



Scientific Background on the Nobel Prize in Chemistry 2014

## SUPER-RESOLVED FLUORESCENCE MICROSCOPY



## Super-resolved fluorescence microscopy

The Royal Swedish Academy of Sciences has decided to award Eric Betzig, Stefan W. Hell and W. E. Moerner the Nobel Prize in Chemistry 2014 for the development of super-resolution fluorescence microscopy.

### 1. Abbe's diffraction limit

Towards the end of the nineteenth century Ernst Abbe (Abbe, 1873) and Lord Rayleigh (Rayleigh, 1896) formulated what is commonly known as the “diffraction limit” for microscopy. Roughly speaking, this limit states that it is impossible to resolve two elements of a structure which are closer to each other than about half the wave length ( $\lambda$ ) in the lateral ( $x,y$ ) plane and even further apart in the longitudinal plane ( $z$ ). In other words, the minimal distances ( $\delta x_{\min}$ ,  $\delta y_{\min}$ ) that, according to Abbe's criterion, can be resolved in the lateral plane are approximated by

$$(\delta x_{\min}, \delta y_{\min}) \approx \lambda / 2$$

Another consequence of the same diffraction limitation is that it is not possible to focus a laser beam to a spot of smaller dimension than about  $\lambda/2$  (Fig. 1).

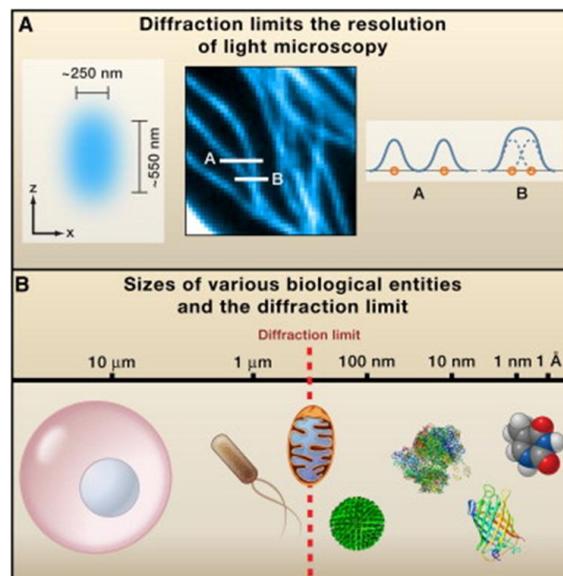


Figure 1: (Huang *et al.*, 2010, *Cell*, 143, 1047 – 57). **A:** (left) Focused laser beam, (middle) structure, (right) resolved (A) and not resolved (B) structural features. **B:** from left to right mammalian cell, *E. coli* cell, mitochondrion, influenza virus, ribosome, GFP, thymine.

In the case of light (optical) microscopy, which is, together with electron microscopy, the most important tool for the imaging of biological structures, this means that two objects within a

distance of between  $400/2=200$  nm (far blue) and  $700/2=350$  nm (far red) cannot be resolved. For electron microscopy, for which the wave length is orders of magnitude smaller, this is no real limitation but this method is very difficult to use on living cells. The length-scale of the *E. coli* cell is about 1,000 nm (1  $\mu\text{m}$ ), i.e. larger than but of similar magnitude as the diffraction limit (Fig. 1). This explains why in the past it has been difficult to image details of the internal structures of living bacteria and, perhaps, why they have been considered to be “primitive” organisms with little internal structure. A more precise definition of Abbe’s limit can be based on the point-spread function (PSF) of fluorescence microscopy. That is, when fluorescence is emitted from a point source, due to Abbe’s diffraction limit it will be spread out in space and appear as a near-Gaussian function in the image plane of a microscope. The Fourier transform of a point source (Dirac functional) is uniform throughout the whole frequency spectrum, while the Fourier transform of the PSF decreases with increasing frequency and becomes zero at a spatial frequency,  $1/\Delta_{\text{min}}$ , in the x- and y-directions (Fig. 2).

