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ISTITUTO ITALIANO
DI TECNOLOGIA

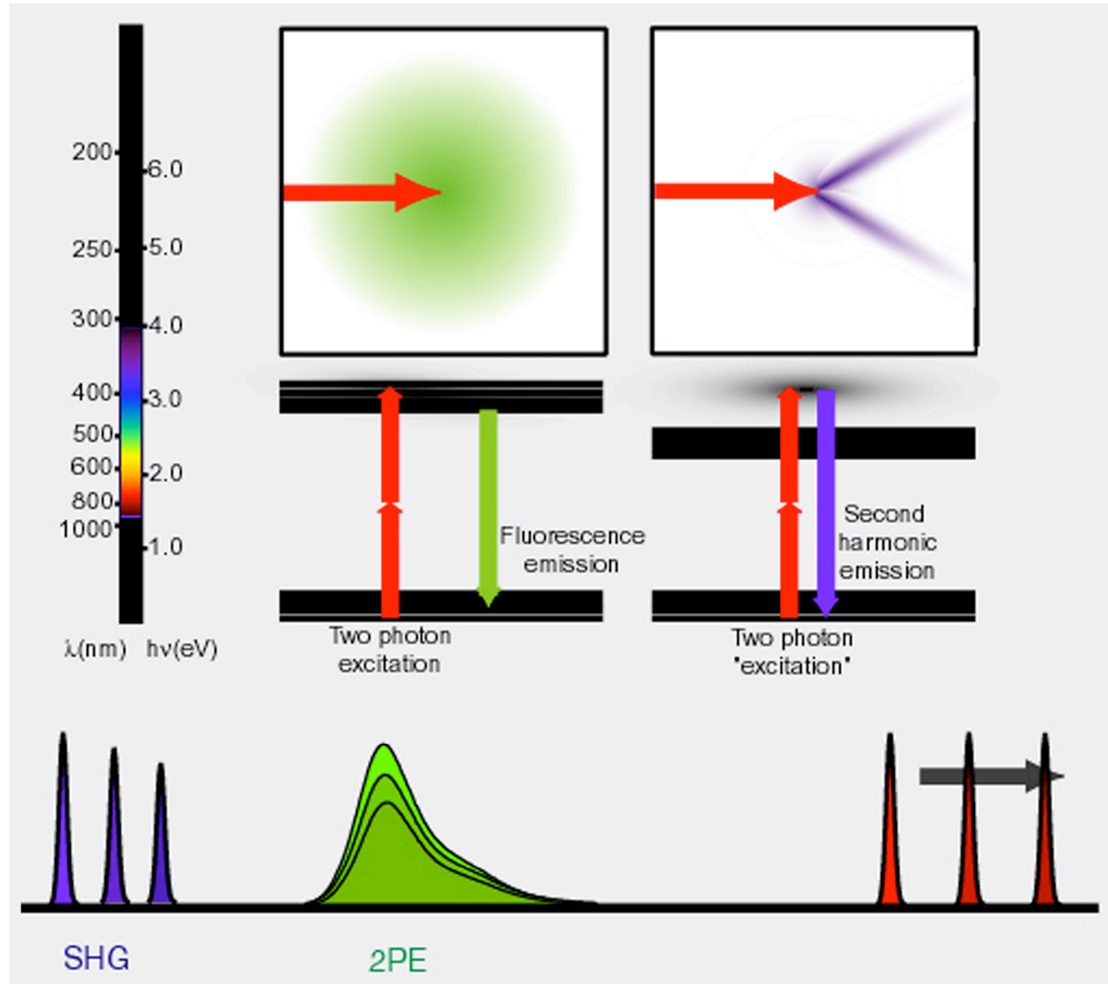
From Microscopy to Nanoscopy
II

Alberto Diaspro

alberto.diaspro@iit.it

SHG imaging

$$\tilde{P}(t) = \varepsilon_0\chi^{(1)}\tilde{E}(t) + \varepsilon_0\chi^{(2)}\tilde{E}(t)^2 + \varepsilon_0\chi^{(3)}\tilde{E}(t)^3 + \ldots$$
$$\equiv \tilde{P}^{(1)}(t) + \tilde{P}^{(2)}(t) + \tilde{P}^{(3)}(t) + \ldots$$



SHG

IR

Coherent Chameleon-XR

λ/2 wave plate

polarizer

Spectrophotometer

PMT

Scanning mirrors

Leica TCS SP5 AOBS scanning head

objective

sample

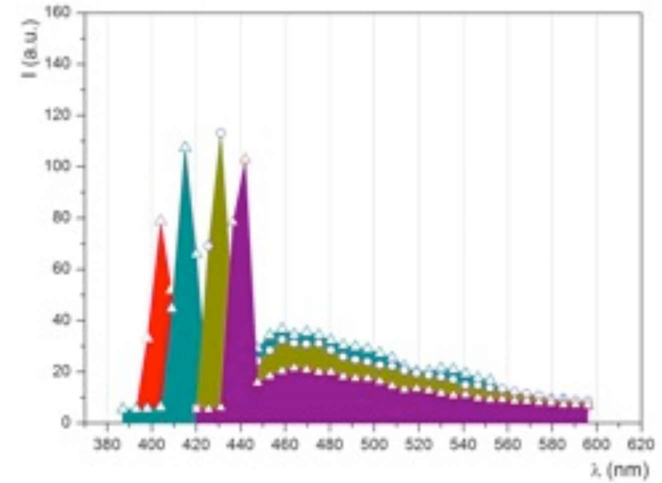
condenser or objective

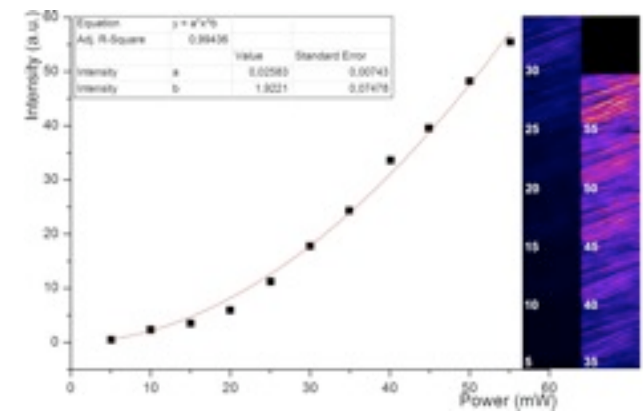
IR filter

Vis filter

mirror

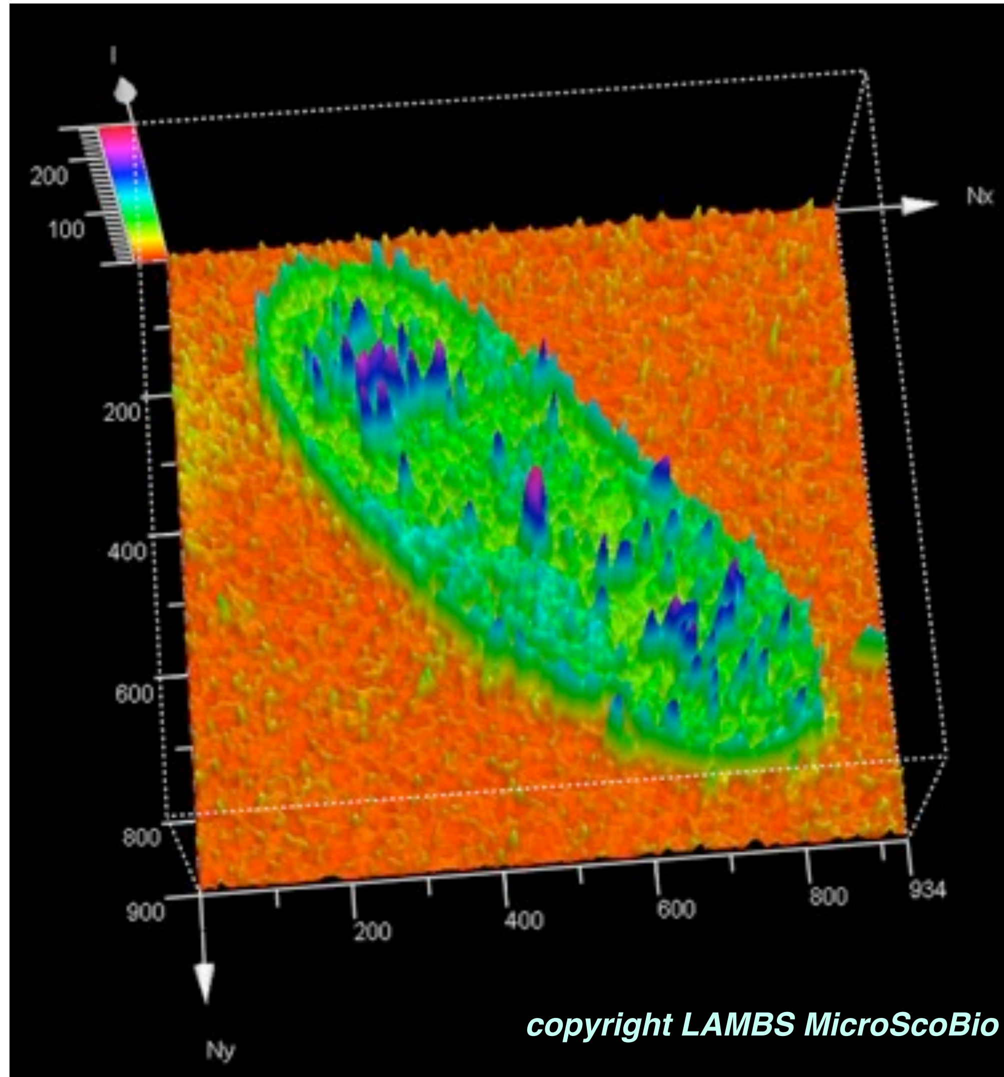
PMT





Paolo Bianchini and Alberto Diaspro (2008) Three-dimensional (3D) backward and forward second harmonic generation (SHG) microscopy of biological tissues. J. Biophoton. 1, No. 6, 443 – 450

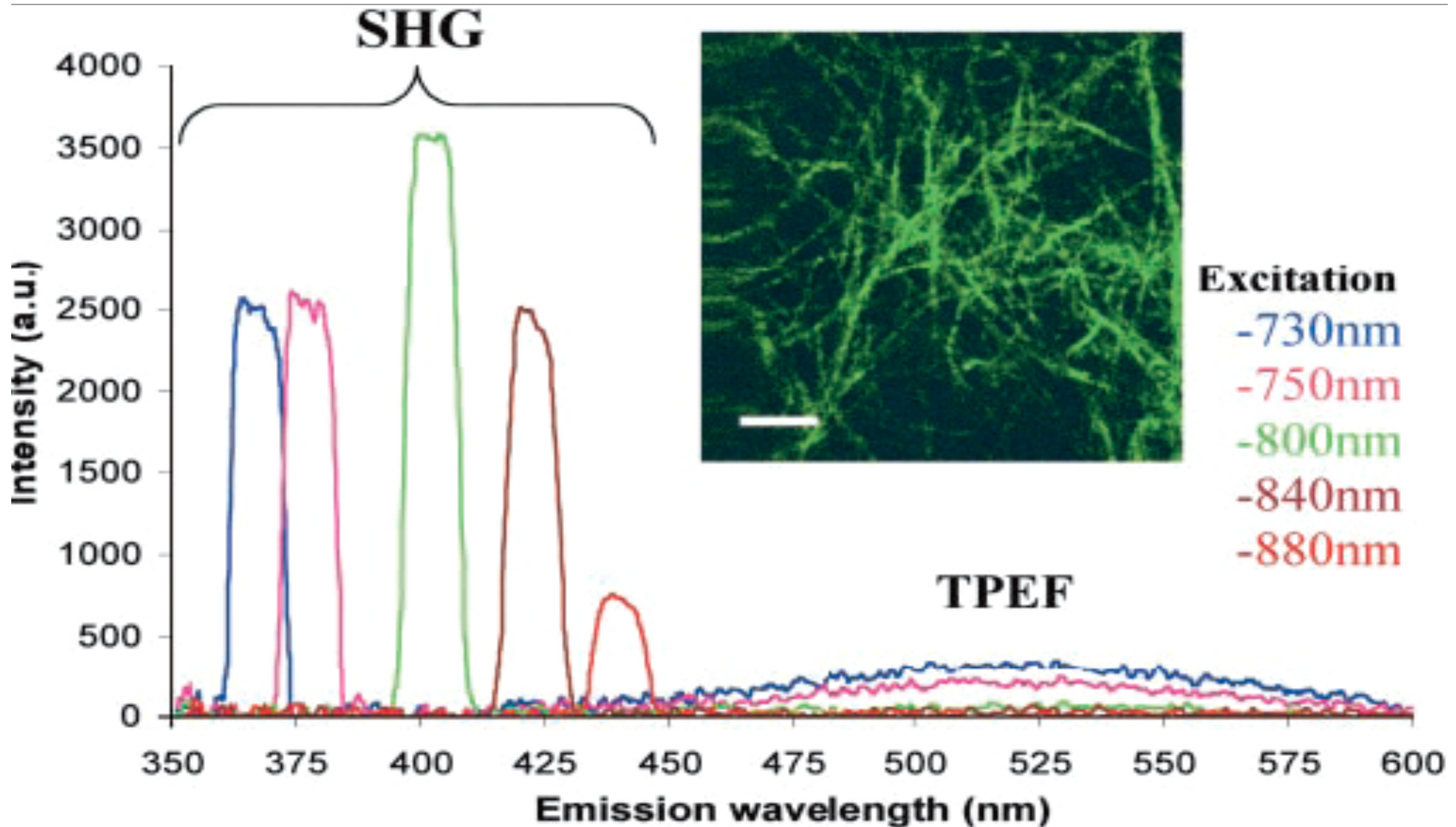
SHG



SHG in the backward pathway: Diaspro, A et al.. (2002), Proc. SPIE, 4622, 24-31.

