SCIENCES SORBONNE UNIVERSITÉ

Super resolution microscopy in life sciences

Imaging beyond the diffraction limit

Philippe GUILLAUD – 2021

M2 Informatique – Imagerie - BIOMED

- Introduction and concepts
- Methods (SIM, PALM/STORM, STED/gSTED)
- Available systems (OMX, LEICA, ZEISS, NIKON)
- Examples

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Introduction

- Since the first studies of biological structures by early pioneers of microscopy like R. Hooke and A. van Leeuwenhoek in the 17th century, technical developments have led to greatly improved image quality but were faced with a limit in optical resolution.

- Based on experimental evidence and basic principles of physics, E. Abbe and Lord Rayleigh defined and formulated this diffraction limited resolution in the late 19th century.

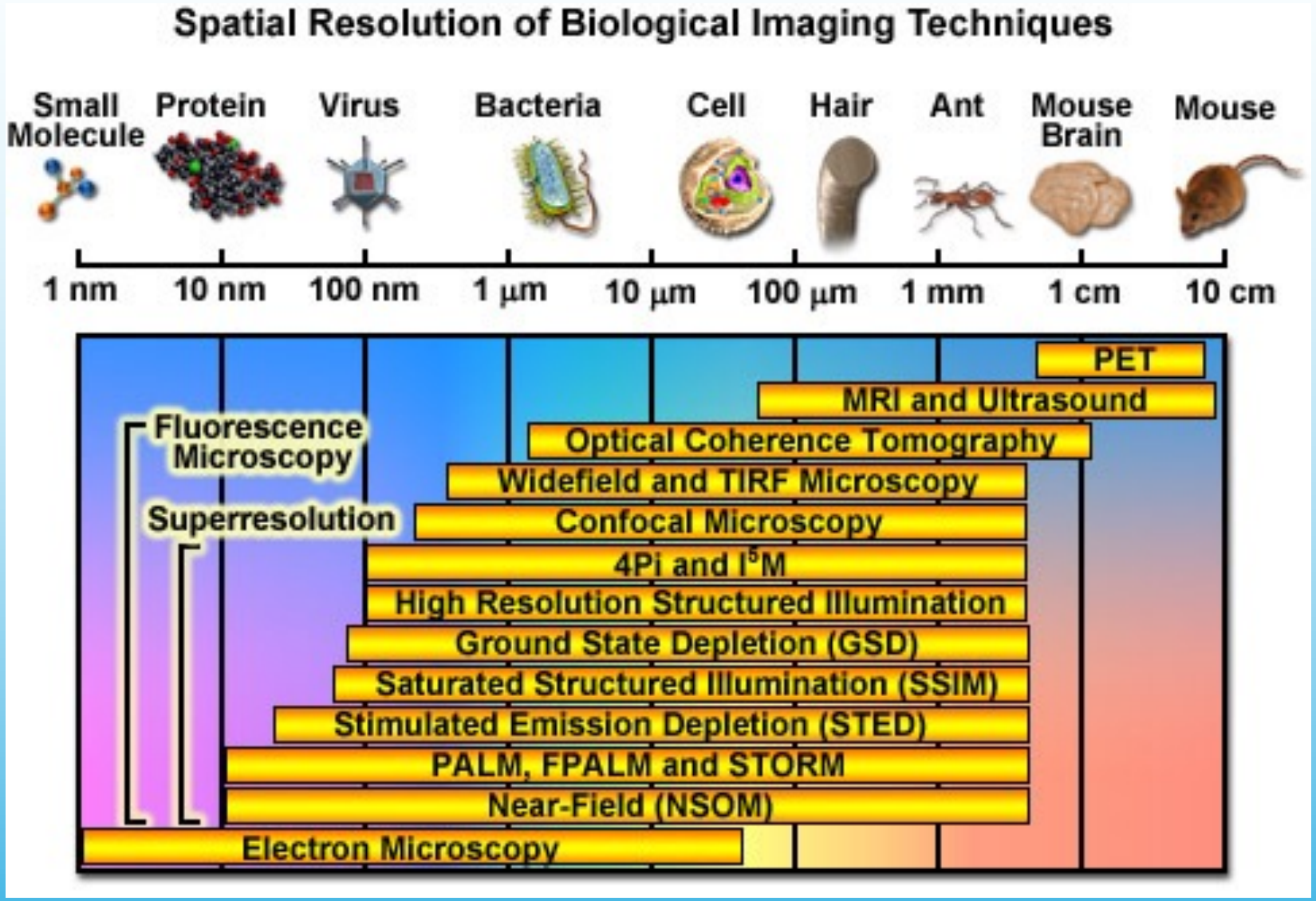
Introduction

- Proposed in 1882 by Ernst Abbe: **$d = \lambda/2NA$**

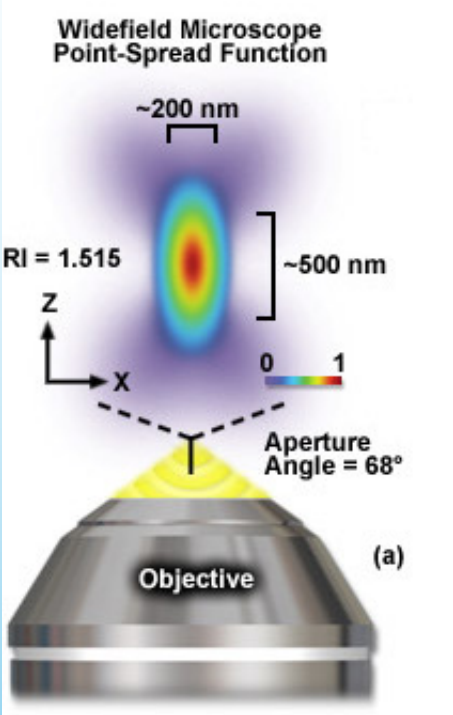
- d represents the diffraction limit of an optical microscope which defines the ability to distinguish between two closely spaced objects in the image plane (around 200 nm)

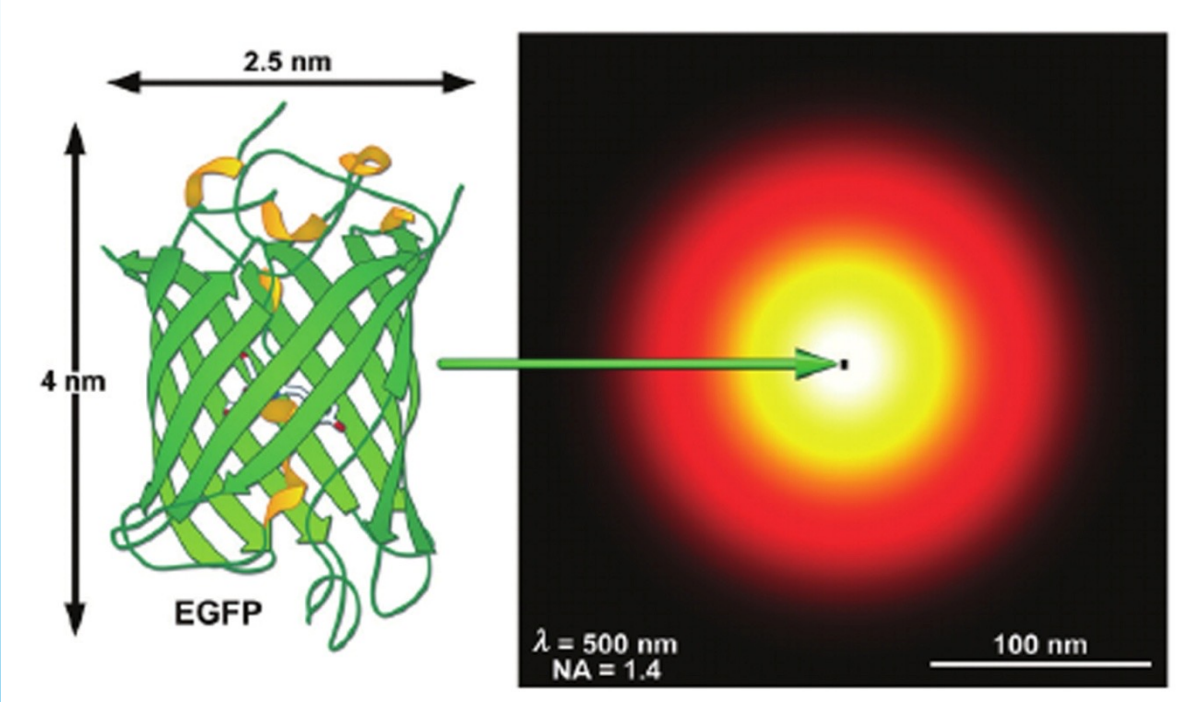
$\Rightarrow$ Much of the fundamental biology of the cell, however, occurs at the level of macromolecular complexes in the size range of tens to few hundreds nm.

Introduction



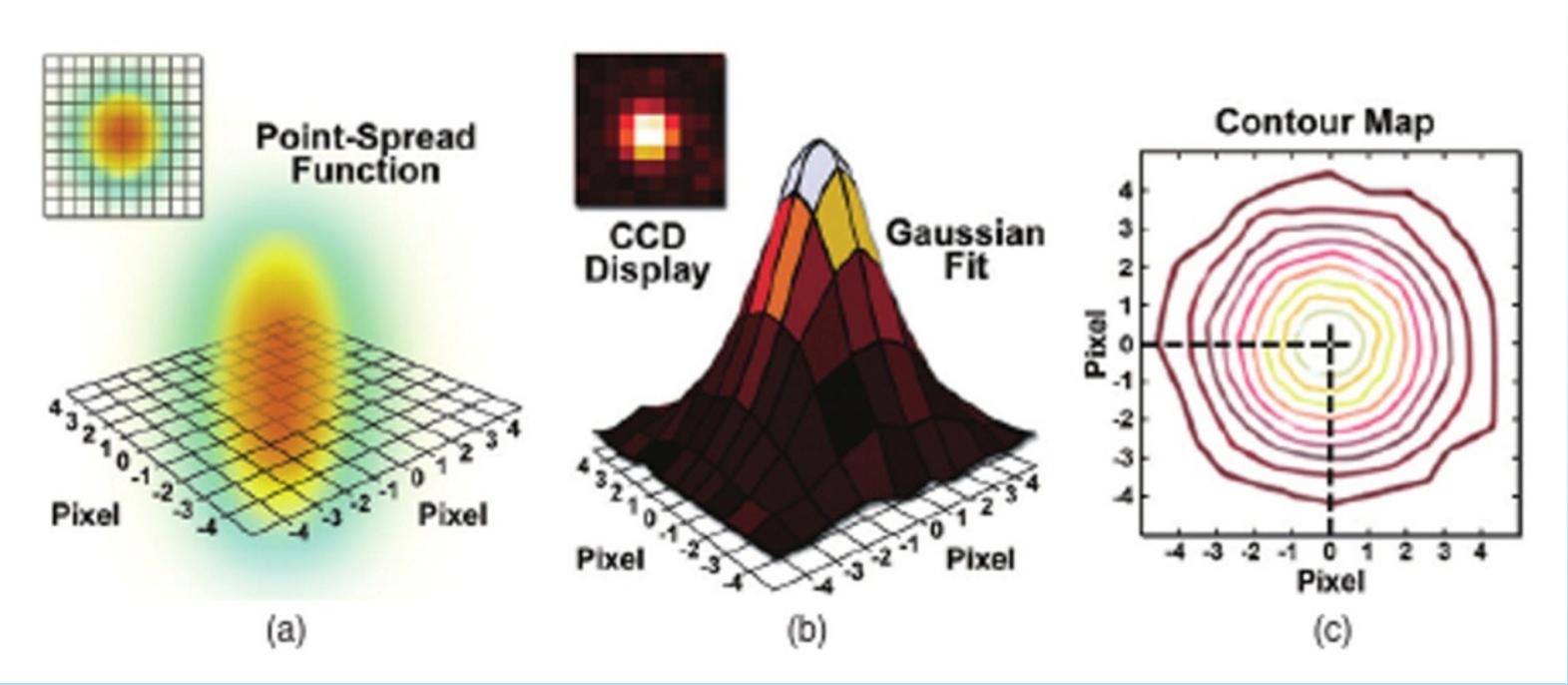
Introduction





The Point Spread Function (PSF) describes how a point-like object is spread out in the image

