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Phase filter enhanced STED-4Pi fluorescence microscopy: theory and experiment

M Dyba, J Keller and S W Hell
Department of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, D-37070 Göttingen, Germany
E-mail: shell@gwdg.de

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Abstract. We study the point-spread and optical transfer-function (OTF) of a stimulated emission depletion (STED)-4Pi fluorescence microscope that provides diffraction unlimited resolution along the optic axis. Our calculations take into account the orientation of the linear transition dipole moment of the fluorescent molecules with respect to that of the focal field. We demonstrate a subdiffraction axial resolution of 44–48 nm for water-immersion lenses, corresponding to a 7–8-fold expansion of the OTF beyond the diffraction barrier of a single lens confocal microscope, which is in excellent agreement with theoretical predictions for the conditions applied. Furthermore, we study phase modifications of the wavefront of the stimulating beam that strengthen weakly transferred frequencies within the OTF support. The enlarged bandwidth enables the separation of objects at 76 nm axial distance.
1. Introduction

Far-field fluorescence microscopy is indispensable for non-invasively visualizing the three-dimensional (3D) structure of transparent samples such as cells. However, its resolution is challenged by diffraction and therefore usually limited to about 200 nm in the focal plane and 500 nm along the optic axis [1]. Several proposals have been made to overcome this limitation including the use of aperture filters [2, 3], as well as the use of entangled photon states [4, 5]. Likewise, the use of multiphoton processes [6]–[9] has occasionally also been connected with super-resolution. But 4Pi [10] and stimulated emission depletion (STED) microscopy [11] have yielded the strongest increase in resolution so far [12]–[14]. While STED microscopy can improve the resolution both in the transverse (x, y) and the axial (z) direction [12], 4Pi microscopy improves the resolution along the optic axis (z) only [14].

The axial resolution of a far-field optical microscope must not be confused with the precision of localization of an object along the z-axis, as is sometimes the case in the literature. The localization accuracy of a distinct object is not prevented by diffraction, because it can be efficiently augmented by reducing noise. By contrast, the ability of a microscope to distinguish axially stacked objects is diffraction limited, as is the resolution in the focal plane. Therefore, increasing the axial resolution necessitates the enlargement of the bandwidth of spatial frequencies transferred from the object to the image along the z-axis. 4Pi microscopy expands this axial bandwidth by coherently adding the spherical wavefronts from two opposing lenses, thus doubling the available aperture size. Making use of simultaneous coherent excitation and fluorescence detection, 4Pi microscopy of type C has so far resulted in an axial resolution of 80–100 nm [14]. Nevertheless, 4Pi microscopy still operates within the realm of diffraction.

In contrast, STED microscopy, which belongs to the family of far-field scanning microscopes that employ a reversible saturable optical fluorescence transition [15]–[18] (RESOLFT), overcomes diffraction altogether. In a RESOLFT type of concept, the subdiffraction resolution is provided by the saturation of an optical transition, and the spatial resolution depends mainly on the saturation level instead of just on the wavelength and on the numerical aperture [16, 19]. Reversibility ensures that the saturation process can be repeated many times at the same site without inducing a lasting alteration of the sample. This is crucial for imaging with a scanning
microscope, since any transition process needs to be repeated many times during each scan. STED microscopy exploits the saturated, in fact exponential, depletion of the excited state of a fluorescence dye by stimulated emission. Reversibility is ensured by the ability of the quenched molecules to be excited over and over again.

In practice, STED and excitation are effected by synchronized ultrafast pulses of light, referred to as ‘STED pulse’ and ‘excitation pulse’, respectively [11, 12]. For efficient depletion to take place, the STED pulse enters the focal region immediately after the excitation pulse has left. Besides, the STED pulse is tuned towards the red side of the emission spectrum, where the probability of re-excitation is low, but that of stimulated emission appreciable. Under these conditions, the intensive STED pulses can de-excite fluorescent molecules excited by the prior excitation pulse through stimulated emission. The excitation energy of the de-excited molecules is carried away by the stimulated photons, but the amplification of the STED pulse is negligible with regard to noise. Separation of the (remaining) fluorescence is also greatly facilitated by the broad spectral range of the fluorescence and by its comparatively long lifetime.

In a single-spot scanning fluorescence microscope, the simplest way of implementing STED for resolution enhancement purposes is to focus the STED pulse into a doughnut shape which forms a zero at about the centre of the excitation spot and has high intensities at its periphery [12]. The subdiffraction sized fluorescence spot resulting from the suppression of fluorescence at the periphery of the excitation spot defines the space in which a marker is allowed to emit fluorescence. Therefore, two distinct objects at subdiffraction distance can be subsequently detected by scanning with such a focal spot through the sample.

The remaining fluorescence spot size and hence the achievable resolution scales in first approximation with the square-root of the power of the STED pulse [19, 20]. The applicable STED pulse power, hence the maximal resolution, is limited by the onset of photo-destructive processes of the fluorescent marker [21]. Under experimental conditions, STED microscopy has so far achieved a lateral resolution of <40 nm on single molecules [22]. The axial squeezing of the fluorescence spot with a doughnut shaped STED focus of ∼620 nm axial FWHM has so far resulted in an axial FWHM of ∼100 nm [12].

A significantly better axial resolution can be attained by STED pulses employing the narrower light distribution around the focal zero resulting from the destructive interference of counterpropagating coherent beams [16, 23]. The latter is accomplished by using the two opposing lenses of a 4Pi type microscope for STED. Thus, in initial experiments, a minimum of ∼160 nm axial FWHM of the focal STED pulse intensity distribution has led to an axial resolution of 33–60 nm. Referred to as STED-4Pi microscopy, this super-resolving imaging mode has been applied to membrane-labelled bacteria [23] as well as to the immuno-fluorescence imaging of microtubules in fixed mammalian cells [24]. Described in a brief experimental report, these initial images displayed have the highest axial far-field optical resolution reported to date [23].

However, the rate for stimulated emission and hence also the size of the fluorescence spot also depend on the orientation of the fluorescent molecules with respect to the light field of the STED pulse. This effect is directly visible with single molecules [25]. For the more common dense labelling of biological samples, the orientation effects are averaged out and the fluorescent spot size is spatially invariant. In this case, the subdiffraction-sized fluorescent spot of the STED microscope can be formally described by an ‘effective’ point-spread-function (E-PSF) whose FWHM is a measure of the resolution, i.e. spatial separation capability, of the system.

In this paper, we present an in-depth comparative theoretical and experimental study of STED-4Pi microscopy. Contrary to previous reports, STED is described by a vectorial theory
that takes into account the orientation of the molecules with respect to the focal field. Comparing measured and calculated data for saturated depletion, we determine the saturation level as a function of the STED beam power and the associated subdiffraction resolution. In addition, we study the role of the wavefront modifying phase function that is used in STED-4Pi microscopy to avoid axial sidelobes of the E-PSF [23]. Resulting from the sinusoidal-like periodic focal structure, the sidelobes are a typical problem with microscopes using two opposing lenses coherently, but can be avoided in a STED-4Pi microscope. We also demonstrate how the fluorescence spot size decreases with increasing intensity of the STED beam and illustrate how the saturation of the stimulated emission eventually breaks the diffraction barrier. Finally, we prove that a STED-4Pi microscope is able to discern features much closer than the diffraction barrier along the optic axis.

