Lecture 2: Super-Resolution Fluorescence Microscopy

The Nobel Prize in Chemistry 2014

Photo: Matt Staley/HHMI

Eric Betzig

Prize share: 1/3

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Stefan W. Hell

Prize share: 1/3

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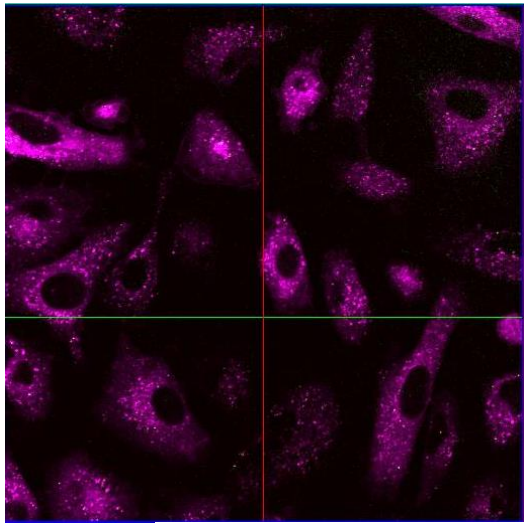
William E. Moerner

Prize share: 1/3

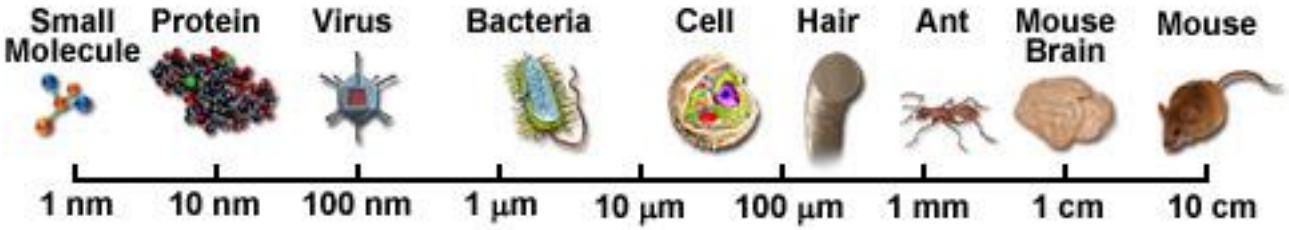
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

Fluorescence microscopy



One of the most widely used tools in modern biomedical research

Used to observe organelles, living cells, tissues and whole organisms.

