

## Achieving Resolution Beyond the Diffraction Barrier

Fluorescence microscopy plays a vital role in the life sciences and the reasons for this are many-fold. The characteristic fluorescence emission wavelength (color) is a perfect tool for specifically and selectively marking and imaging parts of a sample, like for example proteins in a cell. Fluorescence labeling also plays an important role in quantitative studies of the reaction dynamics of macromolecular complexes in solution. It is therefore not surprising that approximately 80 % of all microscopy investigations in biology are based on fluorescence.

It is exactly the interaction between light and fluorescence marker that the researchers from Göttingen use to overcome the diffraction barrier. In regular fluorescence microscopy, the marker molecules are simply excited to a higher energetic state and upon relaxation to the ground state, fluorescence light is emitted. Since the exciting light is subject to diffraction, the smallest spot of fluorescing molecules cannot be smaller than  $\Delta d$  – unless one uses a new physical trick!

This trick consists of transiently transferring the fluorescence marker to a non-fluorescent state with a second focal light whose intensity distribution  $I$  is not homogeneous, but displays a zero at least at one point. The remaining fluorescence signal thus originates almost exclusively from the zero or its immediate surroundings. Moreover, the larger the intensity  $I$ , the smaller is the area around the zero where the fluorescence is possible. As the intensity increases to infinity, the spot would decrease in size to a point. It is obvious that with an intensity that strongly saturates the transition to the non-fluorescent state, the fluorescence spot can be narrowed down without limit. If the transfer to that state is only transient and fully reversible, an accurate image is obtainable by scanning the sample with the sharp spot. The fluorescence registered from the object stems only from this sharp spot and the sharply localized fluorescence emanating from it defines an image data point. These image points are assembled and displayed on a computer.

This general concept can be envisioned in several variations, since transferring a fluorescent molecule almost completely to a non-fluorescent state is best achieved with optically saturated transitions which are involved in the fluorescence. As already mentioned, these transitions need to be reversible to ensure that the marker resorts to its original state. With this new concept, termed RESOLFT (*reversible saturable optical fluorescent transitions*) there is no longer a general resolution limit. If the zero position is created through the objective – which is normally the case - it follows that the size of the fluorescent spot is given by the following law:

$$\Delta d = \frac{\lambda}{2n \sin \alpha \sqrt{1 + I/I_{sat}}}$$

Here  $I_{sat}$  is the characteristic saturation intensity, i.e. a sort of threshold where the fluorescence of a given molecule is with a certain probability (~ 65%) transferred to the non-fluorescent state.  $I_{sat}$  is characteristic for the dye or marker used. Upon increasing the  $I/I_{sat}$

value, the resolution is continually improved. Without the transition (i.e.  $I = 0$ ), we obtain the classical Abbe resolution limit. But now, in contrast, there is no lower limit for  $\Delta d$ , since  $\Delta d \rightarrow 0$  for infinitely large values of  $I/I_{sat}$ . It is therefore clear that also with normal objectives and focused light, it is in principle possible to obtain images that possess molecular resolution.

The first practical example of the RESOLFT concept is STED microscopy (*stimulated emission depletion*) which will be explained in further detail below.

## STED-Microscopy

One can not only excite a molecule, but also abruptly transfer a molecule with light to the ground state (Fig. 2a). In this process, referred to as through stimulated emission and predicted by Einstein in 1917, a large part of the energy of the excited molecule is carried away in the stimulating light beam as a further photon (which is not of importance here). The relaxed molecules can be immediately excited again and further quenched again; the process is therefore reversible and transient. Important for the breaking of the resolution limit is not the quenching as such but more the saturation of the quenching. When a certain threshold of the stimulating light intensity is exceeded (saturation intensity), the fluorescence is negligibly small and the relaxation is almost complete (Fig.2b). This is the case for the measurements shown in Fig.2 at intensities above 1 Gigawatt/cm<sup>2</sup>.

In a typical STED microscope, the exciting and the quenching (depleting) light is simultaneously coupled into the microscope objective (Fig. 3a). Whereas the exciting light (green) has a spot diameter  $>200\text{nm}$  as expected, the depletion light (red) is modified so as to produce a ring at the periphery of the exciting spot and to have a zero in the middle (red ring in Fig.3b). The fluorescence from the periphery of the diffraction limited excitation spot is prevented by the depleting pulse whereas it still exists in the zero and in its immediate neighborhood.

