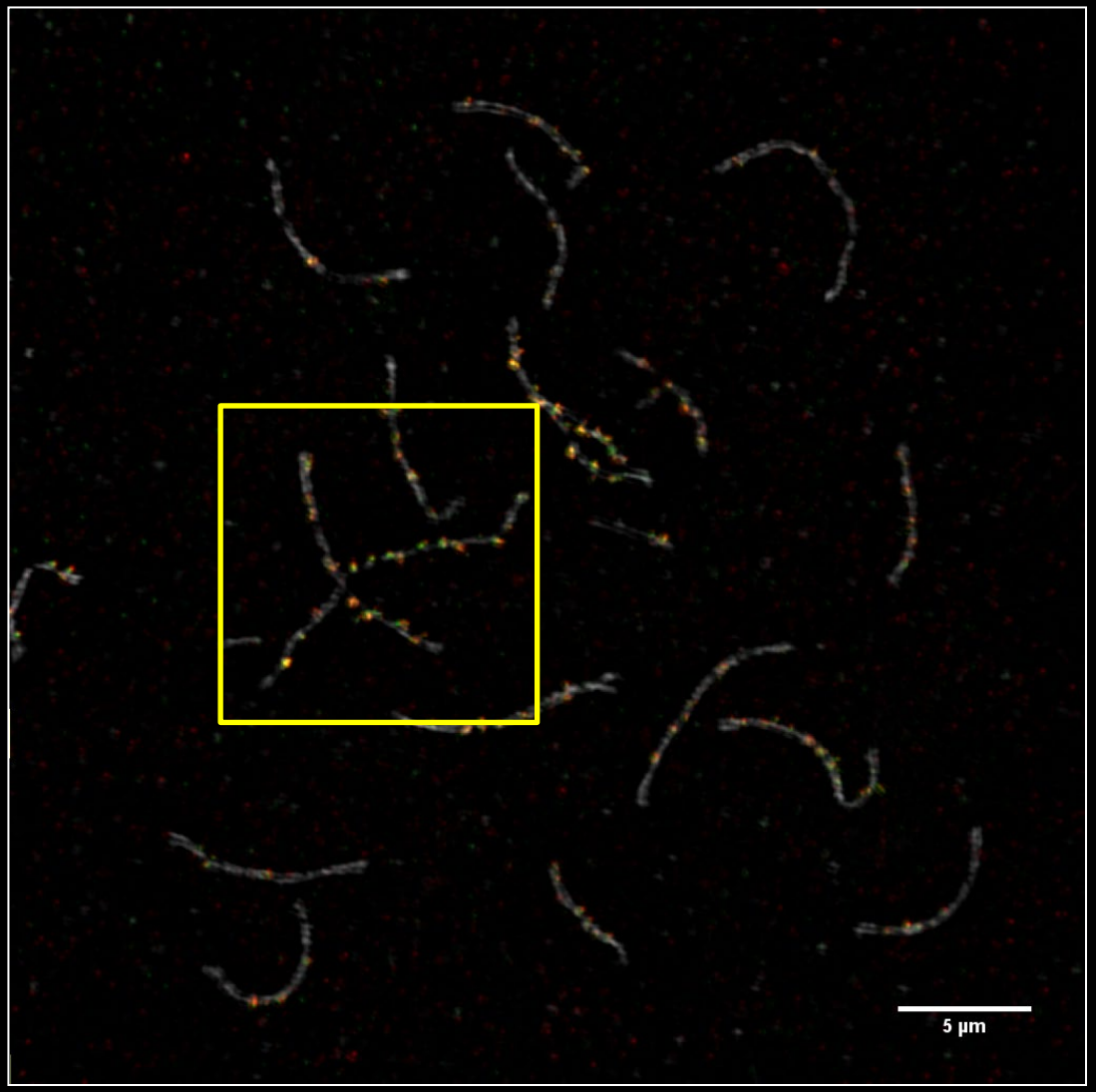
Kanchanawong, Waterman Nature. 2010 Nov 25;468(7323):580–584

