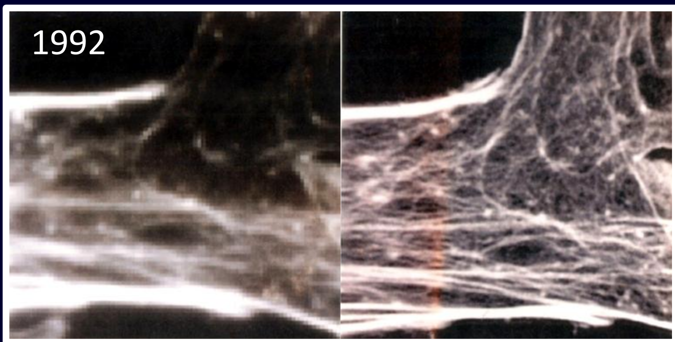
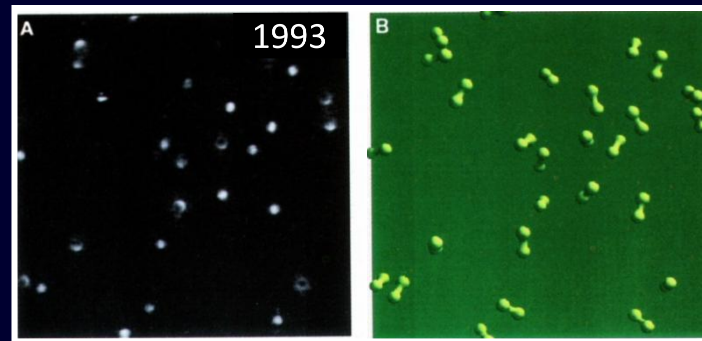
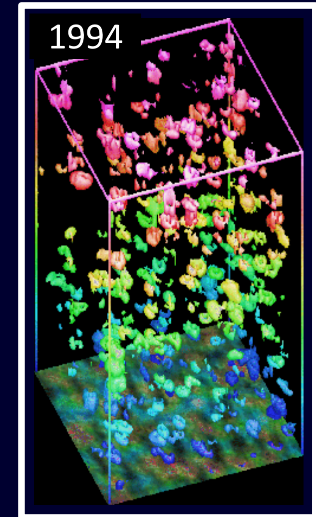
Single Molecules, Cells, and Super-Resolution Optics

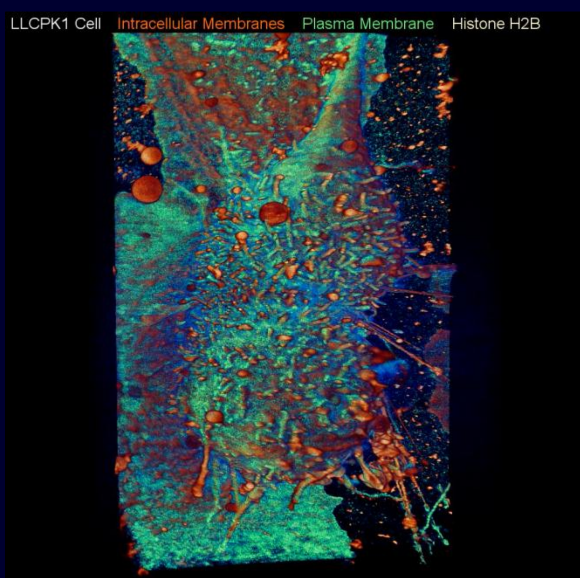
Eric Betzig
Janelia Research Campus, HHMI

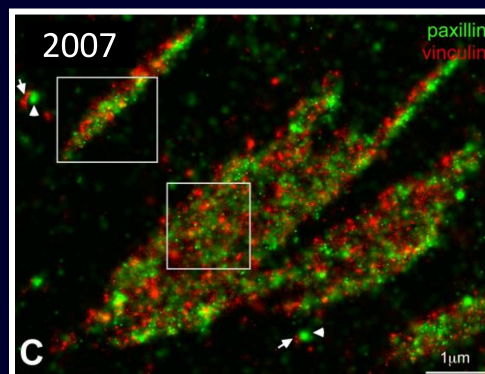


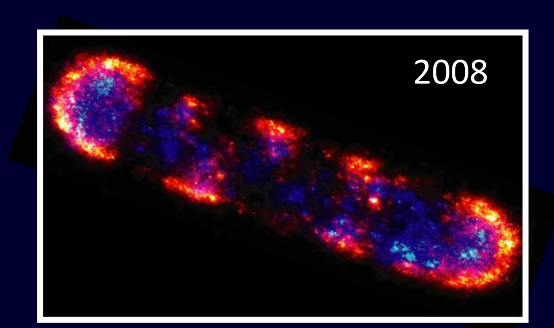


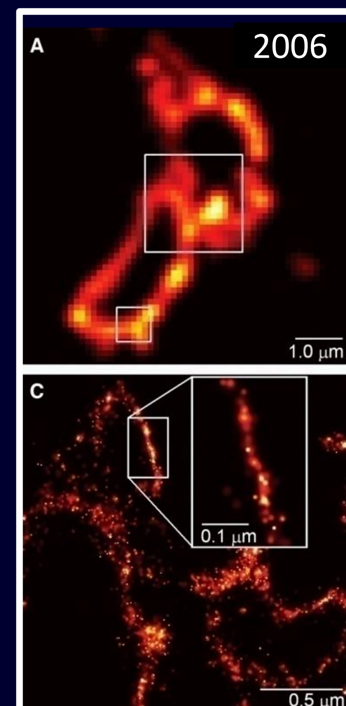


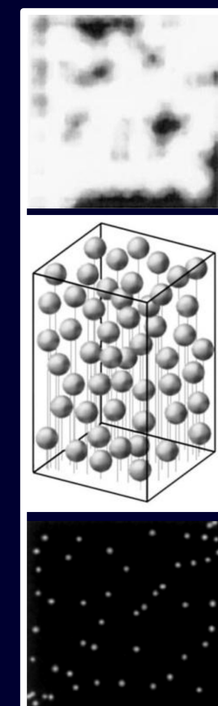
2014









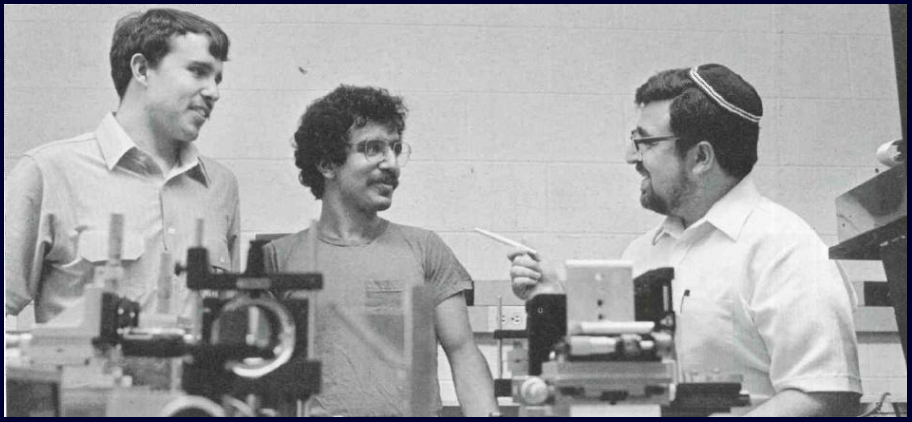


1995

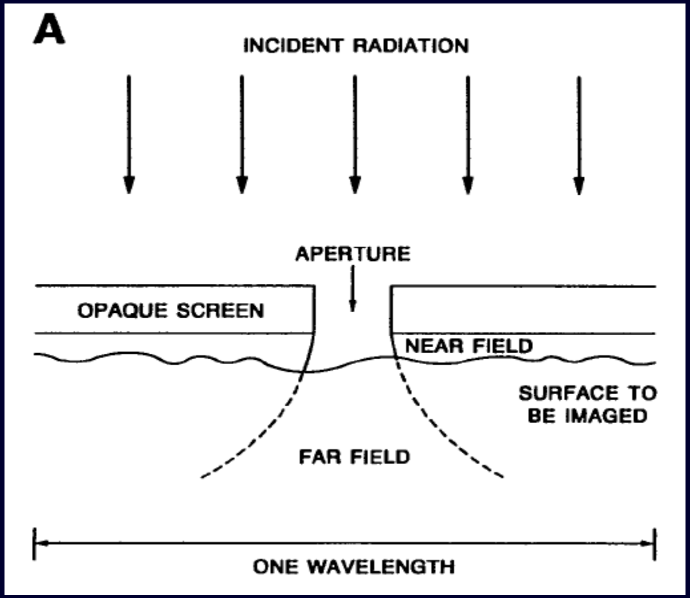
Cornell and the Beginnings of Near-Field Optical Microscopy

Mike Isaacson and his STEM

Me, Alec Harootunian, and Aaron Lewis, 1983



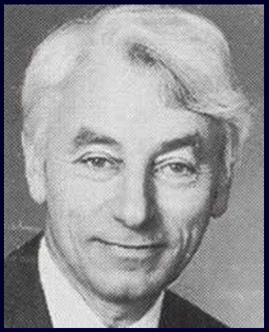
concept



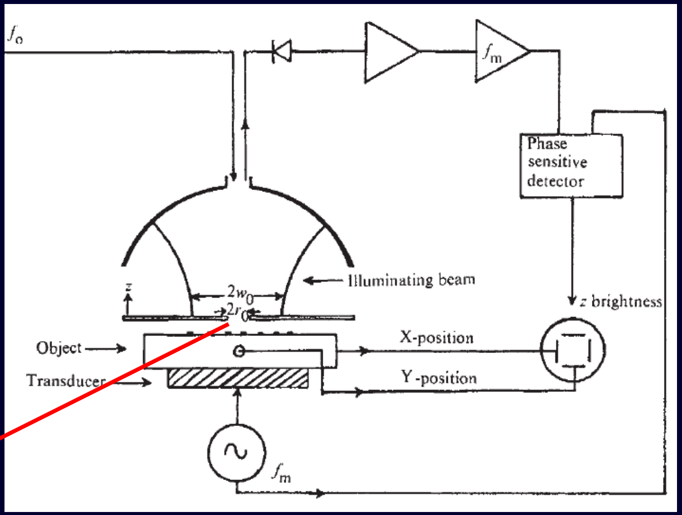
A. Lewis, *et al.*, *Ultramicroscopy* **13**, 227 (1984)

The Long History of Breaking Abbe's Law: Near-Field

Sir Eric Ash



near-field microwave (λ = 3 cm) microscopy



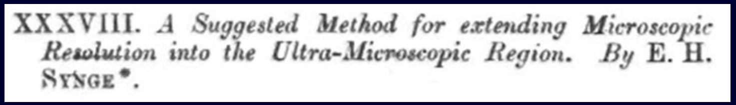
sub-wavelength aperture

object

image

Resolution of 1/60 of the wavelength!

E.A. Ash, G. Nicholls, *Nature* **237**, 510 (1972)



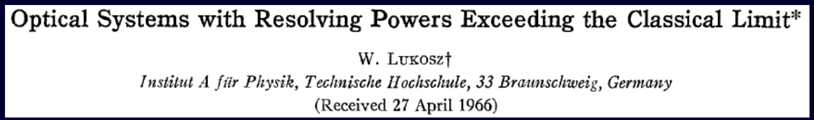
XXXVIII. *A Suggested Method for extending Microscopic Resolution into the Ultra-Microscopic Region.* By E. H. SYNGE*.

Edward "Hutchie" Synge, *Phil. Mag.* **6**, 356 (1928)

- J.A. O'Keefe (1956)
- A.V. Baez (acoustics, 1956)
- C.W. McCutchen (1967)
- U. Ch. Fischer (lithography, 1981)
- D.W. Pohl (1984)
- G.A. Massey (1984)
- J. Wessel (1985)

The Long History of Breaking Abbe's Law: Far-Field

Structured Light


Optical Systems with Resolving Powers Exceeding the Classical Limit*

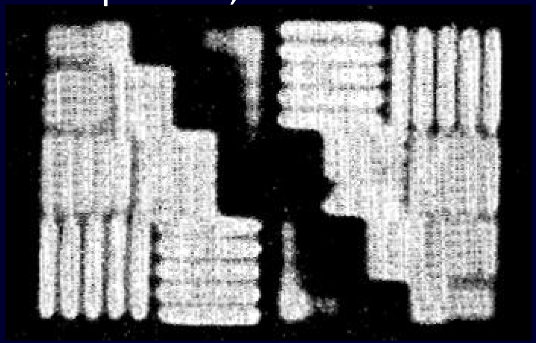
W. Lukosz†

Institut A für Physik, Technische Hochschule, 33 Braunschweig, Germany

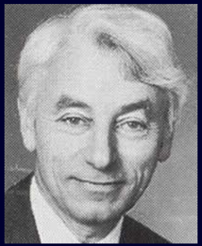
(Received 27 April 1966)

W. Lukosz, *JOSA* **56**, 1463 (1966)

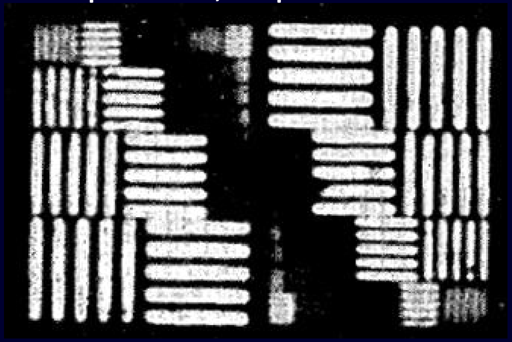
test pattern, conventional



Sir Eric Ash



test pattern, super-resolved



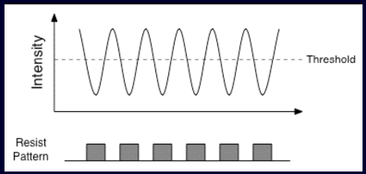
Resolution 3× beyond Abbe's Limit!

A. Bachl, W. Lukosz, *JOSA* **57**, 163 (1967)

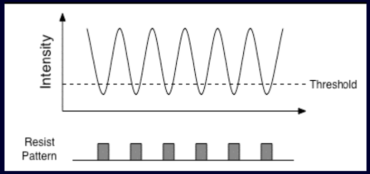
Nonlinear Interaction with Sample

integrated circuit linewidth control

nominal exposure

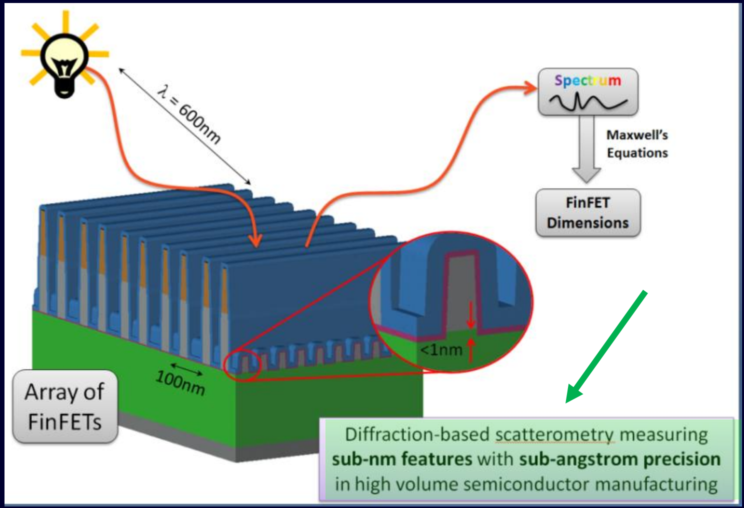


intentional overexposure



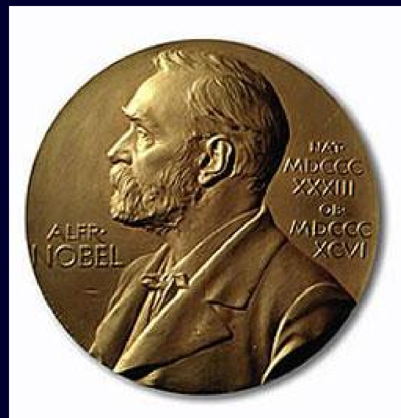
λ

A Priori Information: wafer inspection

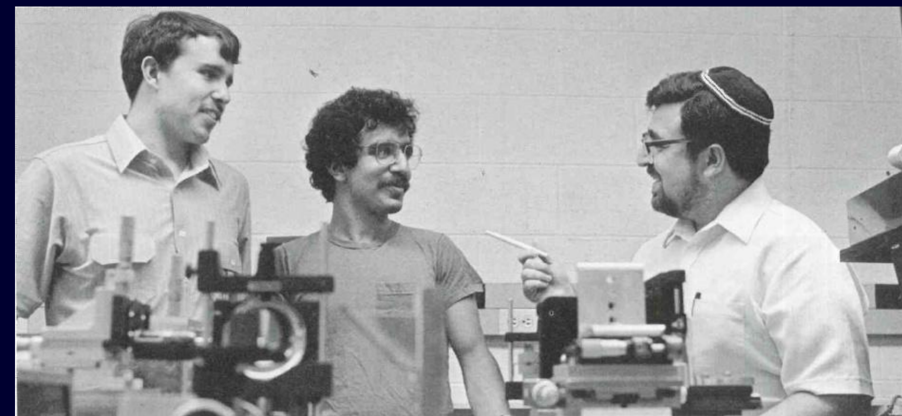


Making Near-field *Optical* Microscopy Work

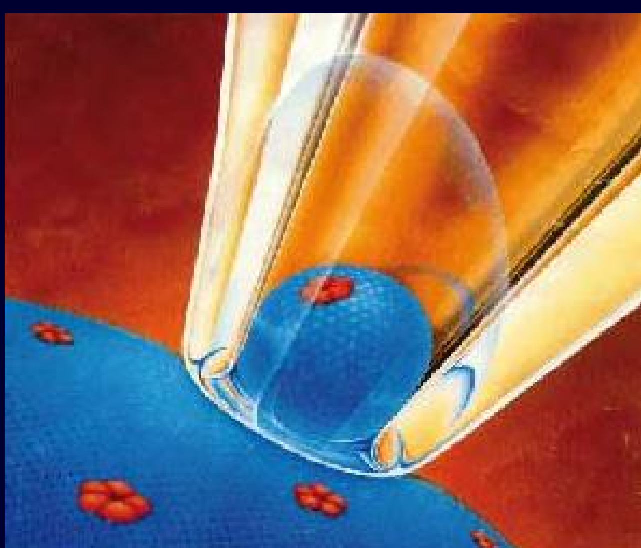
Edwin Neher and Bert Sakmann, Nobel 1991



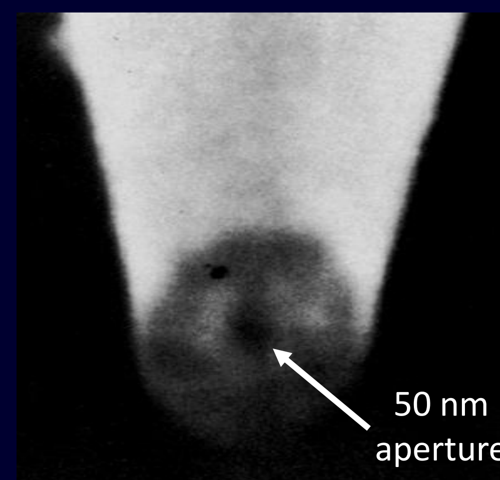
Me, Alec Harootunian, and Aaron Lewis, 1983



patch clamp: single ion channel recording



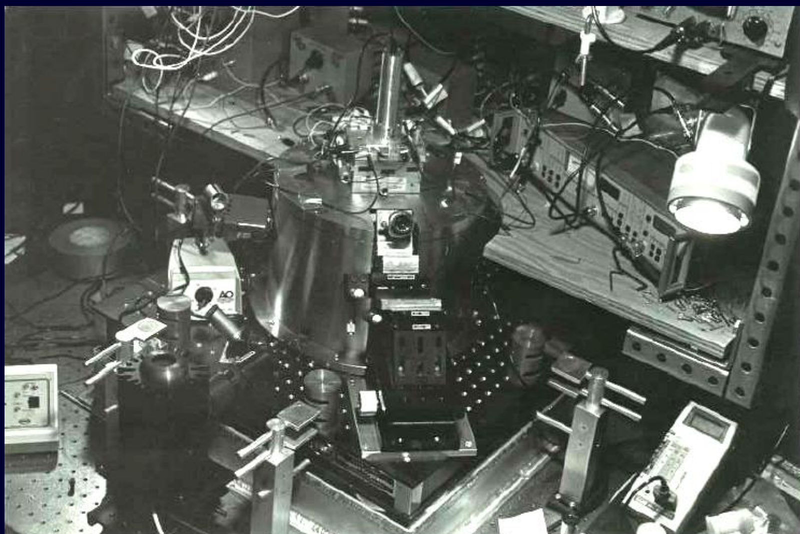
end of aluminum coated pipette



E. Betzig, *et al.*, *Biophys. J.* **49**, 269 (1986)

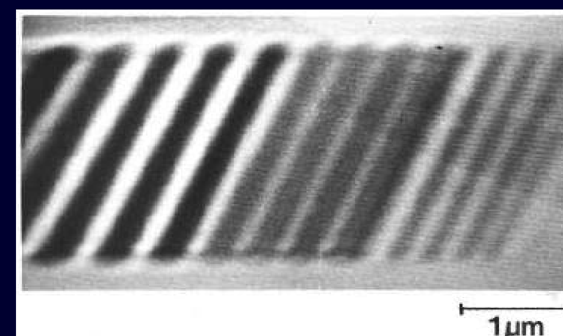
Making Near-field *Optical* Microscopy Work

my near-field scanning optical microscope (NSOM)



