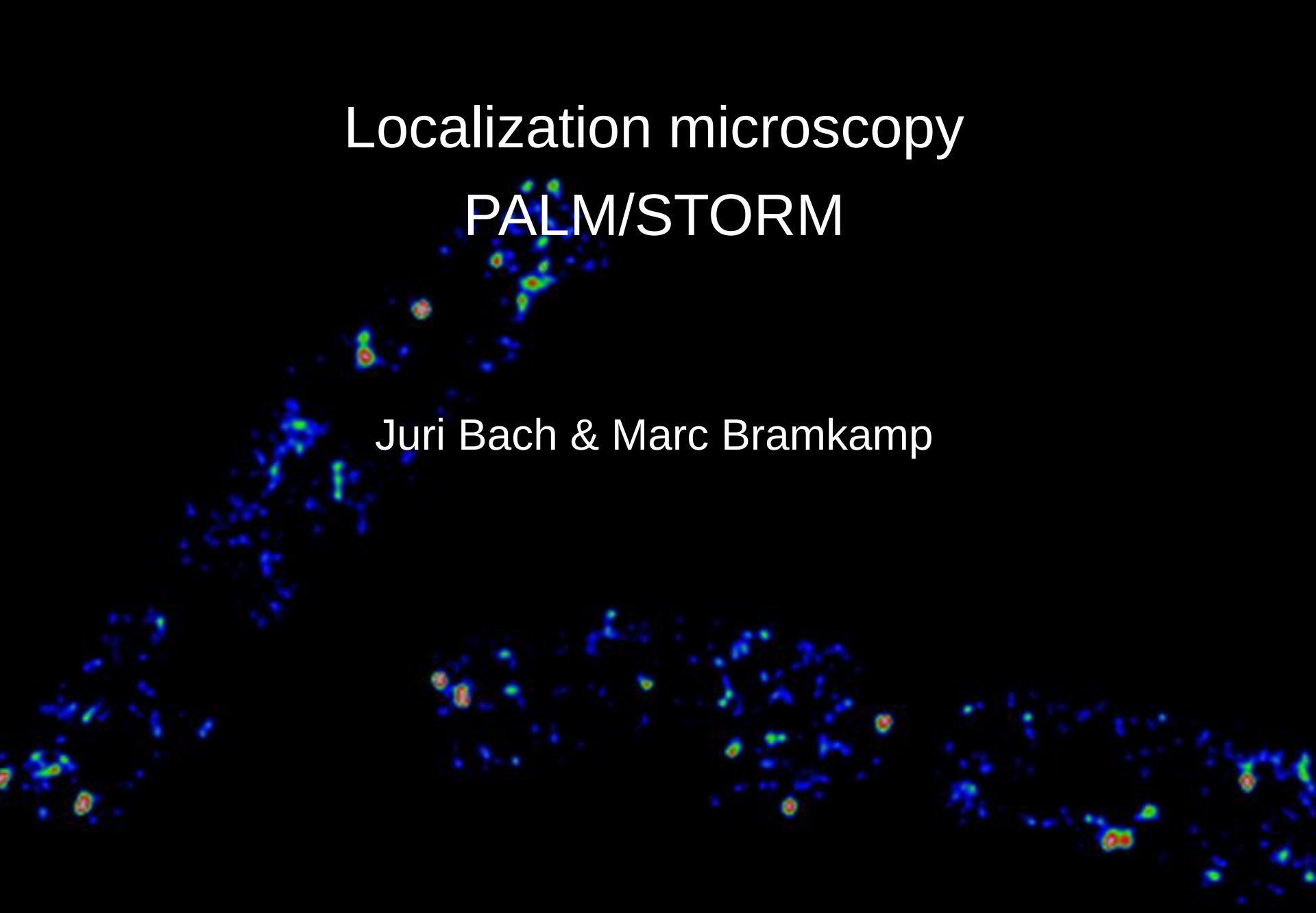
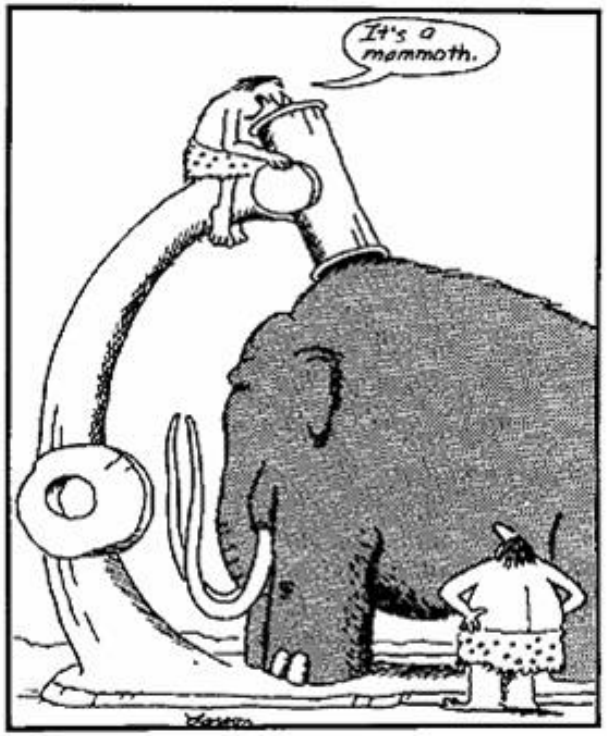
# Localization microscopy
# PALM/STORM

