The Abdus Salam
International Centre for Theoretical Physics

United Nations
Educational, Scientific and
Cultural Organization

IAEA
International Atomic Energy Agency

1932-15

Winter College on Micro and Nano Photonics for Life Sciences

11 - 22 February 2008

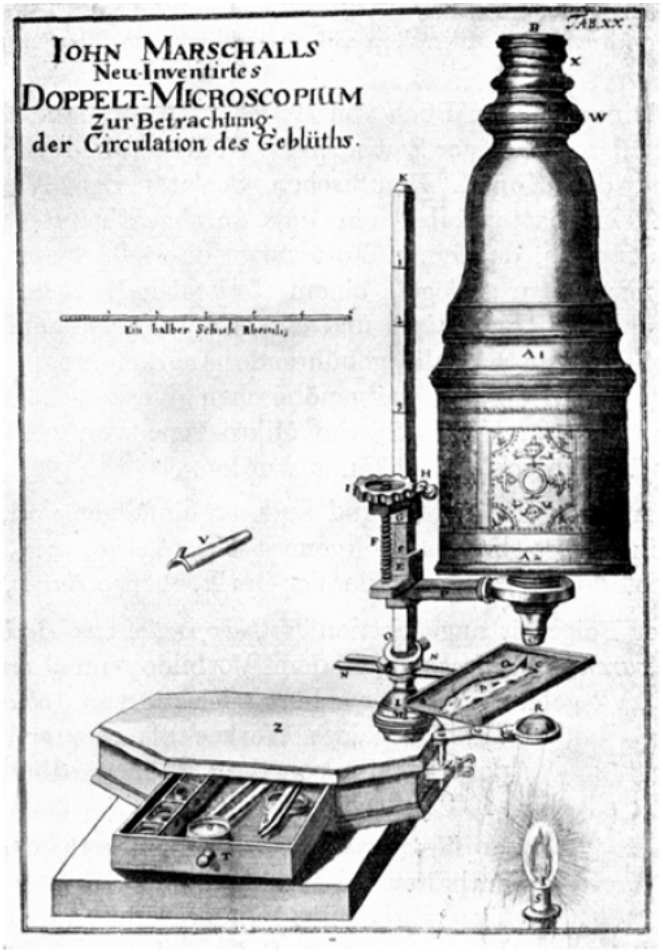
General Overview

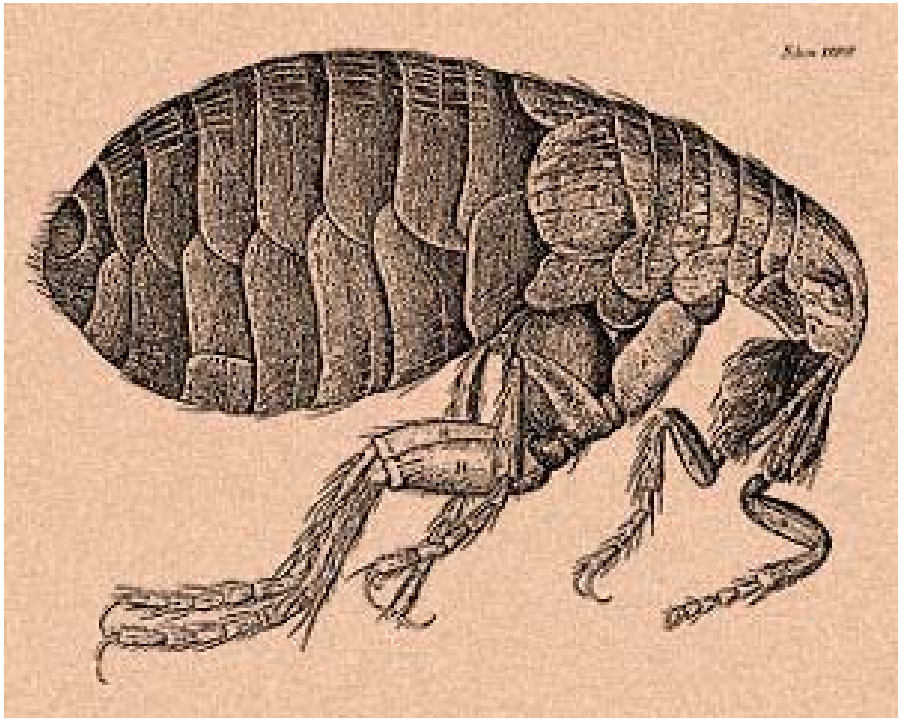
Martina Havenith
Ruhr University Bochum
Bochum, Germany

Microscopy- An Overview

M. Havenith
Ruhr-University Bochum

Microscopy in the 17th century

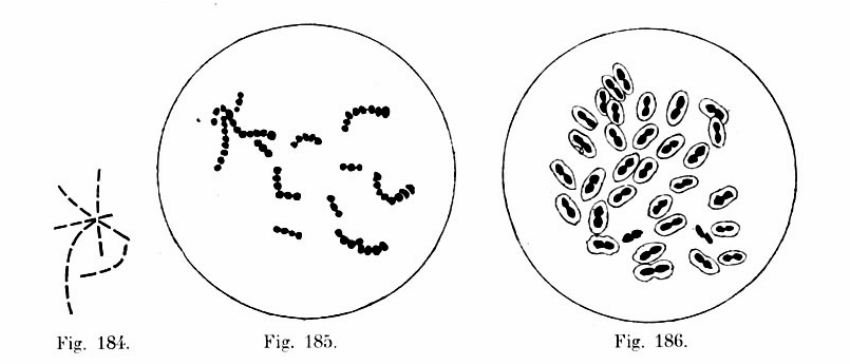




From Robert Hooke, Micrographia (1665)

History of microscopy

Large Microscope of Carl Zeiss (1879) with optical elements according to calculations of Ernst Abbe



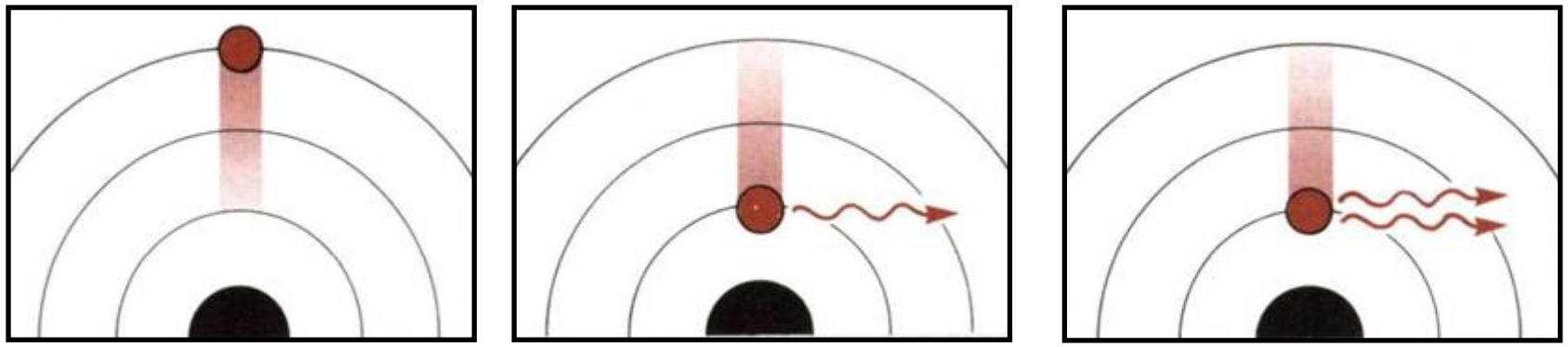
Bacteriae (from Wilhelm Kaiser, “Die Technik des modernen Mikroskopes.“ (1906)

Technological progress in the development of new light sources:

The Laser

Year	Who	
1917	A. Einstein	1. theoret. description of st. emission
1954	Townes, Gordon, Zeiger	1. Maser
1960	Theodore	1. solid state - (Ruby-) Laser
1962	Nathan, Duncke, Bruns, Dill, Lasher	1. diode laser
1970	Hayashi, Panish, Foy, Sumski	continuous operation of diode lasers at room temperature
1991	Haase, Qui, de Puydt Cheng	diode laser with blue Emission

Characteristic properties of a laser



Stimulated emission

Uni directional emission of radiatiom

Radiation can be easily focussed → High intensity

Monochromatic light source

Tunable (frequency selective) radiation source

Short laser pulses are possible: Fsec laser technology

The diode laser

1. K. N. Hall General Electric
2. M. I. Nathan IBM
3. T. M. Quist MIT

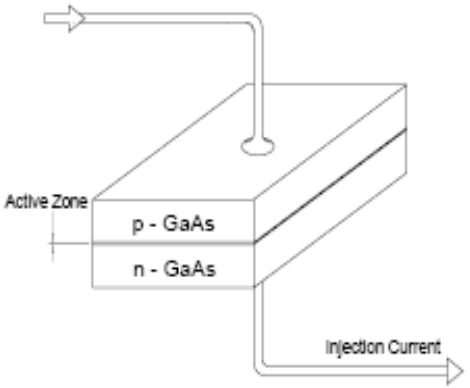


Fig. 15: Simple laser diode around 1962, working at 70 K and with 100 kA/cm^2 in the pulse mode.

AlGaAs: Ga atoms Partially replaced by Al

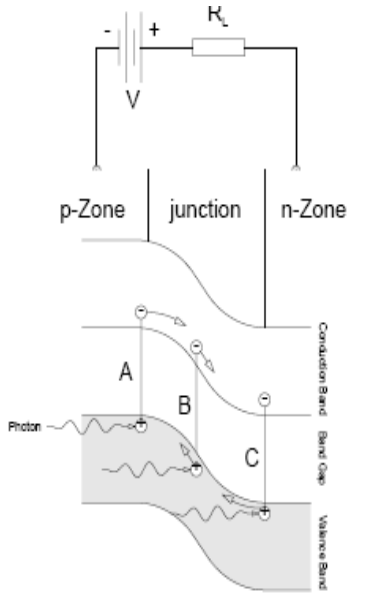
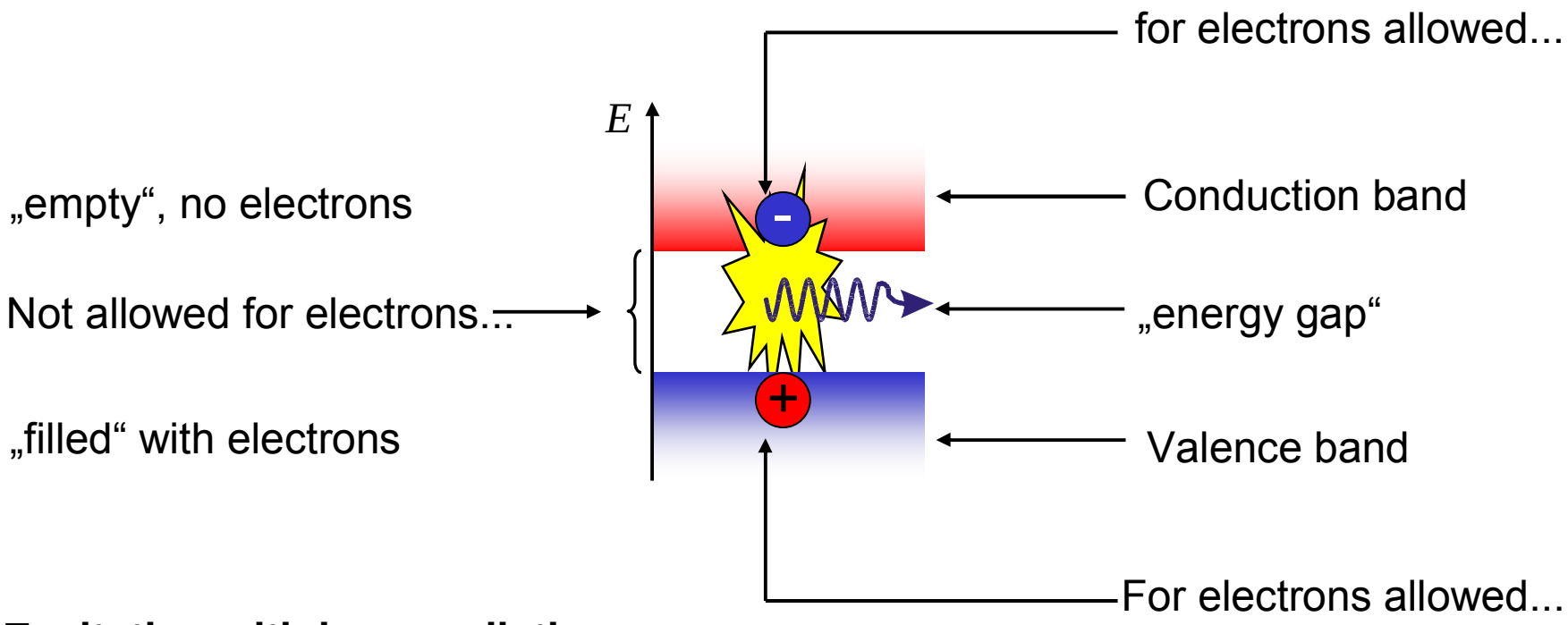


Fig. 25: Absorption of a photon with subsequent transition of the stimulated electron from the valence band to the conduction band

- 1962: 1. diode laser new technology (thin substrates) production in clean rooms otherwise: high losses
- Bell Labs: first operation at room temperature
- Marcet potential low
- 2003: the world wide volume for laser sources in 2003 was 1,3 *10^9 €; for laser systems 3,5 * 10^9€ .
- Until 2010 a yearly increase of 13 % is expected

The diode laser, a compact, cheep and efficient light source

semiconductors have an energy gap...



Excitation with laser radiation:

- energy of the photon → electron of the valence band → conduction band
- an electron is missing in the valence band → „hole“ with positive charge!

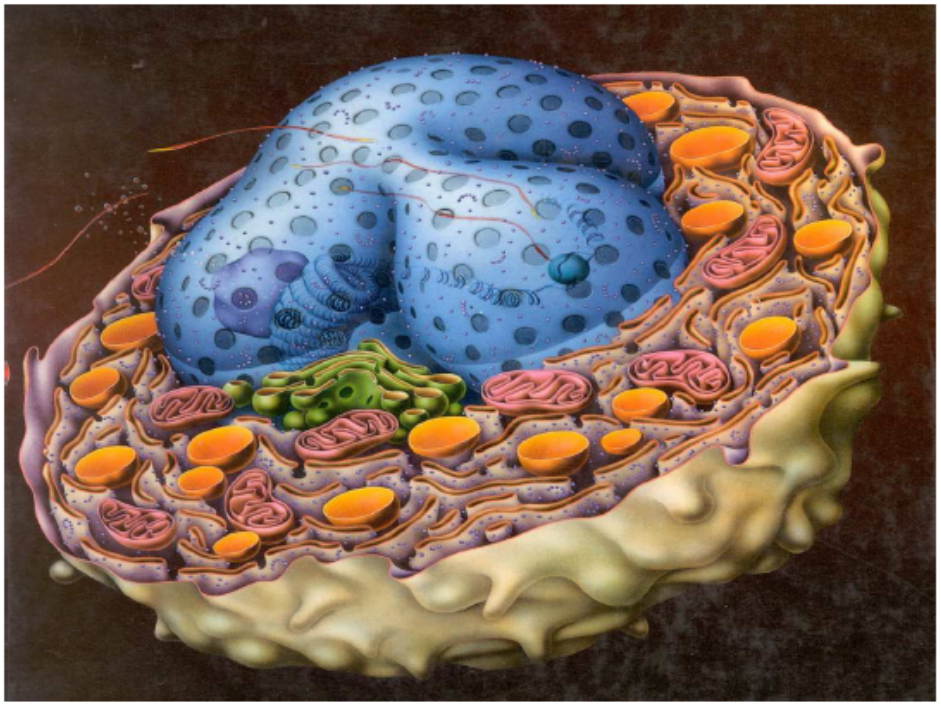
Important:

- The energy of the photon corresponds to the energy gap !
- The frequency depends on the material,temperature, etc…. !