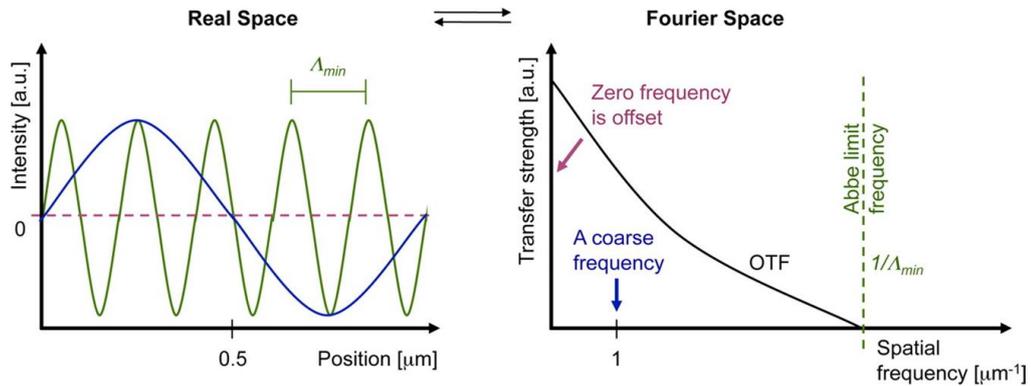


Figure 2: (Schermelleh *et al.*, 2010, *JCB* vol. 190 no. 2 165-175). Left: high (green) and low (blue) spatial frequency wave. Right: corresponding Fourier transform of the PSF (the optical transfer function OTF) of the low (1) and high ( $1/\Delta_{\text{min}}$ ) frequency wave.

Since the Fourier transform of the image of any object is the Fourier transform of the object itself multiplied by the Fourier transform of the PSF of the microscope, the length  $\Delta_{\text{min}}$  defines the limit of structural resolution of the microscope (Schermelleh *et al.*, 2010). Using this parameter to objectively define Abbe’s limit, it is in the lateral plane related to fundamental microscopy parameters as:

$$(\delta x_{\text{min}}, \delta y_{\text{min}}) = \Delta_{\text{min}} = \frac{\lambda}{2n \sin \alpha}, \quad (1)$$

In standard light microscopy the, normally worse, resolution in the axial (z-) direction is often approximated by the expression (Born and Wolf, 2002):

$$\delta z_{\text{min}} \approx \frac{\lambda}{2n (\sin \alpha)^2} \quad (2)$$

Here,  $\alpha$  is half the aperture angle under which the point source is observed and  $n$  is the index of refraction of the microscopy medium. Eq. 1 quantifies Abbe's limit in the lateral plane and huge efforts have been made to optimize its parameters.

## 2. Early wrestling with the diffraction limit of optical microscopy

Early approaches to Abbe's limit are conveniently categorized as far-field and near-field methods.

**2.1 Far-field.** Resolution improvements have been made with confocal (Cremer and Cremer, 1978; Sheppard and Wilson, 1981; Brakenhoff *et al.*, 1985) and multiphoton microscopy (Zipfel *et al.*, 2003; Hell and Stelzer, 2002). Both these methods allow for effective background suppression and have been important also for the establishment of single-molecule spectroscopy (*vide infra*). Considerable improvement of the notoriously bad resolution in the axial direction has been achieved by 4Pi (Hell and Stelzer, 2002; Hell, 2003) and I<sup>5</sup>M (Gustafsson *et al.*, 1995) microscopy, where two objective lenses are used to create an almost spherical focal spot in 3D. Another method, Structured Illumination Microscopy (SIM) commonly uses the interference between two beams to create a sinusoidal pattern in the exciting light (Heintzmann and Cremer, 1999; Gustafsson, 2000). When such a sinusoidal wave interferes with the studied object, light patterns arise in which spatial details below the diffraction limit become visible (Moiré patterns), allowing for an improvement of Abbe's limit confined to a factor of two as long as the emitted fluorescence intensity is a linear function of the intensity of the exciting light. All these far-field approaches may yield an improved resolution, by a factor of two, in relation to the Abbe limit in the lateral plane, and may render a substantial improvement in the axial direction by effectively increasing the aperture angle of the instrument with the help of two objective lenses. Although stretching Abbe's limit of resolution, these techniques remain confined by its prescriptions.

**2.2 Near-field.** Total Internal Reflection Fluorescence (TIRF) is an extensively exploited phenomenon in fluorescence spectroscopy and microscopy (Ambrose, 1956; Axelrod, 1981). Light that is totally reflected at highly inclined angles relative to a glass-medium interface creates an evanescent wave, which penetrates about 100 nm into the medium and can be used to excite fluorescent molecules in the very small volume next to the interface. By this microscopy method the axial resolution is greatly improved in relation to that of standard microscopy but its applicability is strictly confined to near-surface studies, while the interior of whole cells is out of reach.

Near-Field Scanning Optical Microscopy (NSOM, SNOM) is a technique that, in principle, can transcend Abbe's limit indefinitely. It operates without an objective lens and illuminates a studied surface at very close range with light emanating through a narrow aperture, e.g. at the end of a conically shaped glass fiber. By this method the evanescent wave has been limited both laterally and axially to within 20 nm and thus transcended Abbe's limit by an order of magnitude in all dimensions (Betzig and Trautmann, 1992). In a technical *tour de force* the method has successfully been used to detect single fluorophores, making single-molecule microscopy possible (Betzig *et al.*, 1993). In spite of these successes, the implementation of this

technique remains technically cumbersome and its applications have remained limited to surface studies. The scientific and technical breakthrough in super-resolved fluorescence microscopy has instead come in the form of far-field excitation techniques of two main types, as will be described in the next section.

