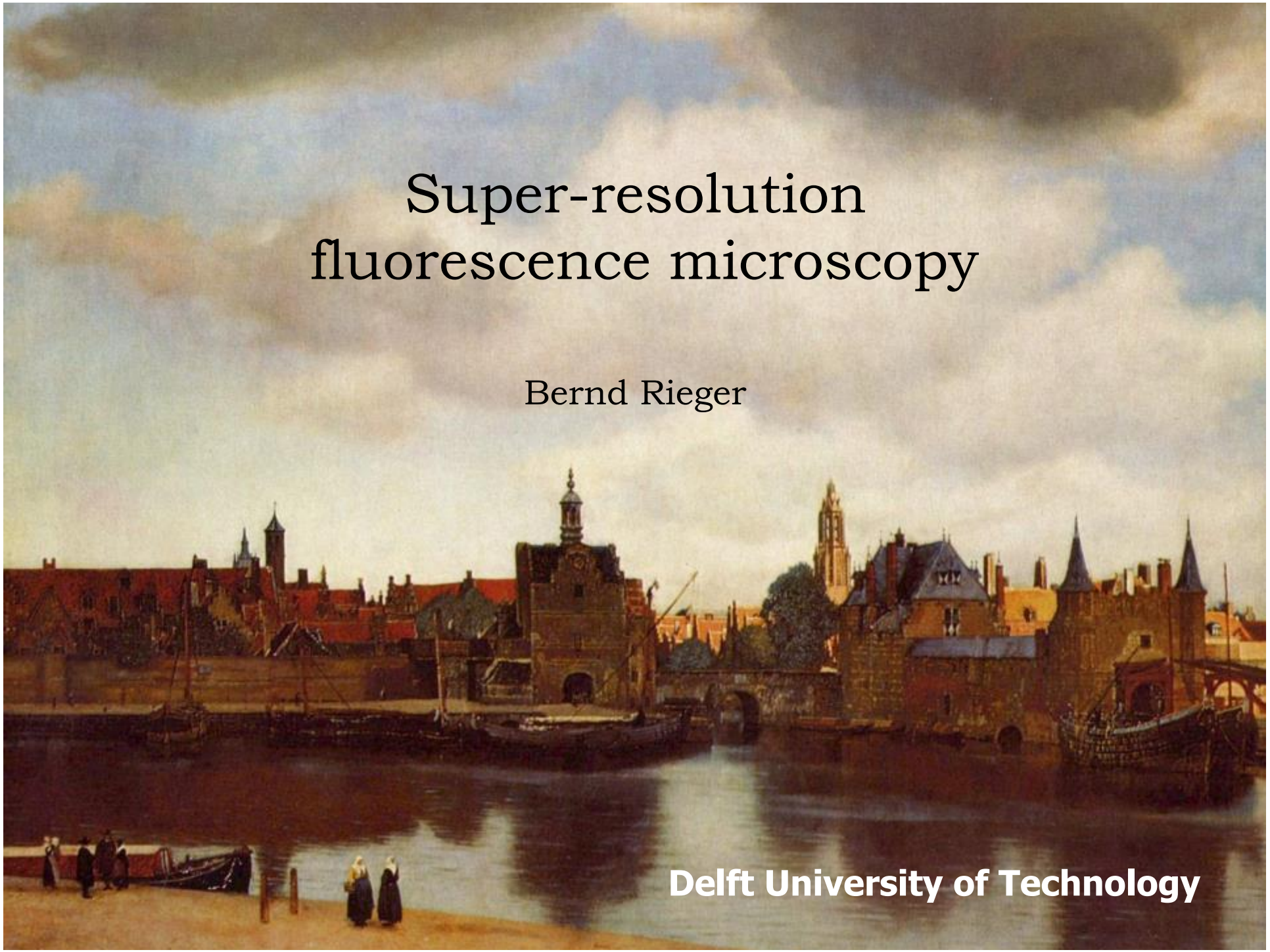


Super-resolution fluorescence microscopy

Bernd Rieger

Delft University of Technology

The Royal Swedish Academy of Sciences has decided to award the

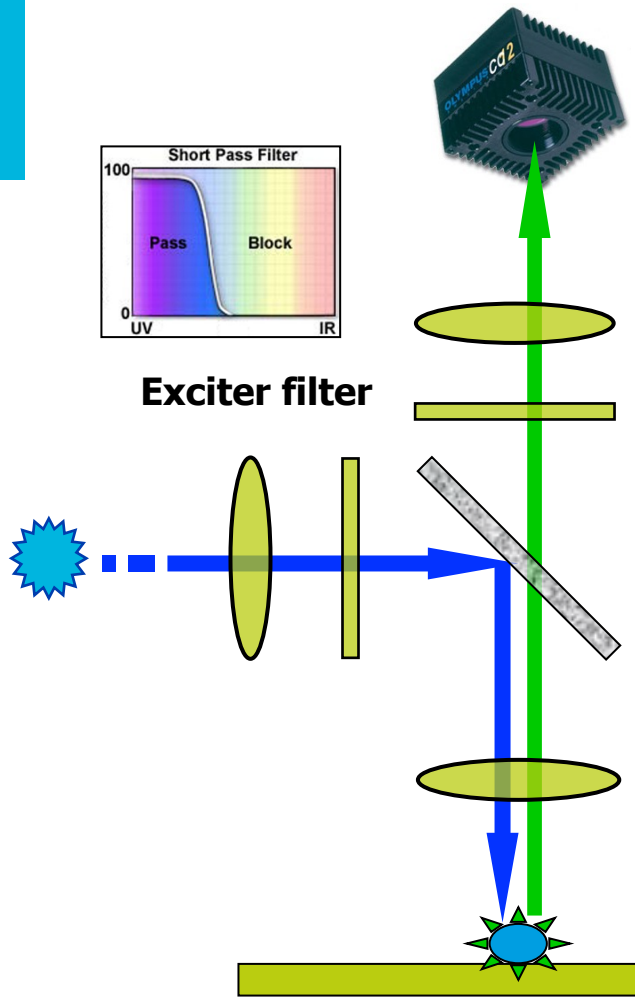
2014 NOBEL PRIZE IN CHEMISTRY

to:

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

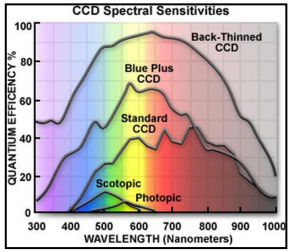
"for the development of super-resolved fluorescence microscopy"

Fluorescence Epi-Illumination



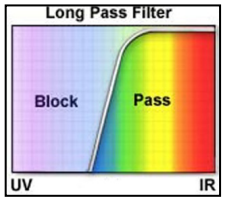
Exciter filter

Digital scientific camera

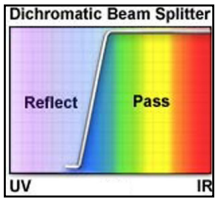


Lenses

Barrier filter

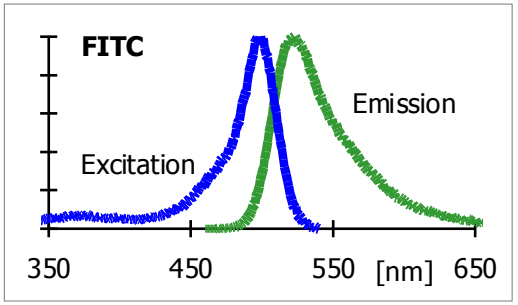


Dichroic mirror

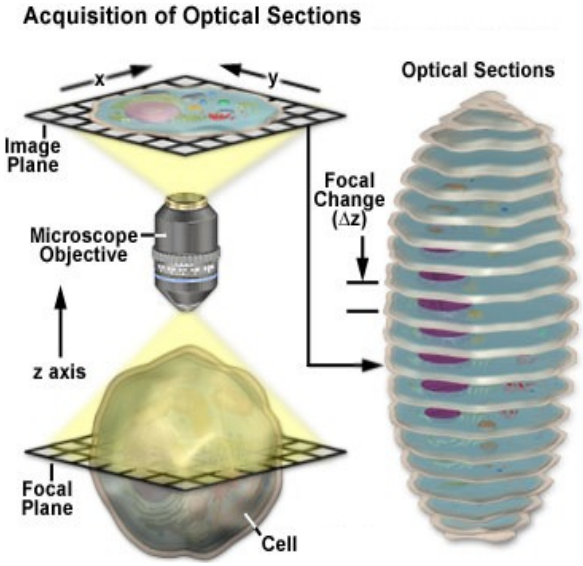


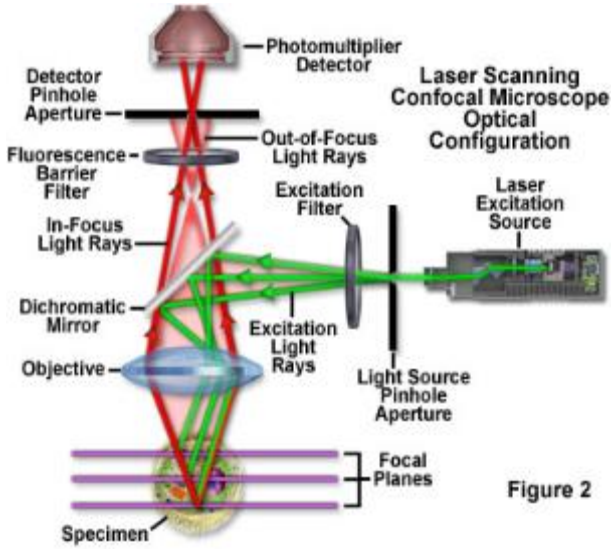
Lenses

Biological Sample

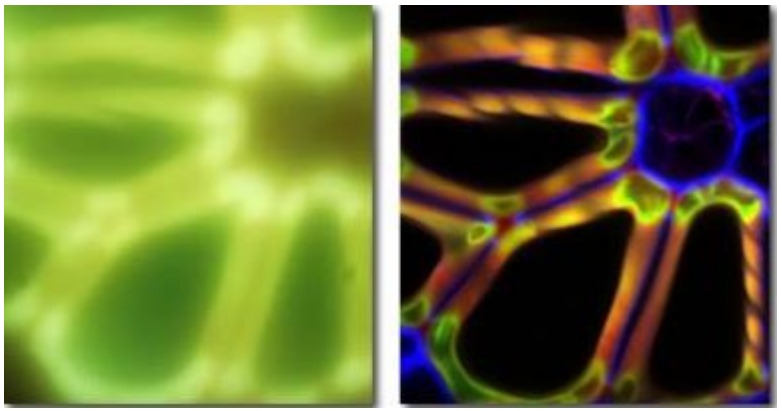


Confocal microscopy of thick 3D-samples





- Scanning microscope with small pinhole on detection side
- Removes hazy background from out-of-focus layers



Widefield & confocal fluorescence microscopy

Widefield:

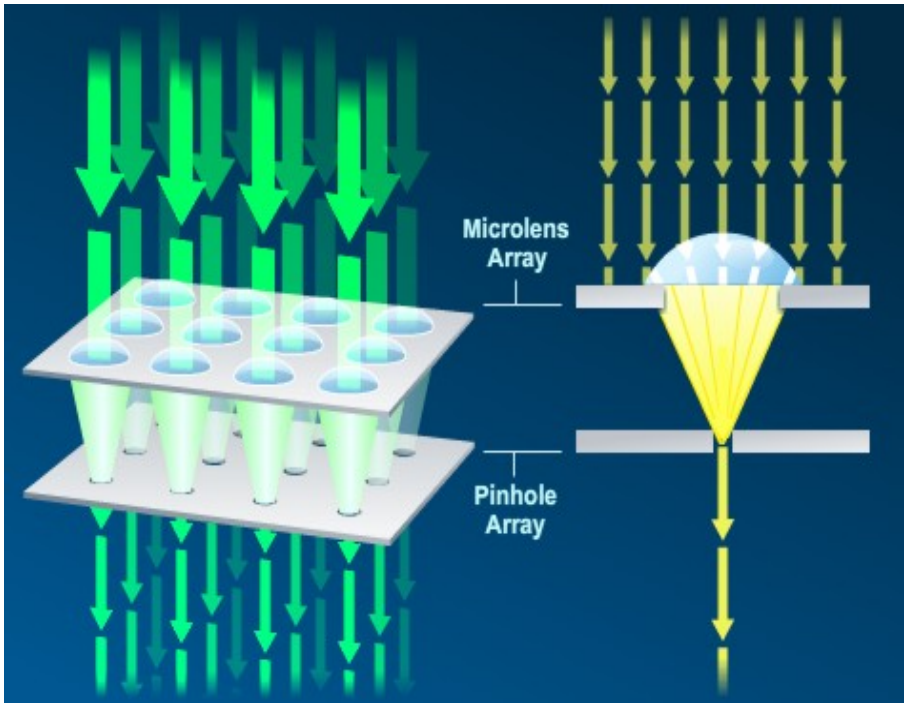
- has uniform illumination
- uses an image sensor for parallel imaging
- is fast
- has poor optical sectioning

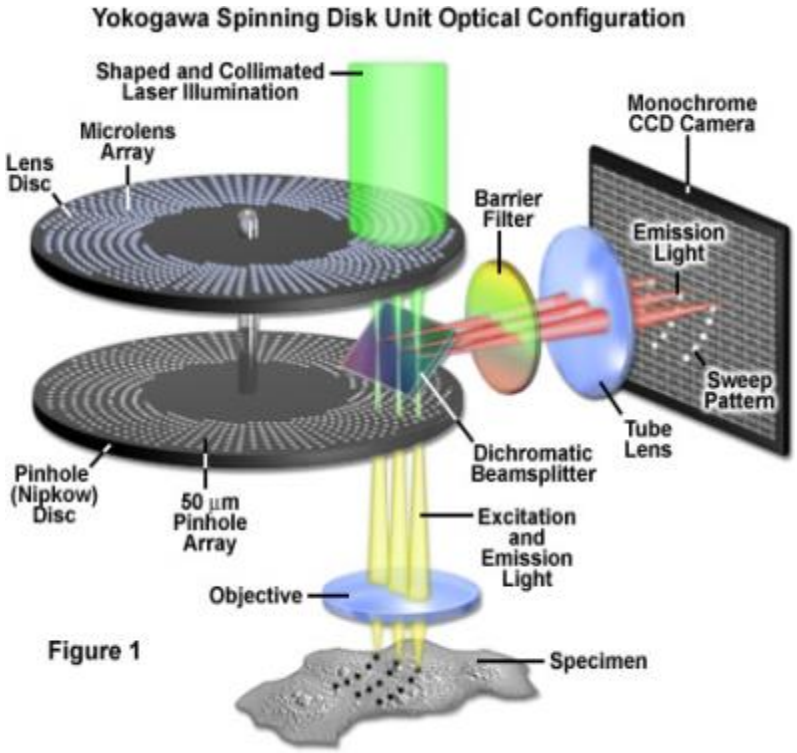
Confocal:

- has single spot illumination
- uses a scanner for sequential imaging
- is slow
- has good optical sectioning

Multi-spot scanning microscopy

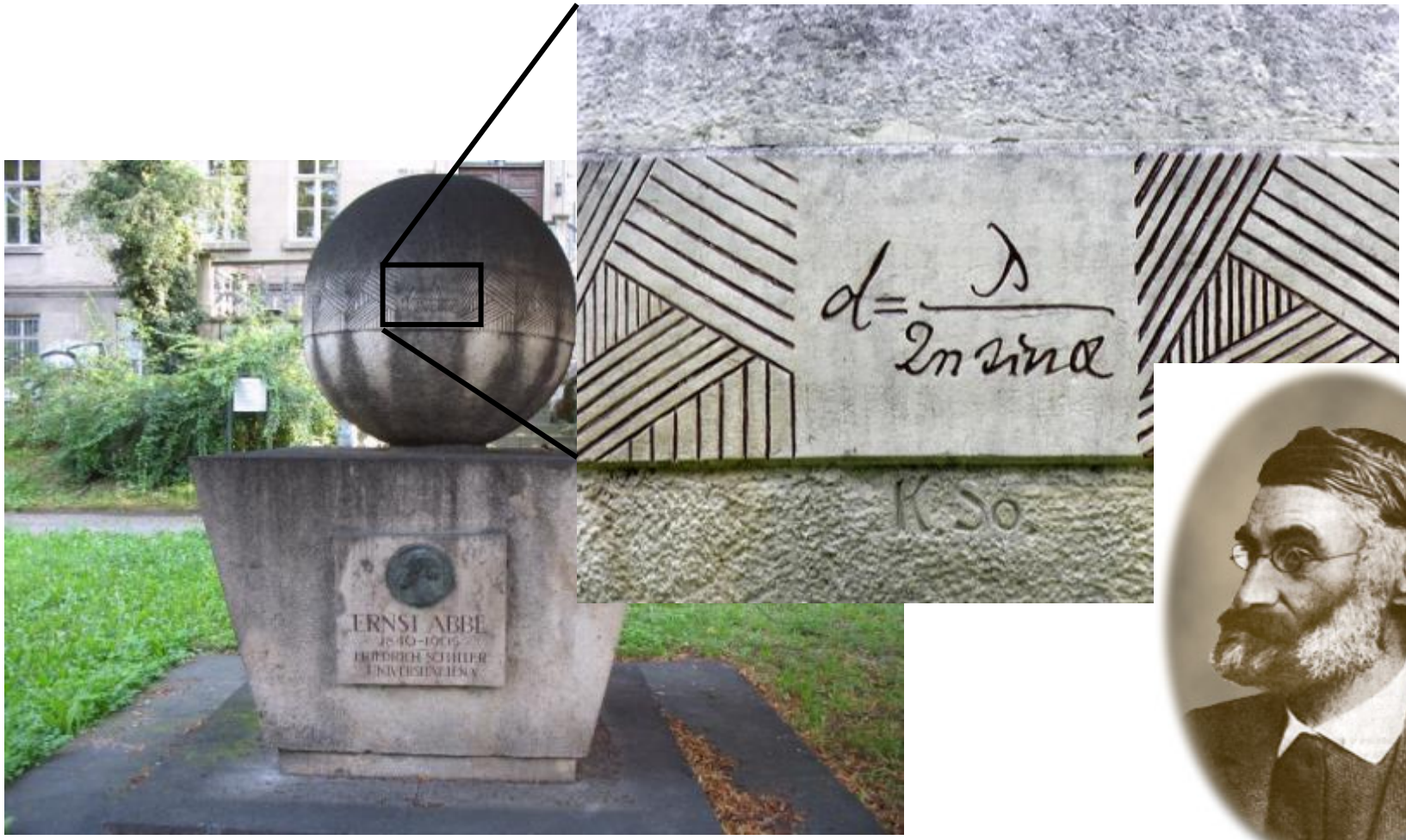
- Array of spots for video-rate imaging with good optical sectioning





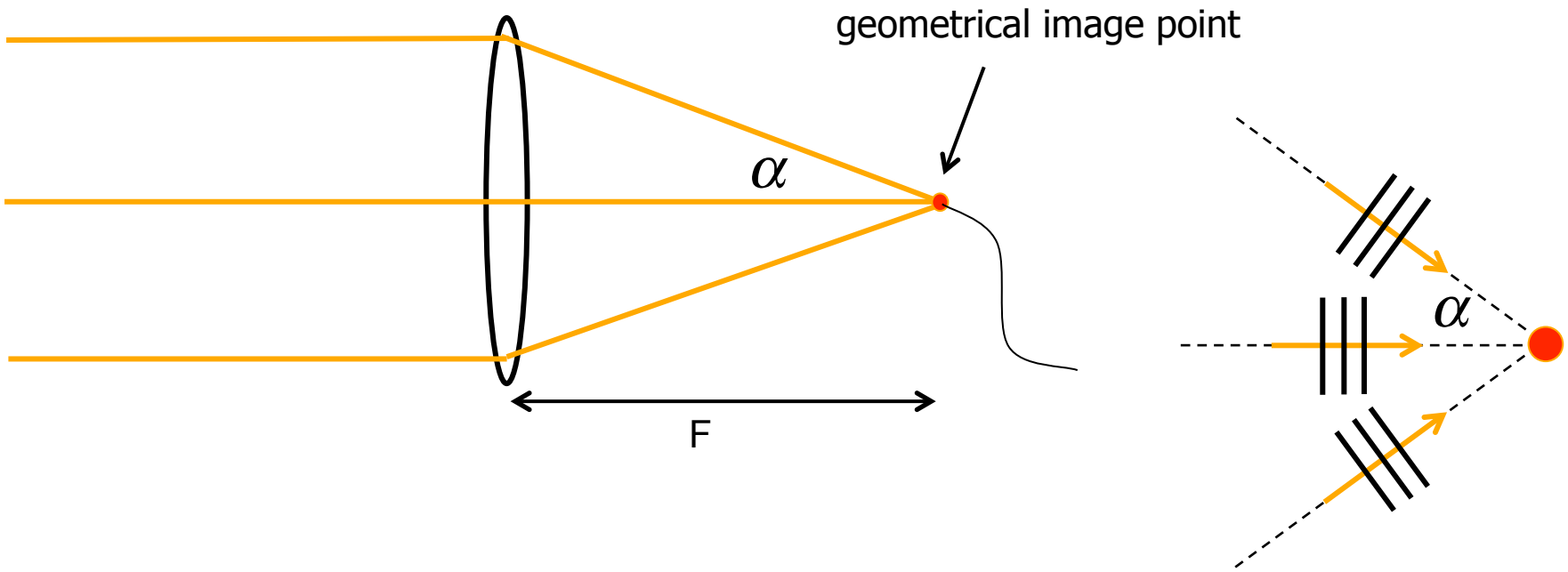
- Drawbacks:
  - light efficiency
  - complex equipment

The diffraction limit.



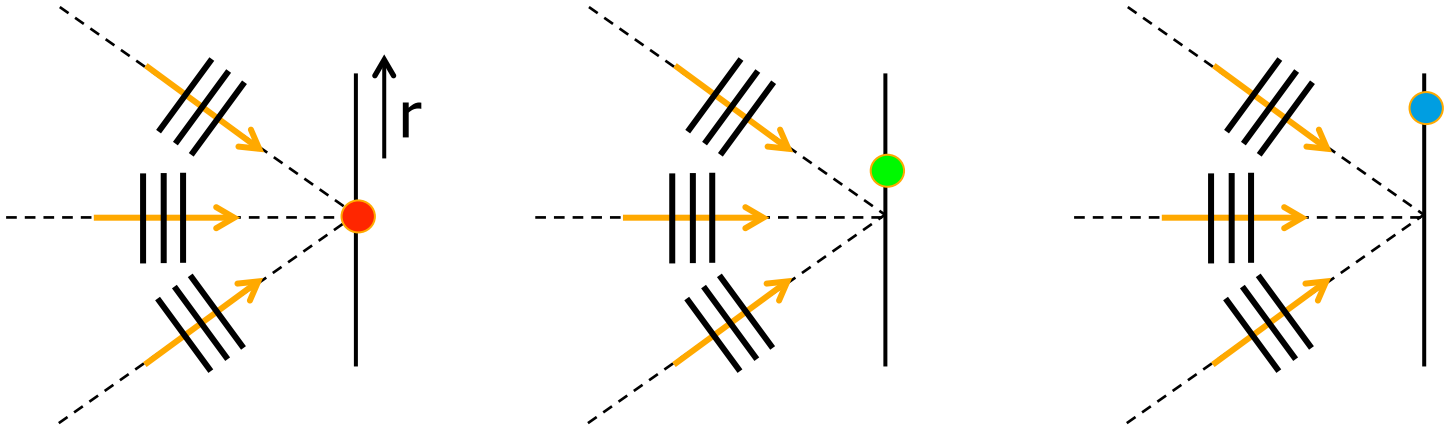
Ernst Abbe
(1840-1905)

Optical image of a point object with a lens



- Rays converging to image point/focus correspond to plane waves propagating at angles $\theta < \alpha$
- Total field is the sum of all these plane waves
- Total intensity depends on constructive/destructive interference

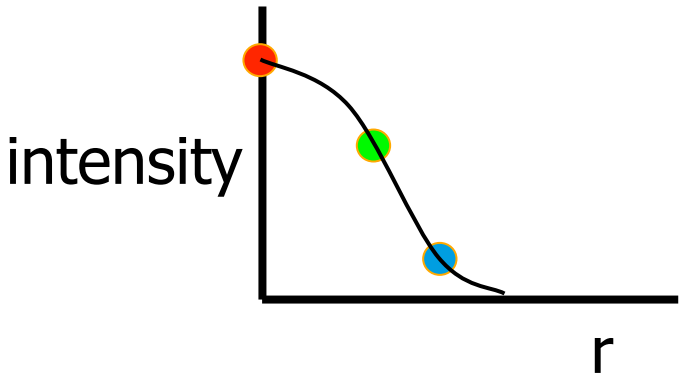
Interference gives a spot



- all waves in phase at the image point
  -> constructive interference/max. intensity

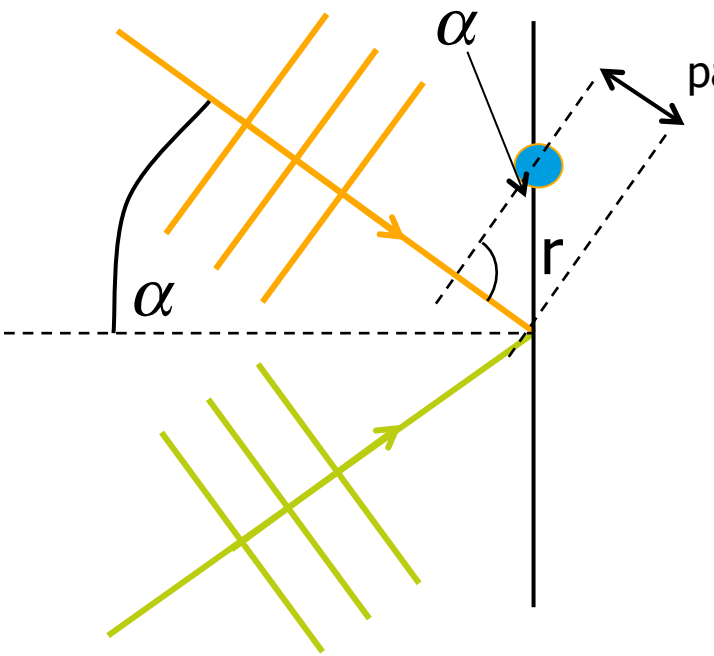
- away from the image point:

  waves no longer in phase
  -> destructive interference builds up
  -> intensity decreases



What is the width of the spot?

- Total intensity near zero if waves at largest angles $\pm\alpha$ have destructive interference:



path length difference $= r \sin\alpha$

- total path length difference between  and at point ● must then be:

$$2r\sin\alpha = \frac{\lambda}{2}$$

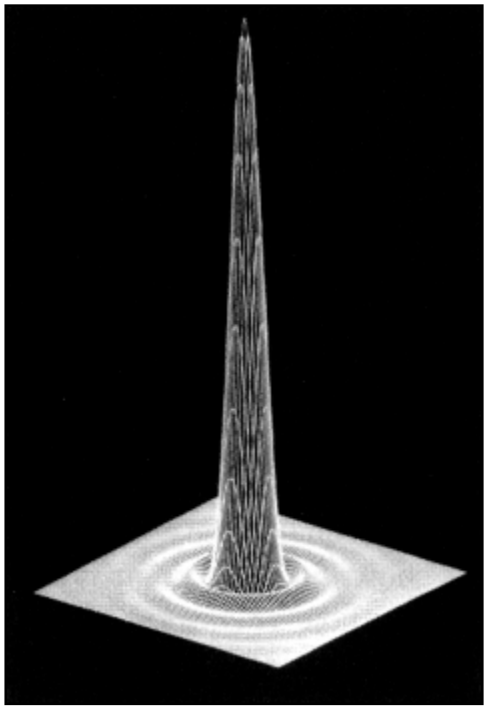
- size of spot at image point:

$$r = \frac{\lambda}{4\sin\alpha}$$

Airy-distribution

- Exact expression for intensity (Point Spread Function) :

$$I(r) = I_0 \left[\frac{2J_1(2\pi NAr/\lambda)}{2\pi NAr/\lambda} \right]^2$$



- with $J_1(x)$ a so-called Bessel-function

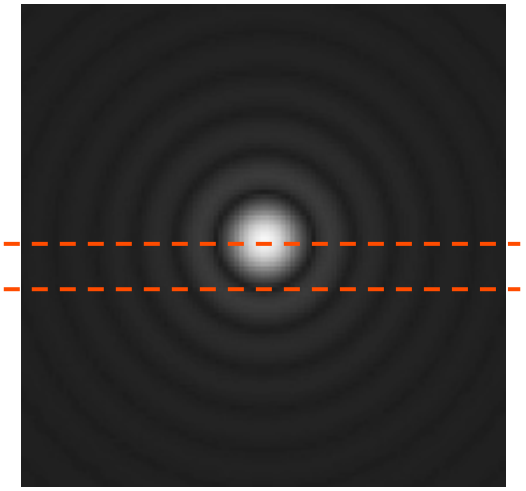
  and the Numerical Aperture is defined by:

$$NA = \sin\alpha$$

- Width

= distance peak to first dark ring:

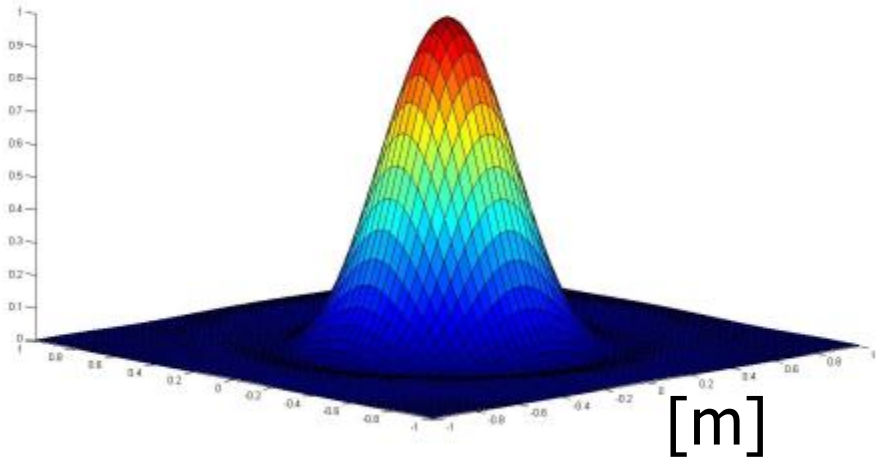
$$\Delta r = 0.61 \frac{\lambda}{NA}$$



Rayleigh

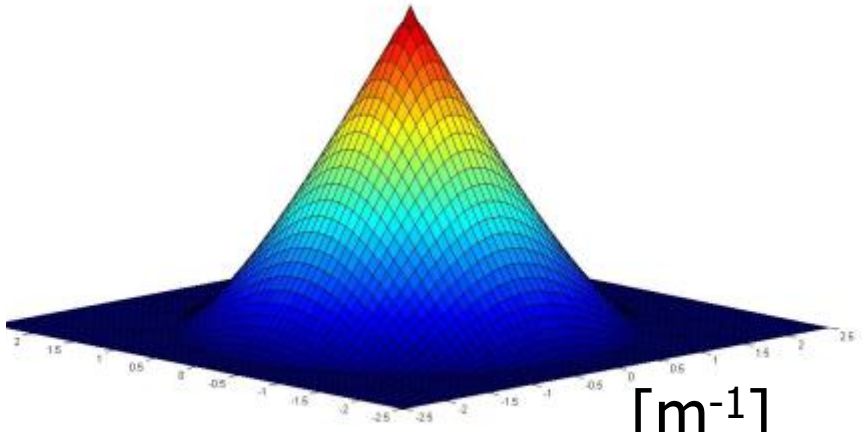
Point Spread Function and Optical Transfer Function

PSF



[m]

OTF

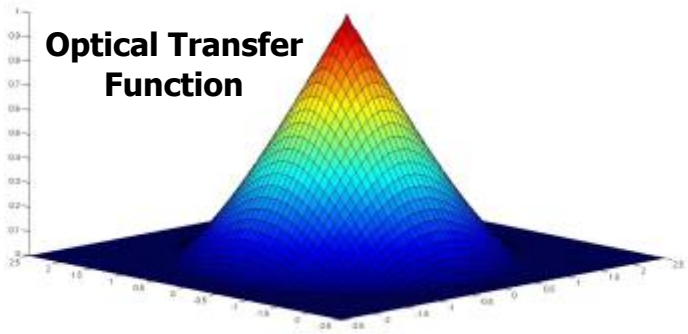


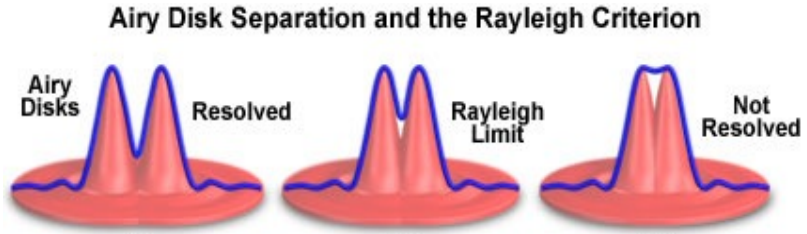
[m^{-1}]

OTF is the Fourier Transform of the PSF

Diffraction limit to resolution

... determined by Abbe or Rayleigh-criterion:



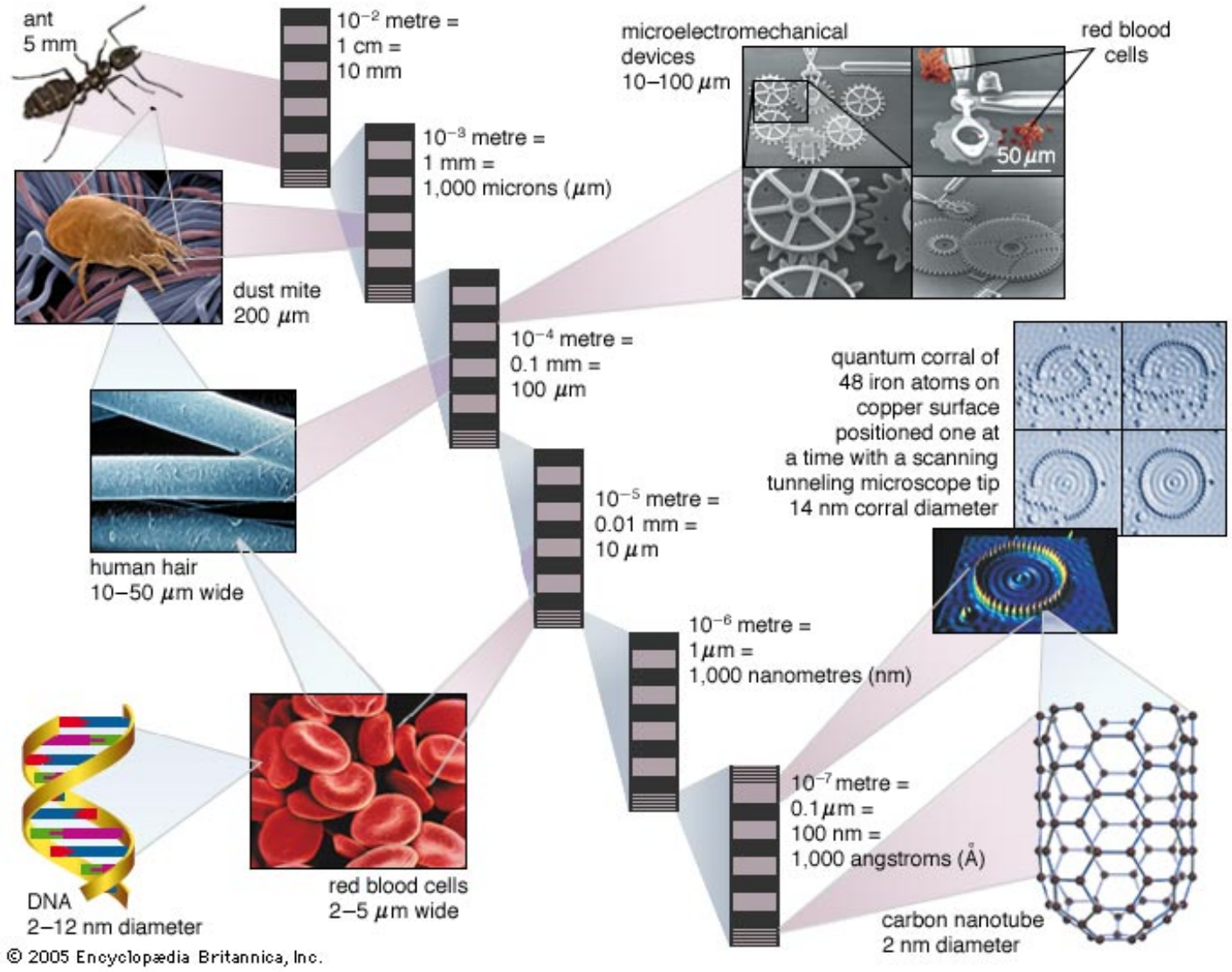


$$d = 0.50\frac{\lambda}{NA}$$

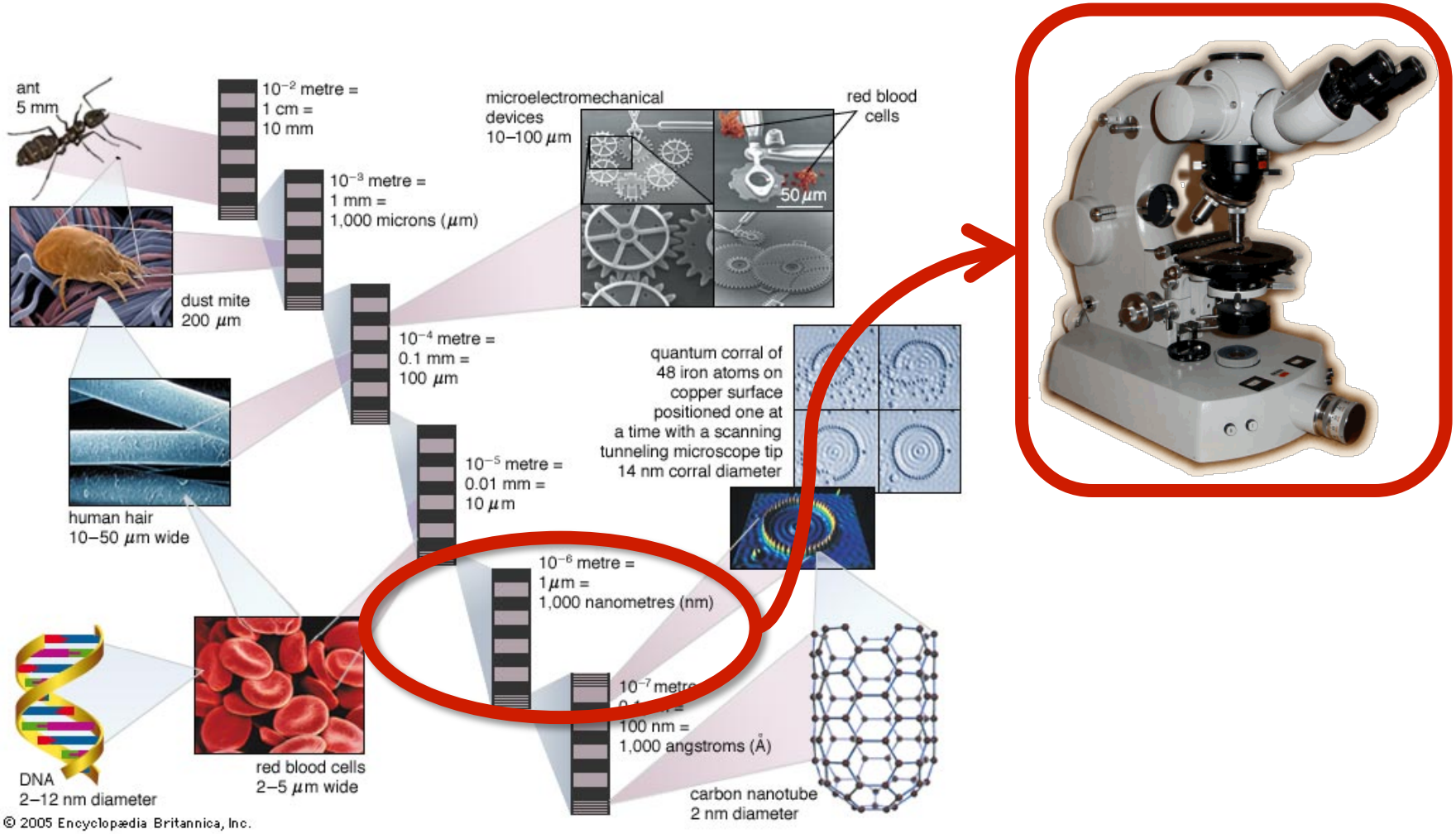
$$d = 0.61\frac{\lambda}{NA}$$

$$d = 0.50\frac{500nm}{1} = 250nm$$

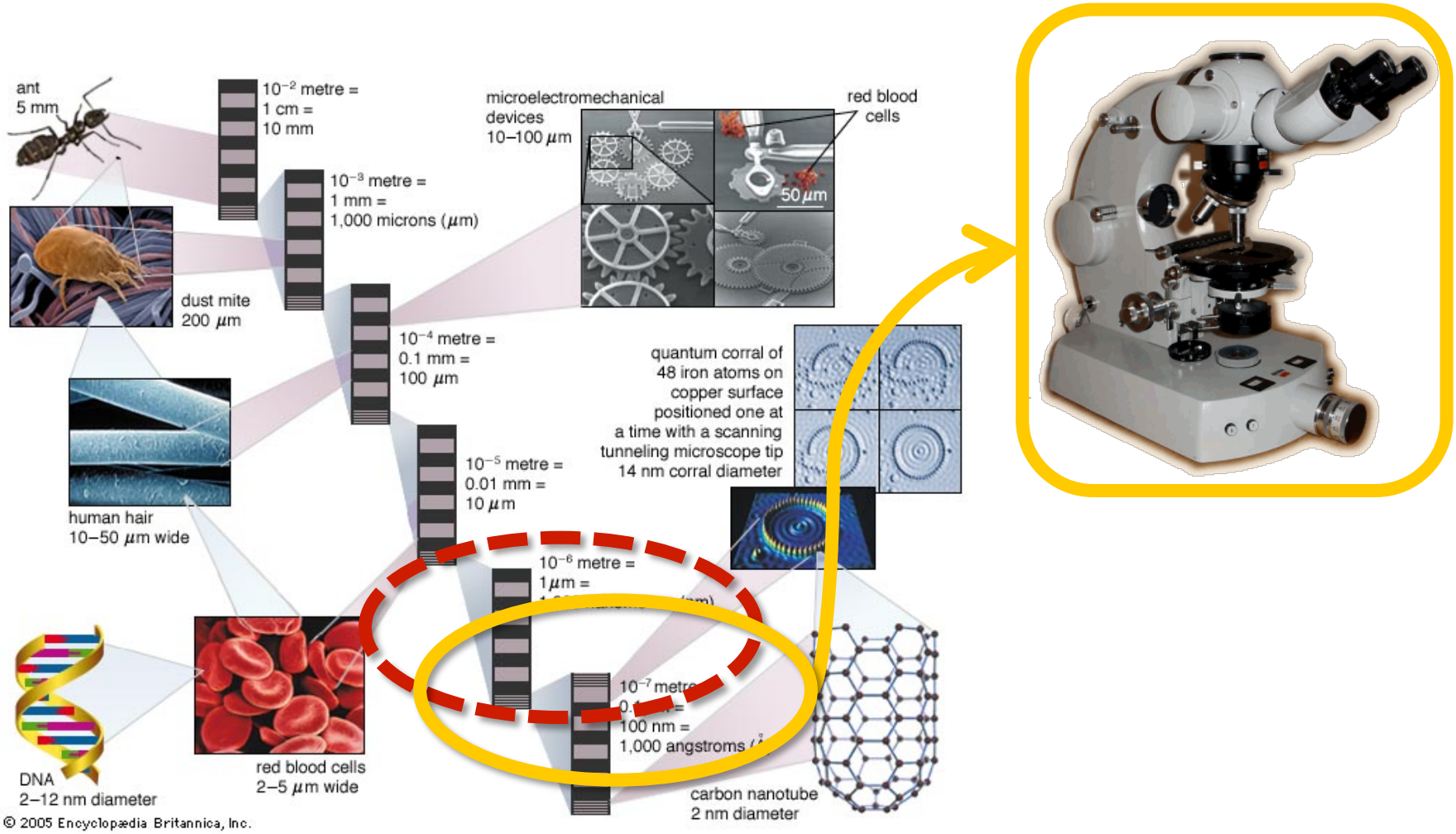
Orders of magnitude



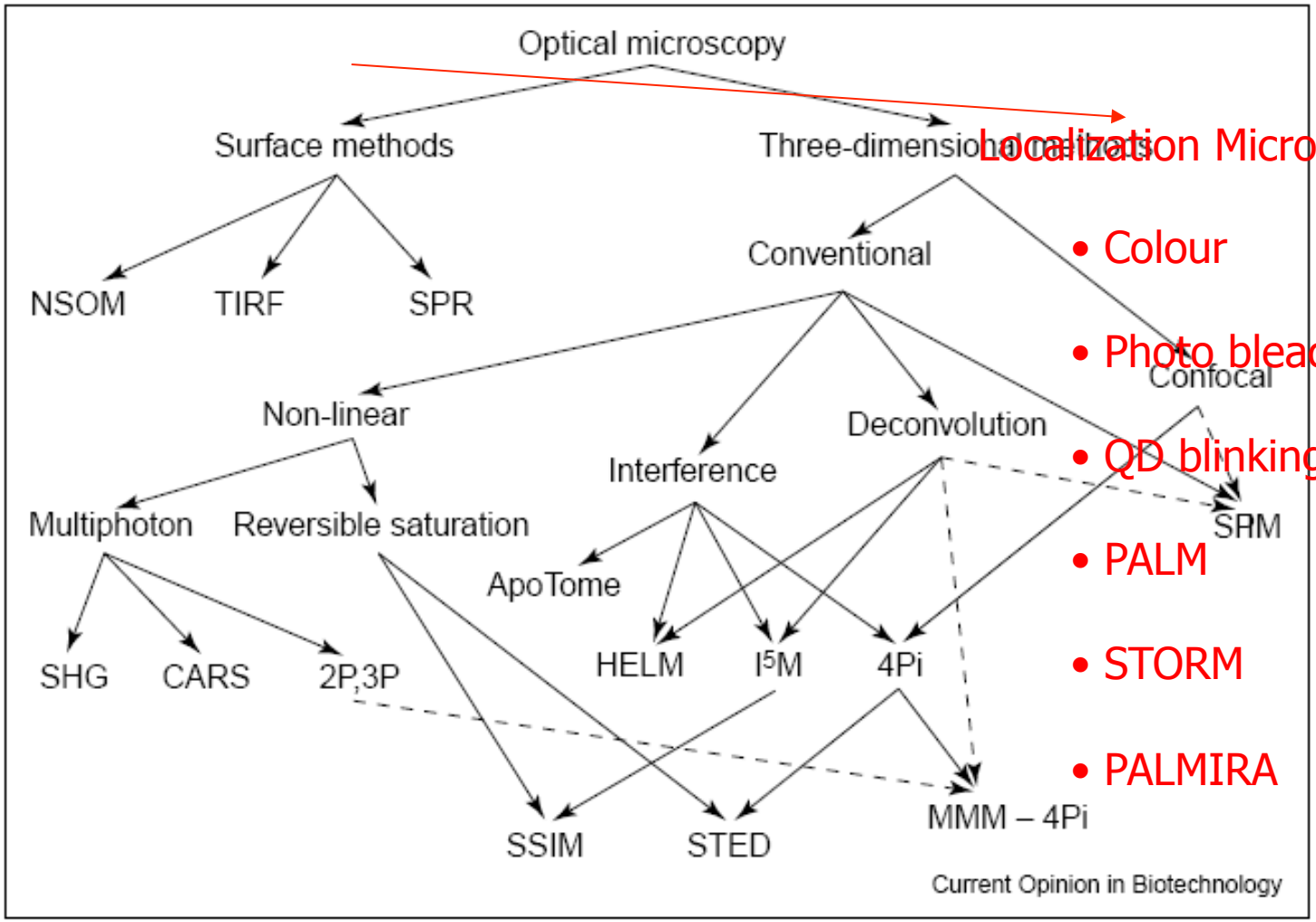
Light microscopy



… to optical nanoscopy!



Zoom in & enhance:
Unravel the subcelluar machineary of life



Localization Microscopy

- Colour
- Photo bleaching
- QD blinking
- PALM
- STORM
- PALMIRA

Garini, Vermolen & Young, From micro to nano: recent advances in high-resolution microscopy, *Current Opinion in Biotechnology*, **16**, 2005

Super-resolution techniques

- Near-field, surface enhanced
- Far-field

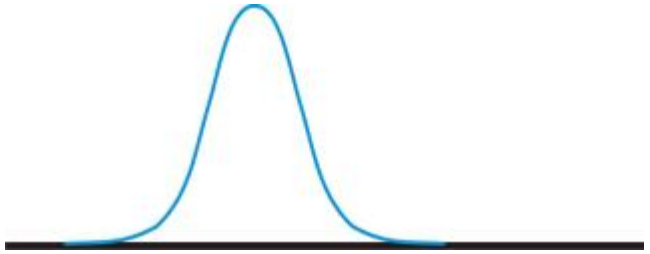
Far-field super-resolution techniques

- Structured Illumintation:
  - Resolution improvement ~2x
  - Optical sectioning, 3D possibility
  - Live cell

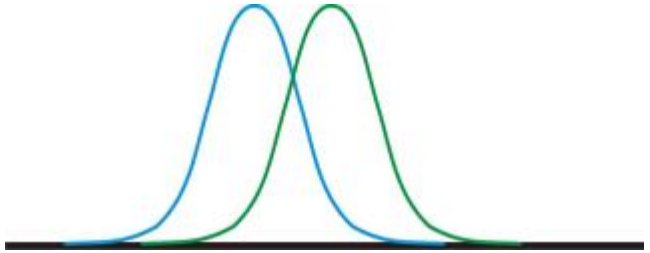
- Fluorescence switching
  - Resolution improvement ~10x
  - Sometimes optical sectioning, 3D possibility
  - Live cell not straight forward

Illumination patterns for resolution increase

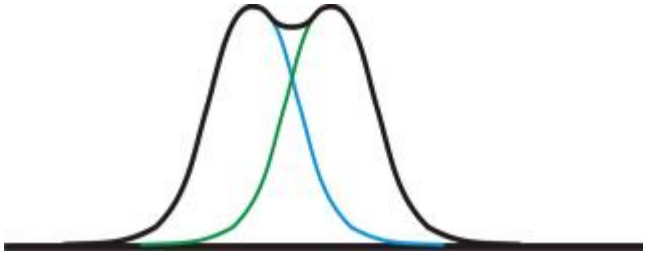
PSF single emitter: →



PSF's two emitters that are close by: →



Resulting image with overlap: →



How should the illumination change to make only one emitter visible?

Structured Illumination Microscopy (SIM)

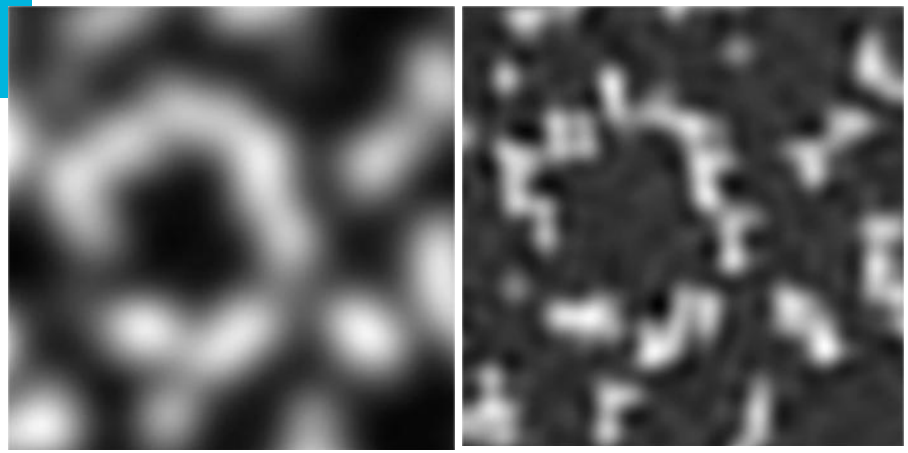
PSF's two emitters that are close by: →

Illumination pattern: →

Resulting images without overlap: →

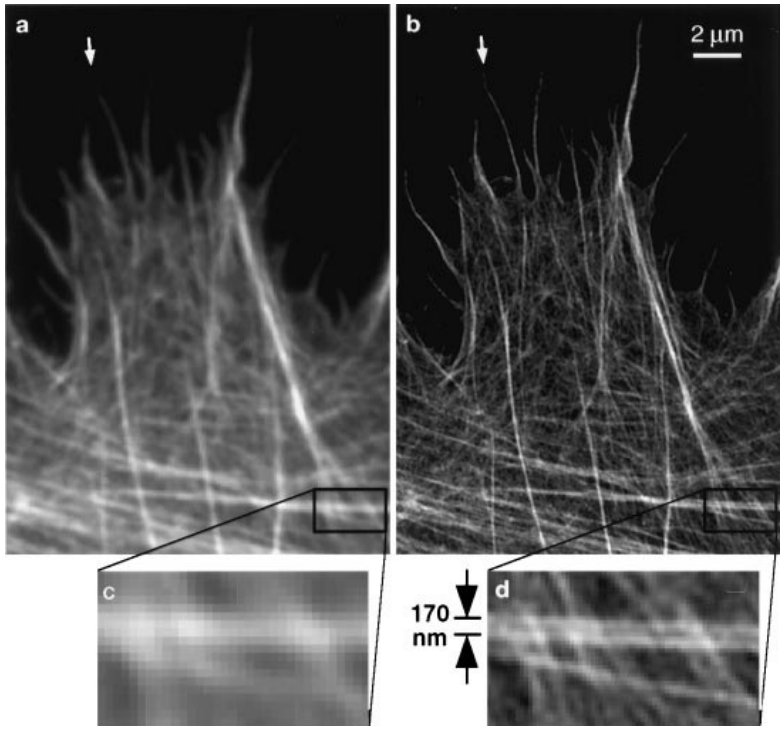
- Periodic illumination pattern (stripes)
- Make multiple images with shifted pattern
- Use computer to construct final image

Proof of enhanced resolution of SIM images



Widefield SIM

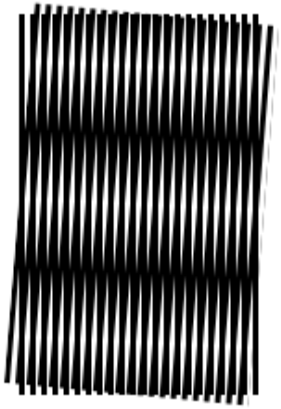
Widefield SIM

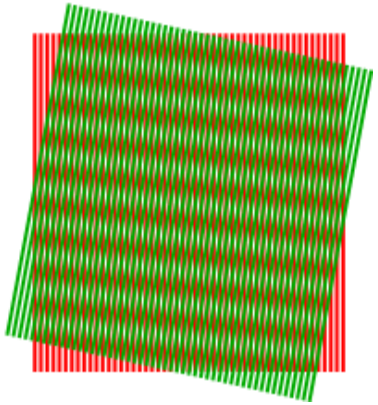


sources: Gustafsson, J. Micr. 2000, Frohn et al. PNAS 2000

Moiré-effect

- Overlay of periodic patterns gives image with larger period
- Can be used to “magnify” small structures
- = “Structured Illumination Microscopy”





Examples Moiré-effect I

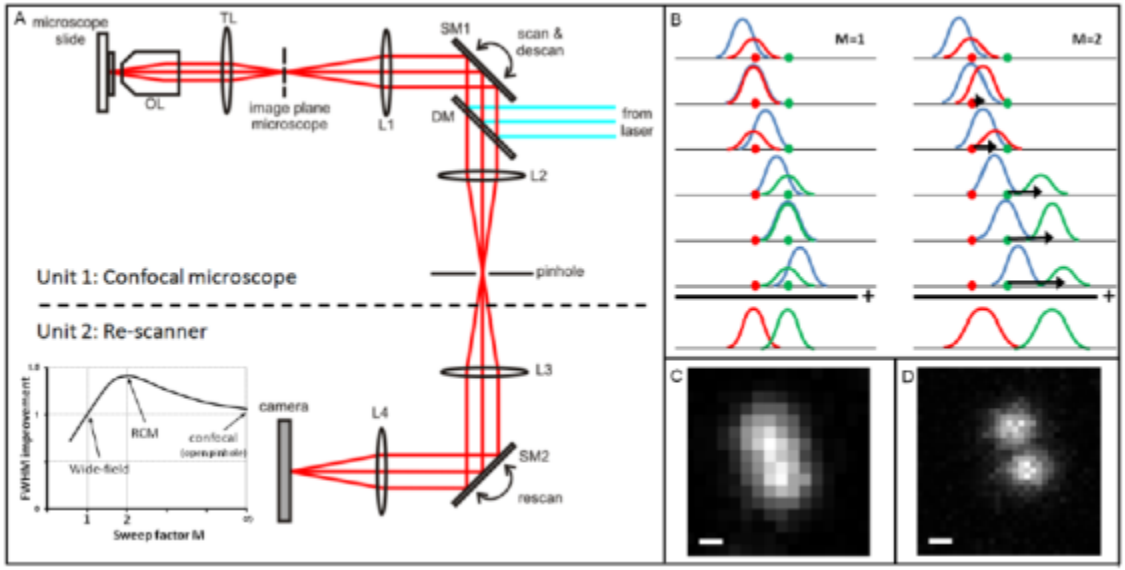
Practical's on Structured Illumination

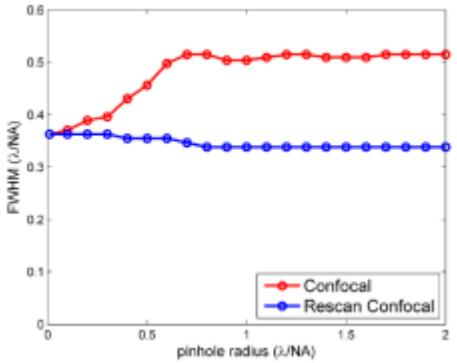
- Offers 2x increased resolution with good signal transfer of all spatial frequencies.

- To make it work, there are many practical problems:
  - projected grid positions must be known *exactly* on the sample
  - several grid positions must be acquired
  - the digital reconstruction adds "structured noise"

- "Blind" structured illumination
  - uses many (~100) unknown patterns generated by e.g. speckles for illumination.
  - reconstruction must compute sample & illumination

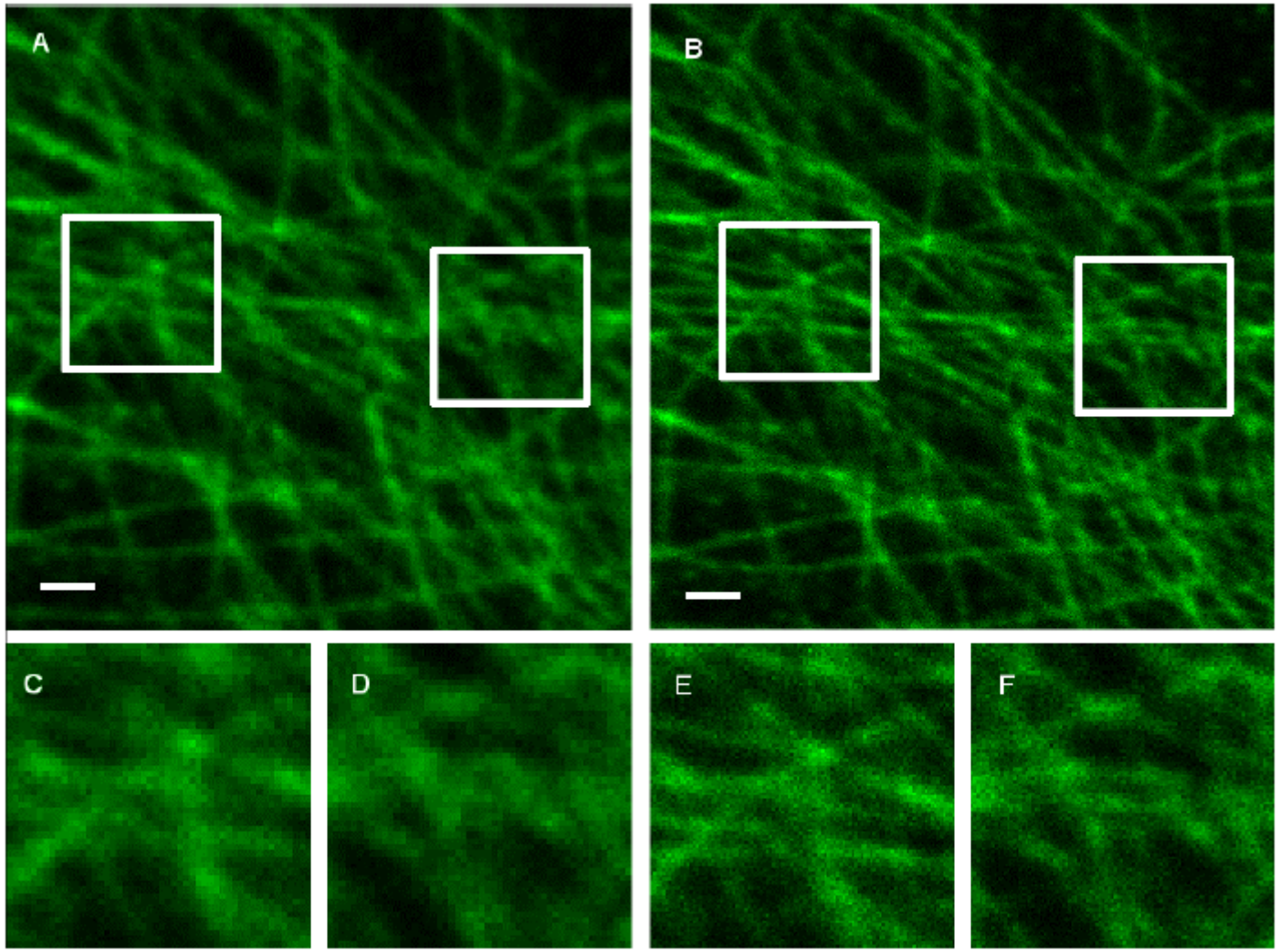
Confocal 2.0





- C.J.R. Sheppard. *Super-resolution in confocal microscopy*. Optik, **1988**
- C.B. Müller and J. Enderlein. *Image scanning microscopy*. Physical Review Letters, **2010**.
- De Luca et al.. *Re-scan confocal microscopy: scanning twice for better resolution*. Biomedical Optical Express, **2013**.
- S. Roth et al. *Optical photon reassignment microscopy (OPRA)*. Optical nanoscopy, **2013**.
- York et al. *Instant super-resolution imaging in live cells and embryos via analog image processing*. Nature Methods, **2013**.

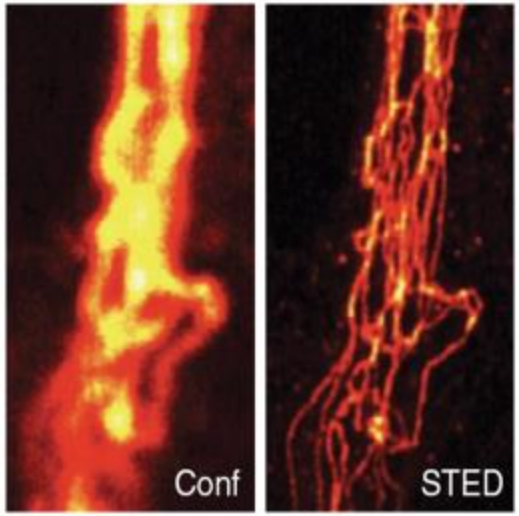
Confocal 2.0

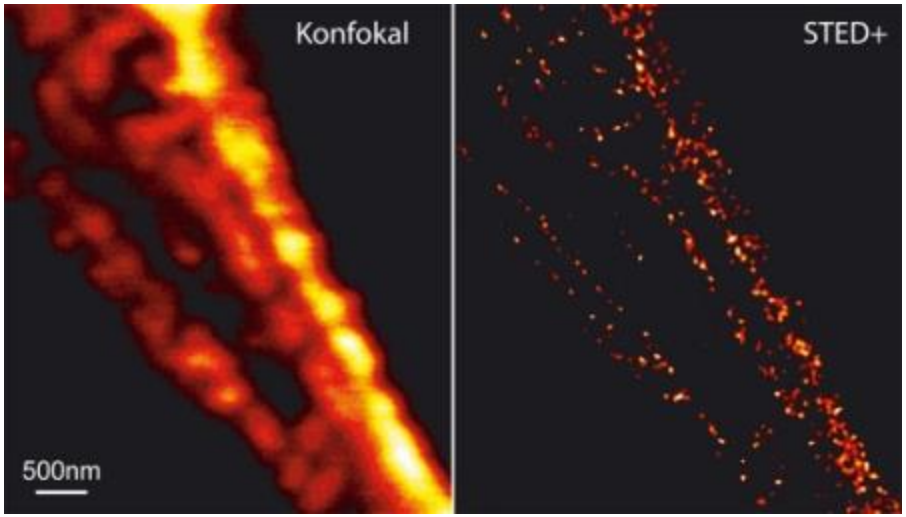


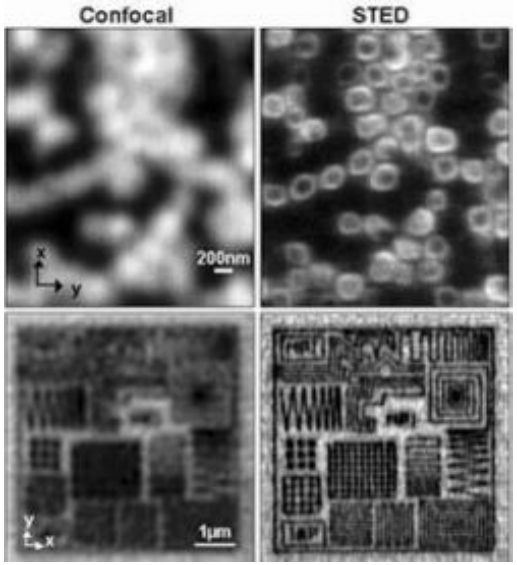
Confocal 2.0

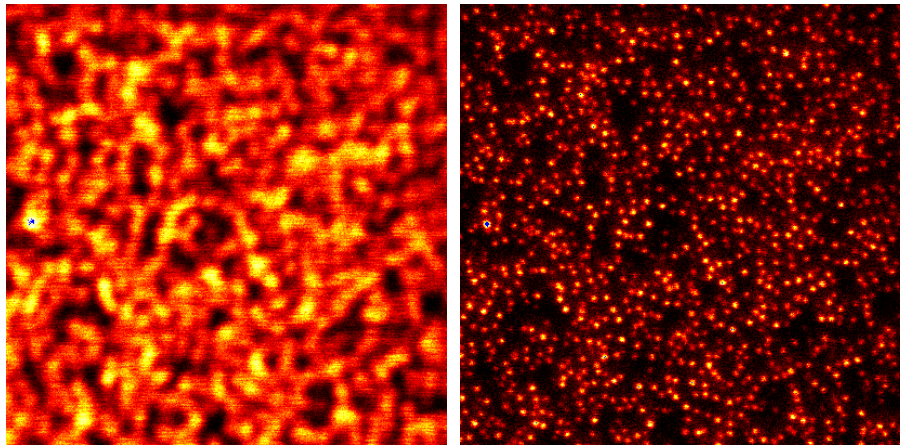
- Offers increased resolution
- Theoretical 2x better, but limited by strong decay of the OTF practically only 1.4x better.
- Very easy to retro-fit on existing confocal microscopes.

STimulated Emission Depletion (STED)







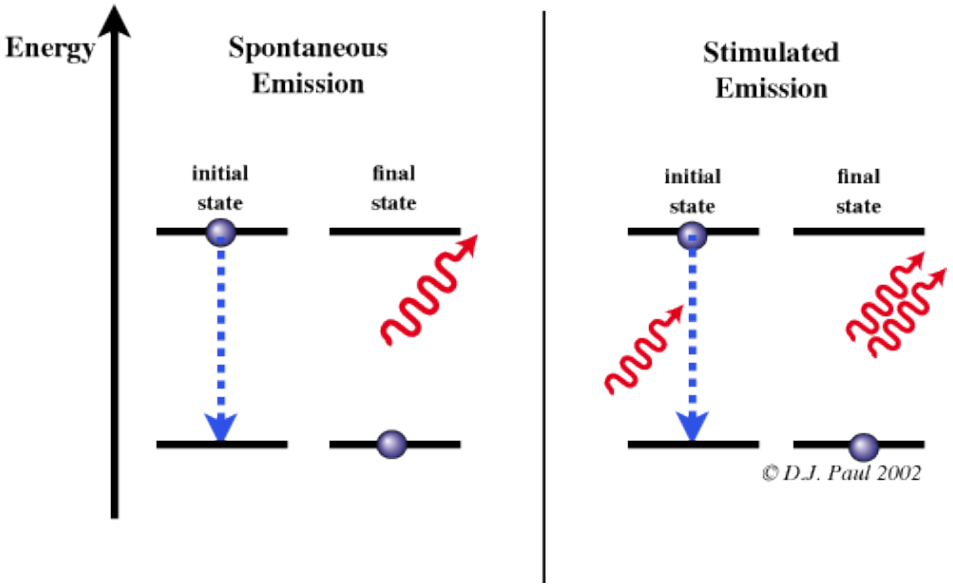


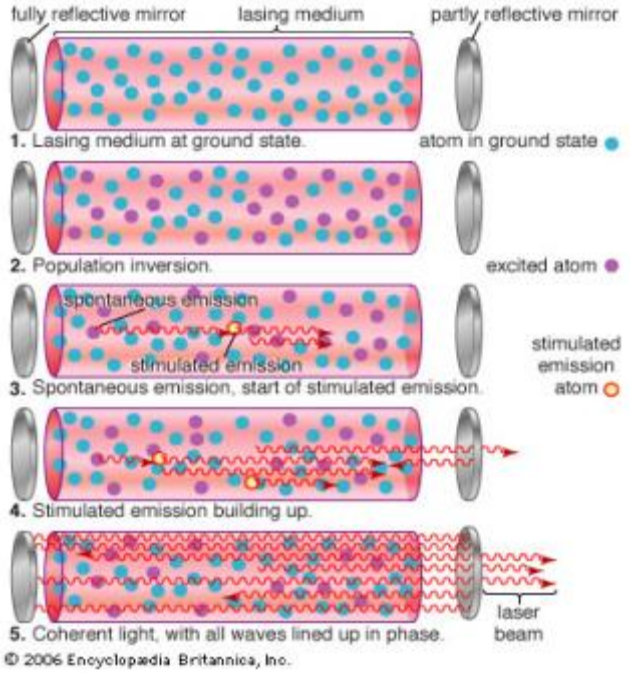
Stimulated emission

Incident photon "knocks out" molecule in excited state

Emitted photon identical to incident photon

(energy, direction of propagation, polarization)





How does this relate to a LASER?

(Light Amplification by **Stimulated Emission** of Radiation)

Application of stimulated emission in STED

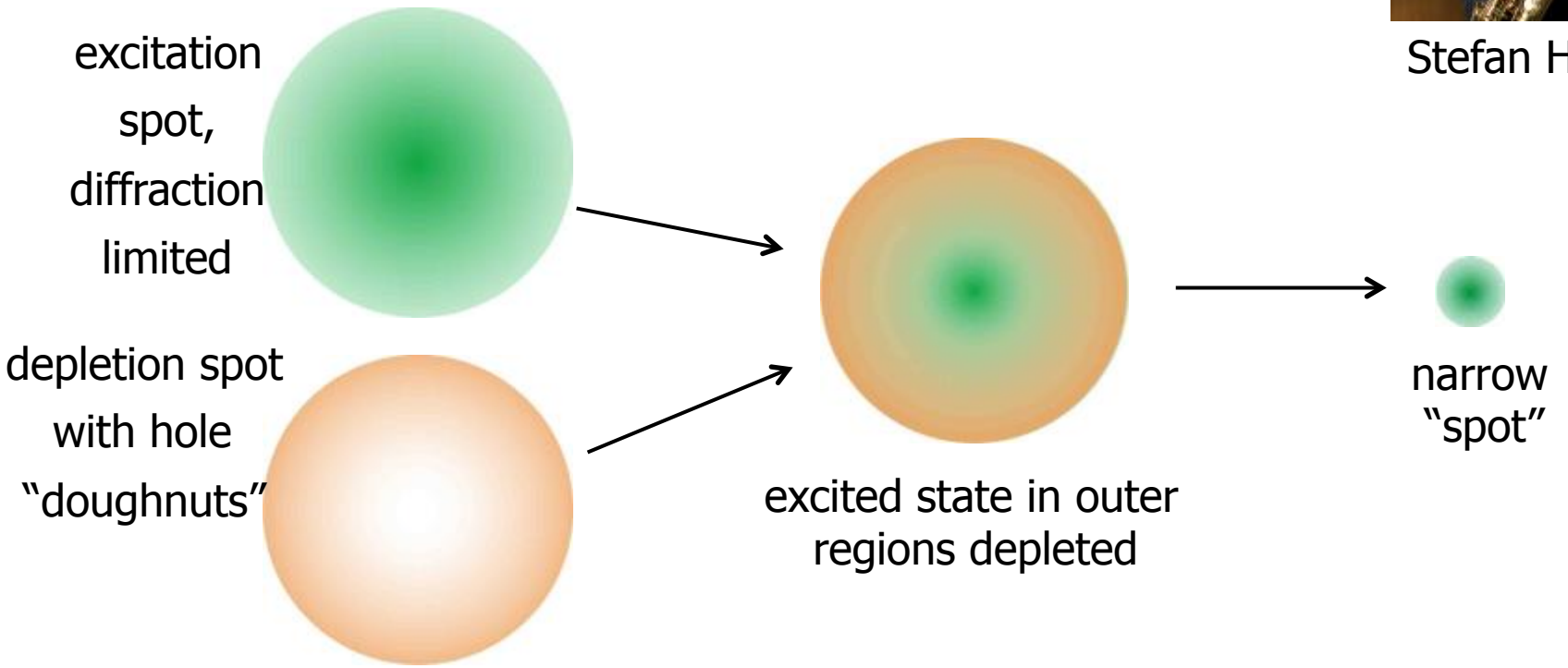
Excite fluorophores with first spot

Illuminate with second ring-shaped spot

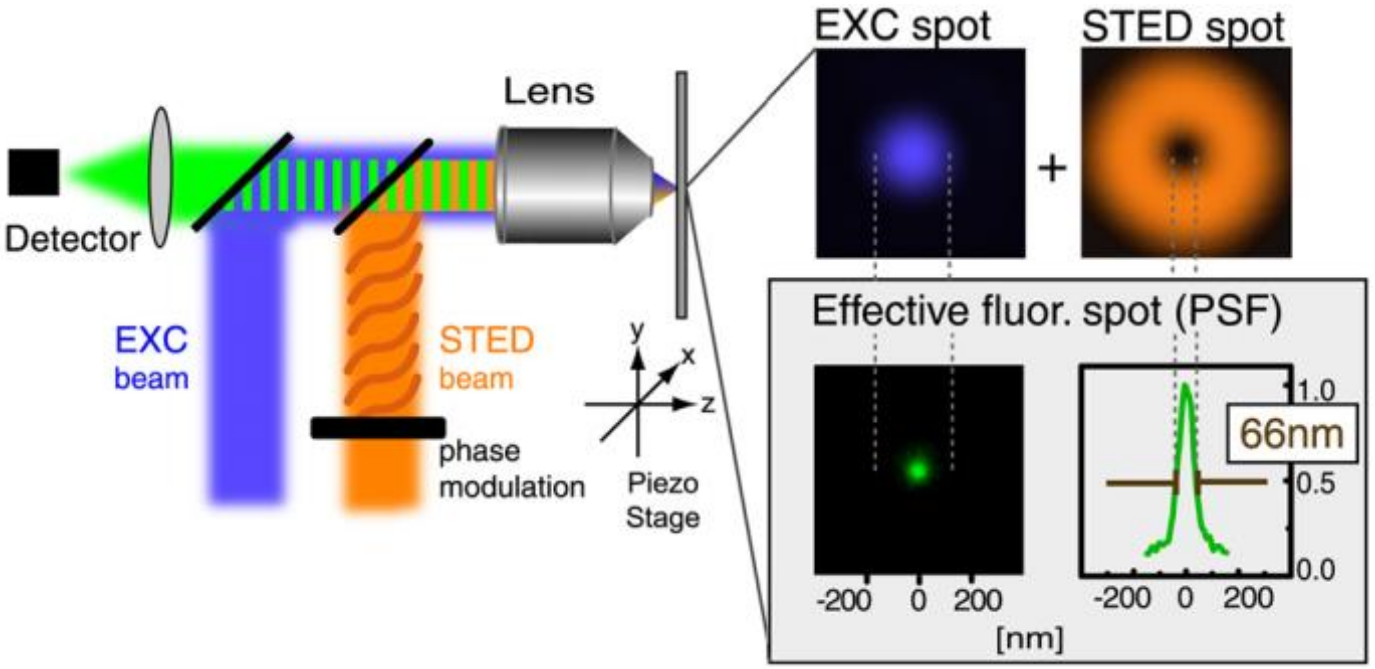
Deplete excited state via stimulated emission

Collect fluorescent light from central "spike"

Stefan Hell



STED-setup



- STED-beam must have bit larger wavelength (Stokes-shift)
- STED-spot must be engineered to "doughnut"-beam/ring-shaped spot
- Needs high powers to fully deplete excited state

source: http://www.mpg.de/english/illustrationsDocumentation/documentation/pressReleases/2006/pressRelease20060412/index.html

What are typical powers needed?

Probability that photon de-excites fluorophore by stimulated emission must be very close to one

$$\#\text{photon-hits} = \text{intensity} \times \text{cross-section} \times \text{excited state lifetime}$$

$$= \frac{P/h\nu}{(\lambda/NA)^2} \times \sigma \times \tau \gg 1$$

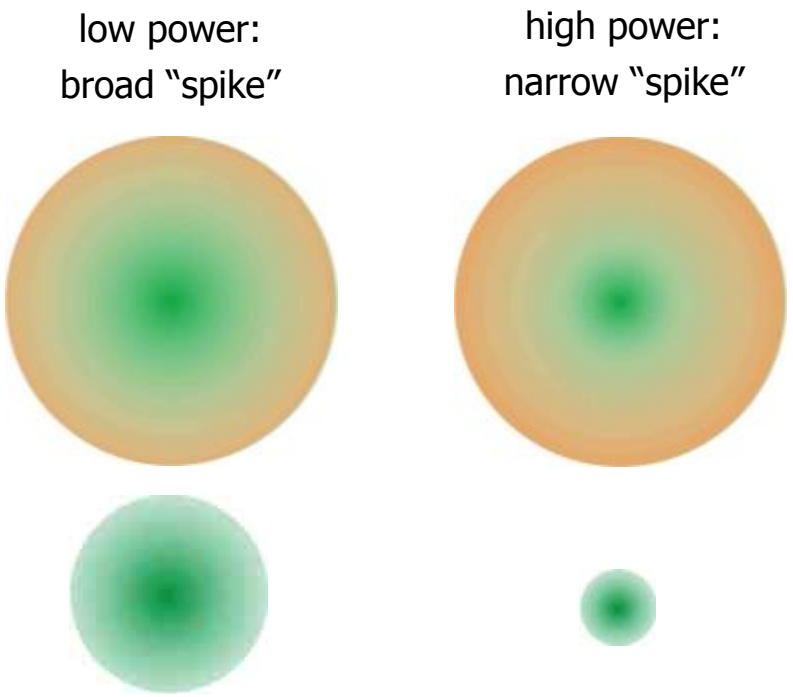
Power must satisfy:

$$P \gg \frac{h\nu}{\sigma\tau}\left(\frac{\lambda}{NA}\right)^2$$

Example (hv=2 eV, λ/NA=0.5 μm, $\sigma=10^{-20}$ m^2, t=10^{-9} sec)

$$P \gg 0.08W$$

Laser powers of several Watt needed!!!



Resolution of STED

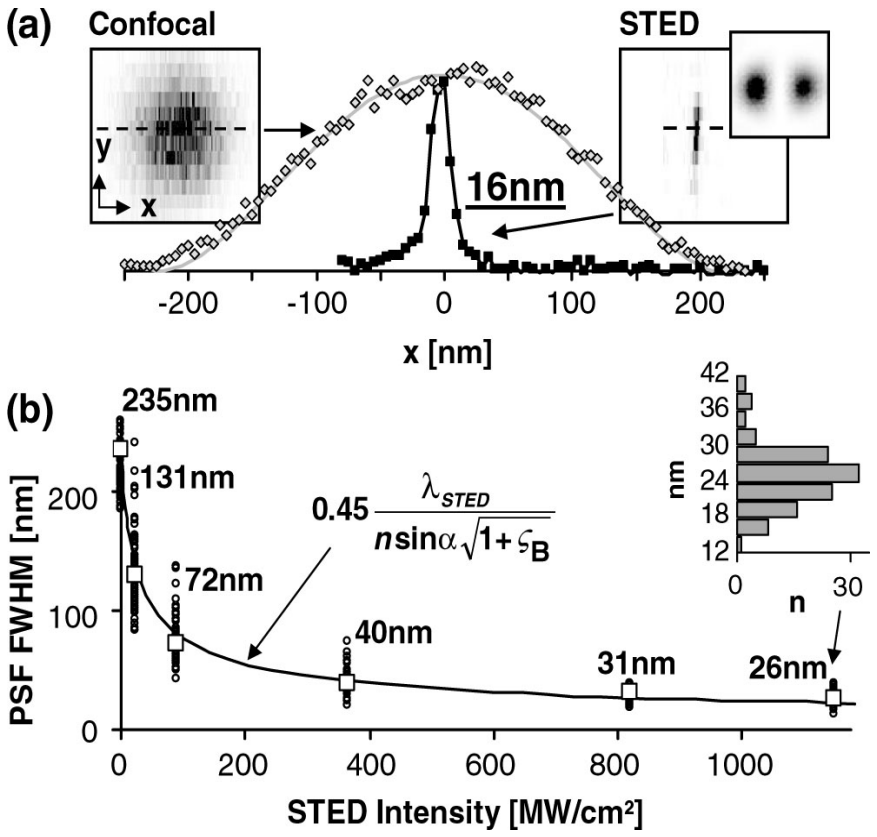
Increase in depletion beam power

=> narrower emission spike

=> better resolution

Modified Abbe-formula:

$$d = \frac{\lambda}{2n \sin\alpha \sqrt{1 + I/I_{STED}}}$$



V. Westphal, S.W. Hell (2005), *Phys. Rev. Lett.* **94**, 143903.

STED

Idea:

- "Switch-off" the emitters in the PSF expect at the very center.