Ultimate goal in Optical Microscopy

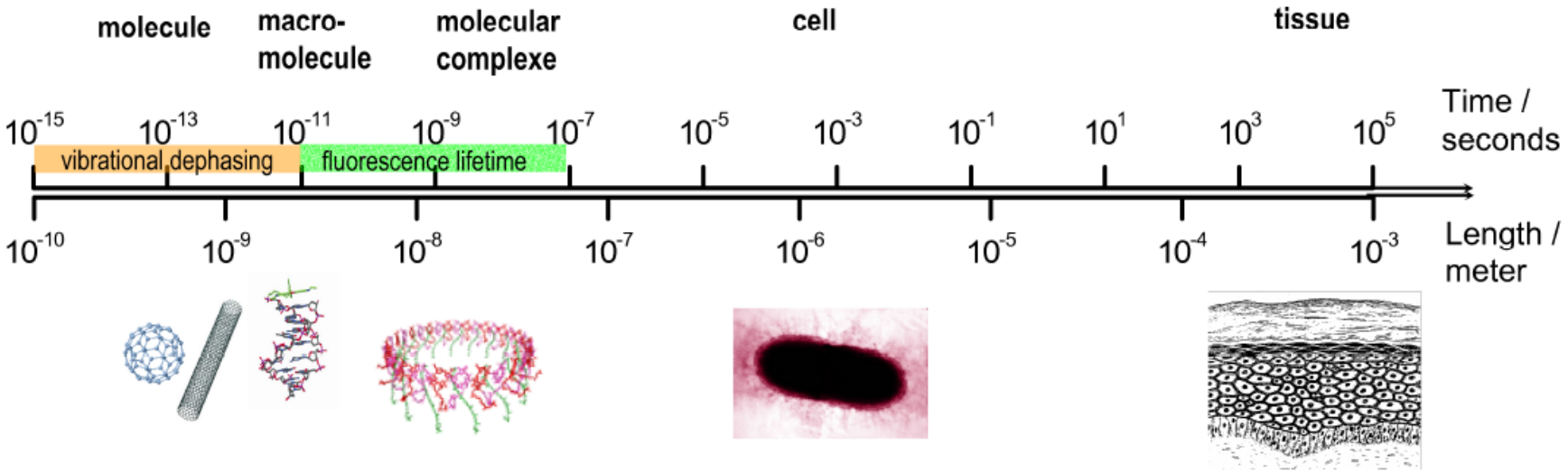
Noninvasive three-dimensional characterization of mesoscopic objects within complex heterogeneous systems in space and time (i.e. living cells and tissue)

- **with high spatial resolution,**
- **with high spectral resolution,**
- **with high temporal resolution,**
- **with high sensitivity,**
- **no sample preparation,**
- **and no system perturbation .**



What has been achieved so far ?

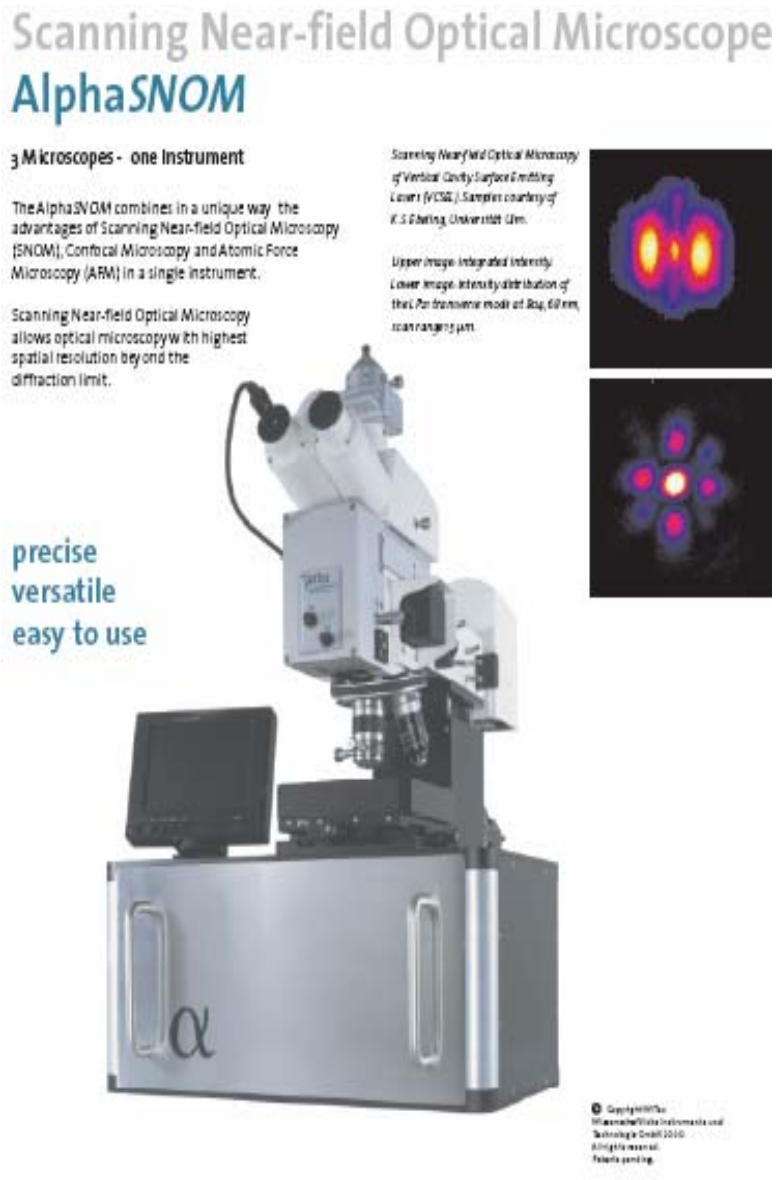
Motivation

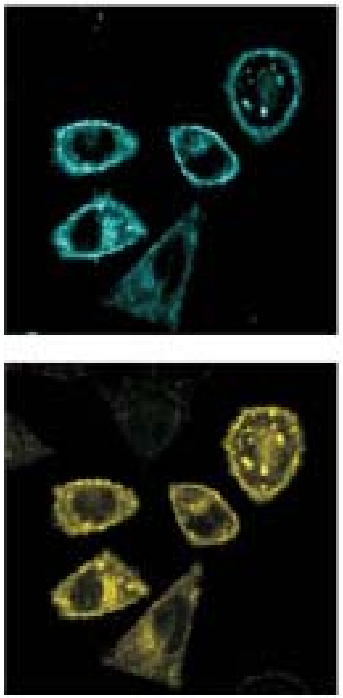

molecule
macro-molecule
molecular complexe
cell
tissue
10^{-15} 10^{-13} 10^{-11} 10^{-9} 10^{-7} 10^{-5} 10^{-3} 10^{-1} 10^{1} 10^{3} 10^{5}
Time / seconds
vibrational dephasing
fluorescence lifetime
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3}
Length / meter

How do we improve the images?

- Fluoresence spectra:
  fluorescence frequency is distinct from excitation frequency
- Fluoresence labels:
  green flurosecence proteins (GFP)
- Improve sensitivity (single molecule detection)
- Come to the optimum set-up in the far field
  push the diffraction limit
- Break the diffraction limit
- Ultimate goal: detection of single molecules with nm resolution

Optical Laser microscopy





Hoffmann, Lohse
Biospektrum 2006

Spezific marked
Adenosin receptor construction

Fluorescence: labeling of specific proteins
SNOM

Localisation of proteins, e.g. Malaria proteins in cells

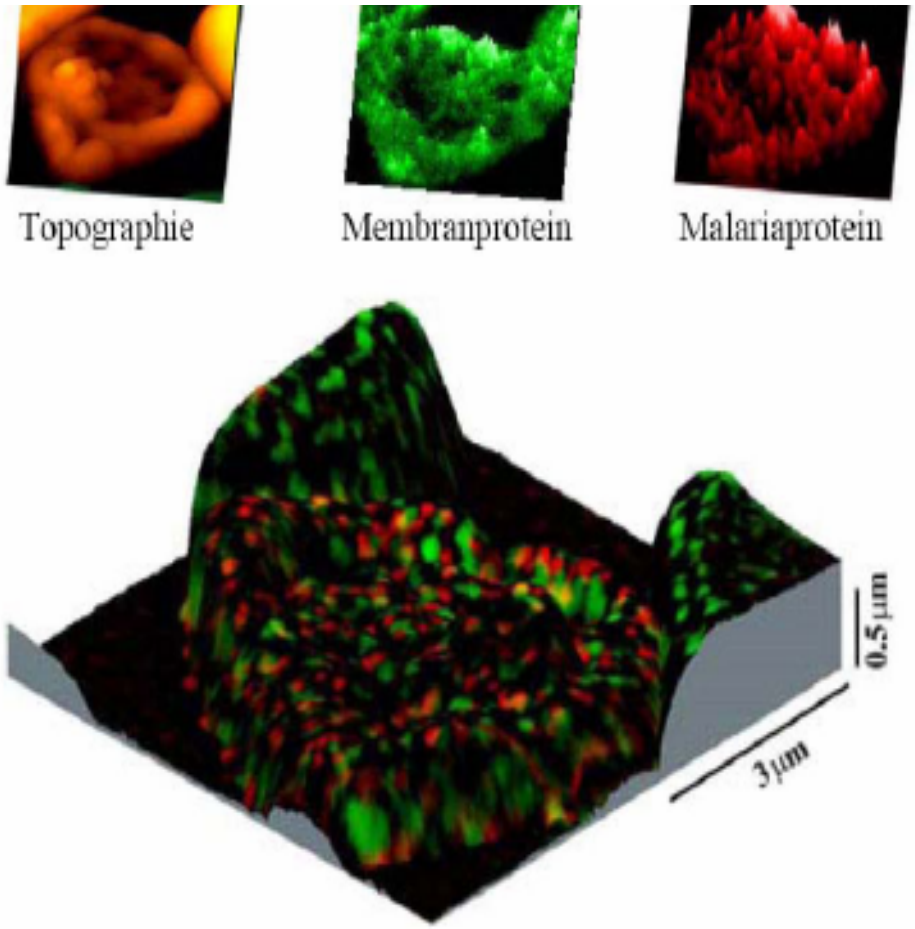


Abb. 12 oben: *Topographie der Zelle (links), Zellaufnahmen des Membranproteins (mitte, markiert mit GFP, Green Fluorescence Protein) und des Malariaproteins (rechts, markiert mit RFP, Red Fluorescence Protein)*

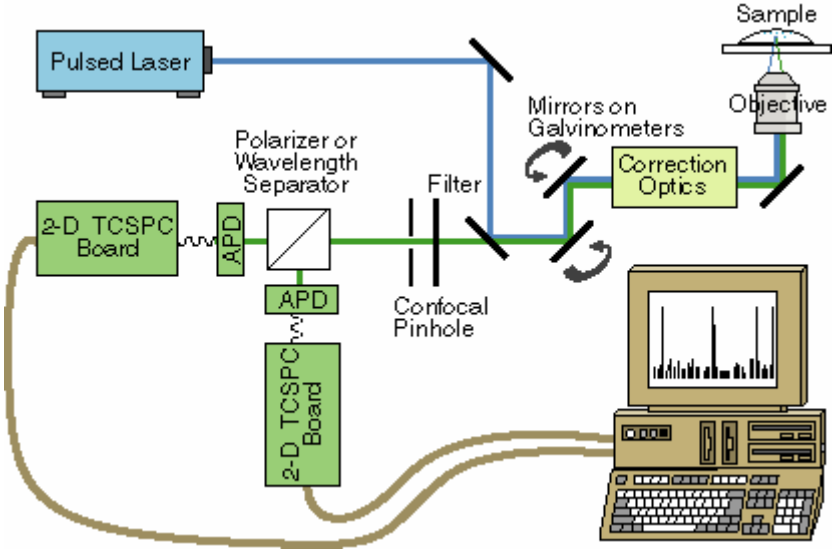
unten: Fluoresenzaufnahme der infizierten Zelle

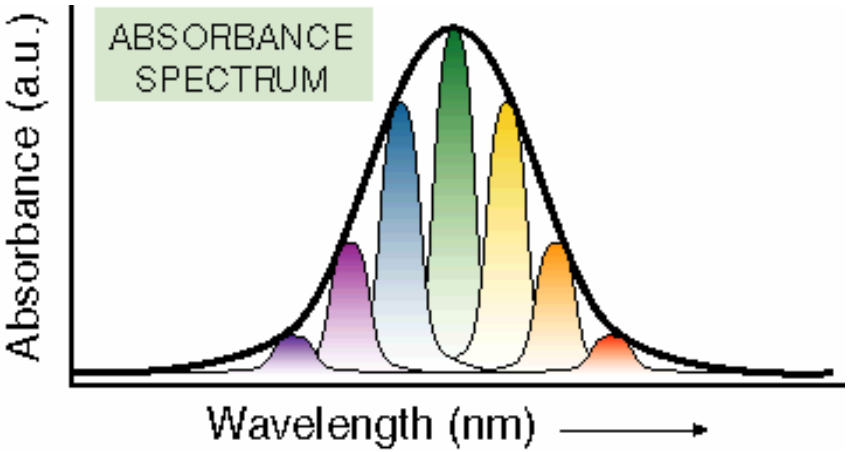
Single Molecule spectroscopy

Looking at the dynamics and spectroscopy of a single biological molecule:

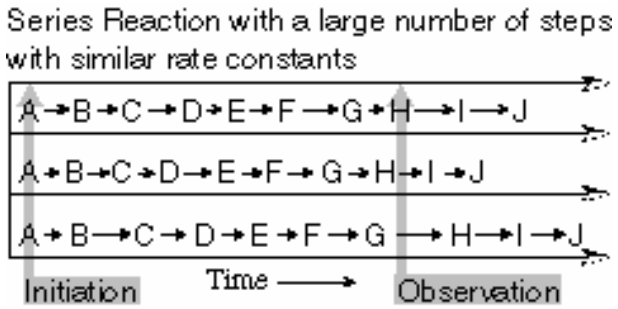
Required:

- sensitive detection system
- High dilution of chromophores
- Photostability of chromophores



