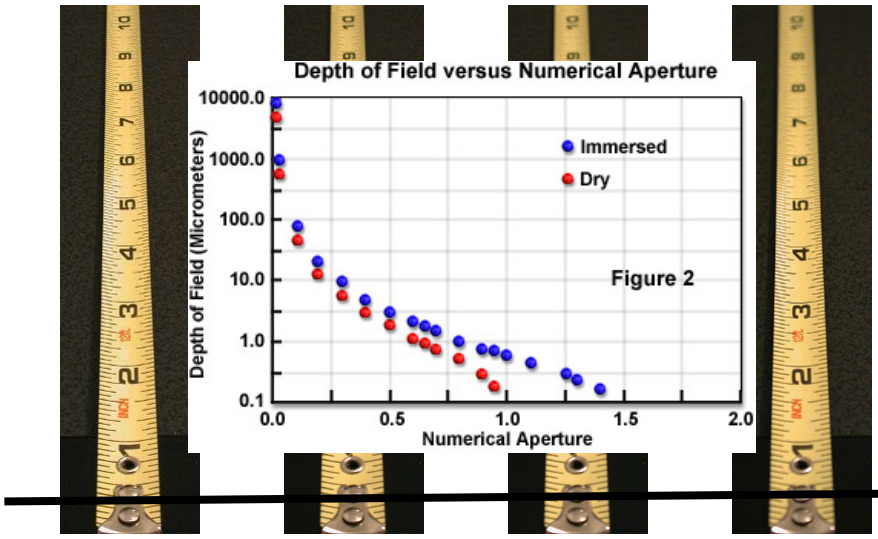
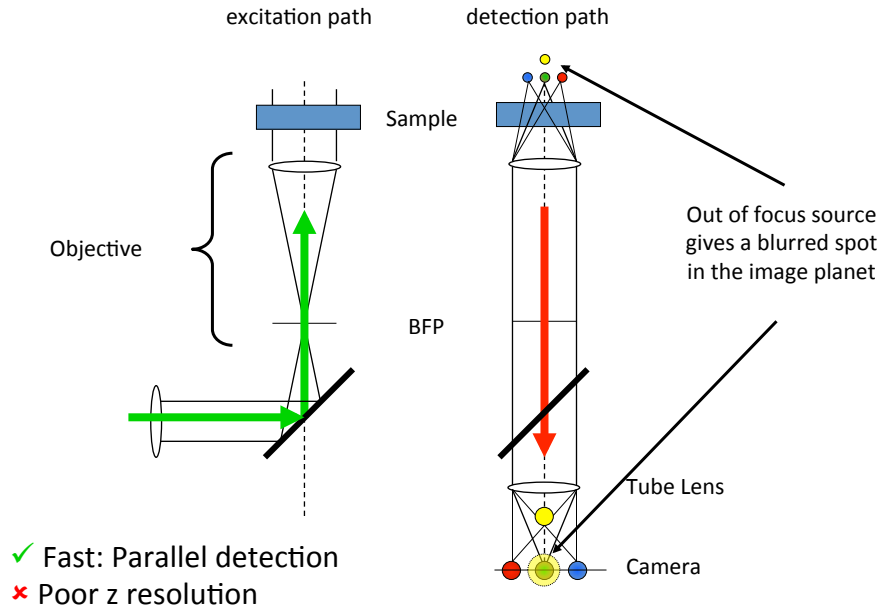
Confocal Microscopy

Depth of Focus



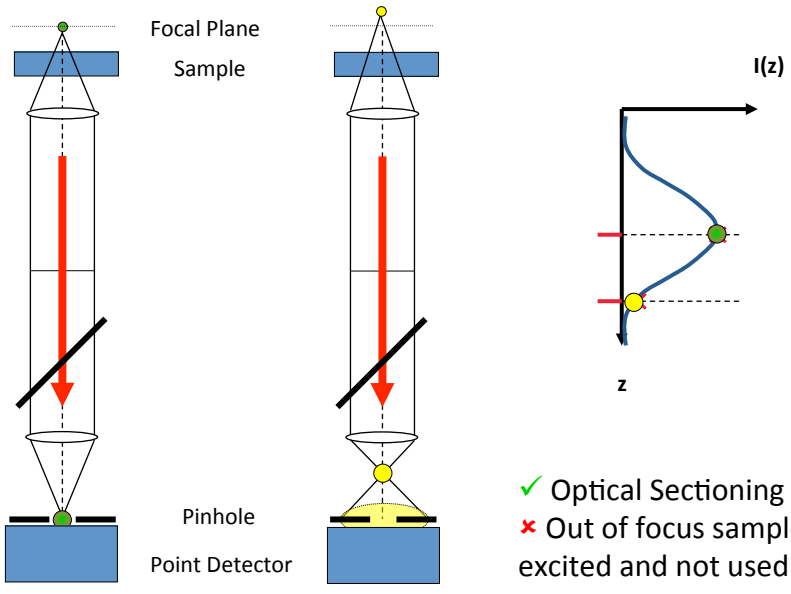
http://www.cs.mtu.edu/~shene/DigiCam/User-Guide/950/depth-of-field.html

Wide Field Microscopy



✓ Fast: Parallel detection
× Poor z resolution

Confocal Principle

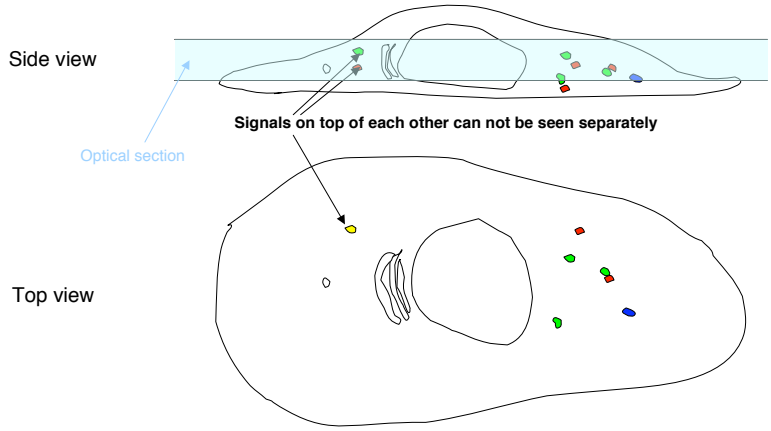


✓ Optical Sectioning
× Out of focus sample is excited and not used

Confocal vs. Widefield

Widefield: *optical section*

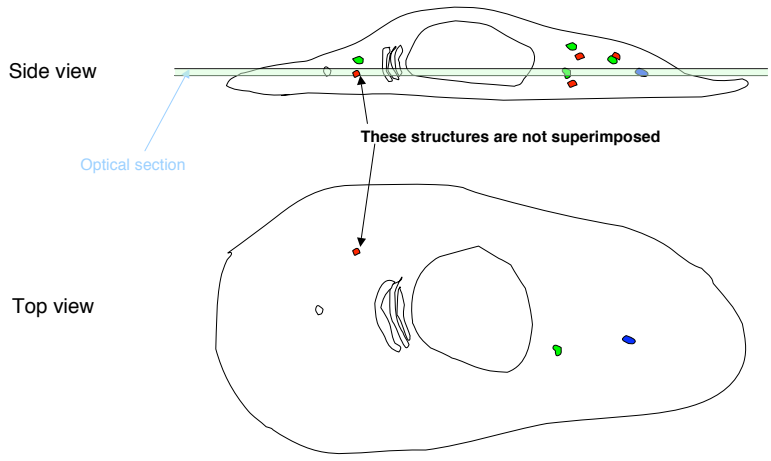
Many signals can not be seen separately!



Confocal vs. Widefield

Confocal *: optical section*

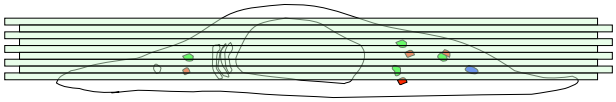
Improved z-resolution allow for more accurate signal discrimination!



3D imaging

Confocal: *"extended focus""*

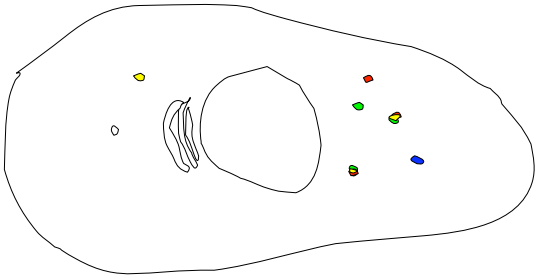
Side view



single optical sections get projected on one plane - the result is an clean image: everything is focused over the hole depth without any out-of-focus-noise.

Projection
(Top view):

Information content of all the sections is projected to one plane.



Confocal examples

Confocal and Widefield Fluorescence Microscopy

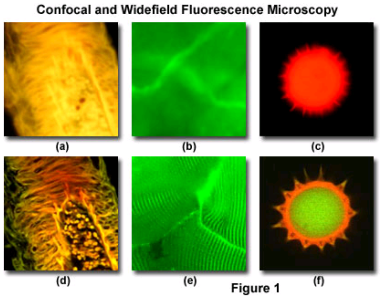
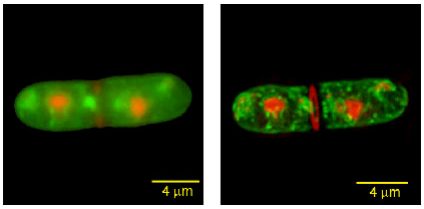


Figure 1



Fission yeast cell: DNA and the medial septum are stained with Hoechst 33342, membranes are visualized using the fluorescent lipophilic dye DiOC6.

