Photoswitching of red cyanine dyes

Fluorescent
650 nm
+ thiol
Cy5 / Alexa 647
photoactivation
Deactivation
360 nm
650 nm
Dark
Fluorescent
hυ
RS⁻
hυ
Dark

Nanooptics 24/1 B. Huang (2009); Dempsey et al. (2009)

Switchable probes commercially available

400 500 600 700 nm

Cyanine dye + thiol system
Alexa647 Cy5 Cy5.5 Cy7
Bates et al., 2005, Bates et al., 2007, Huang et al., 2008

Rhodamine dye + redox system
Atto565 Alexa488 Alexa532 Atto590 Atto655 Atto700 Atto520 Alexa568
Heilemann et al., 2009

Photoactivatible fluorescent proteins
PA-GFP mEosFP2 PS-CFP2 Dendra2 Dronpa PAmCherry EYFP

Reviews:
Lukyanov et al., Nat. Rev. Cell Biol., 2005
Lippincott-Schwartz et al., Trends Cell Biol., 2009

Nanooptics 24/2 B. Huang (2009)

PALM: Photoactivated Localization Microscopy

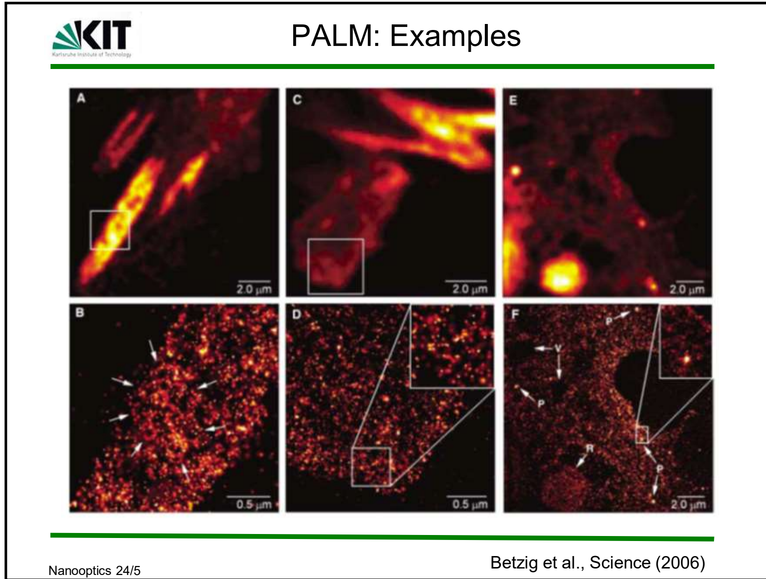
ACTIVATED
BLEACHED
ACTIVATED

Nanooptics 24/3 Betzig et al., Science (2006)