### 3. Discovery of super-resolved fluorescence microscopy

To date there are two far-field principles that lead to fluorescence-based microscopy with a resolution far beyond Abbe's famous limit. The first is here referred to as "super-resolved *ensemble* fluorophore microscopy" and the second as "super-resolved *single* fluorophore microscopy".

The first principle was originally conceived as and implemented by stimulated emission depletion (STED) of fluorescence from all molecules in a sample except those in a small region of the studied object. With saturating stimulated emission, the "active" region can be made arbitrarily smaller than the diffraction-limited size. By scanning the light spot defining the fluorescing region across the studied object and monitoring the fluorescence emission continuously a computer reconstruction of the object can be obtained (Hell, 2000). The principle can also be implemented by Saturated Structural Illumination Microscopy (SSIM) (Gustafsson, 2005). These methods can be used when the fluorescing regions contain ensembles of fluorophores as well as single fluorophores and are therefore generically referred to as "Super-resolved *ensemble* fluorophore microscopy". Their common denominator is that saturation of the excitation peaks in SSIM and saturation of the stimulated emission in STED both create spatial frequencies much higher than those allowed by Abbe's limit (Fig. 2). Their discovery had two distinct phases. The first relates to the theoretical descriptions of the methods and the second to their experimental implementation.

The second principle is based on the *a priori* knowledge that virtually all photons from an object detected at a given time come from single fluorophores that are separated from each other by distances larger than Abbe's limit (Eq. 1). This information is then used to estimate the position of these emitting point sources with a precision that is much higher than that allowed by Abbe's diffraction limit. The second principle is here generically referred to as "Super-resolved single fluorophore microscopy". Its discovery may be described as three distinct steps. The first relates to the discovery of single fluorophore spectroscopy in dense media, the second to the theoretical description of the principle and the third to its experimental implementation.

#### 3.1 Super-resolved ensemble fluorophore microscopy: STED and its progeny

In the beginning of the 1990s, Stefan Hell moved as a post-doc from Germany to the University of Turku in Finland to find space and a possibility to develop his, at the time, controversial idea that it was not only possible but also feasible to transcend Abbe's diffraction limit in far-field light microscopy. In two theoretical papers he demonstrated the principles and outlined, in quantitative terms, the experimental conditions for the ground-breaking novel concept of Stimulated Emission Depletion (STED) microscopy (Hell and Wichmann, 1994) and similar (Hell and Kroug, 1995) techniques. Back in Germany he was eventually able to assemble a sufficiently sophisticated fluorescence microscope to provide the experimental proof-of-principle of STED microscopy (Klar *et al.*, 2000). In STED, two laser beams are used. A low-

intensity laser beam irradiates the fluorophores that define the structure of the studied object. This beam displays a broad focal region as determined by Abbe's diffraction limit. Another, high-intensity STED beam, red-shifted in relation to the first beam, has a zero intensity minimum in the focal region and its intensity grows in all directions from the focus. It rapidly brings fluorophores that have been excited by the first beam, from the vibrational ground state of the first excited singlet state,  $S_1$ , down to a high vibrational energy state of the electronic ground state from which they rapidly move to the vibrational ground state (Fig. 3).

