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# Ground-state-depletion fluorscence microscopy: a concept for breaking the diffraction resolution limit

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**Abstract.** We introduce and study a novel concept in far-field fluorescence microscopy fundamentally overcoming the classical diffraction resolution limit. This is accomlished by reducing the spatial extent of the effective focus of a scanning fluorescence microscope. The reduction is achieved by depleting the ground-state energy of the molecules located in the outer region of the focus. Our theoretical study shows that ground-state-depletion fluorescence microscopy has the potential of increasing the resolution of far-field fluorescence microscopy by an order of magnitude which is equivalent to a lateral resolution of 15 NM.

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For many years, it has been widely considered that far-field optical microscopy had reached its resolution limits. This consideration led to the development of new microscopical techniques such as electron and optical near-field microscopes. These techniques, however, are confined to the observation of the specimen's surface and not able to deliver three-dimensional images of intact translucent specimens, e.g., living plants, animal or human cells. Therefore, overcoming the resolution limits of far-field light microscopy is not only a challenging physical task but also of high interest for biological investigations.

The most important contrast in biological light microscopy is fluorescence, because it can be much more specific and sensitive than reflectance or adsorbance. In a threoretical study introducing the Stimulated-Emission-Depletion (STED) fluorescence microscope, Hell and Wichmann have shown the possibility of obtaining lateral resolutions of 35 nm in the far field [1]. In a STED fluorescence microscope, the diffraction resolution limit is overcome by depleting the excited state of the fluorescing molecules in the outer region of the focus. Depending on the intensity used for the stimulating beam, the extent of the excitation point-spread function of the scanning microscope is effectively reduced by a factor 2–5. The depletion is performed by the effect of stimulated emission. However, depleting excited molecules requires a stimulation rate stronger than the fluorescence decay, but slower than the decay rate of the intrastate vibrational relaxations [1]. For a STED luorescence microscope picosecond lasers are required. In this publication, we introduce the concept of Ground-State-Depletion (GSD) fluorescence microscoy, a method having the potential of achieving far-field lateral resolutions of 10–20 nm with low-power continuous-wave illumination.

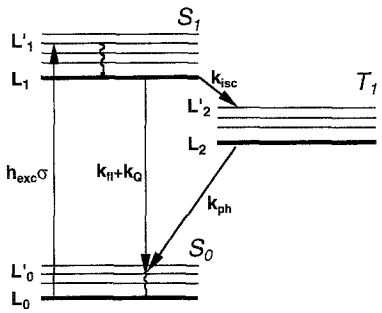


**Fig. 1.** Energy states of a typical dye: $S_0$ is the ground state, $S_1$ is the first singlet state, and $T_1$ the first triplet state

Figure 1 shows the energy states of a typical fluorophore. $S_0$ is the ground state and $S_1$ the first singlet state. As we intend to employ low-power continuous-wave light, we also have to consider the first triplet state $T_1$. $L_0$ is a low vibrational level of $S_0$, $L_1'$ the directly excited Franck-Condon state in $S_1$, $L_1$ the vibrationally relaxed state in $S_1$, $L_1$ the vibrationally relaxed state in $S_1$, $L_2$ a vibrationally relaxed level of $T_1$ and $L_0'$ a vibrationally higher level of the ground state. Since the vibrational relaxations are of the order of picoseconds, one is not able to significantly populate the states $L_0'$ and $L_1'$ with low-power continuous wave-light. We therefore restrict our study to the vibrationally relaxed states $L_0$, $L_1$, and $L_2$.

With an excitation photon flux of $h_{\mathrm{exc}}$, the population probabilities $n_{0,1,2}$ of the fluorophore are given by:

$$\begin{aligned}
\frac{dn_0}{dt} &= -h_{\mathrm{exc}}\sigma n_0 + \left(k_{\mathrm{fl}} + k_{\mathrm{Q}}\right) n_1 + k_{\mathrm{ph}} n_2 \,, \\
\frac{dn_1}{dt} &= +h_{\mathrm{exc}}\sigma n_0 - \left(k_{\mathrm{fl}} + k_{\mathrm{Q}}\right) n_1 - k_{\mathrm{isc}} n_1 \,, \\
\frac{dn_2}{dt} &= +k_{\mathrm{isc}} n_1 - k_{\mathrm{ph}} n_2 \,,
\end{aligned} \tag{1}$$