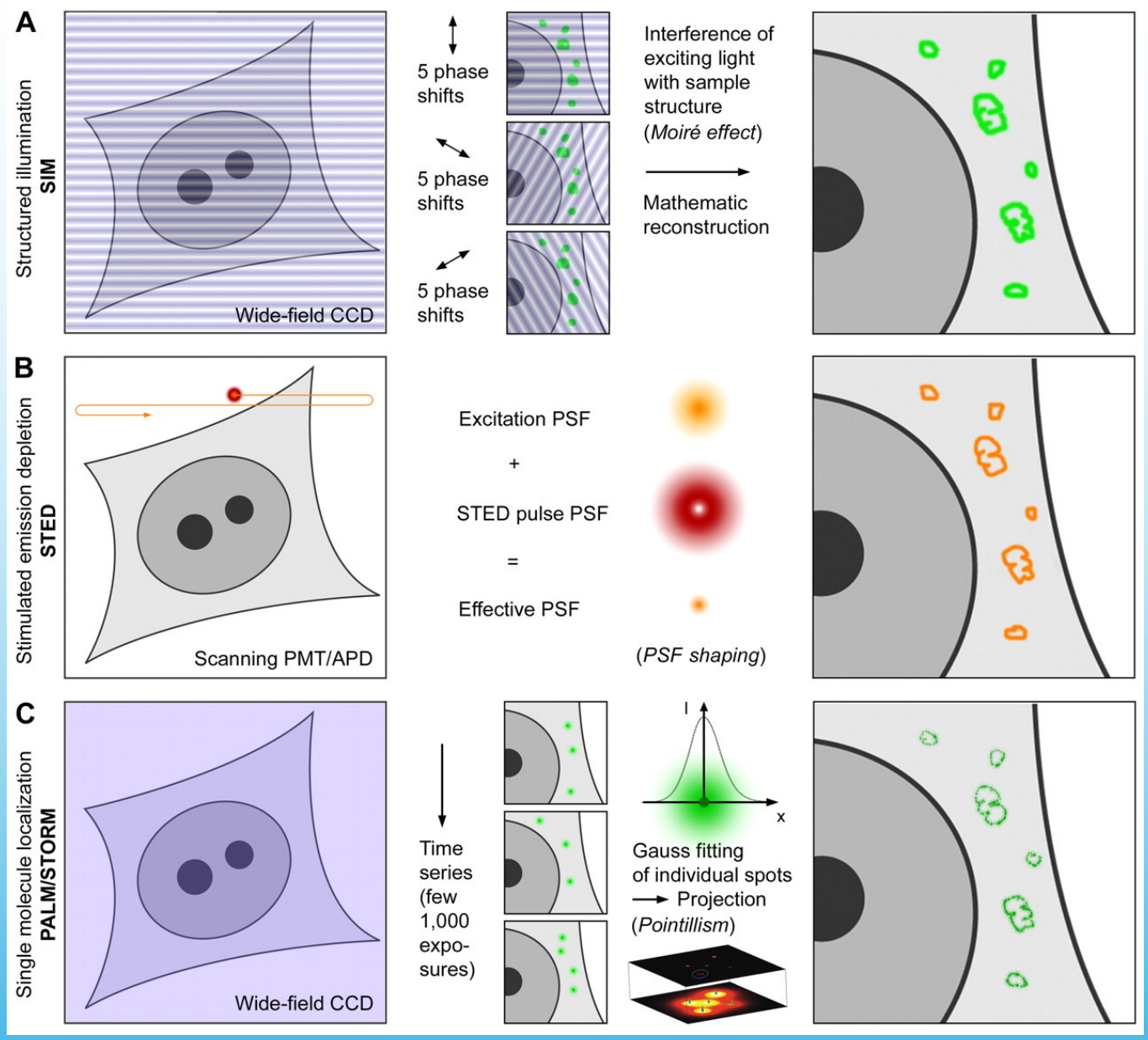
Introduction



The Point Spread Function (PSF) can be mathematically fitted to Gaussian distribution

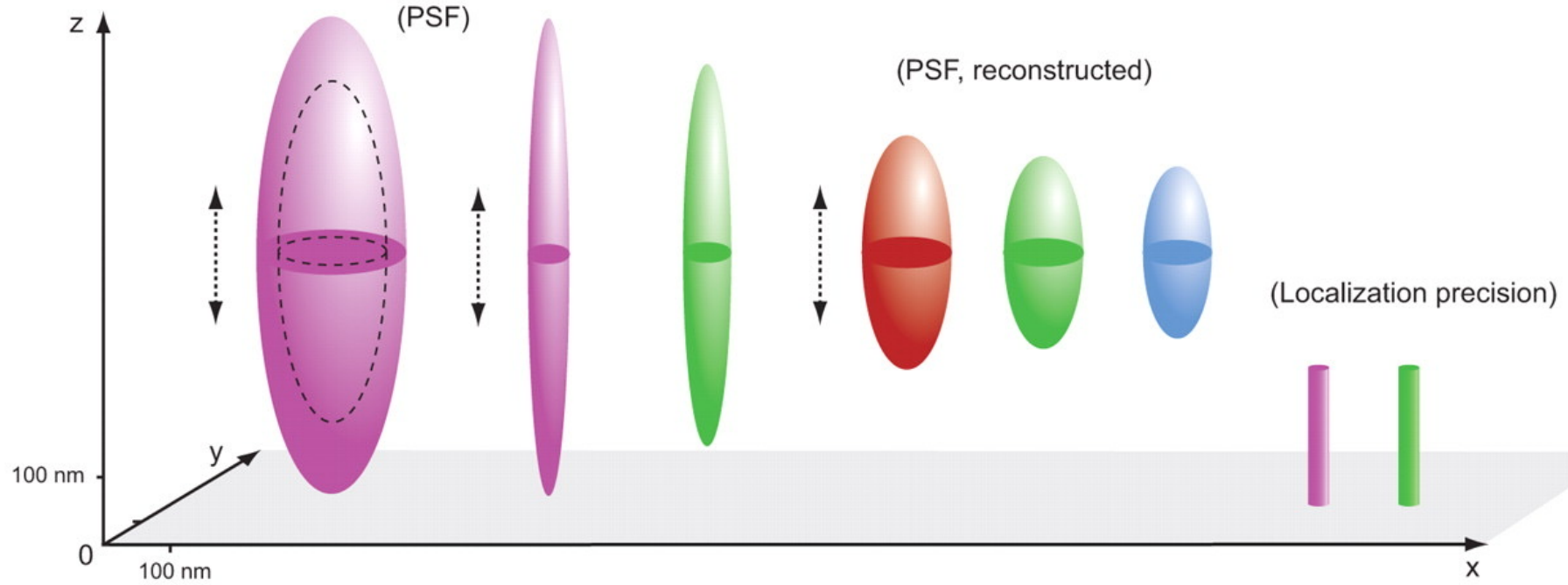
$\Rightarrow$ Methods to affine the Gaussian fit of the PFS in order to obtain a more precise detection of the object's centroid (Deconvolution, FIONA, SIM, PALM/STORM, STED)

Introduction



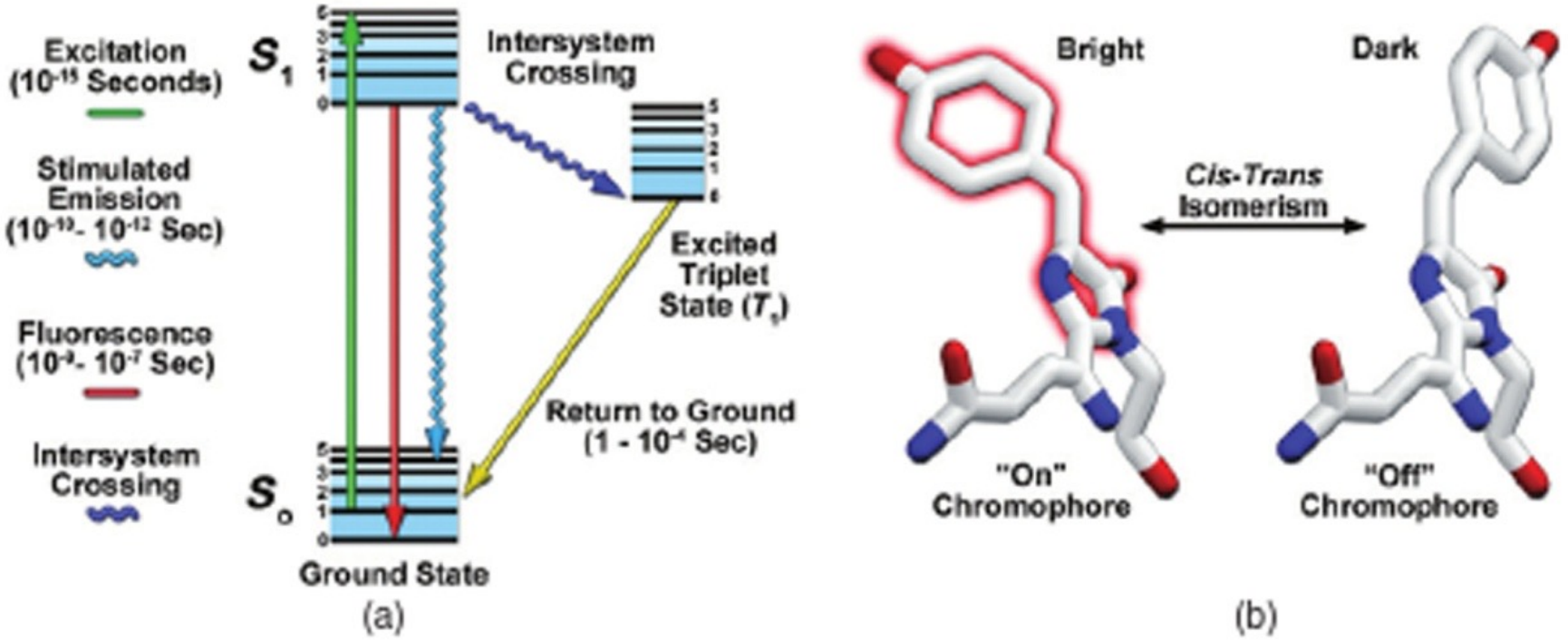
Introduction

	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520



~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Introduction



RESOLFT concept: Reversible Saturable Optical Fluorescence Transitions, described by S. Hell and colleagues in the early 2000

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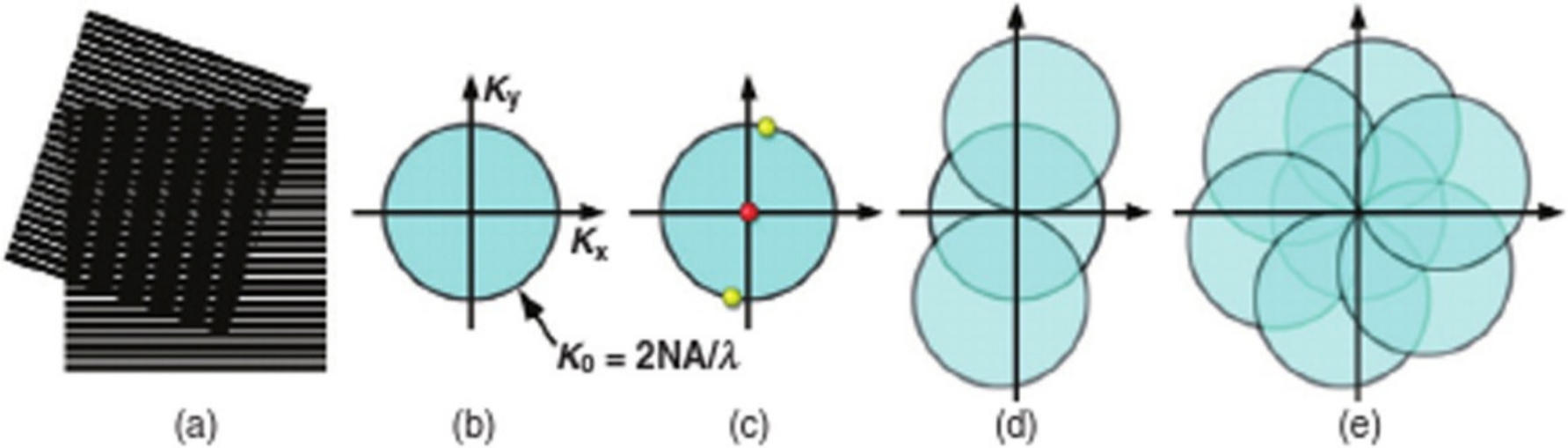
Methods