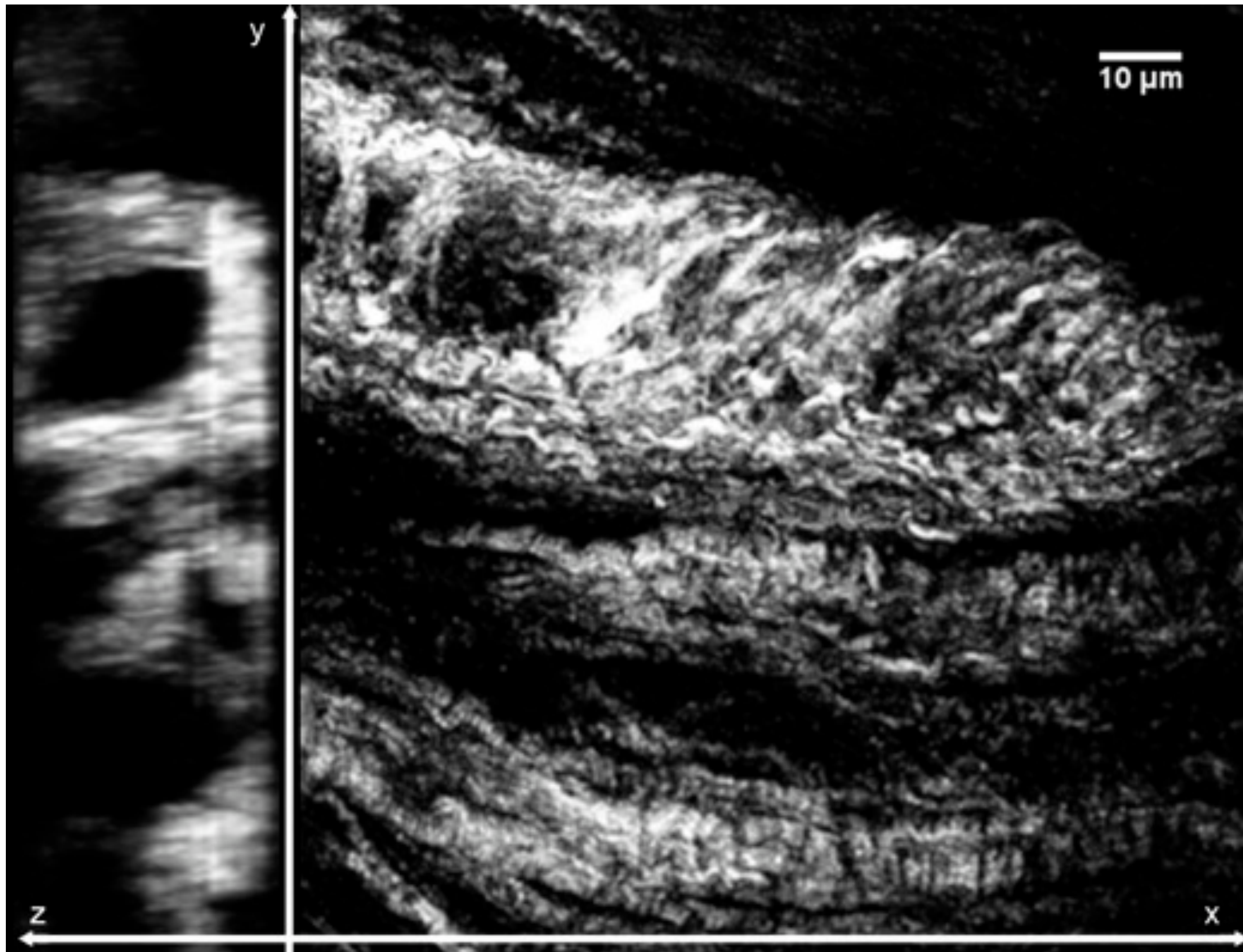
SHG

collagen fibers



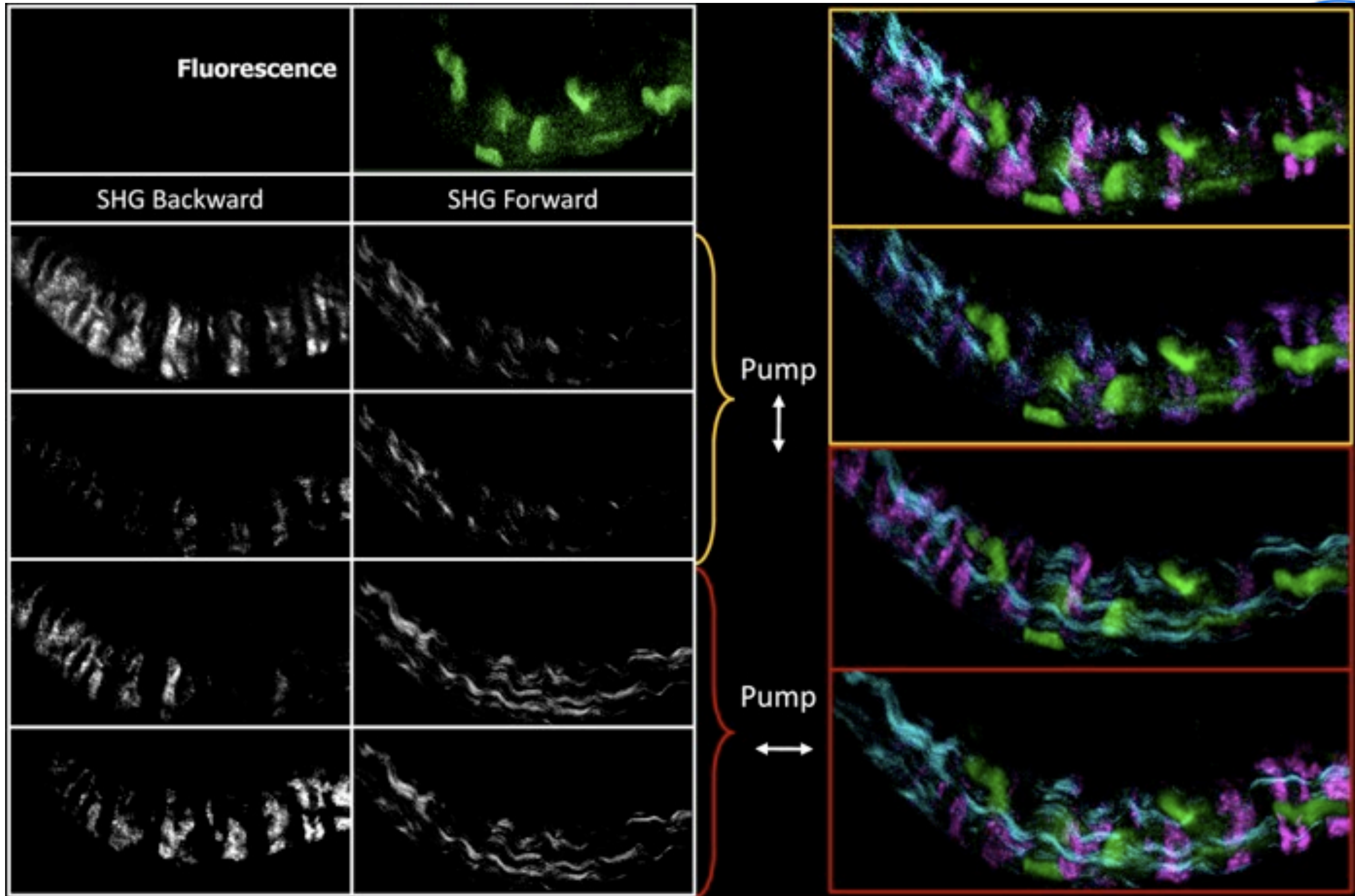
SHG - Zoumi, A.; Yeh, A. & Tromberg, B.J. (2002), Proc Natl Acad Sci U S A 99(17), 11014—11019.

SHG

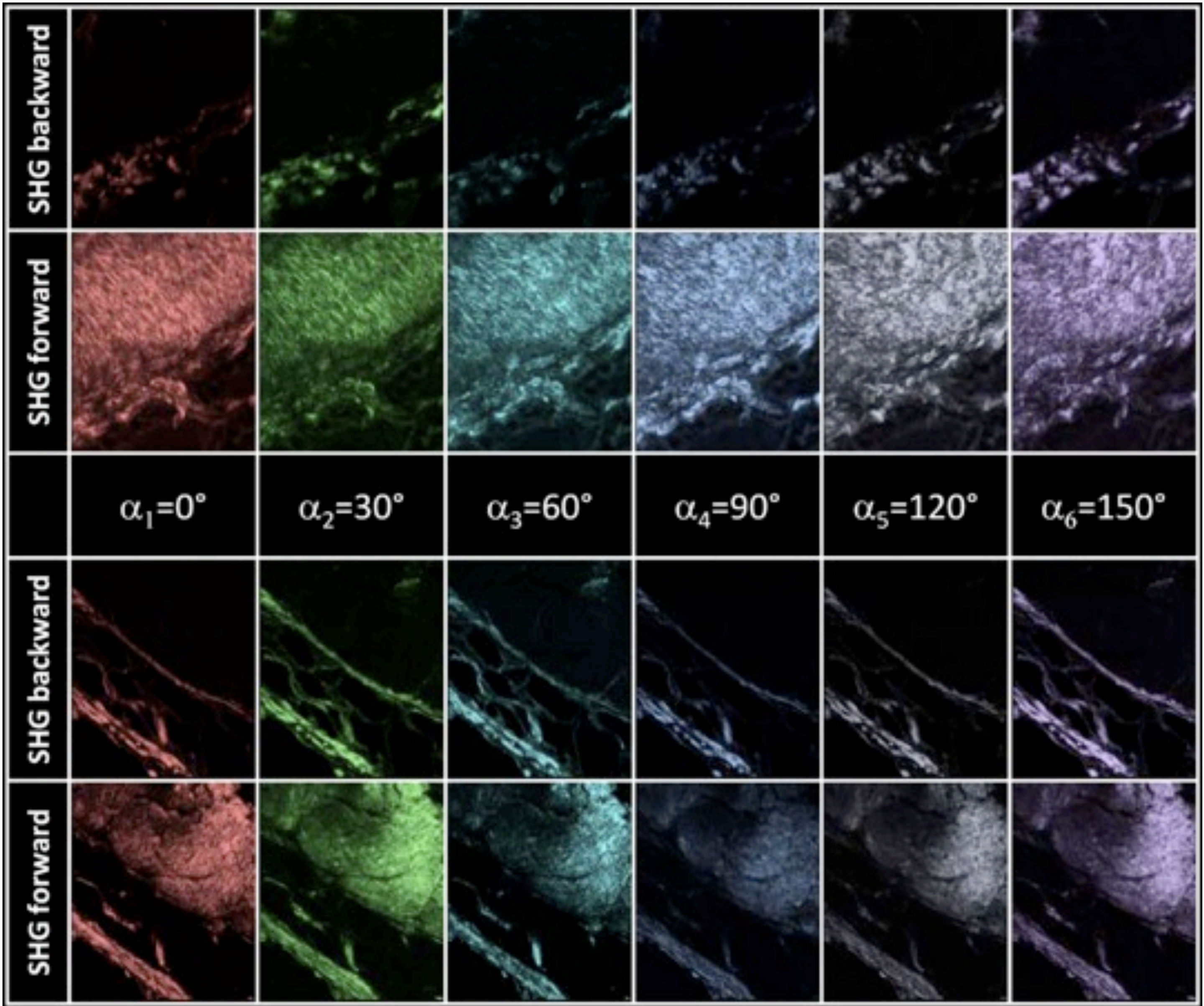


Peritumoral murine blood vessels acquired in the living animal under general anesthesia (ketamine-xylamine, IM). Bianchini and Diaspro (2008) J.Biophotonics