Pollen Grain Serial Optical Sections by Confocal Microscopy

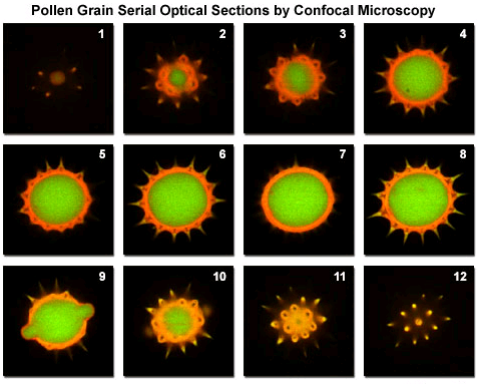
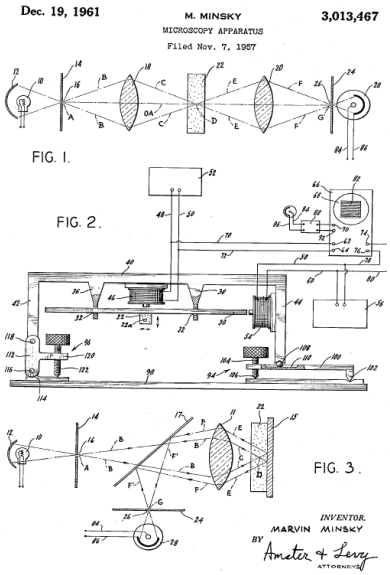


Figure 6

Patent



[…] This high degree of selectivity afforded by the optical system results in a minimum of blurring, increase in signal-to-noise ratio, increase in effective resolution, and the possibility of high resolution light microscopy through unusually thick and highly-scattered specimens.

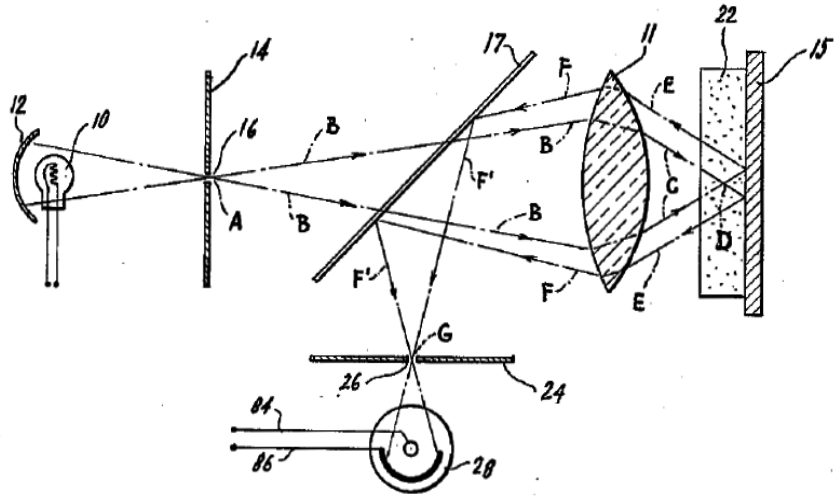
M. Minsky, 1957

US Patent 3,013,467

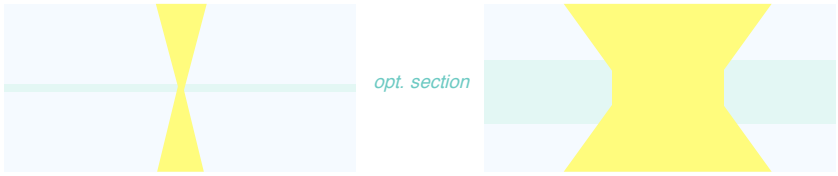
Minsky's claims

- Minimized blurring
- Increase in signal-to-noise ratio
- Increase in effective resolution
- The possibility of high resolution light microscopy through unusually thick and highly-scattered specimens.

Minsky's diagram



Pinhole diameter effects



small pinhole diameter:

-> thin optical section

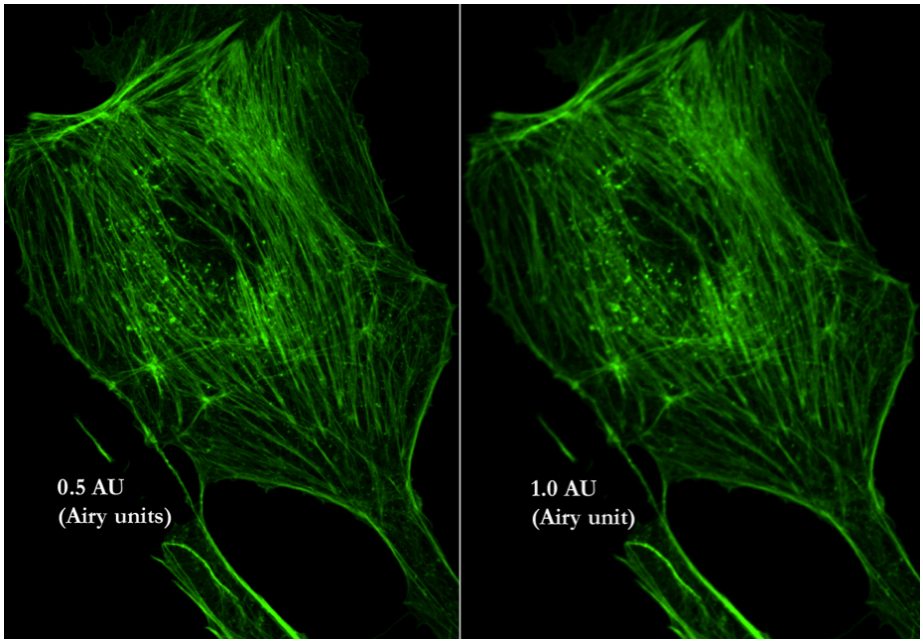
= high z-resolution possible
= low signal strength

big pinhole diameter:

-> thick optical section

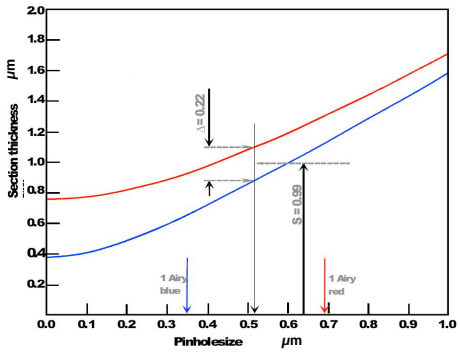
= low z-resolution
= brighter signal

Pinhole diameter effects



Pinhole diameter effects

- The pinhole is optimized **for each objectiv.**
- „**Airy 1**“ is a good start, but NOT an iron rule; play with pinhole to get either more light or more resolution.
- Resolution depend also on **wavelength**; keep in mind if resolution REALLY matters. Leica pinhole values are optimized for medium wavelength.



The pinhole variable determines your z-resolution.

PSF (very short)

The point spread function (PSF) describes 3-D light distribution in an image of a point source (for a given lens). An x-y slice through the center of the wide-field point spread function reveals a set of concentric rings: the so-called Airy disk that is commonly referenced in texts on classical optical microscopy.

Optical units:

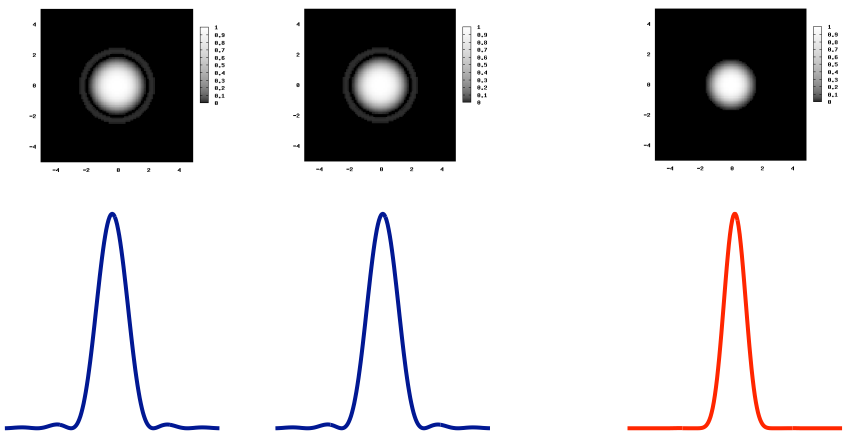
$$v = d \cdot \frac{2\pi}{\lambda} \sin\theta \qquad u = z \cdot \frac{2\pi}{\lambda} \sin^2\theta$$

$$I(0,v) \propto \left| \frac{2J_1(v)}{v} \right|^2 \qquad I(u,0) \propto \left(\frac{\sin(u/4)}{u/4} \right)^2$$