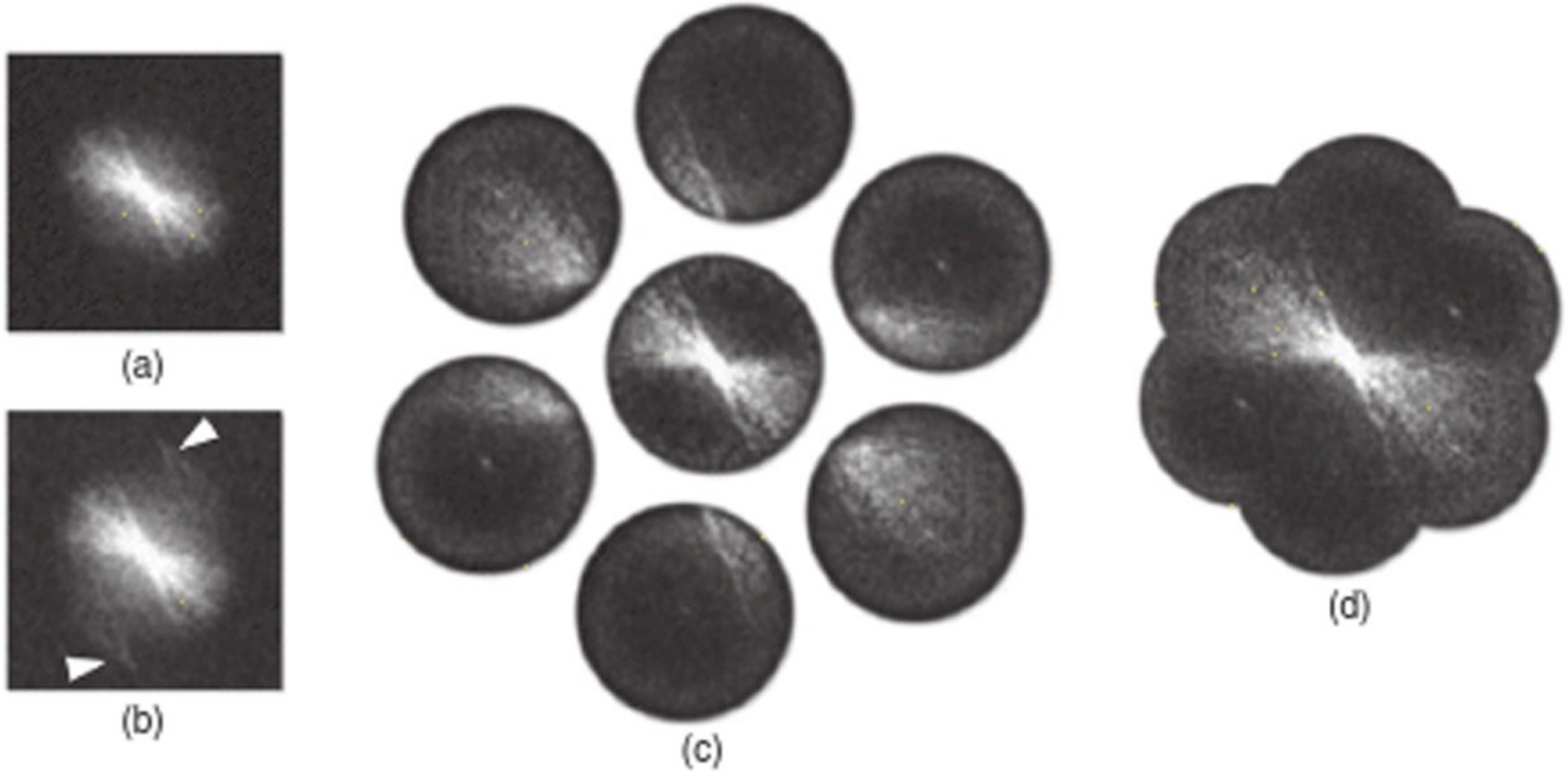
- **STED**: Stimulated Emission Depletion Microscopy (1994-2000).
- **SIM**: Structured Illumination Microscopy (2000-2007).
- **FIONA**: Fluorescence Imaging with One nanometer accuracy (2003).
- **PALM**: Photoactivated Localization Microscopy (2006).
- **STORM**: Stochastic Optical Reconstruction Microscopy (2006).

Structured Illumination: SIM



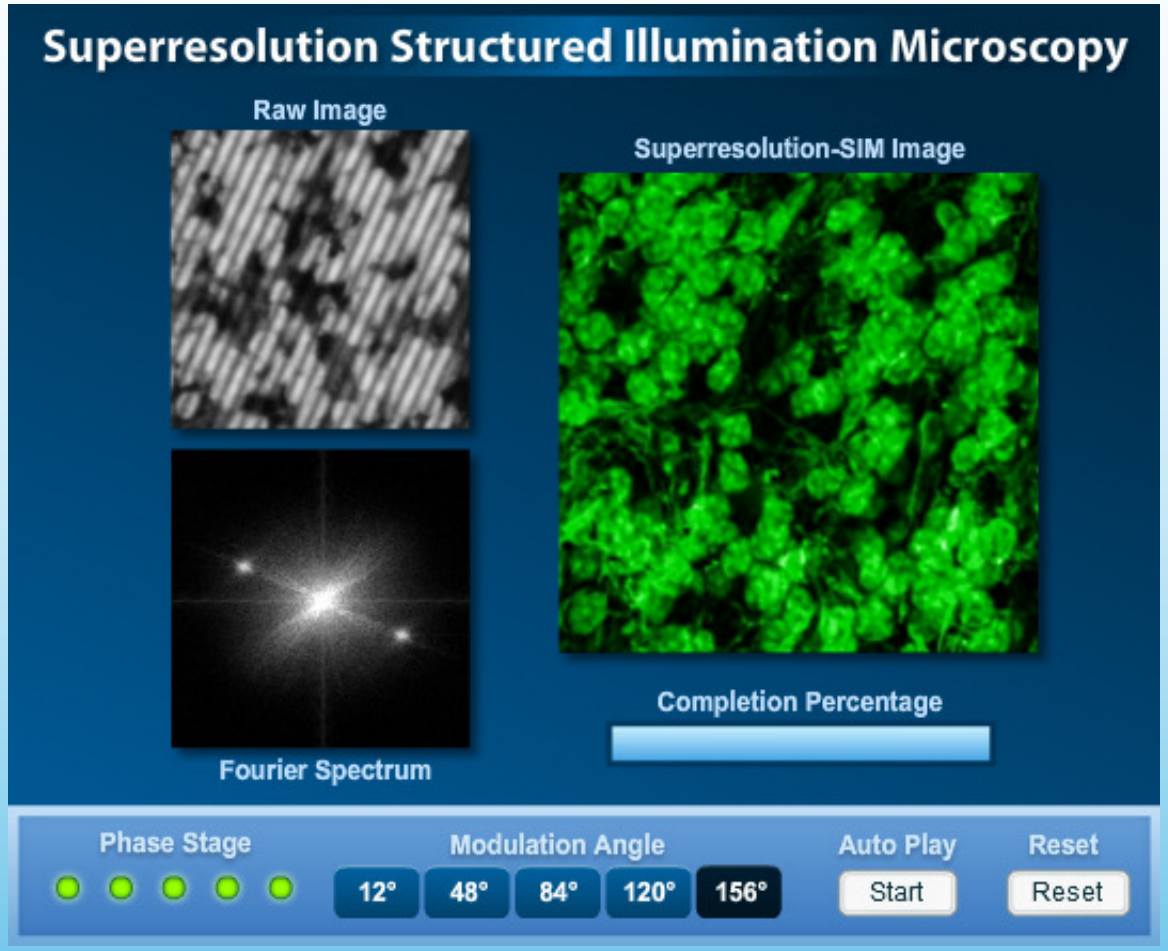
Structured Illumination Microscopy (SIM) The technique involves projecting a series of sinusoidal 'high frequency' striped patterns (optical grating) onto the specimen. Moiré fringes containing information relating to the specimen's sub resolution structure develop when this pattern illuminates finer labeled structures of the sample. This information is extracted by image processing algorithms and a super resolution image is formed by combining multiple images collected from different grating orientations. With SIM, one can expect to roughly increase the resolving power by a factor of two (~100 nm). As the technique is not reliant on the properties of the fluorescent probe and does not requires special sample preparation, it is possible to image most fluorescent labels.

Structured Illumination: SIM

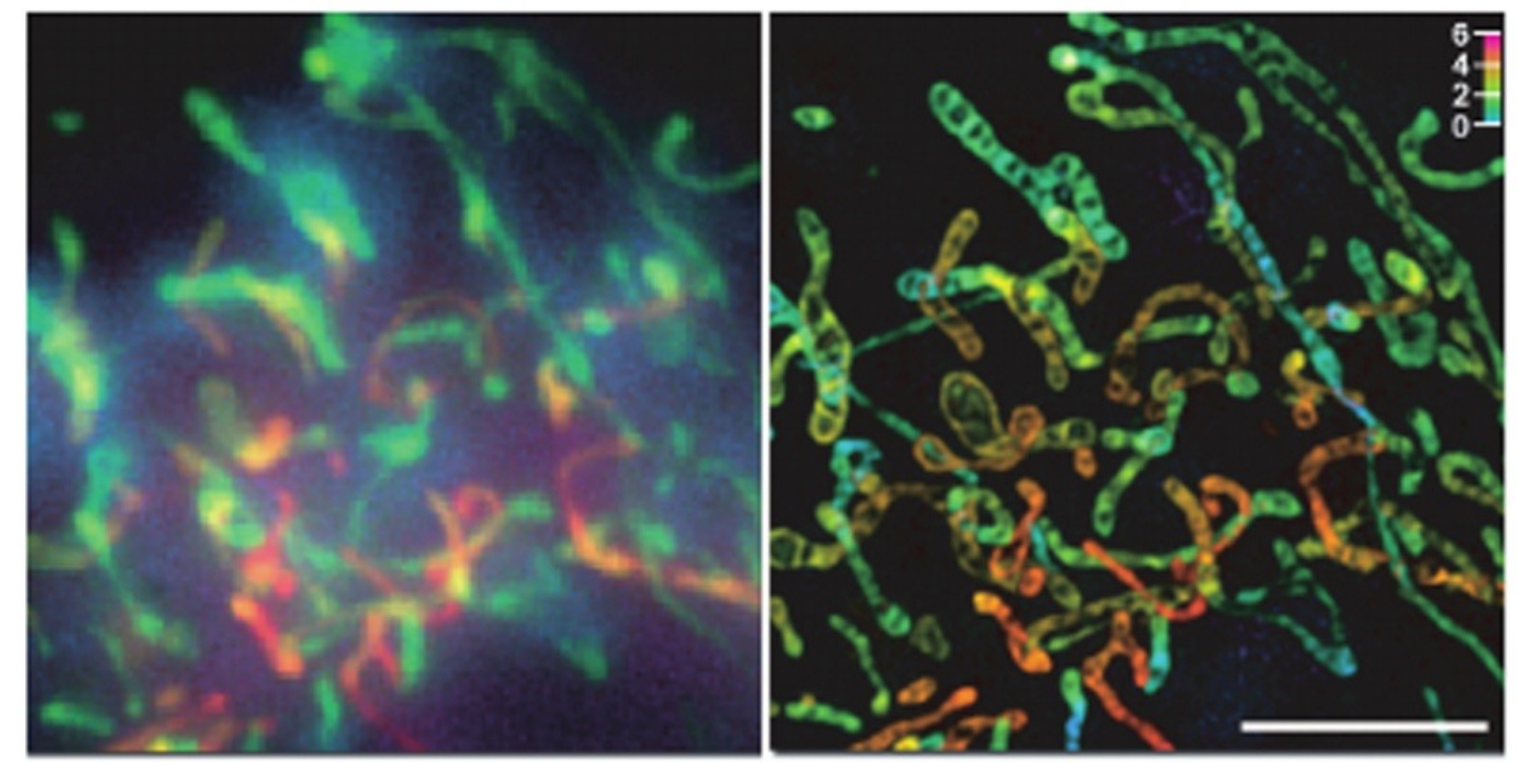


The procedure of reconstructing high frequency information in reciprocal space. Fourier transforms of a microscope image without (a) and with (b) structured illumination. Information is contained in the "observable region" in both cases, but in panel b, moiré effects have displaced high frequency information (arrowheads) and carried it into the observable region. (c) Seven components shown separately as calculated from nine images taken at three azimuthal rotations of the grid pattern. (d) Reconstruction of the image transform extends the visible region outwards by a factor of 2 compared with the original image shown in panel a, allowing a twofold enhancement in resolution.