2. Theory of a STED-4Pi microscope

2.1. Vectorial calculation of the fluorescence depletion

In a single spot scanning STED microscope, stimulated emission is implemented at the outer focal regions of the scanning excitation spot in order to suppress fluorescence. Stimulated emission is performed in a highly saturated way, i.e. strongly dominant over fluorescence emission [11, 12]. For the most efficient implementation, a pico- or sub-picosecond light pulse raises the fluorescent molecule from the ground state $S_0$ to an electronically excited Franck–Condon-state $S_1^{\text{vib}}$ as sketched in figure 1(a). Within the first few picoseconds after the excitation, the molecule non-radiatively relaxes to the fluorescent state $S_1$ [26]. The STED pulse quenches the potentially fluorescing molecule to the ground state $S_0^{\text{vib}}$, thus preventing it from emitting a fluorescence photon. The state $S_0^{\text{vib}}$ further decays to $S_0$ by non-radiative deactivation within $<1$ ps. Featuring $\tau_{\text{STED}} \sim 50–200$ ps, the duration of the STED pulse is selected such that the pulse acts within a fraction of the nanosecond lifetime of the fluorescent state $S_1$, ensuring that basically all excited molecules encounter the STED pulse. On the other hand, $\tau_{\text{STED}}$ is by two orders of magnitude longer than the vibrational decay of the $S_0^{\text{vib}}$. Therefore, the STED pulse stimulates the excited molecules into a state that is mainly empty due to the fast $S_0^{\text{vib}} \rightarrow S_0$ transition, on the whole precluding the molecule’s recurrent re-excitation $S_0^{\text{vib}} \rightarrow S_1$.

This favourable selection of $\tau_{\text{STED}}$ enables us to regard $\tau_{\text{STED}}$ as the smallest temporal unit when treating STED mathematically. Therefore, it is sufficient to rely on the total photon fluence of the STED pulse [23]; dealing with the temporal shape of the intensity of the STED pulse in the focal region is not required in first approximation. For example, the rate for stimulated emission $k_{\text{STED}} = \sigma_{se} h_{\text{STED}}$ is proportional to the stimulated emission cross-section $\sigma_{se}$ and the focal STED pulse fluence $h_{\text{STED}}$ is measured in photons per area per pulse.

At a given pulse duration, stimulated emission can be viewed as a light-driven channel of de-excitation that suppresses the fluorescence exponentially with an increase in the STED pulse fluence. Thus, the normalized probability of a single molecule to reside in $S_1$ after the STED pulse has passed is simply given by $\eta(h_{\text{STED}}) = \exp(-\sigma_{se} h_{\text{STED}})$ [23]. The giant optical nonlinearity provided by this exponential dependence of the fluorescence on the fluence of the STED pulse is the key to overcome the diffraction limit. We note that this nonlinearity is substantially different from that encountered in multi-photon processes, e.g. from the quadratic
Figure 1. (a) Energy level diagram of a fluorescence molecule and optical transitions involved in the STED process. A picosecond laser pulse (green) excites into $S_1^{\text{vib}}$. After fast vibrational relaxation, the red-shifted STED pulse (red) transfers the molecule to $S_0$ before fluorescence emission can occur. (b) The diagram shows the fluorescence suppression $\eta$ by STED as a function of the STED pulse fluence $h_{\text{max}}$ expressed by the saturation factor $\zeta$. The data are recorded with a thin fluorescent layer of Pyridine 2 in the focal plane of a NA = 1.2 water-immersion lens. A standard PSF for STED (745 nm) was superposed with the excitation PSF (554 nm). The line shows the corresponding theoretical curve for an isotropic distribution of dipole orientations. The applied photon fluence of the STED pulse (at the focal point) $h_{\text{max}}$ is fitted to $\zeta = h_{\text{max}}/h_{\text{sat}}$ of the calculation. The estimated saturation fluence $h_{\text{sat}}$ reveals the cross-section for stimulated emission $\sigma_{\text{se}}$ according to the relationship $\eta = e^{-\sigma_{\text{se}}h_{\text{sat}}} = 1/2$ valid for a single molecule with a transition dipole moment that is parallel to the field stimulating the emission. The inset in (b) displays the same data on a semi-logarithmic scale proving the clear deviation of measured curve of $\eta_{\text{layer}}$ from a simple exponential decay, which is in excellent agreement with the prediction from the vectorial theory.

dependence of fluorescence on two-photon excitation which is only capable of providing a small amount of increase in spatial resolution.

The basic principles of STED microscopy can be satisfactorily described by treating $h_{\text{STED}}$ as a scalar. However, to obtain quantitative predictions at strongly focused fields [27], it is beneficial to take into account the polarization of the field of the STED pulse with respect to that of the molecular transition dipoles. The need for a vectorial theory is aggravated by the
The fact that high angle focusing spawns off field components that are orthogonal to the initial light field orientation [28, 29]. The extension to a vectorial description is realized by introducing a vector \( \vec{h}_{STED} \) describing the fluences of the different field components in the \( x, y, z \)-directions. Furthermore, we introduce \( |\vec{h}_{ESTED}| \) as the focal field amplitude with \( h_{STED,j} = A|\vec{h}_{STED,j}|^2 \) with \( j = 1, 2, 3 \) for the \( x, y, z \)-directions and \( A \) denoting a constant considering the appropriate units.

For most fluorescence molecules, the transition dipoles for excitation and stimulated emission are parallel [26]. Therefore, if the molecule does not undergo rotational diffusion in the time interval between the excitation and quenching pulses, we can assume an unchanged transition dipole orientation in space. The de-excitation probability induced by a focal fluence \( \vec{h}_{STED} \) on excited molecules with an initial transition dipole distribution \( d_{exc}(\vartheta, \phi) \) can now be written as

\[
\eta = \frac{1}{\eta_0} \int_0^{2\pi} \int_0^\pi d_{exc}(\vartheta', \phi') \exp\left(-\sigma_{exc}|\vec{n}_{\vartheta,\phi'} \cdot \vec{E}_{STED}|^2\right) d\vartheta' d\phi'
\]  

(1)

with

\[
\eta_0 = \int_0^{2\pi} \int_0^\pi d_{exc}(\vartheta', \phi') d\vartheta' d\phi',
\]

where \( \vec{n}_{\vartheta,\phi} \) is the Cartesian unit vector with an orientation defined by \( \vartheta, \phi \). In the exponent of equation (1), we write the projection of the light field onto the axis of the transition dipole of the molecule. The initial distribution of the orientation of the transition dipoles of excited molecules in a sample \( d_{exc}(\vartheta, \phi) \) in turn depends on the initial orientation of the molecules and on the polarization of the excitation field \( \vec{E}_{exc} \), which is defined in analogy to \( \vec{E}_{STED} \). In the following discussion, we assume an isotropic molecular orientation before excitation, so that we obtain

\[
d_{exc}(\vartheta, \phi) = \sigma_{exc} |\vec{n}_{\vartheta,\phi} \cdot \vec{E}_{exc}|^2,
\]

(2)

where \( \sigma_{exc} \) is the excitation cross-section [26].

