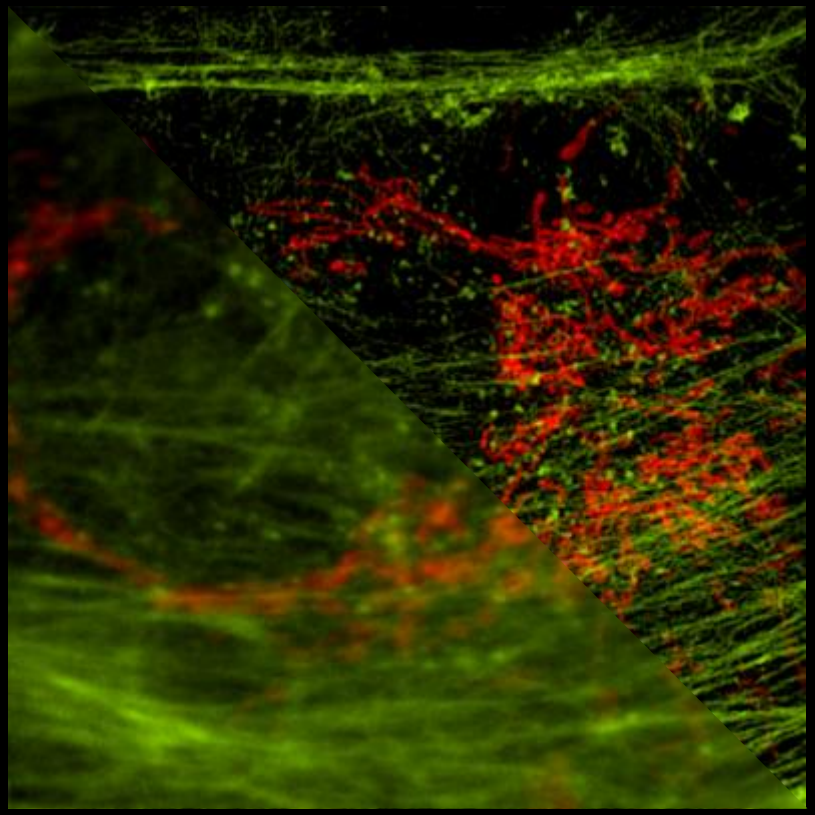
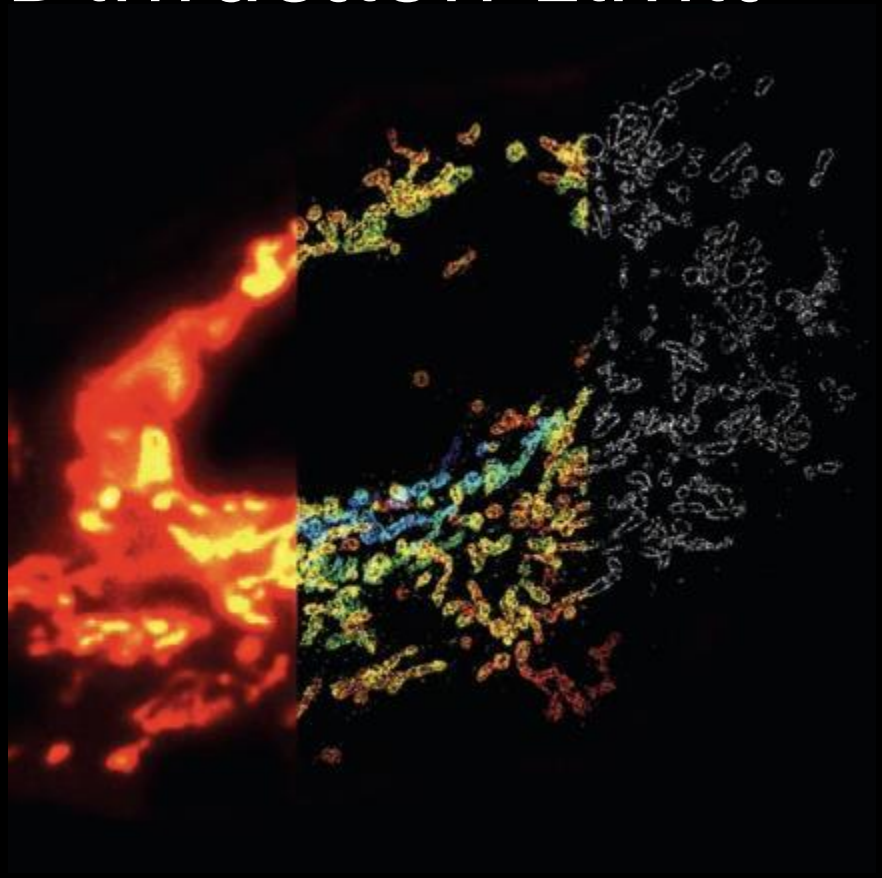
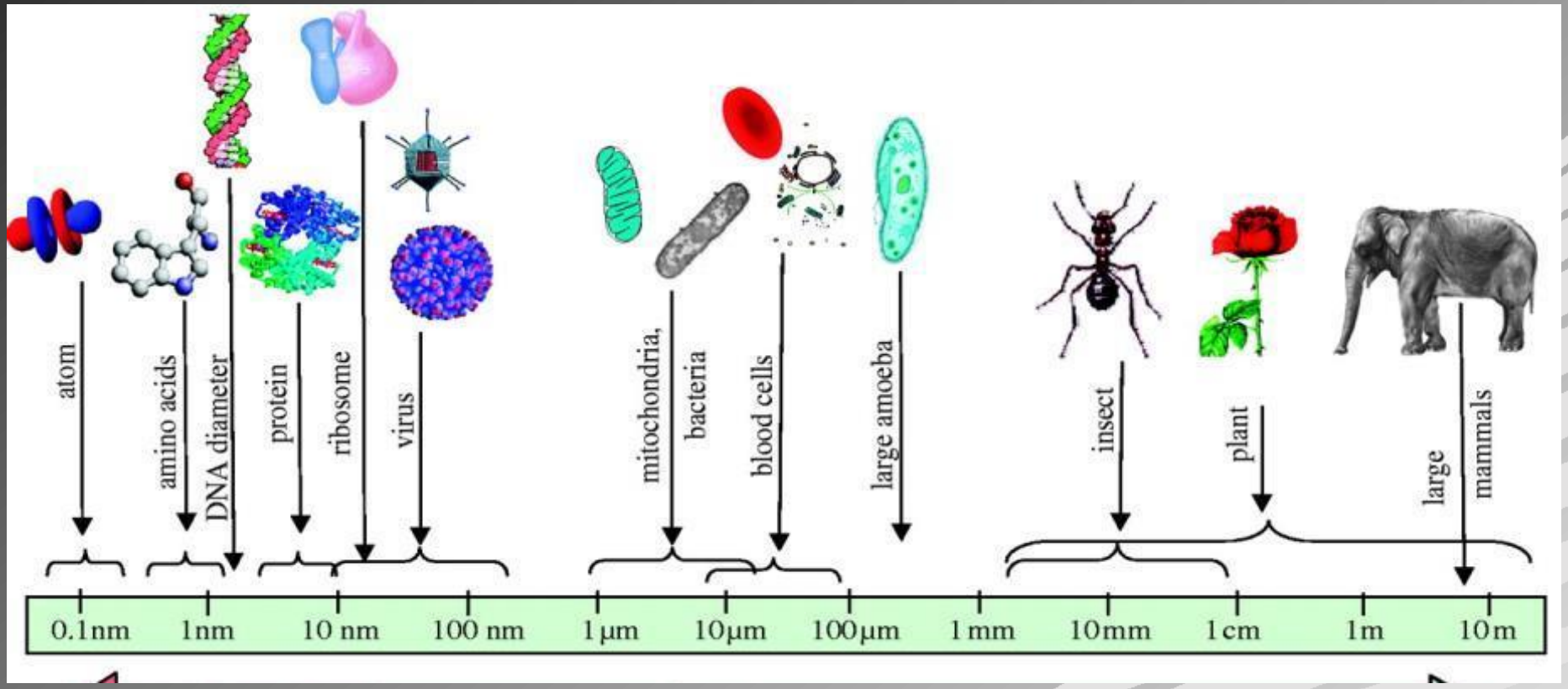
Detection, Resolution, and Imaging Beyond Abbe's Diffraction Limit





Ric Villani
Senior Biosystems Application Manager
Nikon Instruments Inc.
February 22nd, 2016

Imaging at various lengths



PET

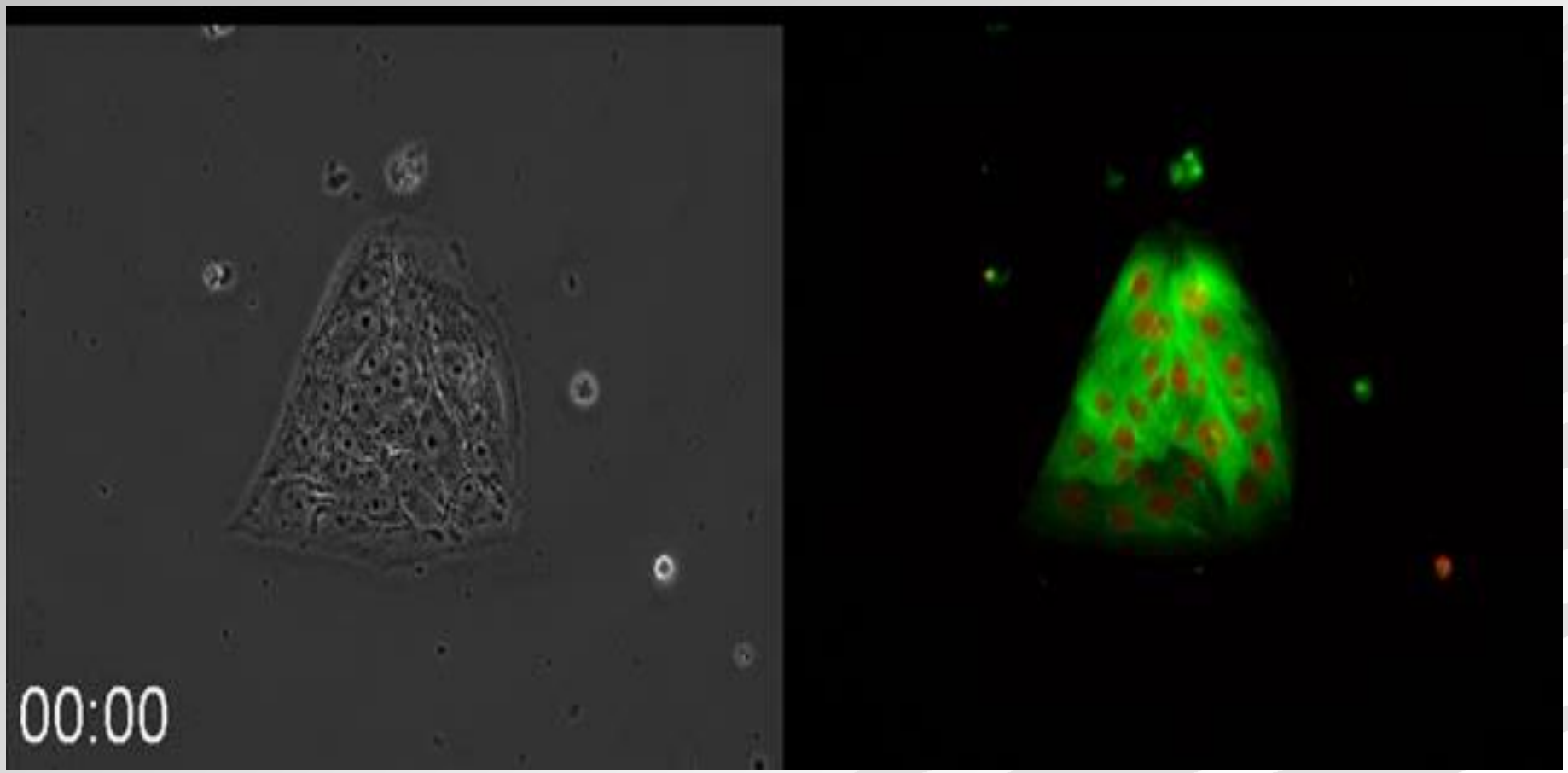
MRI

CT

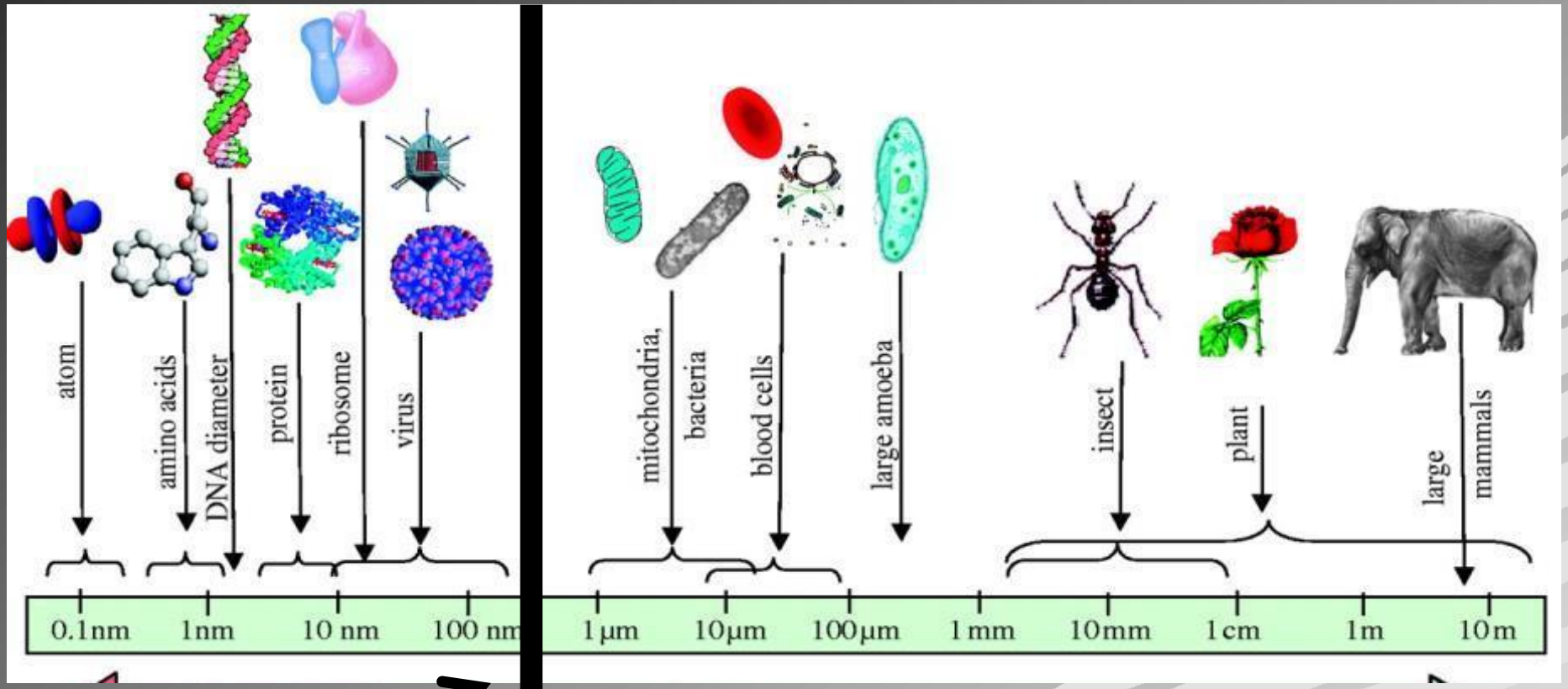
Light Microscopy

EM, SPM

X-ray crystallography, NMR

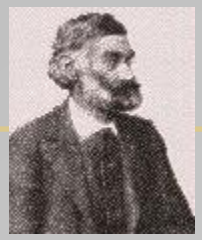

00:00

Imaging at various lengths



200 nm
½ wavelength of light
$\lambda/2$

Light Microscopy



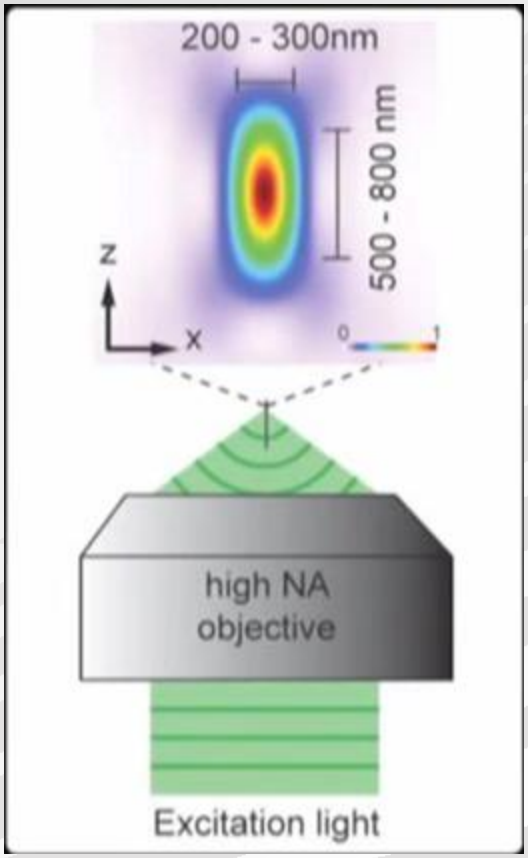
500-800 nm

200 nm

λ

LENS

$$d = \frac{\lambda}{2n \sin\alpha}$$



Verdet (1869)
Abbe (1873)
Helmholtz (1874)
Rayleigh (1874)

Detection Versus Resolution

With the diffraction limit, we can detect, but not resolve beyond a certain point

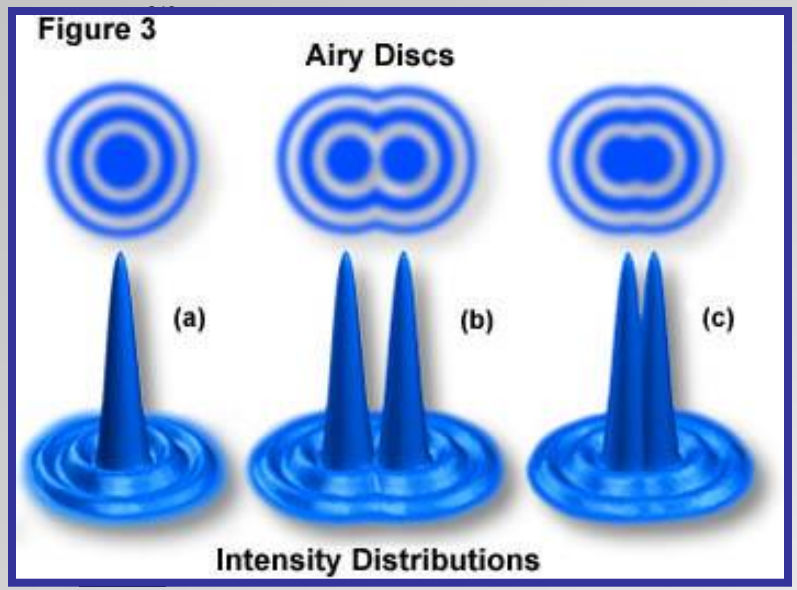
Detect:

- To determine if a structure or substance is present in a sample or not

Resolve:

- To determine the number or size of an object of interest in a sample or relative position of two objects.

Detection Versus Resolution



$$r_{xy} = \frac{1.22\lambda}{(2NA)}$$

$$r_z = \frac{2\lambda \cdot \eta}{(NA_{obj})^2}$$

Sub-resolution light sources are "convolved" by the microscope appearing as "diffraction limited" Airy Disks.

Detection is a function of total brightness of the diffraction volume.

Resolution is a function of Airy Disks separation.

Why are we limited?

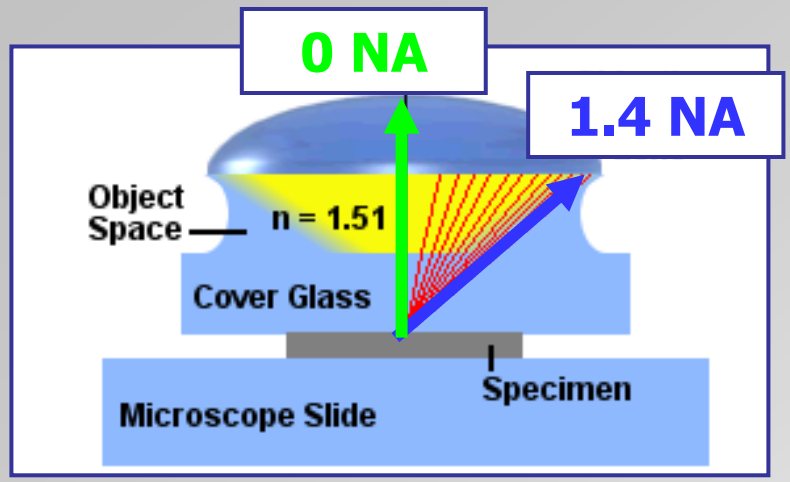
- Abbe's equation has no lower limit

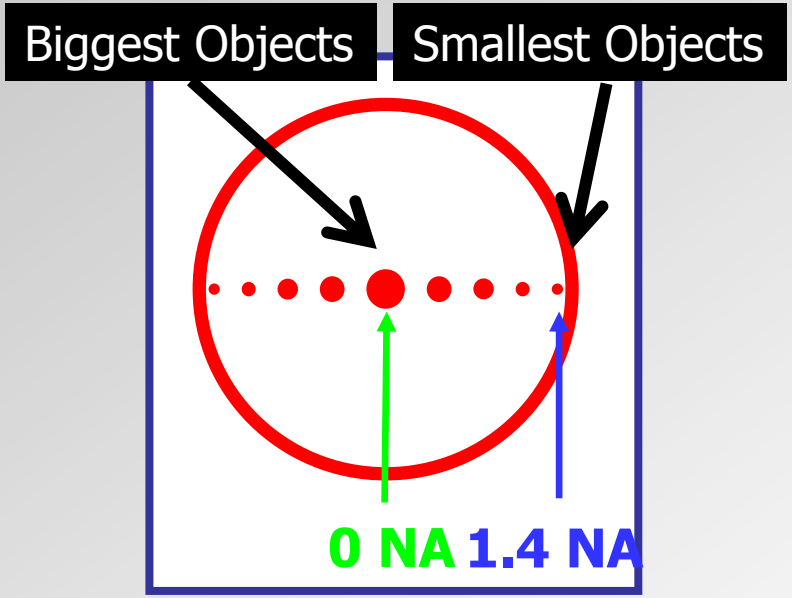
$$r_{xy} = \frac{1.22\lambda}{(2NA)}$$

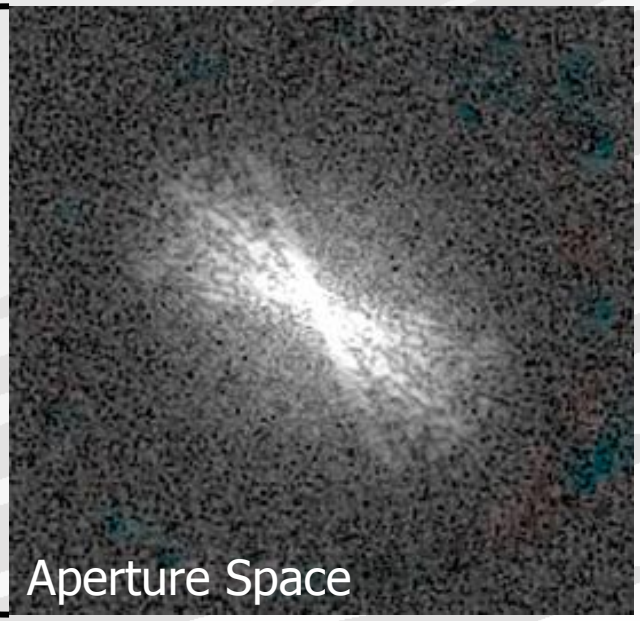
- Better Resolution from:
  - Shorter Wavelength
    - Crown glass transmits only to ~400nm
  - Higher NA's
    - NA of objective typically limited to 1.49NA

Why are we limited in NA?

