

# Fluorescent labeling techniques in biomolecules: a flashback

Harekrushna Sahoo\*

*Received 3rd March 2012, Accepted 18th April 2012*

DOI: 10.1039/c2ra20389h

## **Scheme 1.** Fluorescent

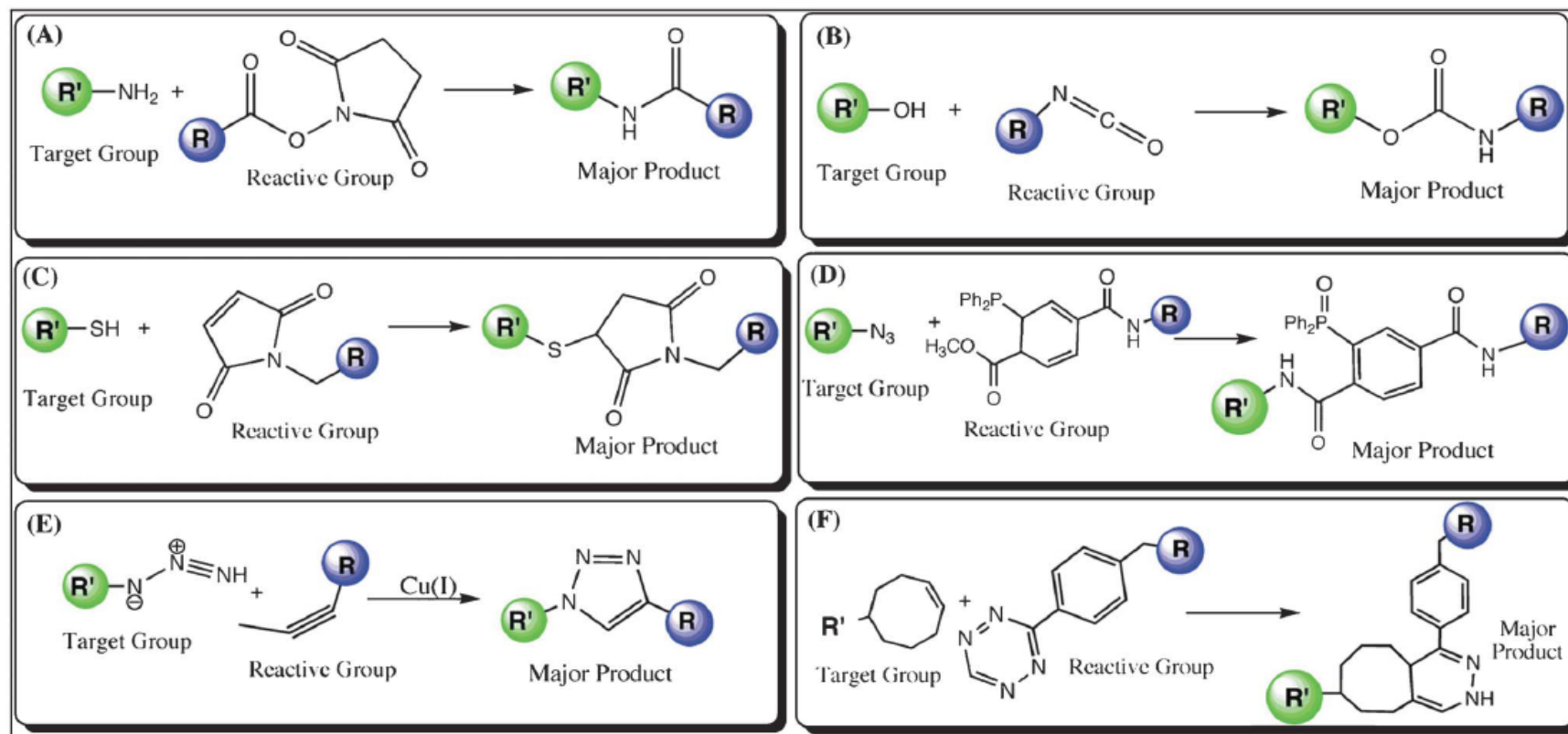
labeling scheme: a schematic diagram showing the labeling

between a biomolecule (green) linked to a tag (light blue) and a fluorophore (navy blue).

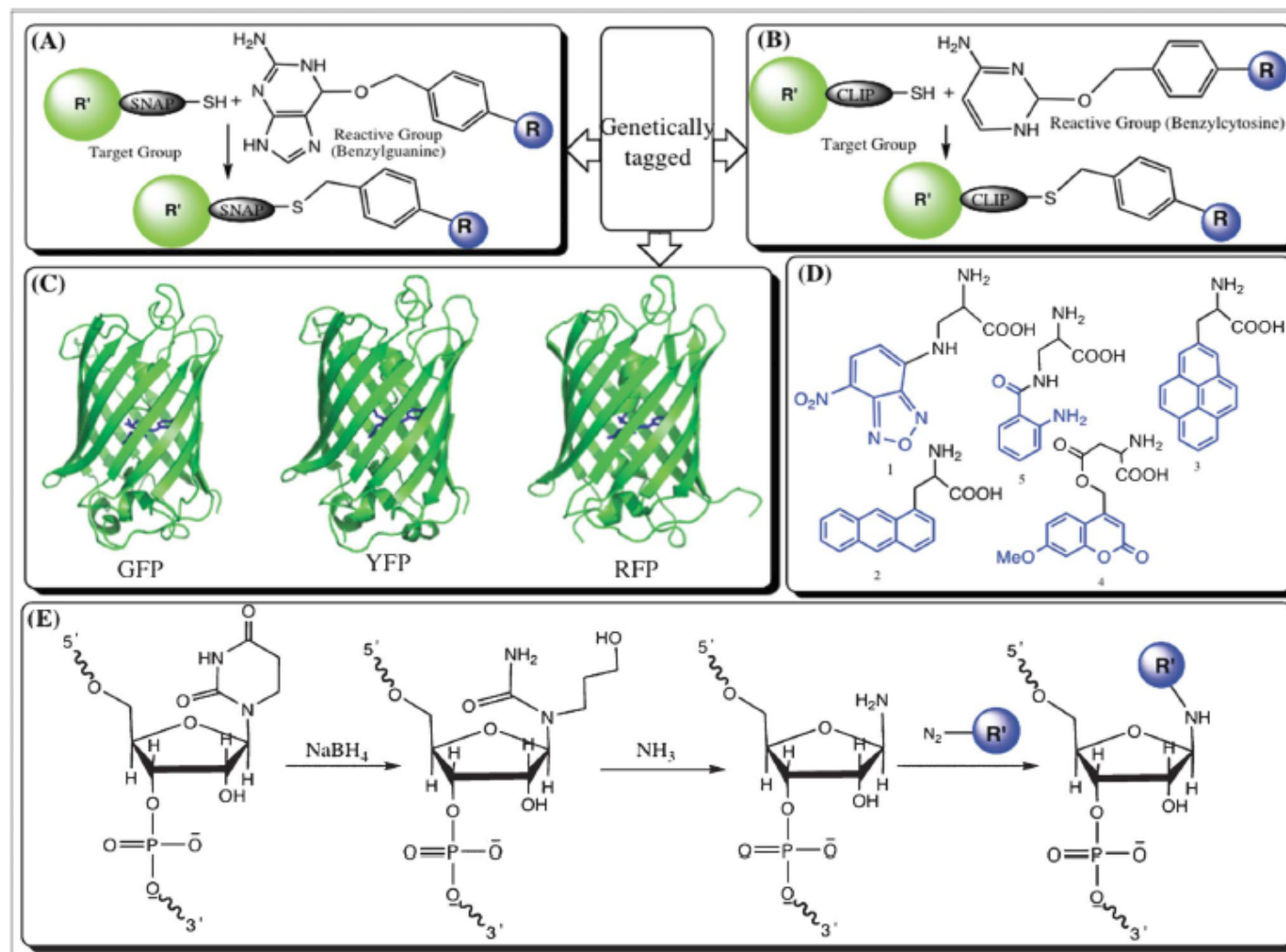


A general list of most commonly used fluorescent methods:

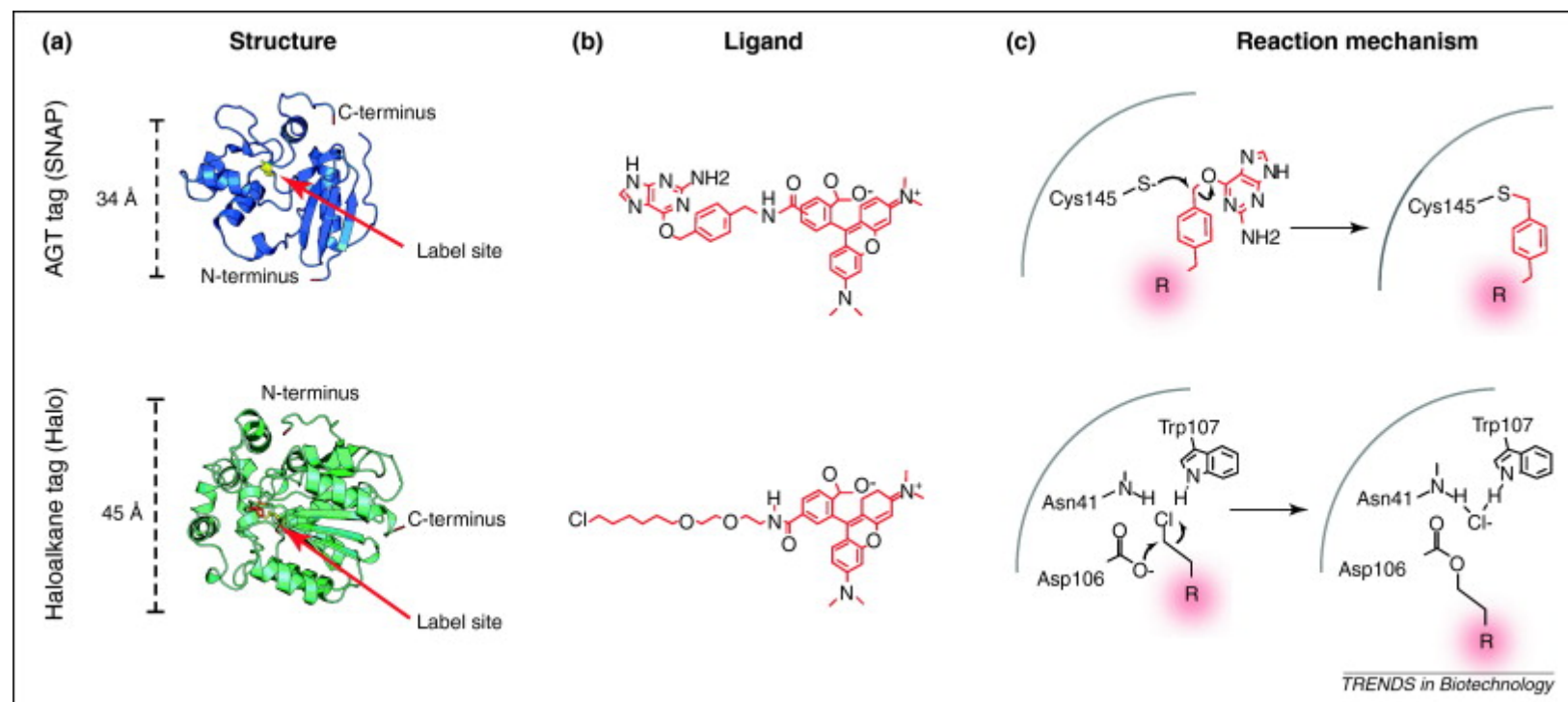
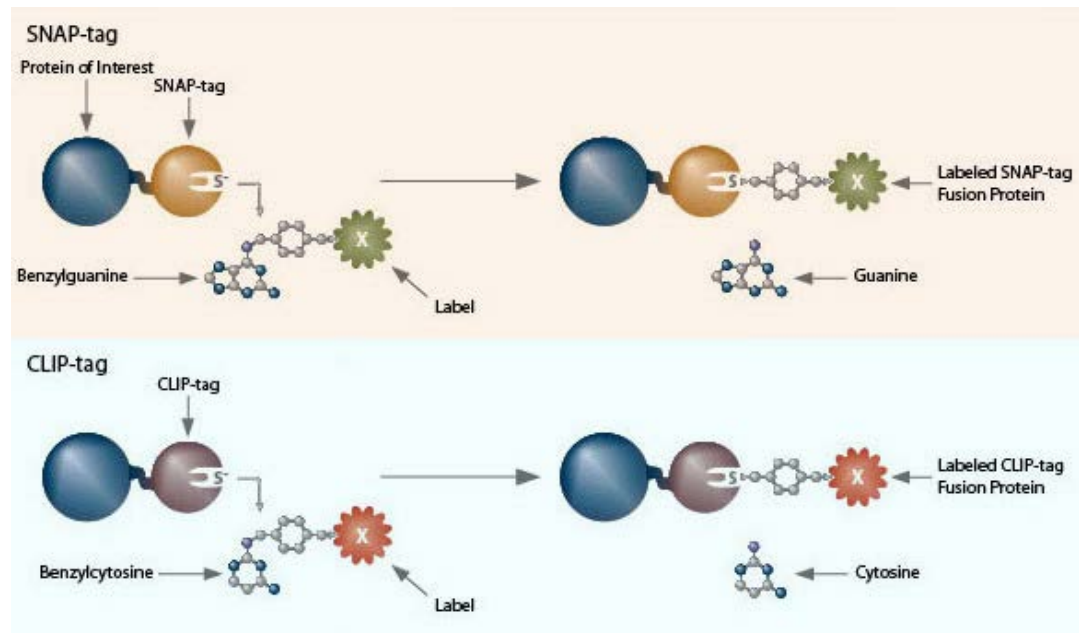
| Fluorescent Methods     |                       |                                              |
|-------------------------|-----------------------|----------------------------------------------|
| Chemical                | Enzymatic             | Tagging                                      |
| Maleimide-Thiol         | Transglutaminase      | Tetracysteine                                |
| NHS ester-Amine         | Sortase               | Histidine                                    |
| Isothiocyanate-Amine    | Cutinase              | Aspartate                                    |
| Alkyl Halide-Hydroxy    | Intein                | Lanthanide                                   |
| Isocyanate-Hydroxy      | Lipoic Acid ligase    | CLIP                                         |
| Tetrazine-Alkene/Alkyne | Biotin Ligase         | Halo                                         |
|                         | Myristoyl Transferase | SNAP                                         |
|                         |                       | Fluorescent proteins<br>(Genetically Tagged) |



**Fig. 1** Schematic diagram of chemical labeling techniques (R' in green represents the biomolecule to be labeled with a target moiety, while R is the fluorophore linked with a reactive group); (A) Amine labeling: an NHS (*N*-hydroxysuccinimide)-ester group couples to the  $\text{-NH}_2$  moiety, (B) Hydroxy labeling: a fluorophore is modified with an isocyanate group, which couples to the  $\text{-OH}$  moiety, (C) Thiol labeling: coupling between a maleimide modified fluorophore and the  $\text{-SH}$  moiety, (D) Azide labeling: Staudinger ligation of azide moiety with phosphine group, (E) Azide labeling: copper(I)-catalyzed cycloaddition of a fluorophore with the azide group linked to a biomolecule, and (F) Tetrazine labeling: A biomolecule labeled with tetrazine undergoes Diels-Alder cycloaddition with a dienophile linked to a fluorophore.



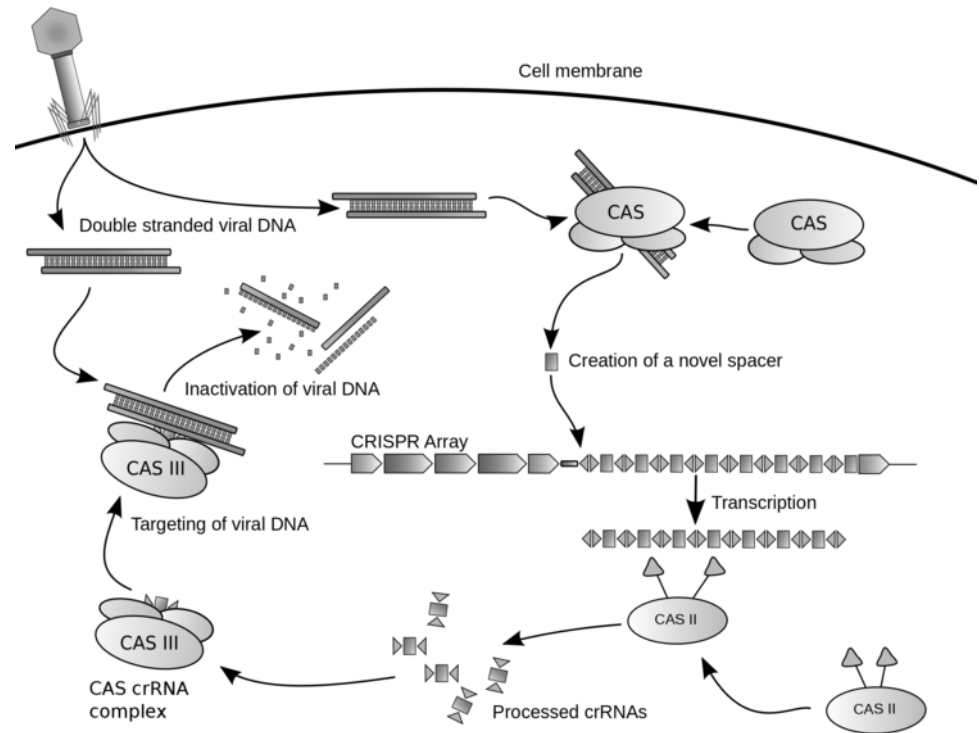
**Fig. 6** Schematic representation of labeling techniques using genetic modifications: (A) SNAP-tag: attachment of benzylguanine conjugated to a fluorophore (R, blue) to the SNAP tag fused to the target protein (R', green); (B) CLIP-tag: attachment of benzylcytosine conjugated to a fluorophore (R, blue) to the SNAP tag fused to the target protein (R', green); (C) Fluorescent protein: Green fluorescent proteins and a few examples of its variants, such as GFP (excitation and emission: 488 and 508 nm), YFP (excitation and emission: 514 and 530 nm) and RFP (excitation and emission: 554 and 585 nm). The blue color represents the chromophoric compound in the protein; (D) Unnatural amino acids: a few examples of widely used fluorescent non-natural amino acids, such as microenvironment sensitive (3-(4-nitrobenzo[c]1,2,5-oxadiazol-7-ylamino)-2-aminopropanoic acid (1), amino acids carrying 7-methoxycoumarin (4) and b-anthraniloyl (5)), 2-amino-3-(anthracen-5-yl)propanoic acid (2), which is less sensitive towards polar and nonpolar solvents and a hydrophobic labeled dye (2-amino-3-(pyren-2-yl)propanoic acid (3)). Moieties in blue indicate the fluorescent groups with a longer excitation wavelength than 320 nm, which thus remove the interference from the intrinsic fluorophores in the protein, and (E) tRNA: Reductive cleavage of dihydrouridine in the presence of  $\text{NaBH}_4$  and subsequent amination to form an amine group at the target site. In the second step, a fluorophore (R in blue) modified with a dihydrazide moiety is coupled to the primary amine group of the target site.





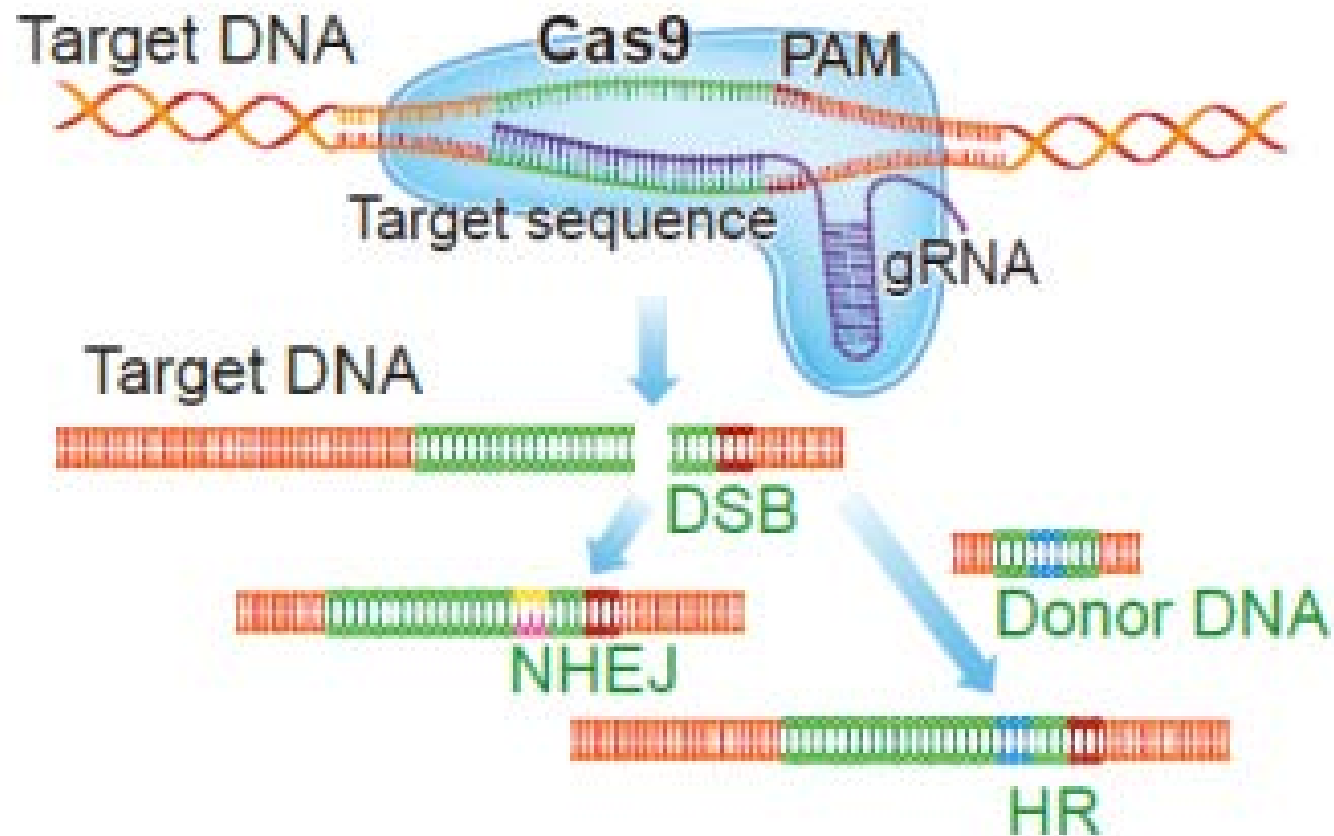
# CRISPR CAS9

**CRISPRs (clustered regularly interspaced short palindromic repeats)** are segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of "spacer DNA" from previous exposures to a bacterial virus or



# Movie

- <https://youtu.be/2pp17E4E-O8>



# **Super-Resolution Microscopy**





# The Nobel Prize in Chemistry 2014



Photo: Matt Staley/HHMI

**Eric Betzig**

Prize share: 1/3



© Bernd Schuller, Max-Planck-Institut

**Stefan W. Hell**

Prize share: 1/3



Photo: K. Lowder via Wikimedia Commons, CC-BY-SA-3.0

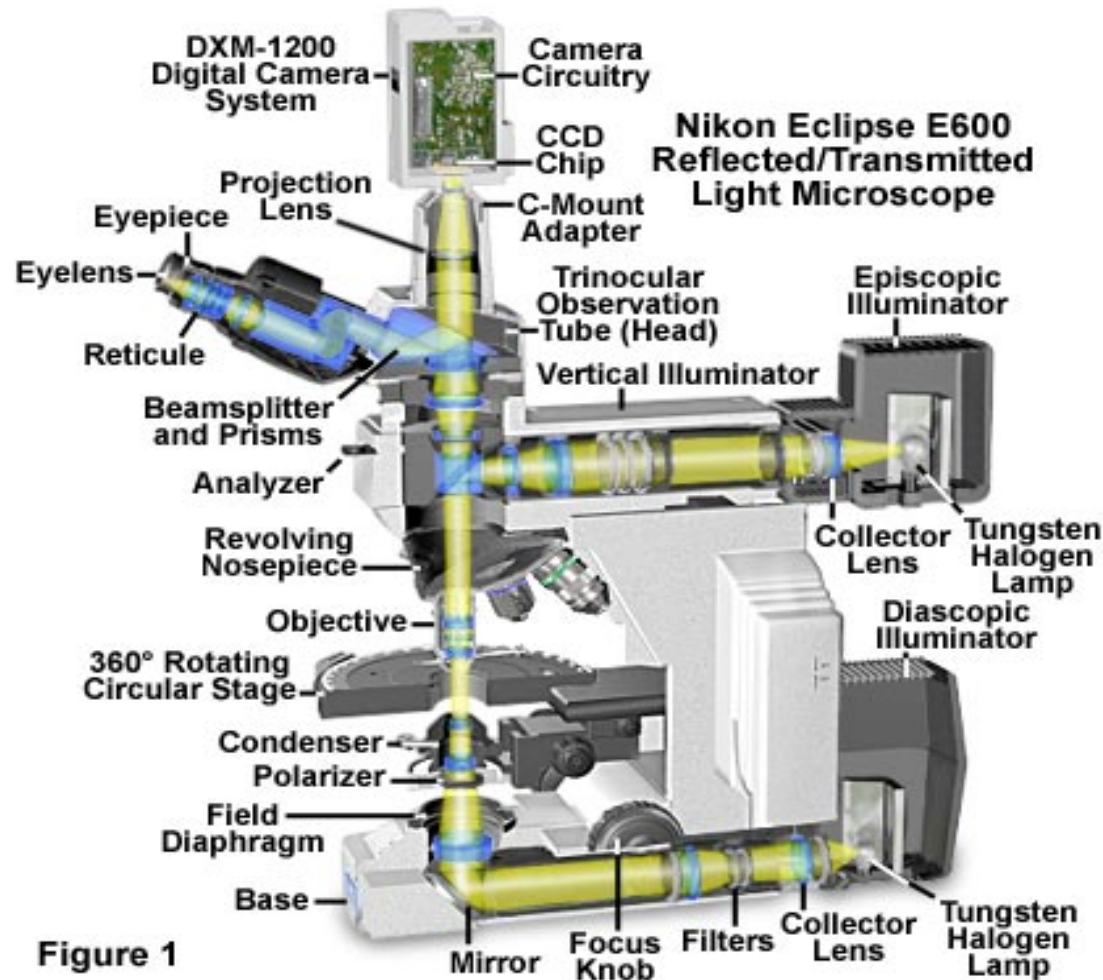
**William E. Moerner**

Prize share: 1/3

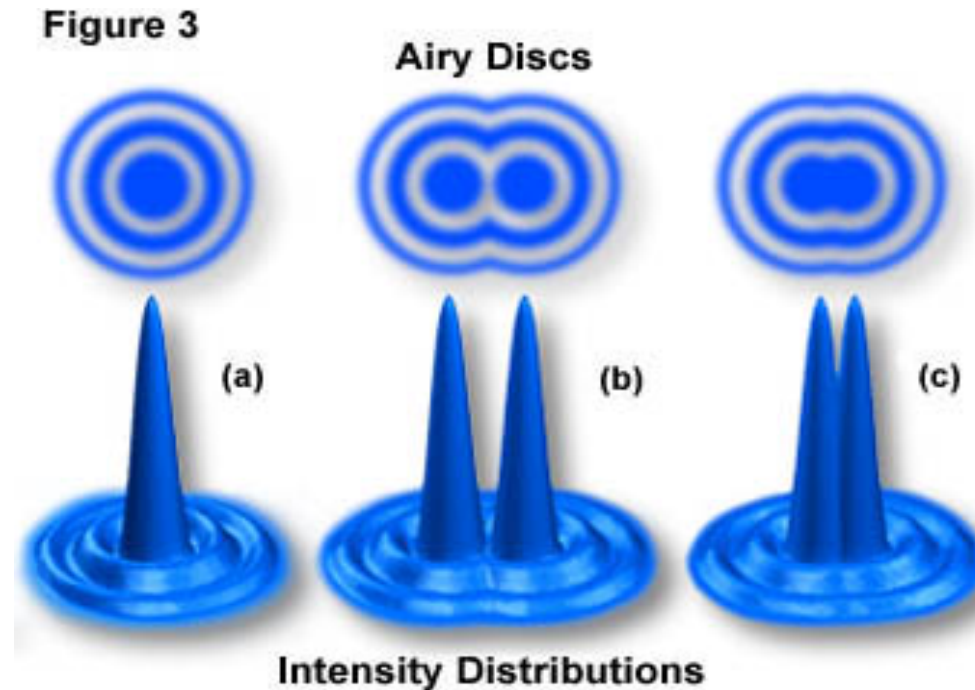
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

Two separate principles are rewarded. One Eric Betzig and William Moerner, working enables the method *stimulated emission depletion (STED) microscopy*, developed by second method, *single-molecule microscopy*. Stefan Hell in 2000.

# Microscope



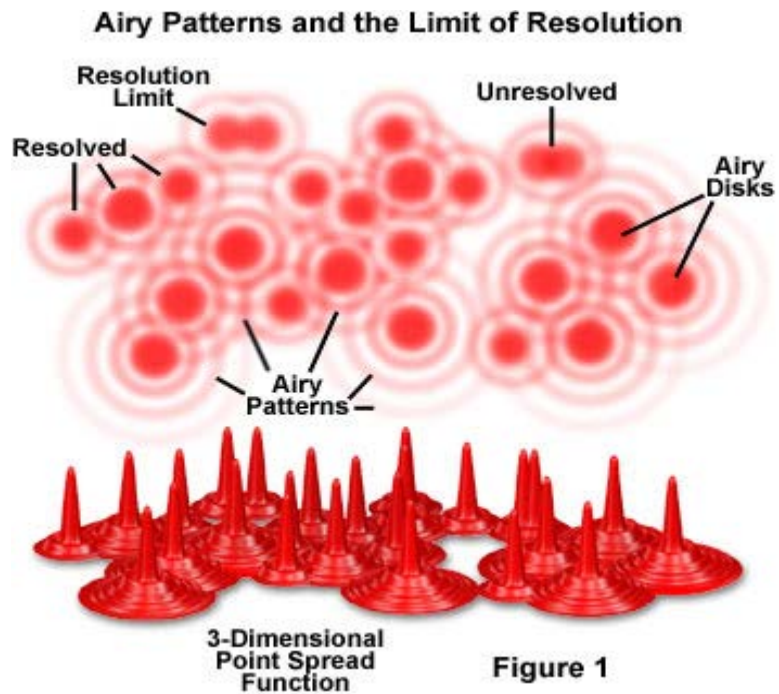
# Resolution



$$\text{Resolution } (r) = \lambda / (2NA) \quad (1)$$

$$\text{Resolution } (r) = 0.61 \lambda / NA \quad (2)$$

$$\text{Resolution } (r) = 1.22 \lambda / (NA(\text{obj}) + NA(\text{cond})) \quad (3)$$



**Figure 1**

| Magnification | Objective Type |                 |               |                 |                 |                 |
|---------------|----------------|-----------------|---------------|-----------------|-----------------|-----------------|
|               | Plan Achromat  |                 | Plan Fluorite |                 | Plan Apochromat |                 |
|               | N.A.           | Resolution (μm) | N.A.          | Resolution (μm) | N.A.            | Resolution (μm) |
| 4x            | 0.10           | 2.75            | 0.13          | 2.12            | 0.20            | 1.375           |
| 10x           | 0.25           | 1.10            | 0.30          | 0.92            | 0.45            | 0.61            |
| 20x           | 0.40           | 0.69            | 0.50          | 0.55            | 0.75            | 0.37            |
| 40x           | 0.65           | 0.42            | 0.75          | 0.37            | 0.95            | 0.29            |
| 60x           | 0.75           | 0.37            | 0.85          | 0.32            | 0.95            | 0.29            |
| 100x          | 1.25           | 0.22            | 1.30          | 0.21            | 1.40            | 0.20            |

N.A. = Numerical Aperture

### Resolution versus Wavelength

| Wavelength (Nanometers) | Resolution (Micrometers) |
|-------------------------|--------------------------|
| 360                     | .19                      |
| 400                     | .21                      |
| 450                     | .24                      |
| 500                     | .26                      |
| 550                     | .29                      |
| 600                     | .32                      |
| 650                     | .34                      |
| 700                     | .37                      |

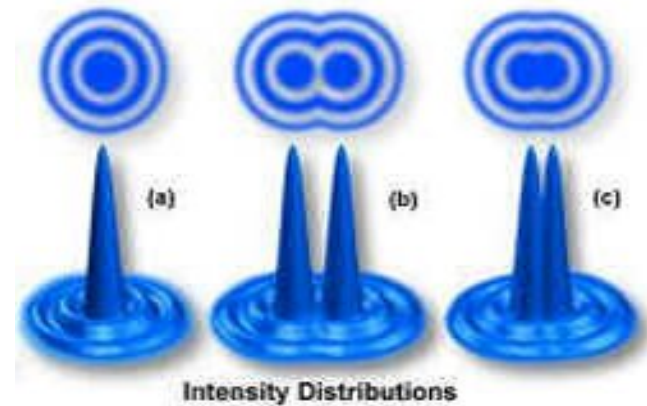
$$\text{N.A.} = 0.95$$

# Diffraction Limit

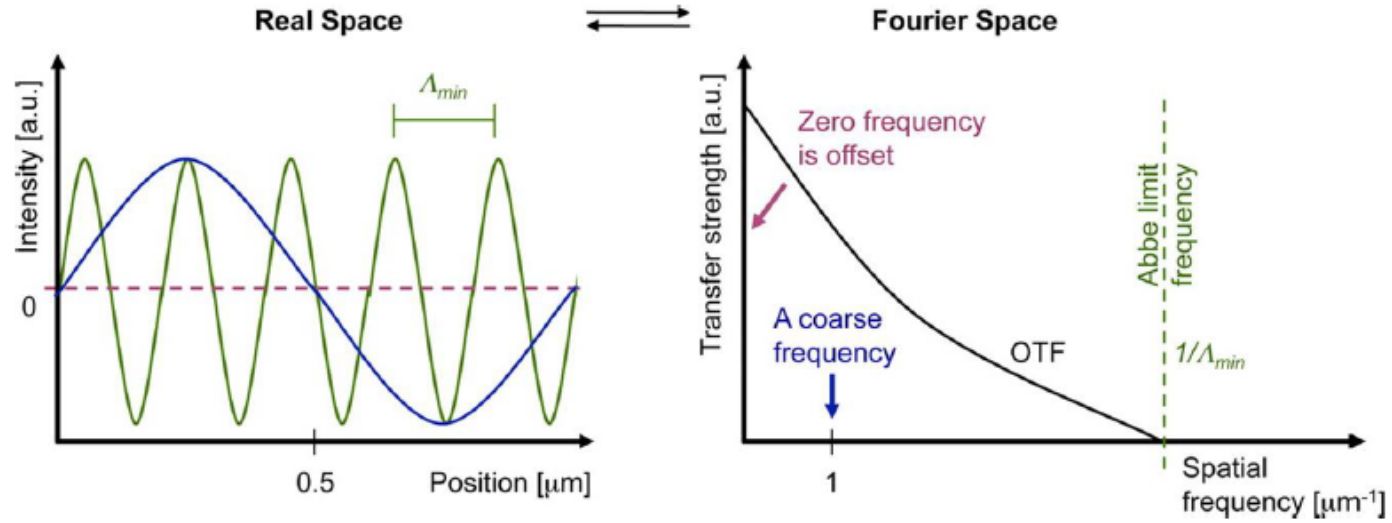


$$d = \lambda / (2n \sin \alpha)$$

$$, k_0 = 2NA / \lambda_{em}$$







For a homogeneous medium, the solution to the homogeneous equation is of the form

$$H_{\omega}(k_r, z) = A^+(k_r) e^{ik_z z} + A^-(k_r) e^{-ik_z z} \quad (2.98)$$

with  $k_z$  being the vertical wavenumber,

$$k_z = \sqrt{k^2 - k_r^2}. \quad (2.99)$$

Since the inverse Hankel transform must be evaluated over a semi-infinite wave-number domain, we have to choose a definition for the square root for  $k_r > k$ . We choose the definition

$$k_z = \begin{cases} \sqrt{k^2 - k_r^2}, & k_r \leq k \\ i\sqrt{k_r^2 - k^2}, & k_r > k. \end{cases} \quad (2.100)$$

# Breaking the Diffraction Barrier: Super-Resolution Imaging of Cells

Bo Huang,<sup>1</sup> Hazen Babcock,<sup>2</sup> and Xiaowei Zhuang<sup>2,3,\*</sup>

