STED microscopy for fluorescent nitrogen vacancy defects in diamond

Achieving 5.8nm resolution using far field optics

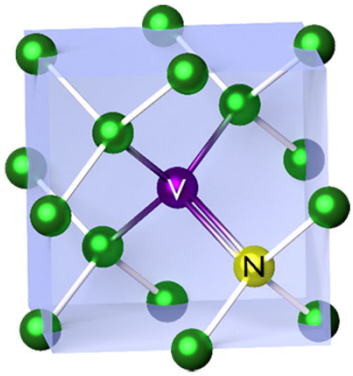
Clemens Wolter

Hello and welcome to my presentation about STED microscopy. We have already learned that STED can break the diffraction limit. Today I‘m going to present a paper that took STED microscopy and pushed the resolution to an extreme. The extreme in this is case is a resolution of 5.8nm which exceeds the wavelength of the used light by two orders of magnitude.

Outline

- What is a Nitrogen-Vacancy Defect?
- Fluorophore Properties
- Why Nitrogen-Vacancy Defects as fluorophores?
- Comparison between confocal and STED images
- Proof of no bleaching
- Maximizing the resolving power by optimizing STED in a single direction

What is a Nitrogen-Vacancy Defect?

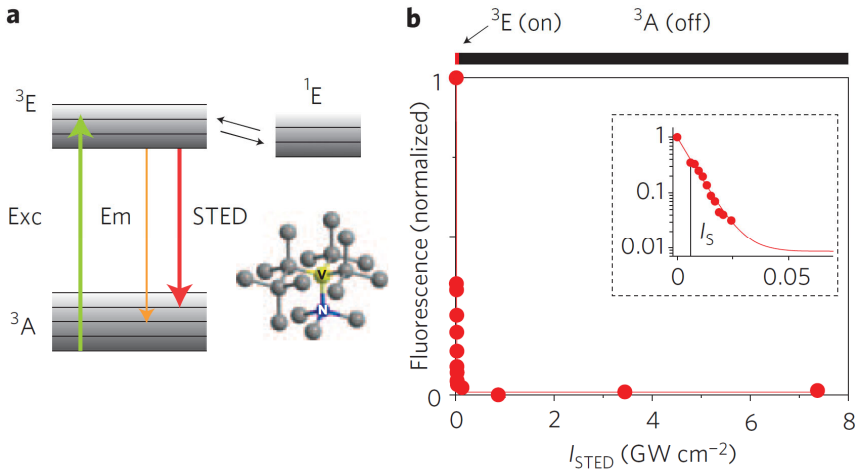


https://commons.wikimedia.org/wiki/File:Nitrogen-vacancy_center.png

Firstly, well have to take a look at what a nitrogen-vacancy defect in the diamond structure is. The nitrogen-vacancy center is a point defect in the diamond lattice. It consists of a nearest-neighbor pair of a nitrogen atom, which substitutes for a carbon atom, and a lattice vacancy.

The interesting part for our purposes is that this defect can be in two different states which are called NV0 and NV-. The transition between these two states emits visible light, that is it is fluorescent and is thus a fluorophore.

Fluorophore Properties



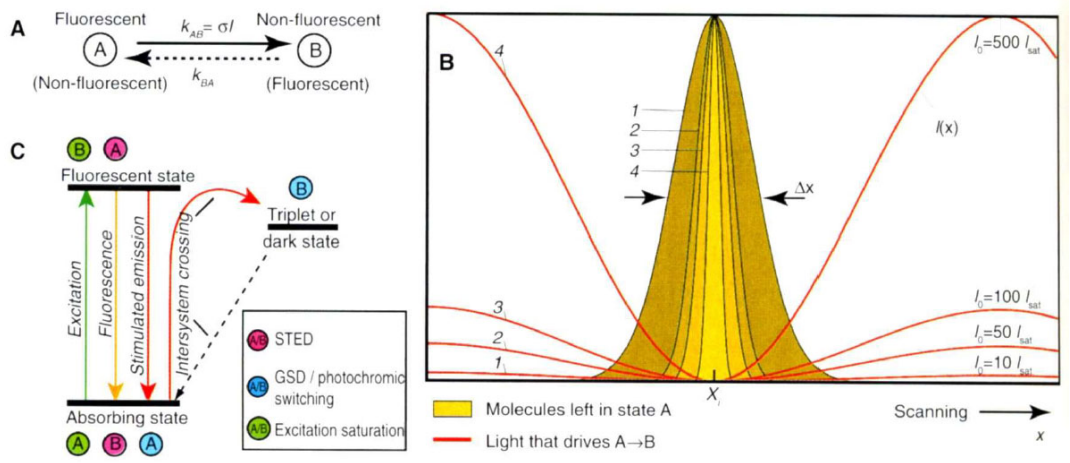
Source: Rittweger et al.

