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Superresolution Microscopy on the Basis of Engineered Dark States

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Recent advances in fluorescence microscopy have broadened our imagination of what is possible using far-field fluorescence microscopy. New concepts such as stimulated emission depletion (STED) microscopy and subdiffraction resolution imaging by subsequent localization of single molecules (i.e. concepts denoted STORM, dSTORM, PALM, FPALM, etc.) are rapidly emerging and provide new ways to resolve structures beyond the 200 nm scale.[1−9] Due to their conceptual and technical simplicity, the latter techniques based on wide-field imaging of single fluorophores have spread enormously. Yet, a severe disadvantage of these techniques is that they rely on photoactivatable or photoswitchable fluorophores limiting their general applicability and multiplexing capacity.[10−14]

The common principle of subdiffraction resolution microscopy by subsequent single-molecule localizations is that only one point-like light source is active for a diffraction limited area at any time. This fluorophore is localized by imaging with a sensitive camera. In the beginning of data acquisition all except a few fluorophores are prepared in the off-state and the number of active fluorophores is kept constant by applying an activating light source that compensates for the loss of fluorophores by photobleaching or switching off. Instead of stroboscopic alternating illumination to prepare the desired number of molecules in the on-state and then read-out and switch-off again, more recent schemes apply simultaneous excitation with both the read-out/switch-off wavelengths and the activating wavelength.[7,15] Here the intensities of the lasers are adapted to balance the rate of switching molecules on and off.

In this communication, we engineer on- and off-states by controlling the photophysics of the fluorophores by electron transfer reactions. We exploit these engineered dark states for subdiffraction resolution fluorescence microscopy of single molecules, actin filaments, and microtubules in fixed cells. This strategy is essentially applicable to all synthetic, single-molecule compatible fluorophores and not restricted to a few photoswitchable or photoactivatable derivatives, since dark states ("blinking") can be induced for every fluorophore, e.g., by increasing the triplet state lifetime through oxygen removal or by inducing radical-ion states. The key issue to using this blinking for superresolution microscopy is to localize a sufficient number of fluorophores within a diffraction limited spot. This number is directly linked to the ratio of the off-times to the on-times of the fluorophores as well as to the data acquisition speed. To be able to resolve as many fluorophores as possible in a diffraction limited spot it is the aim to have very short but bright on-states and long off-states. On the other hand controlling the off-state duration with a balance of fast acquisition and high resolution is desired.[16] To this end, the length of the off-state limits the achievable spatial resolution since due to technical limitations of the camera's acquisition speed the resolution cannot be improved infinitely by reducing on-state duration through high excitation intensities. The natural lifetime of the triplet state typically is in the lower millisecond range in the absence of oxygen and poses an upper limit of the dark state duration. We suggest using electron transfer reactions to extend the range of achievable on- and off-times.[17] With freshly dissolved ascorbic acid (AA) we can extend the off-state lifetimes of many fluorophores severalfold.

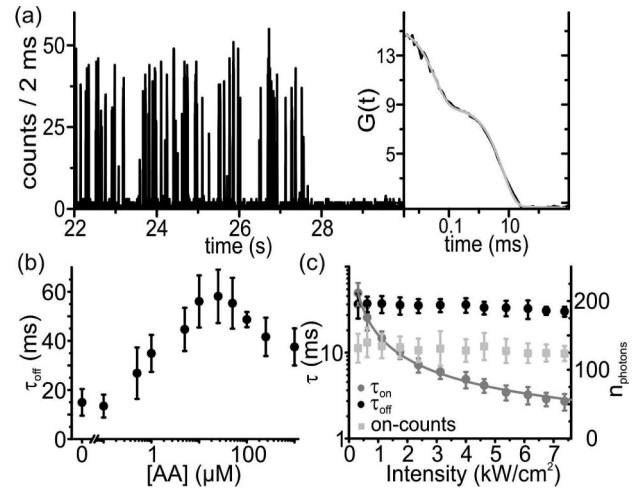

Figure 1. Controlling blinking for superresolution microscopy. (a) Part of a fluorescence transient of single Cy5 in oxygen depleted phosphate buffered saline (PBS) with ascorbic acid (AA, 100 μM, 2 ms binning). Inset shows the autocorrelation function yielding $\tau_{cis-trans} = 70\ \mu s$ and $\tau_{off} = 60$ ms. (b) Dependence of τ_{off} on AA concentration. (c) Dependence of τ_{on}, τ_{off}, and the number of photons detected per on-state (on-counts) on excitation power (c(AA) = 200 μM).