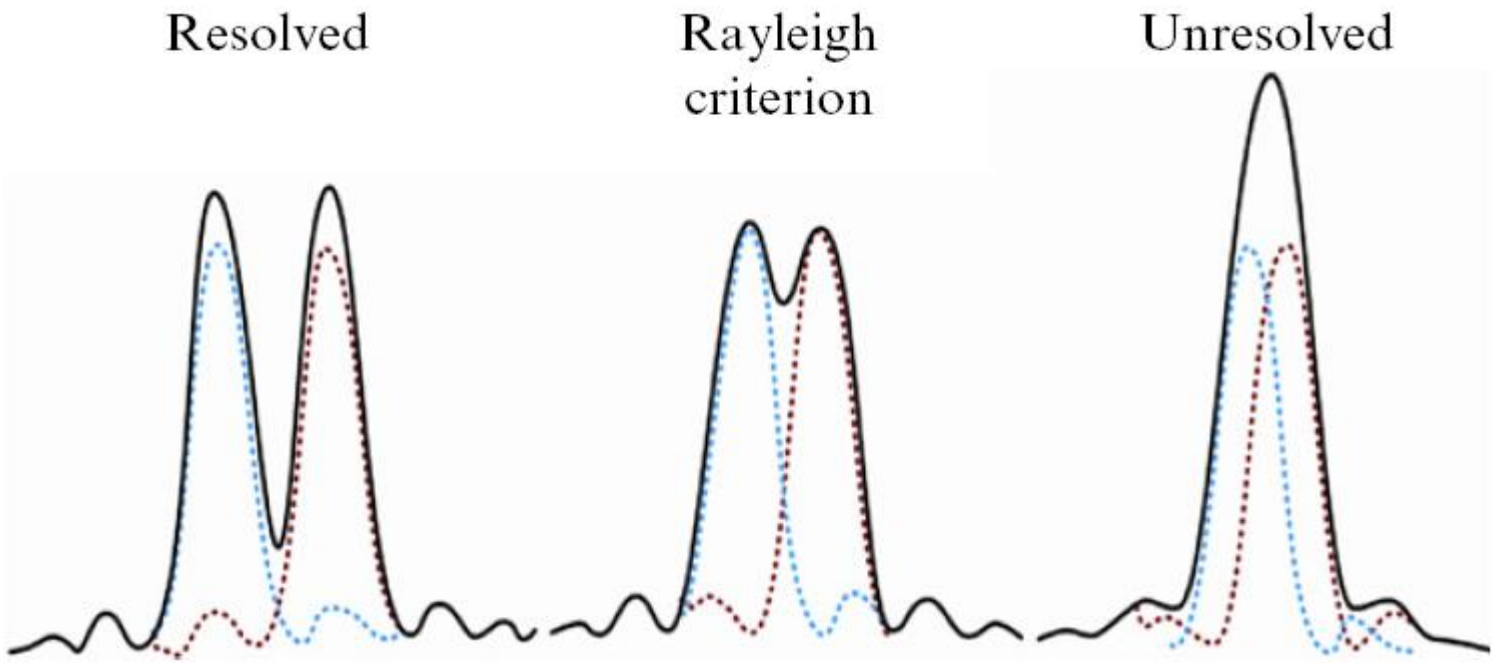


Fluorescence microscopy

Lack resolution for single molecule studies

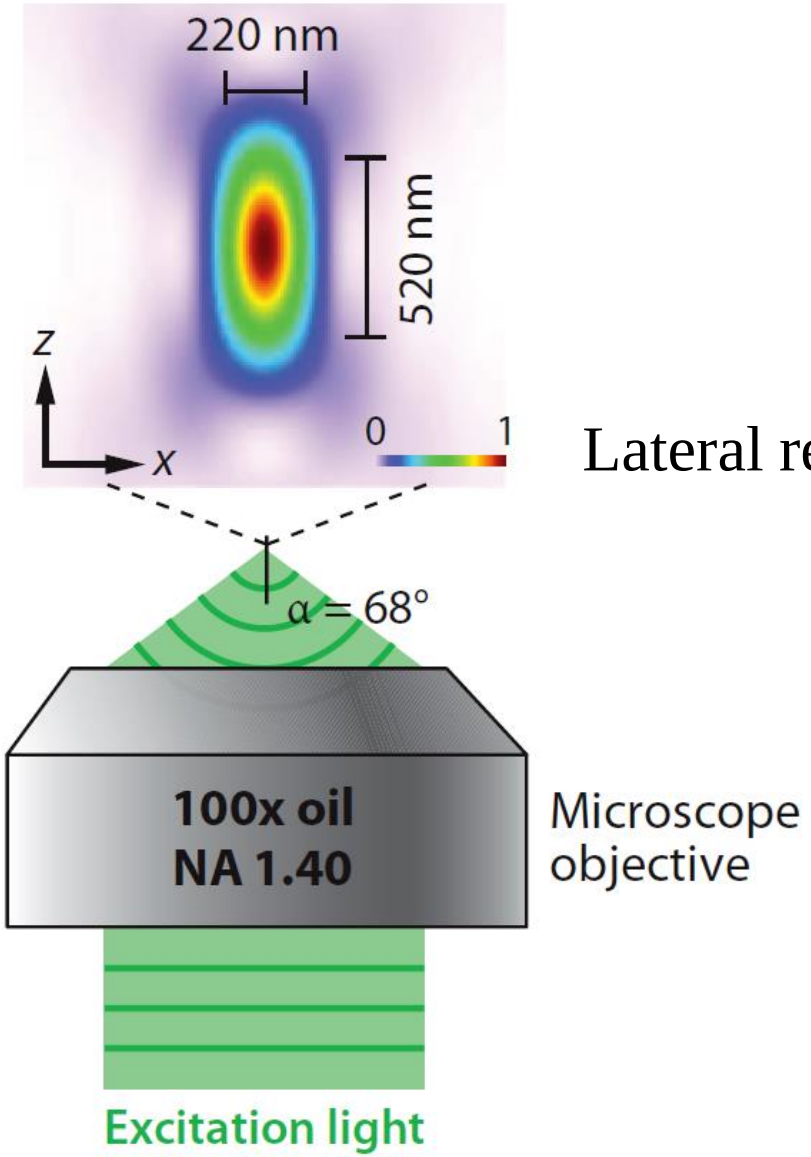
Resolution

Resolution is the minimum distance necessary to distinguish two light emitting particles. If two objects are closer than the diffraction limit (λ/2NA), their PSFs overlap and you cannot tell that they are, in fact, two separate emitting objects.



To get improved resolution, one can try to decrease the width of individual PSFs, or one of the PSFs can be transiently or permanently photobleached, or one can minimize the overlap of the PSFs making them spectrally distinct.

Resolution limit in fluorescence microscopy

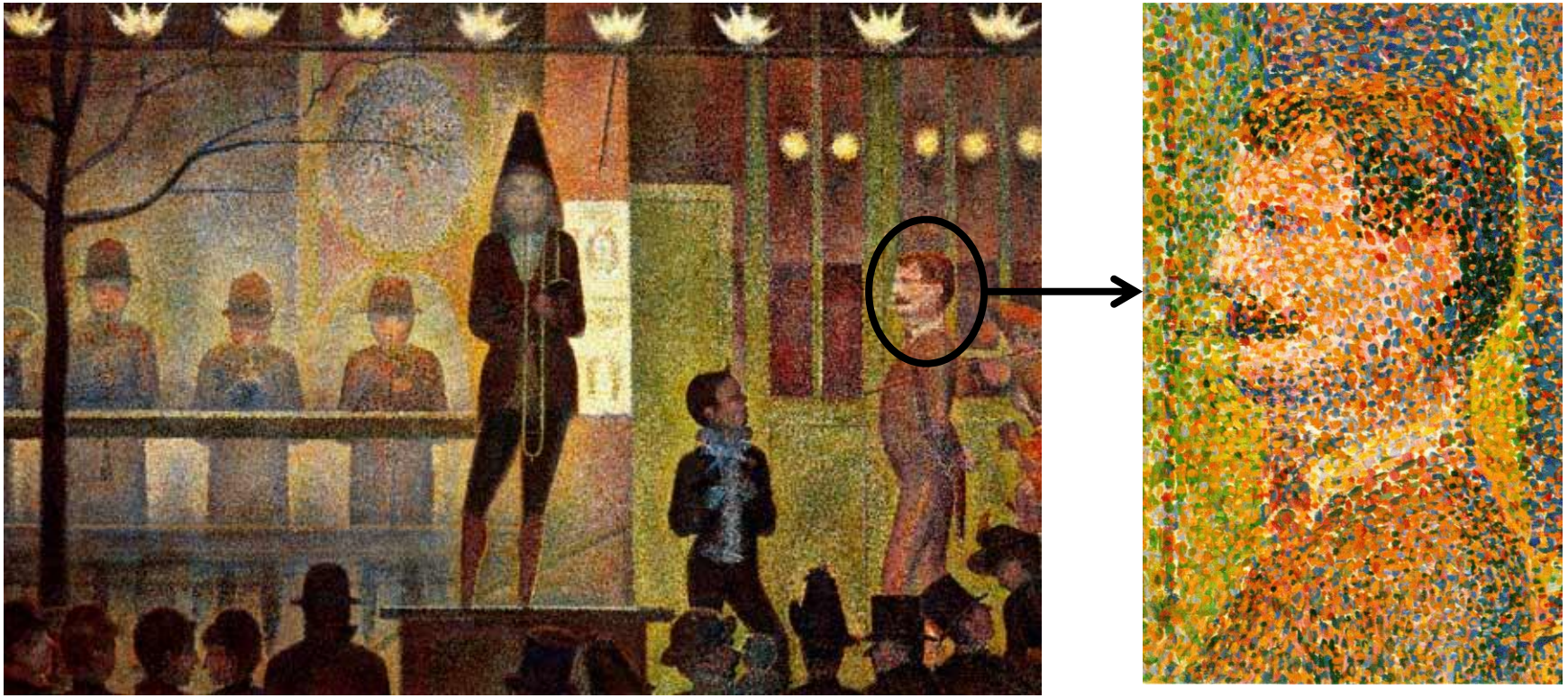


Lateral resolution = $\lambda/2NA$

Improving resolution: I

Images are composed of pixels arranged in unique patterns

Pointillism is a painting technique where small, distinct dots of pure color form an image.

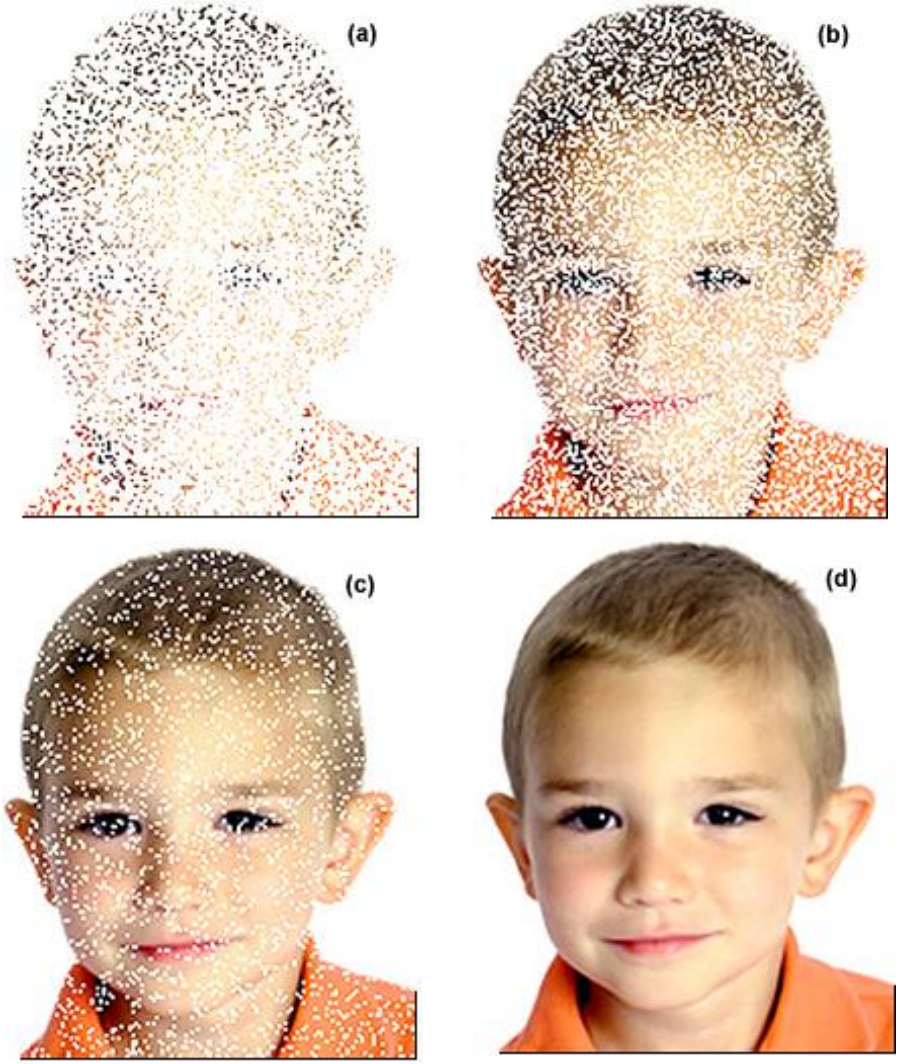


La Parade de Cirque (1889): Georges Seurat

Image can be generated by defining the positions of all dots that compose the structure.