SHG



SHG

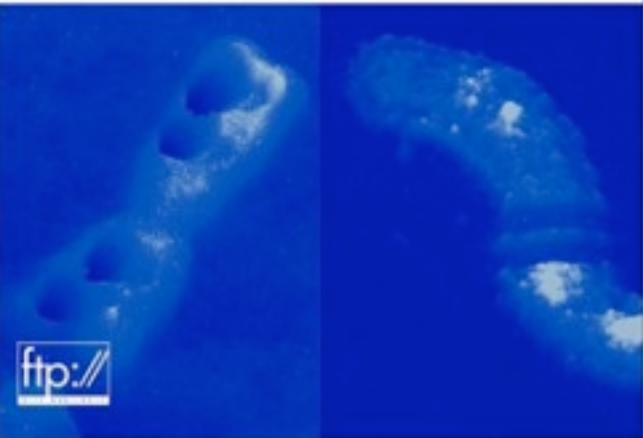


SHG

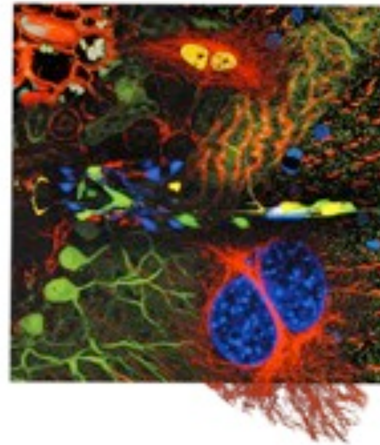
BOOKS

Confocal and Two-Photon Microscopy
Foundations, Applications, and Advances

Edited by Alberto Diaspro



PAOLO SAPUPPO - ALBERTO DIASPRO - MARIO FARETTA



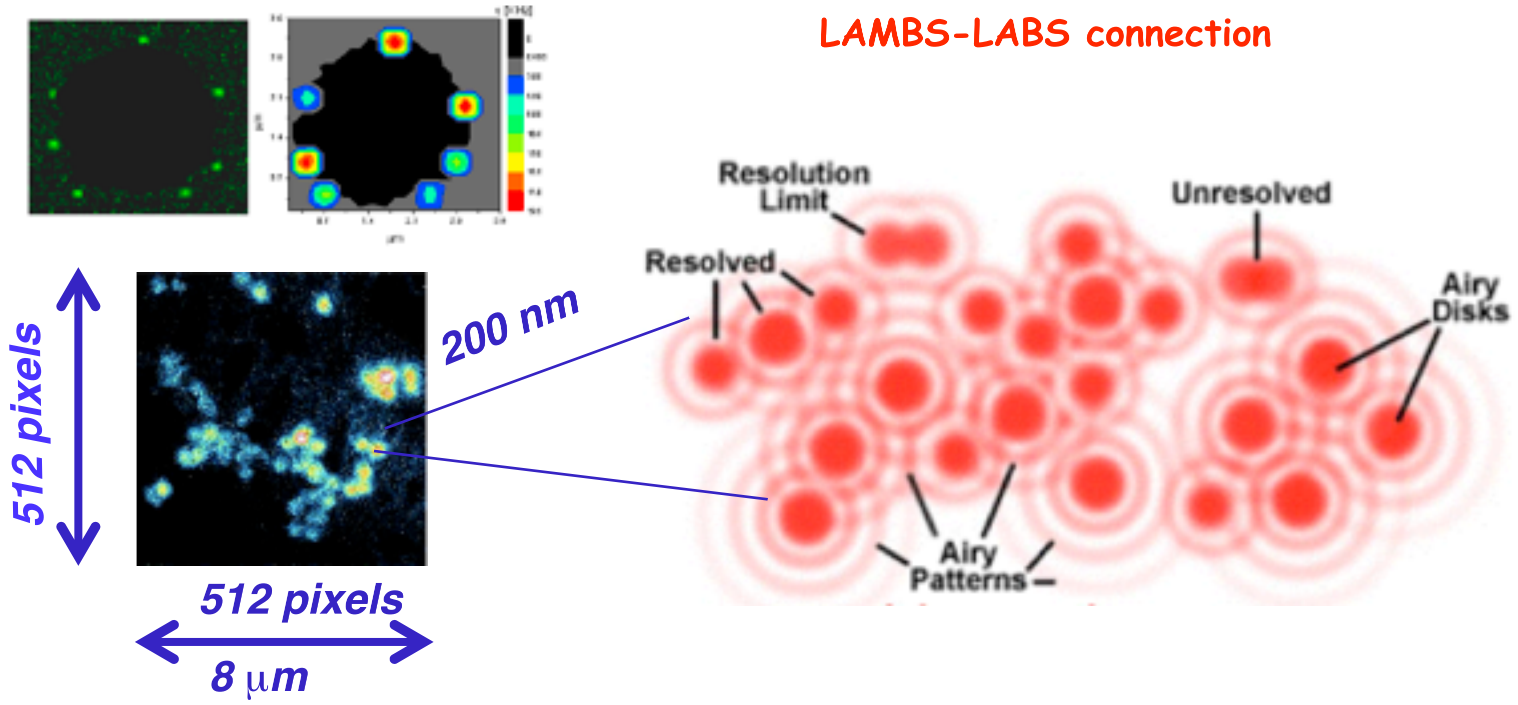
MICROSCOPIA CONFOCALE

Leica
MICROSYSTEMS

NANOSCOPY
AND
MULTIDIMENSIONAL OPTICAL
FLUORESCENCE MICROSCOPY

EDITED BY ALBERTO DIASPRO

FROM MICROSCOPY TO NANOSCOPY

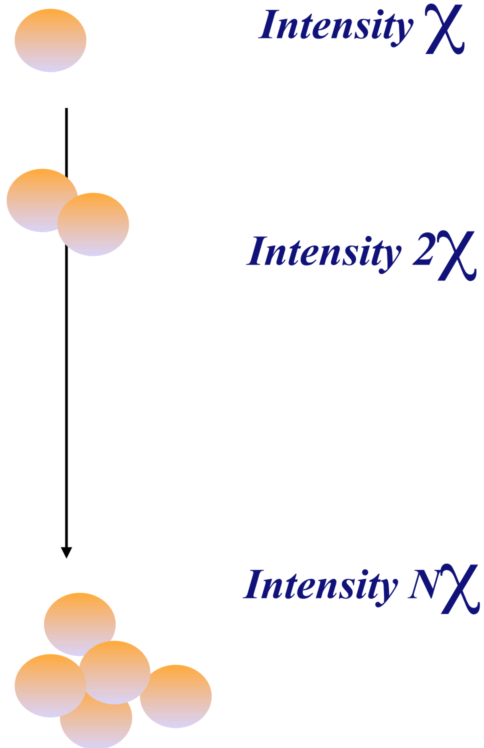


Single molecule structure is still not resolved

Chirico, G., Cannone, F., Beretta, S., Baldini, G., Diaspro, A., Micr. Res. Tech., 55:359-364 (2002)
Chirico G., Cannone F., Baldini G., and Diaspro A. Biophysical J., 84 (2003).
Chirico G., Cannone F., and Diaspro A., J.Phys.D,36 (2003).