Lateral and axial intensity distributions

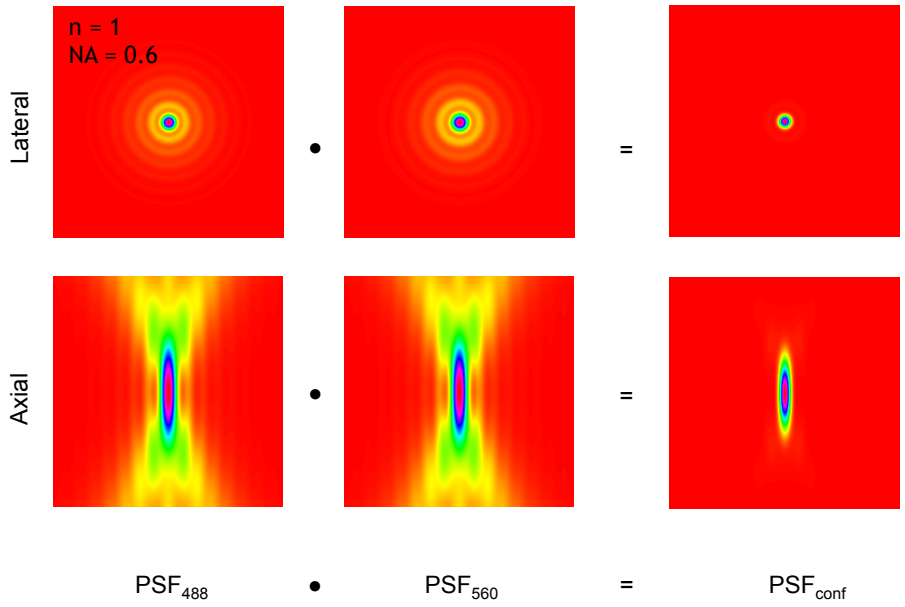
Confocal PSF

Is the convolution of the Excitation PSF and the Emission PSF

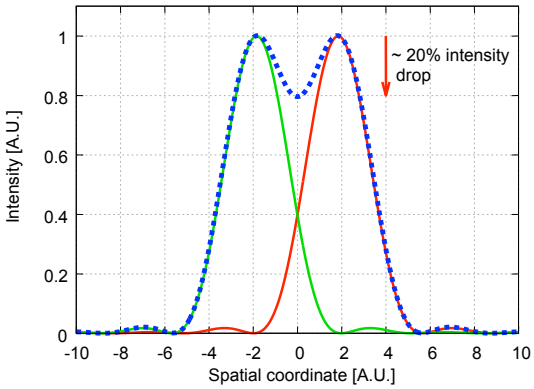


PSF_{exc} • PSF_{det} = PSF_{conf}

Confocal PSF

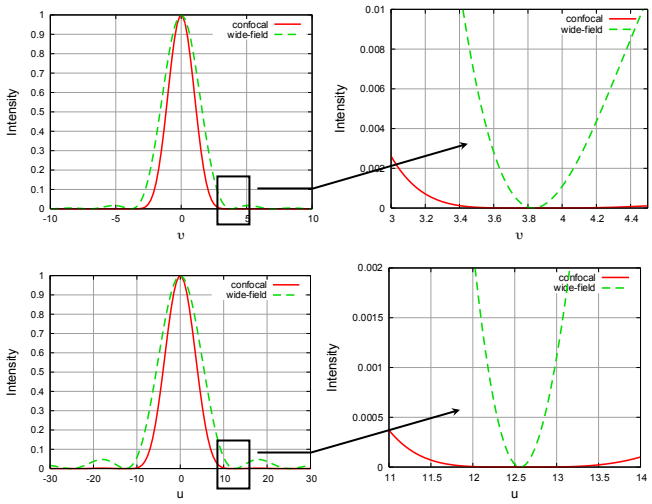


Remember de Resolution Criterion



The **Rayleigh criterion** is the generally accepted, although arbitrary, criterion for the minimum resolvable detail – the imaging process is said to be diffraction-limited when the first diffraction minimum of the image of one source point coincides with the maximum of another.

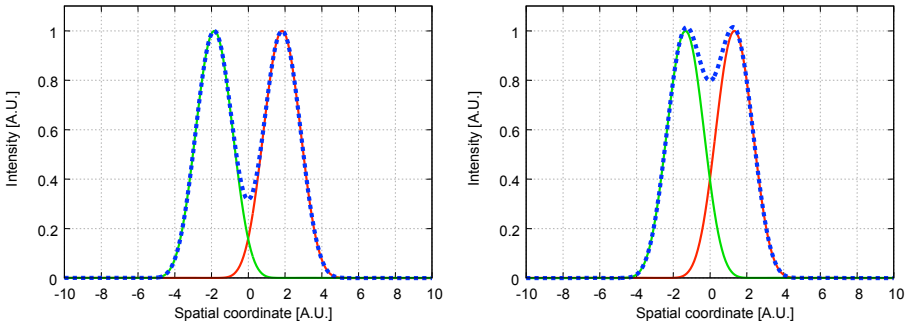
Wide field vs. confocal



$$r_0 = \frac{0.61 \cdot \lambda}{n \sin\theta} = \frac{0.61 \cdot \lambda}{\mathrm{NA}}$$

$$z_0 = \frac{2 \cdot \lambda n}{(\mathrm{NA})^2}$$

Wide field vs. confocal

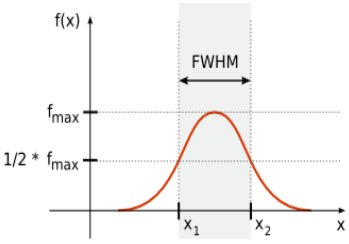


Rayleigh criterion cannot be used directly to define the improvement of resolution in a confocal microscope. The position of the first minimum does not change. The drop in intensity is much larger, though. In fact, we can move two intensity distributions a little bit closer (1.4 times), and still get the required 20% drop in intensity.

Resolution FWHM

$$\mathrm{FWHM}^{r}_{conf} \approx \frac{0.61 \cdot \lambda}{\sqrt{2} \cdot \mathrm{NA}}$$

$$\mathrm{FWHM}^{r}_{conf} \approx \frac{1}{\sqrt{1 + \left(\lambda_{exc} / \lambda_{\mathrm{det}}\right)^{2}}} \frac{0.61 \cdot \lambda_{exc}}{\mathrm{NA}}$$

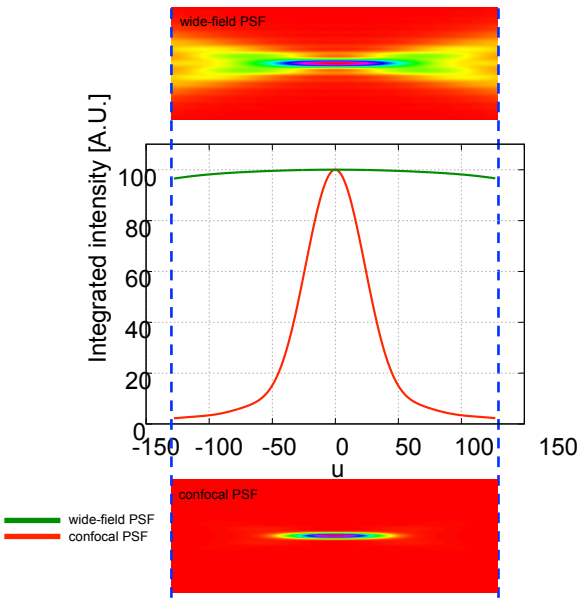


$$\mathrm{FWHM}^{z}_{conf} = \frac{2 \cdot \lambda n}{\sqrt{2} \cdot (\mathrm{NA})^{2}}$$

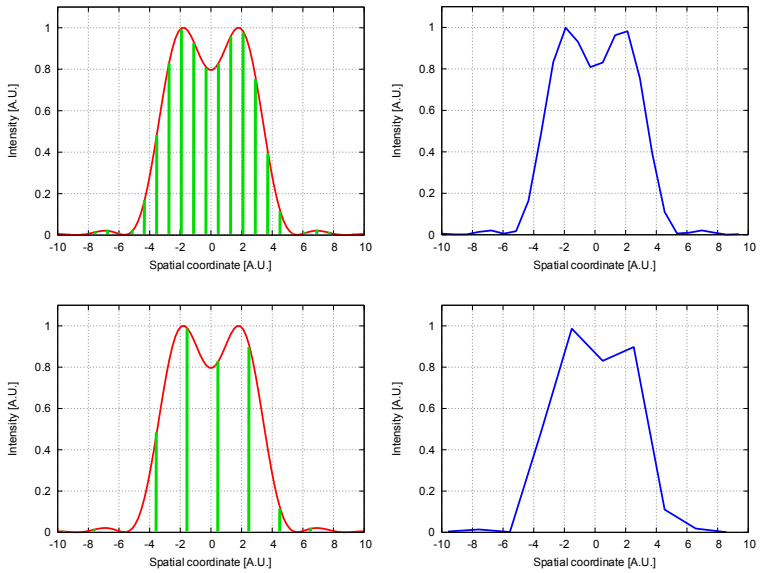
$$\mathrm{FWHM}^{z}_{conf} = \frac{1}{\sqrt{1 - \left(\lambda_{exc} / \lambda_{det}\right)^{2}}} \frac{2 \cdot \lambda_{exc} n}{(\mathrm{NA})^{2}}$$

The full width at half maximum (FWHM) is a parameter commonly used to describe the width of a "bump" on a curve or function. It is given by the distance between points on the curve at which the function reaches half its maximum value.

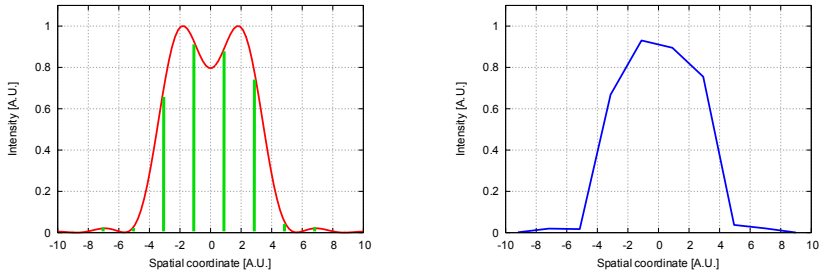
3D sectioning capabilities



Sampling



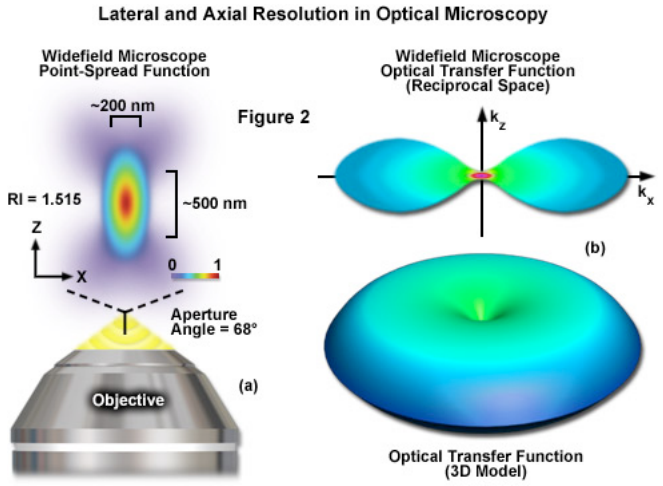
Sampling



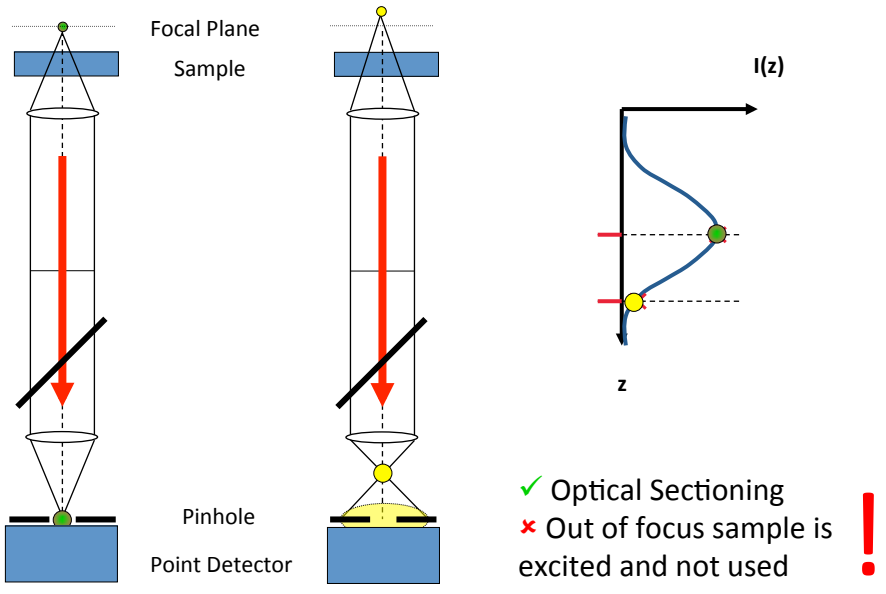
The Nyquist criterion states that, in order to prevent undesired aliasing, one must sample a signal at a rate equal to at least twice its bandwidth.