The Other Side



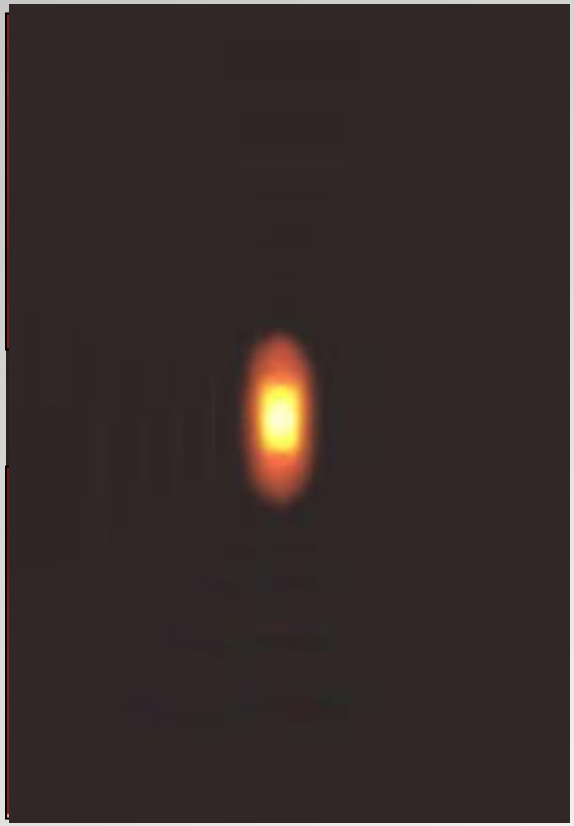




Images From - Mats Gustafsson - UCSF

Approaching the Limit

- The diffraction limit is for a single point in space
- Samples have signal above and below
- Imaging the diffraction volume is obscured.



Confocal: Emission Restriction

MP: Excitation Restriction

TIRF: Sub-Diffraction Excitation

Breaking the Limit

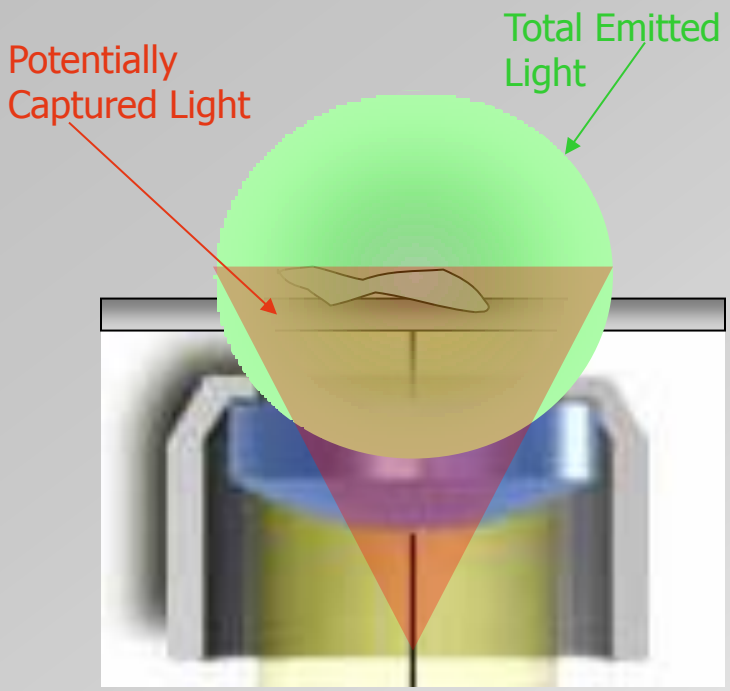
I. Structural Super Resolution "PSF Engineering"

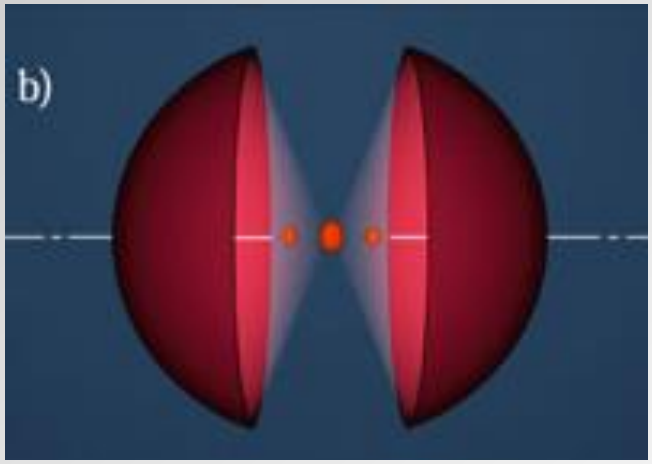
4Pi	
I^5M	Image Interference Microscopy
STED	Stimulated Emission Depletion
SIM	Structured Illumination Microscopy

II. Single Molecule Localization "PSF Mapping"

FIONA	Fluorescence imaging with one-nanometer accuracy
STORM	Stochastic optical reconstruction microscopy
PALM	Photoactivated light microscopy
GSDIM	Ground state depletion & individual molecule return

Opposing Objective Microscopy





- A single lens collects HALF of the potential emitted light & thus HALF of the potential axial information
- Limited collection causes axial stretch seen in PSF
- Destructive interference between light from two objectives produces a less elongated PSF.
- 4Pi and I^5M

http://www.mpibpc.mpg.de/groups/hell/4Pi.htm

4Pi Confocal Microscopy

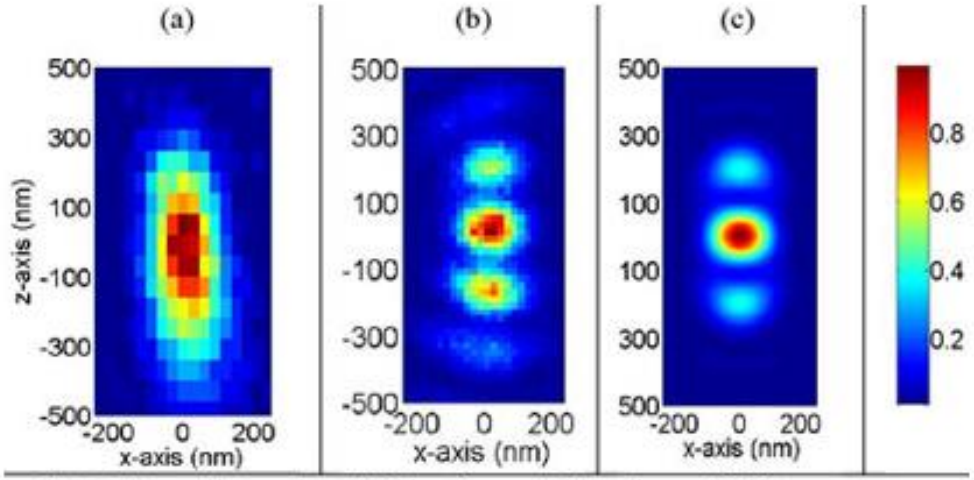
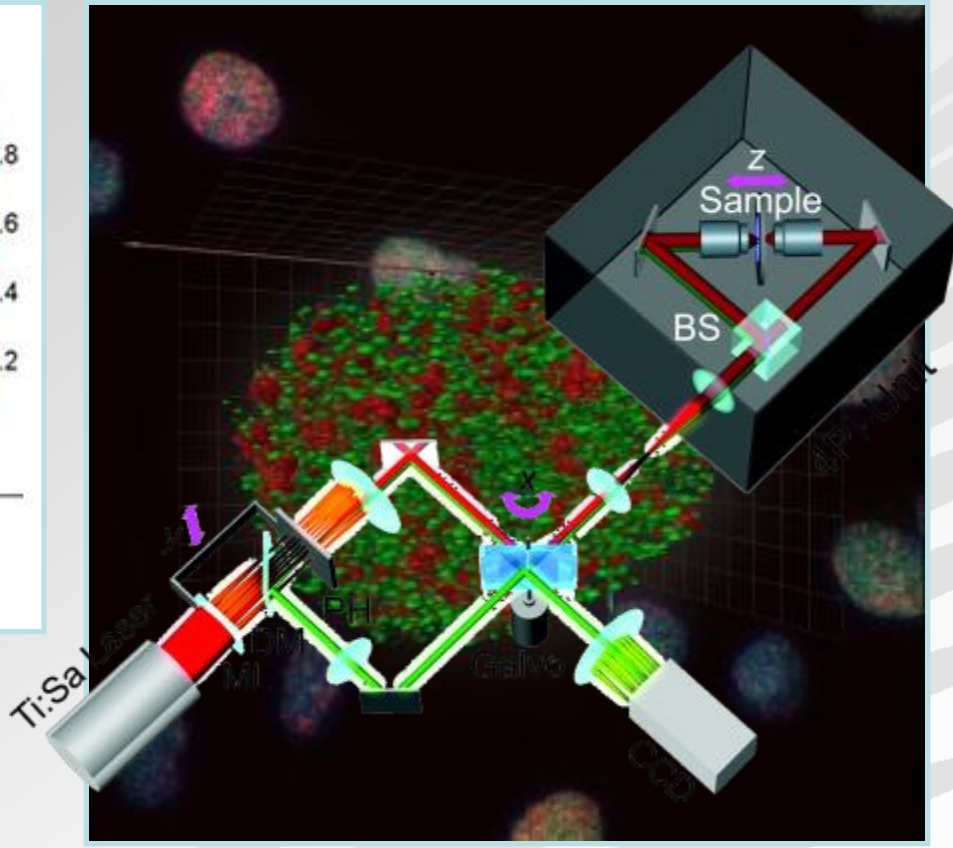


Fig.3. Two dimensional Point Spread Functions (PSF) **(a)** Experimental one objective, **(b)** experimental 4Pi and **(c)** calculated 4Pi PSF in x-z plane.



- XY Resolution ~ 250nm
- Z Resolution ~ 85nm - 150nm

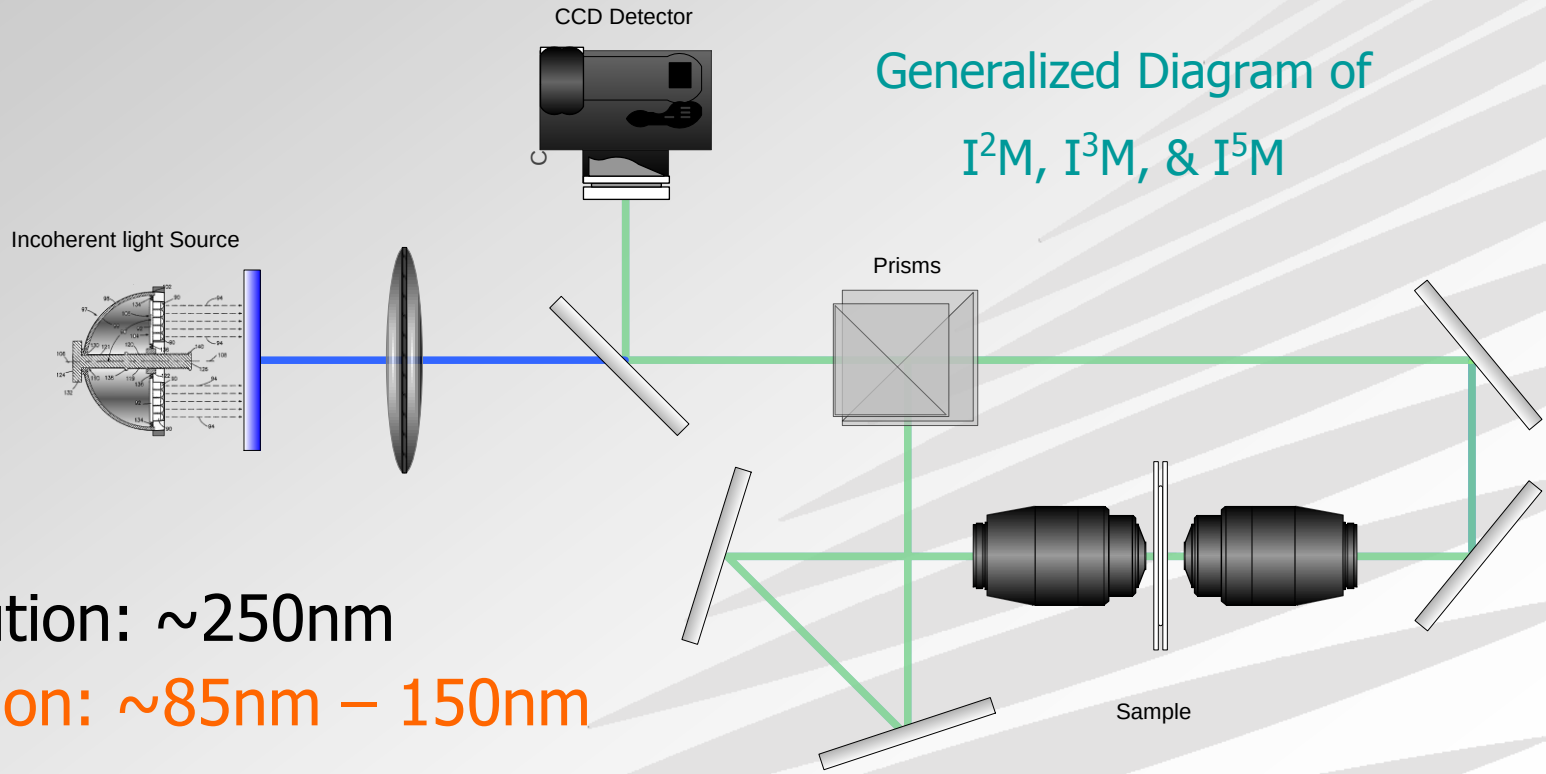
Bennett Goldberg, Anna Swan, Selim Ünlü
High Resolution 4Pi Microscopy

http://www.bu.edu/dbin/ece/web/research/photonics.html

http://www.sciencedaily.com/releases/2006/11/061113180203.htm

Image Interference Microscopy

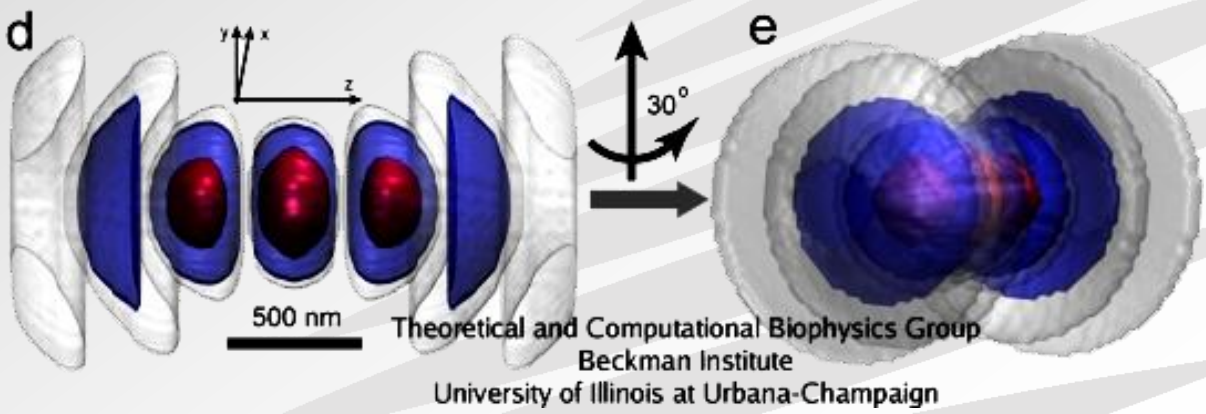
- Image Interference Microscopy is a widefield version of 4Pi.
- This method utilizes full field illumination as opposed to confocal scanning to produce an image.



- XY Resolution: ~250nm
- Z Resolution: ~85nm – 150nm

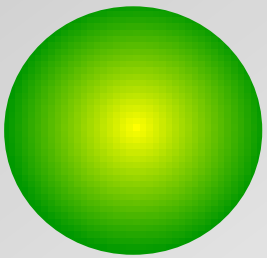
Opposing Objective Methods

- **Systems can yield a 5-9 fold increase in Z resolution**
- No resolution benefit in XY
- Require extremely accurate microscope alignment
- Require mathematical image processing to remove axial “lobes” from the PSF
- Commercially challenging



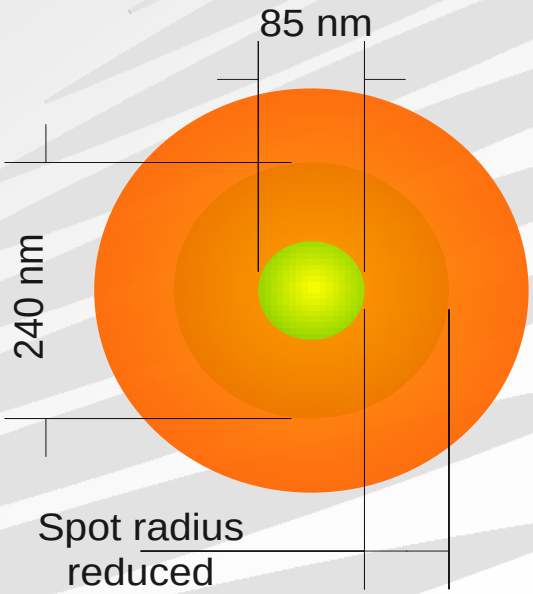
Sub-Diffraction Fluorescence Excitation

- Traditional confocals use the objective to form a diffraction-limited excitation spot
- Excitation with a sub-diffraction spot would produce sub-diffraction emission
- Emission produced could be localized to a smaller volume, increasing resolution

