LECTURE 13
Breaking the resolution limit
Ilan Davis, March 2011

SPIM
STED
4Pi
I5M
PALM / STORM

Breaking the Resolution Barrier

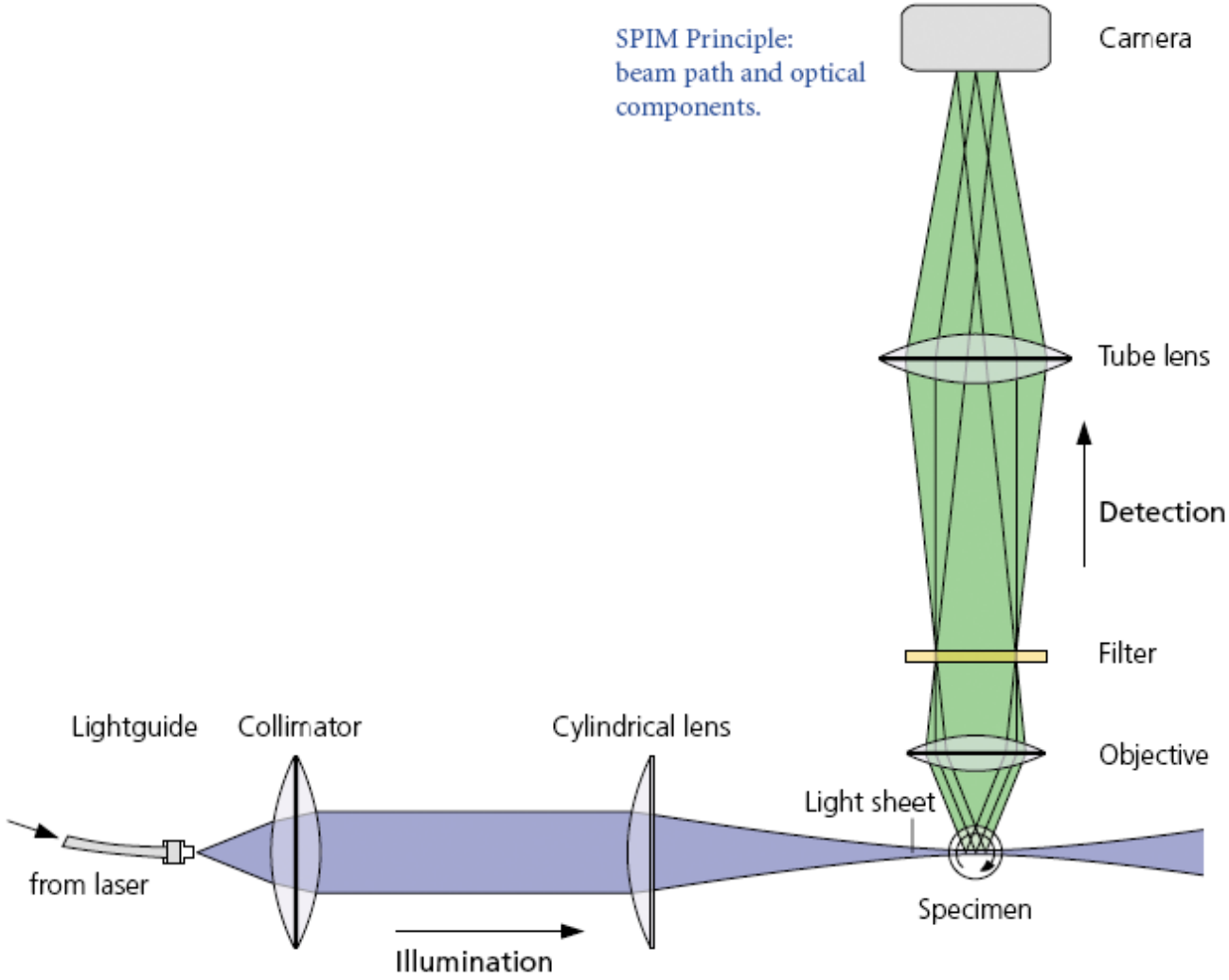
OMX	-Time resolution
Structured Illumination	-XY resolution and some Z
STED	-XY resolution and some Z
SPIM	-Tomography depth penetration
4-Pi	-Z resolution
I5M	-Z resolution

PALM, STORM, PALMERA, FIONA, iPALM

SPIM (Selective Plane Illumination Microscopy)

(or theta microscope)

Huisken J, Swoger J, Del Bene F, Wittbrodt J, **Stelzer EHK** (2004) Optical sectioning deep inside live embryos by selective plane illumination microscopy Science 305: 1007-1009 http://www.embl-heidelberg.de/ExternalInfo/stelzer/frames.html?publications



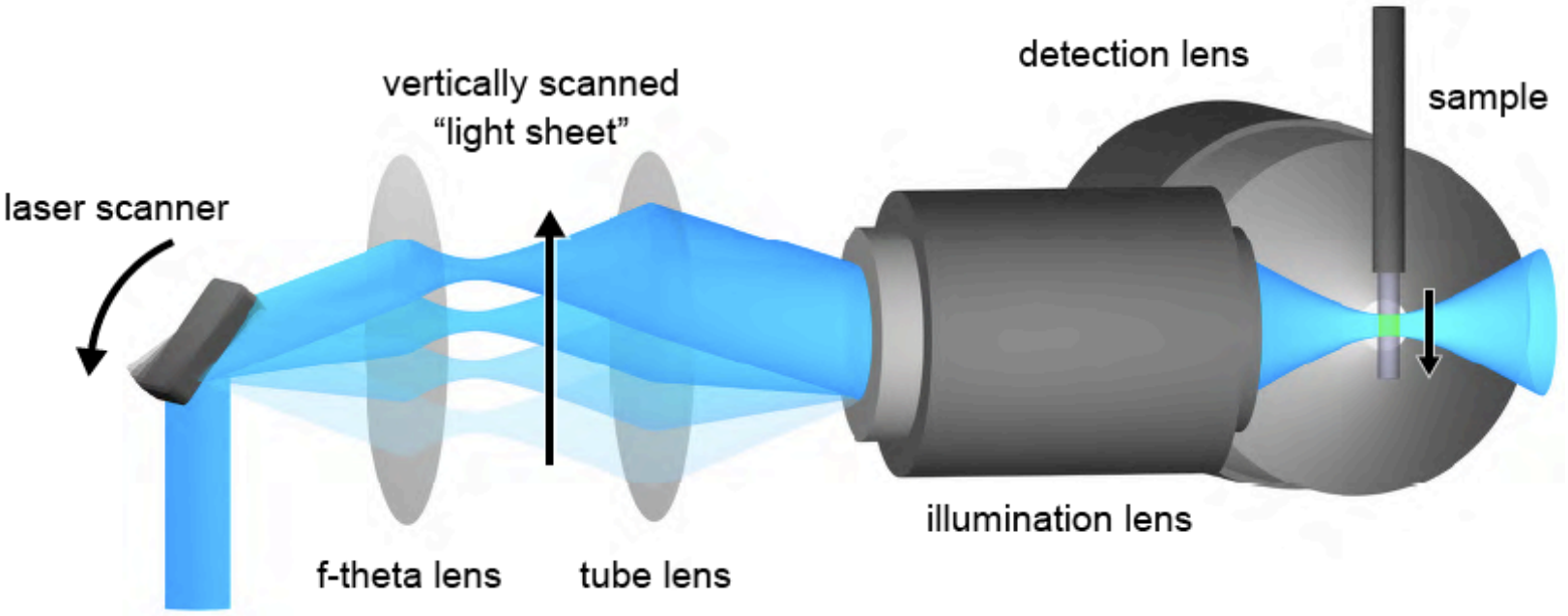
Digital Scanned Laser Light Sheet Fluorescence Microscopy ("Scanning SPIM")

Reconstruction of Zebrafish Early Embryonic Development by Scanned Light Sheet Microscopy

Philipp J. Keller,[1,2]* Annette D. Schmidt,[2] Joachim Wittbrodt,[1,2,3,4]* Ernst H. K. Stelzer[1]

http://www.embl-heidelberg.de/ExternalInfo/stelzer/pdf/Keller_2008b_Science_Reconstruction.pdf