In principle, for the depletion, one wants the narrowest possible ring i.e. a very narrow local intensity minimum with a zero. However the production of the central minimum is also governed by Abbe's law of diffraction, therefore a minimum with a diameter smaller than  $\Delta d$  is not feasible. While the depletion of the molecule is oversaturated, the region where fluorescence is still possible is continuously narrowed down with increasing intensity (Fig.3b). If such a narrow spot were now used to scan across the probe, the sequentially registered fluorescence light (stemming from a much narrower spot than before) would provide a sharper image than is possible with a microscope which uses a diffraction limited and therefore larger spot. Most recent experiments with single molecules as measurement probes show that the size of the spot does indeed follow the new law.

The STED microscope possesses in principle almost all of the advantages of a focusing fluorescence microscope. It is basically able to provide 3D images and works under usual light microscopy conditions. Among the many uses is in research on nanoscale dimension components of a cell as well as with artificial nano-structured materials. An example of the wide range of applications is shown in Fig.4. Both of the top pictures

demonstrate the increase in resolution when imaging fluorescence dye marked pores. The normal mode (the so-called confocal mode) cannot resolve the rings as demonstrated by the STED counterpart.

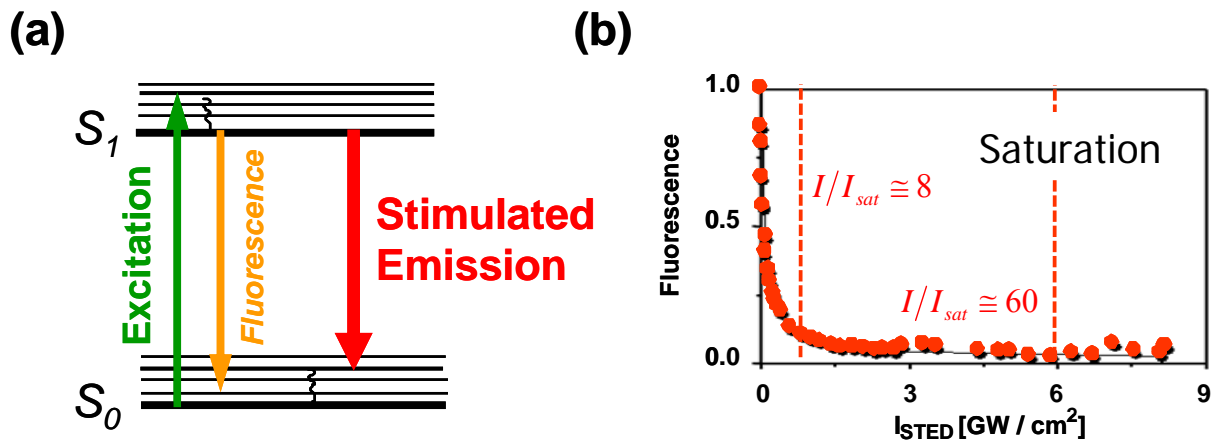
The lower two pictures show the increase in resolution of a lithographic nanostructure of the size that is normally used in the manufacturing of computer chips. The photoresist line structure was written with an electron beam in collaboration with the Institute for X-ray Physics at the University of Göttingen. Even structures that are <80nm apart can be easily discerned whereas the normal optical image shows no details. A mathematical image processing procedure (linear deconvolution) was used for both images to slightly enhance the resolution. However, this processing is not necessary in STED microscopy, only optional.

Since it is possible to further enhance the resolution through optimization of the physical imaging parameters, one can expect to obtain even sharper images with STED microscopy in the future. Very recently, it has been possible to demonstrate that under realistic experimental conditions, the spot size can be reduced up to  $\lambda/50$ , which in this case meant 16nm! The possibility of obtaining resolution of this dimension was until now only reserved for electron microscopy and scanning probe microscopy.

