Fluorescence Microscopy

DOI: 10.1002/anie.201302341

Improved Super-Resolution Microscopy with Oxazine Fluorophores in Heavy Water**

Steven F. Lee, Quentin Vérolet, and Alexandre Fürstenberg**

Advanced fluorescence microscopy techniques including single-molecule and super-resolution imaging require bright and photostable fluorophores that can be selectively attached to biomolecules. There is therefore an ongoing interest in the development of improved chromophores for biology, especially ones that absorb and emit in the near-infrared owing to the reduced cellular autofluorescence and phototoxicity at these wavelengths.[1] Besides photostability, single fluorophore brightness is an essential parameter in localization-based super-resolution imaging techniques such as (F)PALM, STORM, dSTORM, and GSDIM[2] where images are reconstructed from the localizations of individual fluorophores and the precision σ is dictated by the number of photons N detected from every emitter,[3] with $\sigma \propto N^{1/2}$; the brighter the emitter, the more precisely it can be localized and the better the resolution is in the final image. Nonetheless, with very few exceptions, most commercially available fluorophores excitable beyond 630 nm have poor fluorescence quantum yields with values on the order of 0.3 or lower (Table S1 in the Supporting Information), limiting their brightness.

We introduce here a simple, cost-effective, and biocompatible method to enhance the fluorescence quantum yield of a whole class of commonly used commercially available red-emitting fluorophores (oxazines) such as ATTO655, ATTO680, and ATTO700. We demonstrate that their fluorescence quantum yield in heavy water (D_2O, deuterium oxide) is more than twice that water (H_2O). Although enhancements in fluorescence quantum yield in heavy water have been long known,[4] we make use of this effect in single-molecule imaging and show that it leads to the detection of twice as many photons per oxazine molecule and to a corresponding improvement in the mean localization precision, which enables higher resolution in biological subdiffraction imaging.

Oxazines such as cresyl violet and Nile blue are well-studied laser dyes and histological markers used for their excellent photostability and high fluorescence quantum yields in organic solvents.[4b] ATTO655, ATTO680, and ATTO700 belong to a new generation of oxazines compatible with bioimaging owing to their increased water solubility and reactive functionalities that enable directed covalent coupling. These dyes display absorption maxima between 650 and 700 nm, have fluorescence quantum yields in water of

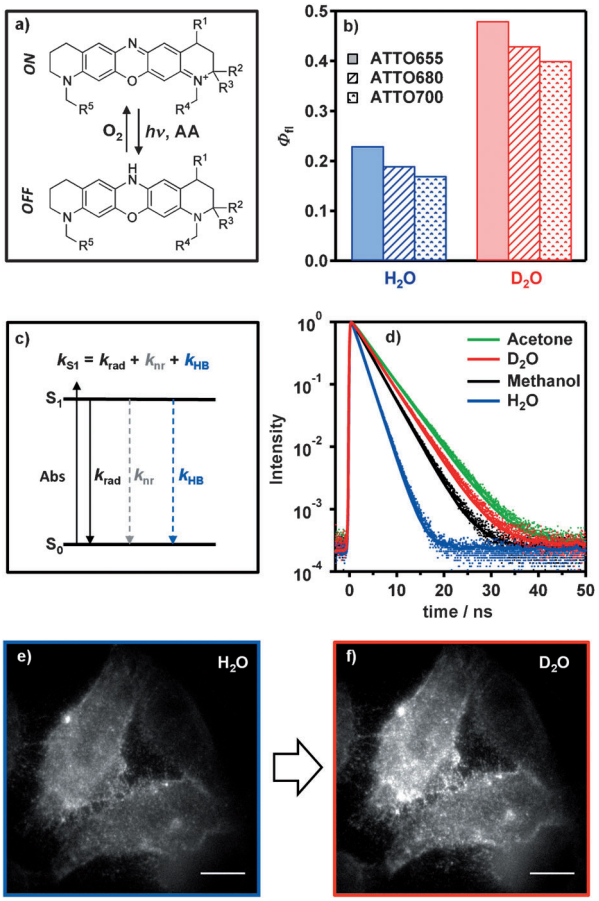

Figure 1. a) Photoswitching scheme of ATTO655 (AA: ascorbic acid).[6b] b) Fluorescence quantum yield of ATTO655, ATTO680, and ATTO700 in H_2O (blue) and in D_2O (red). c) Jablonski diagram for oxazines. In H_2O, hydrogen bonding (k_{HB}) causes strong nonradiative deactivation in addition to the other nonradiative (k_{nr}) and radiative (k_{rad}) pathways. In D_2O and other solvents with weaker hydrogen-bonding capability, the efficiency of this nonradiative process is greatly diminished. d) Fluorescence lifetime of ATTO655 in various solvents. e, f) Fixed CHO cells expressing CCR5 immunostained with ATTO655 and imaged under identical conditions first in H_2O (e) and then in D_2O (f). Images are shown at constant contrast. Scale bar: 5 μm.