Up to now, we have treated \( \vec{E}_{exc} \) and \( \vec{E}_{STED} \) and the corresponding photon fluences \( \vec{h}_{exc}, \vec{h}_{STED} \) as constant vector fields. However, in a high-angle focusing system such as in a high aperture microscope, \( \vec{E}(\vec{r}) \) and \( \vec{h}(\vec{r}) \) are rather complicated functions of space and therefore depend on \( \vec{r} = (x, y, z) \cdot \vec{h}_{exc}(\vec{r}) \) and \( \vec{h}_{STED}(\vec{r}) \) are here referred to as the (fluence-based) excitation and STED-PSF describing the focal light fields. The local de-excitation probability \( \eta(\vec{r}) \) for excited molecules at each point of the PSF can now be calculated as a function of \( \vec{E}_{exc}(\vec{r}) \) and \( \vec{E}_{STED}(\vec{r}) \).

The focal fields can be obtained from a numerical computation based on a high aperture diffraction theory such as the one introduced by Richards and Wolf [29]. Thus, we are now able to calculate the spatial structure of the probability of de-excitation \( \eta(\vec{r}) \) by use of formulae (1) and (2). The optional presence of a point-like confocal detector is readily taken into account by a detection PSF \( h_{det}(\vec{r}) \) describing the normalized probability of detection. Using \( C \) as a normalizing constant, we can finally calculate the effective PSF (E-PSF) \( h_{eff}(\vec{r}) \) of a STED microscope, i.e. the resulting focal region in which fluorescence is permitted [23]:

\[
h_{eff}(\vec{r}) = C|\vec{h}_{exc}(\vec{r})|h_{det}(\vec{r})\eta(\vec{r}).
\]

(3)
In principle, $h_{\text{det}}$ is also a vectorial function, taking into account the vectorial nature of light upon collection. However, the vectorial defocusing effects upon collection are negligible with regard to the orientational depolarization brought about by the rotational diffusion of the molecules (which also occurs after the two pulses have passed). In addition, the vectorial effects of the STED beam are more critical. In particular, for high values of $\vec{h}_{\text{STED}}$, minor changes in the non-principal components of $\vec{h}_{\text{STED}}$ are emphasized due to saturation. In addition, the non-principal components of $\vec{h}_{\text{STED}}$ alter the depth and structure of the focal intensity minima.

We now define the ‘saturation fluence’ $h_{\text{sat}}$ as the fluence at which we obtain $\eta = \exp(-\sigma_{se}|\vec{h}_{\text{STED}}|) = 0.5$ for a single molecule, under the condition that the excitation and STED fields are linearly polarized and parallel to the molecule’s transition dipole. It is convenient to define a ‘saturation factor’ $\zeta = h_{\text{max}}/h_{\text{sat}}$ as a measure of the applied focal fluence, whereby $h_{\text{max}} = \max_F[|\vec{h}_{\text{STED}}(\vec{r})|]$ is defined as the maximum fluence of the STED pulse encountered at a point in the focal region. For example, in a regular PSF that is not doughnut-shaped, $h_{\text{max}}$ is found at the geometric focal point: $h_{\text{max}} = |\vec{h}_{\text{STED}}(0)|$.

To test our vectorial approach to quantifying STED, we first measured the fluorescence suppression $\eta_{\text{layer}}(\zeta)$ encountered with an ultrathin fluorescence layer in the focal plane of a water-immersion objective lens of numerical aperture $NA = 1.2$. We employed collinearly polarized and regularly focused excitation and STED pulses. The smaller wavelengths for excitation ($\lambda_{\text{exc}} = 554 \text{ nm}$) as compared with that for STED ($\lambda_{\text{STED}} = 745 \text{ nm}$) ensured that the excitation spot was fully covered by its STED counterpart. The ultrathin layer was composed of the styryl pyridinium compound Pyridine 2 (1-ethyl-4-(4-(p-dimethylaminophenyl)-1,3-butadienyl)-pyridinium perchlorate, Lambda Physik, Göttingen, Germany). $\eta_{\text{layer}}$ is the overall de-excitation probability averaged over the whole fluorescence layer in the focus, i.e. over the excitation Airy disc in the focal plane and beyond. Figure 1(b) reveals the decrease of $\eta_{\text{layer}}(\zeta)$ with increasing fluence $h_{\text{max}}$ of the STED pulse, measured in photons per area per pulse. The red line represents the vectorially calculated data as a function of the saturation level $\zeta = h_{\text{max}}/h_{\text{sat}}$. This curve is calculated for the above conditions according to formulae (1), (2) and the vectorial PSF formulae by Richards and Wolf.

For $h_{\text{max}} \gg h_{\text{sat}}$ (i.e. $\zeta \gg 1$) the inhibition of the fluorescence signal is expected to become complete: $\eta_{\text{layer}} \to 0$. Indeed, the measurement nicely confirms the nonlinear suppression of the fluorescence. Nevertheless, at the periphery of the STED spot, the depletion cannot be as effective as it is at the focal point ($r = 0$), because the molecules are subject to lower fluences of stimulating light. Hence for a laterally extended layer, the fluorescence decay function $\eta_{\text{layer}}(\zeta)$ falls off less steeply as compared to what is expected from a single molecule at the geometric focal point that is lined up with the light field. The deviation of $\eta_{\text{layer}}(\zeta)$ from a simple exponential function is more apparent in the inset of figure 1(b) showing the same calculated and measured data in a semi-logarithmic plot. The agreement between the calculated values and the measured data proves the validity of our vectorial model for calculating the effect of STED.

Since the cross-section for stimulated emission was unknown, the calculation predicted just the general shape of the curve. The subsequent application of a least-squares fit, however, allowed us to determine the saturation fluence $h_{\text{sat}} = 4.6 \times 10^{15} \text{ cm}^{-2}$ of the dye Pyridine 2 at $\lambda_{\text{STED}} = 745 \text{ nm}$. This value in turn yielded its cross-section for stimulated emission $\sigma_{\text{se}} = 1.52 \times 10^{-16} \text{ cm}^2$.

According to our numerical calculations, the fluorescence at the focal point was down to $\eta \approx 10^{-15}$ which is connected with a saturation factor $\zeta = 47.5$. Thus, the data of figure 1(b) prove that it is possible to achieve very high optical nonlinearities by STED. The residual amount

of fluorescence that is still present at high STED pulse energies originates from the focal periphery and from molecules oriented mainly perpendicular to the polarization of the STED light. The good fit of the data to the calculation shows that the assumptions made about STED as well as the isotropic dipole distribution seem to be well fulfilled. The vectorial theory hence describes STED quite accurately for lenses of high numerical aperture.