- Switching mechanism: Prevent the emitters from emitting.

Idea is around since 1990s, took a long time to make it work

See literature list for more

Grotjohan et al., Nature 2011

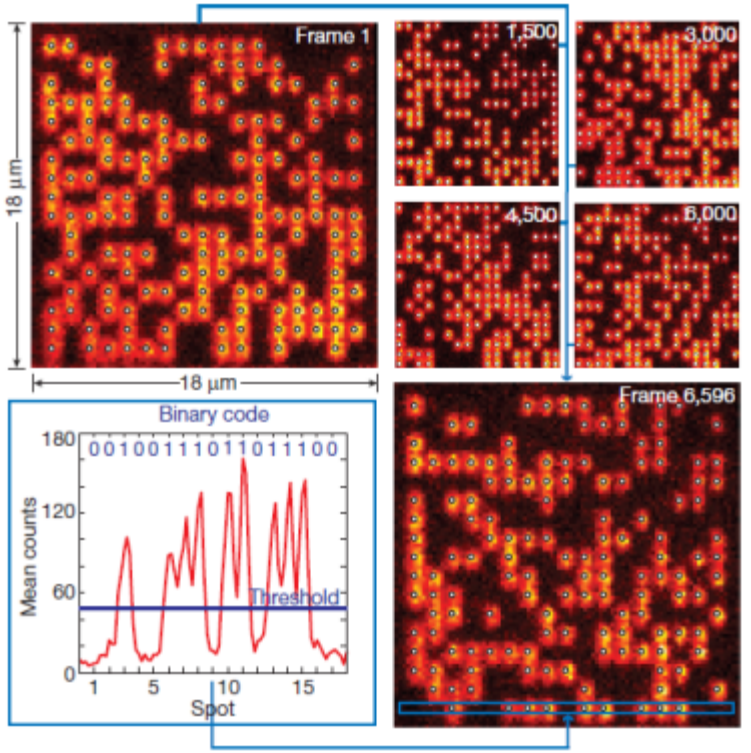
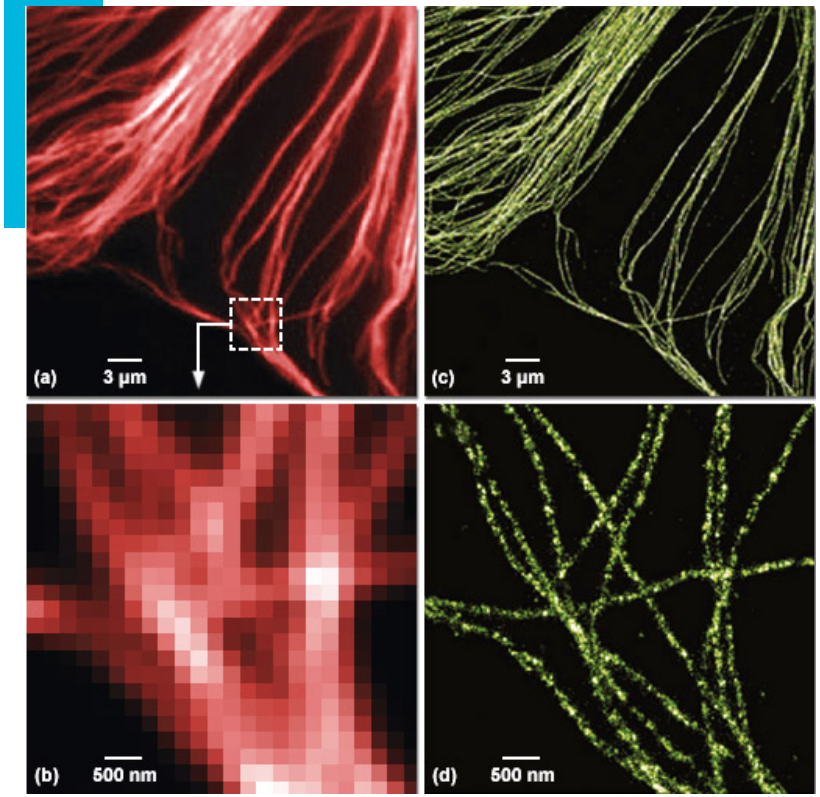
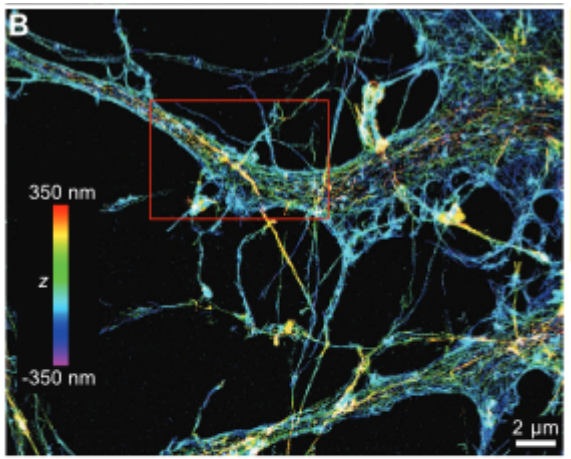
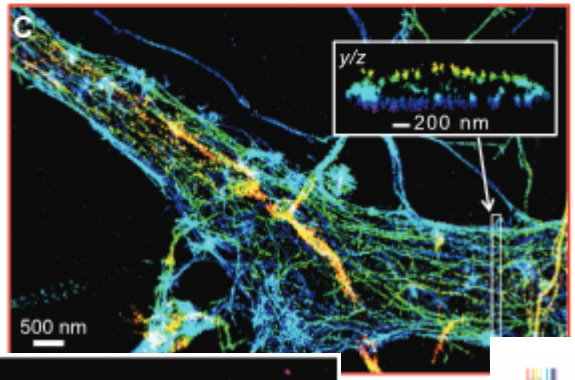


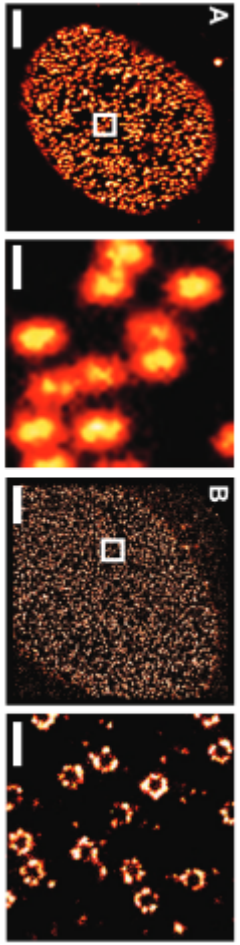
Figure 2 | Rewritable data storage. The text of 25 Grimm's fairy stories (ASCII code; 1.9 Mbits) consecutively written and read on a 17 × 17 µm area of a PAA layer containing rsEGFP, with bits written as spots (representative frames shown). The white dots mark spots that were recognized as set bits ('1's). The graph shows an intensity profile along the indicated area, averaged over three pixels along the *y*-axis. The blue line indicates the threshold used to assign read spots to '0's or '1's.

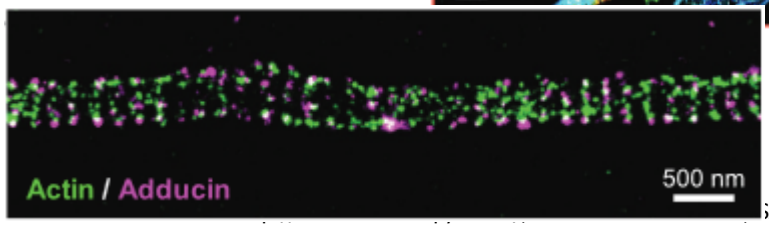
"PALM" and "STORM"

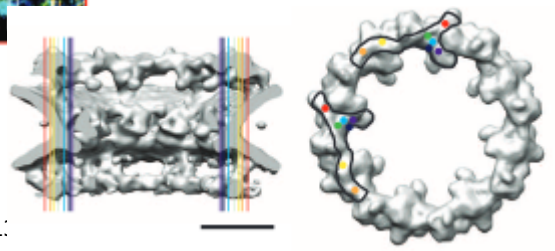






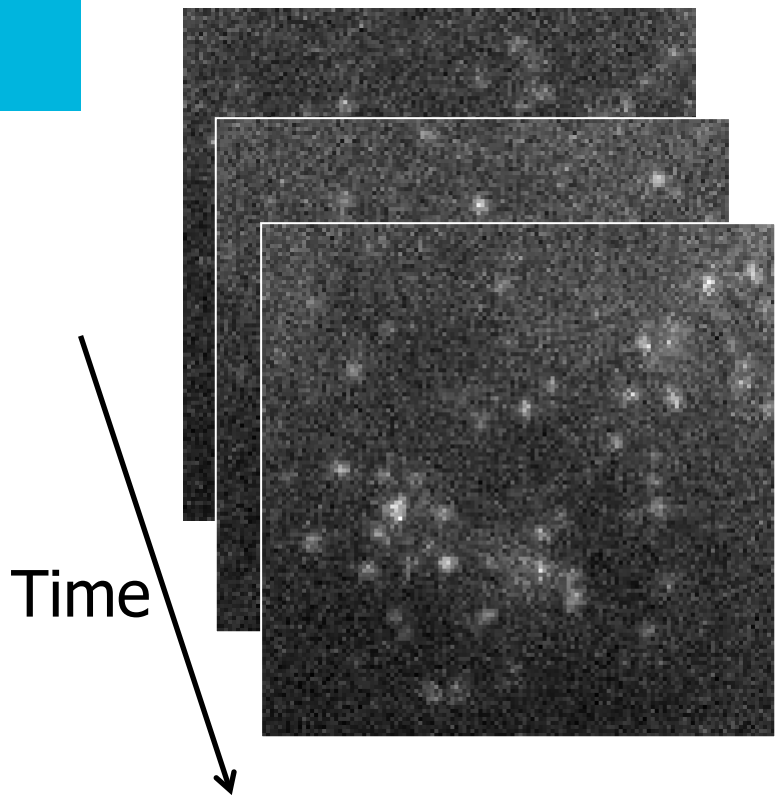




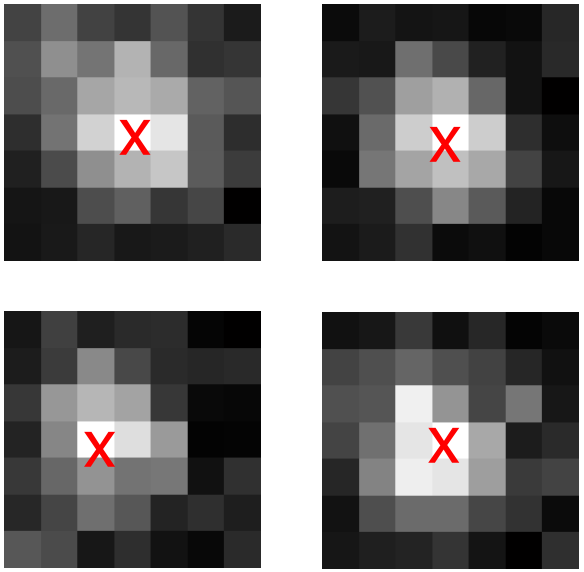


Szymborska 201

Single molecule localization microscopy



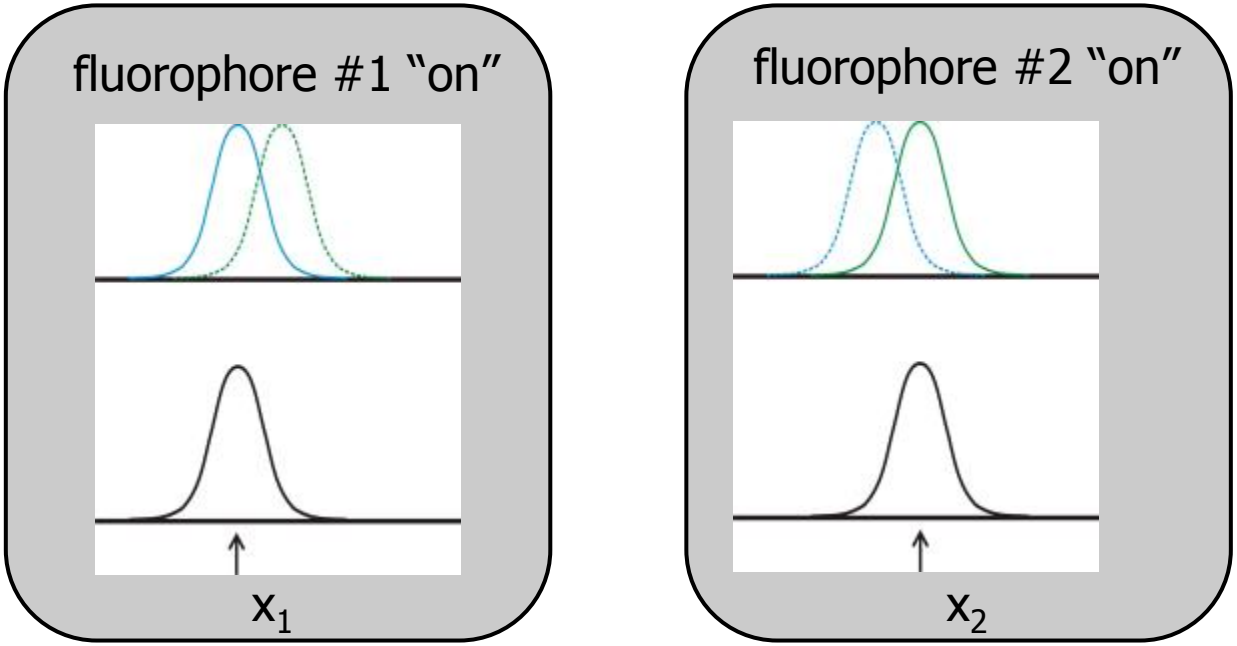
Fit center positions



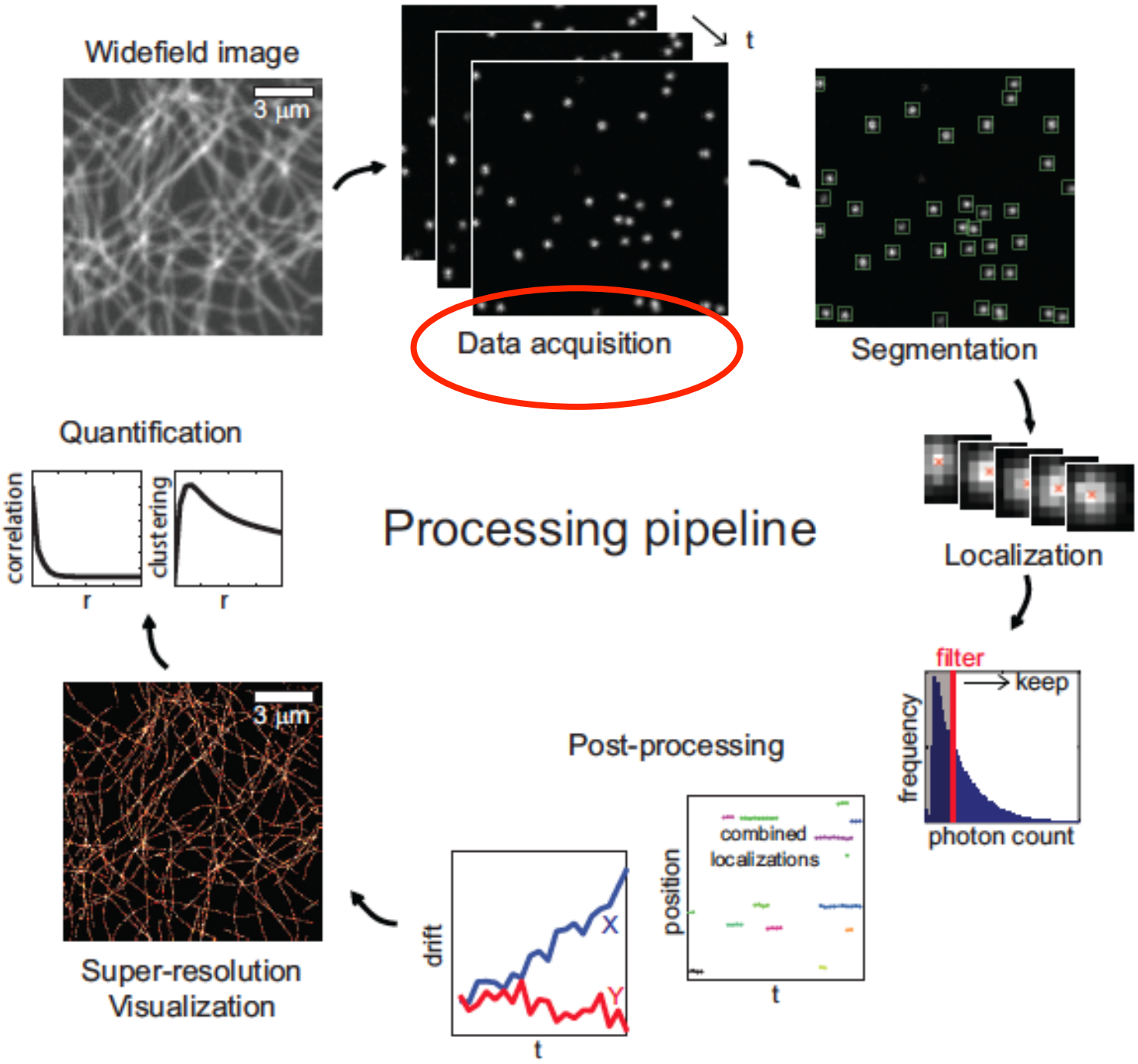
Place “blob” on all measured positions to get the final image

Single emitter localization

Switch fluorophores "on" or "off" **in time**



Good ides iff: Position of single emitter can be determined with high certainty

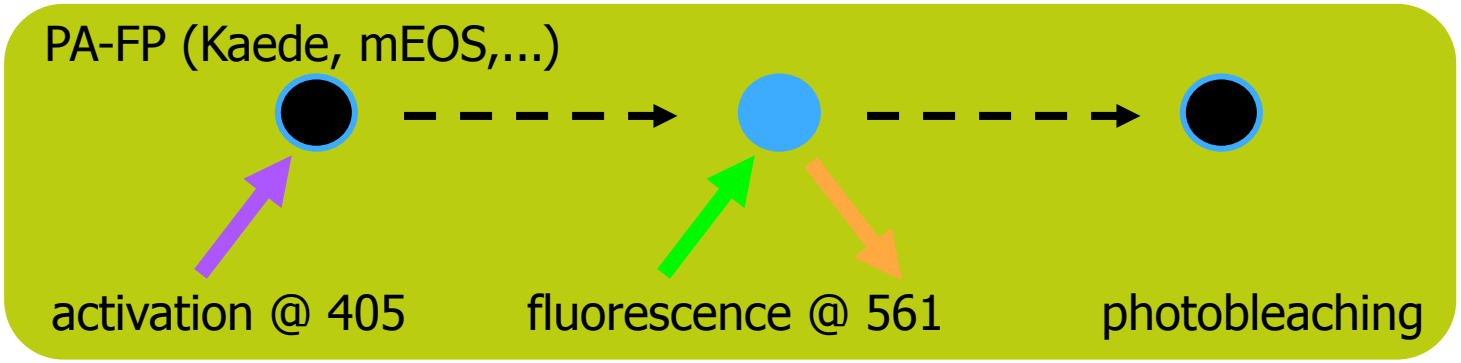

Widefield image
3 µm
t
Data acquisition
Segmentation
Localization
filter
keep
frequency
photon count
Post-processing
combined
localizations
position
t
drift
X
Y
t
3 µm
Super-resolution
Visualization
Quantification
correlation
r
clustering
r
Processing pipeline

Mechanisms for “on” / “off” switching

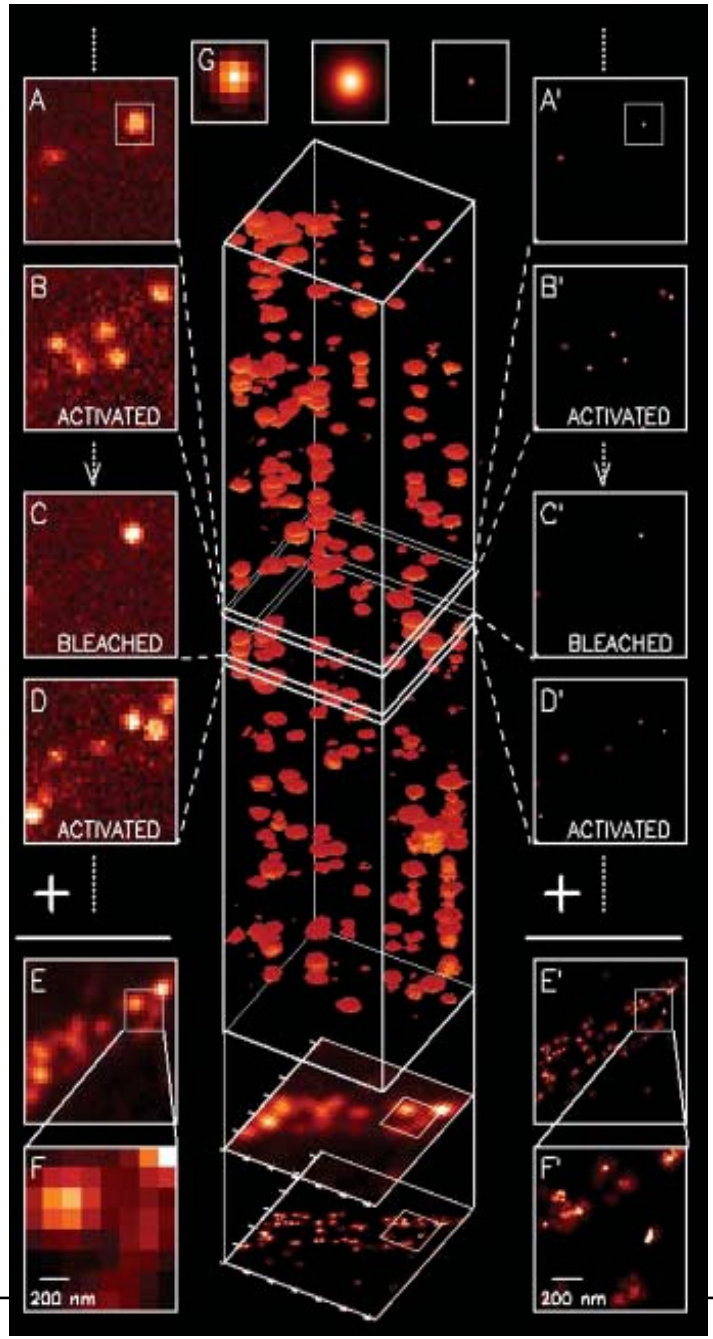
PALM = Photo-Activation Localization Microscopy

Betzig/ H.Hess, Science 2006

S. Hess, Biophysical Journal 2006

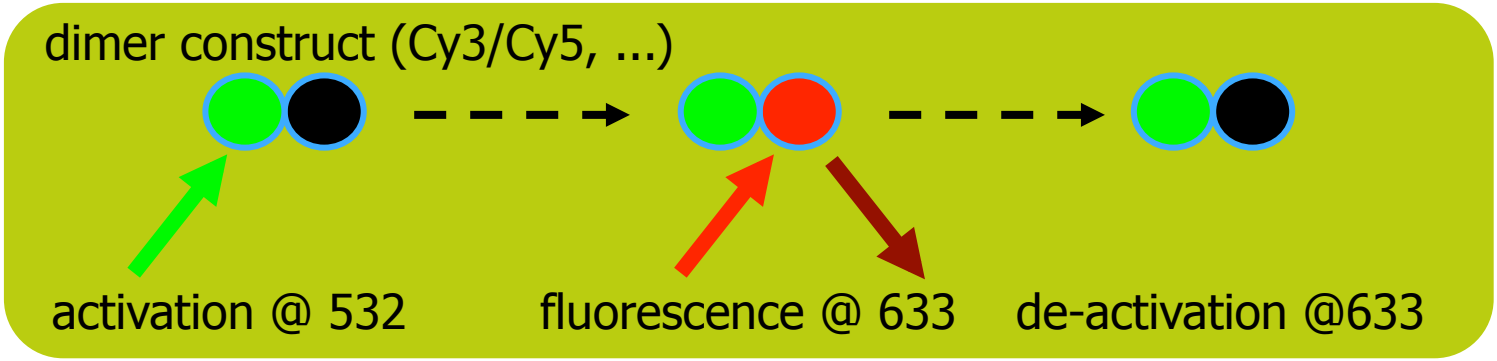


PALM workflow



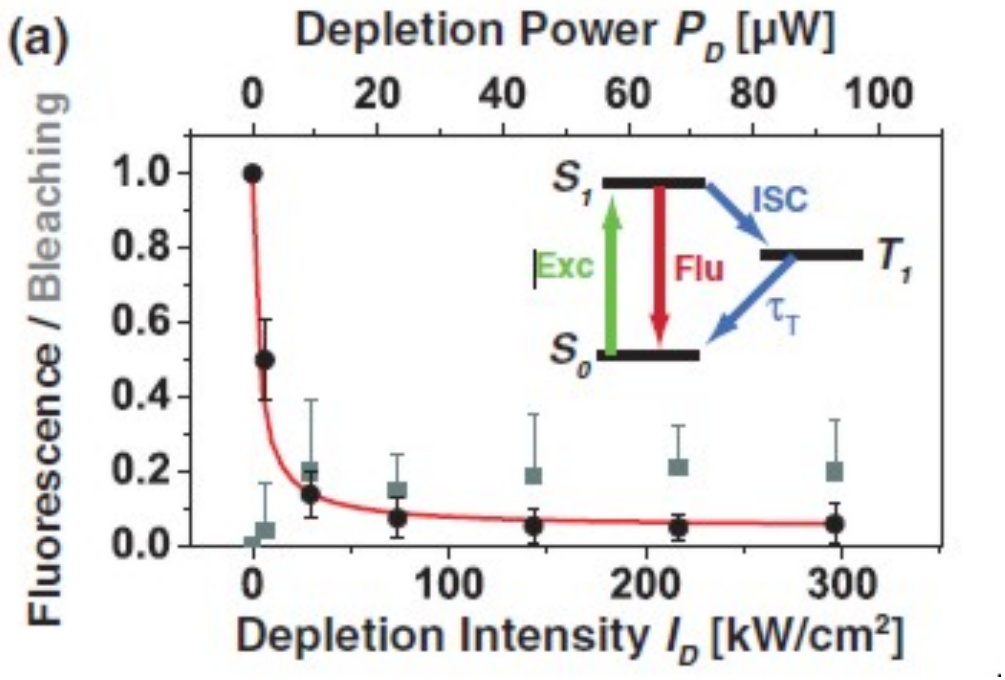
Mechanisms for “on” / “off” switching

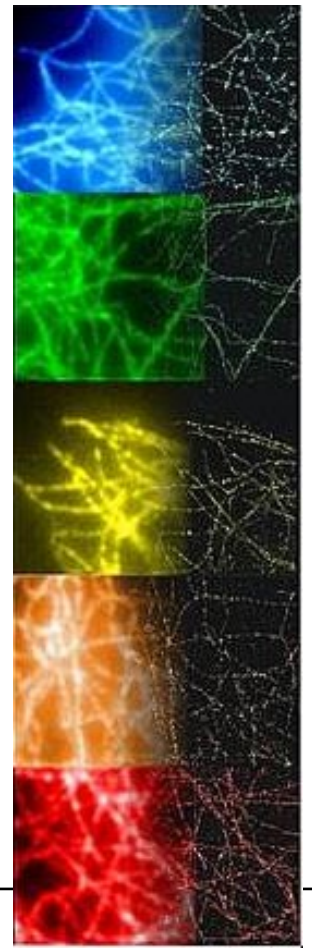
STORM = STochastical Optical Reconstruction Microscopy
(Zhuang, Nat. Methods 2006)



Mechanisms for “on” / “off” switching

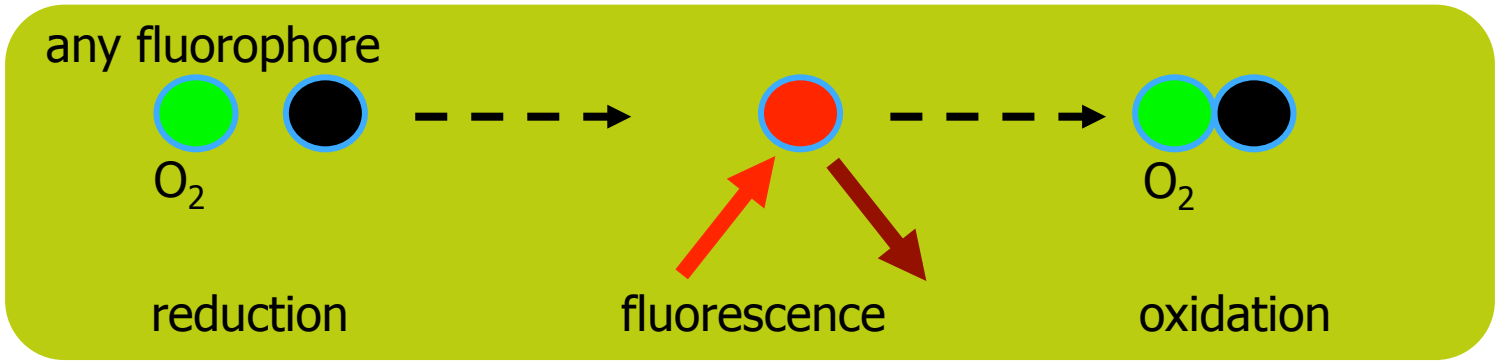
GSDIM = Ground State Depletion followed by single molecule IMaging (Hell, PRL 2007)

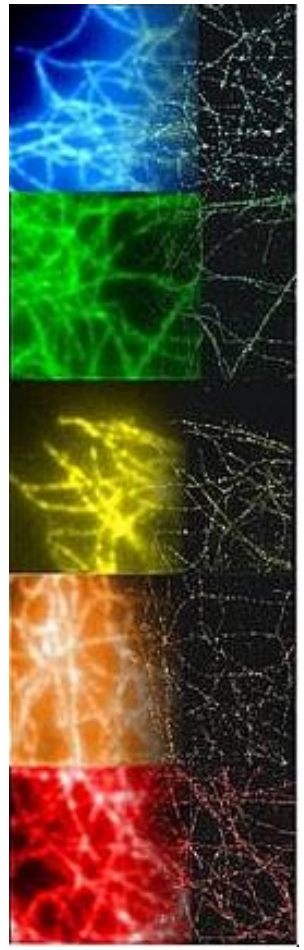




Mechanisms for “on” / “off” switching

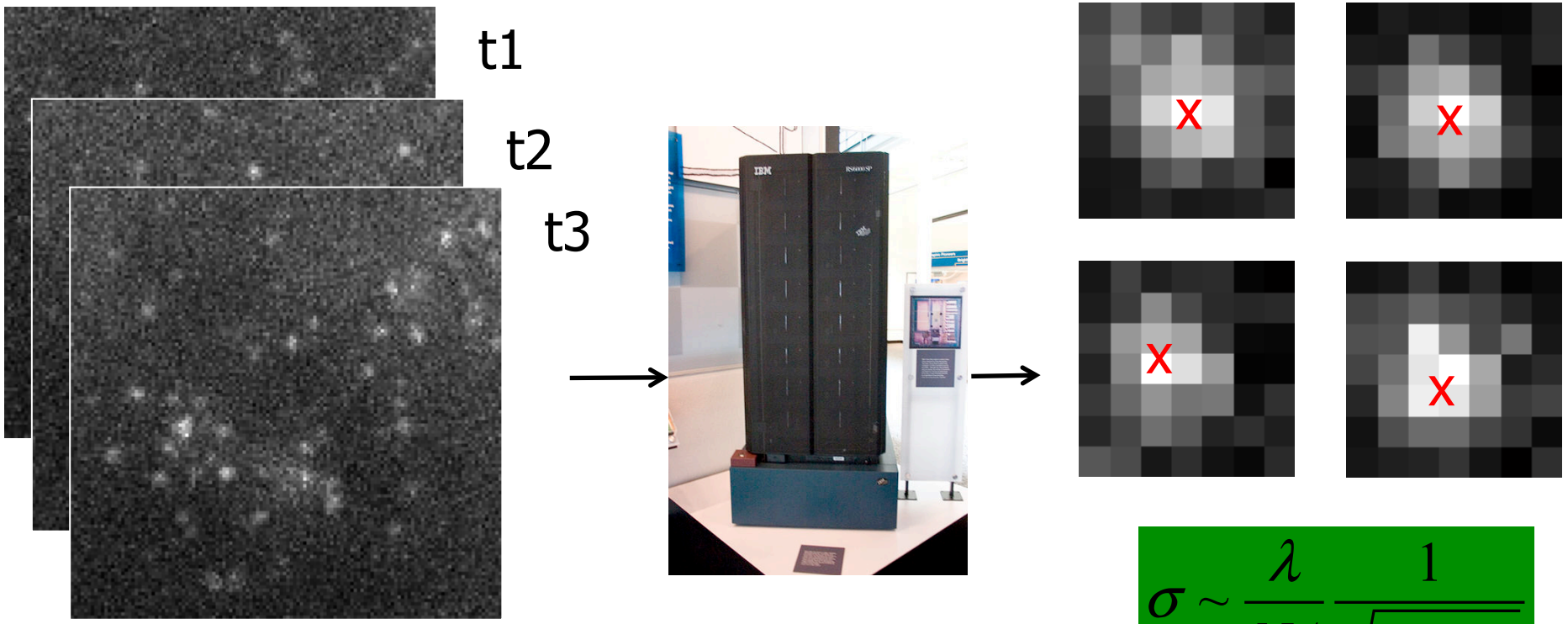
dSTORM = "direct" STORM (Heilemann, Angew. Chem. 2008)



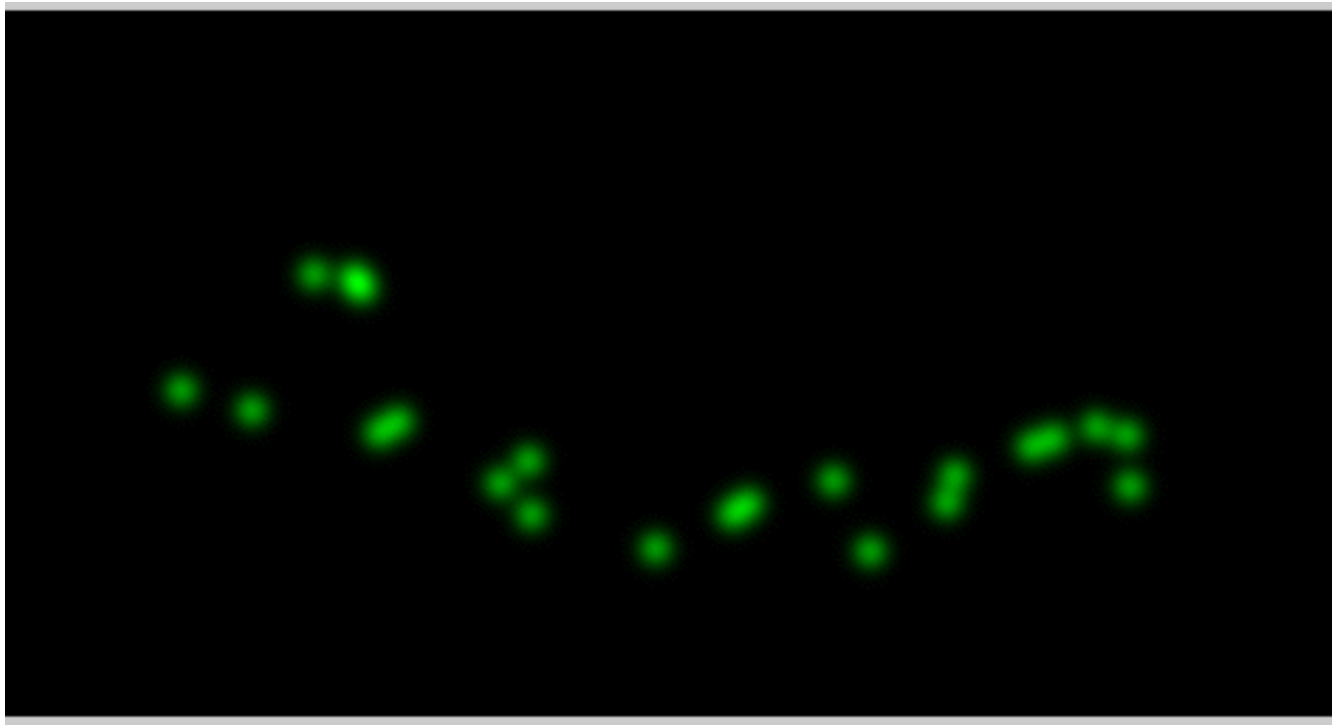


Why is a single emitter better than many?