Synaptonemal complex in 3D



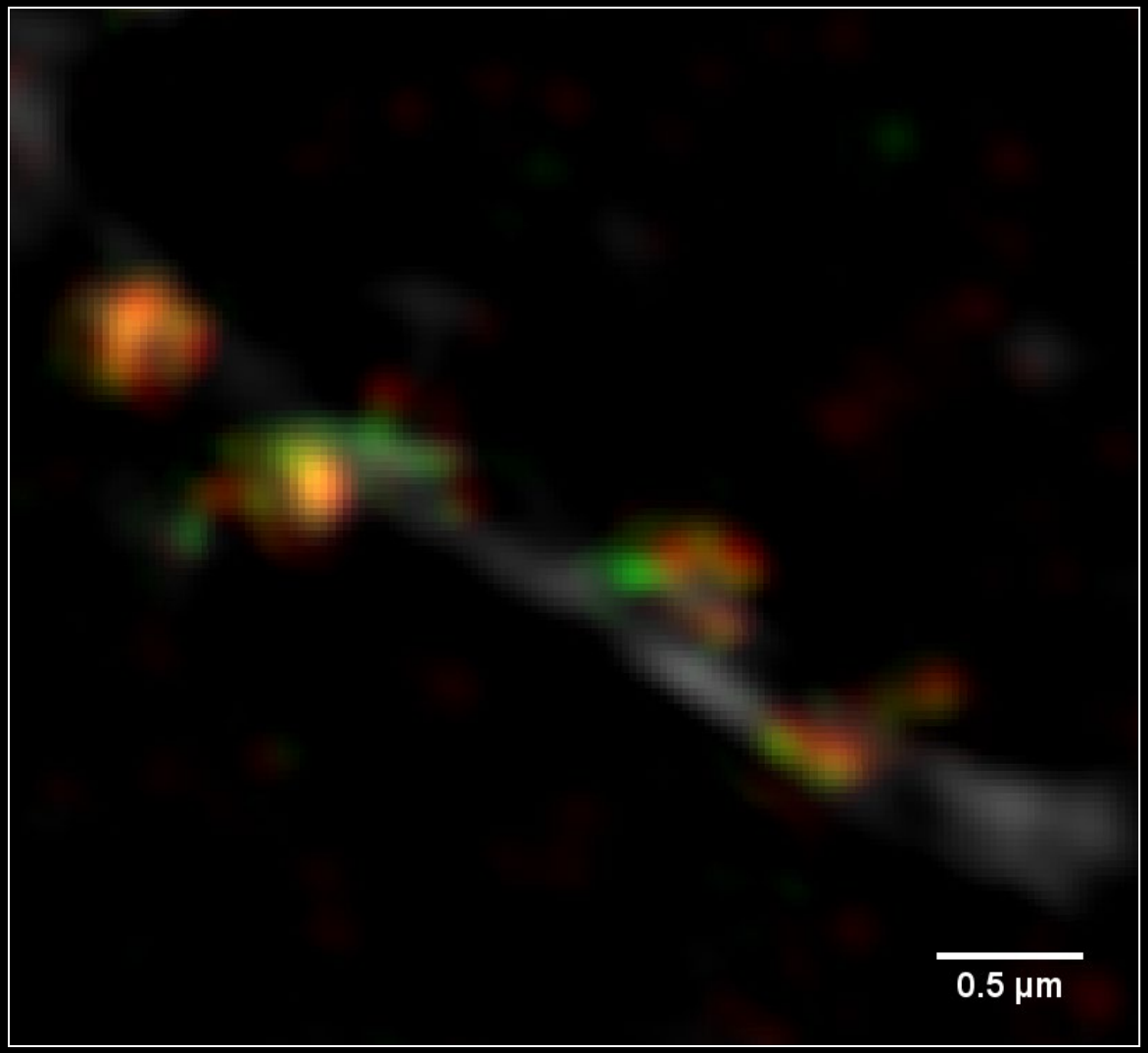


Combining SIM and dSTORM