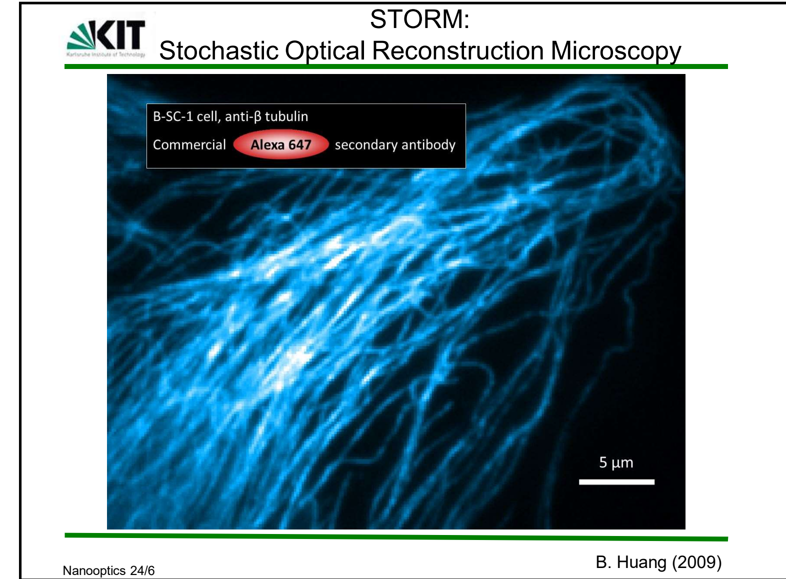
PALM: Examples

A
1.0 μm
B
1.0 μm

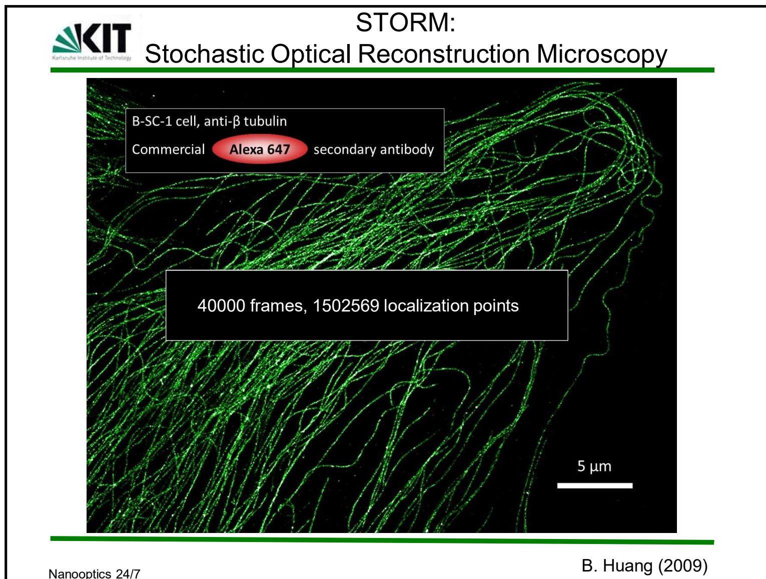
Nanooptics 24/4 Betzig et al., Science (2006)



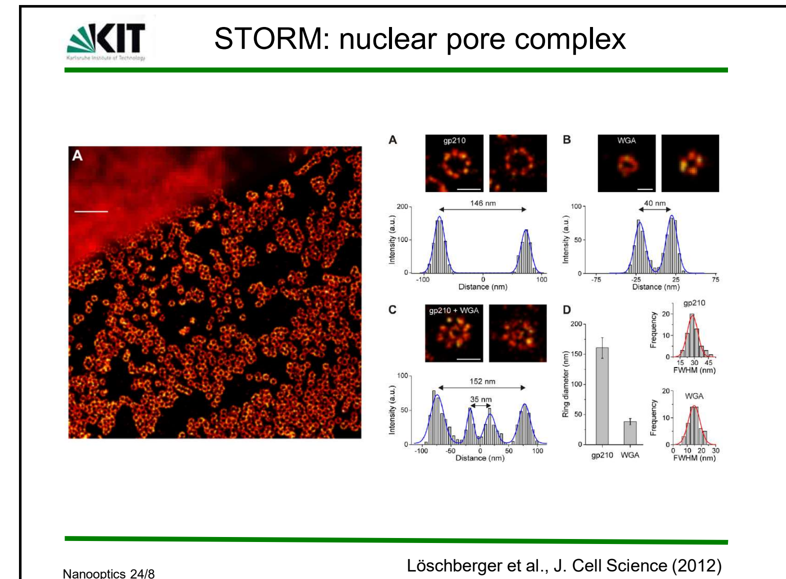
KIT
PALM: Examples
A
C
E
2.0 µm
B
D
F
0.5 µm
Nanooptics 24/5
Betzig et al., Science (2006)



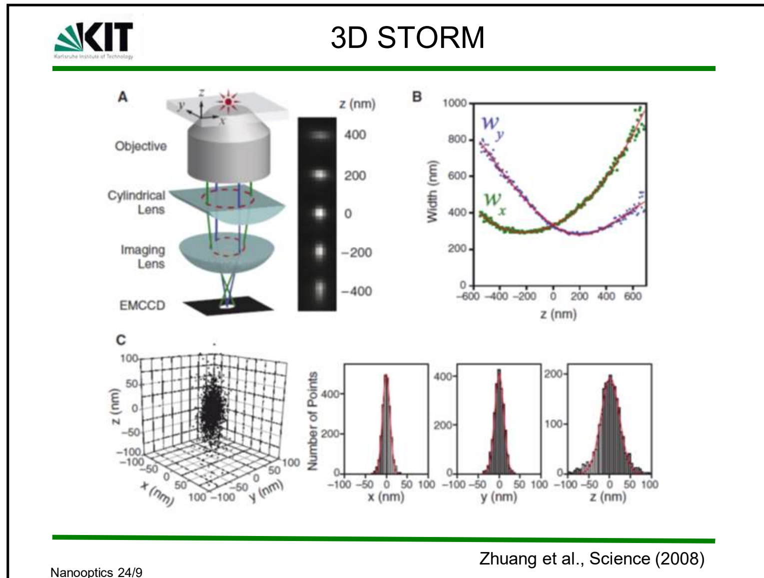
KIT
STORM:
Stochastic Optical Reconstruction Microscopy
B-SC-1 cell, anti-β tubulin
Commercial Alexa 647 secondary antibody
5 µm
Nanooptics 24/6
B. Huang (2009)