Structured Illumination: SIM

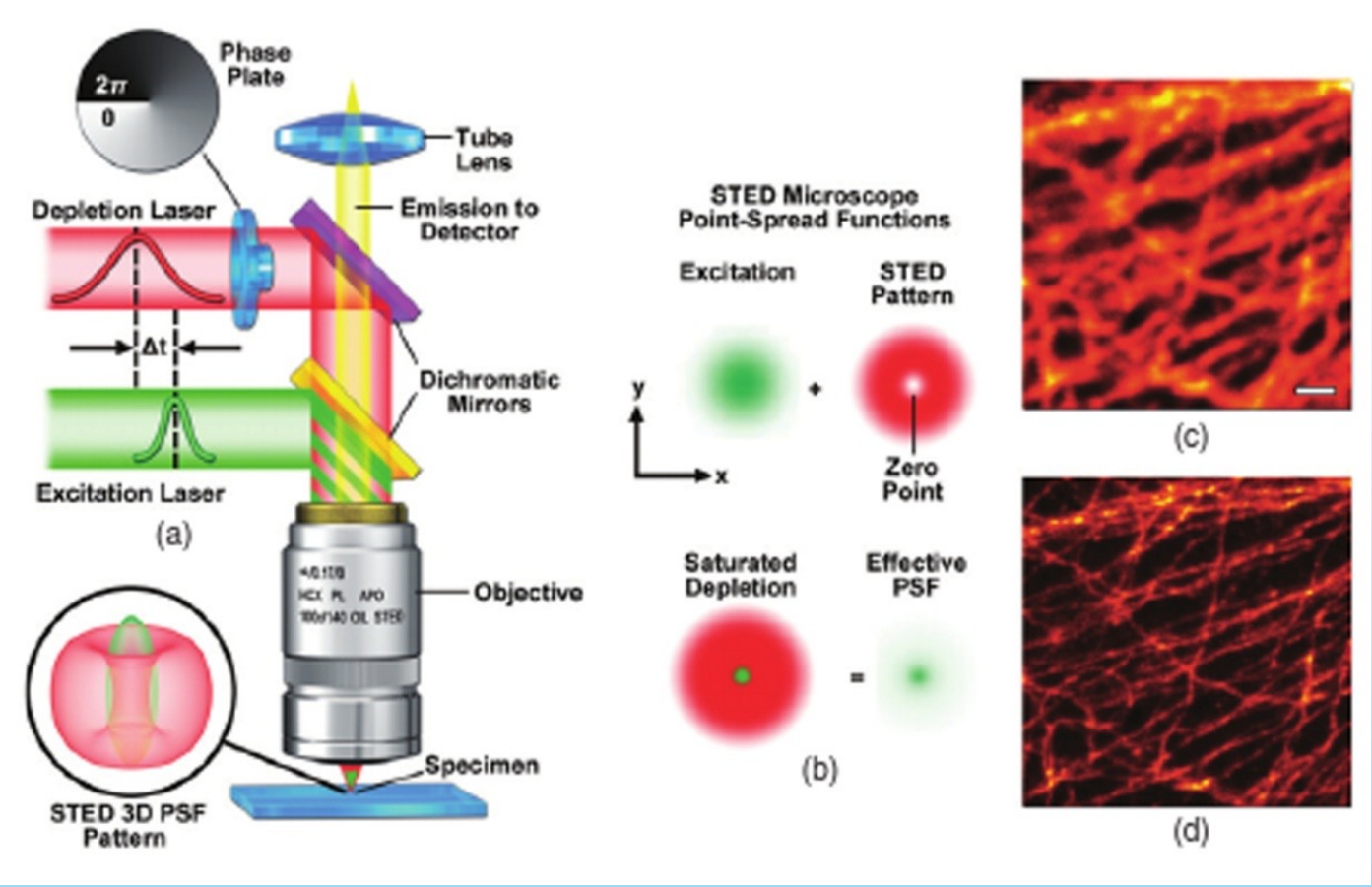


Structured Illumination: SIM



Mitochondria

Stimulated Emission Depletion: STED



STED (Stimulated Emission Depletion) microscopy: In conventional point-scanning confocal microscopy, photons in the excitation laser beam (diffraction limited in size) cause electrons of the dye molecule to become excited from the ground state to a higher energy level. Within a few nanoseconds, before these electrons have chance to relax and emit a photon (the basis of fluorescence), a second red-shifted doughnut-shaped laser beam centered on the same excitation spot, is applied. This second beam drives excited electrons, except for those located in the center of the doughnut, back to their ground state by stimulating emission of a photon of the same wavelength. Thus, molecules located in the hole can fluoresce normally whereas those surrounding cannot. By increasing the power of the depleting laser, the effective diameter of the hole is reduced and with it, the size of the spot from which molecules are allowed to fluoresce.

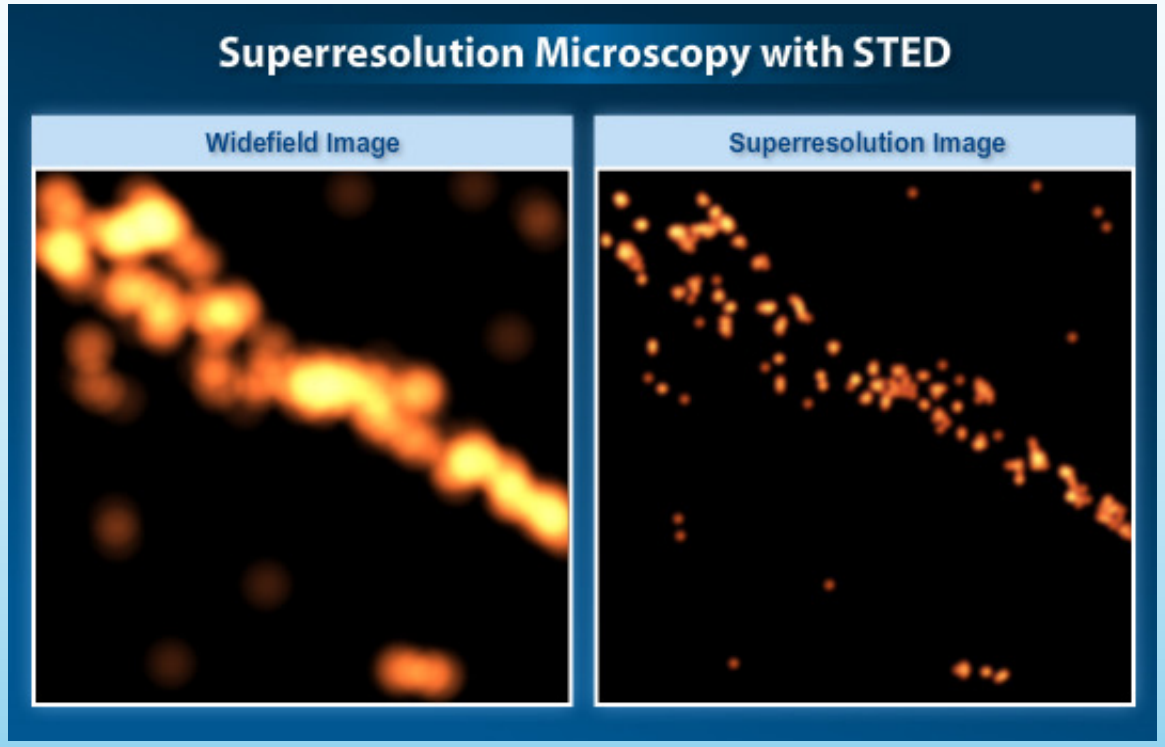
Stimulated Emission Depletion: STED

TABLE 15.2 Fluorescent Probes for STED Microscopy

Probe Name[a]	Ex[b] (nm)	Pulse Width[c] (ps)	STED[d] (nm)	Pulse Width[e] (ps)	Resolution[f] (nm)
Synthetic dyes					
ATTO 425	440	130	532	1,000	70–80
Alexa Fluor 488	488	CW	592	CW	<60
ATTO 532	488	100	615	200	60–70
ATTO 565	532	90	640	90	30–40
Alexa Fluor 594	570	90	700	90	60
ATTO 590	570	90	700	90	30–40
ATTO 633	635	100	750	200	30–40
Fluorescent proteins					
EGFP	490	100	575	200	70
EYFP	490	100	598	300	70
Citrine	490	100	598	300	50

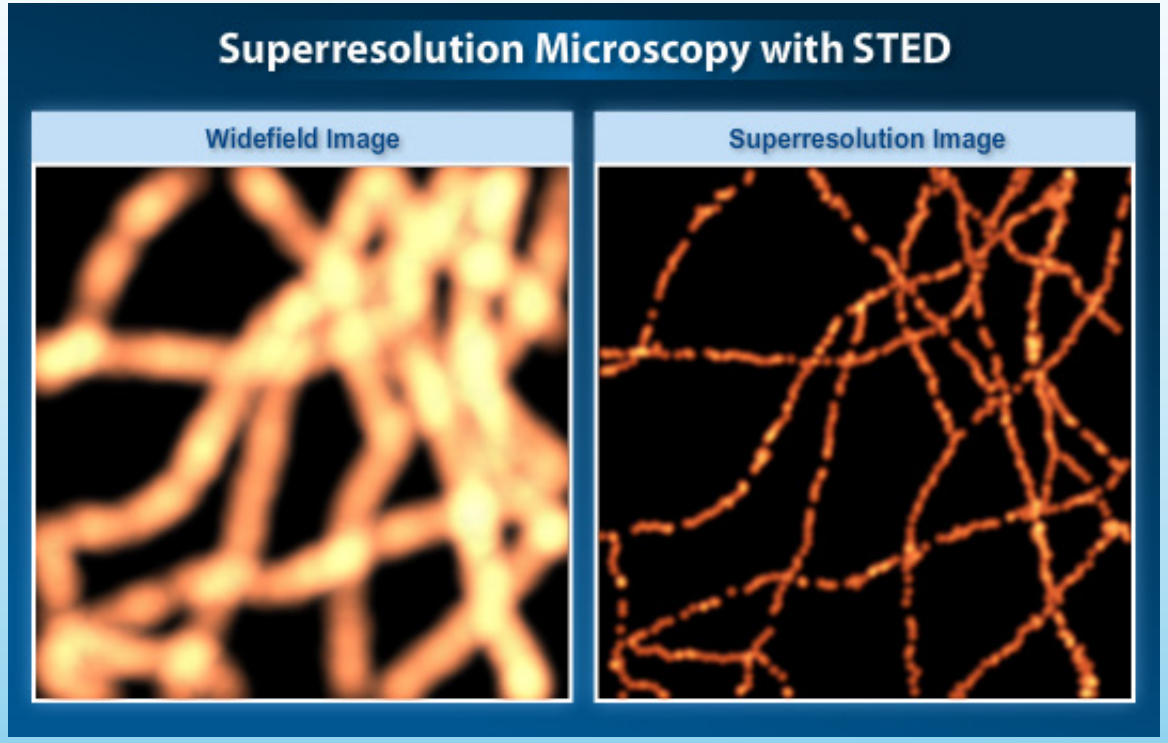
[a]Synthetic fluorescent dyes and fluorescent proteins are listed with respect to their [b]excitation maxima, [c]optimum pulse width, [d]depletion beam wavelength, [e]pulse width of the depletion beam, and [f]resolution. CW, continuous wave laser.