Here you can see a figure taken from the research paper. First, we'll focus on the left part. It shows the discussed states of the nitrogen vacancy. We can see the ground state A3, the fluorescent state E3 and the dark state E1. Additionally, you can see some vibrational states of all the mentioned states. You can also see excitation, spontaneous emission and STED transitions. A transition between A3 and E3 absorbs light with a wavelength of about 532nm. We also see that there is a singlet state E1 next to our triplet state E3.

As an interesting sidenote, nitrogen-vacancy-centers with magnetic quantum number ms = 0 emit more strongly, because their ms =+-1 counterparts have an increased tendency to convert into the dark state E1. This feature allows for the orientation and detection of spin.

In figure b you can see the normalized fluorescence over the STED beam intensity. We can see that the ability of the molecule to fluoresce decreases exponentially with the STED Beam intensity. The nearly rectangular function represents a prefect implementation of STED. The researchers claim that if the STED beam intensity is bigger then 20 MW/cm^2, the nitrogen-vacancy-center is deprived of its ability to fluoresce, because all molecules are in the ground state A3. We can see this represented in the bar above the diagram.

Why Nitrogen-Vacancy defects as fluorophores?



Source: Lecture

Now the question still remains, why would we want to use these nitrogen-vacancy-defects as fluorophores? To answer this question, we'll have to take a look at the basis of STED again. This image is from Dr. Nabers lecture on STED. I will briefly give context to this image even though it was already covered. We have many molecules that can be in two states, the state A which is fluorescent and a state B that is not. Firstly, all molecules are excited into state A. Now Dr. Naber introduced the STED beam which stimulates the emission in the state A. This transition rate is proportional to the intensity of the STED beam. As we have covered on the last slide this transition rate function of the STED beam intensity is nearly rectangular. Thus, one can claim that with a high enough STED beam intensity all Molecules in state A, that are hit with the STED beam transition into state B and are therefore switched off.

Now if the STED beam was everywhere, every Molecule would be switched off. However, the STED-Beam intensity follows the orange curve which is zero at the spot X, thus only the fluorophores close to the minimum are still in state A. Now the orange curve can be scaled to different maximum intensities. A higher maximum intensity also yields a steeper slope of the function which there further confines the region of fluorophores in state A.

However, one should note that even though the intensity in the minimum is claimed to be zero. It is not physically possible to make the intensity exactly zero at this point. At

best the intensity would be below measuring accuracy but this is not the case, there is some measurable rest intensity left. And this rest intensity is also scaled up when scaling the entire function. Still the rest intensity is very small, thus the technique still works.

Why Nitrogen-Vacancy defects as fluorophores?