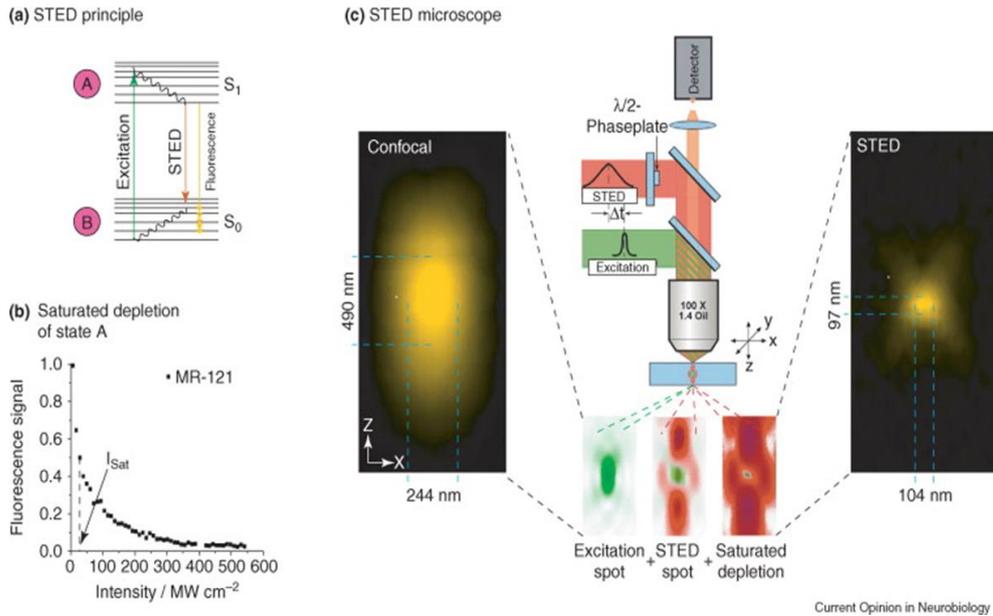


Figure 3: (Hell *et al.*, *Current Opinion in Neurobiology* 2004, 14:599–609). (a) Excitation from the electronic ground state  $S_0$  to  $S_1$  with green light and return to  $S_0$  with the emission of yellow light or by STED with yellow light. (b) Depletion of  $S_1$  with STED. (c) Left: yellow fluorescence density in z- and x-directions in focal plane without STED. Middle: description of STED microscope (top) and (bottom) green excitation without STED, red low-intensity STED and green excitation spot and red high-intensity STED and green excitation spot. Right: yellow fluorescence spot with high intensity STED.

By this arrangement, in conjunction with optimal pulse sequences for the excitation and STED beams, light emission is turned off everywhere except in a small part of the diffraction-limited focal region. The latter region shrinks indefinitely with increasing intensity of the maximal value,  $I_0$ , of the STED beam. The width,  $\Delta_{\min}$ , of the effectively fluorescing region is in the lateral plane approximated by (Hell *et al.*, 2004)

$$\Delta_{\min} \approx \frac{\lambda}{2n \sin \alpha (\sqrt{1 + I_0 / I_{sat}})} \quad (3)$$

It follows from this expression that the resolution limit due to Abbe's criterion has been removed: the resolution measure  $\Delta_{\min}$  approaches zero as  $I_0$  grows indefinitely. Indeed, the illuminated region will approach a Dirac functional with  $I_0$  increasing beyond limit (Fig. 4).

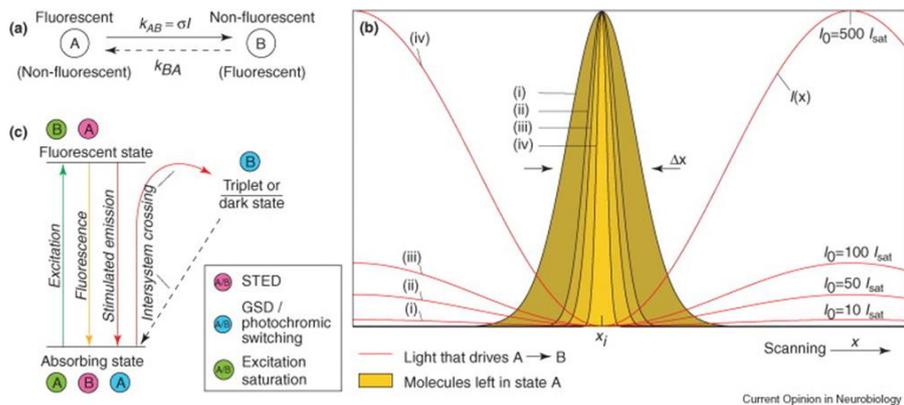


Figure 4: (Hell *et al.*, *Current Opinion in Neurobiology* 2004, 14:599–609). (a) Illustration of the fluorescence depletion principle. (b) Fluorescence density (yellow) as a function of the x-coordinate in the focal plane at STED intensities ( $I(x)$ , red) with different maximal strengths ( $I_0$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

Using STED, the normally diffraction-limited focal spot can be made infinitely small. It is clear that there may be other types of problems, such as photo-damage to biological tissues, with highly intense STED beams. It is important, however, that these obstacles are not caused by a hard physical limit and may therefore be successively removed, e.g. by the introduction of other ground state depletion mechanisms than stimulated emission, which do not require such high intensity (Hell and Kroug, 1995). The outcome of Hell's first experimental demonstration of the STED principle (Klar *et al.*, 2000) is illustrated in Fig. 5, showing how the notorious poor axial resolution is improved from about 500 to 100 nm.