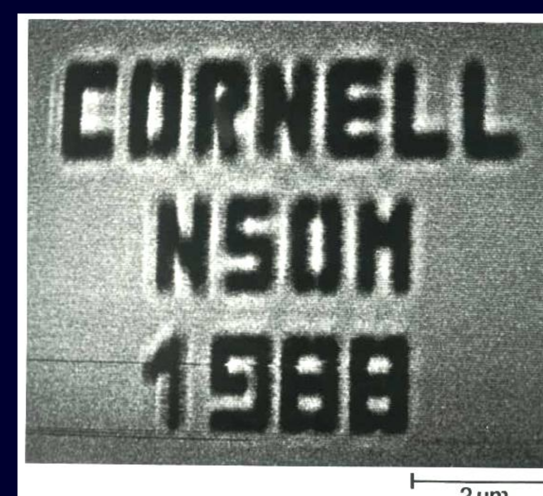
microscope control room

diffraction limited



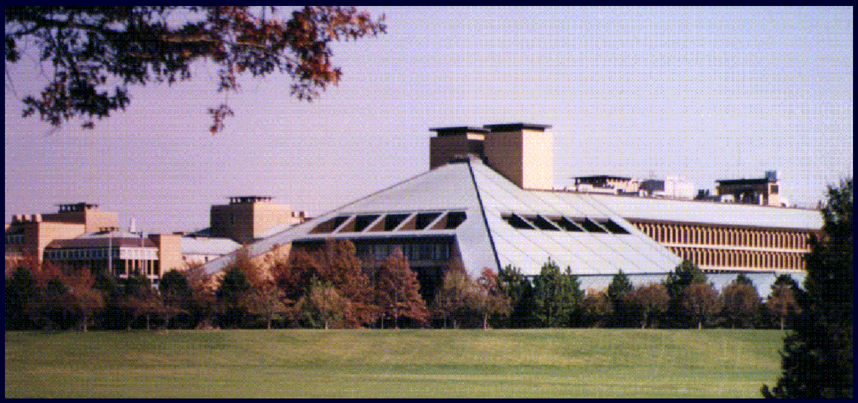
NSOM

NSOM

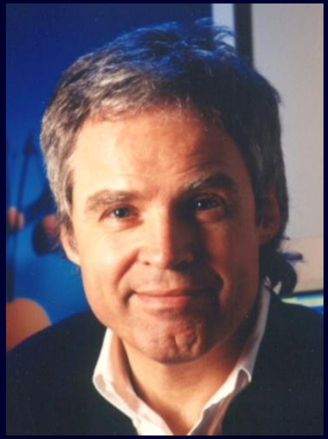


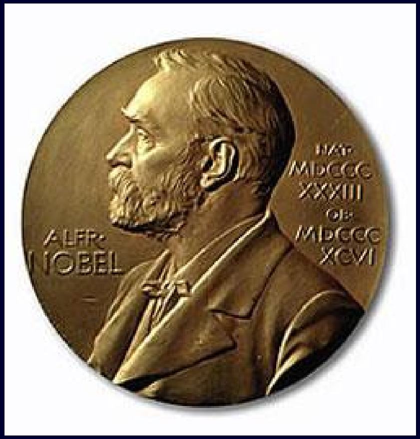
Initial Struggles at Bell Labs

AT&T Bell Labs, Murray Hill, NJ

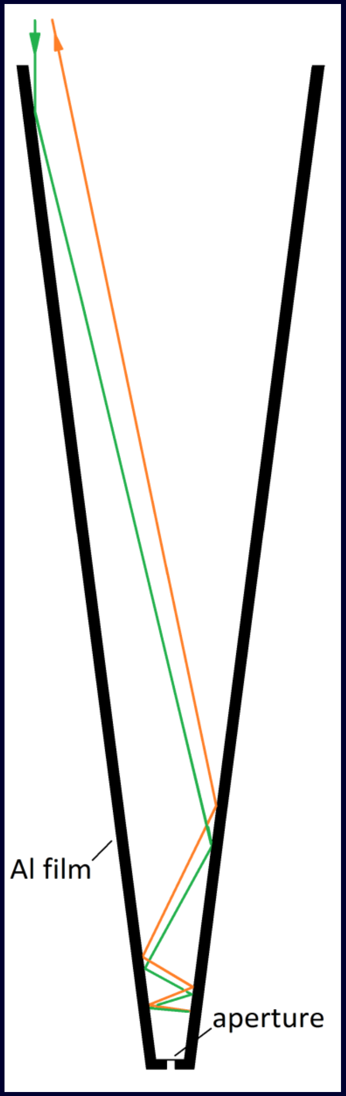


Horst Störmer, 1998 Nobel in Physics

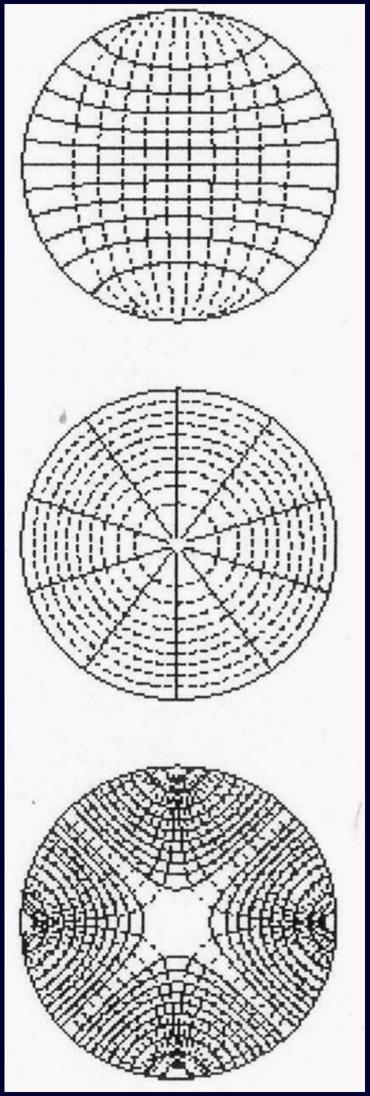




retroreflection in pipette



lowest order waveguide modes at tip



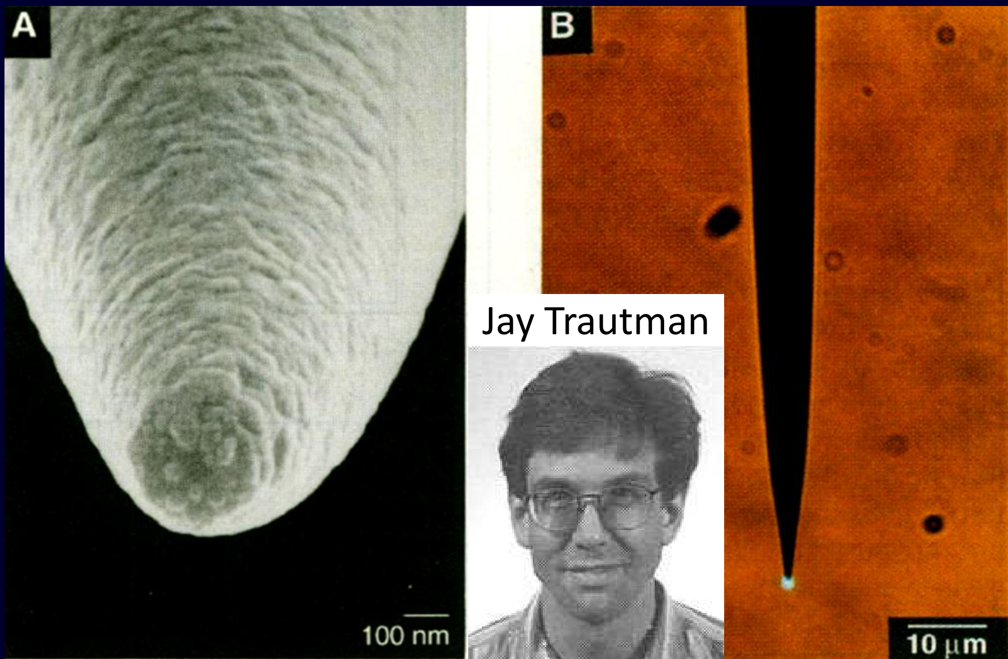
TE_{21}

TM_{01}

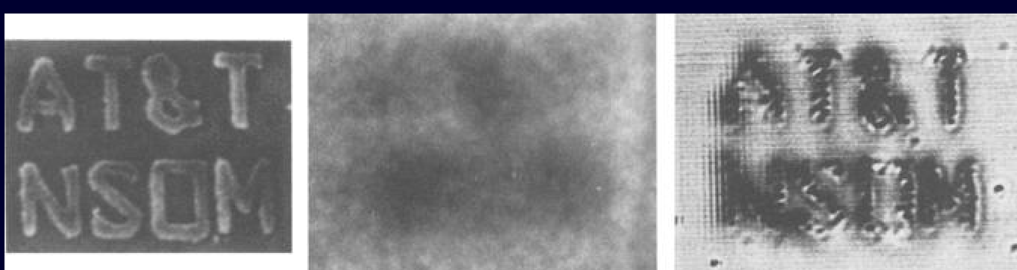
TE_{11}

Making NSOM Routine

adiabatically tapered optical fiber probe



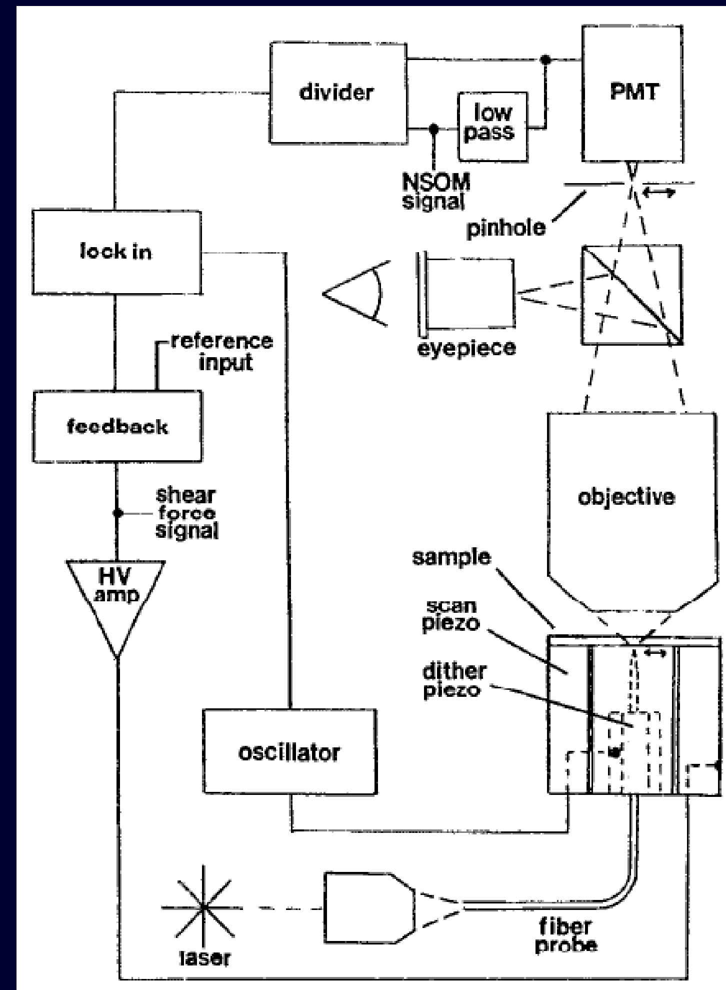
SEM widefield NSOM



1 µm

E. Betzig, J.K. Trautman, *et al.*, *Science* **251**, 1468 (1991)

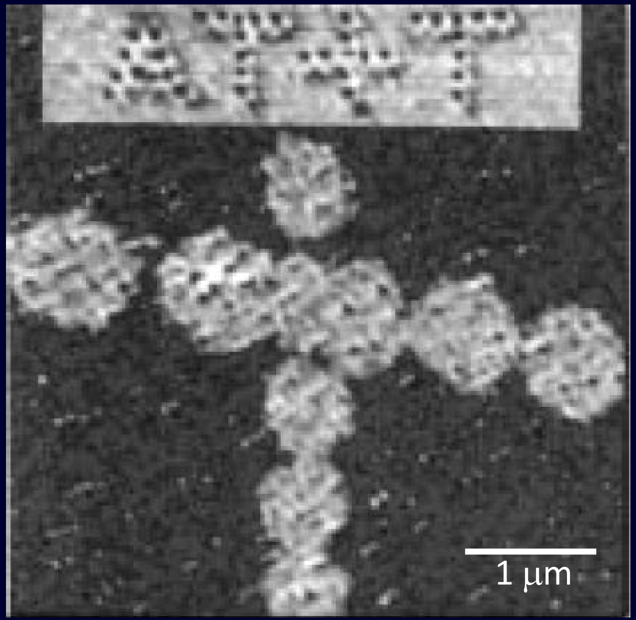
shear force distance regulation



E. Betzig, *et al.*, *Appl. Phys. Lett.* 60, 2484 (1992)

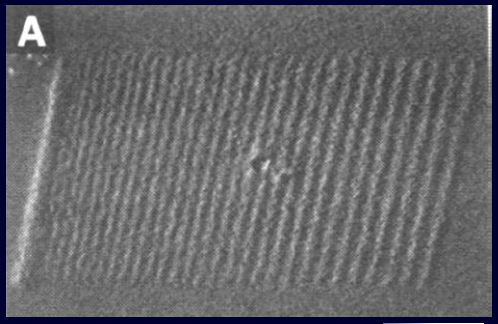
The Golden Age of NSOM

high density data storage

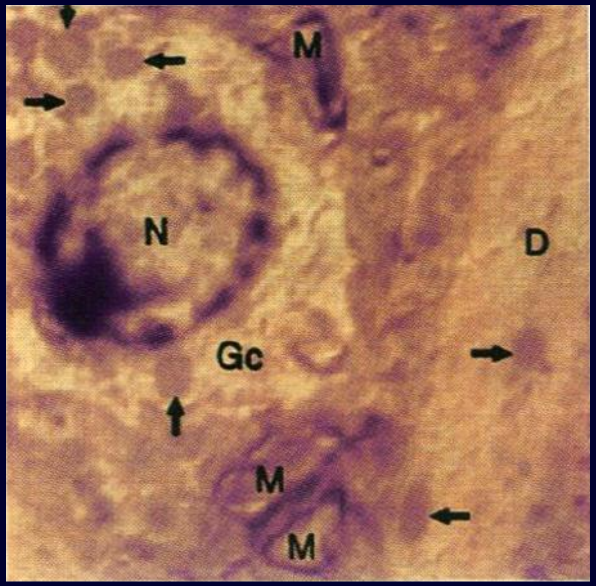


E. Betzig, *et al.*, *Appl. Phys. Lett.* **61**, 142 (1992)

photolithography

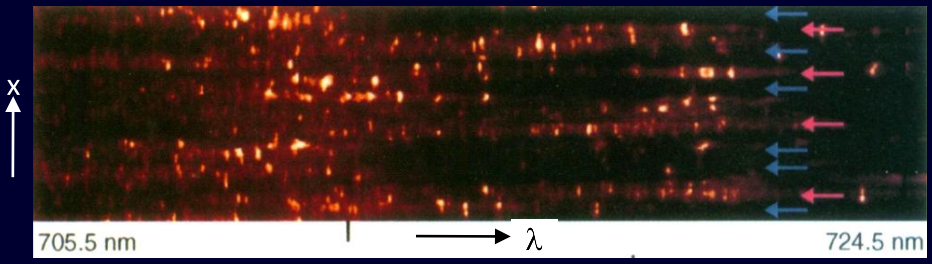


histological section, monkey hippocampus



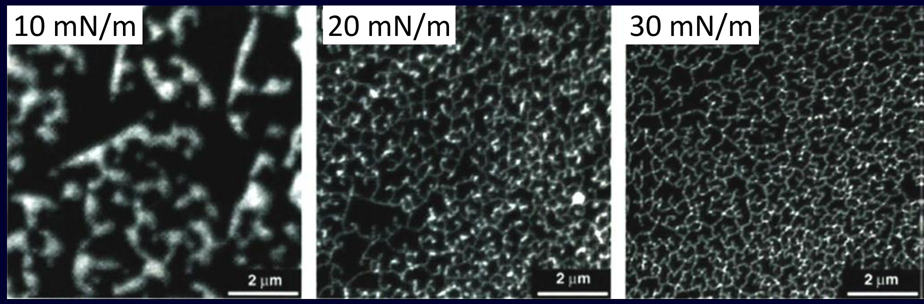
E. Betzig, J.K. Trautman, *Science* **257**, 189 (1992)

nanoscale spectroscopic imaging



H.F. Hess, *et al.*, *Science* **61**, 142 (1994)

fluorescence: phase change in phospholipid monolayers

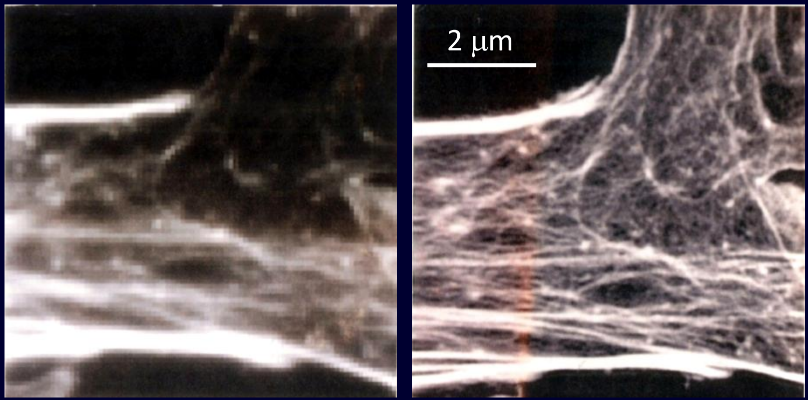


J. Hwang, *et al.*, *Science* **270**, 610 (1995)

Single Molecule Detection (SMD)

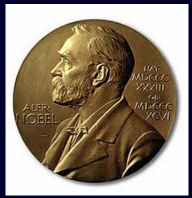
fluorescence: actin, mouse fibroblast cell

widefield NSOM



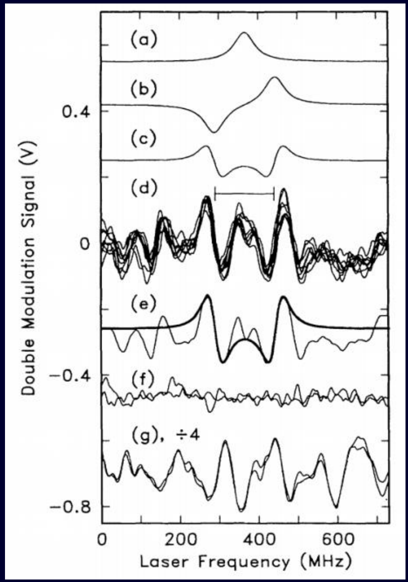
E. Betzig, *et al.*, *Bioimaging* **1**, 129 (1993)

Nobel, 2014



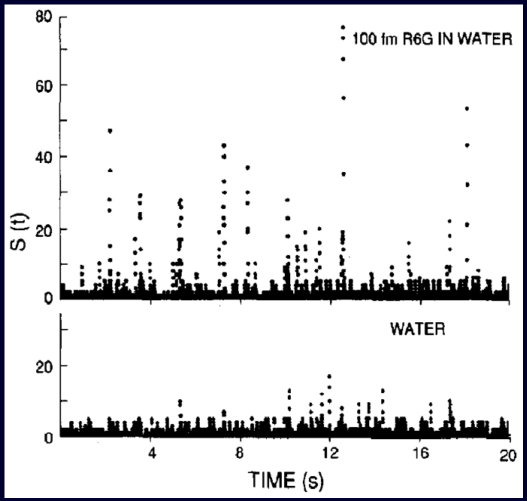
W.E. Moerner

single molecule absorption spectra, 1.6°K



W.E. Moerner, L. Kador, *Phys. Rev. Lett.* **62**, 2535 (1989)

SM fluorescence bursts at room temp



Time gated:

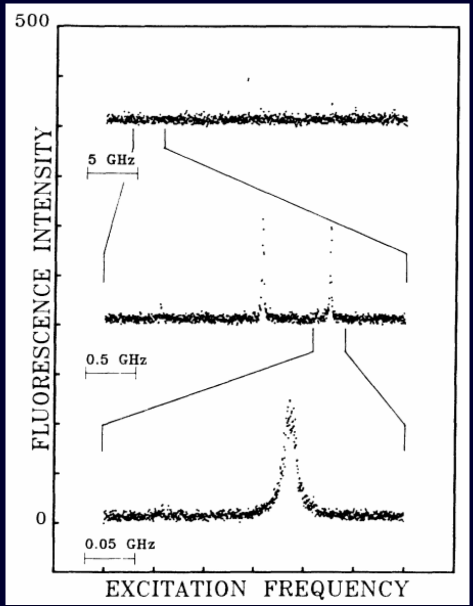
E.B. Shera, *et al.*, *Chem. Phys. Lett* **174**, 553 (1990)