Cell 143, December 23, 2010 ©2010 Elsevier Inc. 1047

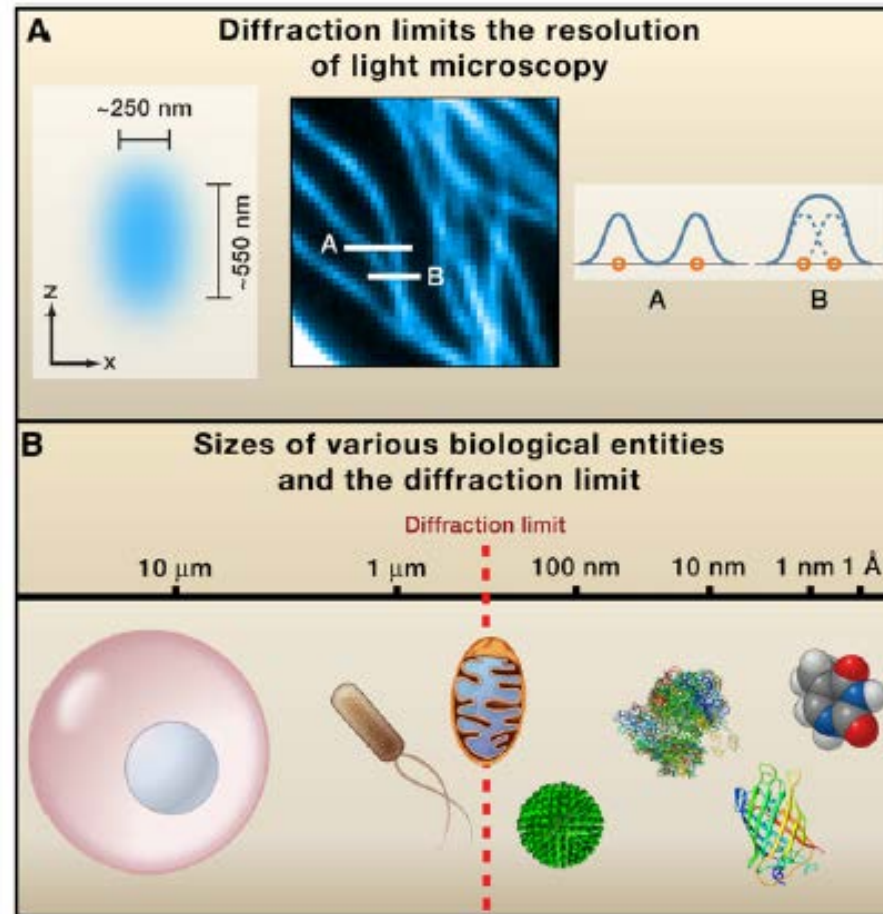


Figure 1. Diffraction-Limited Resolution of Conventional Light Microscopy

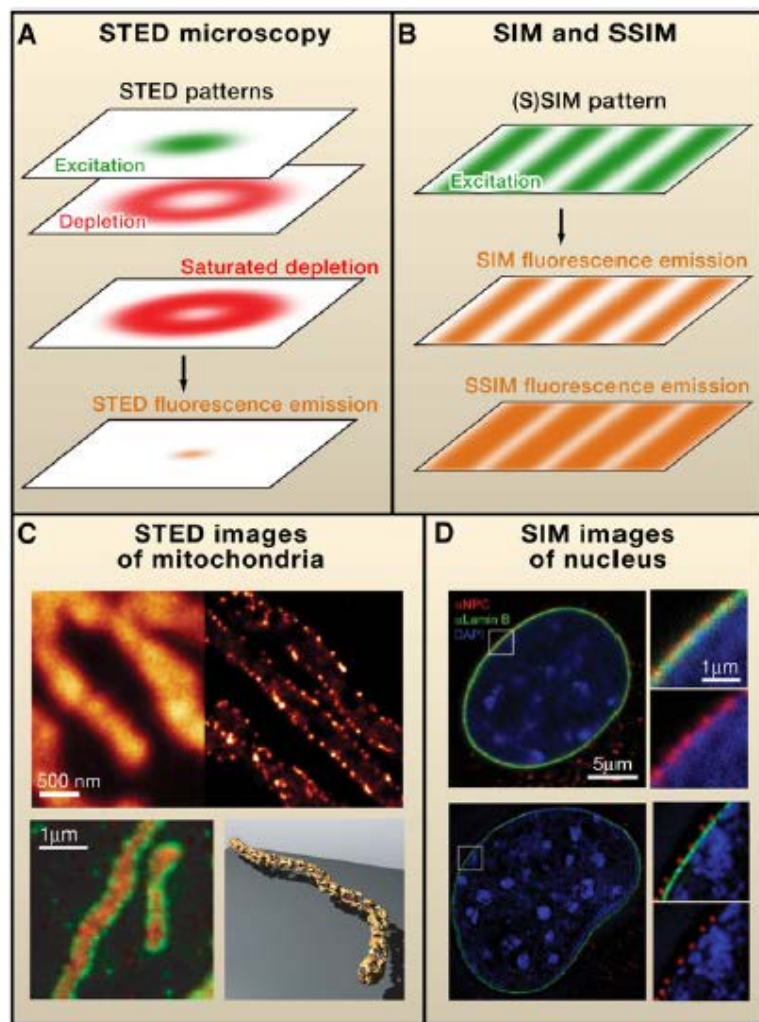


Figure 2. Super-Resolution Fluorescence Microscopy by Patterned Illumination

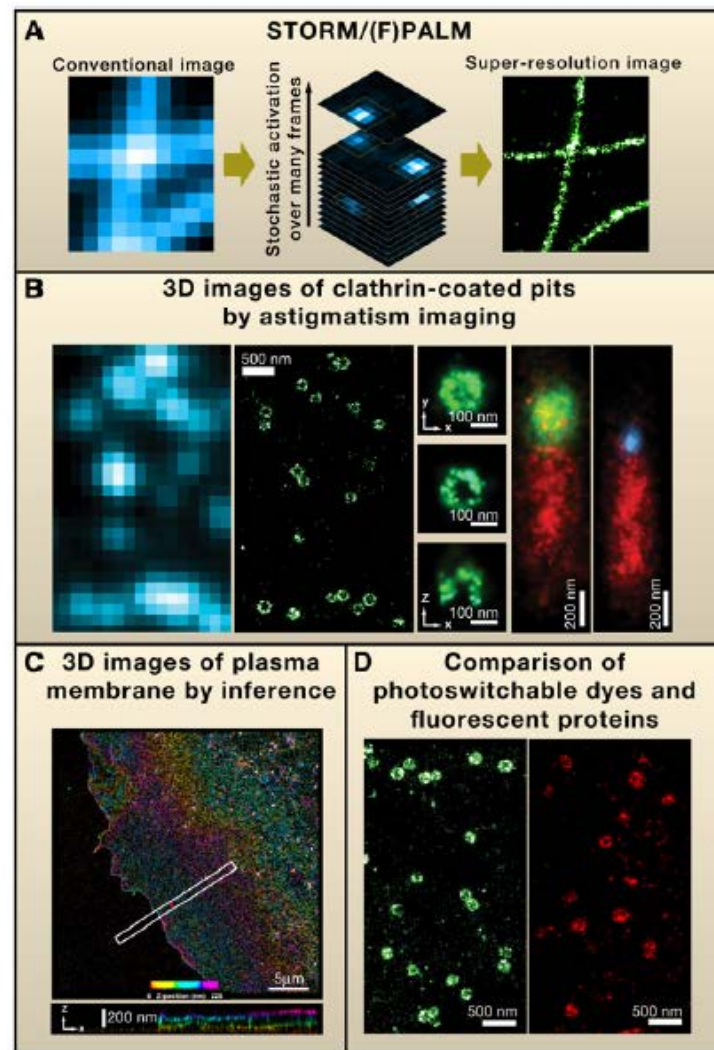
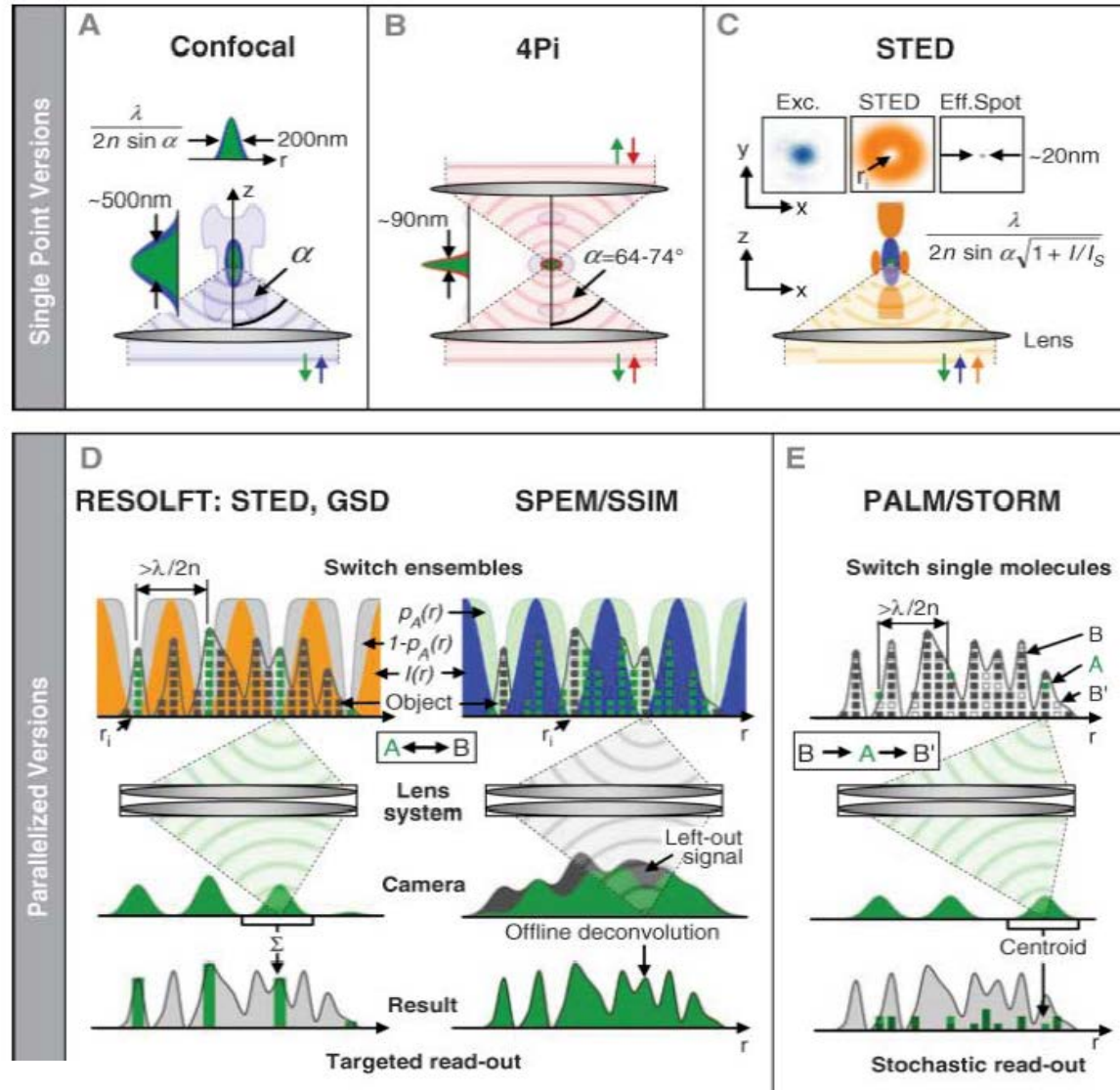
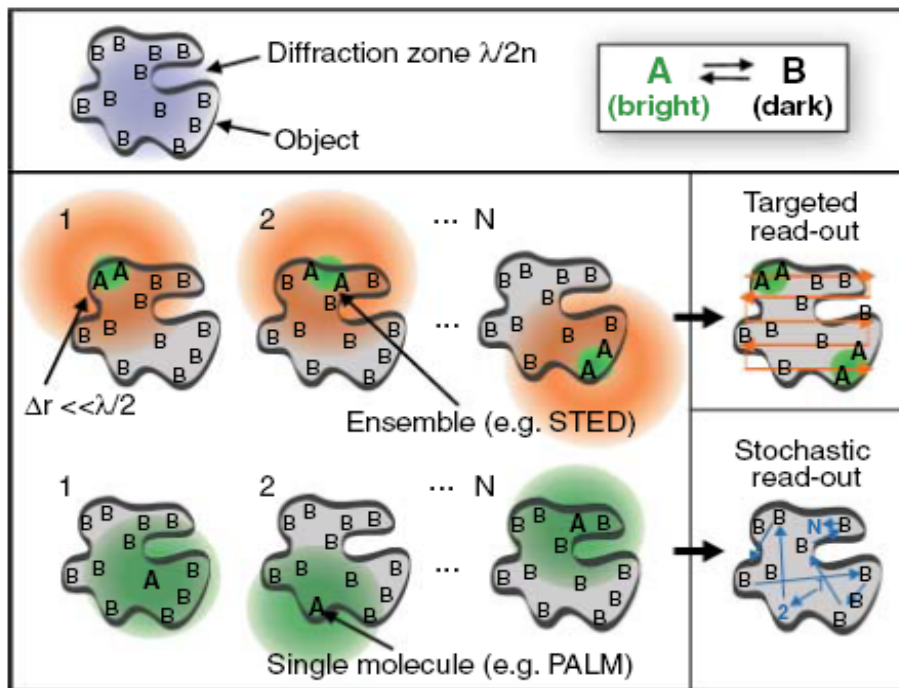


Figure 3. Super-Resolution Fluorescence Microscopy by Single-Molecule Switching

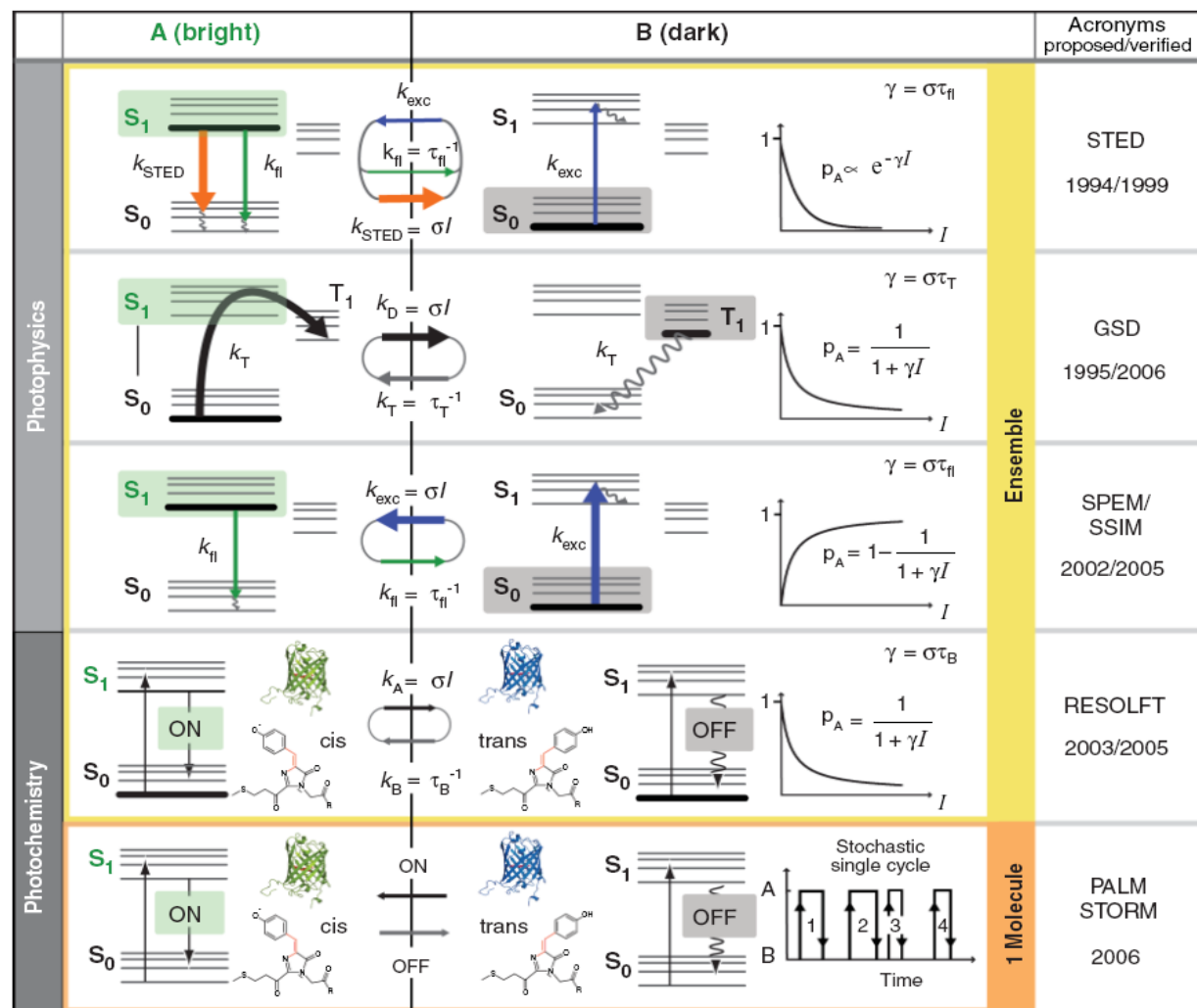


# Super-Resolution Microscopy



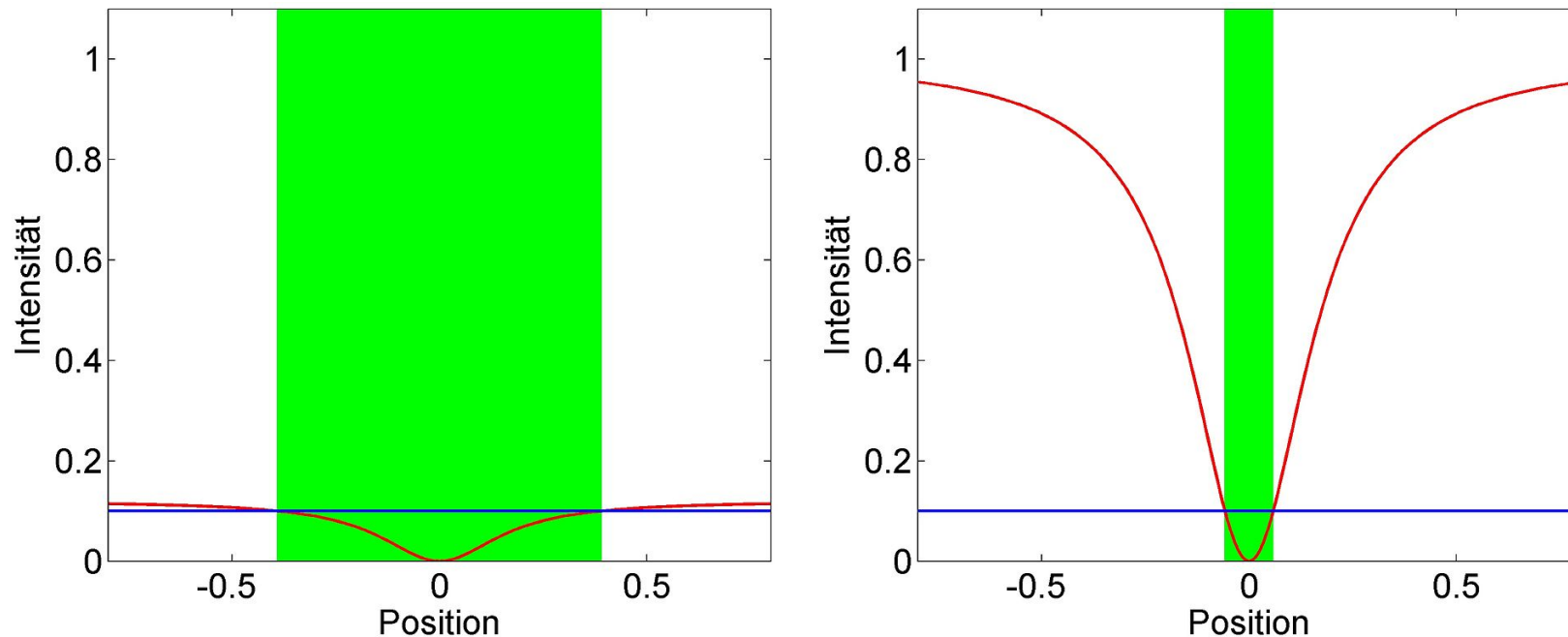


**Fig. 2.** Targeted versus stochastic time-sequential readout of fluorophore markers of a nanostructured object within the diffraction zone whose lower bound is given by  $\lambda/2n$ . A and B denote a bright and a dark state, respectively. In the targeted readout mode, one of the two states (here A) is established at a subdiffraction-sized spot at the position of a zero to read out an unknown number of fluorophore molecules. The image is assembled by deliberate translation of the zero. The zero can also be a groove. In the stochastic readout mode, a single switchable fluorophore from a random position within the diffraction zone is switched to a stable state A, while the other molecules remain in B. The coordinate is calculated from the centroid of the diffraction fluorescence spot measured by a pixelated detector. The coordinate pops up stochastically depending on where the interrogated marker molecule is located.



**Fig. 3.** Bright (A) and dark (B) molecular states used to break the diffraction barrier. Whereas STED, GSD, and SPEM utilize photophysical transitions, the photoswitching version of the RESOLFT scheme, as well as PALM and STORM, exploit photochemical transitions in which atoms are relocated or bonds formed and broken. PALM and STORM rely on measuring single (or at least identifiable) molecules at a time, whereas the other concepts, although compatible with single-molecule imaging, principally read out ensembles. Ensemble techniques rely on reversible transitions between A and B, as indicated by the rates  $k$ . The probability  $p_A$  of being in state A depends nonlinearly on the light intensity applied, as indicated by the equations, ensuring that either A or B is confined to a subdiffraction area at a targeted coordinate in space. The  $e^{-\gamma I}$  and the  $(1 + \gamma I)^{-1}$  dependence entail nonlinearities of infinite order  $(\gamma I)^m$ ;  $m \rightarrow \infty$ . By increasing the lifetime of the chosen states,  $\gamma$  strengthens the nonlinear dependence of  $p_A$ , thus enabling huge nonlinearities at low  $I$ . This is radically different from  $m$ -photon processes that, depending on the concomitant action of  $m$  photons and hence just on  $I^m$ , are firmly limited to order  $m$  (15), which in practice is only  $m < 4$ . Because it operates with single molecules in a known state, the probability concept breaks down in PALM and STORM, but reminiscent of nonlinearity is the optical switching.

# REversible Saturable Optical Fluorescence Transitions (RESOLFT)



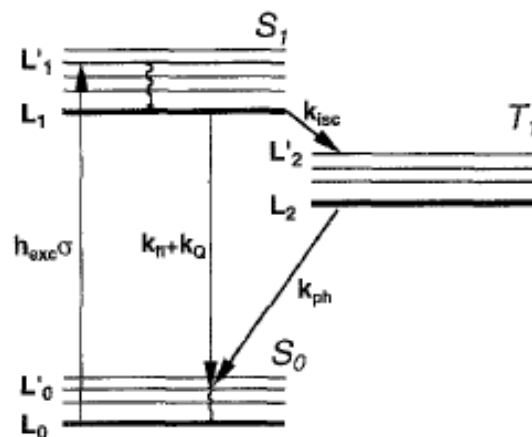
Principle of resolution increase in a RESOLFT microscope. Horizontally a spatial dimensions is drawn. The red line corresponds to the spatially variable intensity of the light beam which drives molecules from a certain state (green) to another state (white). The blue line describes the level of saturation for this transition. Increasing the brightness (right side) of the light beam one can restrict the area, where molecules can reside in their original state (green) to arbitrarily small volumes

## *Rapid communication*

# **Ground-state-depletion fluorescence microscopy: a concept for breaking the diffraction resolution limit**

S.W. Hell, M. Kroug

Department of Medical Physics, University of Turku, and Centre for Biotechnology, P.O. Box 123, FIN-20521 Turku, Finland  
(Fax: +358-21/633-8000, E-mail: STEFAN.HELL@utu.fi)



**Fig. 1.** Energy states of a typical dye:  $S_0$  is the ground state,  $S_1$  is the first singlet state, and  $T_1$  the first triplet state

$$\begin{aligned}\frac{dn_0}{dt} &= -h_{\text{exc}}\sigma n_0 + (k_{\text{fl}} + k_{\text{Q}}) n_1 + k_{\text{ph}} n_2, \\ \frac{dn_1}{dt} &= +h_{\text{exc}}\sigma n_0 - (k_{\text{fl}} + k_{\text{Q}}) n_1 - k_{\text{isc}} n_1, \\ \frac{dn_2}{dt} &= +k_{\text{isc}} n_1 - k_{\text{ph}} n_2,\end{aligned}$$

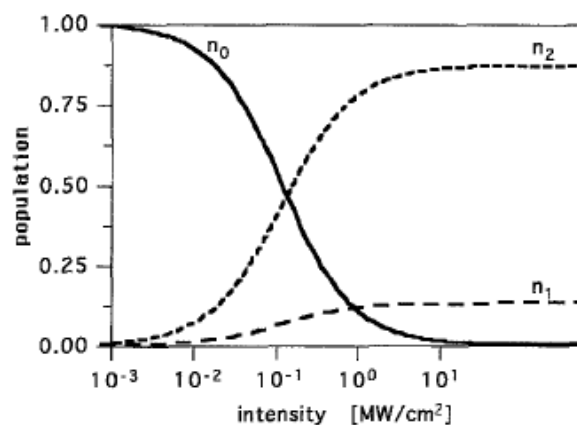


Fig. 2. The population probability of the ground state ( $n_0$ ), the first singlet state ( $n_1$ ), and the triplet state ( $n_2$ ) as a function of the excitation intensity for fluorescein

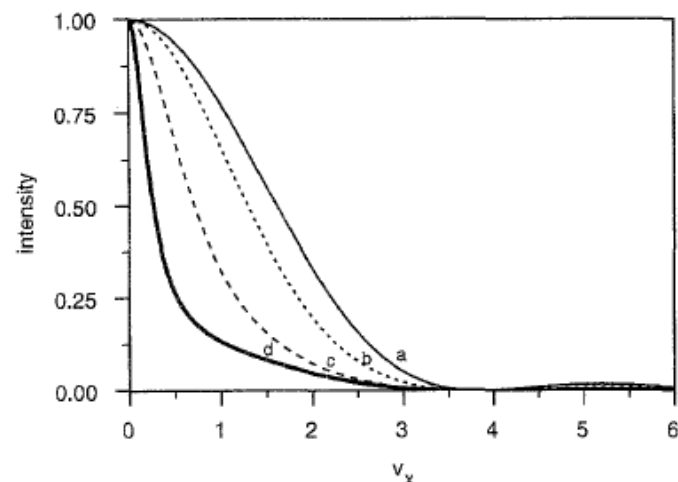


Fig. 4. Calculated effective point-spread function along the axis of the offset for peak intensities of (b) 0.01, (c) 0.1, and (d) 1 MW/cm<sup>2</sup> of the depletion beam as calculated for fluorescein, compared with the point-spread function of a classical scanning fluorescence microscope

tion, as shown in Fig. 5. A comparison of the point-spread-function obtained for a focal intensity of 1 MW/cm<sup>2</sup> with that of a classical scanning fluorescence microscope shows an increase in resolution by a factor of 11. Assuming an excitation wavelength of 400 nm and a numerical aperture of 1.4, one obtains a lateral FWHM of about 15 nm.



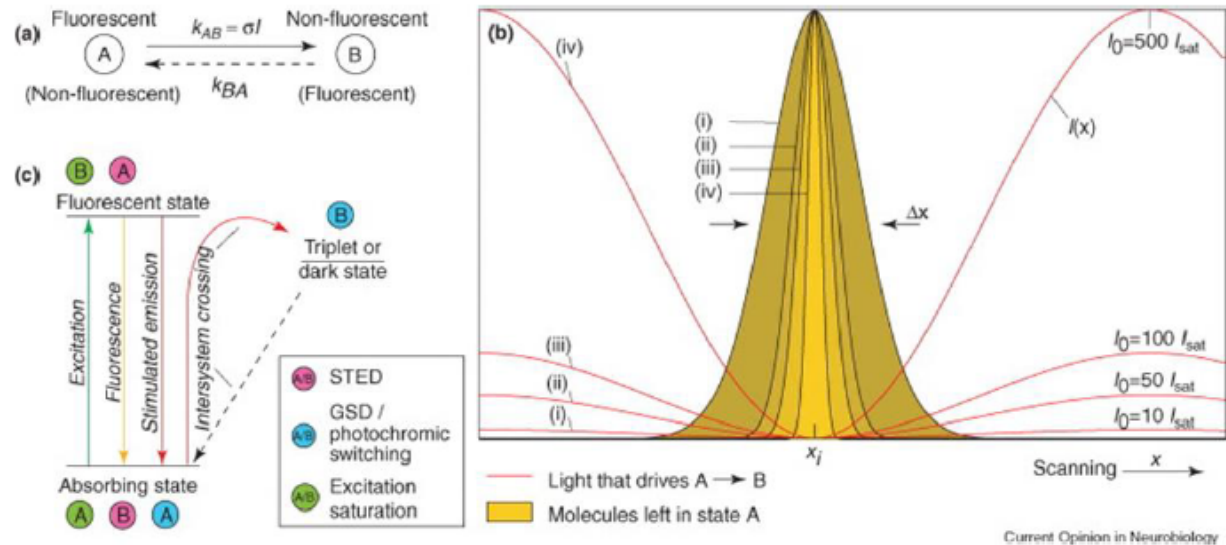
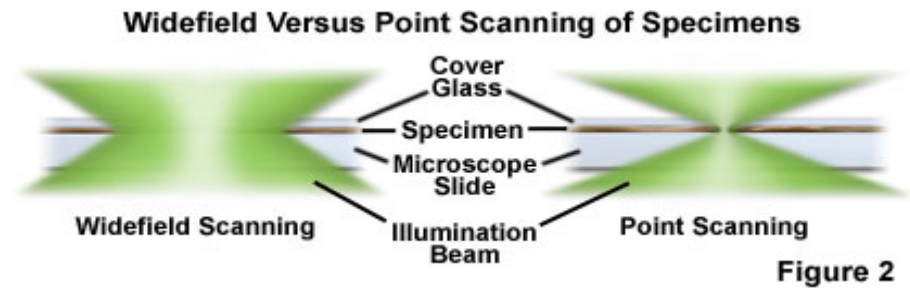
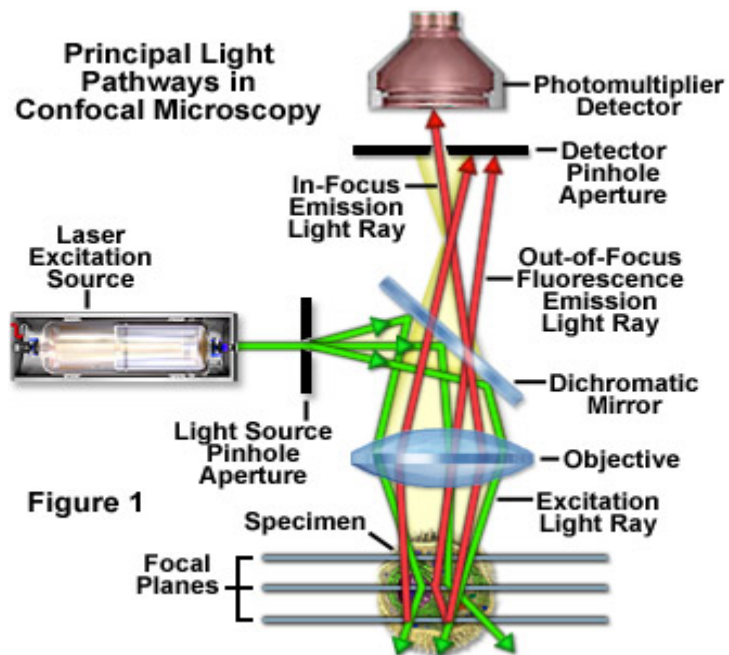


Figure 4: (Hell *et al.*, *Current Opinion in Neurobiology* 2004, 14:599–609). (a) Illustration of the fluorescence depletion principle. (b) Fluorescence density (yellow) as a function of the x-coordinate in the focal plane at STED intensities ( $I(x)$ , red) with different maximal strengths ( $I_0$ ). The fluorescence density (yellow) becomes sharper with increasing  $I_0$ .