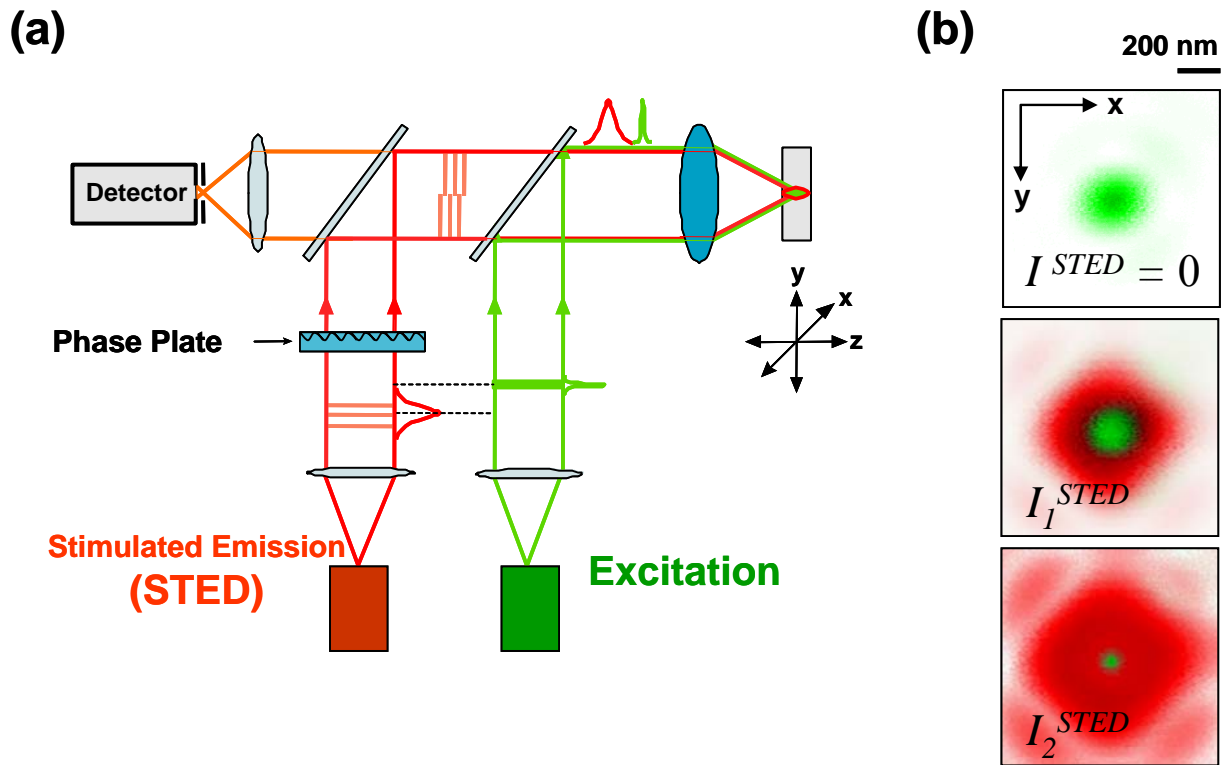
STED is however only one of the variations of the RESOLFT concept (even if the first and at present the best researched). Other very promising variations that saturatingly transfer the dye into a dark state like, for example, in a triplet state or in a dark conformational state show in calculations that a similar high potential exists for breaking the diffraction barrier. The same applies for variations where optically bistable molecules can be reversibly switched from a first to a second state. Even more, since their expected  $I_{sat}$  values are an order of magnitude smaller than that for STED, we can expect resolution of a few nanometers even if the intensity is lower by a factor of 4 to 8. Thus, a door for light microscopy and its applications has been opened which had been thought not possible just a few years ago.