FCS:

R. Rigler, J. Widengren, *Bioscience* **3**, 180 (1990)

SM fluorescence excitation spectrum, 1.8°K

Michel Orrit

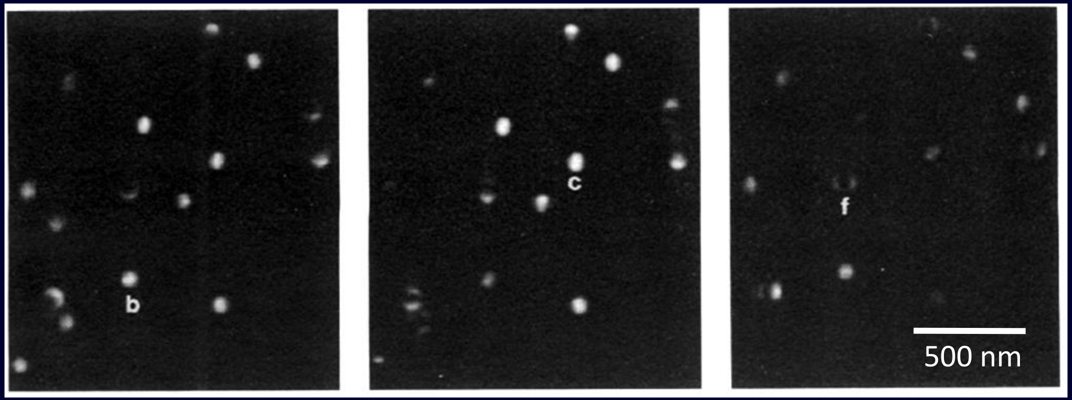


M. Orrit, J. Bernard, *Phys. Rev. Lett.* **65**, 2716 (1990)

NSOM and the Birth of Single Molecule Microscopy

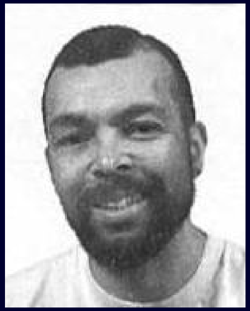
single molecule fluorescence anisotropy

random

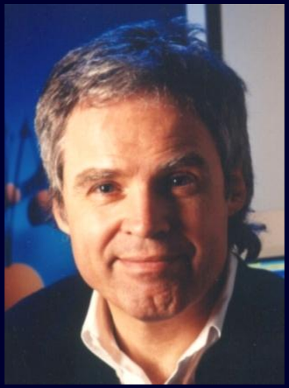


$diI\text{-}C_{18}\text{-}(3)$ molecules on PMMA

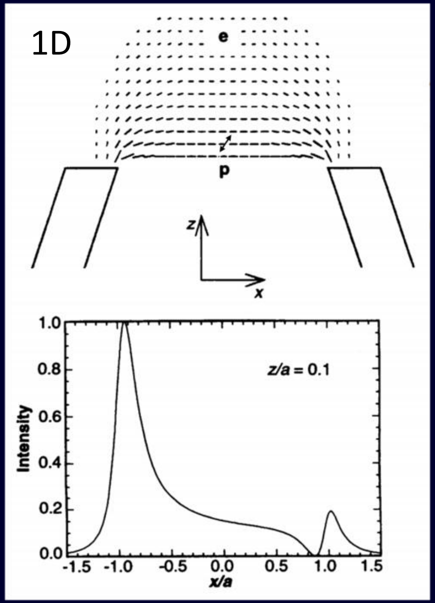
E. Betzig, R.J. Chichester, *Science* **262**, 1422 (1993)

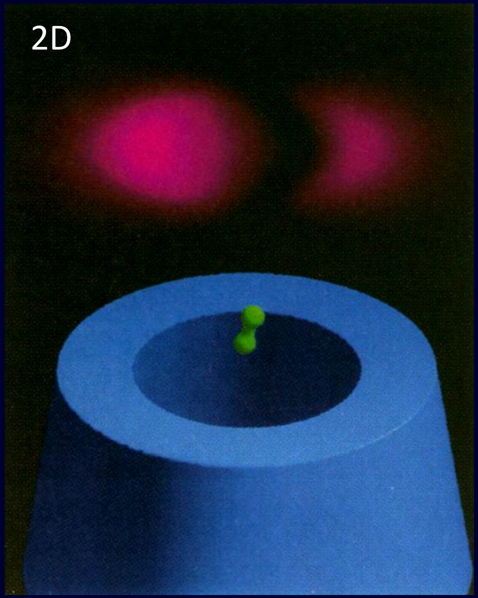


Rob Chichester



Horst Störmer



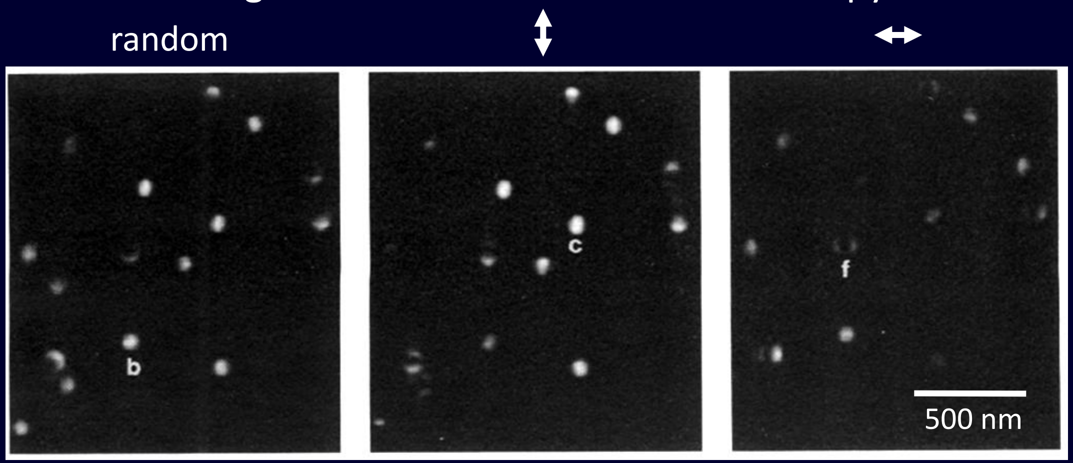


single molecule NSOM signal

$$\left|\mathbf{E}(\mathbf{x})\cdot\mathbf{p}\right|^2$$

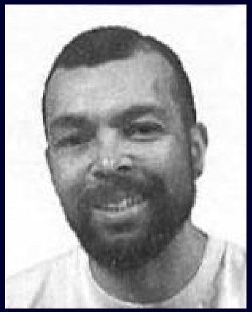
NSOM and the Birth of Single Molecule Microscopy

single molecule fluorescence anisotropy



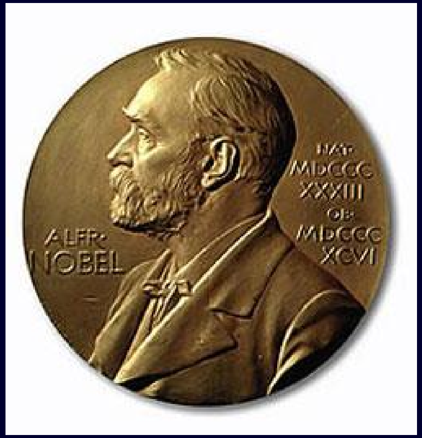
dil-C_{18}-(3) molecules on PMMA

E. Betzig, R.J. Chichester, *Science* **262**, 1422 (1993)



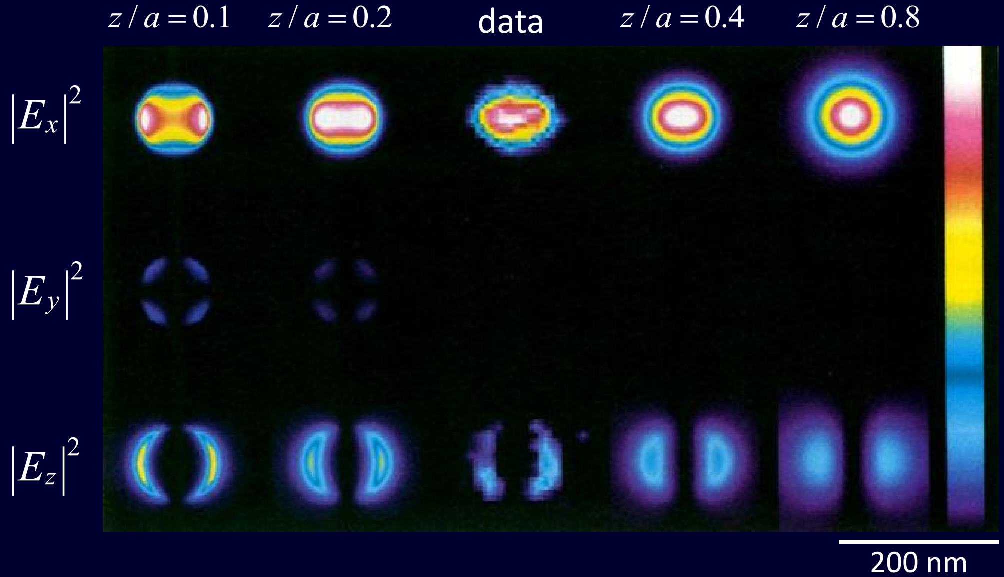
Rob Chichester

Hans Bethe, 1967 Nobel in Physics

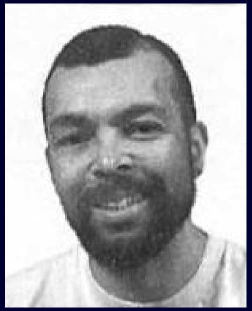


H.A. Bethe, *Phys. Rev.* **66**, 163 (1944)

E fields at aperture: theory vs. experiment



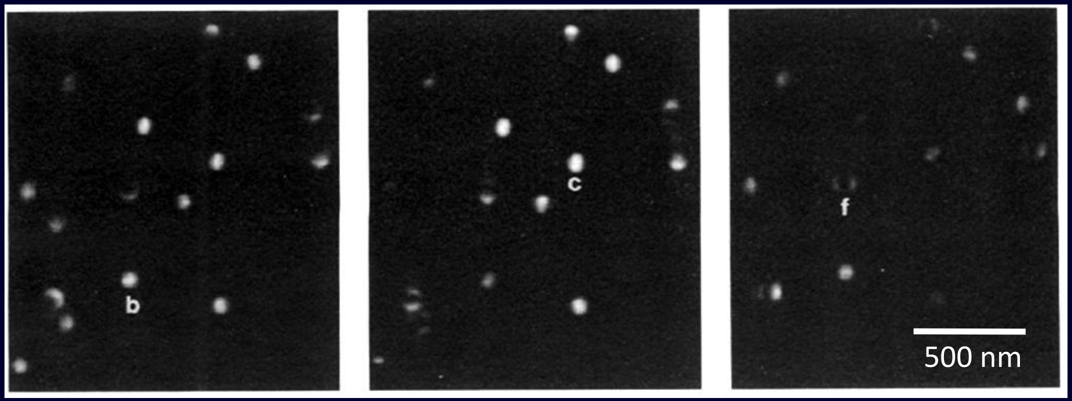
NSOM and the Birth of Single Molecule Microscopy



Rob Chichester

single molecule fluorescence anisotropy

random ↕ ↔

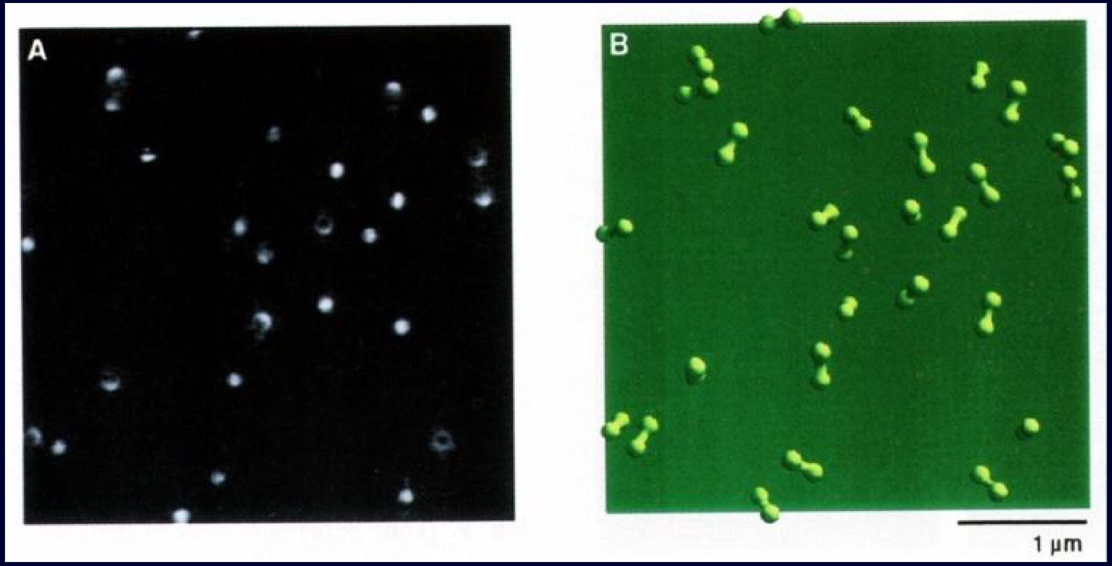


diI-C_{18}-(3) molecules on PMMA

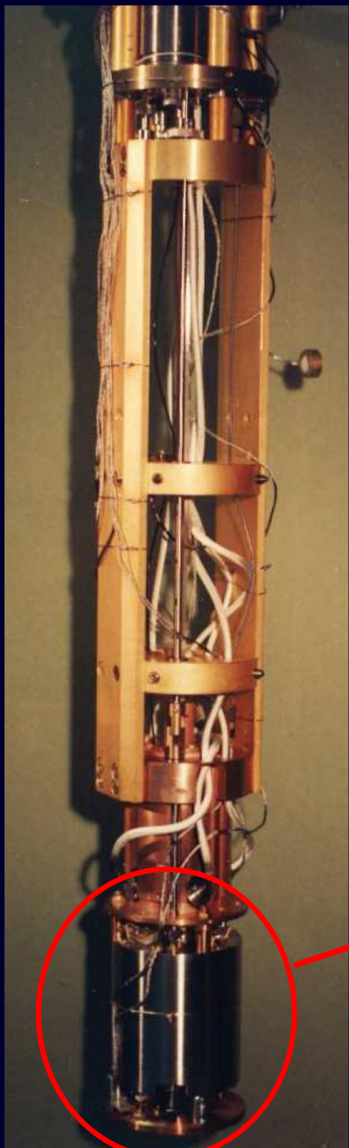
E. Betzig, R.J. Chichester, *Science* **262**, 1422 (1993)

single molecule dipole orientations

- first imaging of single molecules at room temp
- first super-resolution imaging of single molecules
- first measurement of single molecule dipole orientations
- first localization of single molecules to fraction of PSF width (12 nm xy, 6 nm z)

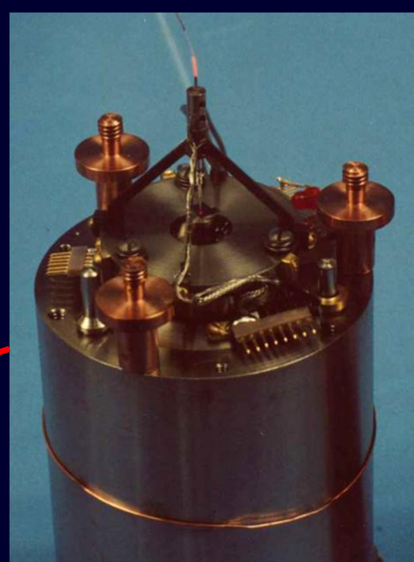


Cryogenic Near-field Spectroscopy

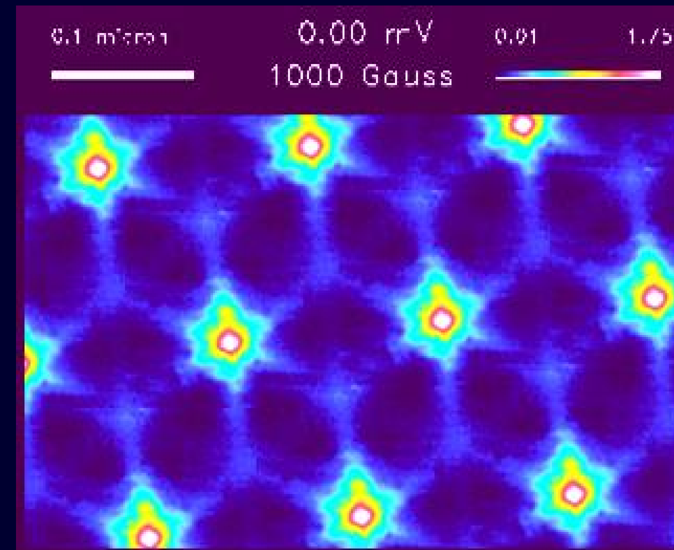


Harald Hess

Harald's low temp STM

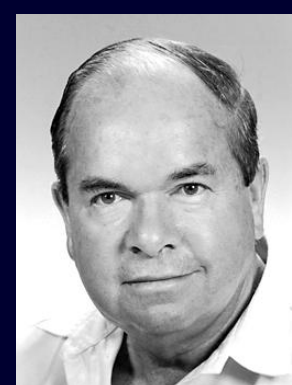


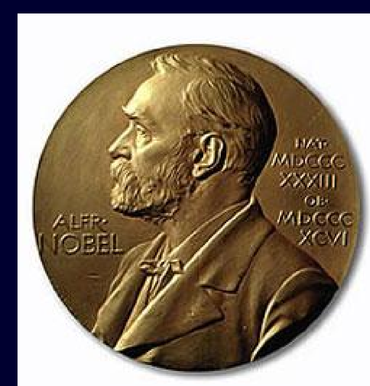
scanning tunnel spectroscopy of
Abrikosov flux lattice in $NbSe_2$



H.F. Hess, et al., *Phys. Rev. Lett.* **62**, 1691 (1989)