# Confocal

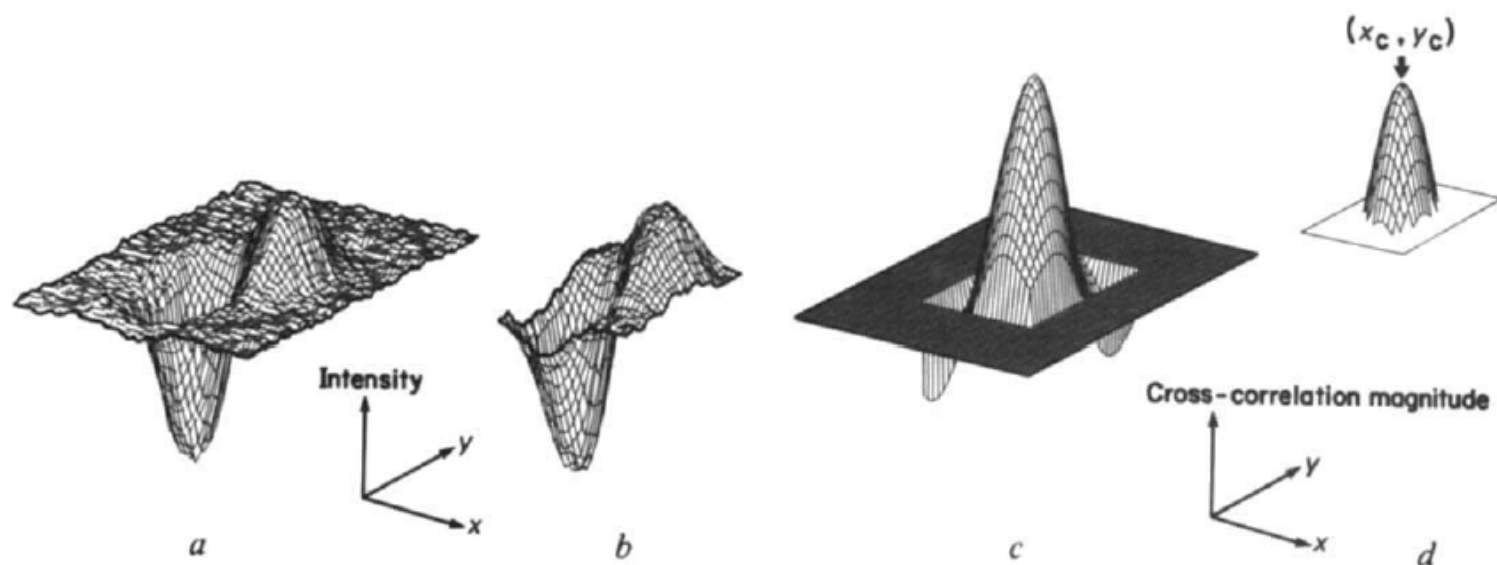


# Tracking kinesin-driven movements with nanometre-scale precision

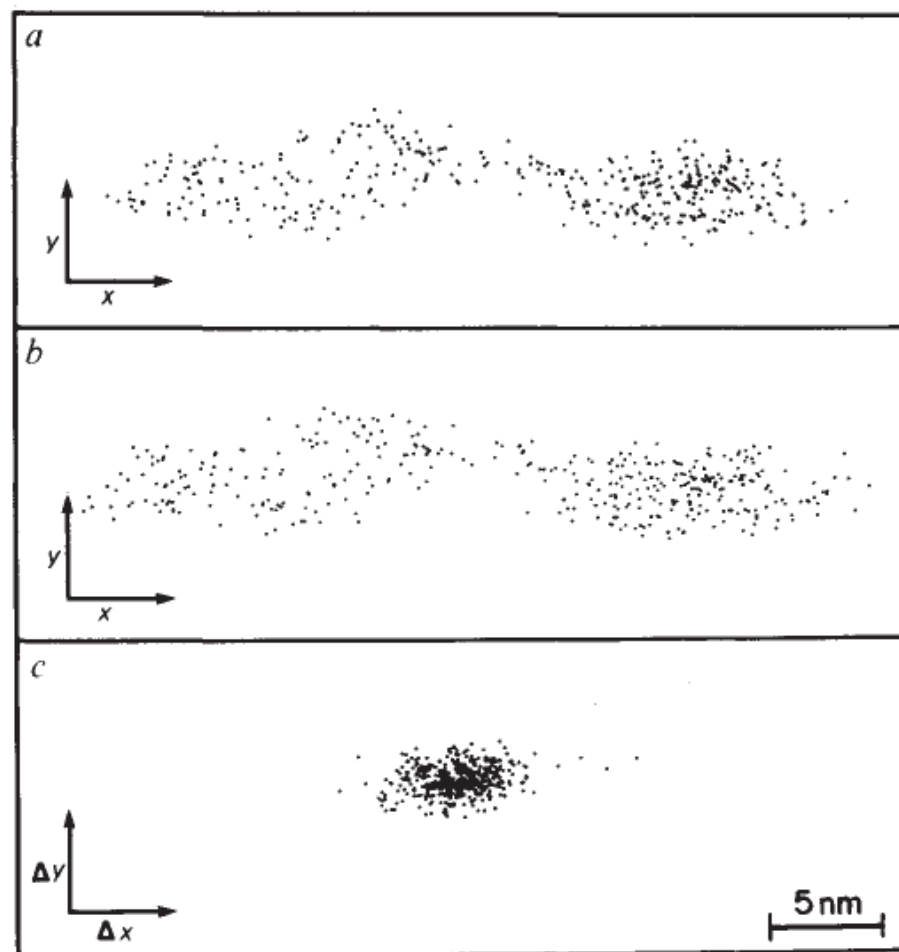
Jeff Gelles\*, Bruce J. Schnapp† & Michael P. Sheetz\*

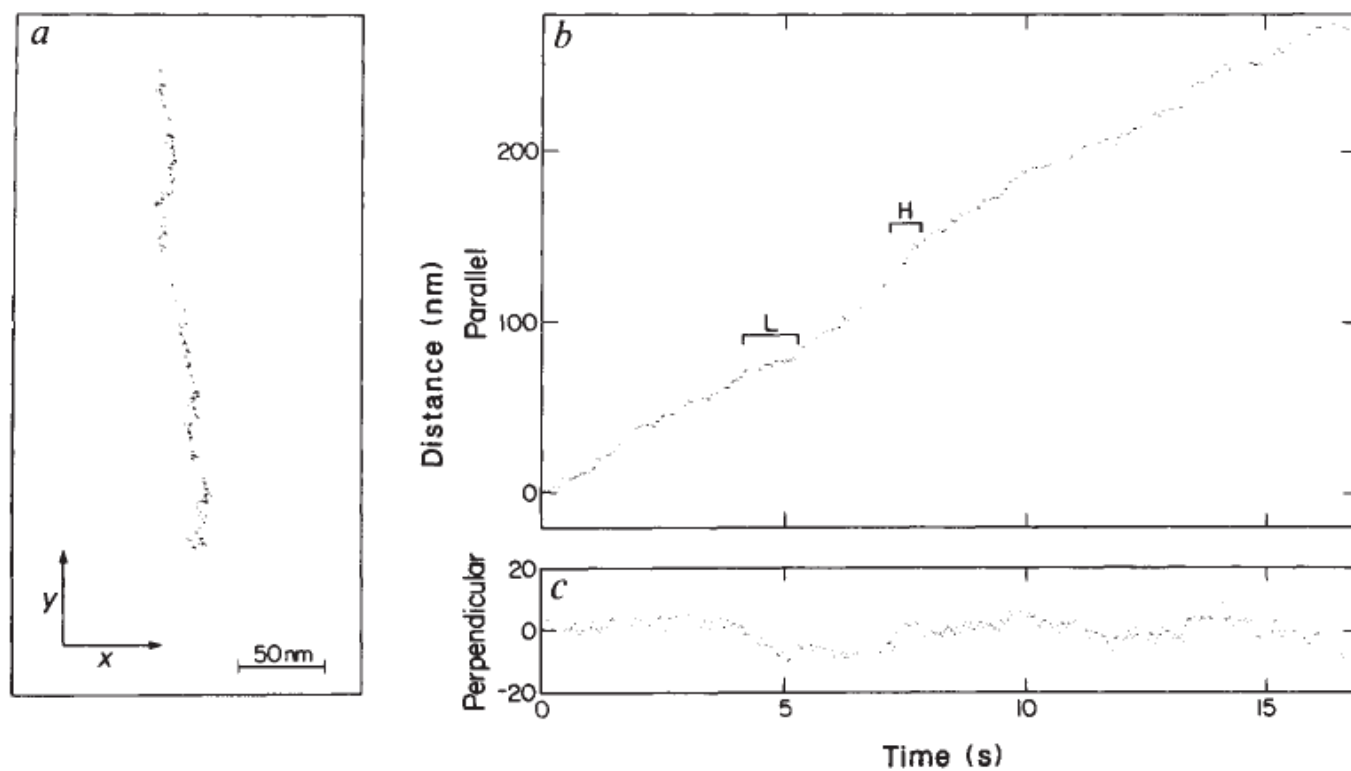
NATURE VOL. 331 4 FEBRUARY 1988

**Fig. 1** Stages in the high-precision measurement of relative bead position in a video frame. *a*, Image; *b*, kernel; *c*, cross-correlation of image and kernel; *d*, centroid calculation. The illustrated sequence of computations derives positional information from the entire bead image rather than from an individual point or edge only, and thereby maximizes the precision of the measurement. *a*, A segment of a digitized video frame containing the video-enhanced DIC microscope image of a bead. The DIC image of a single bead consists of apposed bright and dark areas. The segment consists of a  $49 \times 63$  rectangular matrix of integers,  $I(x, y)$ , each of which represents the recorded light intensity at the point  $(x, y)$  in the image ( $x$  and  $y$  are integers). *b*, A 'kernel' segment  $K(x, y)$ , consisting entirely of a single bead image. The centre of this segment is taken to be the point  $(0, 0)$ . The kernel is used as a template or standard; the effect of the calculations below is to find the position in the frame segment shown in *a* for which the surrounding intensity distribution most closely matches that surrounding the centre of the kernel. When a sequence of consecutive video frames is analysed, a single kernel derived from one of the frames is used in the analysis of all of them. The  $x$  and  $y$  dimensions of the kernel are denoted  $\alpha$  and  $\beta$ , respectively; for the case shown here,  $\alpha = 27$  pixels and  $\beta = 33$  pixels. *c*, Cross-correlation  $C(x, y)$  of the frame segment *a* with a scaled version of the kernel *b*. The cross-correlation calculation



of the frame segment *a* with a scaled version of the kernel *b*. The cross-correlation calculation

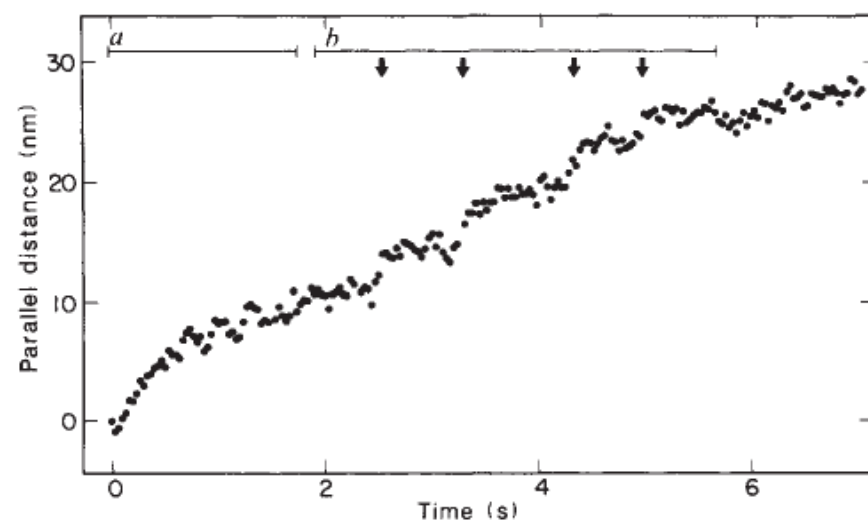




**Fig. 3** Motion of a kinesin-coated bead along a microtubule in the presence of  $10 \mu\text{M}$  ATP. *a*, The bead

**Fig. 4** Motion of a kinesin-coated bead along a microtubule in the presence of  $2.5\ \mu\text{M}$  ATP. The plot shows the projection of the bead position along the microtubule axis as a function of time. The mean velocity along the microtubule axis over the period shown is  $3.9\ \text{nm s}^{-1}$  ( $\sim 0.8\%$  of maximal velocity). Forward movement in region A occurs smoothly with a mean velocity of  $5.6\ \text{nm s}^{-1}$ . Movement in region B occurs in four discrete jumps (arrows) of length  $3.7 \pm 1.7\ \text{nm}$  (mean  $\pm$  s.d.).

**Methods.** The sample was identical to that of Fig. 3, except for a lower ATP concentration ( $2.5\ \mu\text{M}$ ). The data-analysis procedure was identical to that of Fig. 3 except that microtubule orientation was determined manually from the positions of video cursors superimposed on the centre of the microtubule image at distances of  $0.5\ \mu\text{m}$  on either side of the microtubule segment on which the bead motion occurred.





# Localization

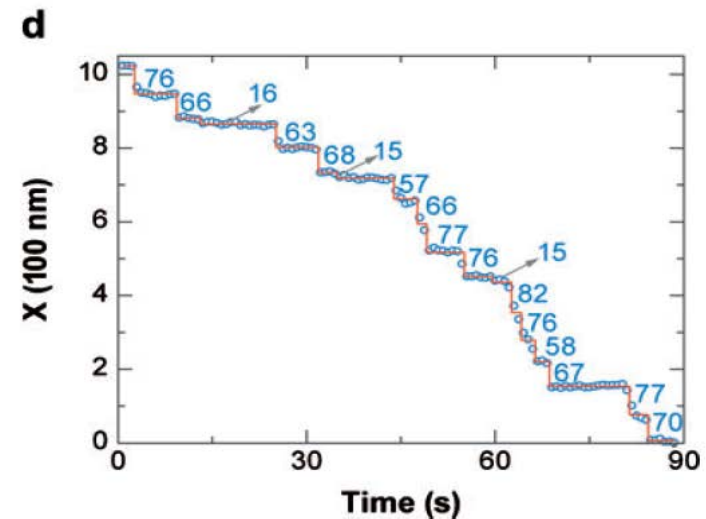
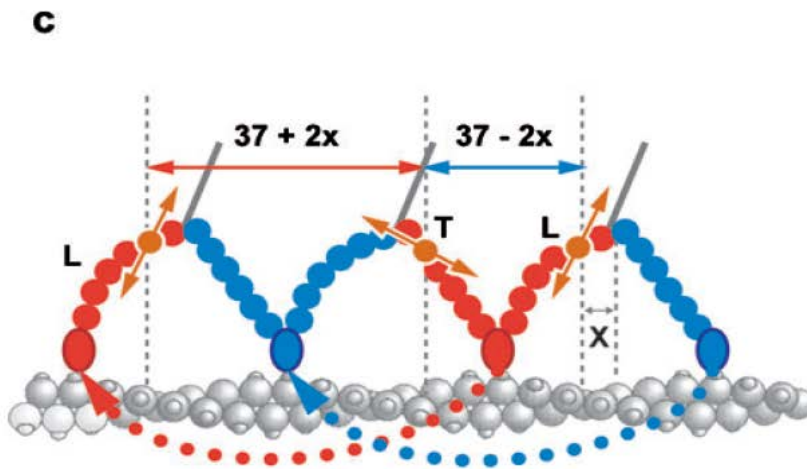
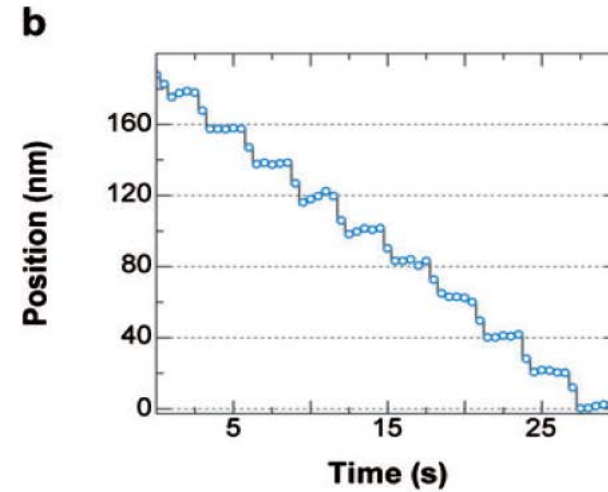
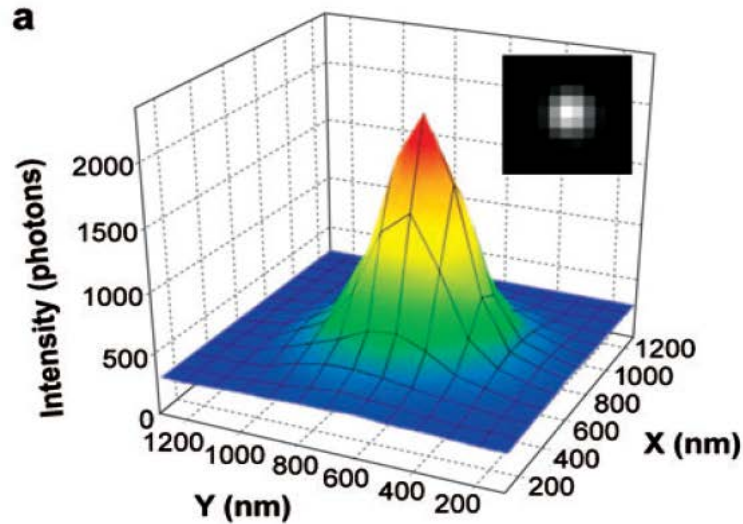
$$I(x, y) = I_{\text{background}} + A.$$

$$\exp \left\{ -1/2 \left[ \left( \frac{x - x_0}{s_x} \right)^2 + \left( \frac{y - y_0}{s_y} \right)^2 \right] \right\},$$

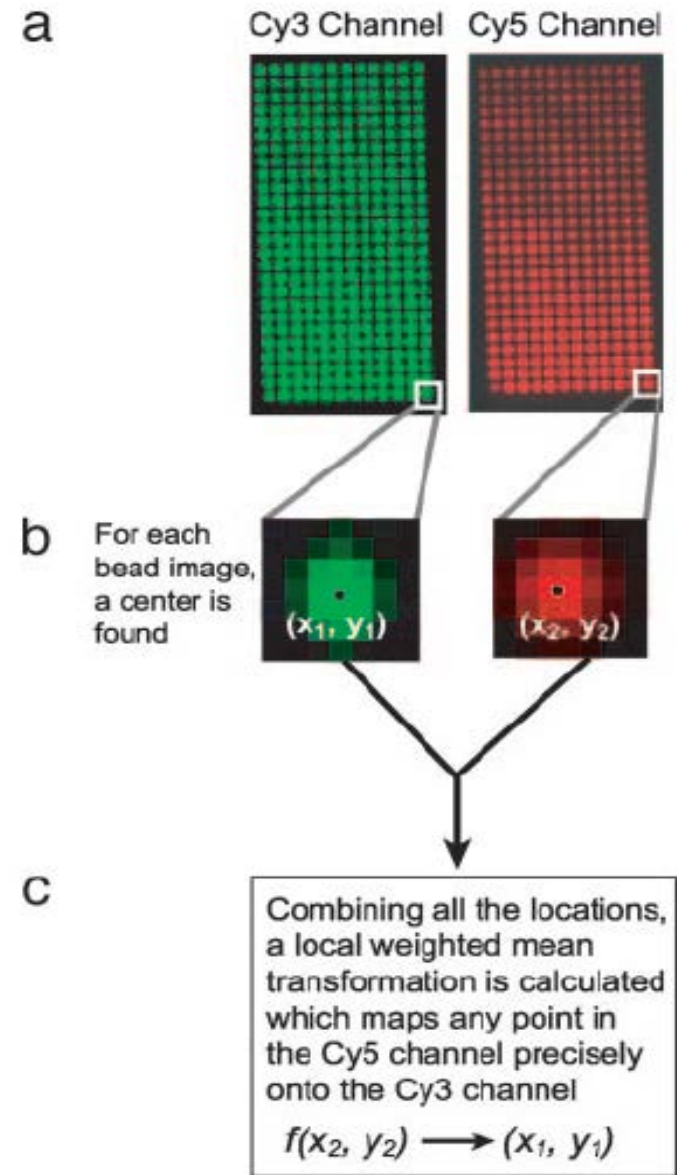
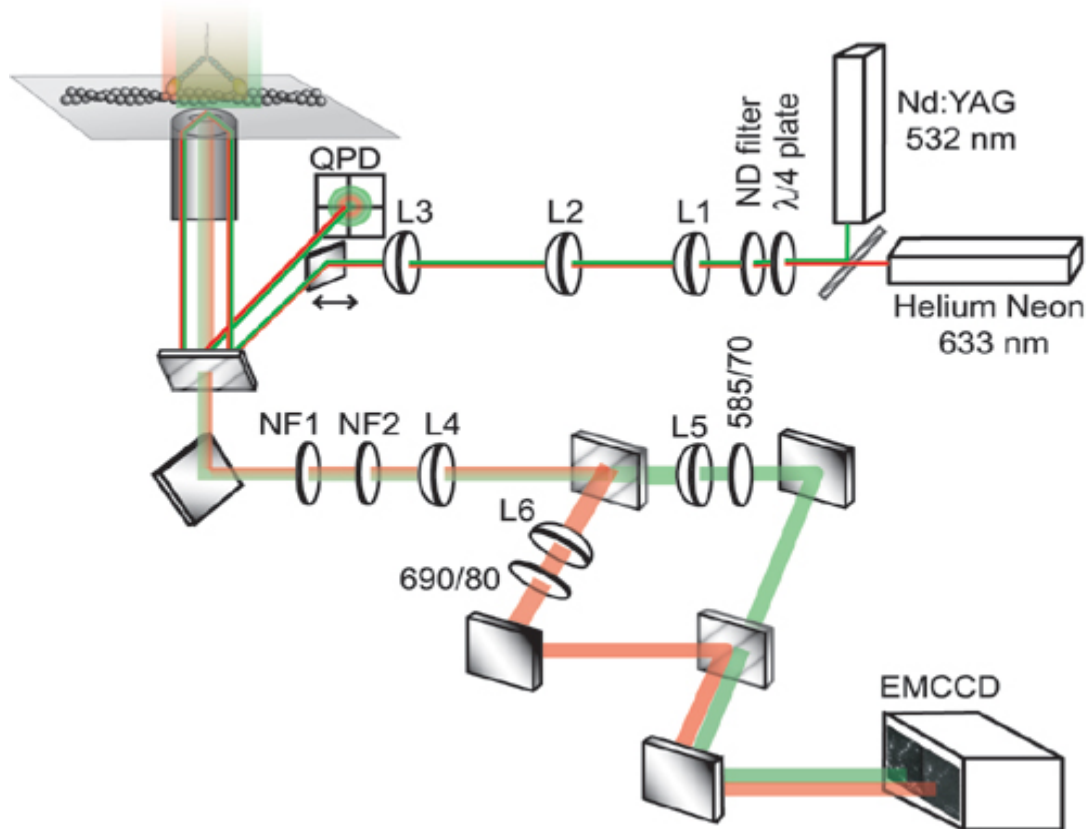
$$\sigma_{x,y}^{\mu}$$

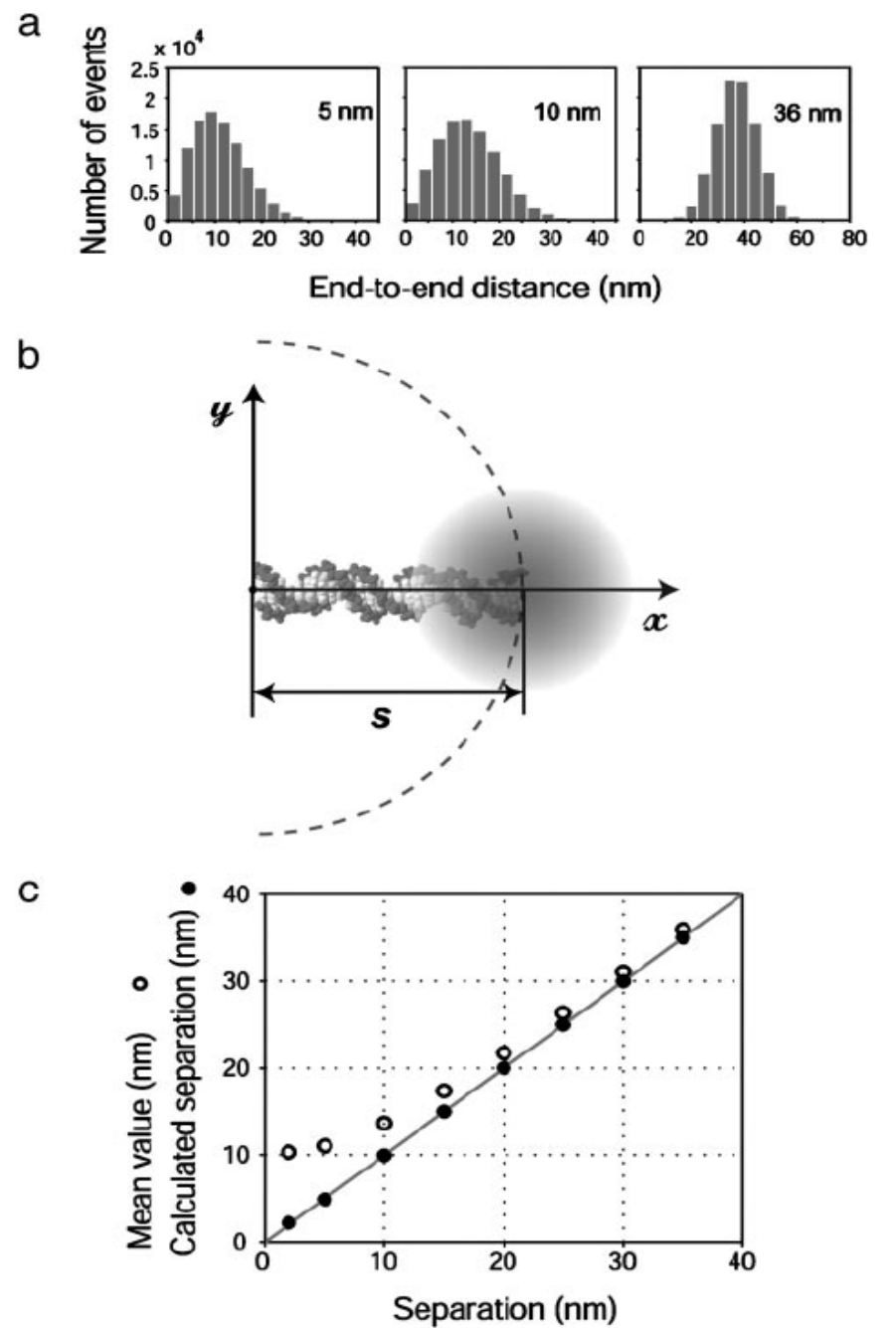
$$= \sqrt{\frac{s_{x,y}^2}{N_{\text{photons}}} + \frac{a^2}{12 \cdot N_{\text{photons}}} + \frac{8 \cdot \pi \cdot s_{x,y}^4 \cdot b^2}{a^2 \cdot N_{\text{photons}}}},$$

# Fluorescence Imaging with One Nanometer Accuracy (FIONA)



# Fluorescence Imaging with One-Nanometer Accuracy (FIONA)

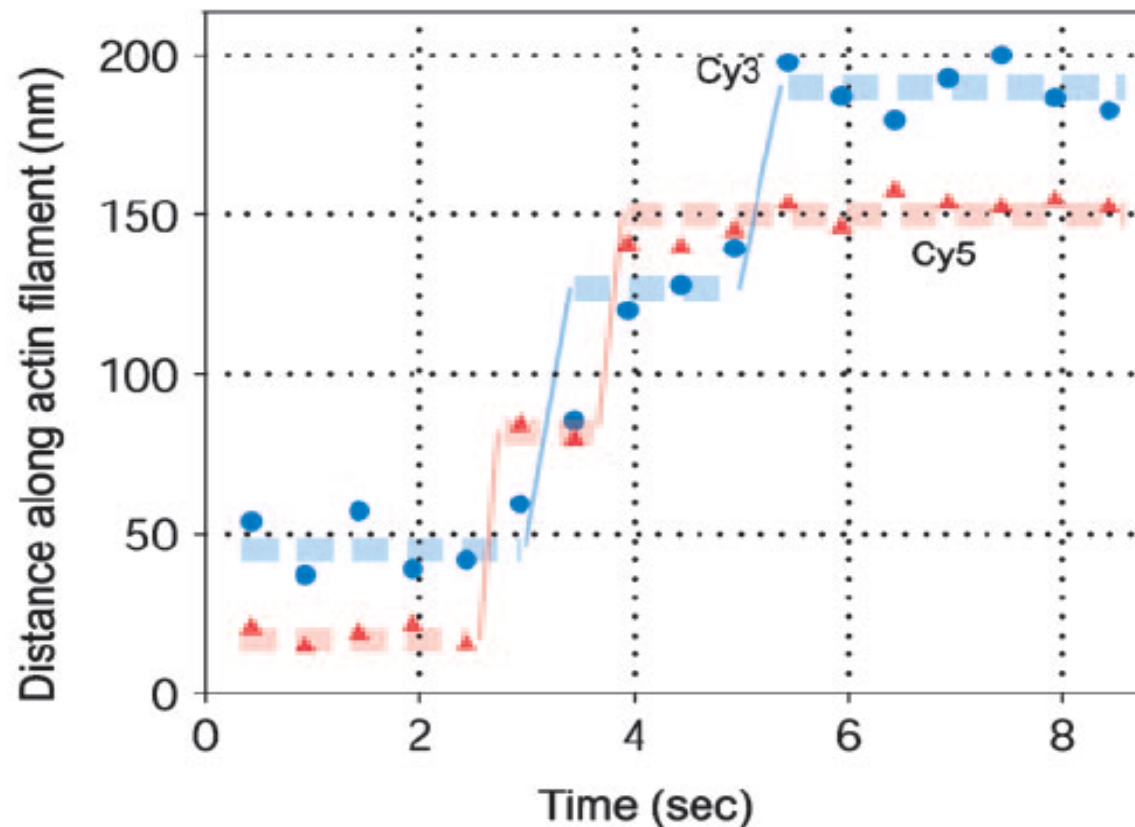




$$f(x, y) = z_0 + A \exp\left(\frac{1}{2}\left[\left(\frac{x - \mu_x}{\sigma_x}\right)^2 + \left(\frac{y - \mu_y}{\sigma_y}\right)^2\right]\right)$$

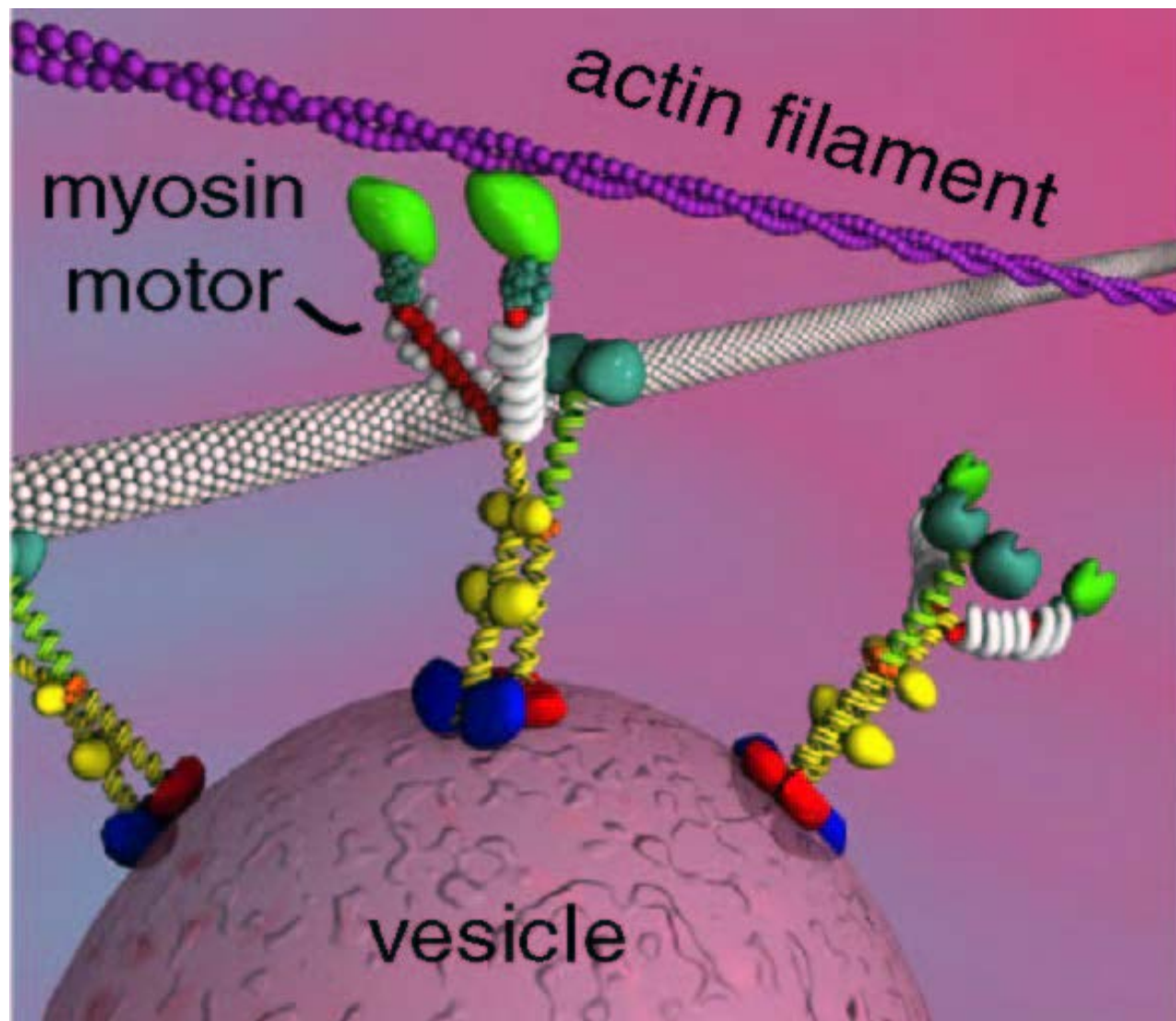
$$\sigma_\mu = \sqrt{(\sigma_x^2 + \sigma_y^2)} / \sqrt{N_\gamma - 1}.$$

**Fig. 2.** Determining the accurate end-to-end distance from the skewed distribution of distance measurements. (a) The distance probability distribution was calculated by means of Monte Carlo simulations. (b) The skew in the histograms toward large values can be understood from a geometric argument. If one end of the DNA molecule is measured to reside at the origin, then only points lying on the circumference of a circle with radius  $s$  and origin  $(0, 0)$  will yield the true end-to-end distance. It is more likely that a point will lie outside of the dashed-line semicircle than inside it, which gives the distance distribution a long tail. (c) Despite the non-Gaussian nature of the distance distribution, the end-to-end separation can be calculated using the geometric mean, variance, and localization errors.

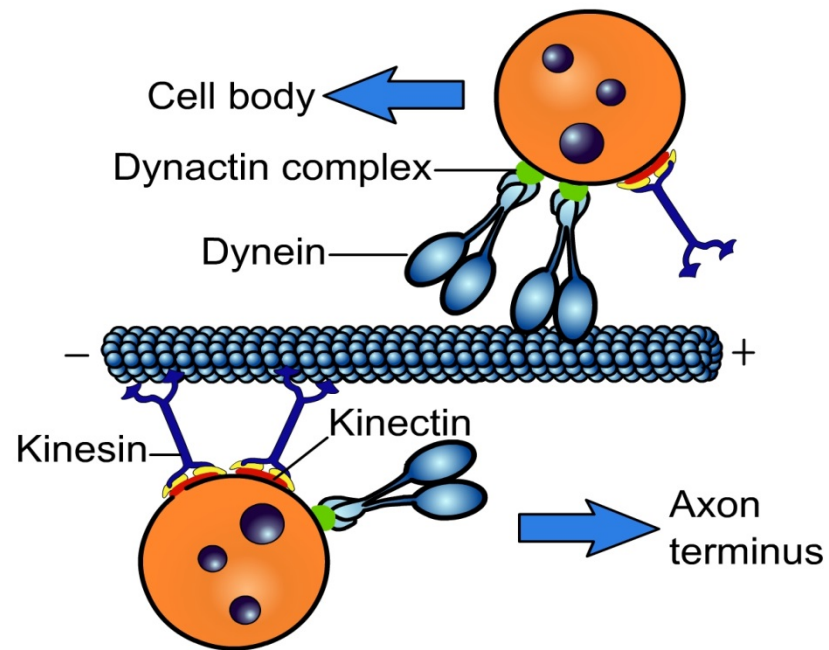
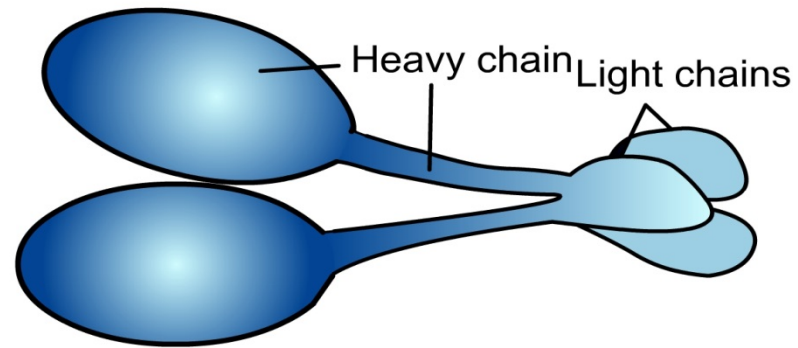


**Fig. 4.** Time trace of a differentially labeled myosin V molecule walking along an actin filament. The labels (Cy3 and Cy5) are covalently attached to calmodulins that were exchanged onto the myosin V molecule. In this trace, both of the fluorescent probe's locations are taking 72-nm steps, indicating that the calmodulins were exchanged close to the motor domain. The alternating positions of the probes provide a direct observation of myosin V's hand-over-hand walking mechanism.