Wide-field microscope	Confocal microscope	Nyquist rate
$\Delta_x = \lambda_{em}/(4n\sin\alpha)$	$\Delta_x^{conf} = \lambda_{em}/(8n\sin\alpha)$	$F_{Nyquist,x} = \frac{1}{2\Delta_x}$
$\Delta_z = \lambda_{em}/(2(n(1-\cos\alpha))$	$\Delta_z^{conf} = \lambda_{em}/(4(n(1-\cos\alpha))$	$F_{Nyquist,x} = \frac{1}{2\Delta_z}$

In the transformed space

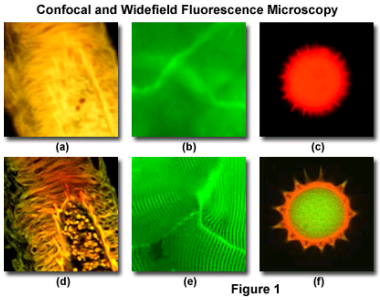


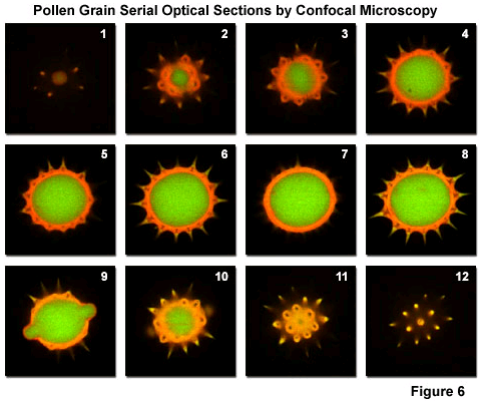
Confocal Principle

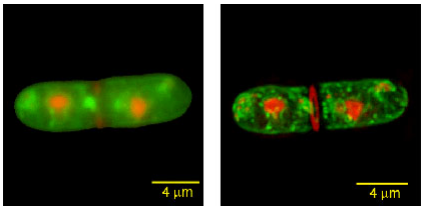


✓ Optical Sectioning
× Out of focus sample is excited and not used

Confocal examples



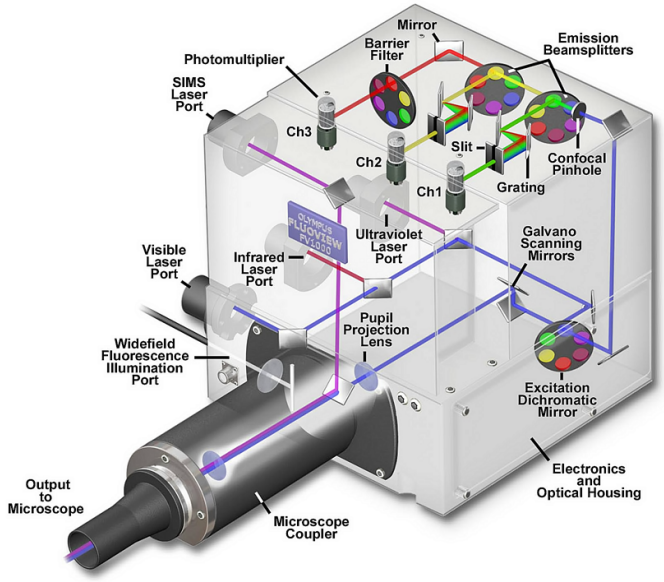




Fission yeast cell: DNA and the medial septum are stained with Hoechst 33342, membranes are visualized using the fluorescent lipophilic dye DiOC6.

Olympus Confocal

Olympus Confocal Head



Confocal light sources

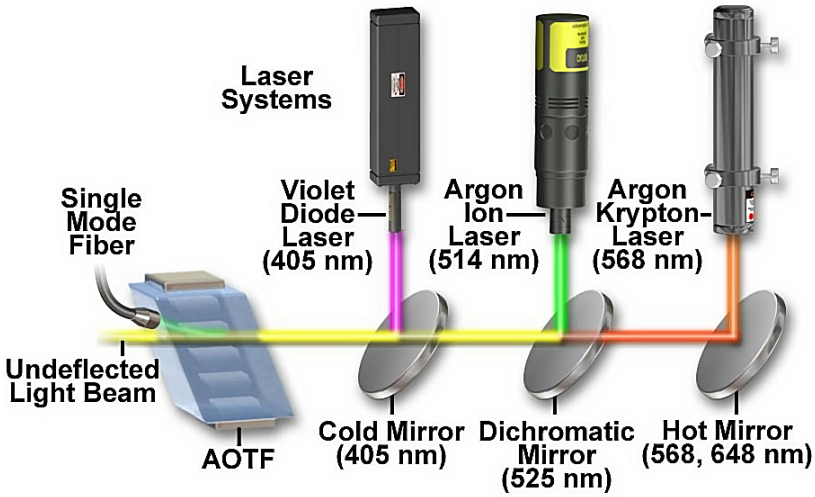
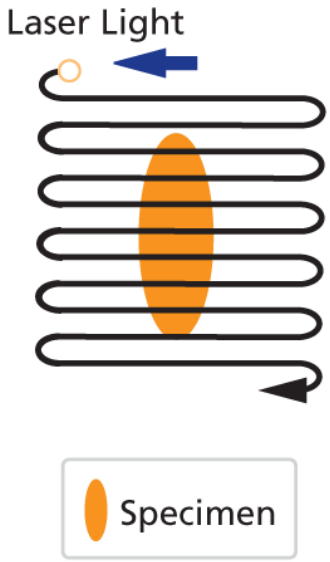
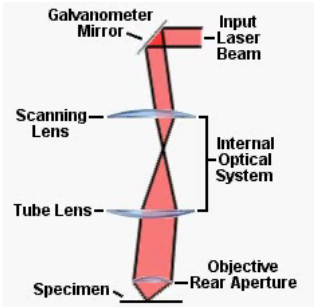
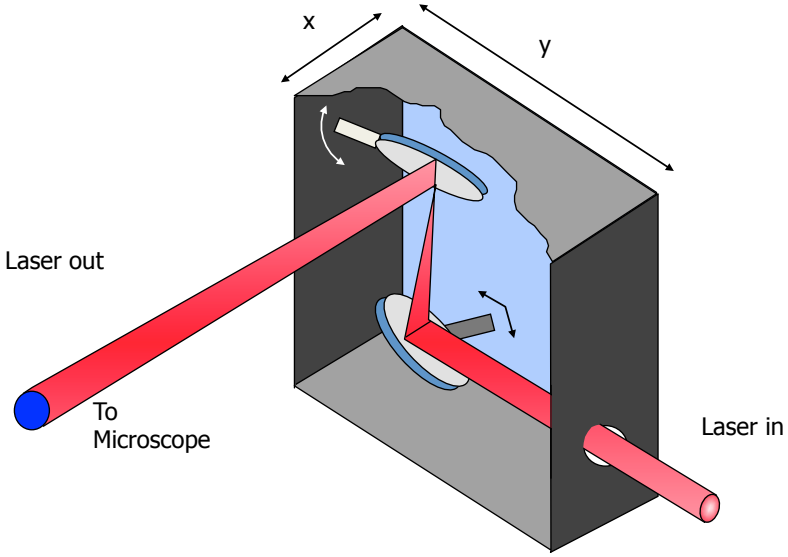


Image formation

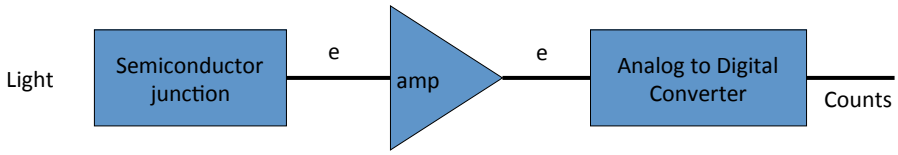




Scanning Galvanometers



Light → Electrons → Counts

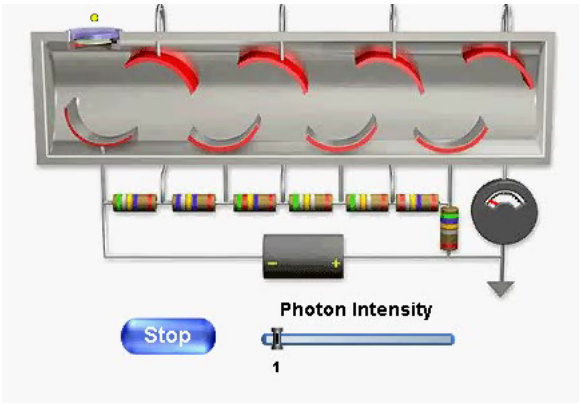


Voltage between Anode & Cathode

Gain
Offset

2^{Bits} = levels
- 8 = 256
- 12 = 4096
- 14 = 16384
- 16 = 65536

Detectors



Photomultipliers, or photomultiplier tubes (PMT) are extremely sensitive detectors of light in the ultraviolet, visible and near infrared. These detectors multiply the signal produced from the incident light to such an extent that detection of single photons is possible.

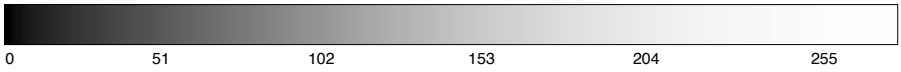
Light → Electrons → Counts

gain and **offset** are used to adjust the detector signal (input) in a way, that a maximal number of grey levels is included in the resulting image (output).

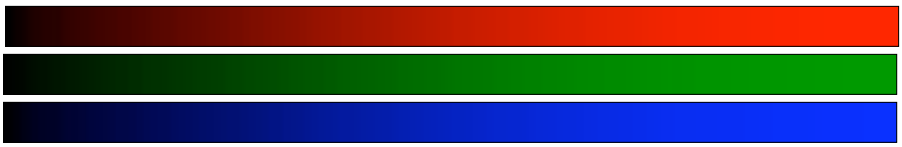
gain
amplifies the input signal by multiplication, which results in a higher gray level value; bright features are brought closer to saturation, general image brightness is increased.

offset
sets the gray level of a selected background to zero; adjust the darkest features in the image to black.