SIM

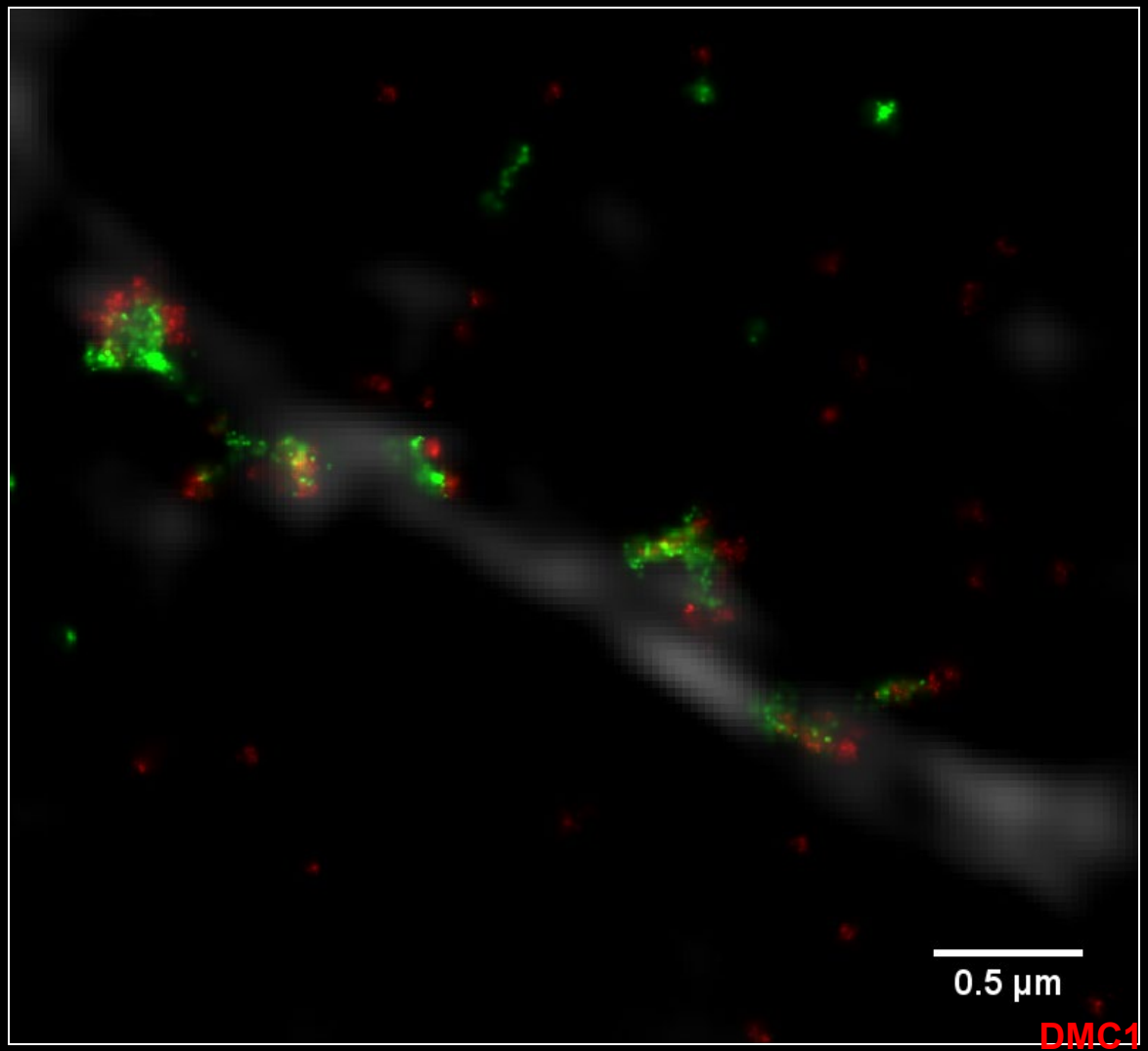
DMC1 Alexa647
SYCP3 Alexa546
RAD51 Alexa488