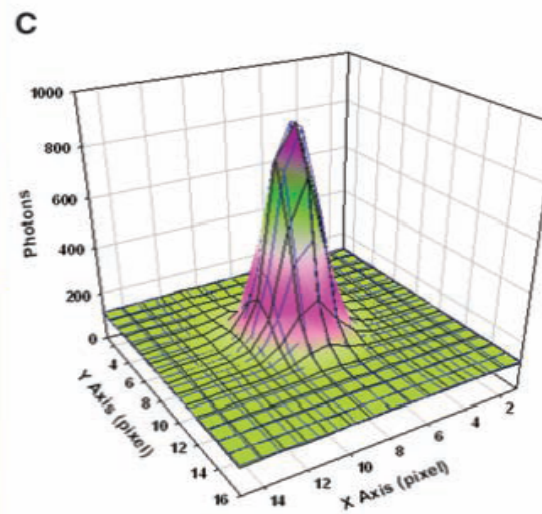
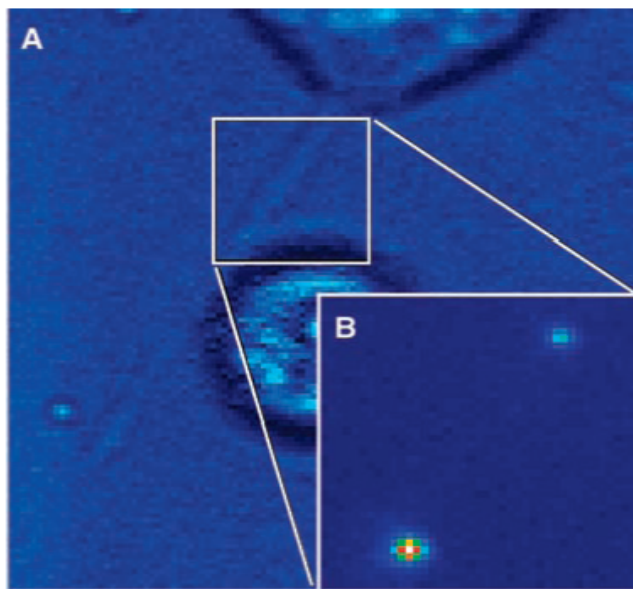
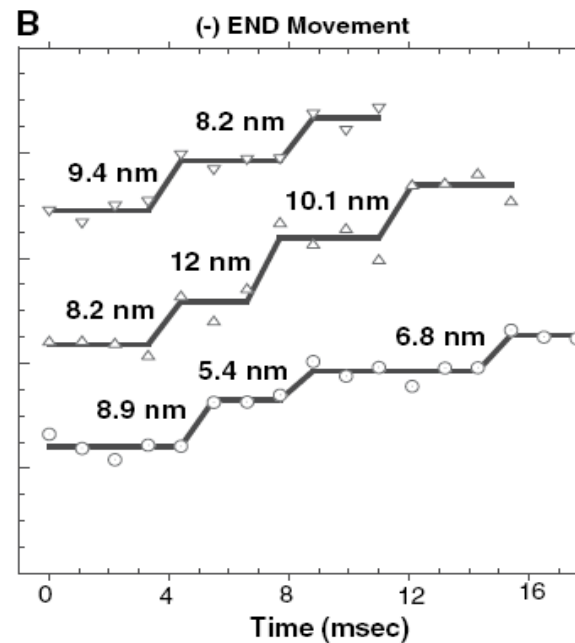
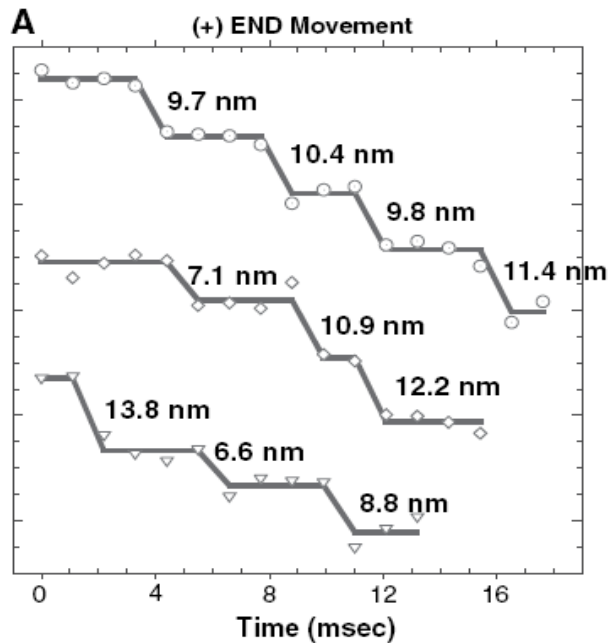
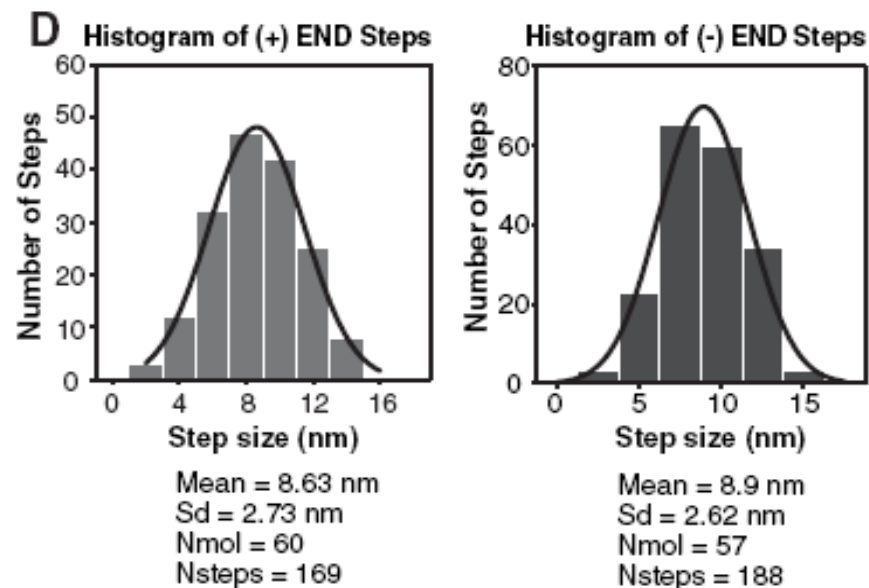
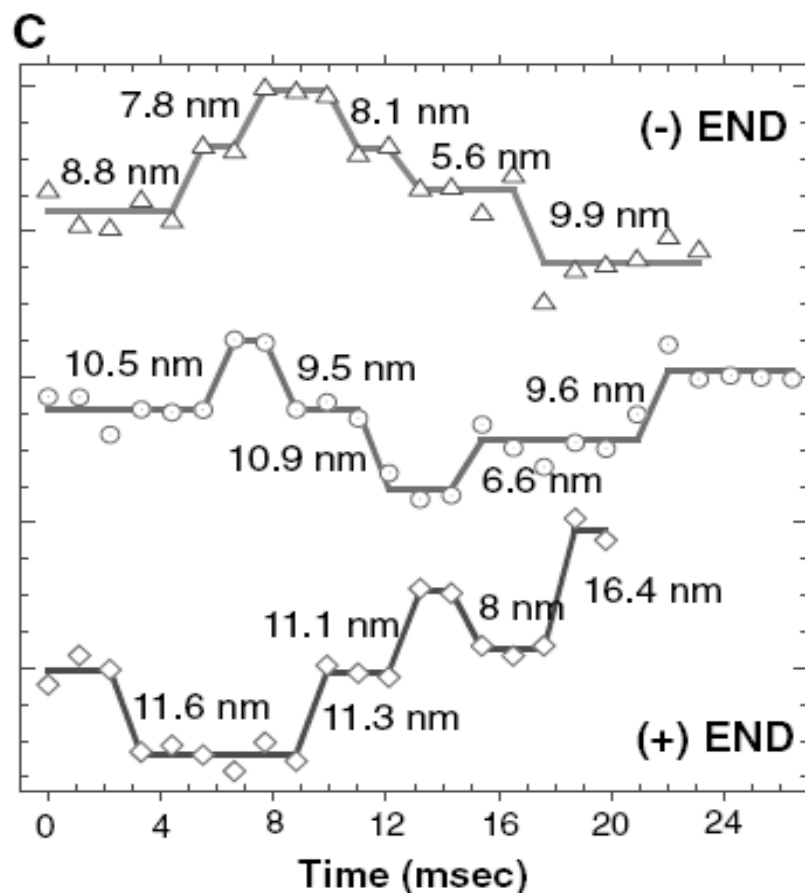


Fig. 1. (A) Bright-field image of a cytochalasin-D-treated S2 cell with a thin process. (B) Fluorescence image of the GFP-labeled peroxisomes within the process. (C) Fluorescence image of a peroxisome can be fit to a two-dimensional Gaussian (correlation coefficient  $r^2 = 0.992$ ), enabling the center to be determined to 1.5 nm within 1.1 ms.

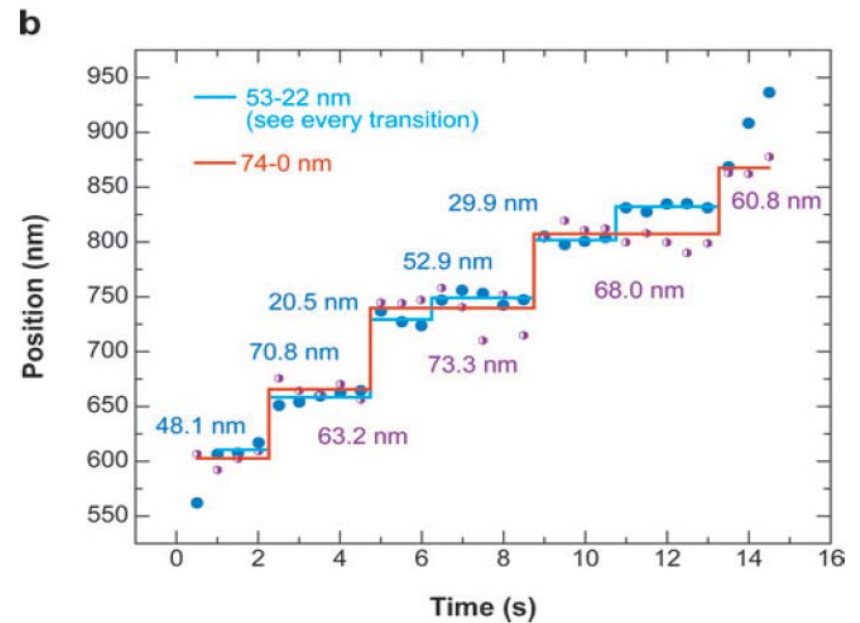
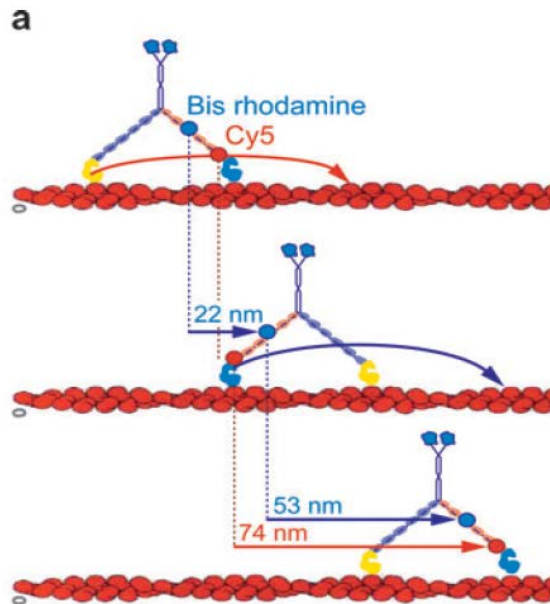
## Kinesin and Dynein Move a Peroxisome in Vivo: A Tug-of-War or Coordinated Movement?





**Fig. 2.** Step-by-step movement of peroxisomes carried by (A) a single kinesin, (B) a single dynein, and (C) a coordination of kinesin and dynein. (D) Histograms of the individual steps of anterograde (kinesin) and retrograde (dynein) movement. A pairwise displacement of kinesin and dynein, showing the multiples of 8-nm displacement, is shown in fig. S6.

# Single-molecule High-Resolution Colocalization (SHREC)



# Proposed method for molecular optical imaging

E. Betzig

Here an alternative approach is proposed in which multiple discrete features within the same focal volume are spatially resolved in two steps (see Fig. 1). First, each feature is identified and isolated through one or more distinguishing optical characteristics. Second, each feature is localized; that is, its spatial coordinates are determined, on a scale that is small compared with the focal volume, either by measurement of the center of the point-spread function (PSF) associated with each feature<sup>2</sup> or by application of a spatial gradient,<sup>3,4</sup> as discussed below. The complete set of coordinates for all features can then be used to reconstruct the final image in which the relative positions of the features are shown.

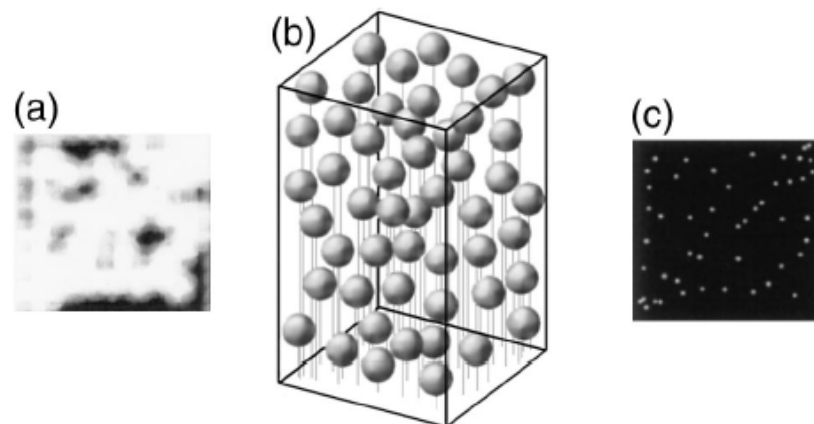
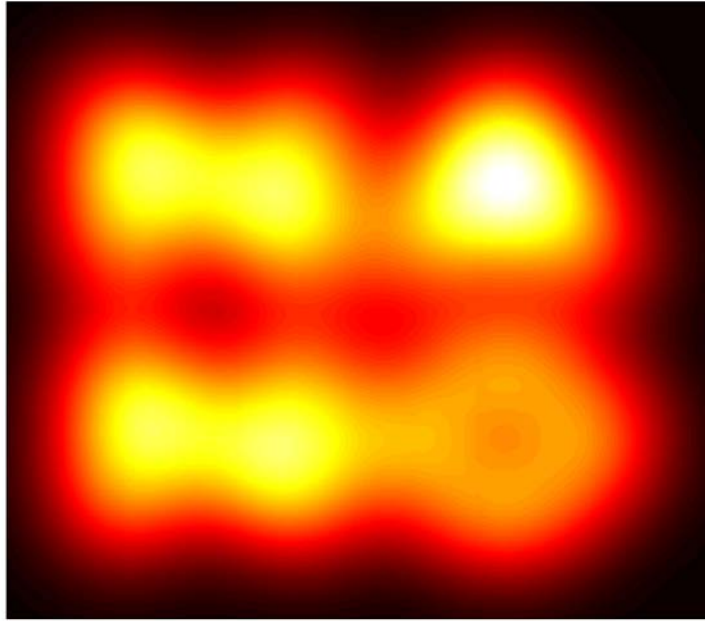


Fig. 1. (a) Field of discrete features as conventionally imaged in  $m$  spatial dimensions with a broad PSF. (b) Same features after isolation in  $m + n$  dimensions on the basis of  $n$  distinguishing optical characteristics. (c) Final image reconstructed at resolution  $\delta \mathbf{x}$  given by the uncertainty in the measured position of each isolated feature. In general,  $|\delta \mathbf{x}| \ll \text{PSF}$ .

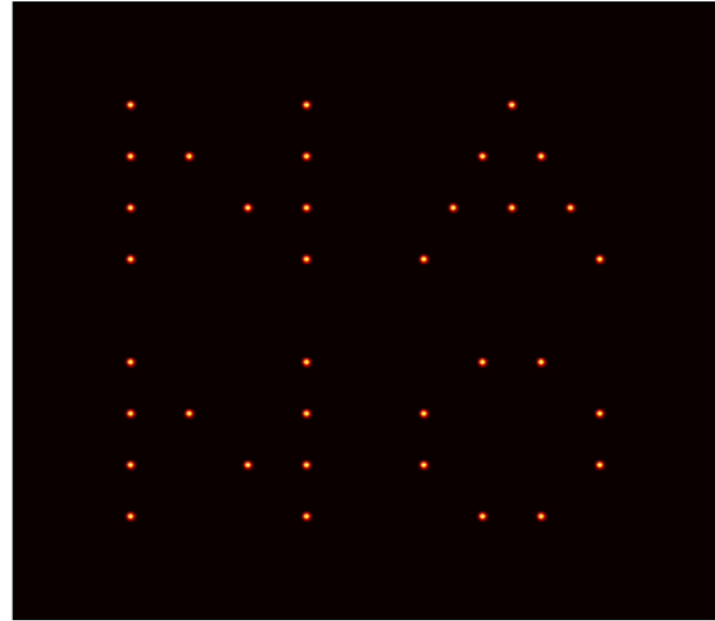
# Photoactivation localization microscopy (PALM)



## Diffraction-limited system:

Lateral resolution  $\Delta xy \approx 0.61 \lambda / \text{N.A.}$   
 $\approx 200 \text{ nm}$

Axis resolution  $\Delta z \approx 2\lambda / \text{N.A.}^2$   
 $\approx 450 \text{ nm}$



## Mean-squared position error:

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

$s$  is the standard deviation of the PSF.

$a$  is the pixel size in the image

$N_m$  is the total number of photons measured from molecule  $m$

$b_m$  is the number of background photons collected in the fitting window



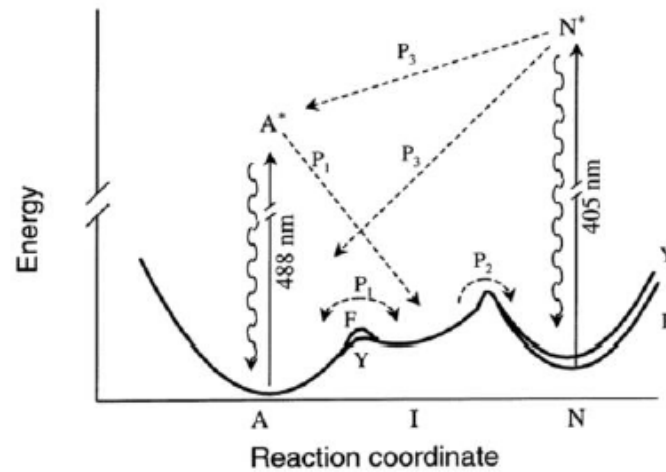
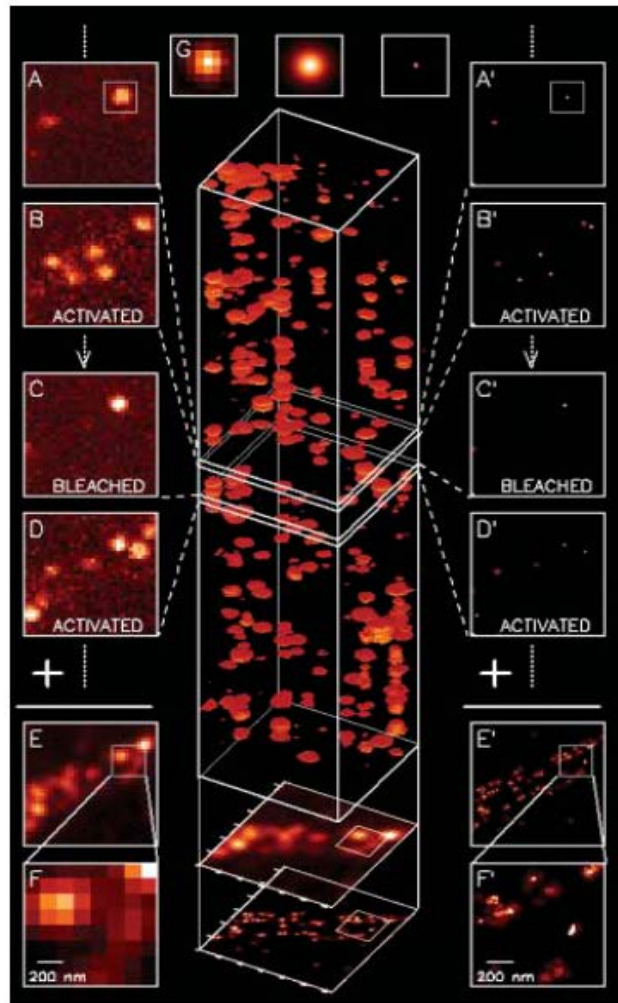
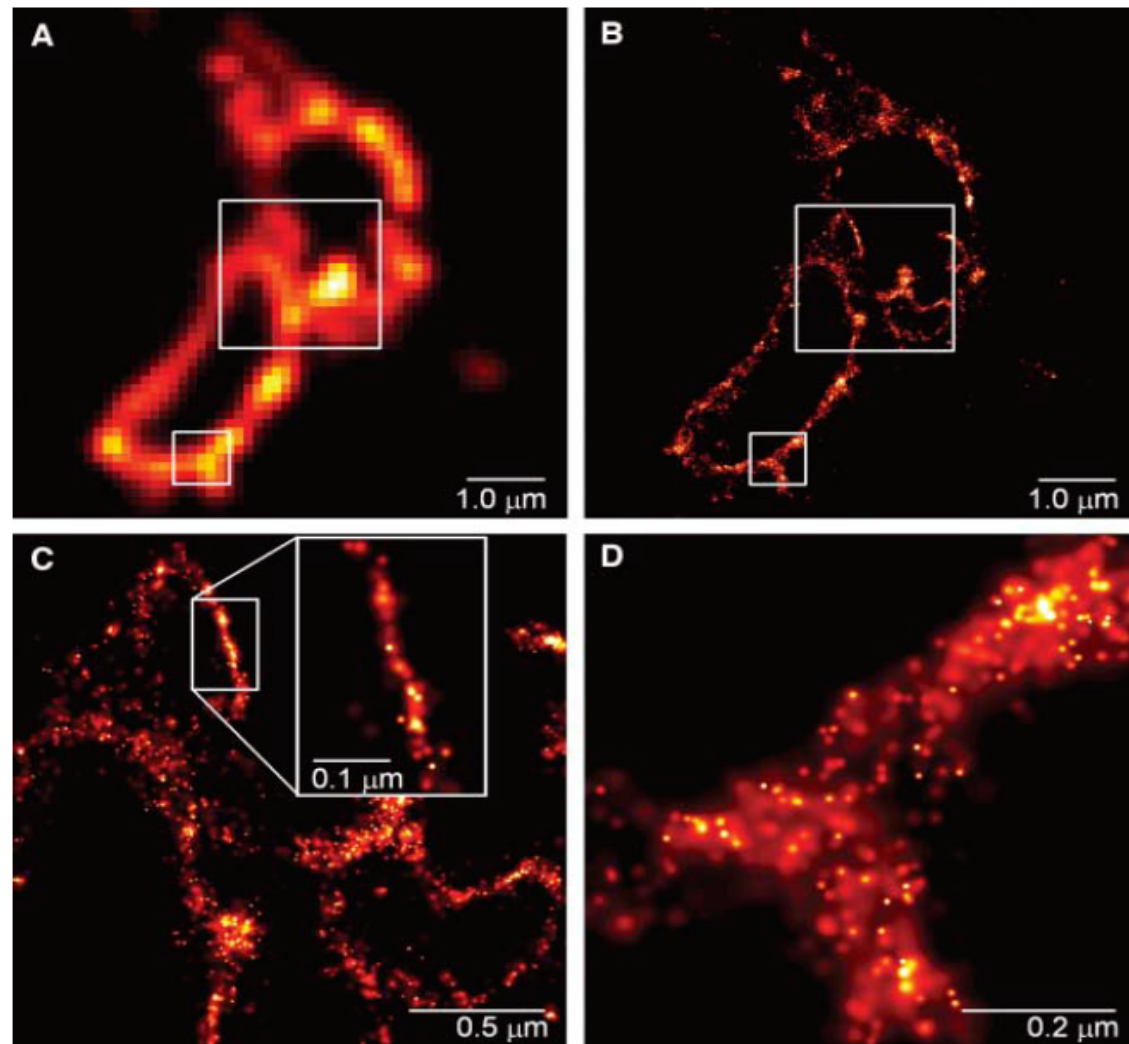


Figure 7: (Dickson *et al.*, 1997, *Nature* 388, 355-358). GFP in state A is excited to A\* and returns to A upon photon emission. When I is reached from A, there is no fluorescence until I spontaneously moves to A: blinking. When I moves to N, there is no fluorescence until N is activated to N\* by 405 nm excitation and GFP returns to A.

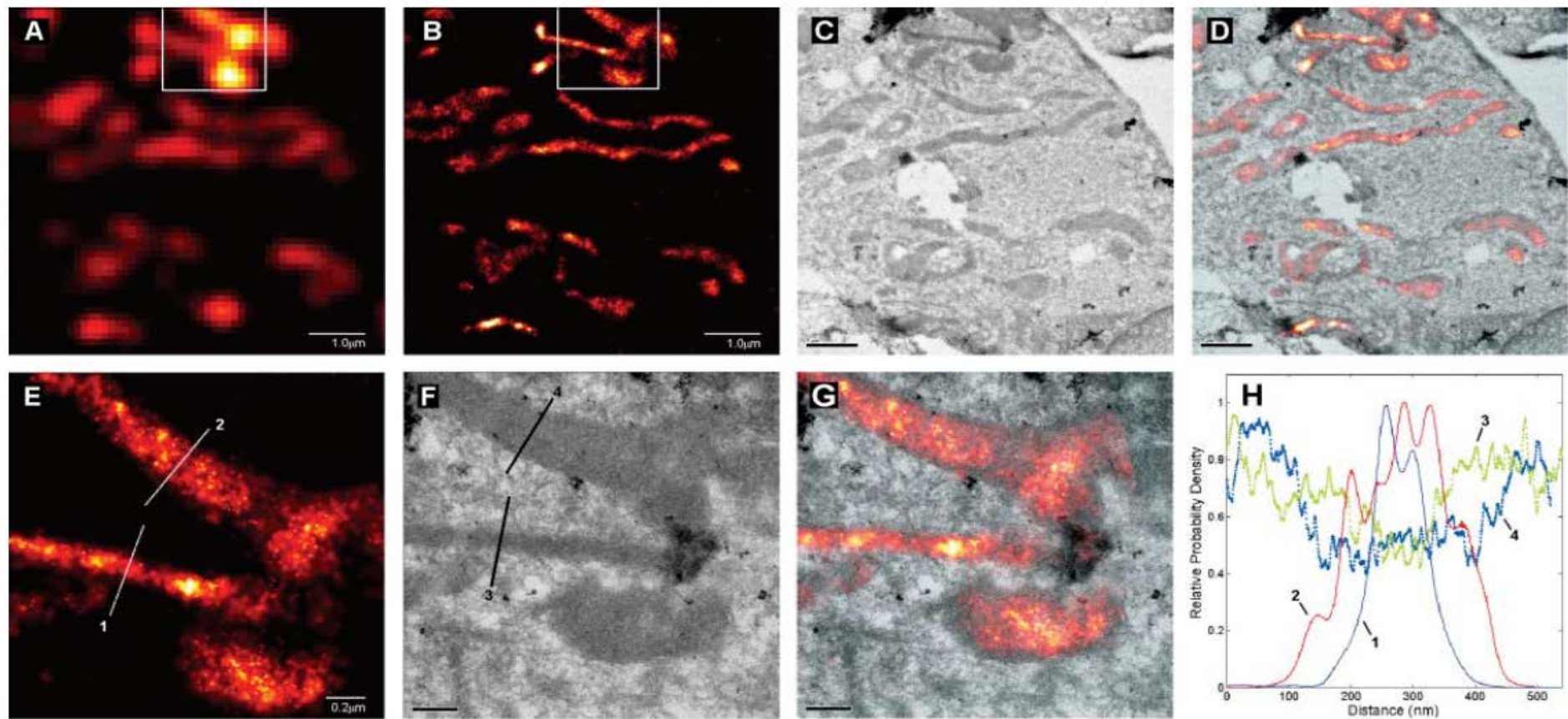
# Imaging Intracellular Fluorescent Proteins at Nanometer Resolution



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



**Fig. 2.** Comparative summed-molecule TIRF (**A**) and PALM (**B**) images of the same region within a cryo-prepared thin section from a COS-7 cell expressing the lysosomal transmembrane protein CD63 tagged with the PA-FP Kaede. The larger boxed region in (**B**), when viewed at higher magnification (**C**) reveals smaller associated membranes that may represent interacting lysosomes or late endosomes that are not resolvable by TIRF. In a region where the section is nearly orthogonal to the lysosomal membrane, the most highly localized molecules fall on a line of width  $\sim 10$  nm (inset). In an obliquely cut region [(**D**), from the smaller boxed region in (**B**)], the distribution of CD63 within the membrane plane can be discerned.



**Fig. 3.** 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. Higher magnification PALM (E), TEM (F), and overlay (G) images within the box in (B) reveal that these matrix re-

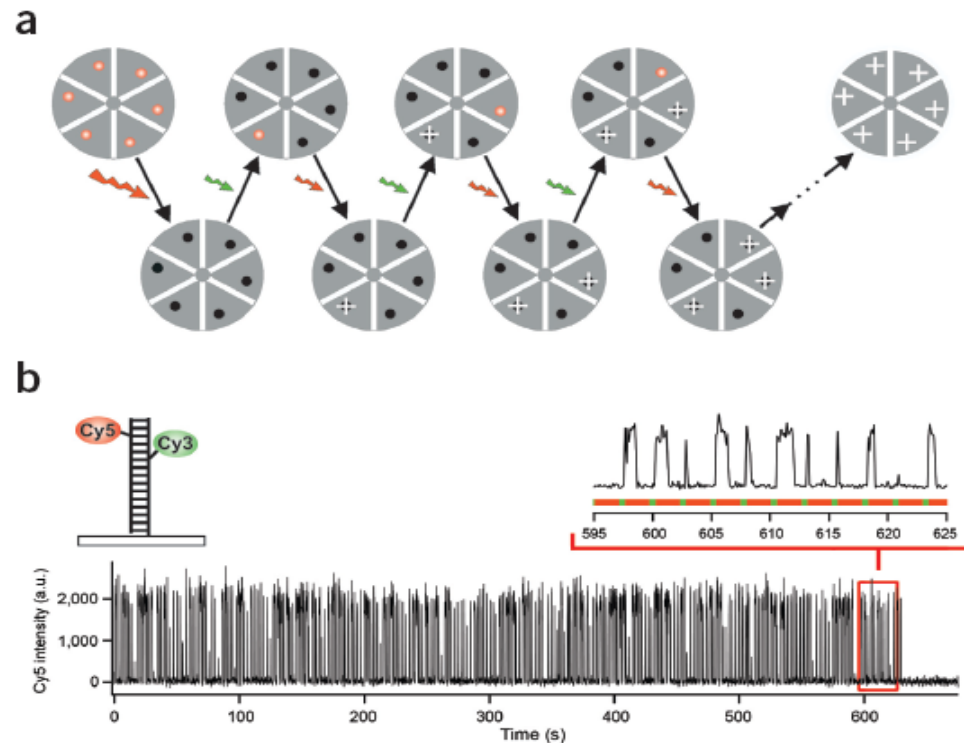
porter molecules extend up to, but not into, the  $\sim 20$ -nm outer mitochondrial membrane. The molecular distribution across two mitochondria along lines 1 and 2 in PALM image (E) are compared in (H) to the TEM signal along lines 3 and 4 in (F) across the same mitochondria. Scale bars:  $1.0\ \mu\text{m}$  in (A) to (D);  $0.2\ \mu\text{m}$  in (E) to (G).

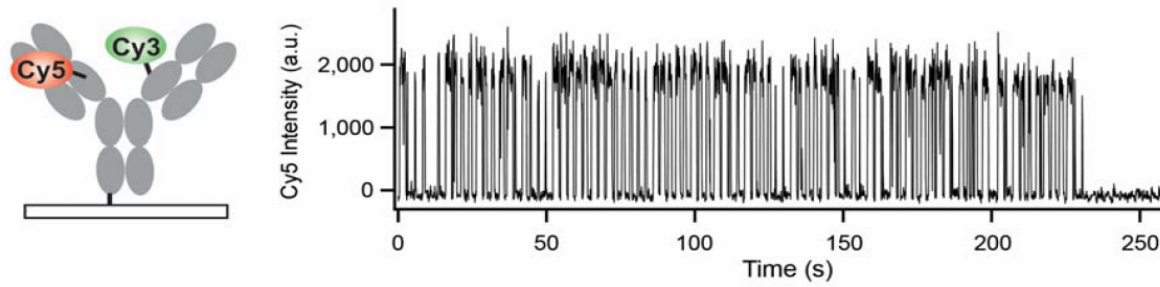


# Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)

Michael J Rust<sup>1,5</sup>, Mark Bates<sup>2,5</sup> & Xiaowei Zhuang<sup>1,3,4</sup>

## NATURE METHODS





: 2.2 : 1 for Cy3 and 0.1 : 1 for Cy5

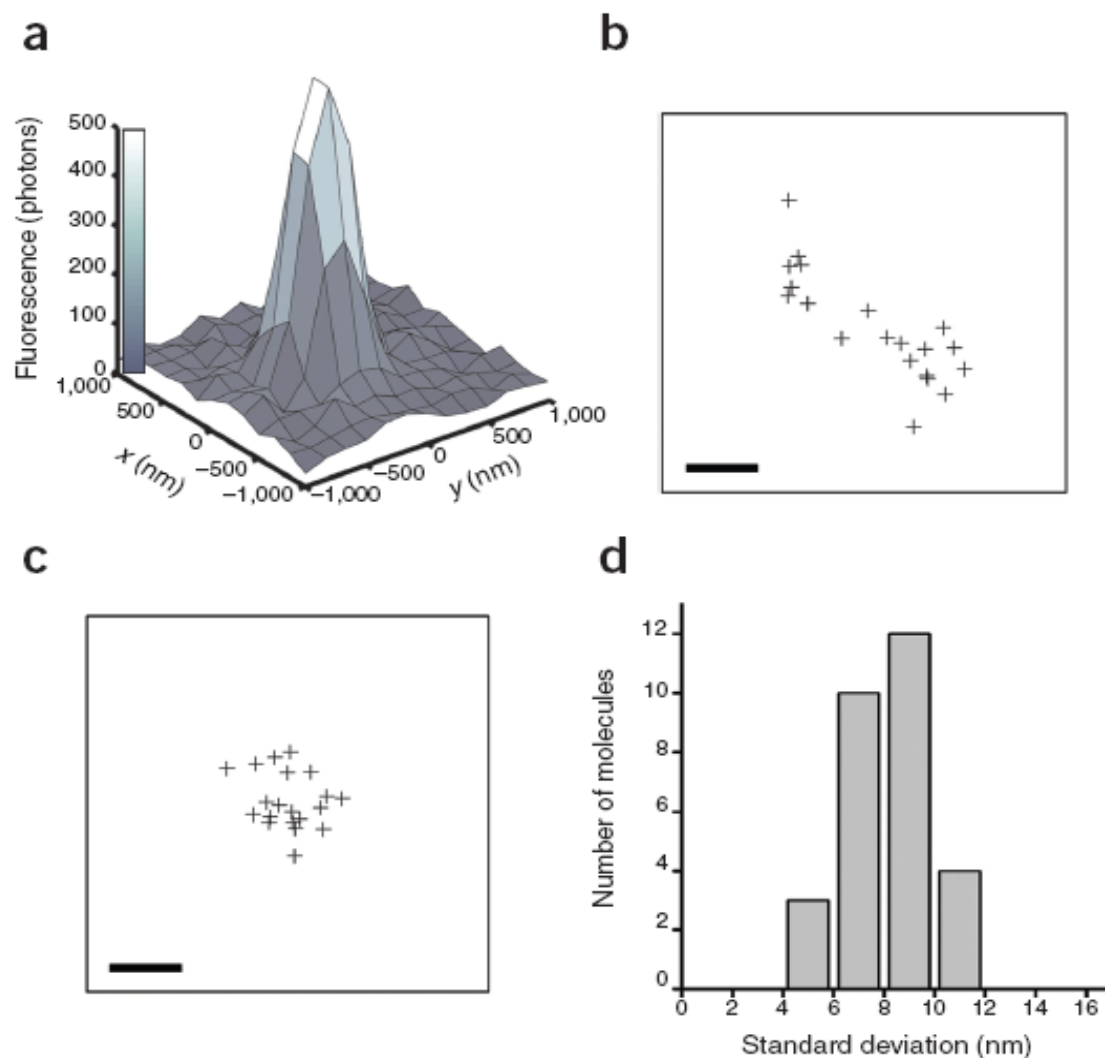
Goat anti-mouse secondary antibody labeled with the cyanine switch exhibits photoswitching behavior similar to switch-labeled DNA. The antibody was labeled with Cy3 and Cy5 (as described in **Supplementary Methods** online) and bound to a quartz slide coated with unlabeled mouse anti-transferrin primary antibody. The trace shows the Cy5 fluorescence intensity detected from a single labeled antibody as it switches on and off until permanent photobleaching occurs after 230 seconds. A red laser (633 nm, 30 W/cm<sup>2</sup>) is used to excite fluorescence from Cy5 and to switch Cy5 to the dark state. A green laser (532 nm, 1 W/cm<sup>2</sup>) is used to switch the Cy5 back to the fluorescent state. The sample was excited with a sequence of alternating green and red laser pulses (0.5 s green followed by 2 s red).

$$I(x, y) = A + I_0 e^{\left[ -(x'/a)^2 - (y'/b)^2 \right] / 2}$$

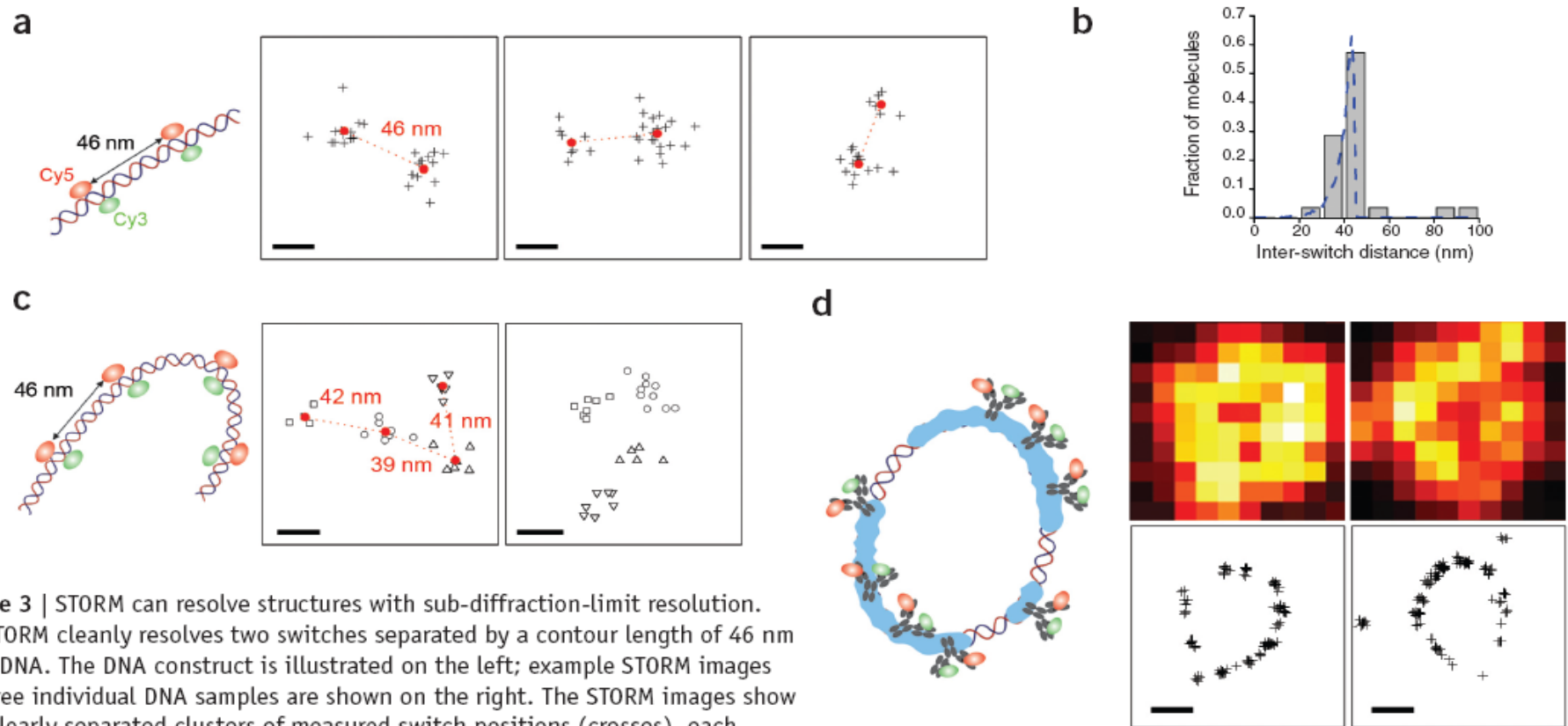
$$x' = (x - x_0) \cos \theta - (y - y_0) \sin \theta$$

$$y' = (x - x_0) \sin \theta + (y - y_0) \cos \theta$$





**Figure 2** | The high localization accuracy of individual switches during each switching cycle defines the intrinsic resolution of STORM. **(a)** The point spread function (PSF) of the emission from a single switch on DNA during a single switching cycle. Fitting the PSF to a two-dimensional Gaussian (not shown) gives the centroid position of the PSF. **(b,c)** The centroid positions of an individual switch determined in 20 successive imaging cycles before **(b)** and after **(c)** correction for sample drift. Scale bars, 20 nm. **(d)** A histogram of the standard deviation of centroid positions. The standard deviation is determined as  $(\sigma_x + \sigma_y) / 2$  for each switch using 20 imaging cycles, where  $\sigma_x$  and  $\sigma_y$  are the standard deviations of the centroid positions in the  $x$  and  $y$  dimensions. This histogram was constructed from 29 switches.