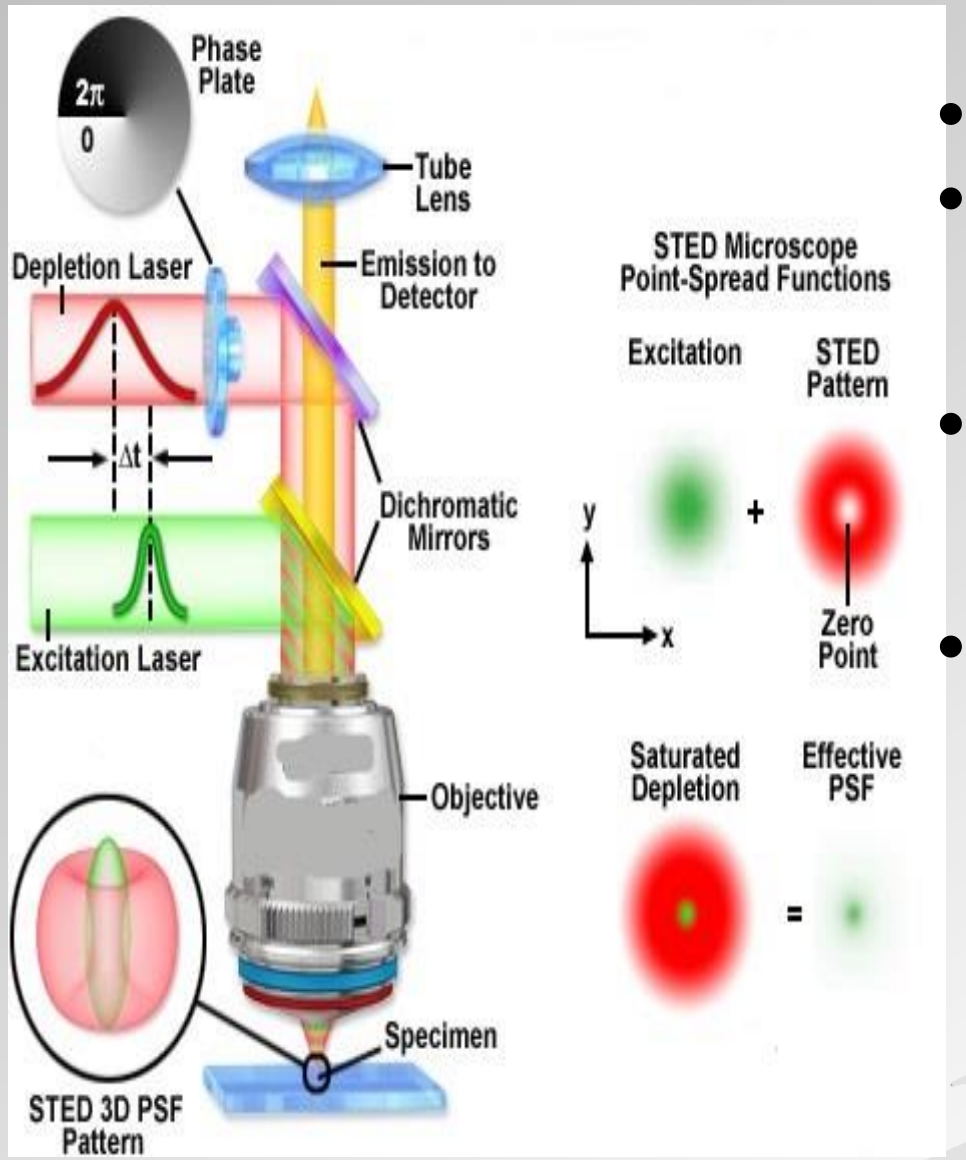


Diffraction Limited Illumination Spot

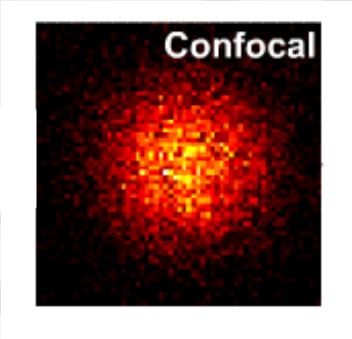
Emission Depletion Source

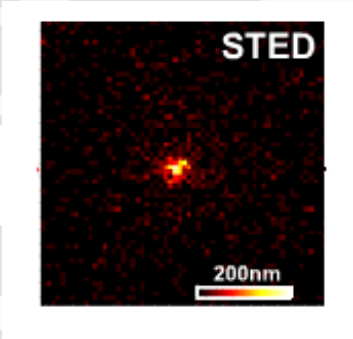


Emission Depletion

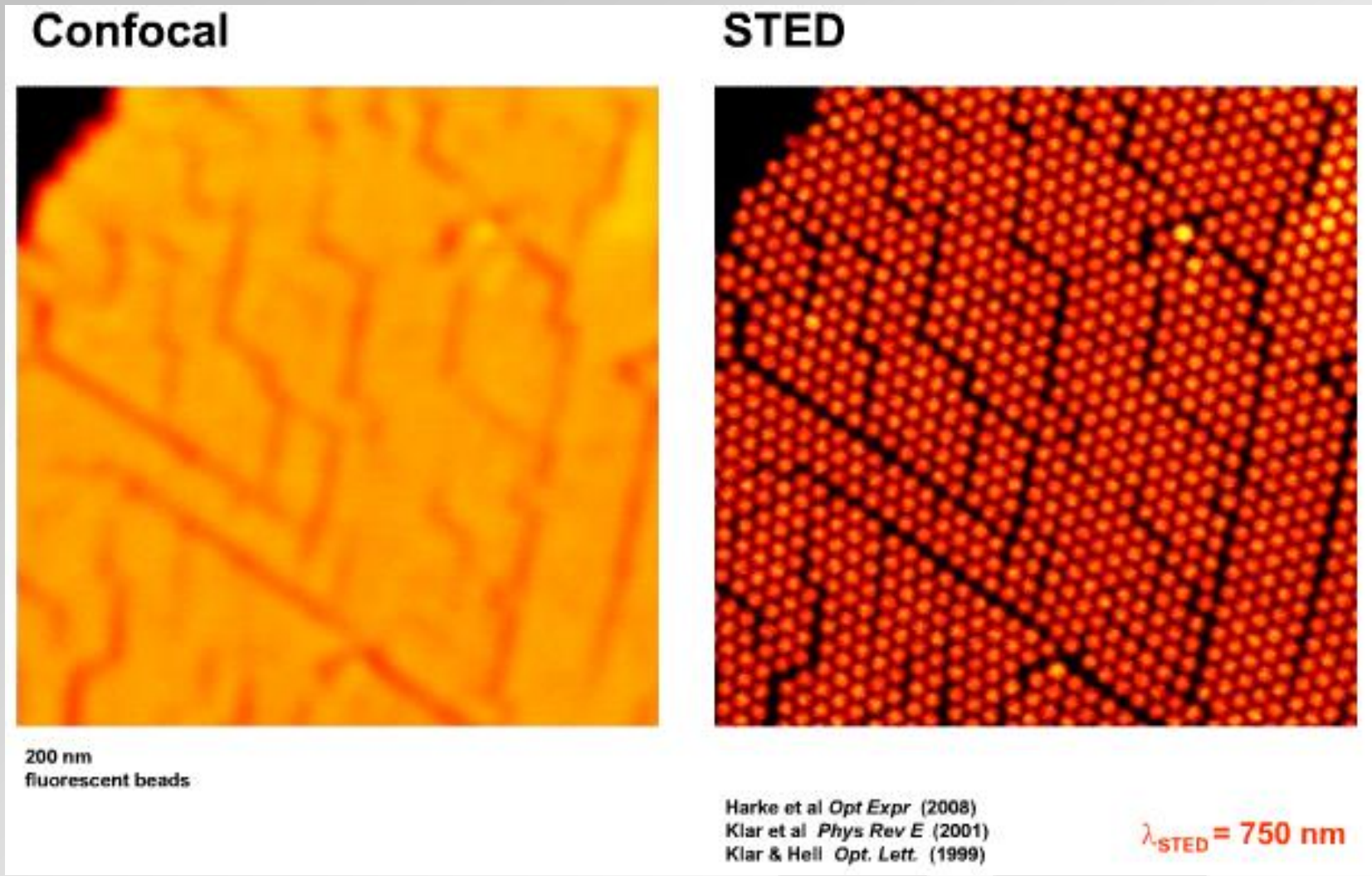


- Normal fluorescence excitation
- Emission depletion doughnut
  - 200 pico-second pulses of light
  - Close to probe's emission
- Emission light donut causes probe to return to ground state without photon emission
- The only emitted photons come from probes illuminated in the donut hole

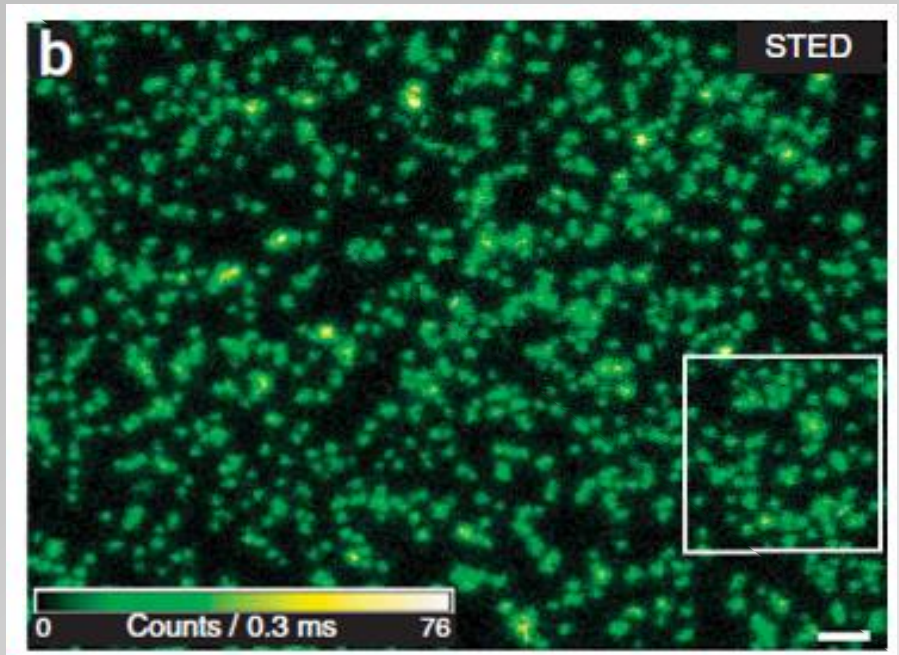




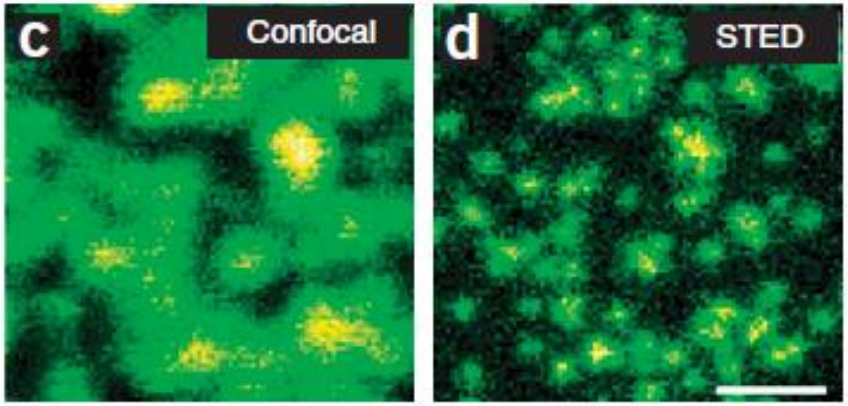
STED Results

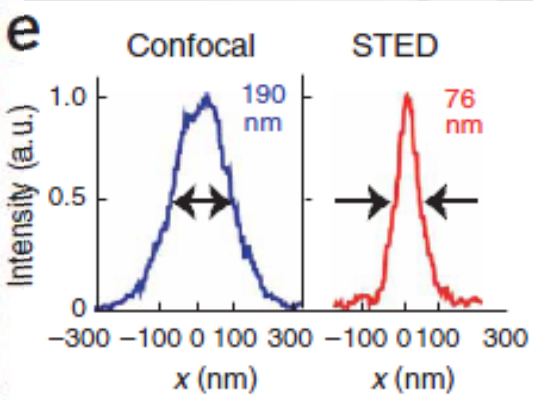


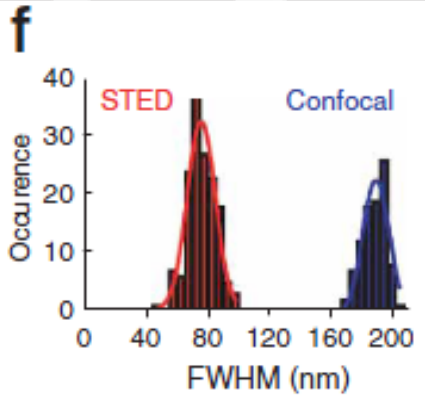
STED Results



- Z Resolution ~ 100nm
- XY Resolution ~100nm
- **High photobleaching**
- **High phototoxicity**



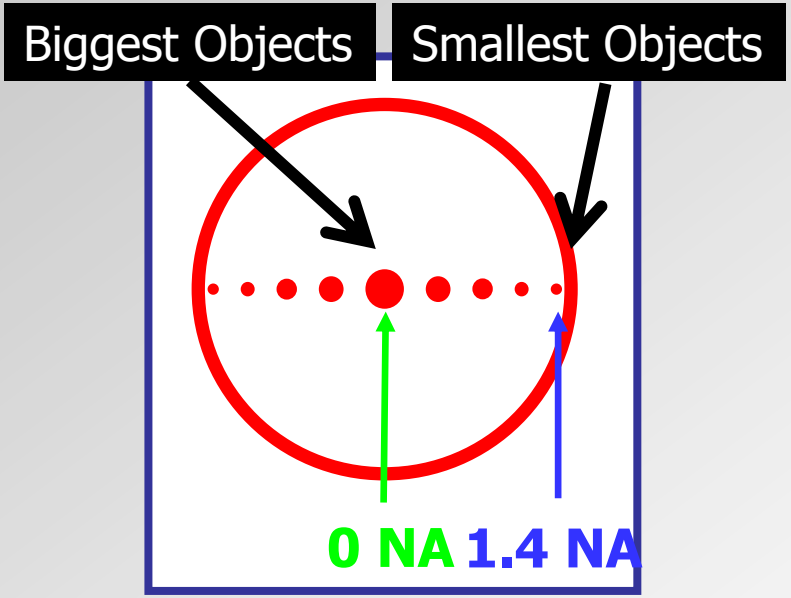


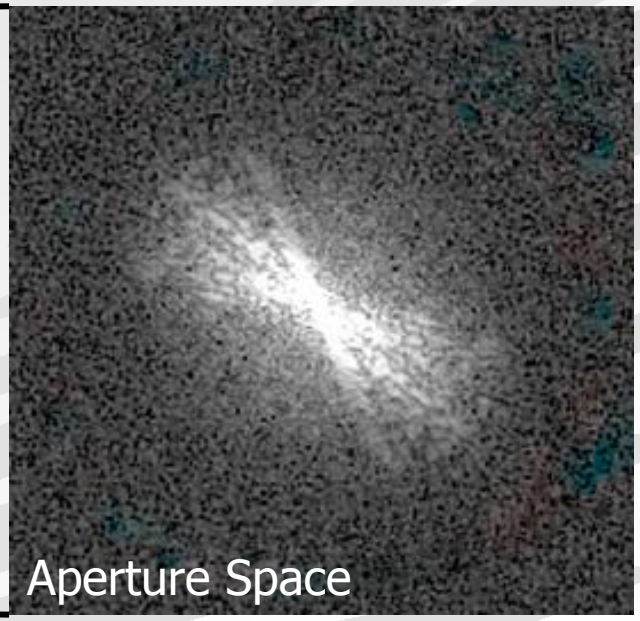


Nanoscale resolution in GFP-based microscopy Katrin I Willig, Robert R Kellner, Rebecca Medda, Birka Hein, Stefan Jakobs & Stefan W Hell
NATURE METHODS VOL.3 NO.9 |SEPTEMBER 2006

Extending Fourier Space

- Resolution is limited by NA
- More back aperture = more NA

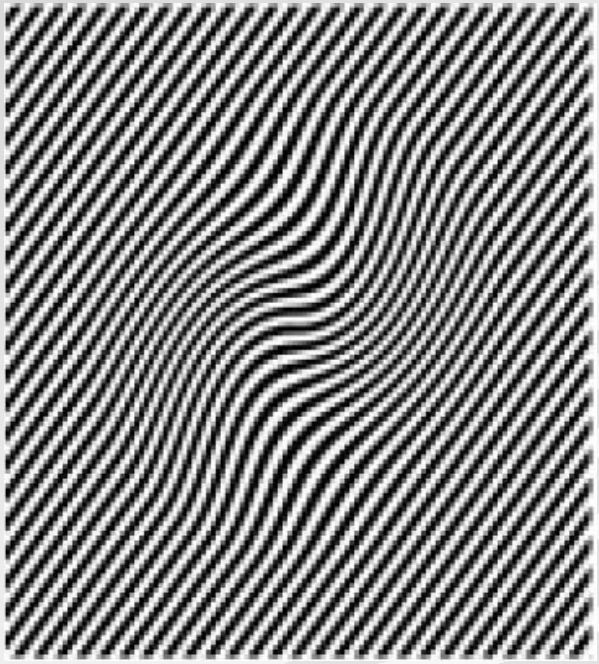




Images From - Mats Gustafsson - UCSF

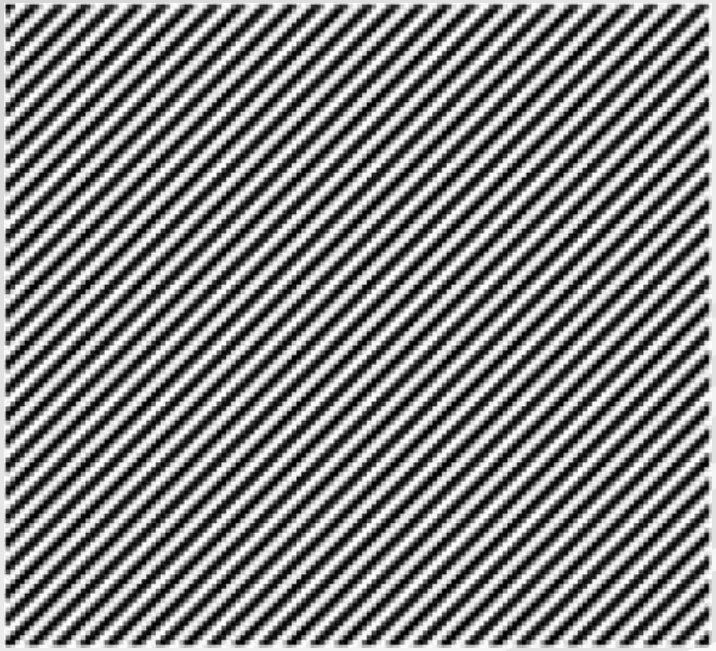
SIM Principle

Unknown Sample Structure



SIM Principle

Unknown Sample Structure
+ Known Illumination Pattern



SIM Principle

Unknown Sample Structure

+ Known Illumination Pattern

Moiré Fringes (Known Structure)

NA of Moire = NA of Sample – NA of Pattern

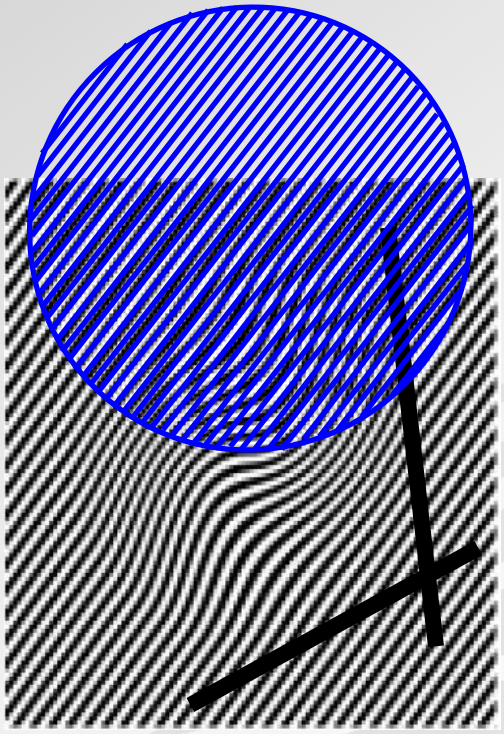
Phase Shifts

Moire Fringes generated at diffraction limit

High spatial frequency (smallest) objects affected most by shift

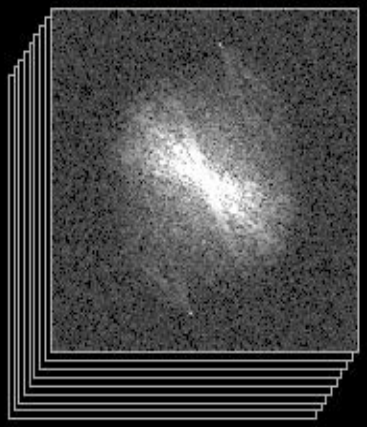
Low spatial frequency (largest) objects affected least by shift

Orientations of pattern and objects matters!

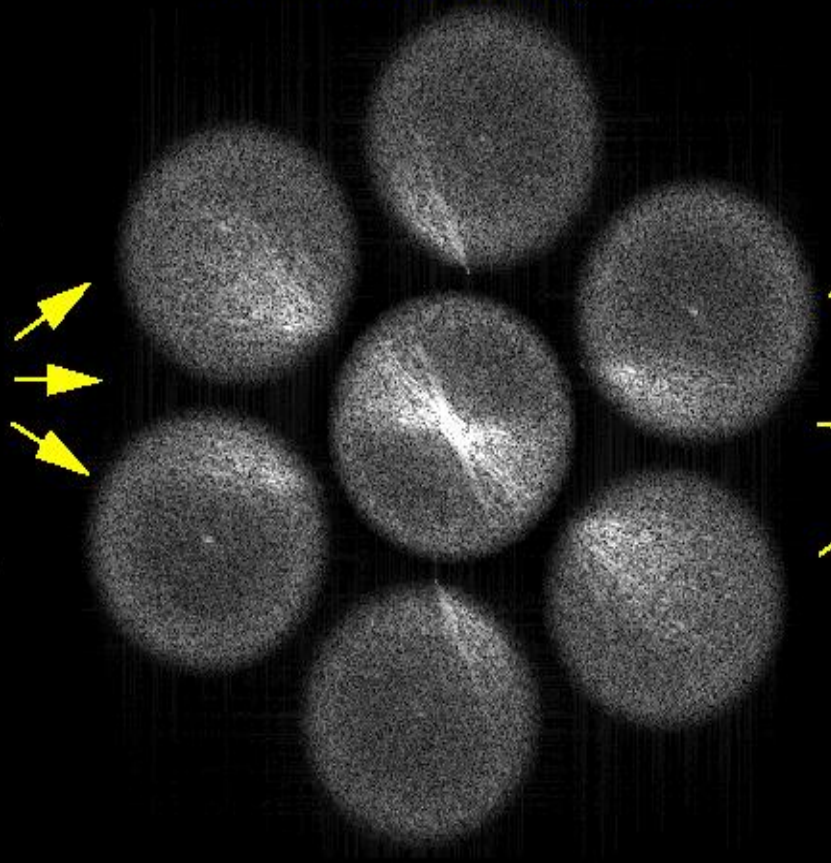


Reconstruction in reciprocal space

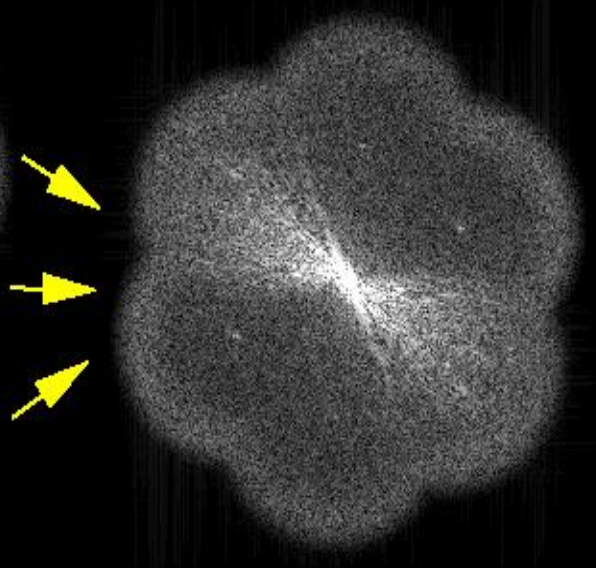
Acquire
images with
3 pattern angles
x 3 phases.