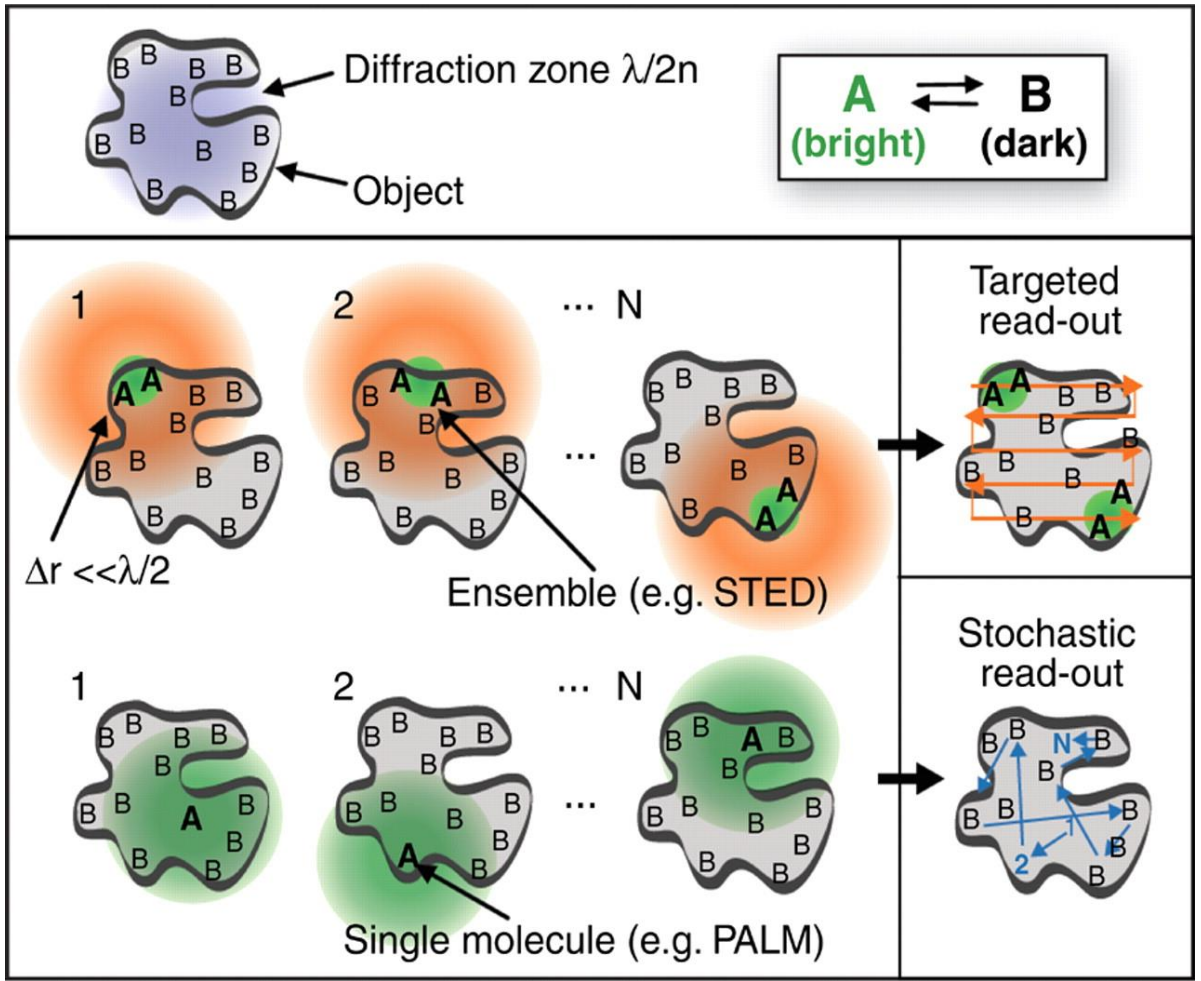
Improving resolution: II

Resolving features in image requires not only localizing points with **high precision** but also with **high density**



Types of super-resolution imaging

1. Super Resolution microscopy by single molecule imaging
2. Super Resolution microscopy by spatially patterned excitation