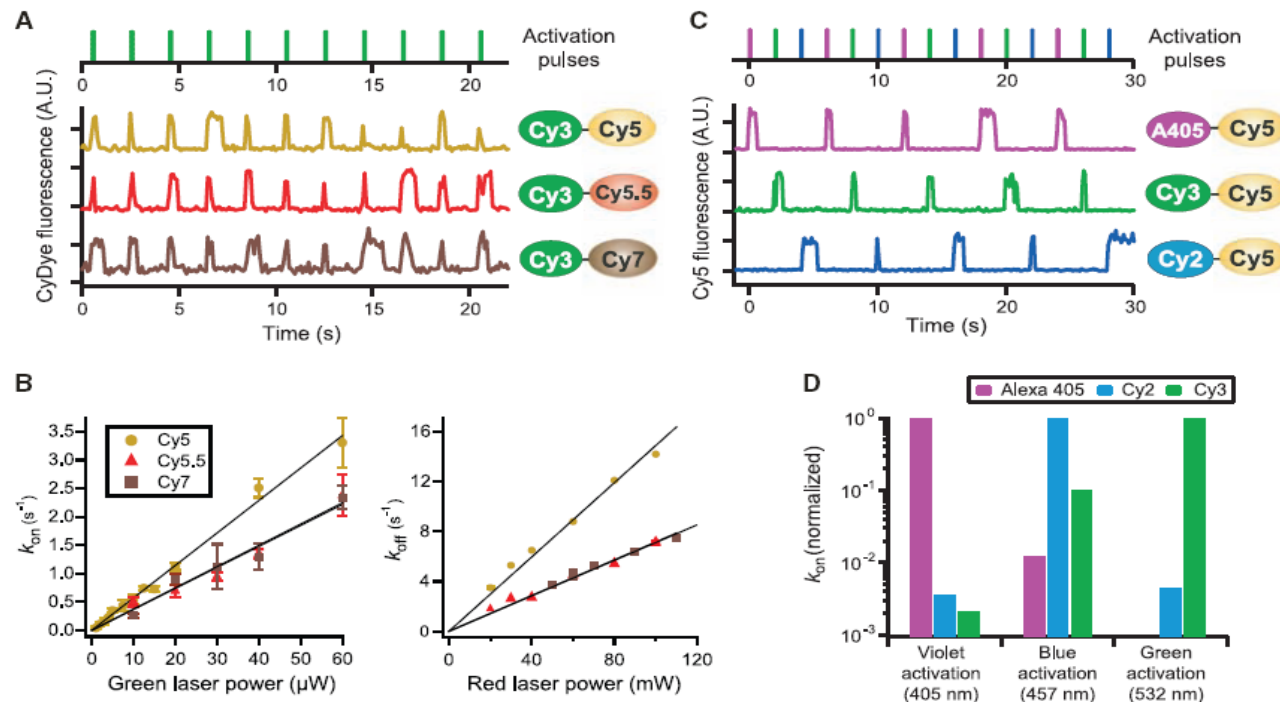


**Figure 3 |** STORM can resolve structures with sub-diffraction-limit resolution. (a) STORM clearly resolves two switches separated by a contour length of 46 nm on dsDNA. The DNA construct is illustrated on the left; example STORM images of three individual DNA samples are shown on the right. The STORM images show two clearly separated clusters of measured switch positions (crosses), each corresponding to a single switch. The center-of-mass position of each cluster is marked by a red dot. The inter-switch distances are 46 nm, 44 nm and 34 nm for these three examples. Scale bars, 20 nm. (b) Comparison between the inter-switch distances measured using STORM (columns) and the predicted distance distribution considering the flexibility of DNA (dashed line). (c) STORM images of four switches attached to a dsDNA, pair-wise separated by a contour length of 46 nm. The measured switch positions are clustered by an automated algorithm and different clusters are indicated by different symbols. Scale bars, 20 nm. (d) STORM images of RecA-coated circular plasmid DNA. Indirect immunofluorescence images with switch-labeled secondary antibody taken by a total internal reflection microscope (top); the reconstructed STORM images of the same filaments (bottom). Scale bars, 300 nm.

# Multicolor Super-Resolution Imaging with Photo-Switchable Fluorescent Probes

SCIENCE VOL 317 21 SEPTEMBER 2007

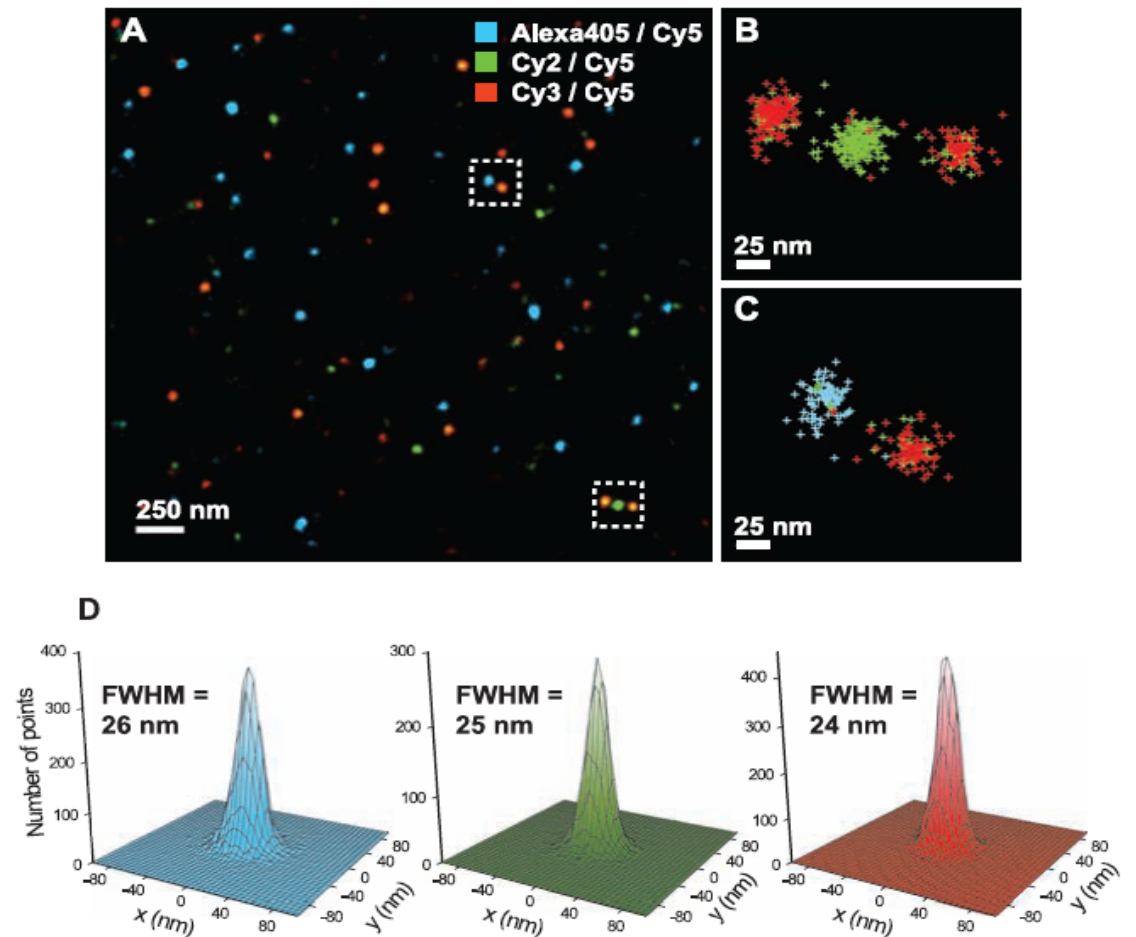
**Fig. 1.** Photo-switchable probes constructed from activator-reporter pairs. **(A)** Spectrally distinct reporters exhibit photo-switching behavior. The lower panel shows the fluorescence time traces of three photo-switchable reporters Cy5 (dark yellow line), Cy5.5 (red line), and Cy7 (brown line), each paired with a Cy3 dye as the activator on a DNA construct. The upper panel shows the green laser pulses (532 nm) used to activate the reporters. The red laser (657 nm) was continuously on, serving to excite fluorescence from the reporters and to switch them to the dark state. Traces were shifted relative to each other for clarity. **(B)** Switching rate constants  $k_{on}$  and  $k_{off}$  of the Cy3-Cy5, Cy3-Cy5.5, and Cy3-Cy7 pairs as a function of green and red laser power. Error bars indicate SEM from about three data sets. The laser power to intensity calibration may vary between different samples because of moderate differences in the laser spot size at the sample. **(C)** The same reporter can be activated by spectrally distinct activators. The lower panel shows the fluorescence time traces of Cy5 paired with three different activators, Alexa 405 (magenta line), Cy2 (blue line), and Cy3 (green line). The upper panel shows the violet (405 nm, magenta line), blue (457 nm, blue line), and green (532 nm, green line) activation pulses. **(D)**

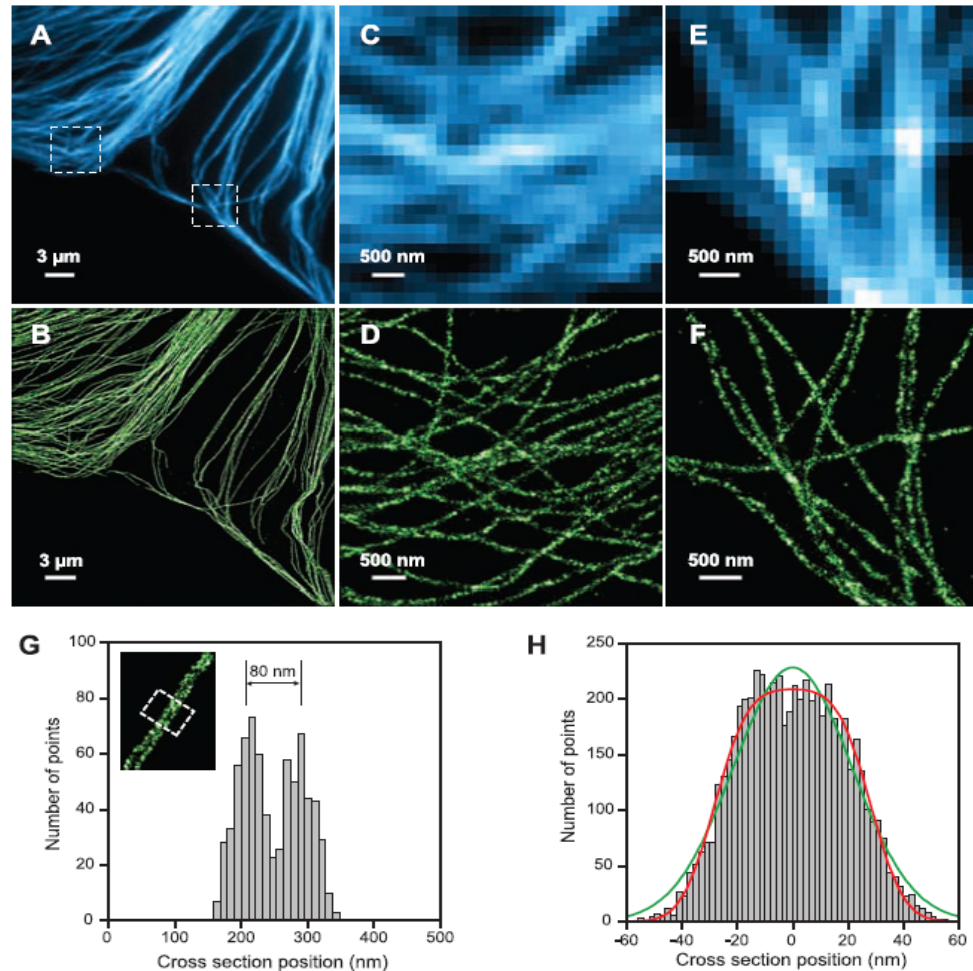


Normalized activation rate constants of the three dye pairs at three activation wavelengths: 405, 457, and 532 nm. The  $k_{on}$  values of Alexa 405-Cy5, Cy2-Cy5, and Cy3-Cy5 were used for normalization at 405, 457, and 532 nm, respectively. The absolute activation rates were rapid for each pair at its corresponding optimal wavelength, with values of  $10 s^{-1}$  or greater at only a few hundred  $\mu W$  of laser power. The activation rate of the Alexa 405-Cy5 pair by the 532-nm laser was too small to be measured reliably.

The laser power to intensity calibration may vary between different samples because of moderate differences in the laser spot size at the sample. The same reporter can be activated by spectrally distinct activators. The lower panel shows the fluorescence time traces of Cy5 paired with three different activators, Alexa 405 (magenta line), Cy2 (blue line), and Cy3 (green line). The upper panel shows the violet (405 nm, magenta line), blue (457 nm, blue line), and green (532 nm, green line) activation pulses. The activation rate of the Alexa 405-Cy5 pair by the 532-nm laser was too small to be measured reliably.

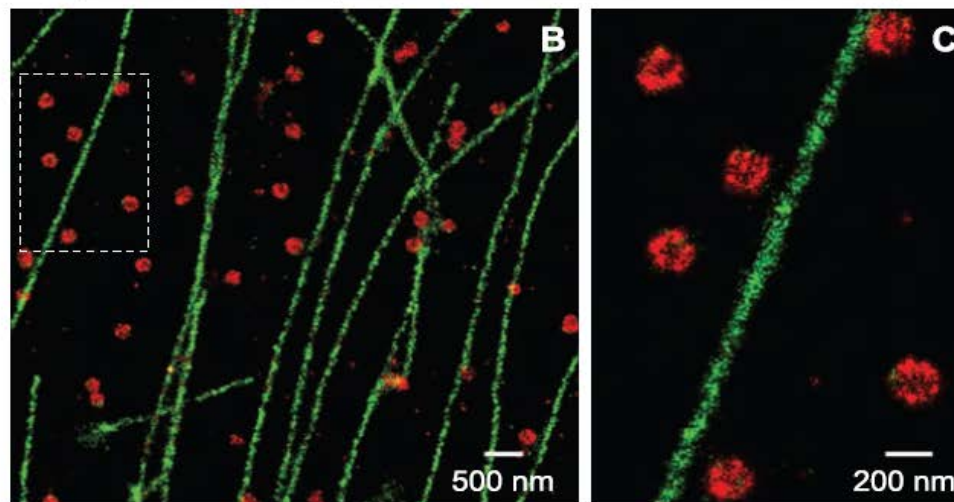
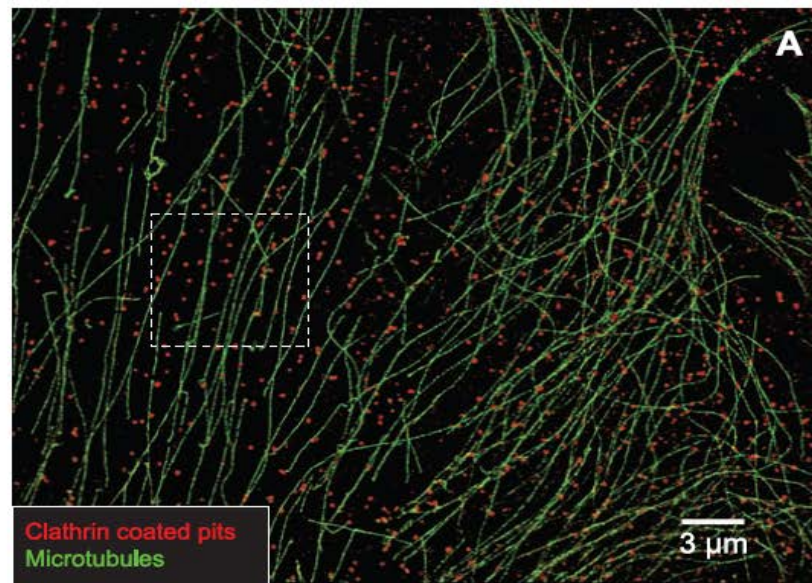
**Fig. 2.** Three-color STORM imaging of a model DNA sample. **(A)** Three-color STORM image of three different DNA constructs labeled with Alexa 405-Cy5, Cy2-Cy5, or Cy3-Cy5 mixed at a high surface density on a microscope slide. The image was plotted by rendering each localization as a Gaussian peak, the width of which was scaled with the theoretical localization accuracy given by the number of photons detected (26). Each colored spot in this image represents a cluster of localizations from a single DNA molecule. A conventional fluorescence image of the same area is shown in fig. S3 for comparison. **(B and C)** Higher-magnification views of the boxed regions in (A) show several examples of closely spaced DNA molecules. Each localization was plotted as a cross, colored according to the following code: If the molecule was activated by a 405-, 457-, or 532-nm laser pulse, the color of the cross was assigned as blue, green, or red, respectively. **(D)** The localization distributions of the blue, green, or red clusters. The two-dimensional histograms of localizations were generated by aligning multiple (50 to 60) clusters by their center of mass. The histograms were fit to a Gaussian profile to determine their FWHM.





**Fig. 3.** STORM imaging of microtubules in a mammalian cell. **(A)** Conventional immunofluorescence image of microtubules in a large area of a BS-C-1 cell. **(B)** STORM image of the same area. **(C and E)** Conventional and **(D and F)** STORM images corresponding to the boxed regions in **(A)**. **(G)** Cross-sectional profiles of two nearby microtubule filaments in the cell. The inset shows the STORM image, and the histogram shows the cross-sectional distribution of localizations within the small regions specified by the white box. **(H)** Cross-sectional profile of a microtubule segment determined from the STORM image. A relatively long segment ( $\sim 7 \mu\text{m}$ ) was chosen to obtain good statistics. The histogram shows the cross-sectional distribution of localizations. The green line is a single Gaussian fit with  $\text{FWHM} = 51 \text{ nm}$ . The red line shows the fit obtained by convolving a rectangular function of width  $= d$  with a Gaussian function of  $\text{FWHM} = r$ . The fit yields  $d = 56 \text{ nm}$  and  $r = 22 \text{ nm}$ , corresponding to the antibody-coated microtubule width and the imaging resolution, respectively.





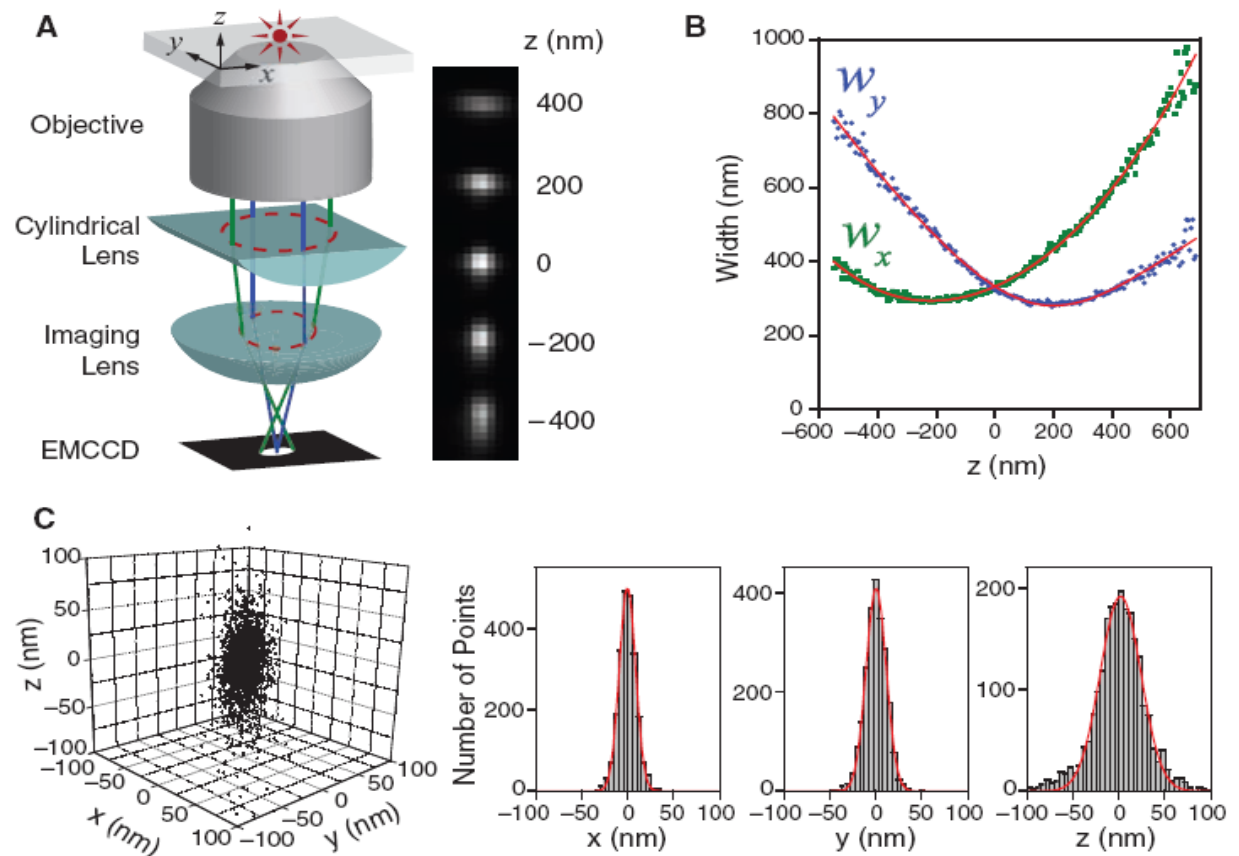
**Fig. 4.** Two-color STORM imaging of microtubules and CCPs in a mammalian cell. **(A)** STORM image of a large area of a BS-C-1 cell. The secondary antibodies used for microtubule staining were labeled with Cy2 and Alexa 647, and those for clathrin were labeled with Cy3 and Alexa 647. The 457- and 532-nm laser pulses were used to selectively activate the two pairs. Each localization was false colored according to the following code: green for 457-nm activation and red for 532-nm activation. **(B)** STORM image corresponding to the boxed region in (A) shown at a higher magnification. **(C)** Further magnified view of the boxed region in (B).



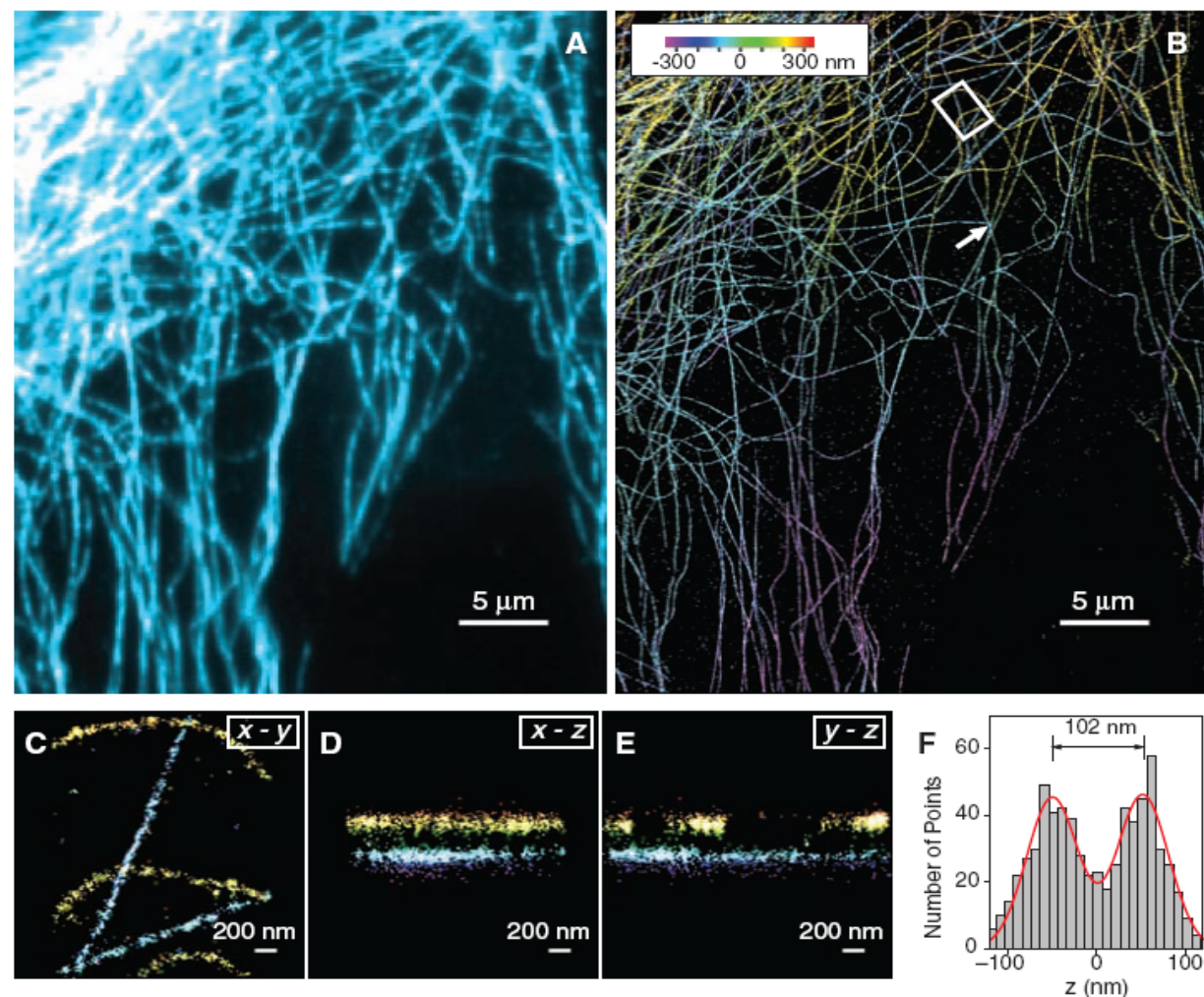
# Three-Dimensional Super-Resolution Imaging by Stochastic Optical Reconstruction Microscopy

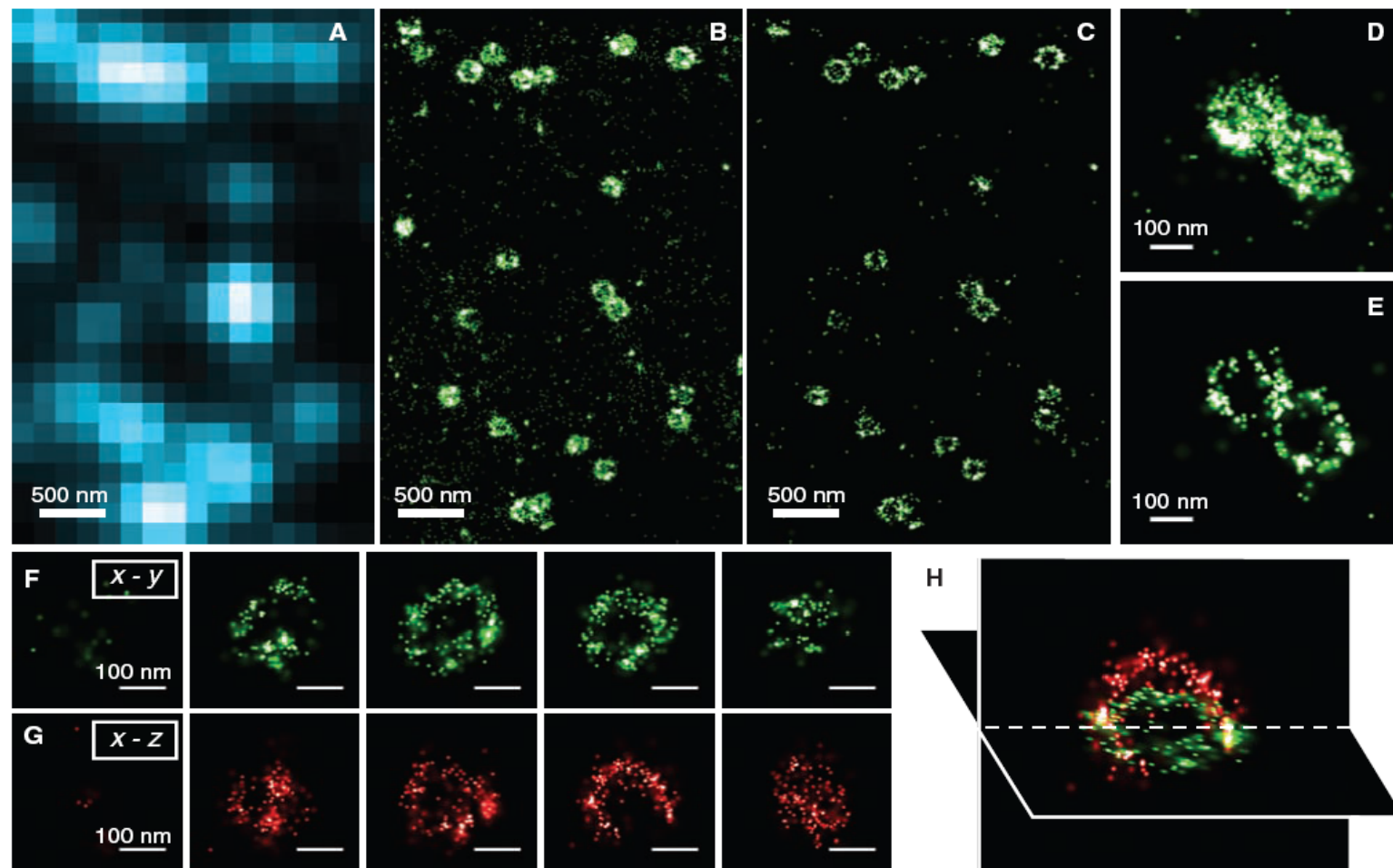
8 FEBRUARY 2008 VOL 319 SCIENCE

**Fig. 1.** The scheme of 3D STORM. **(A)** Three-dimensional localization of individual fluorophores. The simplified optical diagram illustrates the principle of determining the  $z$  coordinate of a fluorescent object from the ellipticity of its image by introducing a cylindrical lens into the imaging path. The right panel shows images of a fluorophore at various  $z$  positions. EMCCD, electron-multiplying charge-coupled device. **(B)** Calibration curve of image widths  $w_x$  and  $w_y$  as a function of  $z$  obtained from single Alexa 647 molecules. Each data point represents the average value obtained from six molecules. The data were fit to a defocusing function (red curve) as described in (27). **(C)** Three-dimensional localization distribution of single molecules. Each molecule gives a cluster of localizations due to repetitive activation of the same molecule. Localizations from 145 clusters were aligned by their center of mass to generate the overall 3D presentation of the localization distribution (left panel). Histograms of the distribution in  $x$ ,  $y$ , and  $z$  (right panels) were fit to a Gaussian function, yielding standard deviations of 9 nm in  $x$ , 11 nm in  $y$ , and 22 nm in  $z$ .



**Fig. 2.** Three-dimensional STORM imaging of microtubules in a cell. **(A)** Conventional indirect immunofluorescence image of microtubules in a large area of a BS-C-1 cell. **(B)** The 3D STORM image of the same area, with the  $z$ -position information color-coded according to the color scale bar. Each localization is depicted in the STORM image as a Gaussian peak, the width of which is determined by the number of photons detected (5). **(C to E)** The  $x$ - $y$ ,  $x$ - $z$ , and  $y$ - $z$  cross sections of a small region of the cell outlined by the white box in (B), showing five microtubule filaments. Movie S1 shows the 3D representation of this region, with the viewing angle rotated to show different perspectives (27). **(F)** The  $z$  profile of two microtubules crossing in the  $x$ - $y$  projection but separated by 102 nm in  $z$ , from a region indicated by the arrow in (B). The histogram shows the distribution of  $z$  coordinates of the localizations, fit to two Gaussians with identical widths (FWHM = 66 nm) and a separation of 102 nm (red curve). The apparent width of 66 nm agrees quantitatively with the convolution of our imaging resolution in  $z$  (represented by a Gaussian function with FWHM of 55 nm) and the previously measured width of antibody-coated microtubules (represented by a uniform distribution with a width of 56 nm) (5).





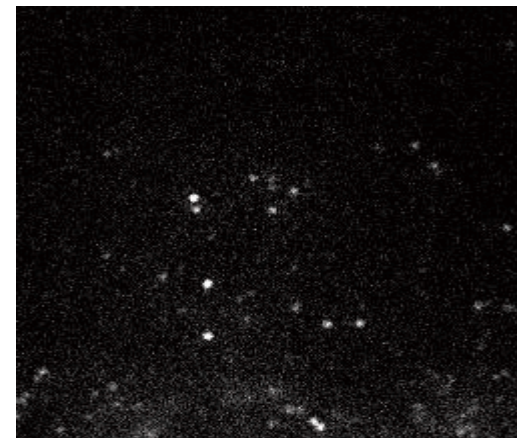
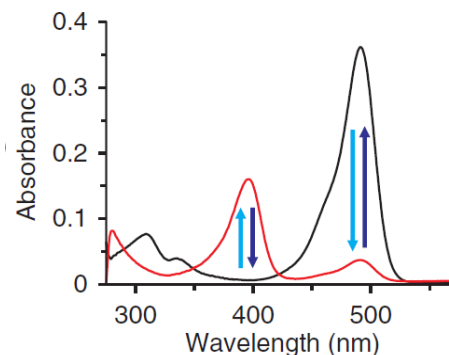
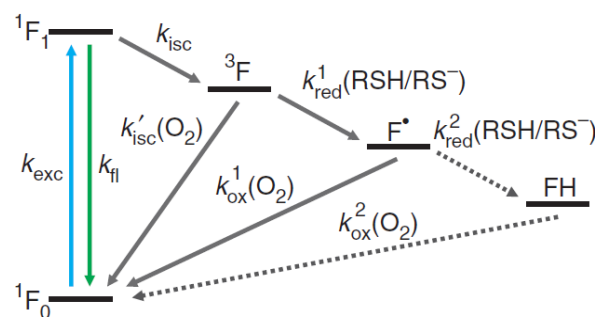
**Fig. 3.** Three-dimensional STORM imaging of clathrin-coated pits in a cell. (A) Conventional direct immunofluorescence image of clathrin in a region of a BS-C-1 cell. (B) The 2D STORM image of the same area, with all localizations at different  $z$  positions included. (C) An  $x$ - $y$  cross section (50 nm thick in  $z$ ) of the same area, showing the ring-like structure of the periphery of the CCPs at the

plasma membrane. (D and E) Magnified view of two nearby CCPs in 2D STORM (D) and their  $x$ - $y$  cross section (100 nm thick) in the 3D image (E). (F to H) Serial  $x$ - $y$  cross sections (each 50 nm thick in  $z$ ) (F) and  $x$ - $z$  cross sections (each 50 nm thick in  $y$ ) (G) of a CCP, and an  $x$ - $y$  and  $x$ - $z$  cross section presented in 3D perspective (H), showing the half-spherical cage-like structure of the pit.