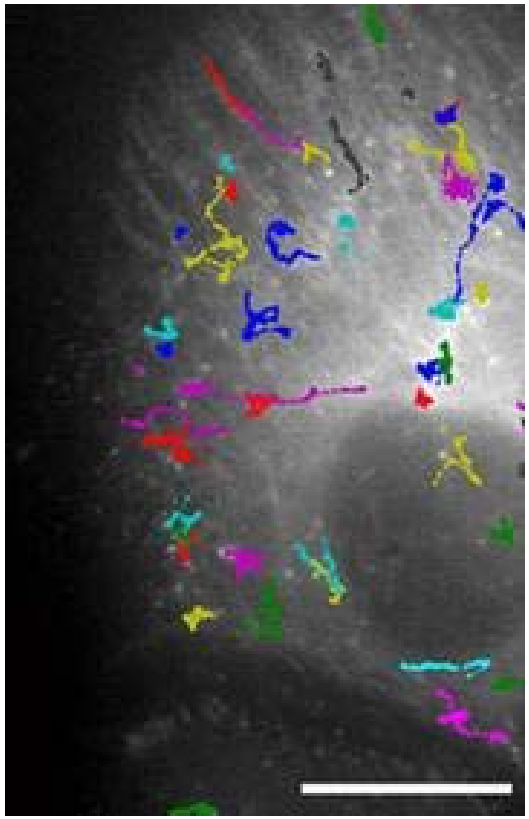


Single molecule versus bulk detection

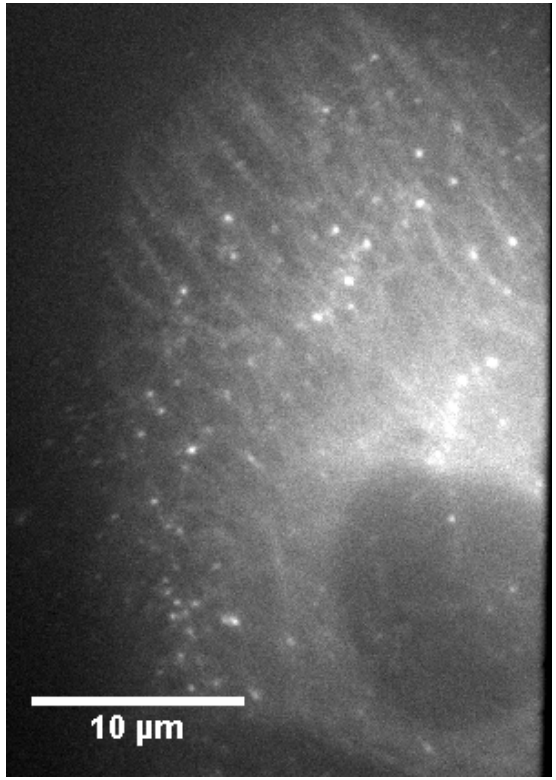


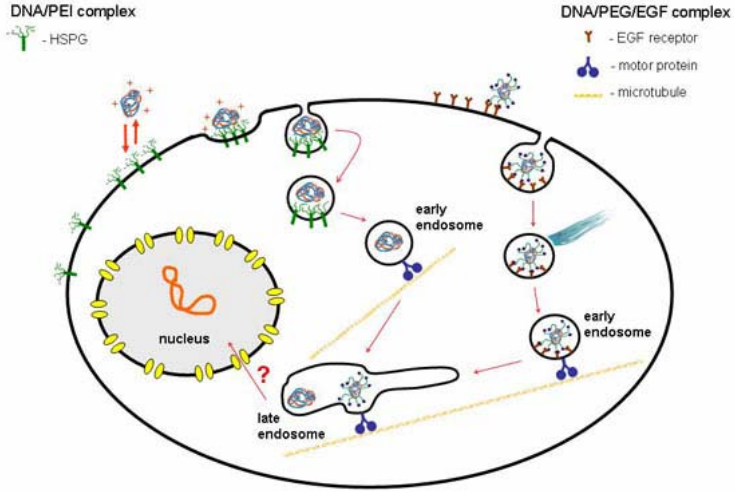
observation of sequential dynamics

Fluorescens microscopy of single particels (Bräuchle)

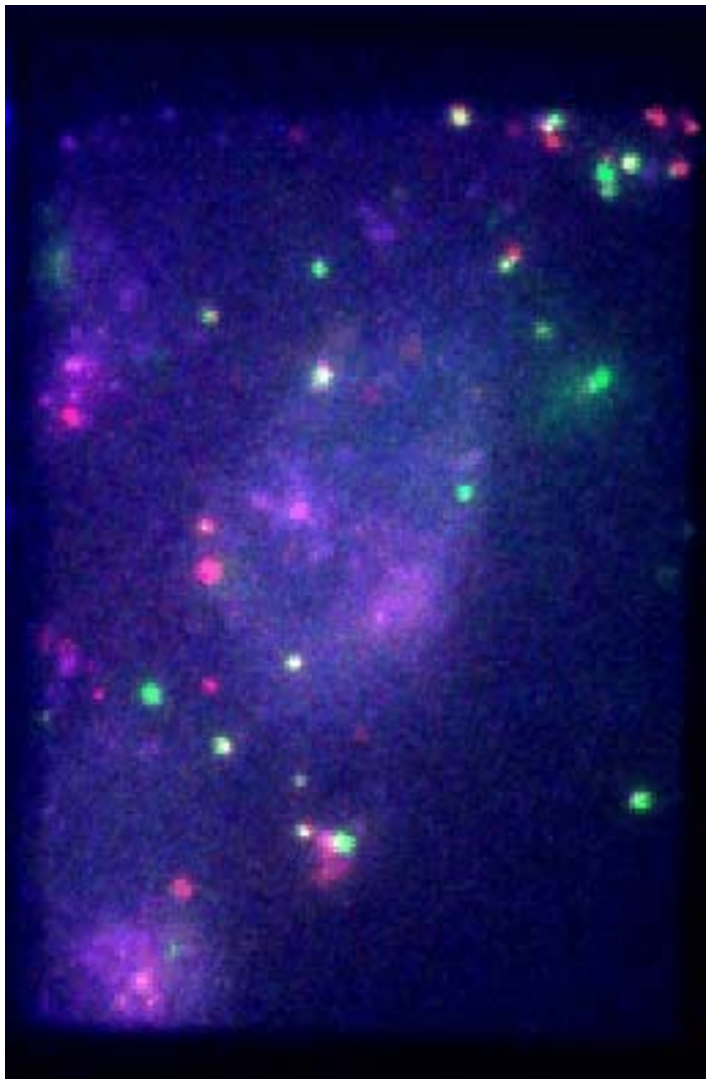


Displayed is the active transport of single labeled particles along the microtubuli
Shown are selected trajecteries





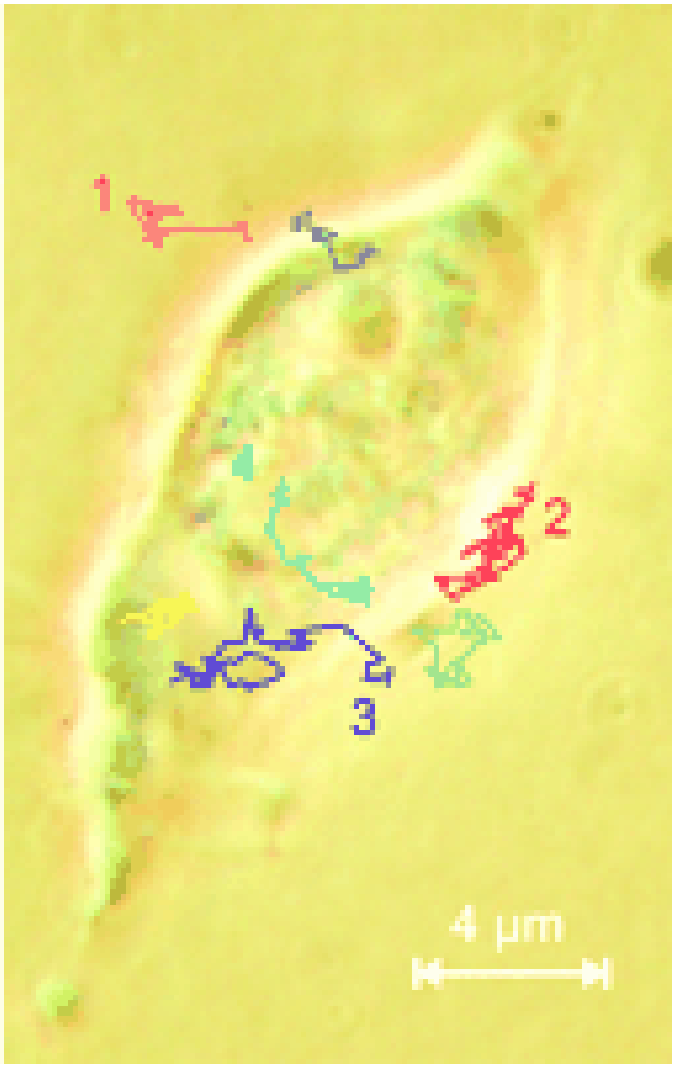
Visualization Of Viruses On Their Infection Pathway In Living Cells



Dual-colored HIV particles within a HeLa cell. Some particles are stationary, others can be seen undergoing transport mediated by cellular motor proteins (C. Bräuchle)

Visualization Of Viruses On Their Infection Pathway In Living Cells

C. Bräuchle



Trajectories of single AAV-Cy5 particles indicating infectious entry pathways of AAVs into a living HeLa cell.
The traces showing single diffusing virus particles were recorded at different times.
They describe various stages of AAV infection, e.g. diffusion in solution (1 and 2), touching at the cell membrane (2), penetration of the cell membrane (3), diffusion in the cytoplasm (3 and 4), penetration of the nuclear envelope (4), and diffusion in the nucleoplasm.

G. Seisenberger ,M.U. Ried, Th. Endreß, H. Büning, M. Hallek, Ch. Bräuchle
***Science* 30 November 2001:**
Vol. 294. no. 5548, pp. 1929 - 1932

Two photon fluorescence microscopy

Principle of fluorescence induced by one-photon absorption (left) and two-photon absorption (right). While the resolution in two-photon fluorescence mciroscopy (2PFM) is less good, photodamage is lower and penetration depth is higher compared to single-photon (confocal) fluorescence microscopy (1PFM). Moreover three dimensional resolution is possible

Two-photon fluorescence microscopy

- Virtual absorption of a photon lasts only 10^{-15} - 10^{-18} s
- Required: very high density of photons
- (0.1 - 10 MW/cm^2) from a ps-to-fs-pulsed light source.
- Multi-photon absorption was predicted in 1930, and the proof-of-principle was performed in the 1960s

Über Elementarakte mit zwei Quantensprüngen

Von Maria Göppert-Mayer

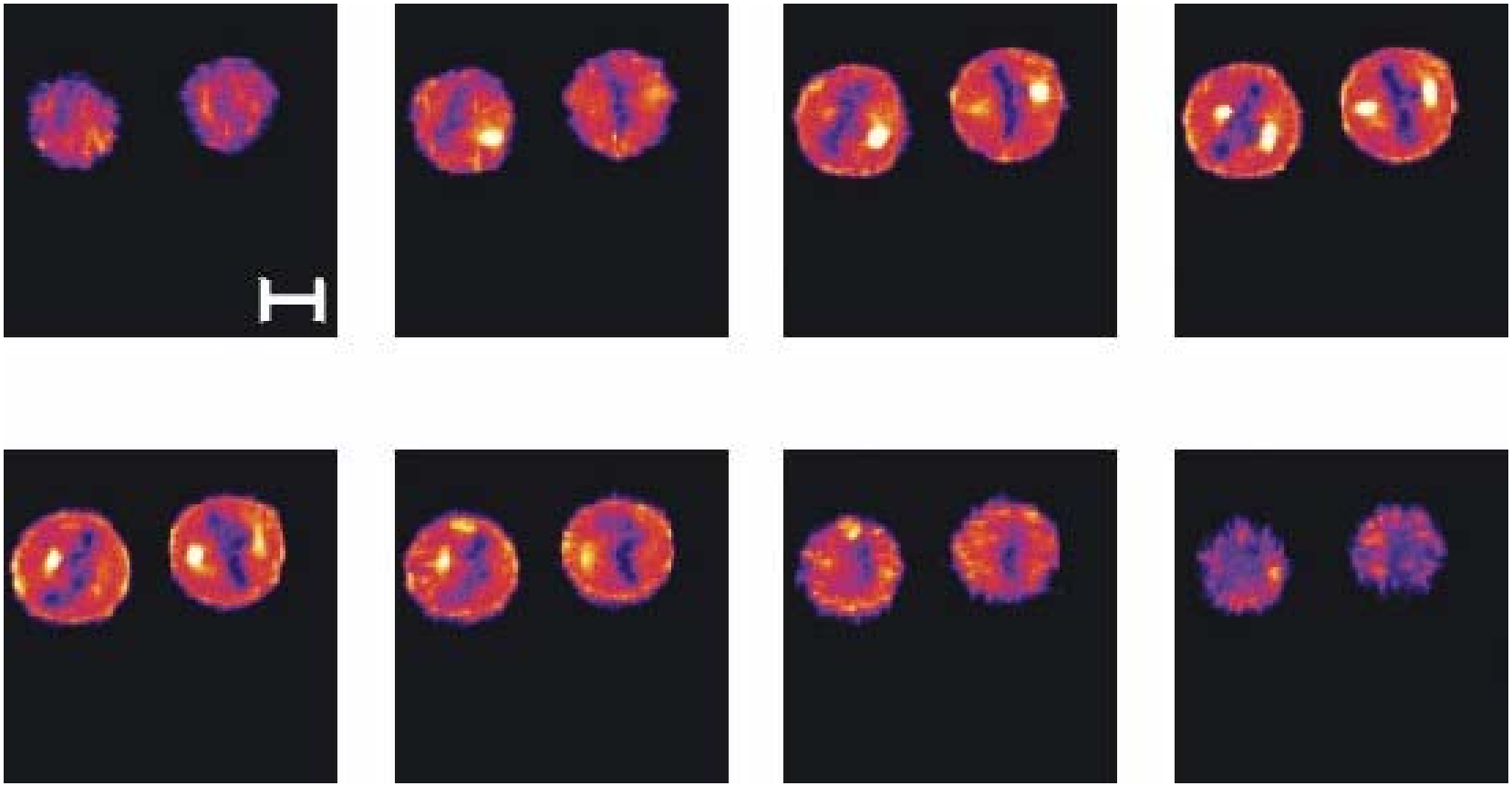
(Göttinger Dissertation)

(Mit 5 Figuren)