Micr. Res. Tech. (2002)
55:359-4

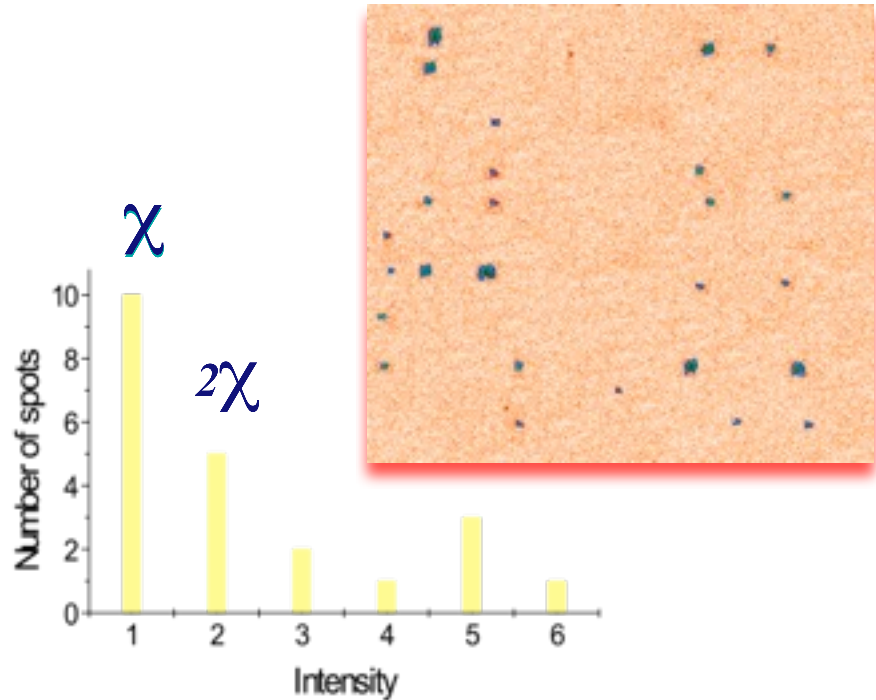
FROM MICROSCOPY TO NANOSCOPY



Intensity χ

Intensity 2χ

Intensity $N\chi$

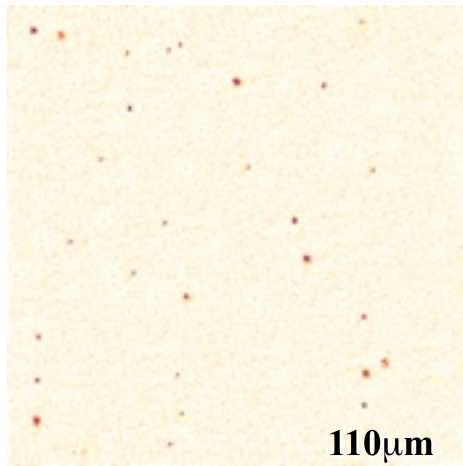


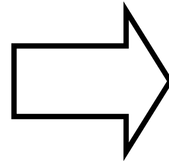
N = number of molecules

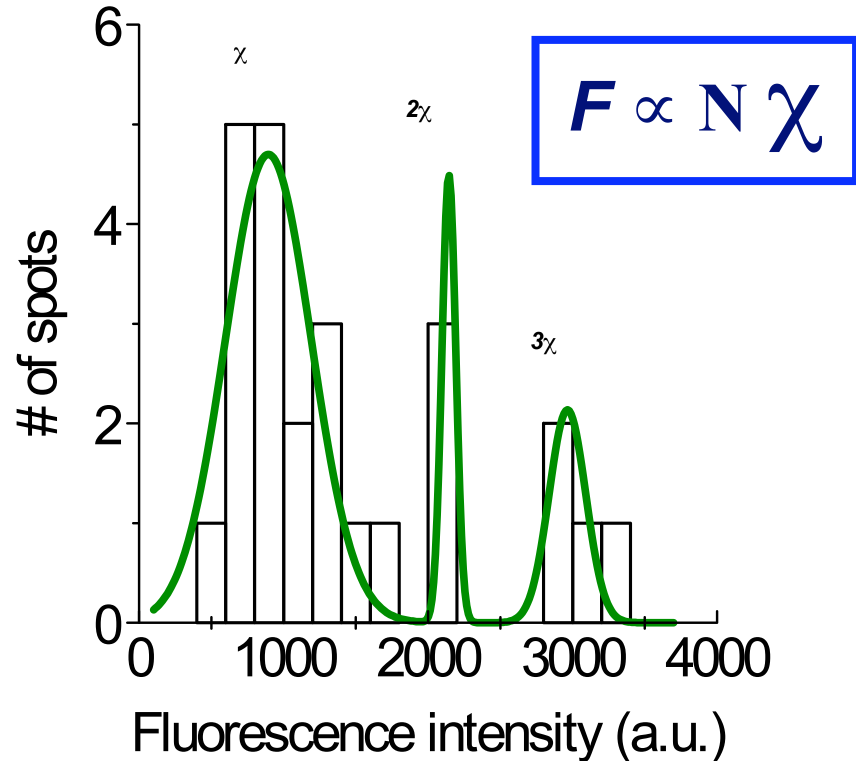
LAMBS-LABS connection

Micr. Res. Tech. (2002) 55:359-4

Fluorescein *c=1μM @ 8mW*







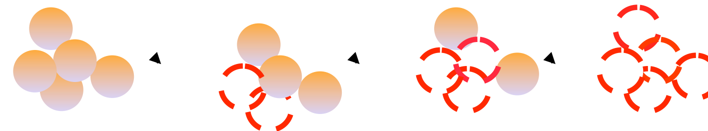
$F \propto N\chi$

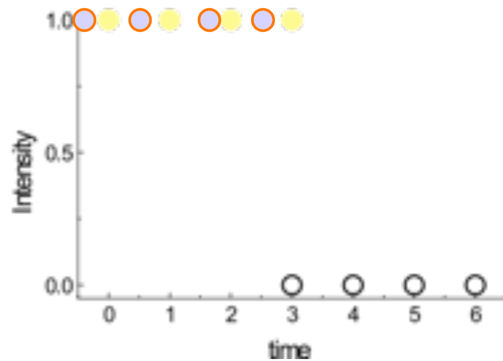
Micr. Res. Tech. (2002) 55:359-4

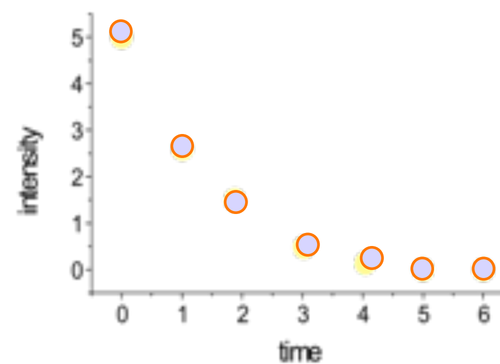
LAMBS-LABS connection

Single Molecule

Aggregates



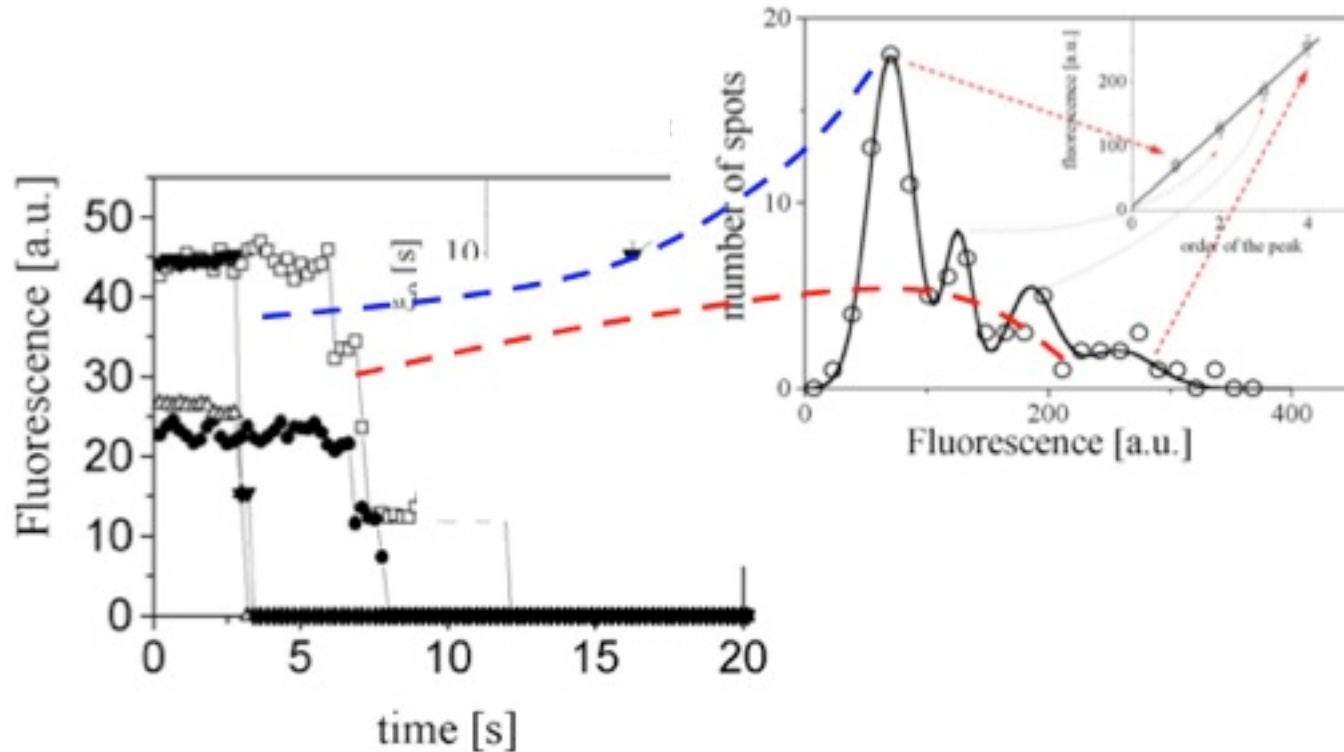




LAMBS-LABS connection

Micr. Res. Tech. (2002) 55:359-4

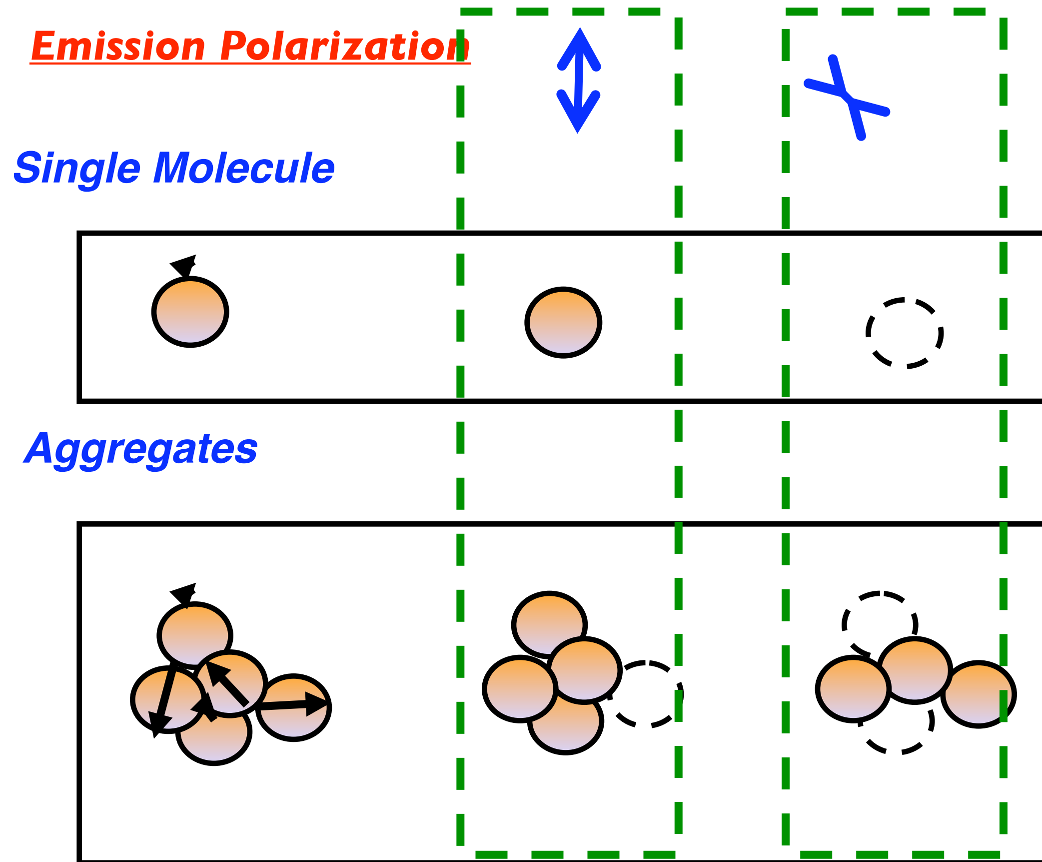
Single Molecule Photobleaching of Sparse Aggregates



LAMBS-LABS connection

Biophys J. 2003 Jan;84(1):588-98

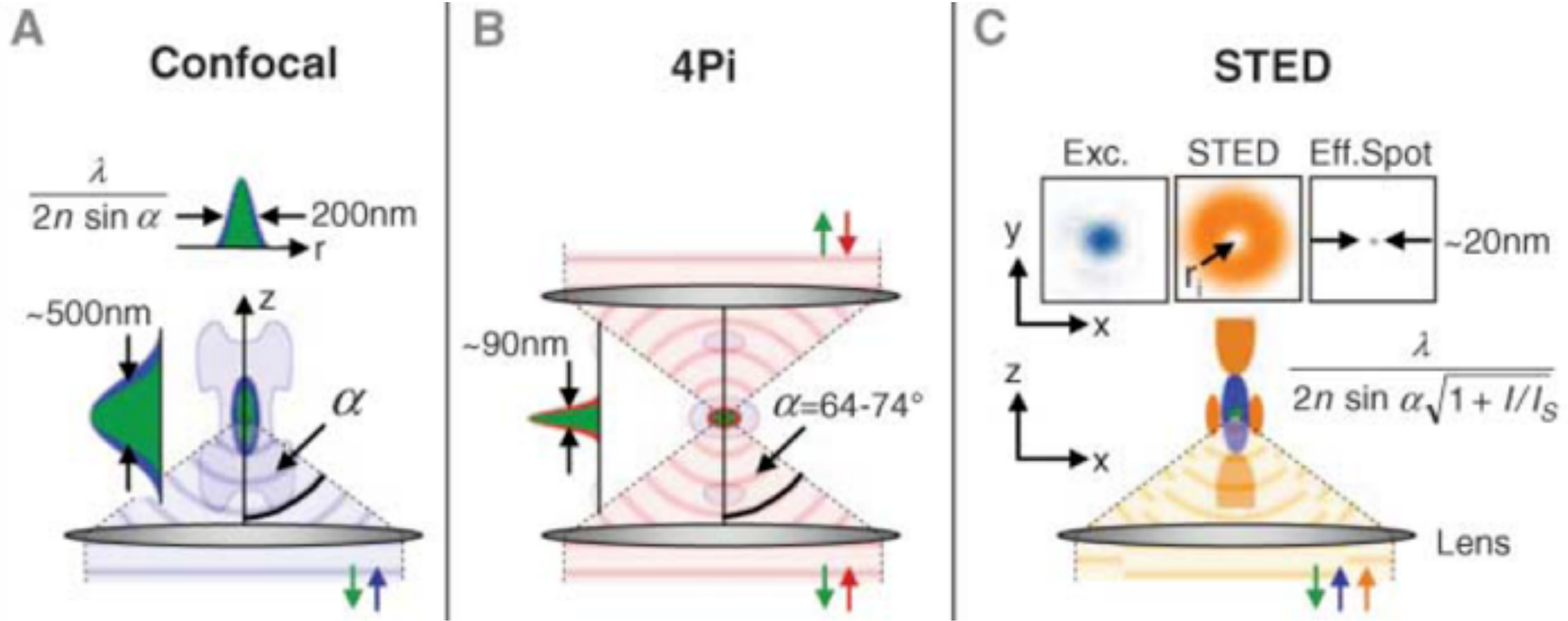
Fluorescence Polarization



LAMBS-LABS connection

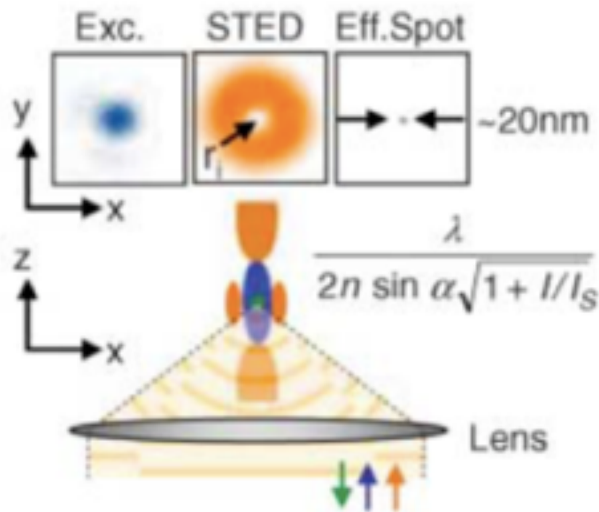
J Biomed Opt. (2003) 8(3): 391-5

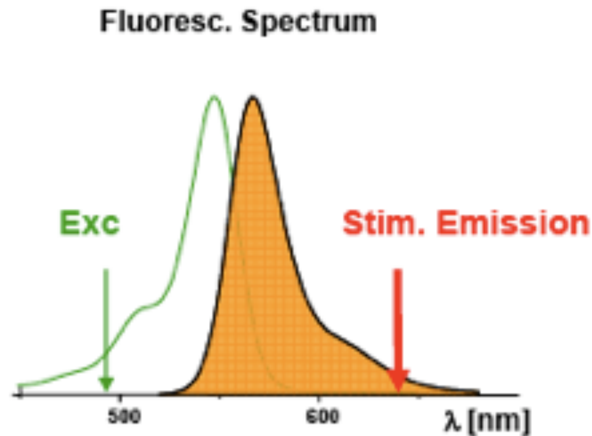
FROM MICROSCOPY TO NANOSCOPY

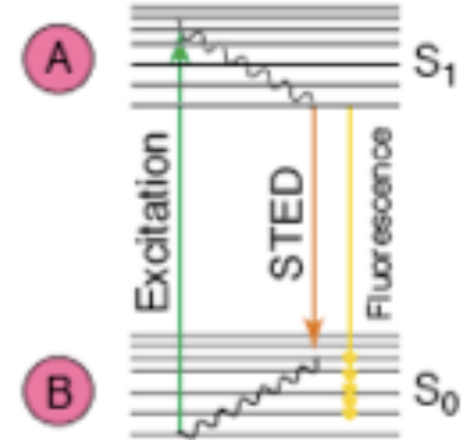


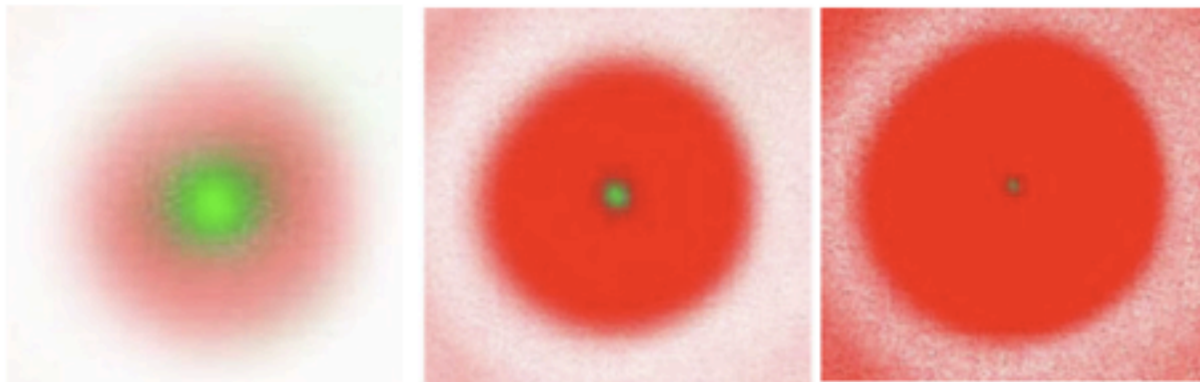
S.W. Hell, et al. *Far-Field Optical Nanoscopy*, Science 316, 1153 (2007)