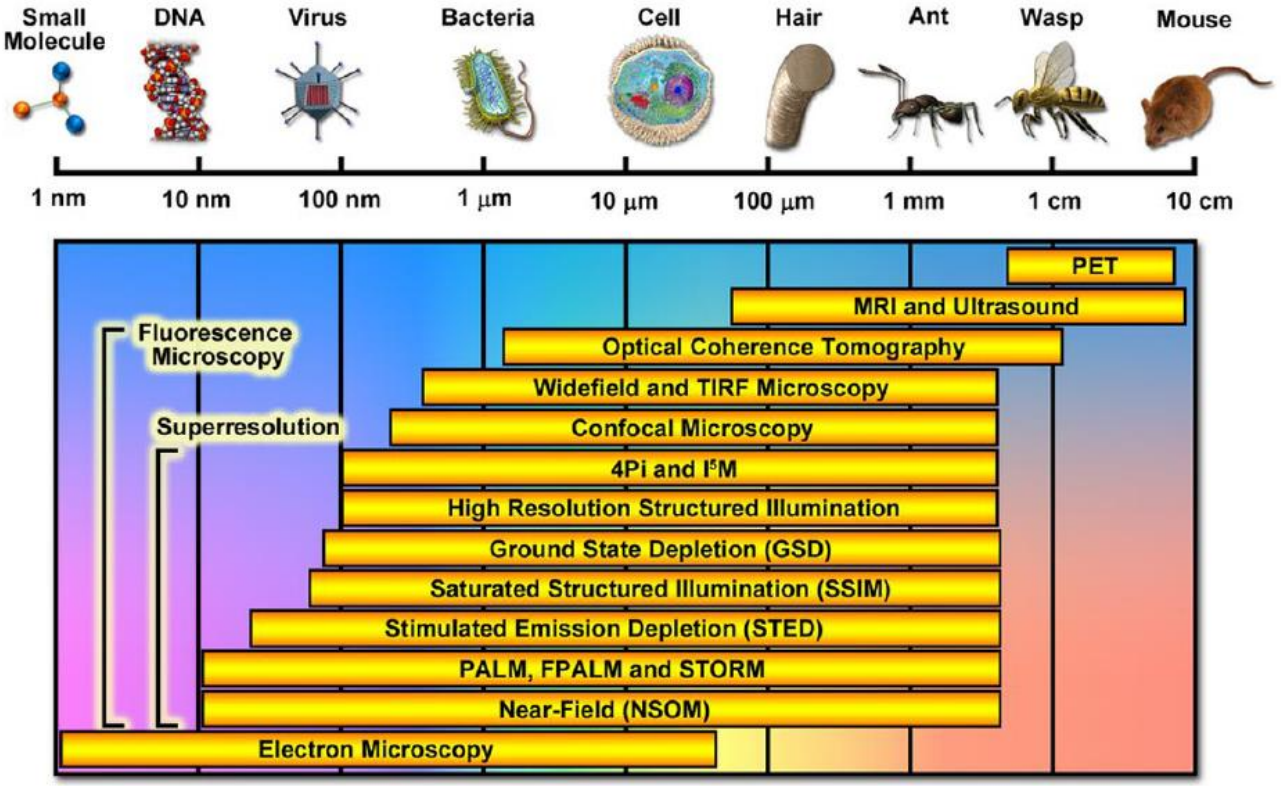


Juri Bach & Marc Bramkamp

Microbiology, LMU Munich

# Resolution




**Allen et al, 2013**

Choosing the right technique for your approach

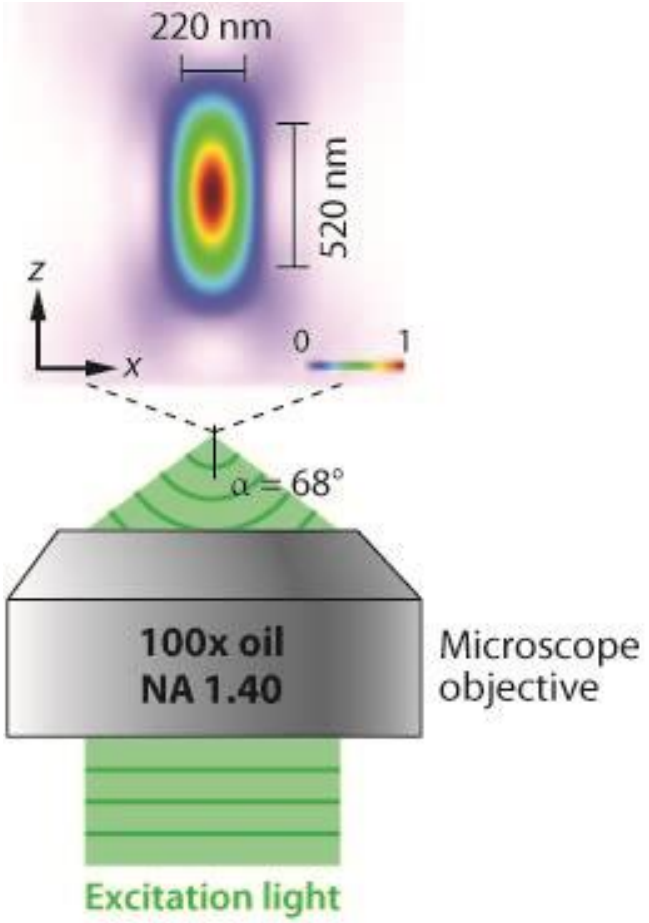
# Resolution

**$\text{Resolution}_{x,y} = \lambda / 2NA$**

**$\text{Resolution}_{z} = 2\lambda / [NA]^2$**

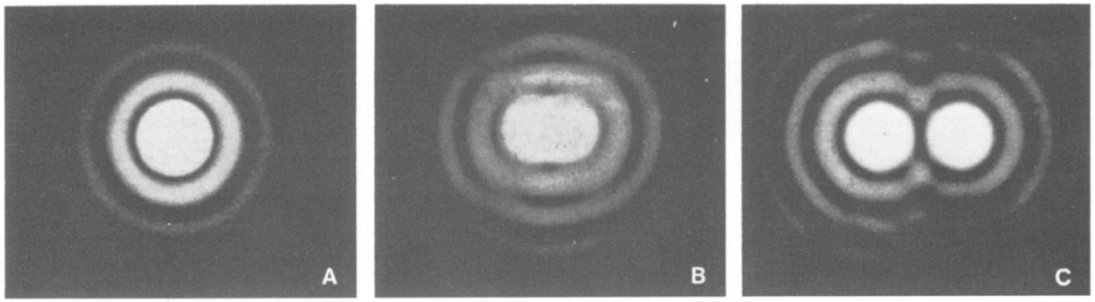
**xy resolution: ~ 220 nm**

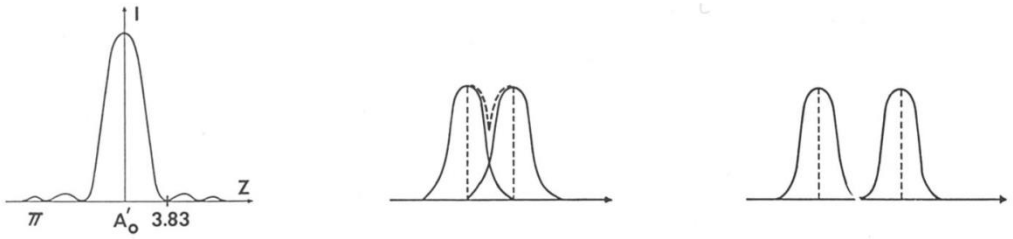
**z resolution (confocal): ~ 520 nm**




**It is physically impossible to resolve smaller objects!**

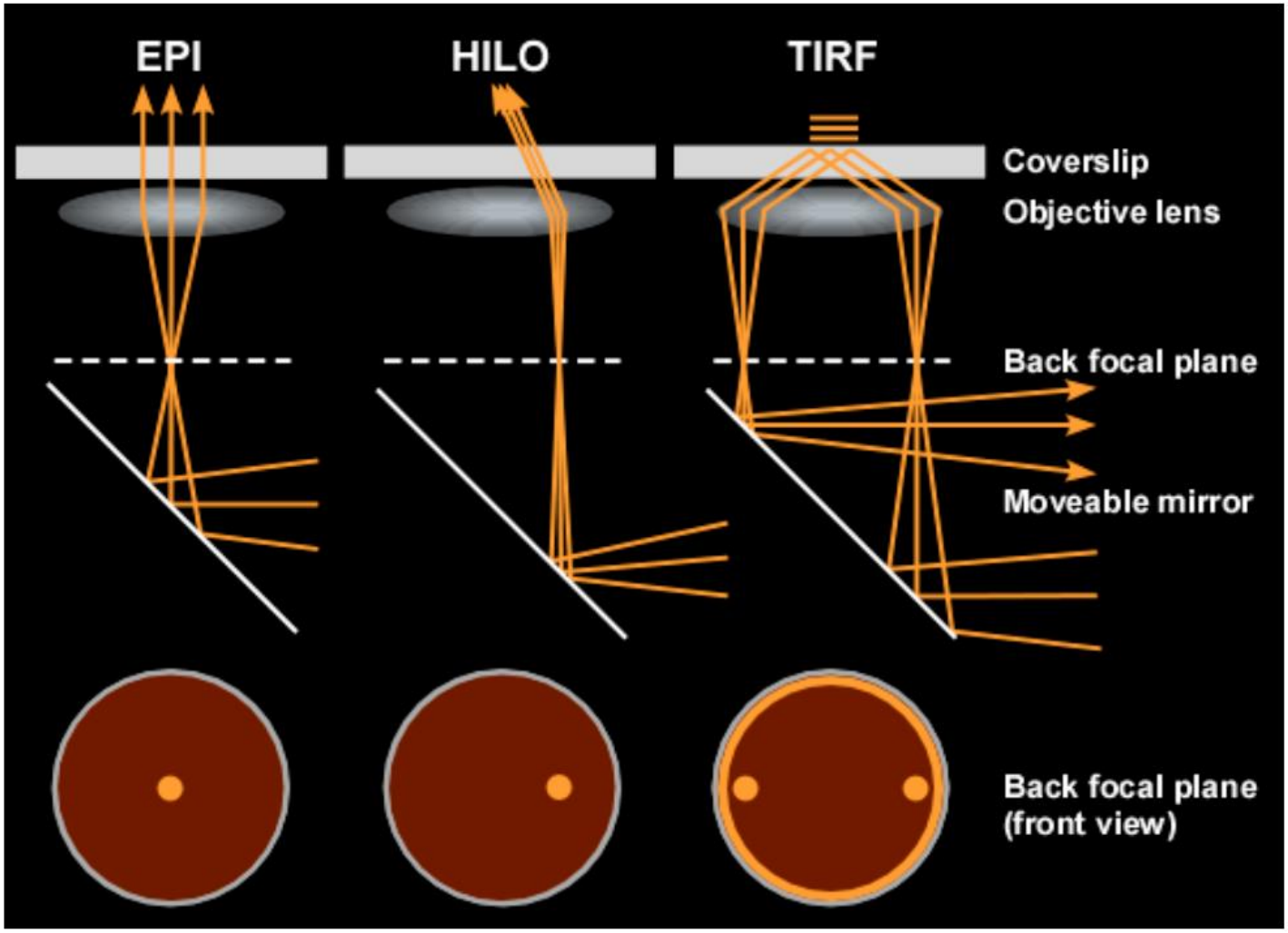
Huang et al., 2009


A
B
C


I
Z
π
A'o
3.83

**How is it possible to resolve complexes smaller than 220 nm by light microscopy?**

# Restricting the z-axis



# Super resolution microscopy

- Stimulated emission depletion **(STED)**
- Saturated illumination microscopy **(SIM)**
- Localization microscopy
  - stochastic optical reconstruction microscopy **(STORM)**
  - (fluorescence) photo-activation localization microscopy **(f)PALM**
- **Various others…**

**Nobel Prize in Chemistry 2014 for surpassing the limitations of the light microscope**

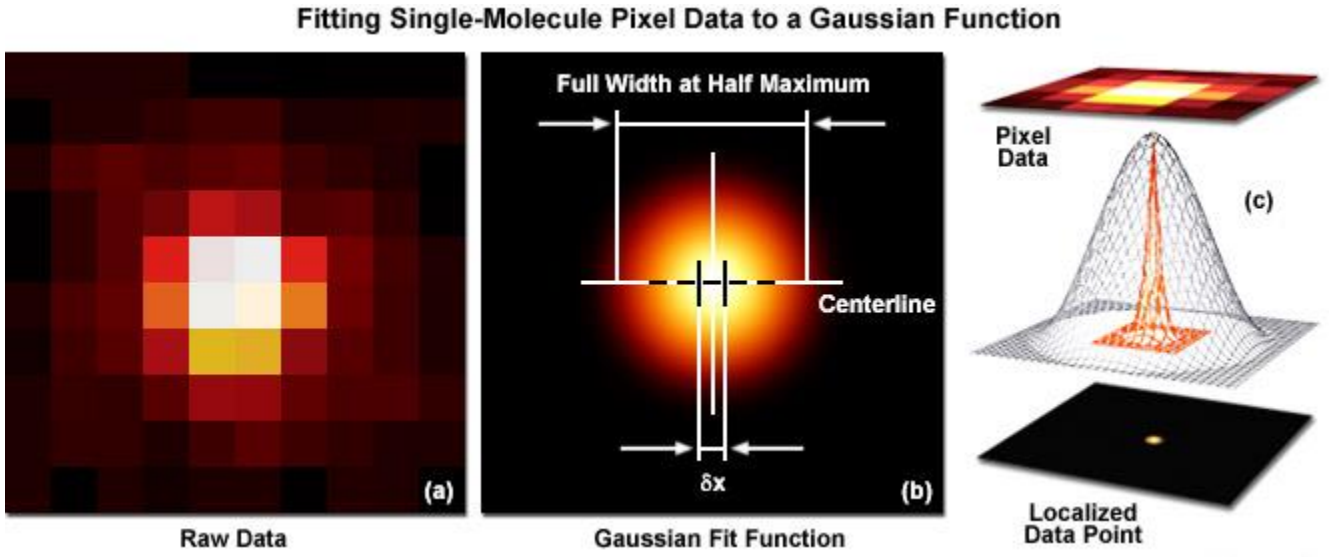
**Eric Betzig, Stefan W. Hell, William E. Moerner**

# Localization microscopy

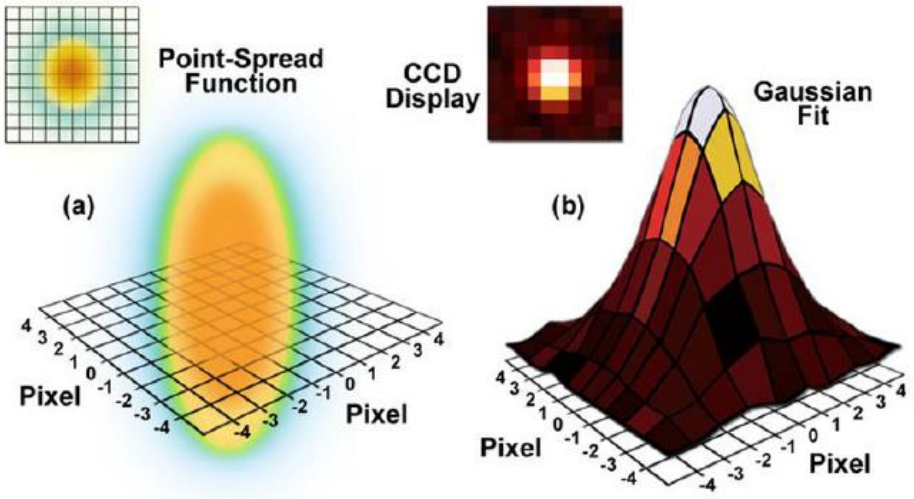
- STORM = Stochastic  Optical Reconstruction Microscopy (Zhuang 2006)
- PALM = Photoactivated Localization Microscopy (Betzig & Hess 2006)
- FPALM = Fluorescence Photoactivation Localization Microscopy (Hess 2006)
- PALMIRA (Hell 2007), GSDIM (Hell 2008), dSTORM (Sauer 2008), SMACM (Moerner 2008)
- PAINT (Hochstrasser 2006), SPRAYPAINT (Moerner 2011),SOFI (Weiss 2009)
- And others…

# The principle of single molecule localization fluorescence microscopy

- Single molecules can be localized with high precision
- Serial imaging makes it possible to reconstruct the positions of all labeled molecules




Zeiss

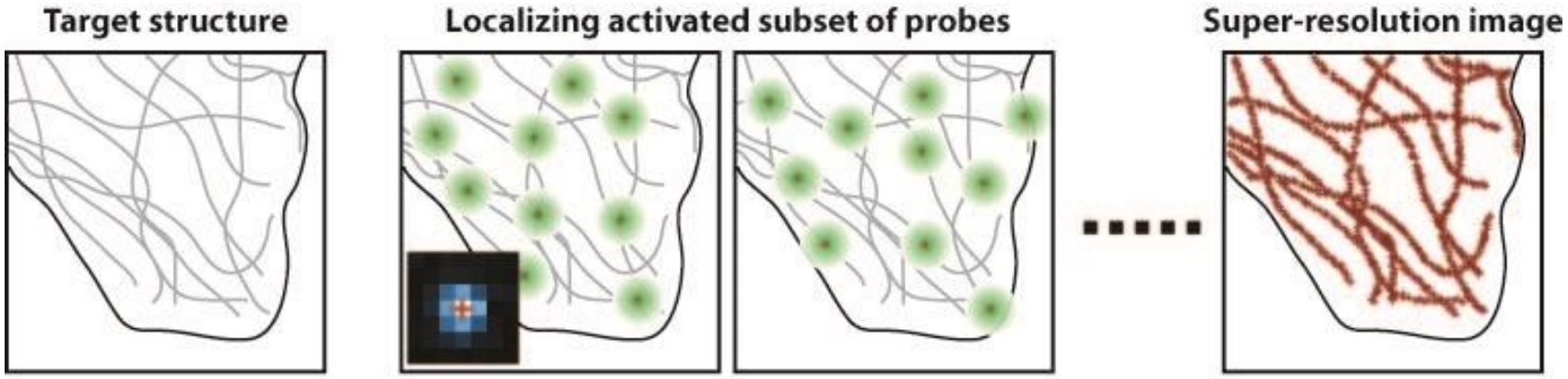



Allen et al, 2013

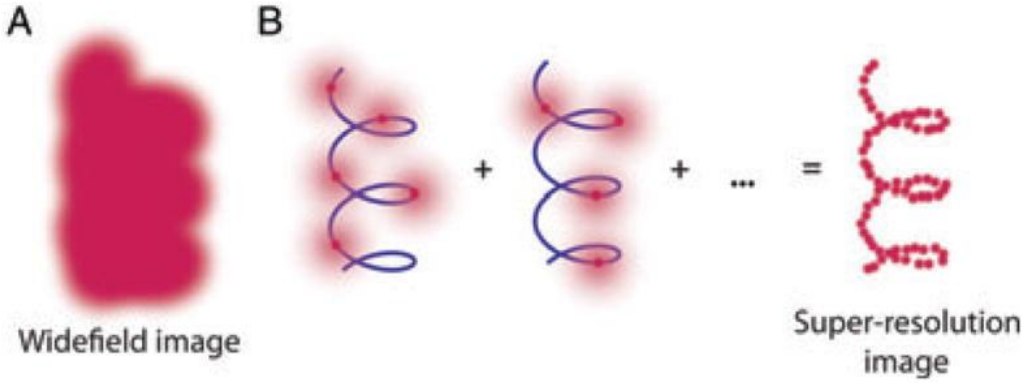
# The principle of single molecule localization fluorescence microscopy