Stimulated Emission Depletion: STED



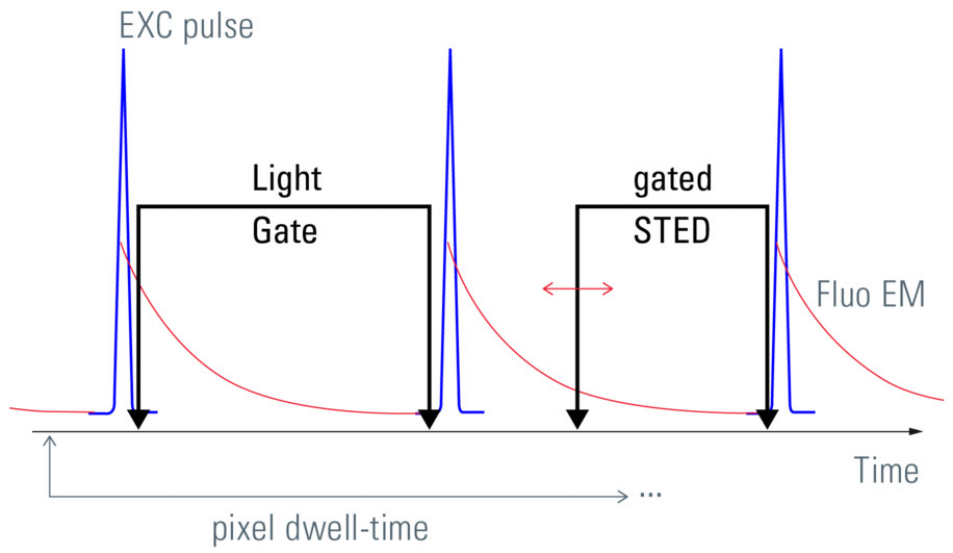
Vesicles

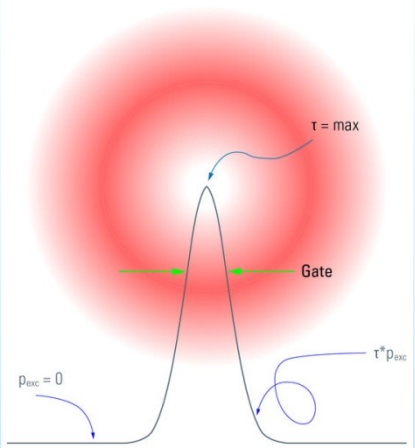
Stimulated Emission Depletion: STED

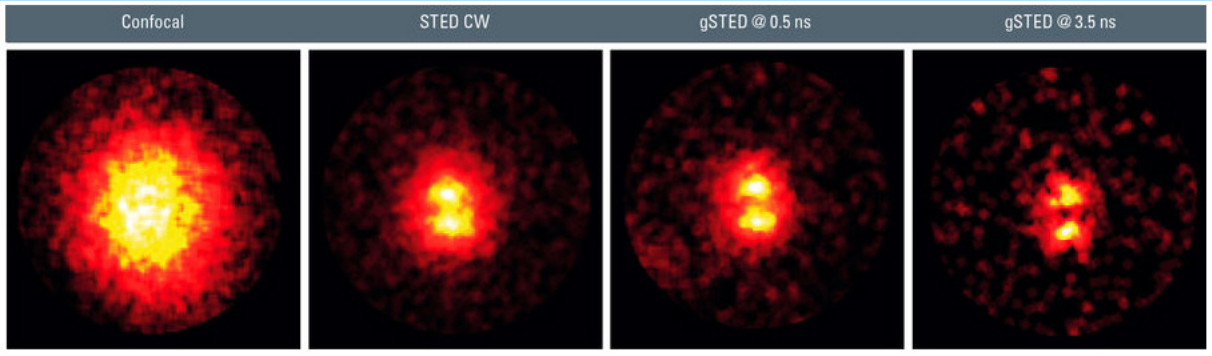


Microtubules

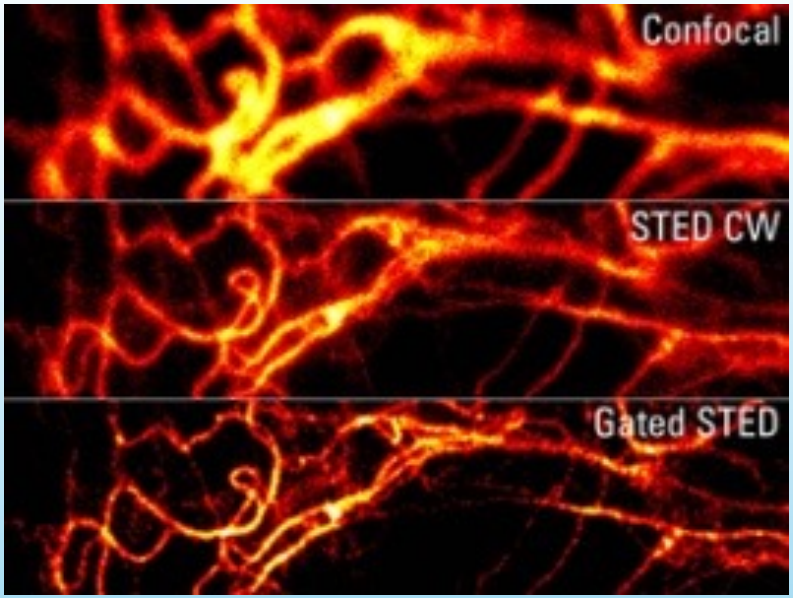
Gated Stimulated Emission Depletion: gSTED



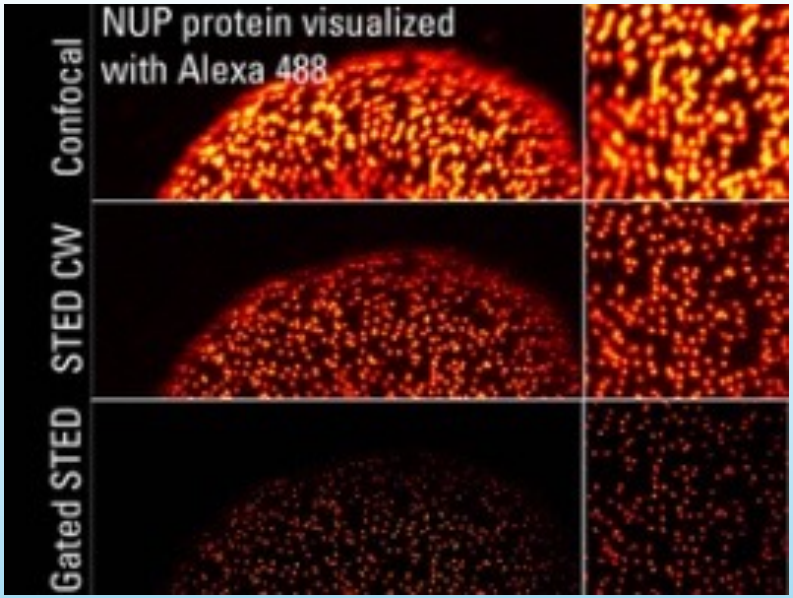




Gated Stimulated Emission Depletion: gSTED

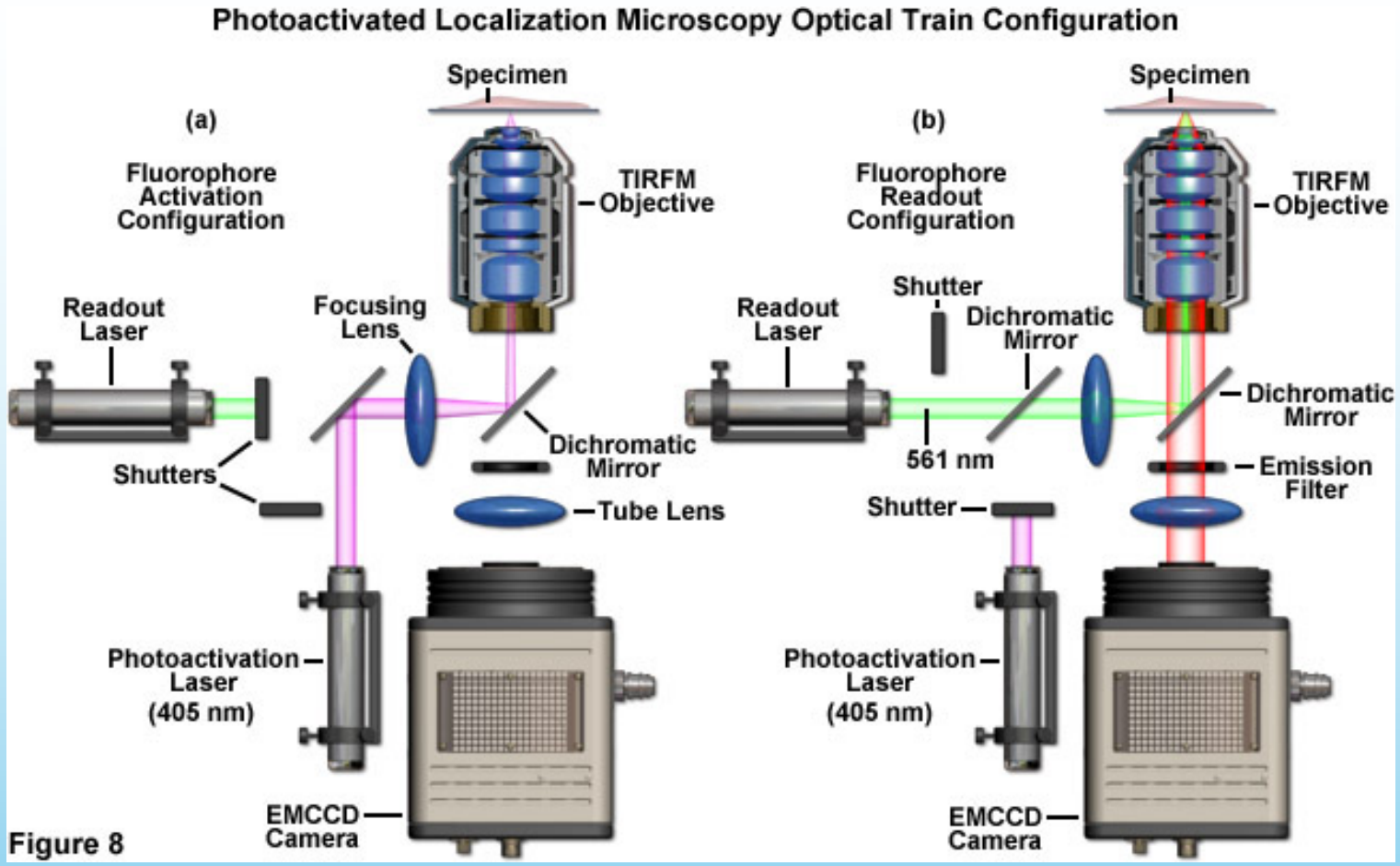


Keratin filaments



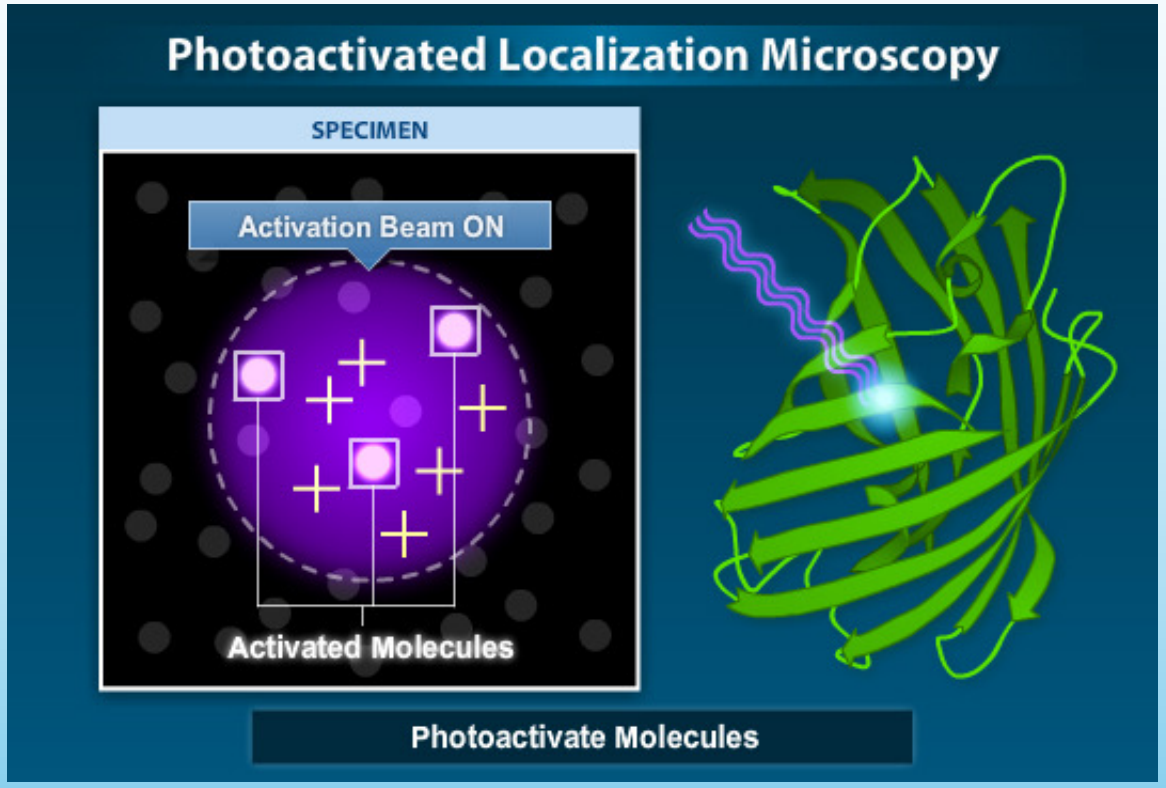
Nuclear protein

Single Molecule localization: PALM/STORM

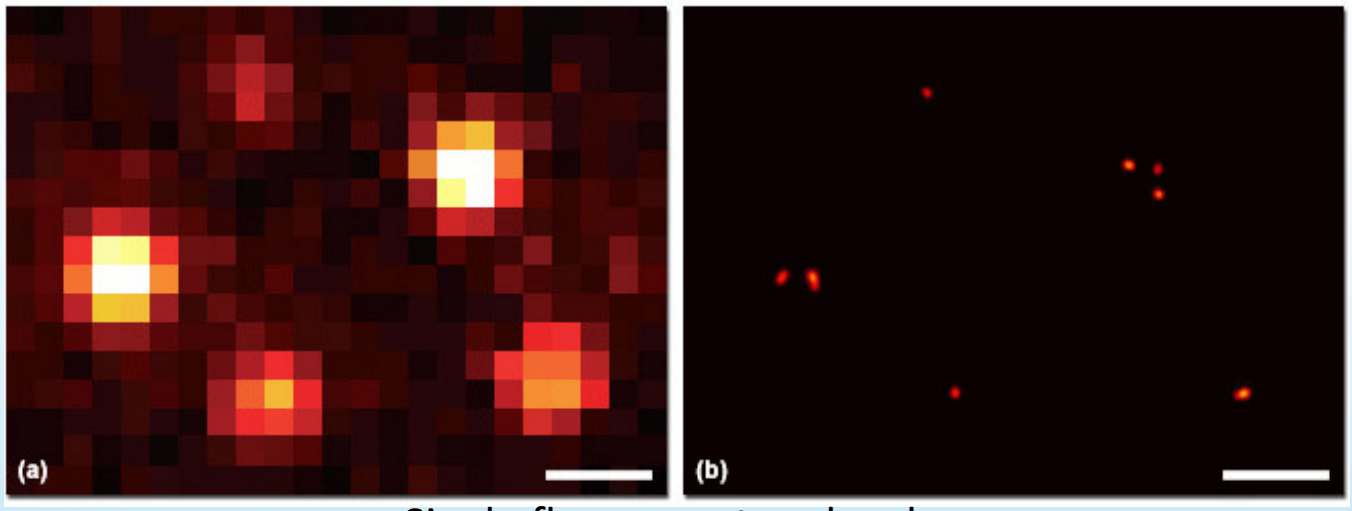


Pointillism microscopy: In PALM (Photo Activation Localisation Microscopy), STORM (Stochastic Optical Reconstruction Microscopy) and GSD microscopy techniques, the resultant image is formed from a number of individual dots; in this case, each dot represent a single fluorescing molecule. These approaches exploit the properties of the fluorophore, in particular its ability to be photoactivated, bleached or photoswitched. The essence of the technique is to switch individual fluorescence molecules on and off and to image them using a camera. The center point of each molecule can then be calculated computationally and its location recorded; the process is repeated hundreds and in most cases thousands of times to form the final image.

