Advanced Microscopy Techniques

- Wave Optics and Microscopy Resolution
- 4Pi Confocal Laser Scanning Microscopy
- Non-linear Techniques (no resolution limit)
  - Photoswitchable/-activatable fluorophores
  - STED microscopy
  - Blinking Q-dots (first realization of localization microscopy)
  - PALM / STORM / FPALM
  - Localization Microscopy
  - ...

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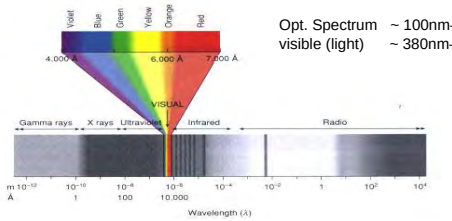
The EM-Spectrum

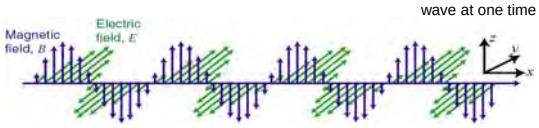


Opt. Spectrum ~ 100nm–1mm
visible (light) ~ 380nm–780nm

An Electromagnetic Wave

Has an electric and a magnetic component (field).

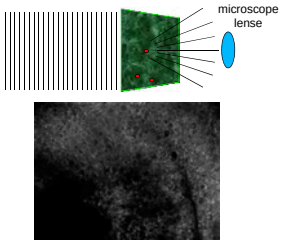
snapshot of the wave at one time



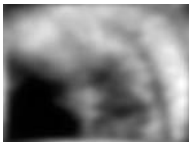
The electric field, the magnetic field, and the direction of the wave (so called k-vector) are all perpendicular:

$$\vec{E} \times \vec{B} \propto \vec{k}$$

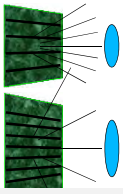
Light propagation - diffraction -



Causes of image degradation: Blur



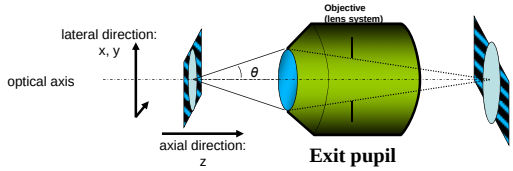
Light propagation - diffraction -



Causes of image degradation: Blur

finer structures (smaller distances) ⇒ higher diffraction
first order of diffraction needs to be imaged i.e.,
needs to pass through lens

Definitions



- Maximal angle for detection θ
- Numerical aperture
  $NA = n \sin\theta$
- Rayleigh resolution criteria for a circular aperture
  $\Delta x = 0.61\ \lambda / NA$

Immersion objective lenses

- more light is detected

$NA = n \cdot \underbrace{\sin\theta}_{<1}$

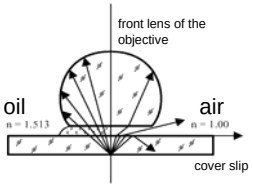
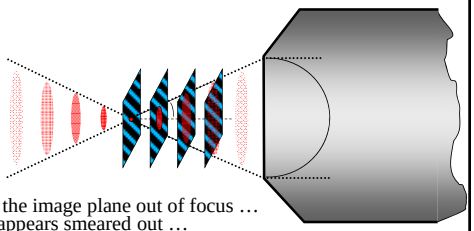


image formation



when moving the image plane out of focus …
… a point appears smeared out …
… and the intensity gets lower and lower

Fundamental Limitation of Optical Resolution (O.R.*) in Epifluorescence Microscopy

Standard GFP = Green fluorescent Protein

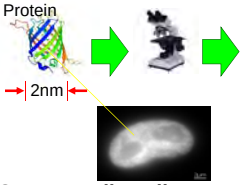
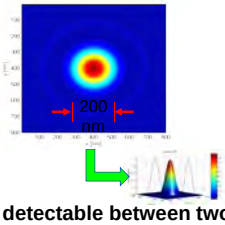


Image of a 'Point Source' (M = 1)