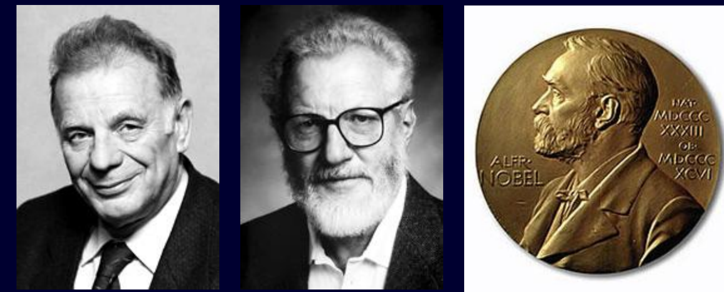
Alexei Abrikosov, 2003 Nobel in Physics

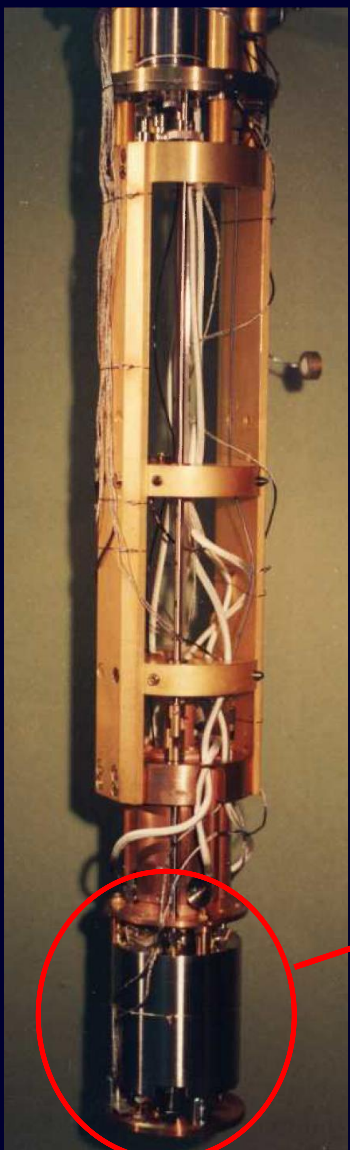




Cryogenic Near-field Spectroscopy

Alferov & Kroemer, 2000 Nobel in Physics

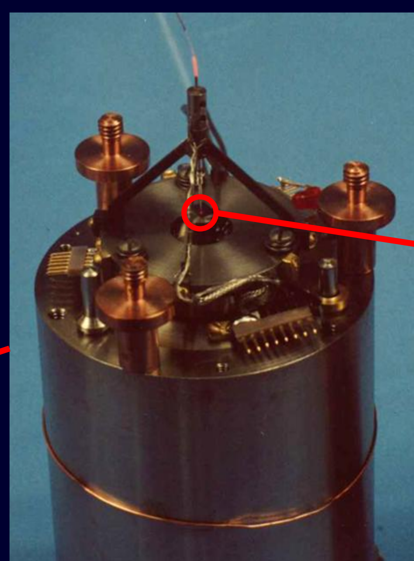




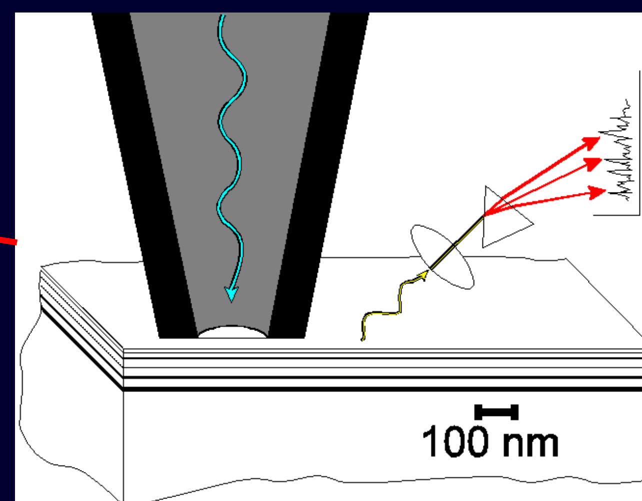
Harald Hess

semiconductor laser diode

Harald's low temp STM



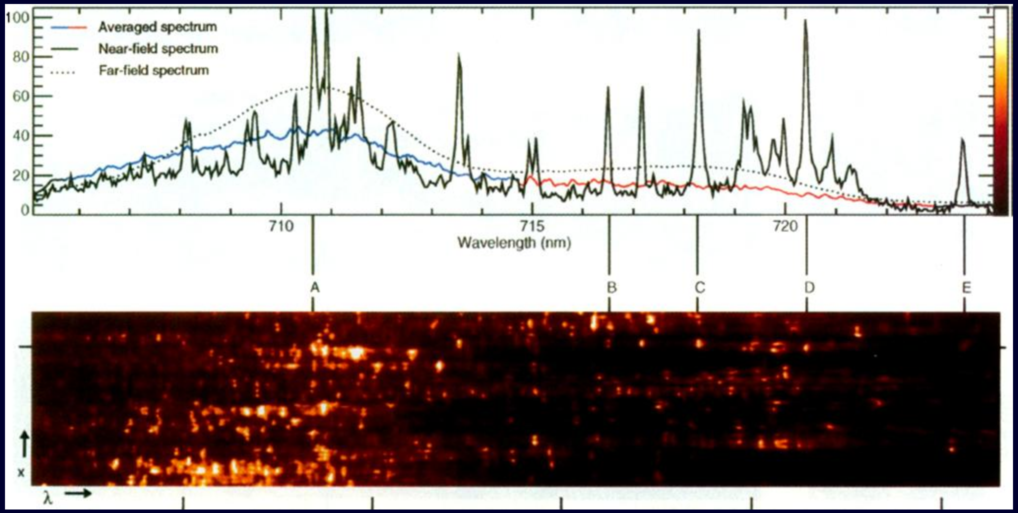
NSOM fiber probe



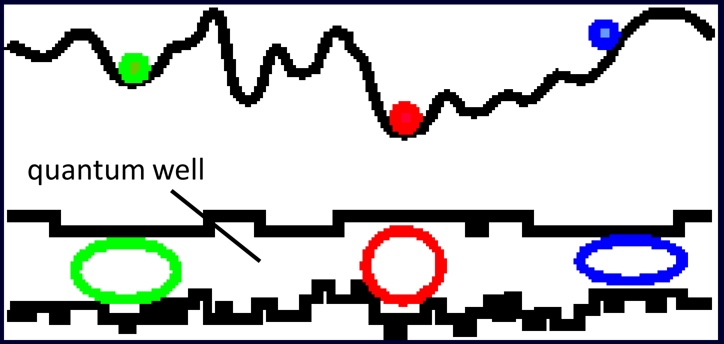
GaAs / AlGaAs multiple quantum well

Cryogenic Near-field Spectroscopy

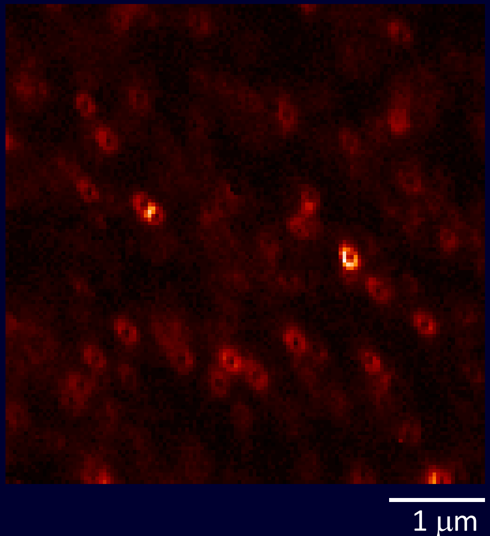
single exciton transitions, 23Å quantum well, 2°K



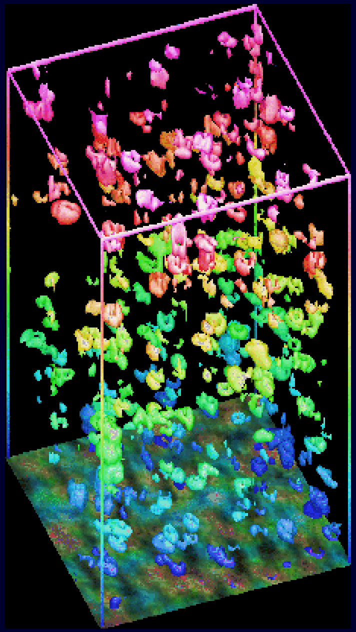
exciton energy variations due to interface roughness



exciton recombination sites scrolling from λ = 700 to λ = 730 nm



isolation of discrete sites in *x,y,λ* space



H.F. Hess, E. Betzig, *et al.*, *Science* **264**, 1740 (1994)

My First Mid-Life Crisis

NSOM engineering limitations:

- poor yield during manufacture
- fragile probes
- topographical artifacts
- weak signals
- probe tips get hot
- large probe tip (0.25 μm)

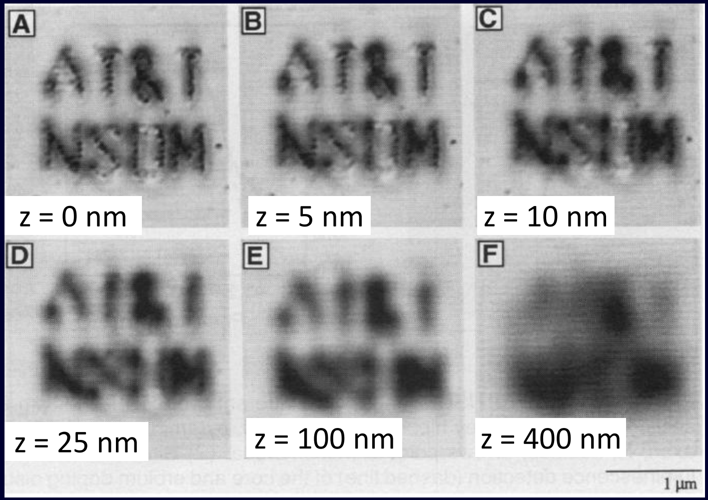
Cells aren't flat!

3D lattice light sheet microscopy,
D. Mullins, T. Ferrin, E. Betzig, *et al.*

NSOM fundamental limitations:

- probe perturbs fields at sample
- complex contrast mechanisms
- nonlinear image formation - artifacts

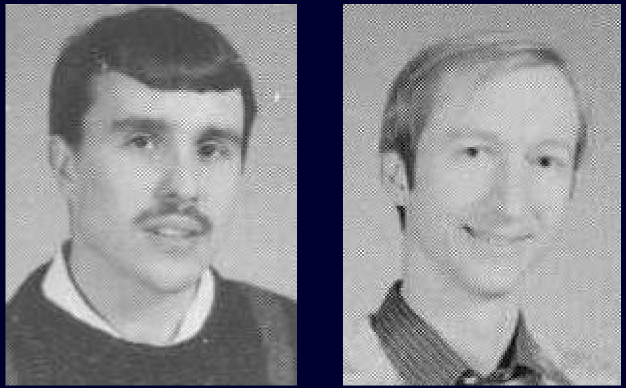
- the near-field is VERY, VERY short



E. Betzig, J.K. Trautman, *Science* **257**, 189 (1992)

My First Mid-Life Crisis

me and Harald, 1989

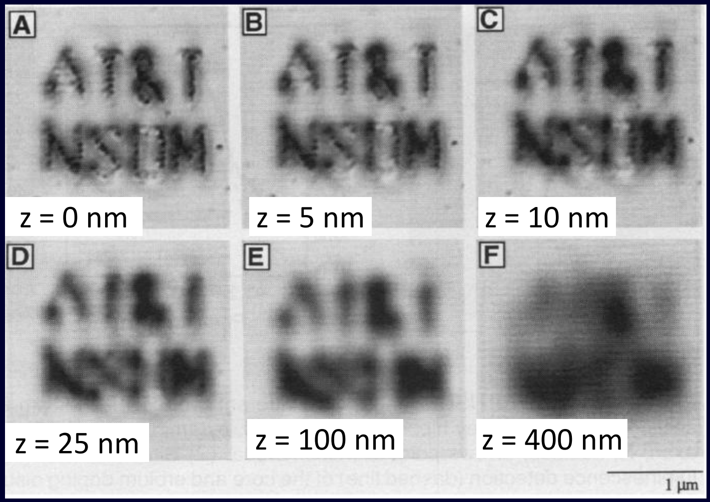


me and Harald, 1994

NSOM fundamental limitations:

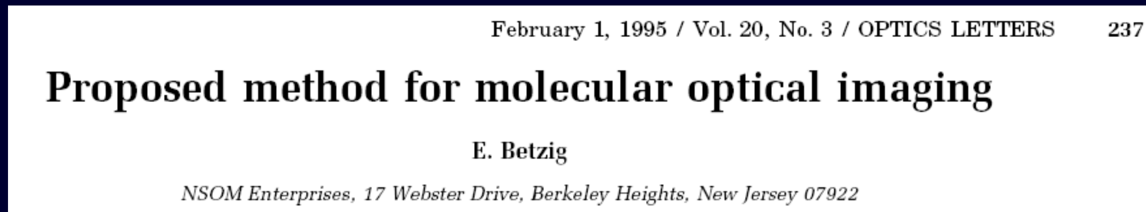
- probe perturbs fields at sample
- complex contrast mechanisms
- nonlinear image formation - artifacts

- the near-field is very, very short



E. Betzig, J.K. Trautman, *Science* **257**, 189 (1992)

Multidimensional Localization Microscopy

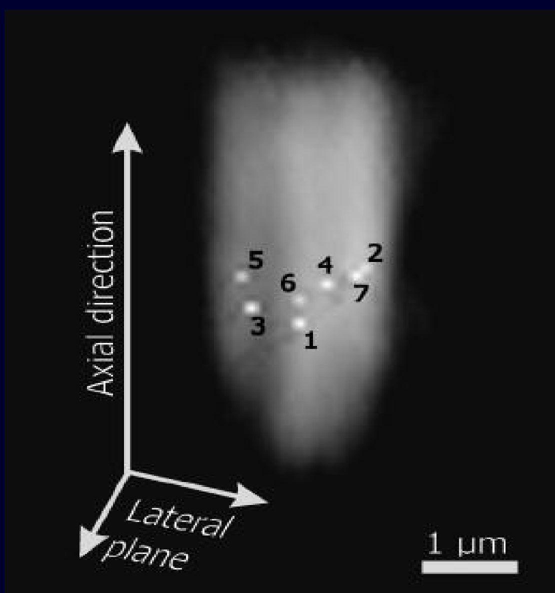

February 1, 1995 / Vol. 20, No. 3 / OPTICS LETTERS 237

Proposed method for molecular optical imaging

E. Betzig

NSOM Enterprises, 17 Webster Drive, Berkeley Heights, New Jersey 07922

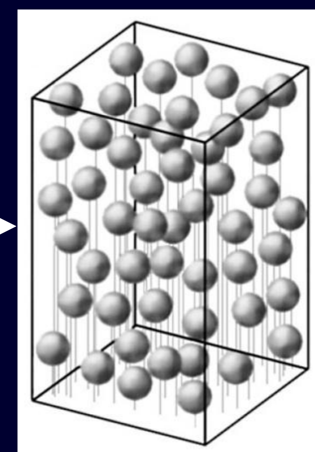
spectral isolation



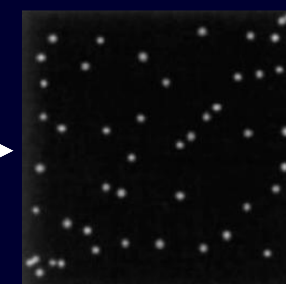
A.M. van Oijen, *et al.*, *JOSA* A16, 909 (1999)

higher dimensional isolation

original image



localization



Photobleaching: X. Qu, *et al., PNAS* 101, 11298 (2004)
M.P. Gordo, *et al., PNAS* 101, 6462 (2004)

Lifetime: M. Heilemann, *et al., Anal. Chem.* 74, 3511 (2002)

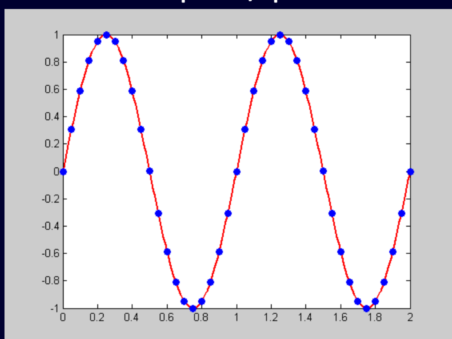
Blinking: K.A. Lidke, *et al., Opt. Express* 13, 7052 (2005)

Spatial Resolution and the Nyquist Criterion

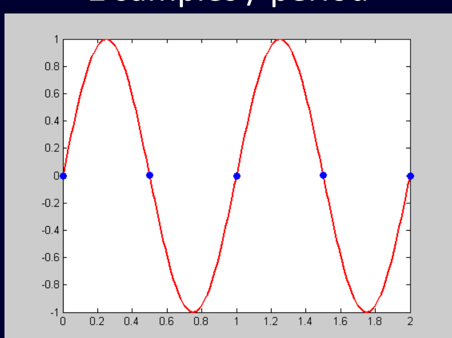
Nyquist criterion:

Sampling interval must be at least twice as fine as the desired resolution

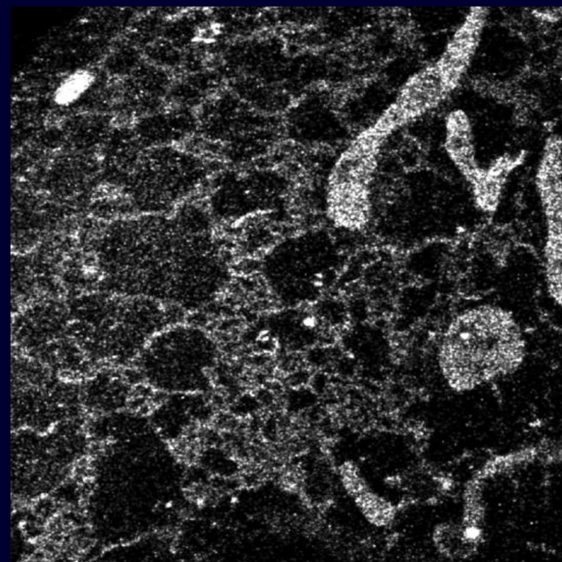
20 samples / period



2 samples / period



initial molecular density



4× greater molecular density

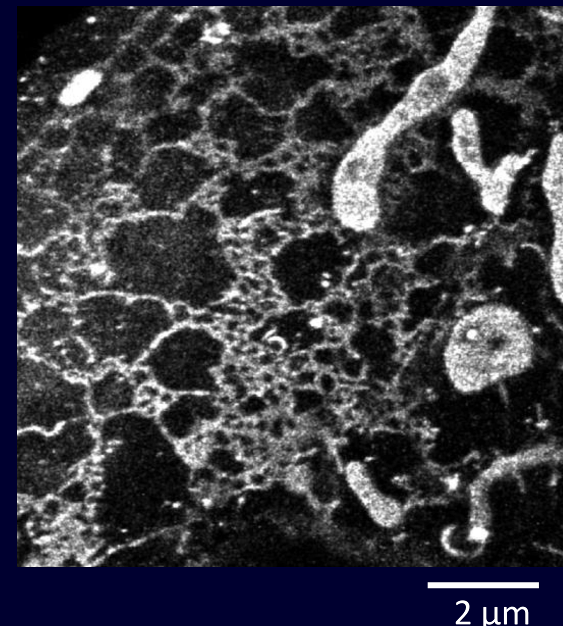


Image Dimensionality	Molecules Required per Diffraction Limited Region for 20 nm Resolution
1D	25
2D	500
3D	2.9×10^4

Diffraction Limited Region:
0.25 µm dia, 0.6 µm long

And Now for Something Completely Different

Flexible Adaptive Servohydraulic Technology (FAST)

- moves 4000 kg load at 8g acceleration
- positioning precision to 5 µm

Robert Betzig

My Second Mid-Life Crisis

Searching for a New Direction

me in Joshua Tree National Park

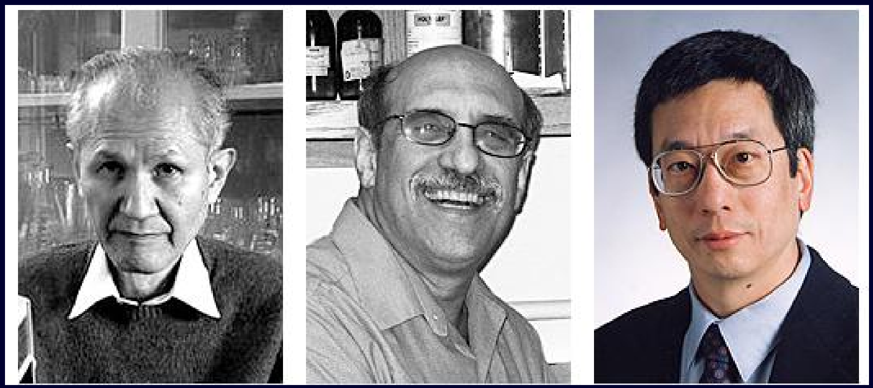
Harald in Sedona, Arizona

me in Oahu, Hawaii

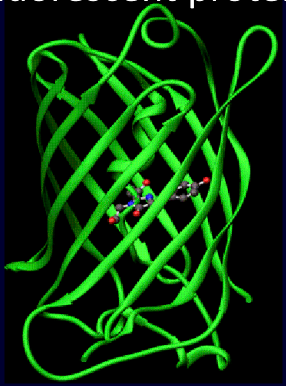
Harald in Yosemite National Park

Fluorescent Proteins Revolutionize Biological Imaging

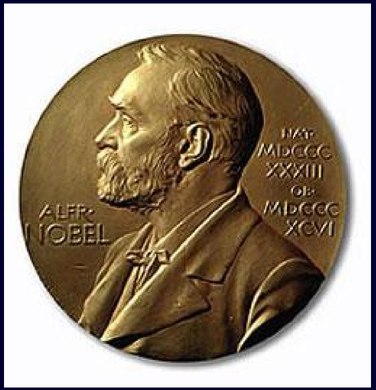
Shimomura, Chalfie, & Tsien



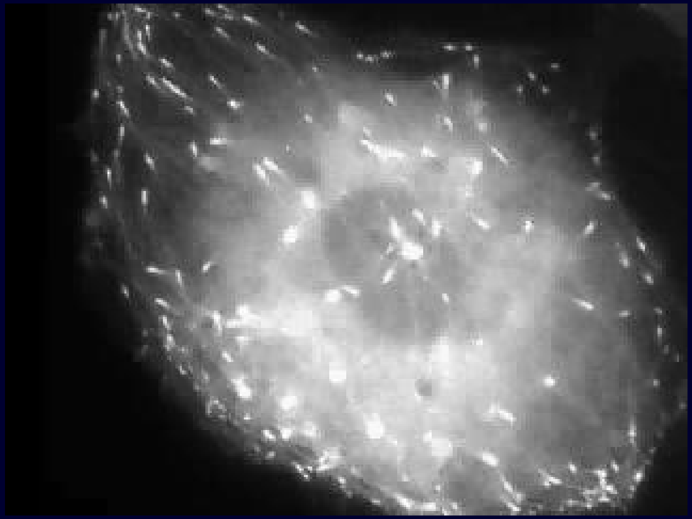
1994: green fluorescent protein



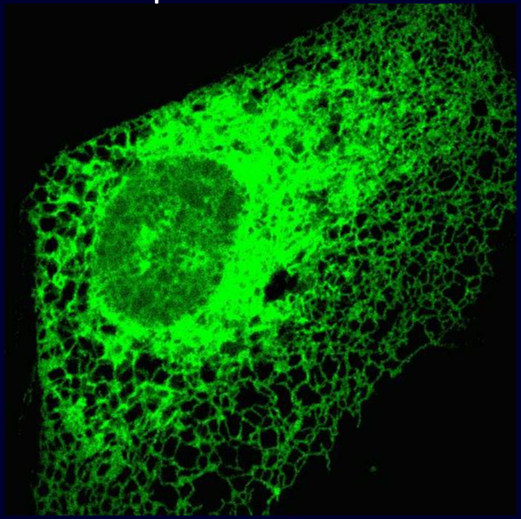
2008: Chemistry Nobel



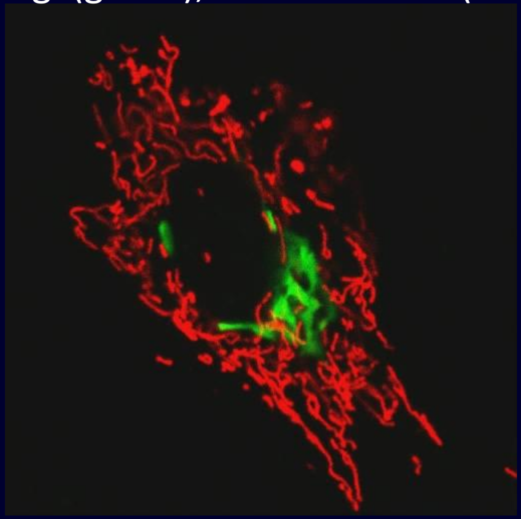
microtubule ends



endoplasmic reticulum

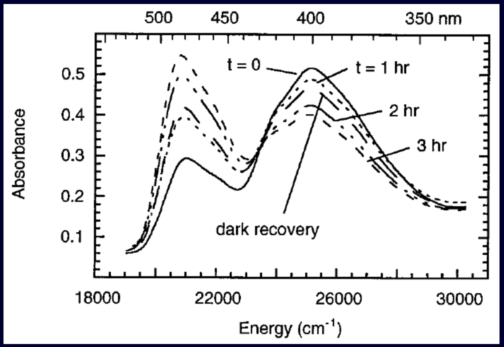


golgi (green), mitochondria (red)