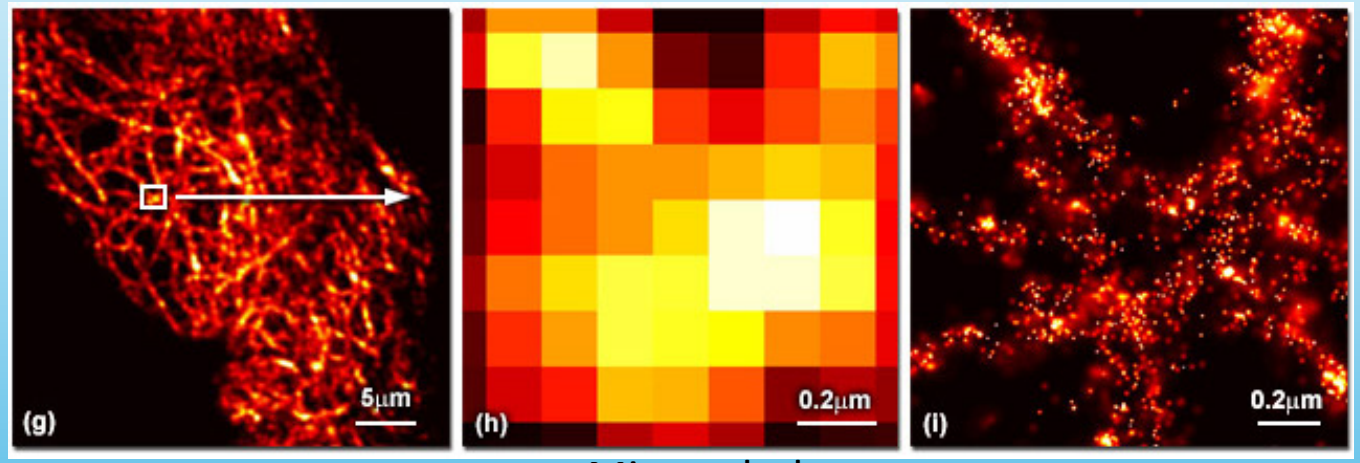
Single Molecule localization: PALM/STORM



Single Molecule localization: PALM/STORM



Single fluorescent molecule



Microtubules

Comparison of the different methods

Table I. **Super-resolution light microscopy methods**

	Near-field		Far-field					
Principle	Small aperture scanning (no lens)	Evanescent wave illumination	Wide-field + deconvolution	Confocal laser scanning	Moiré effect with structured illumination		PSF shaping with saturated emission depletion	Photoswitching and localization of single molecules (pointillism)
Acronym	**SNOM/NSOM**	**TIRFM**		**CLSM**	**SIM** (HELM, PEM) **3D-SIM**	**SSIM** (SPEM)	**STED/CW-STED**	**PALM/FPALM/STORM/ dSTORM/PALMIRA**
Illumination-emission dependence	Linear	Linear	Linear	Linear	Linear	Non-linear	Non-linear	Linear
Detector	Scanning PMT/APD	Wide-field CCD/ CMOS	Wide-field CCD/ CMOS	Scanning PMT/ APD	Wide-field CCD/ CMOS	Wide-field CCD/ CMOS	Scanning PMT/APD	Wide-field CCD/CMOS
XY-resolution	20–120 nm	200–300 nm	180–250 nm	180–250 nm	100–130 nm	50 nm	20–100 nm	20–50 nm
Z-resolution	10 nm (near-field range)	100 nm (near-field range)	500–700 nm	500–700 nm	250–350 nm	N.D.	560 nm (CW-STED) to 700 nm (100 nm with z-phase mask)	100 nm (TIRF) 20–30 nm (3D-STORM, TIRF) 75 nm (BP-FPALM, in plane)
Serial z-sectioning	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Z stack range	N.A.	N.A.	100 µm	100 µm	10–20 µm	N.A.	>20 µm	100 nm – few µm (BP-FPALM)
Dyes	Any	Any	Any	Any	Most conventional dyes (photostable)	Dyes require special characteristics	Dyes require special characteristics (CW-STED works with many conventional dyes)	Dyes require special characteristics
Simultaneous colors	2	3	>3	>3	3	1	2	2
Temporal resolution for 512 × 512 image	s-min	ms	ms	ms-s	ms-s	s-min	ms-min	s-min
Energy load/ intensity	Low	Low	Low	Medium	Medium	High	Medium-high	Medium-high
Live-cell imaging	Yes	Yes	Yes	Yes	Restricted (2D-TIRF)	No	Restricted	Restricted
Postprocessing required	No	No	Yes	No	Yes 9–25 raw images per slice	Yes ~100 raw images per slice	No	Yes >1,000 raw images per slice
Notes	No intracellular imaging	Restricted to region near the coverslip	Risk of artifacts; better for sparse samples		Reconstruction bears risk of artefacts	High excitation required; reconstruction bears risk of artefacts	Complex instrumentation; photobleaching	May require TIRF setup for best performance; labeling density is critical; performs better on particles and filaments as on volume stains
Dual lens implementation			**I^{5}M**	**4Pi**	**I^{5}S**		**4 Pi-STED/iso-STED**	**iPALM**
Z-resolution			70 nm	80 nm	100 nm		20–100 nm	10 nm (depth ~200 nm)

N.A., not applicable; N.D., not described.

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Systems

- Zeiss: Elyra-PS.1 (PALM/BALM/STORM and SIM)
- Leica: STED-CW/gSTED and GSD
- Nikon: STORM and SIM
- Applied Precision: DeltaVision OMX v4 (PALM/BALM/STORM and SIM)




Systems

Leica TCS SP8 with gSTED

Systems

Nikon N-SIM/N-STORM

Systems

Zeiss Elyra PS1

Systems

API OMX v4 Blaze

Systems

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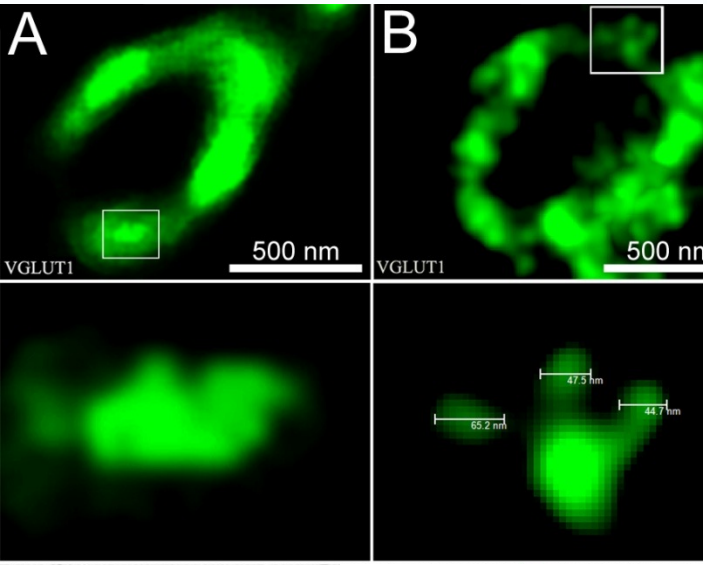
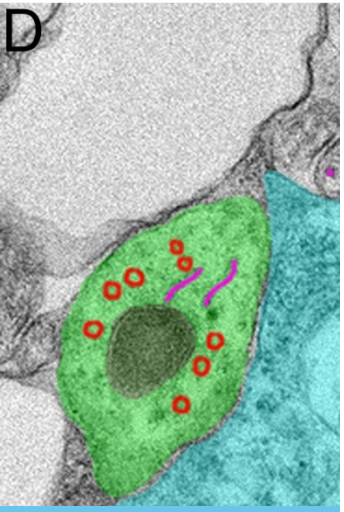
Examples