- Single molecules can be localized with high precision
- Biological specimens typically contain many labeled molecules
- By imaging only a few molecules at a time, it is possible to reconstruct the positions of all the labeled molecules
- This can be achieved by switching fluorescent molecules between “on” and “off” states

# The principle of single molecule localization fluorescence microscopy

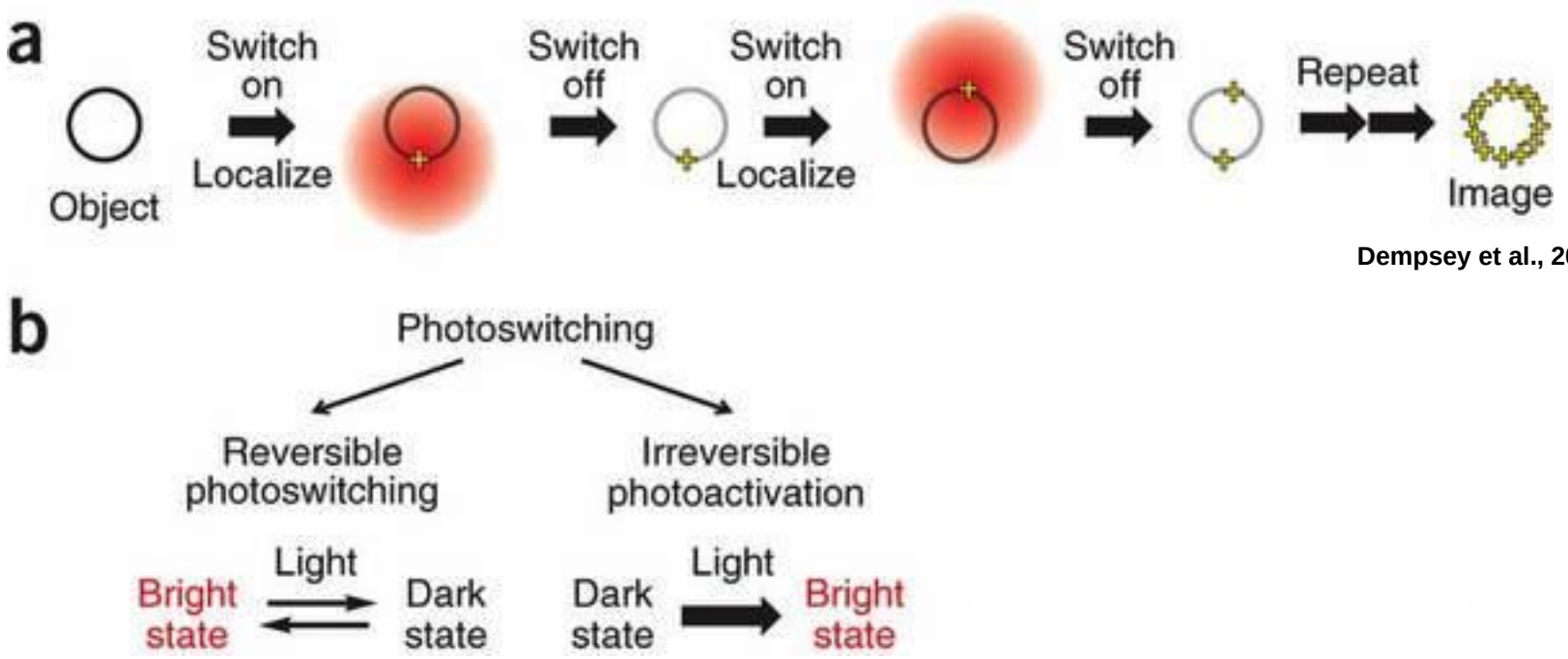



Huang et al., 2009




Moerner, 2006

# Fluorophores for single molecule localization fluorescence microscopy

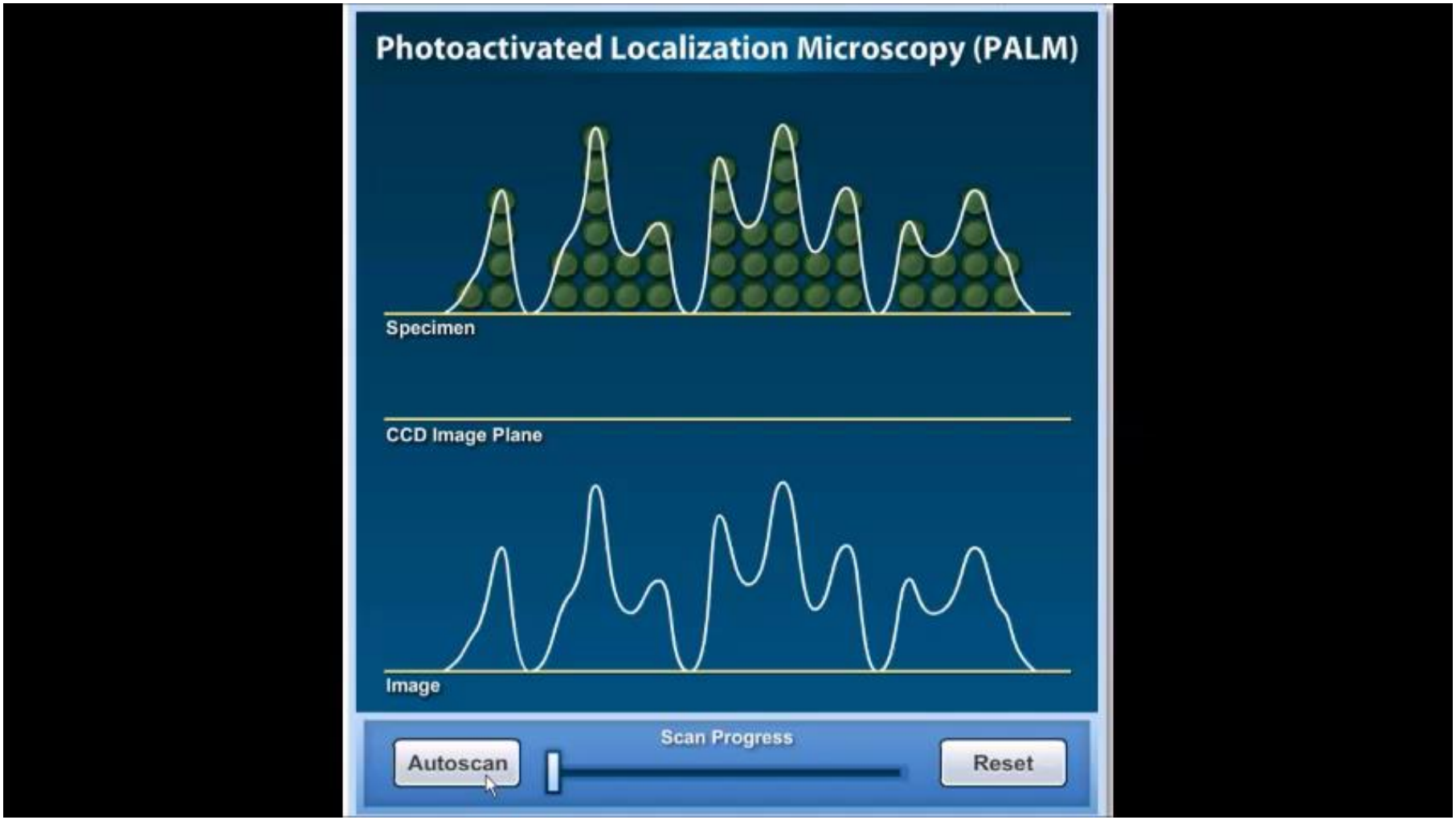



Dempsey et al., 2011

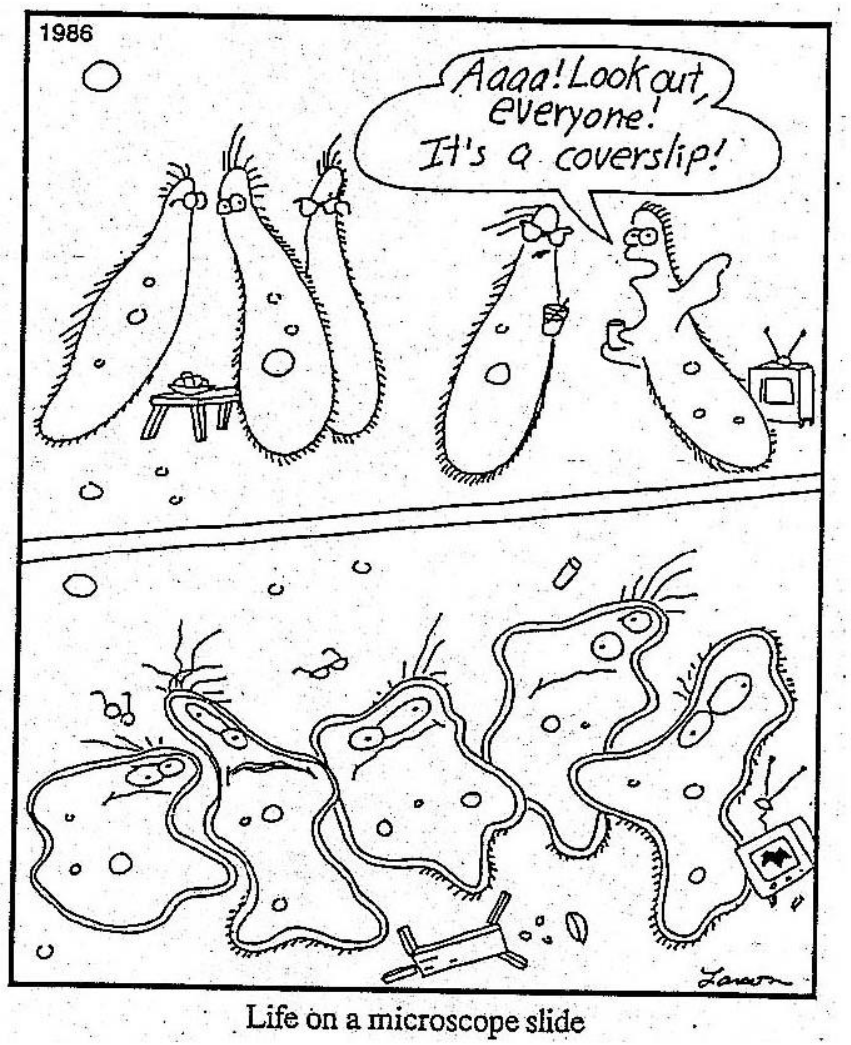
Photoswitchable dyes and fluorescent proteins