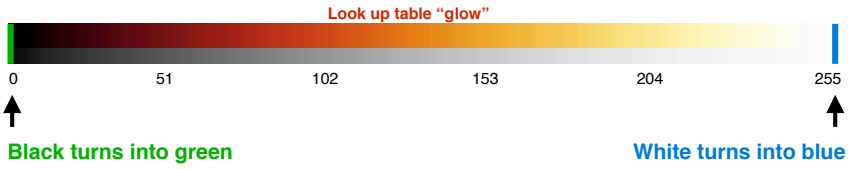
Look up Tables

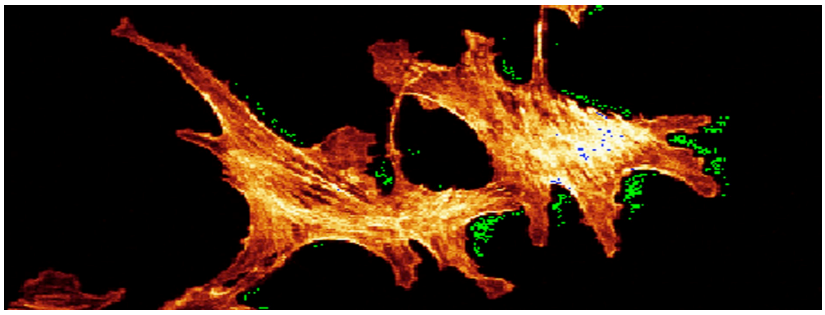


Detected intensity values are displayed as gray levels. The display range of a typical 8-bit monitor covers 256 gray levels. The full range of the LUT is utilized if an image shows all shades of gray between black (=0) and white (=255). The gray levels might be presented in pseudo-colors.



Hi-Lo Look up tables





In the olympus FV1000 is Blue-Red

Detector

Ideal

- ✓ Detect all photons
- ✓ Perfect reading
- ✓ Give 0 counts if dark
- ✓ Fast

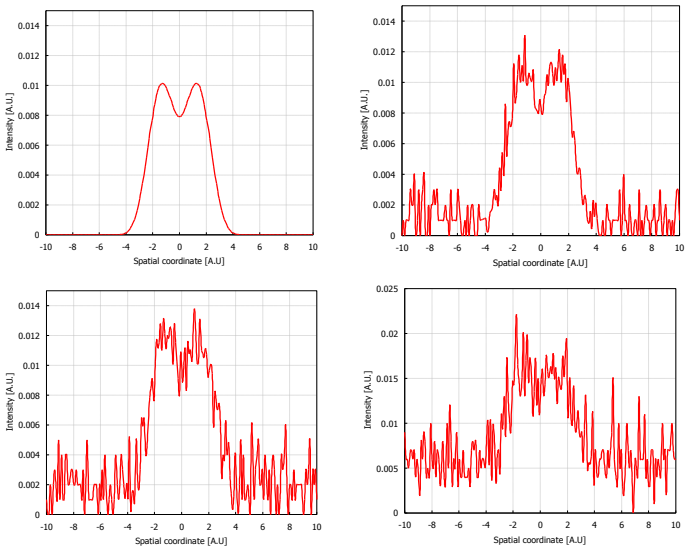
Real

- × Quantum efficiency < 1
- × Readout noise
- × Dark electrons
- × Limited bandwidth

Even in the case of a perfect detector **count noise** is present

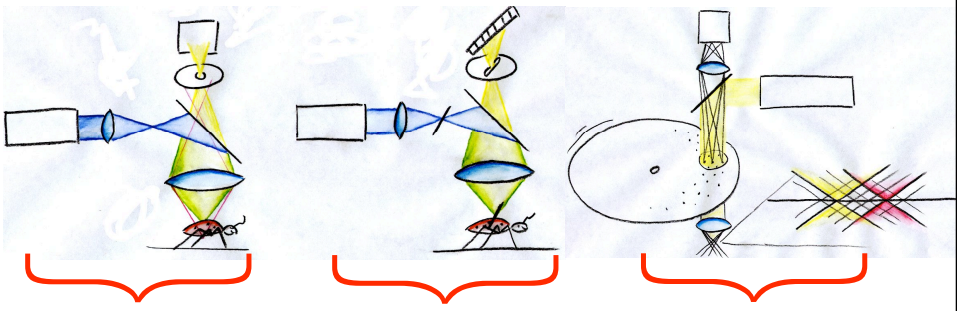
$$SNR = \frac{N}{\sqrt{N}} = \sqrt{N}$$

SNR



The influence of Poisson noise on two intensity distributions separated spatially according to the Rayleigh criterion.

Parallel confocal

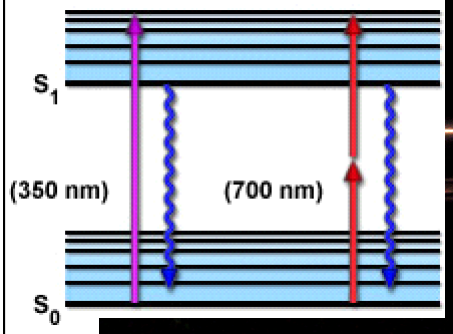


point confocal | slit confocal | spinning disc confocal (Nipkov)

Best resolution and out-of-focus suppression as well as highest multispectral flexibility is achieved only by the classical single point confocal system !

Other techniques
(lo que viene en la 2da mitad)

Two photon principle



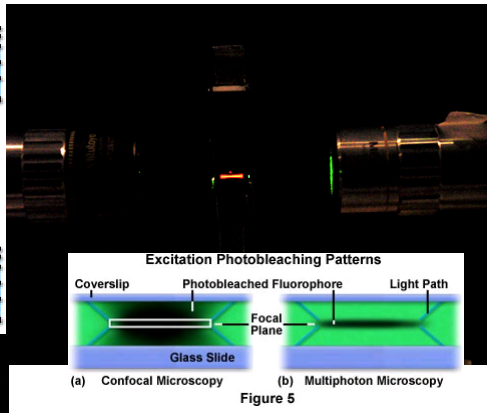
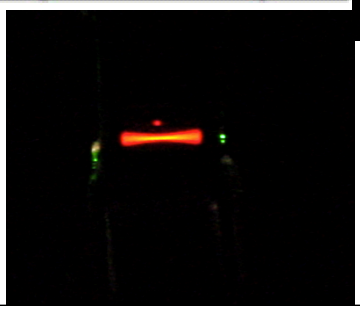
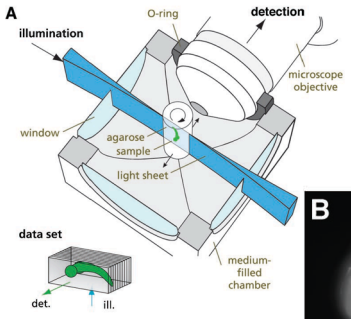


Figure 5

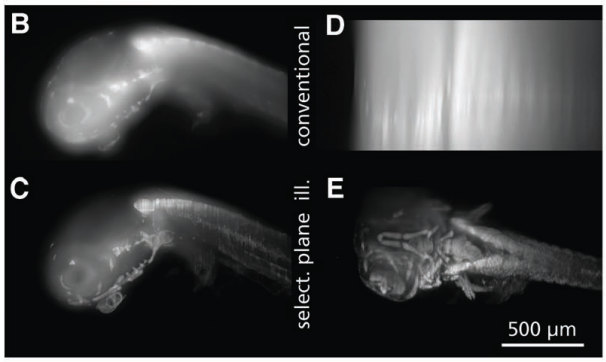


✓ Optical Sectioning
✓ Out of focus sample is not excited
✓ Higher wavelength, higher penetration depth.
× Multiphoton Bleaching can be significant.

Single Plane Illumination Microscopy



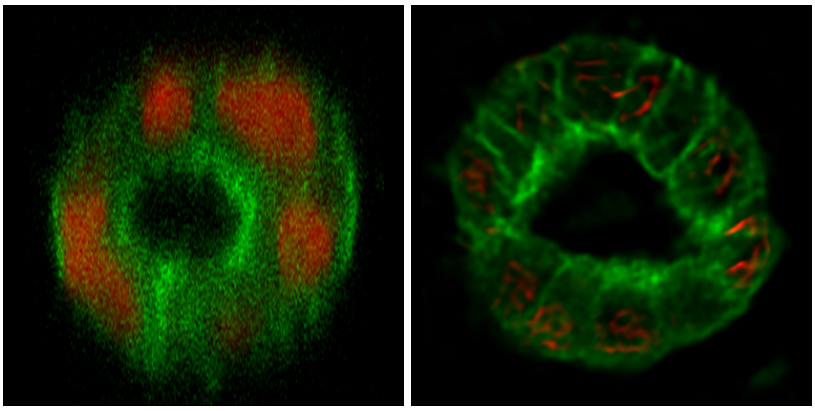
- Illuminate with thin sheet of light
- Detect fluorescence with wide-field detector
- Scan sample to obtain 3D stacks
- Rotate sample for multiple views



Huisken *et al. Science*, 305:1007-1009, 2004.