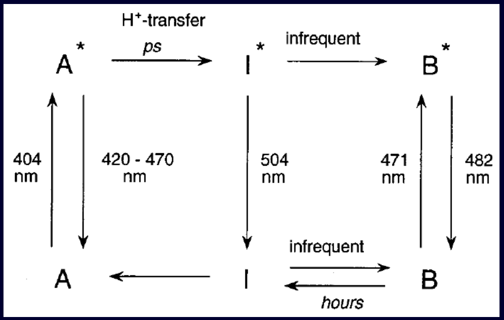


Switching Behavior in Green Fluorescent Protein

488 nm absorption increase under 398 nm illumination



proposed mechanism

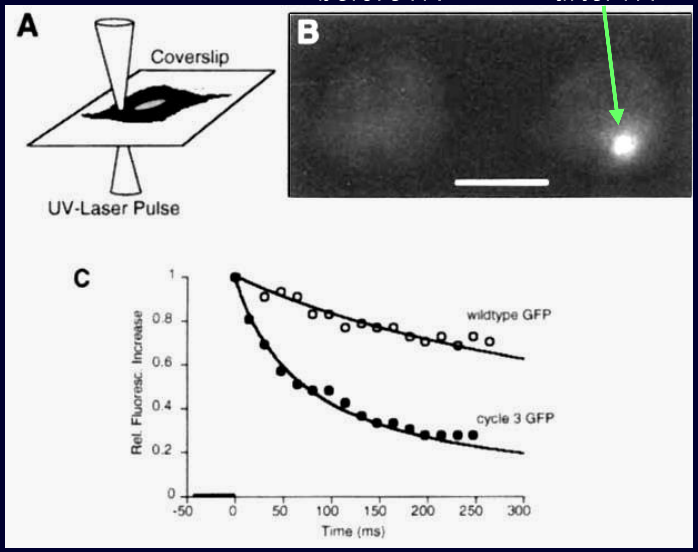


M. Chattoraj, et al., *PNAS* **93**, 8362 (1996)

in vivo UV photoactivation (PA) of wtGFP

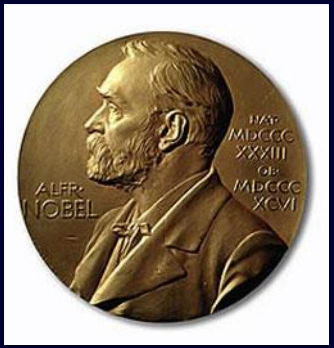
before PA

after PA

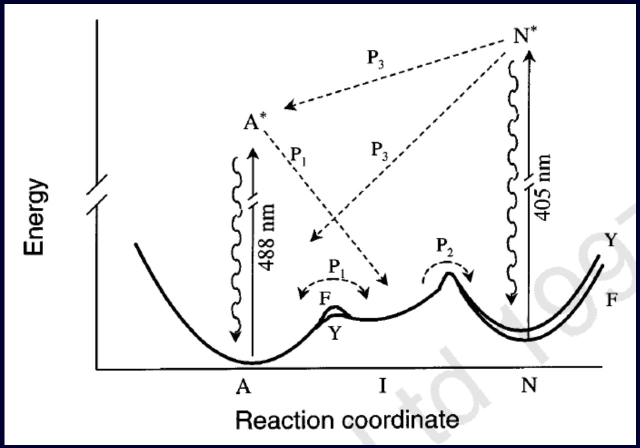


H. Yokoe, T. Meyer, *Nat. Biotech.* **14**, 909 (1996)

W.E. Moerner, 2014 Nobel in Chemistry



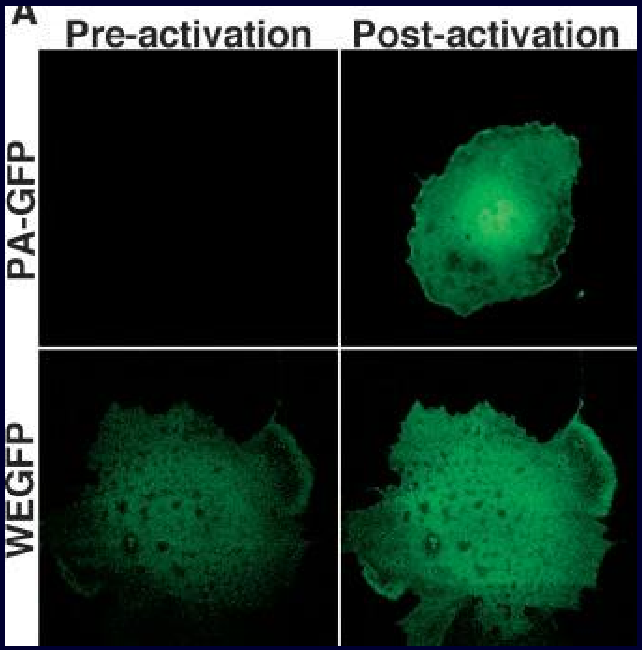
photoactivation energy diagram



R.M. Dickson, *et al.*, *Nature* **388**, 355 (1997)

Directed Mutagenesis of Photoactivated Fluorescent Proteins (PA-FPs)

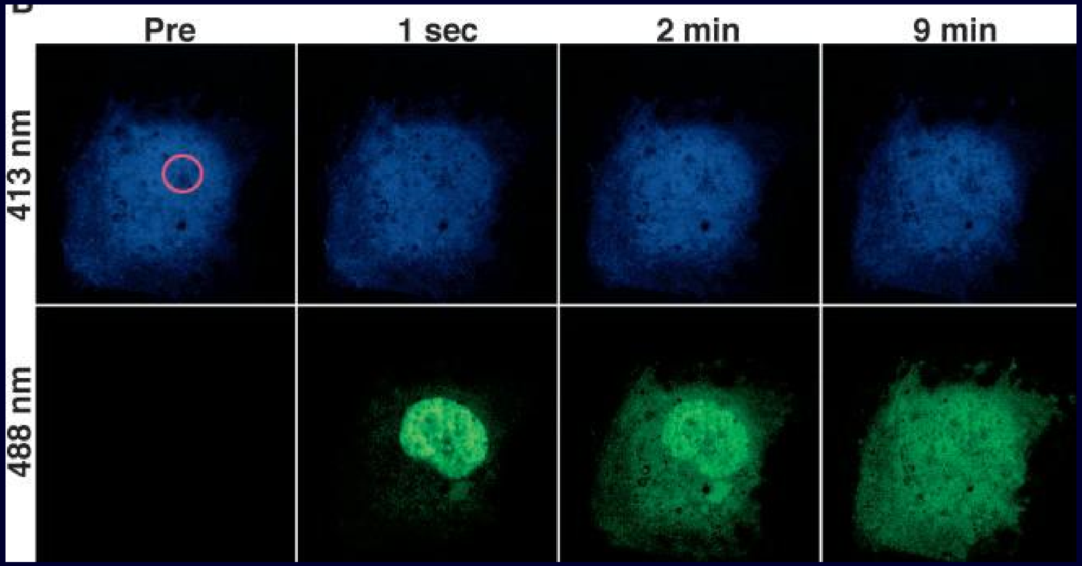
increased on/off contrast of PA-GFP



Jennifer Lippincott-Schwartz

George Patterson

pulse chase: nuclear vs cytosolic diffusion



G.H. Patterson, J. Lippincott-Schwartz, *Science* **297**, 1873 (2002)

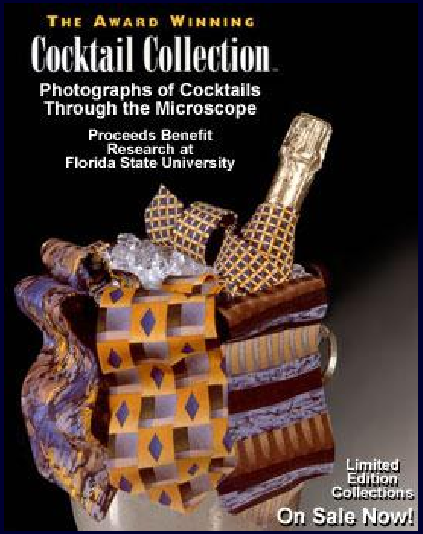
A Fateful Trip

Greg Boebinger

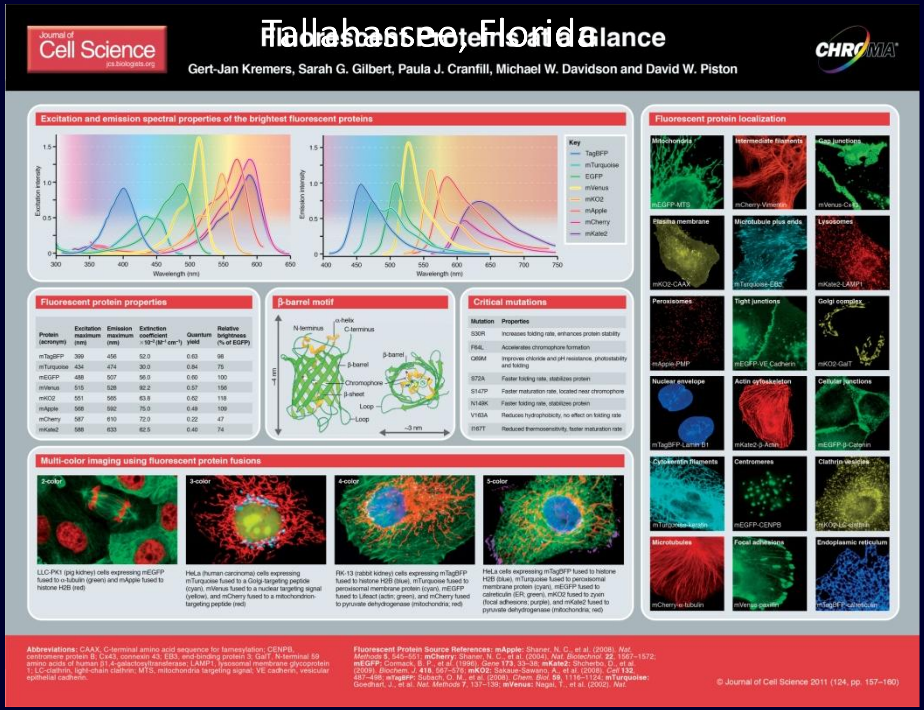
National High Magnetic Field Lab

Mike Davidson

Neckties®



Tallahassee, Florida



website tutorials

Zeiss

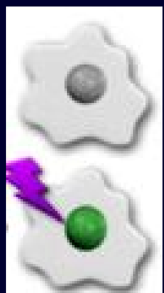
Olympus

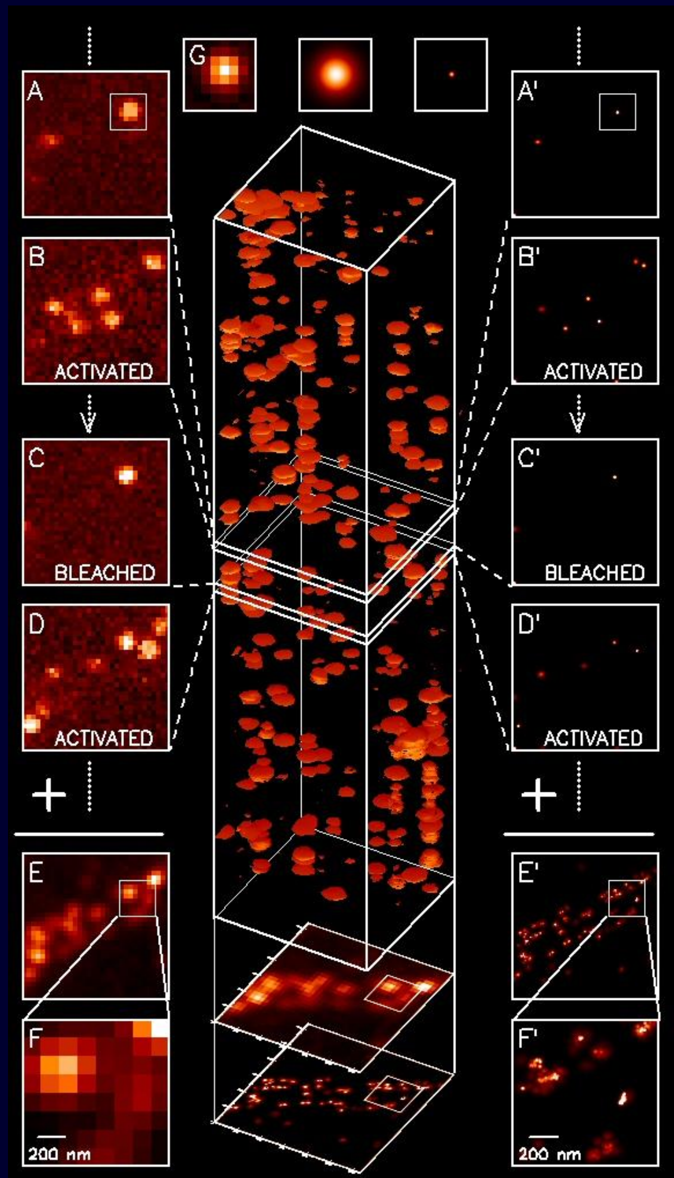
MICROSCOPY RESOURCE CENTER

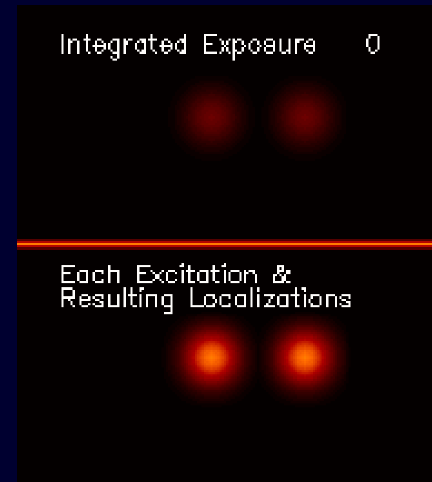
Nikon

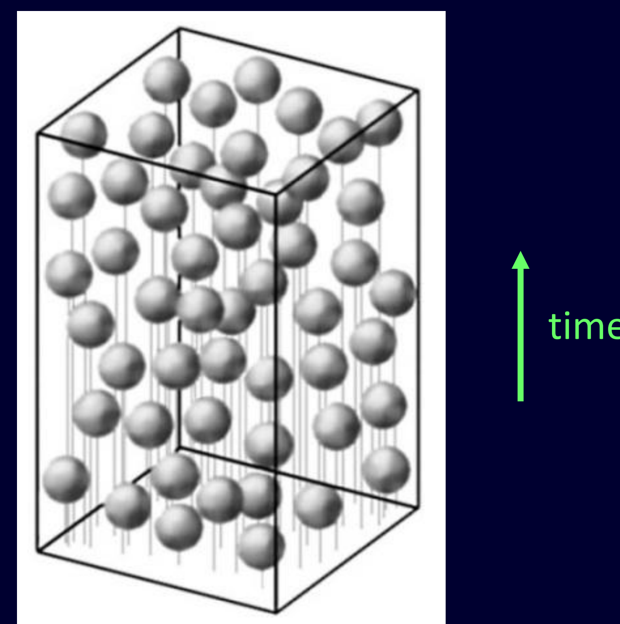
MICROSCOPY U
THE SOURCE FOR MICROSCOPY EDUCATION

Finding the Missing Link









E. Betzig, *et al.*, *Science* **313**, 1642 (2006)

La Jolla Labs

Assembling the Rest of the Team

Rob Tycko, NIDDK

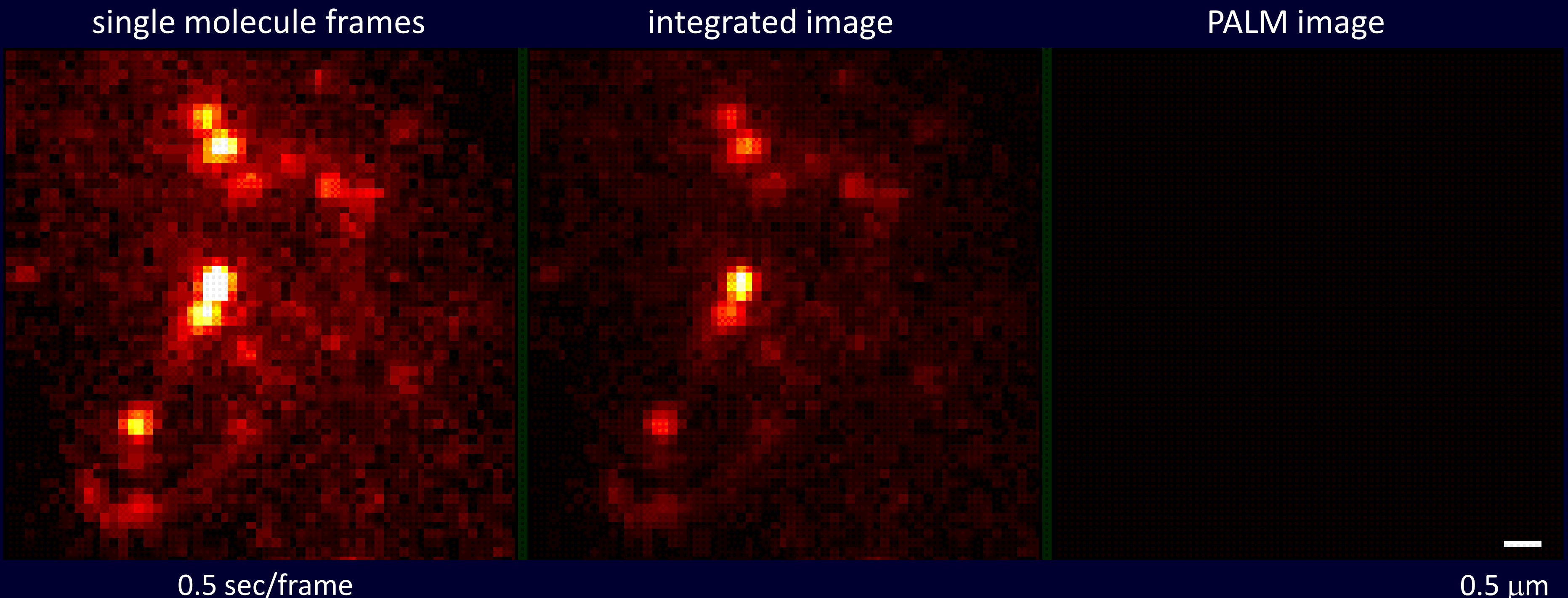
Jennifer Lippincott-Schwartz

George Patterson

the microscope in the darkroom in Jennifer's lab

Photoactivated Localization Microscopy (PALM)