SIM

DMC1 Alexa647
SYCP3 Alexa546
RAD51 Alexa488

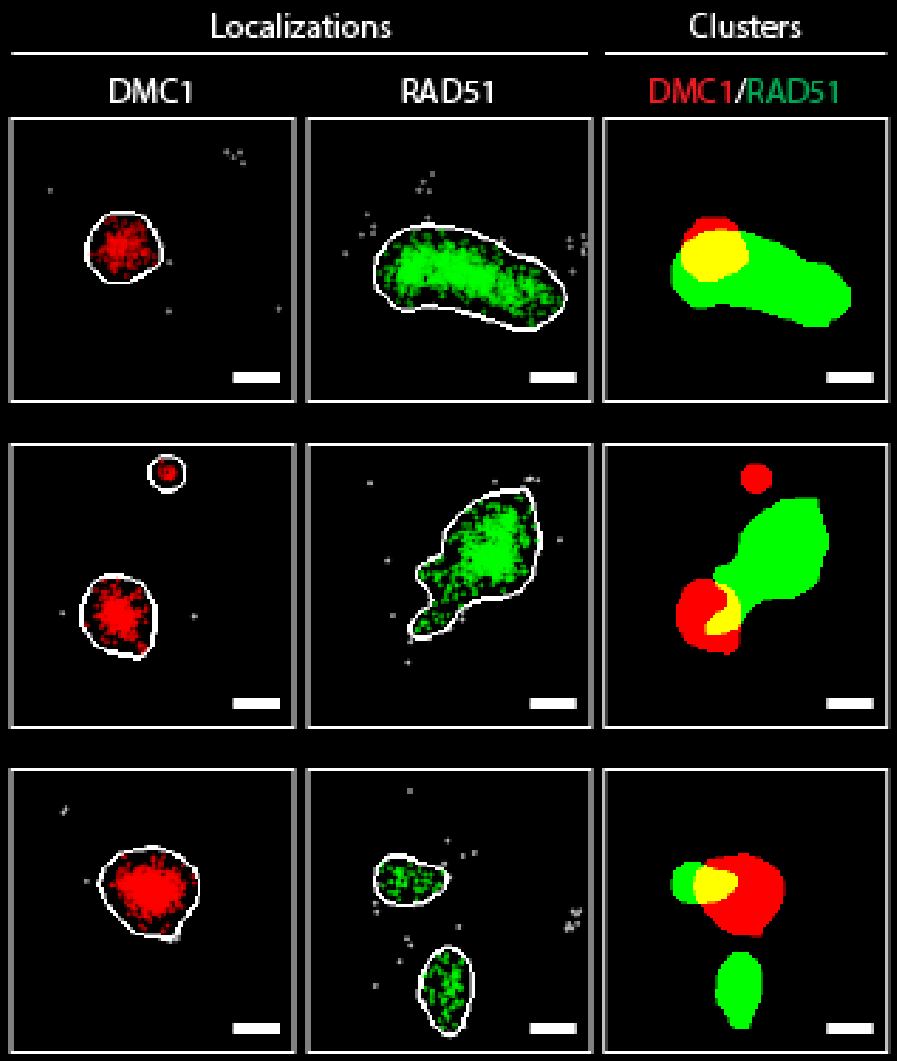
Dual-color STORM



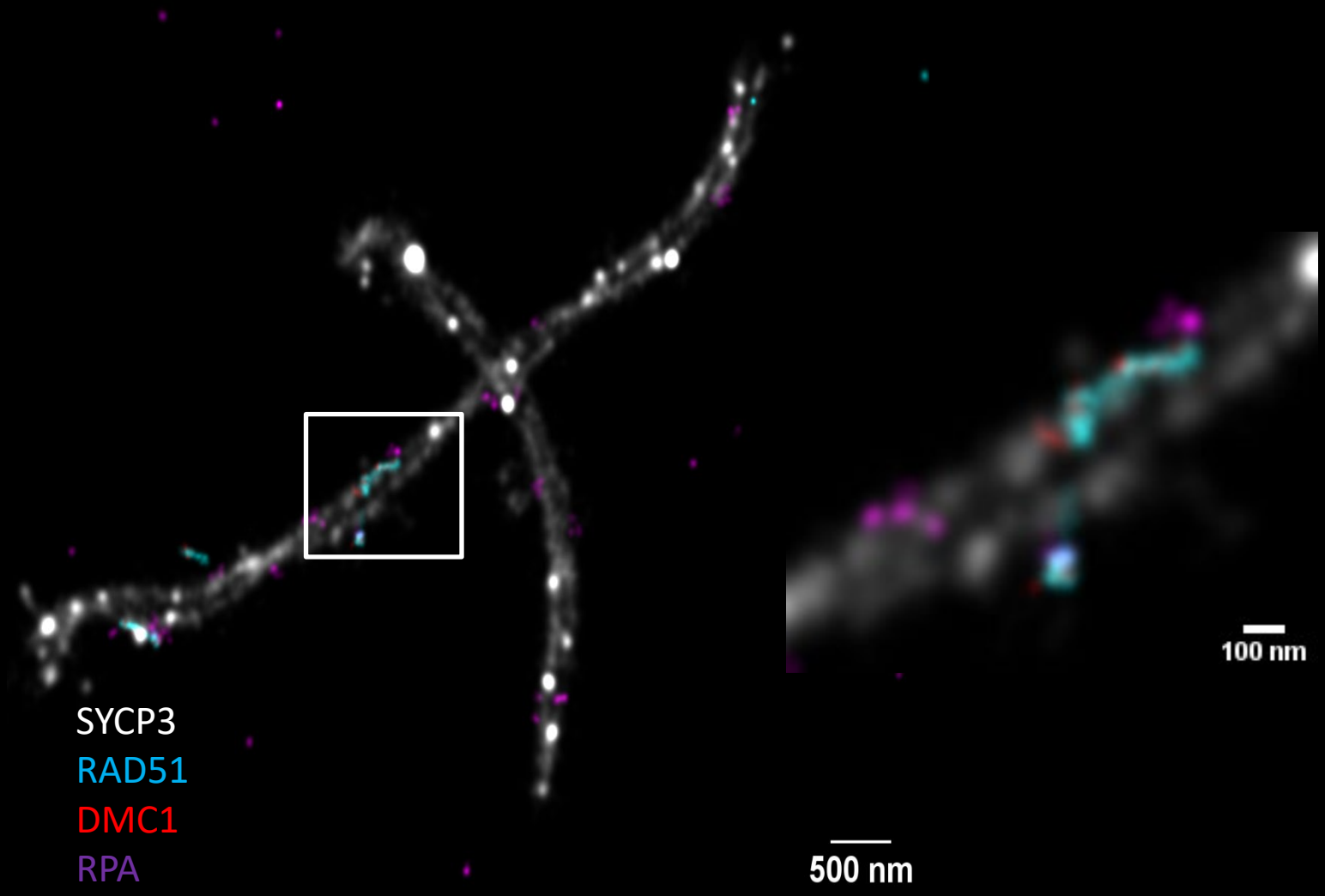
dSTORM

DMC1 Alexa647 (dSTORM)
SYCP3 Alexa546 (SIM)
RAD51 Alexa488 (dSTORM)