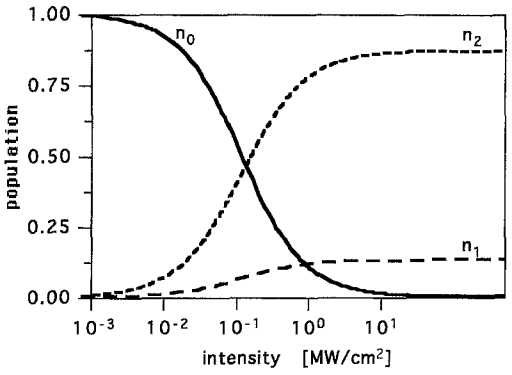


**Fig. 2.** The population probability of the ground state ($n_0$), the first singlet state ($n_1$), and the triplet state ($n_2$) as a function of the excitation intensity for fluoresceine

with $\sum_i n_i = 1$. The parameters $k_{fl} = 1/\tau_{fl}$ and $k_Q = 1/\tau_Q$ are the fluorescence and quenching rate, respectively, $k_{isc} = 1/\tau_{isc}$ is the inter-system crossing rate from the excited singlet to the triplet state, $k_{ph} = 1/\tau_{ph}$ is the decay rate of the triplet state. The rates are the reciprocal values of the respective lifetimes $\tau$. Having lifetimes of $\tau_{ph} = 1\mu s - 1$ ms, the triplet decay rate $k_{ph}$ is the slowest rate involved in the process. When switching on the continuous-wave excitation light, we can assume that a stationary state has been reached after $t \approx 5\tau_{ph}$. The population probabilities $n_i$ of the fluorophore do not undergo any further changes: $dn_i/dt = 0$, and reach the values:

$$n_0 = \frac{k_{ph}(k_{fl} + k_Q + k_{isc})}{D},$$
$$n_1 = \frac{h_{exc}\sigma k_{ph}}{D}, \qquad (2)$$
$$n_2 = \frac{h_{exc}\sigma k_{isc}}{D},$$

with $D = (h_{exc}\sigma + k_{fl} + k_Q)(k_{ph} + k_{isc}) + k_{isc}(k_{ph} - k_{fl} - k_Q)$.

Figure 2 displays the population probabilities $n_i$ as a function of the excitation intensity for fluoresceine which is one of the most frequently used dyes for biological fluorescence labelling. The averge life times for the energy states of fluoresceine are $\tau_{fl} + \tau_Q = 4.5$ ns, $\tau_{isc} = 100$ ns, and, $\tau_{ph} = 1$ $\mu$s [2], where an excitation wavelength of 488 nm was assumed. Figure 2 reveals that for an intensity higher than 10 MW/cm$^2$, 87% of the fluorophore molecules are in the long-lived triplet state, $T_1$, 13% are in the singlet state, and the ground state is depleted. An intuitive explanation is that for high intensities, the molecules undergo fast circular processes from $S_0$ to $S_1$ and back to $S_0$. After each loop, a fraction of $k_{isc}/(k_{isc} + k_{fl} + k_Q)$ is caught in the long-lived triplet state, ultimately depleting the ground state. The ground state remains depleted as long as the excitation beam is switched on, and for its average lifetime $\tau_{ph}$ after it has been switched off.

As a next step, we investigte how the effect of ground-state depletion is employed for increasing the resolution in a far-field fluorescence light microscope. For a uniformly illuminated lens, the intensity distribution in the focal region is given by the Airy intensity distribution:

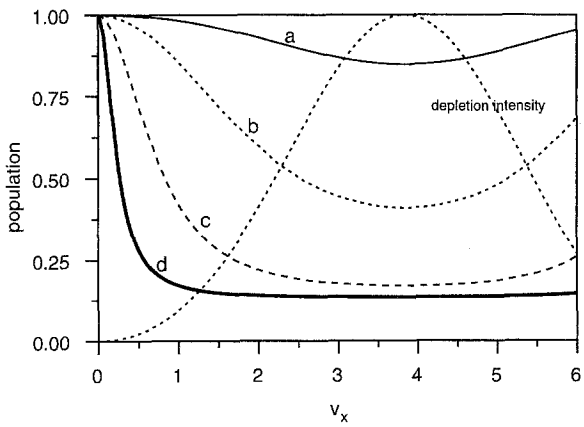


**Fig. 3.** The effect of the depletion beam $h_{depl}(v)\,|_x$ (*dashed*) on the probability of the dye not to be in the triplet state, $1 - n_2(v)$, for the maximum of $h_{depl}(v)\,|_x$ of (*a*) 0.001, (*b*) 0.01, (*c*) 0.1 and (*d*) 1 MW/cm$^2$