Sub-cellular resolution in a large living sample

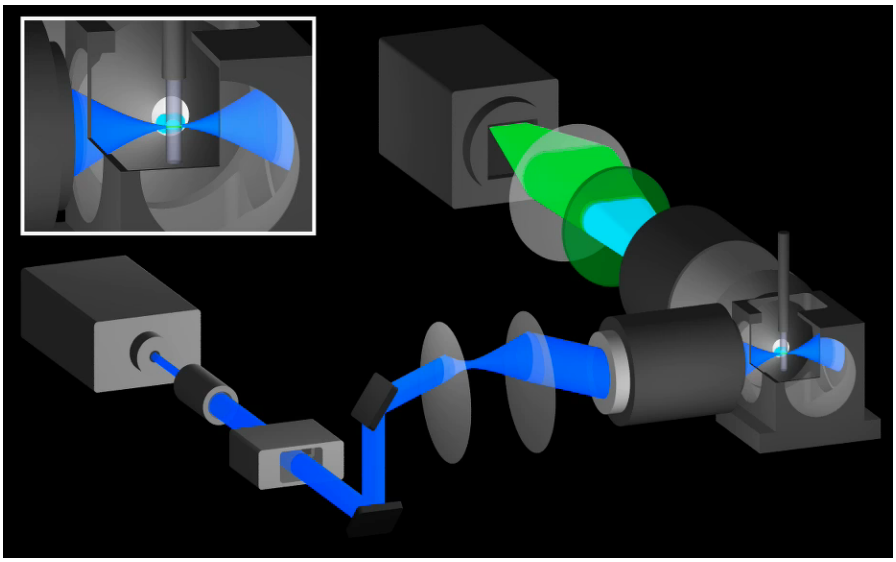
Confocal SPIM



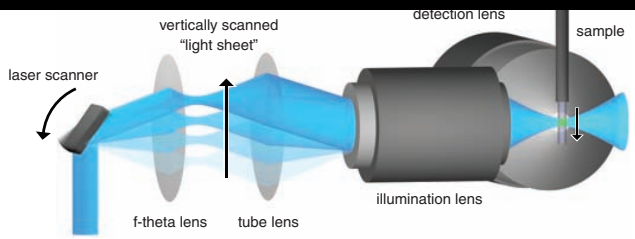
MDCK cyst
Actin-GFP; DRAQ5 nuclear staining
~35 μm diameter; image stack size: 480·480·480 voxels

Verveer *et al.,* Nature Methods, 4, 311-313, 2007

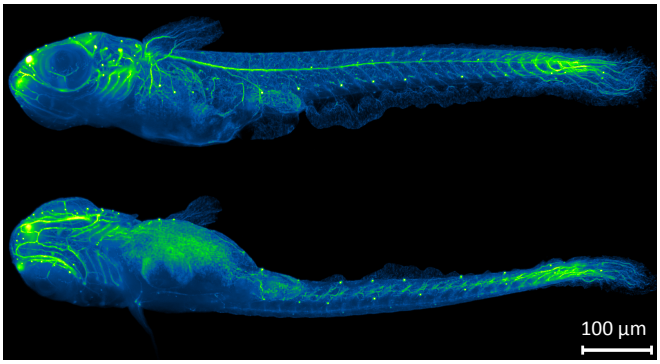
Digital scanned laser light sheet fluorescence mic



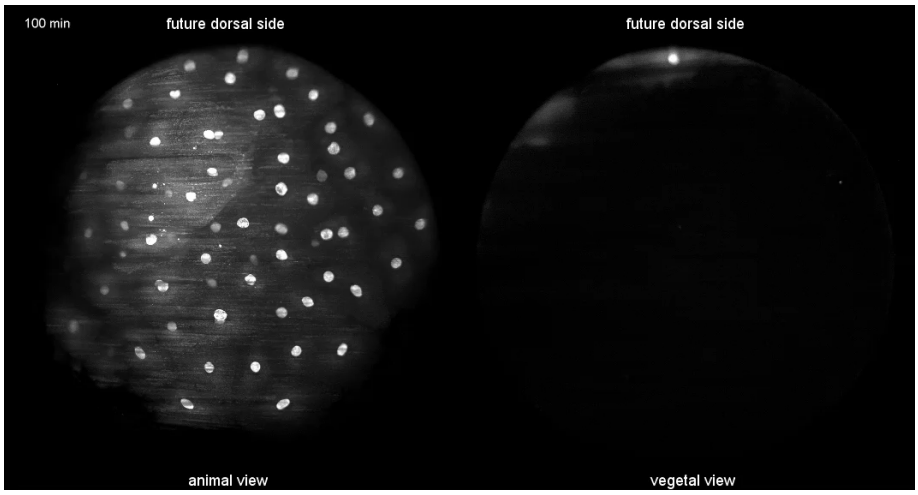
Digital scanned laser light sheet fluorescence mic.



- Scan a thin beam of light through the sample
- Detect perpendicular to the illuminated slice

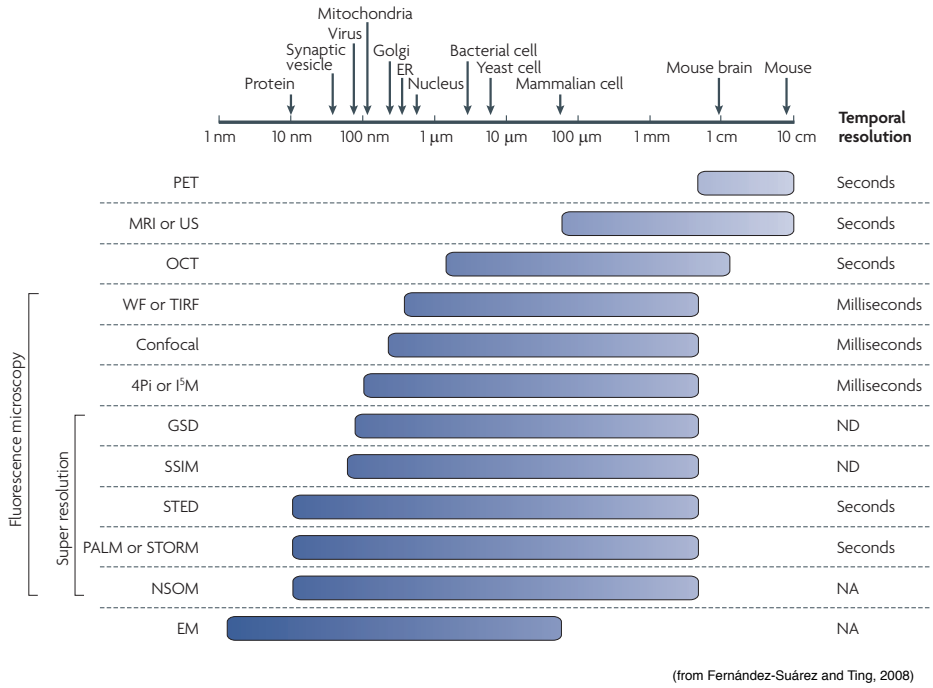


Keller *et al. Science*, 322:1065-1069, 2008.



DSLM time-lapse multi-view recoding of zebrafish embryonic development, with a view on both the animal and vegetal hemispheres. The wild-type zebrafish embryo was injected with H2B-eGFP mRNA at the one cell stage. Imaging was started in the 64-cell stage.

Keller *et al. Science*, 322:1065-1069, 2008.

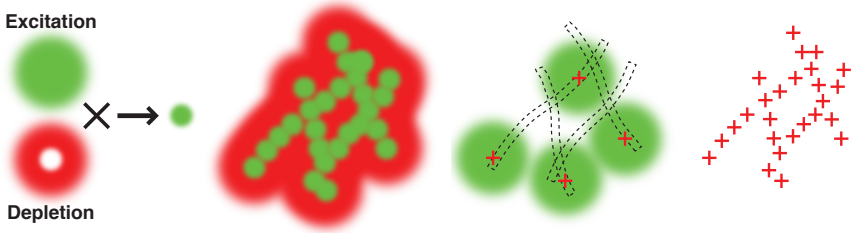


(from Fernández-Suárez and Ting, 2008)

Summary

Conventional microscopy

Structured illumination

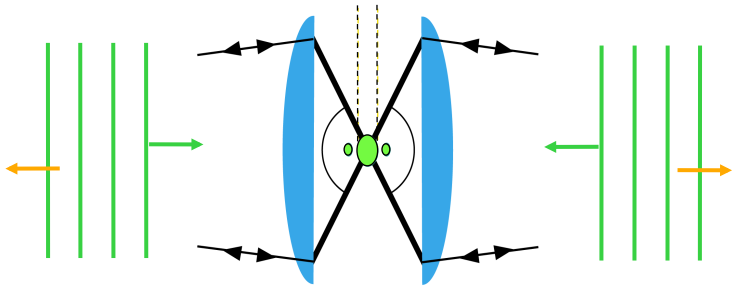


STED

PALM/STORM

4π Microscopy

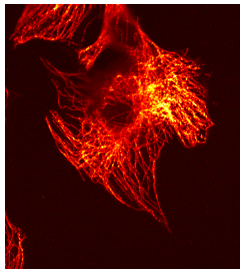
70 - 140 nm



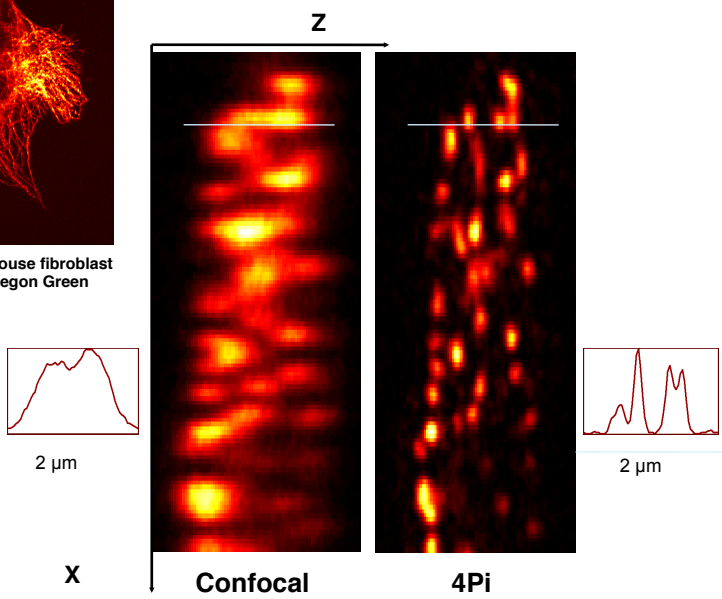
$$\vec{E}^{4Pi}(r,z,\varphi) = \vec{E}_1(r,z,\varphi) + \vec{E}_2(r,-z,\varphi)$$

Coherent illumination and/or fluorescence detection

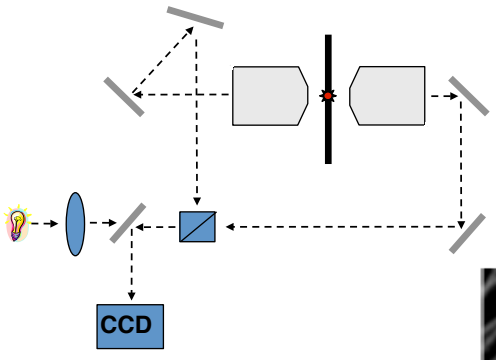
4π Microscopy



Microtubules, mouse fibroblast
Immunofluor, Oregon Green



I5M microscopy



Comparison to 4-Pi:
- Full field method
- requires more extensive post-processing