### 2.2. Effective point spread function of the STED-4Pi microscope

The vectorial focusing theory by Richards and Wolf [29] allows an accurate prediction of the PSF of a high angle lens, which is readily expanded to 4Pi microscopy as well [30]. Thus, we can calculate the fields $\vec{E}(\vec{r})$ and the focal intensity distributions $h(\vec{r})$ for excitation, for STED, and for fluorescence detection of a confocalized STED-4Pi microscope. For given focal fields $\vec{E}_{exc}(\vec{r})$ and $\vec{E}_{STED}(\vec{r})$, equations (1) and (2) directly yield the spatial structure of the fluorescence suppression $\eta(\vec{r})$. Equation (3) consequently enables the calculation of the E-PSF $h_{eff}(\vec{r})$.

Figure 2 displays axial sections $h(x, z)$ of the intensity PSFs that are involved in a STED-4Pi microscope. The parameters for the calculation are identical with those used in the experiment, i.e. for depletion of Pyridine 2 at $\lambda_{exc} = 554 \text{ nm}$, $\lambda_{STED} = 745 \text{ nm}$, $\lambda_{det} = 680 \text{ nm}$. The PSFs are calculated for water-immersion ($n = 1.333$) objective lenses with the maximum available semi-aperture angle $\alpha = 64^\circ$ and $x$-polarized excitation and STED beams. The panels represent $xz$-sections of the different PSF, with $z$ denoting the optic axis.

Figure 2(a) depicts the excitation PSF along with the $z$-profiles of the excitation and of the detection PSF. The diameter of the image of the detection pinhole in the focal plane of the lens was selected to be 360 nm. The fluorescence emission was assumed to be unpolarized. The resulting confocal PSF is displayed in panel (b). The graphs show the axial profile $h_{conf}(x = y = 0, z)$ as well as the signal response of the (confocal) microscope to an ultrathin fluorescent $xy$-plane that is scanned in the $z$-direction. The latter is referred to as the $z$-response $V(z) = \int \int h_{eff}(x, y, z) \, dx \, dy$. For a confocal microscope, $h_{eff}$ is given by the confocal E-PSF, of course. In this case, $V(z)$ is a fixed function for a given optical setup. In contrast, in a STED microscope, the E-PSF and hence also $V(z)$ depend on the STED fluence $h_{max}$ applied, as well as on the exact shape of the central minimum of $h_{STED}(\vec{r})$.

The STED-PSF, displayed in figure 2(c), is produced by coherently adding the focal fields from two opposing lenses of a 4Pi microscope. In order to produce a narrow minimum, the phase difference between the counterpropagating focal fields was set to destructive interference at the focal point. As a result, the focal interference pattern consists of a series of peaks and valleys with distances slightly larger than $\lambda_{STED}/2n$. Fluorescence suppression is expected to occur throughout the focal region except at the central minimum as well as at the satellite minima along the optic axis. Panel (d) shows the calculated effective PSF $h_{eff}$ of the STED-4Pi microscope as anticipated for a saturation factor $\varsigma = 40$. The associated profile $h_{eff}(x = y = 0, z)$ along the optic axis and the $z$-response $V(z)$ illustrate the fundamental increase in axial resolution as compared to that of a confocal fluorescence microscope.

Comparison of figures 2(c) and 2(d) also illustrates how the STED-PSF $h_{STED}$ carves itself into the focal region. In a way, $h_{eff}$ can be seen as an imprint of $h_{STED}$. However, due to the strongly nonlinear suppression, the structures of the maxima and minima in $h_{eff}$ are no longer approximated by a sinusoidal as in the case of $h_{STED}$. Rather, the fluorescence peaks are sharpened.
Figure 2. Calculation of the PSF involved in a STED-4Pi microscope. (a, b) The excitation, detection and confocal PSF. The images represent an axial section in the $xz$-plane parallel to the polarization of the excitation light. The profiles $h(x = y = 0, z)$ give the intensity distribution along the optic axis. Panel (c) depicts the intensity STED-PSF produced by the (regular) 4Pi microscope and the corresponding axial profile $h(x = y = 0, z)$. In panel (d) the effective PSF according to formula (3) is shown for a saturation level of $\zeta = 40$. Panels (e) and (f) represent the same data for the phase filter enhanced STED-4Pi microscope. The phase filter modulates the incident wavefront as depicted in the inset of panel (e). Comparison of (c) and (e) exhibits the effect of the phase filter. The $z$ response profiles $V(z)$ in panels (b), (d) and (f) should be directly compared with the experimental data.

and confined to the region close to $h_{\text{STED}} = 0$ as a direct consequence of the non-linearity. The larger $h_{\text{max}}$, i.e. the applied STED pulse power, the more confined is the area of potential fluorescence, since even minor fluences of $h_{\text{STED}}$ close to the minima already lead to a substantial depletion. This continual reduction of the peak size with increasing $h_{\text{max}}$ leads to subdiffraction fluorescent spots. So, while the axial FWHM of the confocal microscope amounts to 535 nm in our case, a saturation factor of $\zeta = 40$ in STED-4Pi microscopy yields a central peak with an axial FWHM of 47 nm (figure 2(d)). The FWHM of the $z$-response $V(z)$ is 59.5 nm. The latter value is directly compared to experimental data later in this paper.

Featuring secondary and tertiary minima, the classical ‘destructive-phase’ 4Pi-PSF in figure 2(c) also produces fluorescence sidelobes, similar to those in standard 4Pi microscopy. However, the difference from the latter is that we may now eliminate these lobes physically.
by altering the STED-PSF in such a way that the higher order minima of zero intensity are washed out. Because of the nonlinear relationship between $\eta$ and $h_{\text{STED}}$, an increase of the STED pulse fluence in the minima by even a small amount eliminates the side lobes effectively. Such a destruction of the higher-order zero intensity region is achieved by departing from the pure spherical shape of the focused wavefronts of the STED beam [23]. In fact, a symmetrical aberration of the wavefronts of both lenses leaves the central zero intensity point untouched while raising the fluence at the side minima.

To this end, we have introduced a stepwise phase delay on the illumination pupil function of both objective lenses, formally described by $\Psi(\vartheta) = m\pi\theta(\vartheta - P\alpha)$ [23]. Here, $\theta$ denotes the Heaviside step-function and $\vartheta$ the (polar) aperture angle. The parameters $m = 0.56$ and $P = 0.57$ give the amount of delay and the polar coordinate of the phase step on the polar aperture angle $\vartheta$ of the used lenses of $\text{NA} = 1.2$ with semi-aperture angle $\alpha = 64^\circ$. The aberrated wavefronts are schematically depicted in the inset of figure 2(d). The resulting ‘phase filter enhanced’ STED-PSF of figure 2(e) demonstrates that while the central minimum is fully maintained, the secondary and tertiary side minima are strongly elevated. As a result, the corresponding effective PSF, shown in figure 2(f), features a single pronounced main maximum.