- Resolution in STED is governed by

$$\Delta r \approx \frac{\lambda}{2n \sin\alpha \sqrt{1 + I_{max}/I_s}}$$

- Increasing I_{max} increases resolution

$$\frac{I_{max}}{I_s} \to \infty \leftrightarrow \Delta r \to 0$$

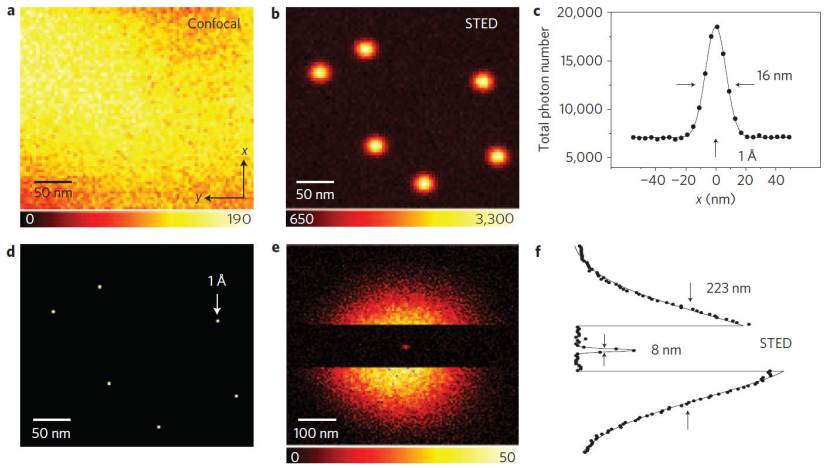
- Organic fluorophores bleach for high intensities
- Nitrogen-Vacancies do not bleach

→ They are ideal candidates for a fluorophore

We have seen that the points spread is reduced for higher STED beam intensities. A lower point spread also increases the resolution of our microscope. As we can see from looking at the approximation of the STED resolution, the resolution increases with a higher Imax. Therefore, in the limit of Imax/Is to infinity we get infinite resolution.

Thus, the reasonable next step would be to use the highest available intensity and get extreme resolution. However, high intensities can bleach our organic fluorophores as used mostly in STED.
The great benefit in the Nitrogen-Vacancies is that they do not bleach. Thus, we can use extremely high STED beam intensities. Making the nitrogen-vacncy-centers ideal candidates for STED.

Comparison between confocal and STED images

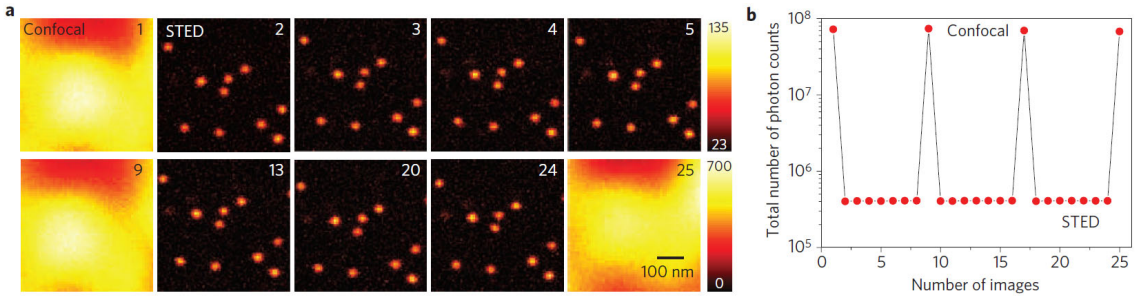


Source: Rittweger et al.

To see how ideal our fluorophore actually is, we'll have a look at the first result shown in the research paper.
A: The upper left image A, shows the result when scanning the sample with a confocal microscope. Its very clear that the image does not actually contain any information at this scale.
B: The upper middle image B shows the same area with the scale of the sample as image A. We can see the nitrogen vacancy centers very bright and clear. This image was taken with a STED beam intensity of already 1.4 Gigawatt/cm^2.
C: In image C on the upper right, we see zoomed in version of image B. What was done is, to zoom onto a single nitrogen-vacancy center and sum the photon counts along the y direction. Thus, we can fit a 1d dimensional gaussian on the data and calculate the FWHM Full width at half maximum of 16nm.
D: However, we can also fit a bivariate gaussian directly to the nitrogen vacancy centers and thus calculate the coordinates of the nitrogen-vacancy centers to determine their positions. Further calculations then allow to calculate the spread of each point with a precision of up to a single Angstrom. Comparing image D to image A really shows the vast information gain achieved through STED compared to confocal imaging.
E: In the lower middle image E an image centered around of the nitrogen-vacancy centers at a larger scale. The image was recorded by initially using a confocal microscope and then switching the STED beam on for a small region. Again, this image highlights the vast information gain from Confocal to STED imaging. For this image the STED beam

intensity was higher with about 3.7 GW/cm^2 which allows for an even smaller point spread of 8nm.
F: On the lower right we can see image F, which is the same representation as image C. This image highlights the very small point spread of only 8nm. Another effect comes into play here. The intensity of the peak is (even relatively speaking) much lower in this picture than in previous pictures. This is because our STED beam is not exactly zero in the minimum and our fluorescence function is not exactly rectangular. Thus some Molecules are switched off in the center, lowering the peak accuracy.