Photoactivatable and photoconvertable fluorescent proteins

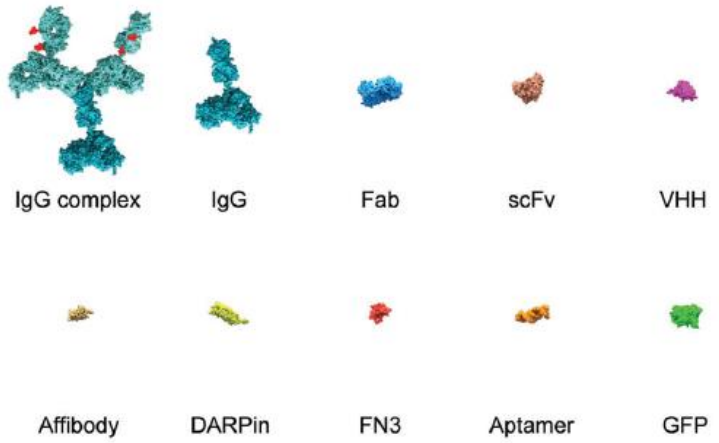
# Principle of localization fluorescence microscopy



# Treat your sample well




| Name | Molecular weight | Linear size | Composition |
|---|---|---|---|
| IgGs | ~150 kDa | ~15 nm | Protein |
| Fabs | ~50 kDa | ~9 nm | Protein |
| scFvs | ~30 kDa | ~6 nm | Protein |
| VHHs (nanobodies) | ~15 kDa | ~3 nm | Protein |
| Affibodies | ~6 kDa | ~2 nm | Protein |
| DARPins | ~10–30 kDa | ~2–3 nm | Protein |
| Fibronectin III (FN3, Monobodies) | ~10 kDa | ~2 nm | Protein |
| Aptamers | ~10–20 kDa | ~2–3 nm | Single stranded DNA or RNA |




Fornasiero and Opazo, 2015

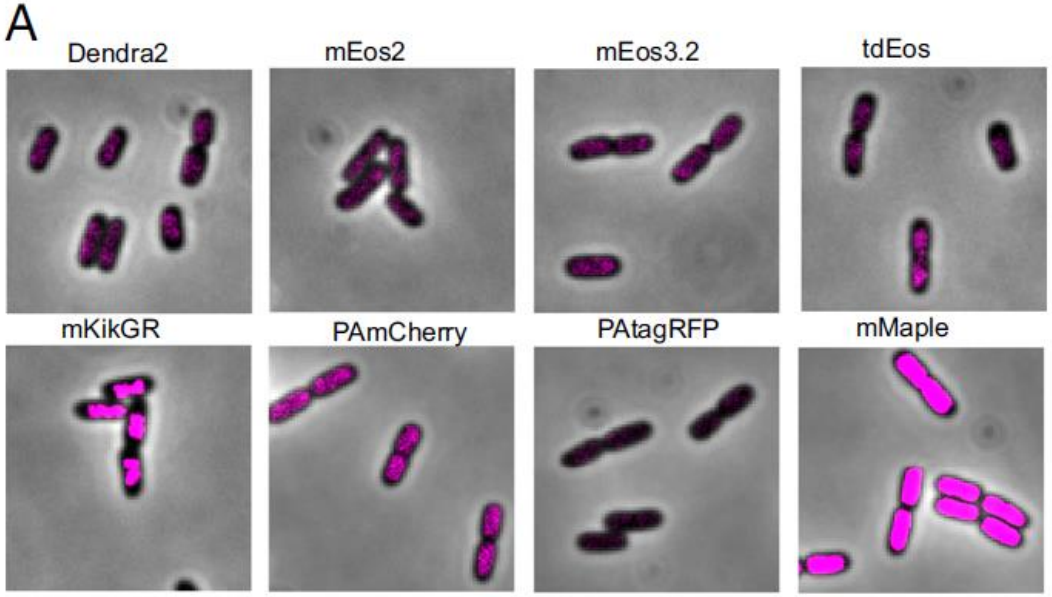
# Numerous fluorophores / dyes already exist

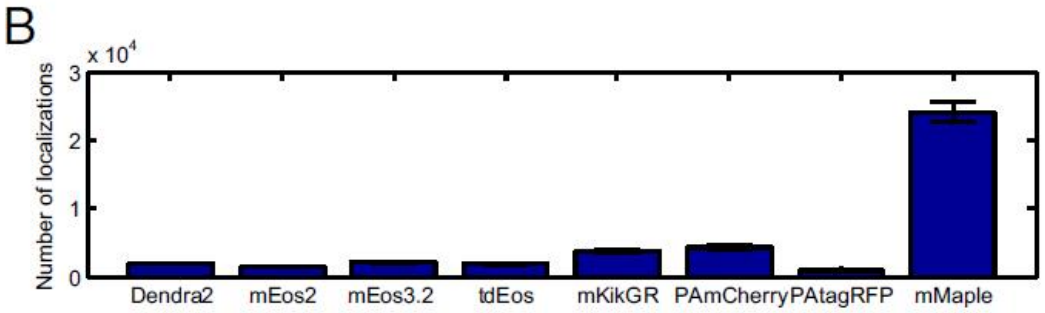
| Protein (Acronym) | Ex (nm) | Em (nm) | EC ($\times 10^{-3}$) | QY | *N* photons emitted | Contrast ratio | Quaternary structure | Brightness (% of EGFP) |
|---|---|---|---|---|---|---|---|---|
| **Photoactivatable fluorescent proteins** | | | | | | | | |
| PA-GFP (N) | 400 | 515 | 20.7 | 0.13 | 70 | NA | Monomer | 8 |
| PA-GFP (G) | 504 | 517 | 17.4 | 0.79 | 300 | 100 | Monomer | 41 |
| PS-CFP2 (C) | 400 | 468 | 43.0 | 0.20 | ND | NA | Monomer | 26 |
| PS-CFP2 (G) | 490 | 511 | 47.0 | 0.23 | 260 | 1500 | Monomer | 32 |
| PA-mCherry1 (R) | 564 | 595 | 18.0 | 0.46 | ND | 4000 | Monomer | 25 |
| PA-TagRFP (R) | 562 | 595 | 66.0 | 0.38 | 500 | 550 | Monomer | 75 |
| **Photoconvertible fluorescent proteins** | | | | | | | | |
| mKikGR (G) | 505 | 515 | 49.0 | 0.69 | ND | NA | Monomer | 101 |
| mKikGR (R) | 580 | 591 | 28.0 | 0.63 | 970 | 400 | Monomer | 53 |
| tdEos (G) | 506 | 516 | 34.0 | 0.66 | ND | NA | Tandem dimer | 165 |
| tdEos (R) | 569 | 581 | 33.0 | 0.60 | 750 | >4000 | Tandem dimer | 59 |
| mEos2 (G) | 506 | 519 | 56.0 | 0.74 | ND | NA | Monomer | 140 |
| mEos2 (R) | 573 | 584 | 46.0 | 0.66 | 500 | >2000 | Monomer | 90 |
| Dendra2 (G) | 490 | 507 | 45.0 | 0.50 | ND | NA | Monomer | 67 |
| Dendra2 (R) | 553 | 573 | 35.0 | 0.55 | ND | 300 | Monomer | 57 |
| **Photoswitchable fluorescent proteins** | | | | | | | | |
| Dronpa | 503 | 517 | 95.0 | 0.85 | 120 | <1000 | Monomer | 240 |
| Dronpa-3 | 487 | 514 | 58.0 | 0.33 | ND | ND | Monomer | 56 |
| rsFastLime | 496 | 518 | 39.1 | 0.77 | ND | ND | Monomer | 89 |
| Padron | 503 | 522 | 43.0 | 0.64 | ND | ND | Monomer | 82 |
| bsDronpa | 460 | 504 | 45.0 | 0.50 | ND | ND | Monomer | 67 |
| KFP1 | 580 | 600 | 59.0 | 0.07 | ND | ND | Tetramer | 12 |
| mTFP0.7 | 453 | 488 | 60.0 | 0.50 | ND | ND | Monomer | 89 |
| E2GFP | 515 | 523 | 29.3 | 0.91 | ND | ND | Monomer | 79 |
| rsCherry | 572 | 610 | 80.0 | 0.02 | ND | ND | Monomer | 5 |
| rsCherryRev | 572 | 608 | 84.0 | 0.005 | ND | ND | Monomer | 1 |
| **Photoconvertible/photoswitchable fluorescent proteins** | | | | | | | | |
| IrisFP (G) | 488 | 516 | 52.2 | 0.43 | ND | ND | Tetramer | 67 |
| IrisFP (R) | 551 | 580 | 35.4 | 0.47 | ND | ND | Tetramer | 50 |
| **Synthetic fluorophores** | | | | | | | | |
| Cy5 | 649 | 664 | 250.0 | 0.28 | 6000 | ND | NA | 208 |
| Cy5.5 | 675 | 694 | 190.0 | 0.23 | 6000 | ND | NA | 130 |
| Cy7 | 747 | 767 | 200.0 | 0.28 | 1000 | ND | NA | 167 |
| Alexa Fluor 647 | 650 | 665 | 240.0 | 0.33 | 6000 | ND | NA | 236 |
| ATTO 532 | 532 | 553 | 115.0 | 0.90 | ND | ND | NA | 308 |
| Rhodamine B | 530 | 620 | 105.0 | 0.65 | 750 | ND | NA | 203 |
| C-Rhodamine | 545 | 575 | 90.0 | 0.90 | ND | ND | NA | 241 |
| C-Fluorescein | 494 | 518 | 29.0 | 0.93 | ND | ND | NA | 80 |