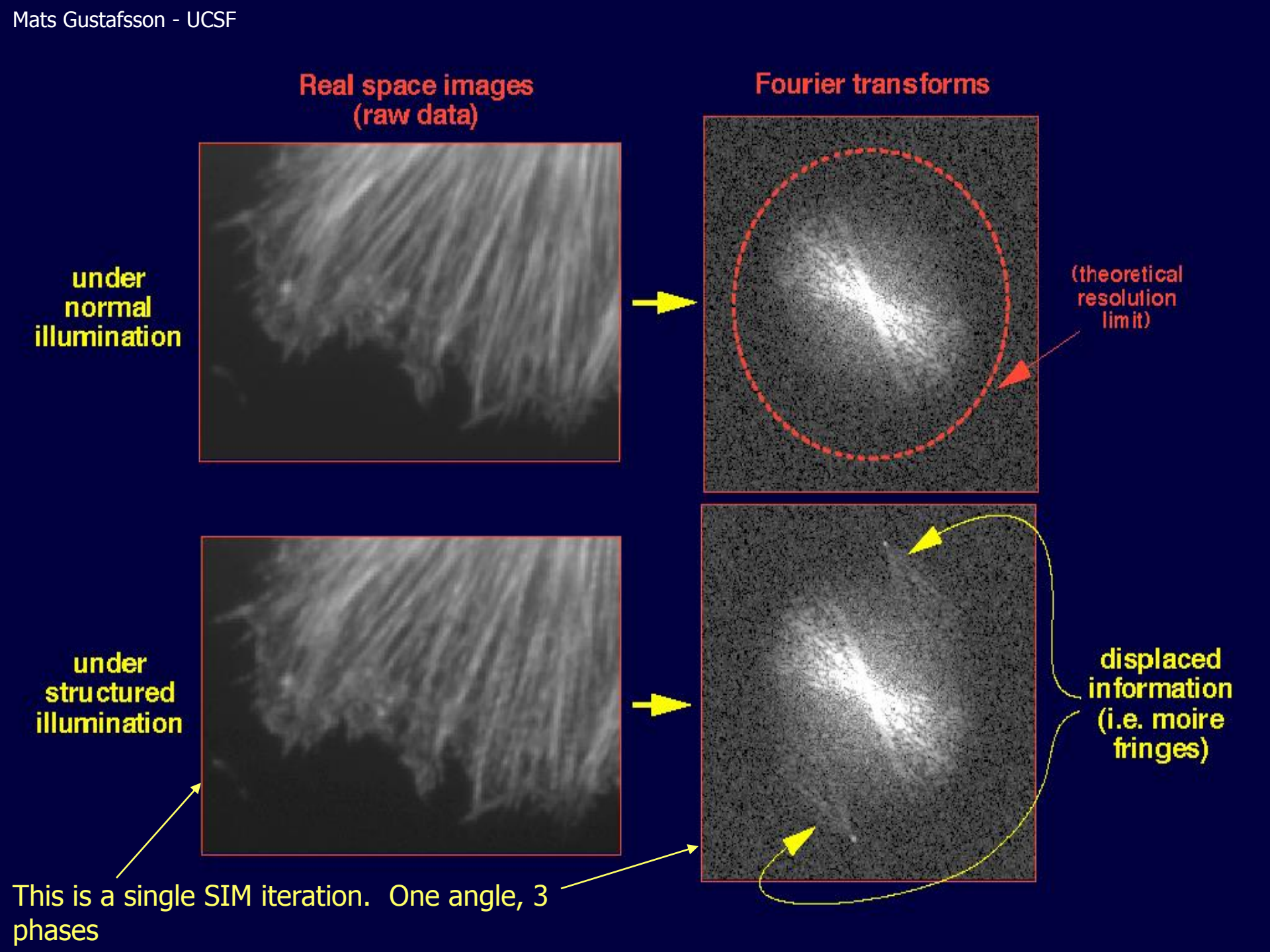
Separate
the 7 information components



Reassemble
into an extended
resolution image



Mats Gustafsson - UCSF



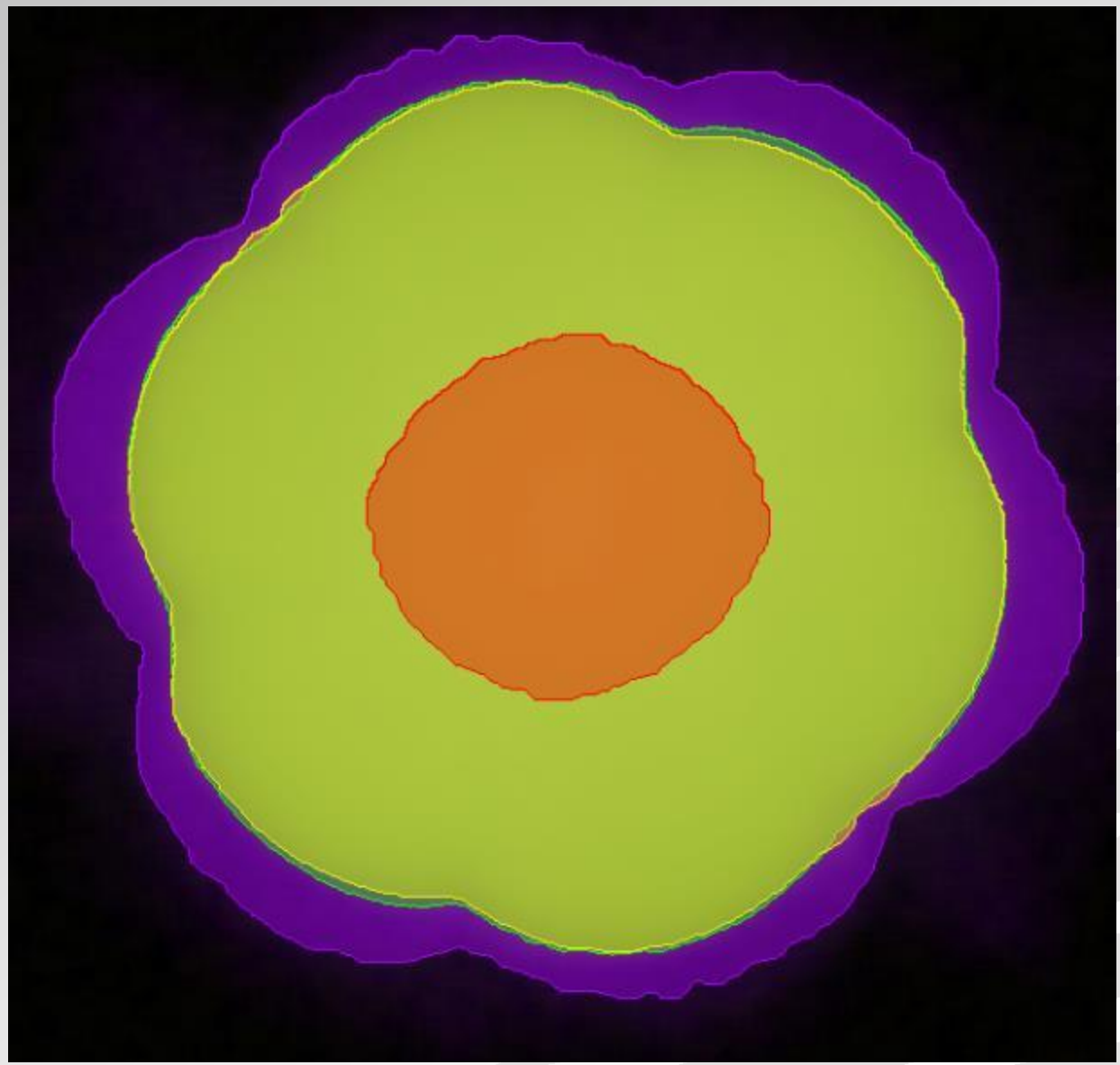
Fourier Space Size Comparisons

TIRF SIM

2D SIM

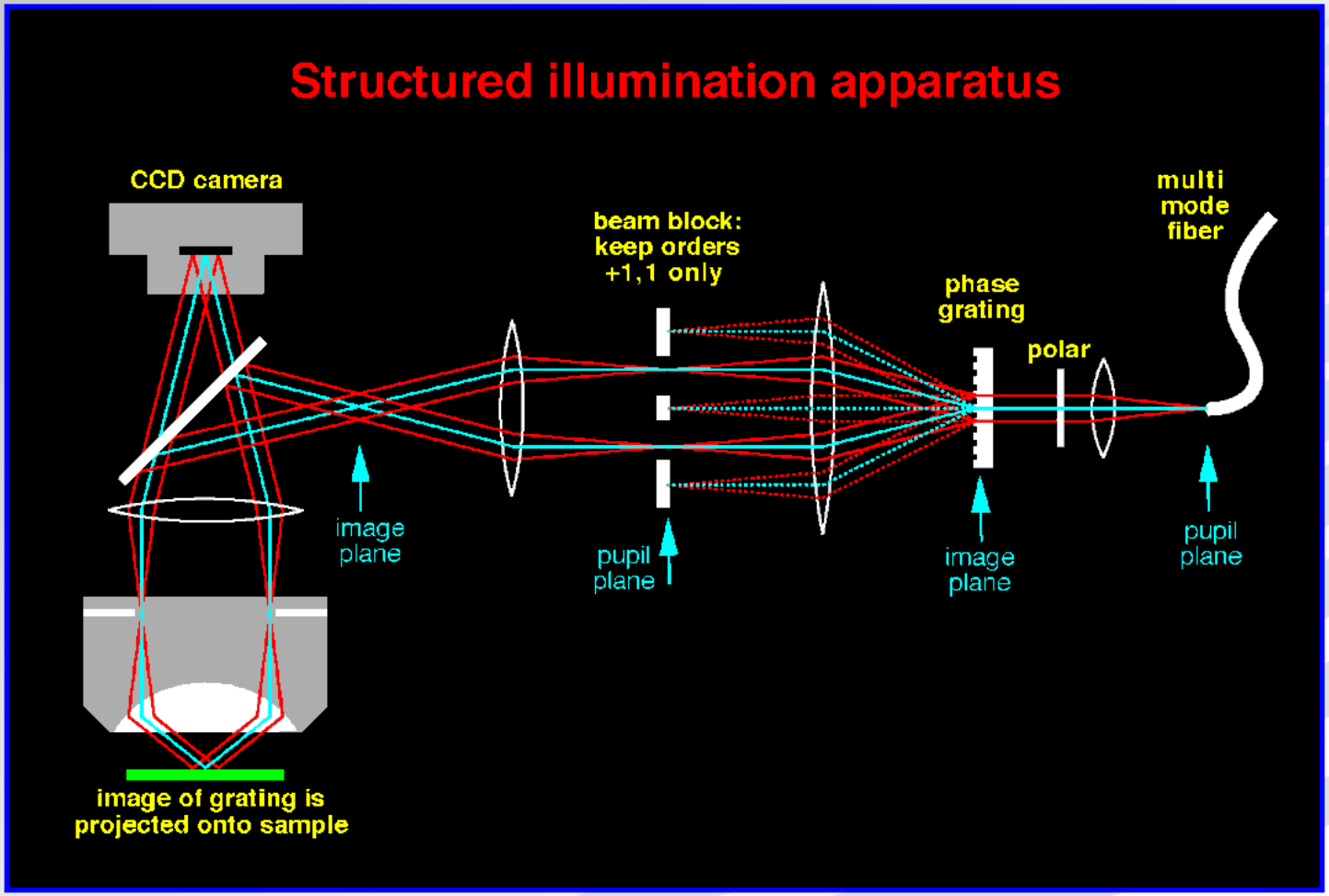
3 D SIM

Wide-Field

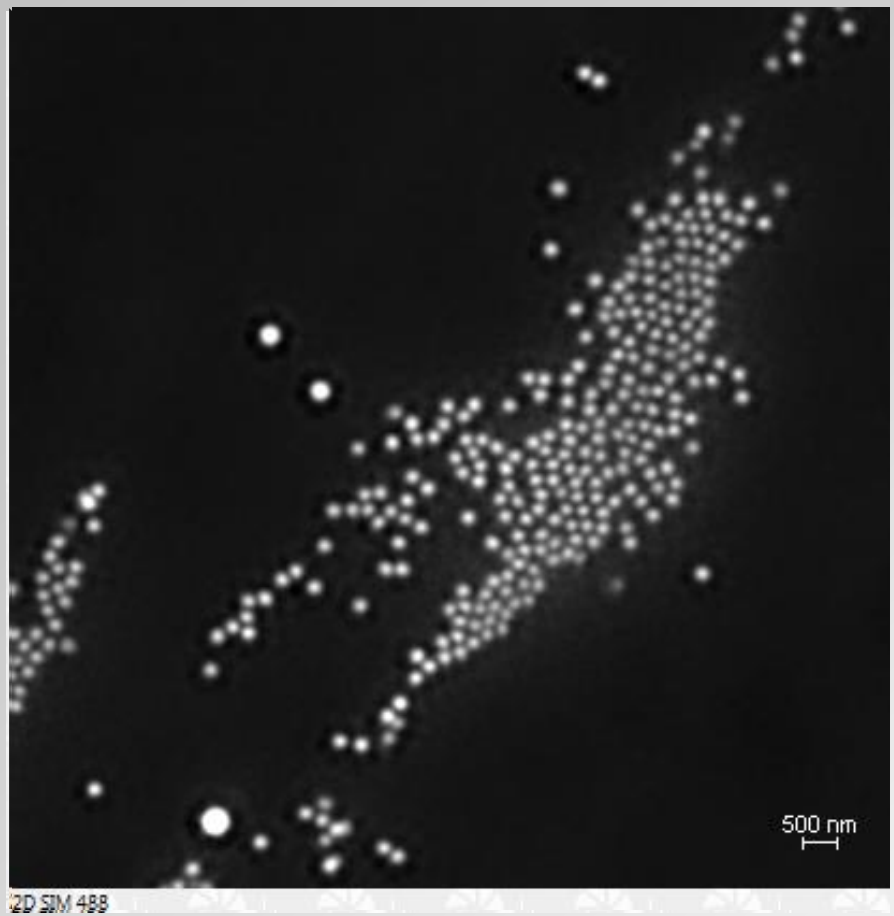


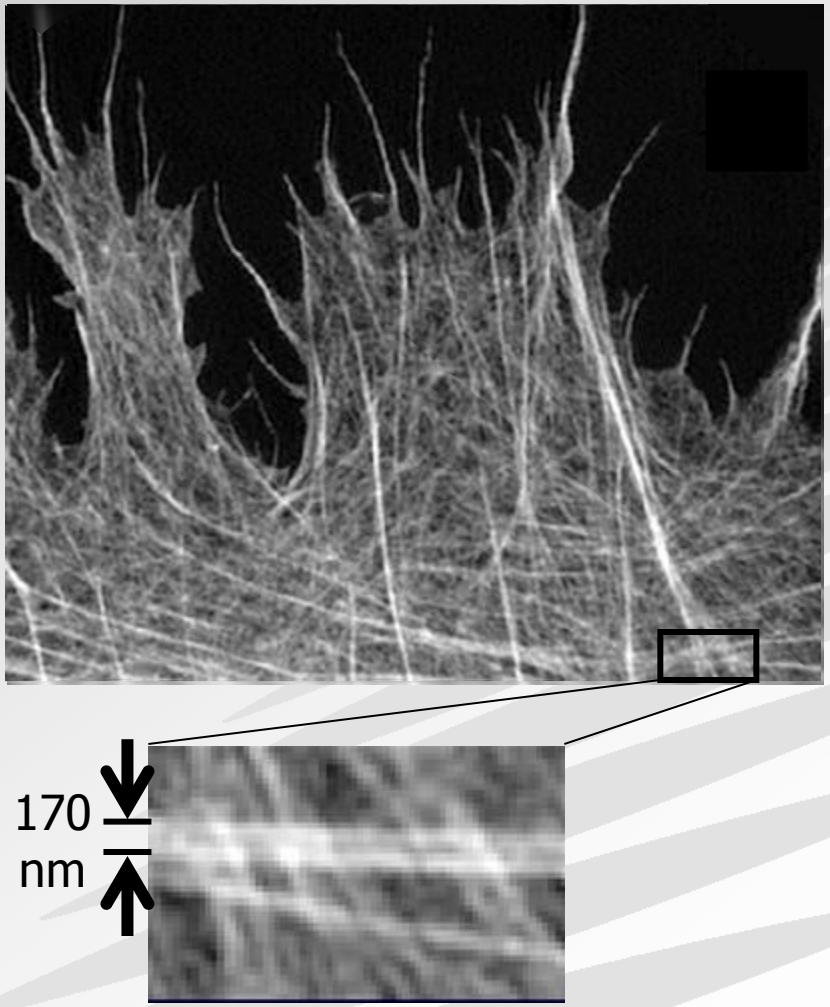
2D SIM

- Suitable for thin samples.
- Can be combined with TIRF to limit Z optical section



2D SIM Results



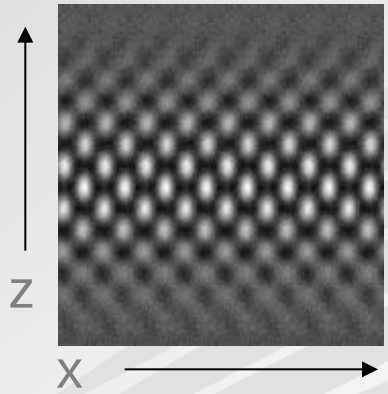


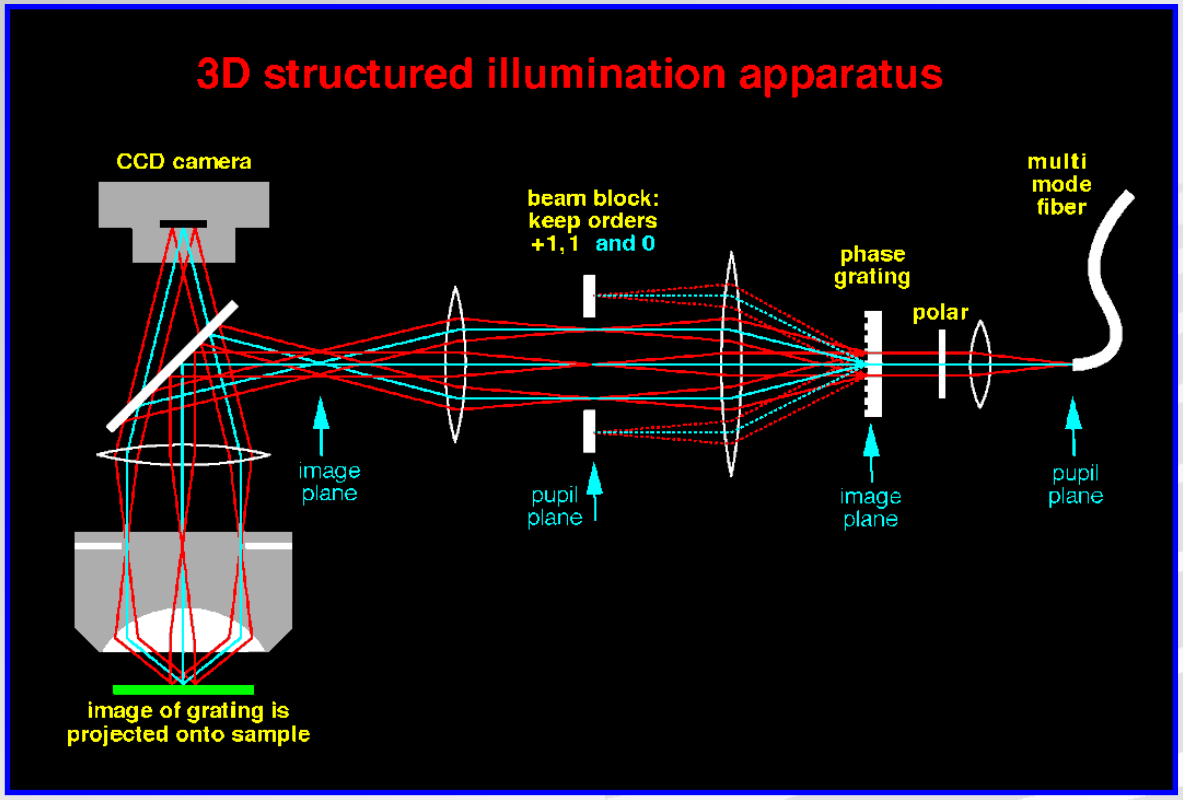
- 200nm beads 488nm
- 2D SIM vs Wide-field