Einleitung

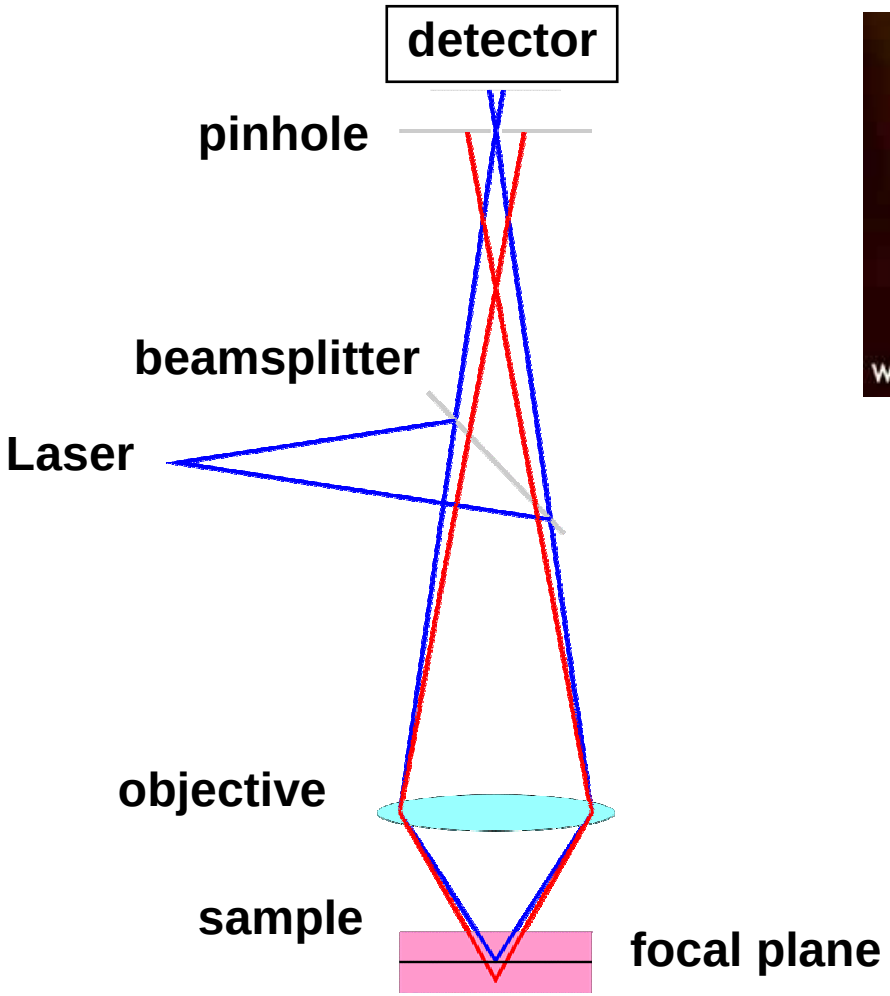
Der erste Teil dieser Arbeit beschäftigt sich mit dem Zusammenwirken zweier Lichtquanten in einem Elementarakt

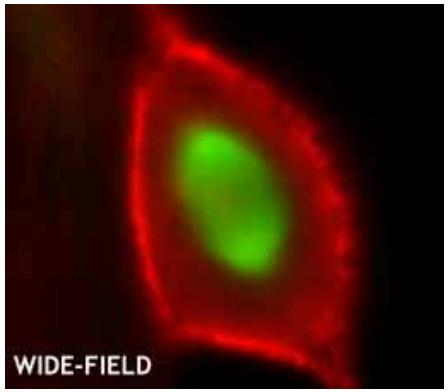
Two photon microscopy

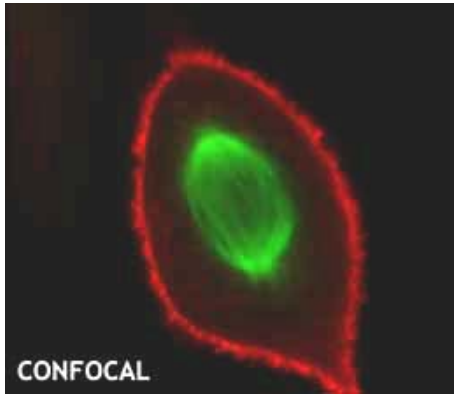


2PFM images of two dividing HEK293 cells
(blue = low intensity; red = medium intensity; yellow = high intensity)

Confocal Microscopy







Confocal Microscopy:

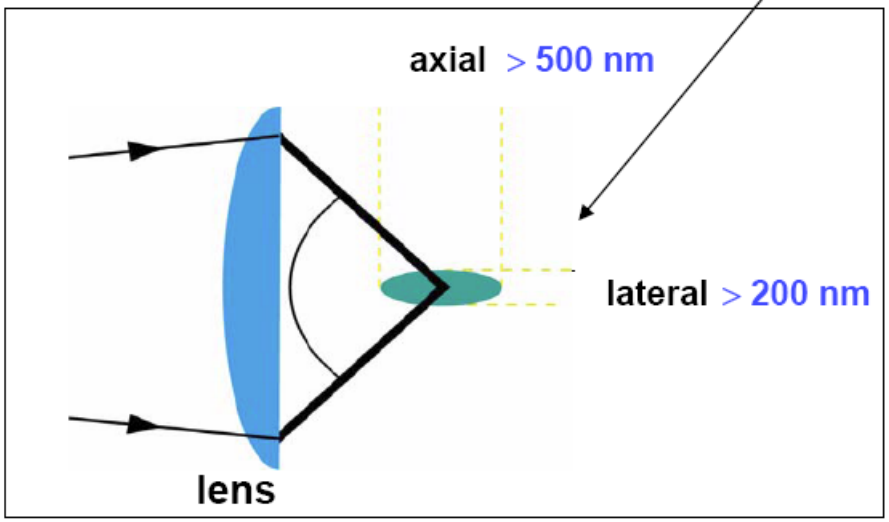
- much smaller background
- 3-D information
- slightly higher resolution
- **but NO chemical sensitivity**

Diffraction resolution limit

Resolution of conventional optical microscope is limited by diffraction

Ernst Abbe
1840 - 1905

Abbe's equation $\Delta x \approx \frac{\lambda}{2n \sin \alpha}$

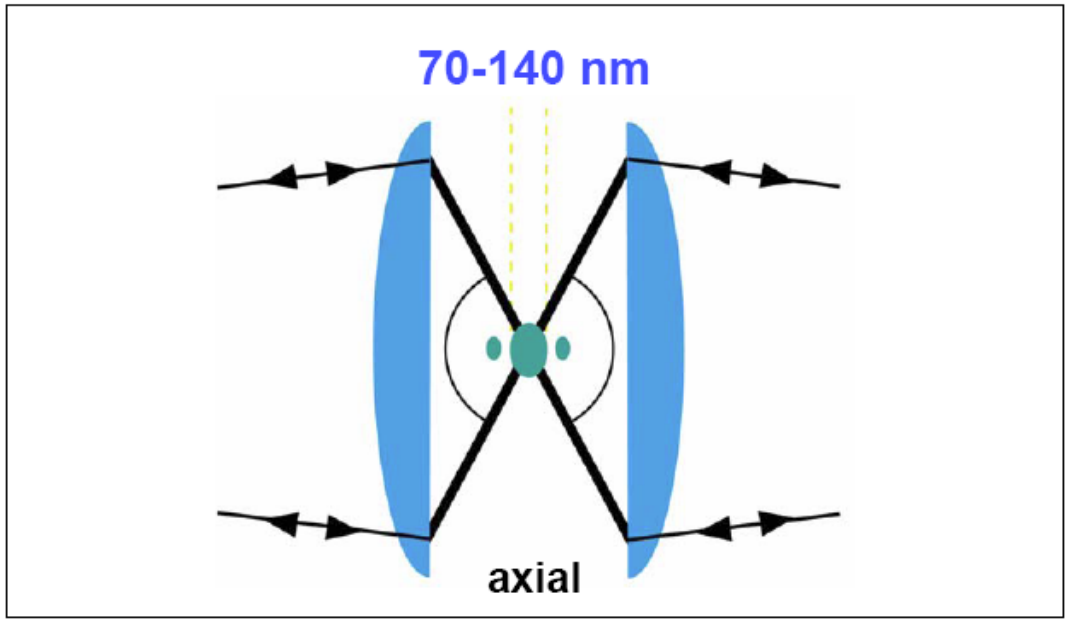


$\Delta z = \lambda$

Max Planck Society

4Pi-Microscopy: sharper in z

Coherent Illumination and/or detection



2 lenses

... but still diffraction limited

Pushing the diffraction limit:

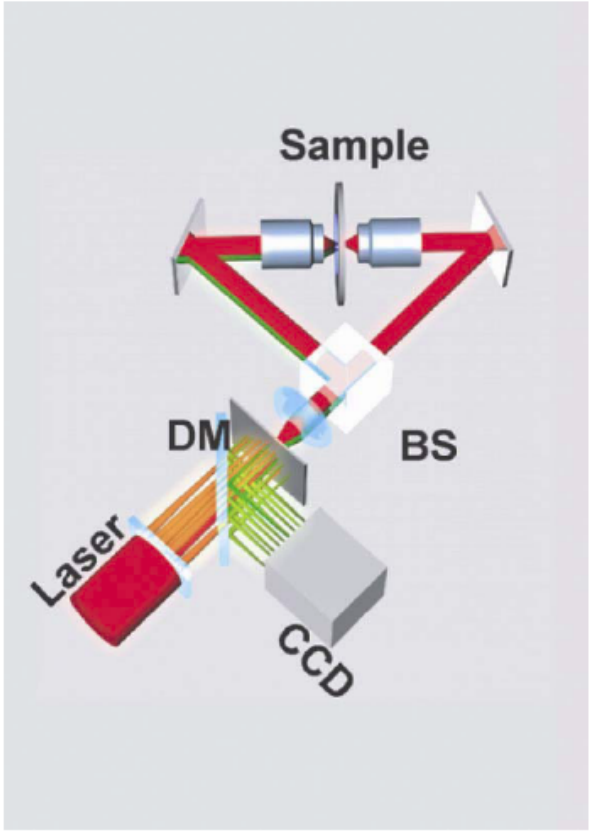
$\Delta r = \lambda/3n$

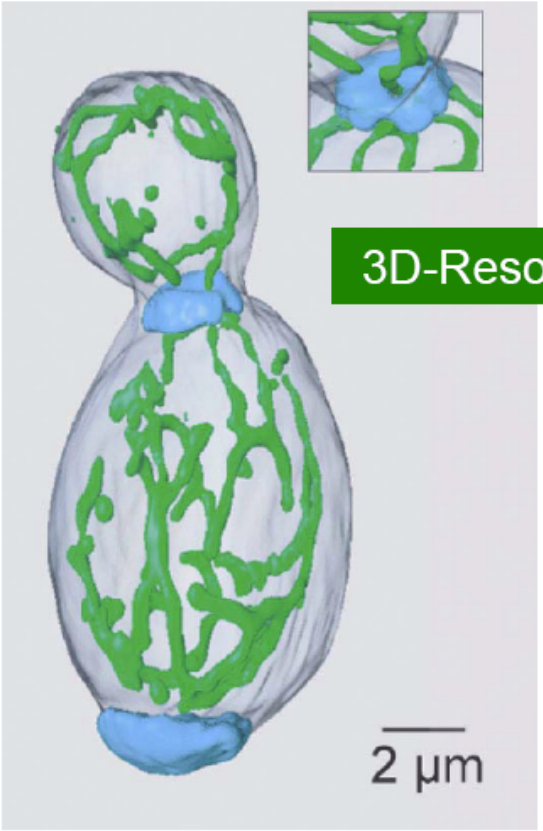
S.W. Hell (1990), *Europ. Patent* OS 0491289.
S.W. Hell, et al. (1992), *Opt. Commun.* **93**, 277.
M. Schrader, et al. (1998), *Biophys. J.* **75**, 1659.
H. Gugel, et al. (2004), *Biophys. J.* **87**, 4146.

Max Planck Society

MMM-4Pi-Microscopy: Multiple spots

16-64 foci for faster scanning





3D-Resolution: 100 nm

Mitochondrial network of live yeast

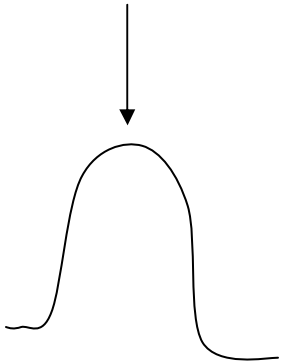
A. Egner, S. Jakobs, S.W. Hell (2002), *PNAS* **99**, 3370.
A. Egner, et al. (2004), *J. Struct. Biol.* **147**, 70.

Principles of superresolution

- improved resolution by numerical methods in case of high dilution
- assumption of Poisson process of photon detection from a single nano-emitter
- Dilution by photo bleaching; distinct colors
- Use of nanodots as emitters

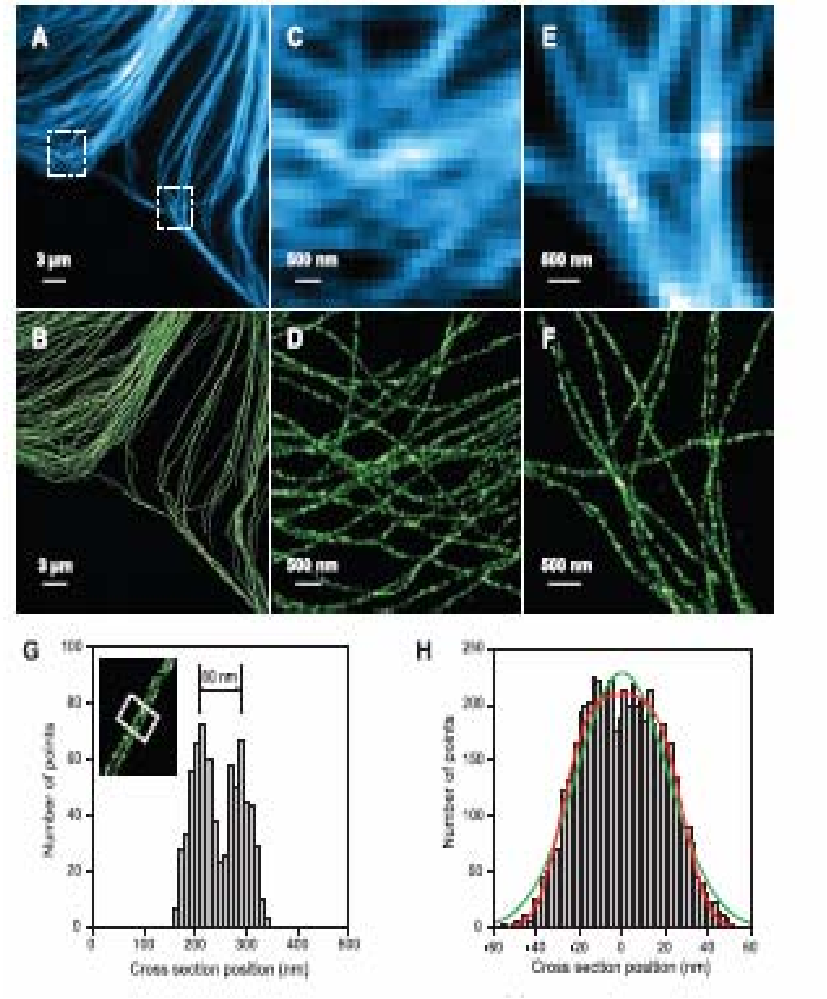
z-position can be determined:

$\Delta r \sim \lambda/2\ (N)^{-1/2}$



STORM: Stochastic Optical Reconstruction Microscopy

STORM image of microtubules in mammalian cell



M. Bates et al., Science 317, 1749 -1753 (2007)

A: conventional immuno-fluorescence image

B: STORM image

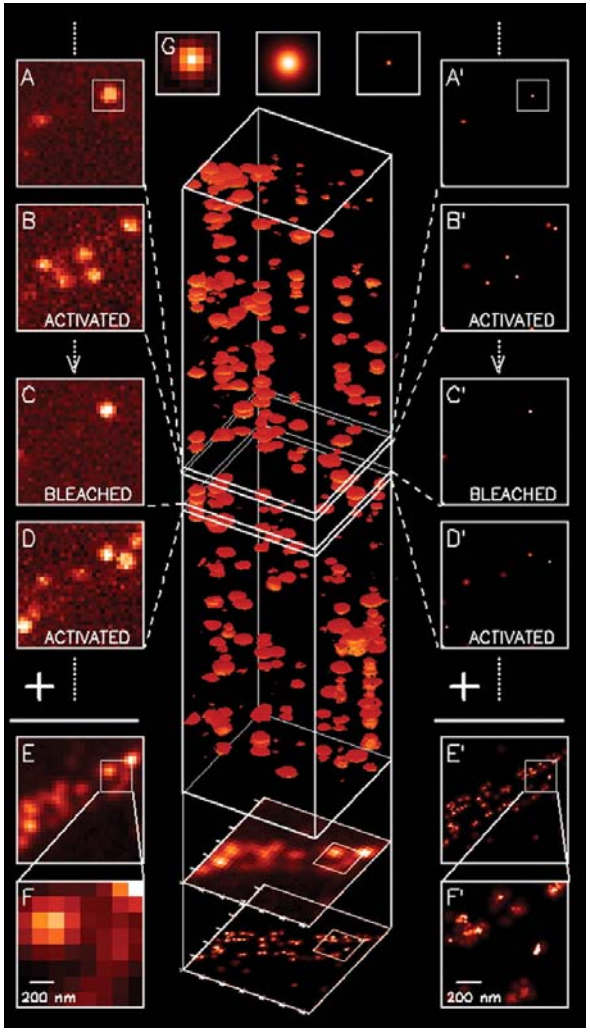
C, D conventional image zoomed boxes of A

D,E: STORM image

G: cross section of nearby microtubuli filaments

H: cross section of microtubuli Segment fit by a Gaussian

The principle behind PALM



E. Betzig et al., Science 313, 1642 -1645 (2006)

Published by AAAS

Subset of PA-FP molecules are attached to proteins of interest

First pictures were taken when only few proteins were activated

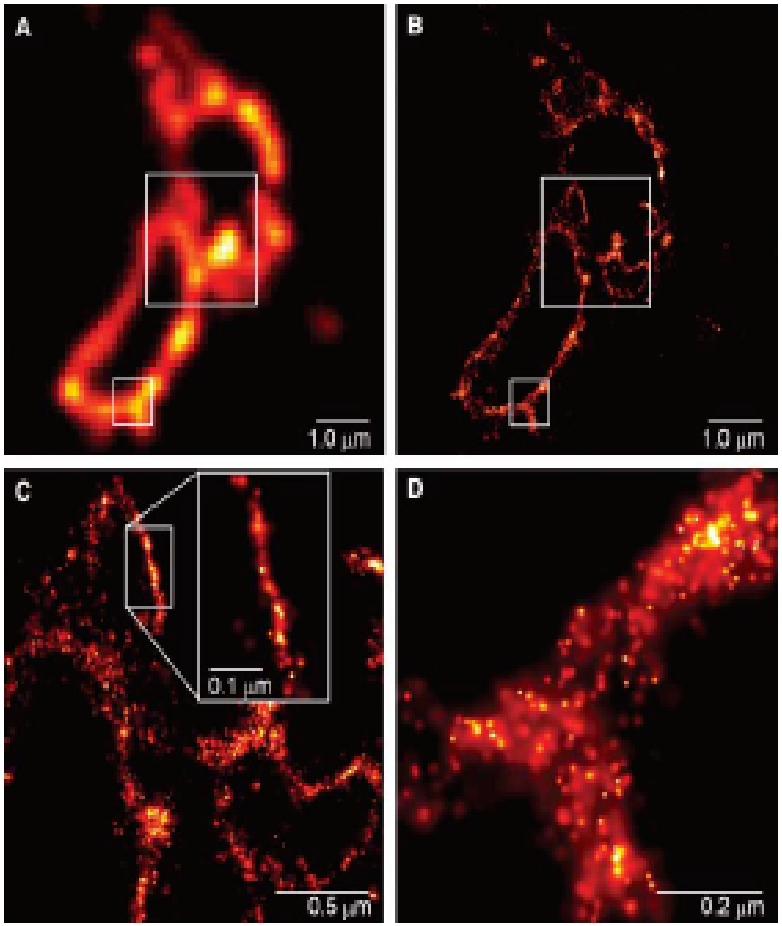
Second laser activates inactive photoactivable fluoresecent proteins (PA-FP)

Overal density higher (A→B)

Cycle of photoactivation, Measurement, bleaching yields sum (E+F)
and I(x,y,t) : middle
→ PALM picture

Science
AAAS

Application to life science



Comparison:

Thin section of a COS-7 cell expressing the lysosomal transmembrane protein CD63 tagged with the PA-FP

A: Total internal reflection fluoresence

B: PALM image of the same region

C: zoom of the large box in B
Small associated membranes : interacting lysosomes; not resolvable in A

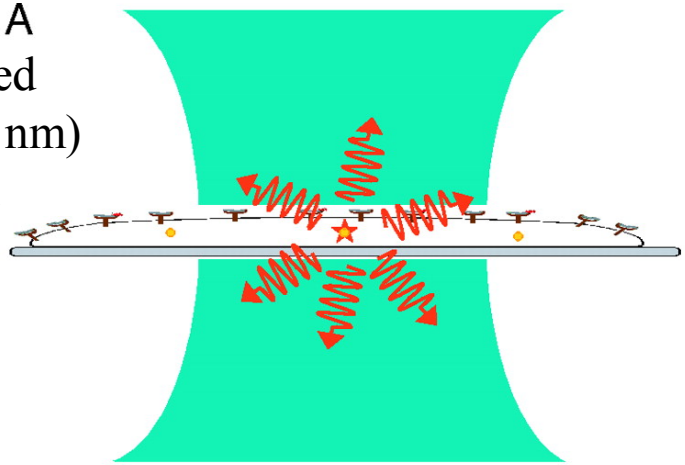
D: zoom of small box: distribution of CD63 within membrane

E. Betzig et al., Science 313, 1642 -1645 (2006)

Overview of superresolution imaging

A

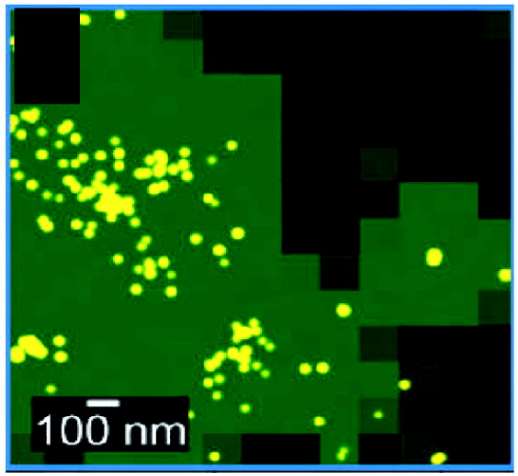
diffraction limited
laser beam (200 nm)
irradiating a cell



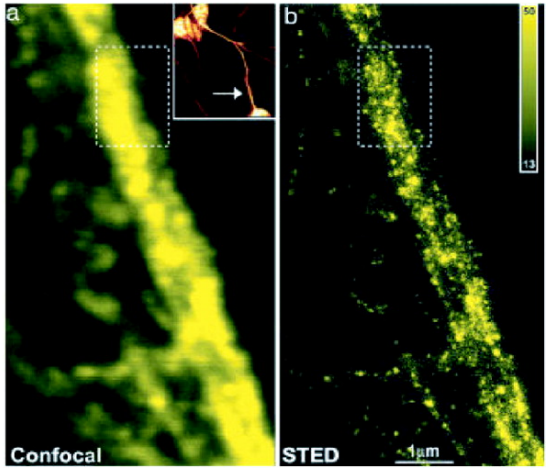
B

fluoresence image
of a bacterial cell
containing a single
protein fusion

C



D

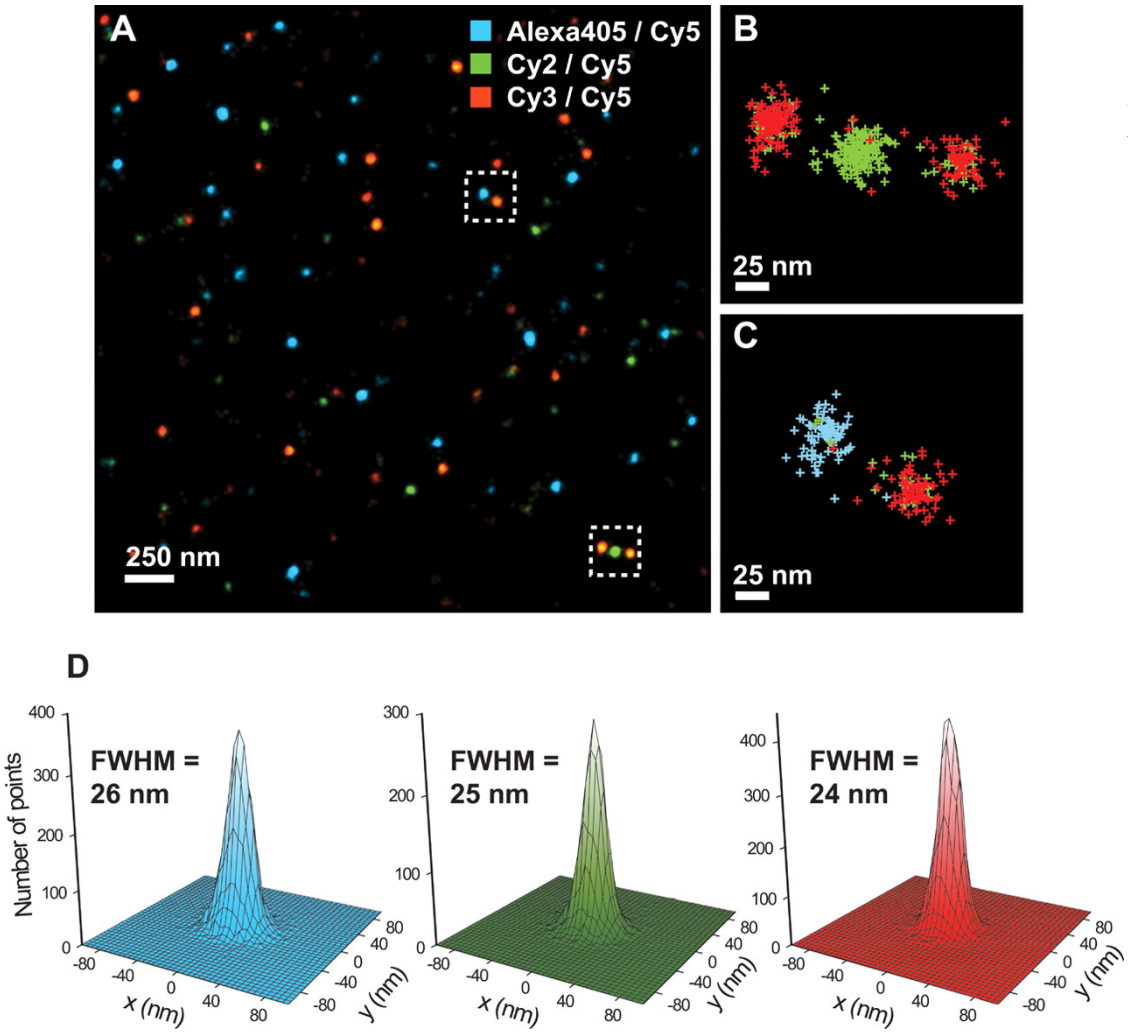


Fluoresence PALM image
Photo activated localization microscopy

In comparison: confocal image and STED

Moerner, W. E. (2007) Proc. Natl. Acad. Sci. USA 104, 12596-12602

Multicolor superresolution imaging: Three-color STORM imaging of a model DNA sample



chromatically distinguishable photo switchable reporters can be activated by one dye

Switch between bright and dark state for each possible

A: STORM image
Depending on activation laser the dots are red, green, blue

B,C: zoom of boxes

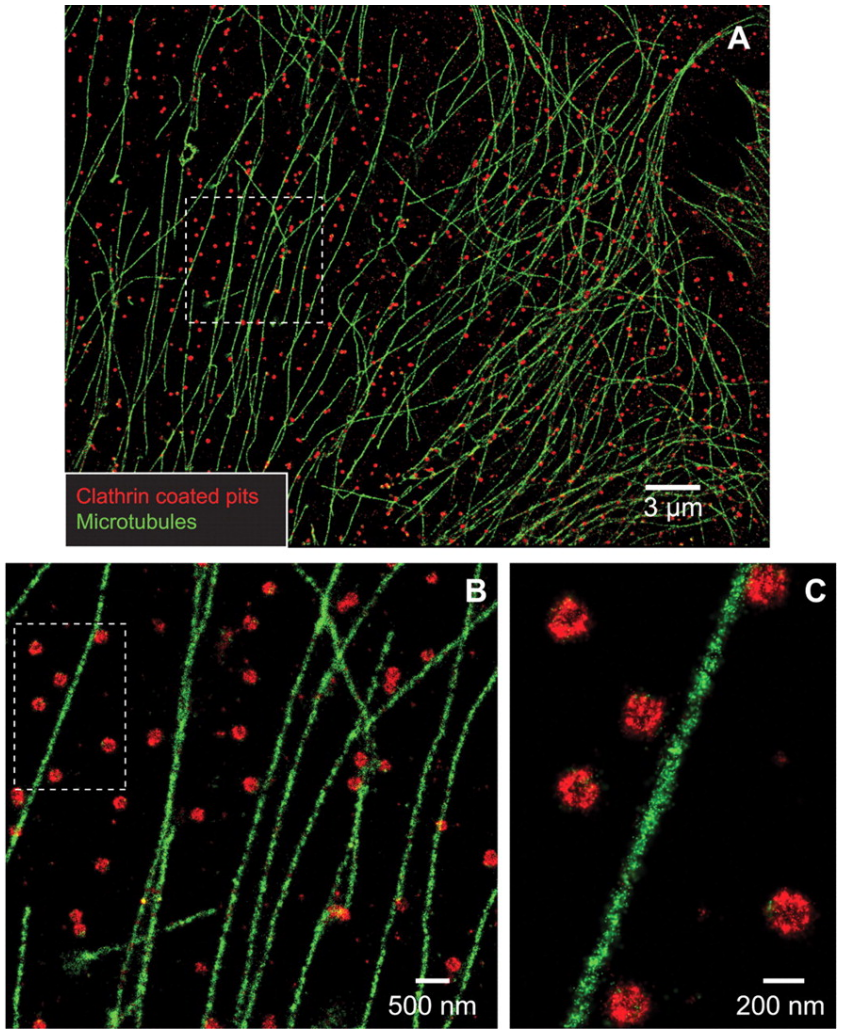
D localization distribution, Each was fit to a Gaussian