FROM MICROSCOPY TO NANOSCOPY









Effective resolution depends on depletion-intensity

no limitation: $d \rightarrow 0$

Rolf Borlinghaus

S.W. Hell, et al. Far-Field Optical Nanoscopy, Science 316, 1153 (2007)

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Fluorescent Dyes Used in STED Microscopy

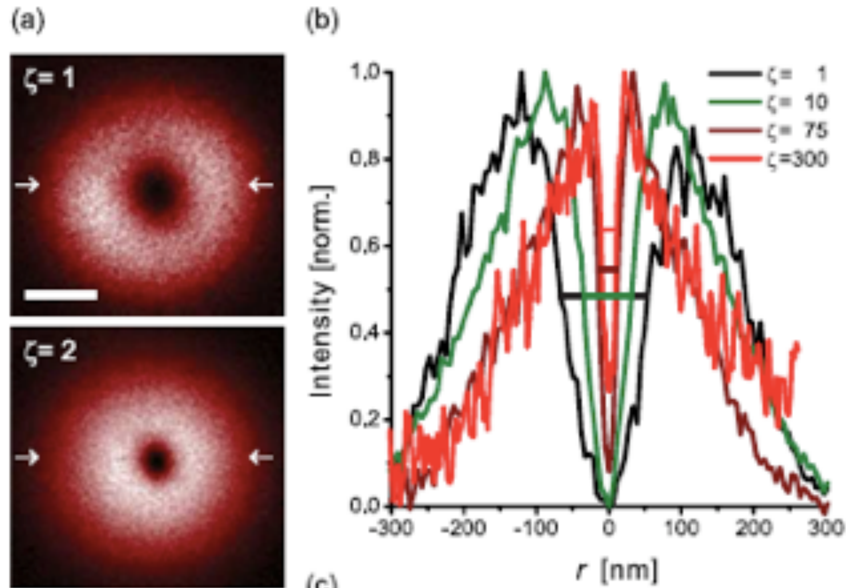
lambs

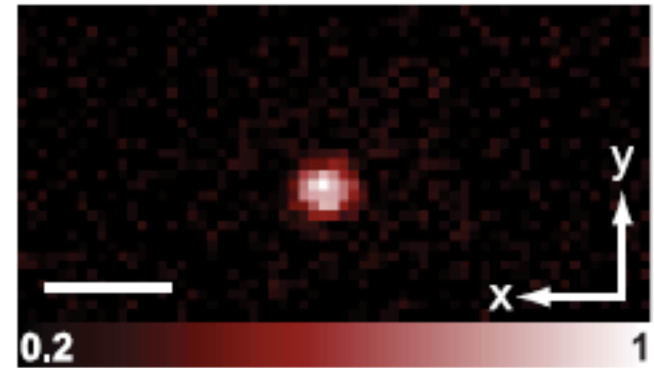
The following dyes have been successfully used in STED microscopy:

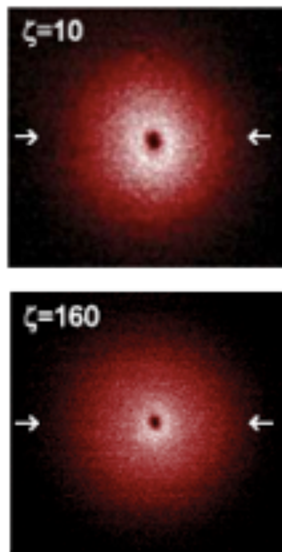
Dye name (Manufacturer / Distributor)	Exc. Wavelength	Exc. Pulse Length	STED Wavelength	STED Pulse Length	Repetition Rate	Avg. STED Power	Peak Irradiance	Pulse Energy	Reported Spatial Resolution (Direction)
ATTO 532 (ATTO-TEC GmbH)	470 nm	100 ps	615 nm	200 ps	80 MHz	14 – 18 mW			66 – 72 nm (xy)
ATTO 532 (ATTO-TEC GmbH)	470 nm	80 ps	603 nm	280 ps	250 kHz	0.5 mW		2 nJ	<25 nm (xy)
ATTO 532 (ATTO-TEC GmbH)	488 nm	100 ps	615 nm	200 ps	80 MHz	16 mW			60 – 70 nm (xy)
Chromeo 488 (Actif Motif)	488 nm	140 ps	602 nm	~ 160 ps	250 kHz	0.6 mW			< 30 nm (xy)
Chromeo 488 (Actif Motif)	488 nm	< 100 ps	590 nm	200 – 300 ps	80 MHz	40 mW			60 – 70 nm (xy)
DY-485XL (Dyomics GmbH)	488 nm	< 100 ps	647 nm	~ 200 ps	72 MHz	(20 + 3) mW			40 – 45 nm (xyz)
GFP	490 nm	100 ps	575 nm	200 ps	80 MHz	7.2 mW			~ 70 nm (xy)
ATTO 565 (ATTO-TEC GmbH)	532 nm	~ 90 ps	640 – 660 nm	~ 90 ps	1 – 2 MHz				30 – 40 nm (xy)
ATTO 565 (ATTO-TEC GmbH)	532 nm	cw	647 nm	cw	cw	114 mW			~ 60 nm (xy)
MR 121 SE (Roche Diagnostics)	532 nm	10 ps	793 nm	107 ps	76 MHz	10.4 mW			~ 50 nm (z)
NK51 (ATTO-TEC GmbH)	532 nm	< 100 ps	647 nm	~ 200 ps	72 MHz	(20 + 3) mW			40 – 45 nm (xyz)
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)
Pyridine 2 / LDS 722 (Exciton, Radiant Dyes GmbH)	554 nm	250 fs	760 nm	13 ps	76 MHz				33 nm (z)
Pyridine 2 / LDS 722 (Exciton, Radiant Dyes GmbH)	554 nm	250 fs	745 nm	50 – 200 ps		12.2 mW			44 nm (z)
RH 414 (Invitrogen Corp.)	554 nm	250 fs	745 nm	13 ps	76 MHz	8.78 mW			30 nm (z)
ATTO 590 (ATTO-TEC GmbH)	570 nm	~ 90 ps	690 – 710 nm	~ 90 ps	1 – 2 MHz				30 – 40 nm (xy)
ATTO 633 (ATTO-TEC GmbH)	630 nm	~ 90 ps	735 – 755 nm	~ 90 ps	1 – 2 MHz				30 – 40 nm (xy)
ATTO 633 (ATTO-TEC GmbH)	635 nm	100 ps	750 nm	~ 200 ps	76 MHz			~ 1.5 nJ	40 nm (xy)
ATTO 647N (ATTO-TEC GmbH)	635 nm	cw	750 nm	cw	cw	423 mW			~ 50 nm (xy)
ATTO 647N (ATTO-TEC GmbH)	635 nm	100 ps	780 nm	300 ps	250 kHz		700 MW/cm^2		~ 65 nm (xy)
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	781 nm	303 ps	40 MHz	10.1 mW			40 nm (x)
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	775 nm	300 ps	76 MHz		800 MW/cm^2		16 nm (x)
JA 26 (K.H. Drexhage, Siegen University)	635 nm	68 ps	780 nm	300 ps	80 MHz	90 – 100 mW			47 nm (xy)
JA 26 (K.H. Drexhage, Siegen University)	637 nm	54 ps	778 – 785 nm	303 ps	40 MHz				120 – 140 nm (xy)

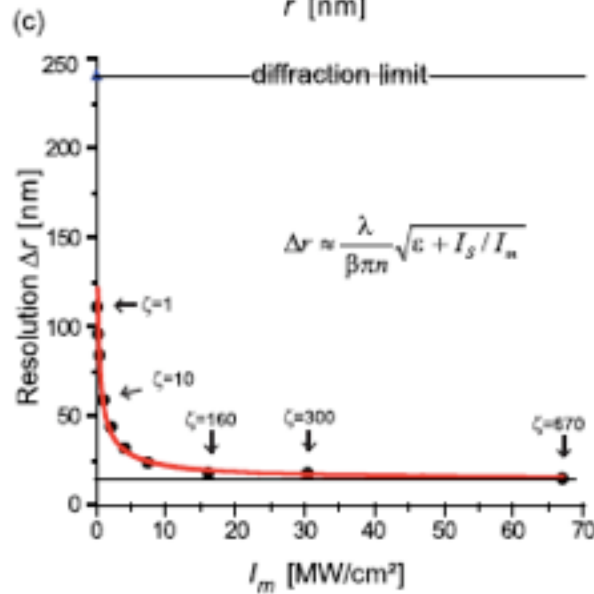
Compilation: Lars Kastrup <lkastru@gwdg.de>