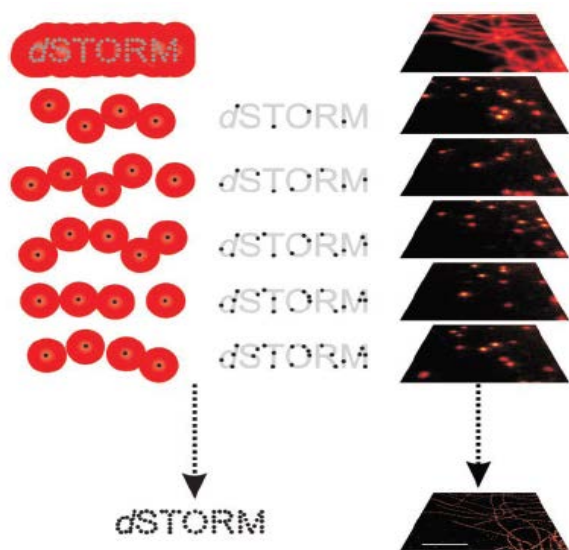


# Direct stochastic optical reconstruction microscopy (dSTORM)

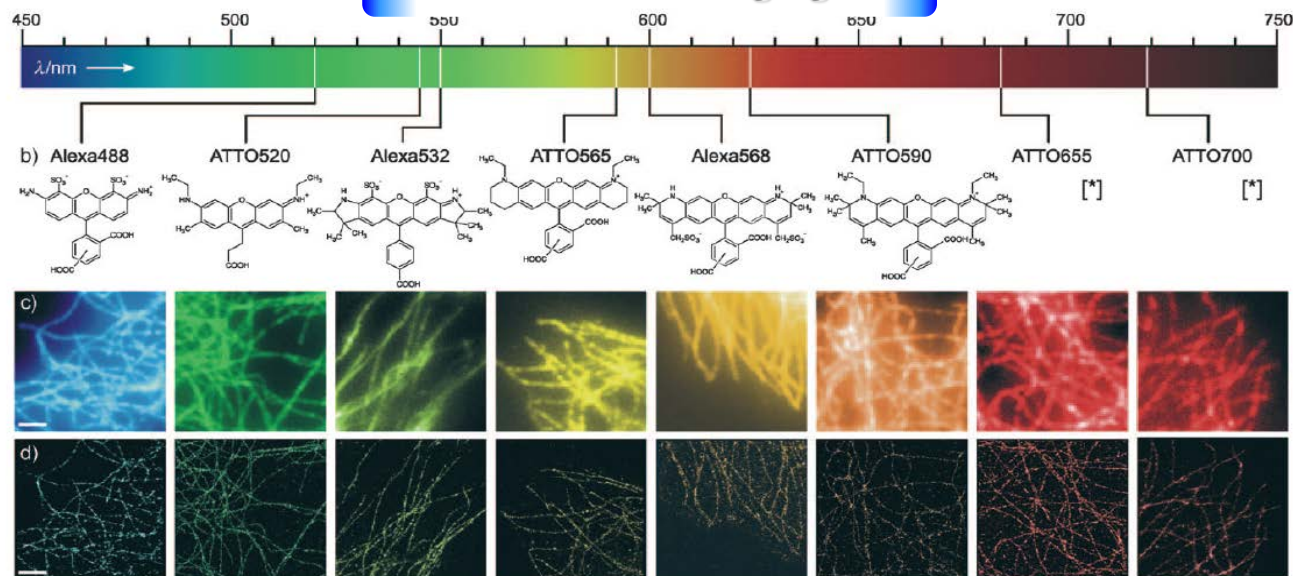
## Reversible photoswitching mechanism of standard fluorophores



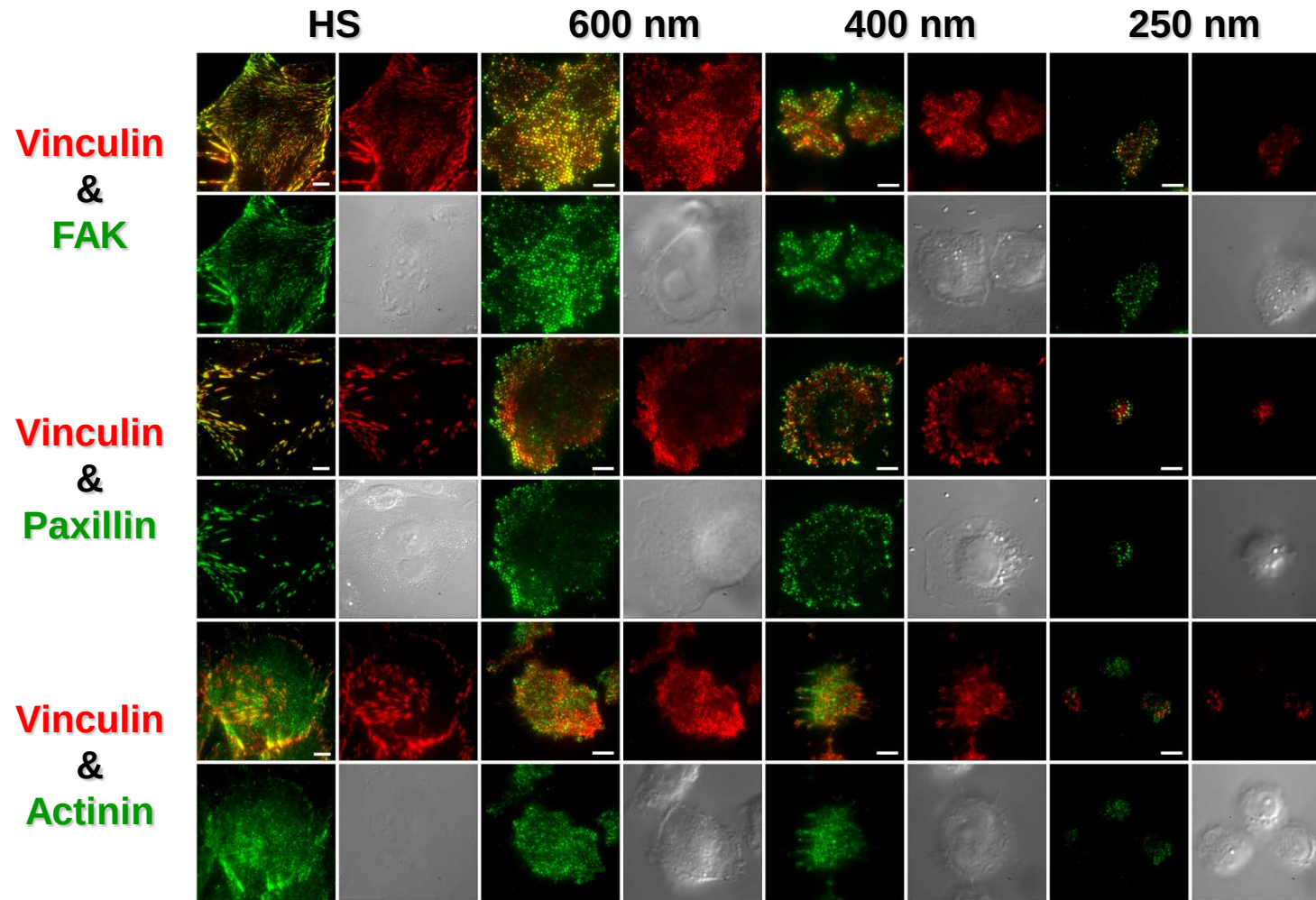
## Localization imaging concept



## Multicolor imaging



# Focal adhesion complexes on HS and pattern ECM surface

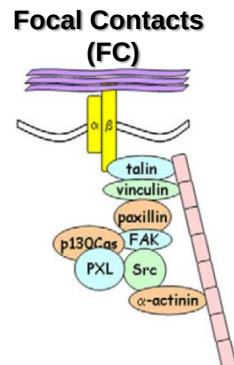
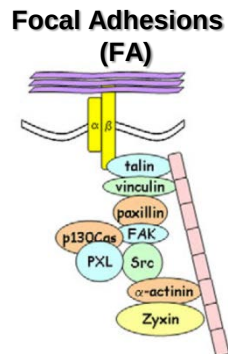
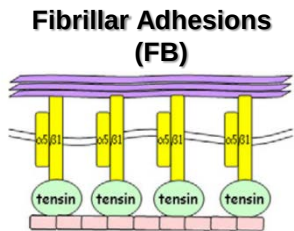
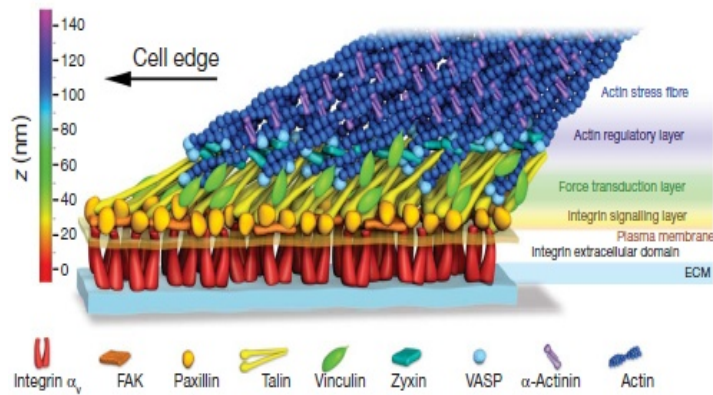




# Multicolor superresolution fluorescence imaging

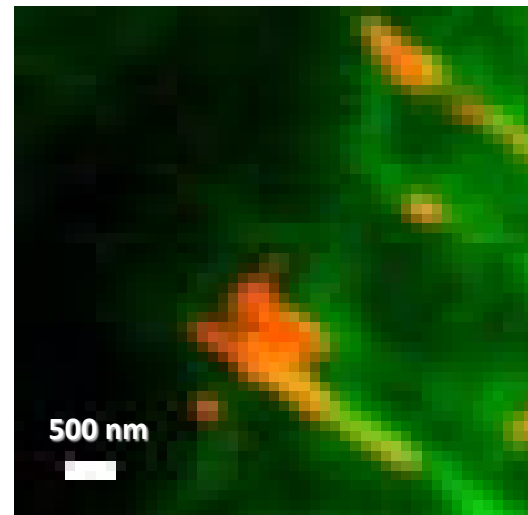
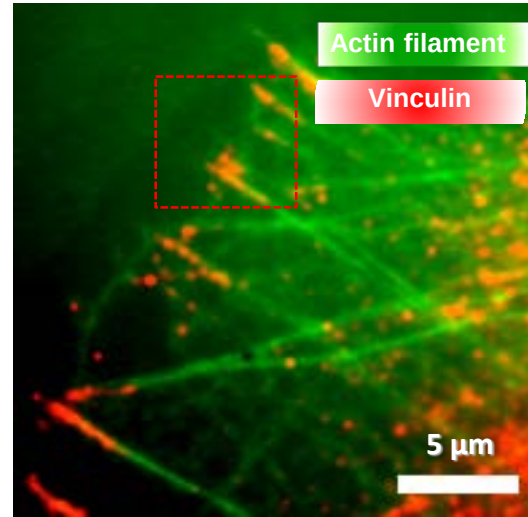
## Objective

1. Characteristic distance between focal adhesion complexes.
2. Cross-correlation between different focal adhesion complexes.
3. Number of focal adhesion complexes at different adhesion types.

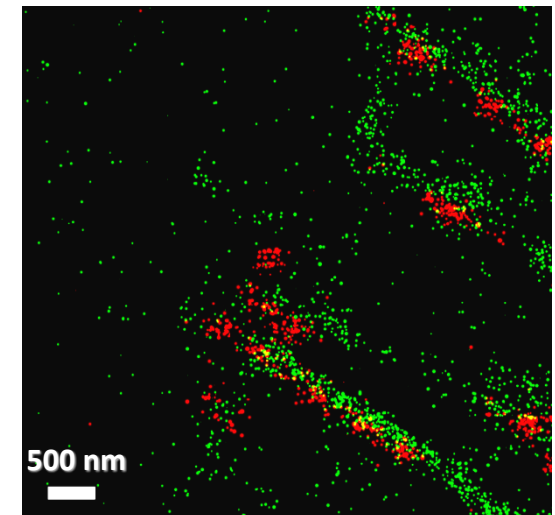
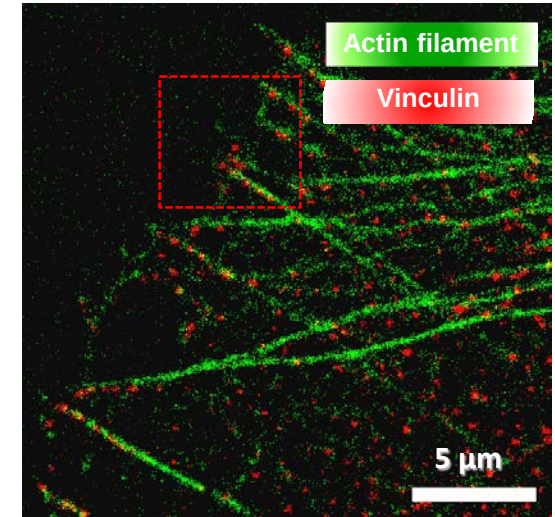


J. Cell Sci. (2010).

## TIRF images



## Super-resolution images





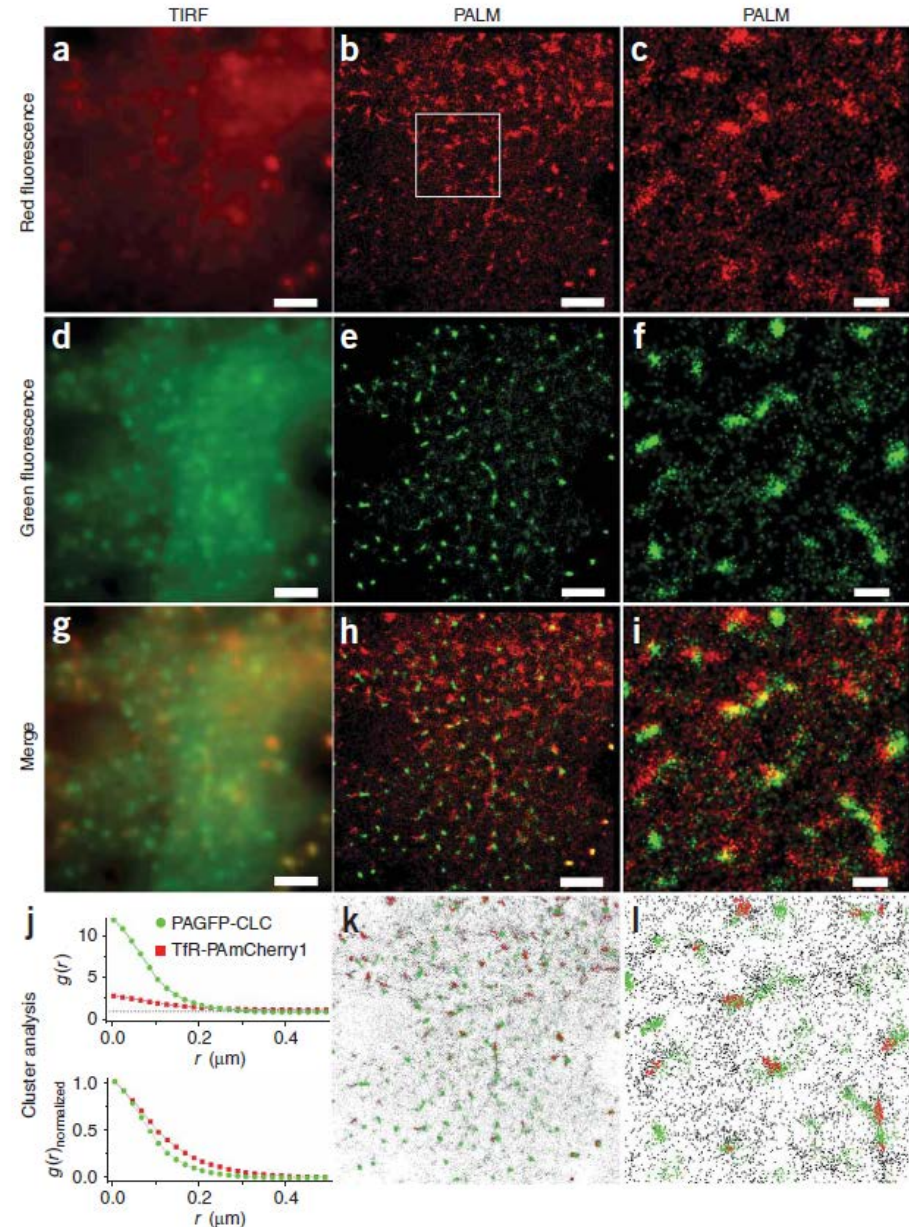
# Pair correlation analysis

$$g(r) = \left\langle \frac{\rho(r)}{\bar{\rho}} \right\rangle$$

$\rho(r)$  is the density of molecules at distance.

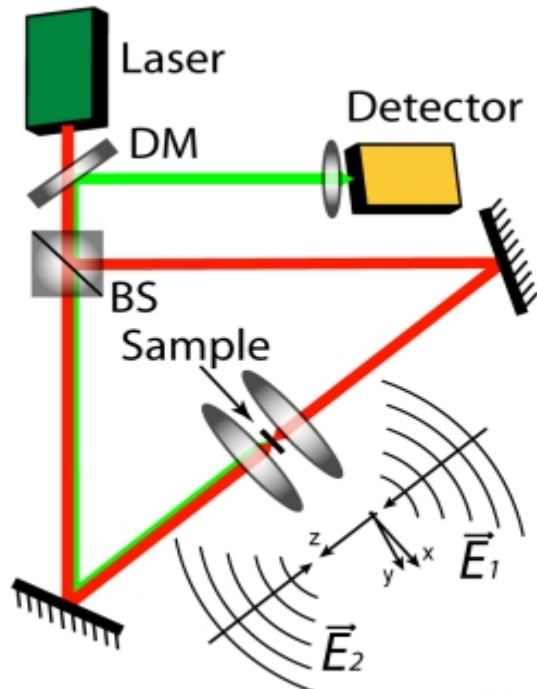
$r$  from a given molecule.

$\bar{\rho}$  is the average density of molecules, with this ratio averaged over the entire ensemble of molecules.

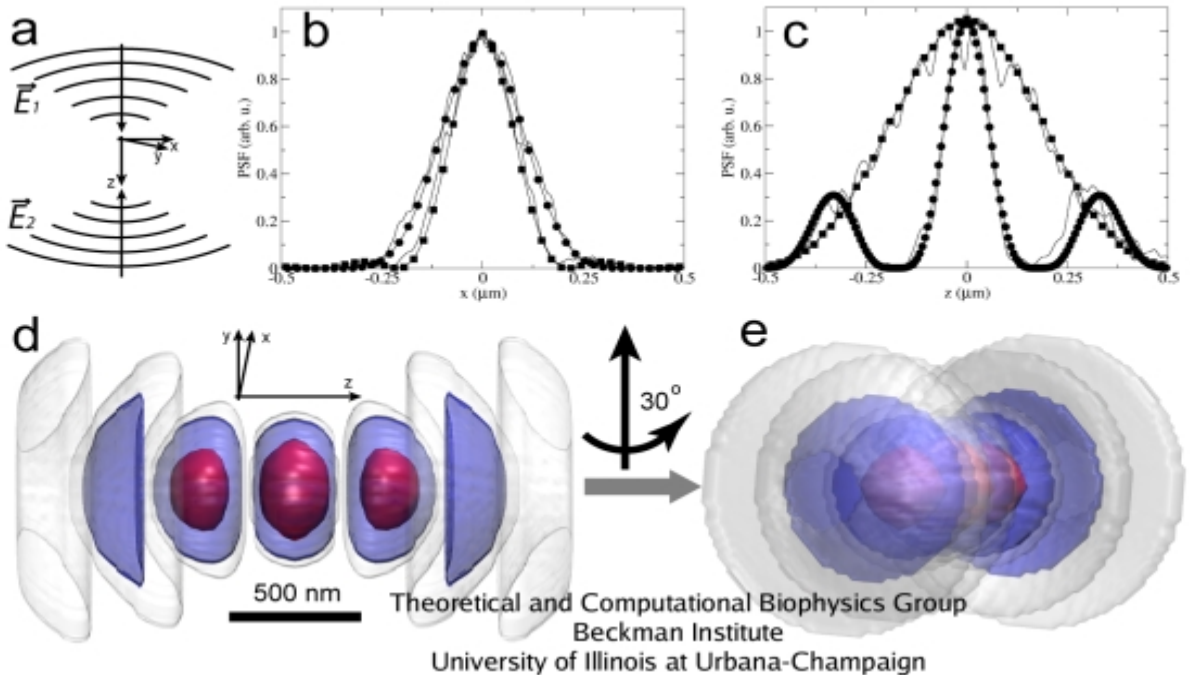


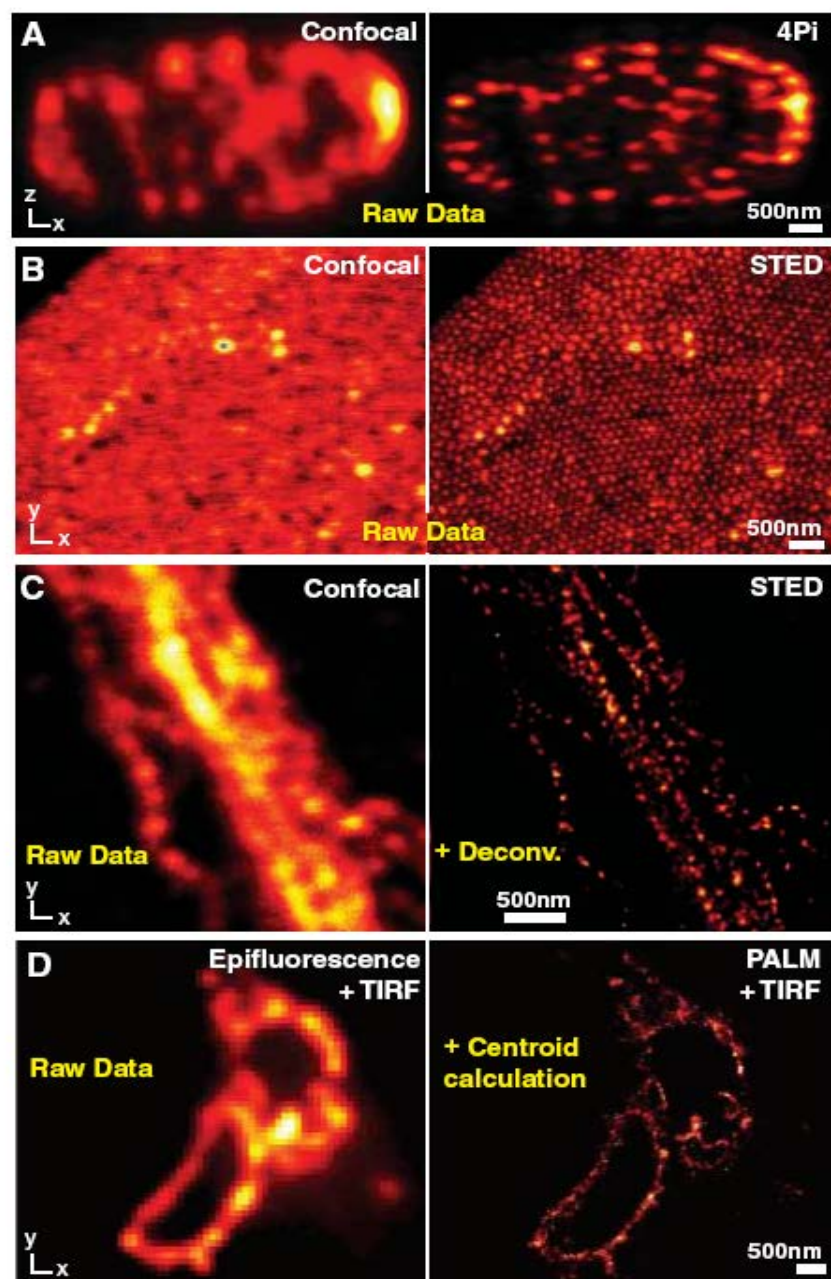
# 4 Pi

Schematic of the 4Pi microscope



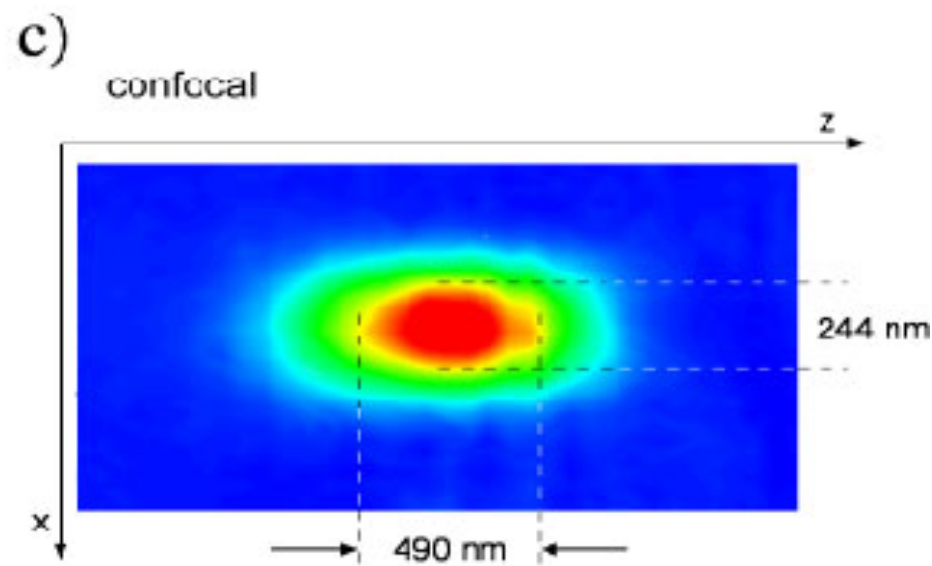
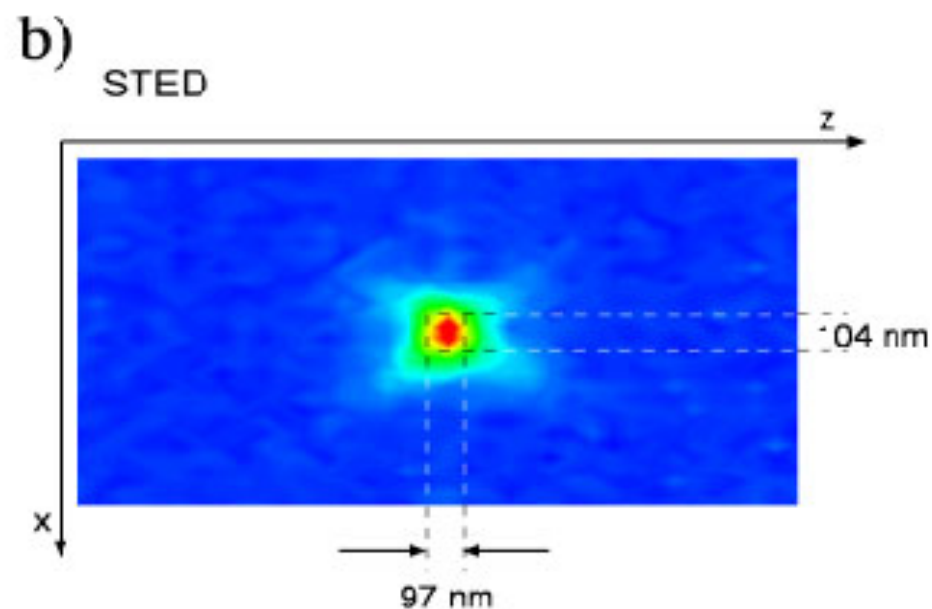
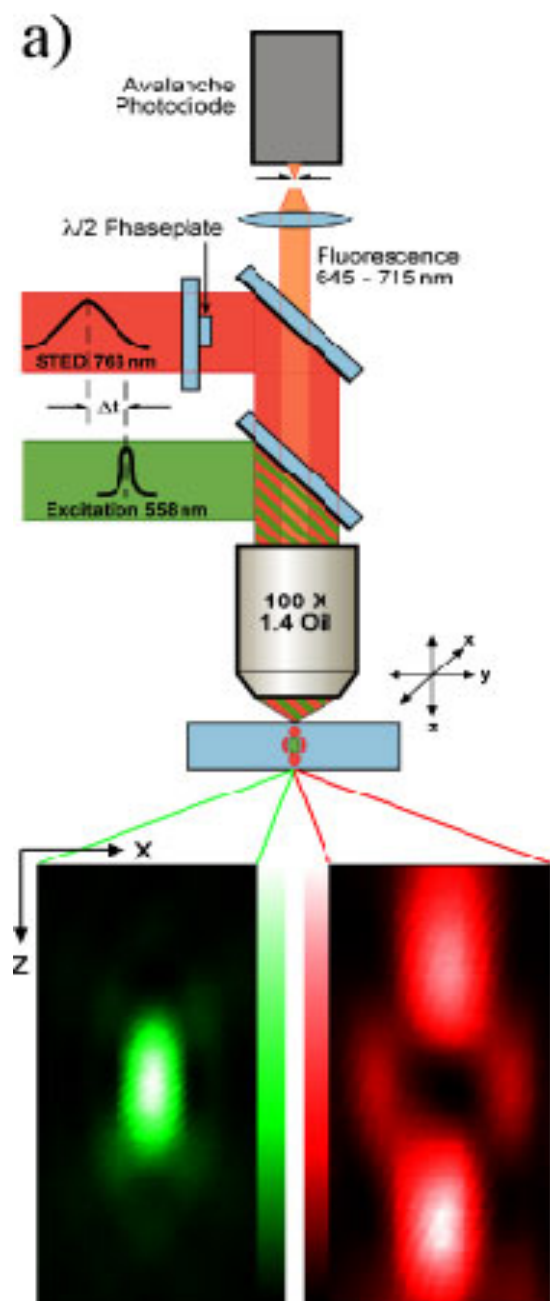
Theoretical and Computational Biophysics Group  
Beckman Institute  
University of Illinois at Urbana-Champaign

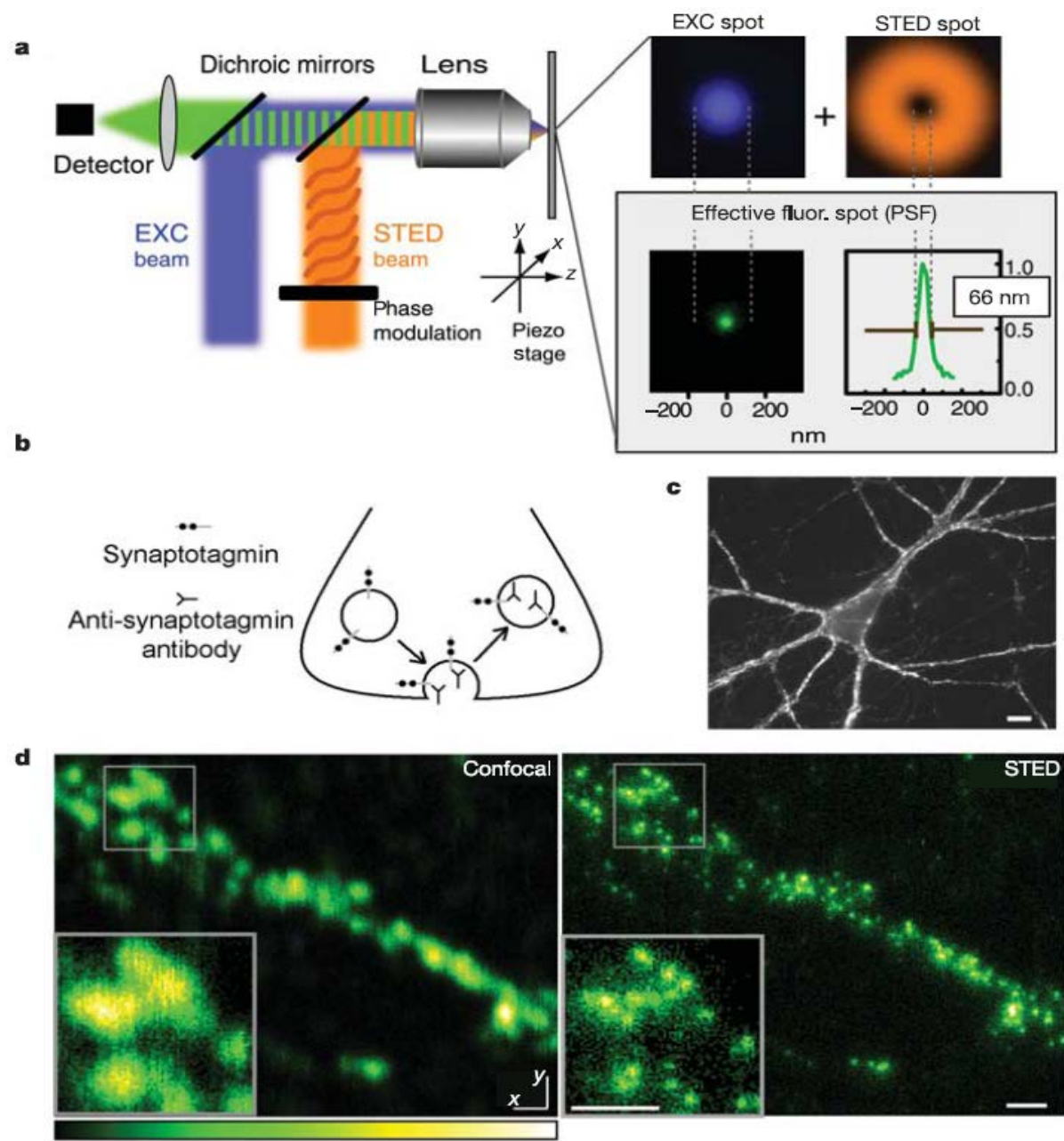




**Fig. 4.** Side-by-side comparisons. **(A)** Confocal versus 4Pi axial (xz) image of microtubules in a neuron: 4Pi image displays 140-nm z resolution; lens of  $\alpha = 74^\circ$  and with two-photon excitation at 800 nm. The plain 4Pi image is due to a narrow solitary peak without lobes; mathematical lobe-removal is not required. **(B)** Unlike the confocal reference, the STED image reveals the spatial order of self-assembled fused silica nanobeads containing a fluorescence core (45). **(C)** Neurofilaments in human neuroblastoma recorded in the confocal mode (left) and with STED after nonlinear deconvolution (right) displaying a focal plane resolution of 20 to 30 nm (39). **(D)** Epifluorescence versus PALM recording of a cryoprepared section from a mammalian cell expressing a lysosomal transmembrane protein tagged with a photoswitchable protein; both images were recorded with a TIRF setup. PALM resolution ranges between 20 and 60 nm, whereas individual protein localizations can be 2 nm (12).