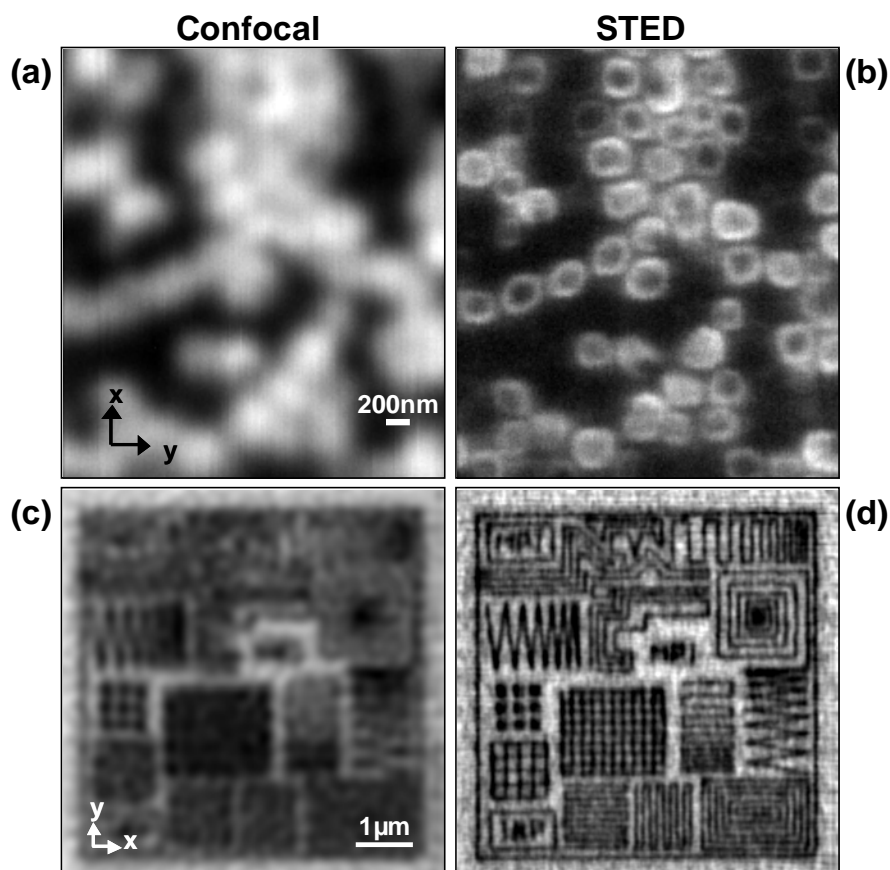
**Fig.1** The formula from Ernst Abbe (1840-1905) expressing the maximum possible resolution from a light microscope on a monument erected by the University of Jena in Abbe's memory.



**Fig.2** Fluorescence extinction by stimulated emission. (a) Energy schematic of a fluorescent molecule: Light at the appropriate wavelength can quench the excited fluorescent state  $S_1$  back to the ground state  $S_0$  via stimulated emission. (b) The fluorescence from  $S_1$  decreases exponentially with increasing intensity  $I_{STED}$  of the stimulating light. Beyond the first shown threshold, the relaxation is almost complete i.e. saturated, and the fluorescence almost completely extinguished (STED: *stimulated emission depletion*). Larger intensities of the stimulating light mean a larger degree of saturation  $I/I_{sat}$ .



**Fig.3** STED microscopy: Typical schematic of a STED microscope with excitation and depletion beams, phase plate, detector and objective. In the focal plane (x,y) of the objective, the excitation (green) pulse produces a disc of about 250nm diameter (b, right top) that is overlapped with the temporally synchronous depletion (red) pulse (b, right middle). The phase plate tailors the depletion pulse such that it is not a disc but a central zero. At low STED saturation, the depletion pulse is only effective at the outer edges of the excitation focus. With increasing intensity, only a smaller part is excluded from depletion through stimulated emission (b, right bottom), so that the focal part where fluorescence is still possible, i.e. the fluorescent spot, is squeezed well below the diffraction limit. Scanning a fluorescence marked object in x,y with this smaller spot delivers images with resolution well below the diffraction limit.



**Fig.4** Images with resolution at and beyond the diffraction limit shown in the left and right column, respectively. Upper row: Pores in a porous membrane marked with a fluorescent dye shown with normal resolution cannot be discerned as such (a). The same imaging carried out parallel with STED microscopy clearly brings out the structure to light (b). The term *confocal* indicates that the reference images (a,c) were recorded in the confocal microscopy imaging mode which currently is the best resolving diffraction limited fluorescence microscopy method. Lower row: Fluorescence dye marked nanostructures produced by electron beam lithography in a polymer shown with normal resolution (c) and then using STED (d). The raw data in (c) and (d) after imaging was subjected to linear deconvolution to mathematically slightly enhance the resolution. In spite of this, the image in (c) does not show the structures, whereas the images with the STED microscope can resolve lines of 80 nm width and 40nm separation between the lines (d). Thus, the optical imaging is moving into domains that were until now reserved for the electron microscope. Data was taken from [2] and [3].