SUBDIFFRACTION RESOLUTION IN FAR-FIELD FLUORESCENCE MICROSCOPY

LIMITE DE ABBE

La résolution des microscopes optiques est limitée par les effets de diffraction. On ne peut normalement pas observer de détails plus petits qu'à peu près un micromètre.

STEFAN HELL

Stefan Hell, physicien allemand, développe dans les années 90 un nouveau type de microscope, le STED. Il reçoit le prix Nobel de Chimie en 2014.

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Subdiffraction resolution in far-field fluorescence microscopy

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We overcame the resolution limit of scanning far-field fluorescence microscopy by disabling the fluorescence from the outer part of the focal spot. Whereas a near-UV pulse generates a diffraction-limited distribution of excited molecules, a spatially offset pulse quenches the excited molecules from the outer part of the focus through stimulated emission. This results in a subdiffraction-sized effective point-spread function. For a 1.4 aperture and a 388-nm excitation wavelength spatial resolution is increased from 150 ± 8 nm to 106 ± 8 nm with a single offset beam. Superior lateral resolution is demonstrated by separation of adjacent Pyridine 2 nanocrystals that are otherwise indiscernible. © 1999 Optical Society of America

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Abbe's work[1] on the role of diffraction greatly influenced the art of microscopy in this century and spurred the development of techniques such as electron and scanning probe microscopy in which the use of light was abandoned.[2] Scanning probe microscopy also triggered near-field optical microscopy,[3] in which a sharp tip is used to confine the interaction of a sample with light to a subdiffraction spot. In spite of their success these techniques have not replaced the far-field light microscope as the most widely applied microscope in biology. The reason is that, besides involving more-sophisticated specimen handling, electron and probe microscopes are confined mainly to the imaging of surfaces. To date, noninvasive imaging in three dimensions has been achieved only with focused light.[4] Fluorescence is particularly important, since it allows specific observation of many cellular compartments and proteins. Evidently, far-field fluorescence microscopy featuring resolution beyond the diffraction limit would be highly attractive.

In this Letter we report what we believe to be the first evidence of pronounced lateral resolution beyond the diffraction limit. This resolution is obtained by use of the concept of stimulated-emission depletion (STED).[5] In this concept the fluorescent molecules that are excited by the outer part of the focus are deprived of their ability to emit a fluorescence photon by exposure to a second beam that induces stimulated emission. The role of the stimulated emission is to force the molecules into the ground state immediately after they are excited. As a result the remaining fluorescence stems from a region that is narrower than the diffraction-limited excitation focus. This effect is equivalent to narrowing the effective point-spread function (E-PSF) of the microscope and to a model case of point-spread function engineering.[5]

Our experiment (Fig. 1) is based on a UV-confocal scanning fluorescence microscope that, owing to the short wavelength and confocality, already has a high classical resolution. By employing an oil-immersion lens of 1.4 numerical aperture (Leica 100× Planapo), we also use the largest aperture available. The images are obtained by scanning of the sample with a piezo stage. The excitation and the stimulating beams both originate from a mode-locked Ti:sapphire laser (Mira 900, Coherent) operating at 766 nm. For this purpose the laser is split into two beams. Whereas the first beam is frequency doubled to 383 nm and used for excitation, the fundamental beam is used for stimulated emission. We refer to them as the UV and the STED beams, respectively. Both beams are spatially filtered, expanded, and recombined at a dichroic mirror that is transmissive at 383 nm and reflective at 766 nm. We ensure that both beams have the same polarization by introducing a $\lambda/2$ plate into the STED beam. The beams illuminate the back aperture of the objective lens with planar wave fronts.