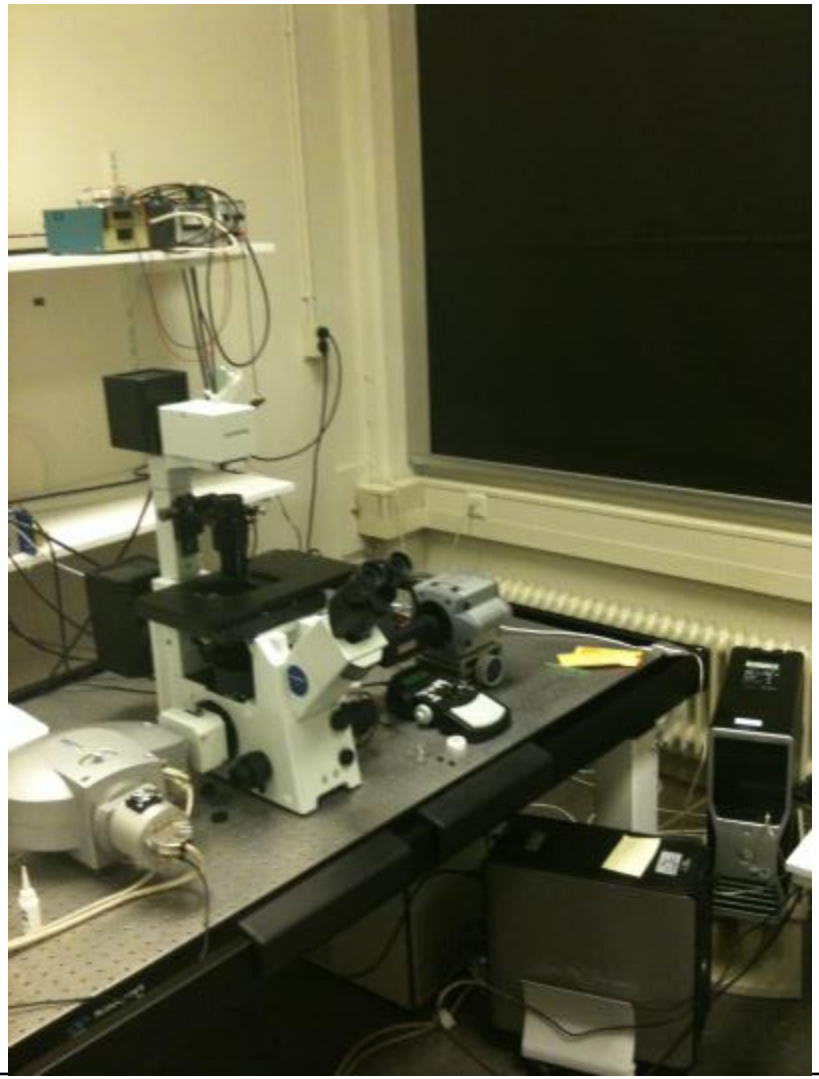
- Single emitters' positions are estimated with nanometer precision

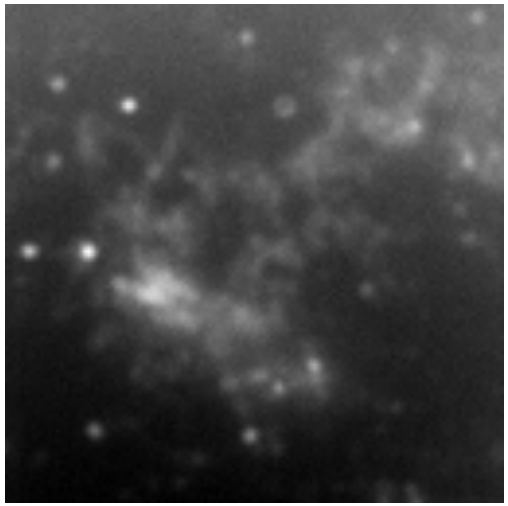


$$\sigma \sim \frac{\lambda}{NA} \frac{1}{\sqrt{n_{photons}}}$$

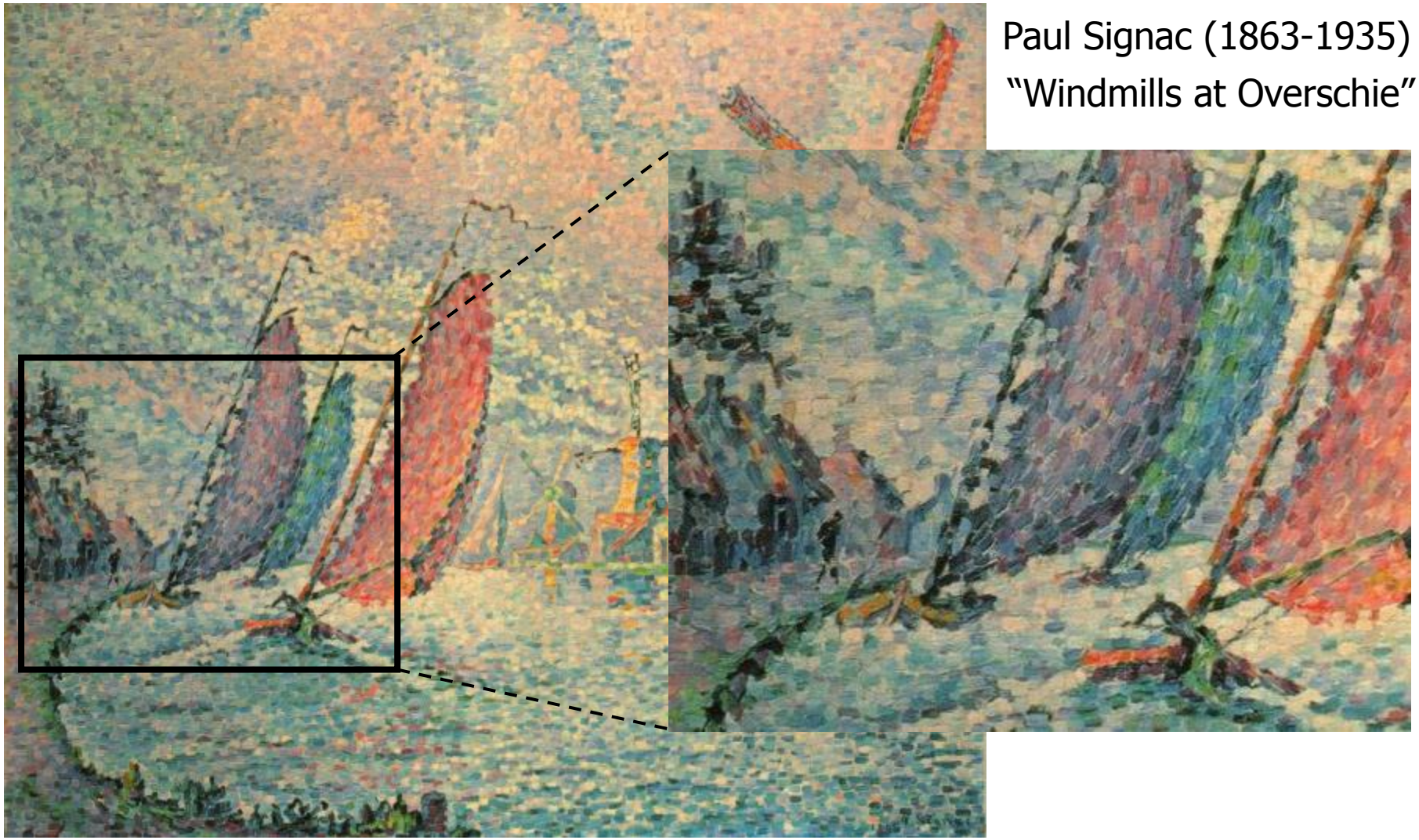


A real acquisition on a microscope





Pointillism in art



Paul Signac (1863-1935)

"Windmills at Overschie"

source: http://www.brown.edu/Courses/CG11/2007/Kelly_Robinson/pointillism.htm

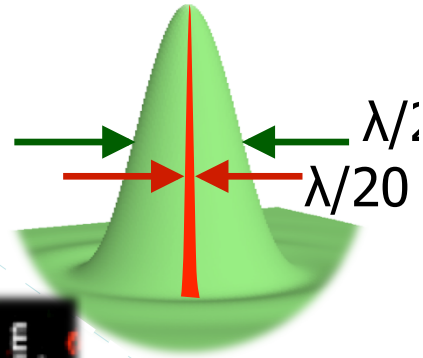
Dithering for paper printing




http://en.wikipedia.org/wiki/Dither

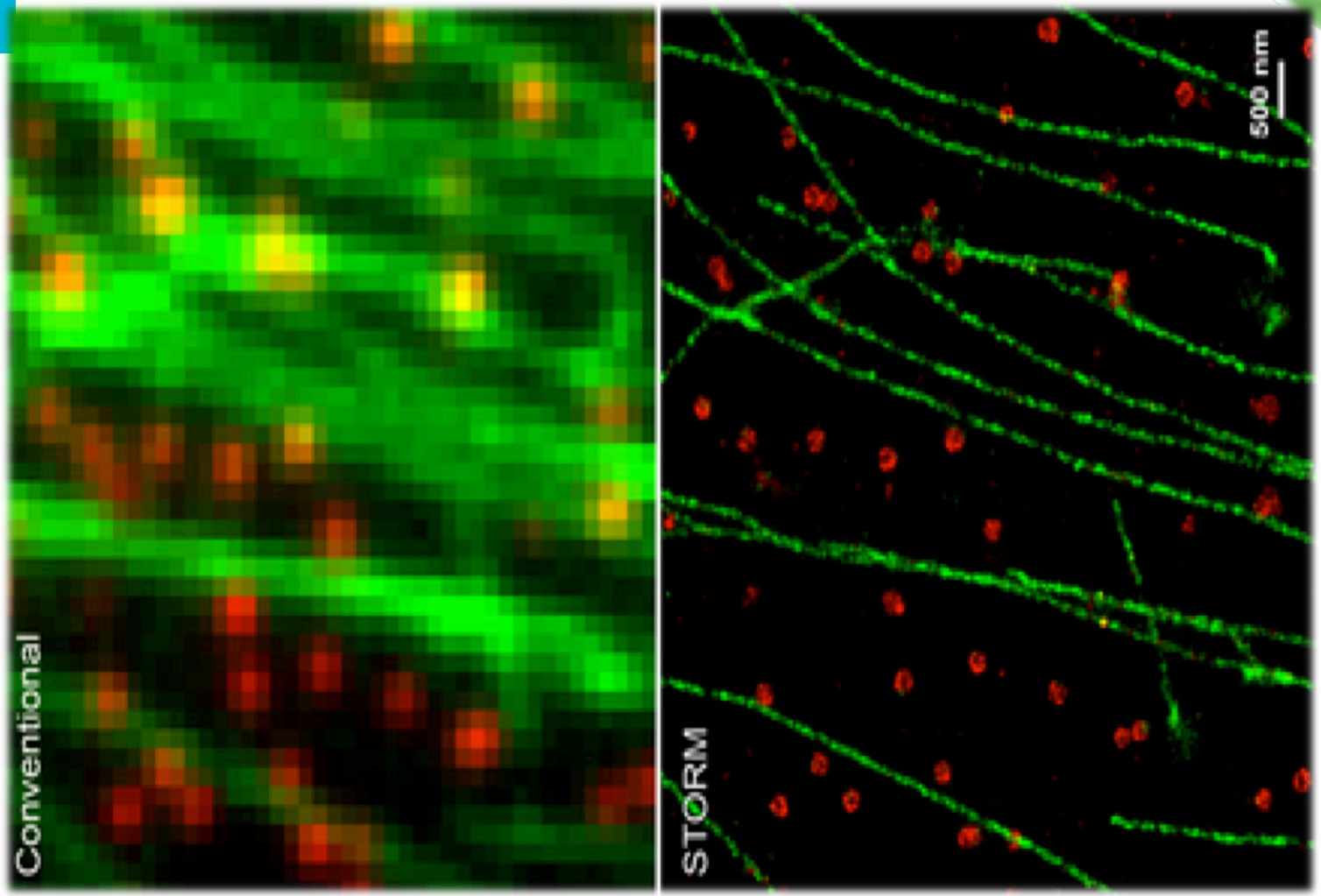
World press photo 2012

Resolution improvement 10-fold



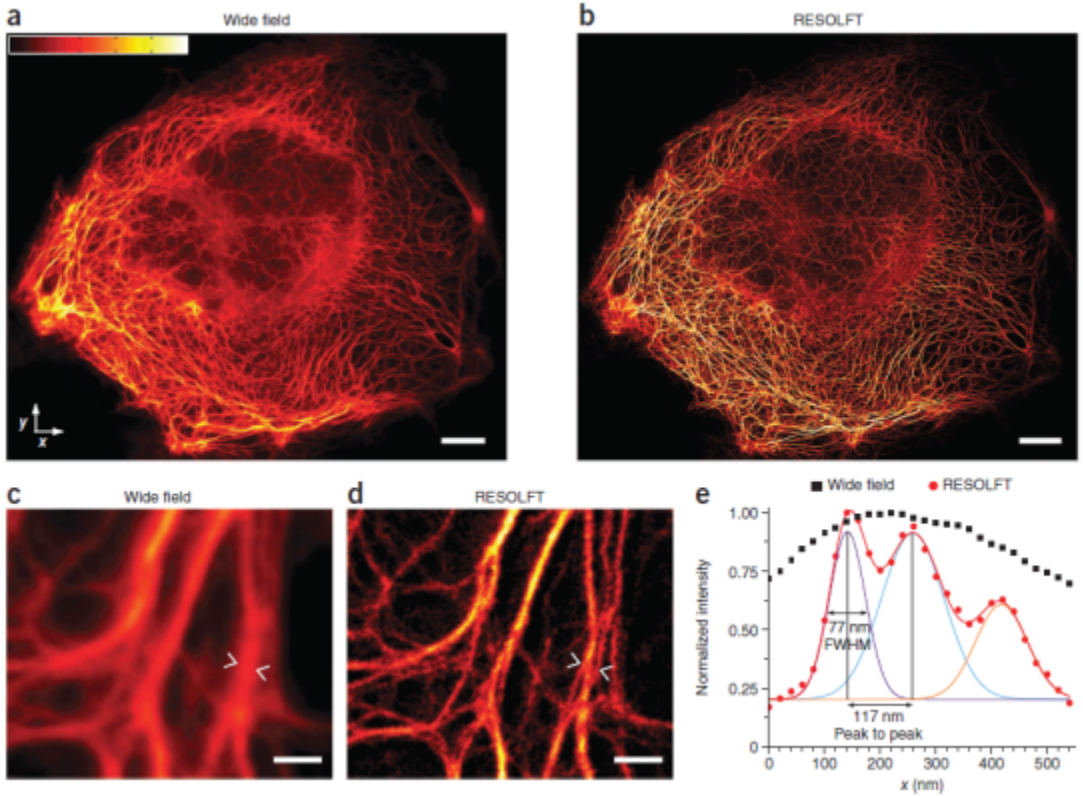
Conventional

Super-resolution



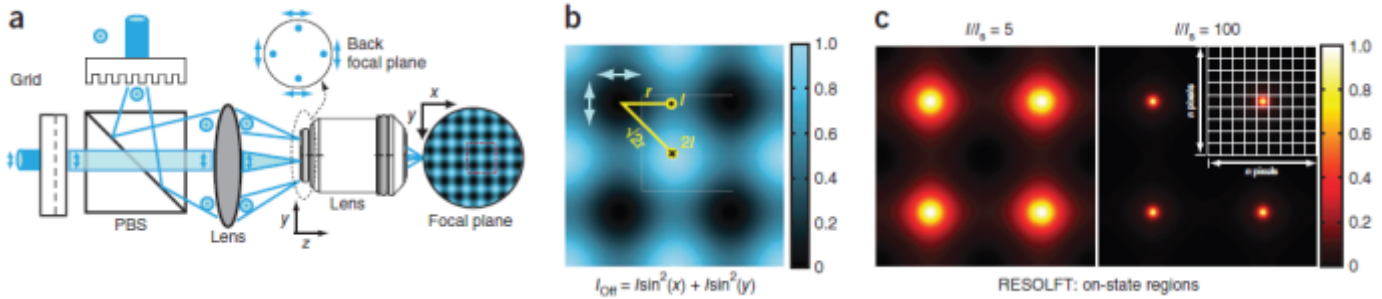
Bates et al., **Science** 317, 2007

Nanoscopy with more than 100,000 'doughnuts'

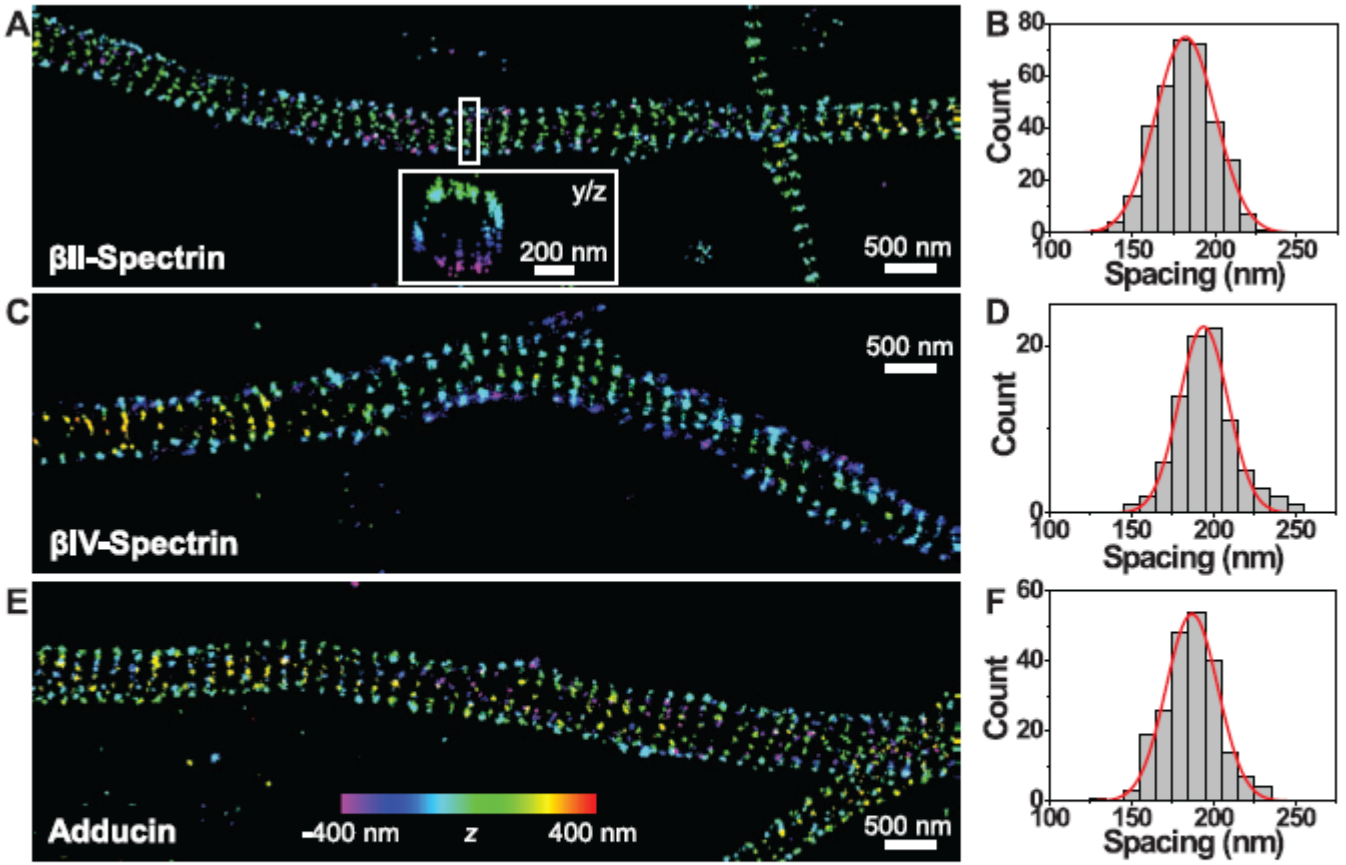


RESOLFT
=REversible Saturable Optical Fluoresece Transitions

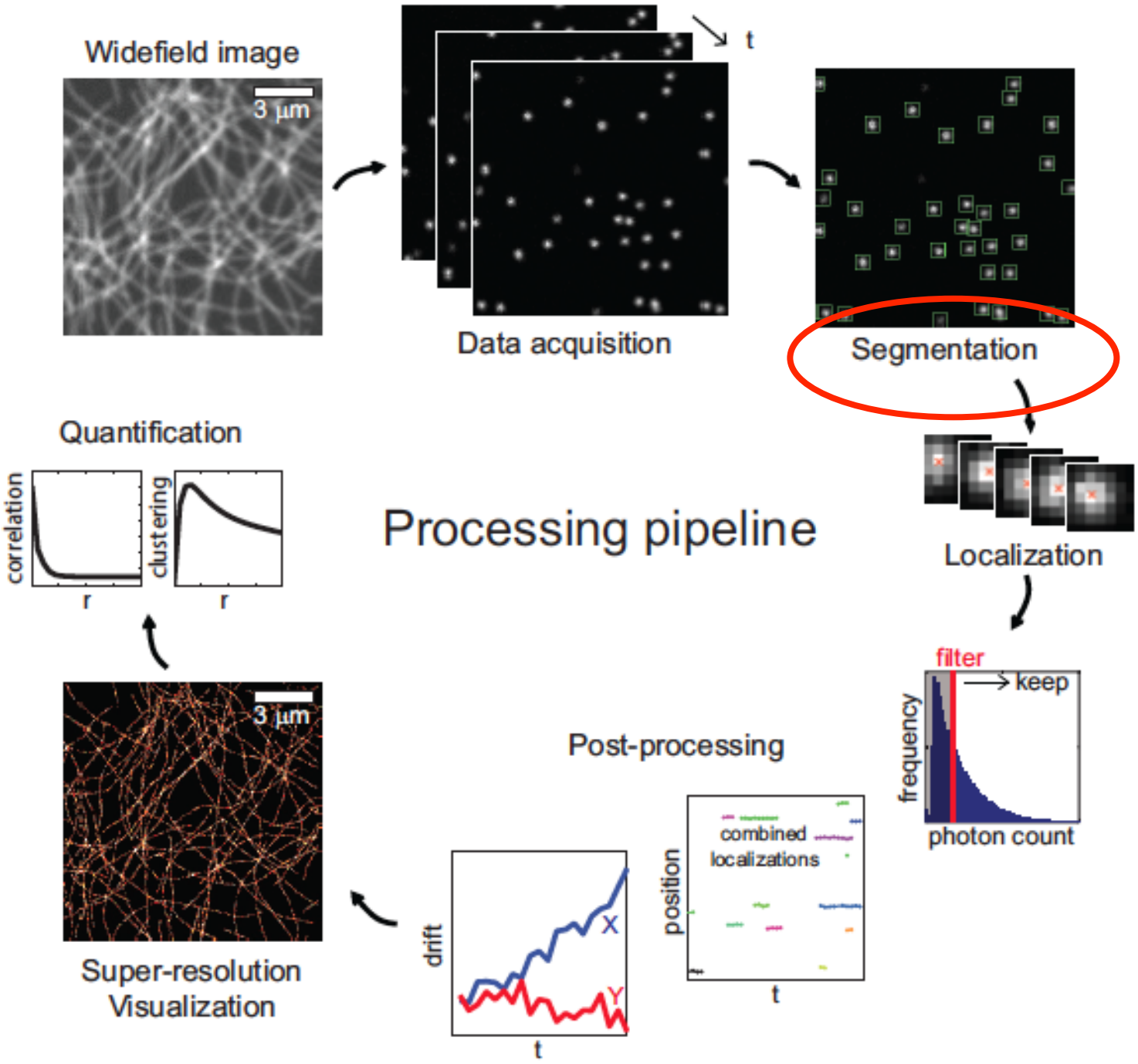
= nonlinear structured illumintation



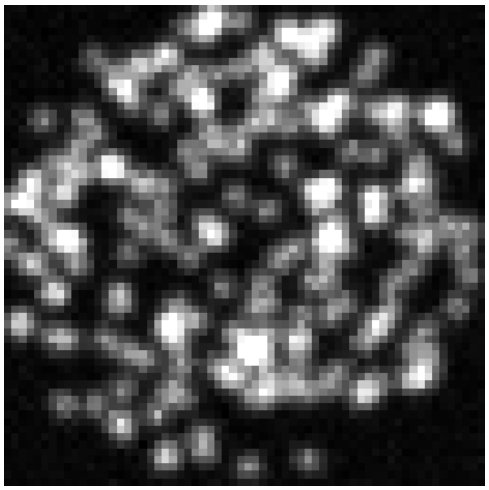
Super-resolution ~10-20 nm



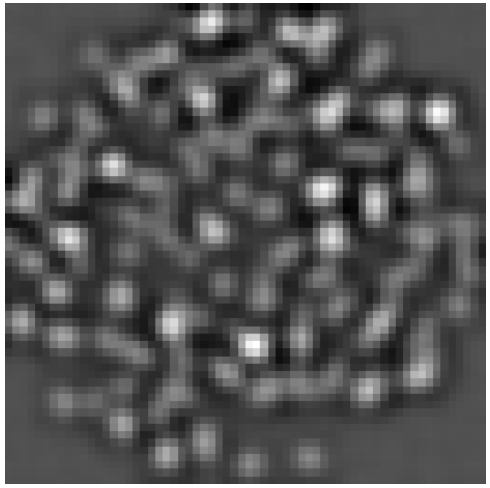
K.Xu, G. Zhong, X. Zhuang, *Actin, spectrin, and associated proteins form a periodic cytoskeletal structure in axons*, **Science** 339: 452, 2013.


Widefield image
3 µm
t
Data acquisition
Segmentation
Localization
filter
keep
frequency
photon count
Post-processing
combined localizations
position
t
drift
X
Y
t
3 µm
Super-resolution Visualization
Quantification
correlation
r
clustering
r
Processing pipeline

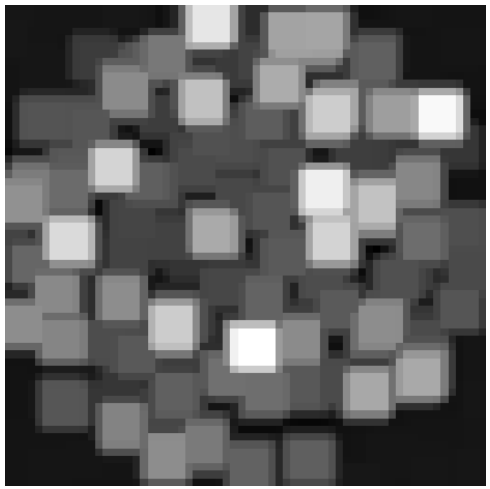
Segmentation



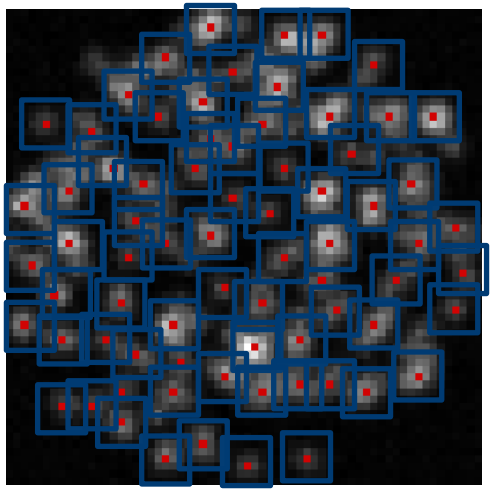
Series of Uniform Filter operation

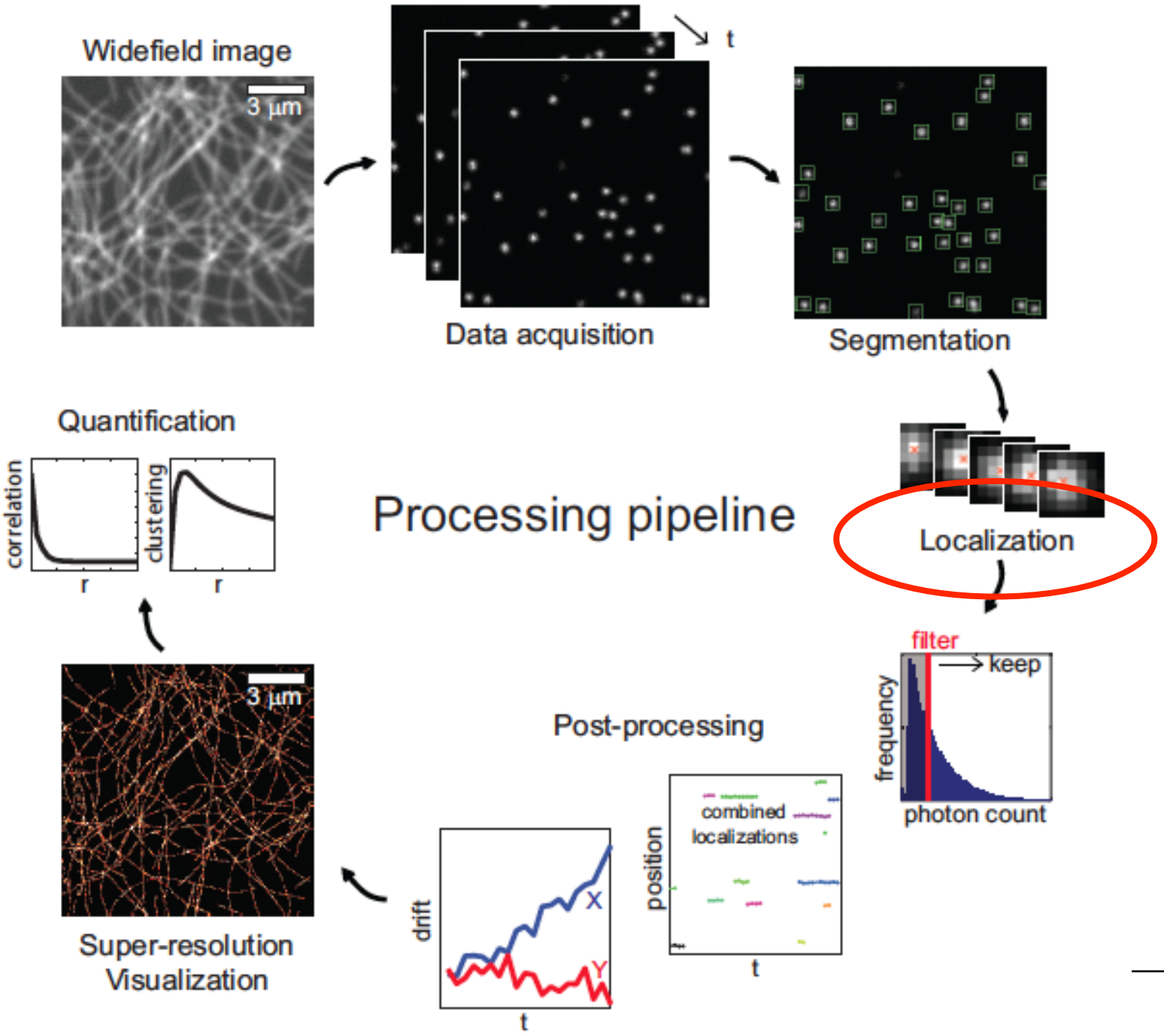


Maximum Filter

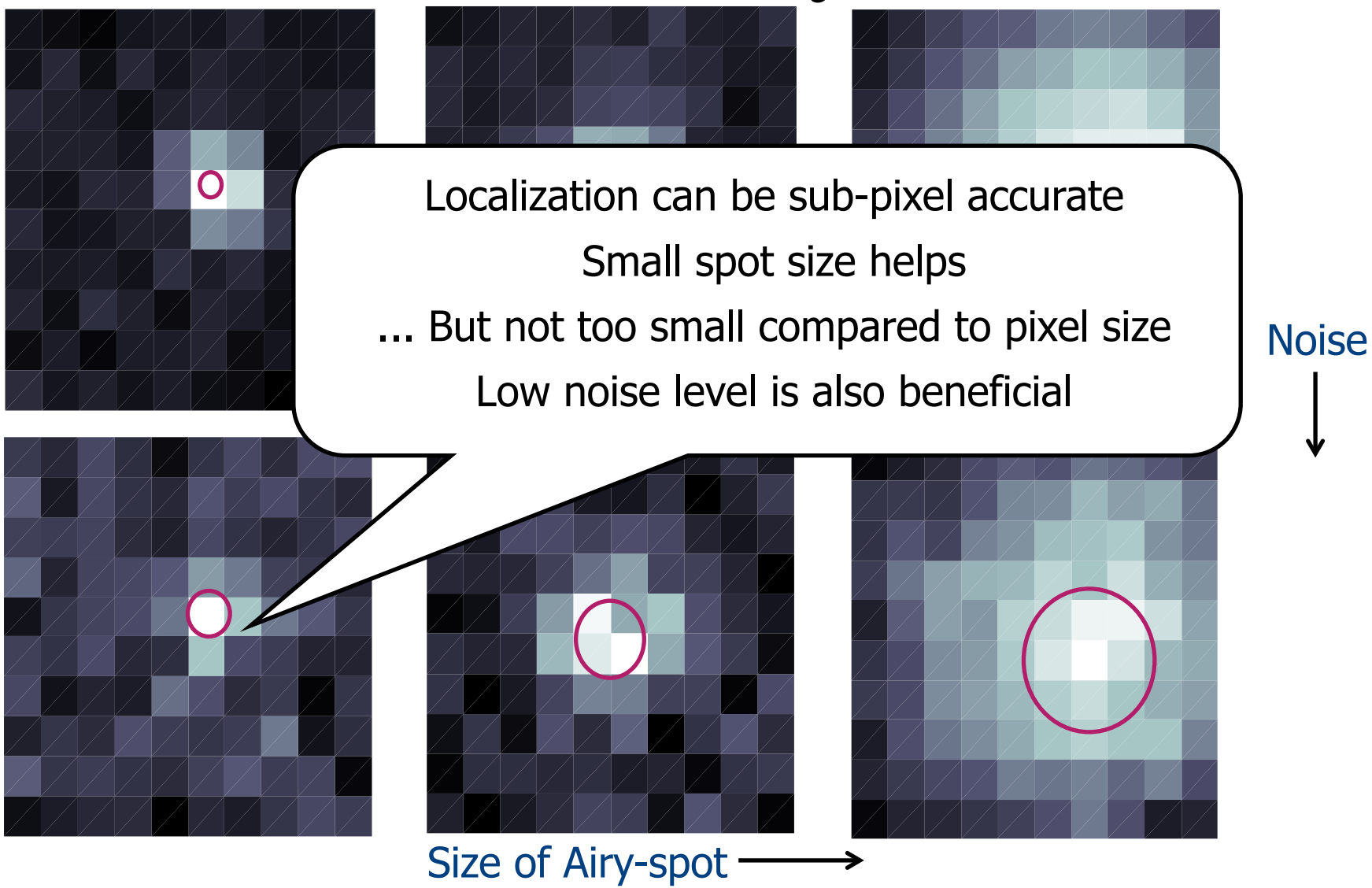


Local maximum




Widefield image
3 µm
t
Data acquisition
Segmentation
Localization
filter
keep
frequency
photon count
Post-processing
combined
localizations
position
t
drift
X
Y
t
3 µm
Super-resolution
Visualization
Quantification
correlation
r
clustering
r
Processing pipeline

Localization uncertainty



Localization uncertainty: rule-of-thumb

- Position estimate by each photon with standard deviation:

$$\sigma \approx \frac{\lambda}{NA}$$

- Independent estimates for N_{ph} photons
- The overall standard deviation is then:

$$\Delta x \approx \frac{\lambda}{NA\sqrt{N_{ph}}}$$

typical number (λ = 488 nm, NA = 1.25, $N_{photons}$ = 400)
Δx = 20 nm

Imaging Model

2D finite pixel Gaussian PSF

$$\mathrm{PSF}(x,y) = \frac{1}{2\pi\sigma^2} e^{\frac{-(x-\theta_x)^2-(y-\theta_y)^2}{2\sigma^2}}$$

$$\mu_k(x,y) = \theta_{I_0} \int_{A_k} \mathrm{PSF}(u,v)\mathrm{d}u\mathrm{d}v + \theta_{bg}$$

$$\boxed{\mu_k(x,y) = \theta_{I_0} \Delta\mathrm{E}_x(x,y)\Delta\mathrm{E}_y(x,y) + \theta_{bg}}$$

$$\Delta\mathrm{E}_x(x,y) \equiv \frac{1}{2}\mathrm{erf}\left(\frac{x-\theta_x+\frac{1}{2}}{2\sigma^2}\right) - \frac{1}{2}\mathrm{erf}\left(\frac{x-\theta_x-\frac{1}{2}}{2\sigma^2}\right)$$

$$\Delta\mathrm{E}_y(x,y) \equiv \frac{1}{2}\mathrm{erf}\left(\frac{y-\theta_y+\frac{1}{2}}{2\sigma^2}\right) - \frac{1}{2}\mathrm{erf}\left(\frac{y-\theta_y-\frac{1}{2}}{2\sigma^2}\right)$$

Ignore:
vectorial effect, aberrations
dipole orientation, readout noise

Gaussian is OK for free rotating emitter

Parameters:

q_x: x-position
q_y: y-position
q_{I0}: emission rate
q_{bg}: background count rate
q_s: spot width

μ_k: expected count in pixel *k*

Probability and likelihood

Probability of observing pixel values $\{n_k|k = 1,...,N_{pix}\}$ given average rate $\{\mu_k|k = 1,...,N_{pix}\}$ is given by Poisson law:

$$P\left(\{n_k\}\middle|\{\mu_k\}\right) = P\left(n_1\middle|\mu_1\right)P\left(n_2\middle|\mu_2\right)...P\left(n_{N_{pix}}\middle|\mu_{N_{pix}}\right)$$

$$P\left(n_j\middle|\mu_j\right) = \frac{e^{-\mu_j}\mu_j{}^{n_j}}{n_j!}$$

The reverse is also true: *Likelihood* that $\{\mu_k|k = 1,...,N_{pix}\}$ corresponds to ground truth given the observed pixel values $\{n_k|k = 1,...,N_{pix}\}$ is:

$$L\left(\{\mu_k\}\middle|\{n_k\}\right) = L\left(\mu_1\middle|n_1\right)L\left(\mu_2\middle|n_2\right)...L\left(\mu_{N_{pix}}\middle|n_{N_{pix}}\right)$$

$$L\left(\mu_j\middle|n_j\right) = \frac{e^{-\mu_j}\mu_j{}^{n_j}}{n_j!}$$

MLE optimization problem statement

The unknown parameters are found for the maximum in the likelihood $L(\{\mu_k\}|\{n_k\})$ as a function of the unknown parameters $(q_1,q_2,q_3,q_4) = (x_0,y_0,\sigma,N)$.

Maximum of $L(\{\mu_k\}|\{n_k\})$ = maximum of log-likelihood:

$$\log\left(L\left(\{\mu_k\}|\{n_k\}\right)\right) = \sum_{k=1}^{N_{pix}} \log\left(L\left(\mu_k|n_k\right)\right)$$
$$= \sum_{k=1}^{N_{pix}} \left(n_k \log\left(\mu_k\right) - \mu_k - \log\left(n_k!\right)\right)$$

Then we must solve:

$$0 = \frac{\partial \log\left(L\left(\{\mu_k\}|\{n_k\}\right)\right)}{\partial \theta_j} = \sum_{k=1}^{N_{pix}} \left(\frac{n_k}{\mu_k} - 1\right) \frac{\partial \mu_k}{\partial \theta_j} \quad \text{for } j = 1,2,3,4$$

Finding the optimum

Using that:

$$\frac{\partial \log(L)}{\partial \theta_j} = \sum_{k=1}^{N_{pix}} n_k \frac{\partial \log(\mu_k)}{\partial \theta_j} - \frac{\partial \left(\sum_{k=1}^{N_{pix}} \mu_k \right)}{\partial \theta_j} \approx \sum_{k=1}^{N_{pix}} n_k \frac{\partial \log(\mu_k)}{\partial \theta_j} - \frac{\partial N}{\partial \theta_j}$$

$$\log(\mu_k) = -\frac{(x_k - x_0)^2 + (y_k - y_0)^2}{2\sigma^2} + \log(N) - 2\log(\sigma) - \log(2\pi)$$

we find:

$$N = \sum_{k=1}^{N_{pix}} n_k, \quad x_0 = \frac{1}{N} \sum_{k=1}^{N_{pix}} n_k x_k, \quad y_0 = \frac{1}{N} \sum_{k=1}^{N_{pix}} n_k y_k$$

$$\sigma^2 = \frac{1}{2N} \sum_{k=1}^{N_{pix}} n_k \left((x_k - x_0)^2 + (y_k - y_0)^2 \right)$$

Localization uncertainty

2nd derivative at optimum is measure for uncertainty:

$$\frac{\partial^2 \log(L)}{\partial x_0^{\,2}} = \sum_{k=1}^{N_{pix}} n_k \frac{\partial^2 \log(\mu_k)}{\partial x_0^{\,2}} = -\frac{N}{\sigma^2}$$

This gives an uncertainty in emitter location:

$$\Delta x_0 = \left[-\frac{\partial^2 \log(L)}{\partial x_0^{\,2}} \right]^{-1/2} = \frac{\sigma}{\sqrt{N}}$$

Likelihood and CRlB

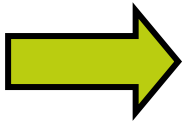
$$L(\vec{x}|\theta) = \prod_k \frac{\mu_k(x,y)^{x_k} e^{-\mu_k(x,y)}}{x_k!}$$ Poisson process

Cramer-Rao lower bound & Fisher information matrix

$$var(\hat{\theta}) \geq I(\theta)^{-1}$$

$$I_{ij}(\theta) = E\left[\frac{\partial \ln(L(\vec{x}|\theta))}{\partial \theta_i} \frac{\partial \ln(L(\vec{x}|\theta))}{\partial \theta_j}\right]$$

$$I_{ij}(\theta) = \sum_k \frac{1}{\mu_k(x,y)} \frac{\partial \mu_k(x,y)}{\partial \theta_i} \frac{\partial \mu_k(x,y)}{\partial \theta_j}$$



Use CRlb to estimate localization uncertainty

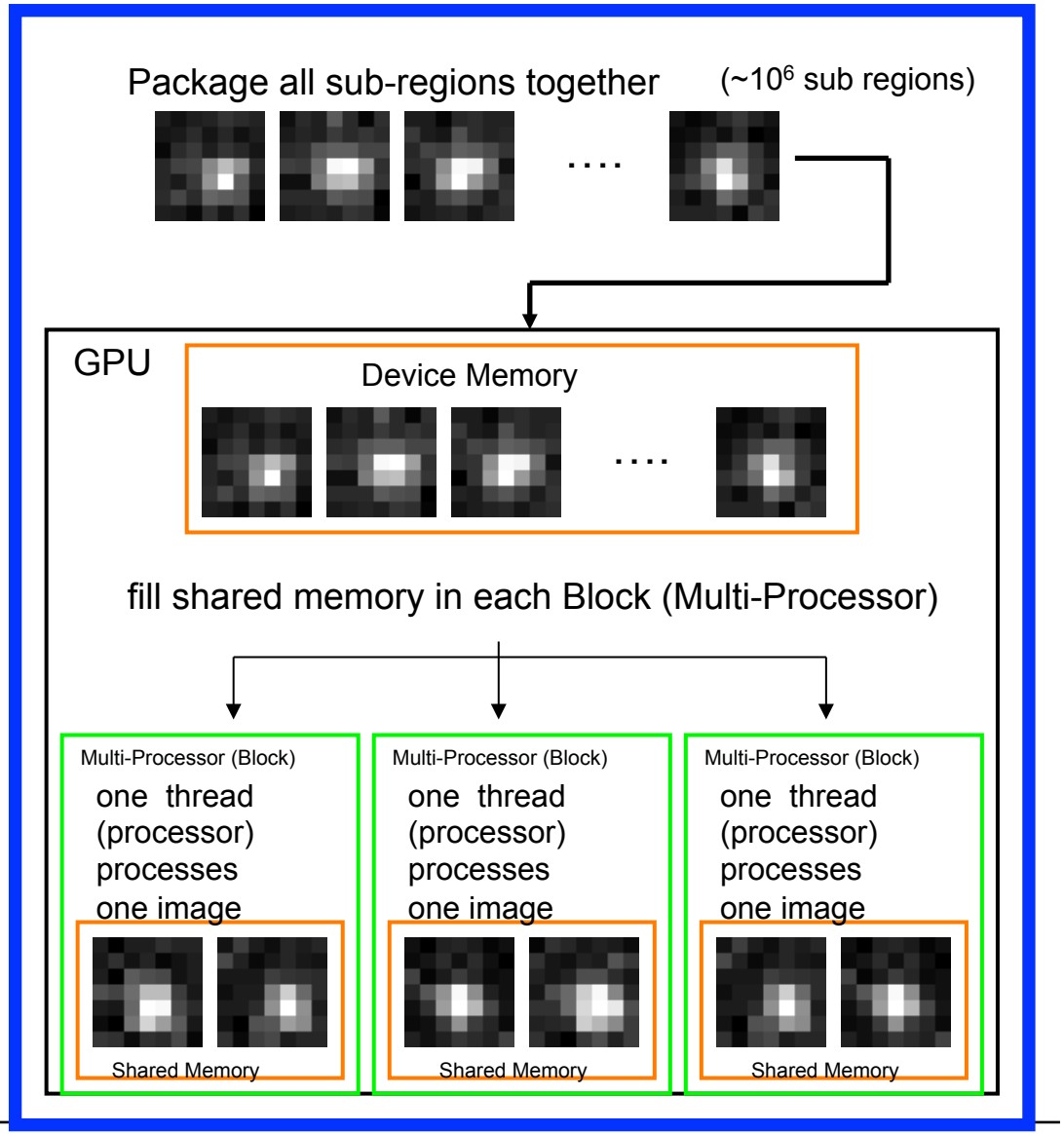
(CRLB: Minimum variance of an estimated parameter)