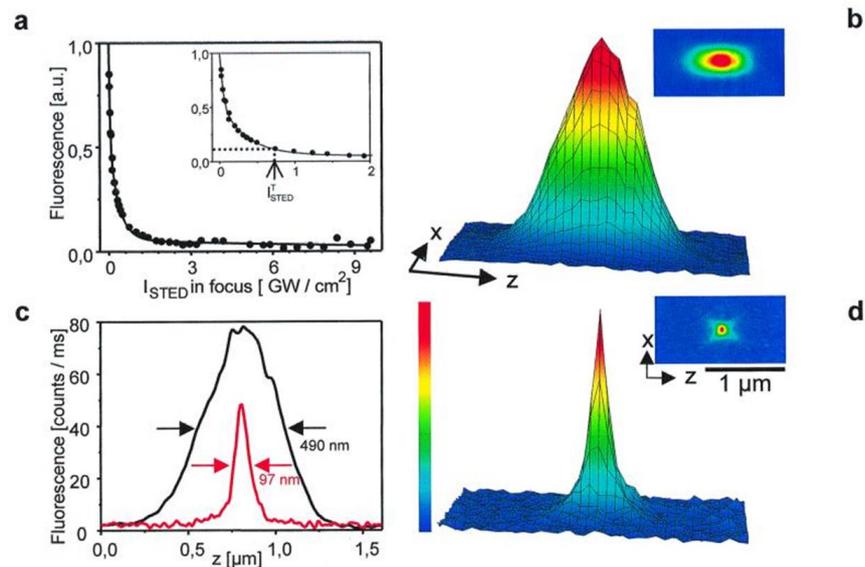


Figure 5: (Klar *et al.*, 2000, *PNAS*, 97, 8206-8210). (a) Nonlinear decrease in fluorescence intensity with increasing STED intensity,  $I_{\text{STED}}$ . Illustration of fluorescence intensity spot in  $x$ - and  $z$ -directions in the absence (b) and presence (d) of STED. Measured fluorescence distribution in the  $z$ -direction in the absence (black) and presence (red) of STED.

Five years later M. Gustafsson demonstrated super-resolution with SSIM techniques (Gustafsson, 2005). His work was prematurely terminated by his tragic passing in 2011.

### 3.2 The arduous road to single-fluorophore detection in dense media

Although the existence of molecules and their stochastic modes of action had long been recognized (Einstein 1905), the view that chemical reactions are parts of a dynamic continuum obeying deterministic laws has been remarkably persistent.

This illusion was shattered by the experimental demonstration of fluctuations around the equilibrium state of a DNA-bound fluorescent dye (ethidium bromide). W. W. Webb, with his collaborators E. Elson and D. Magde, developed the technique of fluorescence correlation spectroscopy (FCS) and could use the fluctuation-dissipation theorem to estimate the rate constants that govern the kinetics of the interaction between the fluorescent dye and double-helical DNA under ambient conditions (Magde *et al.*, 1972; 1974; Elson and Magde, 1974). Around the same time, R. Rigler and collaborators suggested the use of fluorescence intensity fluctuations to gain knowledge about rotational molecular motions and fluorescence life times (Ehrenberg and Rigler, 1974).

A few years later, T. Hirschfeld used polyethyleneimine to tag antibodies ( $\gamma$  globulin) with a large number of fluorophores (80-100 per protein molecule) (Hirschfeld, 1976). He then used TIRF microscopy at ambient temperature to observe the diffusion as well as the mechanically driven movement of the multi-labeled antibodies through a small volume illuminated by an Ar laser. These results, obtained with near-field excitation and far-field detection microscopy,

defined the beginning of the long and arduous road to reach single fluorophore detection in dense media.

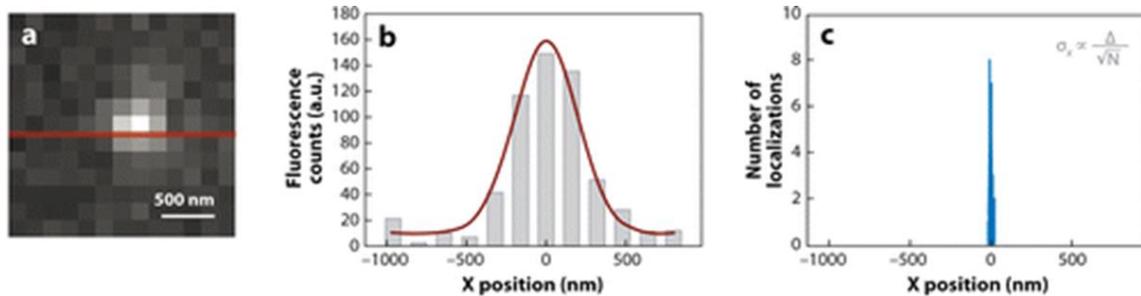
The first observation of a single fluorophore in a dense medium was made in W. E. Moerner's laboratory (Moerner and Kador, 1989). They measured absorption spectra of the statistical fine structures of inhomogeneously broadened optical transitions of single pentacene molecules in *p*-terphenyl crystals at liquid helium temperature (4 K). Moerner's result was revolutionary, not the least because he managed to detect single fluorophores by their photon absorption rather than emission. His results demonstrated that it was indeed possible to measure single fluorophores and thereby greatly inspired the single-molecule field. The following year, M. Orrit used the very same experimental system as Moerner to measure the fluorescence of single fluorophores. As expected, the signal-to-noise ratio was greatly increased in relation to the absorption-based observations (Orrit and Bernard, 1990). The same year, R.A. Keller and R. Rigler with collaborators detected single fluorophores in liquid medium at room temperature (Shera *et al.*, 1990; Rigler and Widengren, 1990). To suppress background radiation, Rigler used confocal microscopy and Keller used pulsed excitation in conjunction with delayed photon detection. In 1993, E. Betzig used scanning near-field microscopy to study single fluorophores on an air-dried surface with super resolution (Betzig and Chichester, 1993). In 1994, R. A. Keller and collaborators (Ambrose *et al.*, 1994) as well as S. Xie and collaborators (Xie and Dunn, 1994) measured excited state life times of single fluorophores and in an influential study the same year S. Nie, D. Chiu and R. Zare demonstrated detection of single fluorophore diffusion with confocal microscopy (Nie *et al.*, 1994). In 1995, Yanagida documented detection of single, fluorescently labeled ATP molecules interacting with single myosin molecules with a TIRF microscope; another landmark in single-molecule spectroscopy (Funatsu *et al.*, 1995).