The UV pulses have a duration of ~200 fs, which is 3–10 times shorter than the vibrational relaxation and 4 orders of magnitude shorter than the fluorescence relaxation of an organic fluorophore that is adequately described by a four-level model.[6] Within 1 ps after excitation, the molecules relax to a low vibrational level of the excited state S_1, which is the actual fluorescent state. The STED pulses are chosen so that they efficiently quench this state by stimulated

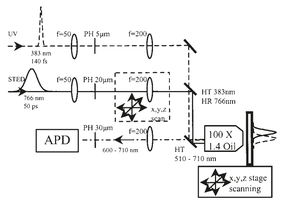

Fig. 1. Setup: frequency-doubled (UV) and fundamental (STED) beams from a Ti:sapphire laser are cleaned, passed through pinholes (PH's), expanded, and combined at a dichroic mirror (HT383/HR766). After the beams are reflected from mirror HT510–710, they overfill the rear aperture of the lens. The STED beam is shifted with respect to the UV beam by a piezo-movable tube lens. APD, avalanche photodiode.

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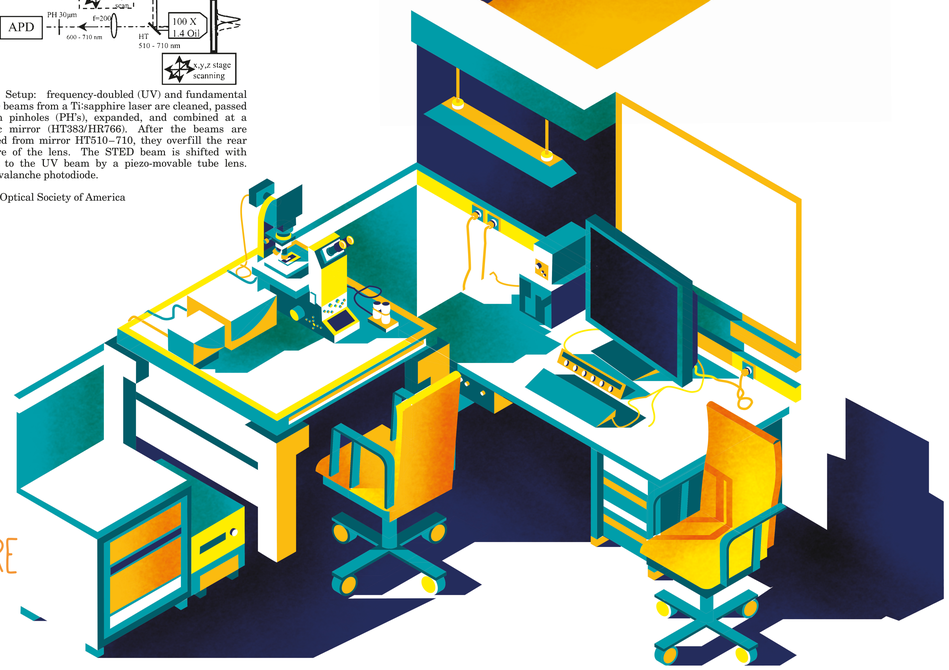
LE MICROSCOPE STED ET SES LASERS

LE LABORATOIRE



"A découvert", une autre façon de mettre en scène l'article scientifique.
réalisé par J. Sictonich (Ecole Estienne) en coll. avec l'équipe "La Physique Autrement"
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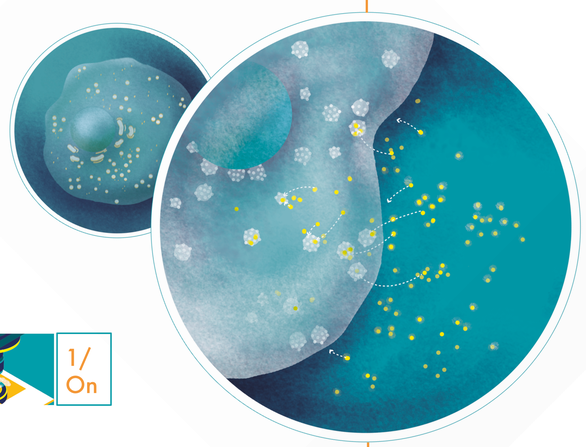
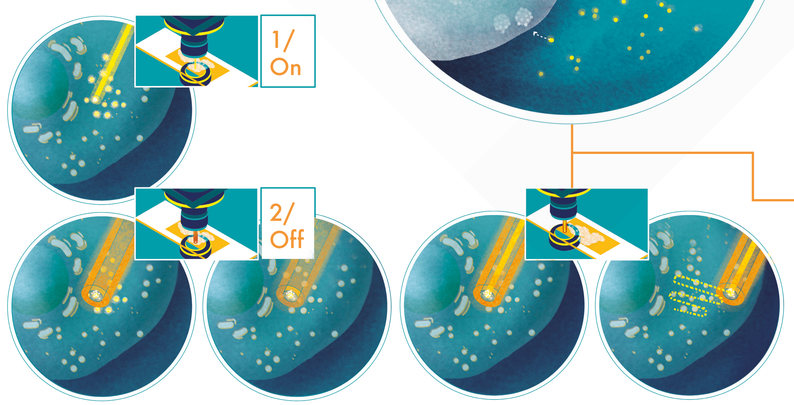
UN LASER EN FORME DE DONUTS