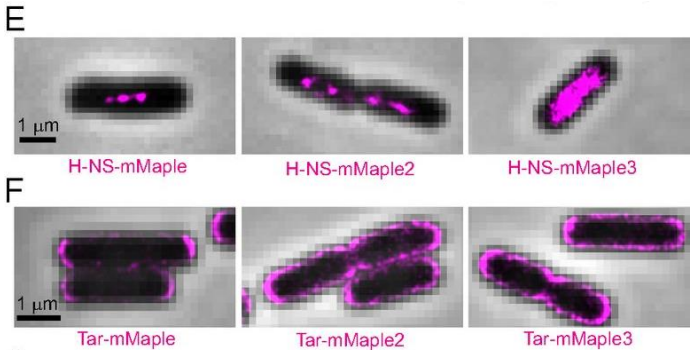
**Allen et al., 2013**

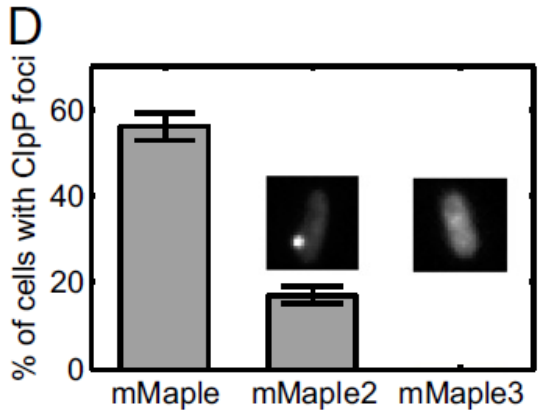
# Fluorophores make the difference

Localization of HU-PAFPs in *E. coli*




Wang et al., 2014

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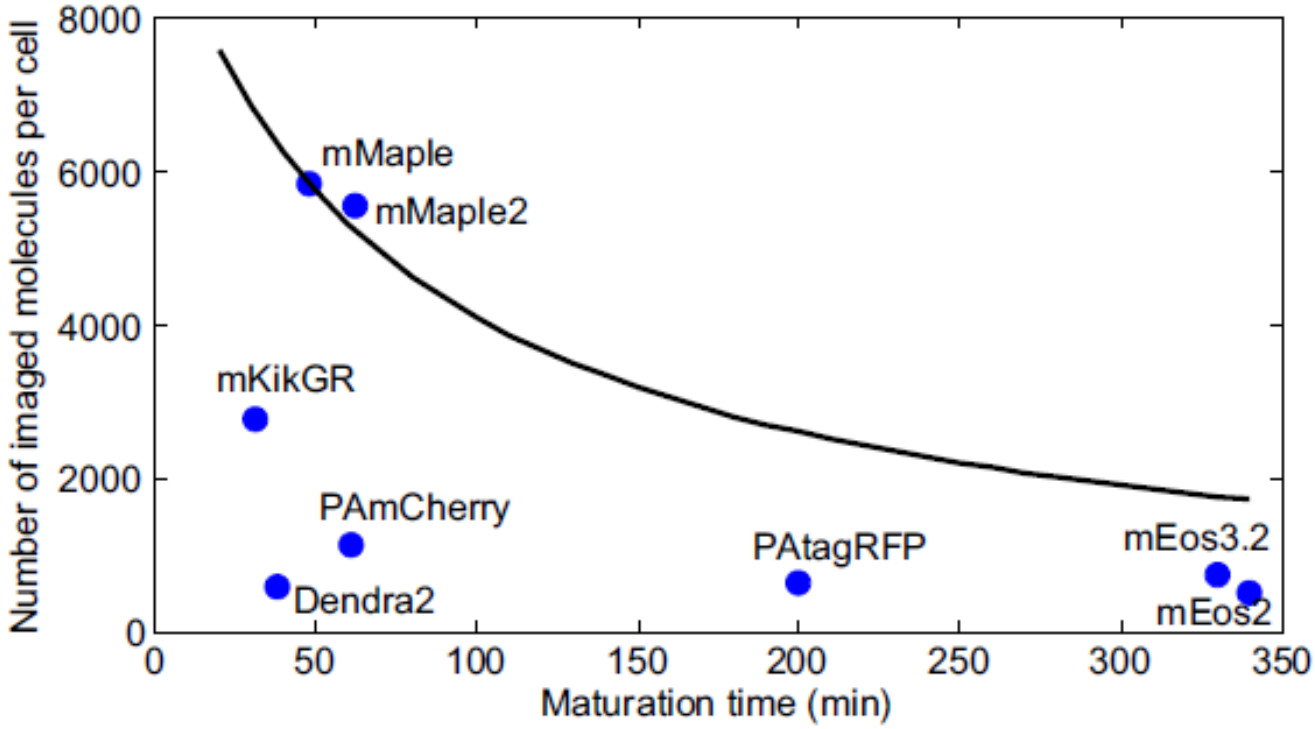
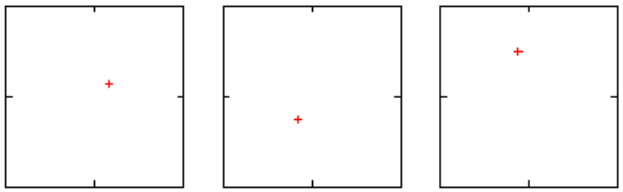


# Fluorophores make the difference

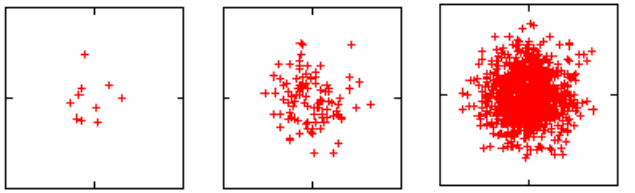
Localization accuracy is proportional to the number of photons detected from a single fluorescent molecule



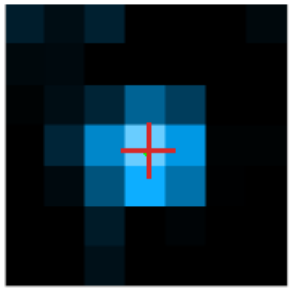
1 photon

$$d \approx \frac{\lambda}{2\,NA}$$

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



10 photons 100 photons 1000 photons



**N** is the number of photons gathered
**a** is the pixel size of the imaging CCD detector
**b** is the standard deviation of the background (which includes background fluorescence emission combined with detector noise)
$\mathbf{s_i}$ is the standard deviation or width of the distribution (in direction **i**)

**Zeiss**

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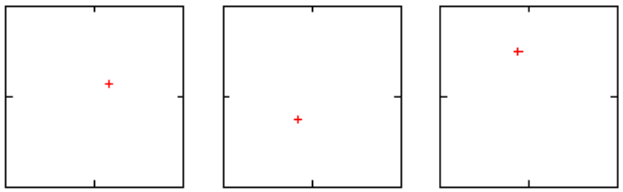

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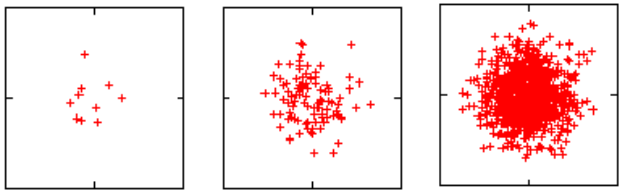

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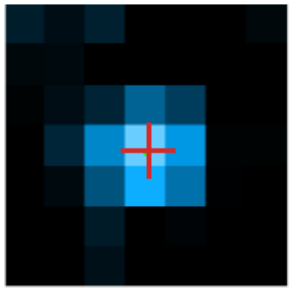

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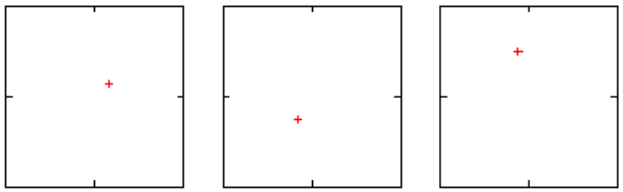

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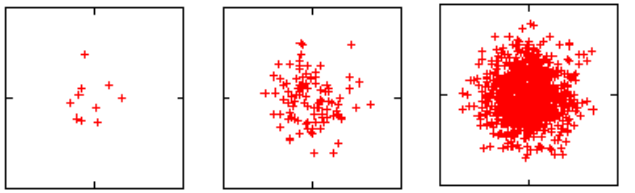

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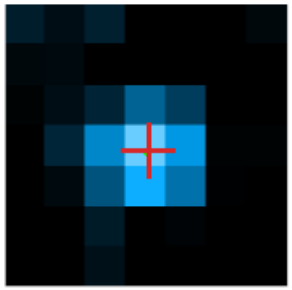

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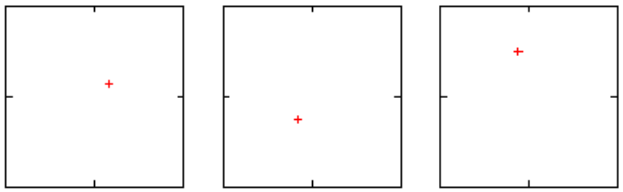

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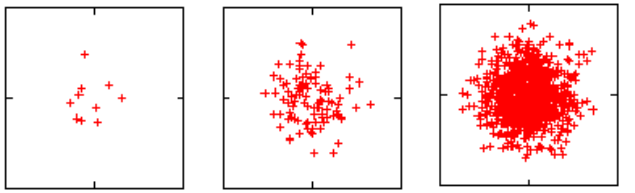

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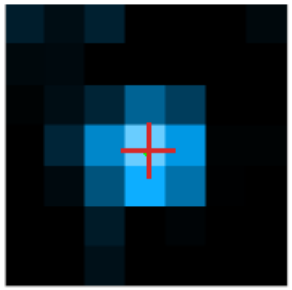

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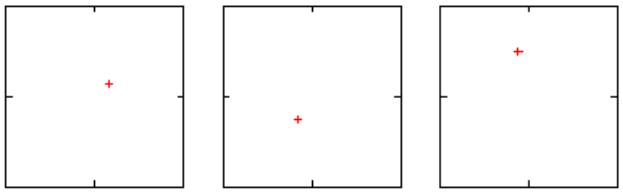

1 photon

$$d \approx \frac{\lambda}{2\,NA}$$

**photon noise** **finite pixel size detector** **background noise**

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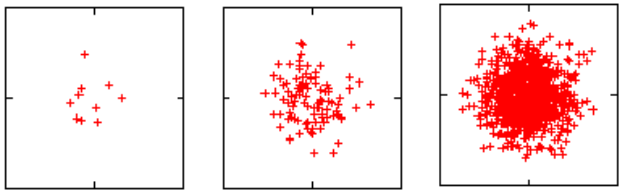

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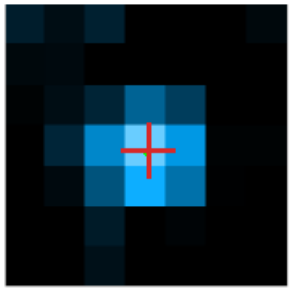

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$$d = \frac{1}{\sqrt{N}} \cdot \frac{\lambda}{2NA}$$

Zeiss

# Fluorophores make the difference

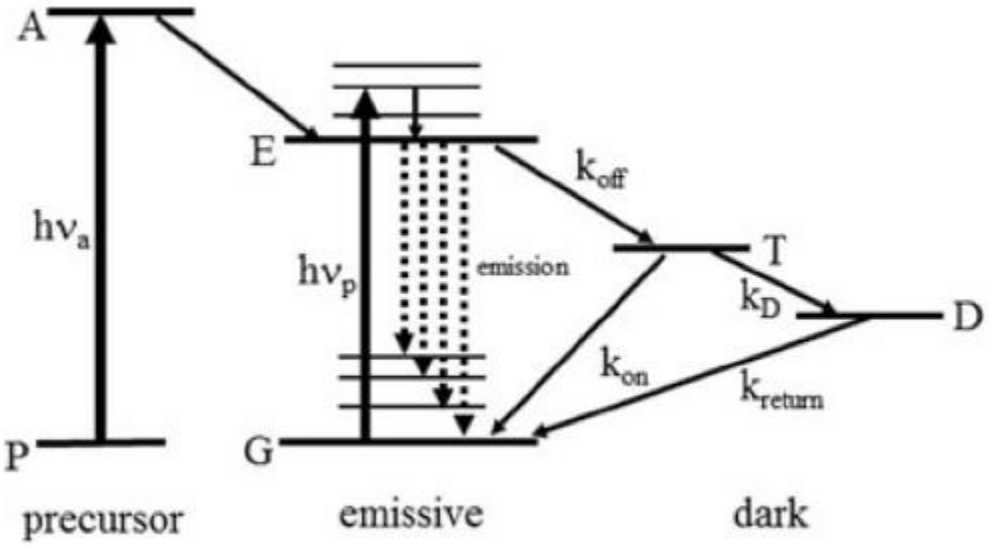
| PAFP | Preactivation/postactivation emission wavelength, nm* | Photon no. | On–off switching rate ratio | ClpP clustering† | No. of localizations per cell‡ | Maturation time, min§ |
|---|---|---|---|---|---|---|
| Dendra2 | 507/573 | 686 | $4.2 \times 10^{-6}$ | – | 1,810 | 38 |
| mEos2 | 519/584 | 745 | $2.9 \times 10^{-6}$ | + | 1,290 | 340 |
| mEos3.2 | 516/580 | 809 | $2.6 \times 10^{-6}$ | – | 1,950 | 330 |
| tdEos | 516/581 | 774 | $3.2 \times 10^{-6}$ | – | 1,800 | 330 |
| mKikGR | 515/591 | 599 | $4.1 \times 10^{-6}$ | + | 3,800 | 31 |
| PAmCherry | —/595 | 706 | $7.8 \times 10^{-6}$ | + | 4,200 | 61 |
| PAtagRFP | —/595 | 906 | $5.7 \times 10^{-6}$ | – | 760 | 200 |
| mMaple | 505/583 | 798 | $1.9 \times 10^{-6}$ | + | 24,000 | 48 |
| mMaple2 | 506/582 | 783 | $1.0 \times 10^{-6}$ | + | 21,000 | 62 |
| mMaple3 | 506/583 | 675 | $6.2 \times 10^{-7}$ | – | 12,300 | 49 |
| PAGFP | —/517 | 313 | $1.3 \times 10^{-3}$ | – | | <10 |
| PSCFP2 | 468/511 | 223 | $8.1 \times 10^{-6}$ | + | | |
| Dronpa | —/517 | 262 | $5.8 \times 10^{-4}$ | – | | 25 |
| mGeosM | —/514 | 248 | $4.9 \times 10^{-4}$ | + | | <10 |