The axial profile $h_{\text{eff}}(x = y = 0, z)$ represented by the solid line in figure 2(f) features sidelobes <1%, an amount that is negligible at the typical imaging noise levels. The $z$-response $V_{\text{eff}}(z)$ displays somewhat larger sidelobes (<9%) that appear as weak modulations of a shallow background. These lobes stem from residual values of the effective PSF $h_{\text{eff}}$ located off-axis $(x, y \neq 0)$. Integrating the signal over the whole plane, the $z$-response accumulates these values resulting in this apparent background. In a practical imaging situation, the $z$-response $V_{\text{eff}}(z)$ quantifies the ability of the microscope to axially separate two infinitely extended stacked planes, which is by definition the most challenging situation in 3D-imaging [1]. In contrast, the axial profile $h_{\text{eff}}(x = y = 0, z)$ quantifies the potential of a microscope to axially separate two points. Thus, any two ultrathin axially stacked objects to be resolved will fall in between the two extreme cases, underscoring the superb axial resolution of the phase-modulated STED-4Pi microscope (figure 2(f)).

Importantly, the introduction of $\Psi(\vartheta)$ leaves the central peak basically unchanged. For the same saturation factor $\varsigma = 40$, the FWHM of the central peak also amounts to 48 nm in real space for the conditions given above. $V(z)$ exhibits an axial FWHM of 59.5 nm. So in essence, the applied phase aberration hardly bears any influence on the achieved resolution. It basically smears out the zero intensity points of higher order by redistributing the fluence away from the two main peaks. As a side effect, this redistribution also reduces the fluence in the two main maxima of the STED-PSF, and hence also the saturation factor $\varsigma$. However, this reduction can be readily compensated for by increasing the total power of the STED beam. For the $\Psi(\vartheta)$ specified above, the same saturation factor $\varsigma = 40$ is attained by increasing the STED beam power by a factor of 2.36. An important consequence of the phase filter is that $h_{\text{eff}}$ becomes so well-behaved that in many cases super-resolved images can be recorded without a need for further image processing. The resulting image is simply formally

$$d(\vec{r}) = h_{\text{eff}}(\vec{r}) \otimes o(\vec{r}),$$

whereby $o(\vec{r})$ denotes the object function, i.e. the spatial concentration of fluorescent marker molecules in the object and $\otimes$ represents the convolution operator.
Figure 3. The OTF of the confocal and STED-4Pi microscope were obtained by a Fourier transform of figures 2(b), (d) and (f). The panels (a)–(c) show the frequency amplitudes at a logarithmic scale and look-up table. The zero frequency is located at the centres. Note the massively extended axial frequency band in STED-4Pi microscopy, which are evidenced in the axial profiles (d) as well. The application of the phase filter removes the oscillations of the OTF.

2.3. Optical transfer function of the STED-4Pi microscope

A more comprehensive analysis of the super-resolving power of the STED-4Pi microscope is attained by analysing its optical transfer function (OTF) $H_{\text{eff}}(\vec{k})$ which is derived from the Fourier transform $\tilde{F}$ of the effective PSF, $H_{\text{eff}}(\vec{k}) = \tilde{F}(h_{\text{eff}}(\vec{r}))$ [1, 31, 32]. The amplitude of the OTF $|H_{\text{eff}}(\vec{k})|$ quantifies the strength with which the spatial frequencies of the object $O(\vec{r})$, $\vec{k}_i = 2\pi/\lambda_i$, are transferred to the image. $k_i = 2\pi/\lambda_i$ denotes the spatial frequency components, where $\lambda_1, \lambda_2, \lambda_3 = x, y, z$. Thus, the complex image in the frequency domain, $D(\vec{k}) = \tilde{F}(d(\vec{r}))$, is simply given by

$$D(\vec{k}) = H_{\text{eff}}(\vec{k})O(\vec{k}). \quad (5)$$

The support of $H_{\text{eff}}(\vec{k})$, i.e. all $\vec{k}$ for which $|H_{\text{eff}}(\vec{k})| > 0$, determines the spatial frequencies of the object present in a given image. In a diffraction-limited system, such as a confocal fluorescence microscope, this support is finite. This means that there is a cutoff frequency $k_{\text{lim}}$ where $|H(\vec{k})| = 0$ for $|\vec{k}| > |k_{\text{lim}}|$. In other words, the imaging system has a finite frequency bandwidth [1, 32]. Figure 3(a) shows the calculated OTF amplitude $|H(\vec{k})|$ of a confocal fluorescence microscope. In an ideal reflection-type confocal setup with a point detector and $\lambda_{\text{exc}} = \lambda_{\text{det}} = 554$ nm, the axial cutoff frequency is $k_{\text{lim}} = 17 \mu$m$^{-1}$ [33]. For the conditions of figure 3 that conform to experimental conditions (i.e. $\lambda_{\text{exc}} = 554$ nm, $\lambda_{\text{det}} = 680$ nm, and an effective pinhole diameter of 360 nm), $k_{\text{lim}}$ reduces to $15.3 \mu$m$^{-1}$. This limit is tightly connected with the finite aperture of the lens; frequencies above that limit are not present in the image.
$|H(\vec{k})|$ is shown in figures 3(a)–(c) for the confocal fluorescence, as well as for the STED-4Pi microscope with and without the phase pupil filter, respectively. They were numerically calculated by a Fourier transform of the 3D intensity PSF shown in figures 2(b), (d) and (f). Whereas the support of the OTF of the confocal microscope is limited to a comparatively narrow region, its STED-4Pi counterparts ($\zeta = 40$) are significantly larger. This becomes most evident in the corresponding axial profiles $|H(k_x = k_y = 0, k_z)|$. The axial bandwidth of the OTF is increased by a factor of approximately 8 in both STED-4Pi cases, meaning that frequencies are passed to the image that could not be present in a diffraction-limited technique. Moreover, it is evident that along with the continual increase of the saturation factor $\zeta \to \infty$, the axial bandwidth is continually expanded as well, i.e. $k_{\text{lim}} \to \infty$.

The effect of the phase filter $\Psi(\vartheta)$ becomes evident by comparing figures 3(b) and 3(c). $\Psi(\vartheta)$ does not have any influence on the support of the OTF, but on the relative strength with which the frequencies are transmitted within the support. Without the phase filter, the OTF exhibits a pronounced oscillatory modulation in the $k_z$-direction, originating from the periodic axial sidelobes. For example, at the spatial frequency corresponding to the double sidelobe distance from the main peak, the OTF has its first minimum. Since this axial frequency is only weakly transferred by the system, the axial images are also modulated along the optic axis. Applying the phase filter $\Psi(\vartheta)$ reduces the sidelobes, weakening the axial modulation and enhancing the specific frequencies.