Dans le microscope, un laser UV fait fluorer les molécules. Un deuxième laser d'une autre couleur éteint une partie de ces molécules. En déplaçant ces lasers, on peut ainsi « allumer » une petite zone de l'échantillon.

FIXATION DES PROTÉINES DE LA FLUORESCENCE DANS LA CELLULE





BALAYAGE DU LASER STED

emission. First, to allow vibrational relaxation into the fluorescent state we use an optical delay that ensures that the STED pulses arrive at the focus a few picoseconds after the UV pulses. Second, as the excited molecules are quenched into a higher vibrational level of the ground state S_0, reexcitation by the STED pulse is avoided by stretching the STED pulses to ~54 ps, using a grating. This stretching allows the fast vibrational relaxation drain to dump the molecules into a low vibrational level of S_0.

The passband of the dichroic filter that couples the two beams into the objective is 510–710 nm, allowing the back-emitted fluorescence to be detected by a counting avalanche photodiode. The wavelengths of the UV and the STED pulses as well as the fluorescence are shown in Fig. 2(b). The power used is 2.0 μW for the UV beam and 28.3 mW for the STED beam. We note that the excitation intensity is well below saturation and that the STED intensity is in the benign energy region for biological imaging. Owing to the high magnification of the objective, we can precisely displace the STED beam with respect to the UV beam by scanning a tube lens on a piezo stage.

To investigate the resolution increase and the STED effect we prepared a sample consisting of randomly distributed nanocrystals of the fluorophore Pyridine 2, characterized in Figs. 2(a) and 2(b). We prepared the nanocrystals by diluting a saturated solution in ethanol by a factor of 10 and spreading a few drops on the cover glass. Nanocrystals formed during the evaporation of the ethanol. The nanocrystals were covered with a glycerol-based mounting medium [100-mM Tris-HCl (pH 8.5), 9% Mowiol 4-88 (Hoechst), 25% glycerol]. In addition, we prepared a thick, fluorescent layer by mixing the solution with the mounting medium in a 1:1 ratio. We then determined the efficiency of the stimulated-emission depletion by spatially and temporally overlapping the UV and the STED foci in the layer. Through interruption of the STED beam by a chopper [Fig. 2(c)] we found that the STED beam was able to reduce the total fluorescence to 3% of its maximum value. In a separate measurement (data not shown) we verified that for such strong depletion the relationship between the depletion efficiency and the STED-beam intensity is nonlinear. This relationship favors a sharp depletion edge at the outer part of the focus.