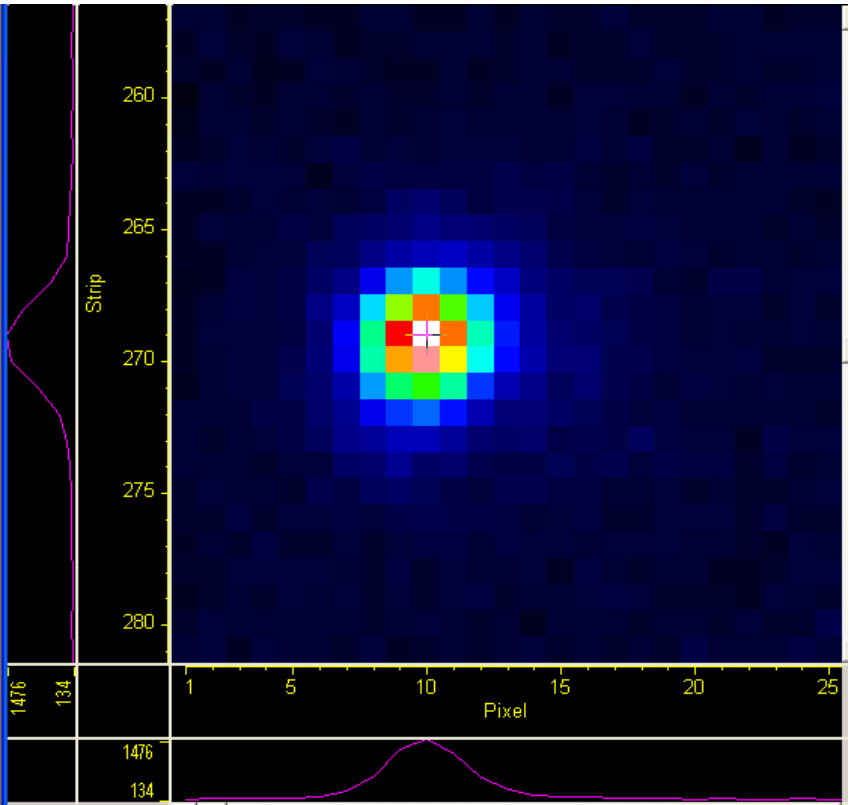
Section 1

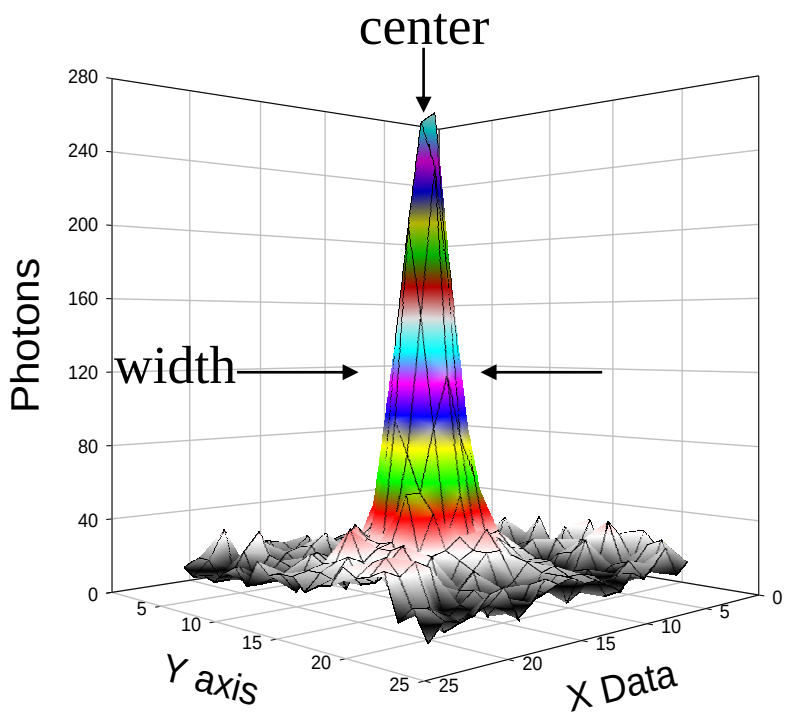
SUPER RESOLUTION MICROSCOPY BY SINGLE MOLECULE IMAGING

Super Resolution microscopy by single molecule imaging

A biological structure is ultimately defined by the positions of the molecules that build up the structure. It is thus conceivable that super-resolution fluorescence microscopy can also be achieved by determining the position of each fluorescent probe in a sample with high precision.

$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^2 b^2}{a^2 N^2} \right)} \approx \frac{s_i}{\sqrt{N}}$$





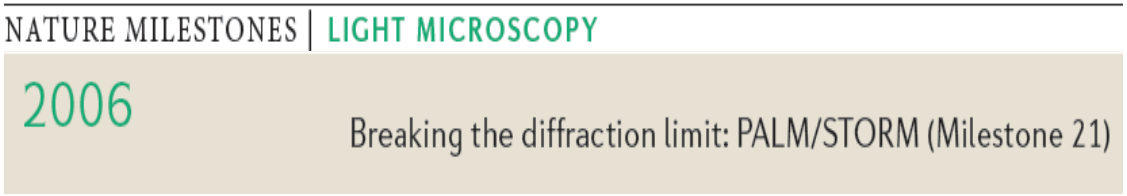
Super Resolution microscopy by single molecule imaging

Use photoactivatable or photoswitchable fluorescent probes that can be activated at different time points by light at a wavelength different from the imaging light. Individually image, localize, and subsequently deactivate the fluorophore.

1. Stochastic Optical Reconstruction Microscopy (STORM)

2. Photoactivated Localization Microscopy (PALM)

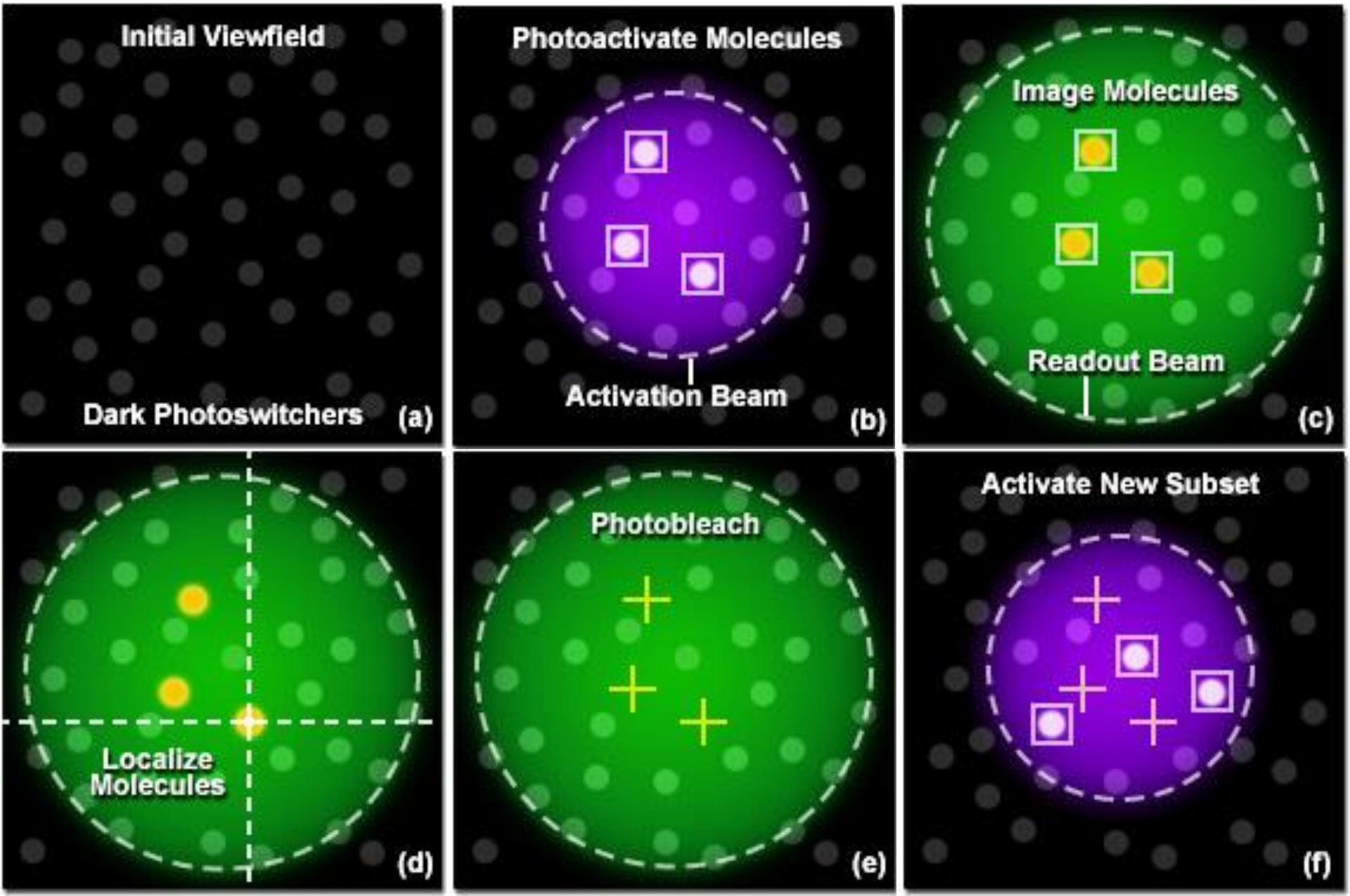
3. Fluorescence Photoactivation Localization Microscopy (FPALM)