M. Bates et al., Science 317, 1749 -1753 (2007)

Published by AAAS

Science
AAAS

Two-color STORM imaging of microtubules and CCPs in a mammalian cell



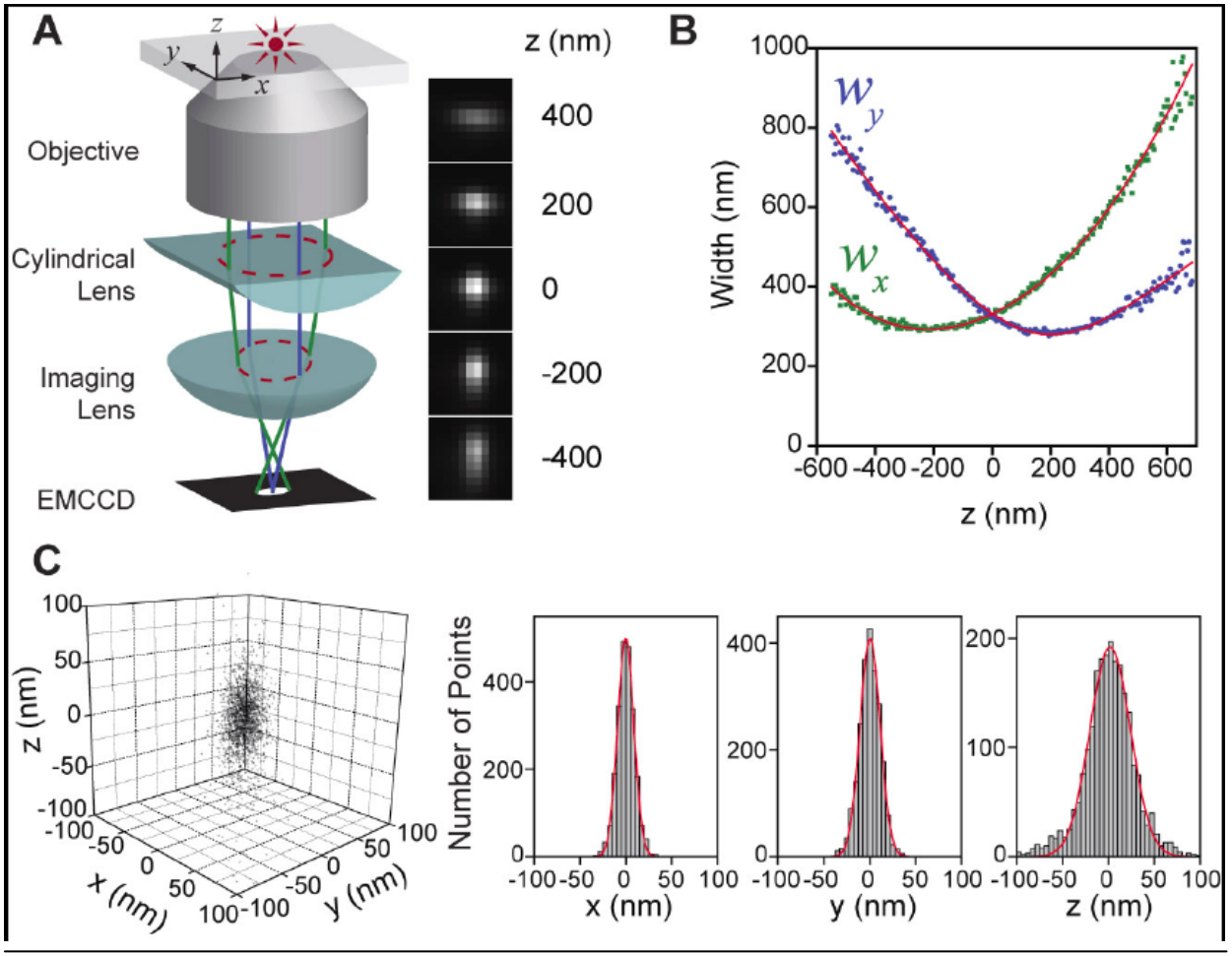
STORM image:

Secondary antibodies used
for microtubuli staining were
labeled with Cy2 ;
those for clathrin with Cy3

Shown: microtubuli and and
Clathrin coated pits:
cellular structures used for
receptor mediated endocytosis

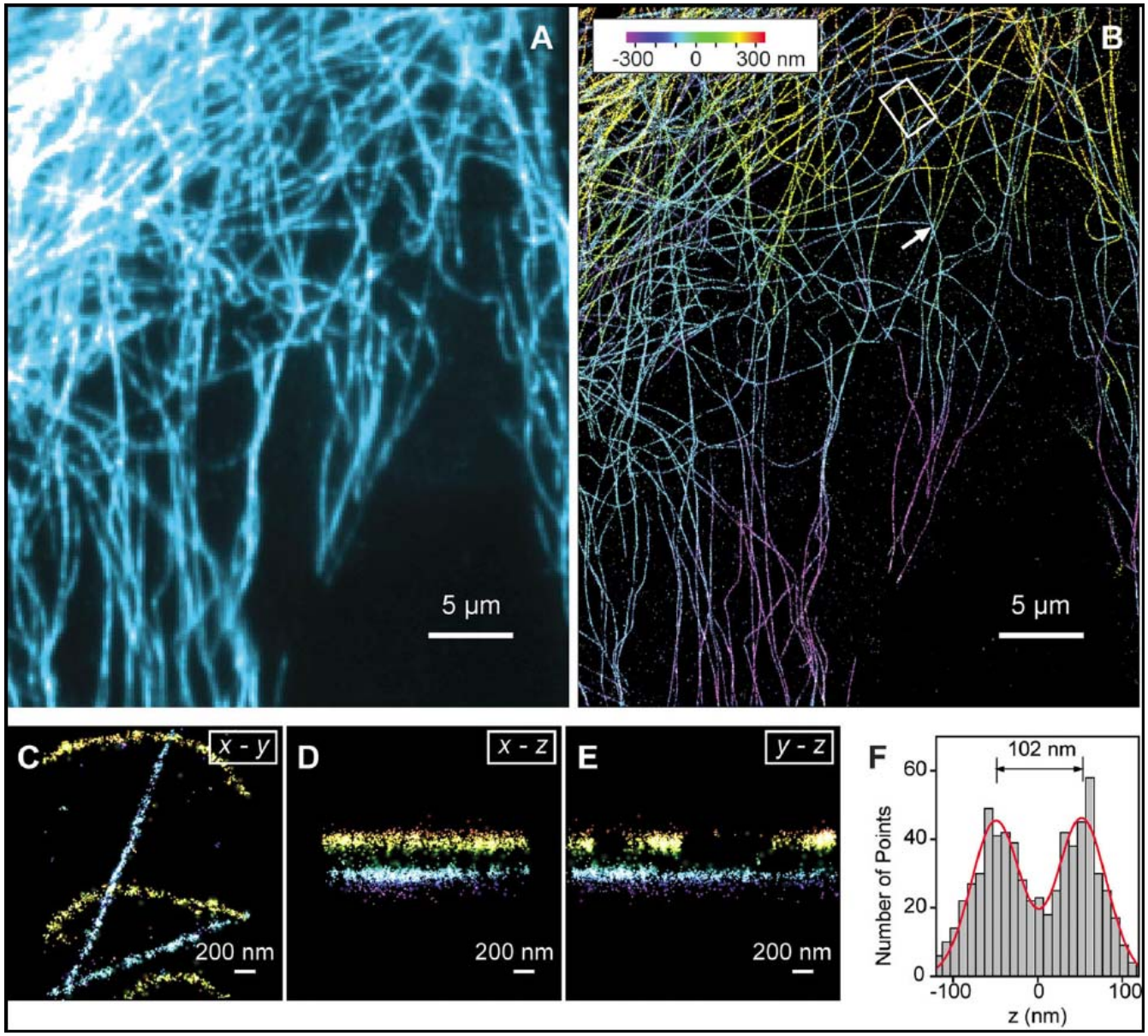
M. Bates et al., Science 317, 1749 -1753 (2007)

3D-STORM



Huang et al. Science Express 1153529 (2008)

3D STORM



A: conventional Image

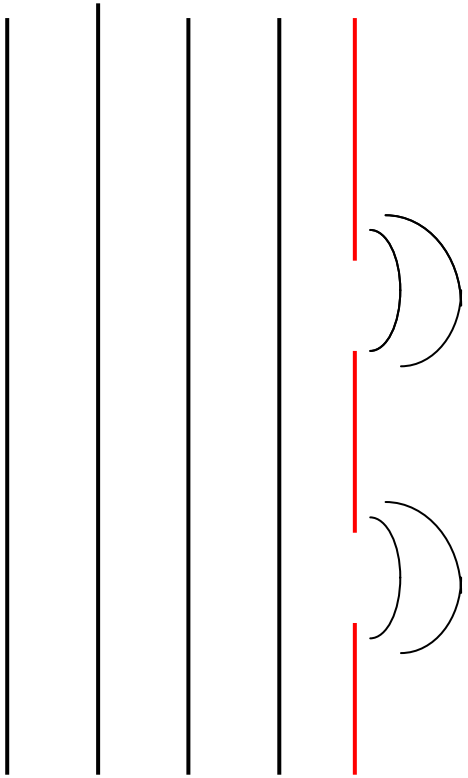
B: 3D STORM Image color=area

C-E zoom of box 5 microtubuli

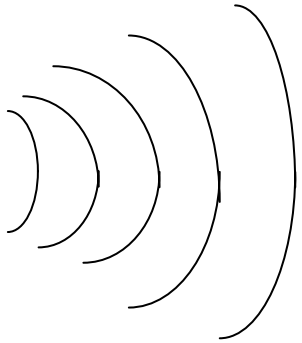
F histogramm Showing the z- coordinates

Huang et al. Science Express 1153529 (2008)

Scanning nearfield optical microsocopy (SNOM): Breaking the diffraction limit



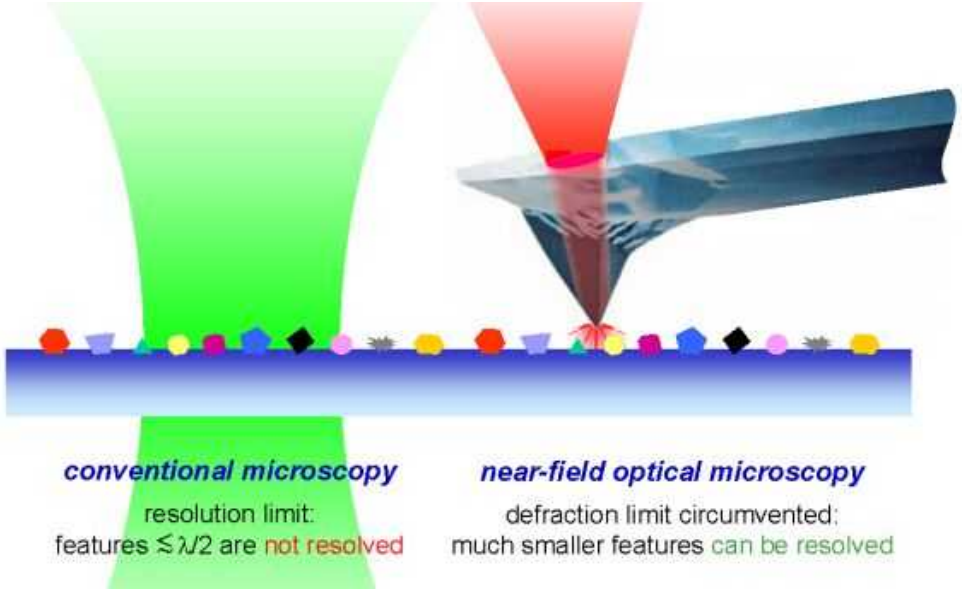
diffraction limit for plane waves
Abbe‘s equation

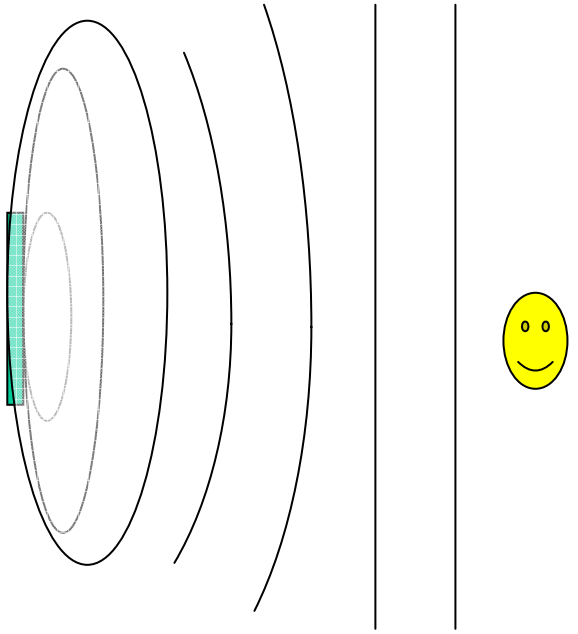


Use of distinct wave fronts:
Abbe‘s limit not valid anymore

Infrared Near field microscopy
Chemical imaging beyond the wave length limit

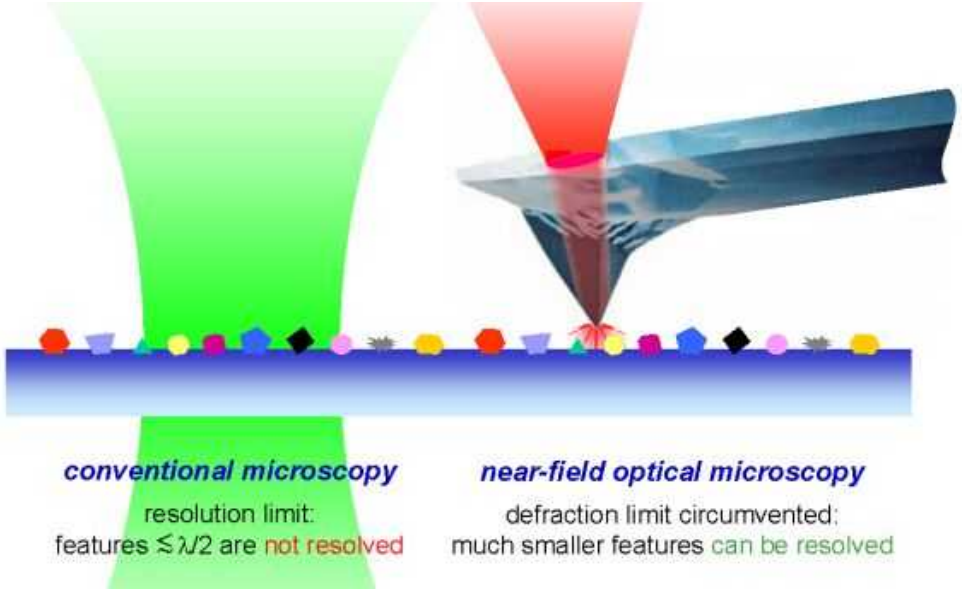
Aperture Free SNOM

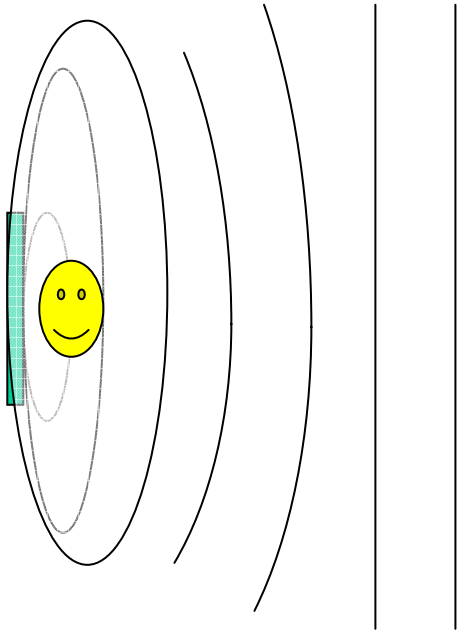




Far field
Plane wave

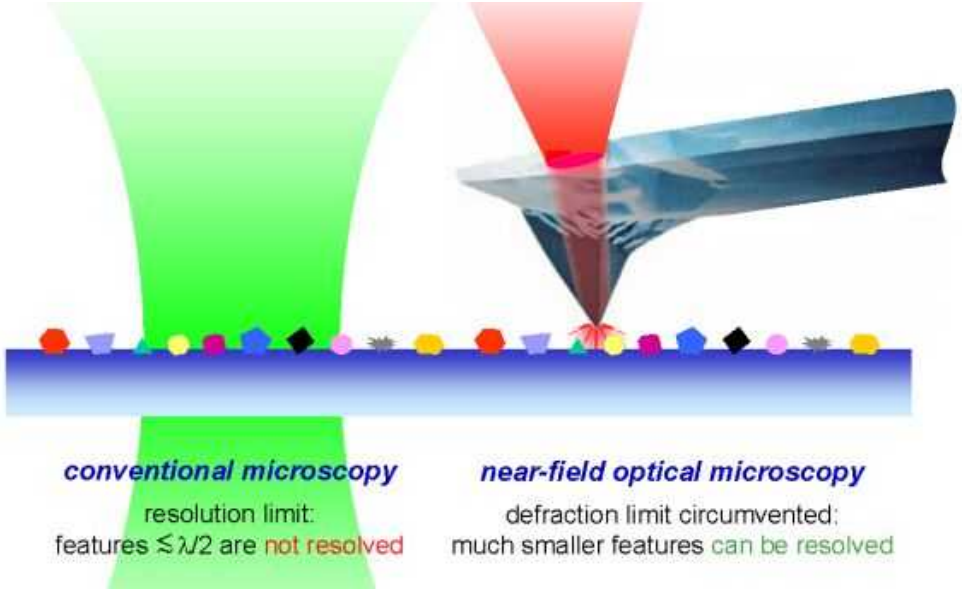
Infrared Near field microscopy
Chemical imaging beyond the wave length limit