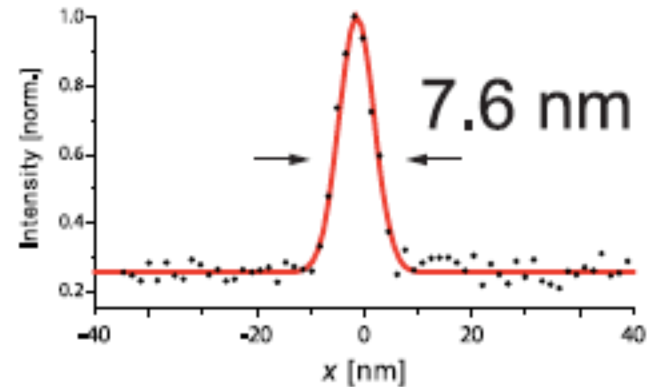
Last change: 25-Jun-2008 03:24 PM







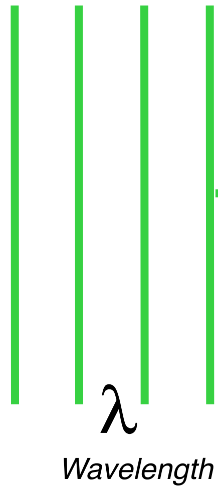


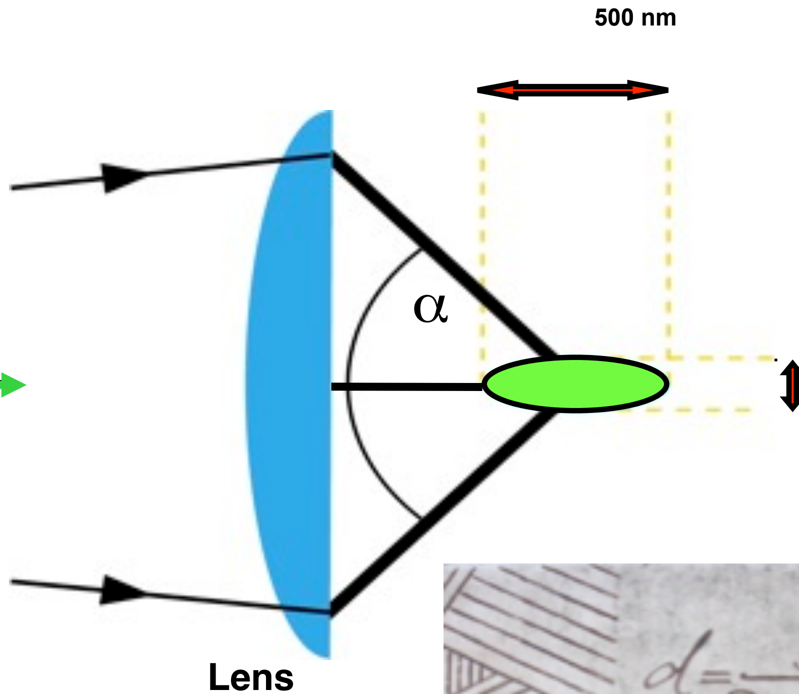


E. Rittweger, D. Wildanger and S. W. Hell EPL, 86 (2009) 14001

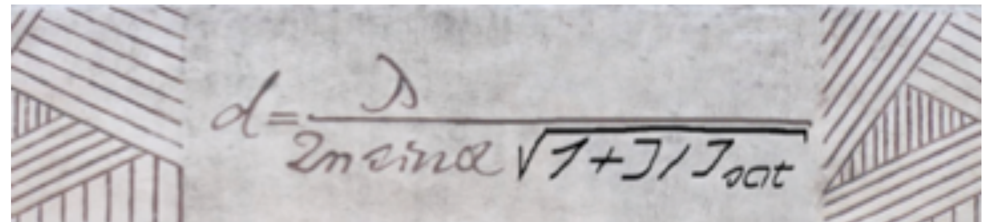
FROM MICROSCOPY TO NANOSCOPY

ABBE, E (1873) ARCHIVE F. MIKROSKOP.ANAT. 9, 413-420



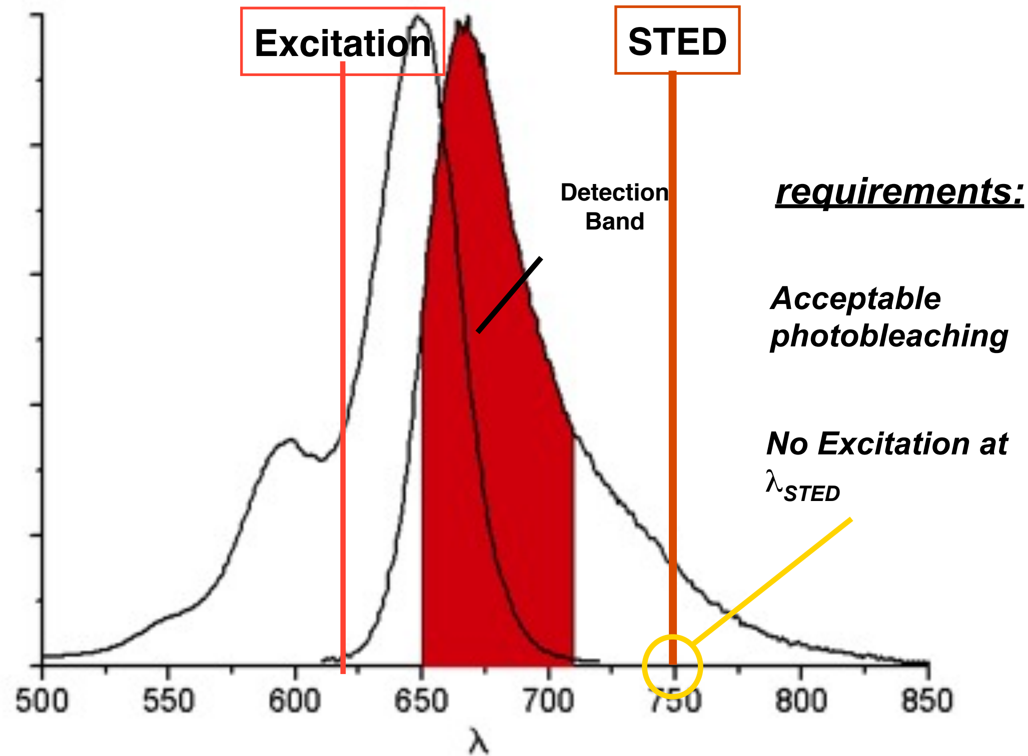


$$\Delta x = \frac{\lambda}{2n\sin\alpha}$$



S.W. Hell, et al. Far-Field Optical Nanoscopy, Science 316, 1153 (2007)

Slide credit: Stefan W. Hell, Max Planck Institute for Biophysical Chemistry, Department of NanoBiophotonics Göttingen



requirements:

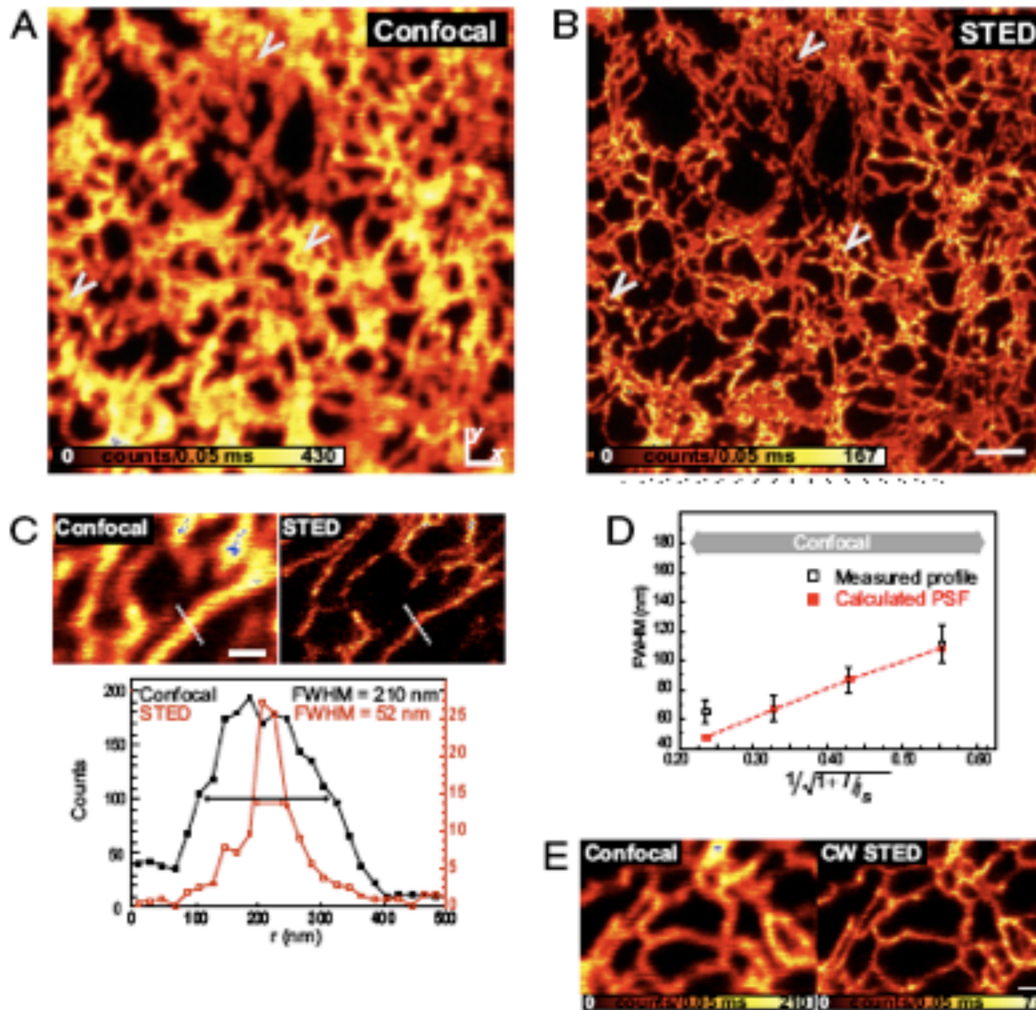
Acceptable photobleaching

No Excitation at λ_{STED}

Selected, optimized dyes and wavelength needed

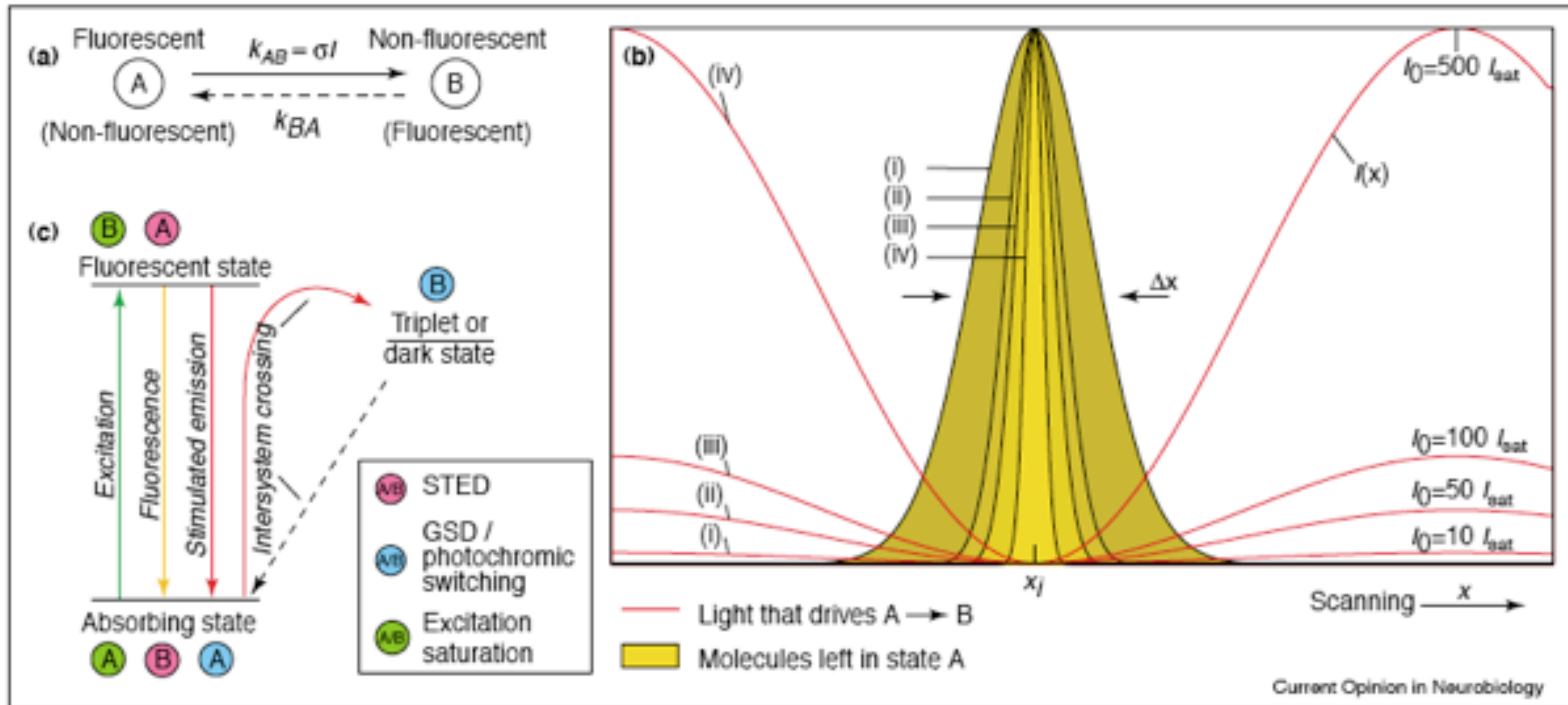
STED

FROM MICROSCOPY TO NANOSCOPY



Subdiffraction-resolution imaging of the ER in a living mammalian cell - YFP tag.

Hein, B., K. I. Willig, S. W. Hell (2008): "Stimulated emission depletion (STED) nanoscopy of a fluorescent protein-labeled organelle inside a living cell". Proc. Natl. Acad. Sci. USA 105 (38), 14271-14276.