In principle, the demodulation can also be carried out by a mathematical post-processing of the image, in which the weaker frequencies are artificially amplified [31]. In general, however, the physical removal of the lobes is preferable, because of the limitations to the mathematical processing set by noise. The comparison of the OTFs in figures 3(a)–(c) show that the super-resolving power of the STED-4Pi microscope is achieved solely by the saturated transition of STED; it stems neither from the phase filter nor from the optional deconvolution.

3. Experimental results and discussion

3.1. Experimental setup

The setup shown in figure 4 consists of a standard 4Pi microscope, where the STED beam (of 745 nm wavelength) is focused through both arms of the 4Pi interferometer [23]. The fluorescent sample is placed at the common focus of the two opposing lenses, but contrary to a 4Pi microscope, excitation and detection are performed through a single lens, $L_1$, only. For this purpose, a train of 250 fs pulses at 554 nm wavelength are directed via mirror $M_1$, a beamsplitter (BS) and the dichroic mirror $DC_2$ to $L_1$. The pair of water immersion objective lenses feature the largest available numerical aperture, $NA = 1.2$, and a free working distance of 80 $\mu$m. The fluorescence is imaged onto a confocalized detector, providing optical sectioning in the non-STED imaging mode as well. The imaging is performed by scanning the sample through the fixed focus.

The phase filter is realized by a phase delaying layer of MgF$_2$ that is coated on a glass substrate (see the inset of figure 4). A key issue is that this substrate is placed prior to the BS to ensure symmetrical phase fronts entering both objective lenses (see the inset of figure 2(e)), keeping the STED-PSF zero at the focal point ($h_{\text{STED}}(0) = 0$). The optical performance of the confocal and the STED-4Pi mode can be compared simply by blocking the STED beam. Removing the phase filter enables the study of the phase function $\Psi(\vartheta)$ on the STED-4Pi setup.
3.2. Measurement of the z response and the axial transfer function

The $z$-response $V(z)$ can be measured with a thin fluorescent layer on a cover slip, created by exploiting the property of some styryl compounds to adsorb at glass surfaces from solution [34]. By placing a 20 µM aqueous solution of Pyridine 2 between two coverslips, a bright layer emerges at the cover slips surface similarly as described for other fluorophores [35]. The low quantum yield of Pyridine 2 in water ensures that the fluorescence of the solution itself is favourably low.

The axial scans of such layers are presented in figures 5(a), (c) and (e). The confocal $z$ response (a) has a FWHM of 806 nm, which is 26% above the theoretical value of 640 nm. Nevertheless, it is a value that is typically achieved with state-of-the-art water immersion lenses of NA = 1.2 [36]. A peak fluence of $h_{\text{max}} = 1.92 \times 10^{17}$ cm$^{-2}$ in the STED-4Pi microscope, corresponding to $\xi = 42$ with Pyridine 2, reduced the width of the central fluorescent peak by a factor of $\sim 18$ compared to the confocal mode. The FWHM of the STED-4Pi response amounted to $44 \pm 3$ and $48 \pm 3$ nm, without (c) and with phase filter (e), respectively. These values are in remarkable agreement with the respective theoretical values of 47.2 and 48.4 nm (figure 2).

Furthermore, the comparison of the measured with the calculated $V(z)$, shown in figures 2(d) and (f), reveal that the form of the measured functions $V(z)$ is well reproduced by our vectorial computations.

Equally evident in the data is the sidelobe reducing effect of the phase filter. Applying $\Psi(\theta)$ reduces the side lobes from $\sim 50\%$ down to $20\%$ as evidenced in figure 5(c) and (e). However, in the measurement, they did not disappear completely, as anticipated from the theoretical data.
Figure 5. Experimental $z$ responses $V(z)$ recorded in the confocal mode (a) and STED-4Pi mode at a saturation level $\varsigma = 42$. (c), (e) These profiles can directly be compared to the theoretical $z$-responses of figure 2. They exhibit a 17–18 times decreased axial width. The Fourier transforms along the $z$-axis showing the axial frequency transmission are given in the right-hand panels (b), (d) and (f). These curves reveal the gain in axial bandwidth in good agreement with the theoretical results of figure 3(d).

(figure 2(d)). This deviation may either be due to imperfections of the implementation of the phase filter or to asymmetric aberrations in the optical pathways, some of which may have been induced by the sample itself. Amounting to only 20% of the main peak, the sidelobes...
can readily be eliminated by linear deconvolution of the recorded image data, as has been demonstrated already in the STED-4Pi imaging of biological cells [24]. In the absence of $\Psi(\theta)$, the lobes approach the 50% level which is known to be close to the maximum tolerable height when transferring spatial frequencies representing the lobe periodicity to the image. So, in spite of the slight discrepancy between calculation and experiment, the sidelobe suppression originating from the application of $\Psi(\theta)$ is effective and critical for the performance of the system.

The improvement brought about by the phase filter is best demonstrated by calculating the Fourier transform of the measured $V(z)$. The results are displayed in figures 5(b), (d) and (f) representing the experimental OTF profiles $|H_{\text{eff}}(k_x = k_y = 0, k_z)|$ of the confocal, STED-4Pi, and phase filter enhanced STED-4Pi microscope, respectively. These profiles indicate the ability of the respective microscope to distinguish stacked planes at a given periodic spatial distance. They actually represent the measured counterparts to the curves in figure 3(d). The latter show that for the used lenses and wavelengths, the confocal axial transfer function reaches its theoretical axial cutoff frequency at $k_{\text{lim}} = 15.3 \mu m^{-1}$. Due to the slightly broader experimental FWHM of 806 nm, the experimentally derived OTF data of figure 5(b) exhibits a slightly lower frequency range of up to $\sim 12 \mu m^{-1}$, which is still in good agreement with the theoretical expectation. The uncertainty in the experimental cutoff value basically stems from the noisy OTF amplitude encountered at higher spatial frequencies. These values need to be contrasted with the frequency band of the STED-4Pi microscope that supports a bandwidth of about 90 $\mu m^{-1}$ above the noise level. Thus the predicted expansion of the axial frequency bandwidth by a factor of $\sim 8$ over the confocal microscope is fully confirmed in the experimentally derived OTF.

We note that in figures 3(d) and 5 both the measured and the theoretical $|H_{\text{eff}}(k_x = k_y = 0, k_z)|$ are normalized such that the integral over $k_z$ remains constant; in other words, $|H_{\text{eff}}(k_x = k_y = 0, k_z)|$ is not normalized to the zero frequency (DC) value. As a result, due to the reduction of the overall signal, the zero frequency (DC) amplitudes are lower in the STED-4Pi recordings as compared to those in the confocal reference. The reduced DC frequency of the STED-4Pi data, i.e. the reduced overall signal, are attributed to the fact that in real space the fluorescence signal is originating from a smaller volume. This reduction therefore is a direct consequence of increased spatial resolution.