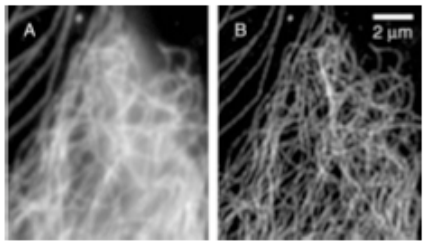
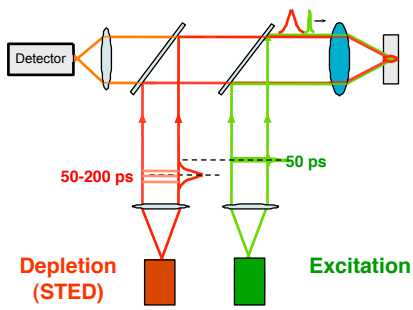
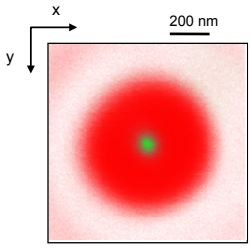


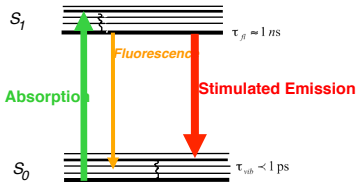
Fig. 4. XY views of fluorescently labelled microtubules in PtK2 tissue culture cells: (A) Conventional microscopy (single section), (B) I^{5}M using a linear inverse filter followed by iterative deconvolution (reprojected).

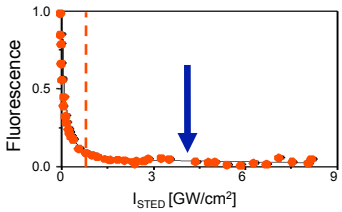
STED microscopy





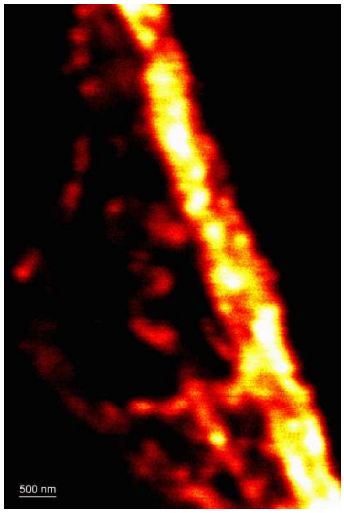
The stronger the STED beam the narrower the fluorescent spot

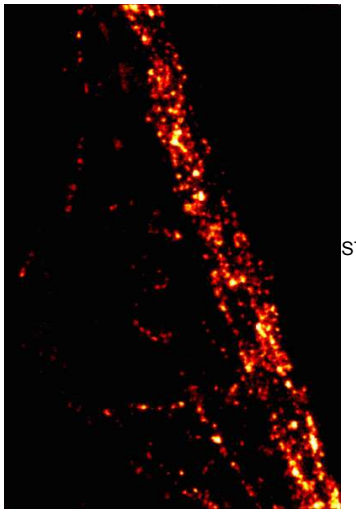




STED microscopy

Confocal

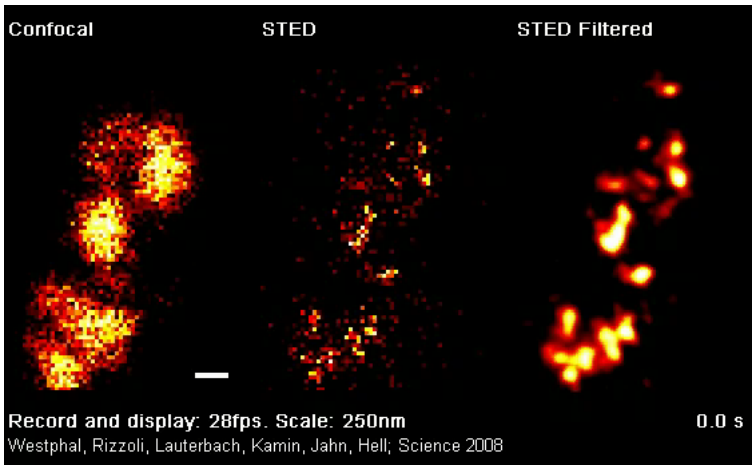




STED

Heavy subunit of neurofilaments in neuroblastoma

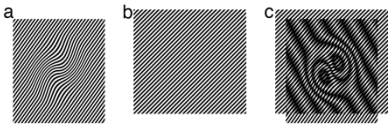
STED microscopy



Real-time (28 frames per second) movie of a 2.5 μm-long area in a hippocampal axon in culture. The individual spots represent single labeled synaptic vesicles.

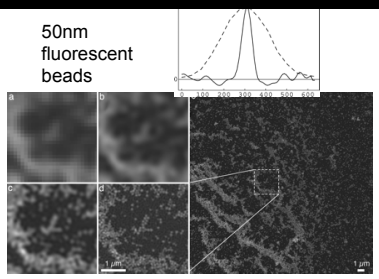
Structured Illumination microscopy

Resolution extension through Moire effect

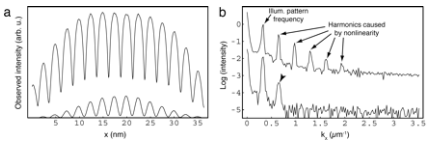


Sine wave pattern generated by a laser illumination
Captured at 5 phases and 3 angles and multiple Z sections

50nm fluorescent beads

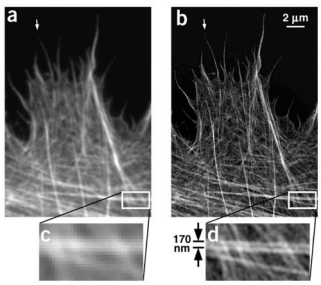


Non linear pattern -by saturation

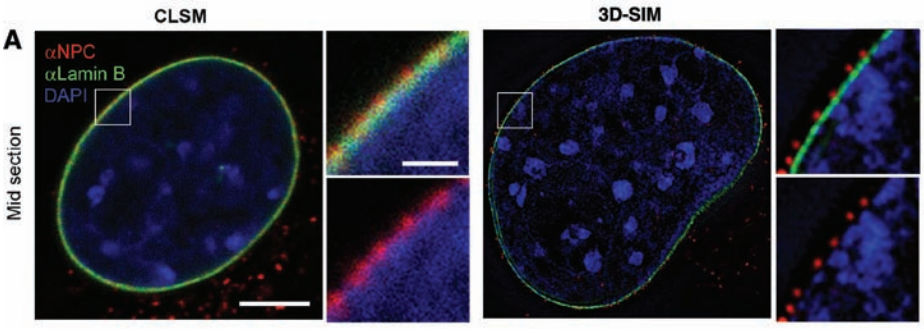


Comparison to STED
- Full-field method
- requires many images and extensive reconstruction

Actin in Hela Cell



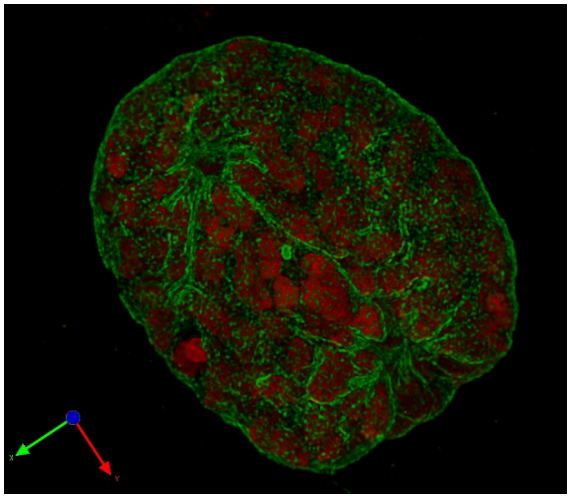
Structured Illumination microscopy



Simultaneous imaging of DNA, nuclear lamina, and NPC epitopes by 3D-SIM. C2C12 cells are immu- nostained with antibodies against lamin B (green) and antibodies that recognize different NPC epitopes (red). DNA is counterstained with DAPI (blue).

Schermelleh *et al.*, *Science* 320, 1332-1336 (2008)

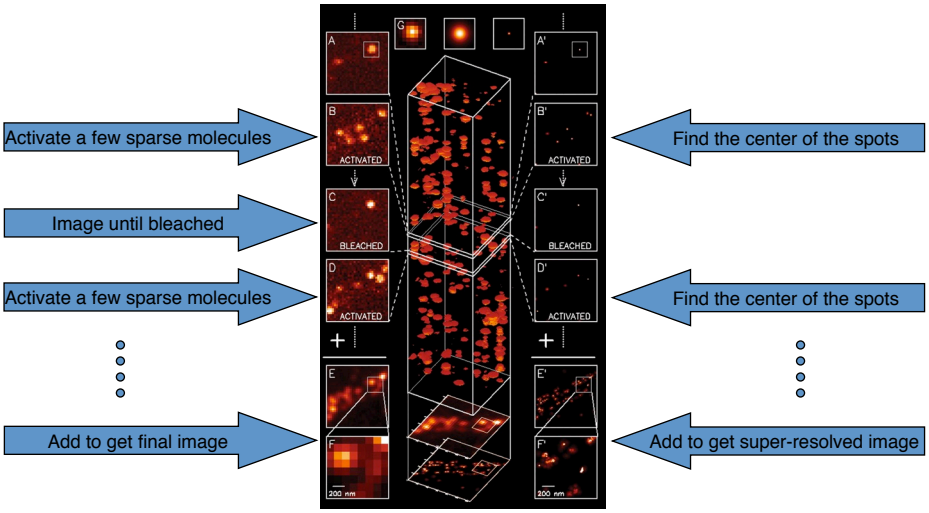
Structured Illumination microscopy



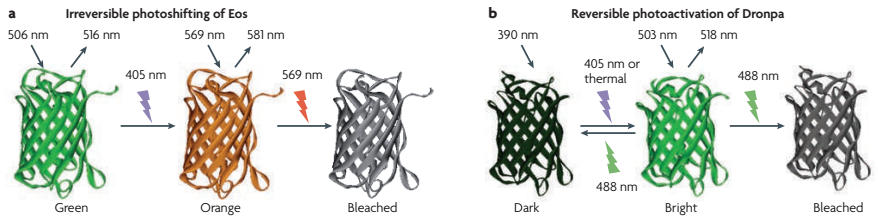
3D volume rendering of a prophase nucleus imaged with 3dSIM. C2C12 cell is immunostained with antibodies against Lamin B and counterstained with DAPI (red).