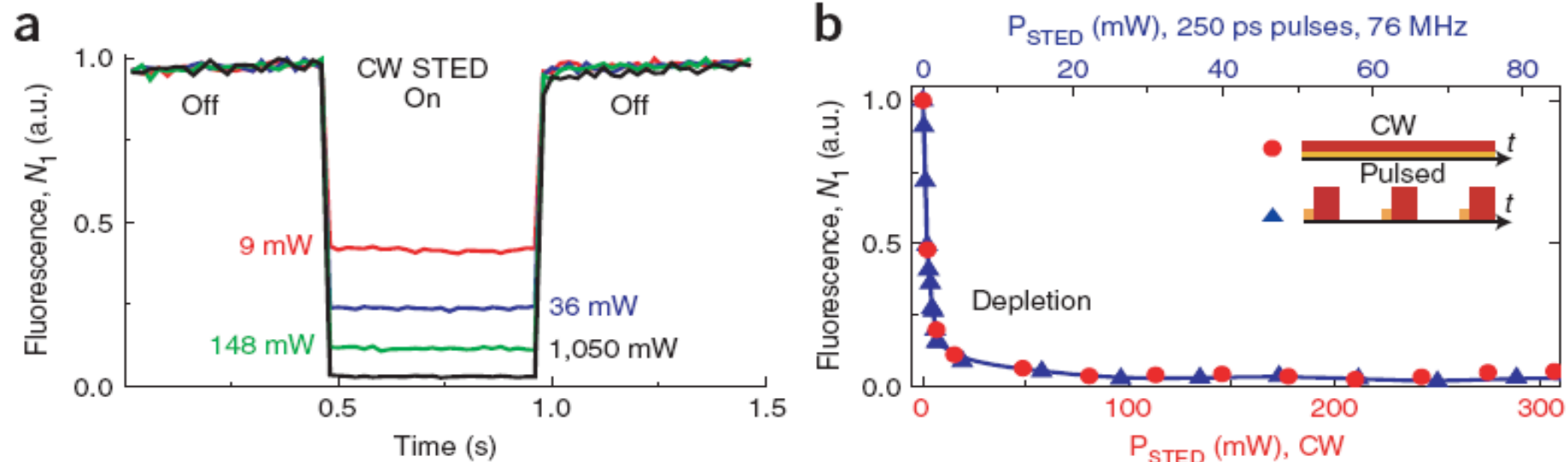


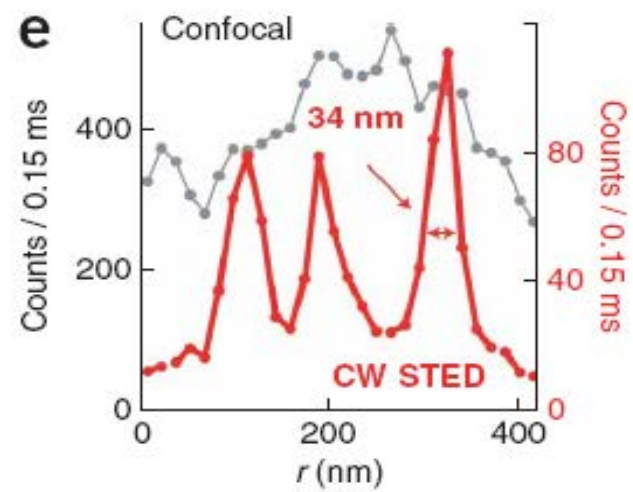
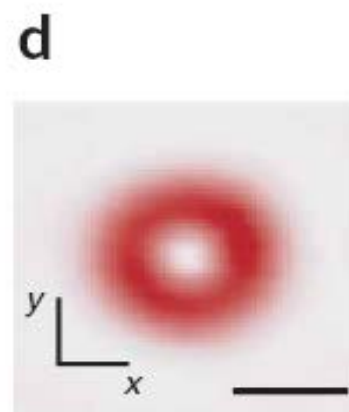
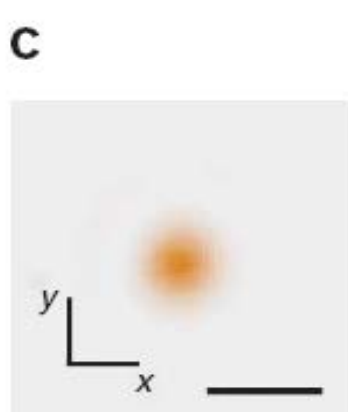
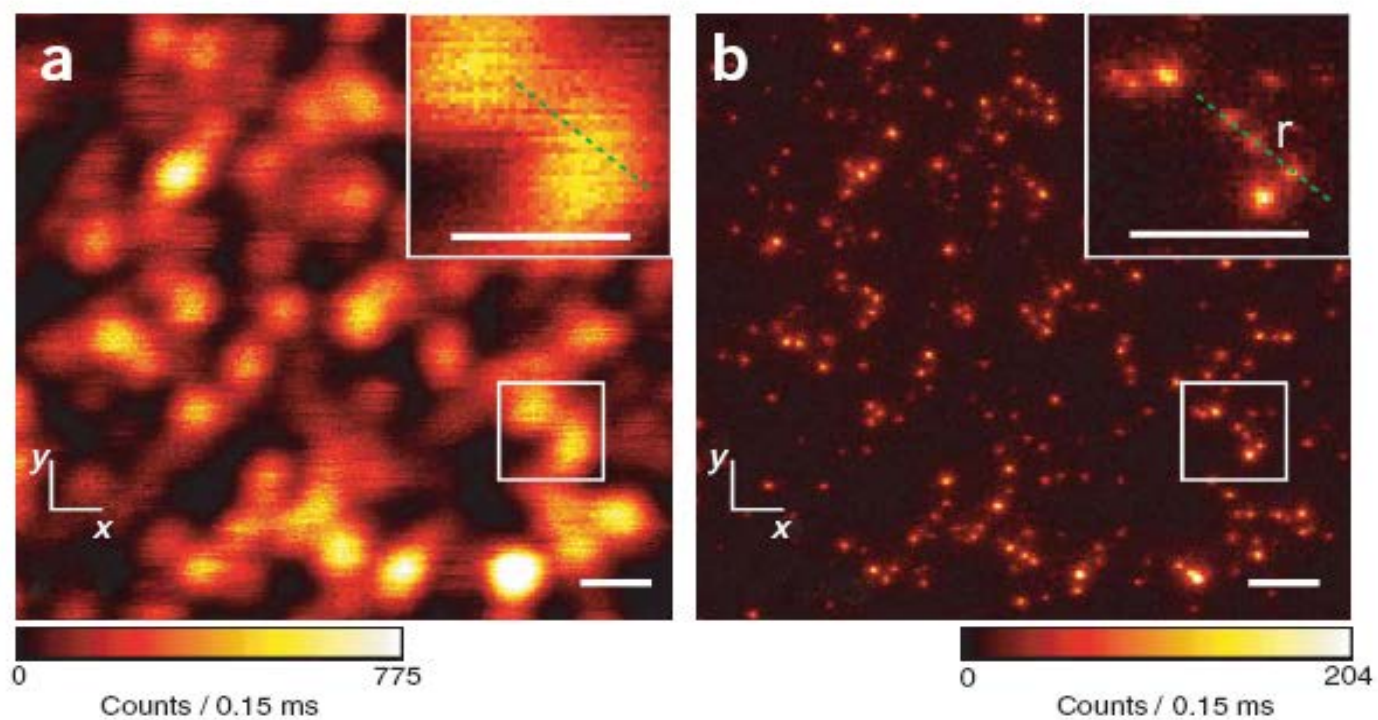


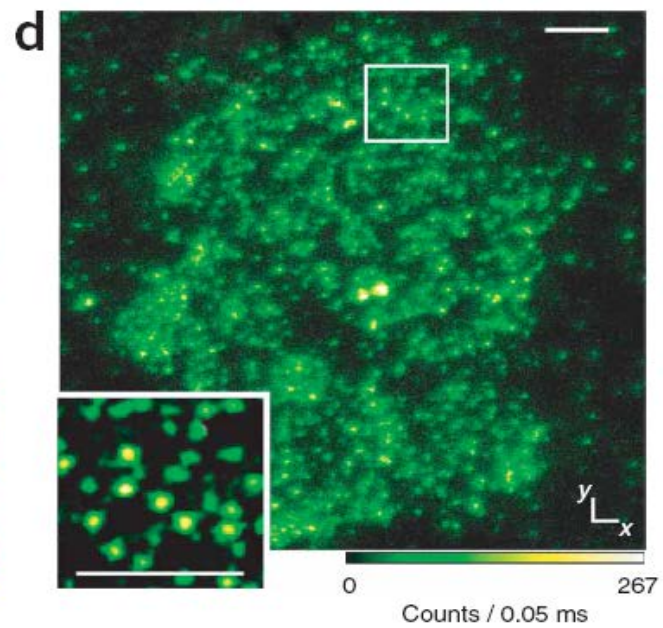
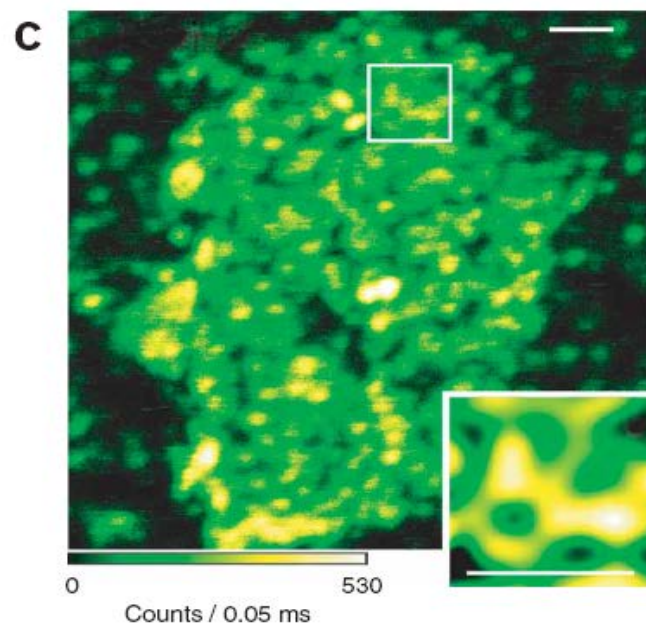
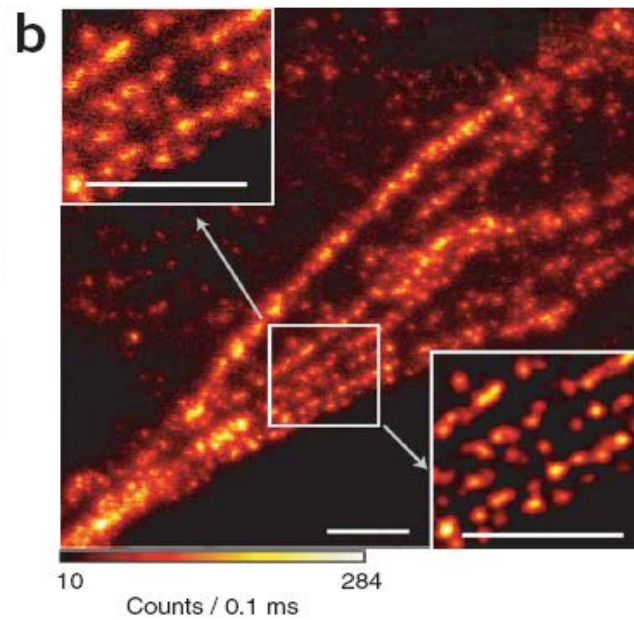
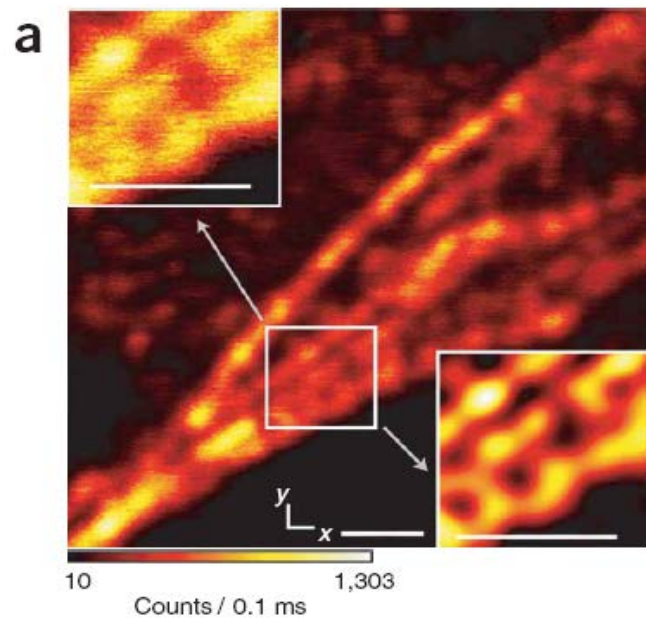




# STED microscopy with continuous wave beams





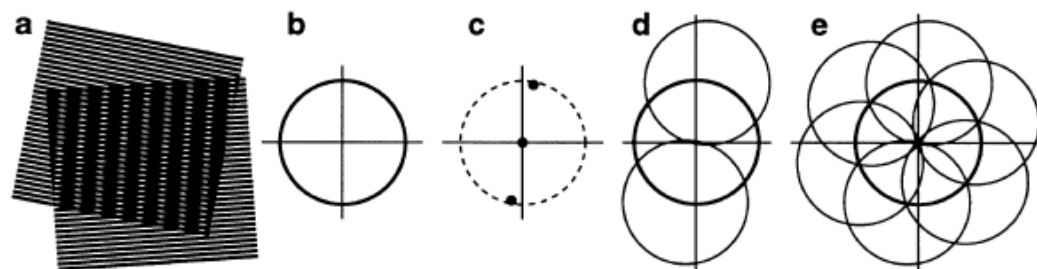


SHORT COMMUNICATION

## Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy

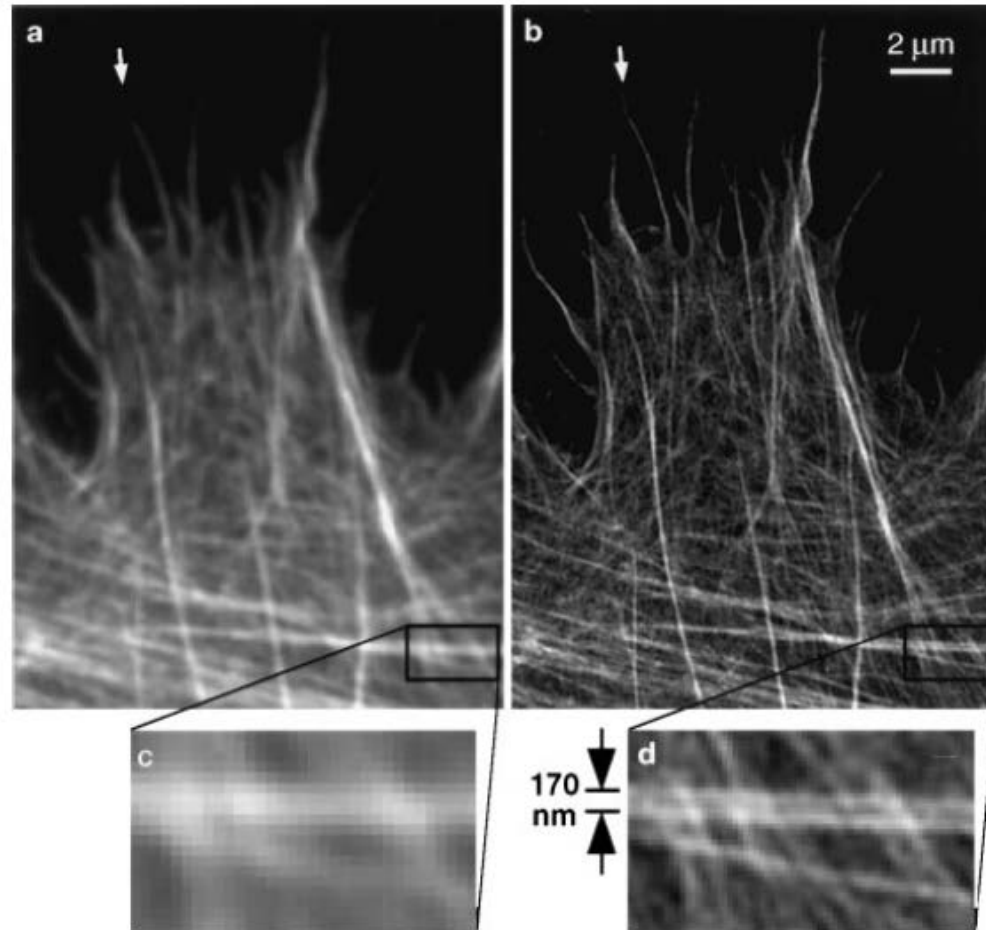
M. G. L. GUSTAFSSON

*Department of Biochemistry and Biophysics, University of California San Francisco,  
San Francisco, California 94143-0448, U.S.A.*



**Fig. 1.** Concept of resolution enhancement by structured illumination. (a) If two line patterns are superposed (multiplied), their product will contain moiré fringes (seen here as the apparent vertical stripes in the overlap region). (b) A conventional microscope is limited by diffraction. The set of low-resolution information that it can detect defines a circular 'observable region' of reciprocal space. (c) A sinusoidally striped illumination pattern has only three Fourier components. The possible positions of the two side components are limited by the same circle that defines the observable region (dashed). If the sample is illuminated with such structured light, moiré fringes will appear which represent information that has changed position in reciprocal space. The amounts of that movement correspond to the three Fourier components of the illumination. The observable region will thus contain, in addition to the normal information, moved information that originates in two offset regions (d). From a sequence of such images with different orientation and phase of the pattern, it is possible to recover information from an area twice the size of the normally observable region, corresponding to twice the normal resolution (e).





**Fig. 4.** The actin cytoskeleton at the edge of a HeLa cell, as imaged by (a, c) conventional and (b, d) structured illumination microscopy. (c, d) Enlargements of the boxed areas in (a) and (b), respectively. Fibres separated by less than the resolution limit of the conventional microscope are well resolved using structured illumination (d). The apparent widths (FWHM) of the finest protruding fibres [small arrows in (a, b)] are lowered to 110–120 nm in (b), compared to 280–300 nm in (a).



# Nonlinear structured-illumination microscopy: Wide-field fluorescence imaging with theoretically unlimited resolution

PNAS 102 (September 12, 2005) 14111-14116

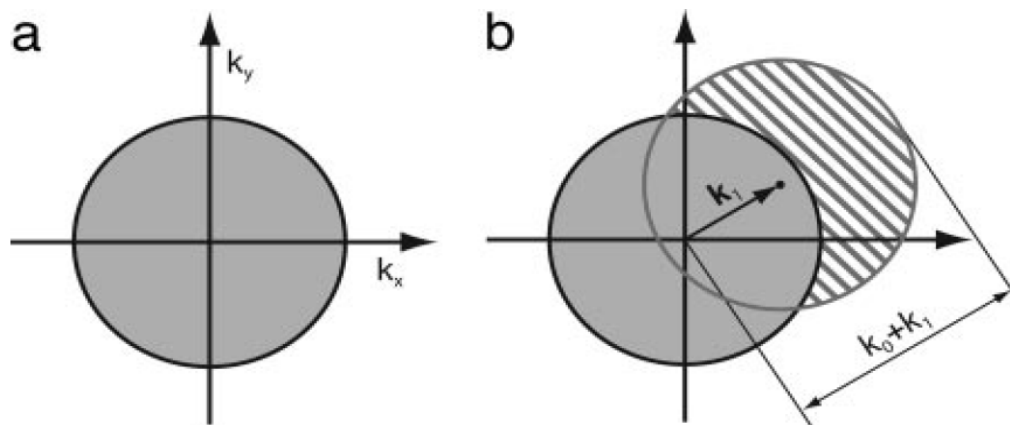
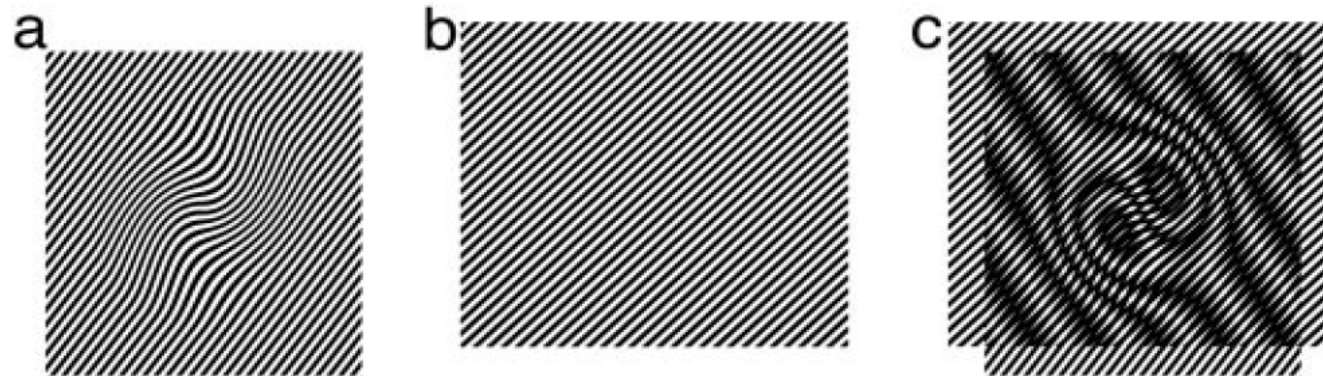
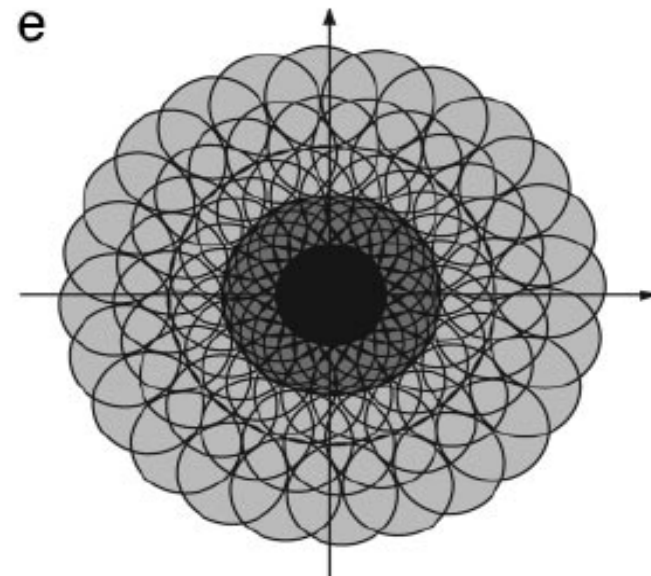
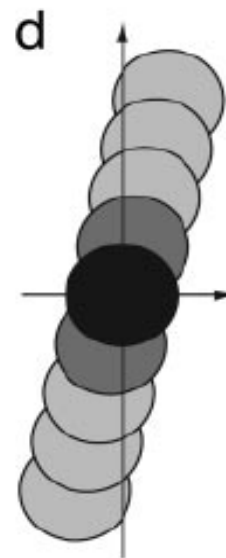
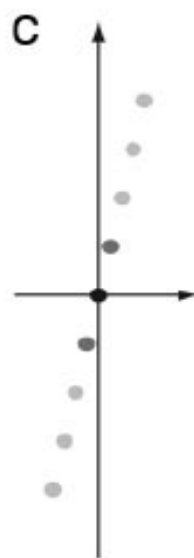
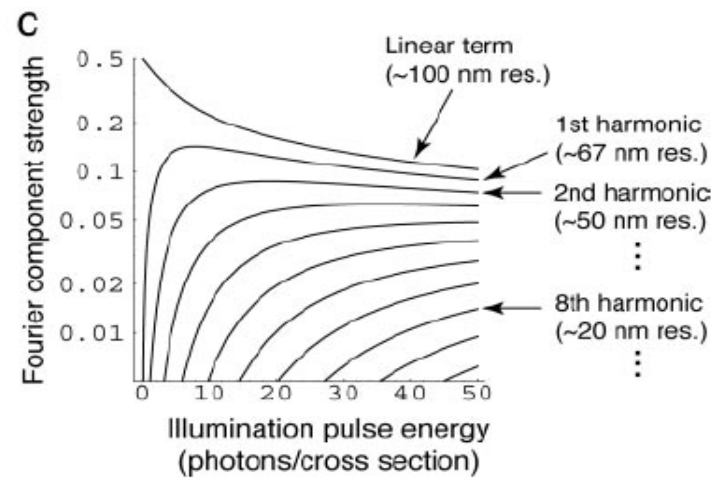
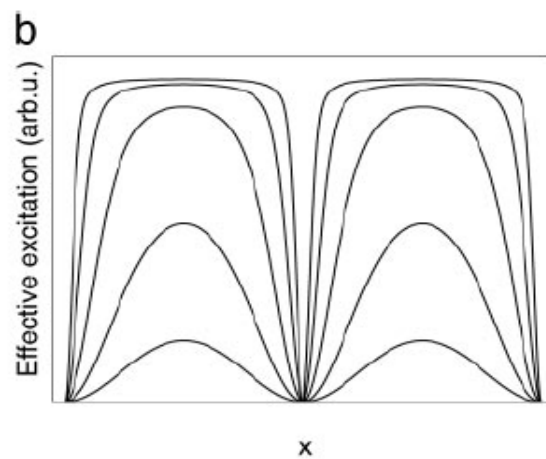
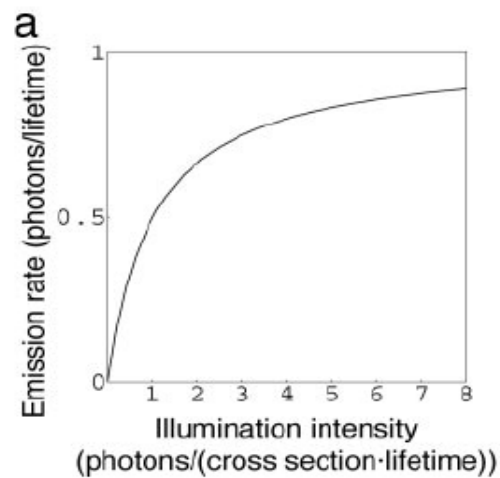


Fig. 2. Structured-illumination concept. (a) The set of sample spatial frequencies that can be observed by the conventional microscope defines a circular observable region of radius  $k_0$  in frequency space. (b) If the excitation light contains a spatial frequency  $k_1$ , a new set of information becomes visible in the form of moiré fringes (hatched circle). This region has the same shape as the normal observable region but is centered at  $k_1$ . The maximum spatial frequency that can be detected (in this direction) is  $k_0 + k_1$ .

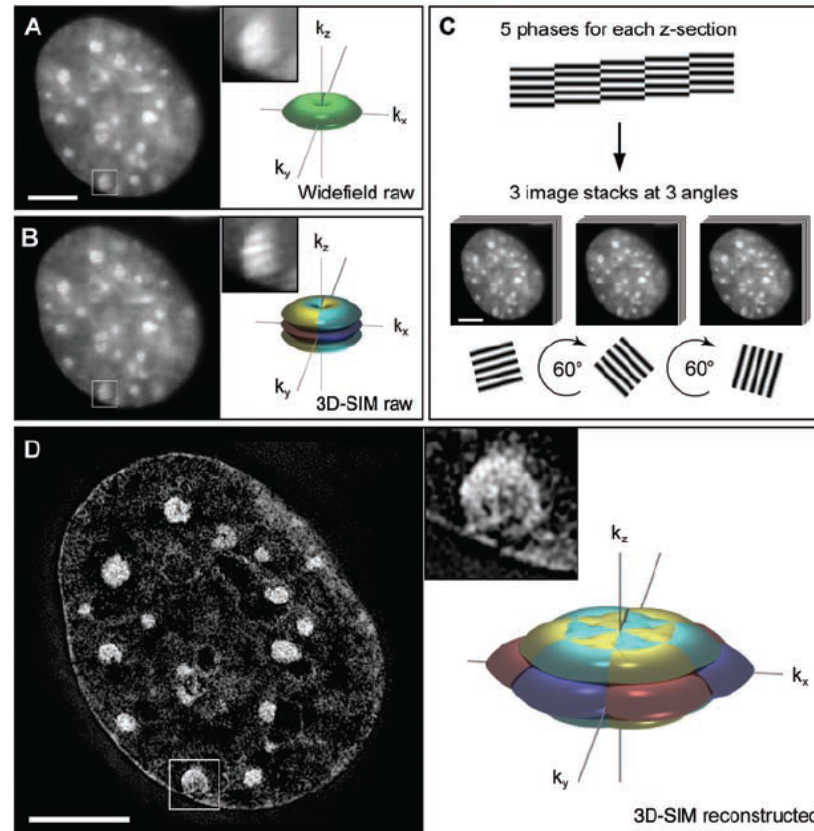


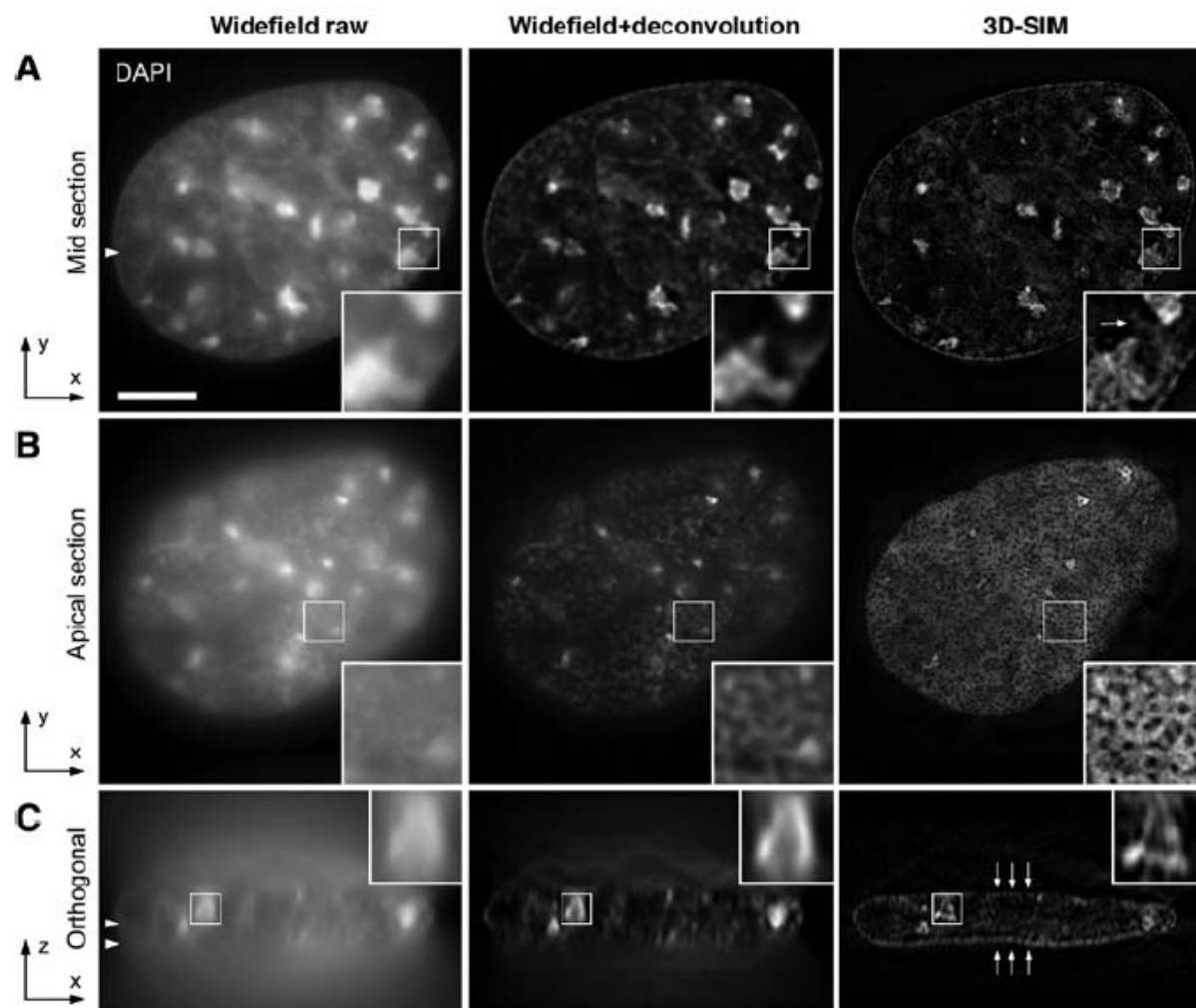
# Subdiffraction Multicolor Imaging of the Nuclear Periphery with 3D Structured Illumination Microscopy

6 JUNE 2008 VOL 320 SCIENCE

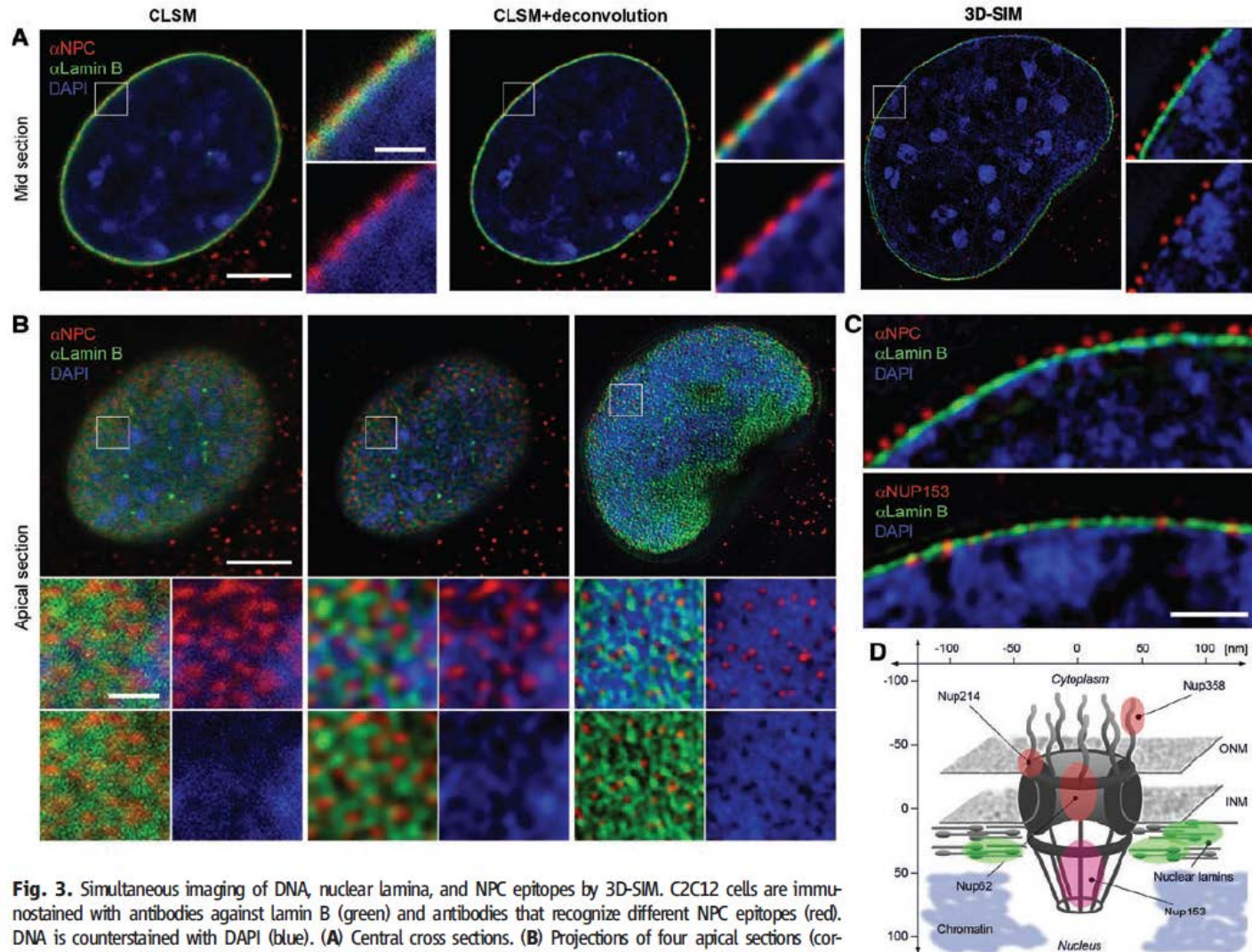
Lothar Schermelleh,<sup>1\*</sup> Peter M. Carlton,<sup>2\*</sup> Sebastian Haase,<sup>2,4</sup> Lin Shao,<sup>2</sup>  
Lukman Winoto,<sup>2</sup> Peter Kner,<sup>2</sup> Brian Burke,<sup>3</sup> M. Cristina Cardoso,<sup>4</sup> David A. Agard,<sup>2</sup>  
Mats G. L. Gustafsson,<sup>5</sup> Heinrich Leonhardt,<sup>1\*†</sup> John W. Sedat<sup>2\*†</sup>

**Fig. 1.** Subdiffraction resolution imaging with 3D-SIM. (A and B) Cross section through a DAPI-stained C2C12 cell nucleus acquired with conventional wide-field illumination (A) and with structured illumination (B), showing the striped interference pattern (inset). The renderings to the right illustrate the respective support of detection in frequency space. The axes  $k_x$ ,  $k_y$ , and  $k_z$  indicate spatial frequencies along the  $x$ ,  $y$ , and  $z$  directions. The surfaces of the renderings represent the corresponding resolution limit. The depression of the frequency support ("missing cone") in  $z$  direction in (A) indicates the restriction in axial resolution of conventional wide-field microscopy. With 3D-SIM, the axial support is extended but remains within the resolution limit. (C) Five phases of the sine wave pattern are recorded at each  $z$  position, allowing the shifted components to be separated and returned to their proper location in frequency space. Three image stacks are recorded with the diffraction grating sequentially rotated into three positions  $60^\circ$  apart, resulting in nearly rotationally symmetric support over a larger region of frequency space. (D) The same cross section of the reconstructed 3D-SIM image shows enhanced image details compared with the original image (insets). The increase in resolution is shown in frequency space on the right, with the coverage extending two times farther from the origin. Scale bars indicate  $5\ \mu\text{m}$ .