Figure 5(f) also proves that applying $\Psi(\theta)$ maintains the bandwidth enlargement. In accordance with the expectation, the strong periodic modulation of $H_{\text{eff}}(k_x = k_y = 0, k_z)$ in the regular STED-4Pi mode is favourably reduced in figure 5(f). Thus the filter ensures transmission of the complete frequency band while eliminating the transmission gaps that are present otherwise.

To vividly illustrate the breaking of the diffraction resolution limit, we both calculated and measured the $V(z)$ profiles of the phase filter enhanced STED-4Pi microscope for stepwise increased power of the STED beam. The latter is best quantified by $h_{\text{max}}$, or alternatively by the saturation factor $\varsigma$. We started from $\varsigma = 0$, which is equivalent to the confocal case, increasing the degree of saturation up to $\varsigma = 30.4$ equivalent to $h_{\text{max}} = 1.39 \times 10^{17} \text{ cm}^{-2}$. For our set of optical conditions, the latter was achieved by focusing beams of 3.45 mW time-averaged STED power through each lens.

The resulting series of profiles $V(z)$ has been assembled in a movie. Each frame represents an experimental profile $V(z)$ together with its calculated counterpart. At $\varsigma < 1$, one basically reduces the fluorescence at the location of the two interference maxima of the STED-PSF above and below the focal plane. At $1 < \varsigma < 2$, the fluorescence originating from the location of the
Figure 6. Increasing the saturation factor $\zeta$ in a STED-4Pi microscope: axial narrowing of the width of the central fluorescent focal peak (a), the signal reduction caused by residual STED light at $\vec{r} = 0$ (b), and the height of the fluorescence peaks at the first-order minimum of the STED-PSF (c). The diagrams show parameters that are derived from the data shown in the supplementary movie. The values are measured and calculated using data similar to those shown in figures 2 and 5, respectively, but for saturation factors ranging from $\zeta = 0$ (confocal limit) to $\zeta = 67$. The effect of the increasing $\zeta$ on the axial resolution is evidenced in the movie. The inset of (a) shows the calculated and experimental decrease in FWHM on a double-logarithmic plot. Note that both in theory and in the measurement the axial resolution increases with $1/\sqrt{\zeta}$.

maxima is inhibited so that the fluorescence is possible only in the area of the STED-PSF valley. At this stage, the shape of the interference-based STED-PSF produced by the two opposing lenses leaves a ‘negative imprint’ on the standard confocal PSF produced by the lens $L_1$. The peaks of fluorescence that are left over at this stage are still near the diffraction limit. For $\zeta > 2$, the fluorescence peaks are reduced below the diffraction limited extent of $\sim \lambda_{STED}/(2n)$. Besides, due to the phase filter, the side lobes are reduced at high power levels.
After each measurement of the $V(z)$ in the STED-4Pi mode, a confocal reference has been recorded as well by blocking the STED beam. The data were taken consecutively at the same site of the fluorescent layer. After each pair of recordings, the focal spot was moved to unbleached areas of the fluorescence layer. As the layer varies in brightness at different sites of the sample, the individual $V(z)$ pairs also vary in signal, which is also apparent in the movie. In other words, the signal fluctuations apparent from the movie are in the first place due to the layer.

From data as presented in the movie, important parameters can be derived about the performance of a STED-4Pi microscope. Figure 6(a) shows the axial FWHM of $h_{\text{eff}}(\vec{r})$ as a function of the saturation factor, ranging from $\zeta = 0$ to 67. The reduction of the FWHM is due to the saturation of STED, first becoming visible by the squeezing of the main peak of $h_{\text{eff}}(\vec{r})$. With the growing depletion area, the central peak becomes more and more confined towards the central minimum of the STED-PSF. For the highest STED power applied, the axial FWHM reaches less than 50 nm, which corresponds to only 6% of the confocal value. As anticipated for microscopes based on a RESOLFT concept, the achieved spatial resolution at high saturation factors scales with the inverse square-root of $\zeta \,[20, 21]$. Therefore, the double-logarithmic inset of figure 6(a) also depicts a fitted curve (dashed), given by $A\zeta^{-1/2}$, that is asymptotically reached by the simulated data in agreement with the theory. For $\zeta > 8$ also the experimental data verify the square-root dependence in good approximation.

The movie also reveals that the axial sharpening of the focal spot is accompanied by a noticeable signal reduction of the central peak of $h_{\text{eff}}(\vec{r})$ of the STED-4Pi microscope. This reduction is not anticipated from the theory, unless the fluence of the STED-PSF at the central zero does not vanish in full: $h_{\text{STED}}(\vec{r} = 0) \neq 0$. Figure 6(b) displays the ratio between the maximum fluorescence signal measured for the STED-4Pi and the subsequent confocal recording at $z = 0$ in the movie data. For $h_{\text{STED}}(\vec{r} = 0) = 0$, the ratio is expected to be unity irrespective of $\zeta$. The fact that the ratio appears to slightly exceed unity for low STED powers ($\zeta < 5$) is due to photobleaching effected by consecutive recordings, i.e. due to experimental limitations. Clearly pronounced is the signal decrease down to the 40% level observed for $\zeta > 45$. This reduction in signal evidences imperfections of the zero intensity point of the experimentally realized STED-PSF. It can be envisaged as the fluorescence drop of a single molecule at the focal point. It is avoidable by perfecting the zero of the STED-PSF. Therefore, this undesired signal drop must not be confused with the desired signal reduction accompanying the reduction of the focal volume. The latter is unavoidable for high resolution microscopy and is also manifested in our data as a spreading of the signal across the enlarged bandwidth of the OTF, as witnessed in figures 3(d) and 5(b), (d) and (f).

A further important quality parameter of $h_{\text{eff}}(\vec{r})$ is the height of the sidelobes shown in figure 6(c). Whereas the dots represent the relative height of the first sidelobes as compared to the central peak, the continuous line is deduced from numerical calculations. Comparison of the data reveals that the calculated relative lobe height is smaller than the measured one, throughout. However, this does not necessarily reflect an incorrect theoretical prediction of the absolute values of the lobes. Rather, it is more likely that the height of the central peak value is reduced (see figure 2(b)), which inevitably results in larger relative sidelobe heights. So while in the calculation a non-vanishing STED intensity was assumed at $\vec{r} = 0$, the data of both figures 6(b) and (c) consistently support $h_{\text{STED}}(\vec{r} = 0) \neq 0$. Therefore, in order to compensate for the reduction in peak height, we have divided the measured peak height of $h_{\text{eff}}(\vec{r})$ by the measured average signal reduction displayed in panel (b). These corrected experimental results, shown
Figure 7. Axial images ($xz$) recorded perpendicular to the surface of a coverslip that is labelled with the fluorescent dye Pyridine 2. In the confocal version (a), the surface appears as a vertical bar of $\sim 800$ nm axial width (FWHM), whereas a line thinner than 50 nm is present in the STED-4Pi counterpart image of the same area (b). In the case of the STED-4Pi data the ghost peaks resulting from the sidelobes have been removed by a linear Tikhonov deconvolution filter. The bright blobs in the confocal image are due to silica microspheres of $\sim 100$ nm diameter that, adsorbing Pyridine 2 molecules as well, were placed onto the coverslip. In the confocal image, the microspheres just generate a brighter signal due to the increased amount of dye molecules; they cannot be recognized as objects that are distinct from the surface of the cover slip. In contrast, the STED-4Pi microscope clearly separates these objects from the surface layer. The distance between the resulting peaks of 76 nm can also be seen in the axial profile (c). The fact that the peak distance is slightly smaller than the sphere diameter is due to the diffraction-limited lateral resolution of the STED-4Pi-microscope ($\sim 200$ nm). The comparatively broad (pancake-shaped) focal spot of this system averages over various axial distances $<100$ nm offered by the microsphere surface. Nevertheless, the comparison between the two recordings evidences the $\sim 8$-fold increase in axial resolution of the STED-4Pi-microscope over its standard confocal counterpart.