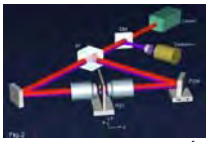
Simulation

λ_{em}=520nm, NA=1.4, n=1.46, Voxelsize =10nm

***O.R. = smallest distance d_{min} detectable between two 'point like' sources d_{min} (FWHM) = 0.5λ_{em} ~ 200 nm**

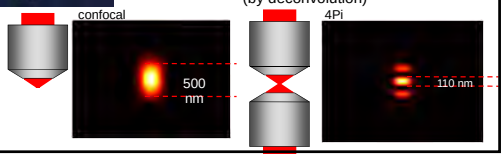
4Pi Microscopy

4Pi principle



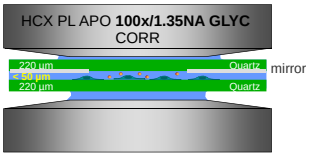
light paths:
Excitation (red)
Emission (blue)

~110nm **axial** resolution
Sidelobes need to be eliminated (by deconvolution)



4Pi Microscope

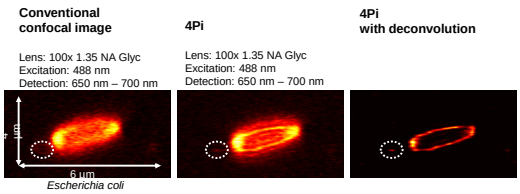
Sample geometry



Consequences:

- Limitation in sample thickness (less than 50µm)
- Active sample surface 0.5cm²

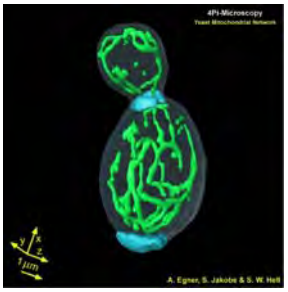
4Pi Resolution Improvement



Escherichia coli

Leica Microsystems, Mannheim, Germany

4Pi image of mitochondria in live yeast



Egner A. *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99** (6), 3370–3375

Non-linear techniques

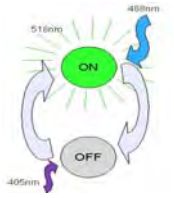
- Structured Illumination Microscopy (widefield)
- STED microscopy (scanning)
- Stochastic imaging (widefield)
  - Blinking Q-dots (first principle of localization microscopy)
  - PALM / STORM / FPALM
  - Localization Microscopy
  - ...

Photoactivatable or Photoswitchable proteins

- Can switch between fluorescent (bright) states and non-fluorescent (dark) states (or between different fluorescence emission colors)
- Switching is done with specific wavelengths
- Creation of nonlinearities at low intensity

Dronpa (Photoactivation)

- GFP-like fluorescent protein
- Fast switching time
- High QE and contrast
- Switches >100 times on single molecule scale

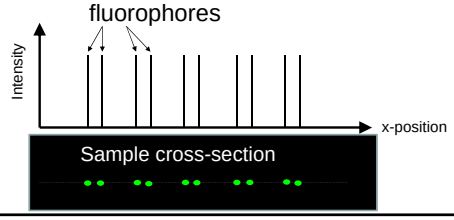


L. Hirvonen

non-linear Structured Illumination

Data acquisition

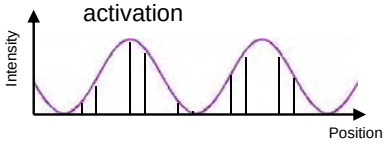
Object: pairs of photo-switchable fluorophores



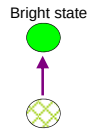
L. Hirvonen

Data acquisition

Sinusoidal activation



Sample cross-section

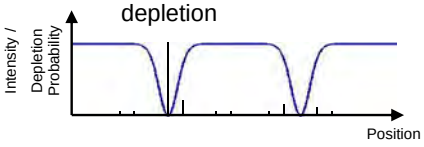


Dronpa

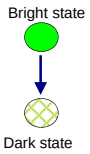
L. Hirvonen

Data acquisition

Nonlinear depletion



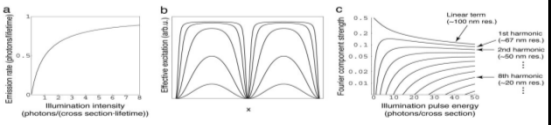
Sample cross-section



Dronpa

L. Hirvonen

Nonlinear structured illumination II



- Saturated excitation
- Pattern of the emission takes on a nonsinusoidal shape, which contains higher harmonics