Images From - Mats Gustafsson - UCSF

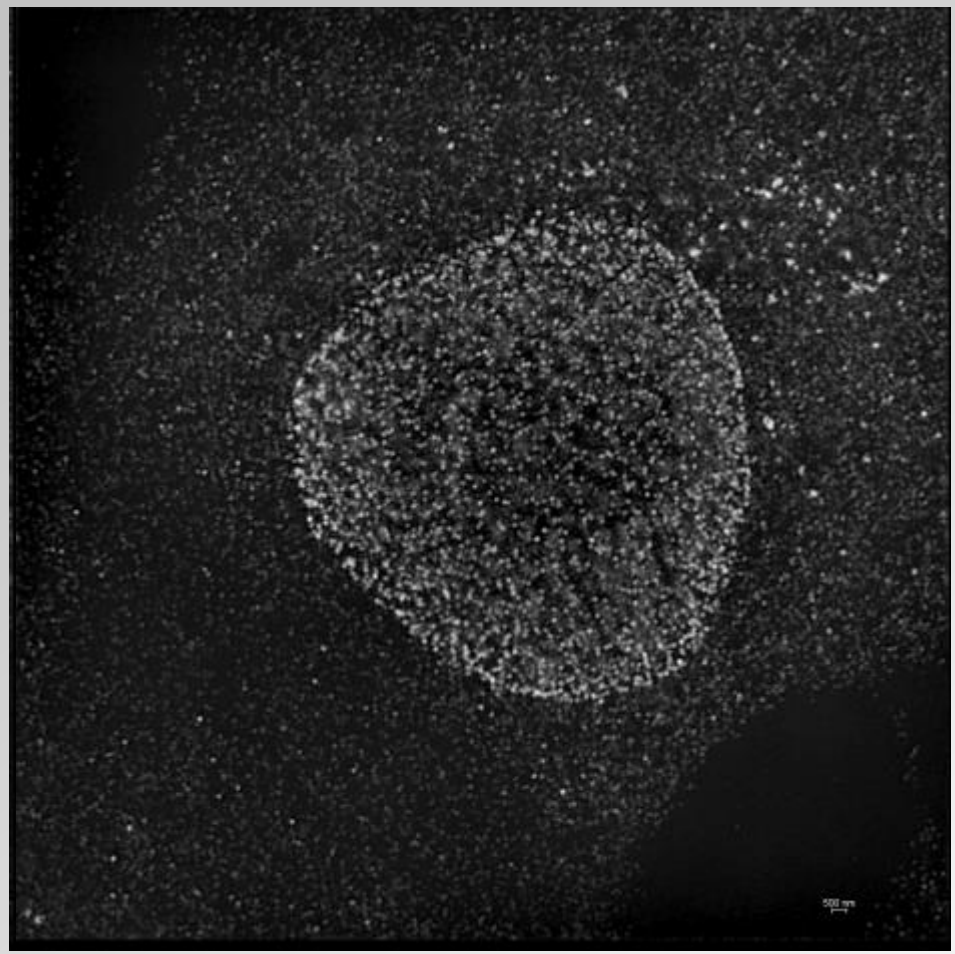
3D SIM

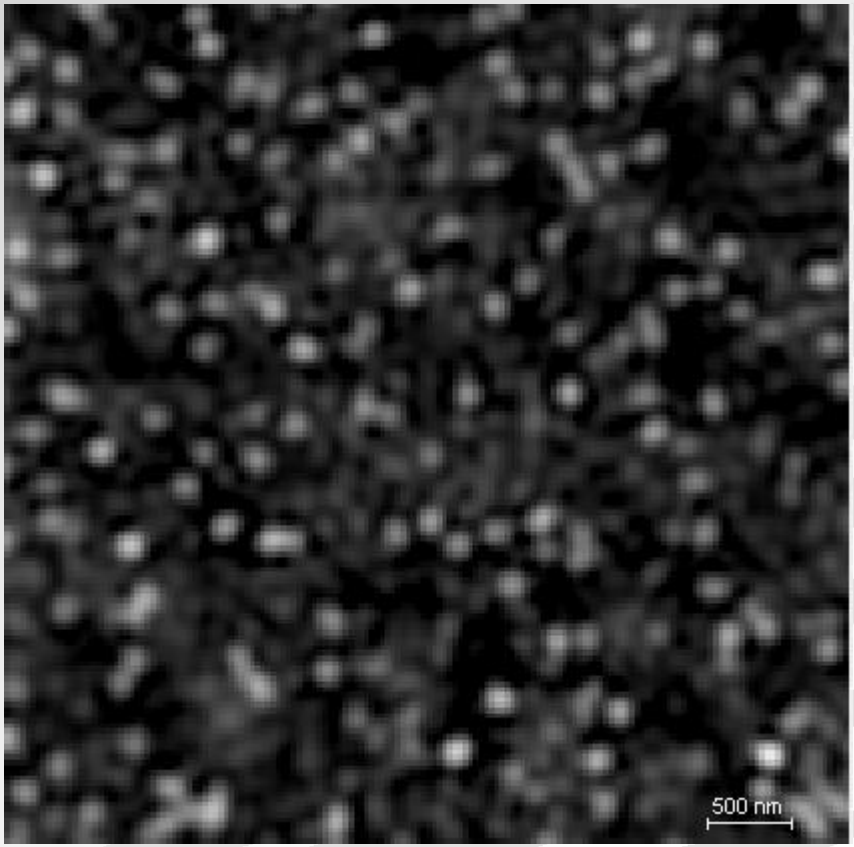
- Similarly, light can be patterned in three dimensions for 3D SIM
- Yields maximum axial information.
- Single Z Plane of 3 grid angles x 5 grid phases (15 images)





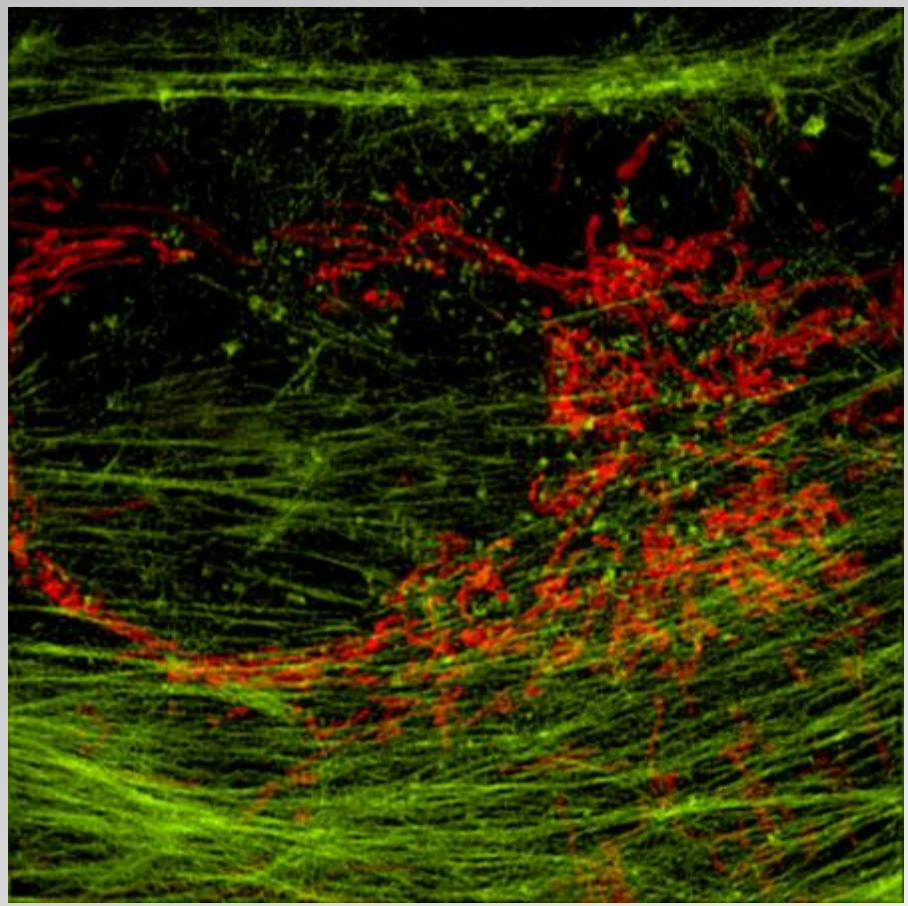
3D SIM Results



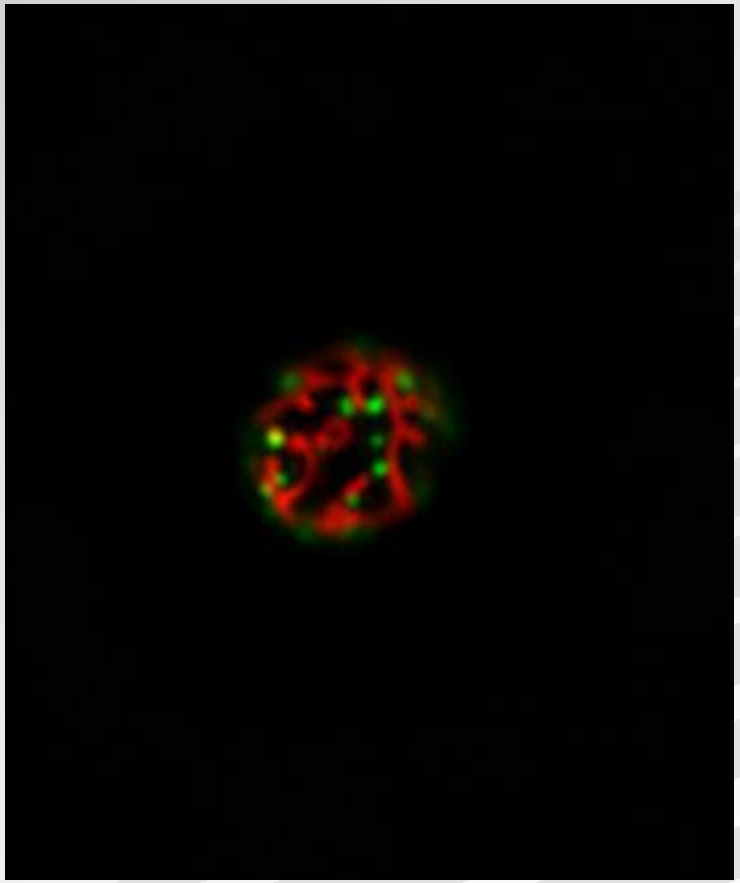


- NuclearPore Complex Protein (AF 488)

3D SIM Results

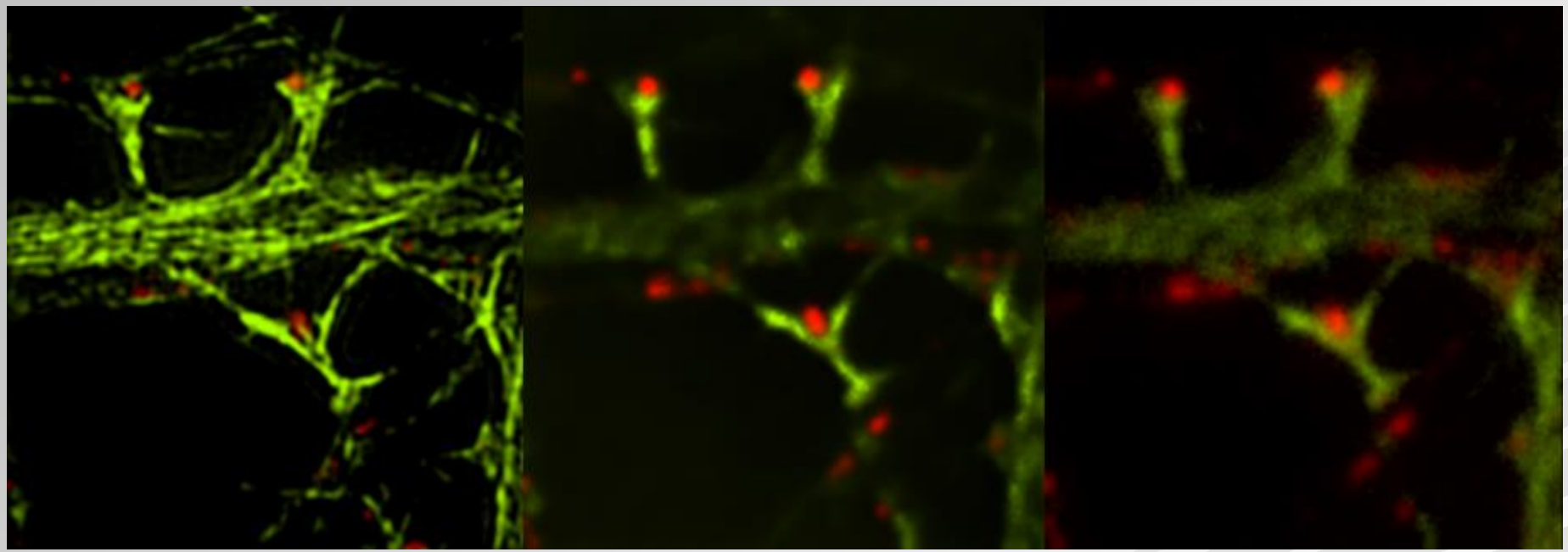


Opossum Kidney Cells
AF 488 phalloidin and
mitotracker red.



- Yeast Cells
- Mitocondria (Red)
- ER (green)

3D SIM vs Widefield Deconvolved



Images courtesy of Bassell Lab – Emory University

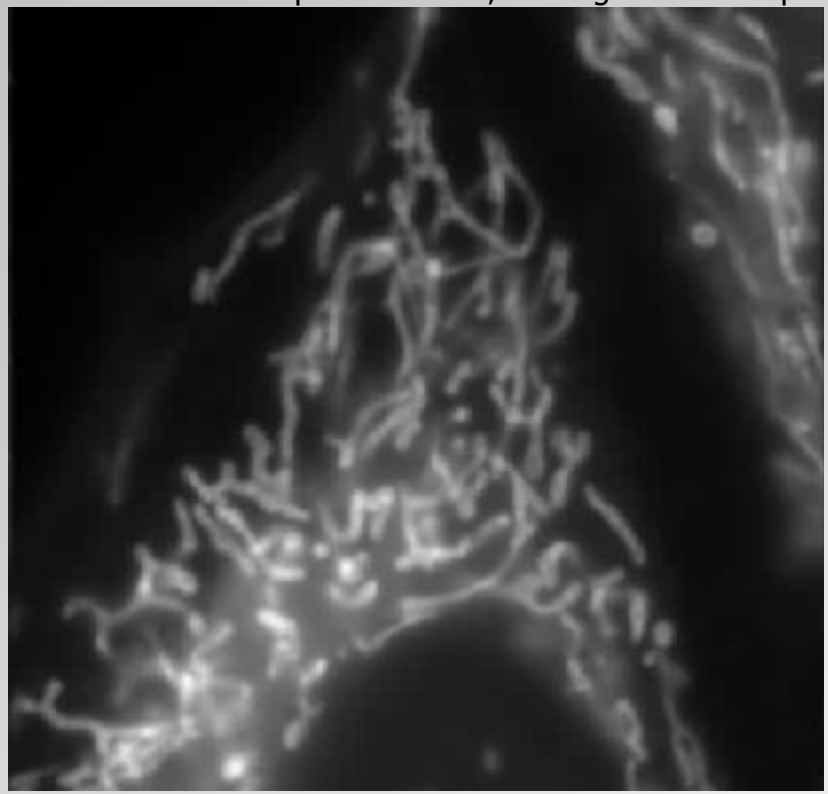
- Alexa 488 labeled microtubules and Alexa 561 labeled synapsin

- 3D SIM Left, Deconvolution Center, Widefield Right

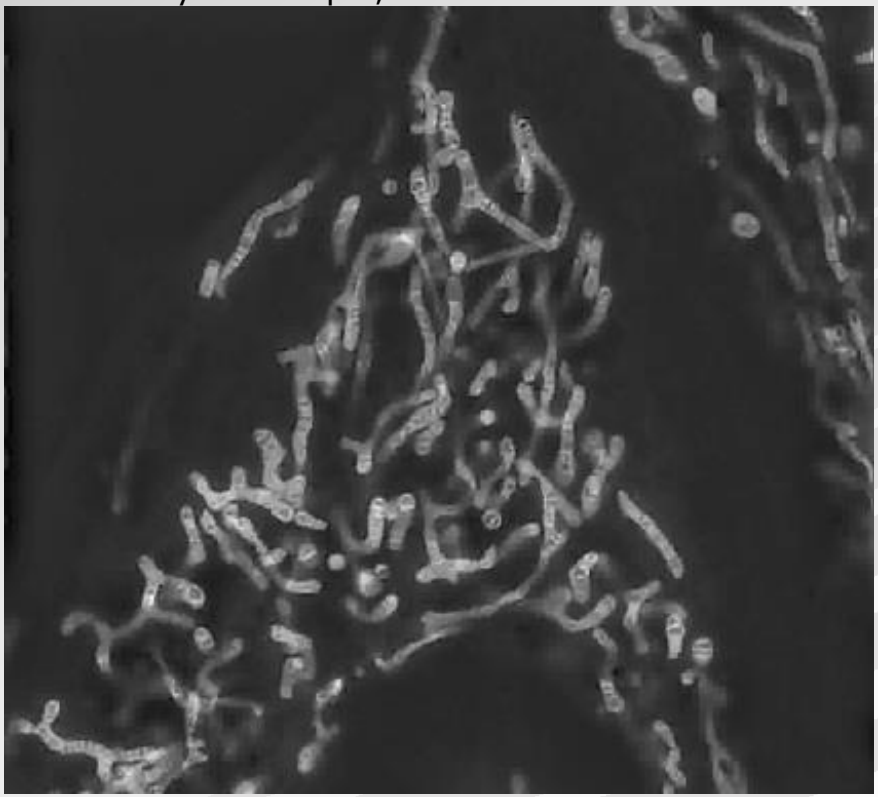
Applications of N-SIM: Live Cell Imaging

Live Cell - NIH3T3 Mitochondria -MitoTracker Red-Timelapse

Exposure: 64ms,15 images. Total acq. time: 1.8s. 5-cycle timelapse, 1s interval

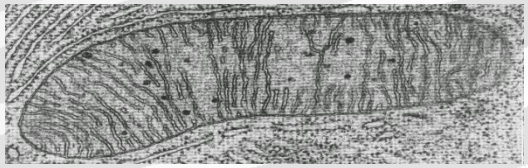


Conventional



N-SIM

Sample thickness up to 20µm

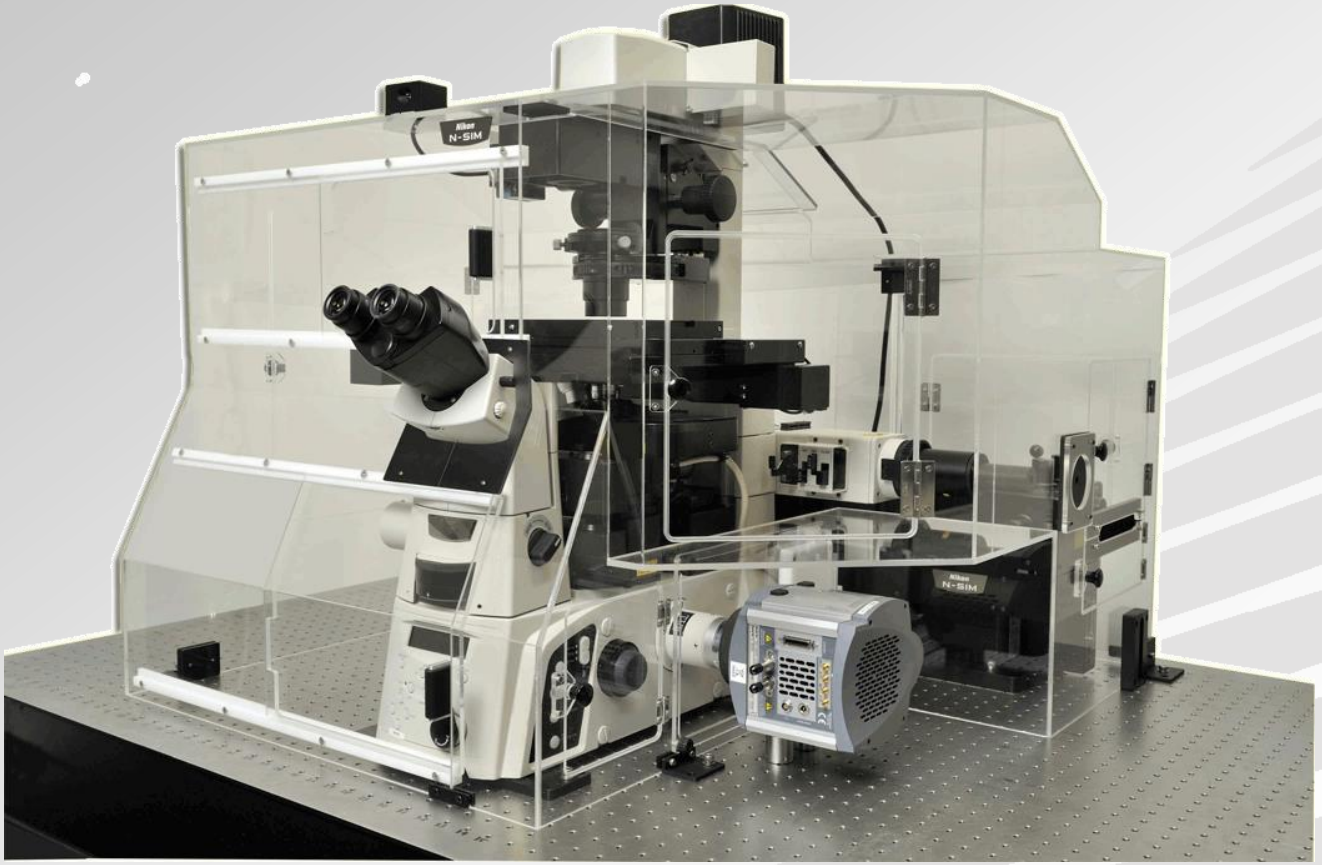


Mitochondrial cristae are now visible

N-SIM

Sim Results

- **XY Resolution: 85-110 nm**
- **Z Resolution: ~300nm**



Breaking the Limit

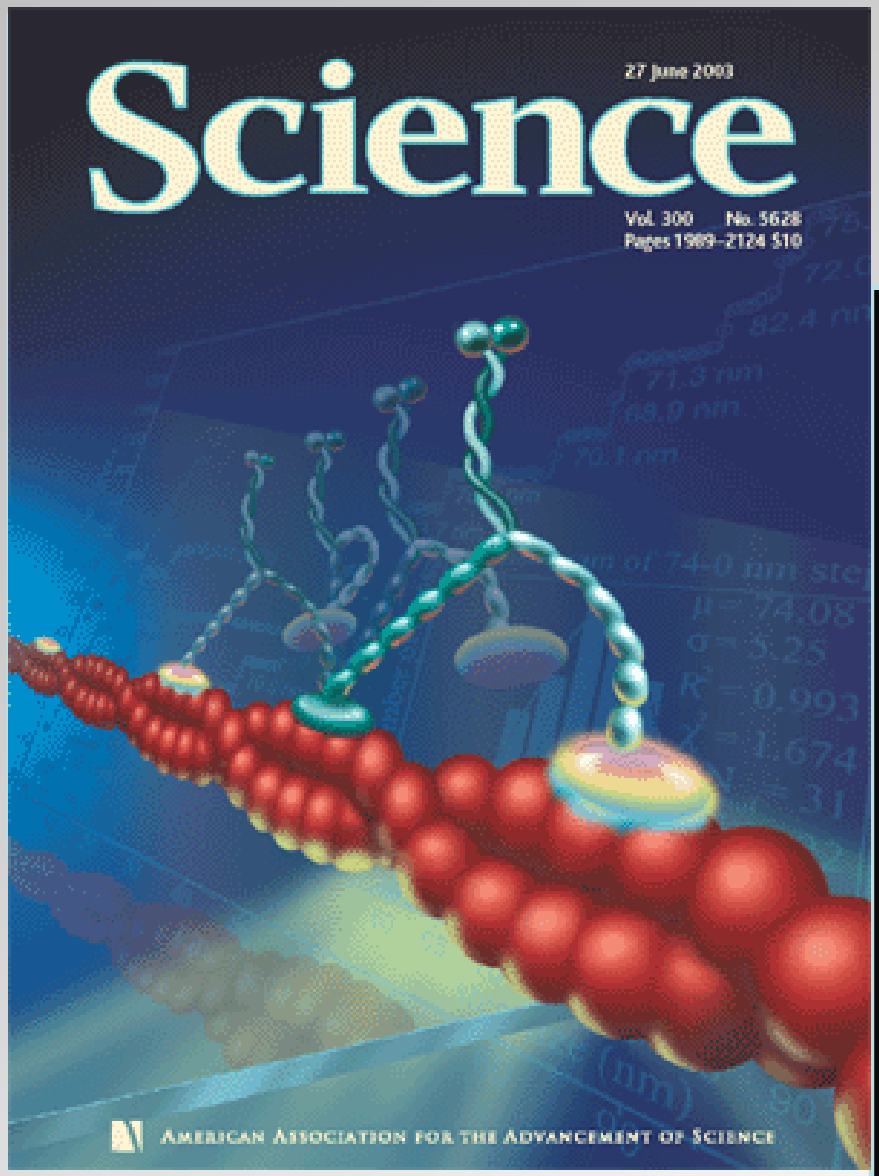
I. Structural Super Resolution "PSF Engineering"