[*] Dr. S. F. Lee
Department of Chemistry, University of Cambridge
Lensfield Road, Cambridge, CB2 1EW (UK)
E-mail: sl591@cam.ac.uk
Q. Vérolet, Dr. A. Fürstenberg
Department of Human Protein Sciences, University of Geneva
CMU, Rue Michel-Servet 1, 1211 Genève 4 (Switzerland)
E-mail: alexandre.fuerstenberg@unige.ch

[**] This project was supported by the Swiss National Science Foundation through Ambizione fellowship PZ00P3_131935 and by Dormeur Investment Service Ltd. We thank Oliver Hartley for lab space, support, and cell lines, Thomas Huber for helpful comments, and Eric Vauthey for useful discussions and for sharing equipment including the TCSPC detection system.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201302341.

between 0.17 and 0.23, and exhibit very little solvatochromism (Figures S1–S3 in the Supporting Information). They have found numerous applications in fluorescence imaging, particularly in dSTORM[5] as they can efficiently undergo photoswitching cycles in the presence of a reducing agent (such as cysteamine or ascorbic acid) which push the molecules into a reduced, non-emissive state, with the dissolved oxygen in the ambient solvent restoring the fluorescent state (Figure 1a).[6]

In order to attain a better understanding of why the quantum yield of these dyes is far below unity, we studied their photophysics in different solvents. We found that both the fluorescence quantum yield (Figure 1b) and the fluorescence lifetime (Figure 1d and Figures S4–S6 in the Supporting Information) of oxazines increased with decreasing capability of the solvent to act as a hydrogen-bond donor: for ATTO655, quantum yields varied from 0.23 in water to 0.39 in methanol and 0.46 in ethanol, reaching values as high as 0.57 in solvents unable to act as hydrogen-bond donors (Table 1). Most interestingly, in D_2O, the quantum yield is 0.48 (Figure 1b), 2.1 times higher than that in H_2O and very close to the maximum values observed. Qualitatively identical observations were made for ATTO680 and ATTO700 (Tables S2 and S3, Figures S5 and S6 in the Supporting Information). Overall, our data indicate that the radiative rate is unchanged for these dyes in all solvents and that a nonradiative decay pathway is selectively operative in hydrogen-bonding solvents (Figure 1c), and exclusively responsible for the reduction in the quantum yield. Hydrogen-bond-assisted nonradiative deactivation of the excited state has been described as a fluorescence-quenching mechanism for many fluorophores including oxazines and is known to be much less efficient in deuterated solvents due to a kinetic isotope effect.[4,7] With other fluorophores commonly used in single-molecule imaging but belonging to different dye families, such as ATTO520, ATTO647N, Alexa Fluor 647, and rhodamine derivatives, we also measured an increase in the fluorescence quantum yield in D_2O, but only between 5 and 30% (Table S4 in the Supporting Information), much lower than the 110% detected with ATTO655. The occurrence of this process in oxazines can be rationalized in terms of charge redistribution after photoexcitation and calculations have suggested that the electronic density significantly increases in the excited state, in particular on the central nitrogen and to a lesser extent on the central oxygen of the oxazine chromophore,[8] leading to a strengthening of existing hydrogen bonds. The chemical structure of these ATTO derivatives shares a common central moiety with an increasing number of conjugated double bonds in the outer rings causing the shift in absorption and

Table 1: Photophysical properties of ATTO655 in various solvents: fluorescence quantum yield (Φ_{fl}), excited-state lifetime (τ_{S1}), radiative rate constant (k_{rad}), and sum of rate constants for nonradiative (k_{nr}) and hydrogen-bond-assisted nonradiative deactivation (k_{HB}).

Solvent	Φ_{fl}	τ_{S1} [ns]	k_{rad} [$\times 10^8$ s^{-1}]	$k_{nr} + k_{HB}$ [$\times 10^8$ s^{-1}]
H_2O	0.23	1.9	1.2	4.1
PBS in H_2O	0.23	1.9	1.2	4.1
D_2O	0.48	3.9	1.2	1.4
PBS in D_2O	0.48	3.9	1.2	1.4
methanol	0.39	3.3	1.2	1.8
ethanol	0.46	3.6	1.3	1.5
2-propanol	0.49	3.8	1.3	1.3
acetone	0.55	4.5	1.2	1.0
acetonitrile	0.57	4.4	1.3	1.0
DMF	0.56	4.1	1.4	1.1
DMSO	0.56	3.9	1.4	1.1