M.Gustafsson

Experimental results

51nm beads

Conventional FWHM ≈ 265nm

linear structured illumination

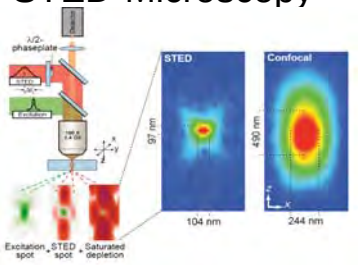
saturated structured illumination FWHM ≈ 59nm

M.Gustafsson

STED

STED-Microscopy

STED
Stimulated
Emission
Depletion



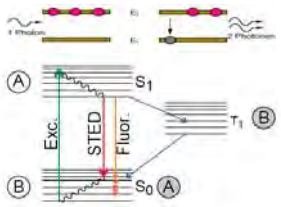
T.A. Klar et.al, PNAS, 97, 2000; MPI Brochure 2003

STED Basics

- Einstein (1917): stimulated emission
- Excitation pulse and STED pulse have an very small (very exact) time difference (Δt)

$$\tau_{vib}\ (fs) \ll \tau_{STED}\ (ps) \ll \tau_{fl}\ (ns)$$

- stimulated emission before fluorescence
- STED pulse needs a very high intensity (10 - 100 MW/cm^{2}s)



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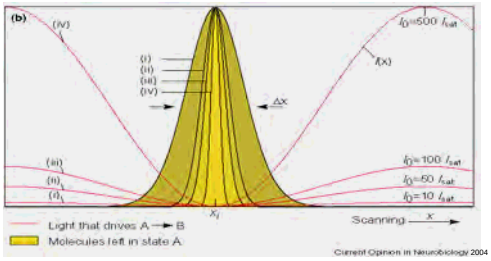
STED-Puls



Current Opinion in Neurobiology 2004

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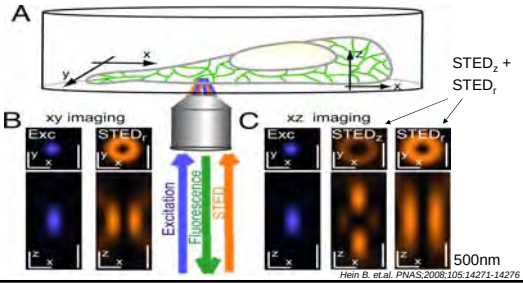
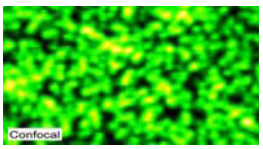
Schematic showing the use of the excitation and deexcitation (STED) beams for 3D STED imaging inside a living cell



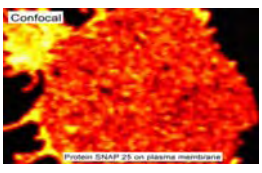
STED$_z$ + STED$_r$

Hein B. et.al. PNAS;2008;105:14271-14276

Confocal vs. STED microscopy
- STED microscopy resolves nanoparticle assemblies -



Confocal versus corresponding STED image of dispersed 40 nm beads.



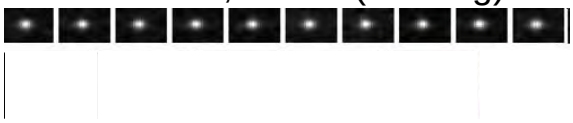
Confocal versus STED image of the nanoscale arrangement of the protein SNAP-25.

K.I Willig et al 2006 New J. Phys. 8 106

Wide field techniques

- Pointilism (blinking, bleaching)
- PALM / STORM / PALMIRA
- SPDM

Pointilism, Part 1 (blinking)



Top:10 frames from a time series acquired with Quantum Dots.