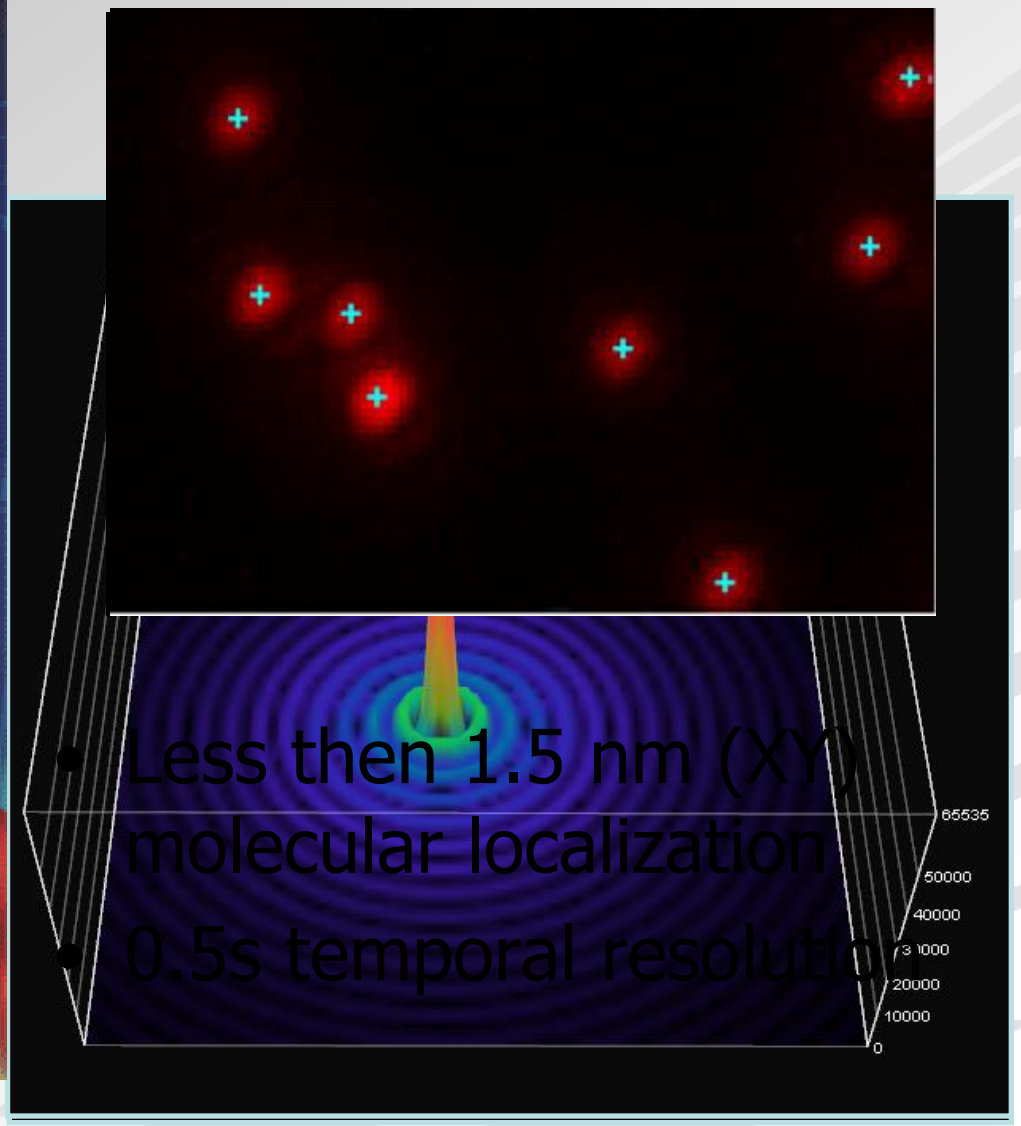
4Pi	
I^5M	Image Interference Microscopy
STED	Stimulated Emission Depletion
SIM	Structured Illumination Microscopy

II. Single Molecule Localization "PSF Mapping"

FIONA	Fluorescence imaging with one-nanometer accuracy
STORM	Stochastic optical reconstruction microscopy
PALM	Photoactivated light microscopy
GSDIM	Ground state depletion & individual molecule return

FIONA: Breaking the Limit





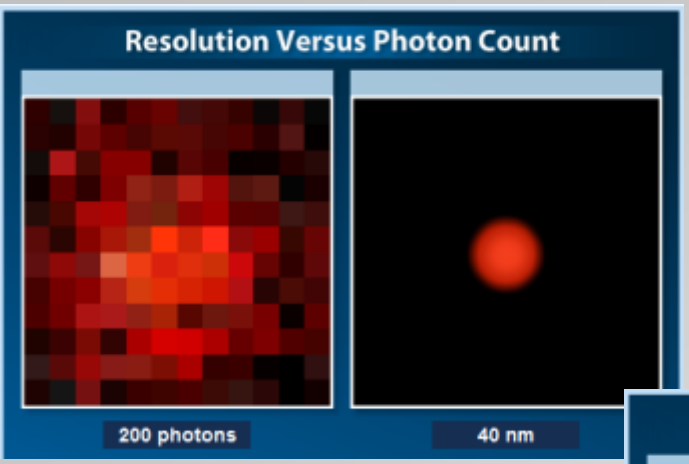
- Less then 1.5 nm (XY) molecular localization
- 0.5s temporal resolution

FIONA

S/N (Center) = width/$\sqrt{N}$

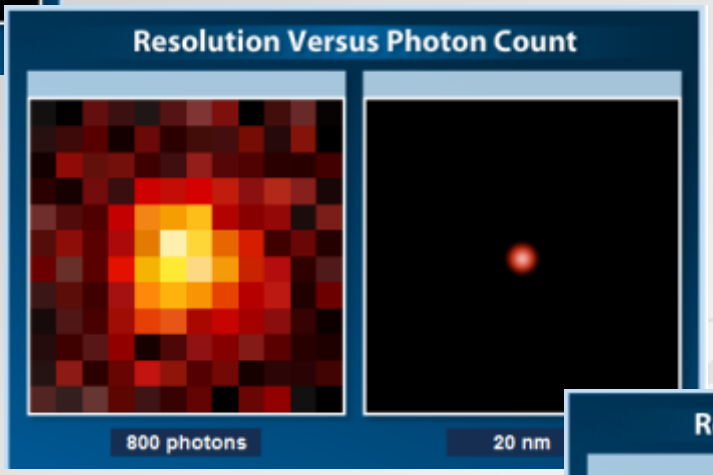
N= number of Photons

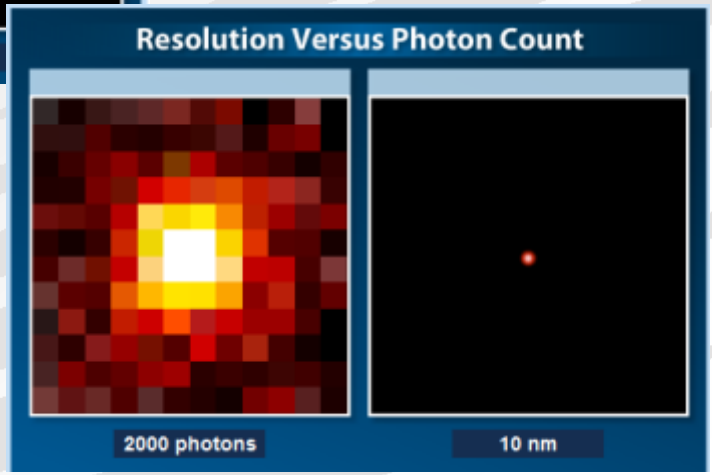
Accuracy of Localization



The Gaussian fit is a probability as to where the single molecule is located.

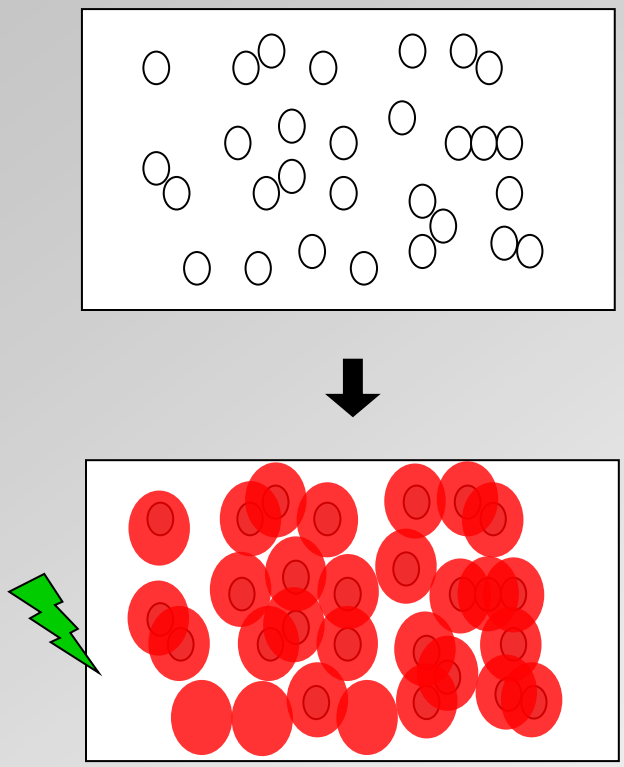
The greater the number of photons the more accurate the localization

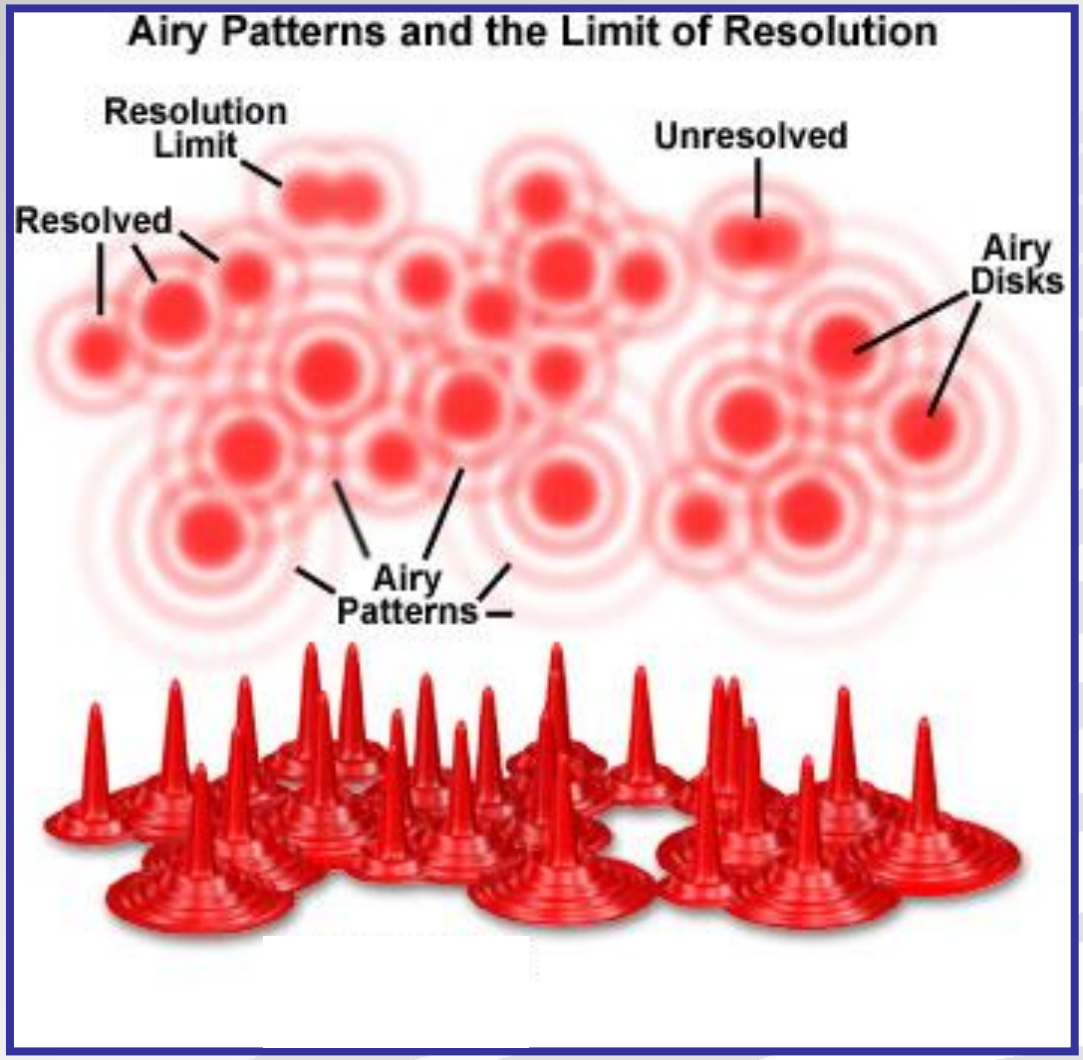




Localization Microscopy Principle

Conventional Fluorescence

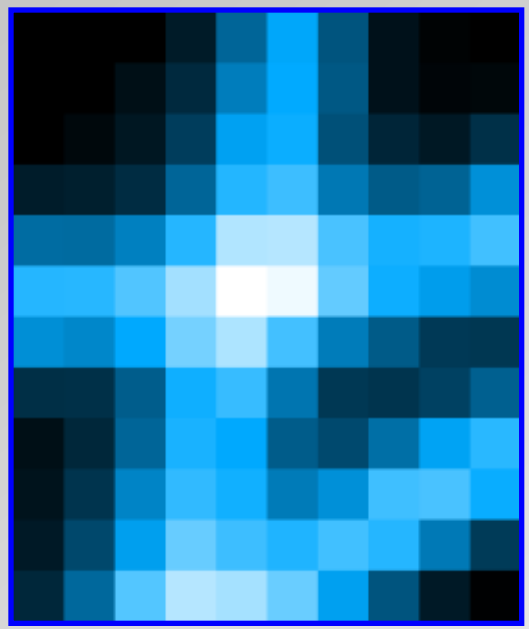




Super-Resolution by Localization

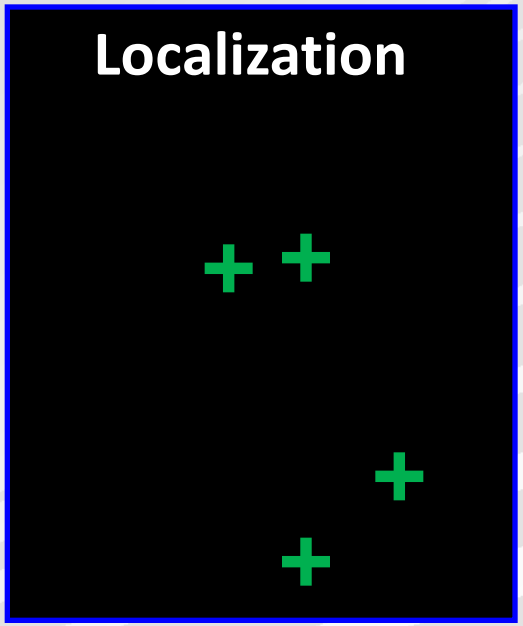
Photo-switchable probes are capable of moving from a "dark state" to an emitting state through the use of high energy light sources.

Conventional fluorescence



Raw images

STORM Image



2x real time

Stochastic Optical Reconstruction Microscopy = STORM

N-STORM

Rust, Bates & Zhuang, Nat. Methods, 2006
Bates, Huang, Dempsey & Zhuang, Science, 2007

Emission Isolation Localization

Spontaneous Activation (d-STORM/GSDIM/PALM*)

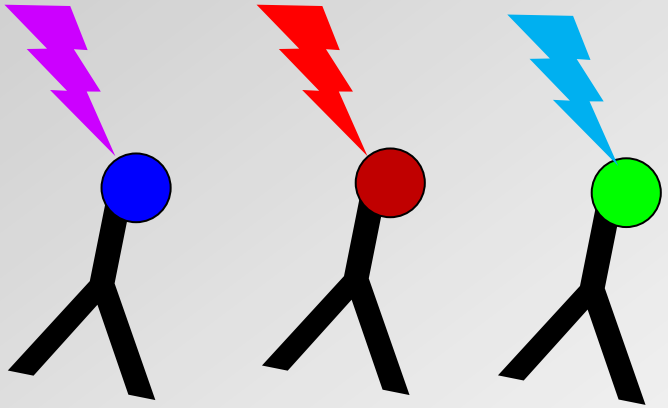
- Reporter(s) kept in the "Dark State"
- Spontaneous activations

Triggered Activation (n-STORM)

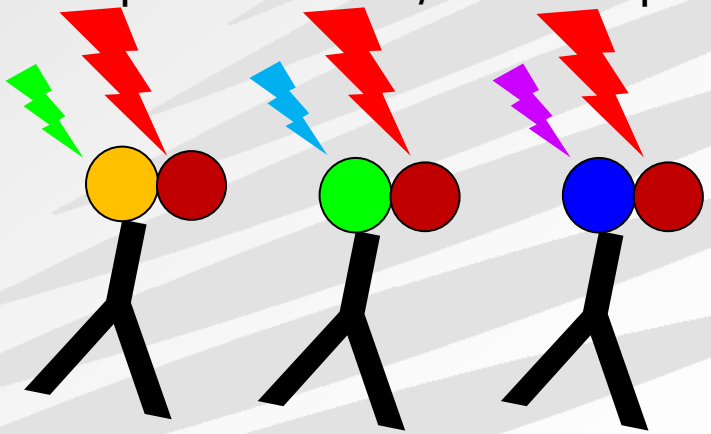
- Reporter kept in the "Dark State"
- Triggered activations

Multi-Color

- Multiple Reporters (no activators)