Keller *et al.* (2008); figure 1



Got you's

"Issues" with light sheet microscopy

1) Sheet quite thick - a few microns

2) Shadowing affect - absorption of light from bright parts of the specimen outside the field of view

Bessel Beam Plane Illumination Microscope
Eric Betzig, Janelia Farm

http://www.nature.com/nmeth/journal/vaop/ncurrent/full/nmeth.1586.html

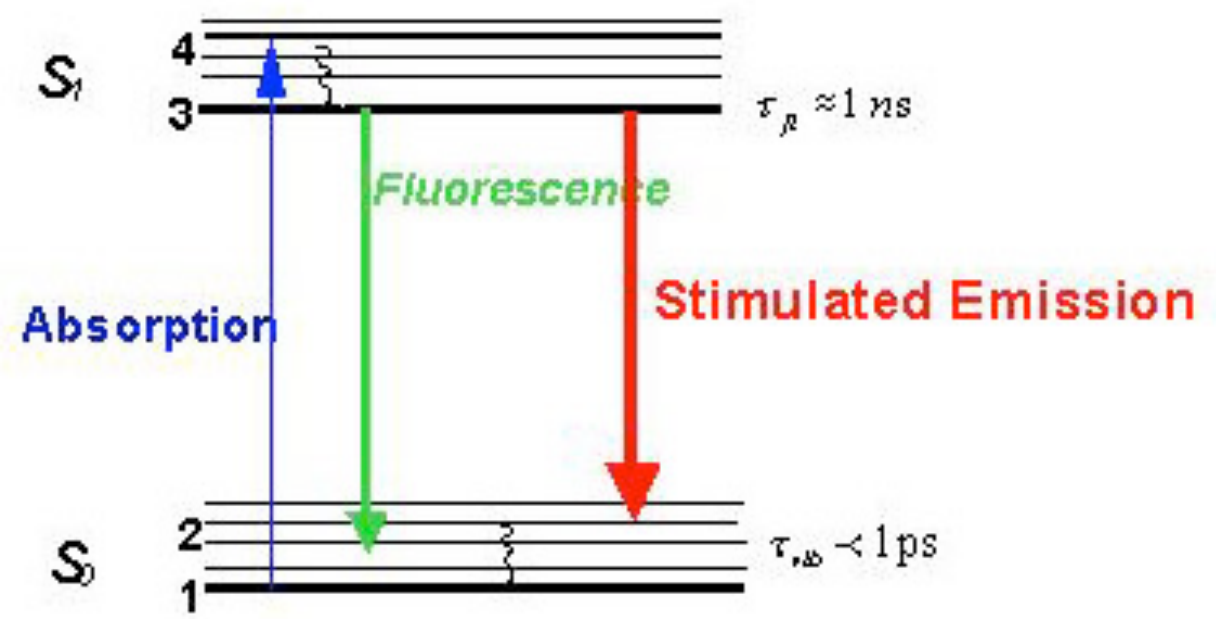
http://www.imaging-git.com/news/bessel-beam-plane-illumination-microscopy

Bessel Beam - thinner sheet
But problem with additional lobe - cured by 2photon

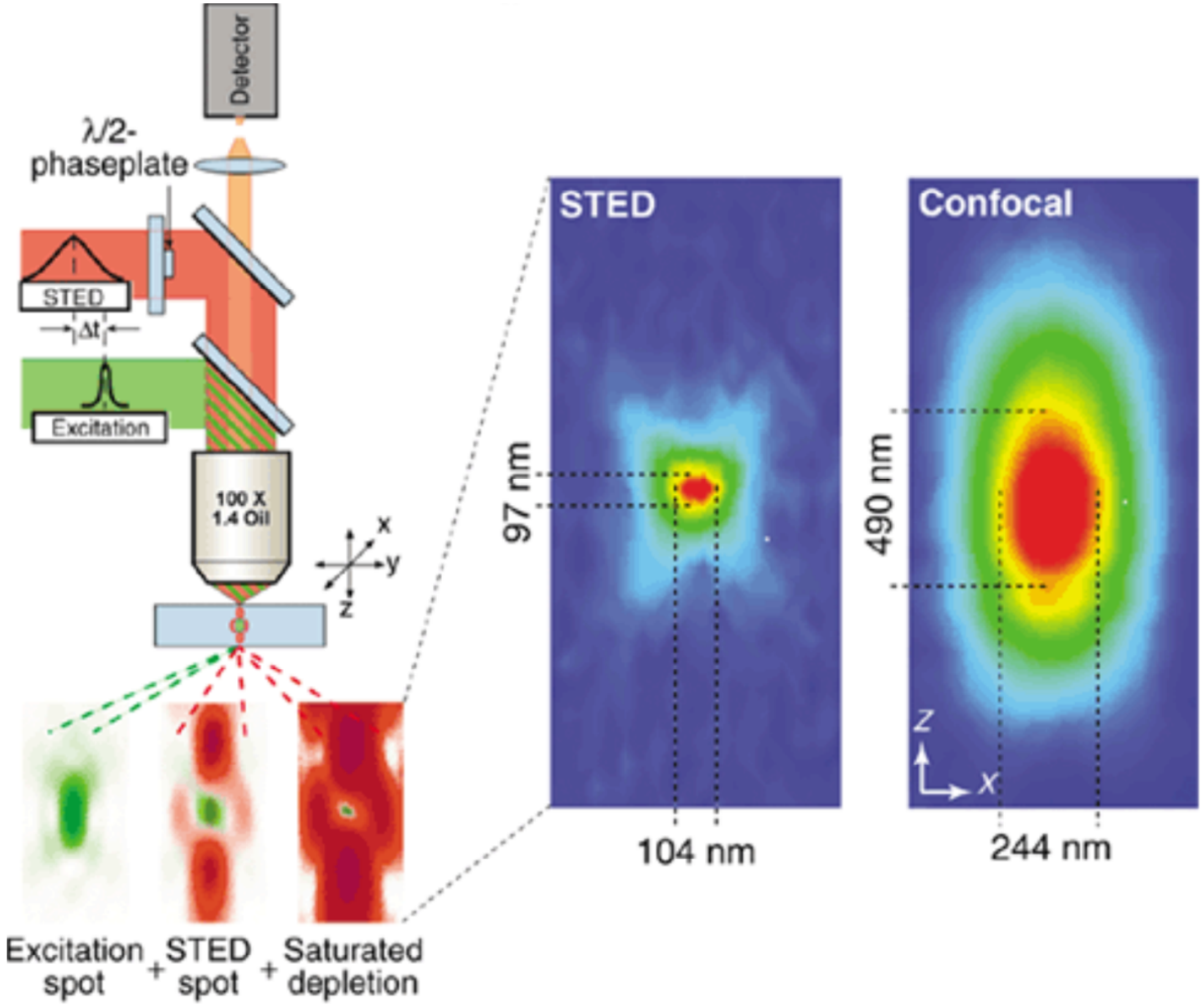
STED (Stimulated Emission Depletion)

Toward fluorescence nanoscopy
Stefan W Hell Nature Biotechnology 21, 1347 - 1355 (2003)
http://www.nature.com/nbt/journal/v21/n11/full/nbt897.html

STED microscopy reveals that synaptotagmin remain sclustered after synaptic vesicle exocytosis
Willig et al. (2006) (Hell) Nature 440, 935
http://www.nature.com/nature/journal/v440/n7086/pdf/440879a.pdf
http://www.nature.com/nature/journal/v440/n7086/pdf/nature04592.pdf



STED (Stimulated Emission Depletion)



Fluorescent Dyes Used in STED Microscopy

The following dyes have been successfully used in STED microscopy:

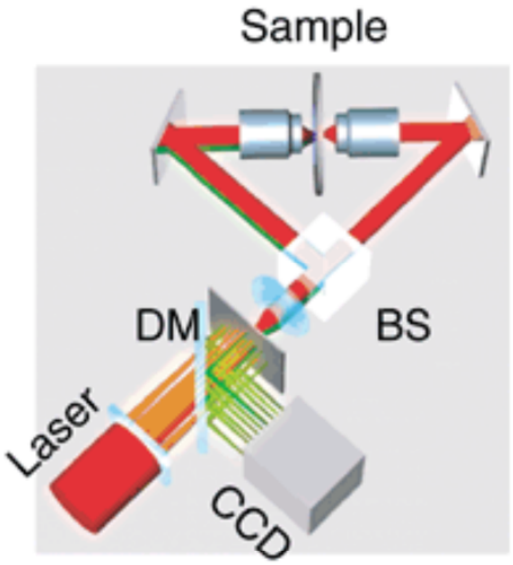
Dye name (Manufacturer / Distributor)	Exc. Wavelength	Exc. Pulse Length	STED Wavelength	STED Pulse Length	Repetition Rate	Avg. STED Power	Peak Irradiance	Pulse Energy	Reported Spatial Resolution (Direction)	Reference(s)
ATTO 532 (ATTO-TEC GmbH)	470 nm	100 ps	615 nm	200 ps	80 MHz	14 – 18 mW			66 – 72 nm (*xy*)	K. Willig et al., *Nature* **440**, 935 (2006) J. Sieber et al., *Biophys. J.* **90**, 2843 (2006) K. Willig et al., *New J. Phys.* **8**, 106 (2006)
ATTO 532 (ATTO-TEC GmbH)	470 nm	80 ps	603 nm	280 ps	250 kHz	0.5 mW		2 nJ	<25 nm (*xy*)	G. Donnert et al., *Proc. Natl. Acad. Sci. USA* **103**, 11440 (2006)
ATTO 532 (ATTO-TEC GmbH)	488 nm	100 ps	615 nm	200 ps	80 MHz	16 mW			60 – 70 nm (*xy*)	D. Fitzner et al., *EMBO J.* **25**, 5037 (2006) R. Kellner, *Neurosci.* **144**, 135 (2007)
Chromeo 488 (Actif Motif)	488 nm	140 ps	602 nm	~ 160 ps	250 kHz	0.6 mW			< 30 nm (*xy*)	L. Meyer et al., *Small*, in print (2008).
Chromeo 488 (Actif Motif)	488 nm	< 100 ps	590 nm	200 – 300 ps	80 MHz	40 mW			60 – 70 nm (*xy*)	*Unpublished Data*
DY-485XL (Dyomics GmbH)	488 nm	< 100 ps	647 nm	~ 200 ps	72 MHz	(20 + 3) mW			40 – 45 nm (*xyz*)	R. Schmidt et al., *Nat. Meth.* **5**, 539 (2008)
GFP	490 nm	100 ps	575 nm	200 ps	80 MHz	7.2 mW			~ 70 nm (*xy*)	K. Willig et al., *Nat. Meth.* **3**, 721 (2006)
ATTO 565 (ATTO-TEC GmbH)	532 nm	~ 90 ps	640 – 660 nm	~ 90 ps	1 – 2 MHz				30 – 40 nm (*xy*)	D. Wildanger et al., *Opt. Expr.* **16**, 9614 (2008)
ATTO 565 (ATTO-TEC GmbH)	532 nm	cw	647 nm	cw	cw	114 mW			~ 60 nm (*xy*)	K. Willig et al., *Nat. Meth.* **4**, 915 (2007)
MR 121 SE (Roche Diagnostics)	532 nm	10 ps	793 nm	107 ps	76 MHz	10.4 mW			~ 50 nm (*z*)	M. Dyba et al., *Nat. Biotech.* **21**, 1303 (2003)
NK51 (ATTO-TEC GmbH)	532 nm	< 100 ps	647 nm	~ 200 ps	72 MHz	(20 + 3) mW			40 – 45 nm (*xyz*)	R. Schmidt et al., *Nat. Meth.* **5**, 539 (2008)
Sulfonated & rigidized rhodamine derivatives (V. Boyarskiy, NanoBiophotonics, MPI Göttingen)	532 nm	100 ps	640 nm	~ 300 ps	80 MHz		40 MW/cm^2		< 90 nm (*xy*)	V. Boyarskiy et al., Chem. Eur. J. 14, 1784 (2008)
Pyridine 2 / LDS 722 (Exciton, Radiant Dyes GmbH)	554 nm	250 fs	760 nm	13 ps	76 MHz				33 nm (*z*)	M. Dyba, S.W. Hell, *Phys. Rev. Lett.* **88**, 163901 (2002)
Pyridine 2 / LDS 722 (Exciton, Radiant Dyes GmbH)	554 nm	250 fs	745 nm	50 – 200 ps		12.2 mW			44 nm (*z*)	M. Dyba et al., *New. J. Phys.* **7**, 134 (2005)
RH 414 (Invitrogen Corp.)	554 nm	250 fs	745 nm	13 ps	76 MHz	8.78 mW			30 nm (*z*)	M. Dyba, S.W. Hell, *Phys. Rev. Lett.* **88**, 163901 (2002)
ATTO 590 (ATTO-TEC GmbH)	570 nm	~ 90 ps	690 – 710 nm	~ 90 ps	1 – 2 MHz				30 – 40 nm (*xy*)	D. Wildanger et al., *Opt. Expr.* **16**, 9614 (2008)
ATTO 633 (ATTO-TEC GmbH)	630 nm	~ 90 ps	735 – 755 nm	~ 90 ps	1 – 2 MHz				30 – 40 nm (*xy*)	D. Wildanger et al., *Opt. Expr.* **16**, 9614 (2008)
ATTO 633 (ATTO-TEC GmbH)	635 nm	100 ps	750 nm	~ 200 ps	76 MHz			~ 1.5 nJ	40 nm (*xy*)	A. Punge et al., *Micr. Res. Techn.*, DOI 10.1002/jemt.20602
ATTO 647N (ATTO-TEC GmbH)	635 nm	cw	750 nm	cw	cw	423 mW			~ 50 nm (*xy*)	K. Willig et al., *Nat. Meth.* **4**, 915 (2007)
ATTO 647N (ATTO-TEC GmbH)	635 nm	100 ps	780 nm	300 ps	250 kHz		700 MW/cm^2		~ 65 nm (*xy*)	G. Donnert et al., *Biophys. J.: Biophys. Lett.*, L67 (2007) V. Westphal et al., *Science* **320**, 247 (2008)
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	781 nm	303 ps	40 MHz	10.1 mW			40 nm (*x*)	V. Westphal et al., *Appl. Phys. B.* **77**, 377 (2003)
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	775 nm	300 ps	76 MHz		800 MW/cm^2		16 nm (*x*)	V. Westphal, S.W. Hell, *Phys. Rev. Lett.* **94**, 143903 (2005)
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	780 nm	300 ps	80 MHz	90 – 100 mW			47 nm (*xy*)	V. Westphal et al., *J. Phys. B: At. Mol. Opt. Phys.* **38**, S695 (2005)
JA 26 (K.H. Drexhage, Siegen University)	637 nm	54 ps	778 – 785 nm	303 ps	40 MHz				120 – 140 nm (*xy*)	V. Westphal et al., *Appl. Phys. Lett.* **82**, 3125 (2003)

Compilation: Lars Kastrup <lkastru@gwdg.de>

Last change: 25-Jun-2008 03:24 PM

4Pi -two objective lenses (S.Hell)