as open circles in panel (c), nicely confirm the experiment especially for high saturation levels $\varsigma > 30$, thereby also supporting the power of the underlying vectorial theory.

The data of figure 6 allow the optimization of a STED-4Pi microscope in terms of the applied STED power. In general, it is advisable to keep the STED power as low as possible in order to avoid unnecessary photodamage [21]. The predicted axial FWHM and lobe height enables the avoidance of unnecessarily high STED pulse fluence. For example, at $\varsigma = 35$, an axial FWHM of $\sim 50$ nm is achieved at a peak signal of $\sim 50\%$ of its confocal counterpart. The relative height of 25–30% of the sidelobes is so low that their effect can be eliminated mathematically [37]. Under experimental conditions, values $\varsigma > 40$ would mainly lower the peak signal without enhancing the axial resolution any further.
Figure 6(a) also shows that for \( \varsigma < 45 \) the experimental FWHM is narrower than its theoretical counterpart. Most likely this traces back to the fact that the central minimum of the STED-PSF is slightly narrower than predicted by the theory. Such a narrowing is indeed anticipated in the presence of residual aberrations reducing the sphericity of the counterpropagating beams [38]. However, the square-root law is still valid for high saturation levels, even if absolute values are lower as predicted by the simulation (see the double-logarithmic inset). All in all, the comparative theoretical–experimental data in figure 6 underscores the key relevance of the central minimum to the optical performance of a STED microscope.

3.3. Axial separation of identically fluorescing objects

To demonstrate the improved axial resolution of the STED-4Pi microscope, we imaged glass microspheres of 100 nm diameter embedded in a Pyridine 2 solution placed on the surface of a coverslip. The fluorescent dye adhered both to the coverslip and to the surface of the microspheres. A typical axial comparison image resulting from an \( xz \)-scan of the sample is given in figure 7. The confocal \( xz \)-image of figure 7(a) shows a blurred vertical line that is occasionally brighter at certain sites, but completely unresolved along the \( z \)-axis. In contrast, in the STED-4Pi \( xz \)-image (b) the glass particles are recognized by a signal that is spatially distinct from the fine fluorescent layer on top of the cover slip. In this implementation of the STED-4Pi microscope, the lateral resolution is not improved; it remains at an estimated 200 nm at the used wavelength. As a consequence, the glass bead signal appears as a laterally flat line structure that is averaged out in the lateral (\( xy \)) direction. Whereas the confocally recorded axial profile does not exhibit any substructure, in its STED-4Pi counterpart, the labelled glass particle is recognized at a distance of 76 nm from the surface by a 35% intensity dip. The \( z \)-profile in panel (c) shows this separation quantitatively.

The super-resolved image was recorded with the regular STED-4Pi microscope by focusing 12.2 mW of average STED power. The concomitant peak fluence was \( J_{\text{max}} = 5.8 \times 10^{17} \text{ cm}^{-2} \). Amounting to \( \varsigma = 127 \), the saturation factor was rather high, indicating that in the experiment of figure 7 the practical resolution limit was ultimately imposed by the non-perfect zero at the focal centre.

4. Conclusion and outlook

We have calculated the effective PSF and OTF of STED-4Pi microscopy using a vectorial theory taking into account the polarization of the light field of the focused STED pulse, as well as the random orientation of the transition dipole for excitation and stimulated emission. The resulting theoretical predictions are found to be in remarkable agreement with the experimental data. The agreement is particularly evident in figure 1 showing the depletion of the fluorescent state by stimulated emission of randomly dispersed molecules in a layer.

Small but noteworthy discrepancies between theory and experiment are found where the mathematical form of the intensity zero of the STED-PSF played a significant role. This is the case at comparatively high levels of saturation. The deviations ultimately stem from the fact that the creation of a central focal minimum is rather sensitive to non-mirror-symmetrical phase aberrations induced by the sample or the optical elements themself. The resulting practical deviation of the intensity minima from theory favourably leads to even narrower central
fluorescence spots. However, this narrowing is bought at the expense of slightly higher sidelobes in the effective STED-4Pi PSF which fortunately always are much below the critical 50% value.

The challenge with an asymmetrically aberrated STED-4Pi PSF is that the resulting non-zero fluence of the central minimum of the STED-PSF reduces the fluorescence signal with increasing STED beam power. Therefore, the residual central fluence of the stimulating beam currently imposes a practical limitation on the attainable resolution. A potential remedy against asymmetric aberrations is a dynamic wavefront correction with active optical elements.

Wavefront engineering, e.g. by liquid-crystal devices, can be conveniently combined with a desired pupil function modification, such as the two-zone phase filter investigated in this work. An important result of our study is that the induced symmetrical aberration does not decrease the support of the OTF of the microscope. Rather, it favourably demodulates the transmission of the axial frequency band.

Last but not least, our calculations and measurements vividly demonstrate an 8-fold expansion of the axial bandwidth of the OTF of the currently realized STED-4Pi microscope over that of its confocal counterpart. The demonstrated 76 nm axial separation between two objects is by about one order of magnitude below the axial distance of \(~760\text{ nm}\) associated with the Rayleigh criterion for 554 nm excitation light of a single lens.

The breaking of the diffraction barrier is brought about by the saturated optical transition involved in STED, which entails no conceptual resolution limits. This is not in contradiction with the fact that for a given saturation factor \(\varsigma\), the resolution still scales with the wavelength \(\lambda_{\text{STED}}\) used for stimulated emission. Therefore, besides increasing \(\varsigma\), the resolution reported here can further be augmented just by using dyes operating at a shorter wavelength \(\lambda_{\text{STED}}\). Since in our experiments \(\lambda_{\text{STED}}\) was about 750 nm, an immediate increase by another 30\% should be possible by applying \(\lambda_{\text{STED}}\sim500\text{ nm}\). Thus, both our theoretical and experimental studies indicate that a much higher axial resolution will be possible in the future. In fact, attaining an axial resolution of \(~20\text{ nm}\) should be possible just by adapting shorter wavelength dyes and/or perfecting the optical system.

References


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