Parallel processing on Graphics Card

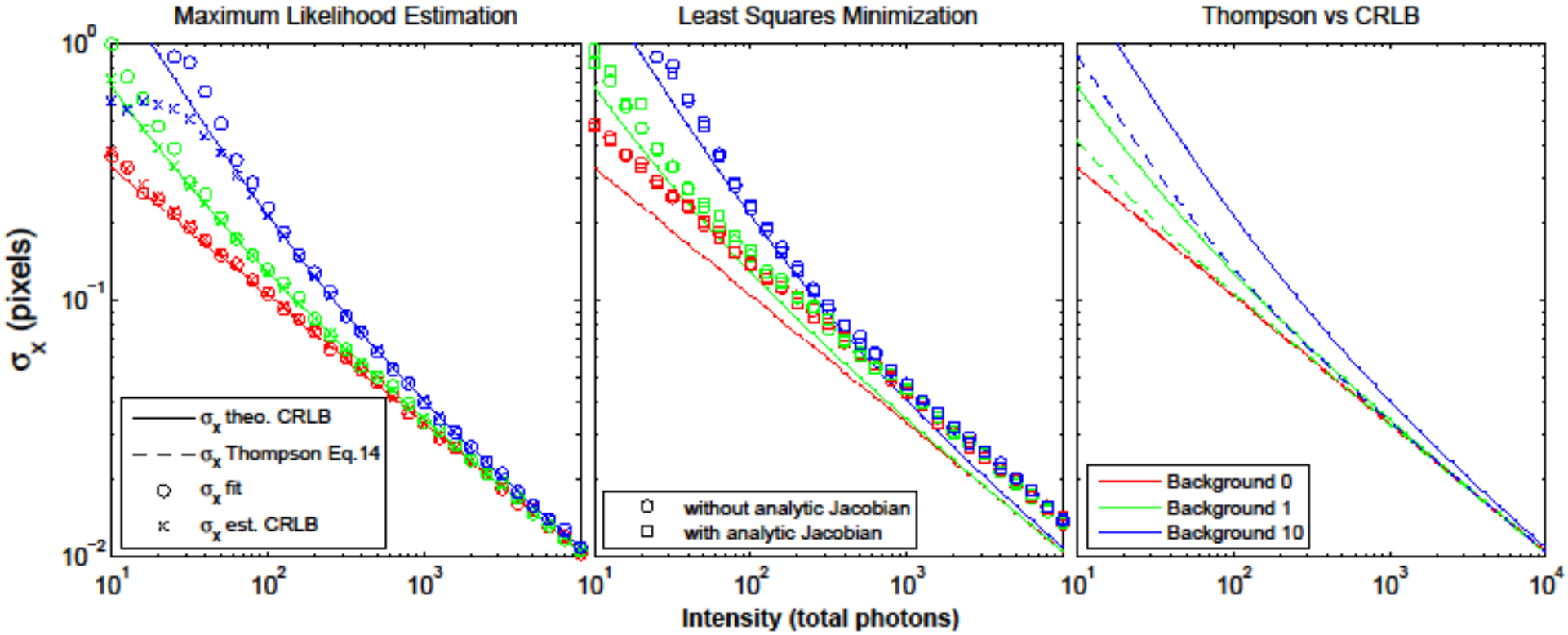
fixed number of iterations
fits GPU's single instruction multiple data strategy (SIMD)

>100 processors on one GPU, price 100-1000€

Size of sub-regions fixed on
$2\sigma_{PSF}$ +1 pixel



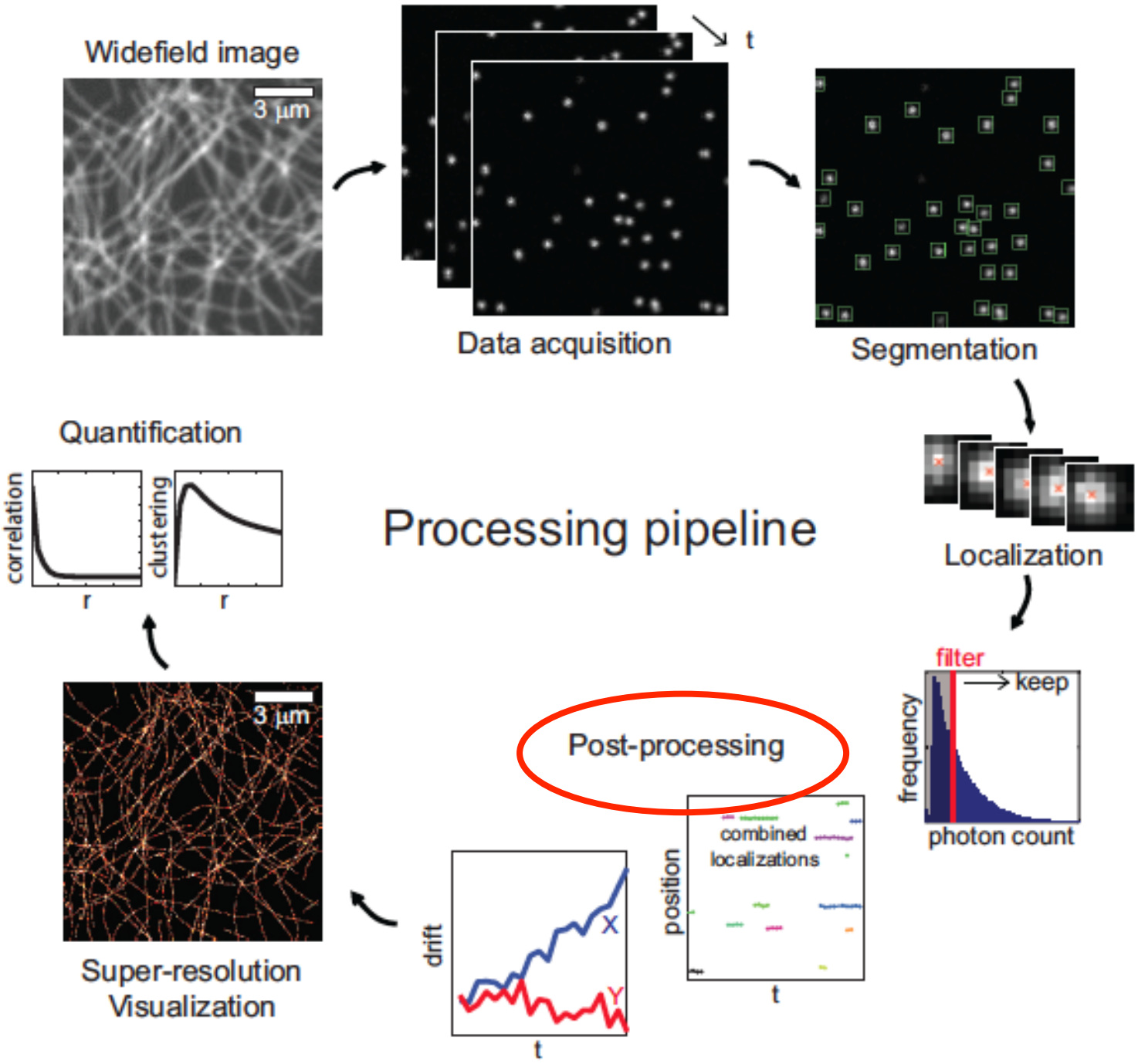
Performance on Simulations



$$\left\langle \left(\Delta x^2\right)\right\rangle = \frac{\sigma_{PSF}^2 + ps^2/12}{N} + \frac{4\sqrt{\pi}\sigma_{PSF}^3\sigma_{bg}^2}{psN^2}$$

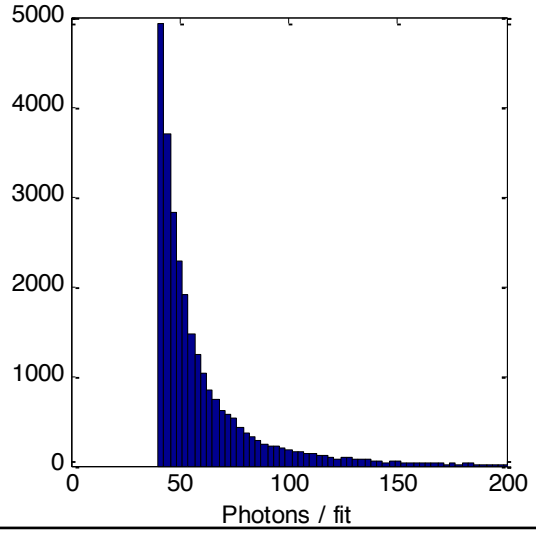
Too optimistic for low counts and any background signal

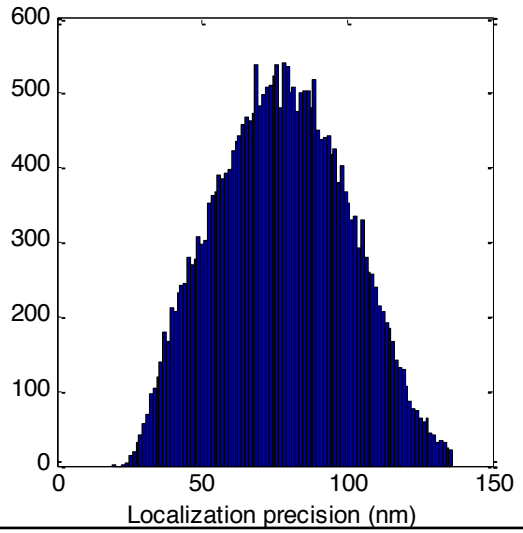
RE Thompson, DR Larson & WW Webb, *Biophysical Journal* 82, 2002

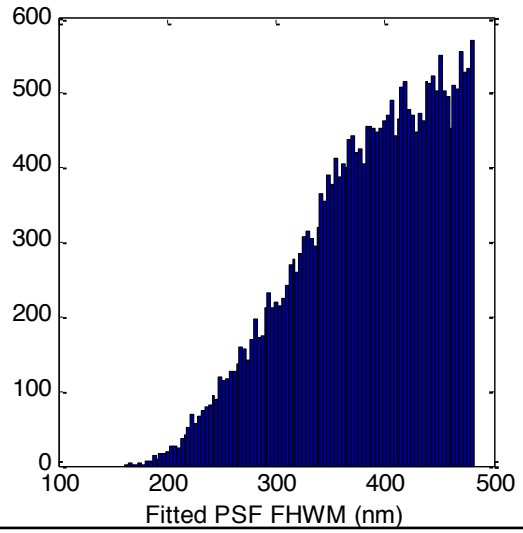

Widefield image
3 µm
t
Data acquisition
Segmentation
Localization
filter
keep
frequency
photon count
Post-processing
combined
localizations
position
t
drift
X
Y
t
3 µm
Super-resolution
Visualization
Quantification
correlation
r
clustering
r
Processing pipeline

Post-processing of localizations (1)

- Filter the localizations based on the **fitted parameters**
  e.g. photon count, precision, width of the PSF

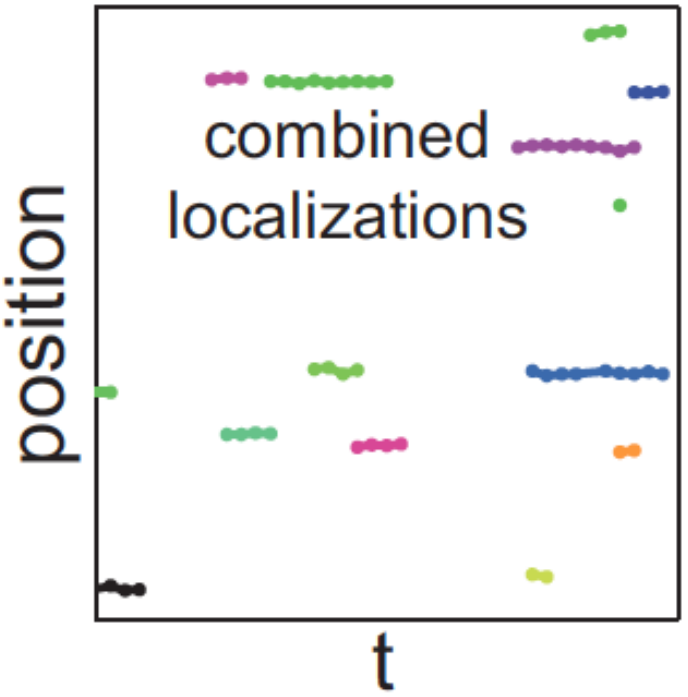






Post-processing of localizations (2)

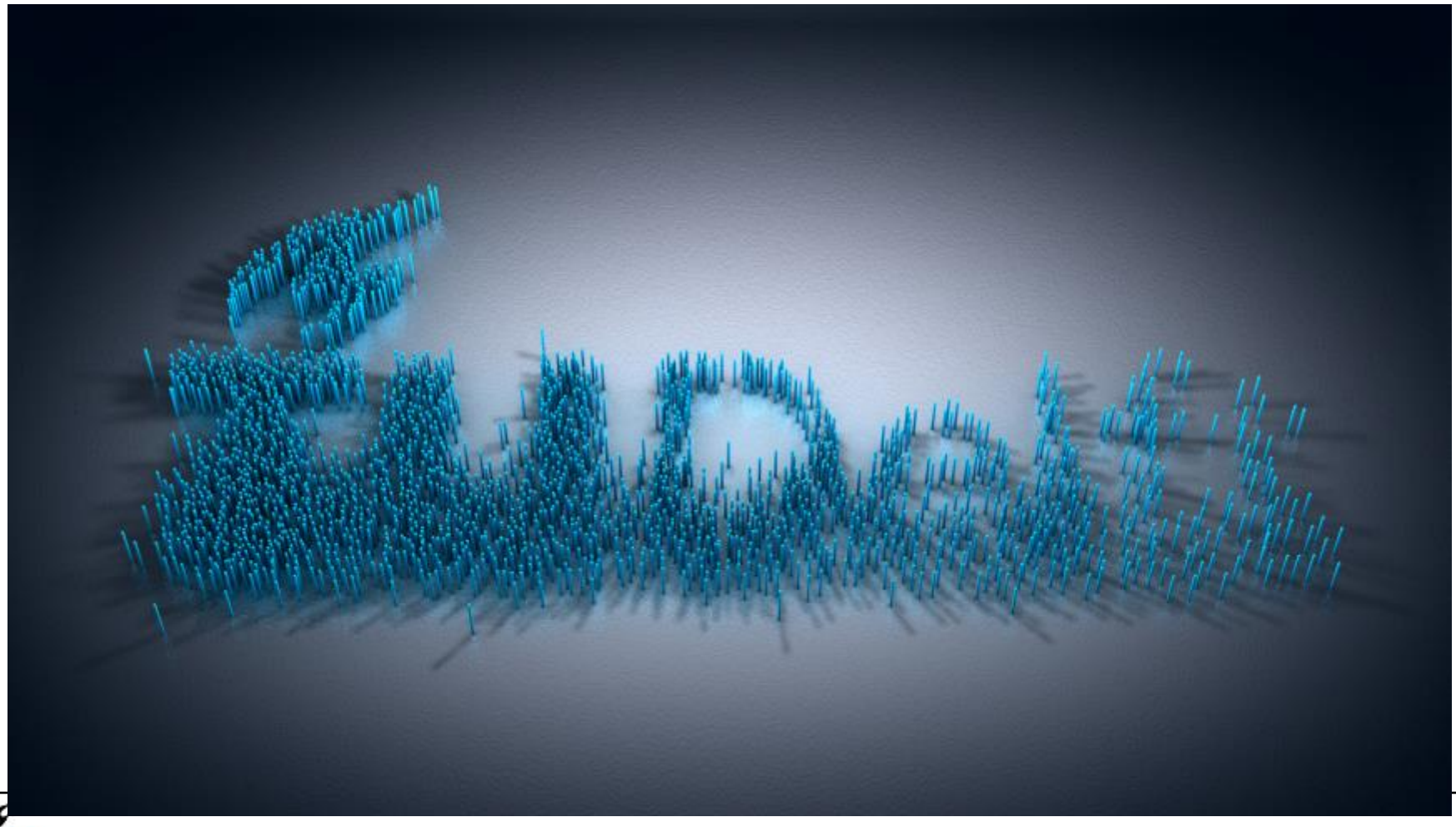
- "Frame connection": Combining spatially close localization from consecutive frames.
- Higher photon count for the individual emitter leads to higher effective localization precision.
- Danger of merging two different emitters into one effective localization.

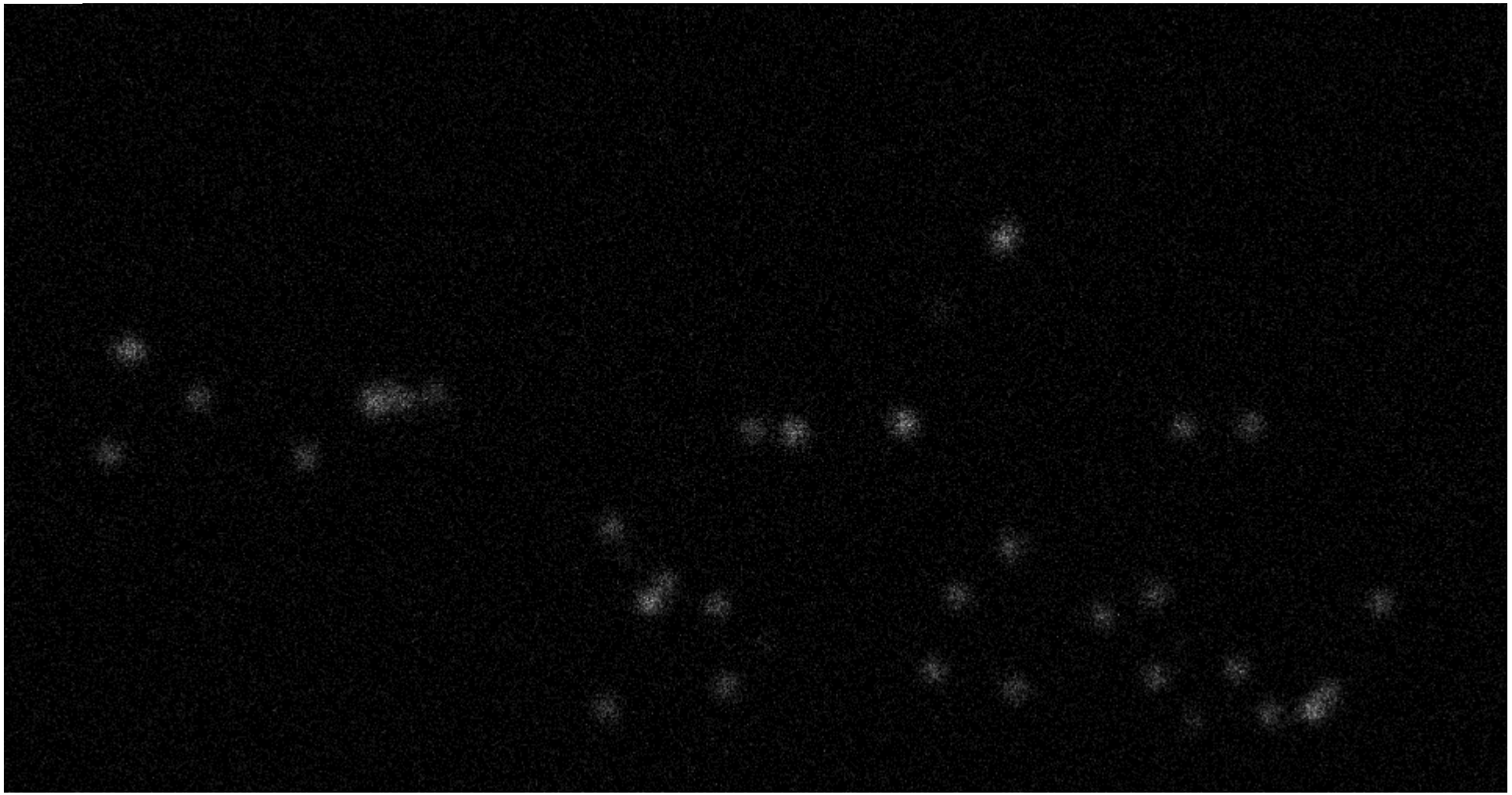


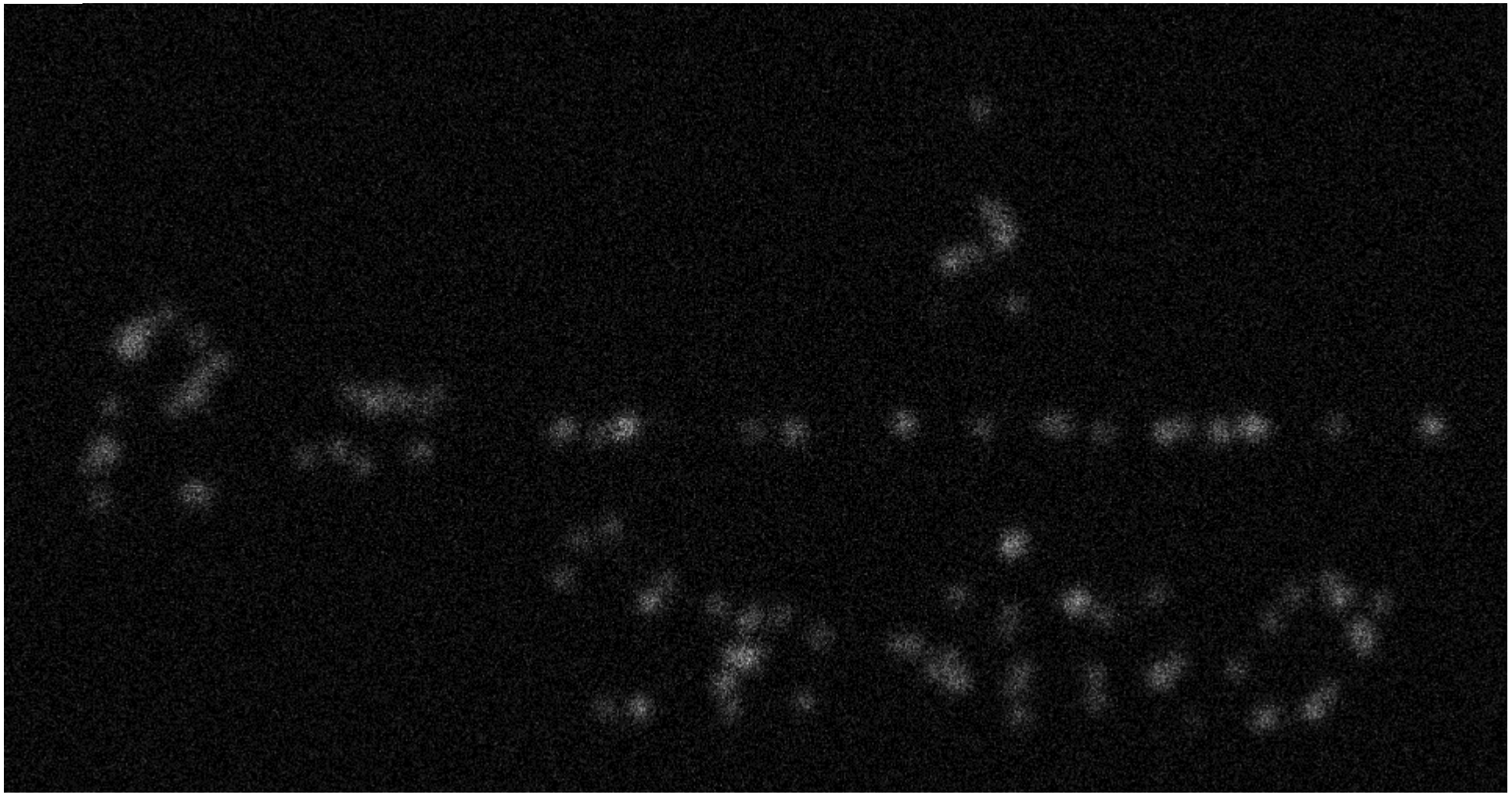
Post-processing of localizations (3)

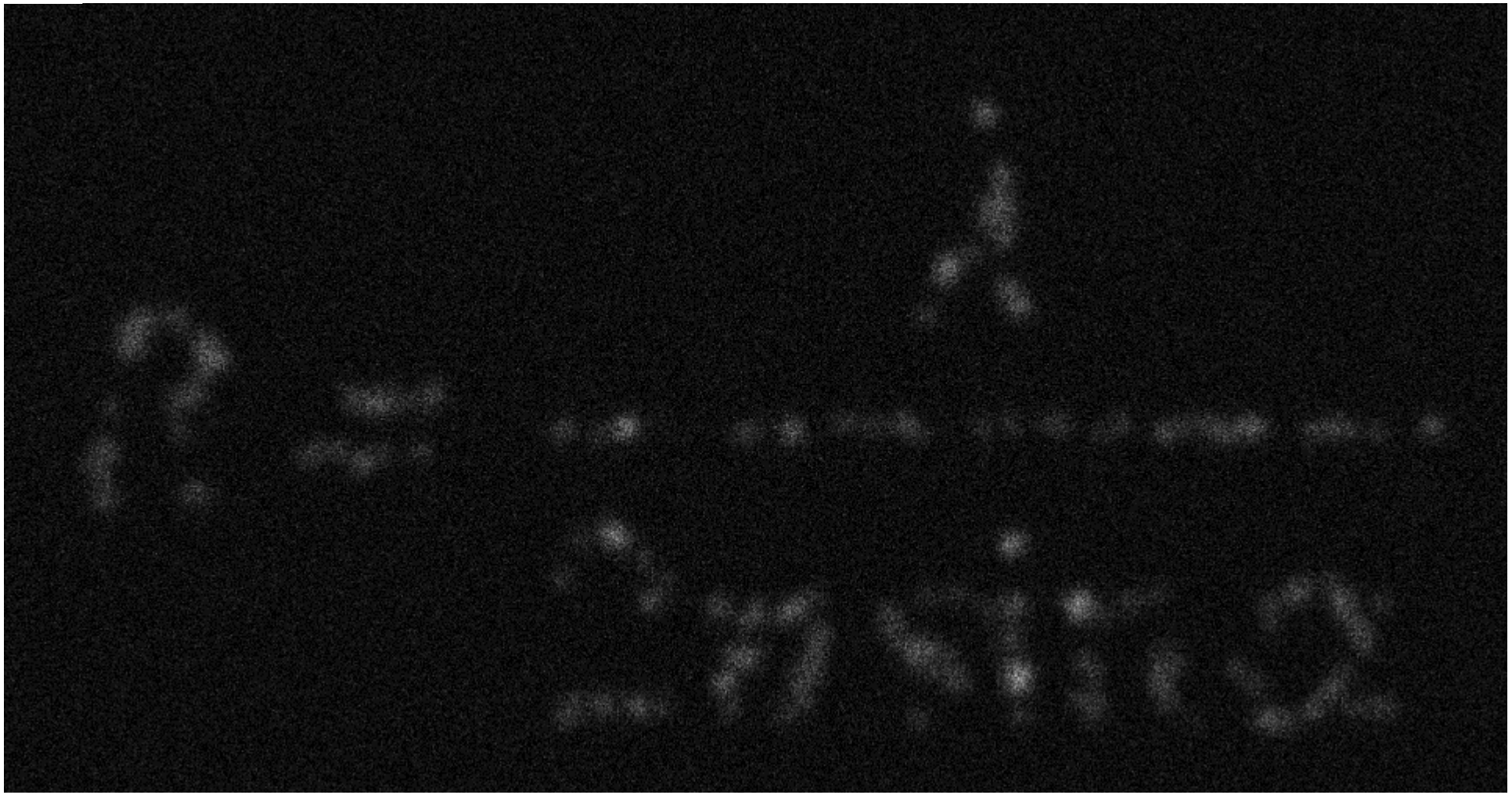
- Drift correction
- Imaging times are typically 5-30 minutes! Drift on the order of the localization precision ~ 10 nm must be avoided!
- 1) Use reference beads for tracking
- 2) Use localizations for cross-correlation based drift correction
- M.J. Mlodzianoski et al. Sample drift correction in 3d fluorescence photoactivation localization microscopy. *Optics Express*, 19(16): 15009-15019, 2011.
- C. Geisler et al., Drift estimation for single marker switching based imaging schemes. *Optics Express*, 20(7):7274-7289, 2012.

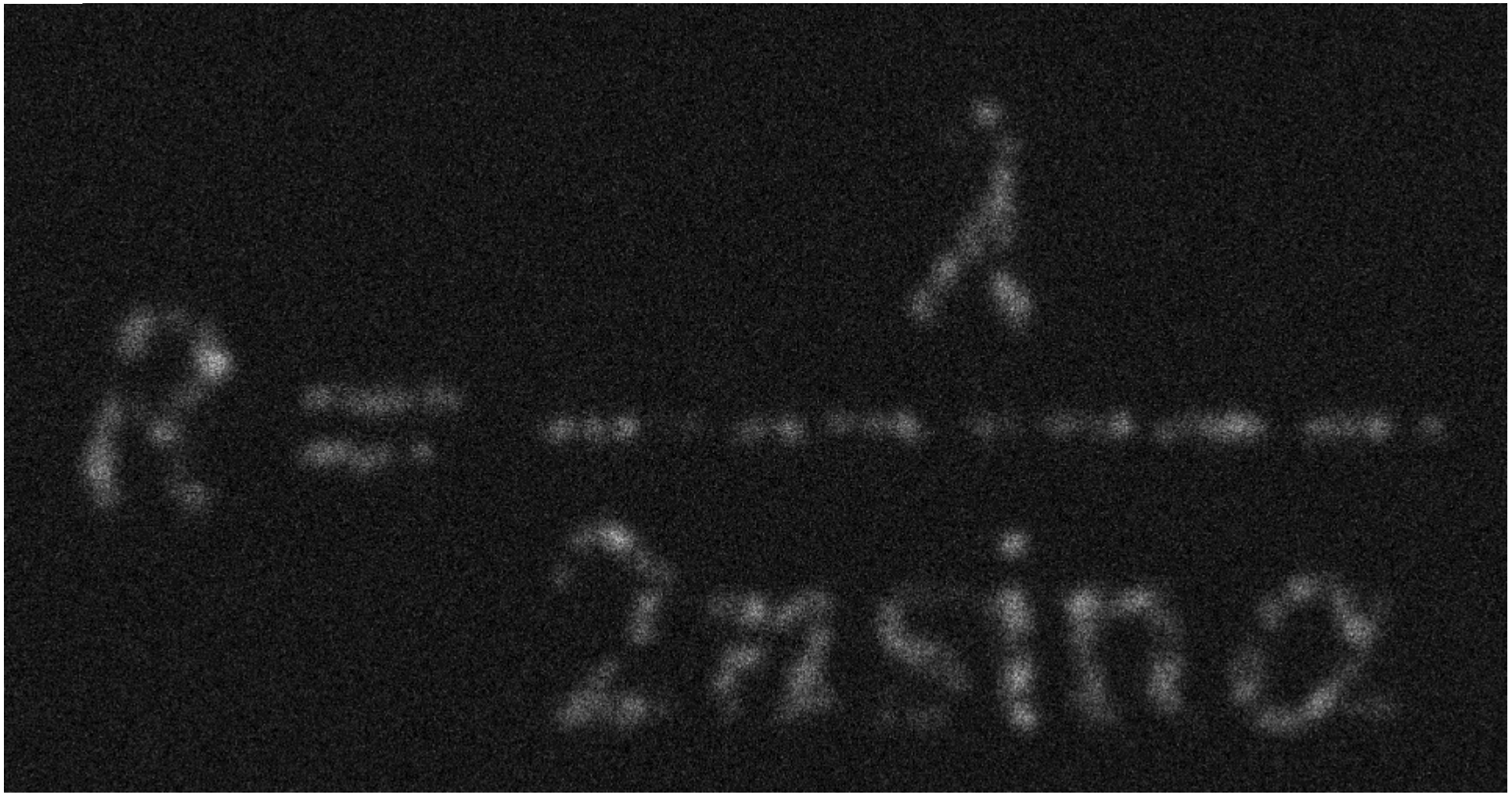
Resolution in localization microscopy











Resolution criteria in super-resolution

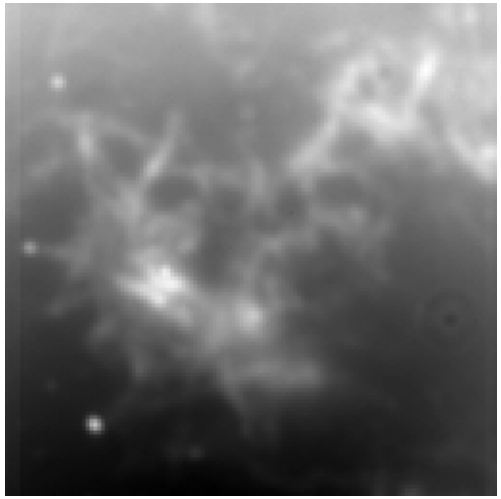
Currently used measures for localization microscopy

- Nyquist sampling density: $2/\sqrt{\rho}$
- Localization precision: σ

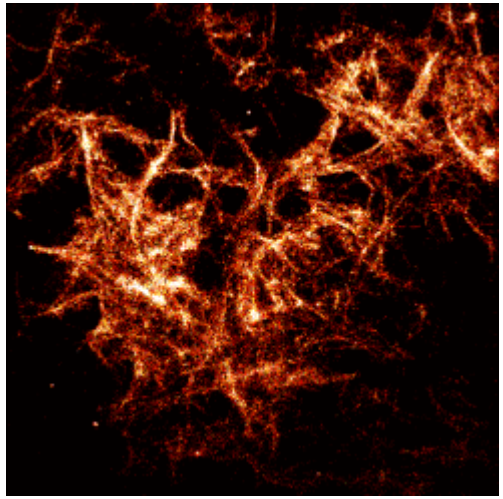
Fourier Ring Correlation (FRC) resolution

Qualitative validity in experiments

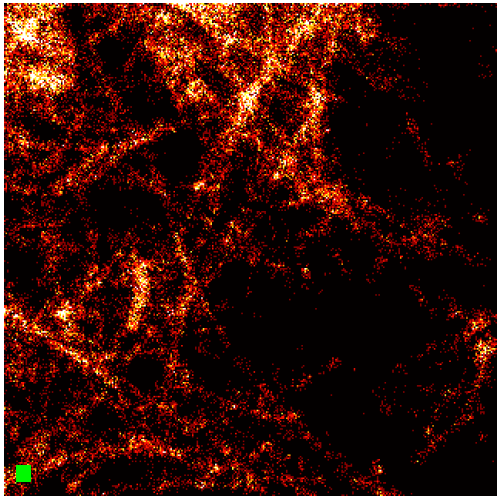
- Actin filaments, Alexa647 coupled to Phalloidin



Widefield



Binned localizations



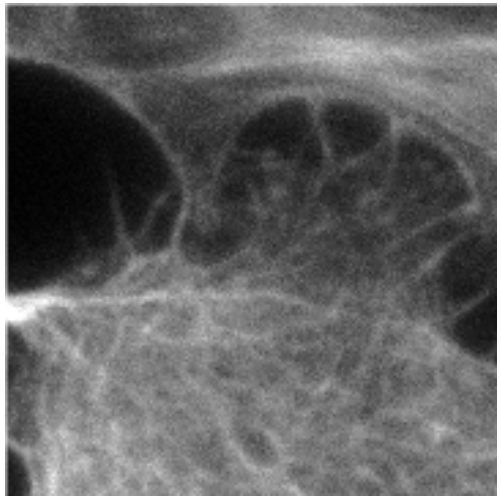
FRC resolution = 100 nm

FWHM loc. unc. ~ 38 nm

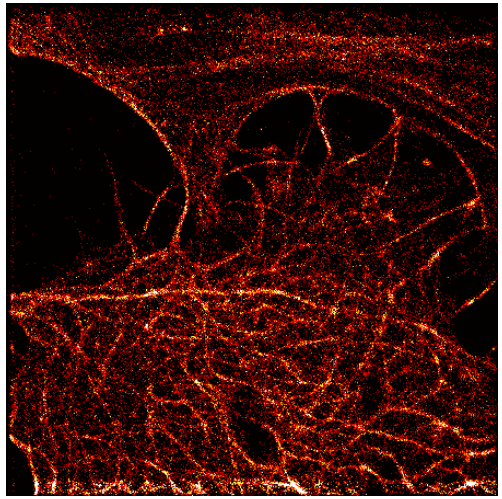
Fourier Ring Correlation (FRC) resolution