As we wanted to rule out that fluorescence reduction is caused by photobleaching or triplet-state quenching, we examined whether fluorescence can be immediately restored after depletion. We divided the UV pulse into two pulses, delayed the second pulse by ~6 ns, and measured the fluorescence by use of time-correlated single-photon counting. The solid curve in Fig. 2(d) shows the signal with only the two UV beams focused into the sample. The exponential decay time is (725 ± 15) ps, which corresponds to the lifetime of Pyridine 2. The dashed–dotted curve shows the dynamics when the STED pulse coincides with the first UV pulse. Although the fluorescence generated by the first UV pulse is quenched, the fluorescence from the second UV pulse remains unaffected, thus demonstrating that Pyridine 2 can be fully reexcited 6 ns after quenching. Fast recovery and nondestructiveness are strong evidence of cool depletion of the excited state by stimulated emission.[8,7]

Next we investigated whether a laterally offset STED beam would reduce the lateral extent of the E-PSF of the microscope. The E-PSF is measured with a subresolution nanocrystal. The resulting images, shown in Fig. 3, are 0.68 μm × 0.68 μm and consist of 72 × 72 pixels. The pixel dwell times were 2 and 4 ms with the STED beam switched off and on, respectively. In Figs. 3 and 4 a uniform background was subtracted and averaging of adjacent pixels was performed. With the STED beam switched off, the microscope featured a confocal E-PSF, as shown in Fig. 3(a). The FWHM along the profile is 150 ± 5 nm, which matches well the predicted value of 145 nm

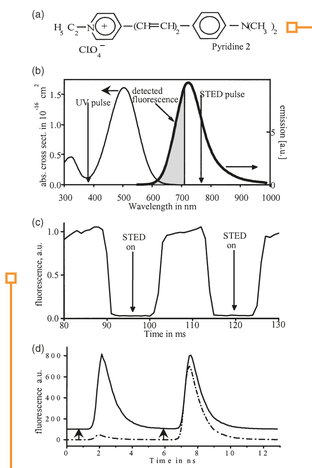
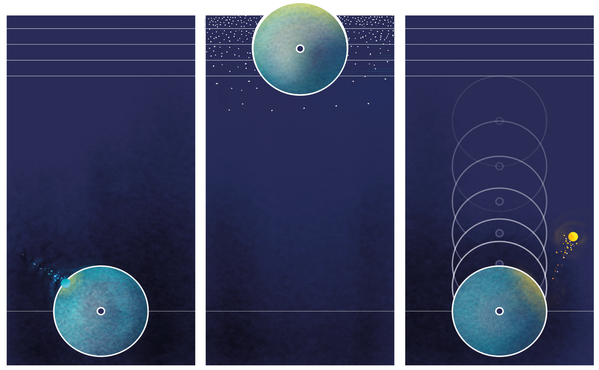


Fig. 2. (a) Chemical structure and (b) spectra of Pyridine 2 and (c) fluorescence with overlapping UV and STED pulses. Periodic interruption of the STED beam with a chopper leads to quenching and recovery. (d) Fluorescence dynamics on the nanosecond scale: Two UV pulses that are 6 ns apart produce two equal fluorescence decays (solid curve). A STED pulse superimposed upon the first UV pulse quenches the excited molecules, which are, however, fully reexcited 6 ns later by the second pulse. (The solid curve is shifted upward.)



PRÉAPARATION DES ÉCHANTILLONS

LE LABORATOIRE OÙ ON PRÉPARE LES ÉCHANTILLONS

LA FLUORESCENCE

Les particules fluorescentes sont excitées par de la lumière UV. Elles ré-émettent de la lumière verte quand elles se desexcitent.

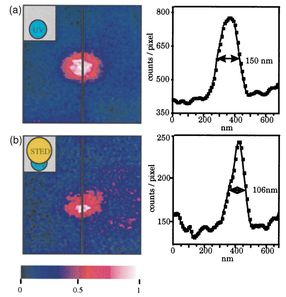


Fig. 3. Effective PSF's of (a) standard UV-confocal and (b) corresponding STED-confocal microscopes with the stimulating beam displaced in the y direction. The profiles on the right reveal a narrower PSF in (b) than in (a).