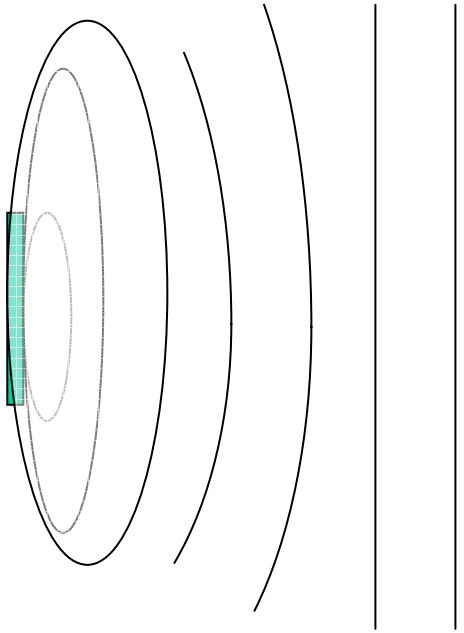


Near field

Infrared Near field microscopy
Chemical imaging beyond the wave length limit



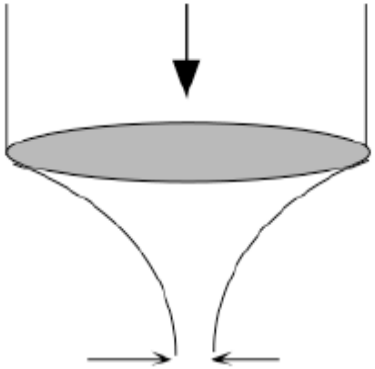
Nano aperature



Nano antenna

Aperture SNOM:

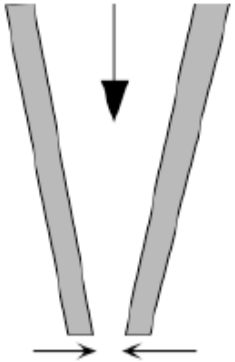
Introduction of new focusing concepts



λ/2

classical

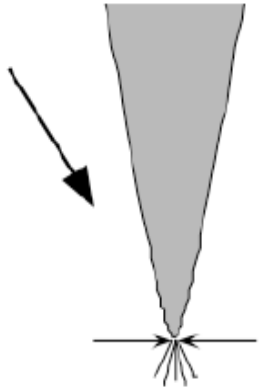
diffraction-limited



λ/10

aperture SNOM

aperture-limited



< 30 nm

scattering SNOM

tip-limited

Near field detection

www.physics.units.it/Ricerca/docXXciclo/Fis02/7_

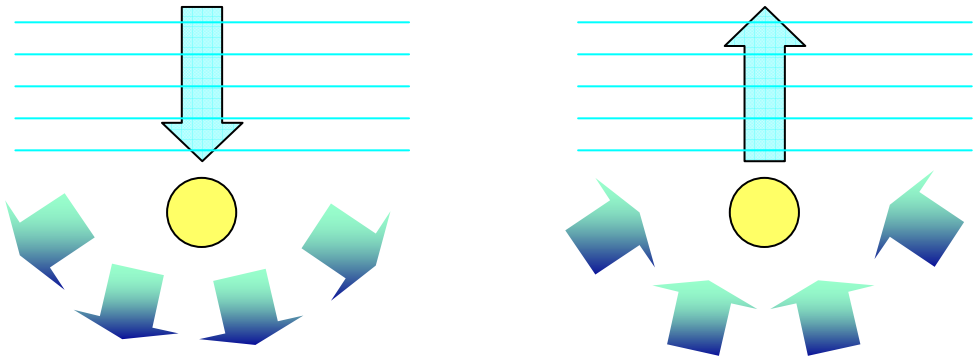
How to detect the near field if it not propagating?

Theorem of reciprocity

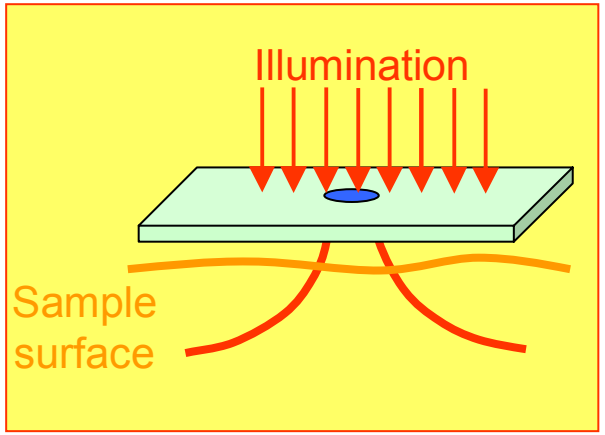
[Time reversibility of the Maxwell equation]

If a plane wave is diffracted into an evanescent wave by a subwavelenght scatterer,

A subwavelenght scatterer should be diffracted into a propagating wave by the same object



Near field detection

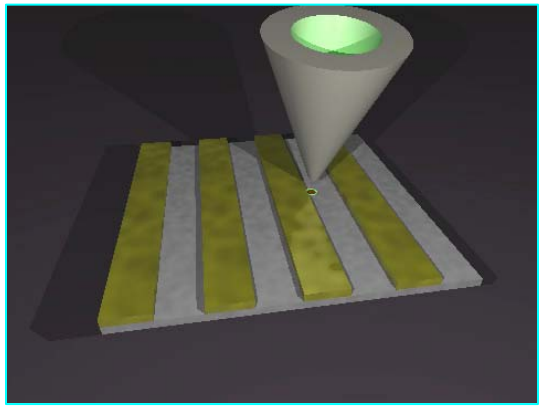


Aperture SNOM

- The light is collected near the sample by a tapered optical fiber with a subwavelenght aperture
- Low light throughput
- Resolution limited to $\lambda/10$

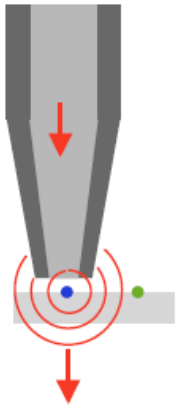
Physical mechanism SPATIAL FILTERING

- True spectroscopic information (including PL, EL, etc)
- Dependence only on the tip geometrical properties
- No dependance on the tip physical properties
- No wavelenght dependence

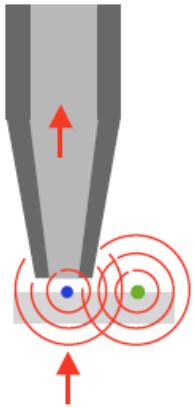


Aperture SNOM operation modes

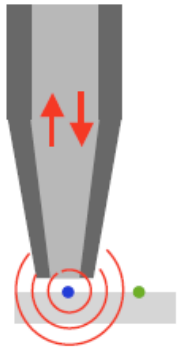
Illumination
in transmission



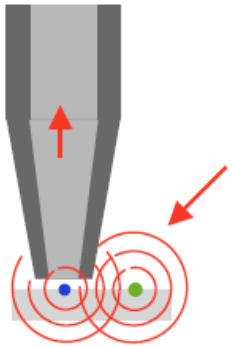
Collection
in transmission



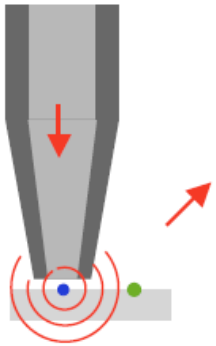
Illumination and collection
in reflection



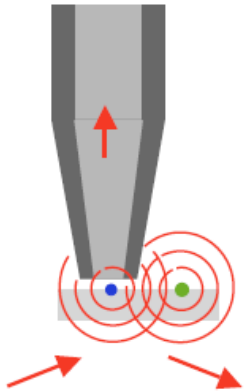
Collection in
oblique reflection



Illumination in
oblique reflection



Collection in
TIR illumination

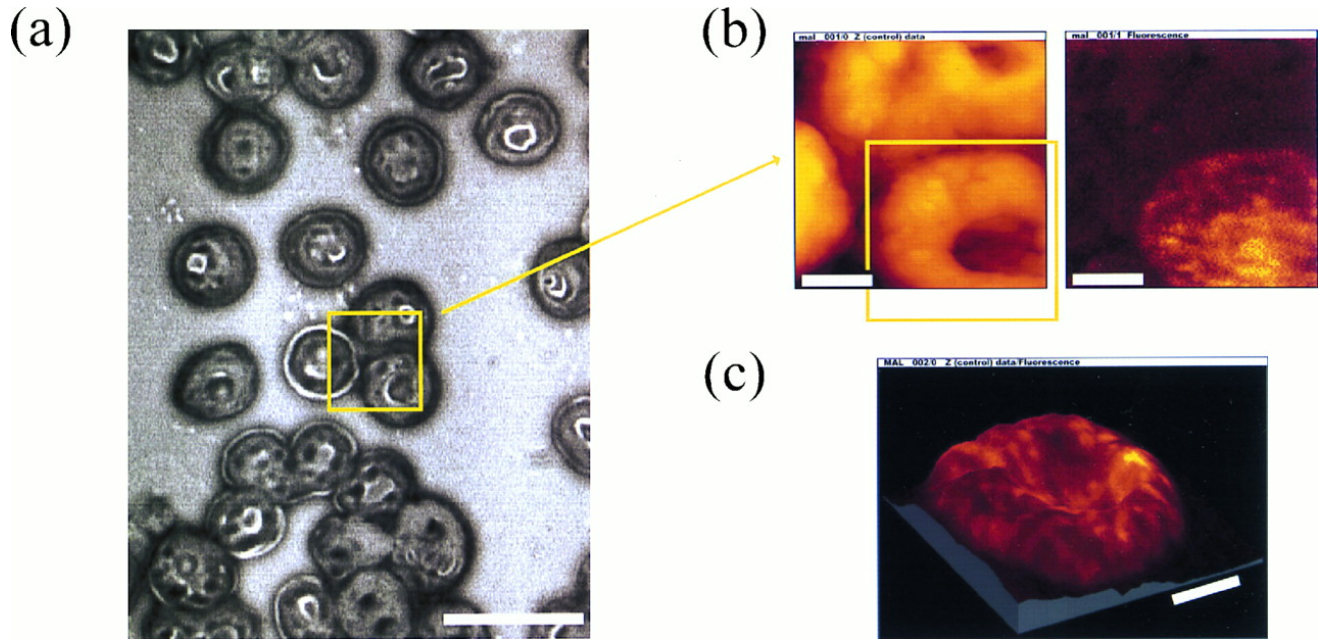


Präsentation F.Keilmann, MPI Martingsried (München) –
Nanofocusing for visible and IR light for microscopy

Aperture SNOM

www.physics.units.it/Ricerca/docXXciclo/Fis02/7_

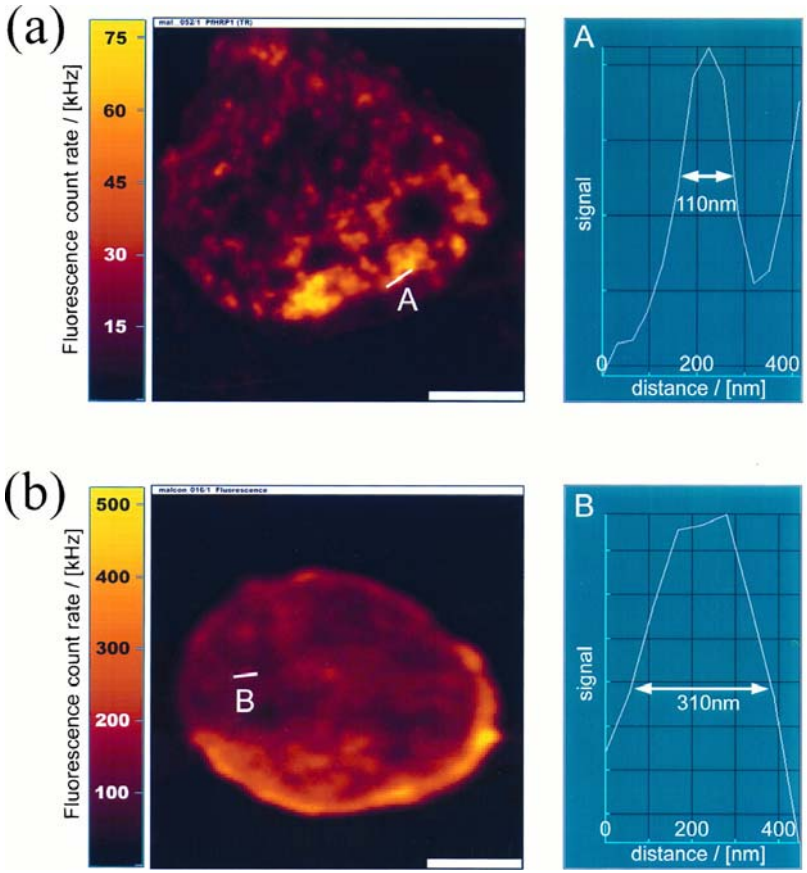
- Application1: blood cell with malaria disease



Study of blood cells infected by malaria's plasmodium falciparium.(PF)
Pf expresses several proteins in particular PfHRP1 and MESA that are fixed on the cell membrane.
Proteins on cell membrane are colored with specific antibody marked with a red and a green fluorophor
Here PfHRP1 is marked red

