Developing STED microscopy for deep imaging

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During the last decade, the development of new techniques in fluorescence microscopy showed that resolution in far-field microscopy is not fundamentally limited by the diffraction barrier as it was believed for more than a century[1]. Among these techniques, especially Stimulated Emission Depletion (STED) microscopy was proven to be readily applicable to important questions on the forefront of various areas of modern biological research[2-4].

It was recently shown that STED microscopy can be used to monitor the activity-dependent changes of the synaptic morphology of dendritic spines in hippocampal brain slices, which makes it a tool with unique properties for neurobiological research[5] (Fig.1). Interestingly, the setup that was used for these measurements still has some potential for improvement, because the oil-immersion objective was not optimized to image in tissue having a low refractive index as the one found in brain slices. As known from confocal microscopy, the index-mismatch between immersion and mounting medium introduces spherical aberrations to the passing wavefronts, which causes significant loss of resolution and signal strength when imaging deeper in the sample[6]. It can be seen from the measurement depicted in Fig.1b), that the STED resolution is always superior to the one achievable with purely confocal microscopy. Nevertheless, the absolute resolution improvement becomes less pronounced when imaging deeper in the sample, raising the question of what could be achieved using adequate optical conditions.

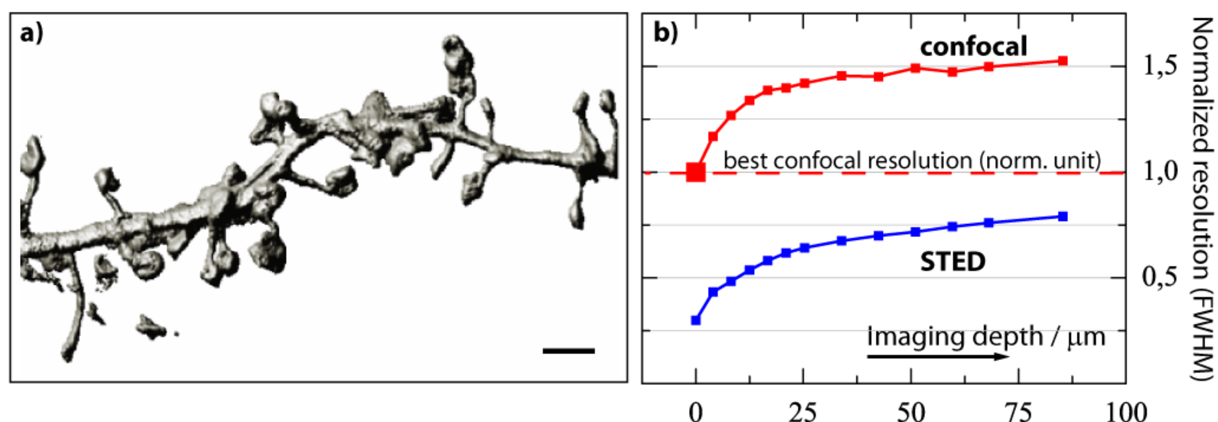

Figure 1: a) 3D volume reconstruction from a STED image stack of a dendritic structure (scale bar is 1µm). b) Imaging resolution with a 100x/1.4 oil-immersion objective in an aqueous sample as a function of imaging depth, normalized to the best value achievable in confocal microscopy. As can be seen, STED clearly maintains its super-resolving margin over the entire working distance. Note that the absolute resolution improvement of STED over confocal microscopy solely depends on the applied STED power.

In order to investigate this question, several microscope objectives optimized for mounting media of lower refractive index were tested for their performance in STED microscopy. A very compact and simple STED microscope was built for this purpose, using just a handful of components and covering no more than 0.6m x 0.6m of table space, including both laser sources (Fig.2). For the STED source,

an inexpensive continuous-wave (cw) fiber laser was chosen, which delivered a maximum optical output power of 1W at 580nm, enabling a four-fold lateral resolution improvement (about 50nm), as measured with fluorescent beads. The excitation light was provided by a picosecond-pulsed diode laser of PicoQuant, operating at 470nm and a repetition rate of 80MHz. This specific combination of wavelengths was also shown to deliver good results for STED microscopy with the fluorescing protein YFP, which makes this simple microscope an interesting option for super-resolution in-vivo experiments. Note that no timing issues between excitation and depletion had to be taken into account due to the cw STED laser, which further reduced the complexity of the setup.