| PAFP | Average no. of blinking events* | No. of imaged molecules per cell† | Expression level by quantitative Western, molecules per cell‡ | Percentage of PAFP imaged, %§ |
|---|---|---|---|---|
| Dendra2 | 1.7 | 1,060 | 27,000 | 3.9 |
| mEos2 | 2.8 | 460 | 47,000 | 1.0 |
| mEos3.2 | 3.0 | 650 | 38,000 | 1.7 |
| tdEos | 3.3 | 270 | 6,600 | 4.0 |
| mKikGR | 1.7 | 2,200 | 82,000 | 2.7 |
| PAmCherry | 1.9 | 2,200 | 61,000 | 3.6 |
| PAtagRFP | 1.7 | 450 | 50,000 | 0.89 |
| mMaple | 2.5 | 9,700 | 49,000 | 20 |
| mMaple2 | 2.7 | 7,700 | 42,000 | 18 |
| mMaple3 | 2.8 | 4,400 | 42,000 | 10 |

Wang et al., 2014

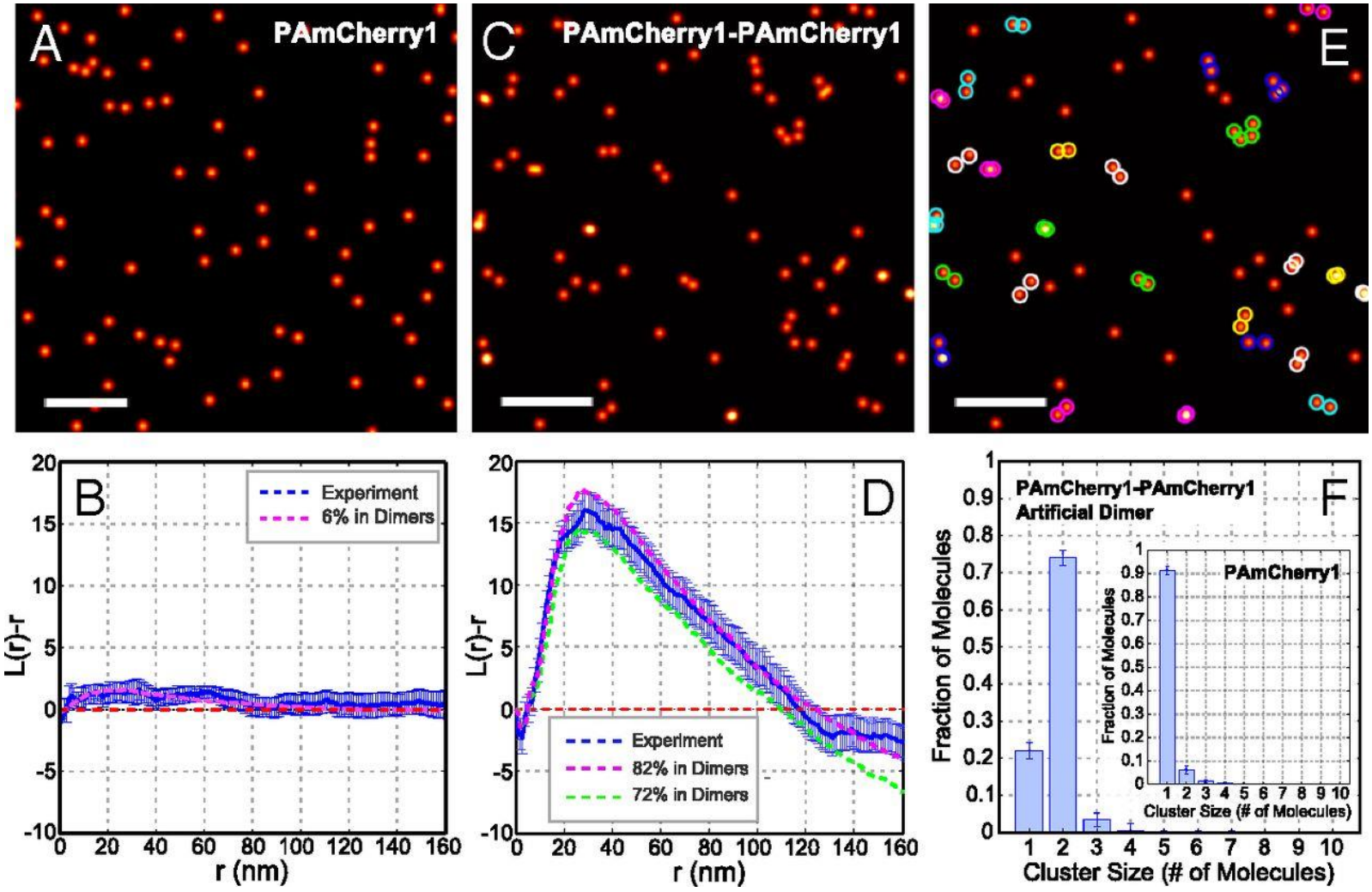
# The dark site of the fluorophore

- PALM (dark, activated, on until bleached)
- Photoswitching (on / off)
  - Isomerizations
  - Photochemical conversion to dark state with optically induced recovery
- Blinking where excitation intensity controls emitting concentration
  - Triplet states
  - Reversible photochem. (EYFP)
  - Redox dark state