Independent component analysis (ICA) decomposition of blinking QD data sets showing the resolution of three QDs in one locus.

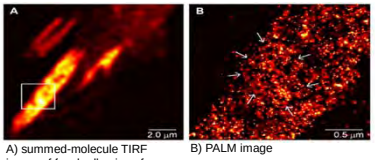
The lower panel shows left to right: time average of the series; ICA-returned component 1; ICA component 2; ICA component 3; color-coded overlay of components 1, 2, and 3; position overlay of 1, 2, and 3.

*Keith Lidke et al. **Optics Express** (2005).*

Photo-Activation Localization Microscopy

(PALM, STORM, FPALM)

1. Photo-activate small fraction of fluorophores
2. Determine positions of single molecules
3. Bleach these molecules
4. Repeat until all fluorophores are measured



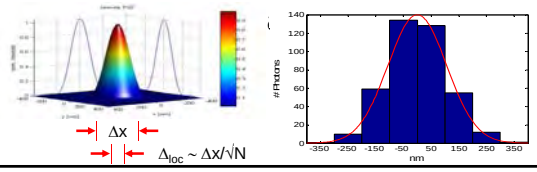
A) summed-molecule TIRF image of focal adhesions for a FoLu cell expressing dEos-tagged vinculin.

B) PALM image

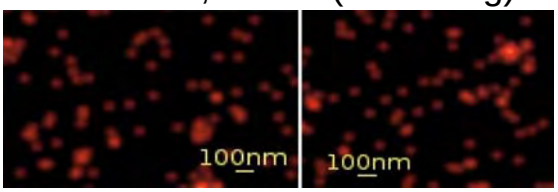
E. Betzig et al., ScienceExpress August 10, 2006

Localization Microscopy: Possible with all modes of microscopes, even with homogeneous widefield illumination & detection

***S*pectrally *A*ssigned *L*ocalisation *M*icroscopy (SALM):**



Pointilism, Part 2 (bleaching)



Positions (x,y) of nuclear pore proteins (p62) in a human MCF7 cell determined with the SMI microscope. The left and right images show two different example areas from p62 protein distribution on the nuclear membrane of the same cell.

*Reymann, J. et al. Chromosome Research **16**, 367-382(2008).*

Localization Microscopy

general principle

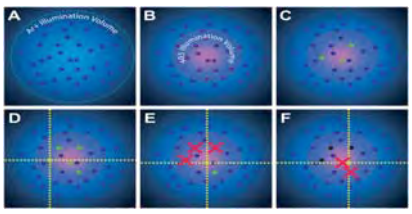
What you do:

- get all fluorophores in the “dark” state
- “switch” only a few of them “on”, so that there is only one molecule per diffraction volume
- detect these signals and “switch” them “off”
- “switch” other fluorophores “on”, detect them ... and so on …
- determine the center of the spots of your detected signals to get the position of each fluorophore

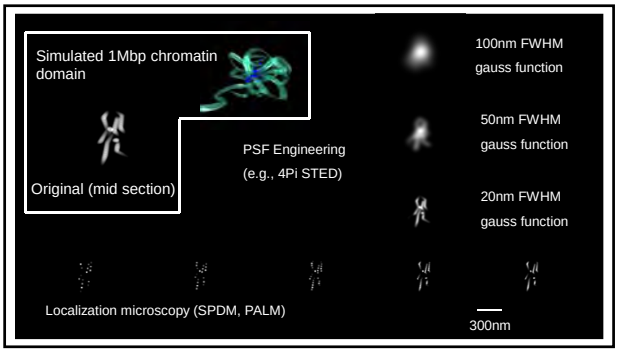
Localisation Microscopy

general principle

What you do:



Hess et al. 2006, Biophysical Journal



Limitations

High resolution ↔ Long acquisition times / Low signal counts

- Higher photobleaching rate
- Phototoxicity
- Signal-to-noise ratio
- 3D is limited
- Live-cell imaging is difficult

Literature

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