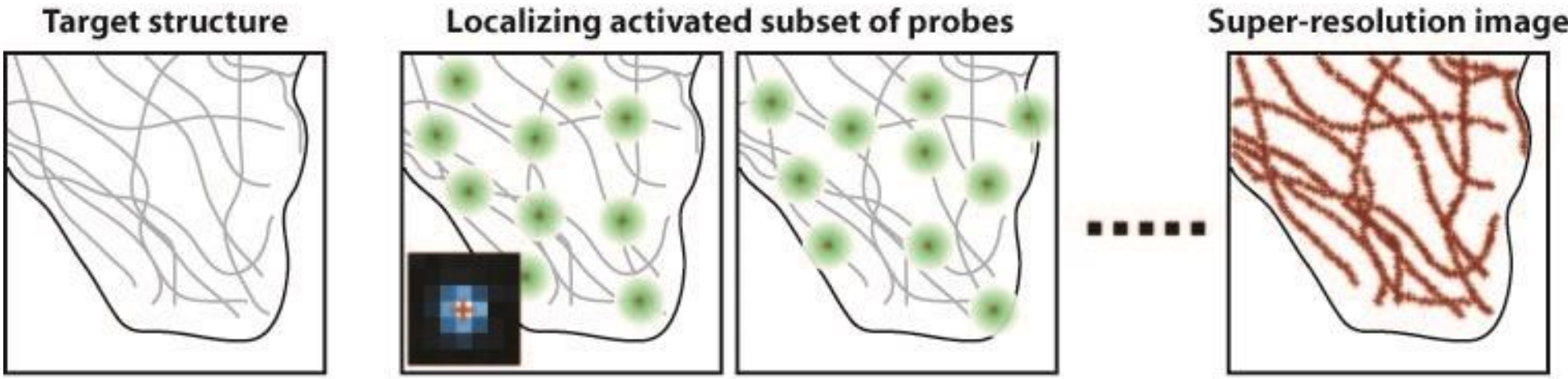
Method of the Year 2008

With its tremendous potential for understanding cellular biology now poised to become a reality, super-resolution fluorescence microscopy is our choice for Method of the Year.

Principle of STORM, PALM and FPALM



Principle of STORM, PALM and FPALM



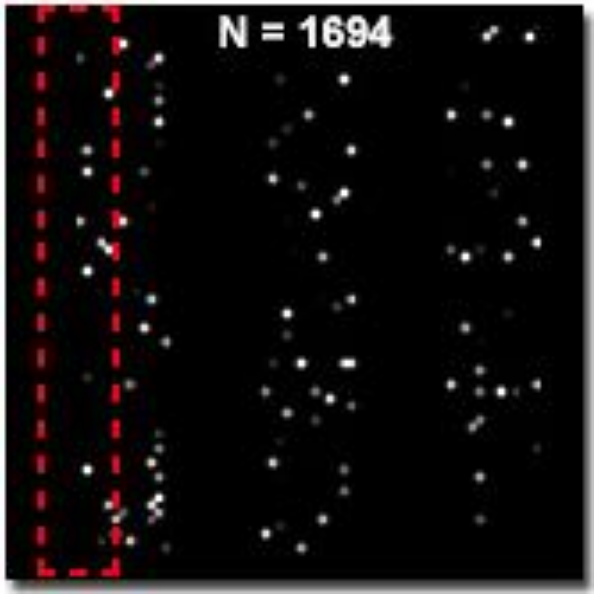
Huang B, et al. 2009.
Annu. Rev. Biochem. 78:993–1016

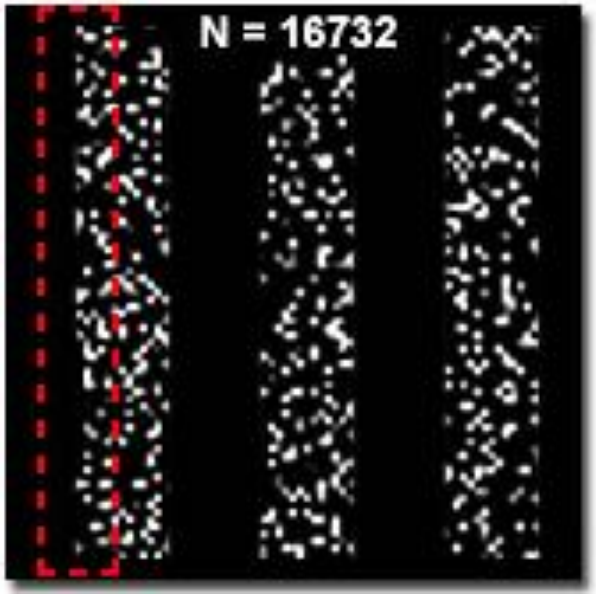
Resolution depends on **LOCALIZATION PRECISION** and **MOLECULAR DENSITY** of fluorescent probes in the specimen.

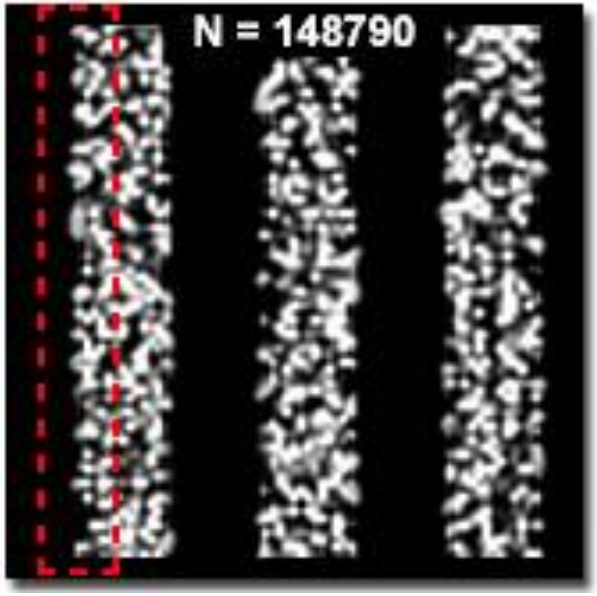
Molecular Density

Besides localization precision, the other key determinant of resolution is the density of labeled molecules in the specimen.

The mean distance between neighboring localized molecules must be at least twice as fine as the desired resolution. To achieve 10-nanometer lateral resolution, molecules must be spaced a minimum of 5 nanometers apart in each dimension to yield a minimum density of 40,000 molecules per square micrometer.







Photoactivatable and Photoswitchable fluorophores

Ideal probes should have

1. Large extinction coefficients at the activation wavelength
2. Large quantum yields at the readout wavelength
3. Reduced tendency for self-aggregation
4. Low but finite photobleaching rate