lysosomes, COS-7 cell, Kaede-tagged CD63

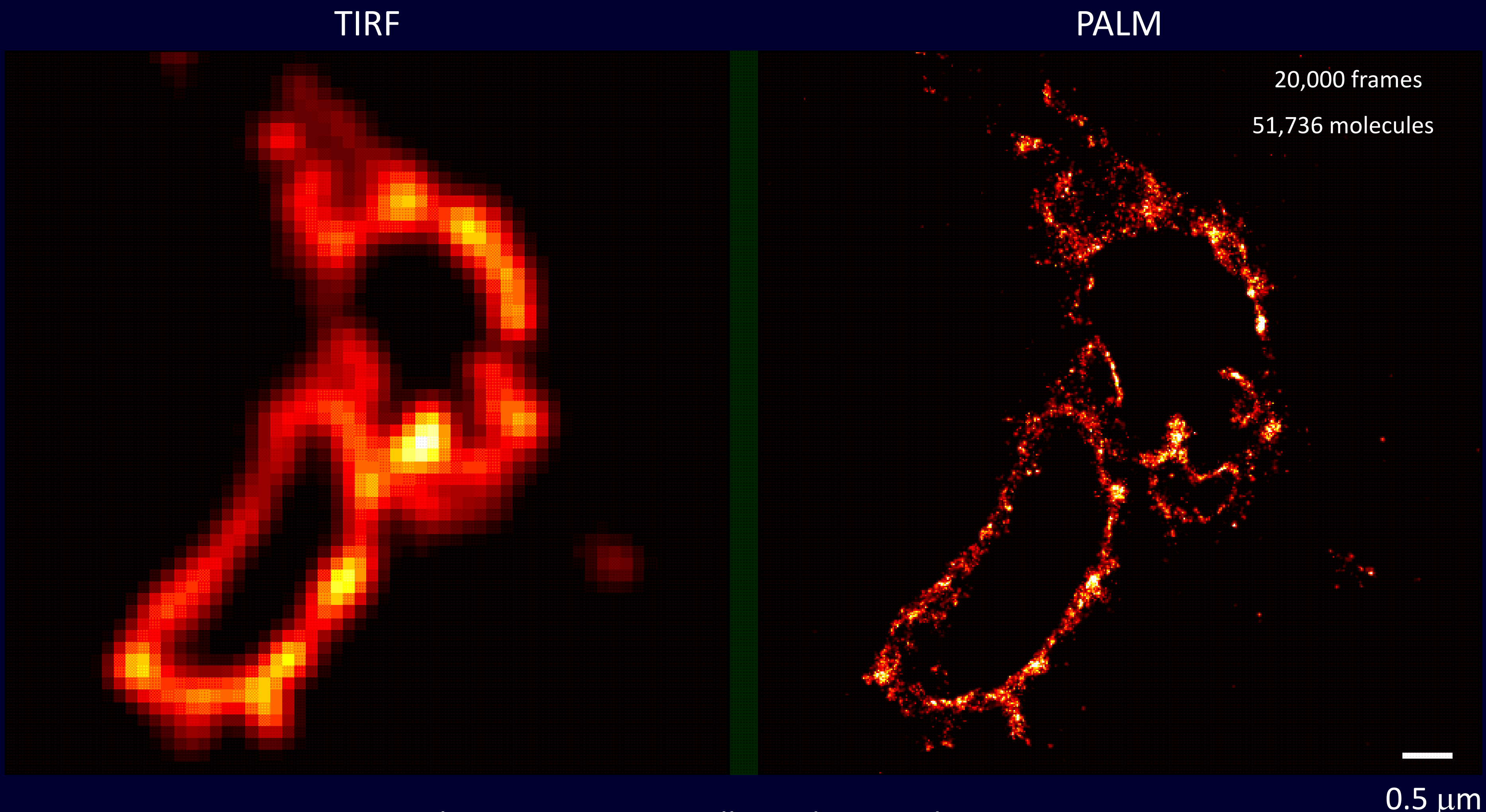


~80 nm cryosection:

- low autofluorescence
- immobile PA-FPs
- image internal organelles

E. Betzig, *et al.*, *Science* **313**, 1642 (2006)

Photoactivated Localization Microscopy (PALM)



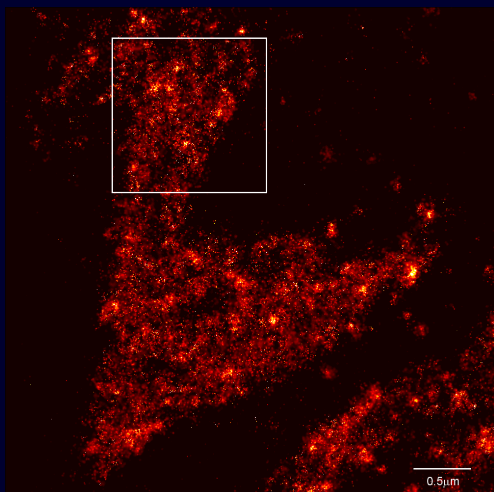
lysosomes, COS-7 cell, Kaede-tagged CD63

E. Betzig, *et al.*, *Science* 313, 1642 (2006)

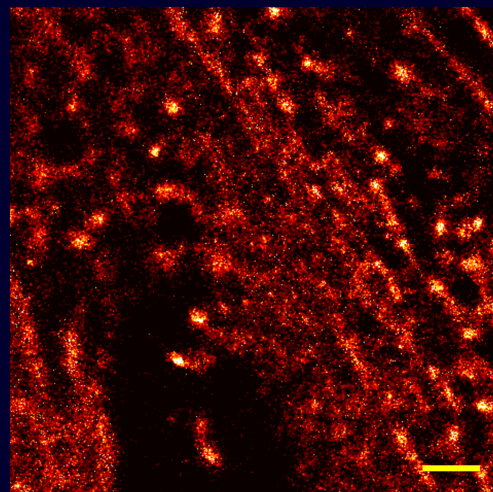
A High On/Off Contrast Ratio is Essential for High Resolution

paxillin, focal adhesions

EosFP > 2000:1

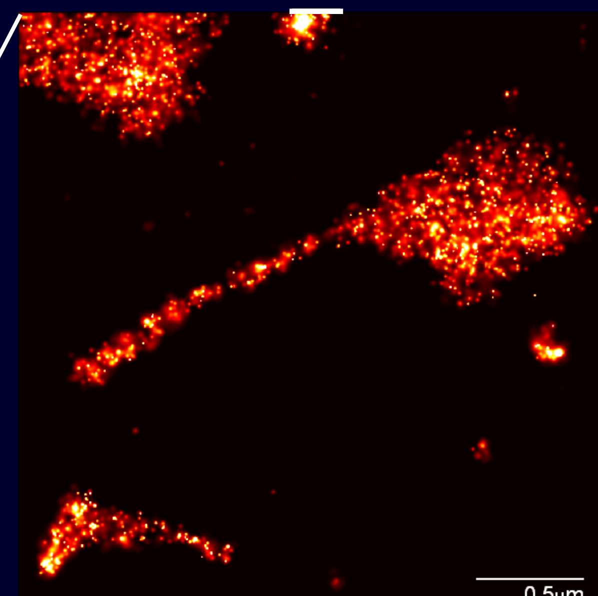


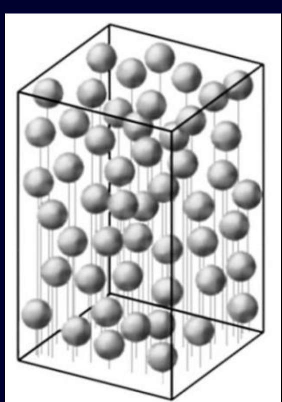
PA-GFP < 75:1



Eos FP and caged Q-rhodamine support Nyquist-defined sub-20 nm resolution

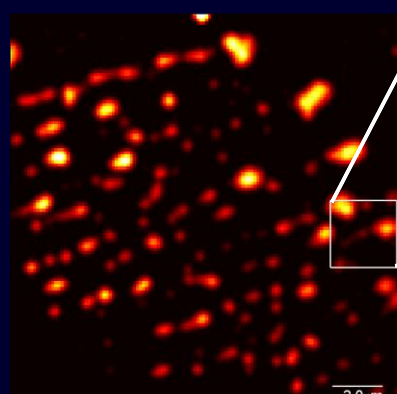
caged Q-rhodamine, > 1000:1





time

diffraction limited TIRF



E. Betzig, *et al.*, *Science* 313, 1642 (2006)

From Rags to Riches, Thanks to HHMI

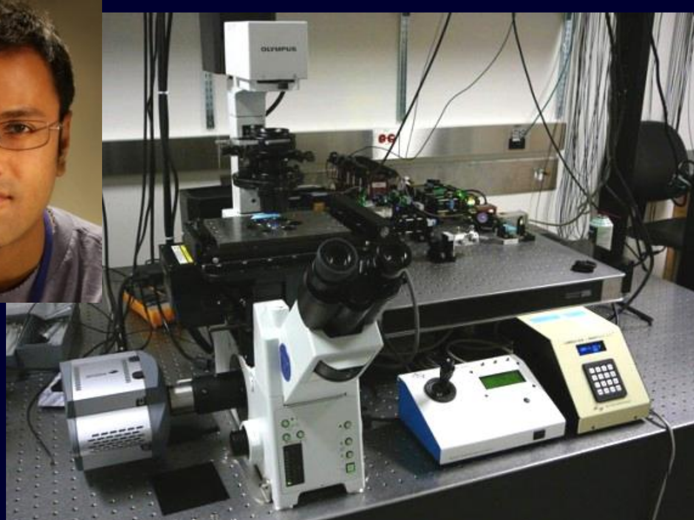
Janelia Research Campus

The Boss: Gerry Rubin

Endless Coffee

Hari Shroff

my PALM

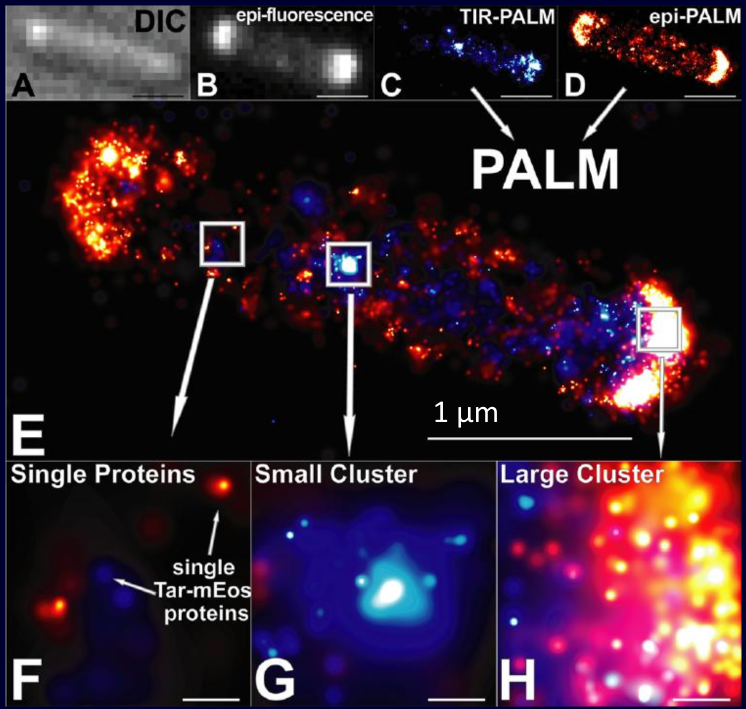


Gleb Shtengel

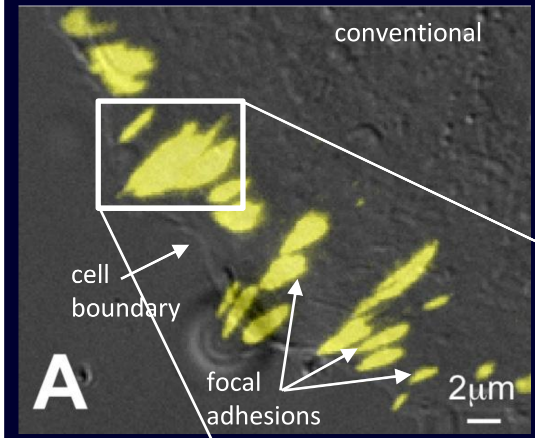
Harald’s iPALM

PALM Application Examples

Chemotaxis Receptors in *E. coli*

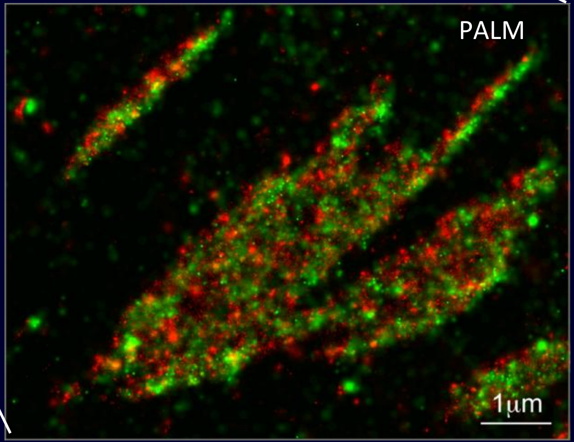


D. Greenfield, *et al.*, *PLoS Biol.* 7, 137 (2009)



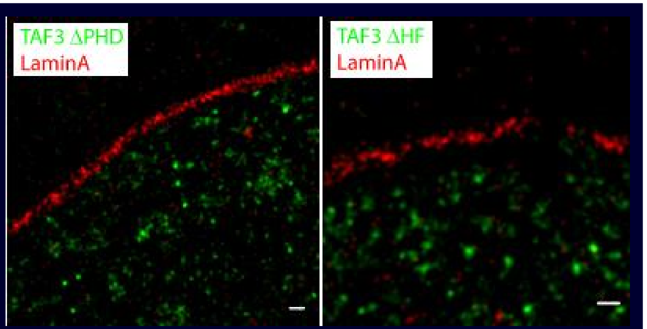
Two-Color Imaging of Focal Adhesion Proteins

Hari Shroff

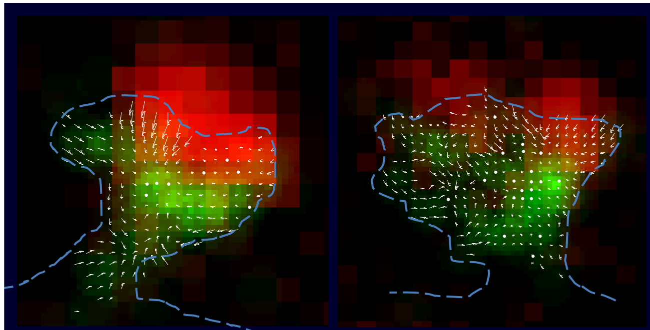


H. Shroff, *et al.*, *PNAS* 104, 20308 (2007)

Regulation of Gene Expression During Myogenesis



J. Yao, *et al.*, *Genes Dev.* **25**, 569 (2011)



Actin Polymerization in Dendritic Spines

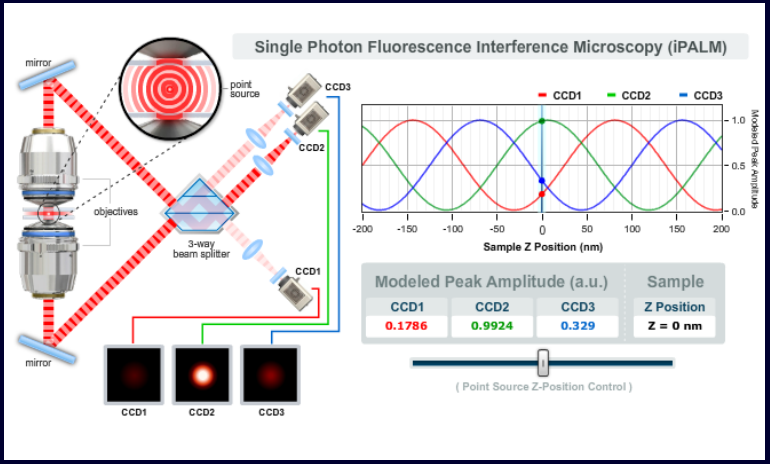
N. Frost, *et al.*, *Neuron* 67, 86 (2010)

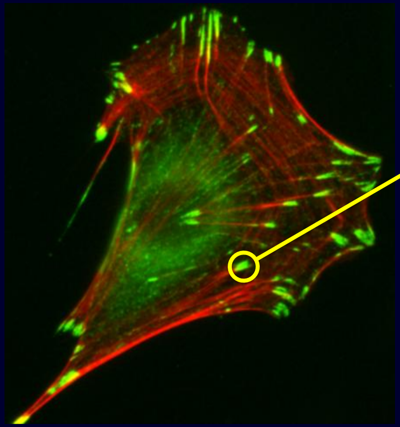
iPALM: Ultrasensitive PALM in 3D

Harald Hess

iPALM schematic

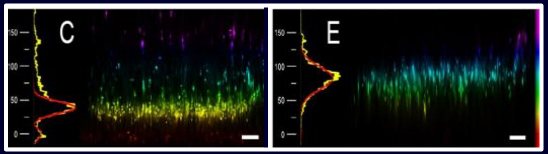
three phase single molecule interferometry



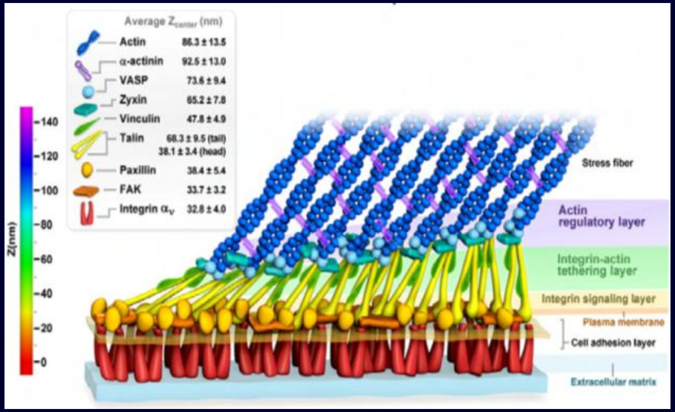


single focal adhesion

iPALM xz view

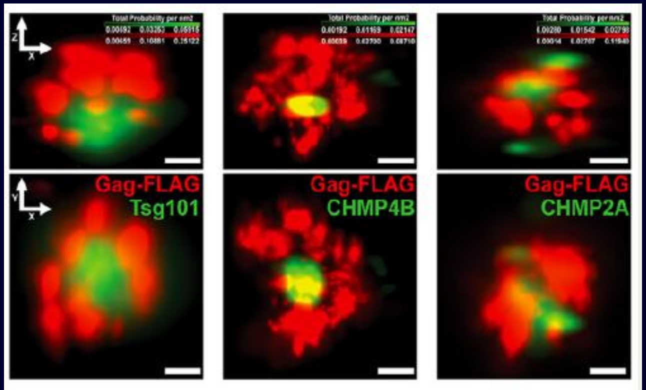


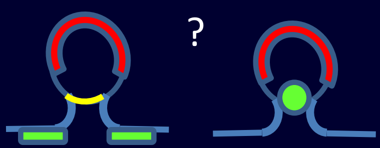
vertical architecture of adhesions

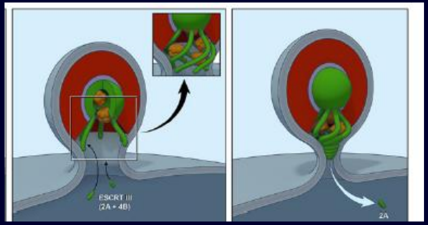


P. Kanchanawong, *et al.*, *Nature* **468**, 580 (2010)

ESCRT machinery at HIV budding sites



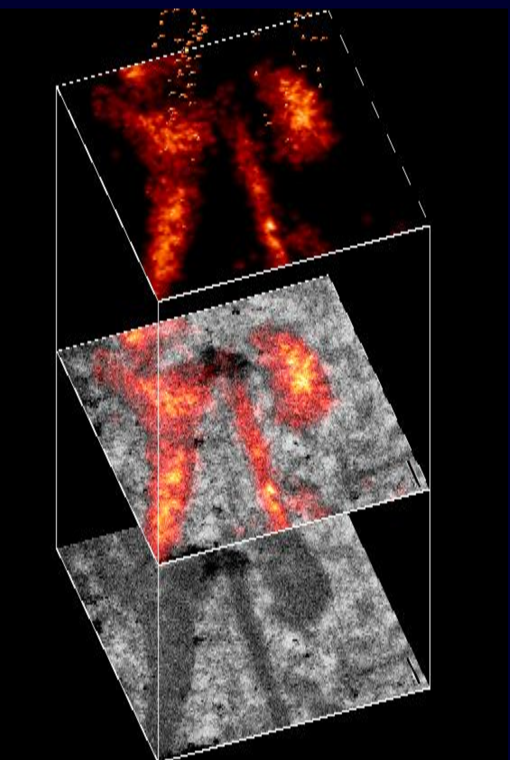




S.B. Van Engelenburg, *et al.*, *Science* **343**, 653 (2014)