**Fig. 3.** Simultaneous imaging of DNA, nuclear lamina, and NPC epitopes by 3D-SIM. C2C12 cells are immunostained with antibodies against lamin B (green) and antibodies that recognize different NPC epitopes (red). DNA is counterstained with DAPI (blue). **(A)** Central cross sections. **(B)** Projections of four apical sections (corresponding to a thickness of 0.5 μm). Boxed regions are shown below at 4x magnification; scale bars indicate 5 μm and 1 μm, respectively. **(A)** CLSM and deconvolution still show partially overlapping signals. In contrast, with 3D-SIM the spatial separation of NPC, lamina, and chromatin and chromatin-free channels underneath nuclear pores are clearly visible. Whereas CLSM fails to resolve close nuclear pores, 3D-SIM shows clearly separated NPC signals at voids of peripheral chromatin and surrounded by an irregular network of nuclear lamina. **(C)** Mid sections comparing stainings with an antibody that mainly reacts with Nup214, Nup358, and Nup62 (αNPC) and one specifically recognizing Nup153 (αNup153). The αNPC signal is above the lamina (140 ± 8 nm), whereas the αNup153 pore signal is at the same level as the lamina (-15 ± 20 nm). Scale bars 1 μm. **(D)** Schematic outline of the NPC, showing the relative position of Nup proteins and surrounding structures. ONM, outer nuclear membrane; INM, inner nuclear membrane.

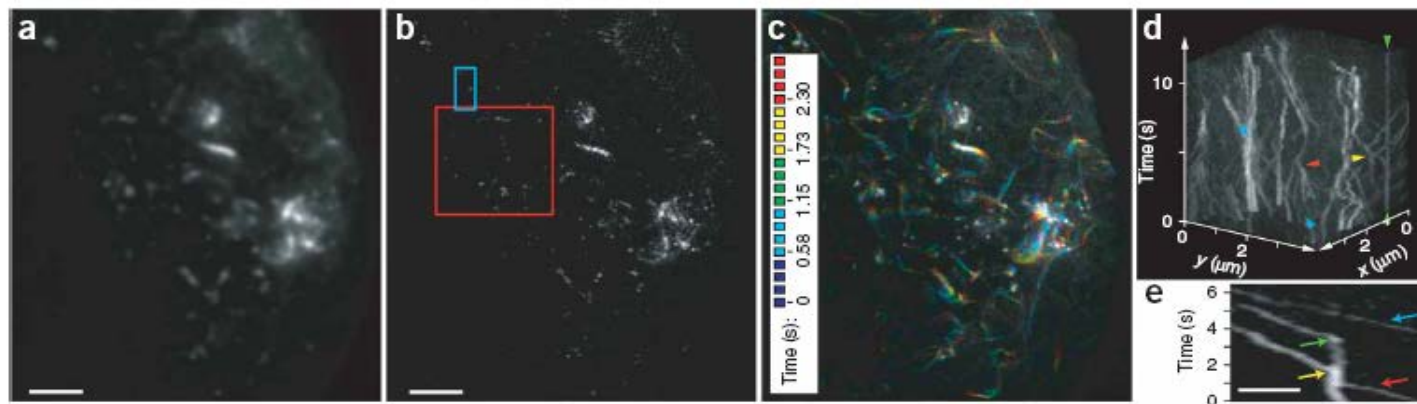
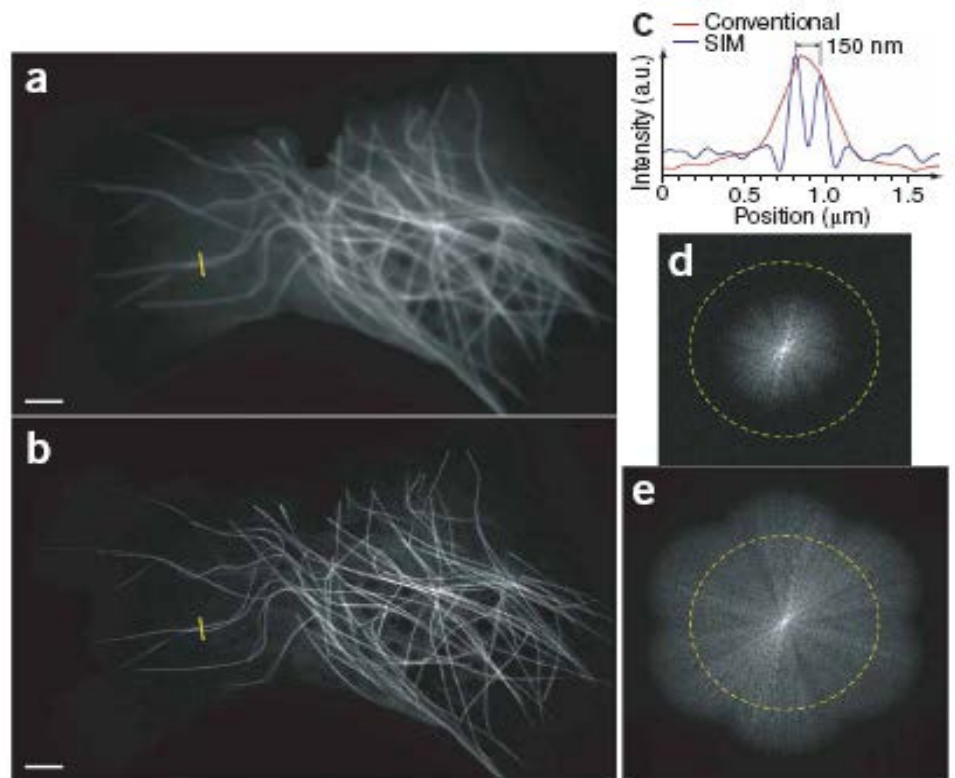


# Super-resolution video microscopy of live cells by structured illumination

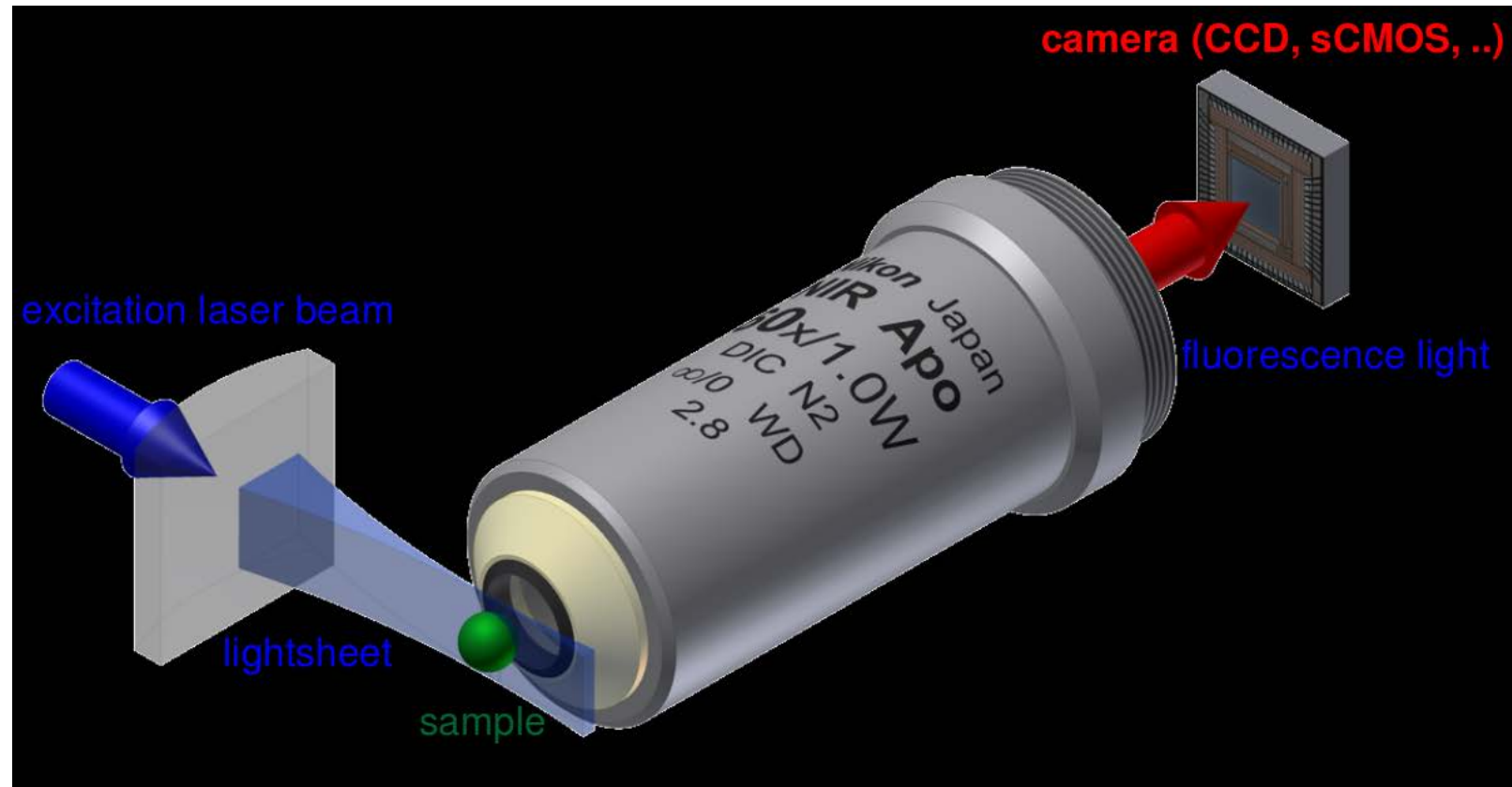
Peter Kner<sup>1,2,7,8</sup>, Bryant B Chhun<sup>1,8</sup>, Eric R Griffis<sup>3,4</sup>,  
Lukman Winoto<sup>5</sup> & Mats G L Gustafsson<sup>5,6</sup>

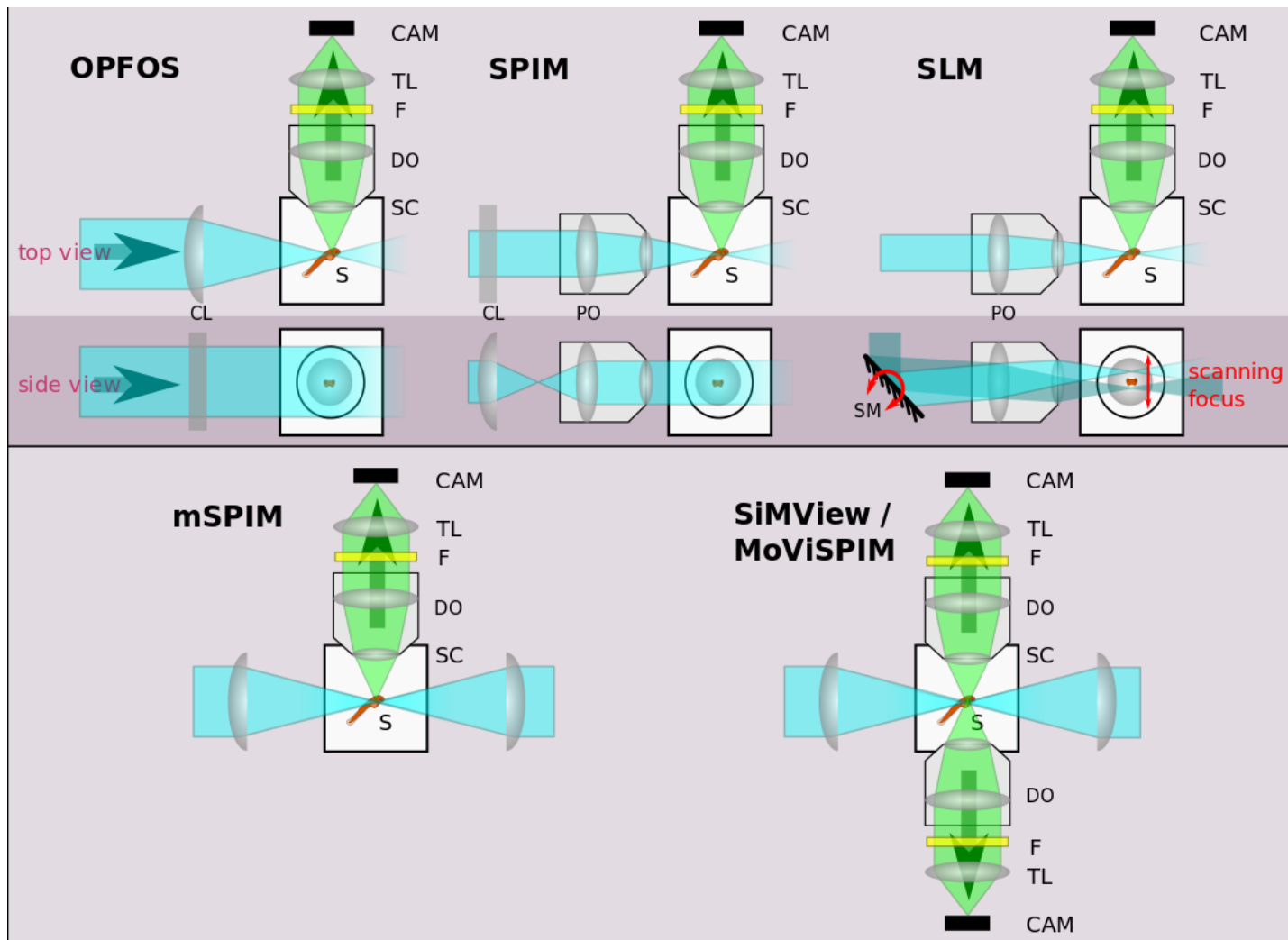
**NATURE METHODS** | VOL.6 NO.5 | MAY 2009 | 339

Structured-illumination microscopy can double the resolution of the widefield fluorescence microscope but has previously been too slow for dynamic live imaging. Here we demonstrate a high-speed structured-illumination microscope that is capable of 100-nm resolution at frame rates up to 11 Hz for several hundred time points. We demonstrate the microscope by video imaging of tubulin and kinesin dynamics in living *Drosophila melanogaster* S2 cells in the total internal reflection mode.



# Light sheet fluorescence microscopy

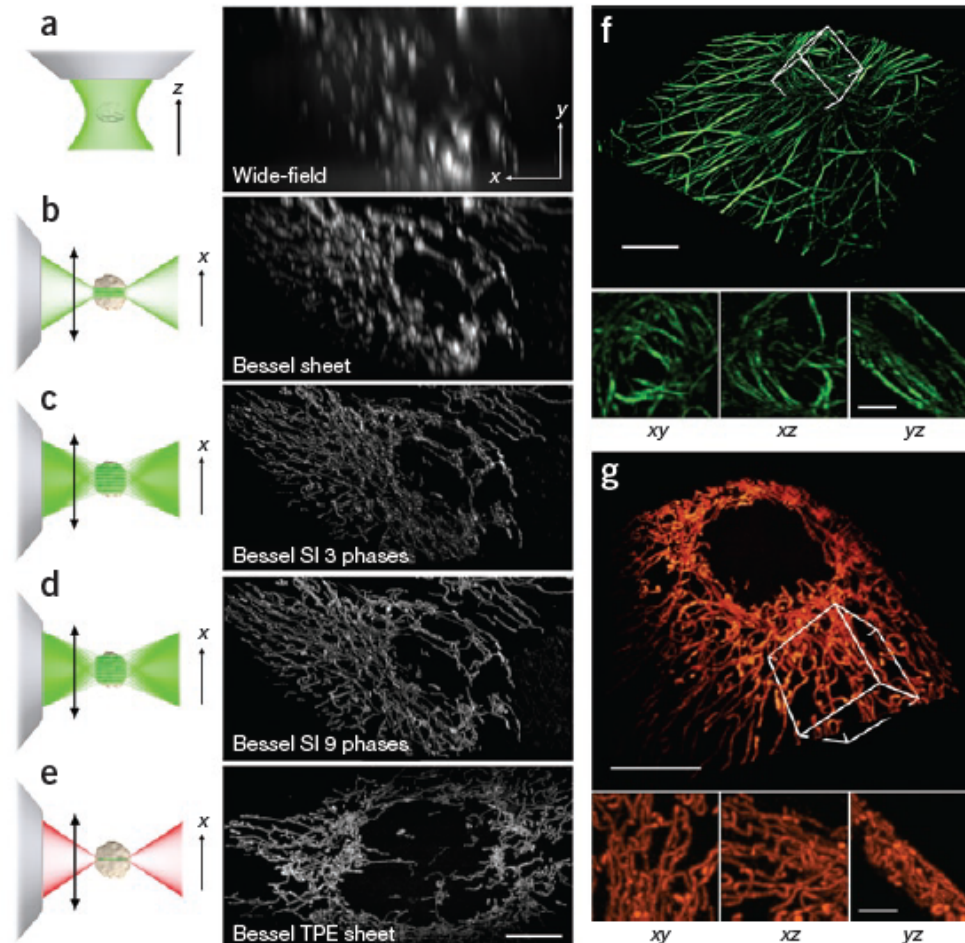




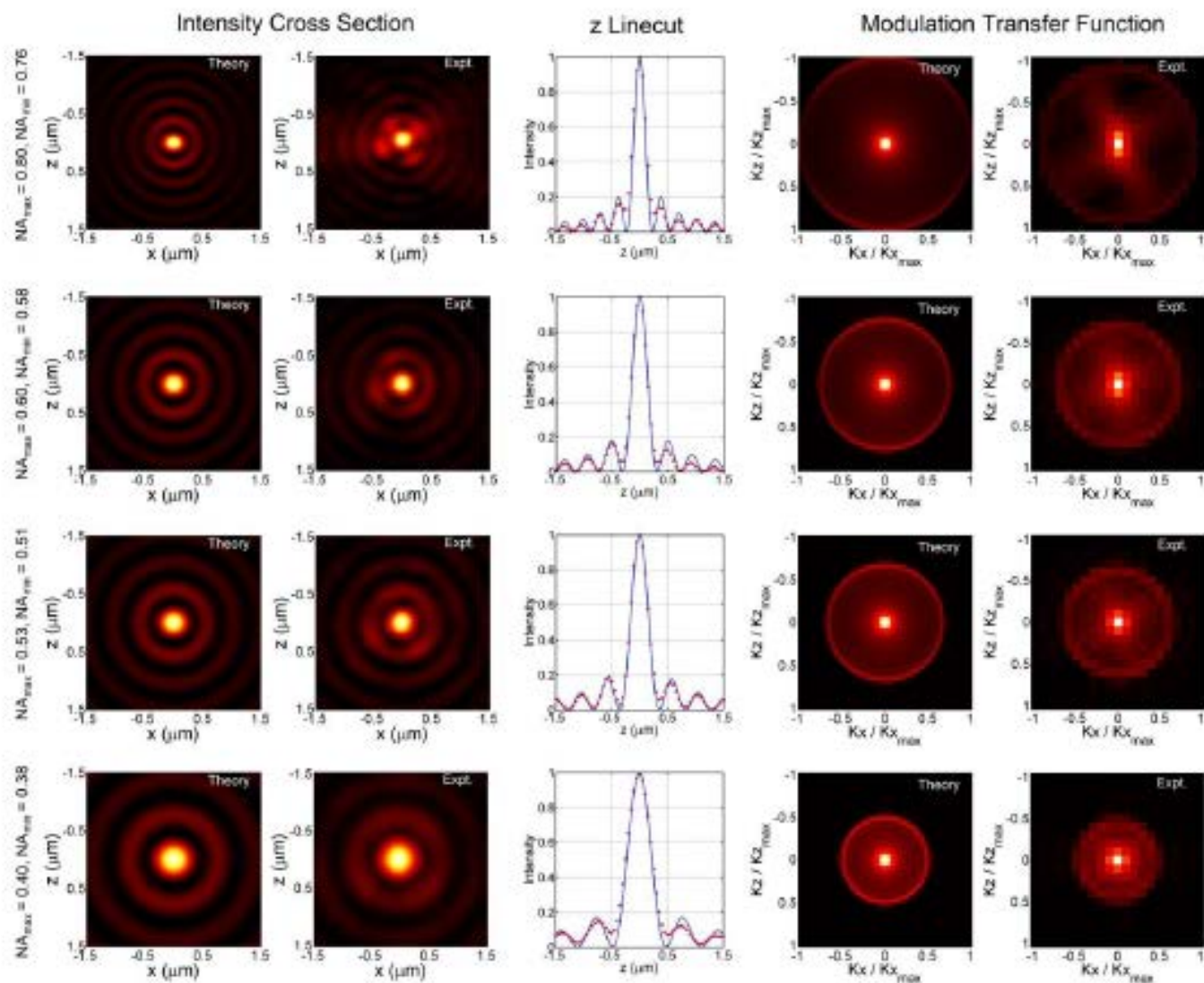
# Rapid three-dimensional isotropic imaging of living cells using Bessel beam plane illumination

Thomas A Planchon<sup>1,6</sup>, Liang Gao<sup>1,6</sup>, Daniel E Milkie<sup>2</sup>, Michael W Davidson<sup>3</sup>, James A Galbraith<sup>4</sup>, Catherine G Galbraith<sup>5</sup> & Eric Betzig<sup>1</sup>

**Figure 2 | Modes of Bessel beam plane illumination microscopy.** (a) Wide-field illumination geometry (left) and maximum-intensity projection (MIP) in the  $x$ - $z$  plane (right;  $z$ , detection axis) from a 3D image stack of a fixed human osteosarcoma cell (U2OS) transfected with plasmids encoding mEmerald fused to human pyruvate dehydrogenase alpha 1 (PDHA1). (b) Bessel sheet mode geometry (left), showing fluorescence excitation from Bessel side lobes (light green) as well as the central peak (dark green), and  $x$ - $z$  plane MIP (right) from same cell as in a. (c,d) Bessel SI mode geometry, showing periodic Bessel beam excitation pattern (left) and  $x$ - $z$  plane MIPs with single-harmonic (c) and multiharmonic (d) excitation (right). (e) Two-photon excitation (TPE) Bessel sheet mode geometry (left), showing infrared excitation (red) of fluorescence in the central peak (green), with negligible fluorescence in side lobes and  $x$ - $z$  plane MIP from a cell (right) similar to those in a–d. (f) Volume rendering in the multiharmonic SI mode (9 phases, 2.4  $\mu\text{m}$  period) of mEmerald-tagged microtubule associated protein 4 (MAP4) in a live U2OS cell. (g) Volume rendering in the TPE sheet mode of mEmerald-labeled mitochondria in a live pig kidney epithelial cell (LLC-PK1 cell line). Insets in f and g show MIPs along orthogonal axes of the cubical volumes shown. Scale bars, 10  $\mu\text{m}$  except 3  $\mu\text{m}$  in insets.

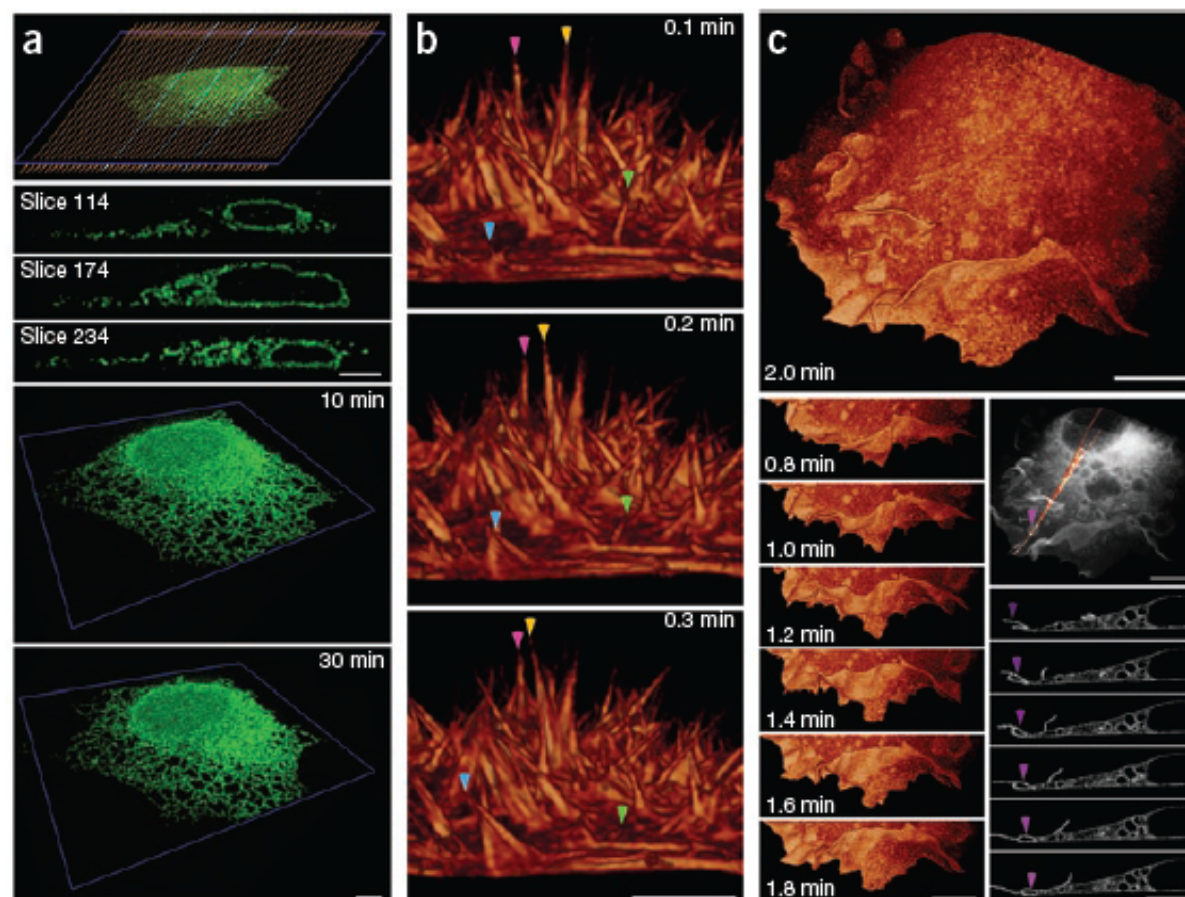


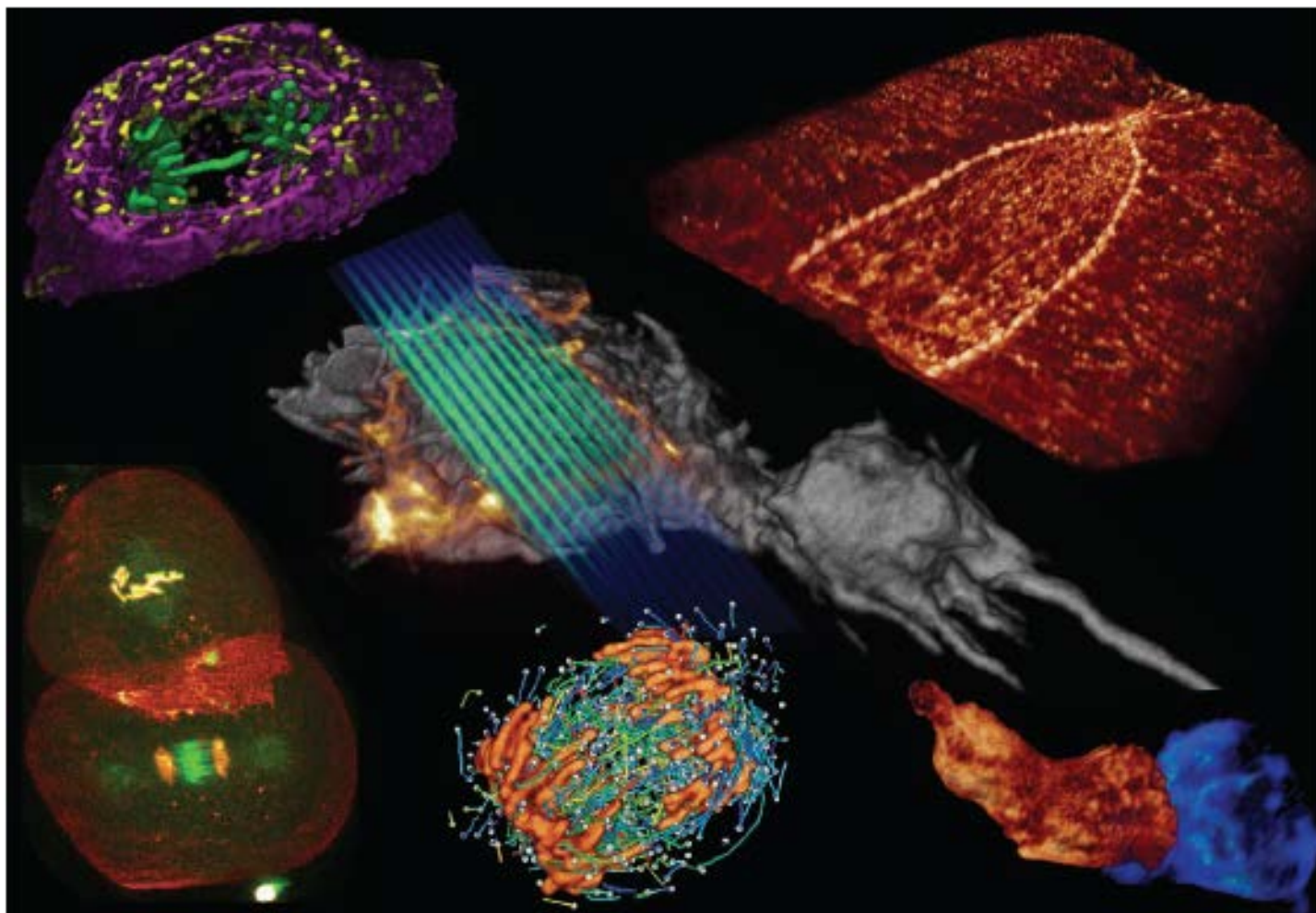






**Figure 4** | Three-dimensional isotropic imaging of live-cell dynamics. (a) Images of ER in a live U2OS cell, visualized in the Bessel multiharmonic 9 phase SI mode, over 45 image volumes: representation of a subset of the 321 image planes (brown lines) comprising each volume (top); images from three indicated image planes; and image volumes after 10 and 30 min of observation. (b) Filopodia at the apical surface of a live HeLa cell, visualized in the Bessel TPE sheet mode over three consecutive image volumes from 100 such volumes taken at 6-s intervals. Filaments that wave (magenta and yellow arrowheads), extend outward (cyan arrowhead) or retract inward (green arrowhead) are marked. (c) African green monkey kidney cell (COS-7) transfected with plasmids encoding mEmerald-c-Src, demonstrating retrograde flow of membrane ruffles (left) and vacuole formation by macropinocytosis (right; arrowheads) in an exemplary plane in a translucent cell view (top). All data were extracted from 73 image stacks taken at 12-s intervals. Scale bars, 5  $\mu\text{m}$  (a,b) and 10  $\mu\text{m}$  (c).



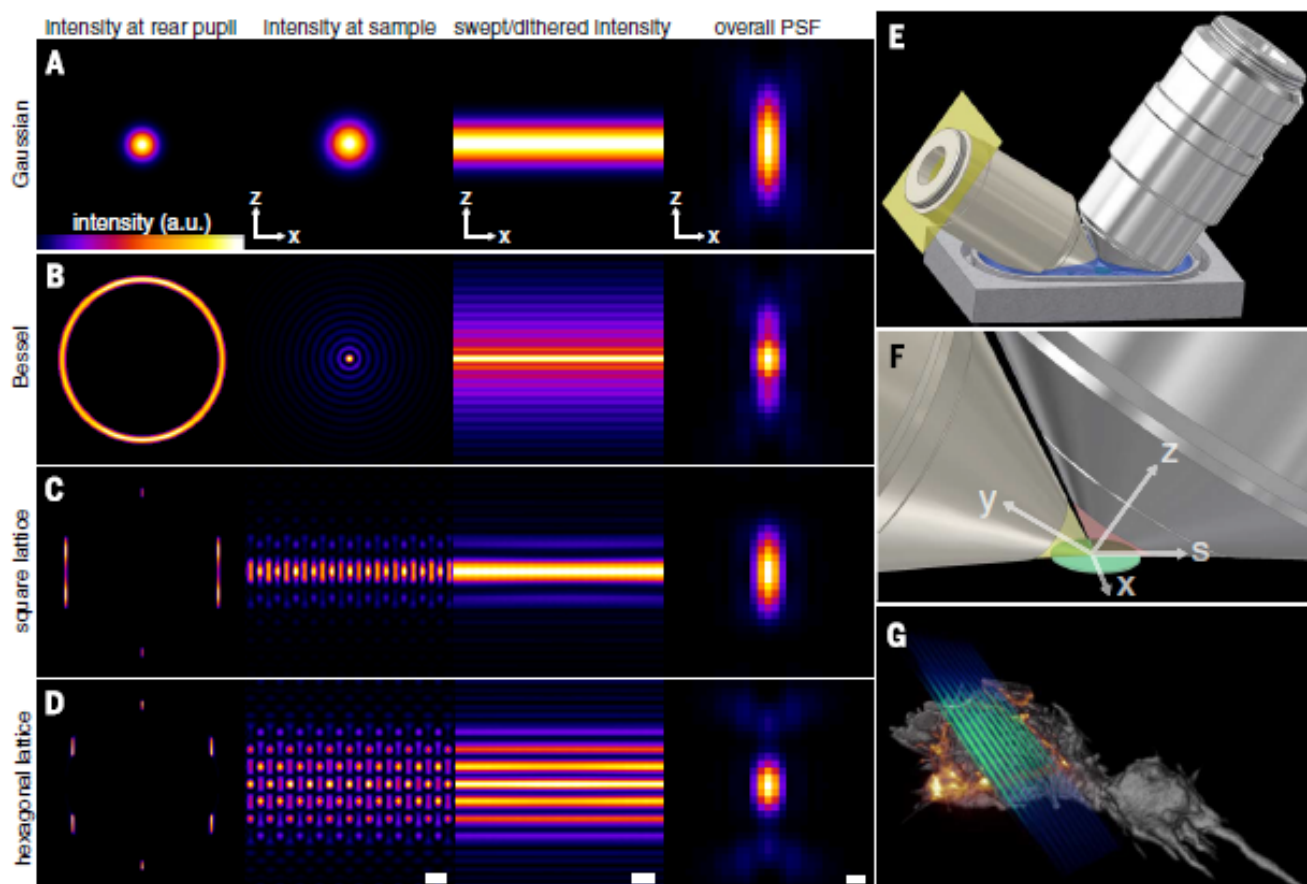


**Lattice light-sheet microscopy.** An ultrathin structured light sheet (blue-green, center) excites fluorescence (orange) in successive planes as it sweeps through a specimen (gray) to generate a 3D image. The speed, noninvasiveness, and high spatial resolution of this approach make it a promising tool for in vivo 3D imaging of fast dynamic processes in cells and embryos, as shown here in five surrounding examples.



**Fig. 1. Methods of light-sheet microscopy.** (A) The traditional approach, where a Gaussian beam is swept across a plane to create the light sheet. a.u., arbitrary units. (B) A Bessel beam of comparable length produces a swept sheet with a much narrower core but flanked by sidebands arising from concentric side lobes of the beam. (C and D) Bound optical lattices (compare with movie S1) create periodic patterns of high modulation depth across the plane, greatly reducing the peak intensity and, as we have found, the phototoxicity in live cell imaging. The square lattice in (C) optimizes the confinement of the excitation to the central plane, and the hexagonal lattice in (D) optimizes the axial resolution as defined by the overall PSF of the microscope. The columns in (A) to (D) show the intensity pattern at the rear pupil plane of the excitation objective; the

cross-sectional intensity of the pattern in the  $xz$  plane at the focus of the excitation objective (scale bar,  $1.0\ \mu\text{m}$ ); the cross-sectional intensity of the light sheet created by dithering the focal pattern along the  $x$  axis (scale bar,  $1.0\ \mu\text{m}$ ); and the  $xz$  cross section of the overall PSF of the microscope (scale bar,  $200\ \text{nm}$ ). (E) Model showing the core of our microscope, with orthogonal excitation (left) and detection (right) objectives dipped in a media-filled bath (compare with fig. S4). (F) Higher magnification view, showing the excitation



(yellow) and detection (red) light cones, which meet at a common focus within a specimen that is either mounted or cultured onto a cover glass within the media. The  $x$ ,  $y$ , and  $z$  directions are indicated. The  $s$ -axis defines the direction the specimen moves from image plane to image plane. (G) Representation of a lattice light sheet (blue-green) intersecting a cell (gray) to produce fluorescence (orange) in a single plane. The cell is swept through the light sheet to generate a 3D image (compare with movie S2).