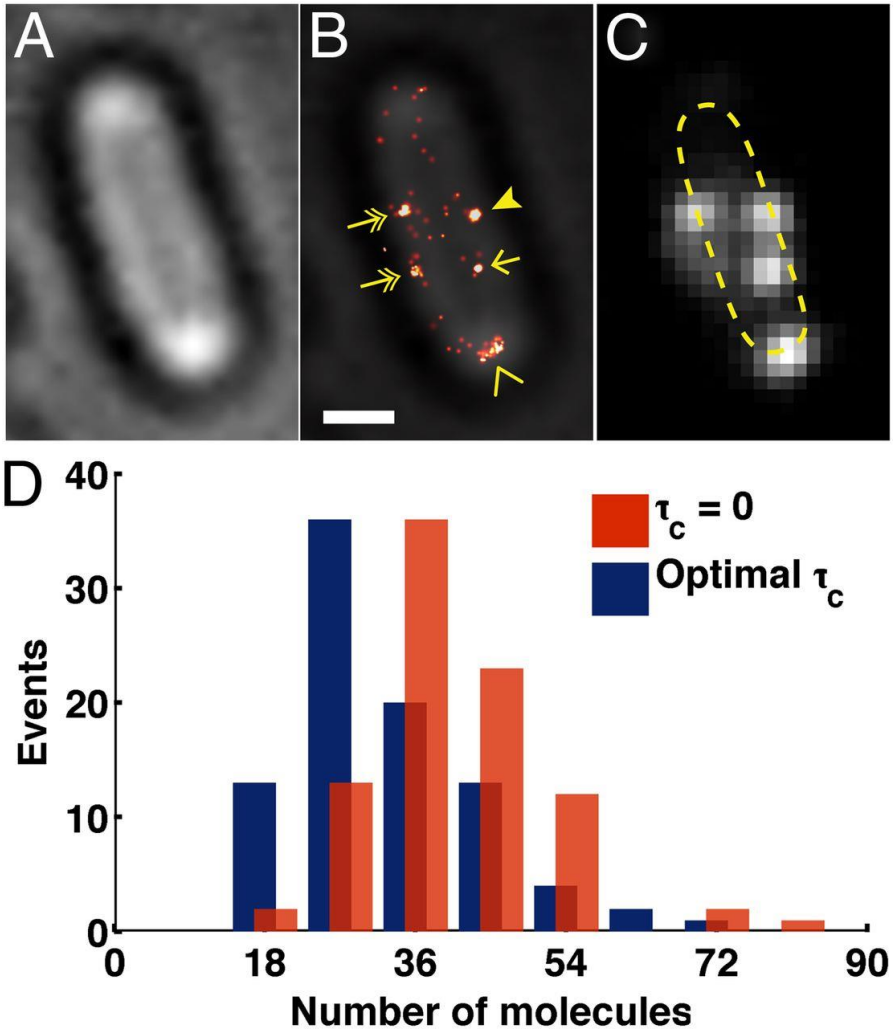

Moerner, 2013

# Determination of cluster size




Nan et al., 2013

# Determination of cluster size

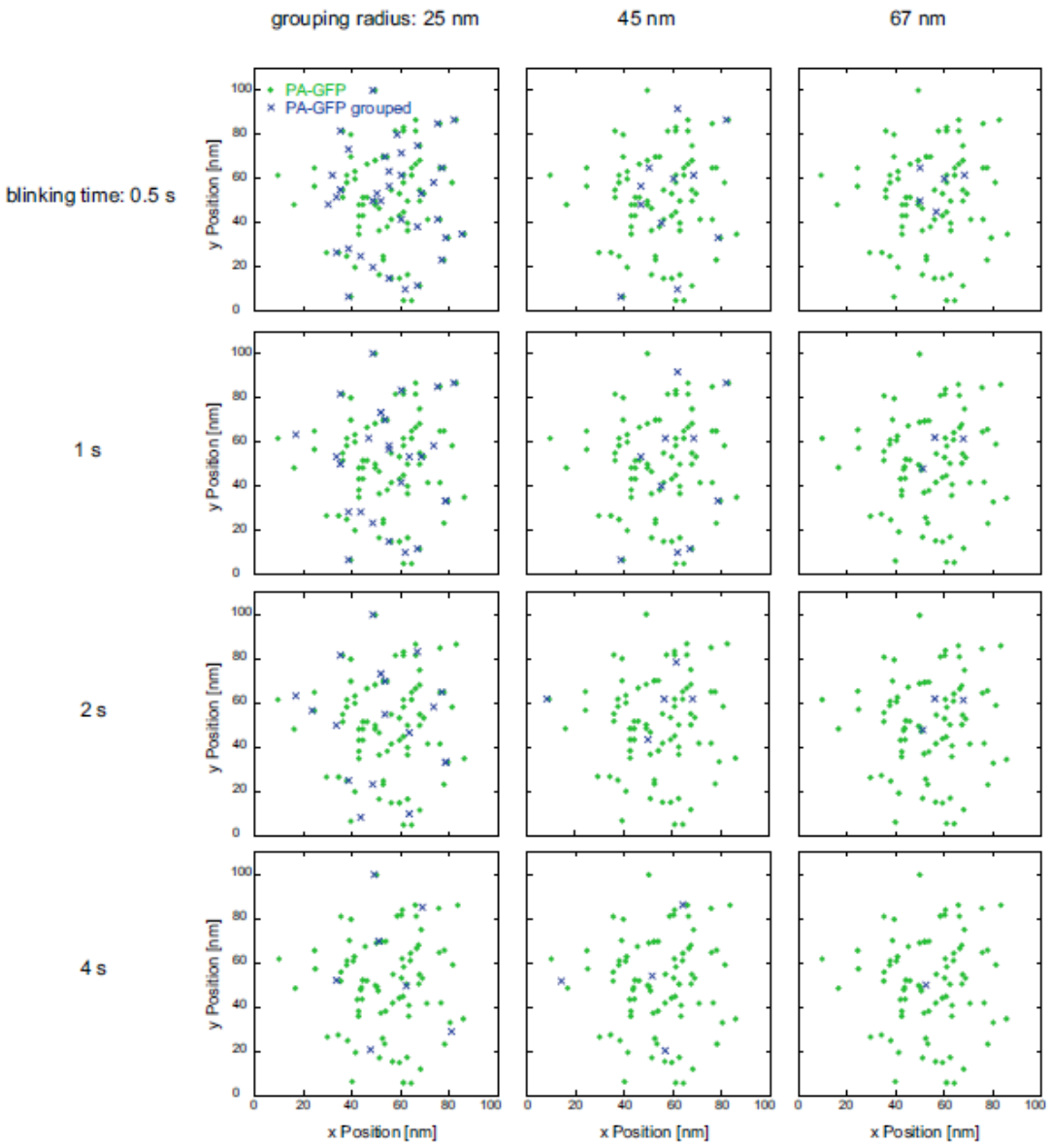



FliM-Dendra2

$\tau_c$: time interval of blinking events

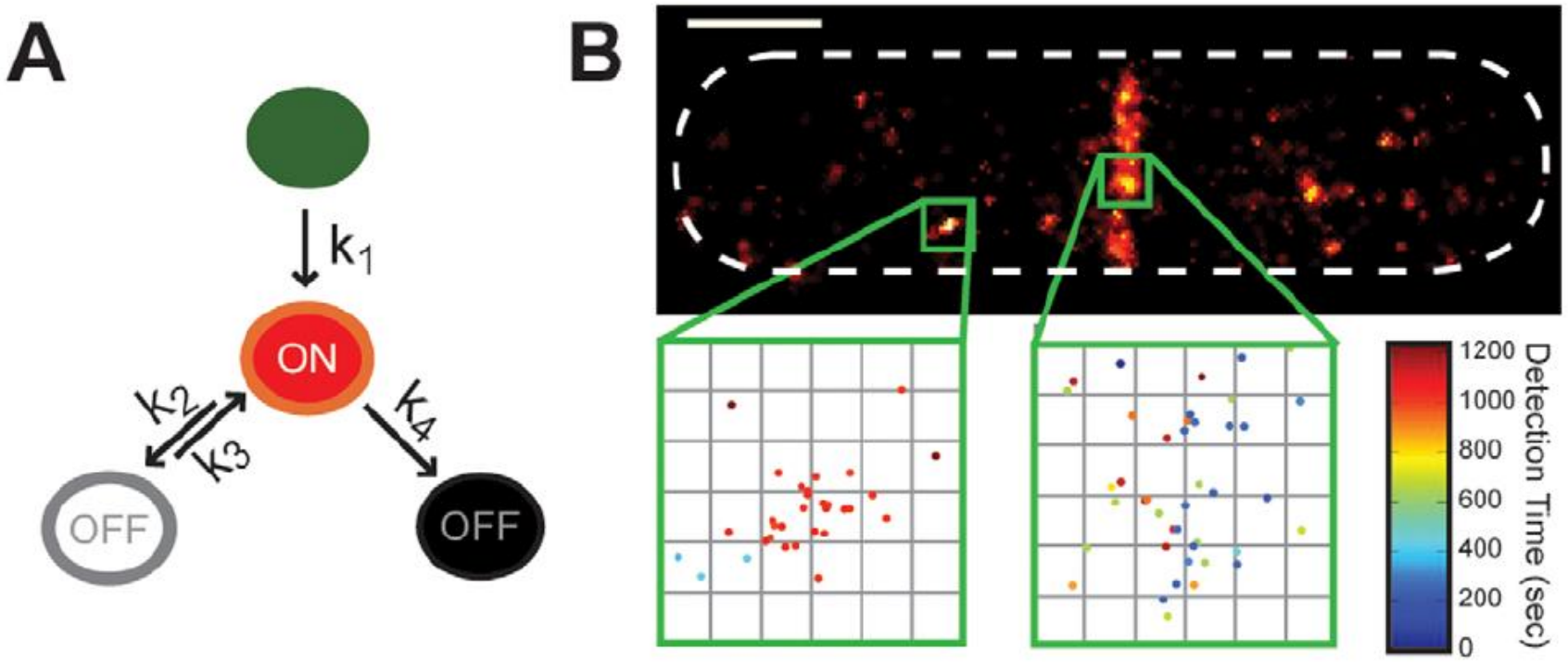
Lee et al., 2012

# Determine blinking characteristics




Renz et al, 2012

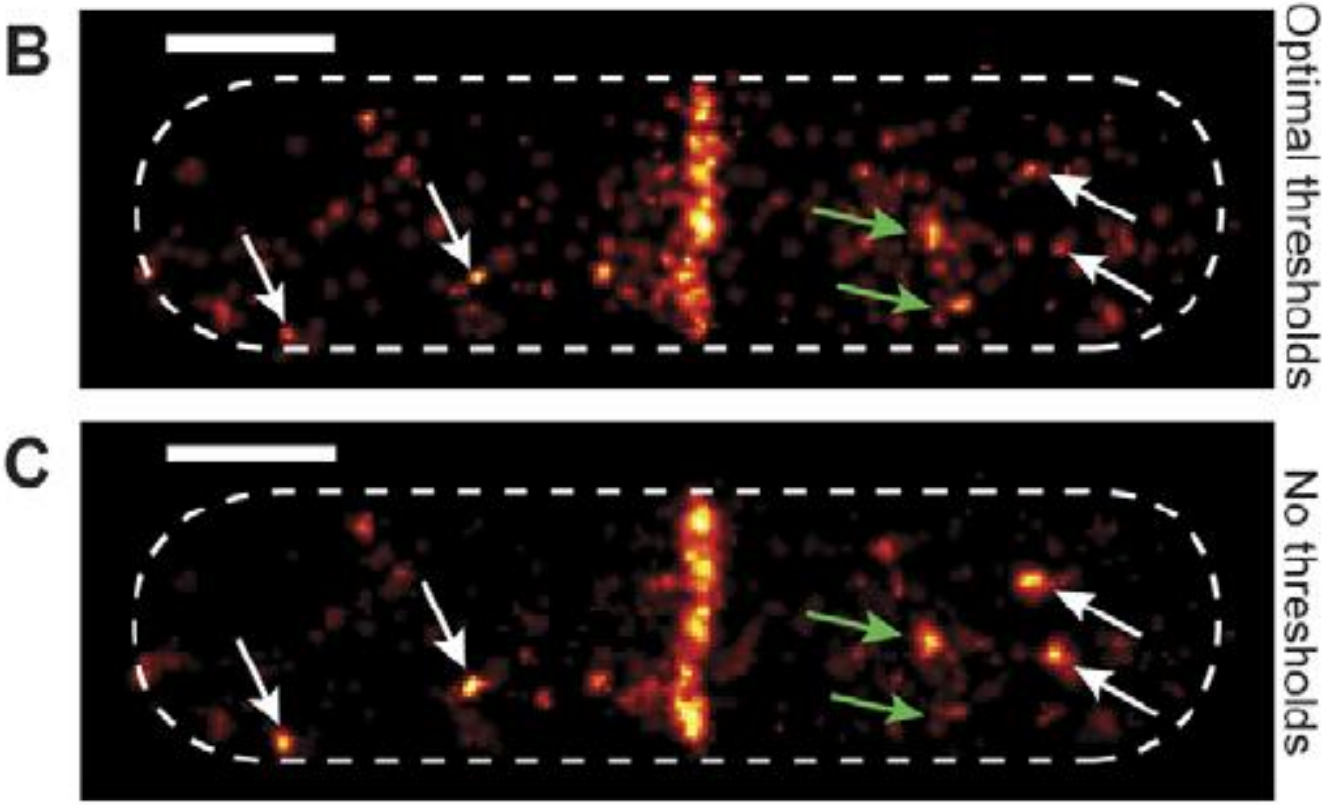
# Determine blinking characteristics




PALM image of *E. coli* FtsZ-mEos2 with conventional clustering thresholds: spots within 167 nm (1 camera pixel) and 50 ms (1 frame) were grouped together and plotted.

Coltharp et al., 2012

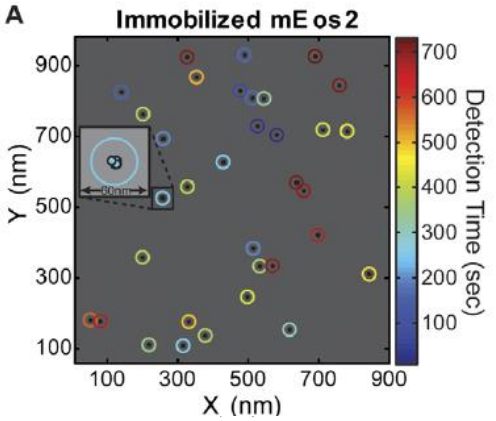
# Determine blinking characteristics



# Determine blinking characteristics

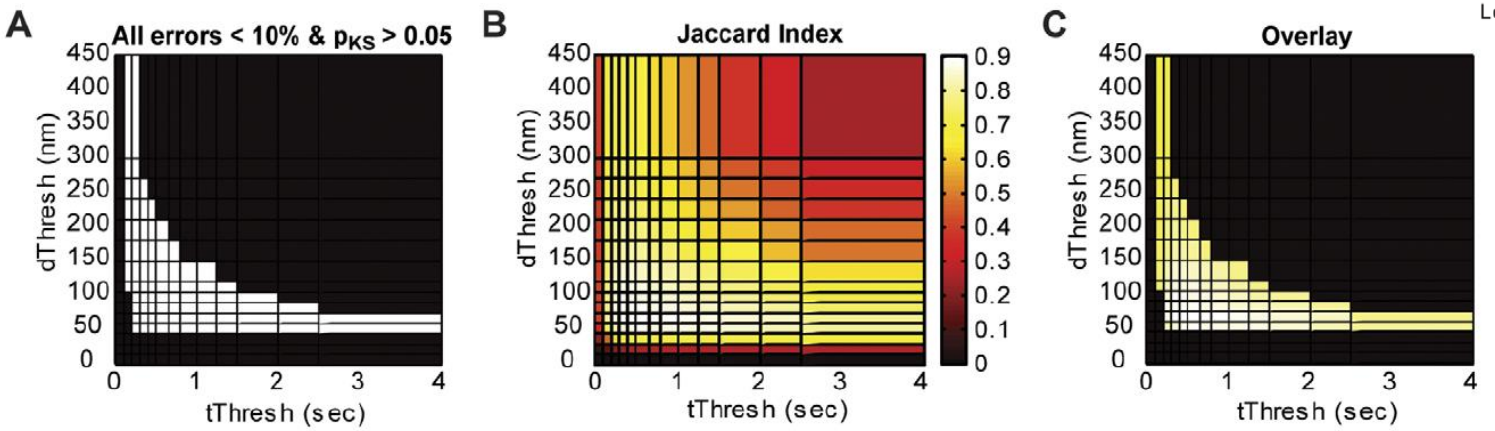
**$D_{tmax}$**

total imaging acquisition time /
number of localizations (detected in the maximum density region)