In summary, these scientific results paved the way for both single-molecule spectroscopy and single-molecule microscopy. A very early discovery of great conceptual impact was the experimental demonstration of fluctuations in chemical reactions, and the application of the fluctuation-dissipation connection to determine reaction rate constants as carried out by Magde, Elson and Webb. Another very early result, anticipating what was to come within the next two decades, was Hirschfeld's demonstration with TIRF illumination and far-field detection of the movement of single antibody molecules with multiple fluorescence labels. A decisive breakthrough was the first detection of single fluorophores, carried out by Moerner under cryo-temperature conditions. This step, showing that the apparently impossible had become possible, paved the way for a host of single-molecule techniques in spectroscopy and microscopy. It inspired fluorescence detection of single fluorophores at cryo-temperature in Orrit's laboratory and became a reference point for single-fluorophore detection under the biologically important conditions of ambient temperature and liquid medium carried out in the laboratories of Keller, Rigler and Yanagida.

### **3.3 Super-resolved single-fluorophore microscopy: PALM, STORM, PAINT and their progeny**

Super-resolved single-fluorophore microscopy should here be understood as a class of techniques for which the super resolution is obtained by the possibility to "super-localize" a

point source of photons. As outlined in section 1 above, photons from a point source will hit the microscope detector with spatial probability density determined by their point spread function (PSF) (Fig. 6).




 Thompson MA, et al. 2012.  
 Annu. Rev. Biophys. 41:321–42

Figure 6: (a) Pixelated fluorescence intensity from single fluorophore in x- and y-directions of the detector. (b) Fluorescence intensity in x-direction (blue bars) fitted to a Normal distribution (red line, Eq. 4). (c). Probability density of the center ( $x=0$ ) of the Normal distribution in (b). The knowledge that there is a single emitter allows the position of the point source to be estimated much more precisely (c) than the width of the PSF (b).

Neglecting background and pixelation, the probability density,  $p(x,y)$ , of photon detection in the lateral plane is approximately Gaussian (Thompson *et al.*, 2012):

$$p(x,y) \approx Ce^{-\frac{(x-\mu_x)^2}{2\Delta^2} - \frac{(y-\mu_y)^2}{2\Delta^2}} \quad (4)$$

$C$  is a normalisation constant,  $\mu_x$  and  $\mu_y$  define the lateral center of the PSF and  $\Delta$  is equal to Abbe's diffraction limit in Eq. 1 above. From the knowledge that the photon distribution on the detector stems from a point source, the center of the point spread function can be estimated with a standard error,  $\Delta_{\min}$ , in both the x and y coordinates, given by (Bobroff, 1996; Webb, 2002):

$$\Delta_{\min} = \frac{\Delta}{\sqrt{N}} = \frac{1}{\sqrt{N}} \frac{\lambda}{2n \sin \alpha} \quad (5)$$