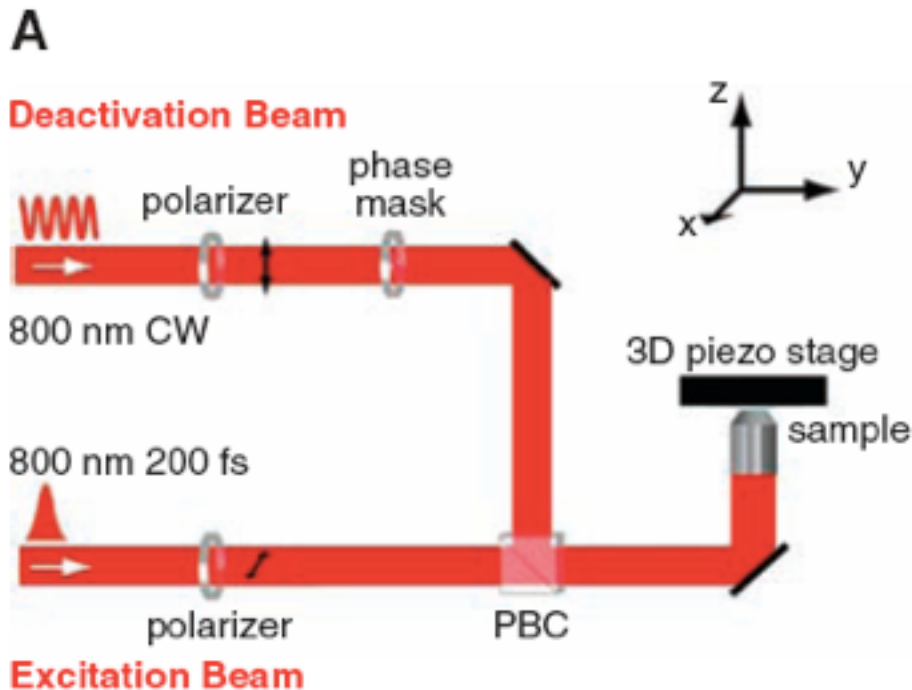


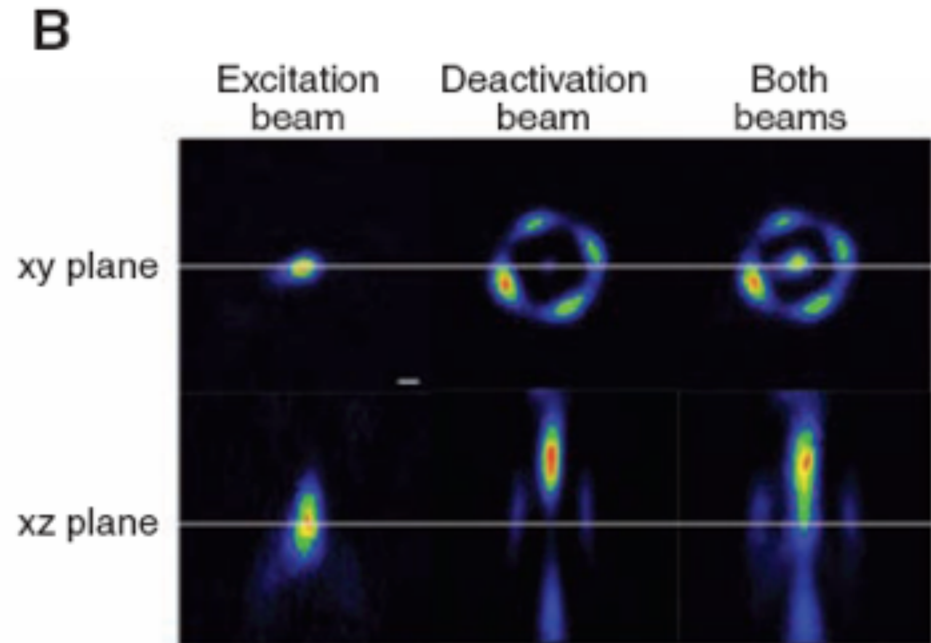
Breaking the diffraction barrier by reversible saturable optical transitions (RESOLFT). Breaking the diffraction barrier by RESOLFT requires **(a)** two states A and B of a label that are distinct in their optical properties. The optical transition A→B takes place at a rate $k_{AB} = \sigma I$ that is proportional to the light intensity I applied. The reverse transition B→A of rate k_{BA} brings the label back to its initial state. **(b)** The profiles i–iv show the spatial region in which the label is allowed to be in state A, if the region is subject to a standing wave of light with peak intensities I_0 = 10, 50, 100, and 500 times I_{sat} and with a zero intensity node at x_j. Increasing I_0 ensures that the region in which the label can reside in A is squeezed down, in principle, indefinitely. If A is the fluorescent state of the label, this ultrasharp region functions as the effective fluorescent spot of the microscope and Δx is the full-width-half-maximum of the state A region. The creation of a fluorescence image requires scanning, that is moving the zero node along the x-axis with subsequent storage of the recorded fluorescence. If B is the fluorescent state, then the ultrasharp regions of state A are dark. In this case, a sort of 'negative image' is recorded. Nevertheless, with suitable mathematical postprocessing, a similar optical resolution can be achieved. In any case, the resolution is no longer limited by diffraction, but only determined by the value of I_0/I_{sat}. **(c)** The simplified energy diagram of a fluorophore depicts possible schemes for implementing saturable optical transitions.

Current Opinion in Neurobiology 2004, **14**:599–609

Achieving λ/20 Resolution by One-Color Initiation and Deactivation of Polymerization

Linjie Li,[1] Rafael R. Gattass,[1] Erez Gershgoren,[1] Hana Hwang,[2] John T. Fourkas[1,3,4,5*]





www.sciencemag.org SCIENCE VOL 324 15 MAY 2009

Achieving λ/20 Resolution by One-Color Initiation and Deactivation of Polymerization

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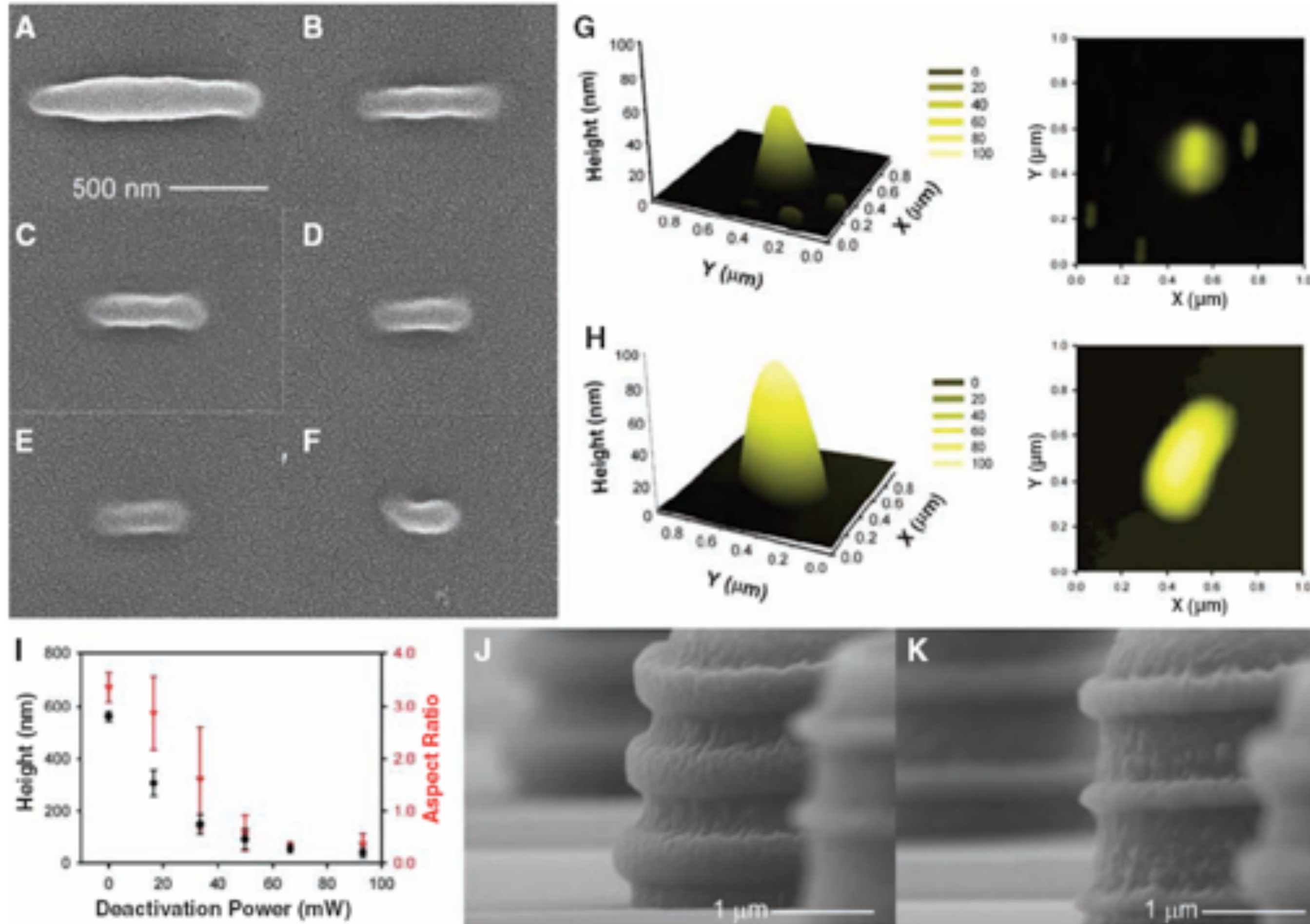
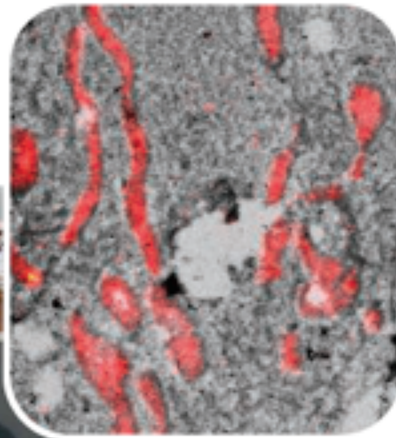
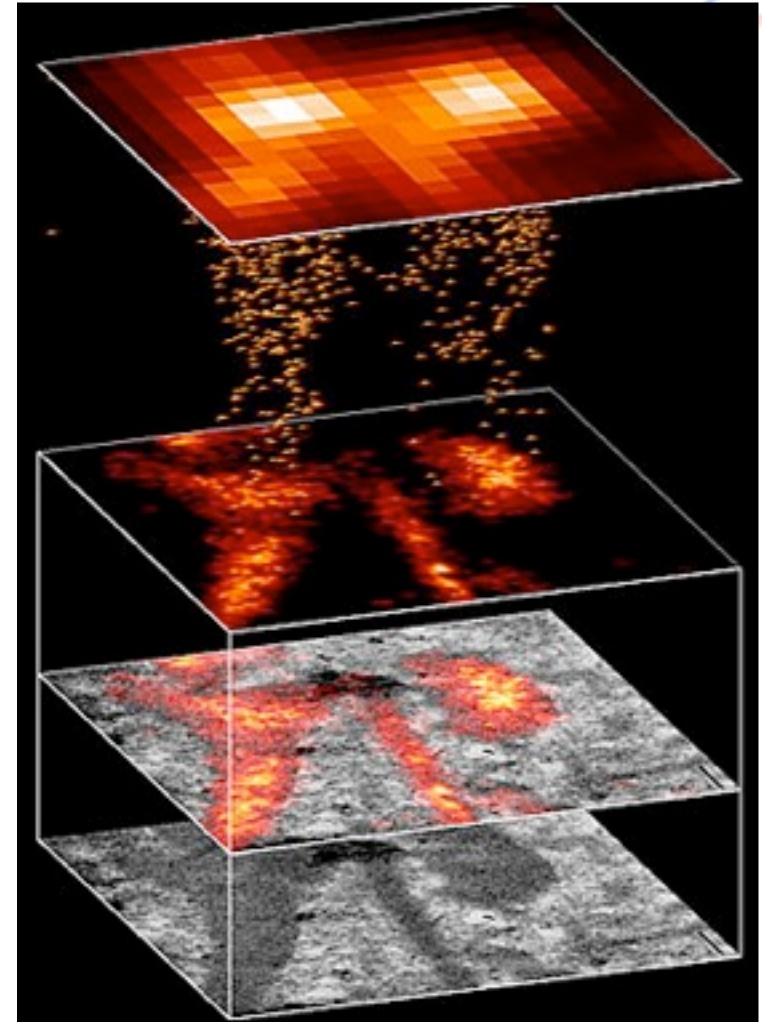


www.sciencemag.org SCIENCE VOL 324 15 MAY 2009

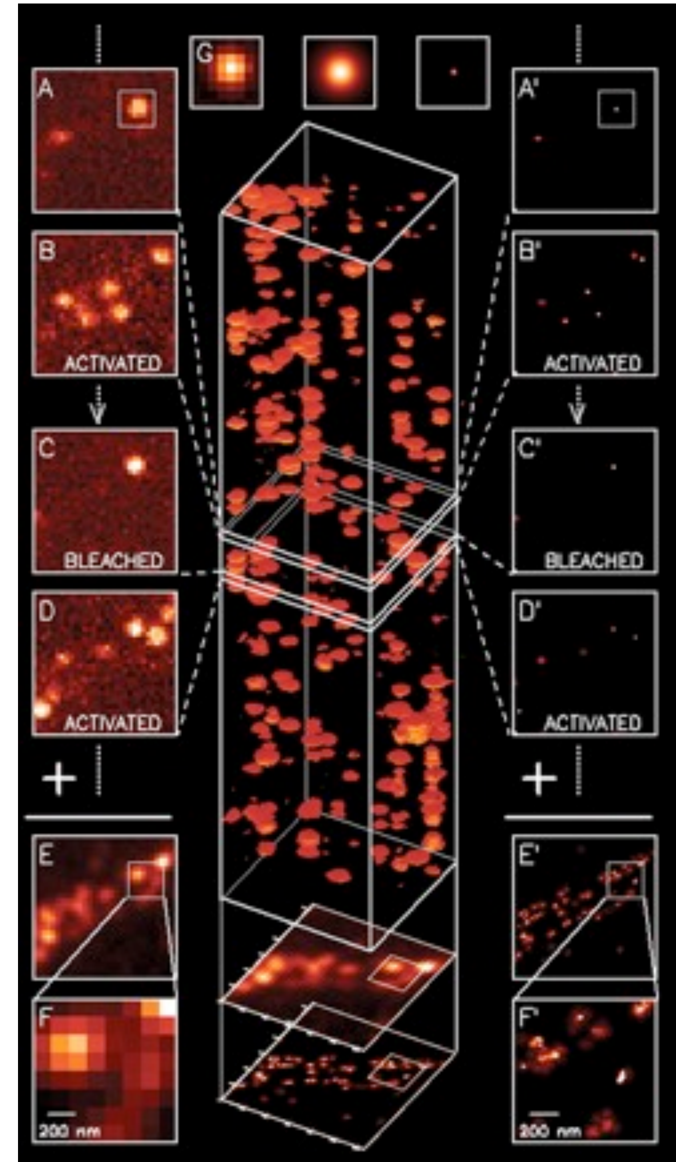
PALM (photoactivated localization microscopy) is an optical method for imaging intracellular proteins at nanometer spatial resolution.