Visualization and Analysis



Multiplex DNA-PAINT Imaging



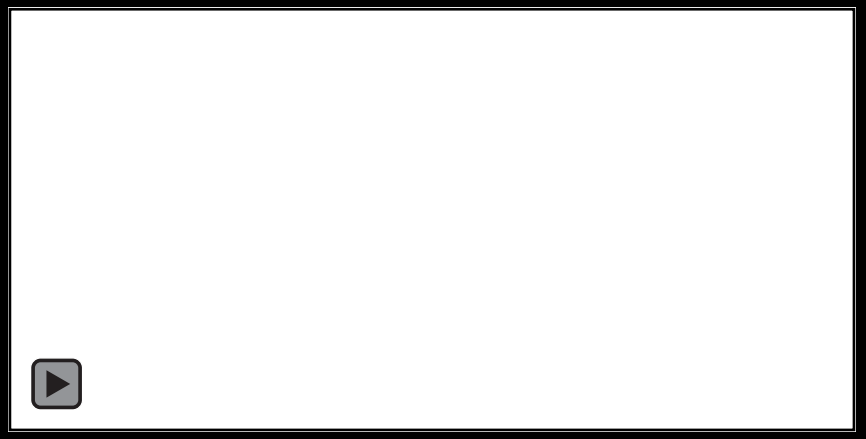
Aditya Mhaskar

Summary

- Basic principle of PALM/STORM/PAINT
  - Observe only a small subset of all fluorescent molecule in every image frame
  - Determine the position of the individual molecules
- Highest achievable x-y resolution using a fluorescent microscope
- Live cells?

Live cell PALM

- Single molecule techniques in live cells
  - Single Molecule tracking
- Study mobility of proteins at single molecule level