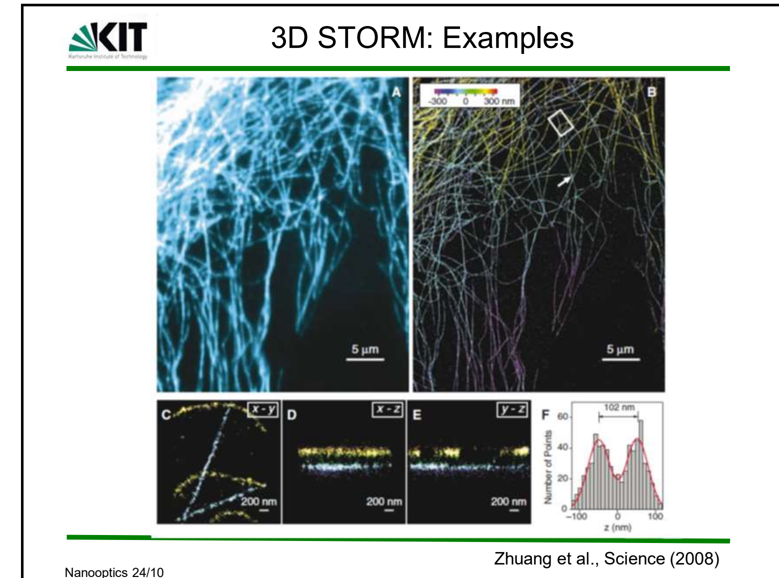


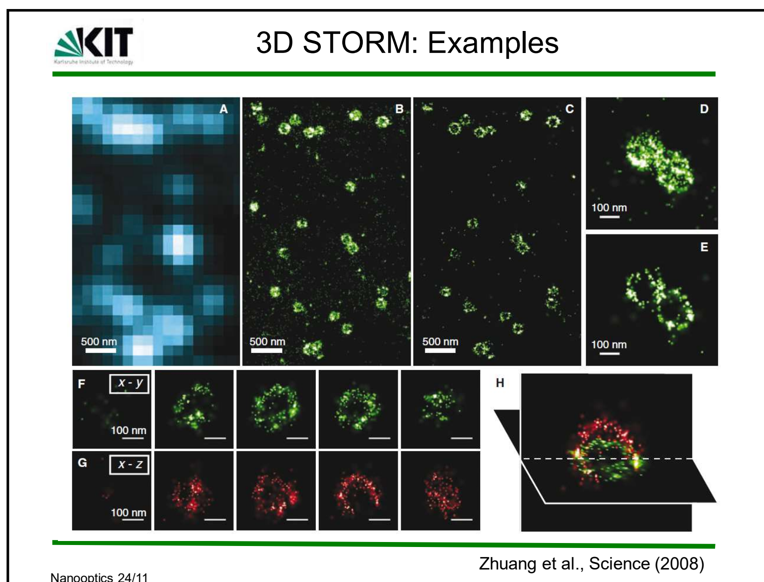
KIT
STORM:
Stochastic Optical Reconstruction Microscopy
B-SC-1 cell, anti-β tubulin
Commercial Alexa 647 secondary antibody
40000 frames, 1502569 localization points
5 µm
Nanooptics 24/7
B. Huang (2009)