Photoactivatable fluorescent proteins

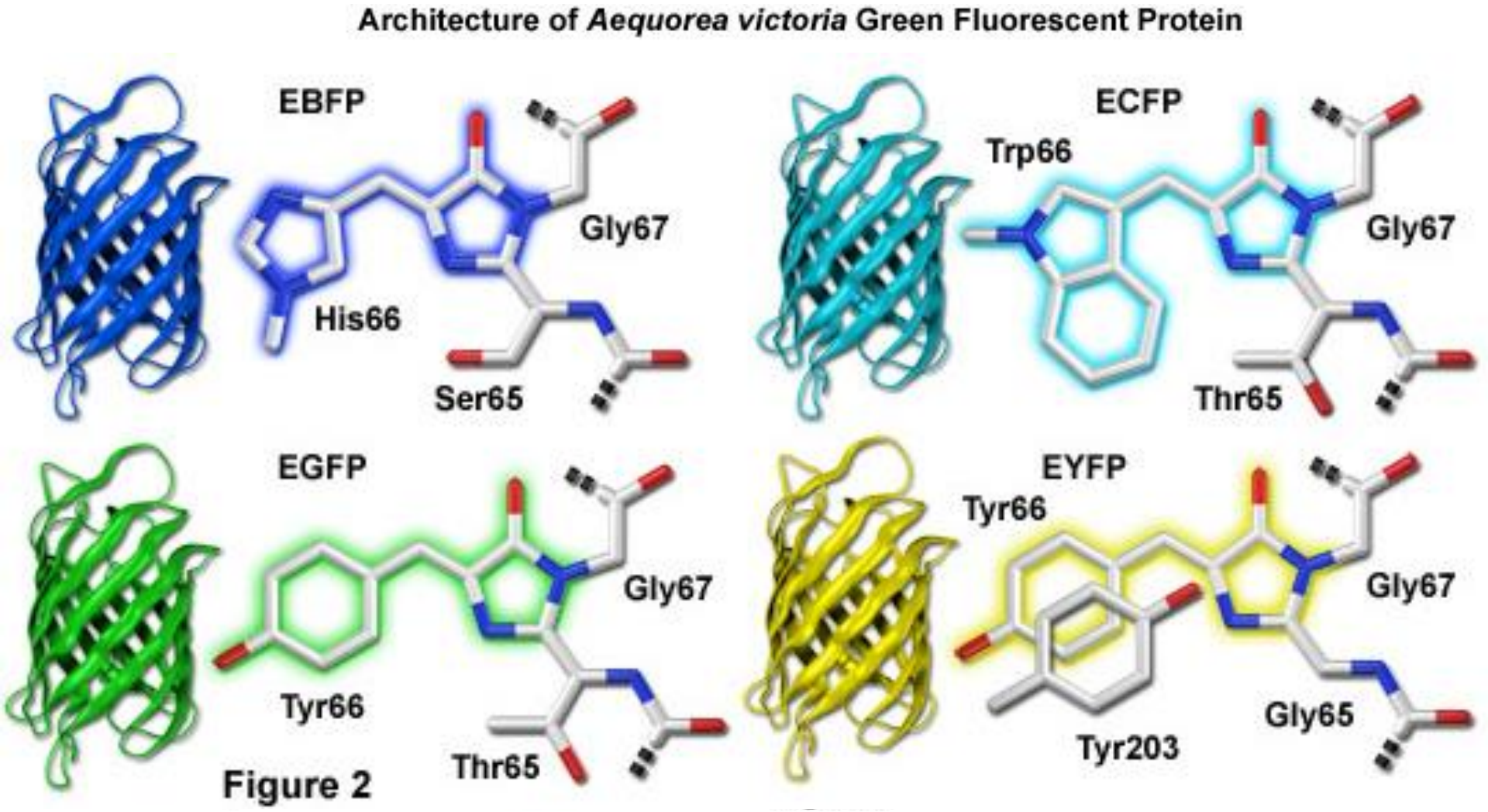


Figure 2

Photoswitchable fluorophores

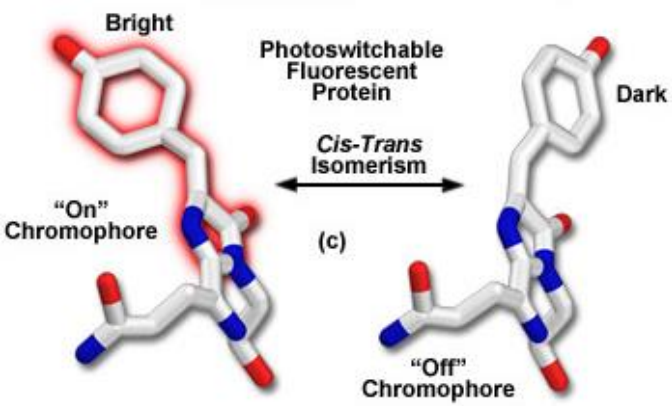


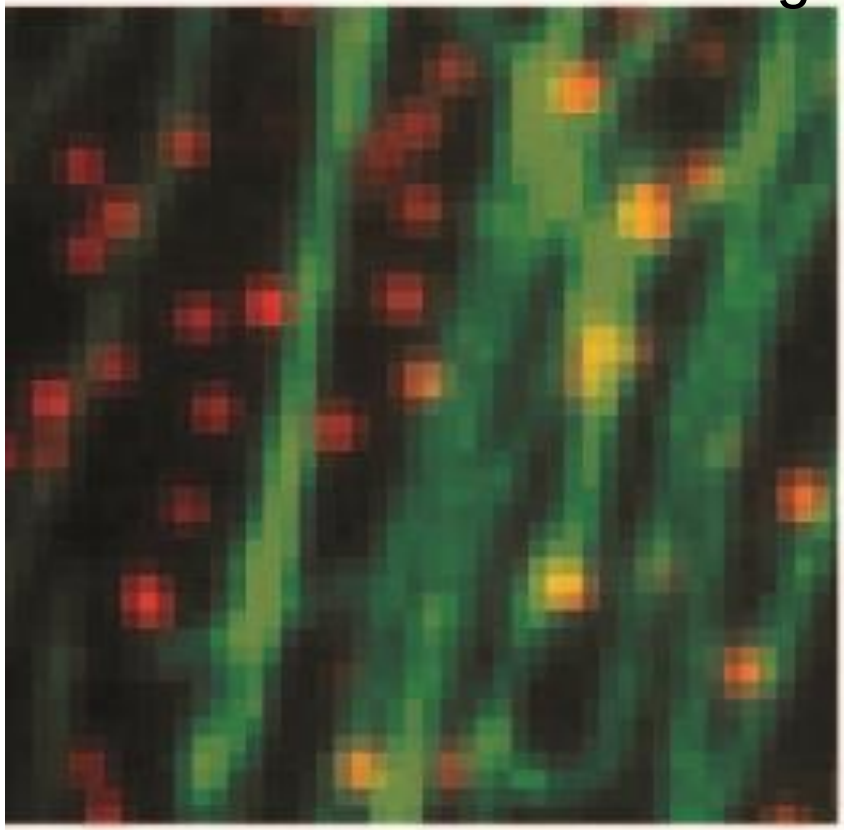
Table 1 Photoswitchable fluorophores used in super-resolution fluorescence microscopy

Fluorophore		Activation wavelength (nm)	Before activation		After activation		Reversible	References
			Ex[a] (nm)	Em (nm)	Ex (nm)	Em (nm)		
Cyan/dark-to-green FP	PA-GFP	405	400	517	504	517	No	(88)
	PS-CFP2		400	468	490	511		(89)[b]
Green-to-red FP	Kaede	405	508	518	572	582	No	(90)
	EosFP	405	505	516	569	581		(91)
	Dendra2	405–488	490	507	553	573		(92)[b]
Dark-to-red FP	PAmCherry	405	NF		564	595	No	(62)
Reversible FP	Dronpa	405	NF		503	518	Yes	(93)
	Dronpa2				486	513		(94)
	Dronpa3				487	514		(94)
	rsFastLime				496	518		(95)
	bsDronpa				460	504		(61)
	EYFP	405	NF		513	527		(66)
Caged dyes	Caged fluorescein	<405	NF		497	516	No	[c]
	Caged Q-rhodamine[d]				545	575		
Cyanine dyes	Cy5 & Alexa 647	350–570[e]	NF		647	665	Yes	(46, 58)
	Cy5.5				674	692		
	Cy7				746	773		
Photochromic rhodamine	SRA545	375	NF		Green	545	Yes[f]	(59, 96)
	SRA552					552		
	SRA577					577		
	SRA617					617		