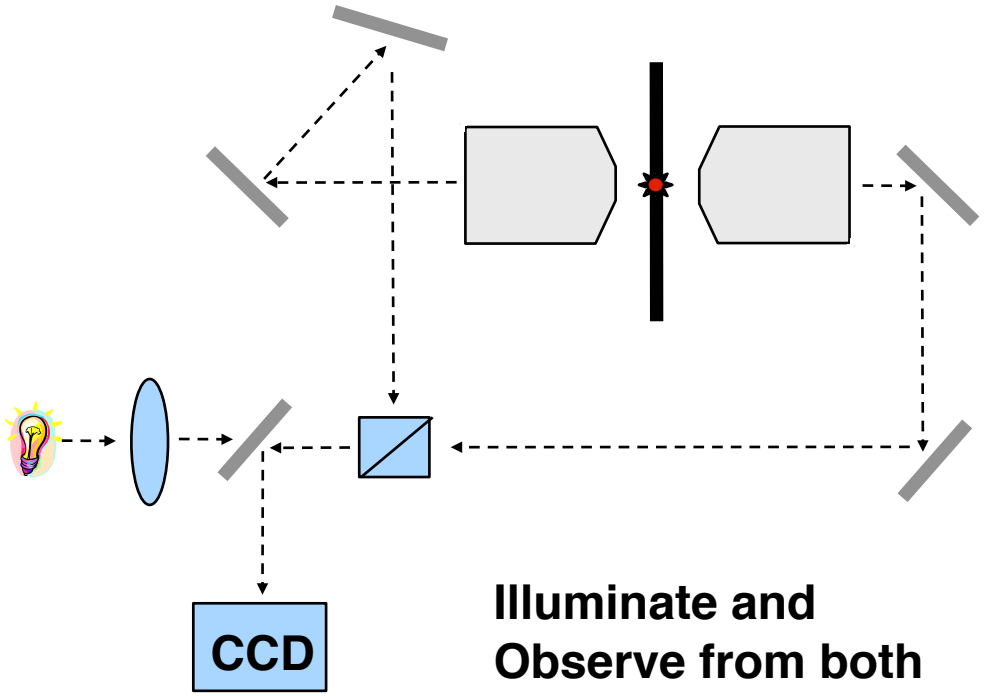
Toward fluorescence nanoscopy
Stefan W Hell Nature Biotechnology 21, 1347 - 1355 (2003)
http://www.nature.com/nbt/journal/v21/n11/full/nbt897.html



Resolution increase in Z

I5M (wide field method)

Gustafsson MG, Agard DA, Sedat JW. I5M: 3D widefield light microscopy with better than 100 nm axial resolution. J. Microscopy 195:10-16, 1999.
http://www.blackwell-synergy.com/links/doi/10.1046/j.1365-2818.1999.00576.x



Illuminate and Observe from both objectives

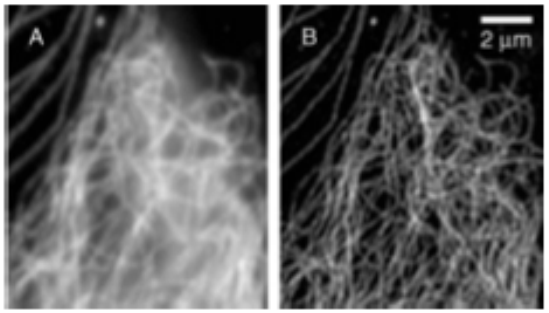


Fig. 4. XY views of fluorescently labelled microtubules in PtK2 tissue culture cells: (A) Conventional microscopy (single section), (B) I^5M using a linear inverse filter followed by iterative deconvolution (reprojected).

Super resolution and precision methods

PALM = Photoactivated localization microscopy / iPALM / PALMERA

STORM = Stochastic optical reconstruction microscopy

FIONA = Fluorescent imaging with one nanometer resolution / DOPI

SHREC = single-molecule high-resolution colocalization

Other abbreviations:

FCS = Fluorescence correlation spectroscopy

FLIM = Fluorescence lifetime imaging

FLIC = Fluorescence interference contrast

FLIP = Fluorescence loss in photobleaching

FRAP = Fluorescence recovery after photobleaching

FRET = Fluorescence resonance energy transfer

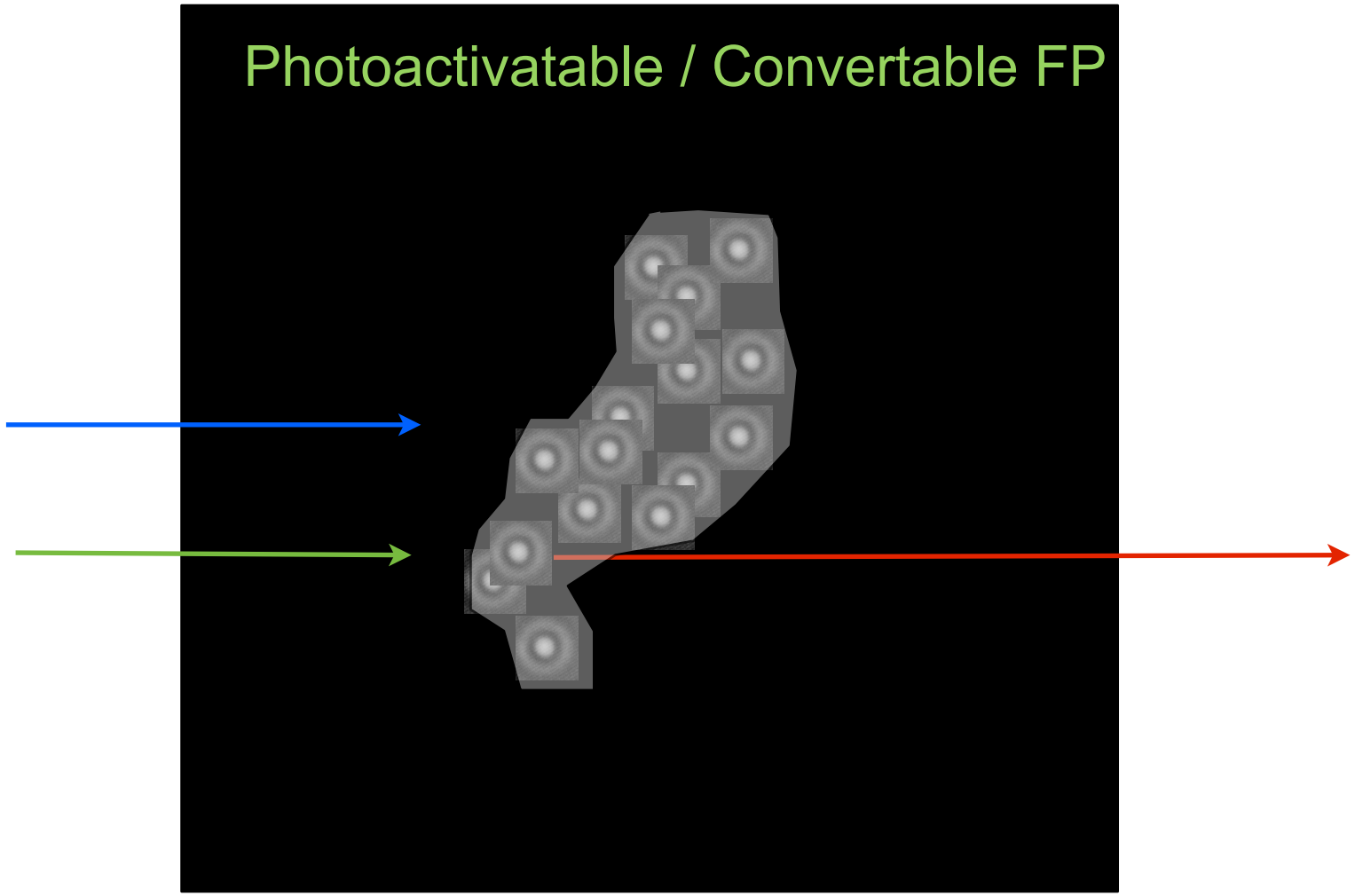
SSTM = Saturated structured illumination microscopy

STED = Stimulated emission depletion

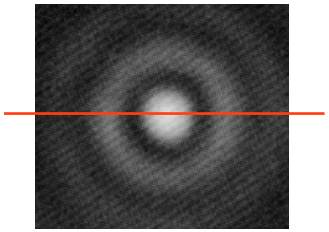
TIRF = Total internal reflection

3D-SIM = 3 dimentional structured illumination microscopy

Photoactivated localization microscopy (PALM)