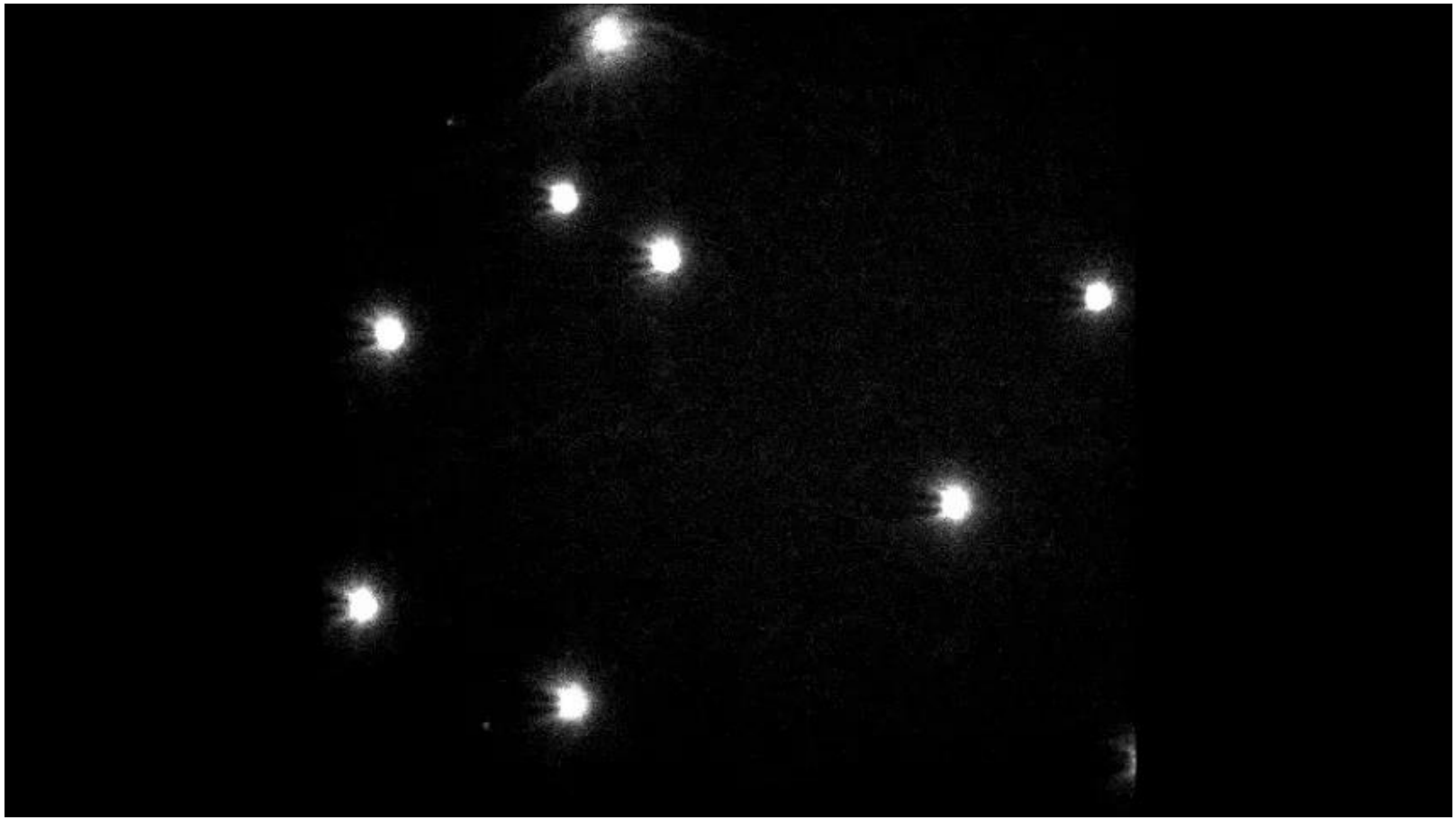

**$N_{ref}$**

$N_{unprocessed}$ / average number of localizations ($\alpha$)
N = number of molecules

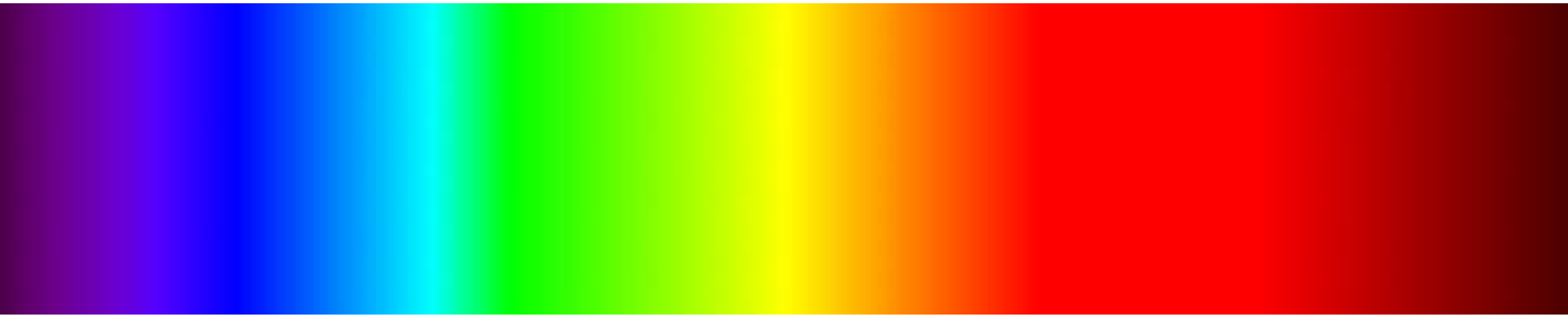



Coltharp et al., 2012

# Drift correction



# Multicolour imaging



**By activation wavelengths**

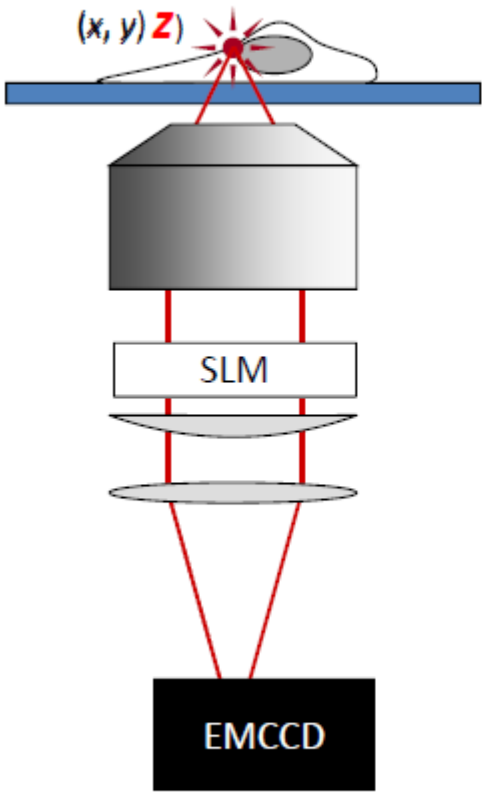
- Dye-pairs
- Crosstalk from nonspecific activation
- Laser sequences
- Single channel detection
- Images naturally aligned

**By emission wavelengths**

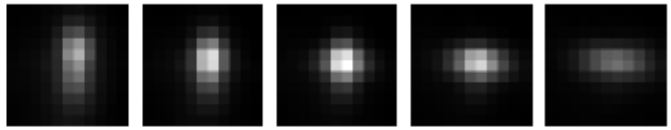
- Simple fluorophores
- Low crosstalk
- Continuous imaging
- Multi-channel detection optics
- Needs nanometer scale image alignment

# Imaging in 3D

## 3D STORM/PALM

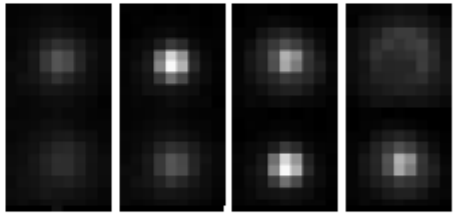



Astigmatic imaging




Huang et al., Science 2008

Bi-plane imaging

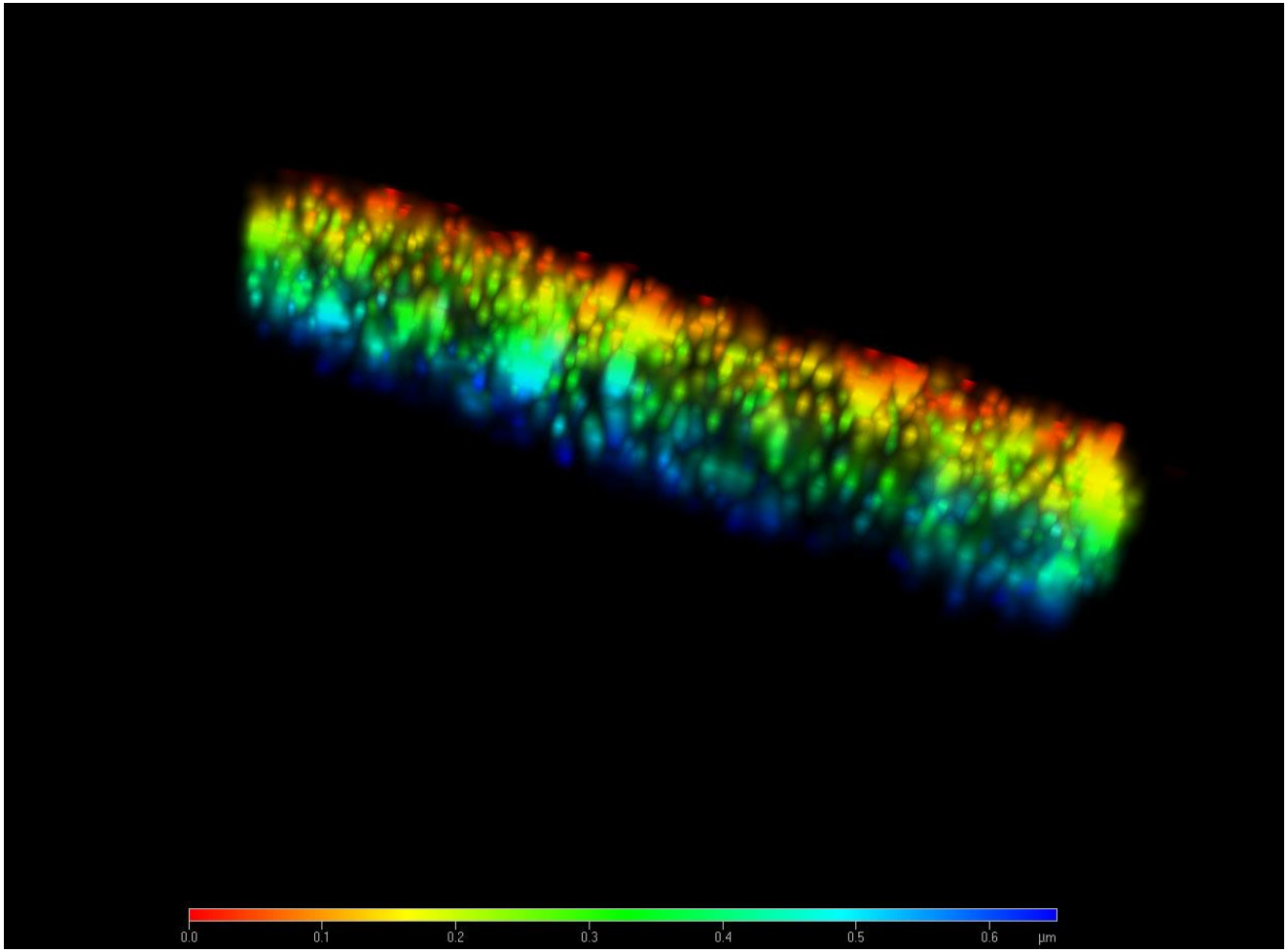


Juette et al., Science 2008

Double-helical PSF


Pavani et al., PNAS 2009

# 3D fPALM



# Single molecule localization super resolution microscopy

## Advantages

- High precision, lateral resolution ~20-40 nm (in vivo)
- Quantitative imaging, visualizing single molecules
- 3D imaging possible
- gentle imaging conditions

## Disadvantages

- Requires special fluorophores / dyes
- Computational heavy
- Long imaging time
  - drift correction required
  - difficult for life cell imaging

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**AG Bramkamp**

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DFG

**Thank you for your attention!**