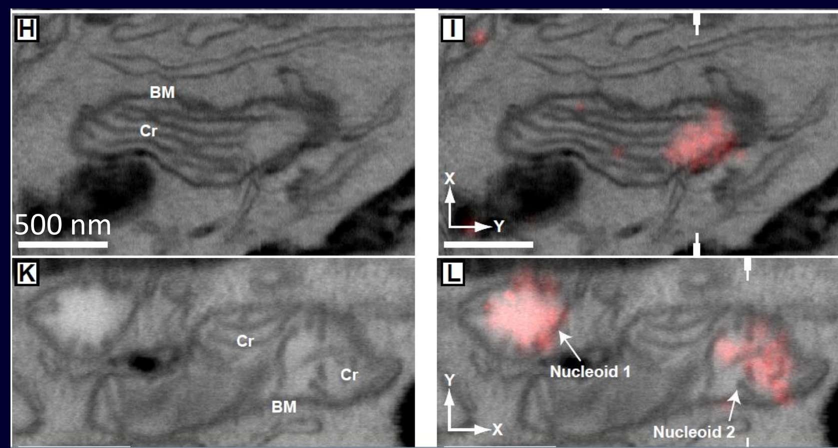
Correlative Electron Microscopy and PALM

first correlative EM with super-resolution: mitochondria

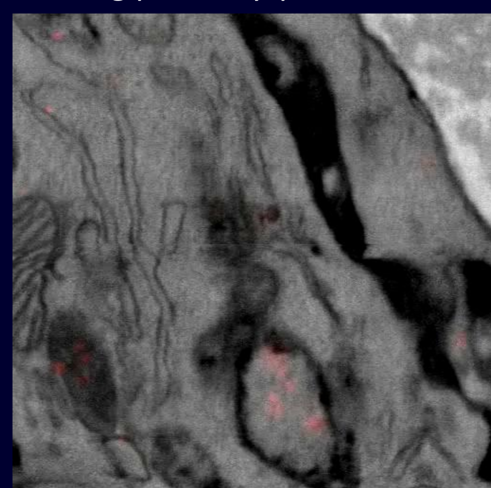


E. Betzig, *et al.*, *Science* 313, 1642 (2006)

3D correlative EM/PALM
mitochondria (B&W – FIB SEM)
mitochondrial DNA (red - iPALM)

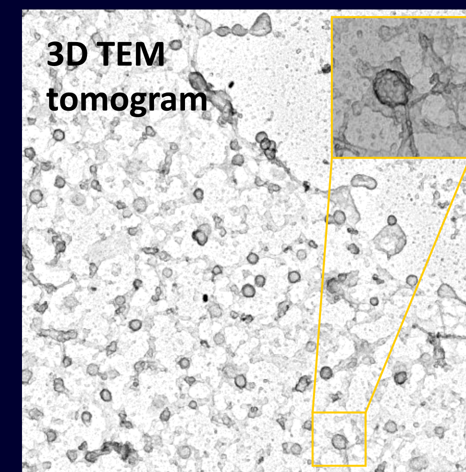


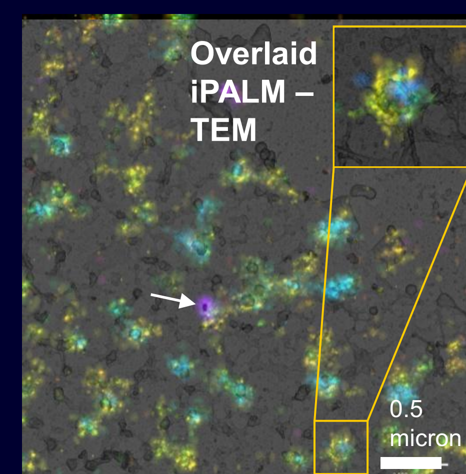
scrolling plane-by-plane thru 3D



B.G. Kopek, *et al.*, *PNAS*, **109,** 6136 (2012)

cell membrane (B&W - TEM)
& clathrin (color - iPALM)



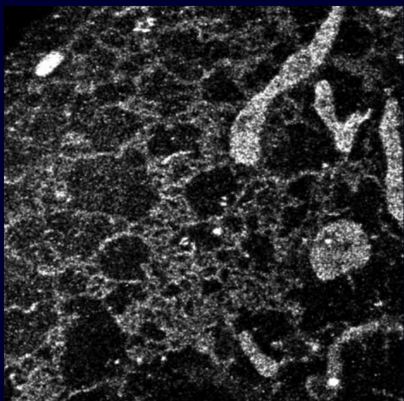


K. Sochaki, et. al, *Nat. Methods*, **11** 305 (2014)

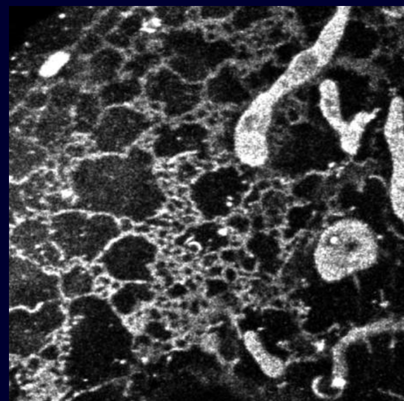
Caveats with Super-Resolution Microscopy: Fixed Cells

extremely high labeling densities required

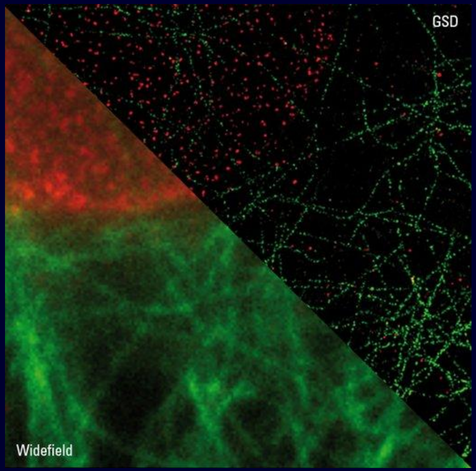
initial density

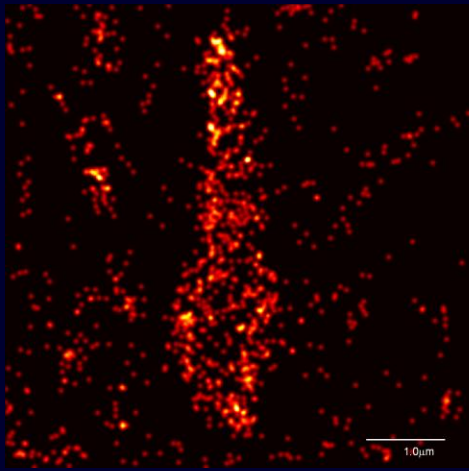


4x higher density

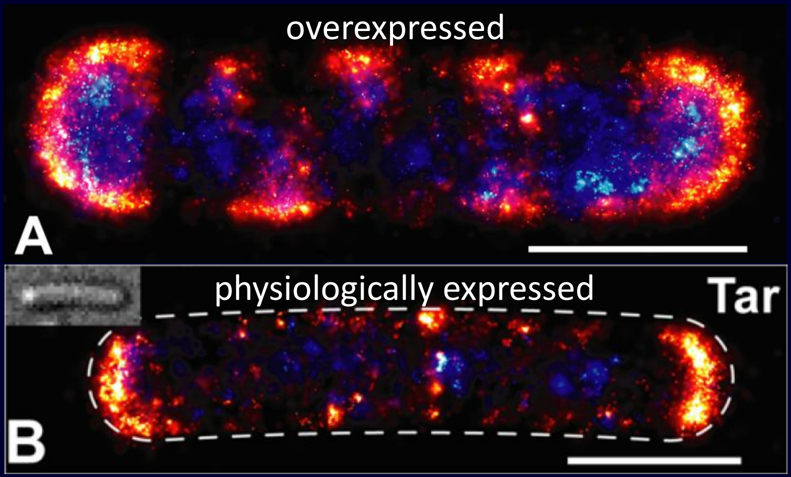


exogenous dyes: limited affinity & high background

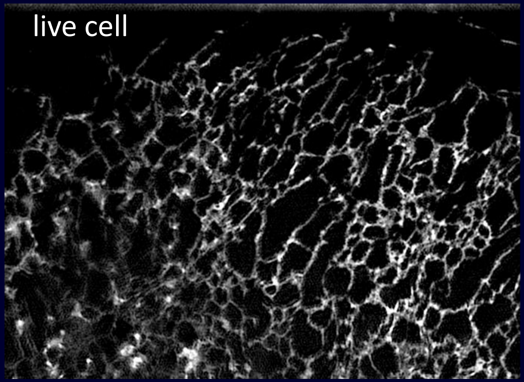


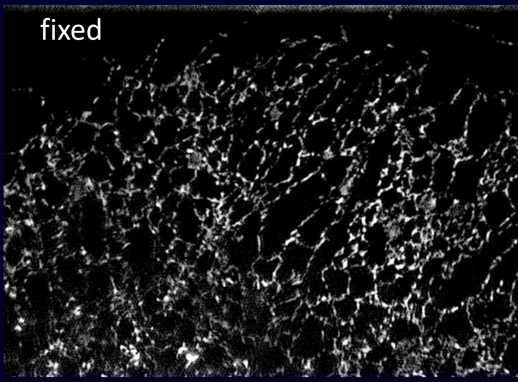


overexpression of protein



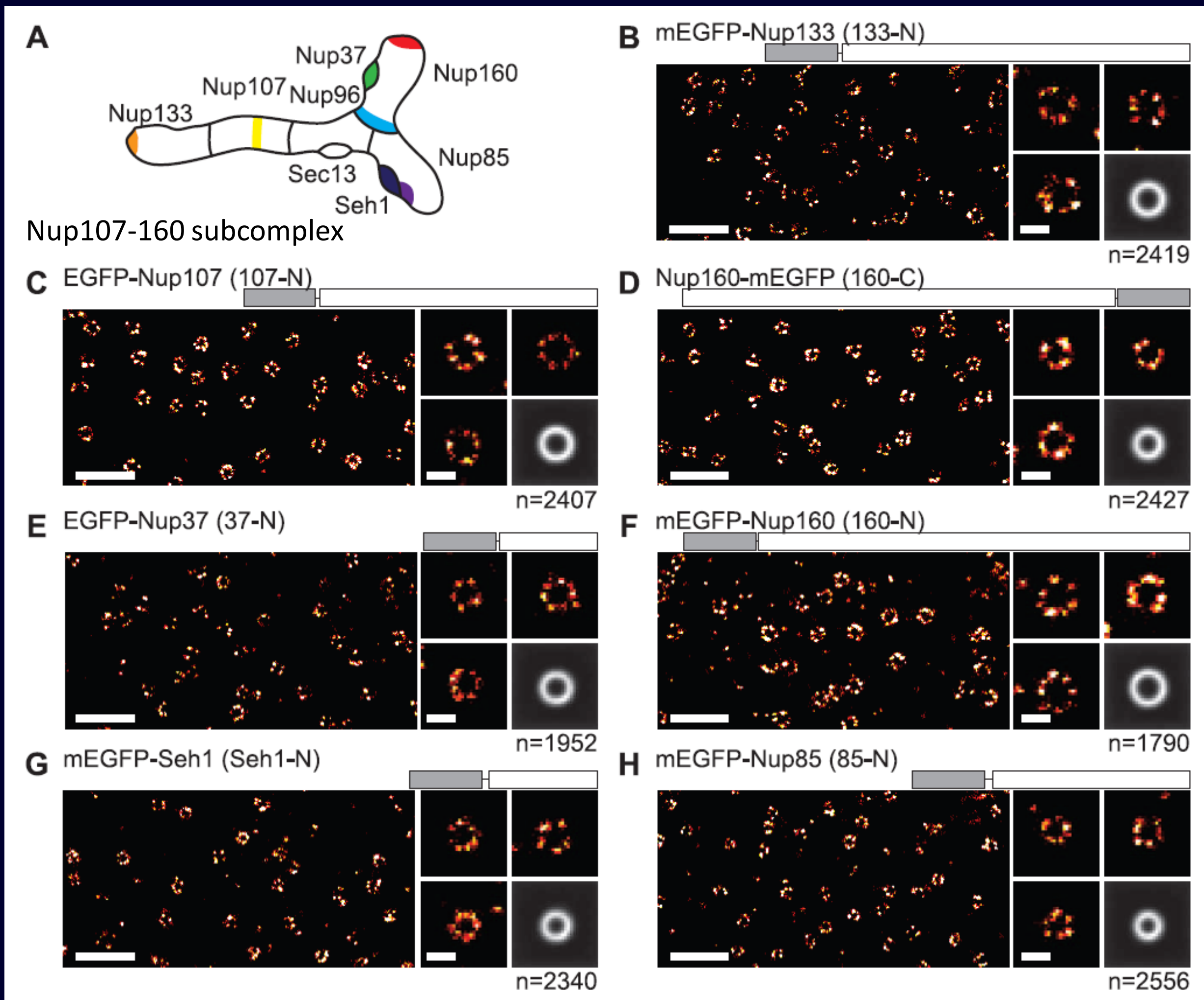
fixation artifacts, endoplasmic reticulum



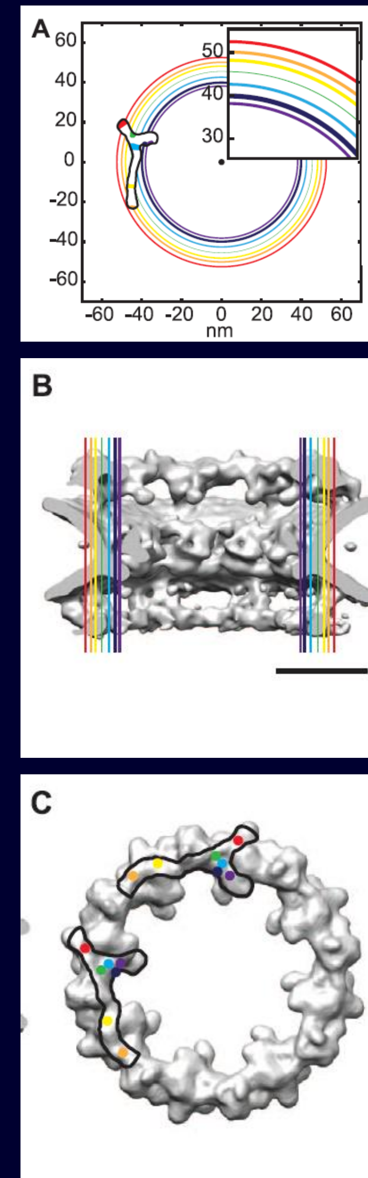


Particle Averaging Improves Resolution of Stereotypic Structures

nuclear pore complex proteins

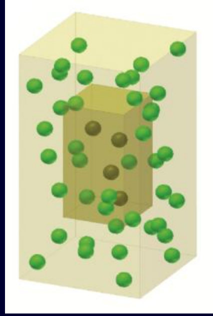


positions determined to < 1 nm



A. Szymborska, *et al*. *Science* 341, 655 (2013)

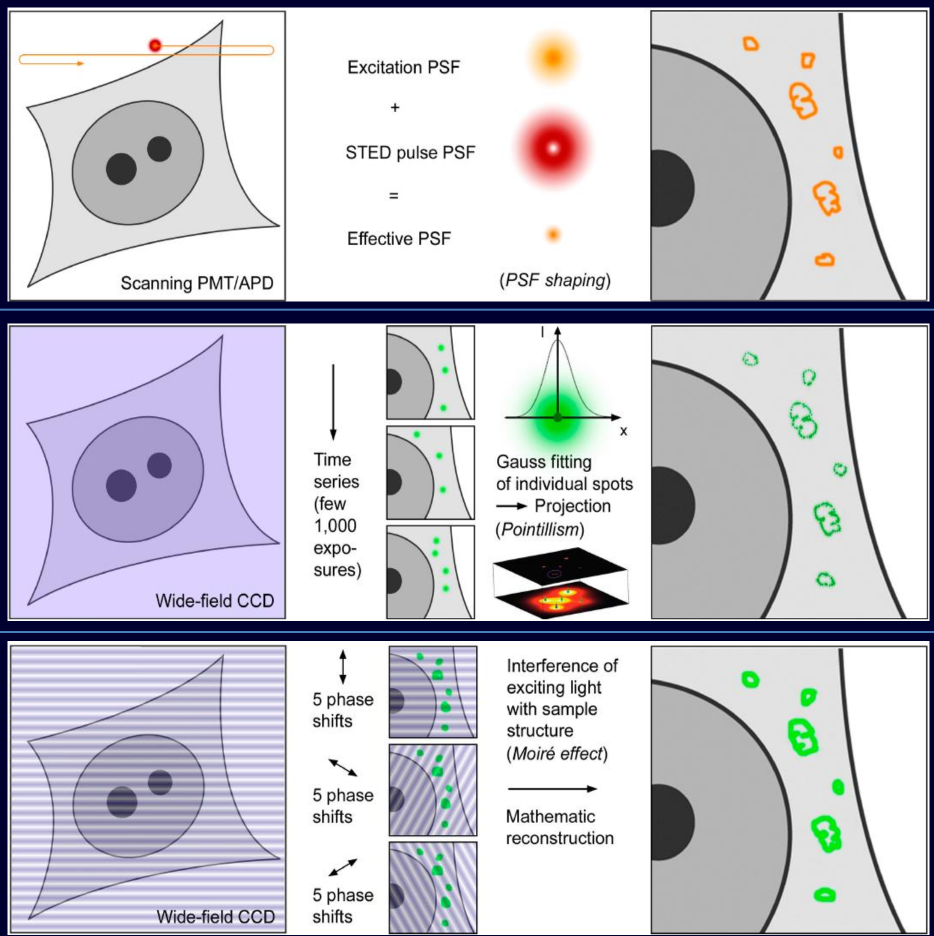
Caveats with Super-Resolution Microscopy: Live Cells



Nyquist criterion:

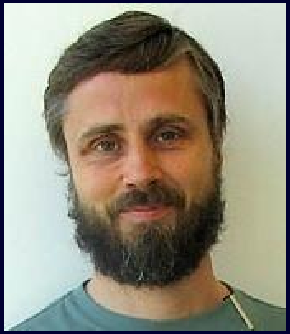
N -fold resolution increase in *D* dimensions → N^D -fold more photons collected

L. Schermelleh, R. Heintzmann, *J. Cell Biol*. (2010)



	reported resolution (nm)	photon increase required	intensity (W/cm²)	acquisition time (sec)
STED / RESOLFT	*xy*: 20 nm	100	10^4 - 10^9	> 60
	xyz: 30 nm	1,070		~1,000
Localization	*xy*: 20 nm	100	10^3 - 10^4	>20
	xy: 10 nm, *z*: 20 nm	14,400		1,500
SIM	*xy*: 100 nm	4	10 - 10^2	0.1 - 1
	xy: 100 nm, *z*: 370 nm	8		~10