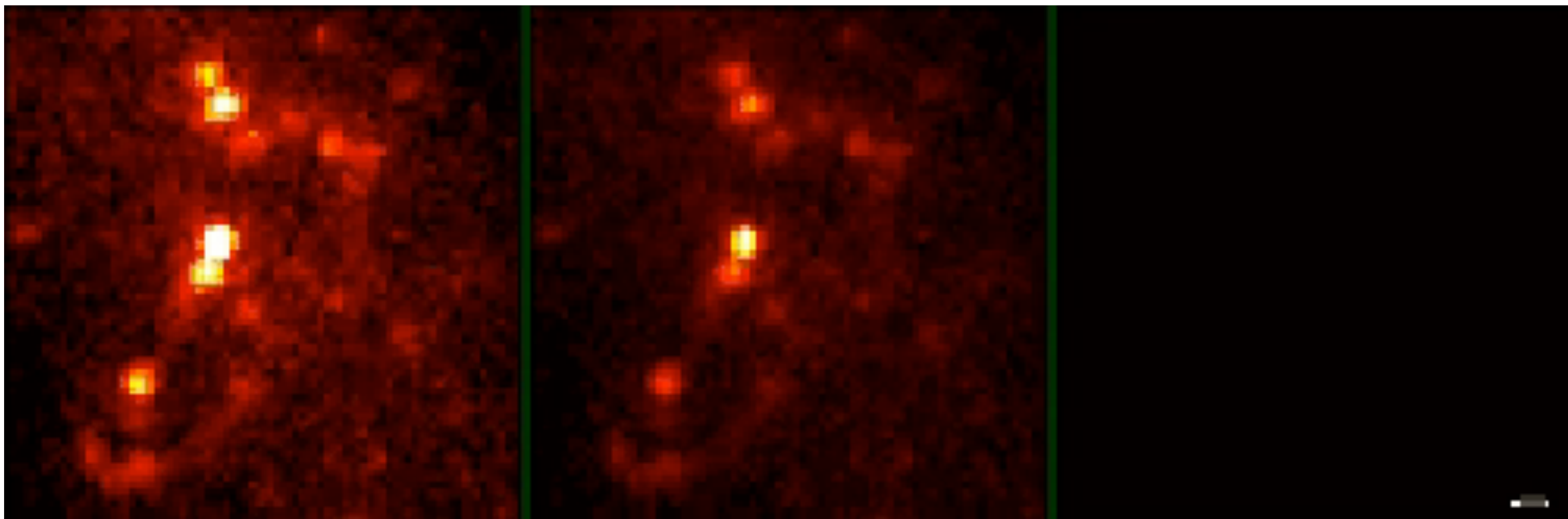




Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess H. Imaging intracellular fluorescent proteins at nanometer resolution. Science. 2006 Sep 15;313(5793):1642-5.

The principle behind PALM. A sparse subset of PA-FP molecules that are attached to proteins of interest and then fixed within a cell are activated (A and B) and then imaged until most are bleached (C). This process is repeated many times (C and D) until the population of inactivated, unbleached molecules is depleted. Summing the molecular images across all frames results in a diffraction-limited image (E and F). However, if the location of each molecule is first determined by fitting the expected molecular image given by the PSF of the microscope [(G), center] to the actual molecular image [(G), left], the molecule can be plotted [(G), right] as a Gaussian that has a standard deviation equal to the uncertainty s(x,y) in the fitted position. Repeating with all molecules across all frames (A' through D') and summing the results yields a superresolution image (E' and F') in which resolution is dictated by the uncertainties s(x,y) as well as by the density of localized molecules.





Partial summed molecule fluorescence image (center) and PALM image (right) constructed during the acquisition of 300 single molecule frames (left) out of 20,000 frames used to construct the images. Scale bar is 0.5 micron.

$$(\sigma_{x,y}{}^2)_m \approx \frac{s^2 + a^2/12}{N_m} + \frac{4\sqrt{\pi}\, s^3 b_m{}^2}{a N_m{}^2}$$

Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess H. Imaging intracellular fluorescent proteins at nanometer resolution. Science. 2006 Sep 15;313(5793):1642-5.

The principle behind PALM.

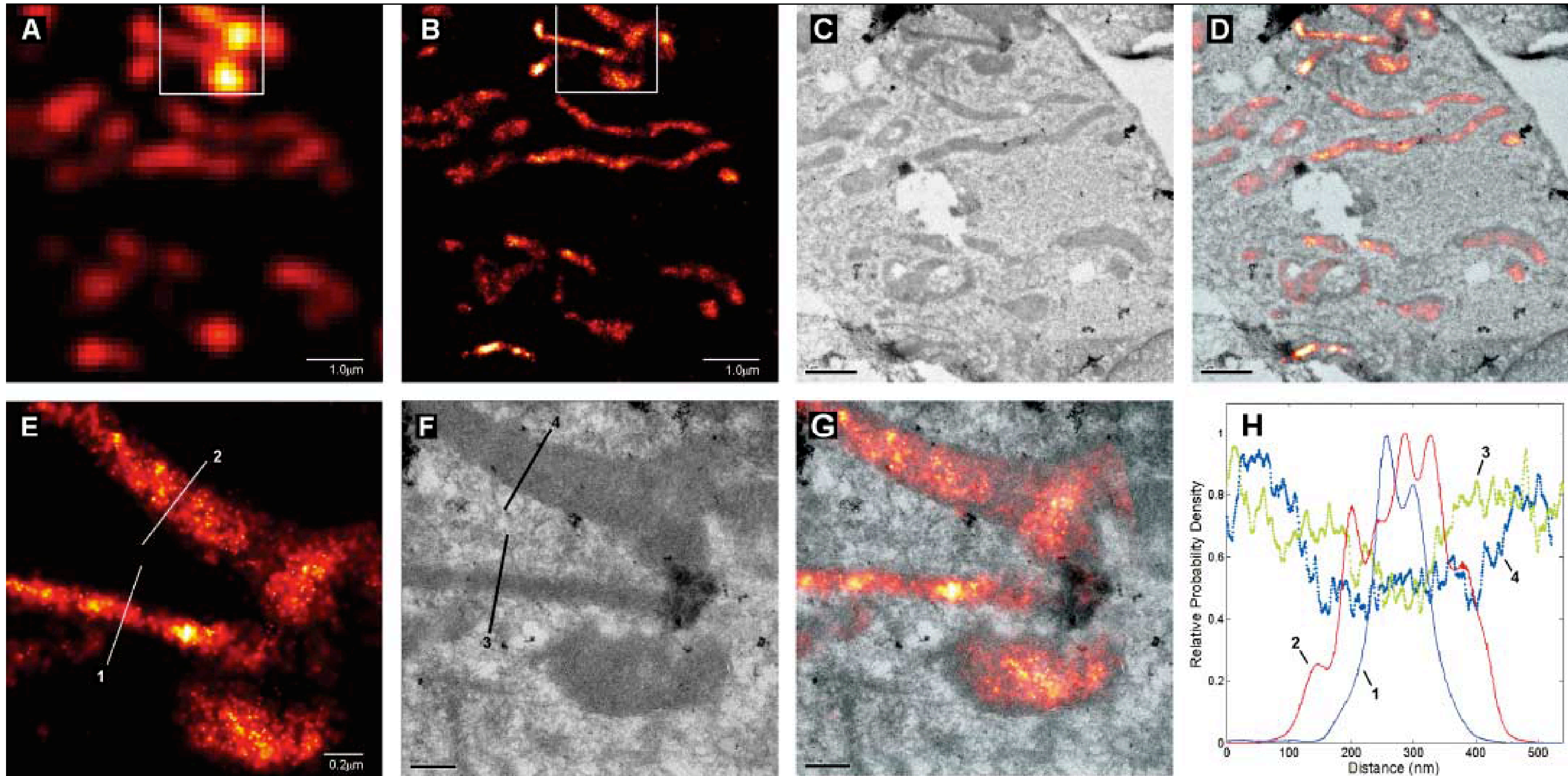
Central to the performance of photoactivated localization microscopy (PALM) is the precise localization of single fluorescent molecules performed by a least-squares fit of an assumed two-dimensional gaussian point spread function (PSF) to each single molecule image.

$$(\sigma_{x,y}{}^2)_m \approx \frac{s^2 + a^2/12}{N_m} + \frac{4\sqrt{\pi}\, s^3 b_m{}^2}{a N_m{}^2}$$

where s is the standard deviation of the PSF, a is the pixel size in the image (taking into account the system magnification), Nm is the total number of photons measured from molecule m, and bm is the number of background photons collected in the fitting windowused for molecule m.
Therefore, PALM design is predicated on achieving the highest possible diffraction limited resolution (i.e., small s) and collection efficiency (high Nm)consistent with minimal background noise bm .

The superresolution image resulting from the sum of all such rendered molecules thus provides a probability density map where brightness is proportional to the likelihood that a PA-FP molecule can be found at a given location.

Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess H. Imaging intracellular fluorescent proteins at nanometer resolution. Science. 2006 Sep 15;313(5793):1642-5.



Comparative summed-molecule TIRF (A), PALM (B), TEM (C), and PALM/TEM overlay (D) images of mitochondria in a cryo-prepared thin section from a COS-7 cell expressing dEosFP-tagged cytochrome-C oxidase import sequence.

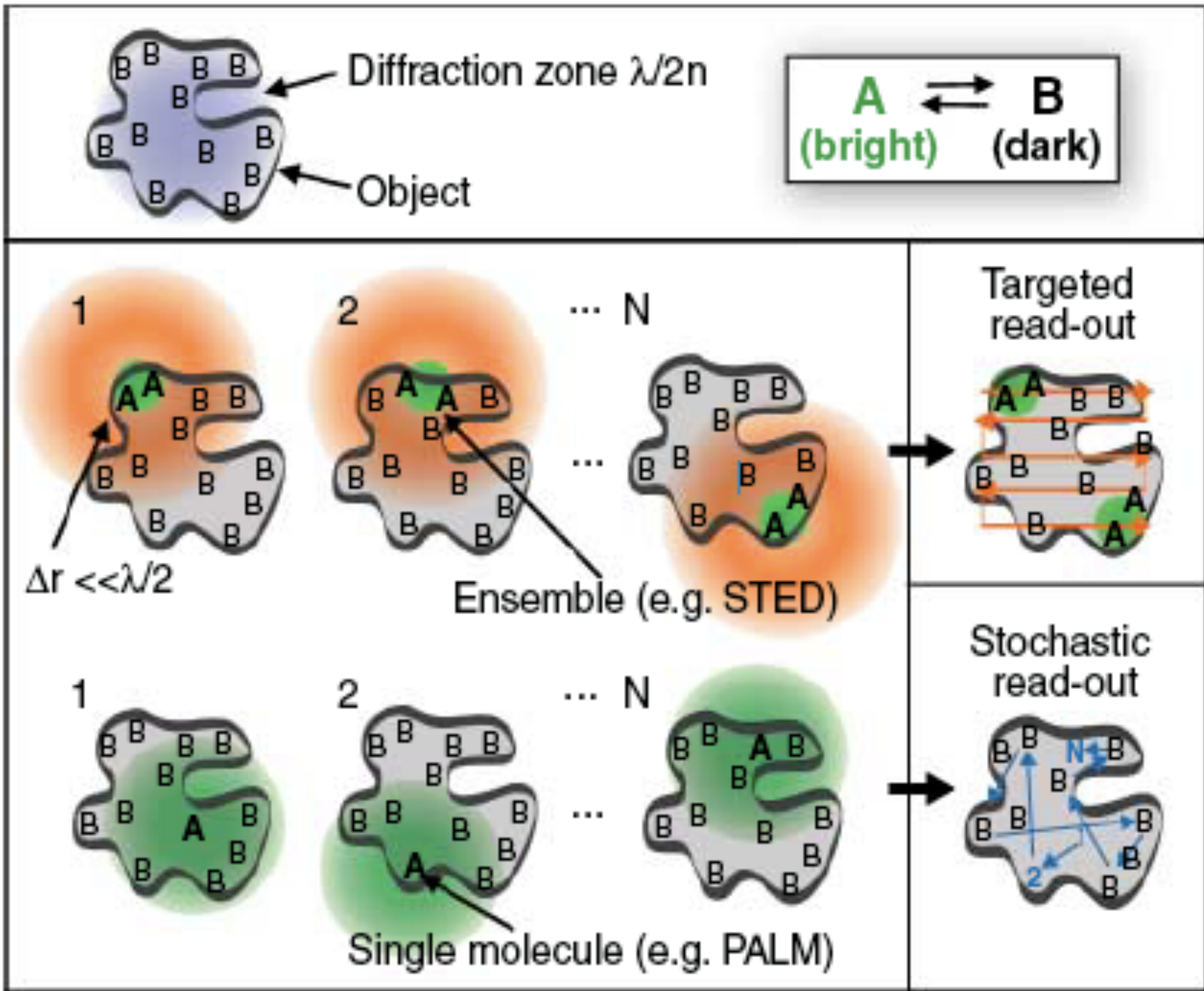
RRB

d-SRB

n-SRB

Vicidomini et al, *Traffic* 9:1828; 2008

CLEM - ‘Work-on-map’ approach



S.W. Hell, et al. Far-Field Optical Nanoscopy, Science 316, 1153 (2007)

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