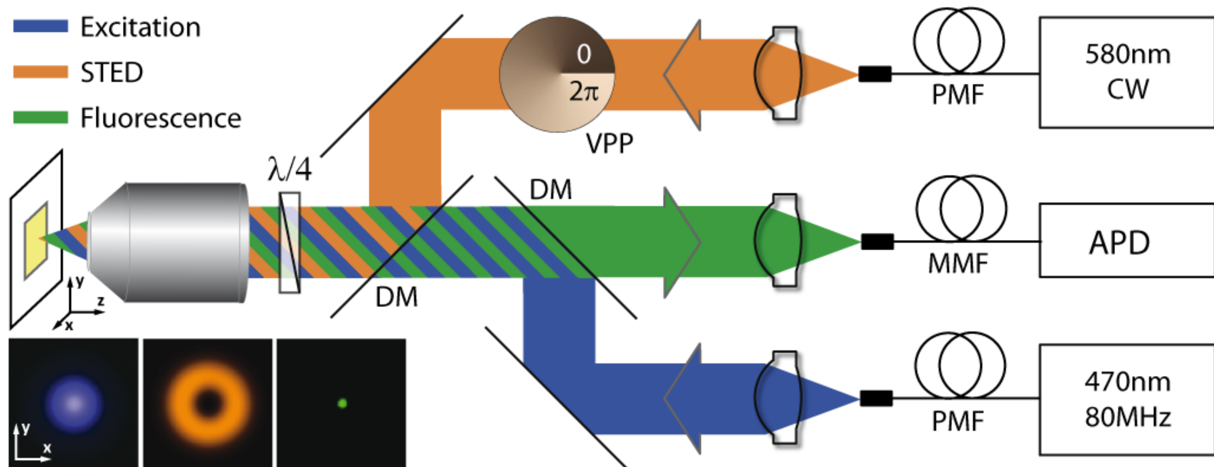

Figure 2: Simple STED microscope which was used to characterize the imaging performance of different microscope objectives. DM: dichroic mirror VPP: vortex phase plate PMF: polarization-maintaining fiber MMF: multi-mode fiber

All measurements were performed with a sample whose refractive index was matched to that of mammalian brain tissue of about 1.375 and a constant STED power in the back aperture of the objective. The two objectives which performed best both featured a correction collar which allowed for correction of spherical aberrations. In order to find the best position of the correction collar at a given imaging depth, it was tuned for greatest brightness of the recorded image. This procedure is known to work well for microscopes having a confocal detection path, and it turned out to deliver good results for the presented STED setup. What remains challenging is the overall alignment quality of the microscope optics, as it shows that the tested objectives are less forgiving concerning non-point-symmetric aberrations such as astigmatism in the STED beam.

The results depicted in Fig.3, which represent preliminary data from my ongoing PhD thesis, are very promising. Not surprisingly, it shows that medium-matched objectives should always be favored over the 100x/1.4 oil objective when imaging deeper than about 10µm under these conditions. Note that the absolute resolution is only stated in order to give an idea of the overall system performance, as it will scale with the applied STED power[7]. The water immersion objective Leica ACS APO 63x/1.15 W CORR was found to deliver constant performance up to a depth of 120µm, whereas the glycerol objective Leica HCX PL APO 63x 1.3 GLYC CORR CS delivered a 10% higher resolution up to a depth of 90µm. Using pure glycerol instead of the standard 80/20 dilution as immersion medium shifted the working range of the glycerol objective down to 150µm. Going even one step further, we performed first measurements using an objective featuring an extremely long free working distance (Leica HCX L APO 20x/1.0), which enabled us to see a more than two-fold improvement in resolution when imaging through 2mm of water (n=1.33). This could be used to introduce tools for

electrophysiological experiments between lens and sample, or to look at truly voluminous samples such as whole-mount embryos.