Centroid analysis



160
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221
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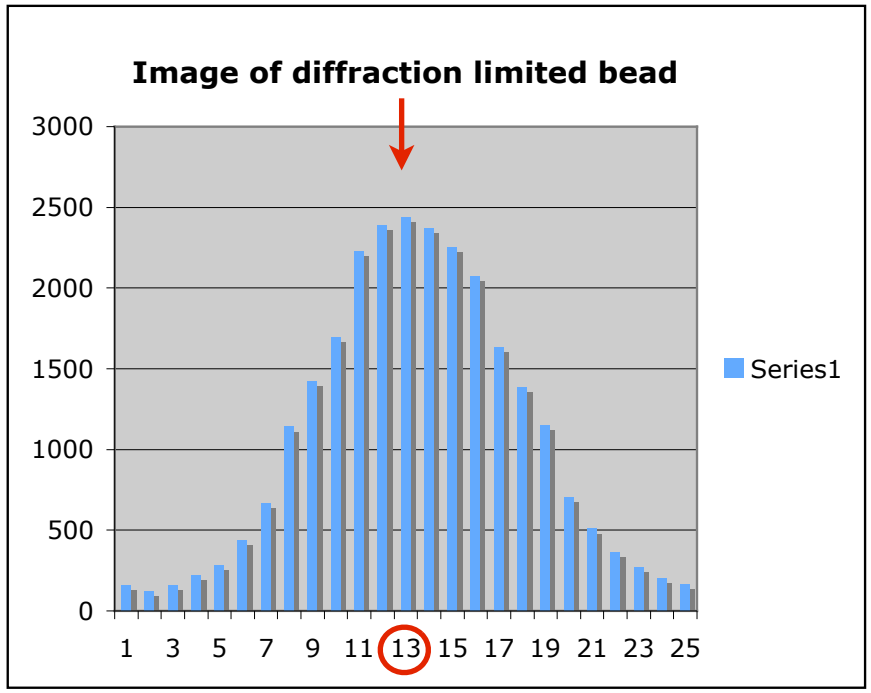
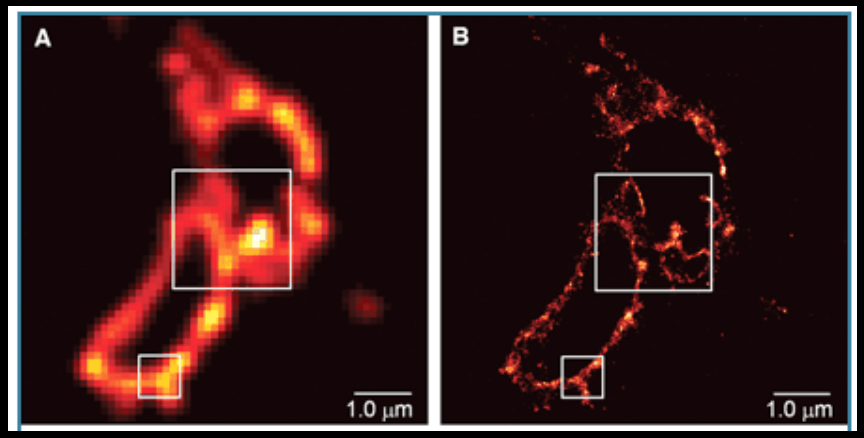
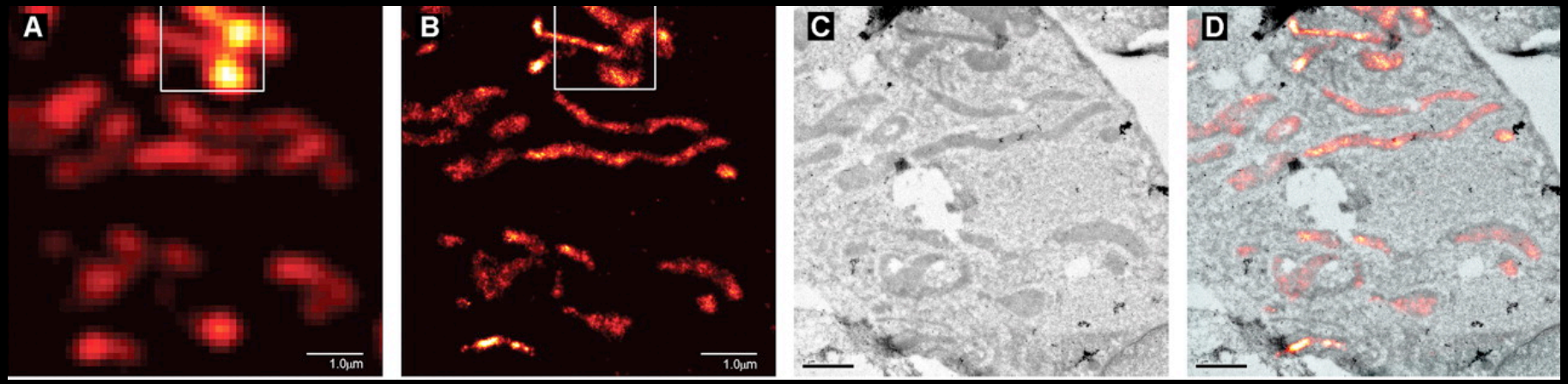


Centroid Analysis
(Intensity weighted centre of mass)

= 13.4999

PALM Developed by Betzig and Hess (Janelia)





Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure

Gleb Shtengel[a], James A. Galbraith[b], Catherine G. Galbraith[c], Jennifer Lippincott-Schwartz[d,1], Jennifer M. Gillette[d], Suliana Manley[d], Rachid Sougrat[d], Clare M. Waterman[e], Pakorn Kanchanawong[e], Michael W. Davidson[f], Richard D. Fetter[a], and Harald F. Hess[a,1]

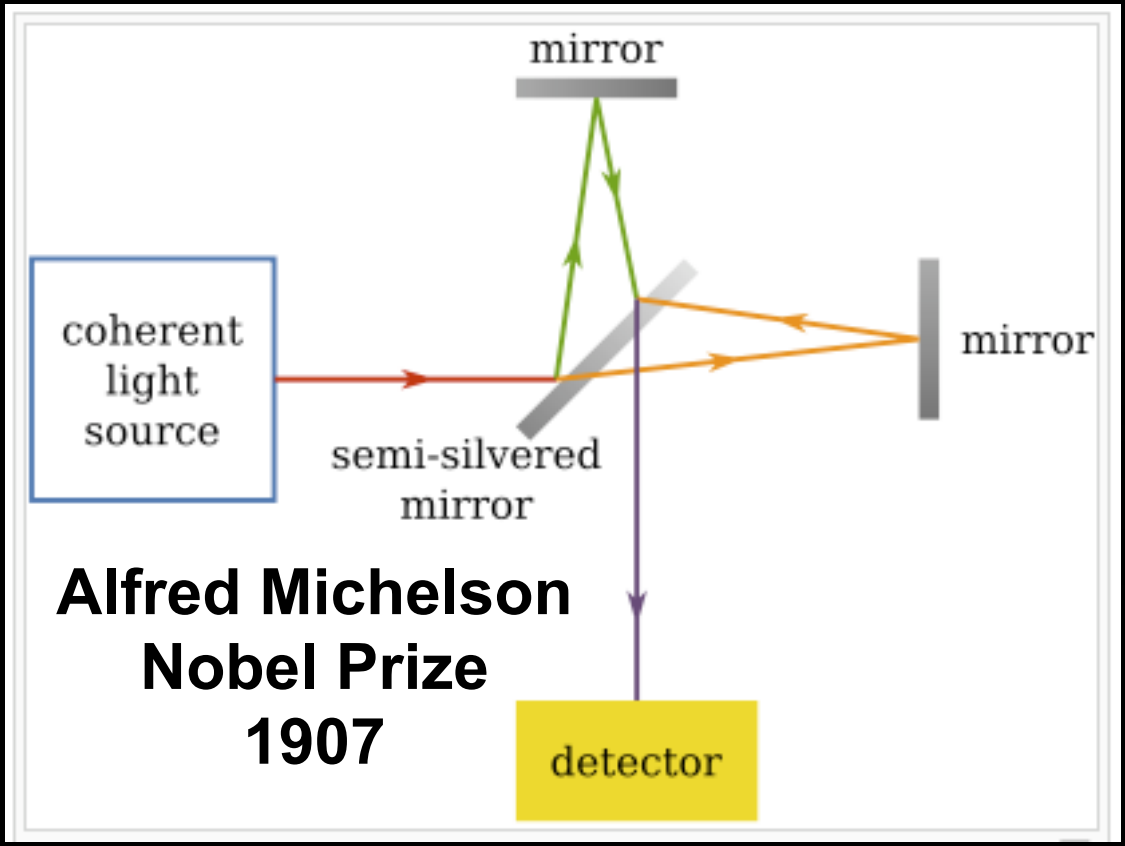
[a]Howard Hughes Medical Institute, Janelia Farm Research Campus, Ashburn, VA 20147; [b]National Institute of Neurological Disorders and Stroke; [c]National Institute of Dental and Craniofacial Research; [d]Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development; and [e]Laboratory of Cell and Tissue Morphodynamics, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and [f]National High Magnetic Field Laboratory and Department of Biological Sciences, Florida State University, Tallahassee, FL 32310