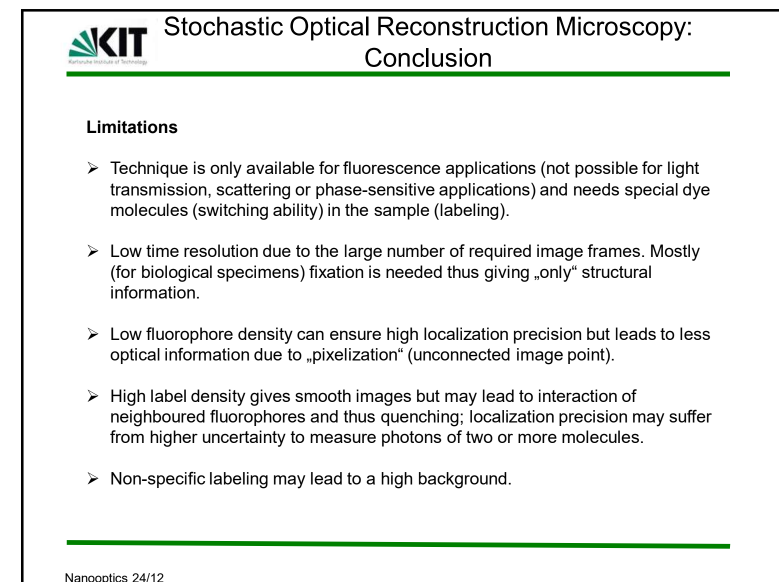


KIT
STORM: nuclear pore complex
A
B
C
D
gp210
WGA
gp210 + WGA
146 nm
40 nm
152 nm
35 nm
Intensity (a.u.)
Distance (nm)
Ring diameter (nm)
Frequency
FWHM (nm)
Nanooptics 24/8
Löschberger et al., J. Cell Science (2012)


3D STORM
Objective
Cylindrical Lens
Imaging Lens
EMCCD
z (nm)
Width (nm)
Number of Points
x (nm)
y (nm)
z (nm)
Zhuang et al., Science (2008)
Nanooptics 24/9


3D STORM: Examples
5 µm
200 nm
102 nm
Zhuang et al., Science (2008)
Nanooptics 24/10


3D STORM: Examples
500 nm
100 nm
Zhuang et al., Science (2008)
Nanooptics 24/11


Stochastic Optical Reconstruction Microscopy: Conclusion
Limitations
Technique is only available for fluorescence applications (not possible for light transmission, scattering or phase-sensitive applications) and needs special dye molecules (switching ability) in the sample (labeling).
Low time resolution due to the large number of required image frames. Mostly (for biological specimens) fixation is needed thus giving „only“ structural information.
Low fluorophore density can ensure high localization precision but leads to less optical information due to „pixelization“ (unconnected image point).
High label density gives smooth images but may lead to interaction of neighboured fluorophores and thus quenching; localization precision may suffer from higher uncertainty to measure photons of two or more molecules.
Non-specific labeling may lead to a high background.
Nanooptics 24/12

4. Nano-optics using far-field optical techniques

4.1 Introduction: single-molecule methods in biology
4.2 Single-molecule tracking (SMT)
4.3 Stochastic optical reconstruction microscopy (STORM)
4.4 4pi microscopy
4.5 Stimulated emission depletion (STED)
4.6 3D laser lithography using STED

4Pi Microscopy

The task:

- Increase resolution in fluorescence microscopy over classical Abbé Limits

$$FWHM_{xy} = \frac{0.4\lambda}{n \cdot \sin\alpha}$$

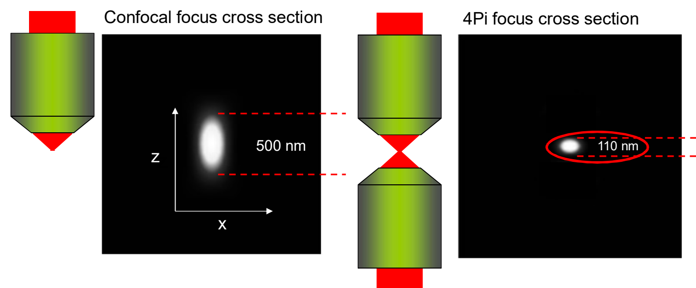
$$FWHM_z = \frac{0.45\lambda}{n(1-\cos\alpha)}$$