Aperture SNOM

- Application 1: blood cell with malaria disease



Comparison between SNOM and confocal microscope images in the same blood cell:

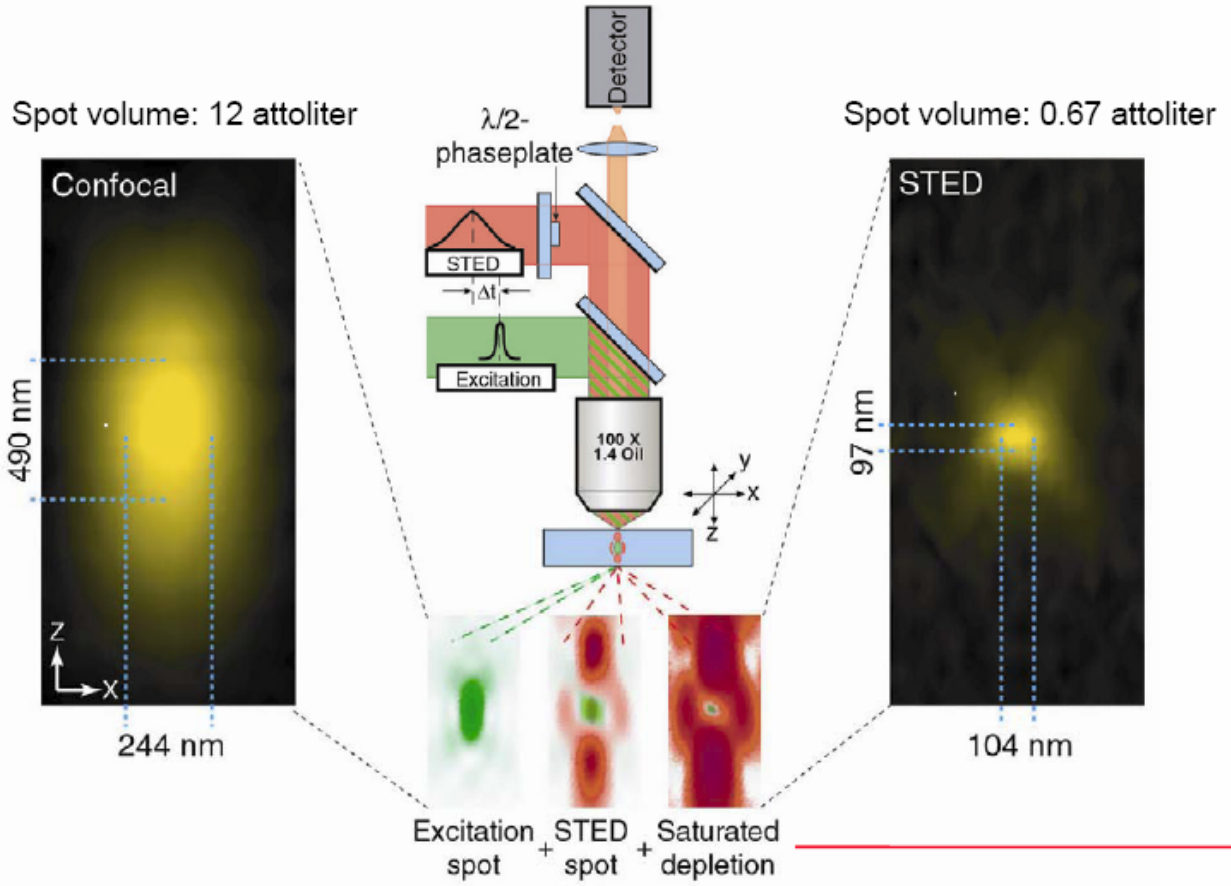
SNOM is sensitive to cell surface

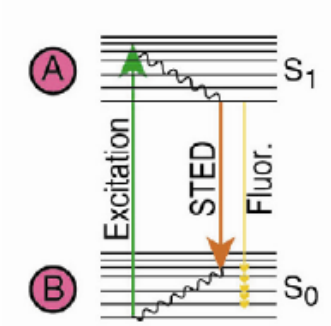
CM images a plane section at the focal plane

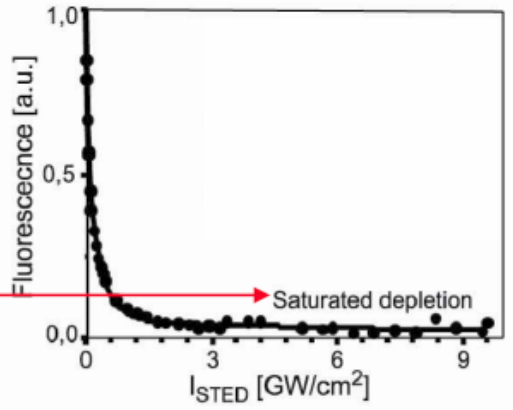
Cellular structure is resolved on the SNOM image but not in CF image

Max Planck Society

STED-Microscopy: Sharper in x, y and z







Beating the diffraction limit:

$\Delta r = \lambda/(2n \sin\alpha\ (1+I/I_{max})^{1/2}$

S.W. Hell, J. Wichmann (1994), *Opt. Lett.* **19**, 780.
T.A. Klar, S. Jakobs, M. Dyba, A. Egner, S.W. Hell (2000), *PNAS* **97**, 8206.
S.W. Hell, M. Dyba, S. Jakobs (2004), *Curr. Opin. Neurobiol.* **14**, 599.

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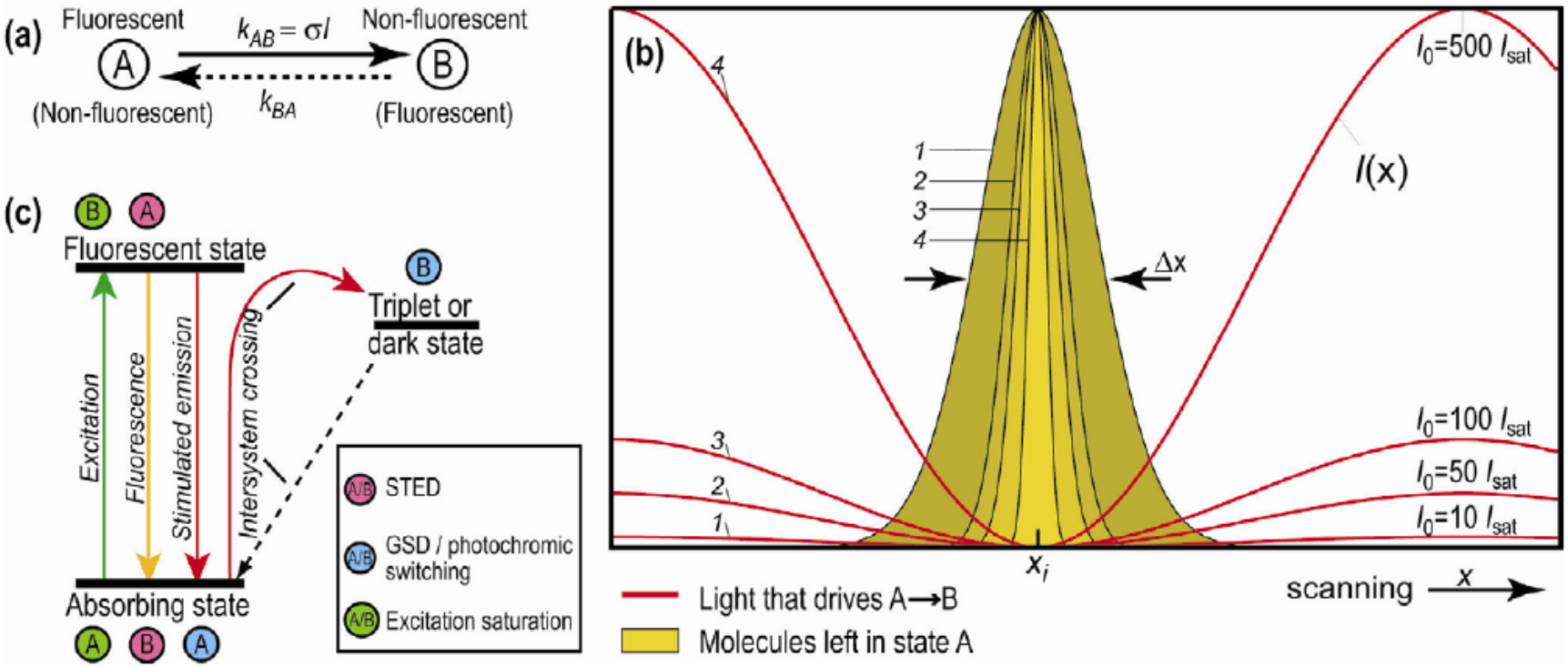
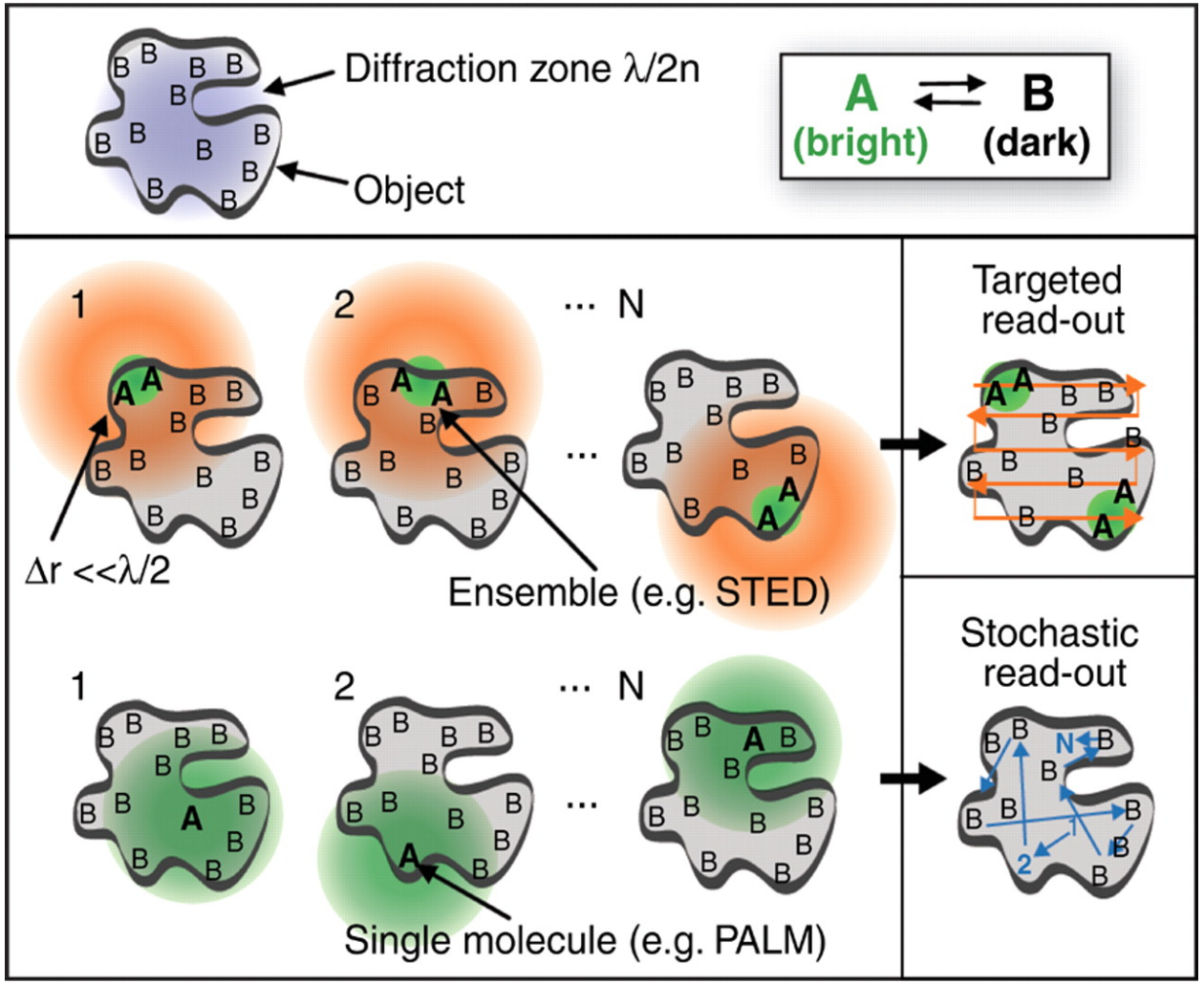
RESOLFT: Reversible Saturable Optical (Fluorescent) Transition



S.W. Hell (2003), *Nature Biotechnol.* **21**, 1347.
S.W. Hell, M. Dyba, S. Jakobs (2004), *Curr. Opin. Neurobiol.* **14**, 599.

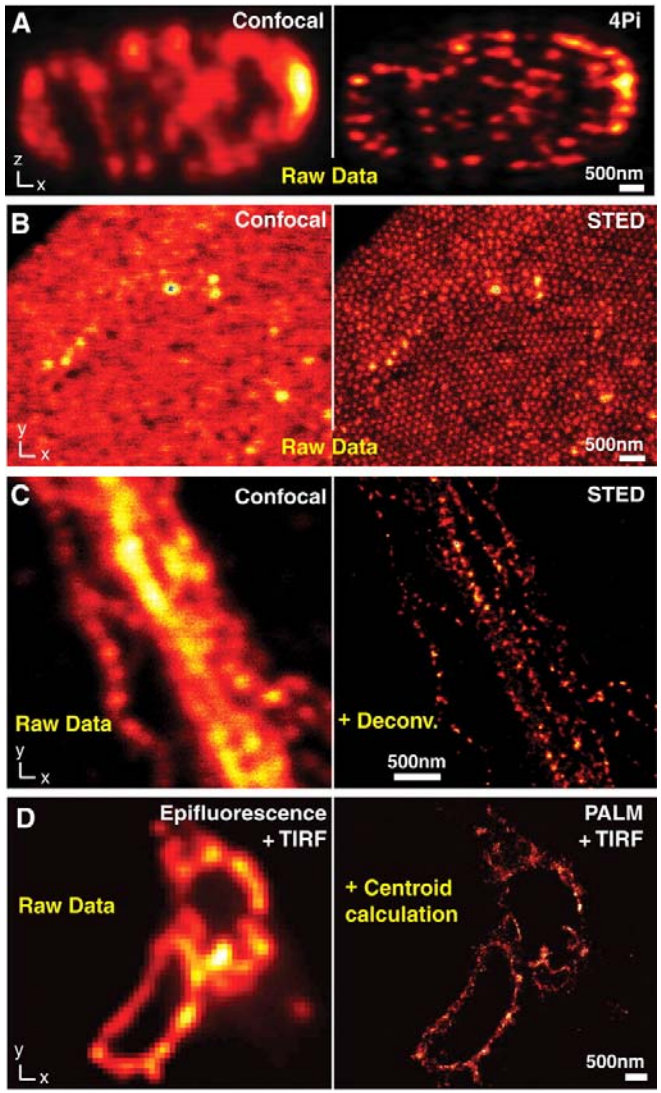
Fig. 2. Targeted versus stochastic time-sequential readout of fluorophore markers of a nanostructured object within the diffraction zone whose lower bound is given by {lambda}/2n



S. W. Hell Science 316, 1153 -1158 (2007)

Published by AAAS

Side-by-side comparisons



S. W. Hell Science 316, 1153 -1158 (2007)