Contributed by Jennifer Lippincott-Schwartz, December 25, 2008 (sent for review November 19, 2008)

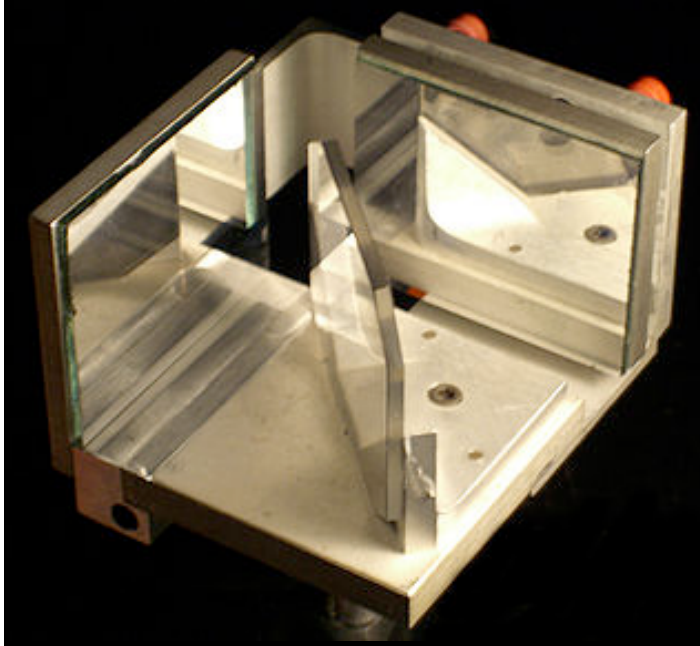
www.pnas.org/cgi/doi/10.1073/pnas.0813131106

To understand iPALM
First you need to understand a few more principles of quantum physics and interferometry

Michelson Interferometer



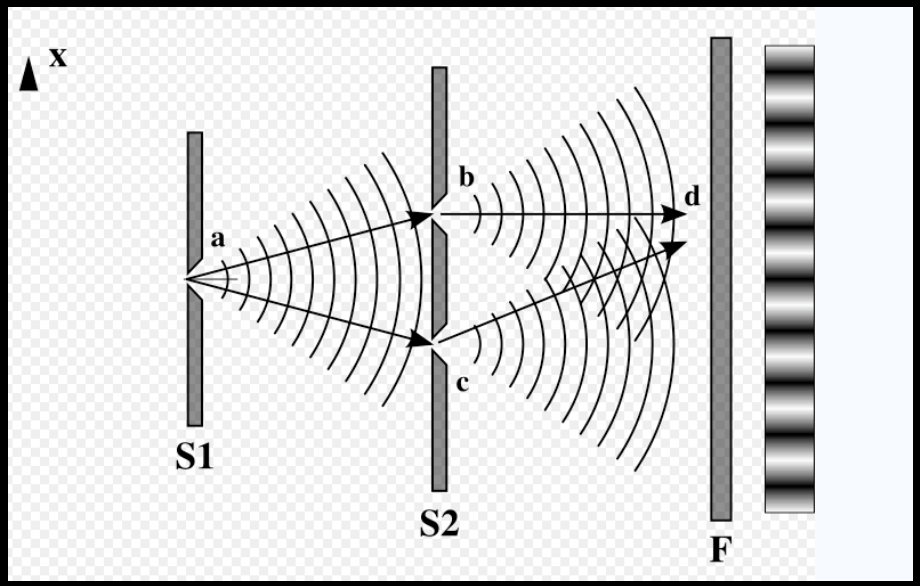
Alfred Michelson
Nobel Prize
1907

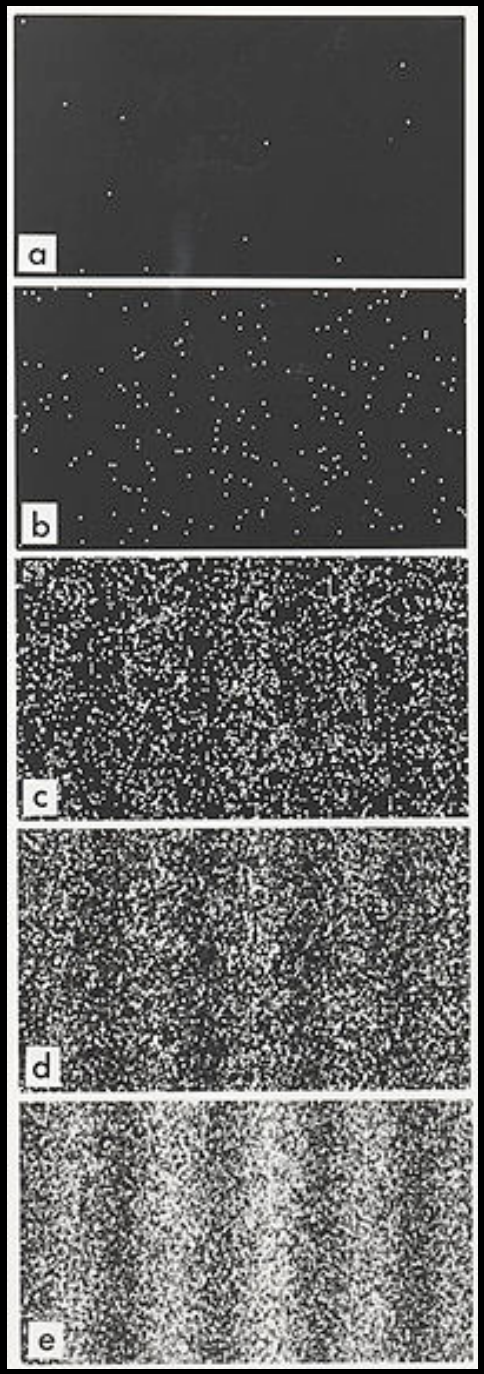


Michelson-Morley experiment to measure speed of light through the “aether”
No difference in speed caused by the movement of the earth through the “aether”