- xy resolution: ~ 200nm
- z resolution (confocal): ~ 500nm

Leica

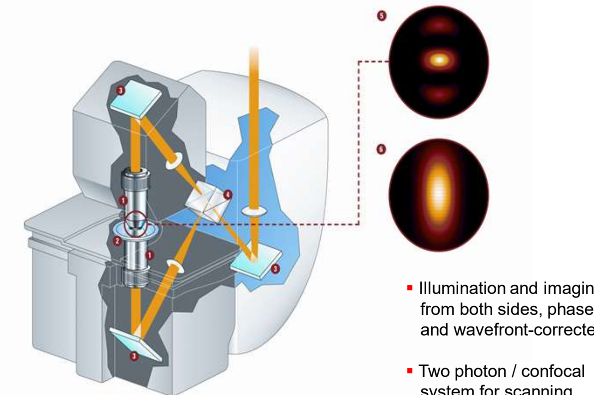
4Pi Microscopy

- 4Pi microscopy combines the Numerical Aperture of 2 opposing objectives through interference
- The 4Pi focus volume is a factor 3 – 5 times smaller than the confocal focus
- The axial resolution at the excitation wavelength of 780 nm is 110nm
- Interference side lobes are removed by linear point deconvolution



Leica

Central element: the 4Pi Interferometer



- Illumination and imaging from both sides, phase- and wavefront-corrected
- Two photon / confocal system for scanning

Leica

4Pi Interferometer controls

Leica

Objectives for 4Pi - outstanding specifications

Specifications:

- Focus aberration of each individual objective: max. 300nm in the range 488nm to 633nm
- Objective pairs must be identical within +/- 50 nm in the range 488nm - 633nm.
- Color magnification error must be less than 1% within the range of 488nm - 633nm.
- Magnification of both objectives must be identical within 0,1%

→ Matching objective pairs need to be selected

Leica

4Pi Microscopy: Sample preparation

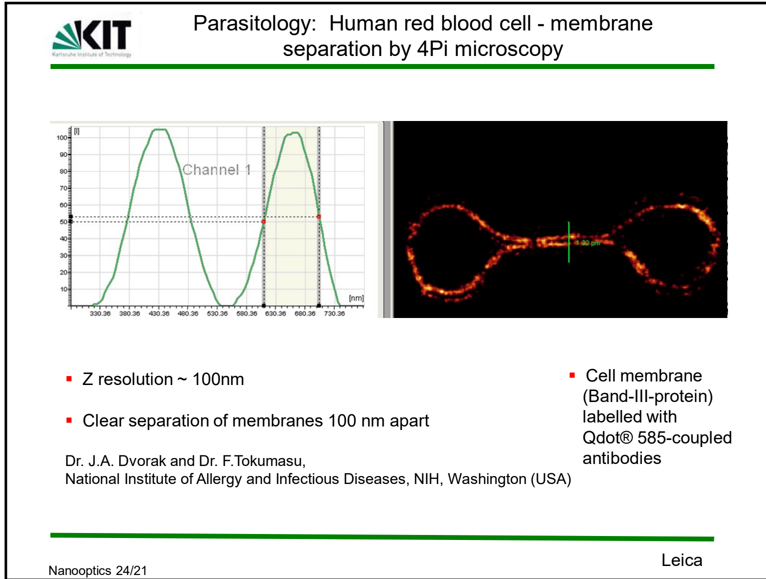
Leica

Performance at theoretical limits

- Calculated (blue) and measured (red) optical resolution for a thin structure (mirror)

Wavelength: 840nm
Objective: 1.4 NA (Oil Immersion)

Leica



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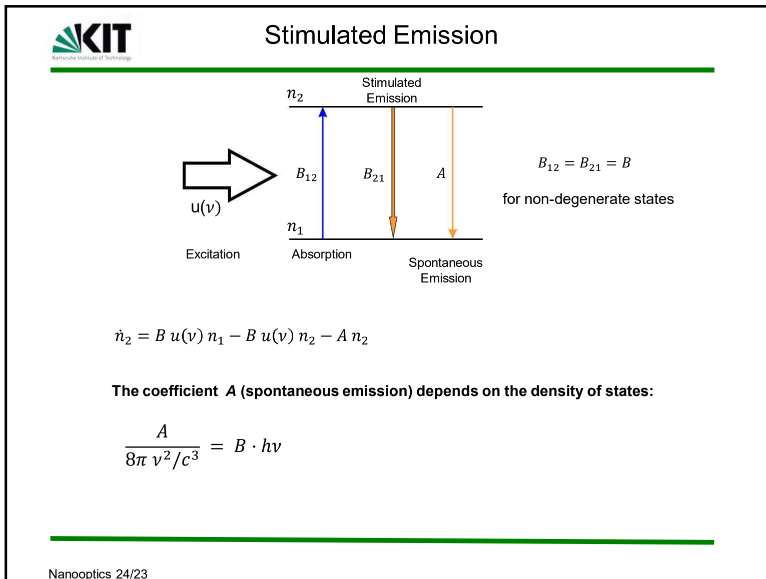