To conclude, STED microscopy seems to be rather robust when imaging deep into a sample if care is exerted regarding the choice of the microscope objective and the alignment of the setup. Continuing from here, the scope of my PhD thesis will be the development of a STED microscope that is optimized for deep imaging in aqueous samples, which could enable new fields in biomedical research to profit from super-resolution imaging.

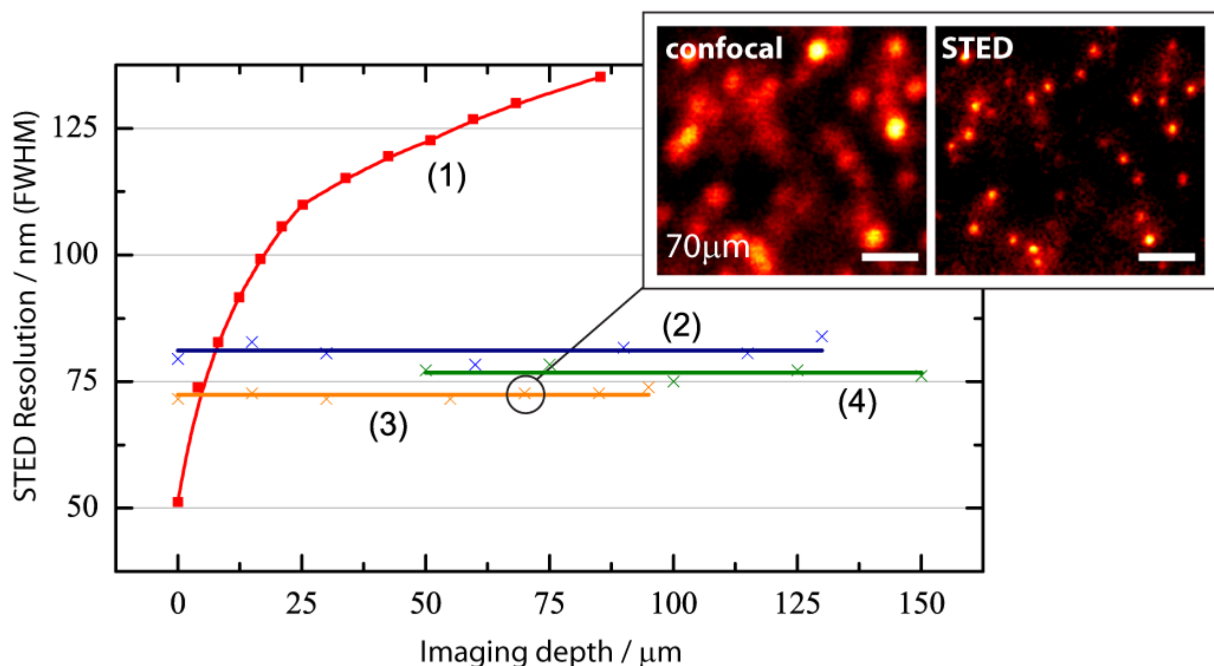

Figure 3: STED resolution measured in a sample with a refractive index of 1.375, depending on imaging depth and microscope objective: 1) Leica HCX PL APO 100x/1.40 oil immersion. 2) Leica ACS APO 63x/1.15 W CORR water immersion with correction collar. 3) Leica HCX PL APO 63x/1.30 GLYC CORR CS with correction collar and 80% glycerol in water as immersion medium. 4) Same as previous but with 100% glycerol as immersion medium. Resolutions were determined with 40nm fluorescing beads and 450mW STED power (cw) in the objective back aperture. Inset: Sample comparing confocal with STED resolution from 3rd series, taken at 70µm depth. (Scale bar is 500nm).

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