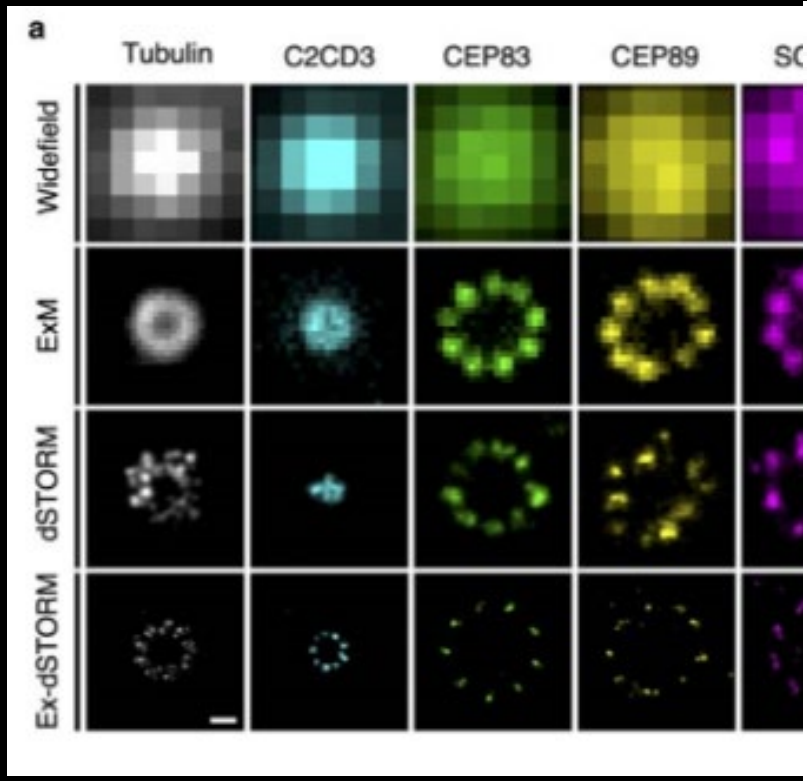


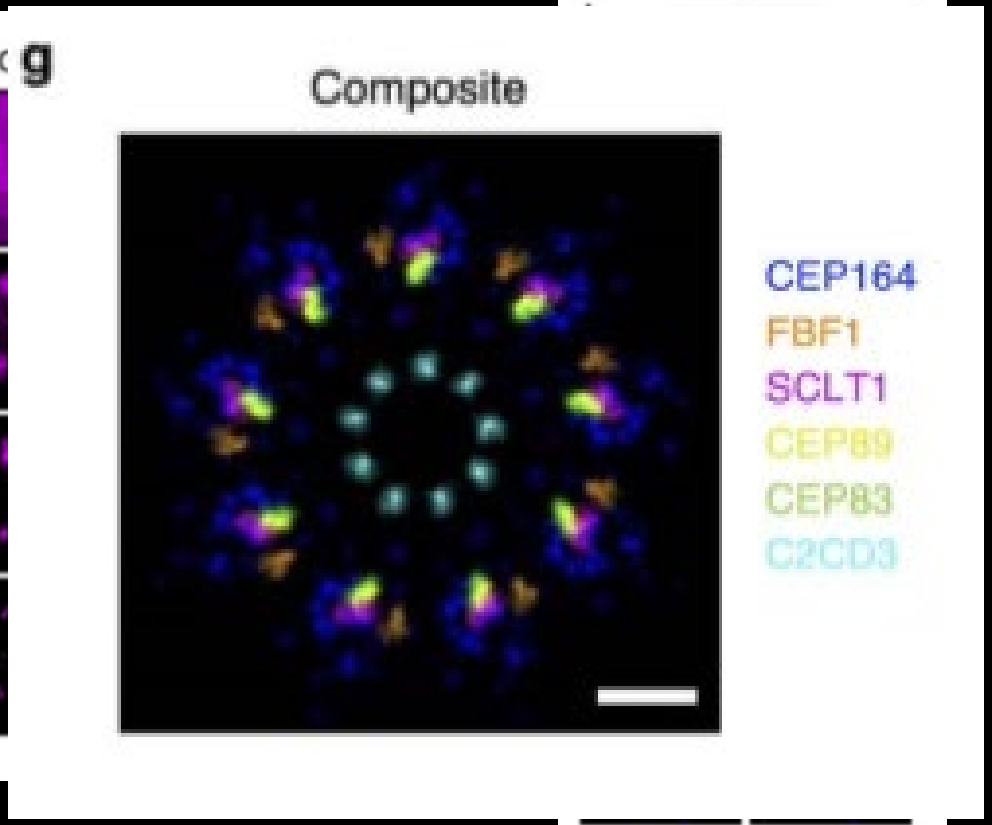
Combining expansion with a super-resolution microscopy

EXPANSION MICROSCOPY

Combining super-resolution techniques

Expansion Microscopy combined with dSTORM





Chang et. al. 2022 BioRxiv Single-molecule localization microscopy reveals the ultrastructural root constitution of distal appendages in expanded mammalian centrioles