$$h(v) = \text{const}\left(\frac{2J_1(v)}{v}\right), \qquad (3)$$

where $J_1$ is the first-order Bessel function, and $v = 2\pi r \text{NA}/\lambda$ is the optical unit in the focal plane, $r$ is the distance from the focal point, NA is the numerical aperture of the lens, and $\lambda$ the wavelength of the excitation light. We assume two beams being symmetrically offset by $\pm\Delta v$ with respect to the geometric focus, say, alone the $x$-axis. For an offset of $\Delta v_x = 1.22\pi$, the first minima of the beams coincide at the geometrical focus, whereas the main maximum of one beam partly overlaps with the first side maximum of the other. The intensity $h_{depl}(v)\,|_x = h_{depl}(v - \Delta v_x) + h_{depl}(v + \Delta v_x)$ of the resulting beam is shown in Fig. 3.

It also shows the effect of the different intensities of $h_{depl}(v)\,|_x$, namely 0.01, 0.1, and 1 MW/cm$^2$ on the probability $1 - n_2(v)$, which is the probability of the dye not to be caught in the triplet state. For lower intensity values, $h_{depl}(v)\,|_x$ leaves a hole in the distribution of about the sample shape as $h_{depl}(v)\,|_x$. For higher intensities, the saturation of the triplet state becomves evident but at $v = 0$, $h_{depl}(v)\,|_x$ has a minimum and the molecules in the closest vicinity of $v = 0$ remain in the ground state. Due to the saturation process, the unaffected regions around $v = 0$ are bordered by steep edges of depletion.

This effect can be exploited for increasing the resolution in far-field fluorescence light microscopy. First, the molecules at the regions surrounding the point of interest (located preferrably at $v = 0$) are exposed to a beam depleting the ground state. After about $\tau_{ph} \approx 1$ $\mu$s, the depletion beam $h_{depl}(v)\,|_x$ is switched off, and after $\tau_{fl} \approx 5$ ns nearly all the molecules from the first singlet state are relaxed. But for a time interval of about $\tau_{ph}/5$, the molecules being caught in the triplet state have still not returned to the ground state. Therefore, the population distribution of excitable molecules is given by $1 - n_2(v)$. When focusing with a further (probing) beam centered at $v = 0$, the effective excitation point-spread function is given by:

$$h_{eff}(v) = h_{exc}(v)[1 - n_2(v)]. \qquad (4)$$

Figure 4 shows the calculated effective point-spread function $h_{eff}(v)$ along the axis of the offset. One notices a decreasing FWHM with increasing maximum intensities of

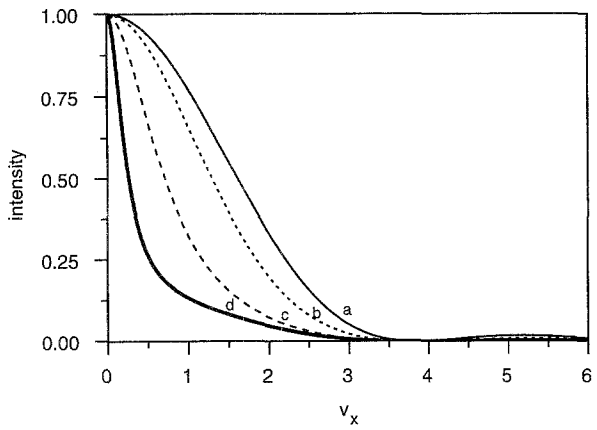


**Fig. 4.** Calculated effective point-spread function along the axis of the offset for peak intensities of (*b*) 0.01, (*c*) 0.1, and (*d*) 1 MW/cm$^2$ of the depletion beam as calculated for fluorescein, compared with the point-spread function of a classical scanning fluorescence microscope