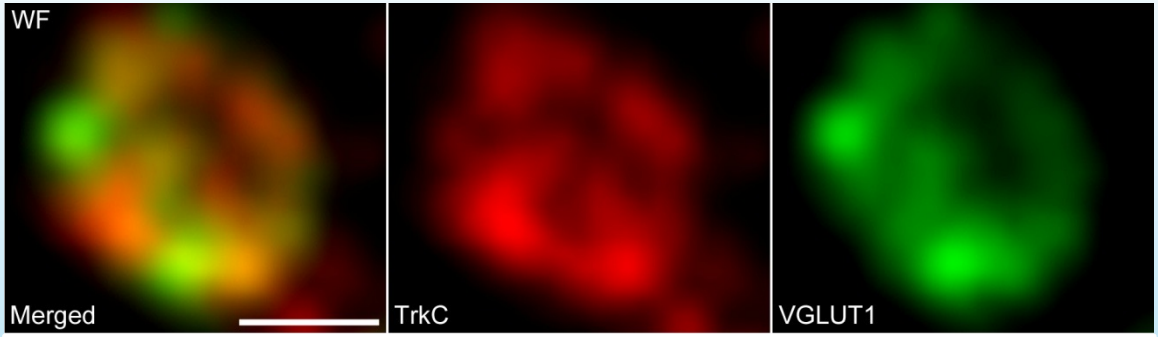


Calyx of Held synapse

Leica MICROSYSTEMS

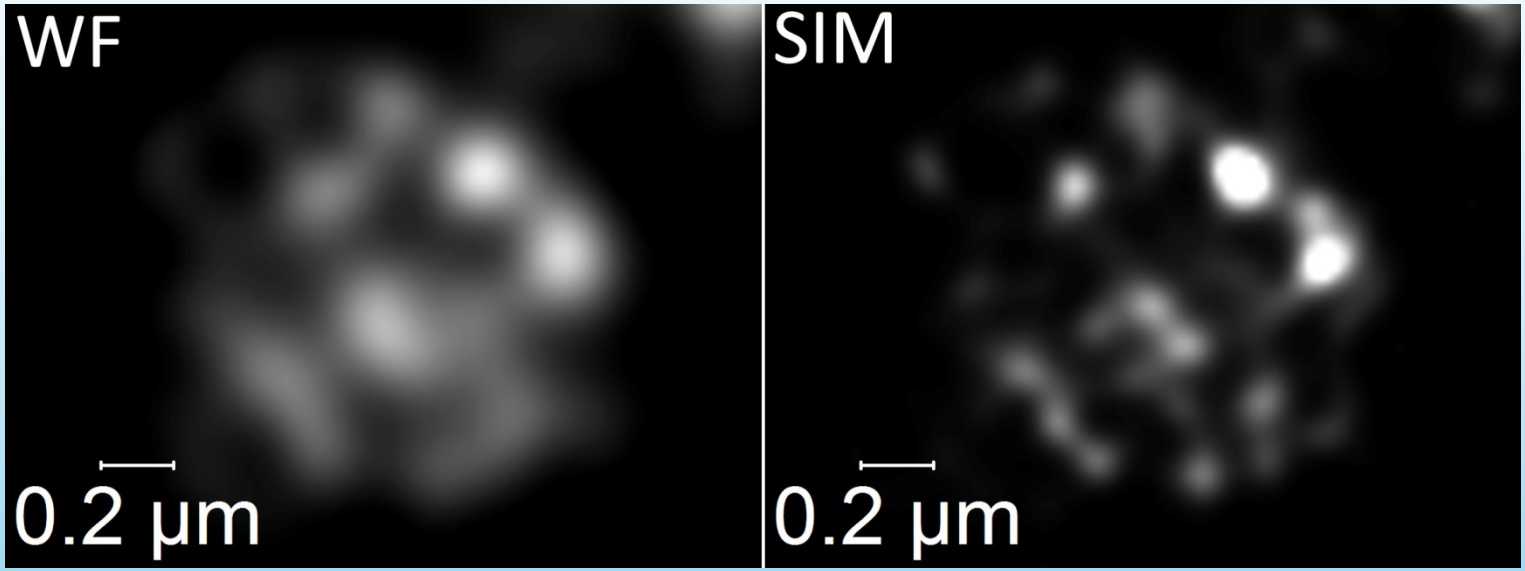
Examples

Calyx of Held synapse



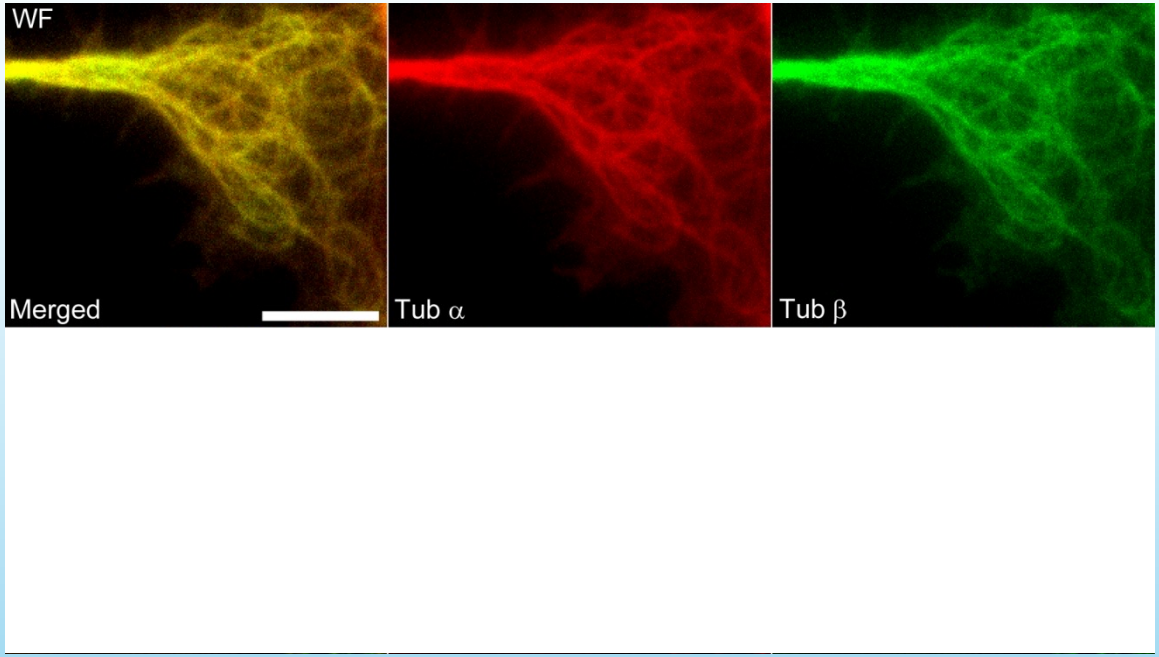
Examples

Calyx of Held synapse

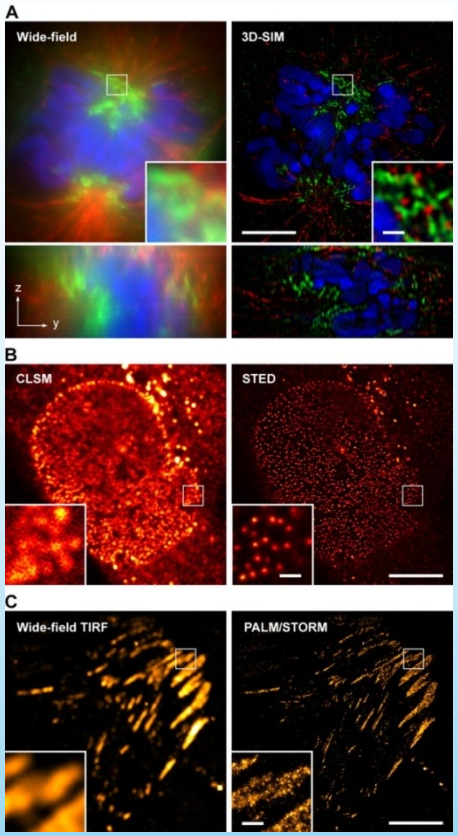


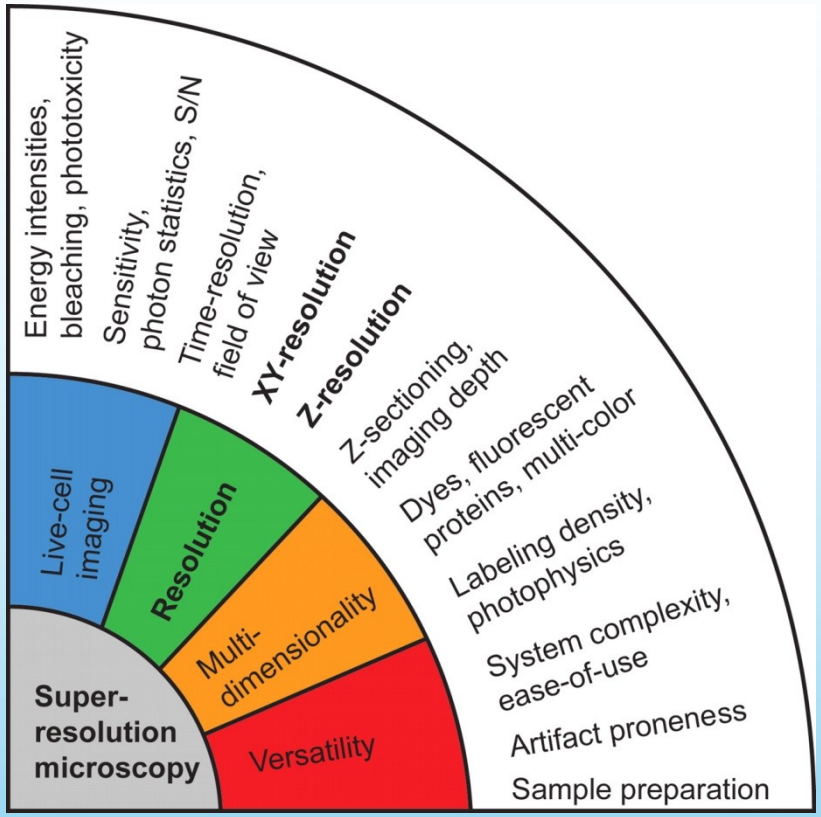
Examples

Neuronal growth cone



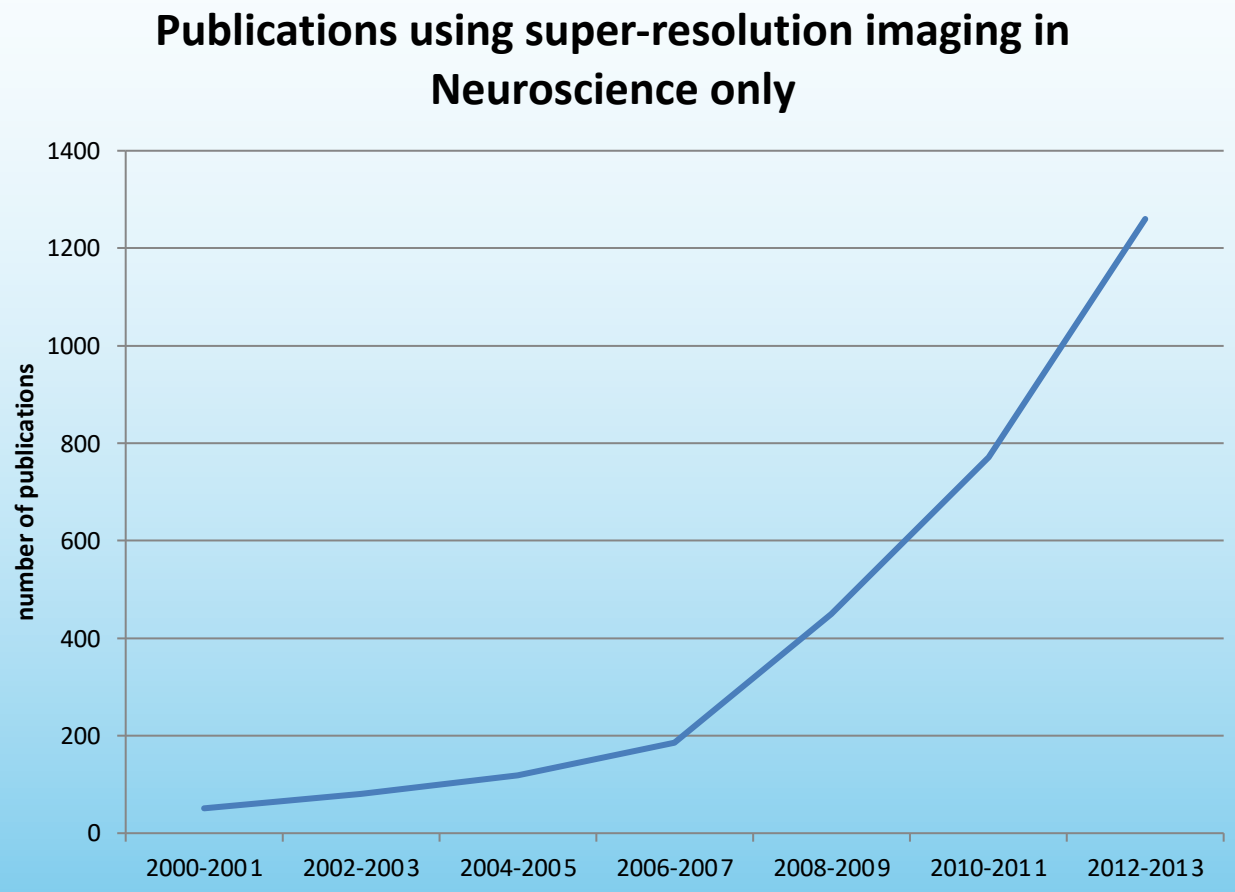
Conclusions





Present trade-offs: At the present time, there is no ideal system available that could combine the highest spatial resolution laterally and axially along with multicolor capabilities and temporal resolution for live-cell imaging.

Conclusions



References

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Far-Field Optical Nanoscopy. *Science 316*: 1153-1158 (2007). An excellent overview of high-resolution fluorescence microscopy techniques that includes discussions of 4Pi, STED, GSD, RESOLFT, SPEM, PALM, and STORM. The author, whose own research in superresolution was instrumental in launching the field, also presents comparative images with the various methodologies.

Patterson, G., Davidson, M., Manley, S. and Lippincott-Schwartz, J.
Superresolution imaging using single-molecule localization. *Annual Review of Physical Chemistry 61*: 345-367 (2010). A review of single-molecule superresolution techniques, including PALM, STORM, and FPALM, including concepts such as contrast ratio and molecular density. The authors discuss the conceptual basis of photoswitch-based imaging and describe the wide variety of fluorescent proteins, synthetic fluorophores, and quantum dots that are excellent candidates for generating images at resolutions of 50 nanometers or less.

Egner, A. and Hell, S. W.
Super-resolved optical sections. *Trends in Cell Biology 15*: 208-215 (2005). A review of high-resolution fluorescence microscopy techniques focused on improvement of axial resolution. In addition to discussions of basic concepts in resolution, the authors also discuss counter-propagating coherent wavefronts and present dramatic three-dimensional reconstructions the Golgi apparatus and mitochondria.

Garini, Y., Vermolen, B. J. and Young, I. T.
From micro to nano: recent advances in high-resolution microscopy. *Current Opinion in Biotechnology 16*: 3-12 (2005). The authors discuss and compare resolution in traditional widefield fluorescence with a variety of high-resolution techniques and attempt to draw comparisons between the different methodologies. Also discussed are interference and structured illumination techniques, nonlinear methods, and high-resolution surface measurements.

References

Gustafsson, M. G. L.

Extended resolution fluorescence microscopy. *Current Opinion in Structural Biology 9*: 627-634 (1999). One of the first comprehensive reviews of high-resolution fluorescence imaging. Described are standing wave microscopy, 4Pi confocal microscopy, and theta, as well as I^5M and structured illumination. Included are several examples of high-resolution imaging and a nice graphical comparison of theoretical resolving powers.

Heintzmann, R. and Ficz, G.

Breaking the resolution limit in light microscopy. *Briefings in Functional Genomics and Proteomics 5*: 289-301 (2006). Targeted in this review are classical and new developments in high-resolution microscopy, and how these methods have been applied in biological research. Among the techniques discussed are widefield fluorescence, confocal, 4Pi, structured illumination, TIRFM, STED, PALM, and STORM.

Hell, S. W.

Toward fluorescence nanoscopy. *Nature Biotechnology 21*: 1347-1355(2003). Filled with superb illustrations and informational text boxes, this comprehensive review article addresses the historical advancements that have made superresolution microscopy a reality. The author thoroughly discusses axial and lateral resolution enhancement techniques and artfully introduces the concept of breaking the diffraction barrier. A large list of pertinent references is included.