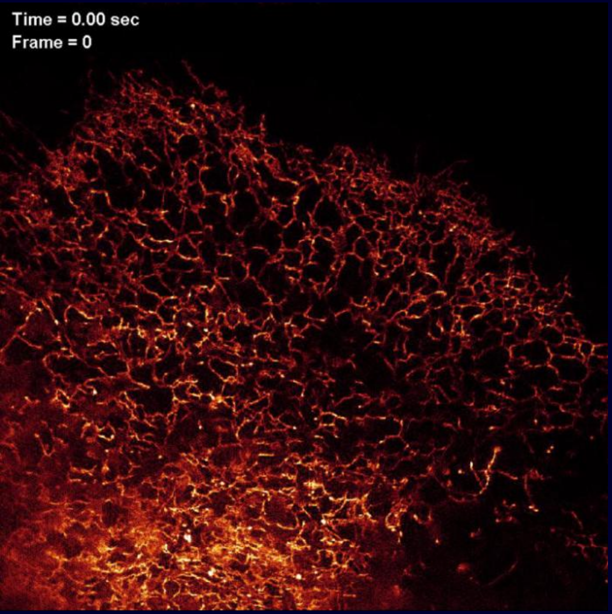
Live Cell Structured Microscopy



Mats
Gustafsson,
1960-2011

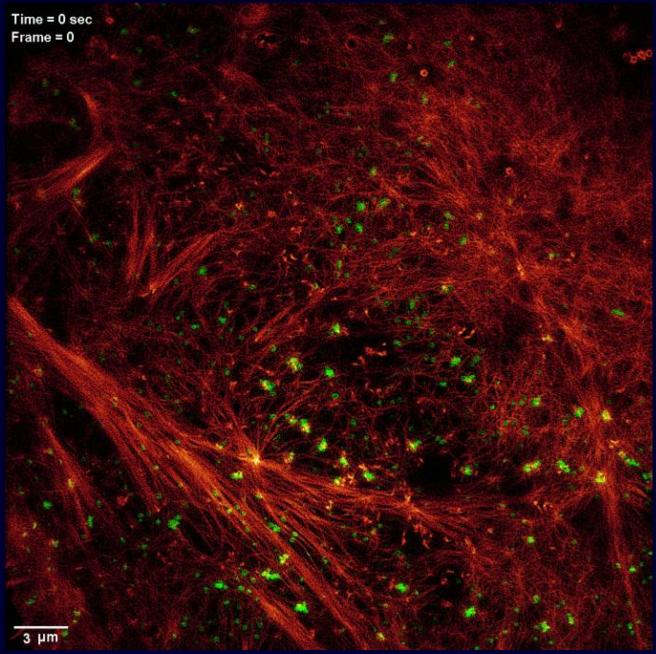
endoplasmic reticulum

2D SIM, 98 nm resolution
0.1 sec acquisition, 1800 frames



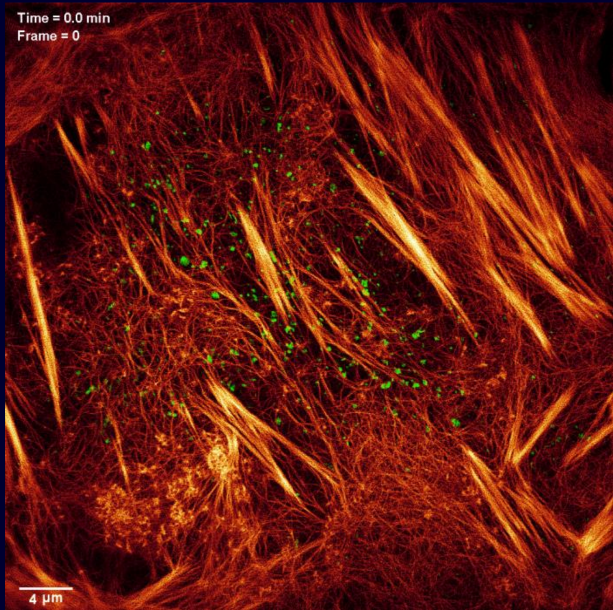
clathrin coated pits and cortical actin

TIRF-SIM, 82 nm resolution
0.5 sec acquisition, 90 frames

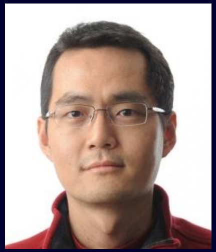


early endosomes and cortical actin

Nonlinear SIM, 62 nm resolution
1.5 sec acquisition, 34 frames



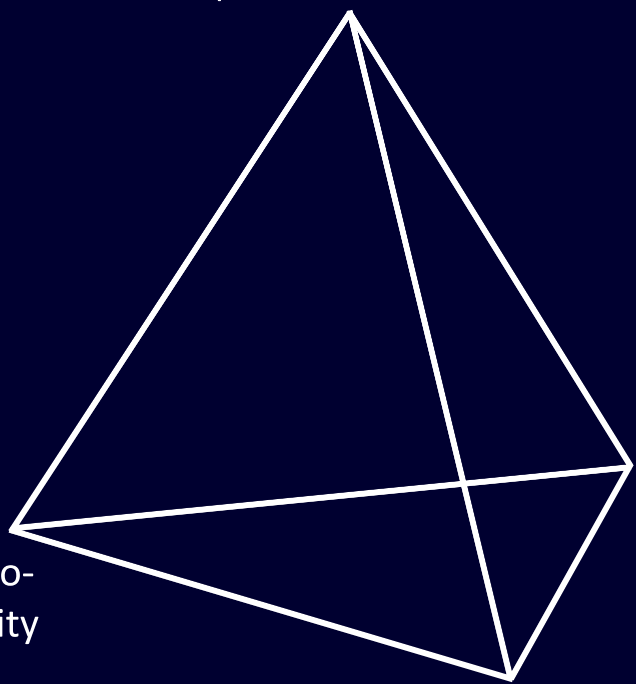
Dong
Li



Lin
Shao

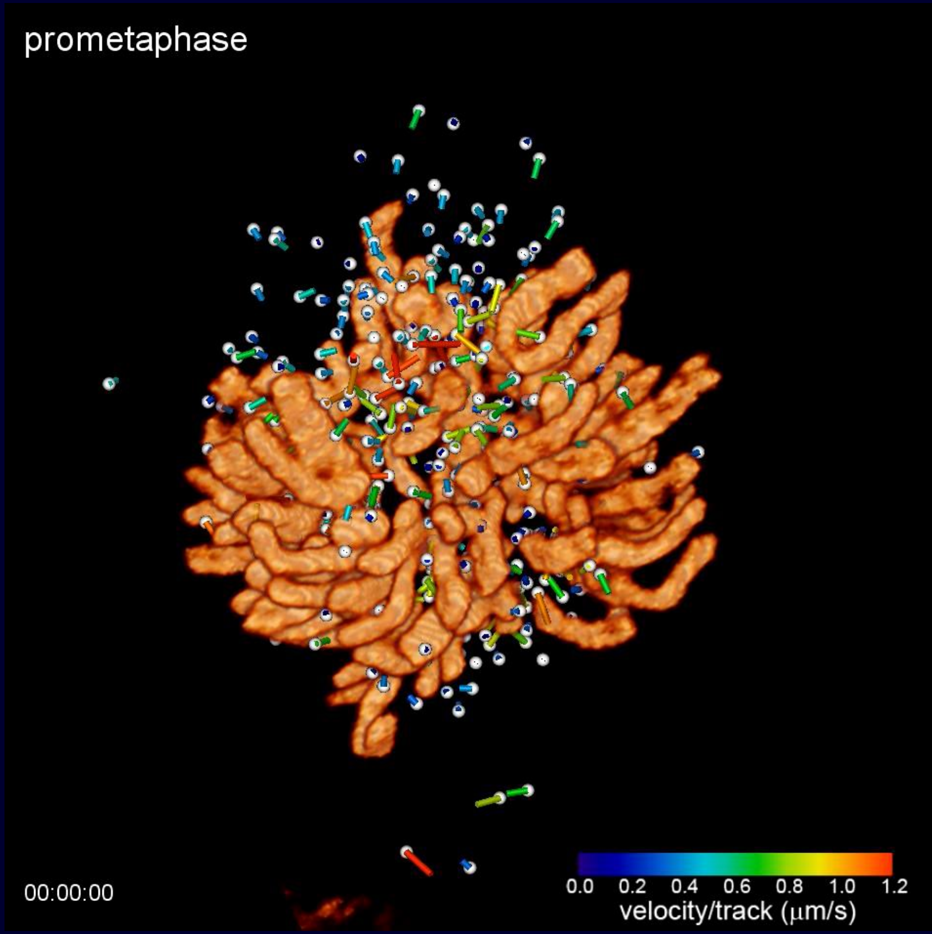
The Challenges and Importance of Studying Live Cell Dynamics

tradeoffs, tradeoffs, tradeoffs



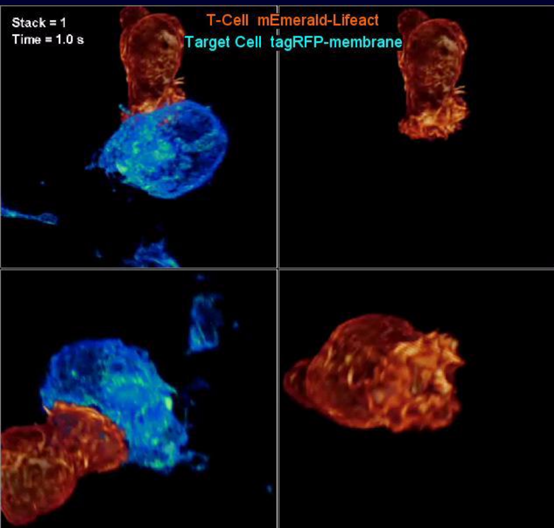
Life is Animate

dividing HeLa cell

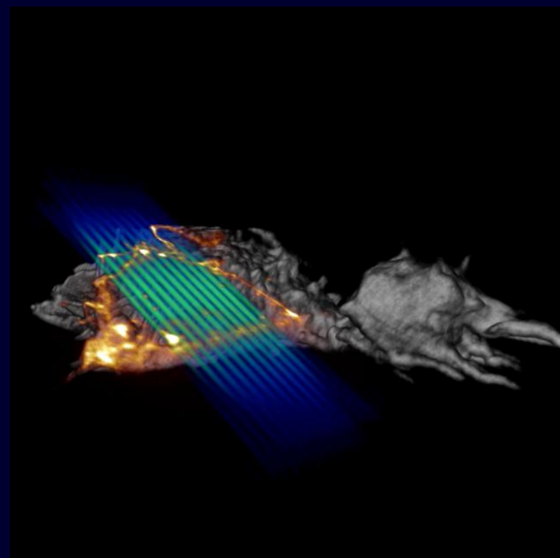


Lattice Light Sheet Microscopy: Non-Invasive 4D Live Cell Imaging

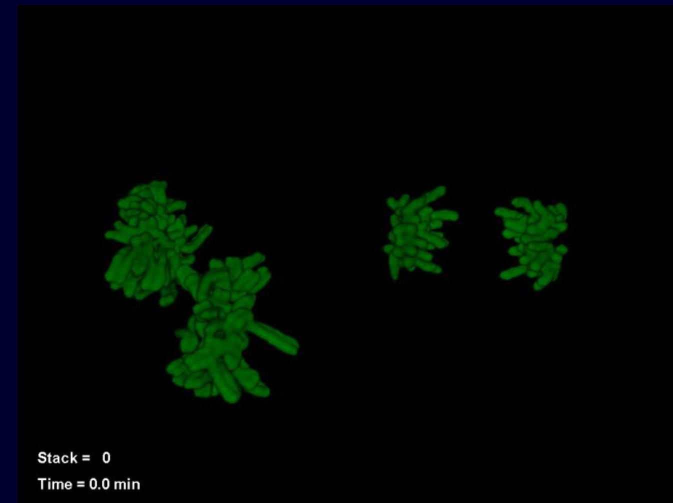
T cell and its target cell



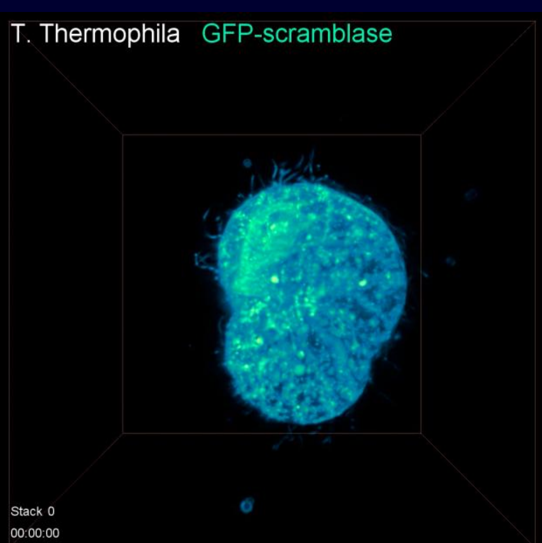
concept



chromosomes, mitos, and ER during mitosis



Tetrahymena thermophila

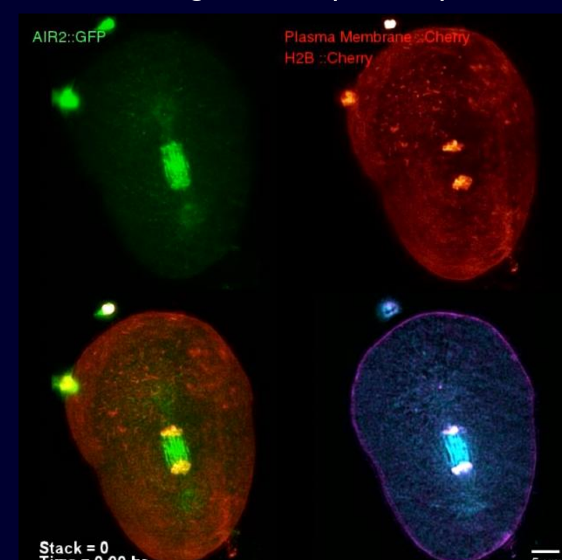


Bi-Chang Chen

Wes Legant

Kai Wang

C. elegans early embryo



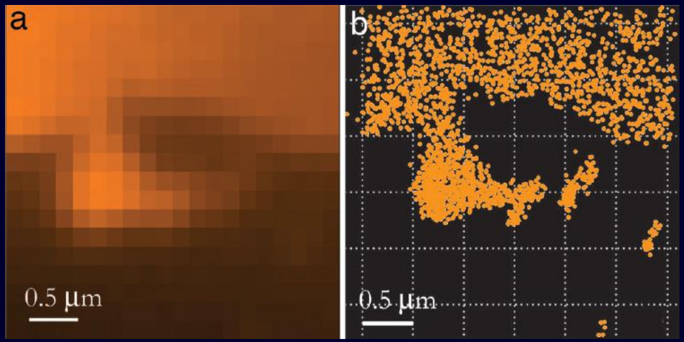
B-C Chen, *et al., Science* **346**,1257998 (2014)

Ultra-High Density 3D Localization Microscopy

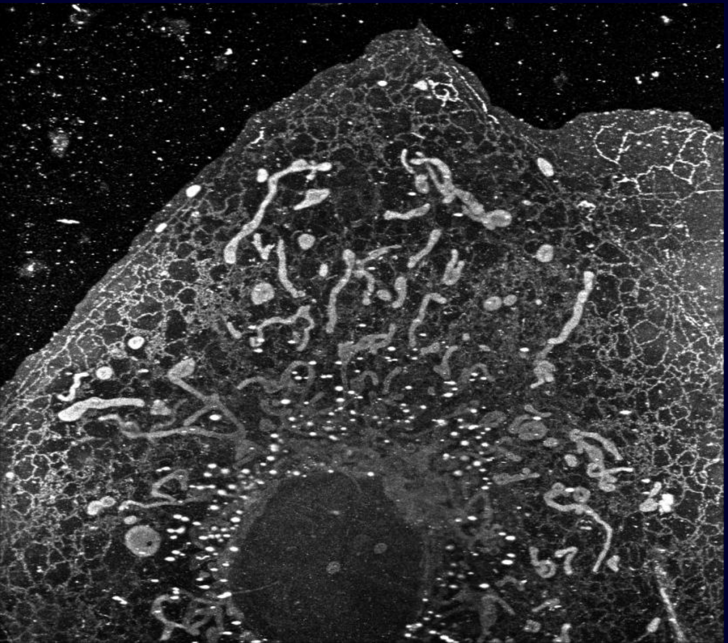
Wesley Legant

Points Accumulation for Imaging in Nanoscale Topography (PAINT)

widefield PAINT

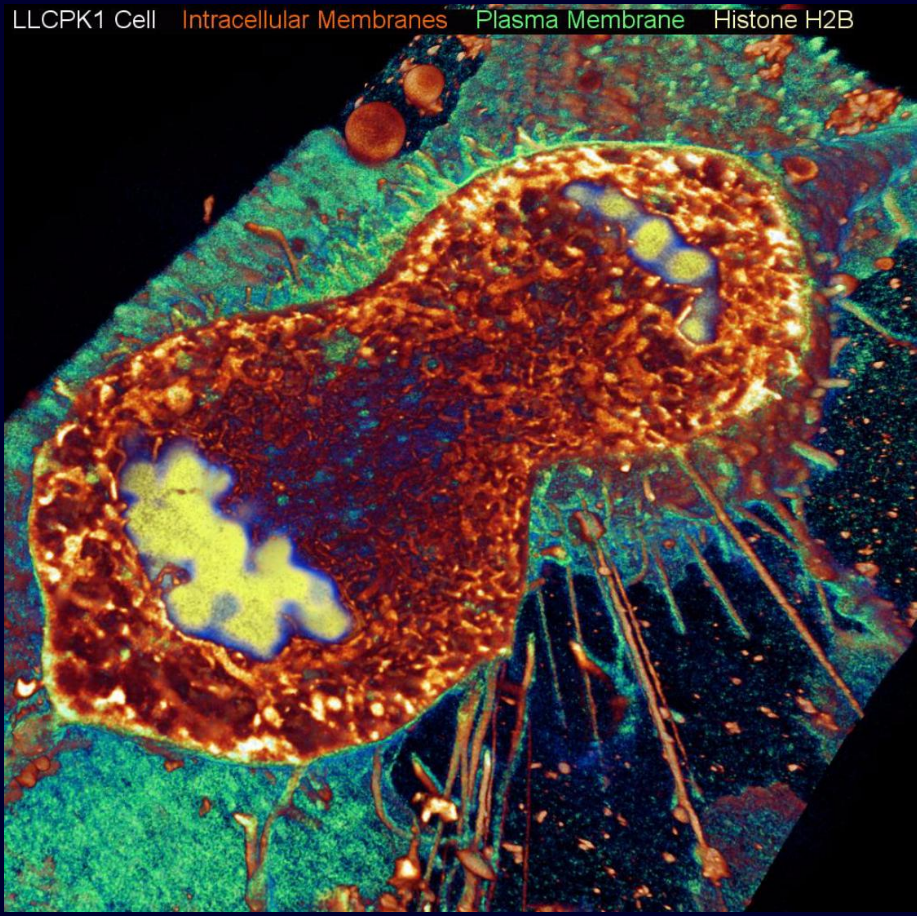


A. Sharonov, R.M. Hochstrasser, *PNAS* 103, 18911 (2006)



intracellular membranes, COS-7 cell

3D PAINT with lattice: dividing cell



over 300 million localized molecules

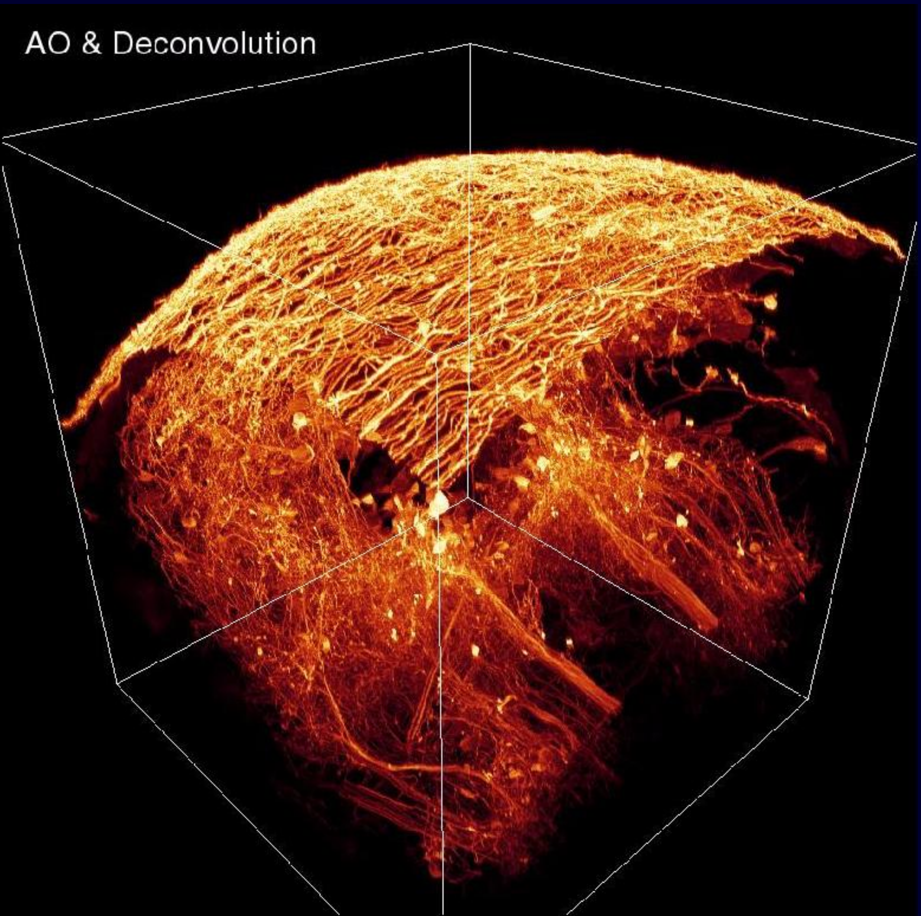
Adaptive Optics (AO): Moving Cell Biology Away from the Cover Slip

non-scattering media: zebrafish embryonic brain

Kai Wang

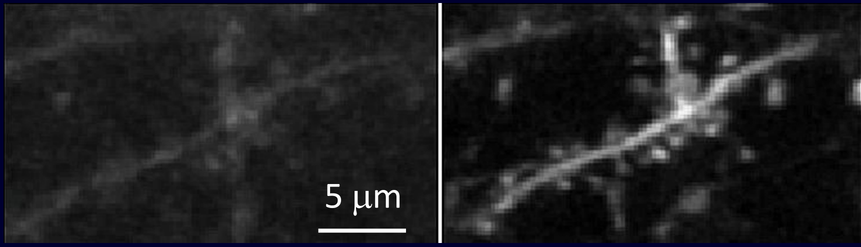
scattering media: mouse visual cortex

Na Ji

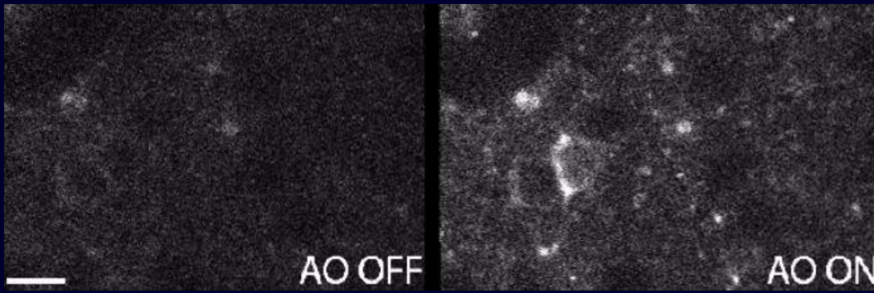


dendritic spines, 600 μm deep

AO off AO on



functional imaging of neural activity, 400 μm deep



The Beauty and Complexity of Living Systems