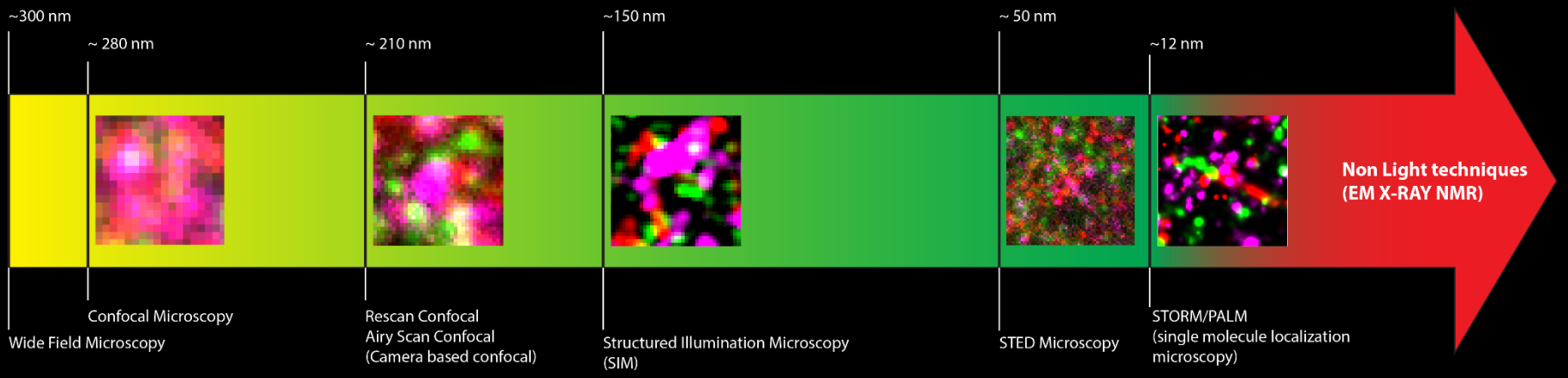
Summary Expansion Microscopy

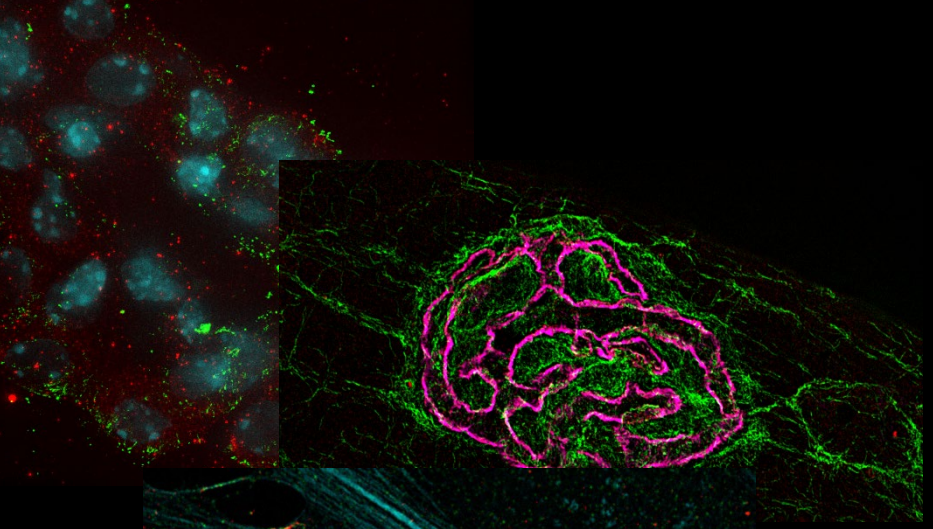
- Sample preparation technique
- Fixed samples
- Multi color
- 3D
- Can be combined with super resolution microscopy

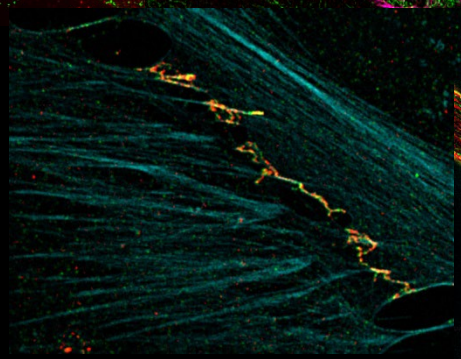
- Difficult to relate back to original sample in measurements
- Limited working distances of lenses, samples get really big!

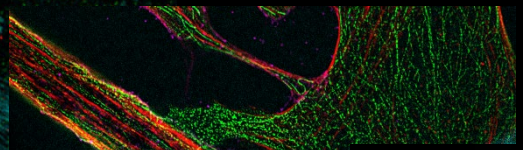
The future is bright and high resolution

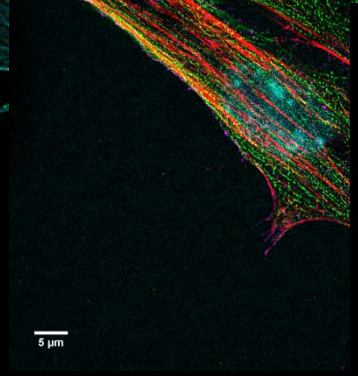
MinSTED
MinFlux
4Pi
PAINT
SIM
4Pi / STORM
ExM
SIM / Lightsheet
ISM
STED
SIMFlux
SMLM
Correlative EM
RCM / PAINT
Rescan confocal