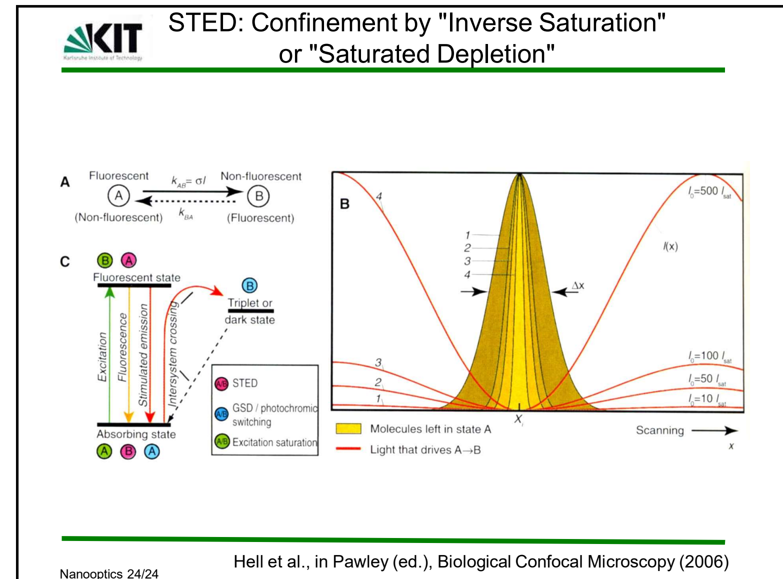

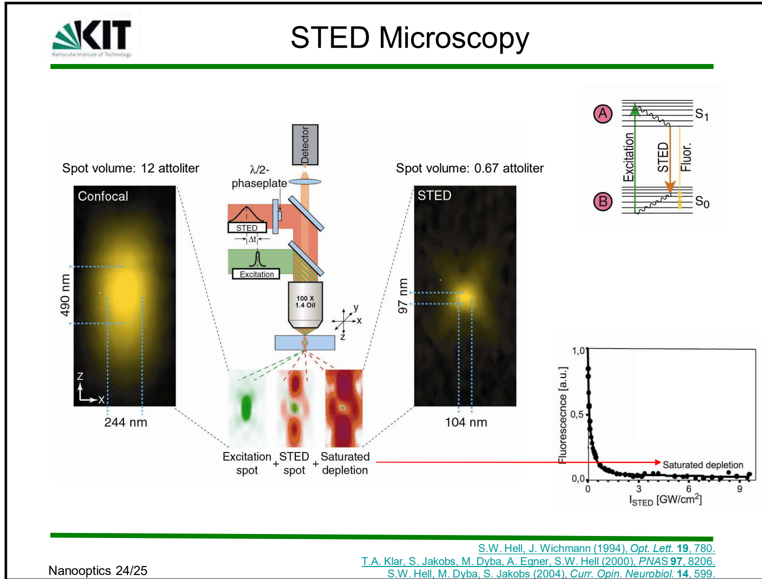

KIT
STED Microscopy
Spot volume: 12 attoliter
Confocal
490 nm
244 nm
λ/2-
phaseplate
Detector
STED
Excitation
100 X
1.4 Oil
Excitation spot + STED spot + Saturated depletion
Spot volume: 0.67 attoliter
STED
97 nm
104 nm
A
B
S1
S0
Excitation
STED
Fluor.
Fluorescecnce [a.u.]
1,0
0,5
0,0
0
3
6
9
Saturated depletion
ISTED [GW/cm2]
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.
Nanooptics 24/25