Figure 1a shows a fluorescence intensity transient of a single Cy5 labeled DNA in PBS after enzymatic removal of oxygen and addition of 100 μM AA as well as the corresponding autocorrelation function with a biexponential fit. The short fluctuation component with an off-time of 70 μs is ascribed to cis−trans isomerization,[10] and the long component of $\tau_{off} = 60$ ms is ascribed to the formation of a radical anion (assuming a neutral fluorophore).[17] In the absence of AA, the long off-state represents the triplet state with $\tau_T = (14 \pm 5)$ ms (Figure 1b). By increasing the AA concentration the lifetime of the long off-state increases up to (60 ± 8) ms since the triplet state is reduced to a radical anion (Figure 1b). At higher AA concentrations the off-states become slightly shorter likely due to an oxidizing impurity.[17]

Next we studied control of the on-state lifetime and the localization precision that can be obtained from the available counts emitted during an on-state (on-counts). The on-state lifetime is reduced by increasing the excitation intensity since dark-state formation is a photoinduced process. The on-times are reduced to ~2 ms already at moderate excitation intensities (Fig. 1c), i.e., a time typical for current CCD camera maximum acquisition speeds. The number of photons detected per-on-time remains constant at ~130 photons (Figure 1c) and is sufficient for a localization precision of single molecules of $\sim s/(n^{1/2}) = 22$ nm, where s represents the standard deviation of the point-spread-function.[18] It

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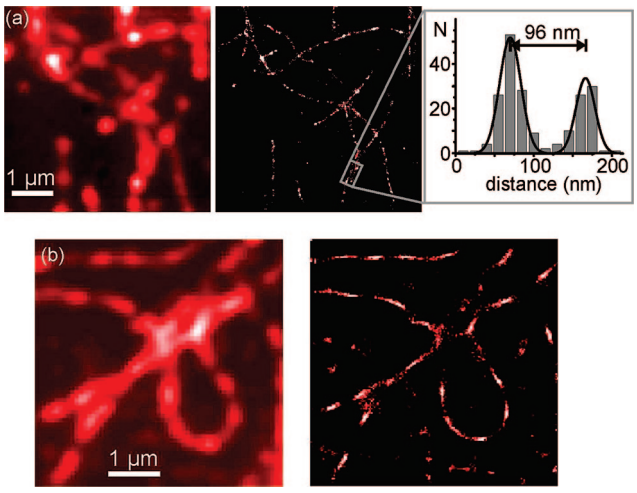

Figure 2. (a) Total internal reflection (left) and "Blink Microscopy" (right) images of actin filaments labeled with Alexa647. A cross-sectional profile of adjacent actin filaments is shown in the right panel. (b) Wide-field (left) and superresolution (right) images of microtubule in fixed 3T3 fibroblast labeled with Alexa647-Fab-fragments.

is also important that the off-times are not affected by the excitation intensity (Figure 1c) since triplet lifetimes are often decreased at higher excitation intensities due to reverse intersystem crossing.[19−22]

Similar control of dark states can essentially be carried out for every fluorescent dye. We found triplet lifetimes and radical anion lifetimes of ATTO565 to go from (6 ± 2) ms to (20 ± 5) ms, of Cy3B from (11 ± 4) ms to (33 ± 12) ms, for Alexa 647 from (13 ± 3) ms to (52 ± 8) ms, and for ATTO647N remaining at (28 ± 7) ms upon addition of AA to oxygen depleted solutions. Accordingly, for all investigated fluorophores dark states of >20 ms are created that can be exploited for "Blink Microscopy".

Recently the trade-off between spatial resolution and acquisition time has been discussed.[16] Using blinking molecules for subdiffraction resolution imaging imposes a lower limit for the temporal resolution as well as an upper limit for the spatial resolution. This is because the duty cycle limits the number of fluorophores that can be distinguished within the area of a diffraction limited spot (simulations and a discussion regarding this issue as well as experimental details are provided as Supporting Information). Simulations based on a regular grid of single molecules show that, for on-times of 1 ms and off-times of 60 ms at a camera frame rate of 1 kHz and a signal-to-noise ratio of 20, a resolution of <50 nm is obtained.[16] The simulations further indicate that the increase of τ_{off} by a factor of 4 using radical ion states instead of triplet states is crucial, since for an off-time of 15 ms a resolution of only ∼100 nm is obtained.

Figure 2a shows images of immobilized actin filaments labeled with Alexa647 recorded after enzymatic oxygen removal and addition of 100 μM AA. A clear signature of individual filaments is resolved in the middle image demonstrating the resolution enhancement. The right panel of Figure 2a shows a cross-sectional profile of two parallel actin filaments at a distance of 96 nm, i.e., a distance not resolvable by conventional fluorescence microscopy. Equivalent photophysical conditions can also be adjusted in fixed cells. Figure 2b shows a wide-field image and the corresponding superresolution image of microtubules labeled with Alexa647-Fab-fragments in a fixed 3T3 fibroblast.

In summary, we demonstrate the applicability of subdiffraction resolution microscopy to essentially all synthetic, single-molecule fluorophores without the need for photoswitchable or photoactivatable fluorophores. Controlling the photophysics of single fluorophores allows engineering dark states of desired duration. Shorter dark states are always achievable through addition of low concentrations of oxidant and enable very fast image acquisition.[17] Using camera integration times of 1 ms, meaningful images are acquired at a rate of >1 Hz depending on the desired resolution. In addition the rate of blinking obeys a monoexponential distribution and is not limited by multistep activation pathways.[10,23] Resolution enhancement based on blinking molecules might also be possible in living cells if a fluorophore with redox properties adapted to intracellular conditions, i.e., redox blinking in living cells, was found.