Wave particle duality of light

Young's diffraction slits





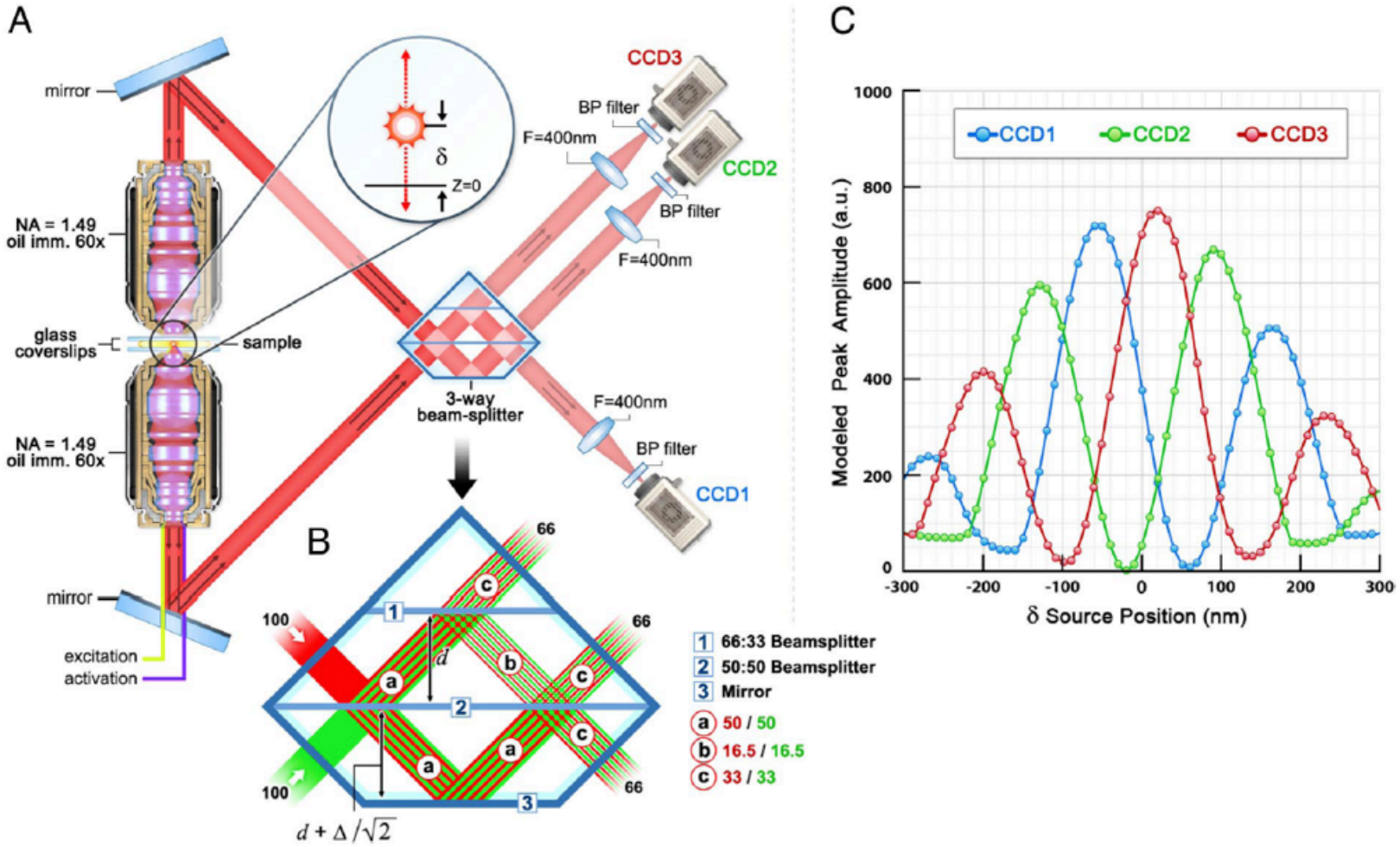


Fig. 1. Schematics and operating principle of multiphase interferometric microscope illustrating how Z-position is resolved. (*A* and *B*) Schematic of the single-photon multiphase fluorescence interferometer. A point source with z-position δ emits a single photon both upwards and downwards. These 2 beams (color coded as red and green in *B*) interfere in a special 3-way beam splitter. (*C*) The self-interfered photon propagates to the 3 color-coded CCD cameras with amplitudes that oscillate 120° out of phase as indicated.

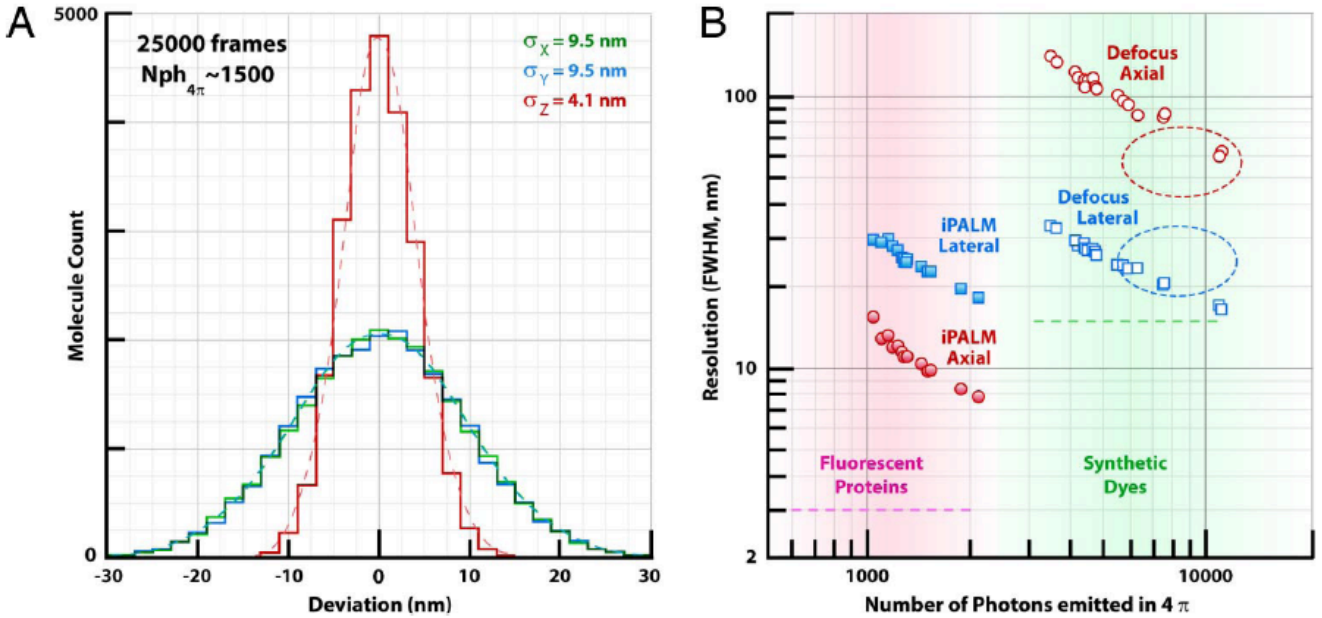


Fig. 2. X, Y, Z resolution of iPALM and its dependence on source brightness, illustrating iPALM's sub-20-nm 3D resolution with endogenous FP labels. (*A*) A histogram of experimentally determined positions from repeatedly sampling (25,000 frames) a source where ≈1,200 photons are detected per frame from ≈1,500 photons emitted into a 4π solid angle. (*B*) Axial (solid red circles) and lateral (solid blue squares) resolution of iPALM determined from FWHM of localization of Au beads of different brightness. Note that the positional FWHM number is 2.4 times larger than σ the variance that is also used to characterize resolution. Axial (empty red circles) and lateral (empty blue squares) resolution of the defocusing method determined from FWHM of localized position of Au beads of different brightness. Large ovals indicate approximately the published results for axial (red) and lateral (blue) resolutions of 3D STORM (2) and BP PALM (3). The typical photon output of fluorescent protein tags and synthetic fluorophores are depicted as pink and green gradients. Also shown (horizontal dashed lines) are addition uncertainties resulting from the displacement between the target protein and the fluorescent probes for different imaging methods.