$N$  is the total number of photons registered by the detector, and it is seen that the spatial resolution of the microscope, taken as its ability to localize a point source,  $\Delta_{\min}$ , is improved from Abbe's original limit by the factor  $1/\sqrt{N}$ . This means that with 100 detected photons from the single point source  $\Delta_{\min}$  is a factor of 10 smaller than Abbe's limit and with increasing numbers of detected photons there is no strict limitation to how much the resolution can be improved. For this, the structure of interest must be so sparsely labeled with fluorophores that their separation is larger than the diffraction limit. This condition, however, leads to yet another problem. According to the sampling theorems by H. Nyquist (1928) and C. E. Shannon (1948) a spatial resolution,  $\Delta_{\min}$ , in the reconstruction of a structure requires that the structure is

uniformly sampled with a spatial frequency higher than  $2/\Delta_{\min}$ . This condition, however, is at odds with the condition that the nearest neighbor distance between labels is larger than Abbe's diffraction limit. In fact, what is required is the dichotomy of a very sparse distribution of fluorescence labels to save the assumption that they are all separable point sources in conjunction with a very dense distribution to fulfill the sampling theorems of Nyquist and Shannon. A conceptual solution to this logical dilemma was first provided by Eric Betzig.

Despite the success of Betzig's work on near-field fluorescence microscopy (e.g. Betzig *et al.*, 1992; 1993) it was becoming increasingly clear that the confinement to surface studies and the cumbersome implementation limited the use of these methods. Betzig decided to leave academic science, but continued to think about super-resolved fluorescence microscopy. He eventually perceived a new way to achieve super-resolution without the shortcomings of near-field microscopy (Betzig, 1995). The solution, he suggested, was to determine the positions of a large number of point sources with distinguishable spectral properties in two steps. In a first step the PSF of each spectral class was determined separately. Making sure that each spectral class formed a sparse set in space, it would be possible to determine the positions of their members with super resolution by estimating the centers of their PSFs with the precision of Eq. 5. By considering the positions of all classes together, a super-resolution image of a densely sampled structure could be obtained. A particular implementation of this method was subsequently used in experiments by G.J. Brakenhoff and collaborators (van Oijen *et al.*, 1998), but an optimal way to experimentally realize Betzig's vision was still lacking.

Two years after Betzig's proposal, Moerner was studying the light emission properties of single molecules of mutants of the green fluorescent protein (GFP) obtained from Roger Tsien's laboratory. The studies (Dickson *et al.*, 1997) were performed at ambient temperature with the protein embedded in an aerated aqueous gel. Unexpectedly, when excited at 488 nm the protein was seen to undergo several cycles of intermittent fluorescence emission: the GFP molecules displayed "blinking" behaviour. After several blinking cycles, the molecules went into a stable dark state. Amazingly, from this dark state they could be re-activated by irradiation at 405 nm. These results demonstrated for the first time that it was possible to optically guide fluorescent proteins between active and inactive states by the sophisticated use of the inherent photochemistry of the protein (Fig. 7).

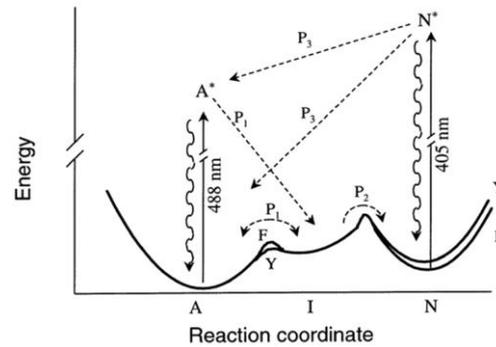


Figure 7: (Dickson *et al.*, 1997, *Nature* 388, 355-358). GFP in state A is excited to A\* and returns to A upon photon emission. When I is reached from A, there is no fluorescence until I spontaneously moves to A: blinking. When I moves to N, there is no fluorescence until N is activated to N\* by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

Moerner's demonstration of blinking and photo activation opened the road to exploring a vast space of GFP mutants for novel optical properties. J. Lippincott-Schwartz engineered a GFP variant with striking properties (Patterson and Lippincott-Schwartz, 2002). This mutant is initially optically inactive. It can, however, be activated by irradiation at 413 nm and then displays fluorescence when excited at 488 nm. Eventually, after intense irradiation at 488 nm the mutant is irreversibly inactivated by photo bleaching.

When Betzig returned to academic science after his post-near-field exile in private industry, he learnt about Lippincott-Schwartz' mutant and realized that it could possibly solve the problem of finding an optimal way to combine sparse sets of fluorophores with distinct spectral properties to a dense total set of fluorophores. The simple solution would be to activate a very small and thus sparse, random subset of GFP mutant molecules in a biological structure by low-level irradiation at 413 nm. Subsequent irradiation at 488 nm would then be used to determine the positions of the members of the sparse subset at super-resolution, according to Eq. 5 above. When the first subset had been irreversibly inactivated by bleaching, a second small subset could be activated and the positions of its members determined at high resolution, and so on until all subsets had been sampled and used to determine the structure under authentic super-resolution conditions. This fulfilled both the condition of only a sparse subset being observed at a time, and the condition of high-frequency (dense) spatial sampling in order to fulfill the Nyquist and Shannon theorems, as illustrated in Fig. 8.