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Supporting Information Available: Experimental methods, results from single molecules, simulations, and a discussion of obtainable resolution. This information is available free of charge via the Internet at http://pubs.acs.org/.

References

(1) Klar, T. A.; Jakobs, S.; Dyba, M.; Egner, A.; Hell, S. W. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8206–8210.
(2) Hell, S. W. *Science* **2007**, *316*, 1153–1158.
(3) Rust, M. J.; Bates, M.; Zhuang, X. *Nat. Methods* **2006**, *3*, 793–795.
(4) Betzig, E.; Patterson, G. H.; Sougrat, R.; Lindwasser, O. W.; Olenych, S.; Bonifacino, J. S.; Davidson, M. W.; Lippincott-Schwartz, J.; Hess, H. F. *Science* **2006**, *313*, 1642–1645.
(5) Huang, B.; Wang, W.; Bates, M.; Zhuang, X. *Science* **2008**, *319*, 810–813.
(6) Hess, S. T.; Girirajan, T. P.; Mason, M. D. *Biophys. J.* **2006**, *91*, 4258–4272.
(7) Heilemann, M.; van de Linde, S.; Schuttpelz, M.; Kasper, R.; Seefeldt, B.; Mukherjee, A.; Tinnefeld, P.; Sauer, M. *Angew. Chem., Int. Ed.* **2008**, *47*, 6172–6176.
(8) Bretschneider, S.; Eggeling, C.; Hell, S. W. *Phys. Rev. Lett.* **2007**, *98*, -.
(9) Bock, H.; Geisler, C.; Wurm, C. A.; Von Middendorff, C.; Jakobs, S.; Schonle, A.; Egner, A.; Hell Stefan, W.; Eggeling, C. *Appl. Phys. B: Lasers and Optics* **2007**, *88*, 161–165.
(10) Heilemann, M.; Margeat, E.; Kasper, R.; Sauer, M.; Tinnefeld, P. *J. Am. Chem. Soc.* **2005**, *127*, 3801–3806.
(11) Lord, S. J.; Conley, N. R.; Lee, H. L.; Samuel, R.; Liu, N.; Twieg, R. J.; Moerner, W. E. *J. Am. Chem. Soc.* **2008**, *130*, 9204–9205.
(12) Flors, C.; Hotta, J.; Uji-I, H.; Dedecker, P.; Ando, R.; Mizuno, H.; Miyawaki, A.; Hofkens, J. *J. Am. Chem. Soc.* **2007**, *129*, 13970–13977.
(13) Bates, M.; Blosser, T. R.; Zhuang, X. *Phys. Rev. Lett.* **2005**, *94*, 108101/108101–108101/108104.
(14) Bates, M.; Huang, B.; Dempsey, G. T.; Zhuang, X. *Science* **2007**, *317*, 1749–1753.
(15) Egner, A.; Geisler, C.; von Middendorff, C.; Bock, H.; Wenzel, D.; Medda, R.; Andresen, M.; Stiel, A. C.; Jakobs, S.; Eggeling, C.; Schonle, A.; Hell, S. W. *Biophys. J.* **2007**, *93*, 3285–3290.
(16) Shroff, H.; Galbraith, C. G.; Galbraith, J. A.; Betzig, E. *Nat. Methods* **2008**, *5*, 417–423.
(17) Vogelsang, J.; Kasper, R.; Steinhauer, C.; Person, B.; Heilemann, M.; Sauer, M.; Tinnefeld, P. *Angew. Chem., Int. Ed.* **2008**, *47*, 5465–5469.
(18) Thompson, R. E.; Larson, D. R.; Webb, W. W. *Biophys. J.* **2002**, *82*, 2775–2783.
(19) Tinnefeld, P.; Buschmann, V.; Weston, K. D.; Sauer, M. *J. Phys. Chem. A* **2003**, *107*, 323–327.
(20) English, D. S.; Harbron, E. J.; Barbara, P. F. *J. Phys. Chem. A* **2000**, *104*, 9057–9061.
(21) Ringemann, C.; Schonle, A.; Giske, A.; von Middendorff, C.; Hell, S. W.; Eggeling, C. *ChemPhysChem* **2008**, *9*, 612–624.
(22) Tinnefeld, P.; Hofkens, J.; Herten, D.-P.; Masuo, S.; Vosch, T.; Cotlet, M.; Habuchi, S.; Muellen, K.; De Schryver, F. C.; Sauer, M. *ChemPhysChem* **2004**, *5*, 1786–1790.
(23) Habuchi, S.; Ando, R.; Dedecker, P.; Verheijen, W.; Mizuno, H.; Miyawaki, A.; Hofkens, J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9511–9516.

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