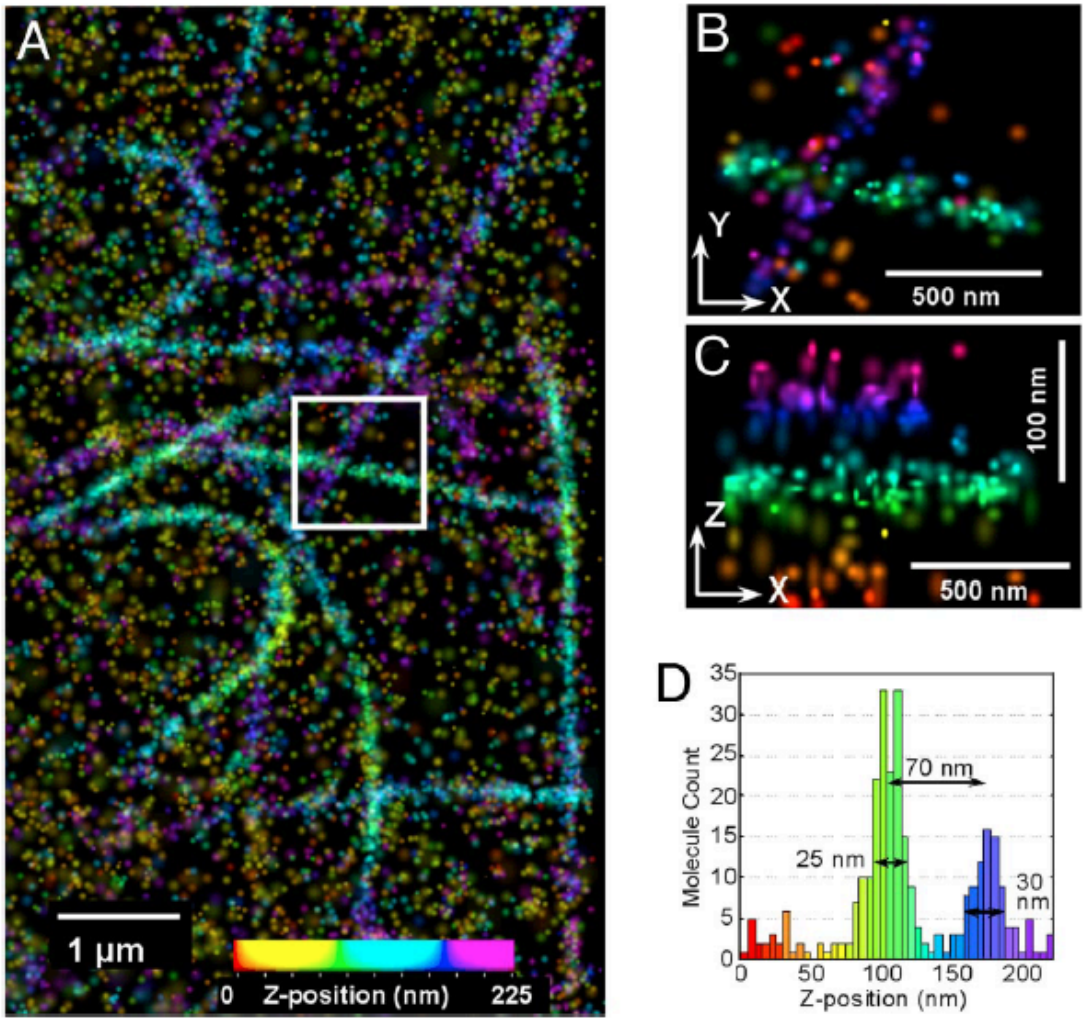


Fig. 3. Superresolution iPALM image of microtubules in a PtK1 cell expressing human α-tubulin fused to *m*-KikGR, rendered with *z* axis color-coding. (*A*) Large area overview. (*B* and *C*) Zoom-in of the area bound by the white box in *X*–*Y* (*B*) and *Z*–*Y* (*C*) projections (*z*-scale is magnified 5×). (*D*) Histogram of *z*-positions of molecules in the boxed region. Each microtubule has a FWHM of 30 nm, and the separation distance of 70 nm between the cyan and purple microtubules is easily resolved.

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Negative Refraction Makes a Perfect Lens

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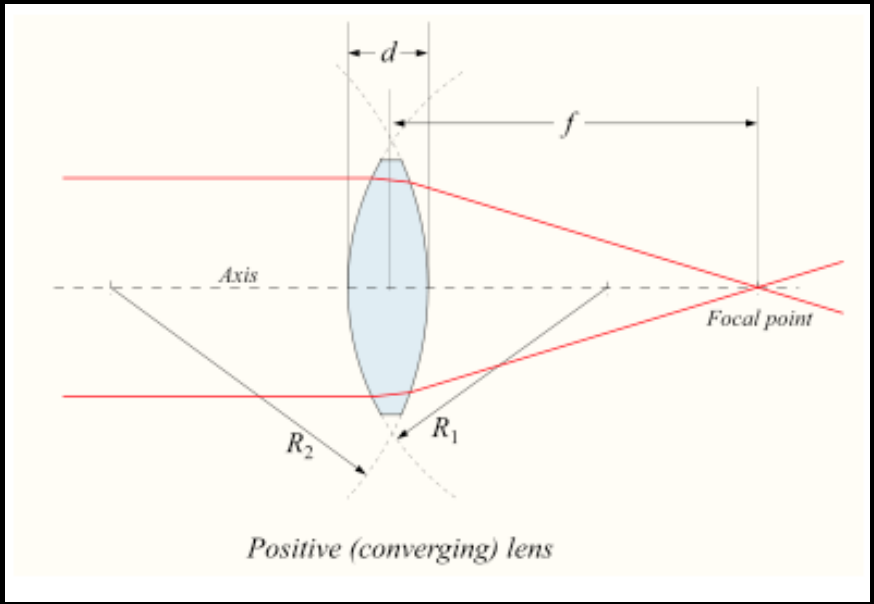
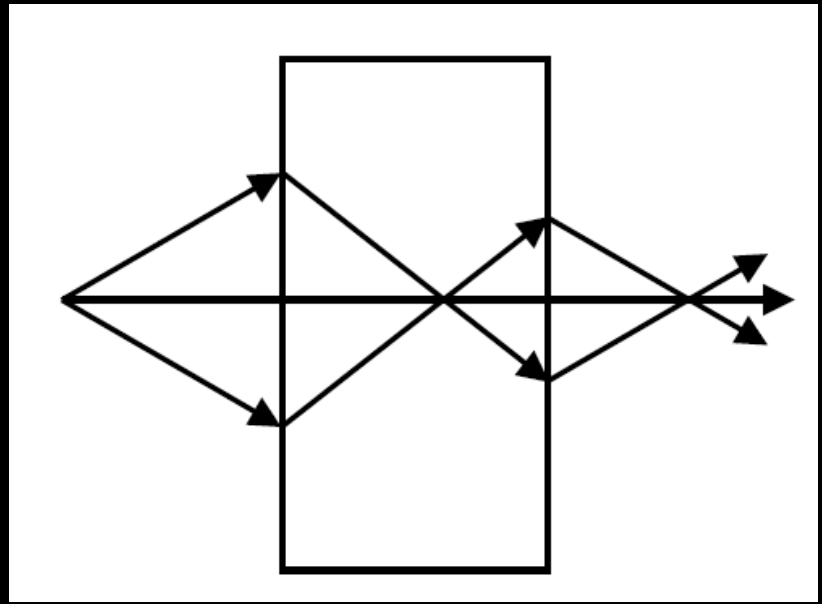
(Received 25 April 2000)

With a conventional lens sharpness of the image is always limited by the wavelength of light. An unconventional alternative to a lens, a slab of negative refractive index material, has the power to focus all Fourier components of a 2D image, even those that do not propagate in a radiative manner. Such "superlenses" can be realized in the microwave band with current technology. Our simulations show that a version of the lens operating at the frequency of visible light can be realized in the form of a thin slab of silver. This optical version resolves objects only a few nanometers across.





60nm sheet of silver

New methods coming in fast and furious !

Chu Nature 2010

0.5 nm precision - closed feedback loops

http://www.nature.com/nature/journal/v466/n7306/full/nature09163.html

Singer Nature 2010

Super-registration - bleed through dichroic

http://www.nature.com/nature/journal/v467/n7315/full/nature09438.html

Which super resolution / precision method to use ?

3D SIM -General method true super resolution - only modest (x2) resolution gain

Live SIM -About to be available from API as OMX Blaze.

Saturated SIM -Not yet available for biology, not clear if will work for biology

SPIM - build your own - Zeiss version - delayed launch?

Betzig Bessel Beam - Too new to know ?

STED - from Leica. Need a physicist and not as good as Hell's instrument

4Pi - from Leica. Absolutely need a physicist. Little reported biology so far.

I5M - same specimen mounting difficulties as 4Pi

FIONA - Relatively straight forward - centroid analysis

PALM/STORM - Simple hardware. Software more complex. Various flavours. Will be widely used. But remember - it is precision of single molecule position calculation - NOT true resolution ! So always has issue of density of labelling. Too dense - slow to capture. Too sparse - true structures not resolved in cell. Efficiency of detection of single molecules - not clear. Difficult to apply to live material

The End