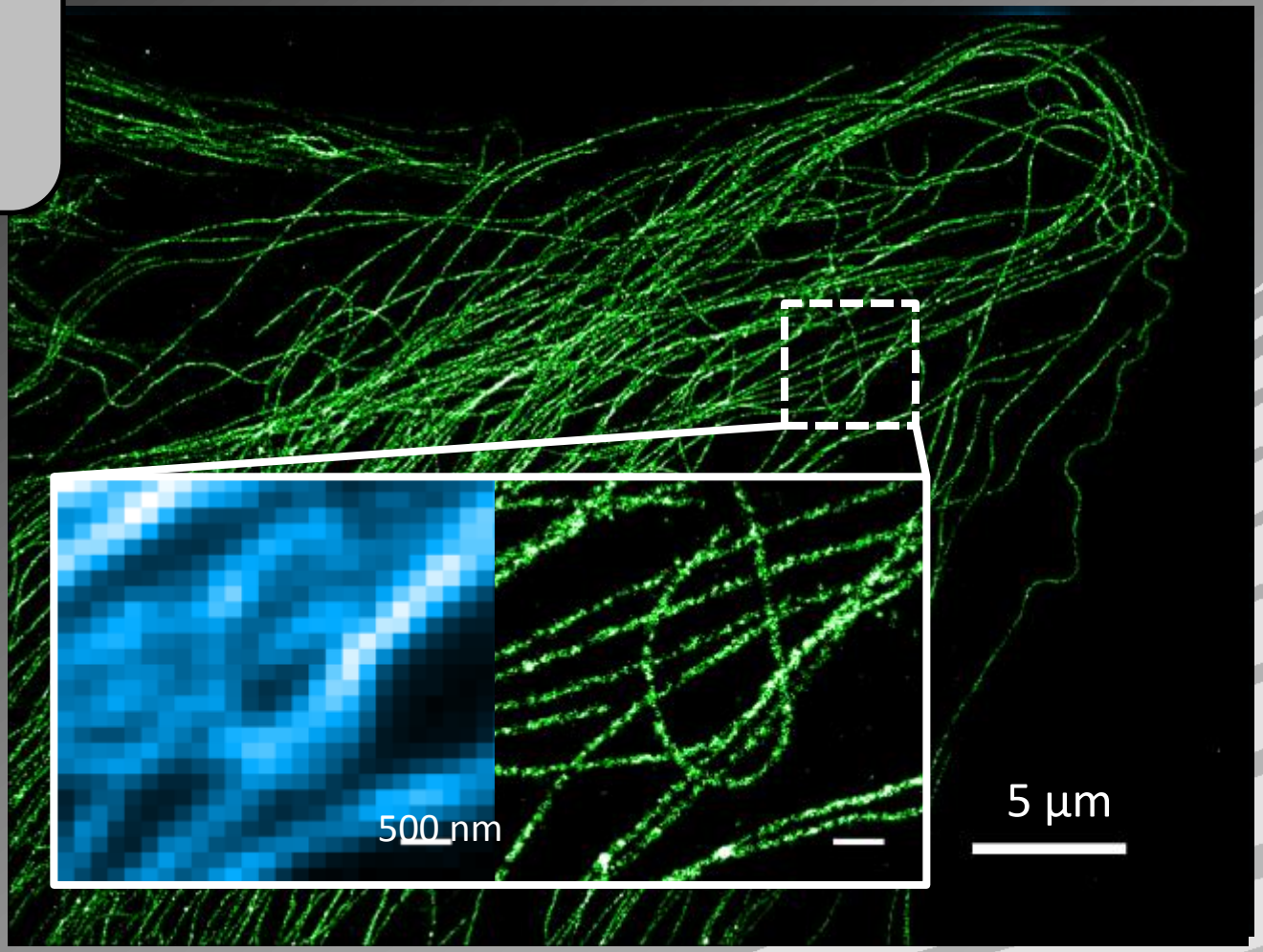


- Multiple Activators / Same Reporter



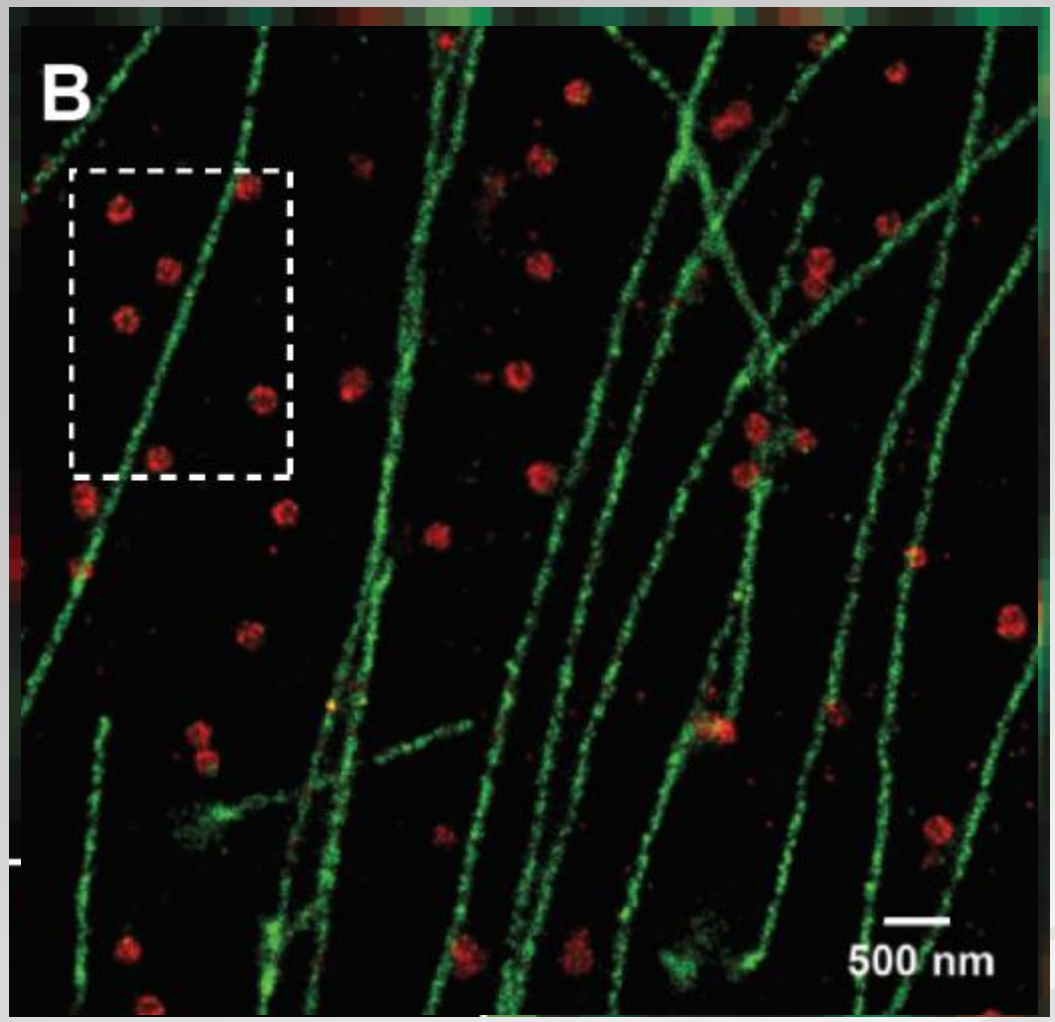
* Genetically encoded probes

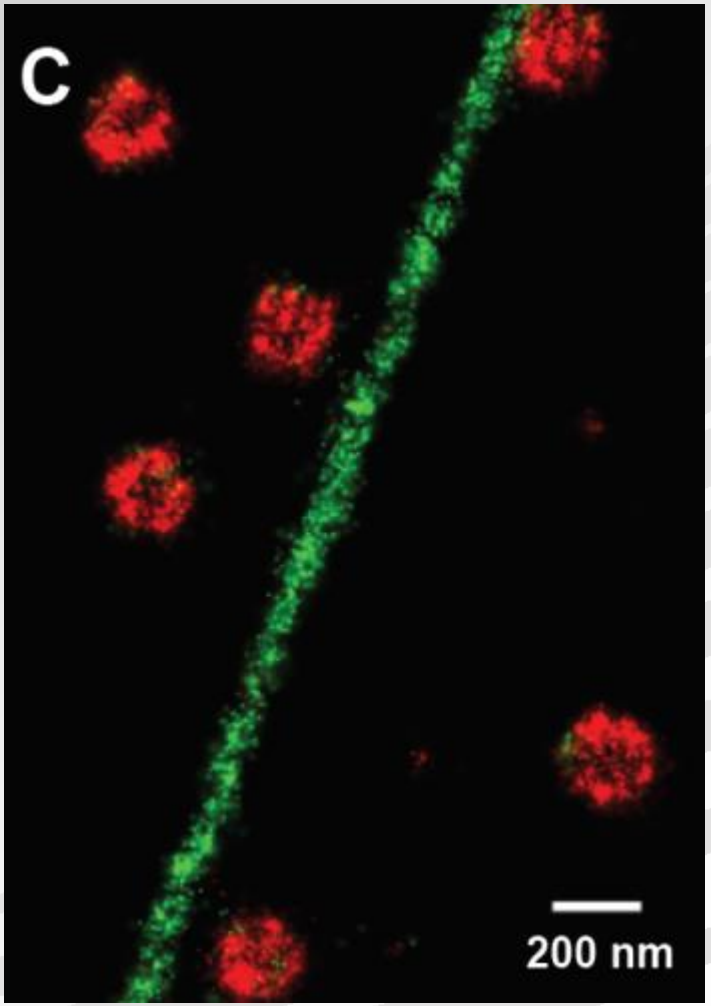
Localization Microscopy



B-SC-1 cell, anti-β tubulin, Alexa 647

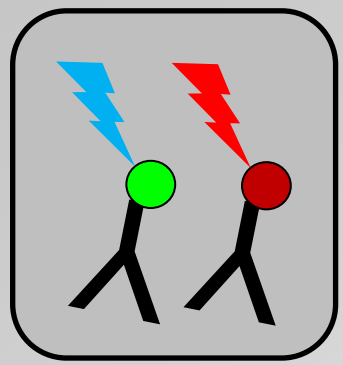
Multiple Color n-STORM

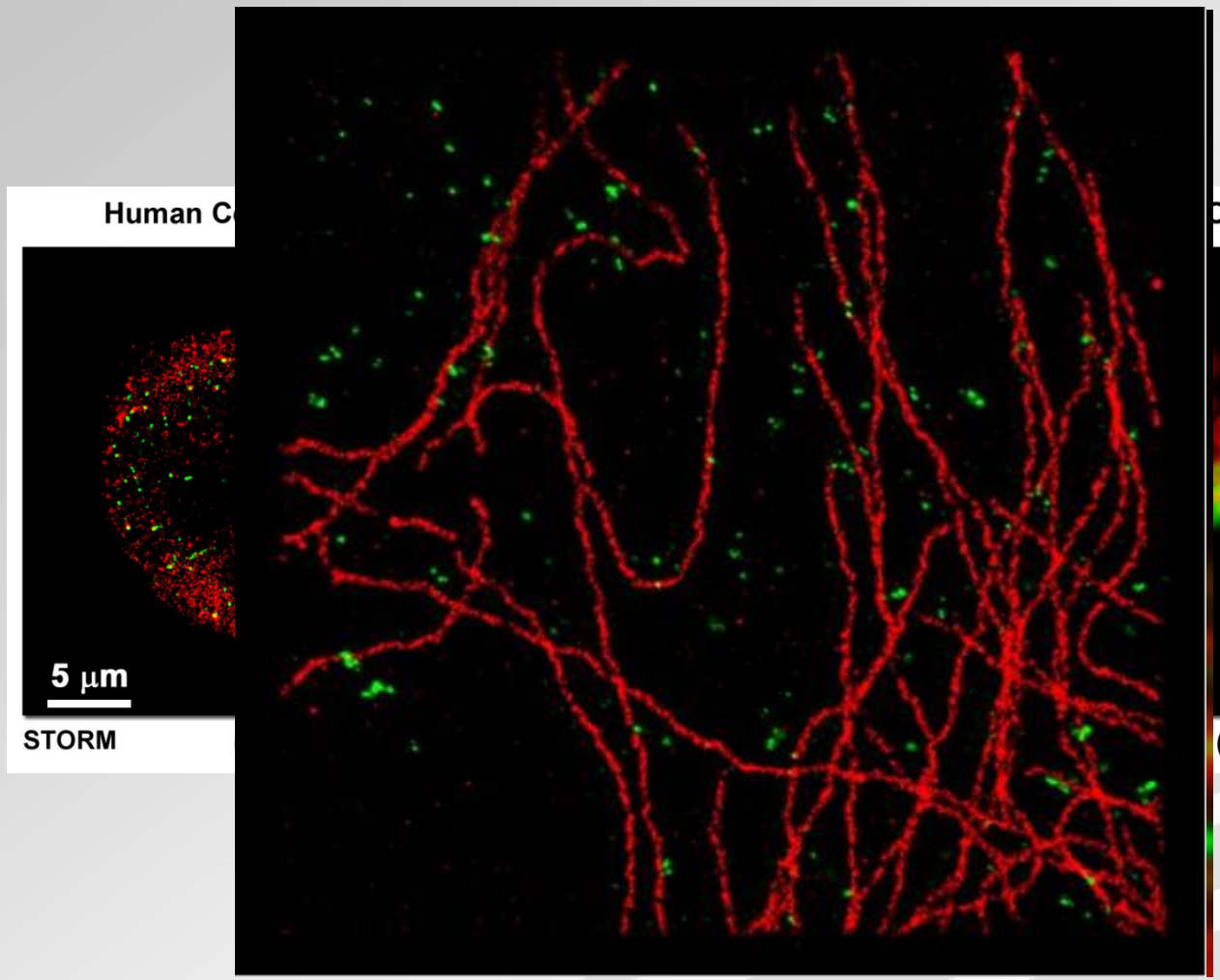




Bates, Huang, Dempsey, Zhuang *Science* (2007)

Multiple Reporters



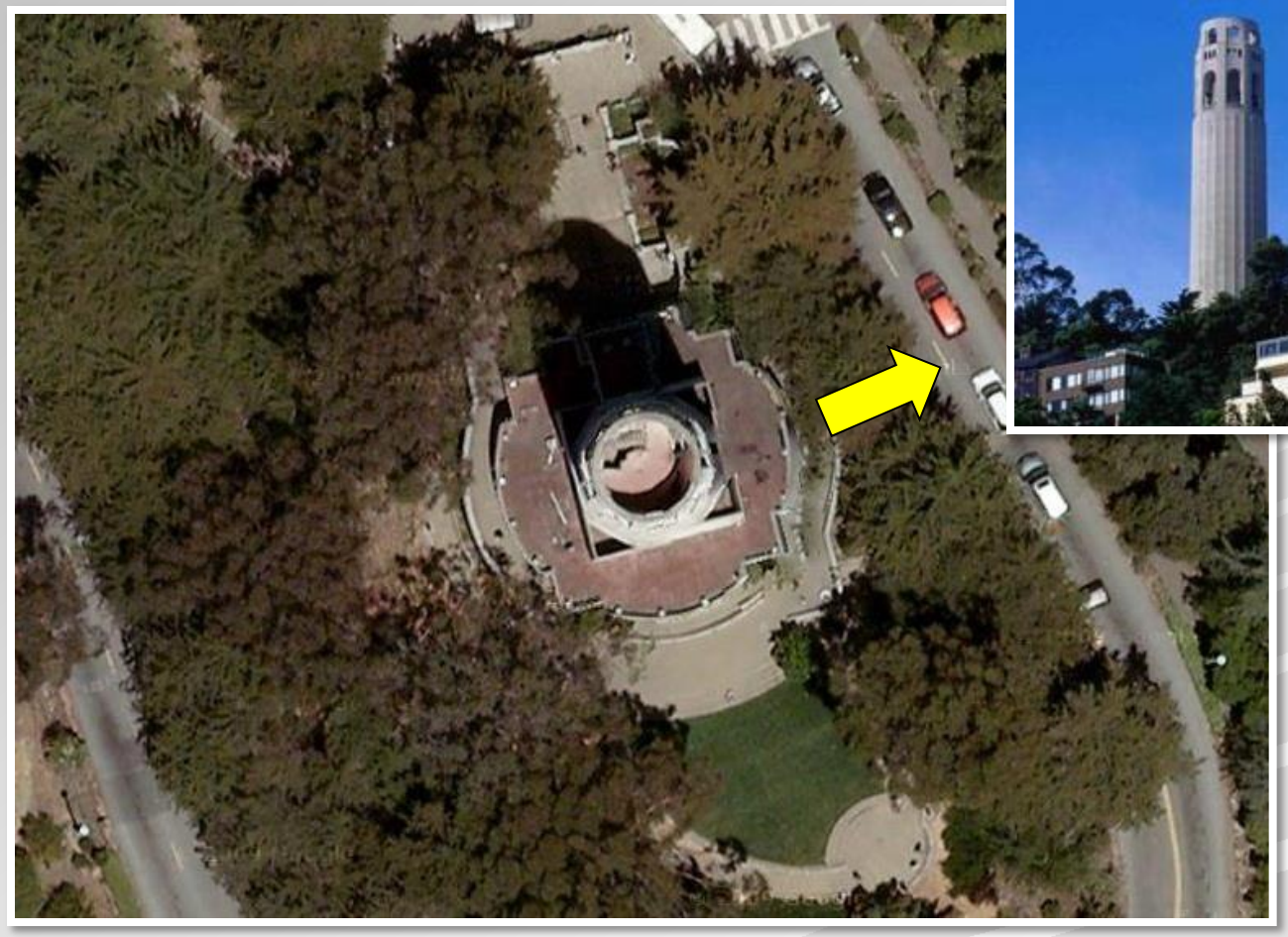


2-color GSDIM: Stephan Hell, Max-Planck Institute for Biophysical Chemistry, Germany

2-color STORM: Michael Davidson, Florida State University

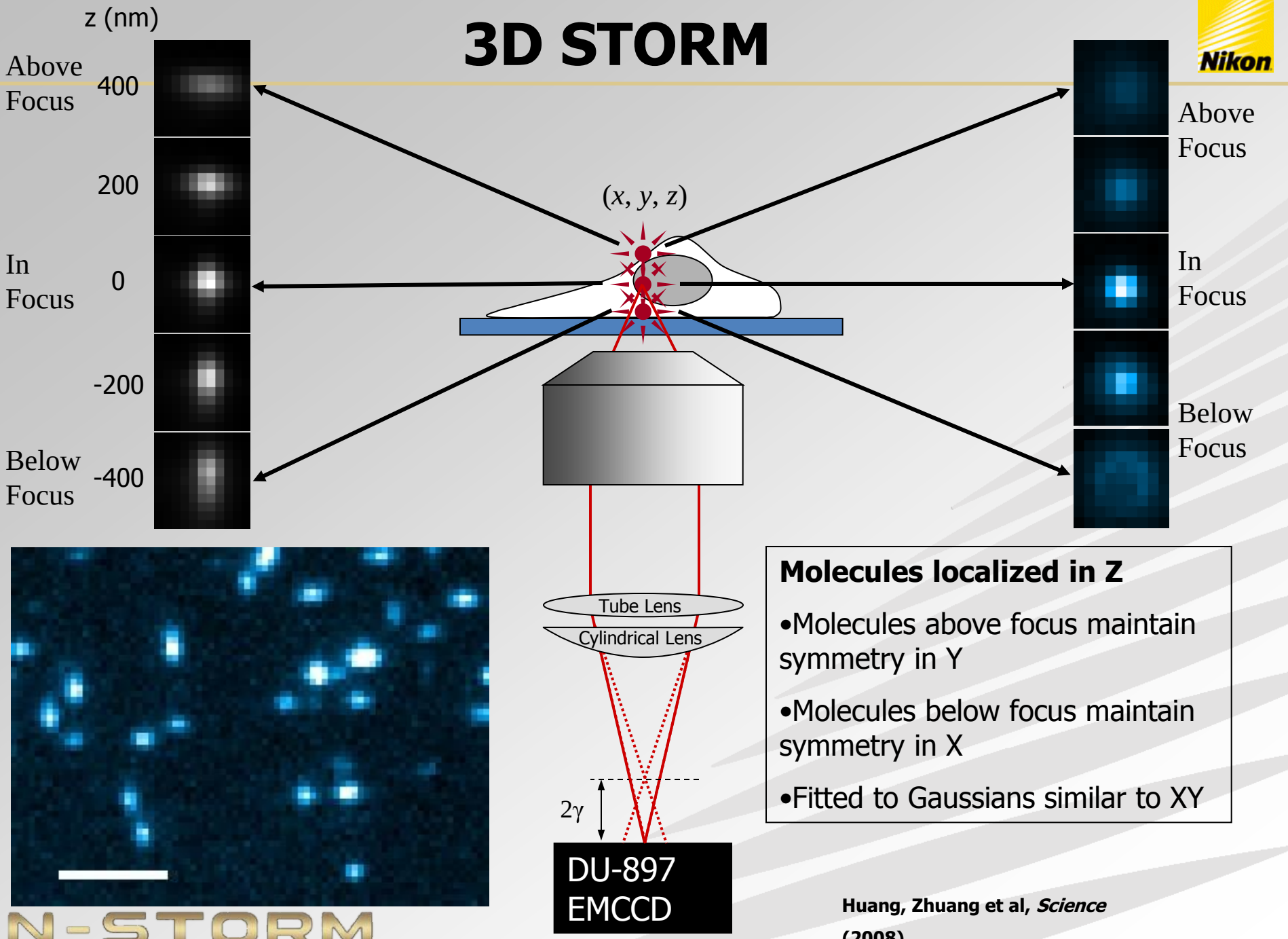
In a 2D world…

Satellite image of ???



Google maps

3D STORM



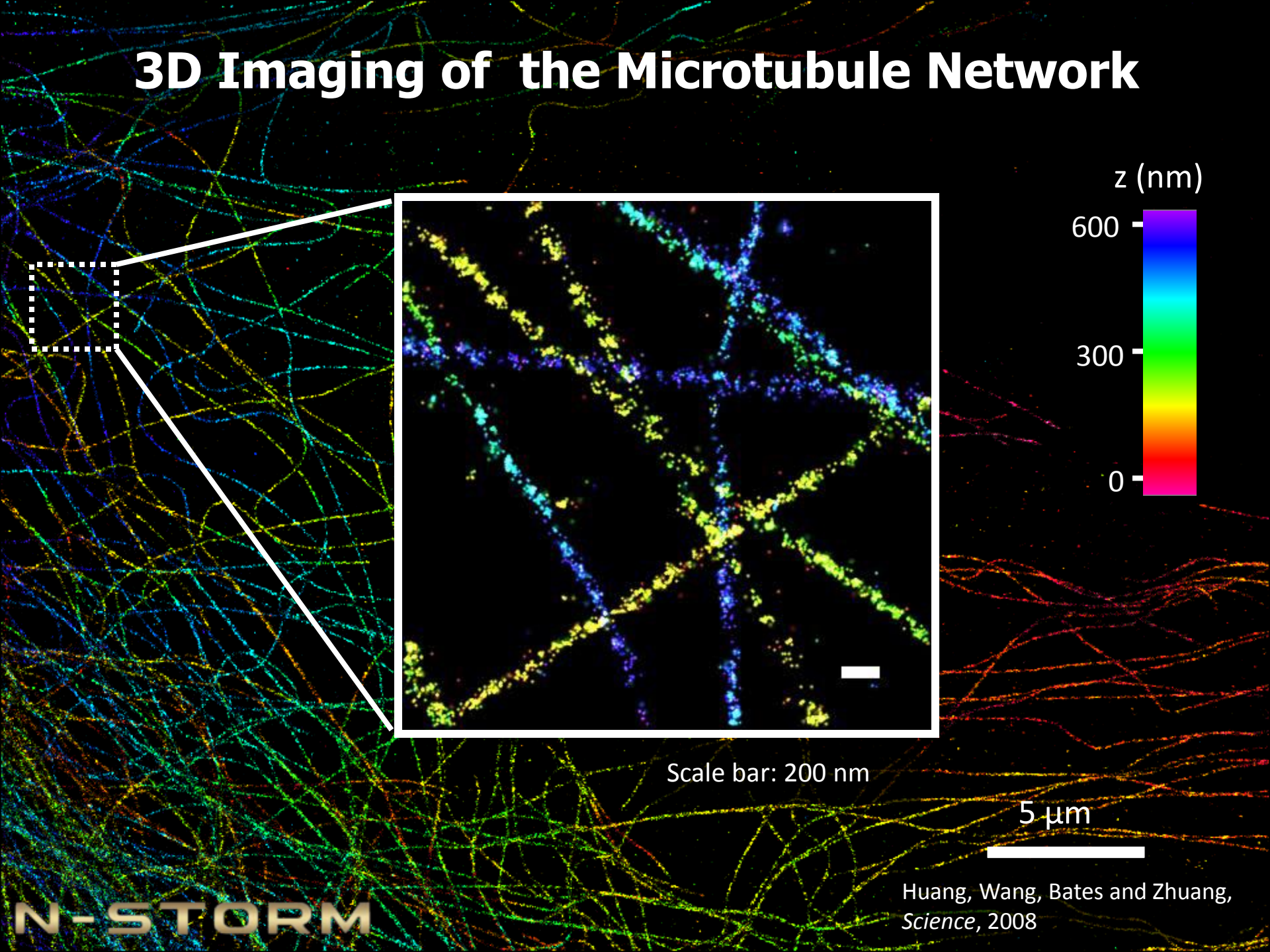
N-STORM

Molecules localized in Z

- Molecules above focus maintain symmetry in Y
- Molecules below focus maintain symmetry in X
- Fitted to Gaussians similar to XY

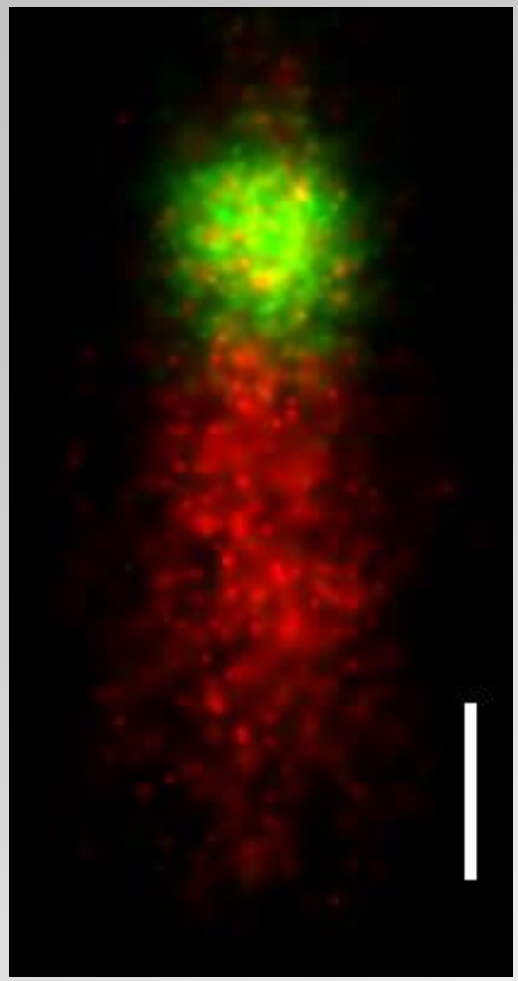
Huang, Zhuang et al, *Science* (2008)