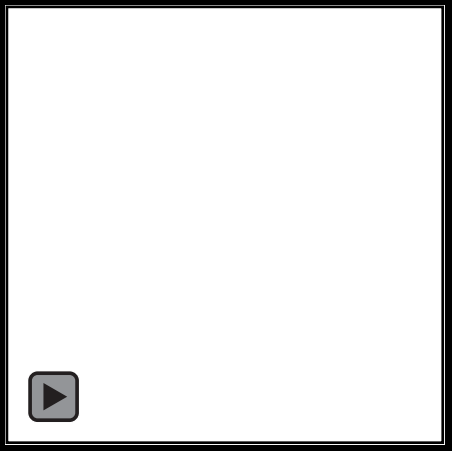




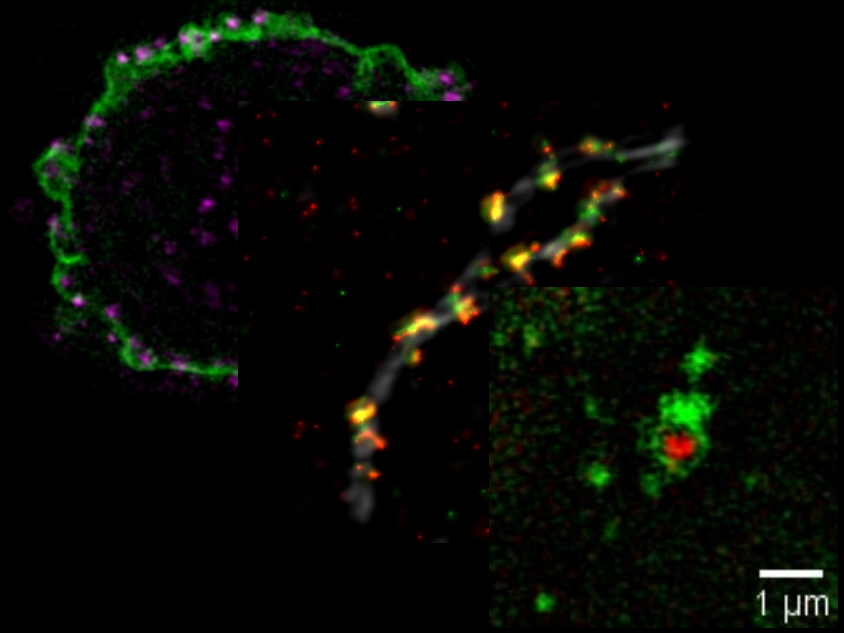




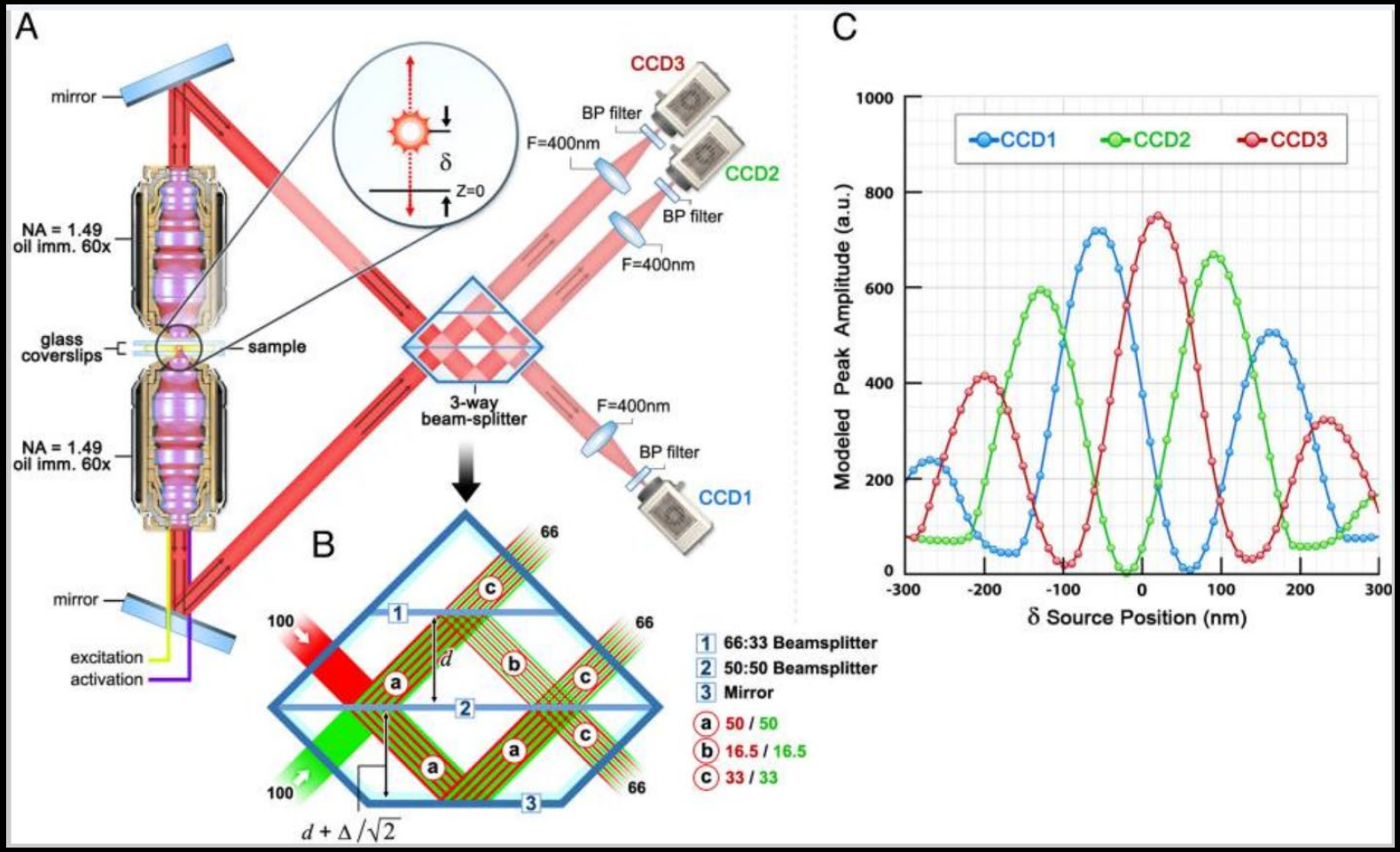




Thank you for your attention



iPALM


A
mirror
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oil imm. 60x
glass
coverslips
sample
NA = 1.49
oil imm. 60x
mirror
excitation
activation
δ
Z=0
CCD3
BP filter
F=400nm
CCD2
BP filter
F=400nm
3-way
beam-splitter
F=400nm
BP filter
CCD1
B
66
100
66
100
66
d
d + Δ/√2
1 66:33 Beamsplitter
2 50:50 Beamsplitter
3 Mirror
a 50 / 50
b 16.5 / 16.5
c 33 / 33
C
CCD1
CCD2
CCD3
Modeled Peak Amplitude (a.u.)
δ Source Position (nm)