Qualitative validity in experiments

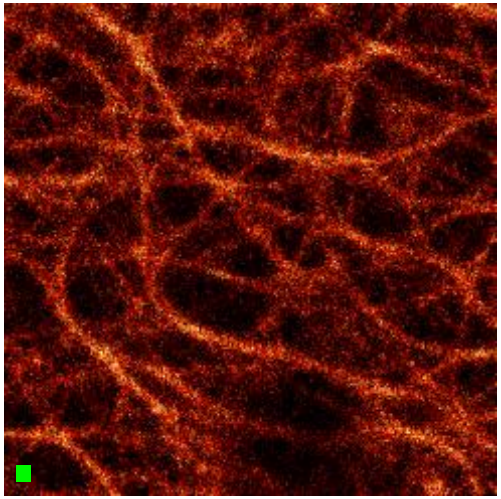
- Actin filaments, Venus4 label



Widefield



Binned localizations

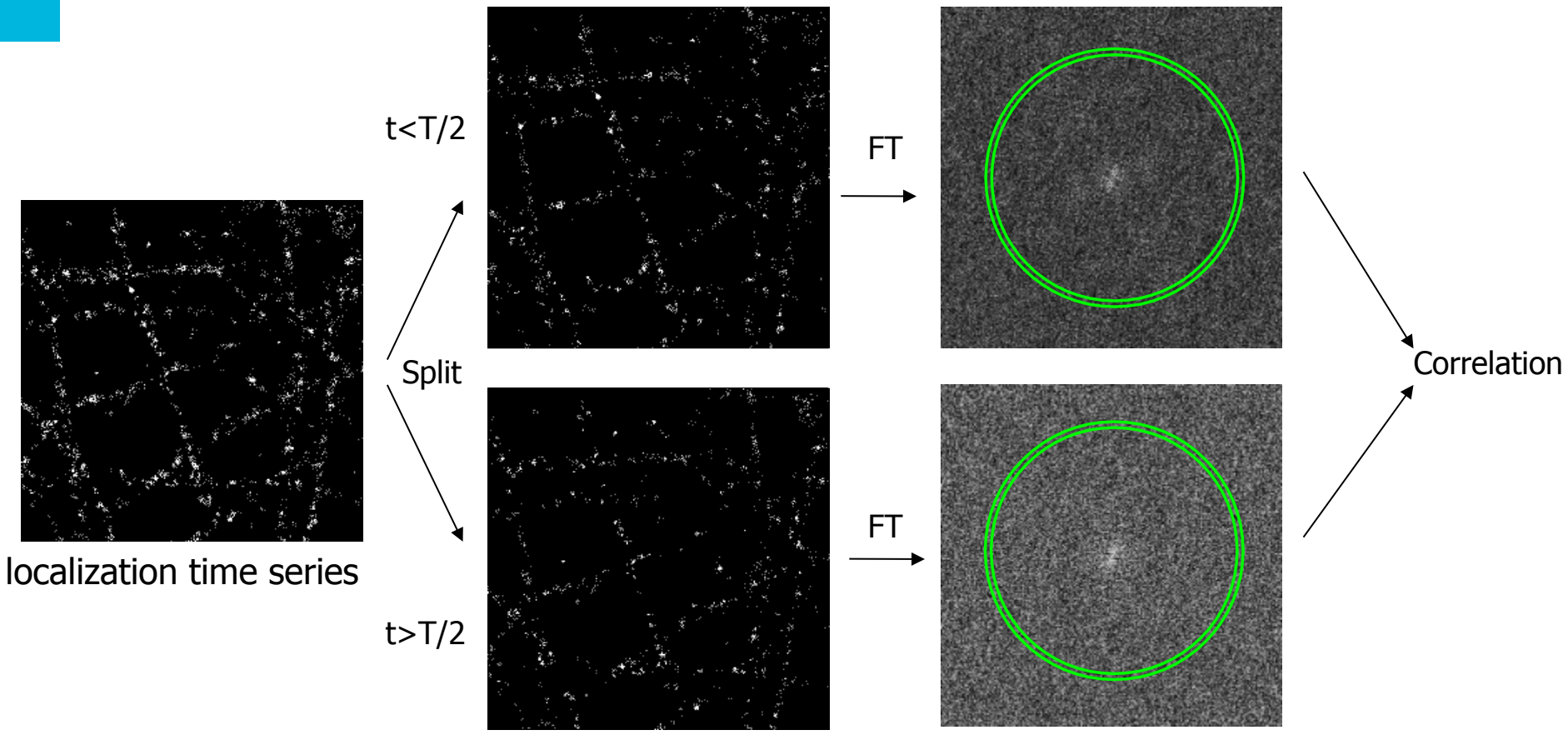


FRC resolution = 130 nm

FWHM loc. unc. ~ 63 nm

Data courtesy of Kees Jalink & Daniela Leyton Puig

Fourier Ring Correlation

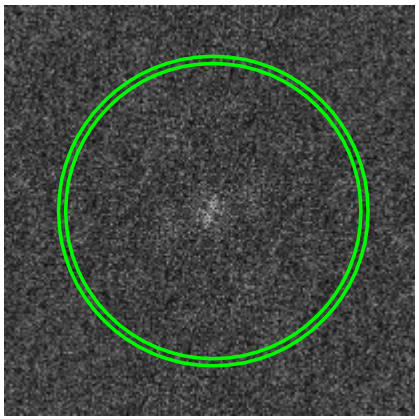


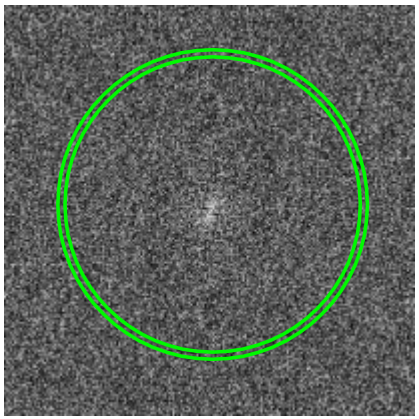
localization time series

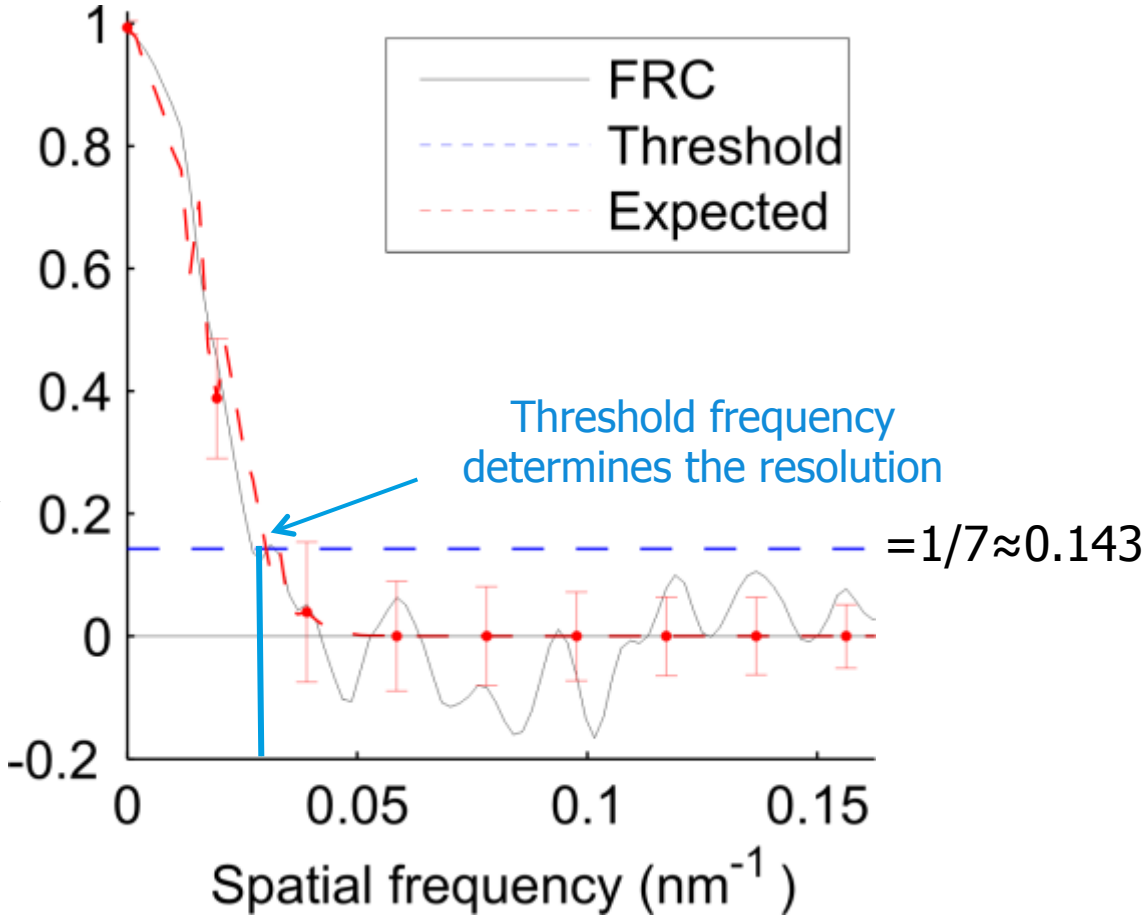
different options for actual splitting of the time series

FRC resolution

$$FRC = \frac{\sum_{ring} \hat{f}_1(\vec{q})\hat{f}_2(\vec{q})^*}{\sqrt{\sum_{ring}\left|\hat{f}_1(\vec{q})\right|^2}\sqrt{\sum_{ring}\left|\hat{f}_2(\vec{q})\right|^2}}$$

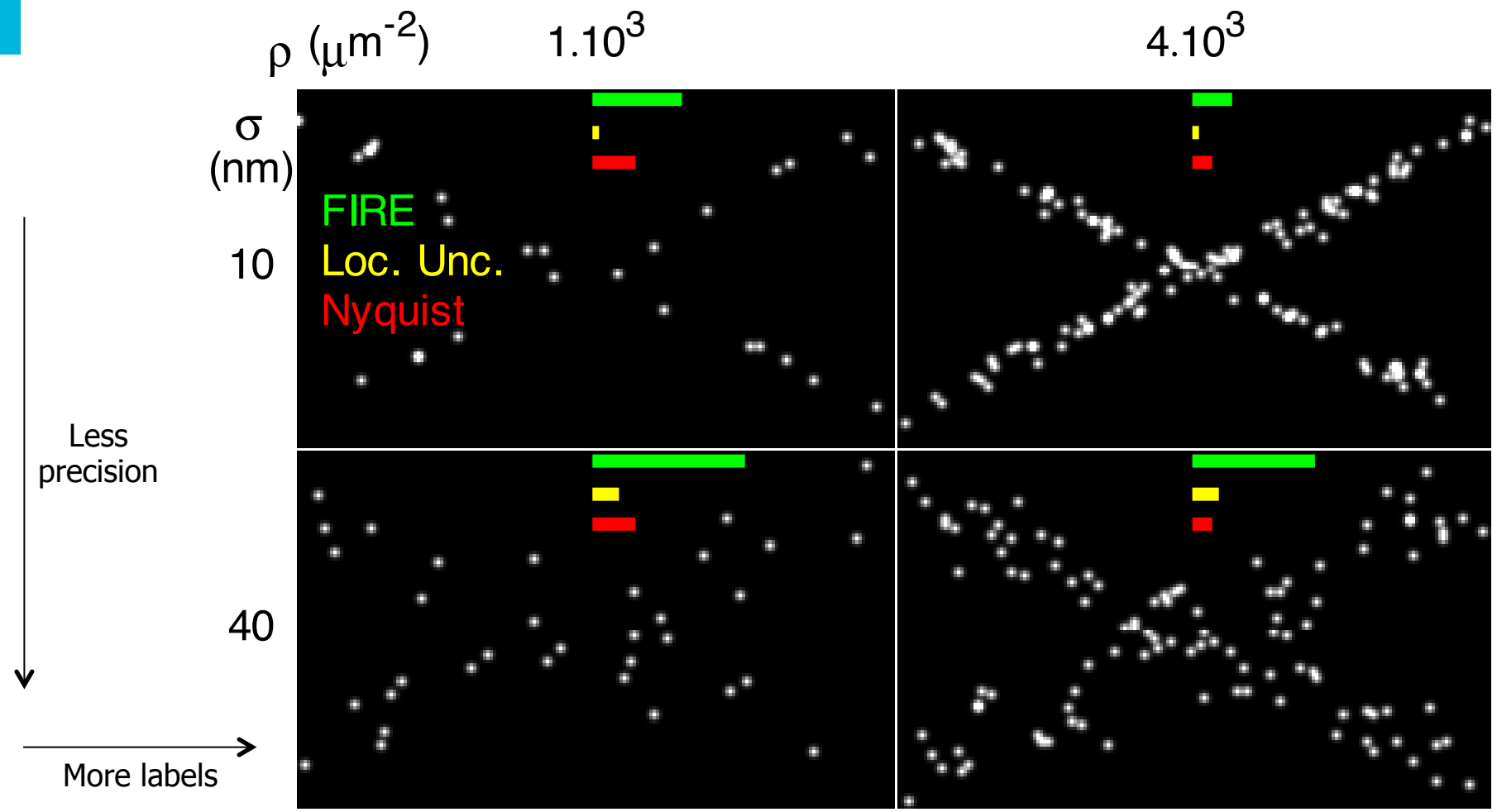






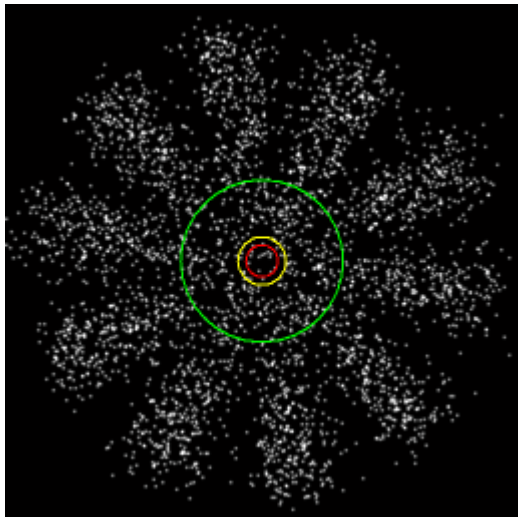
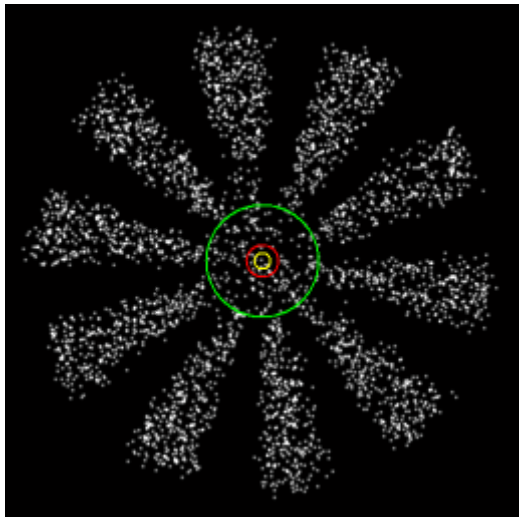
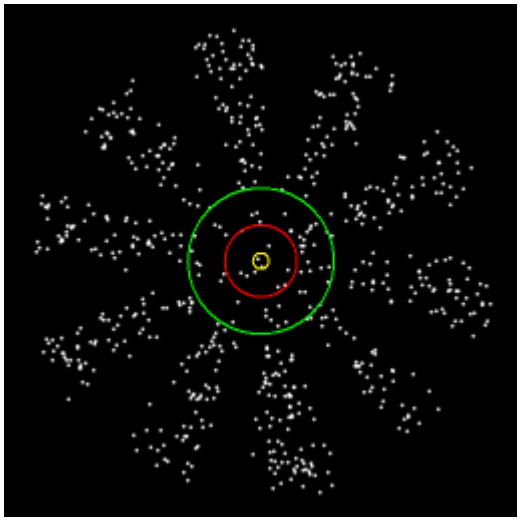
Saxton, Baumeister, *J. Microscopy* (1982);
van Heel, *Ultramicroscopy* (1987); Unser, Trus, Steven, *Ultramicroscopy* (1987)

Qualitative validity in simulations



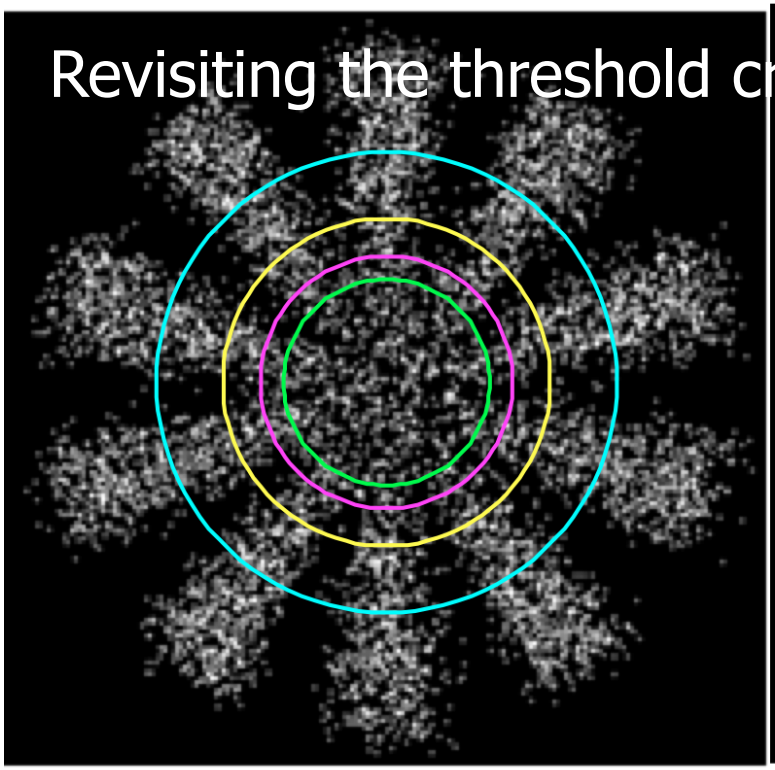
Qualitative validity in simulations

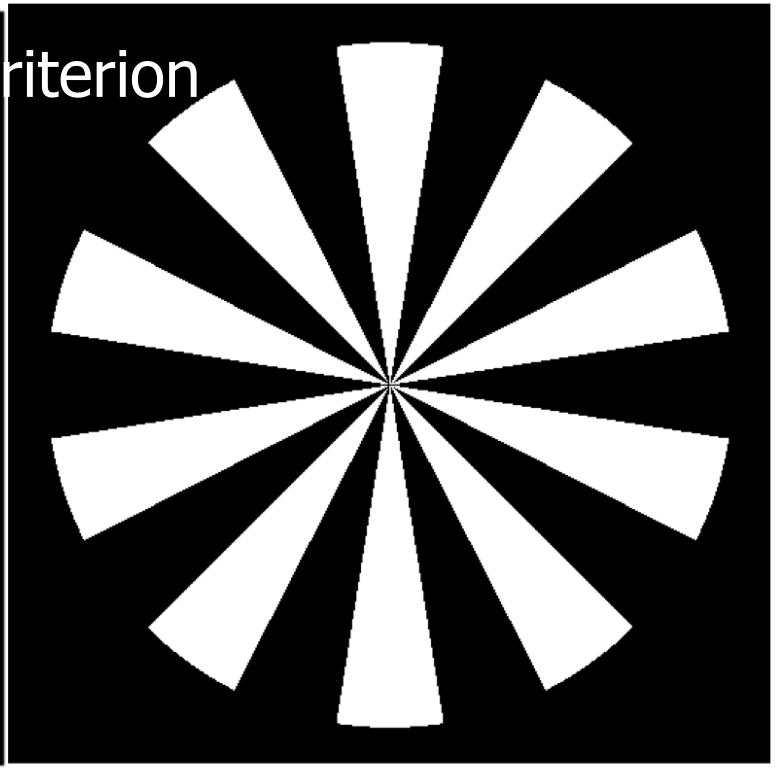
- The green circles show where the distance between the arms is equal to FIRE

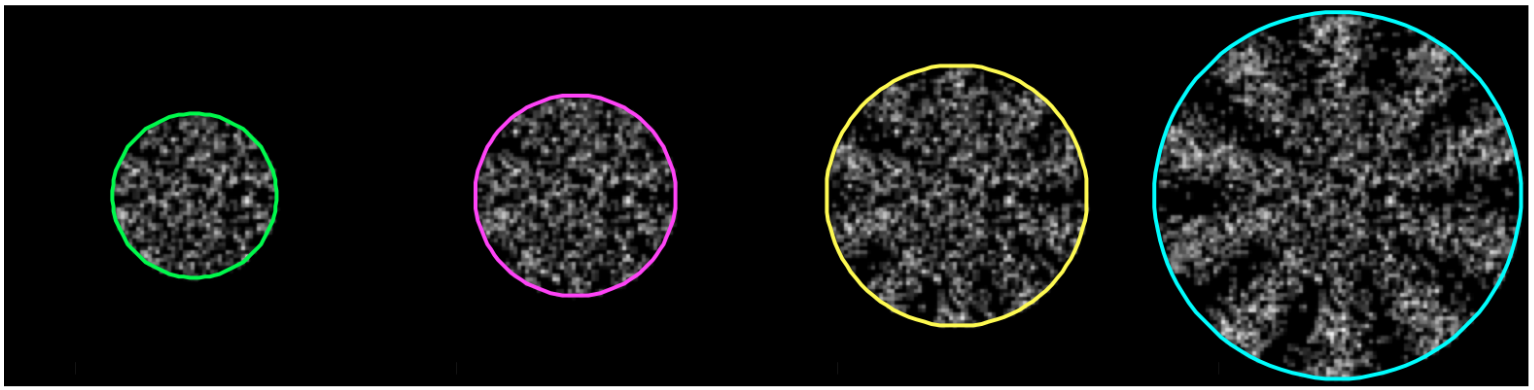




ρ (μm^{-2})	$1.0 \cdot 10^4$	$1.0 \cdot 10^4$	$2.0 \cdot 10^3$
σ (nm)	30	10	10
FIRE (nm)	104	62	91

Revisiting the threshold criterion



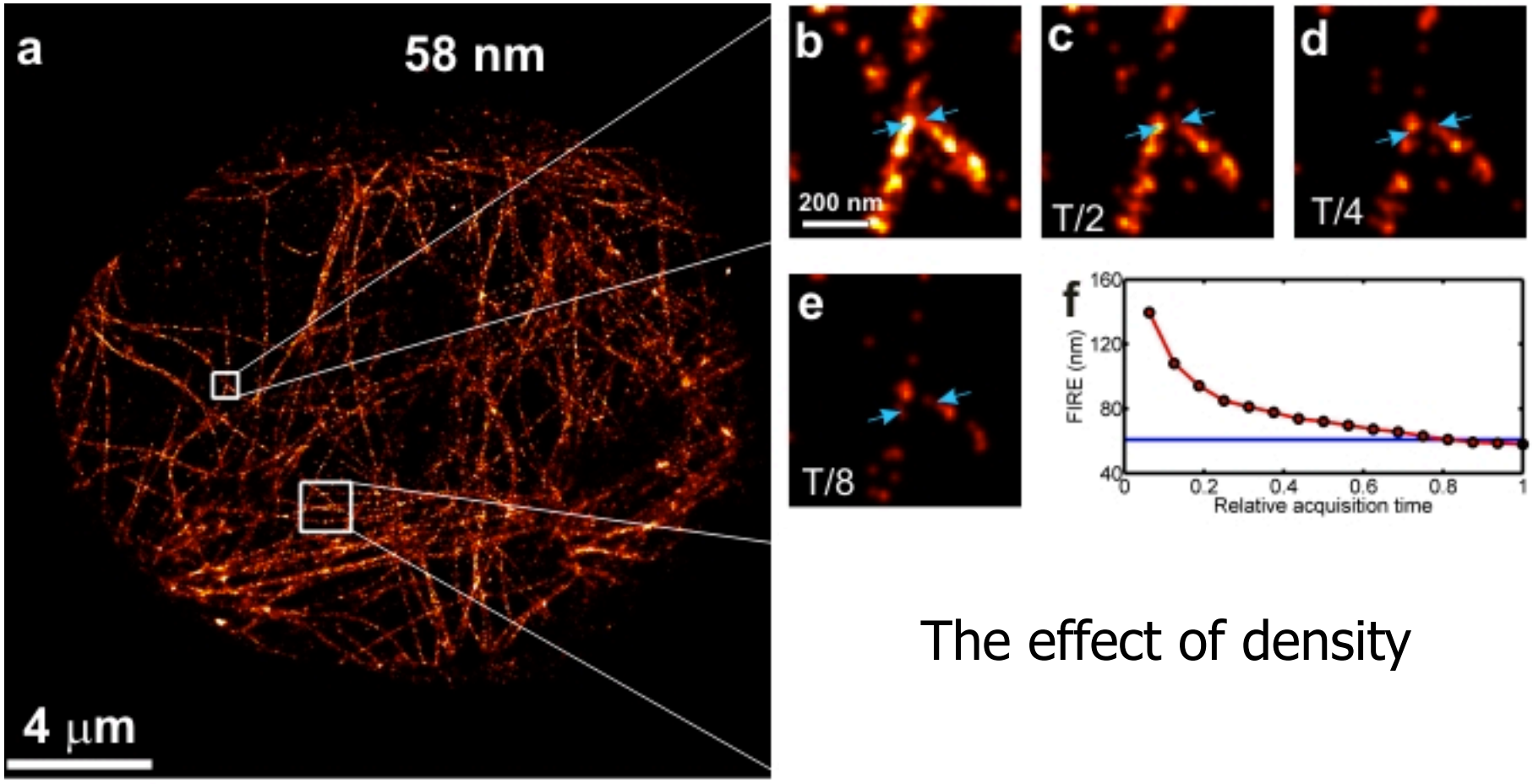




1/7 ½ bit ½ 3sigma

Resolution as a function of acquired frames

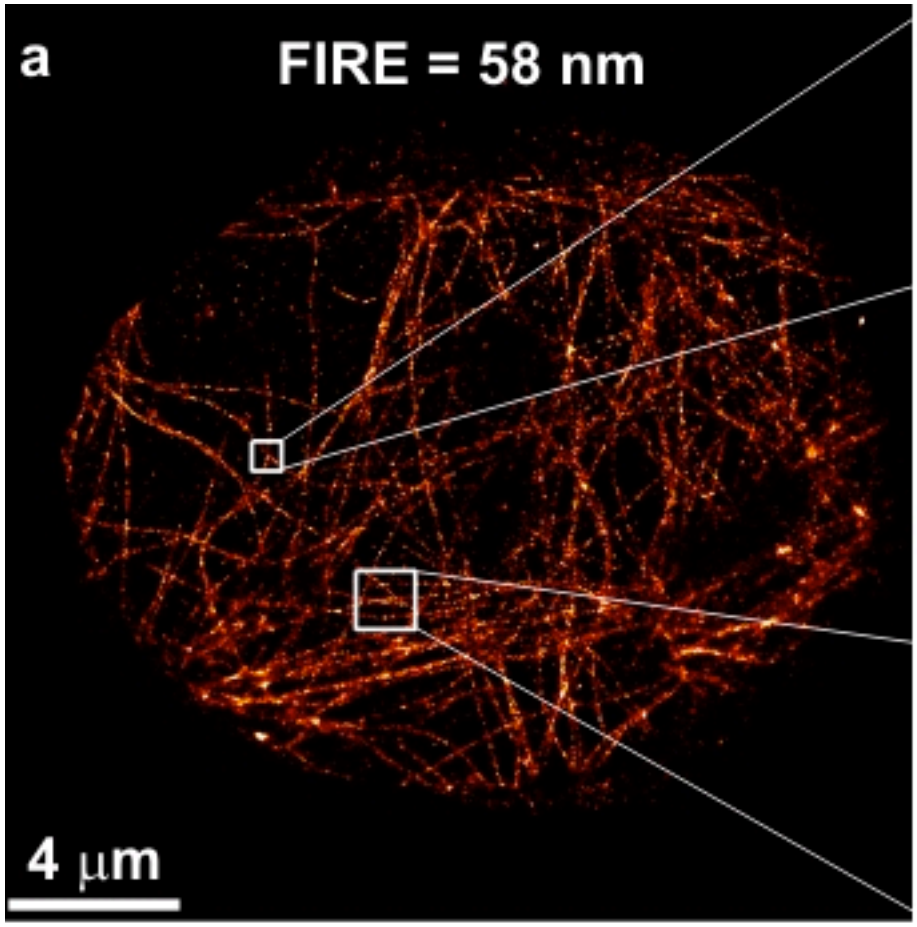
Acquisition of tubulin in HeLa cells with Alexa 647



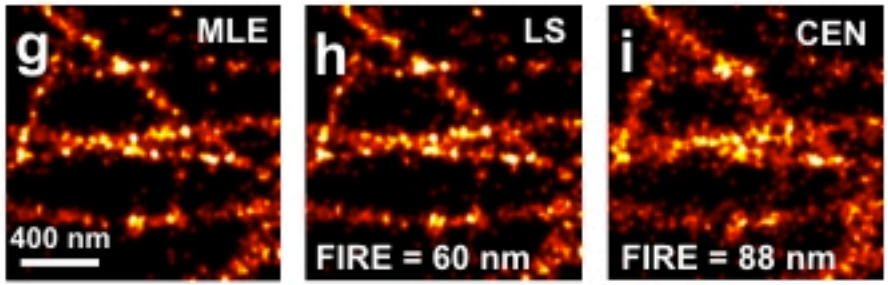
The effect of density

Effect of fitting algorithms

dSTORM acquisition of tubulin in HeLa cells with Alexa 647

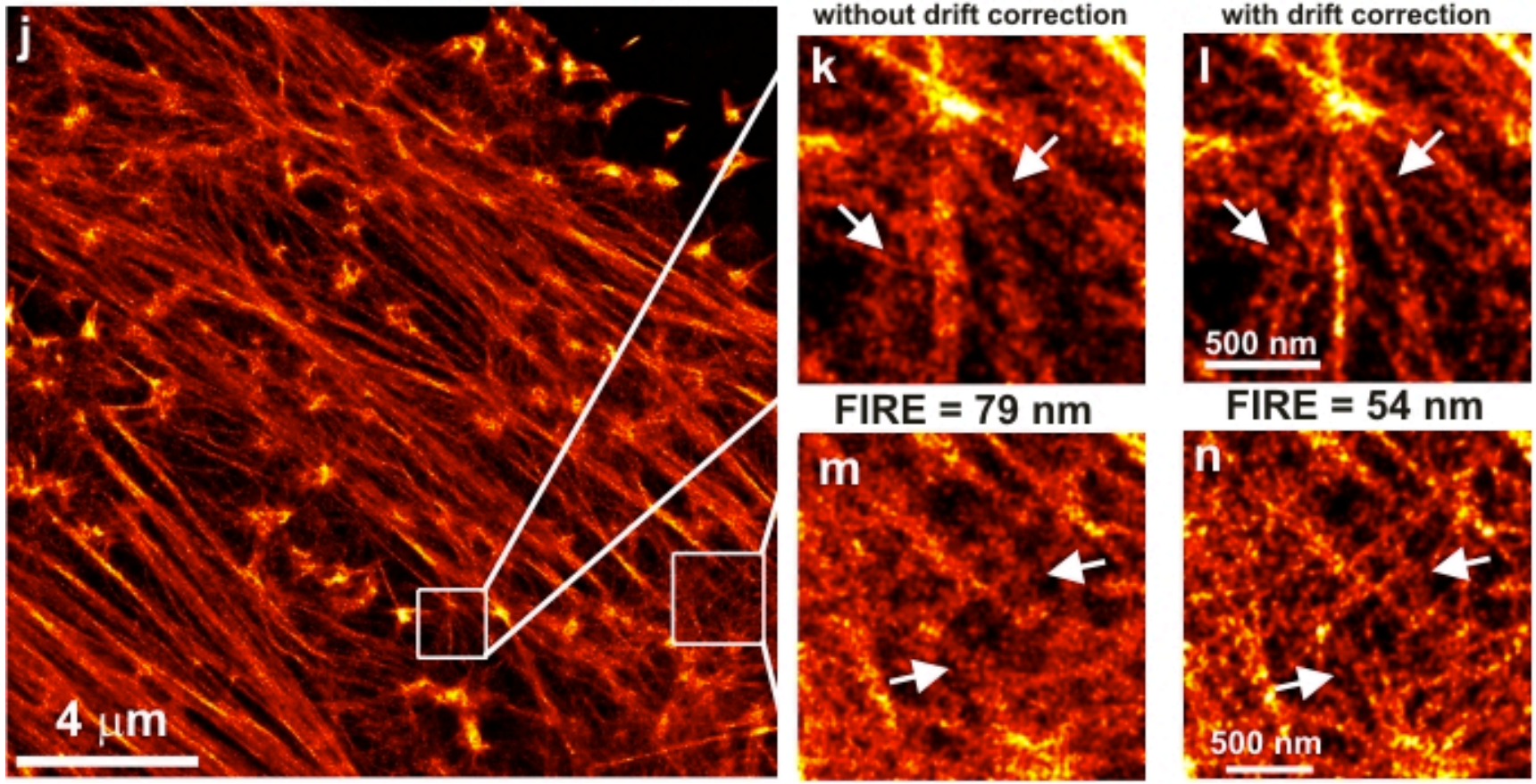


- Maximum Likelihood
- Least Squares
- Centroid



Effect of stage drift

GSDIM acquisition of actin filaments; HeLa cells
(Alex647 coupled to Phalloidin)



Data courtesy of Daniel Leyton Puig & Kees Jalink

Drift corrected ~ 70 nm

Resolution prediction

- Expected value of the correlation curve:

$$\left\langle FRC(q)\right\rangle = \frac{\sum_{\vec{q}\in\text{circle}} \left(Q + N\left|\hat{\psi}(\vec{q})\right|^2\right)\exp\left(-4\pi^2\sigma^2 q^2\right)}{\sum_{\vec{q}\in\text{circle}}\left[2 + \left(Q + N\left|\hat{\psi}(\vec{q})\right|^2\right)\exp\left(-4\pi^2\sigma^2 q^2\right)\right]}$$

Number of localizations

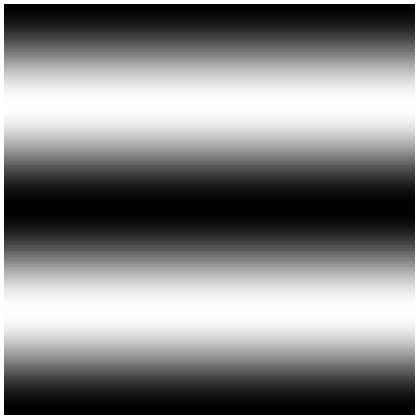
Object shape

Localization precision

Average localizations per molecule;
Q=0, each emitter is only seen once

Resolution prediction

- Sample:

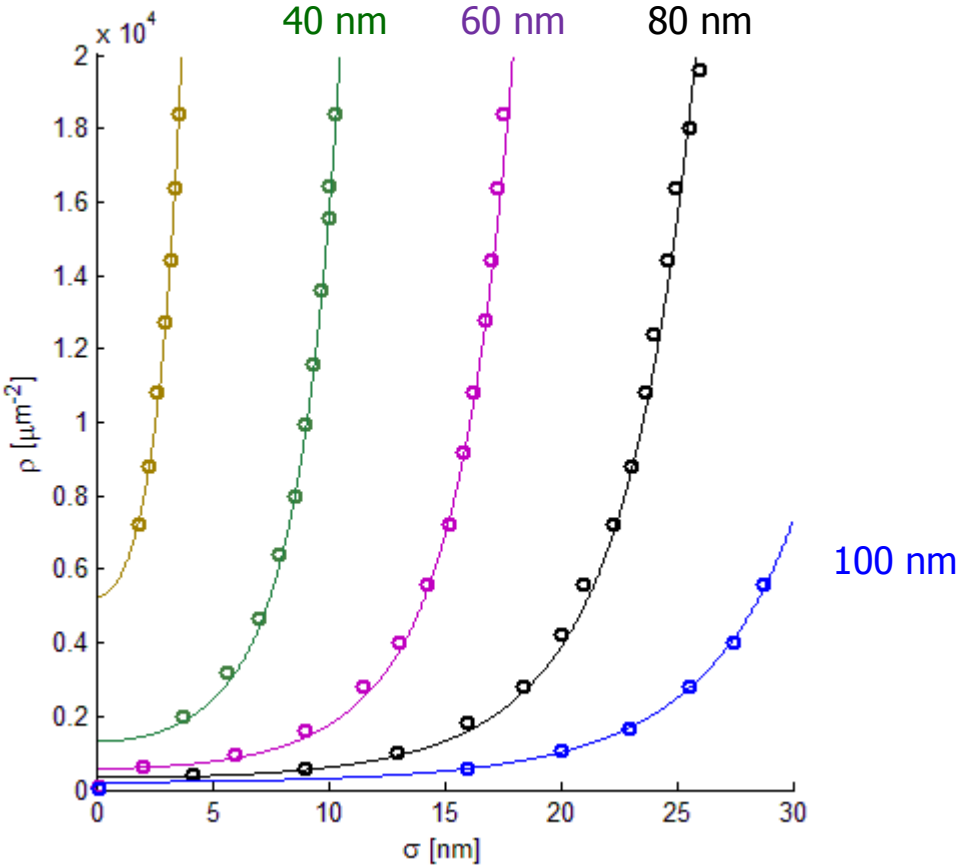


- Expected resolution for 2 sinusoidal lines:

$$\langle FRCresolution \rangle \approx \frac{2\pi\sigma}{\sqrt{W\left(6\pi\rho\sigma^2\right)}}$$

W(x) Lambert W-function

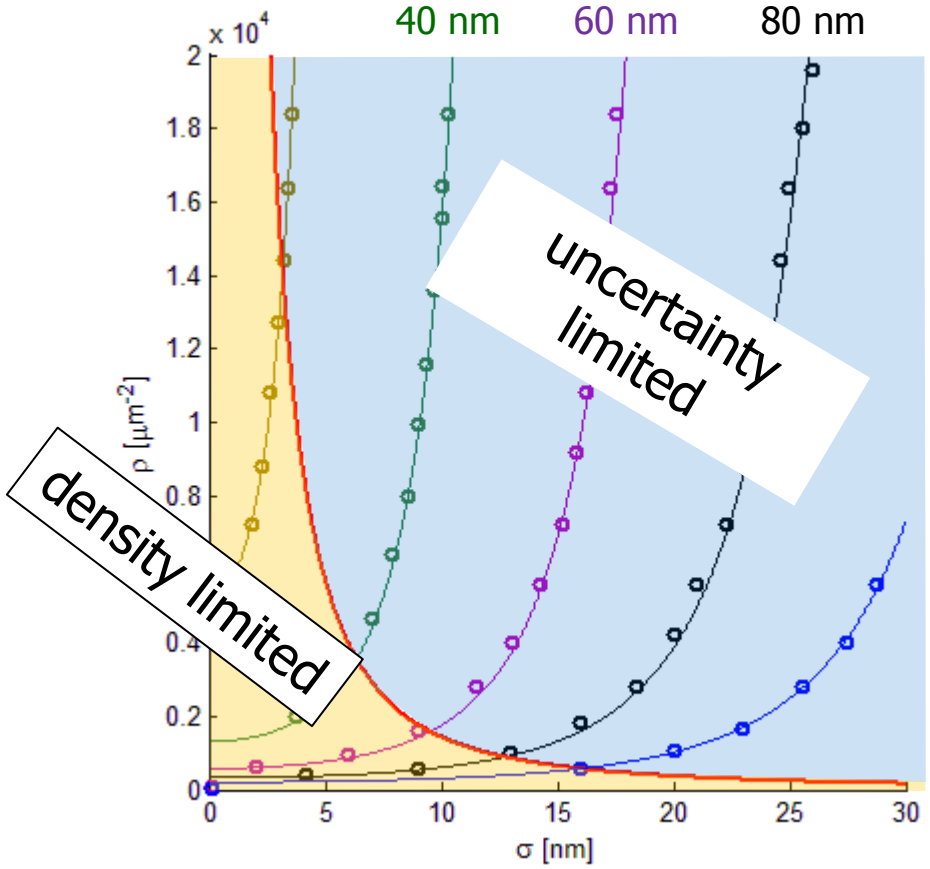
Density or precision limited?



o simulation
- theory

plot lines of constant FRC resolution

Density or precision limited?



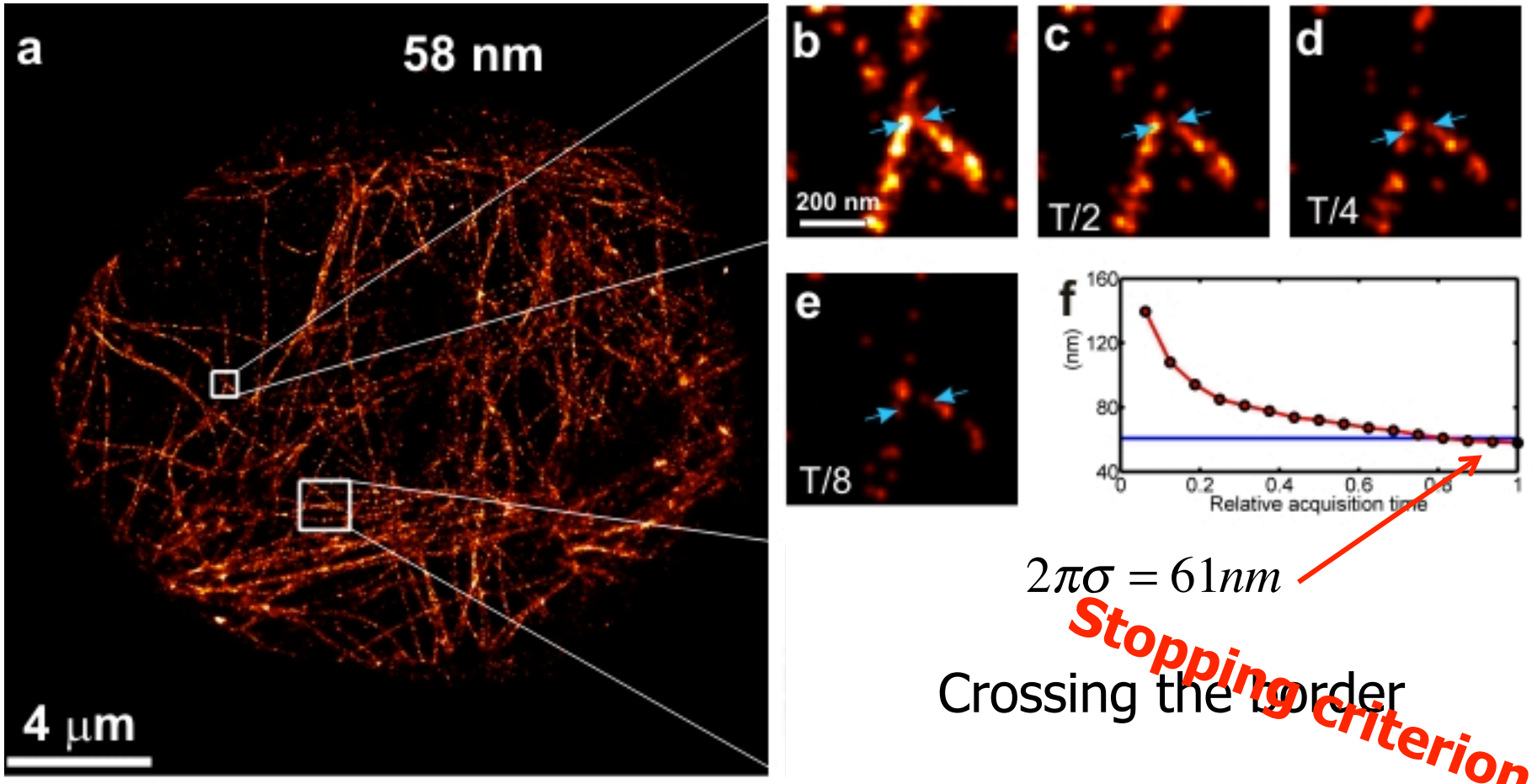
o simulation
- theory

image resolution
= 100 nm

$$FRCresolution = 2\pi\sigma$$

Density or precision limited?