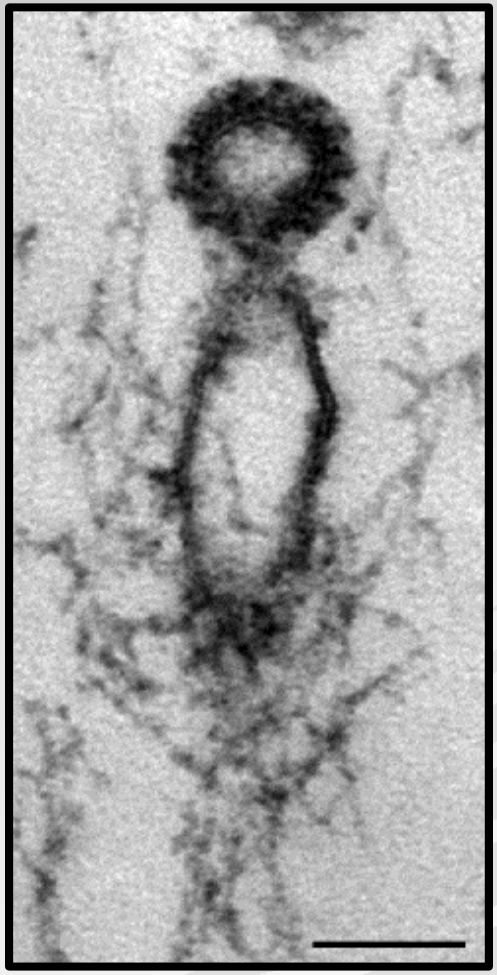
3D Imaging of the Microtubule Network



Huang, Wang, Bates and Zhuang, *Science*, 2008

2 Color 3D STORM





- Clathrin (Green)
- FBP17 – Formin (Red)
- Showing Clathrin's function in endocytosis

Images Courtesy of Dr. Bo Huang - UCSF

Best Dyes for STORM

Dye	Excitation maximum (nm)[a]	Emission maximum (nm)[a]	Extinction (M^{-1} cm^{-1})[b]	Quantum yield[c]	Detected photons per switching event		Equilibrium on-off duty cycle (400–600 s)		Survival fraction after illumination for 400 s		Number of switching cycles (mean)	
					MEA	βME	MEA	βME	MEA	βME	MEA	βME
Blue-absorbing												
Atto 488	501	523	90,000	0.8	1,341	1,110	0.00065	0.0022	0.58	0.99	11	49
Alexa Fluor 488	495	519	71,000	0.92	1,193	427	0.00055	0.0017	0.64	1	16	139
Atto 520	516	538	110,000	0.9	1,231	868	0.0015	0.00061	0.52	0.86	9	17
Fluorescein	494	518	70,000	0.79	1,493	776	0.00032	0.00034	0.51	0.83	4	15
FITC	494	518	70,000	0.8	639	1,086	0.00041	0.00031	0.75	0.9	17	15
Cy2	489	506	150,000	0.12	6,241	4,583	0.00012	0.00045	0.12	0.19	0.4	0.7
Yellow-absorbing												
Cy3B	559	570	130,000	0.67	1,365	2,057	0.0003	0.0004	1	0.89	8	5
Alexa Fluor 568	578	603	91,300	0.69	2,826	1,686	0.00058	0.0027	0.58	0.99	7	52
TAMRA	546	575	90,430	0.2	4,884	2,025	0.0017	0.0049	0.85	0.99	10	59
Cy3	550	570	150,000	0.15	11,022	8,158	0.0001	0.0003	0.17	0.55	0.5	1.6
Cy3.5	581	596	150,000	0.15	4,968	8,028	0.0017	0.0005	0.89	0.61	5.7	3.3
Atto 565	563	592	120,000	0.9	19,714	13,294	0.00058	0.00037	0.17	0.26	4	5
Red-absorbing												
Alexa Fluor 547	550	665	239,000	0.33	3,823	5,202	0.0005	0.0012	0.83	0.73	14	25
Cy5	649	670	250,000	0.28	4,254	5,873	0.0004	0.0007	0.75	0.83	10	17
Atto 647	645	669	120,000	0.2	1,526	944	0.0021	0.0016	0.46	0.84	10	24
Atto 647N	644	669	150,000	0.65	3,254	4,433	0.0012	0.0035	0.24	0.65	9	39
Dyomics 654	654	675	220,000	–	3,653	3,014	0.0011	0.0018	0.79	0.64	20	19
Atto 655	663	684	125,000	0.3	1,105	657	0.0006	0.0011	0.65	0.78	17	22
Atto 680	680	700	125,000	0.3	1,656	987	0.0019	0.0024	0.65	0.91	8	27
Cy5.5	675	694	250,000	0.28	5,831	6,337	0.0069	0.0073	0.87	0.85	16	25
NIR-absorbing												
DyLight 750	752	778	220,000	–	712	749	0.0006	0.0002	0.55	0.58	5	6
Cy7	747	776	200,000	0.28	852	947	0.0003	0.0004	0.48	0.49	5	7.6
Alexa Fluor 750	749	775	240,000	0.12	437	703	0.00006	0.0001	0.36	0.68	1.5	6
Atto 740	740	764	120,000	0.1	779	463	0.00047	0.0014	0.31	0.96	3	14
Alexa Fluor 790	785	810	260,000	–	591	740	0.00049	0.0014	0.54	0.62	5	2.7
IRDye 800 CW	778	794	240,000	–	2,753	2,540	0.0018	0.038	0.6	1	3	127

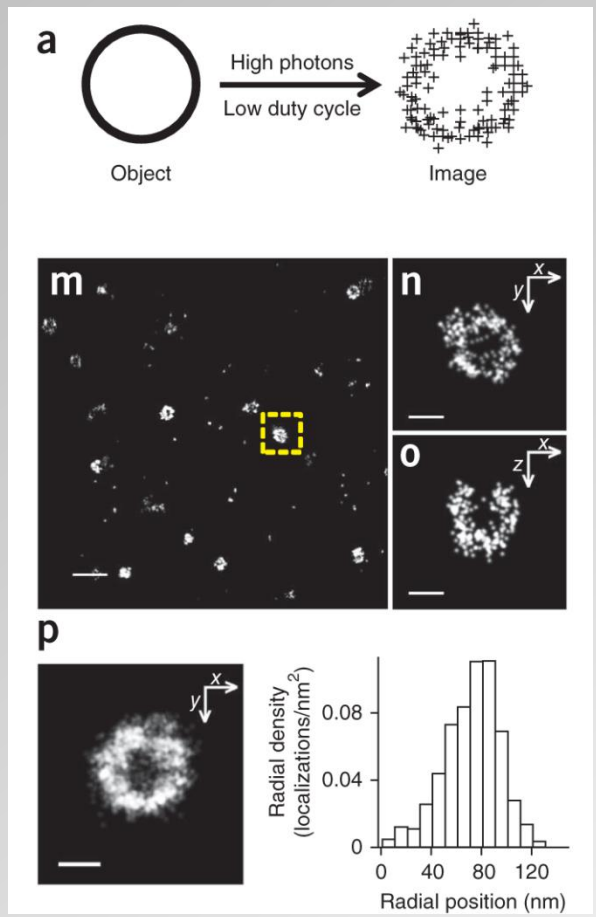
Dempsey et al., 2011

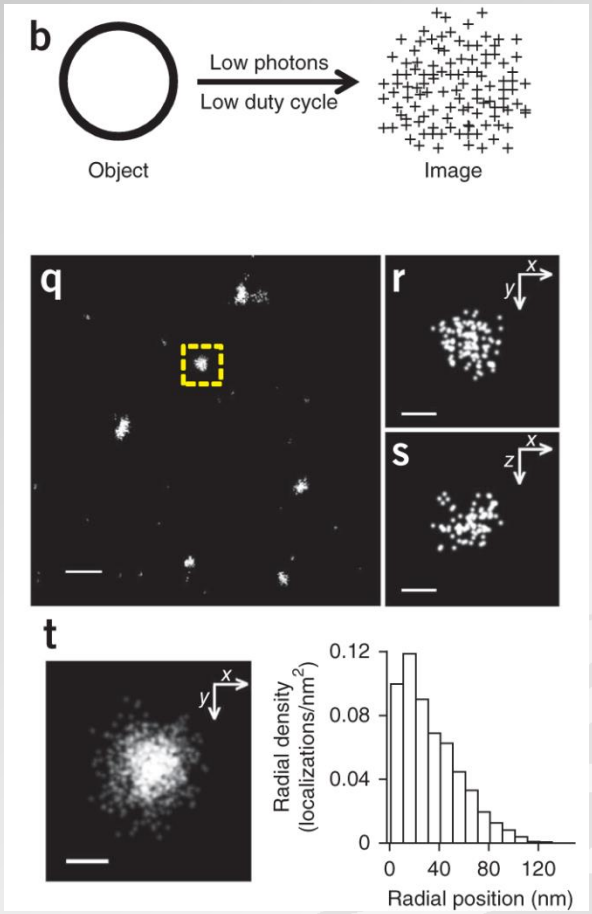
STORM Results

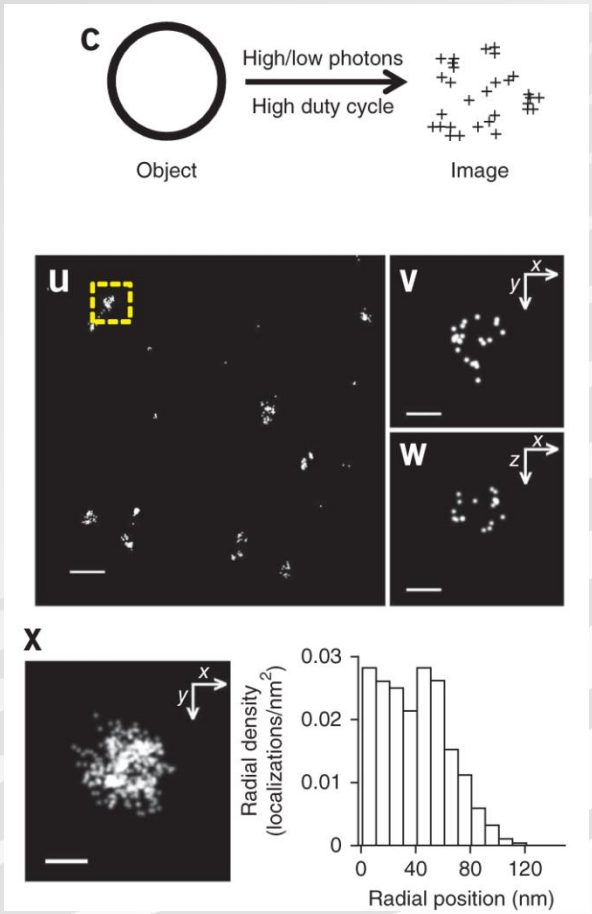
- **XY Resolution: 20-30 nm**
- **Z Resolution: ~50-60 nm**

Do Dyes Matter?!

Alexa 647 | Atto 655 | Cy5.5







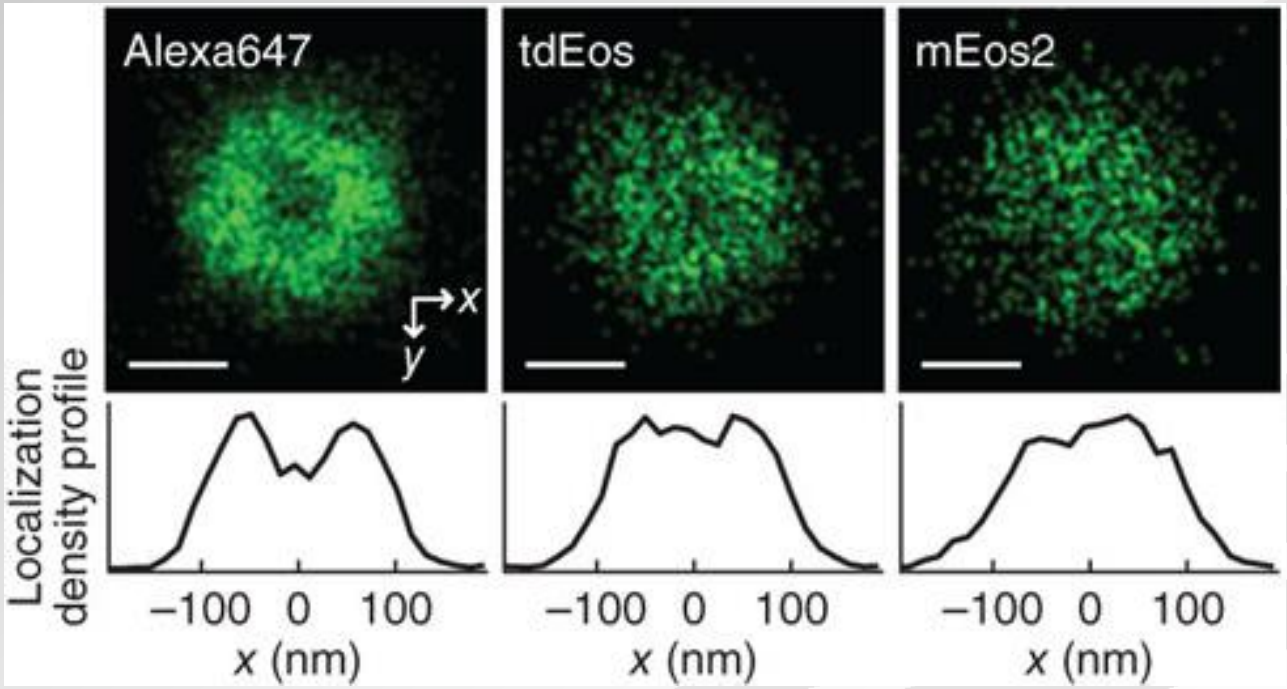
Dempsey et al, Nature Methods, 2011

STORM/GSDIM vs PALM

- Uses photo-switchable synthetic, non-genetically encoded dyes to temporally separate individual fluorophores
- More photons per switch = better localization accuracy

4,000-5,000 photons/switch

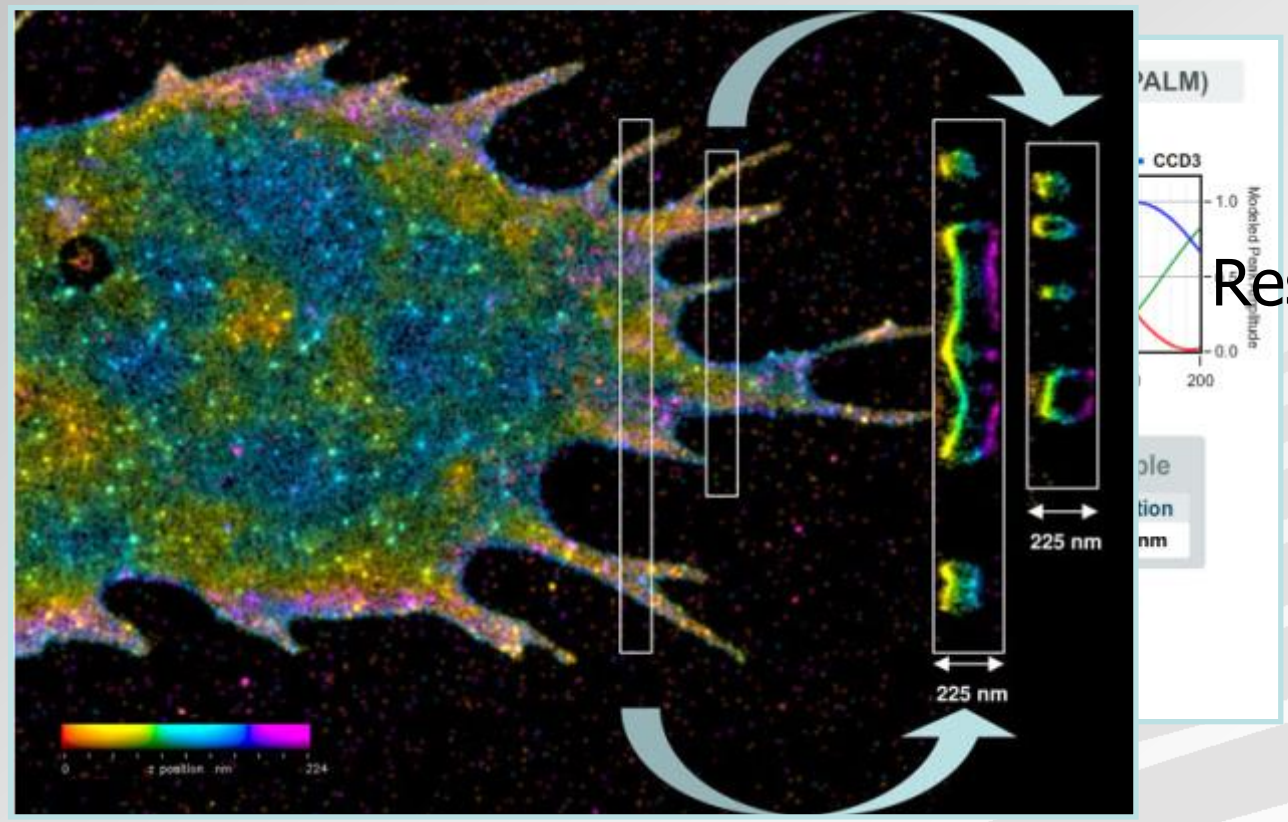
~1,200 photons per switch



Jones et al., 2011, Nat. Methods

iPALM

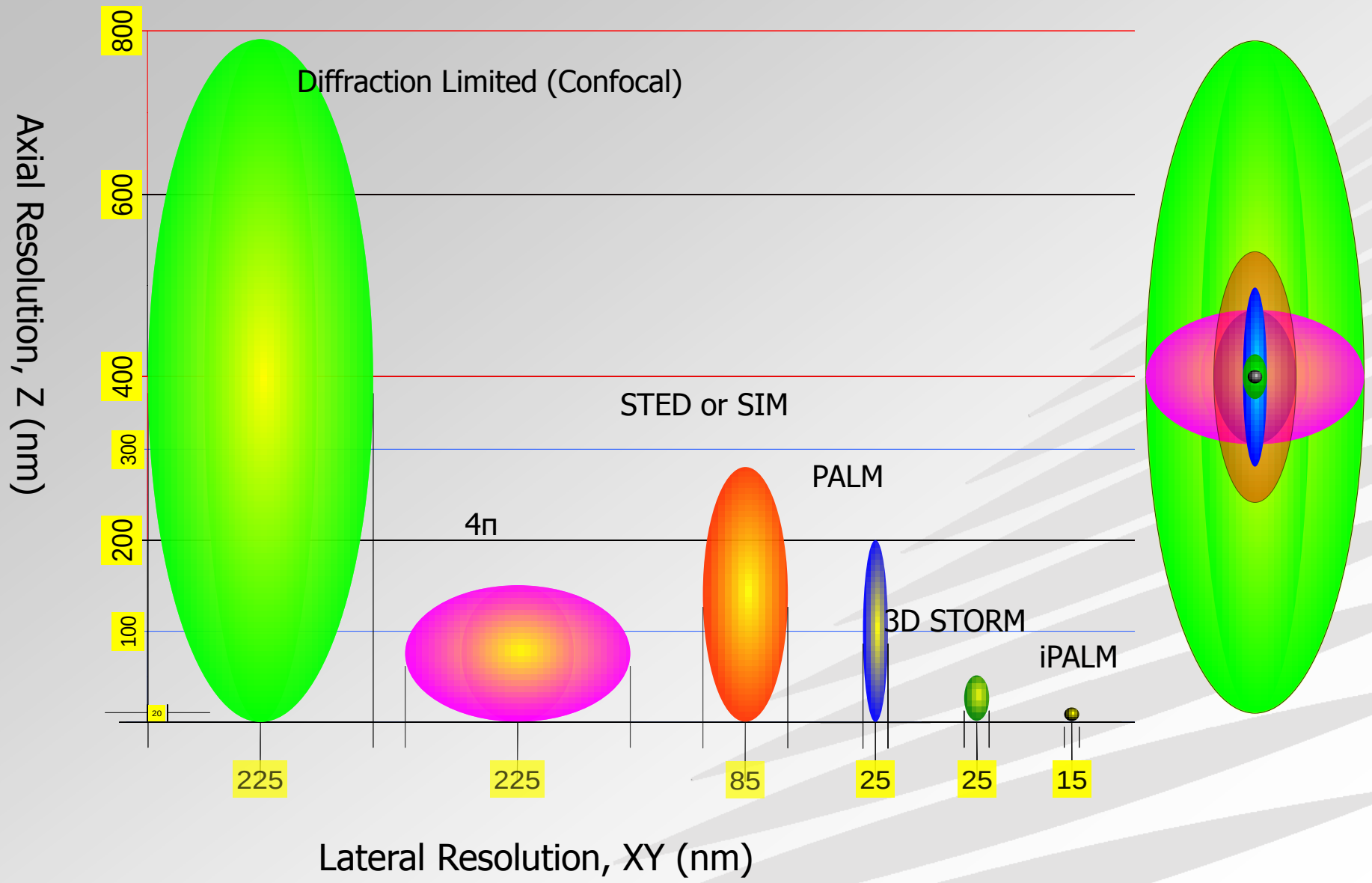
- Interferomety Photo-activation Localization Microscopy
  - Uses 2 opposing objective and 3 cameras simultaneously with interferomety principles to achieve high accuracy Z localization



Resolution Achieved:
XY ~10-20nm
Z ~10-20nm

Dr. Harold Hess
HHMI – Janelia Farm
http://www.hhmi.org/news/hess20090202.html

Volumetric comparison



One Last Thought?

Thank You