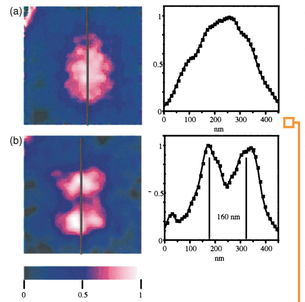


Fig. 4. Images of adjacent nanocrystals in (a) the standard UV-confocal and (b) the STED-confocal microscopes. Comparison of the images and the profiles reveals significantly improved spatial resolution in the far field.

for aberration-free focusing, also indicating that the observed crystal can be regarded as a nearly pointlike source. Figure 3(b) shows the E-PSF with the STED beam switched on. The E-PSF is laterally offset by 390 ± 10 nm in the y direction, as depicted in the inset of Fig. 3(b). As a result, in the y direction the FWHM is reduced to 106 nm; this corresponds to $\lambda/3.6$ and is clearly beyond the Abbe limit.

The measurement in Fig. 4 demonstrates that the sharper E-PSF results in better spatial separation of adjacent nanocrystals. In this case the offset between the UV and the STED beams was 270 ± 10 nm. Whereas Fig. 4(a) shows that the UV-confocal setup is not able to discern the crystals, Fig. 4(b) shows that the STED-confocal arrangement resolves the objects.

A single offset beam proved sufficient for substantial improvement of lateral resolution in one direction. Extending the STED area with a second beam in the x or the z direction or applying a doughnut-shaped beam is expected to improve the resolution in the other directions also. The large disparity in wavelength between the excitatory and the depleting pulses, which results from the use of a near-IR beam and its second harmonic, adversely affects the resolution improvement. Visible beams would involve weaker longitudinal chromatic aberrations and lead to more-efficient narrowing of the focus. Therefore we expect that employing visible light, for example, from an optical parametric oscillator, will make possible a greater relative improvement of the resolution.

In summary, by implementing the concept of STED microscopy, we have achieved lateral resolution beyond the diffraction limit. The technique is noninvasive, and the moderate intensities appear not to cause photodamage to the sample. The STED concept should be applicable to any organic molecule. In fact, this concept can be considered for any application in which the lower excited state of a four-level system is effective. Therefore future applications may well include subdiffraction resolution in pump–probe spectroscopy, three-dimensional photochemistry, and data storage.