Acquisition of tubulin in HeLa cells

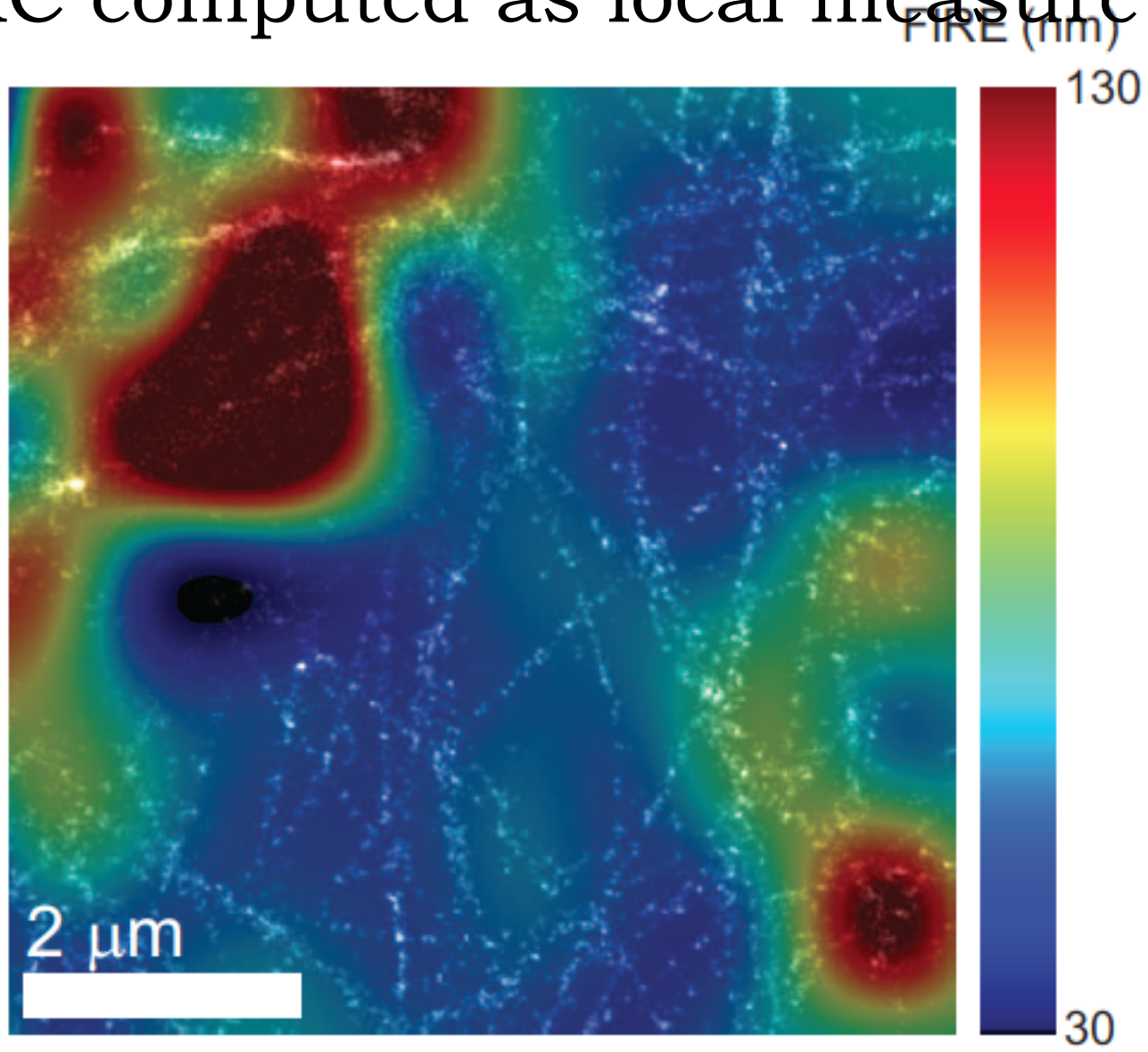


$2\pi\sigma = 61nm$

Stopping criterion

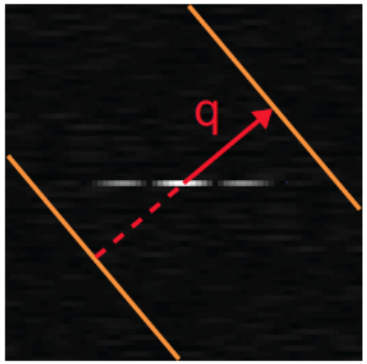
Crossing the border

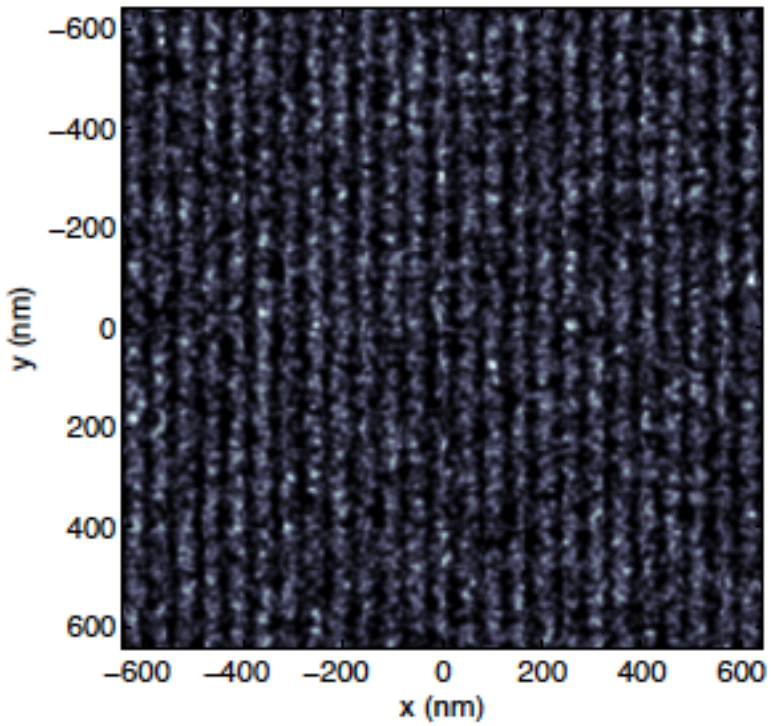
FRC computed as local measure

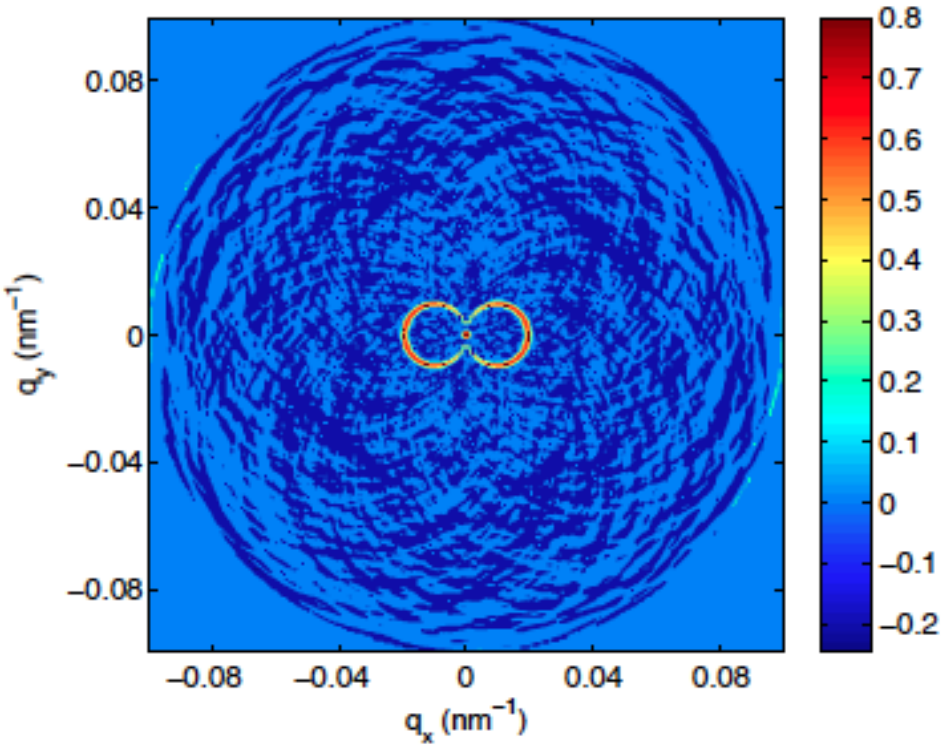


Anisotropic imaging

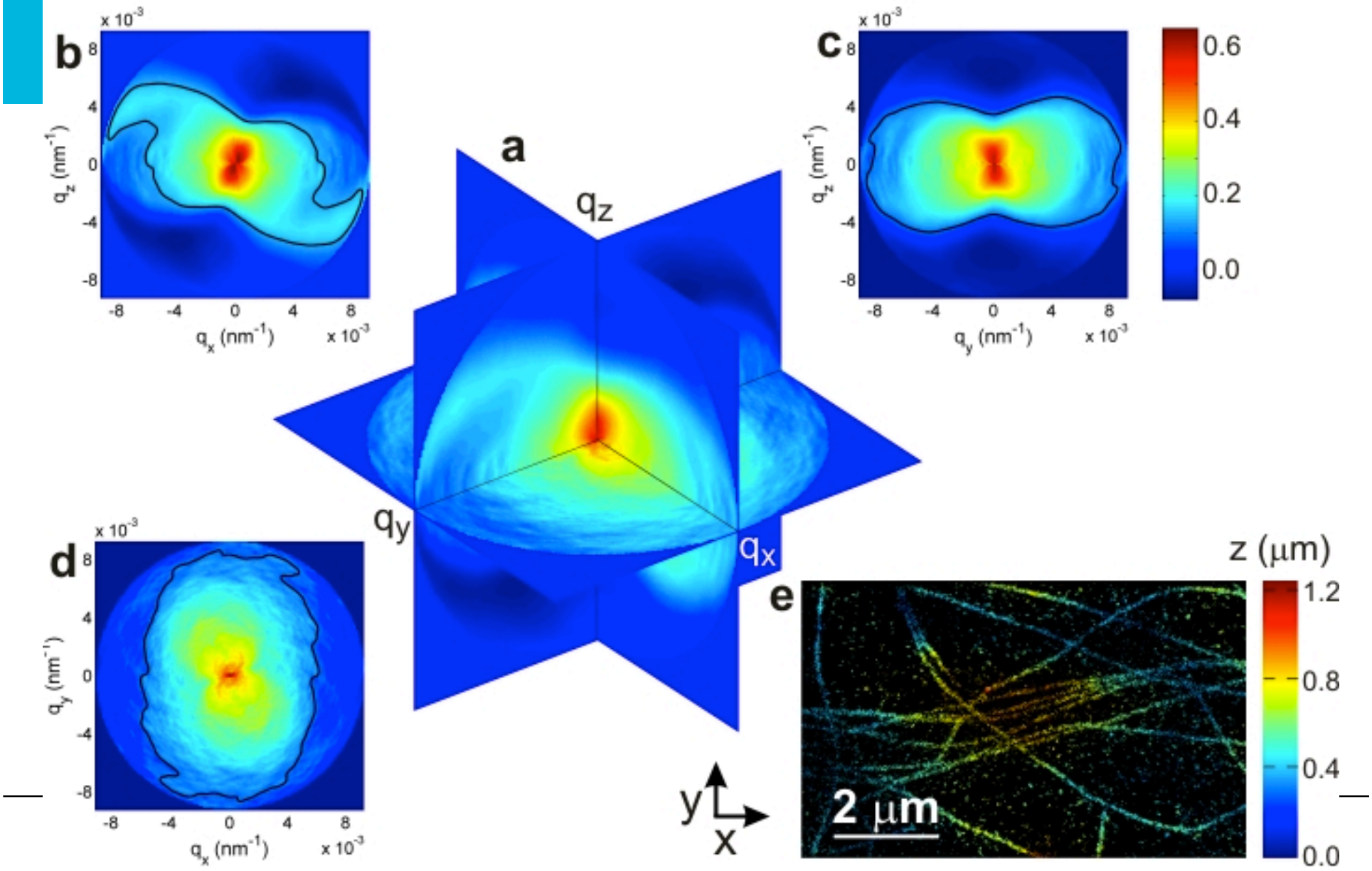
- Generalization of FRC to anisotropic data by correlating over lines (2D) or planes (3D) instead of rings or spherical shells



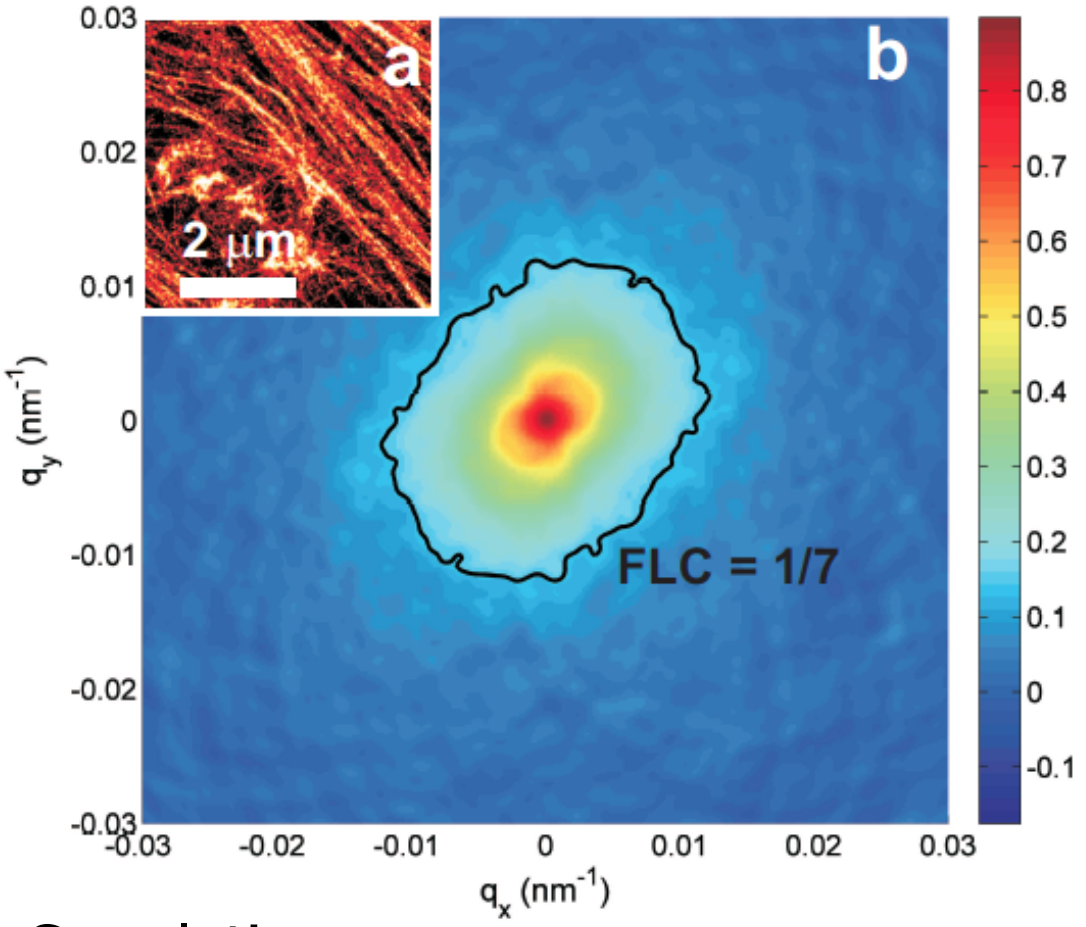




Anisotropic imaging in 3D



Anisotropic imaging in 2D



Fourier Line Correlation

Relation to classical resolution

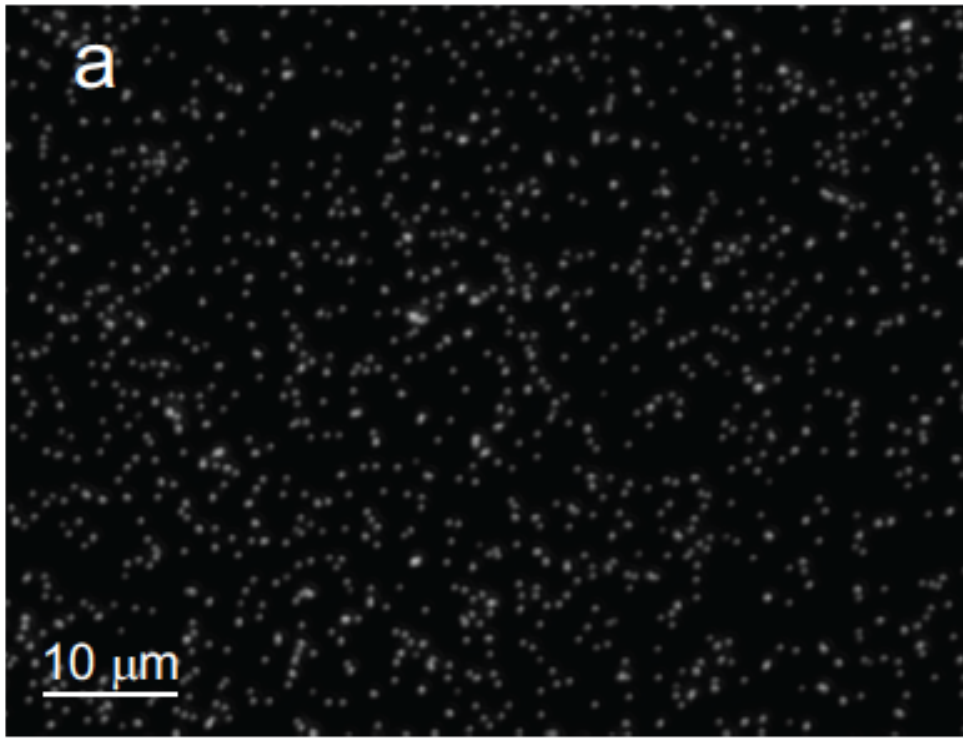
- In localization microscopy, FIRE does not reduce exactly to the Nyquist resolution in the limit of high photon counts
  - For two lines:

$$\lim_{\sigma \to 0} \langle FIRE \rangle = \sqrt{\frac{2\pi}{3\rho}} \approx 1.45 R_{Nyquist}$$

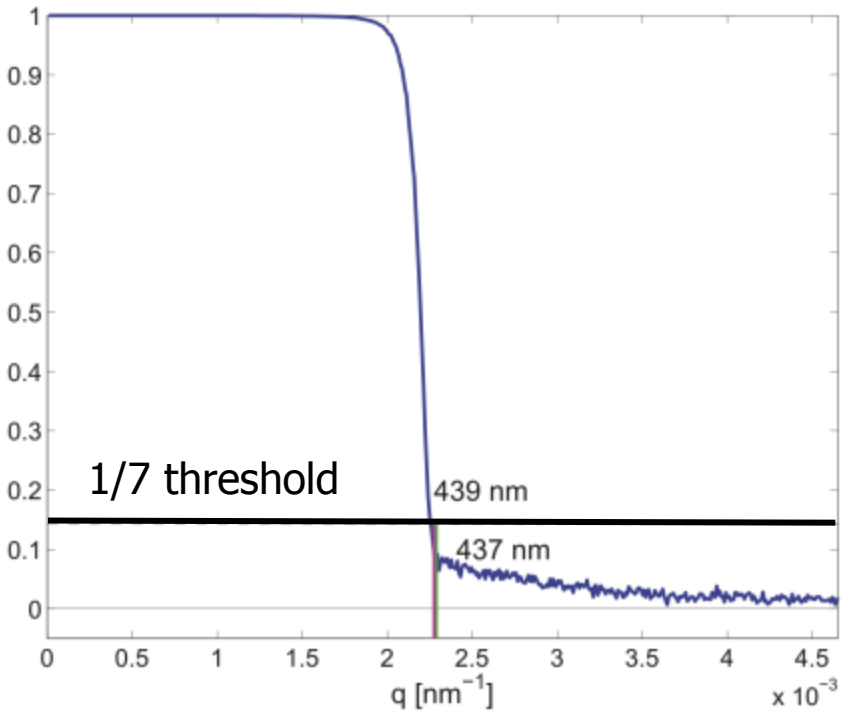
- In widefield microscopy, FIRE reduces to Abbe limit for low noise conditions

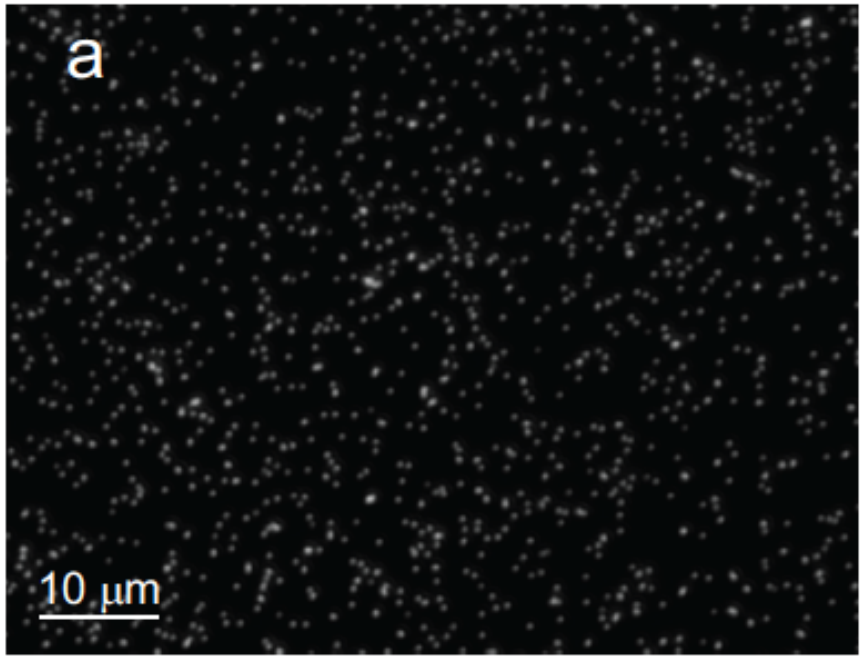
FRC resolution in widefield microscopy

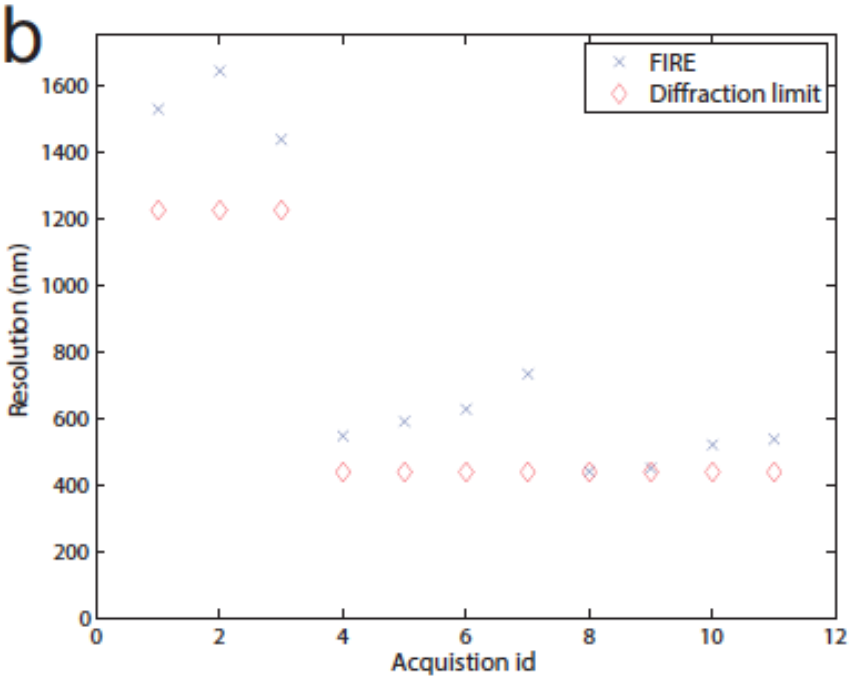
Widefield acquisition of 200 nm fluorescent beads;
just repeated imaging of the same sample

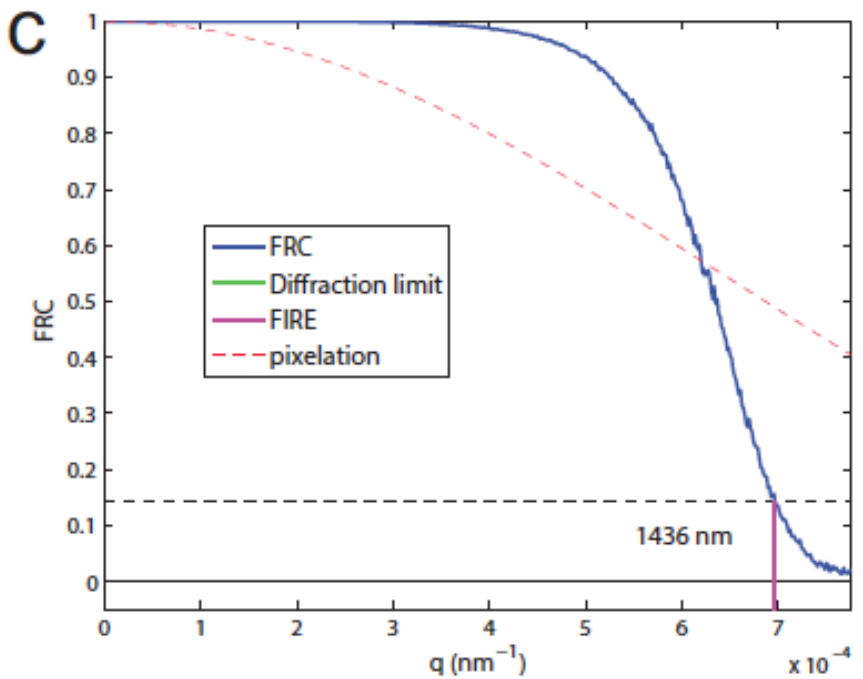


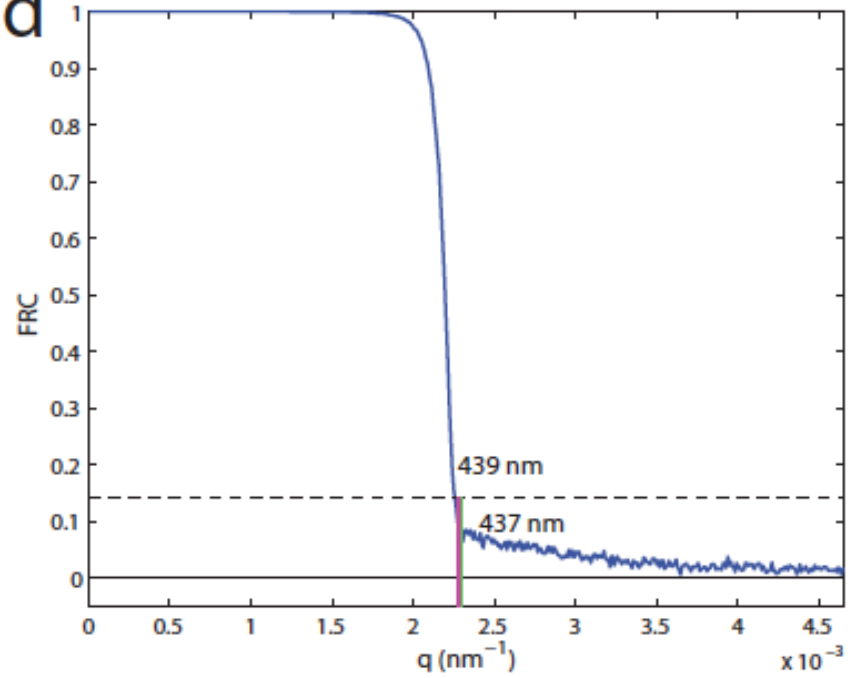
60x 0.7 NA lens
0.49x Nyquist pixel size




a
10 µm


b
FIRE
Diffraction limit
Resolution (nm)
Acquistion id


c
FRC
Diffraction limit
FIRE
pixelation
1436 nm
q (nm^{-1})
x 10^{-4}


d
FRC
439 nm
437 nm
q (nm^{-1})
x 10^{-3}

Estimated spurious correlation Q via model

- Idea: use the expected FRC to estimate the spurious term

$$\left\langle FRC(q)\right\rangle = \frac{\sum_{\vec{q}\in\text{circle}}\left[Q\exp\left(-4\pi^2\sigma^2q^2\right)+N\left|\hat{\psi}(\vec{q})\right|^2\exp\left(-4\pi^2\sigma^2q^2\right)\right]}{\sum_{\vec{q}\in\text{circle}}\left[2+\left(Q+N\left|\hat{\psi}(\vec{q})\right|^2\right)\exp\left(-4\pi^2\sigma^2q^2\right)\right]}$$

Depends on average localizations per detected emitter

Number of localizations

Object shape

Localization precision

Plan:

- Only calculate the numerator
- Divide the exponential away (with estimation)

Estimated spurious correlation Q via model

- Idea: use the expected FRC to estimate the spurious term

$$\left\langle FRC(q)\right\rangle = \sum_{\vec{q}\in\text{circle}} \left[Q\exp\left(-4\pi^2\sigma^2 q^2\right) + N\left|\hat{\psi}(\vec{q})\right|^2 \exp\left(-4\pi^2\sigma^2 q^2\right)\right]$$

Number of localizations — Object shape — Localization precision

Plan:

- Only calculate the numerator
- Divide the exponential away (with estimation)

Estimated spurious correlation Q via model

- Idea: use the expected FRC to estimate the spurious term

$$\propto Q + N\left|\hat{\psi}\left(\vec{q}\right)\right|^2$$

Number of localizations — Object shape — Localization precision

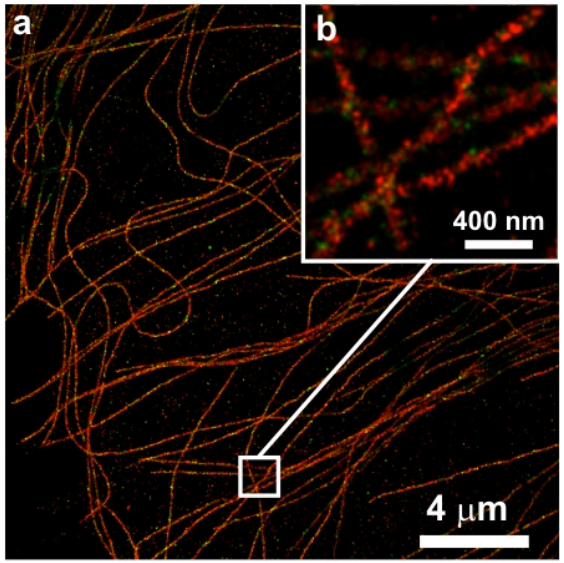
Plan:

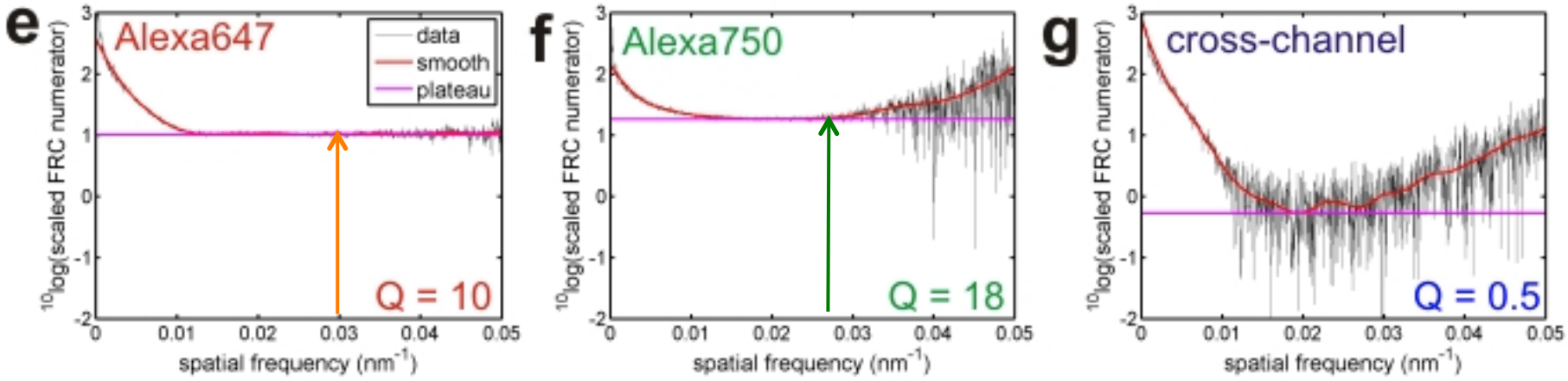
- Only calculate the numerator
- Divide the exponential away (with estimation)

Two color data of tubulin

Scaled FRC numerator curves

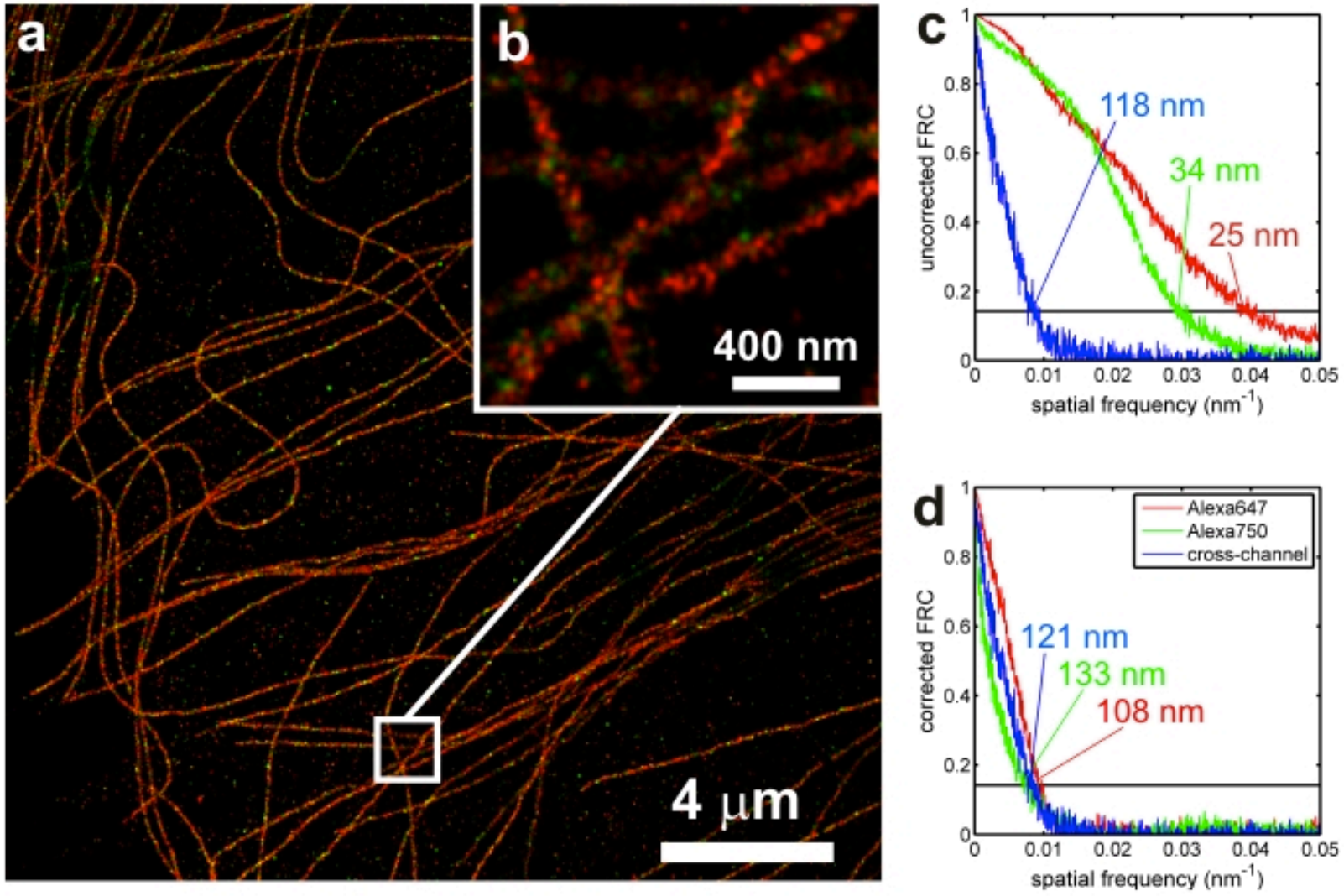
$$\propto Q + N\,|\hat{\psi}(q)|^2$$



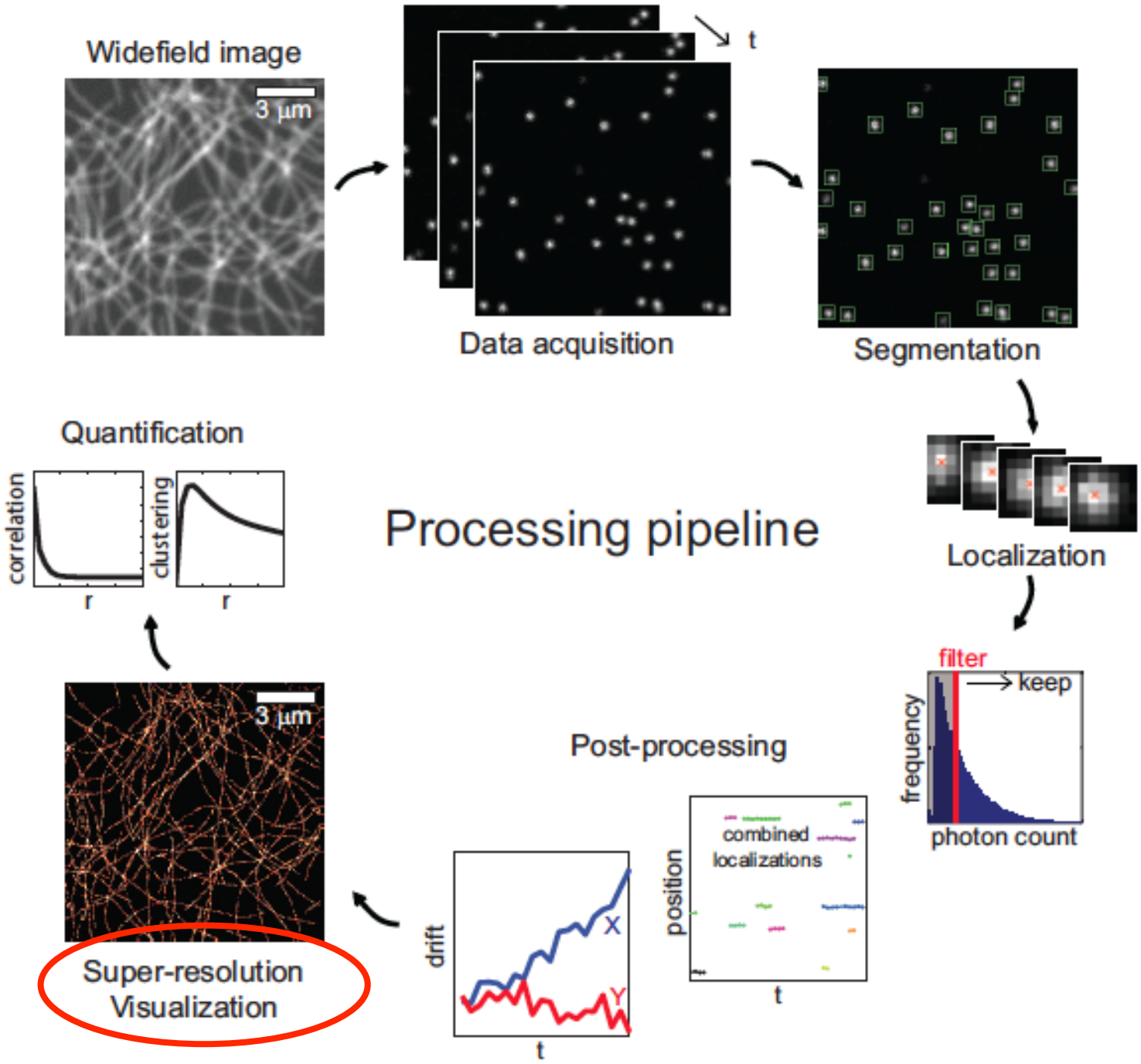


Data courtesy of Mark Bates

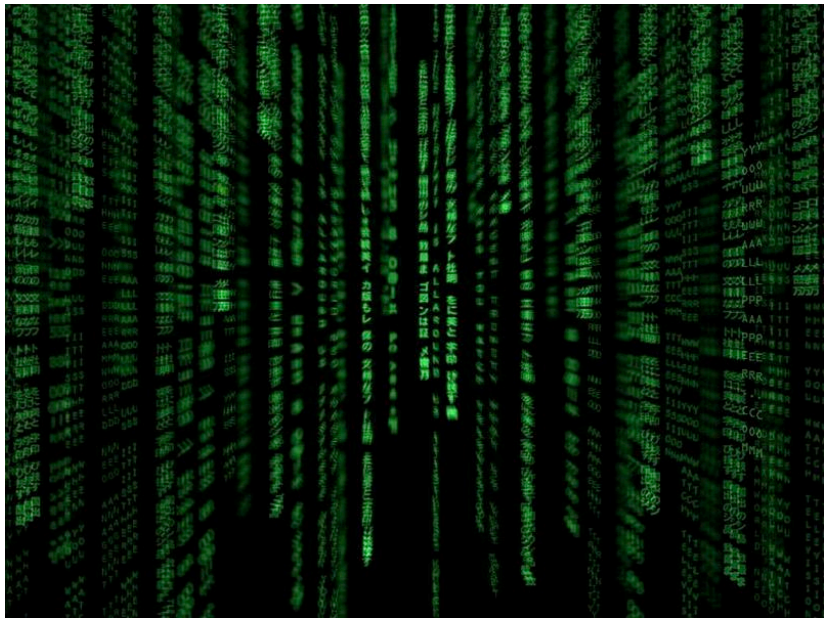
Two color data of tubulin



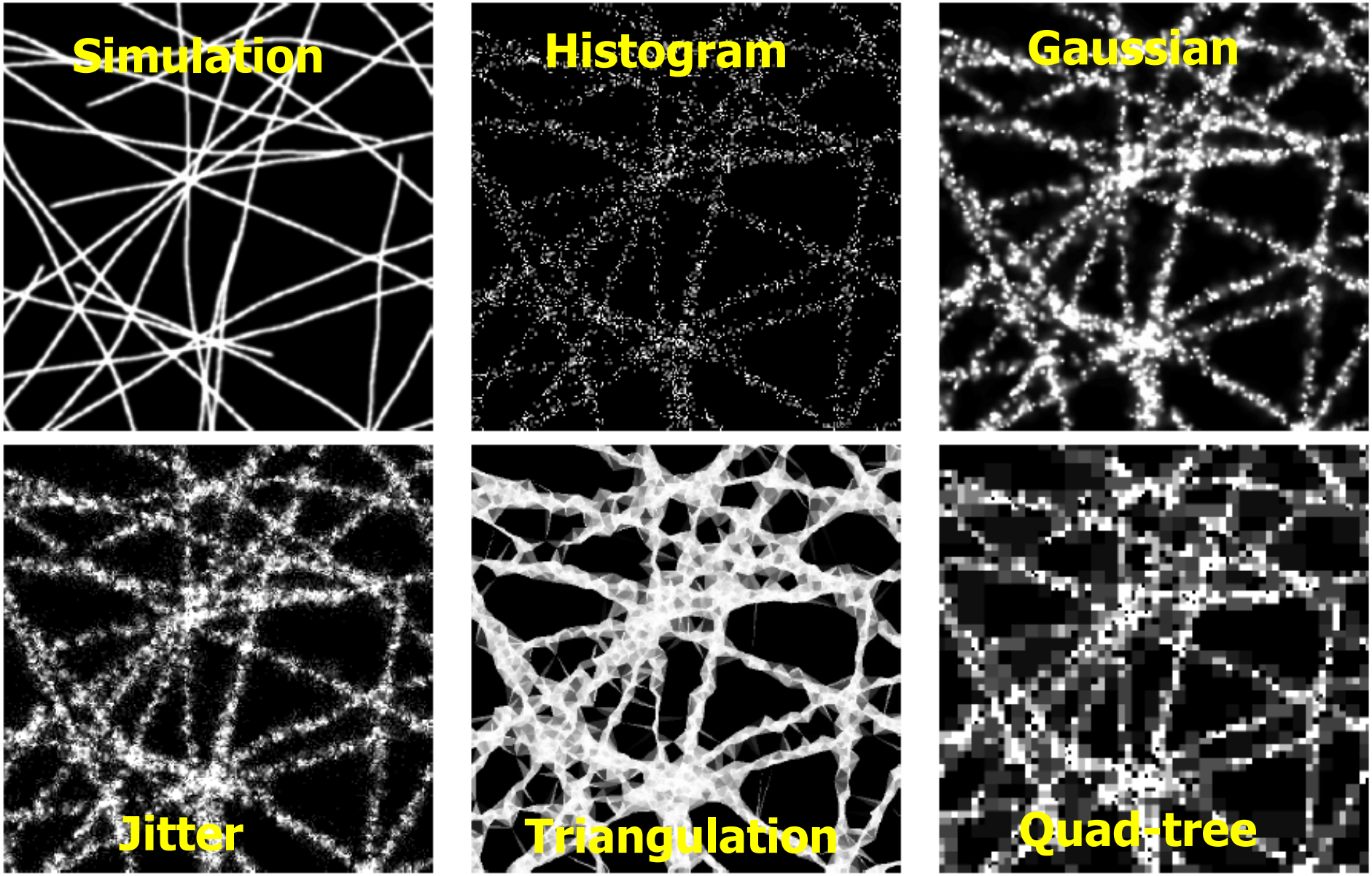
Data courtesy of Mark Bates


Widefield image
3 µm
t
Data acquisition
Segmentation
Localization
filter
keep
frequency
photon count
Post-processing
combined localizations
position
t
drift
X
Y
t
3 µm
Super-resolution Visualization
Quantification
correlation
r
clustering
r
Processing pipeline

How to display localization images anyway?