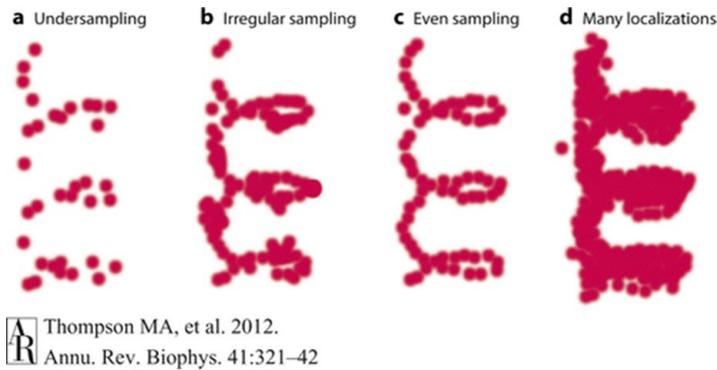


Figure 8: (a) Low frequency spatial sampling, (b) Biased sampling, (c) Uniform sampling, (d) Oversampling.

In collaboration with Lippincott-Schwarz and H. F. Hess, Betzig (Betzig *et al.*, 2006) expressed fusions of a photoactivatable GFP (PA-GFP Kaede), similar to that described above (Patterson and Lippincott-Schwartz, 2002) and a lysosomal transmembrane protein (CD63) to obtain the super-resolved structure of a thin section of a fixed mammalian lysosome (Fig. 9).

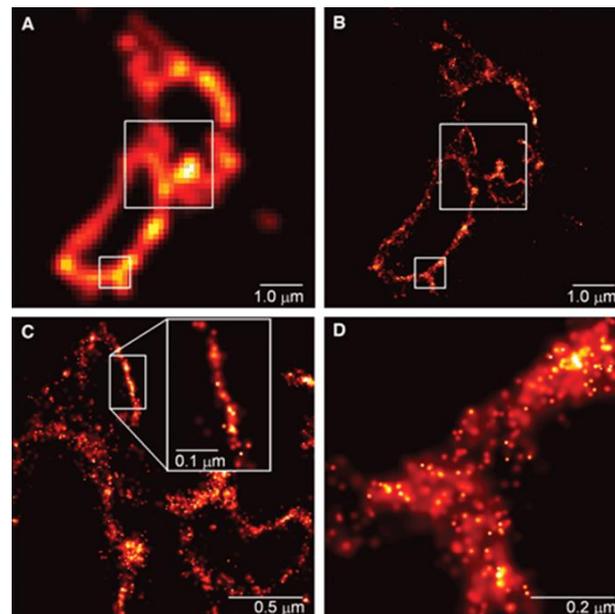


Figure 9: (Betzig *et al.*, *Science*, 2006, 313, 1642-1645). Distribution of GFP-labeled lysosome protein (CD63) without (A) and with (B) PALM. Large box in B at higher magnification (C). Small box in B at higher magnification (D). Note that the scale bar in D is close to Abbe's limit.

They called their method PALM (Photoactivated Localization Microscopy). Later the same year X. Zhuang and collaborators (Rust *et al.*, 2006) and S. T. Hess and collaborators (Hess, 2006) published super-resolved fluorescence microscopy studies based on similar principles and named them STORM (Stochastic Optical Reconstruction Microscopy), and fPALM (fluorescence-PALM), respectively. In addition, R. M. Hochstrasser's group published yet

another strategy to achieve super-resolution (Sharonov and Hochstrasser, 2006). It takes advantage of changes in fluorescence parameters (shift in emission spectrum) of a fluorescence label (Nile Red) as induced upon its binding to an intracellular target (hydrophobic membrane). The method was named PAINT (Points Accumulation for Imaging in Nanoscale Topography). Since then many schemes to control active fluorophore concentrations have been developed (Thompson *et al.*, 2012).

#### 4. After the discovery

The history of super-resolved fluorescence microscopy is short. The ensemble-fluorophore STED-microscopy was implemented in the year 2000 and single-fluorophore based methods in the year 2006. In spite of this, the rapidly developing techniques (e.g. Sahl and Moerner, 2013) of super-resolved fluorescence microscopy are already applied on a large scale in major fields of the biological sciences, like cell biology, microbiology and neurobiology (e.g. Huang *et al.*, 2010). At this point there is all reason to forecast that this development, already producing hosts of novel and previously unreachable results, will accelerate over the next decades. This development is expected to revolutionize biology and medicine by, not the least, eventually allowing for realistic, quantitative descriptions at nano-scale resolution of the dynamics of the complex, multidimensional molecular biological processes that define the phenotypes of all life forms.

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