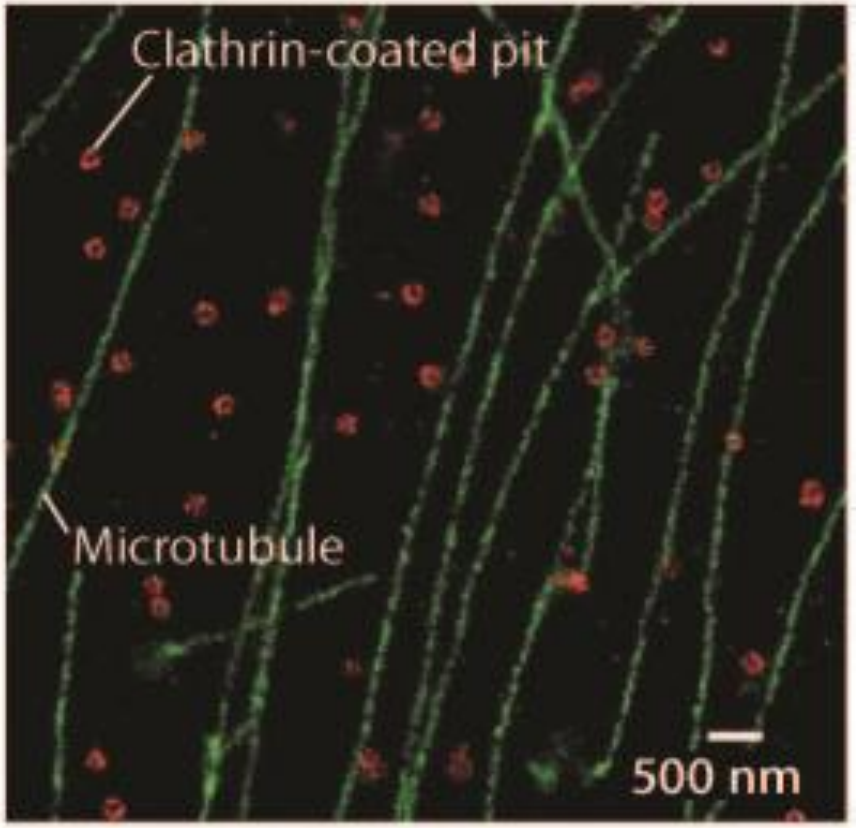
[a] Abbreviations: Em, emission; Ex, excitation; FP, fluorescent protein; NF, nonfluorescent.
[b] Information available from Evrogen.
[c] Information available from Invitrogen.
[d] Commercial product of reactive fluorophore discontinued.
[e] Depending on the attached activator dye.
[f] Thermal relaxation to the dark state.

STORM images

Confocal Fluorescence Image



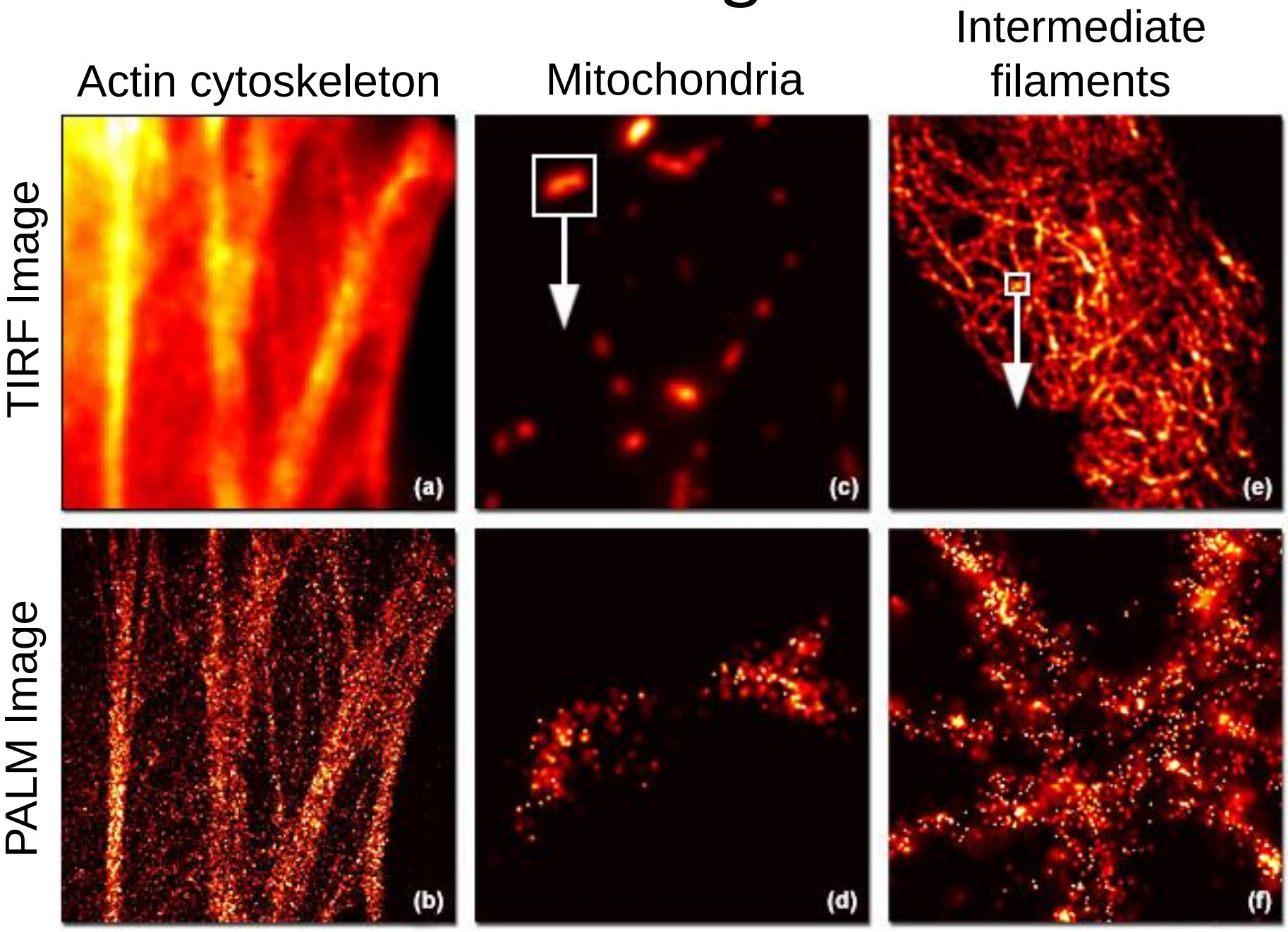
STORM Image



Bates et. al. (2007) Science, 317, 1749

Huang et. al. (2008) Science, 319, 810

PALM images

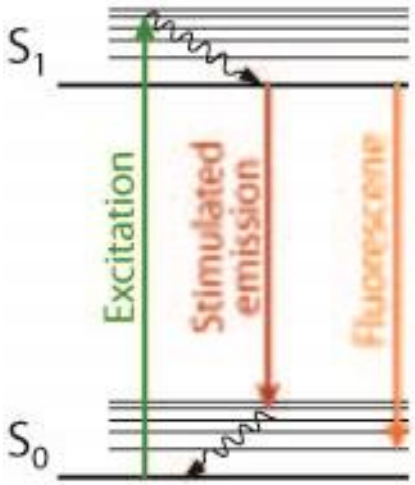

Actin cytoskeleton
Mitochondria
Intermediate filaments
TIRF Image
PALM Image
(a)
(b)
(c)
(d)
(e)
(f)