Hell, S. W.

Microscopy and its focal switch. *Nature Methods 6*: 24-32 (2009). An excellent review article by one of the foremost pioneers and leaders in the field. Professor Hell provides an overview of the different techniques based on point-spread function engineering and single molecule localization, and discusses how all superresolution methodology shares a common denominator: fluorophore photoswitching.

References

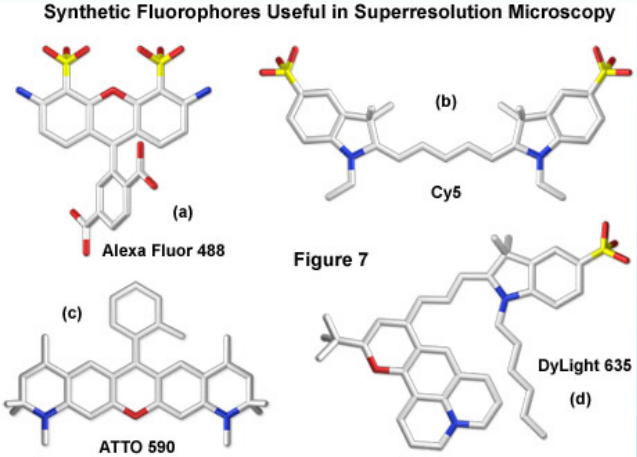
Lippincott-Schwartz, J. and Manley, S.

Putting super-resolution fluorescence microscopy to work. *Nature Methods 6*: 21-23 (2009). Focusing on the practical aspects of using superresolution microscopy to unravel complex biological problems, Drs. Lippincott-Schwartz and Manley carefully describe many of the pitfalls that may be encountered when trying to conduct and interpret images from this new technology.

Huang, B., Bates, M. and Zhuang, X.

Super-resolution fluorescence microscopy. *Annual Review of Biochemistry 78*: 993-1016 (2009). A review article from the laboratory where STORM localization microscopy originated. The authors describe basic concepts in resolution, point-spread function engineering techniques, and single molecule localization microscopy.

Appendix



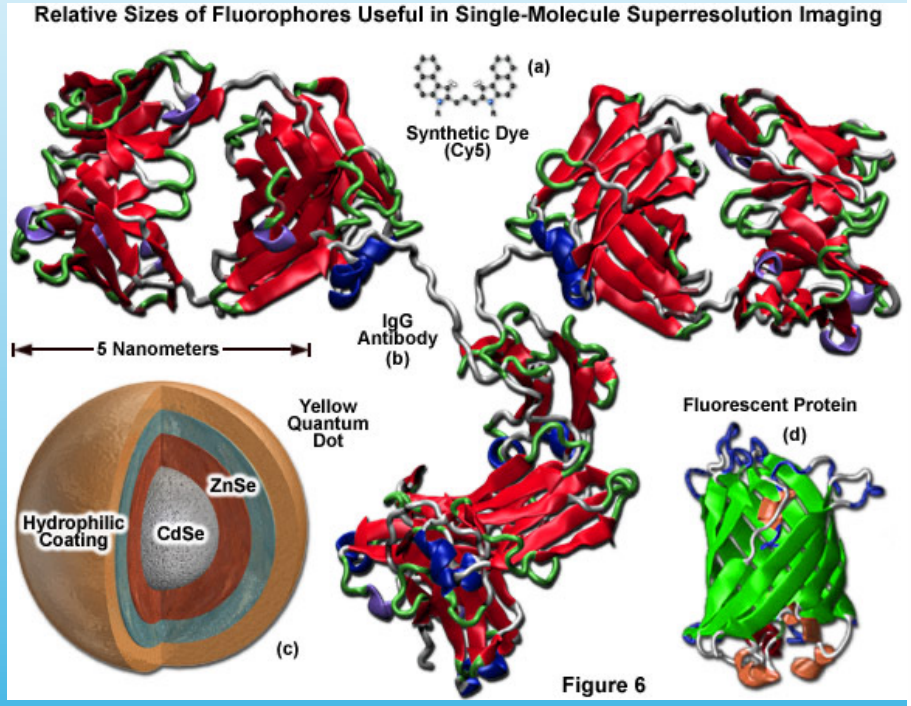


TABLE 15.1 Properties of Fluorescent Probes for Single Molecule Imaging

Probe Name[a]	Ex[b] (nm)	Em[c] (nm)	EC[d] ($\times 10^{-3}$)	QY[e]	N Photons[f]
Photoactivatable fluorescent proteins					
PA-GFP (N)[g]	400	515	20.7	0.13	70
PA-GFP (G)	504	517	17.4	0.79	300
PS-CFP2 (C)	400	468	43.0	0.20	NR
PS-CFP2 (G)	490	511	47.0	0.23	260
PA-mCherry1 (R)	564	595	18.0	0.46	1300
Photoconvertible fluorescent proteins					
tdEos (G)	506	516	34.0	0.66	NR
tdEos (R)	569	581	33.0	0.60	750
mEos2 (G)	506	519	56.0	0.74	NR
mEos2 (R)	573	584	46.0	0.66	500
PSmOrange (O)	548	565	113.3	0.51	NR
PSmOrange (FR)	635	662	32.7	0.28	350
Photoswitchable fluorescent proteins					
Dronpa	503	517	95.0	0.85	120
rsFastLime	496	518	39.1	0.77	200
Padron	503	522	43.0	0.64	NR
bsDronpa	460	504	45.0	0.50	NR
rsCherry	572	610	80.0	0.02	NR
rsCherryRev	572	608	84.0	0.005	NR
Synthetic fluorophores					
Alexa Fluor 488	495	519	71.0	0.92	1000
ATTO 488	501	523	90.0	0.80	1200
Cy3B	559	570	130.0	0.67	2000
Alexa Fluor 568	578	603	91.3	0.69	2000
Alexa Fluor 647	650	665	240.0	0.33	4000
Cy5	649	670	250.0	0.28	5000
Cy7	747	767	200.0	0.28	1000
DyLight 750	752	778	220.0	0.25	750
C-Rhodamine[h]	545	575	90.0	0.90	1100
C-Fluorescein	494	518	29.0	0.93	525

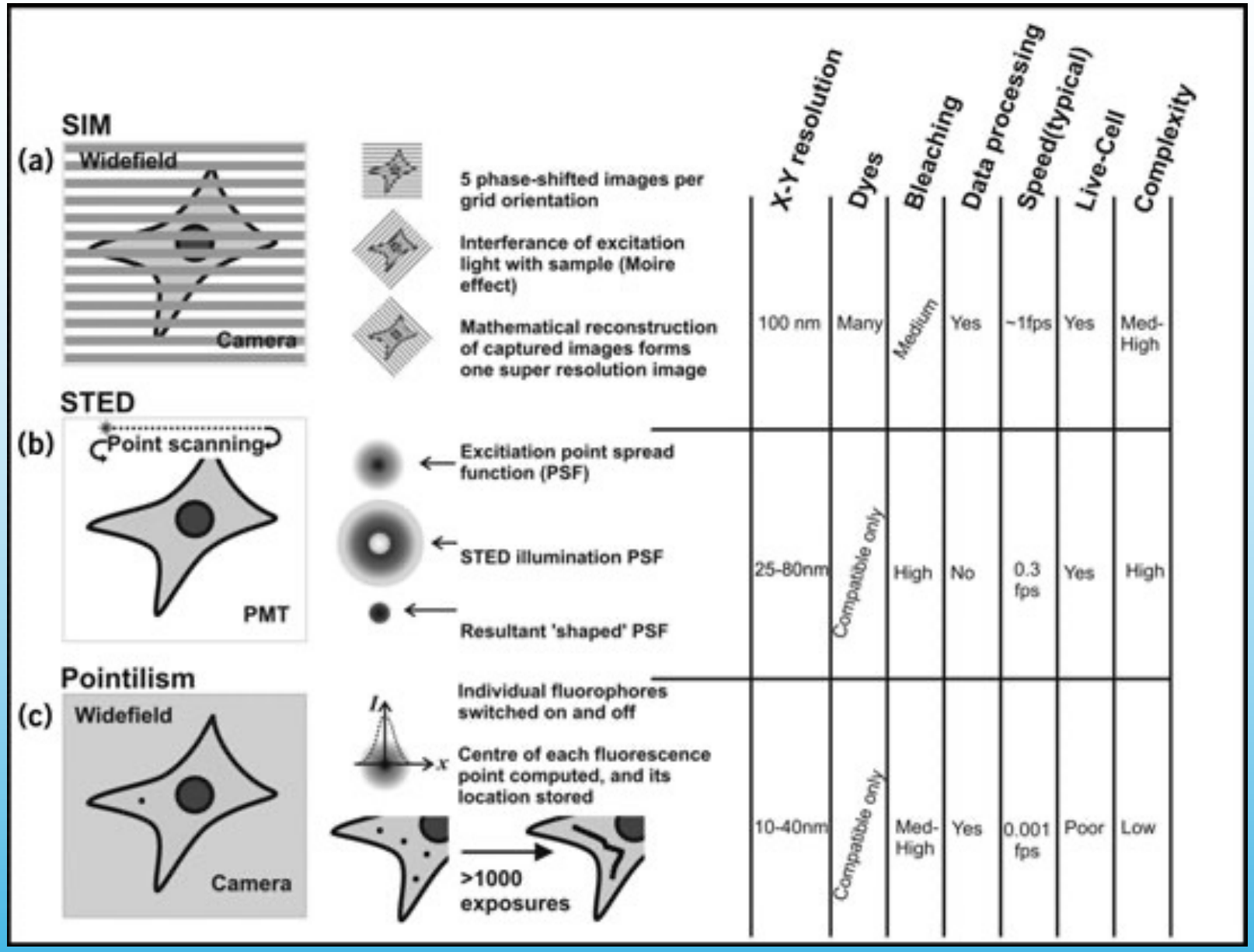
[a] The common name and/or acronym for each fluorophore.
[b] Peak excitation (Ex).
[c] Peak emission (Em) wavelengths.
[d] Molar extinction coefficient (EC).
[e] Quantum yield (QY).
[f] Number of photons emitted per molecule per switching cycle (N Photons) are indicated.
[g] Terminology: (N) native, (C) cyan, (G) green, (O) orange, (R) red, (FR) far-red.
[h] C-Rhodamine and C-Fluorescein refer to caged derivatives.
NR, not reported.

Appendix

Table 1 A comparison among super-resolution techniques

	SIM	STED	PALM and STORM
Principle	Moiré pattern by spatially structured illumination[7]	PSF shrinking by stimulated emission depletion[10]	Photoactivation/photoswitching and localization of single fluorescent molecules[13–15,19]
Lateral resolution	~100 nm[91]	30–50 nm[11]	10–40 nm[19,77]
Axial resolution	<300 nm[92]	~30–600 nm[93,94]	~10–50 nm[77,78,95]
Probes	Common organic dyes	Specific organic dyes	Genetically encoded photoactivatable/ photoswitchable proteins (PALM) Cyanine activator and reporter dyes (STORM) Photoswitchable organic dyes (directSTORM)
Preparations	Fixed samples *In vitro*[91] *In vivo*[20]	Fixed samples *In vitro*[21,23] *In vivo*[60]	Fixed samples *In vitro*[22,96,97]
Speed	Middle[91]	High[23]	Low[22]
Advantages	Live three-dimensional imaging[9,98] 3- and 4-color imaging[8] Larger field of view at comparable frame rates	Live three-dimensional imaging[21] 2- and 3-color imaging[11,24] Deep tissue[61] and *in vivo*[60] imaging Intrinsically optical; no data processing required	Live three-dimensional imaging[22] 3- to 6-color imaging[18,99,100] Single-particle tracking (sptPALM)[62]
Disadvantages	Limited lateral resolution Complex data processing Possible artifacts	Limited axial resolution, improved with isoSTED[41] High photobleaching High labeling density required	Cross-talk between fluorophores Complex data processing High labeling density required Possible artifacts

Appendix


SIM
(a)
Widefield
Camera
5 phase-shifted images per grid orientation
Interferance of excitation light with sample (Moire effect)
Mathematical reconstruction of captured images forms one super resolution image
STED
(b)
Point scanning
PMT
Excitiation point spread function (PSF)
STED illumination PSF
Resultant 'shaped' PSF
Pointilism
(c)
Widefield
Camera
Individual fluorophores switched on and off
Centre of each fluorescence point computed, and its location stored
>1000 exposures
X-Y resolution
Dyes
Bleaching
Data processing
Speed(typical)
Live-Cell
Complexity
100 nm
Many
Medium
Yes
~1fps
Yes
Med-High
25-80nm
Compatible only
High
No
0.3 fps
Yes
High
10-40nm
Compatible only
Med-High
Yes
0.001 fps
Poor
Low