Proof of no bleaching



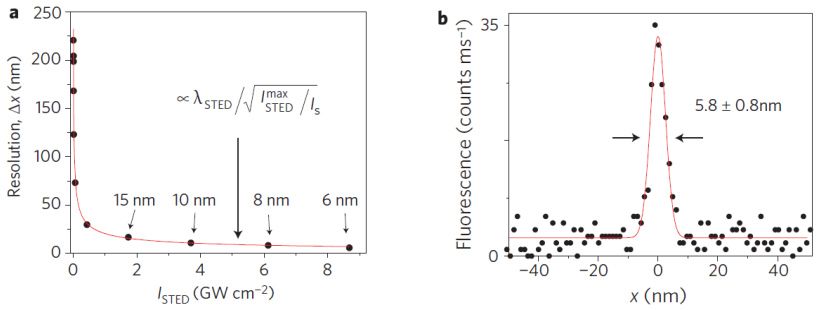
Source: Rittweger et al.

The initial claim and argument towards using the nitrogen-vacancy-centers in diamond was that these fluorophores have virtually no bleaching. To test this hypothesis the researchers took a specific area of the sample and scanned it many times using STED and from time-to-time confocal microscopy. The produced images are shown in figure A. Firstly, we can see that the sample is very stable, all nitrogen-vacancy centers remain in the same spot and there is no obvious blinking or bleaching of any of imaged nitrogen-vacancy-centers. There is virtually no change in brightness and the about 100-fold resolution increase compared to confocal microscopy.

The remarkable photostability is also highlighted in the right Figure B. This shows the number of photon counts for each image. We can see very clearly that the photon count is (close to) exactly the same for every image of the same type. As the number of counted photons does not change during each imaging of the sample this is strong evidence that there is no bleaching in the nitrogen vacancy centers even though all STED images are taken at very high intensities. Therefore, the earlier claim that nitrogen vacancies are ideal candidates for fluorophores is supported by our experimental evidence.

To conclude this experiment demonstrates far-field optical nanoscopy recording without photobleaching. Which was the main drawback and argument against the use of STED in the early stages.

Maximizing the resolving power by optimizing STED in a single direction



Source: Rittweger et al.

Lastly to test the maximum resolution power, the researchers looked at optimizing STED in a single direction. They used a focal STED beam spot with a y-oriented central zero-line with steep peaks along the x-axis and focused on a single Nitrogen-Vacancy-centre. This was done for different STED beam intensities as shown in figure A. The curve of resolution over the intensity follows an inverse square root law. The points plotted are the (FWHM) full widths at half maximum of each gaussian. The gaussian distribution of the maximum intensity is shown in figure B. Using STED beam intensities of around 8.6 GW/m^2 allowed to squeeze the point distribution function to a (FWHM) full width at half maximum of 5.8nm in a single direction. Which is the praised resolution of the beginning of this presentation which exceeds the wavelength of the used light by two orders of magnitude.

Sources

- Dr. Nabers Lecture
- Rittweger et al.: STED microscopy reveals crystal colour centres with nanometric resolution (PUBLISHED ONLINE: 22 FEBRUARY 2009; DOI: 10.1038/NPHOTON.2009.2)
- Hell, et al.: Far-Field Optical Nanoscopy (Science 316, 1153 (2007); DOI: 10.1126/science.1137395)
- Wikipedia contributors. 2023. Nitrogen-vacancy center — Wikipedia, The Free Encyclopedia. https://en.wikipedia.org/wiki/Nitrogen-vacancy_center. [Online; accessed 10-Feburary-2023]

Lastly here you can see my sources.