Section 2

SUPER RESOLUTION MICROSCOPY BY SPATIALLY PATTERNED EXCITATION

Stimulated Emission Depletion Microscopy

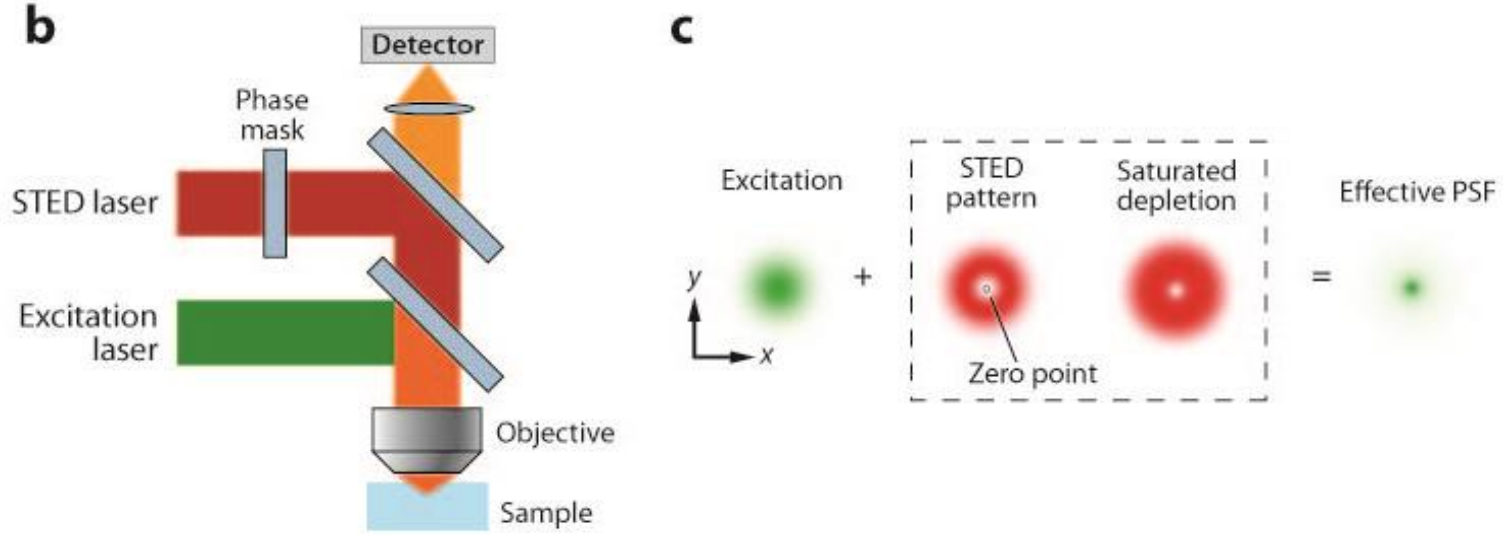
A ground state (S_0) fluorophore can absorb a photon and jump to the excited state (S_1). Spontaneous fluorescence emission brings the fluorophore back to the ground state. Stimulated emission happens when the excited-state fluorophore encounters another photon with a wavelength comparable to the energy difference between the ground and excited state. This process effectively depletes excited-state fluorophores capable of fluorescence emission



Science 2007 316:1153

Stimulated Emission Depletion Microscopy

To sharpen excitation PSF, the STED laser has a pattern with zero intensity at the center of the excitation laser focus and nonzero intensity at the periphery. However, this spatial pattern also limited by the diffraction of light.

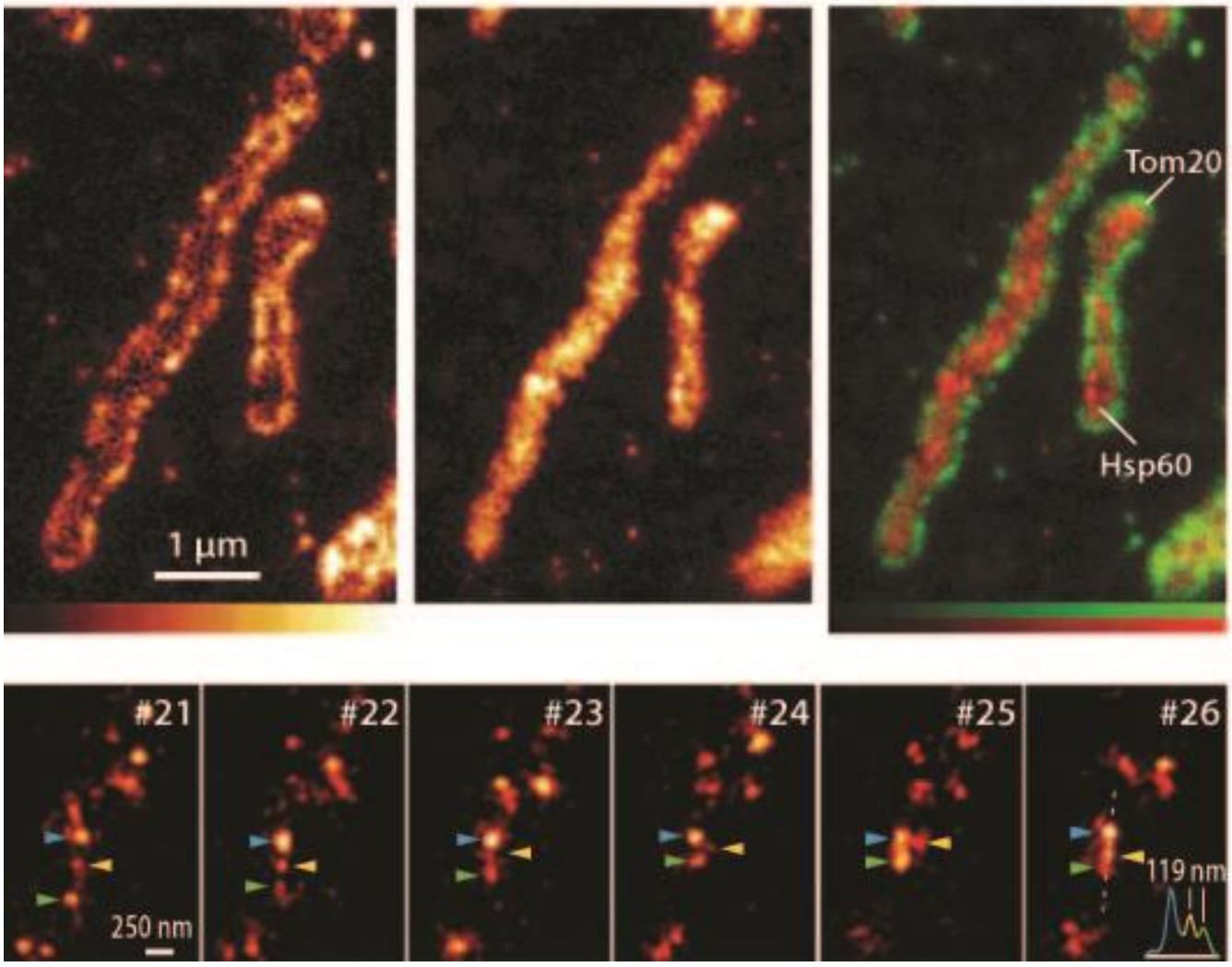


The dependence of depleted population on the STED laser intensity is non-linear when the saturated depletion level is approached. By raising the STED laser power, the saturated depletion region expands without strongly affecting fluorescence emission at the focal point. The size of the effective PSF is

$$\Delta_{eff} \approx \frac{\Delta}{\sqrt{1 + I/I_S}}$$

Science 2007 316:1153

2D & 3D STED images



Schmidt et. al. (2008) Nature Methods, 5, 539
Westphal et. al. (2008) Science, 320, 246