- Localization microscopy produces data but no images
- Estimated fit parameters:
  - x, y, (z) position
  - localization uncertainty
  - intensity
  - background
  - goodness of fit/ Fisher information
- Reconstruction of the object in the Nyquist sense is not considered part of the visualization process



Histogram binning [Egner et al. *Biophysical Journal* 2007]
Jittered histogram binning [Krizek et al. *Optics Express* 2011]
Gaussian rendering [Betzig et al. *Science* 2006]
Delaunay triangulation & Quad-tree visualization [Baddeley et al. *Microscopy and Microanalysis* 2010]

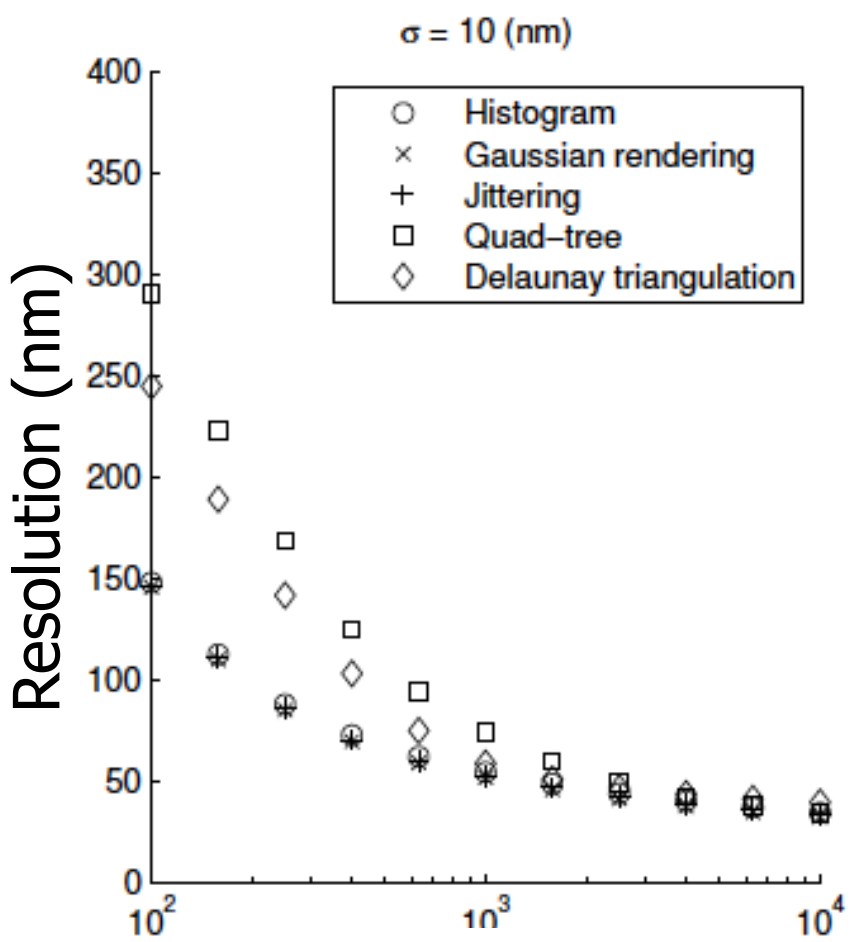
Considerations for visualization

- Intuitive interpretation:

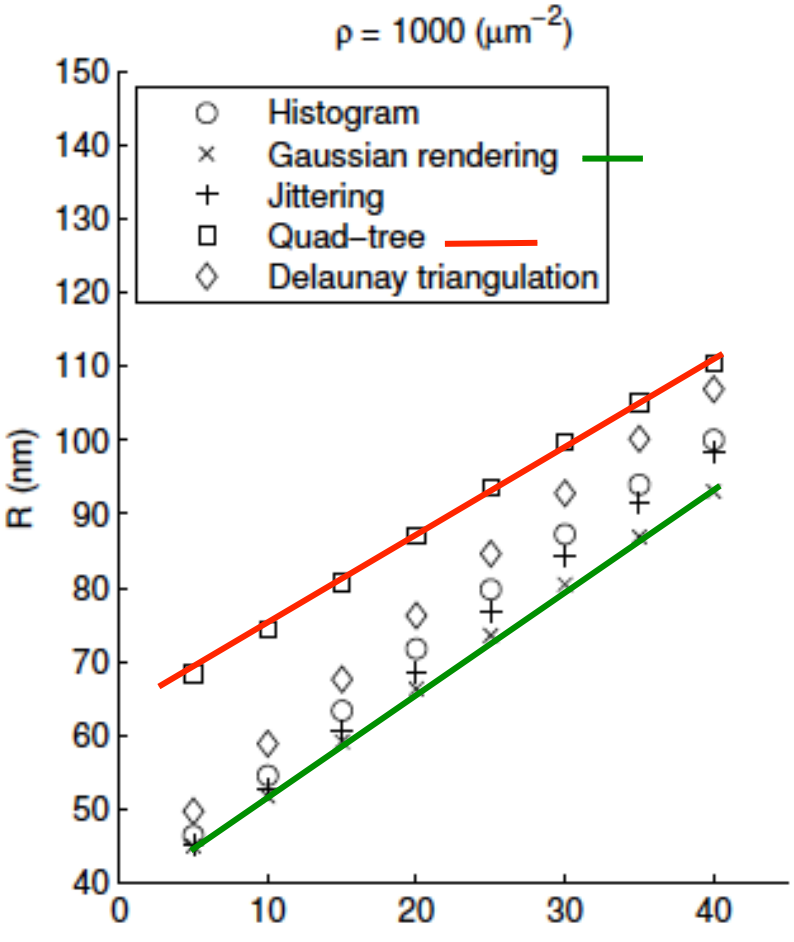
  Linearity of intensity values with emitter density

  [not preserved by scattergram, Triangulation, Quadtree]

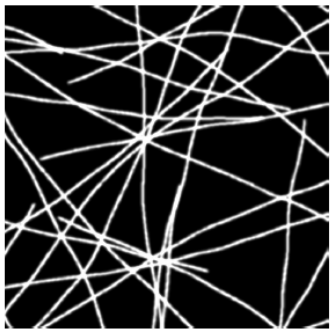
- Give the **best possible image resolution**.



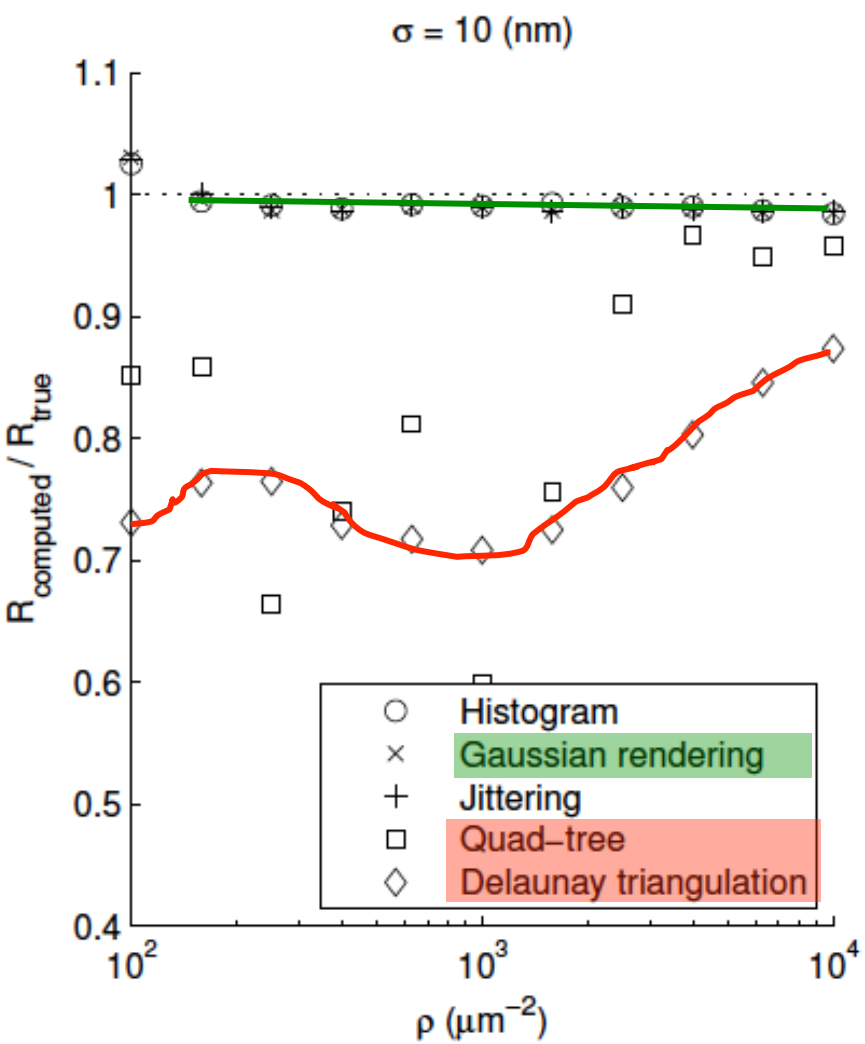
Density



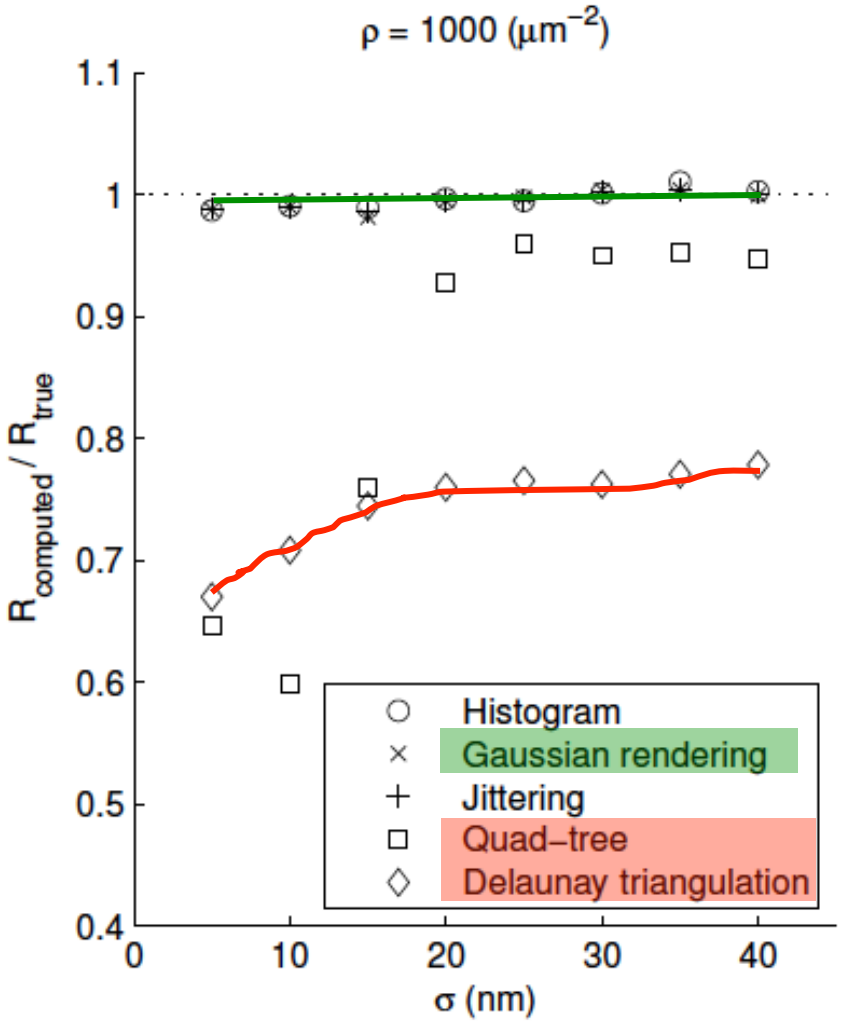
Localization uncertainty



Simulation of ground truth



Density



Localization uncertainty

Why is Gaussian better than histogram binning?

- Compute expected FRC

$$FRC = \frac{\sum_{ring} \hat{f}_1(\vec{q})\hat{f}_2(\vec{q})^*}{\sqrt{\sum_{ring}\left|\hat{f}_1(\vec{q})\right|^2}\sqrt{\sum_{ring}\left|\hat{f}_2(\vec{q})\right|^2}}$$

$$\left\langle \hat{g}(q) \right\rangle \approx \frac{1}{N}\hat{\psi}(q)\sum_j \exp(-4\pi^2\sigma_j^2 q^2)$$

$$\left\langle \hat{h}(q) \right\rangle \approx \frac{1}{N}\hat{\psi}(q)\sum_j \exp(-2\pi^2\sigma_j^2 q^2)$$

Object: set of points

$$\psi(r) = \sum_{j=1}^{N}\delta(r - r_j^{em})$$

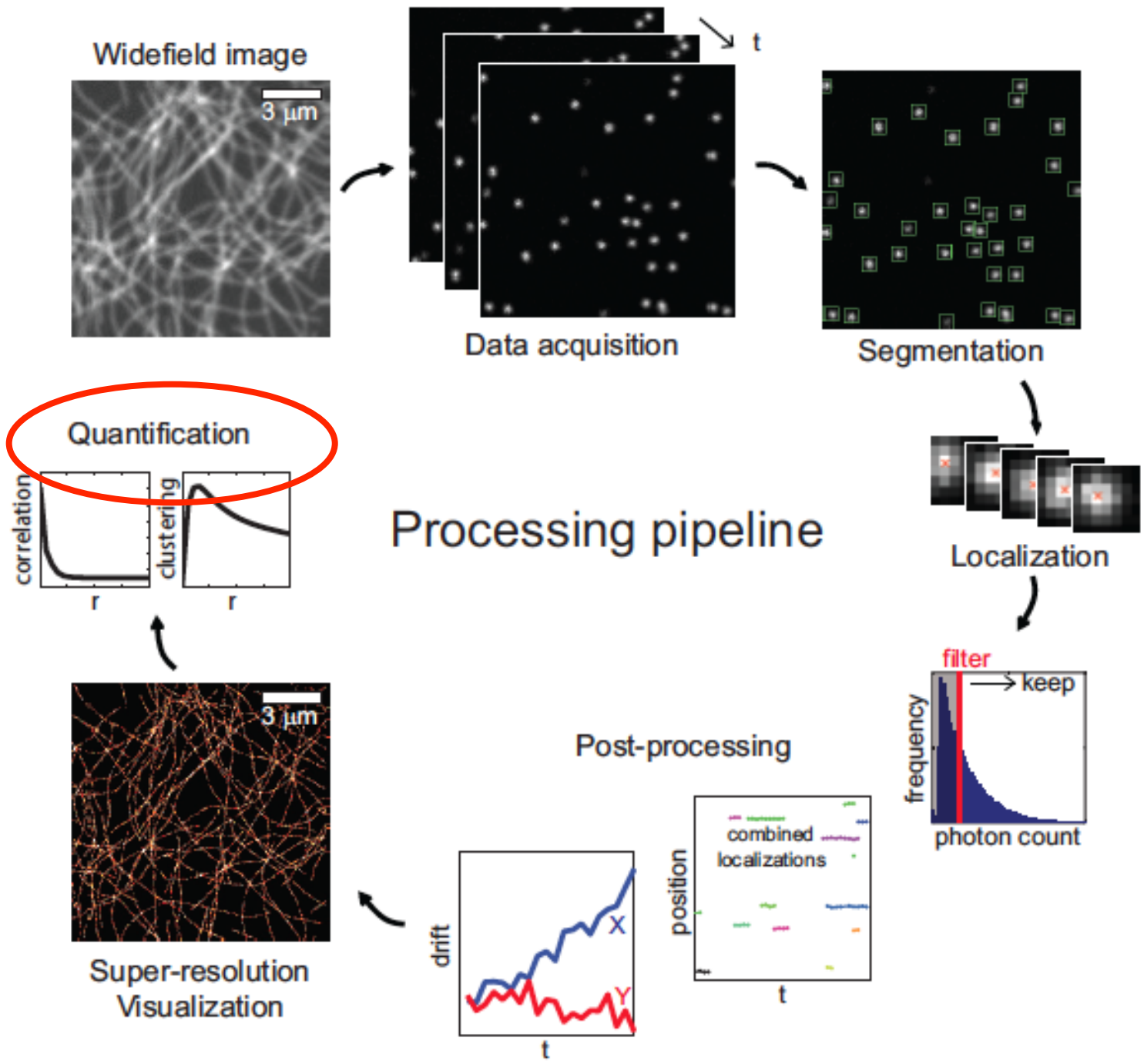
$$\left\langle FRC_g \right\rangle - \left\langle FRC_h \right\rangle = \left\langle e^{-4\pi^2\sigma^2 q^2} \right\rangle - \left\langle e^{-2\pi^2\sigma^2 q^2} \right\rangle^2 \geq 0$$

- Gaussian weights localizations to *q* depending their σ
  Imprecise localizations decrease faster as function of *q*

(ignoring pixelation effect on histogram)

Conclusions: visualization

- Gaussian rendering is best, especially if the localization error is large (theoretical proven)
- Gaussian rendering only better than histogram binning if each emitter is rendered with its own localization uncertainty
- Gaussian, jittering and histogram binning give typically same resolution
- Quad-tree and triangulation give irregular bias and should not be used. Especially for low densities.

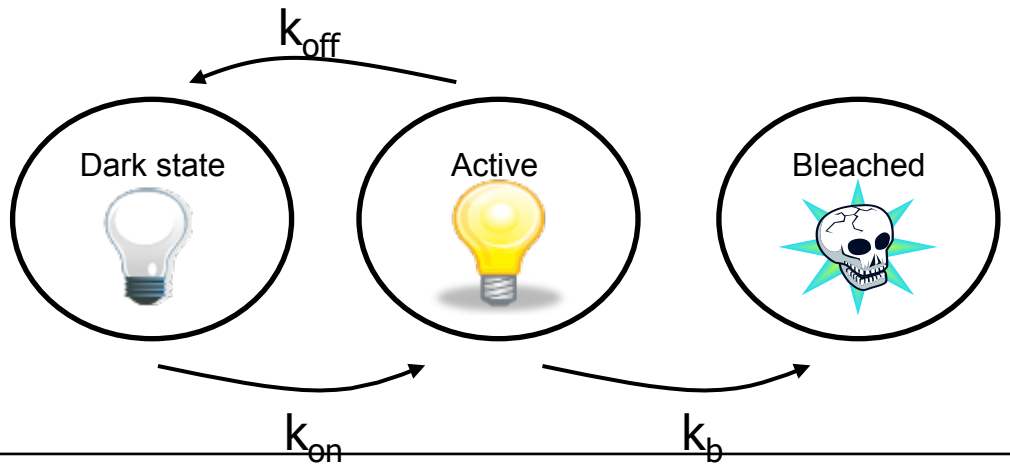

Widefield image
3 µm
t
Data acquisition
Segmentation
Localization
filter
keep
frequency
photon count
Post-processing
combined localizations
position
t
drift
X
Y
t
3 µm
Super-resolution Visualization
Quantification
correlation
r
clustering
r
Processing pipeline

From Q to emitter counting

$$Q = \langle M \rangle + \left(\frac{Var(M)}{\langle M \rangle} - 1 \right)$$

- Q can be used to estimate <M> = the average number of times one molecule is localized

- This requires a model for emitter switching:



model also used by: Lee et al, PNAS 109, 43, 2012

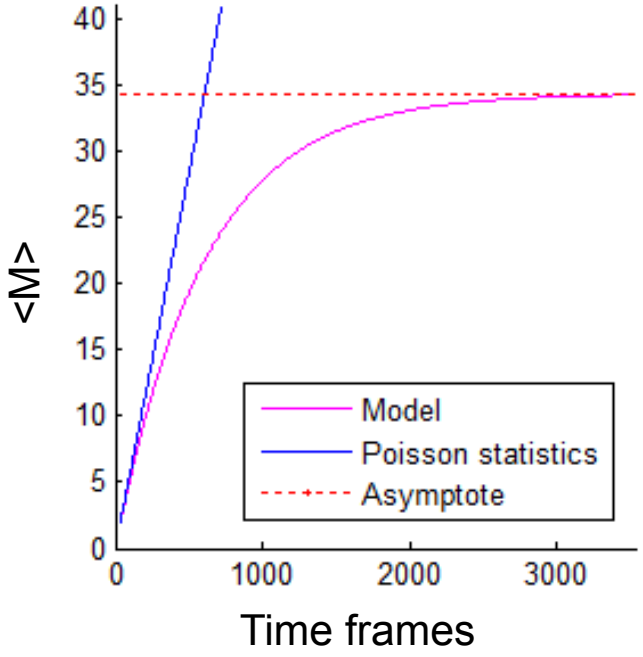
Statistical switching model

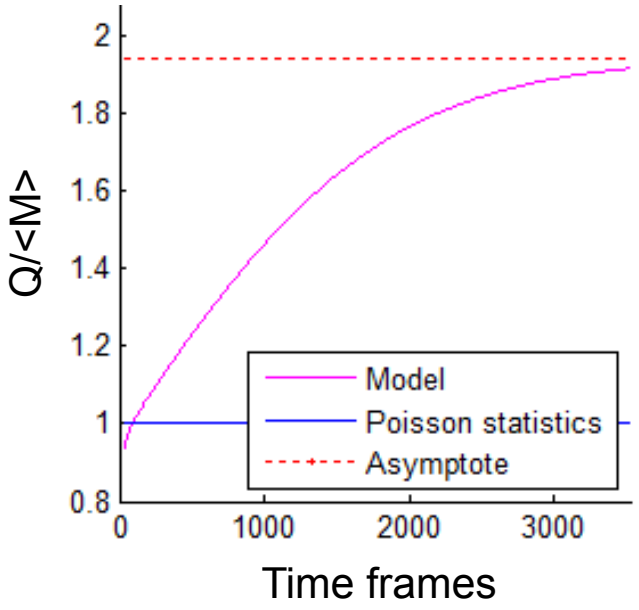
Expected number of localizations per emitter M vs. Q

- For $k_{off} >> k_{on}$:

$$\langle M \rangle = \left(1 + \frac{k_{off}}{k_b}\right)\left(1 - \exp\left(-k_{eff} t\right)\right)$$

$$Q = 2\frac{k_{off}}{k_b}\left(1 - \frac{k_{eff} t}{\exp\left(k_{eff} t\right) - 1}\right) \approx f\left(k_{eff} t\right) \cdot \langle M \rangle$$

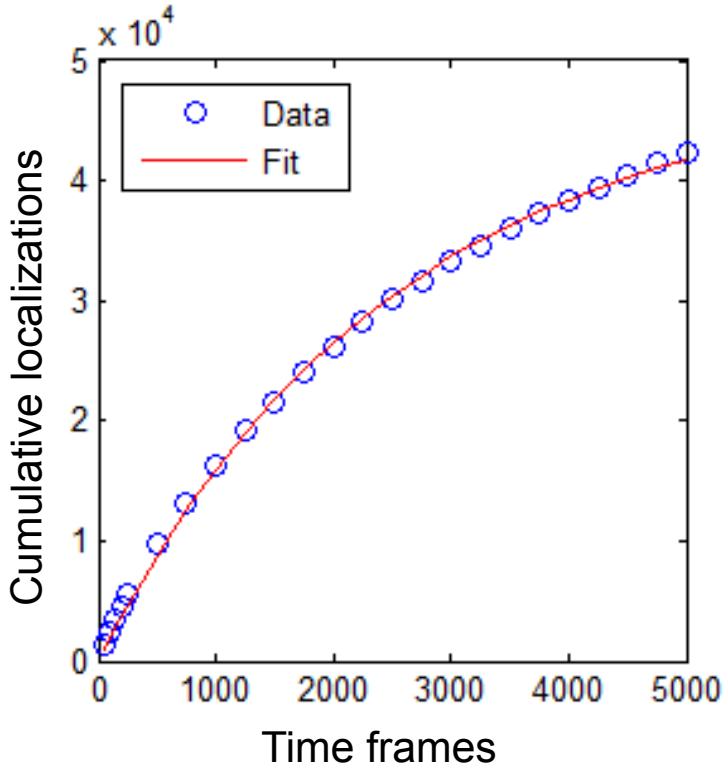




$$k_{eff} = \frac{k_{on} k_b}{k_{on} + k_{off} + k_b}$$

The unknown rate constant

k_{eff} is estimated from cumulative localizations



$$\langle M \rangle = \left(1 + \frac{k_{off}}{k_b}\right)\left(1 - \exp\left(-k_{eff}\, t\right)\right) \propto N$$

Fit model:

$$y = a\left(1 - \exp\left(-bt\right)\right)$$

Results:

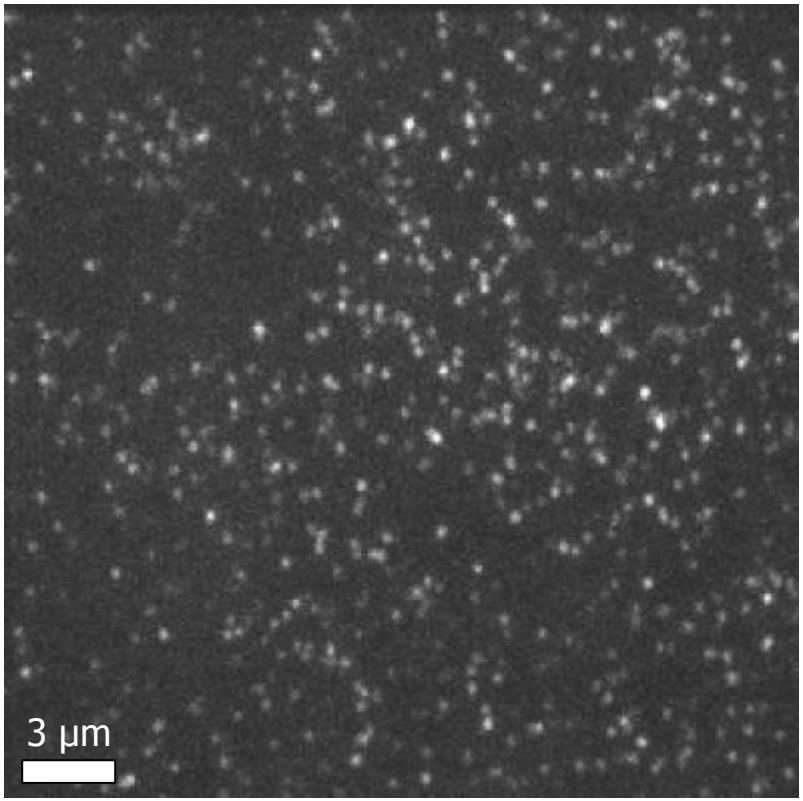
a = 4.8 10^4

b = 4.0 10^{-4} ⇨ k_{eff}

R^2 = 0.9987

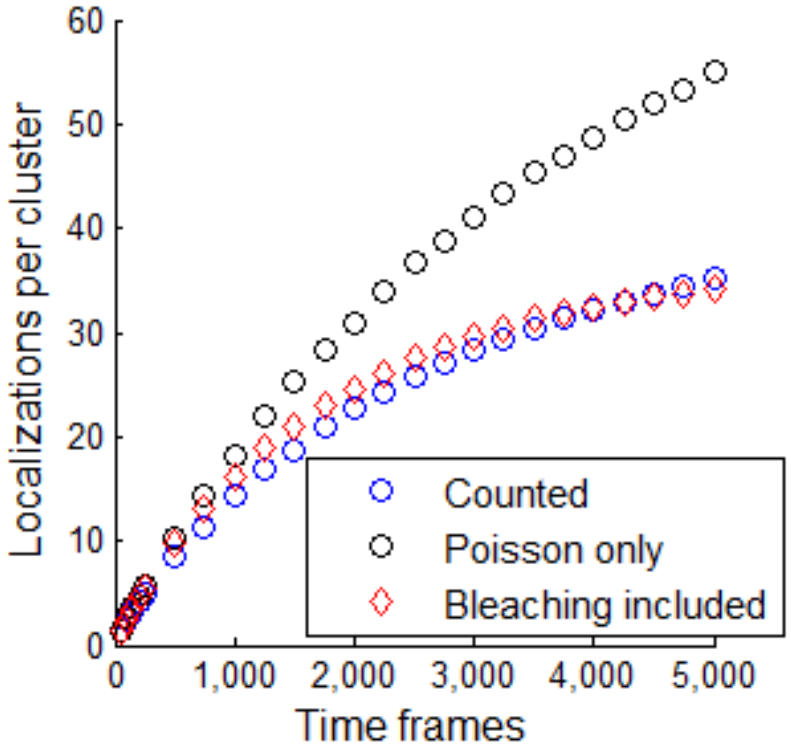
Control experiment with ground truth

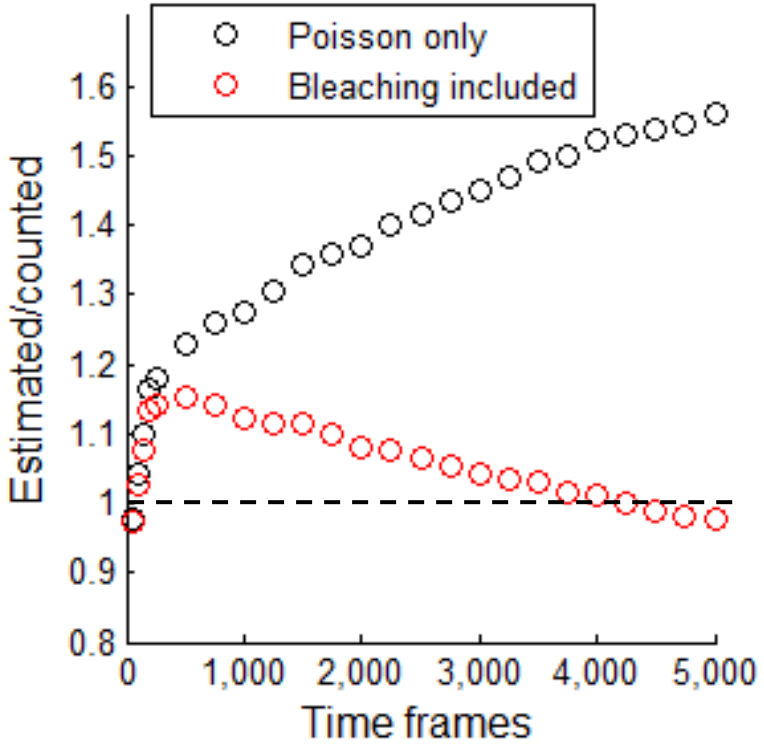
Sparse Alexa 647 labeled antibodies on a glass surface



Control experiment with ground truth

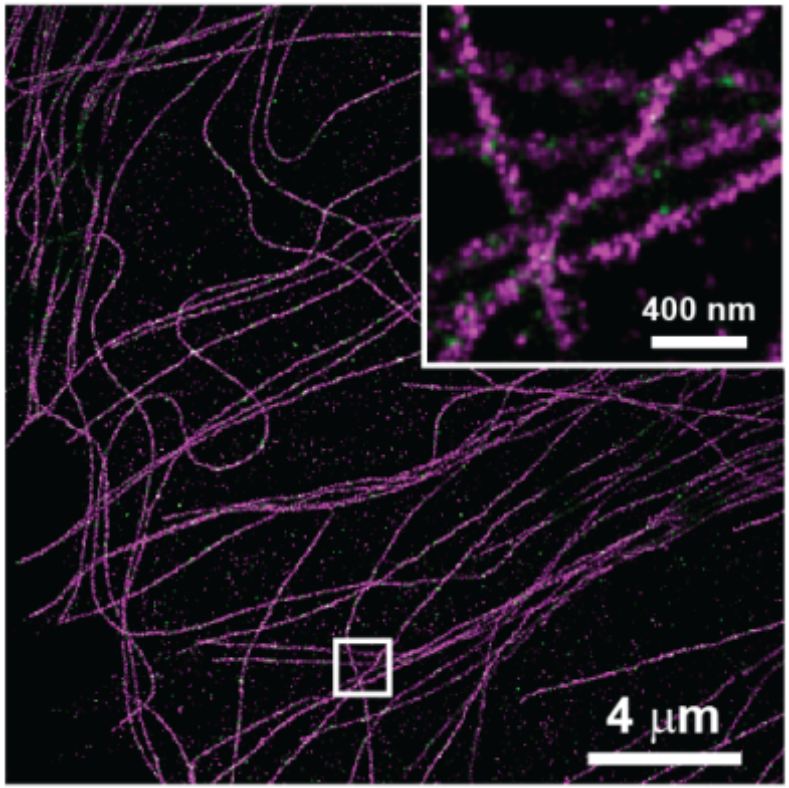
Photobleaching effects are correctly included



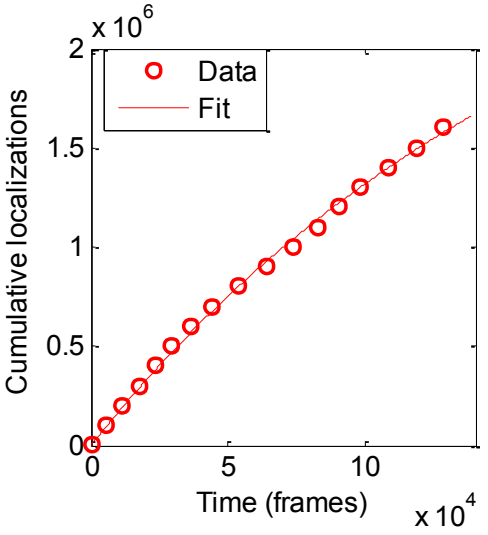


Application to dual color data

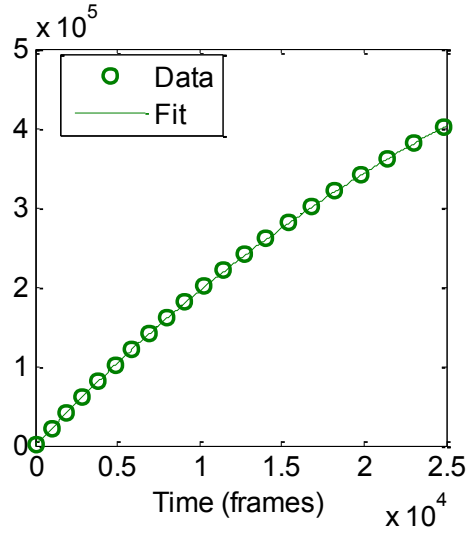
Estimating the unknown rate constant



Alexa 647

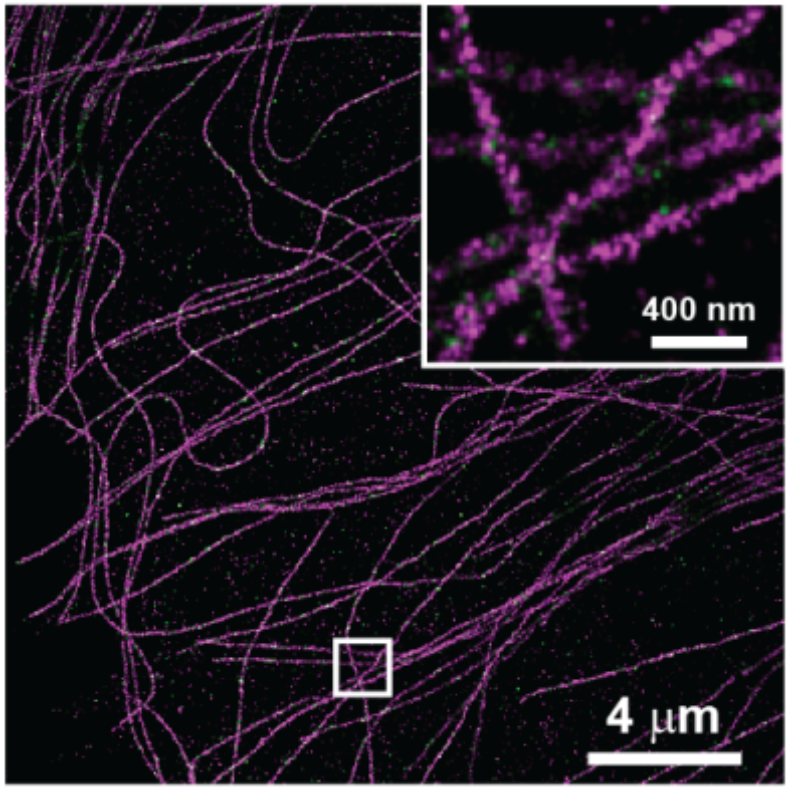


Alexa 750



Application to dual color data

of localizations per emitter and all rate constants



Alexa 647	Alexa 750
$\langle M \rangle = 7.8$	$\langle M \rangle = 11$
$k_{eff} = 3.3 \cdot 10^{-4} s^{-1}$	$k_{eff} = 5.6 \cdot 10^{-4} s^{-1}$
$k_{on} = 1.8 \cdot 10^{-3} s^{-1}$	$k_{on} = 2.8 \cdot 10^{-2} s^{-1}$
$k_{off} / k_b = 10$	$k_{off} / k_b = 19$

Conclusions

- FRC Image Resolution is proposed as a image based resolution measure for localization microscopy
- Sensitive to labelling density, localization precision, sample shape
- Can deal with multiple localizations per emitter

- Counting of average localizations per emitter
  - ✓ Without prior knowledge of sample structure
  - ✓ Without a calibration experiment for rate constants
- Neglecting photobleaching leads to overestimation
  - Also for pair-correlation approach
- Caveats:
  - Transition rates are assumed constant
  - False positive localizations
  - Blinking/bleaching model may be too simplistic