Photo-activated localization microscopy

Principle: successively activate GFP-tagged molecules and localize them precisely



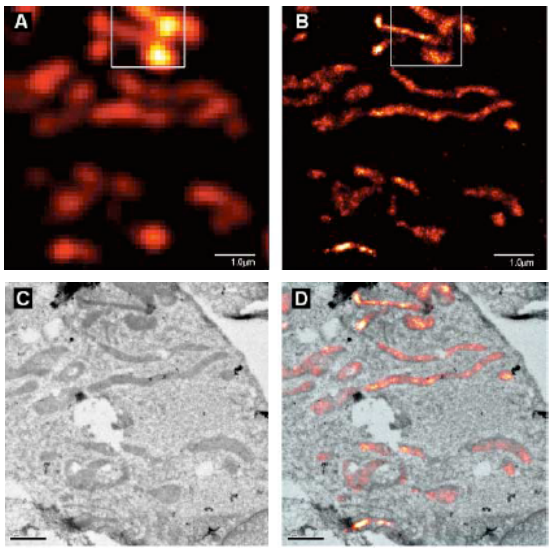
E. Betzig *et al.*, *Science* 313, 1642 -1645 (2006)



Fluorescent protein	Activating light	Quenching light	Pre/post colours	λ_{ex} (nm)	λ_{em} (nm)	ε_{abs} ($M^{-1}cm^{-1}$)	η_{fl} (%)	Contrast	*N*	Oligomeric state	Refs
Irreversible photoactivatable fluorescent proteins											
PA-GFP	UV-violet	NA	Dark/green	504	517	17,400	79	200	ND	Monomer	51,61,62
PA-RFP1-1	UV-violet	NA	Dark/red	578	605	10,000	8	ND	ND	Monomer	60,61,64
Photoshiftable fluorescent proteins											
PS-CFP2	UV-violet	NA	Cyan/green	490	511	47,000	23	>2,000	260	Monomer	48,61,69, 112
Kaede	UV-violet	NA	Green/orange	572	582	60,400	33	2,000	~400	Tetramer	22,61,65
KiKGR	UV-violet	NA	Green/red	583	593	32,600	65	>2,000	ND	Tetramer	61,66
Monomeric Eos	UV-violet	NA	Green/orange	569	581	37,000	62	ND	~490	Monomer	22,48,52, 53,61,68
Dendra-2	Blue	NA	Green/orange	553	573	35,000	55	4,500	ND	Monomer	68,112
Reversible photoactivatable fluorescent proteins											
FP595	Green	450 nm	Dark/red	590	600	59,000	7	30	ND	Tetramer	56,70
Dronpa	UV-violet	488 nm	Dark/green	503	518	95,000	85	ND	120	Monomer	48,59,61
Padron	Blue	405 nm	Dark/green	503	522	43,000	64	ND	ND	Monomer	71

(from Fernández-Suárez and Ting, 2008)

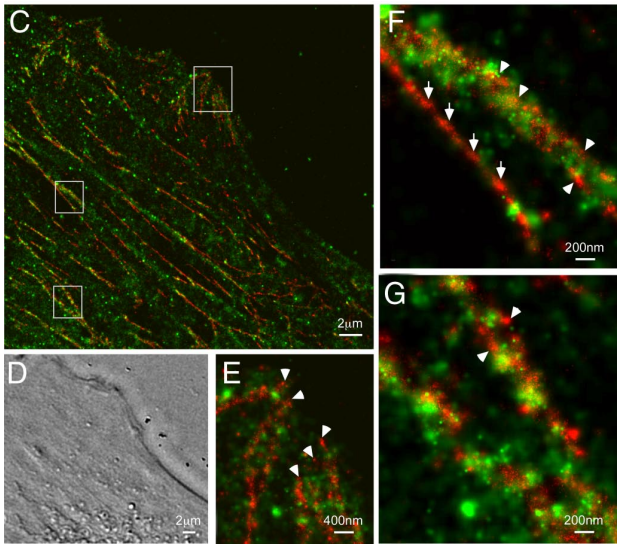
Comparative widefield, PALM, TEM, and PALM/TEM overlay images of mitochondria in a cryo-prepared thin section from a COS-7 cell expressing dEosFP-tagged cytochrome-C oxidase import sequence



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

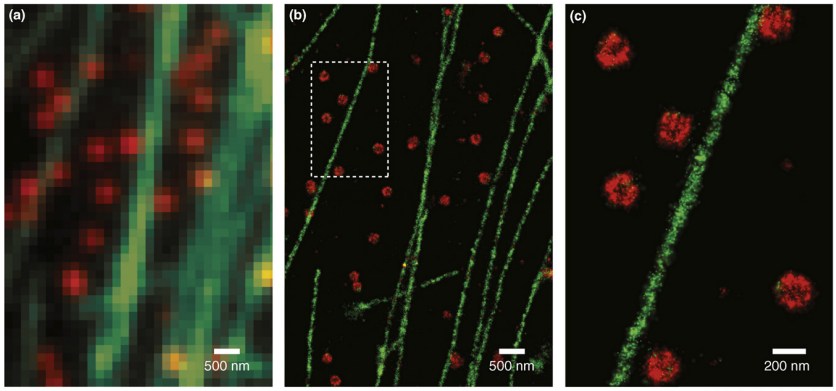
Dual color Photo-activated localization microscopy

Nanostructural organization of cytoskeletal actin and the adhesion protein paxillin in an HFF-1 cell.



Stochastic Optical Reconstruction microscopy

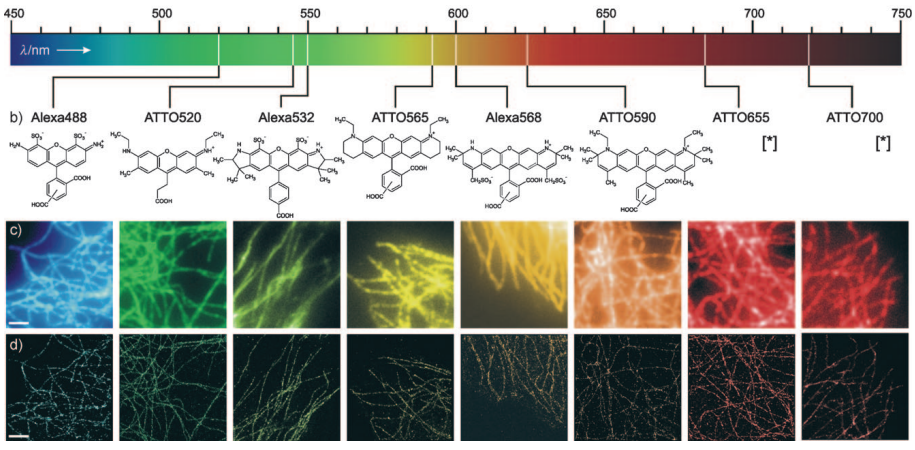
- STORM = PALM using organic fluorophores
- Originally using tandem fluorophores
- Later also with single fluorophores (dSTORM)



Conventional immunofluoresence (a) and STORM (b) images of microtubules (green) and clathrin-coated pits (red). Antibodies used for microtubule staining were labeled with Cy2 and Alexa 647. For clathrin labeling the antibodies were labeled with Cy3 and Alexa 647

Bates *et al.*, (2006)

Stochastic Optical Reconstruction microscopy

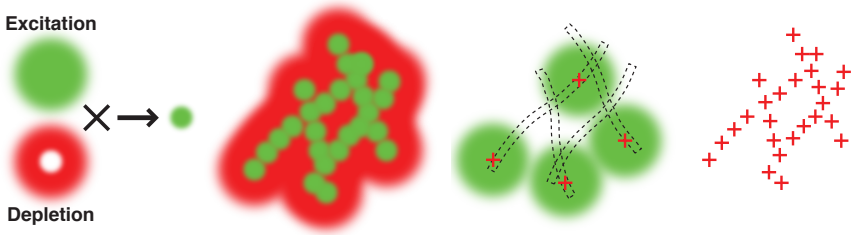


(from Heilemann, 2009)

Summary

Conventional microscopy

Structured illumination



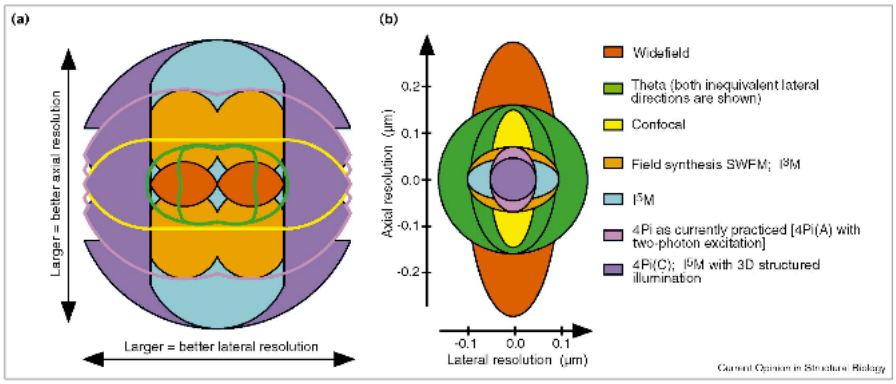
STED

PALM/STORM

Illumination Detection-Schemes

	x-y	z	€	# Groups
Wide Field	λ	>> λ	20 k	Thousands
Confocal	λ	λ	200 k	Hundreds
Two photons	λ	λ	500 k	A few Hundreds
4π, STED	<< λ	<< λ	1000 k	1

In basic configuration


(a)
Larger = better axial resolution
Larger = better lateral resolution
(b)
Axial resolution (μm)
0.2
0.1
0.0
-0.1
-0.2
-0.1
0.0
0.1
Lateral resolution (μm)
Widefield
Theta (both inequivalent lateral directions are shown)
Confocal
Field synthesis SWFM; I3M
I5M
4Pi as currently practiced [4Pi(A) with two-photon excitation]
4Pi(C); I5M with 3D structured illumination
Current Opinion in Structural Biology