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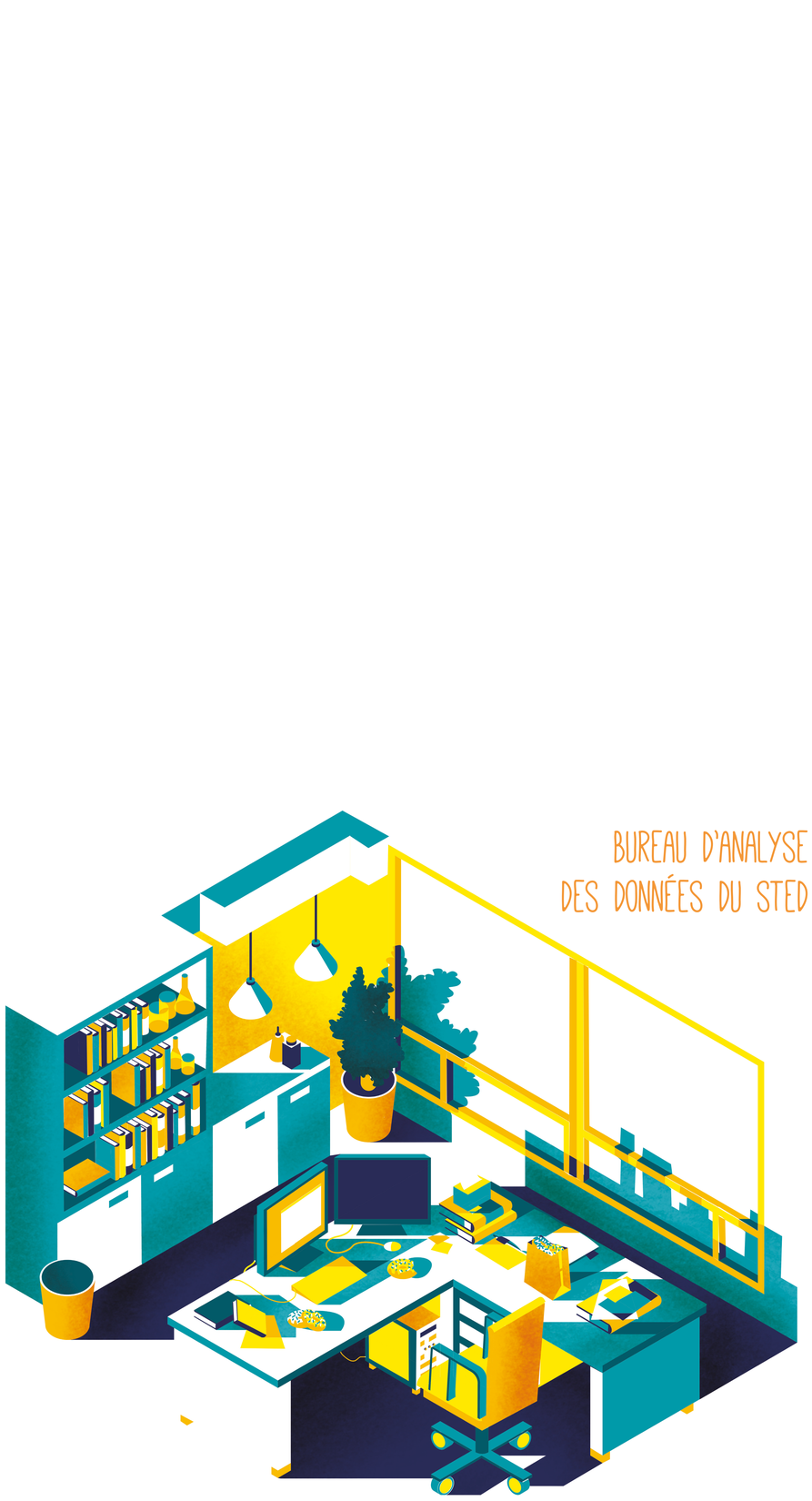
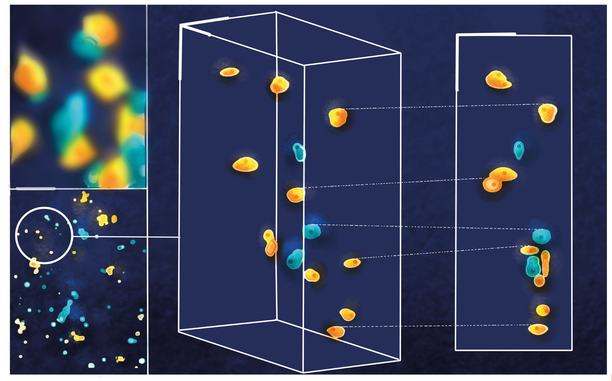
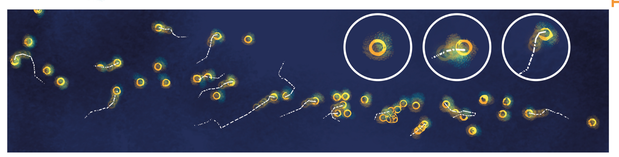


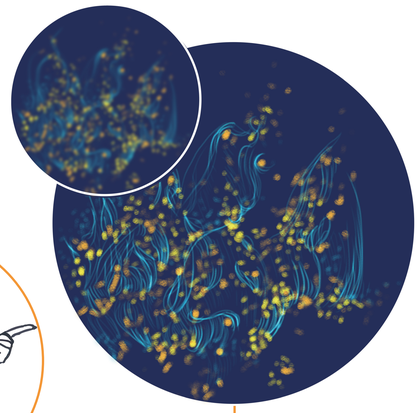
BUREAU D'ANALYSE DES DONNÉES DU STED

EN 3D & EN MOUVEMENT

Depuis cet article, les microscopes super-résolus ont fait beaucoup de progrès.
On peut maintenant faire des images en 3D ou suivre le mouvement d'objets biologiques en direct.







UNE SUPER-RÉSOLUTION

Ici, deux échantillons nanométriques peuvent être distingués seulement grâce au STED. Ce bien meilleur pouvoir de résolution permet d'observer d'infimes détails dans la matière.