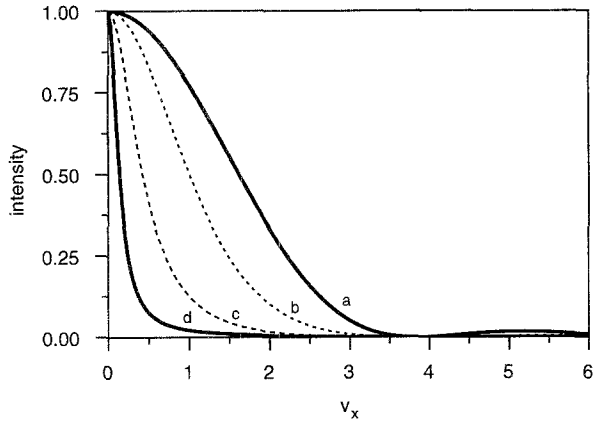


**Fig. 5.** Calculated effective point-spread function along the axis of the offset for peak intensities of (*b*) 0.01, (*c*) 0.1, and (*d*) 1 MW/cm$^2$ of the depletion beam as calculated for a dye having similar parameters as in Fig. 4 but a (fast) intersystem crossing rate of (10 ns)$^{-1}$

$h_{\mathrm{depl}}(v)\,|_x$. For maximum intensities $h_{\mathrm{depl}}(v)\,|_x$ of 0.01, 0.1, and 1 MW/cm$^2$, one obtains 2.6, 1.44, and 0.52 in optical units. For $h_{\mathrm{depl}}(v)\,|_x$= 1 MW/cm$^2$, the resolution is considerably enhanced, the FWHM is 6 times smaller than that of a classical scanning fluorescence microscope. However, one can also notice that the width of the peak is not significantly reduced at the bottom. This is because the ground state is not entirely depleted in our example using fluorescein.

As the depletion efficiency depends primarily on $k_{\mathrm{isc}}/(k_{\mathrm{isc}}+k_{\mathrm{fl}}+k_{\mathrm{Q}})$, for a fluorophore having a faster intersystem crossing time, e.g., of $k_{\mathrm{isc}}$ = (10 ns$^{-1}$) [3], the ground state can be depleted down to 2%. This leads to an efficient reduction of the extent of the effective excitation point-spread function, as shown in Fig. 5. A comparison of the point-spread-function obtained for a focal intensity of 1 MW/cm$^2$ with that of a classical scanning fluorescence microscope shows an increase in resolution by a factor of 11. Assuming an excitation wavelength of 400 nm and a numerical aperture of 1.4, one obtains a lateral FWHM of about 15 nm.

When illuminating a 1.4 NA oil immersion lens with a power of 1 mW, an average focal intensity of 1 MW/cm$^2$ is obtained. Therefore, the intensities required for GSD fluorescence microscopy are easily achievable in practice. The low power is an advantage of GSD fluorescence microscoy. An intrinsic limitation of GSD fluorescence microscopy is that the maximum pixel rate will be determined by the relaxation of the dye from the triplet state, i.e., for recording the neighbouring point, one has to wait until all the molecules are in the ground state again. This delay is approximately $5\tau_{\mathrm{ph}} \approx 5\mu$s, thus determining the maximum recording speed to about 200 kHz, which is of the same order of that of a standard beam scanning confocal microscope. A further aspect that might play a role is that the molecules in the long-lived triplet state undergo chemical reactions, thus causing a bleaching of the fluorescence molecules. As these phenomena depend on the environment, a carefully chosen chemical environment might be advantageous or required for the proper performance of the dye. Selecting a dye that shows the most favourable conditions for ground-state depletion is a useful approach in the practical development of a GSD fluorescence microscope. To obtain an equivalent resolution increase in the $y$-axis, one would use an additional and similar pair of depletion beams. One could also imagine especially prepared focal-intensity distributions of depletion beams that are concentric around the geometric focal point. Furthermore, point-like spatial detection (confocal operation) is advantageous since it provides three-dimensional imaging. The latter is particularly interesting for biological imaging.

In conclusion, we have introduced and theoretically studied the concept of ground-state depletion microscopy for increasing the resolution of a far-field fluorescence microscope. Our calculations show that is possible to fundamentally overcome the diffraction resolution limit in a scanning fluorescence microscope. We found that for carefully selected conditions of a fluorophore it is possible to increase the resolution of a far-field fluorescence light microscope up to an order of magnitude.

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