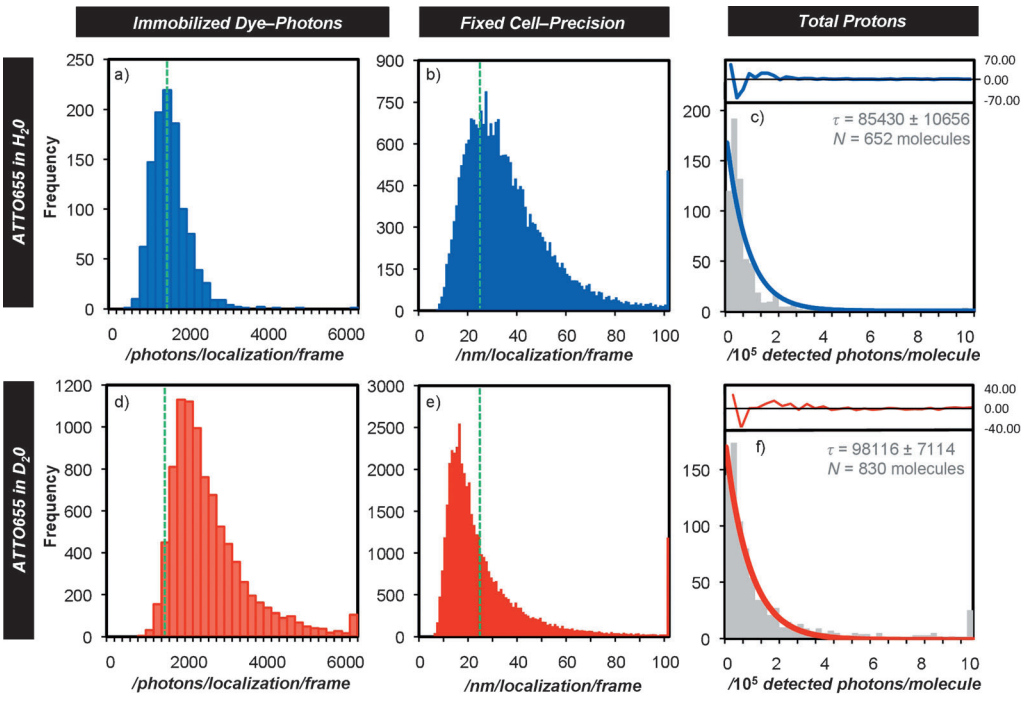

Figure 2. Single-molecule characterization of ATTO655 in both H_2O (top row) and D_2O (lower row). a,d) Number of photons detected from single ATTO655 molecules immobilized on a glass surface for both H_2O (blue) and D_2O (red). b,e) Distribution of localization precisions from dSTORM experiments in fixed cells immunostained with ATTO655 in both H_2O (blue) and D_2O (red). c,f) Total number of photons detected before photobleaching from immobilized ATTO655 molecules imaged in H_2O (blue) and in D_2O (red) and fitted to a single exponential decay with the residuals shown in the upper panels. Where appropriate a dashed green line at the peak frequency bin in H_2O is added to guide the eye.

emission wavelengths.[9] Hydrogen-bond-assisted nonradiative deactivation therefore seems generally operative with both oxazines and other dye classes in aqueous environments but the quenching of fluorescence can be strongly reduced with oxazines by replacing H_2O by D_2O.

The usefulness of this phenomenon was demonstrated by imaging fixed Chinese hamster ovary (CHO) cells stably expressing the C-C chemokine receptor 5 (CCR5),[10] a membrane protein belonging to the G protein coupled receptor family. CCR5 was stained with a primary antibody labeled with ATTO655 and imaged under identical conditions by standard wide-field fluorescence microscopy in H_2O (Figure 1e) and in D_2O (Figure 1f). The image was significantly brighter for the sample in D_2O. Importantly, small amounts of water do not critically affect the process (Figure S7 in the Supporting Information) with a simple removal and replacement of the solvent being sufficient to effect a large increase in brightness. Similarly the presence of salts which contain ^1H atoms such as those in biological buffers have no effect on the fluorescence quantum yield in D_2O due to the relatively low proportion of ^1H atoms, compared to D in the solvent (Figure S8 in the Supporting Information).

To quantify whether the increase in fluorescence quantum yield corresponded to a larger number of photons from individual fluorophores, we covalently immobilized ATTO655 on a glass surface and imaged isolated emitters under dSTORM conditions (Figure S9 in the Supporting Information). As expected, the number of photons detected (Figure 2a,d) in D_2O was approximately twice as high as in H_2O (where the number is consistent with other literature reports[11]) and a corresponding improvement in the localization precision was observed (Figure S10 in the Supporting Information). In fixed cells, similar observations were made (Figure 2b,e) but the complex cellular environment resulted in a broadening of the intensity distribution histogram. Nonetheless, this led to both an increase in the number of photons detected (Figure S11 in the Supporting Information) and to an improved experimentally determined localization precision in D_2O (Figure 2e). An analysis of the fluorescence duty cycle for both solvent conditions did not show significant changes under camera exposure times typical of dSTORM imaging (Figure S12 in the Supporting Information). The total number of photons detected before photobleaching (roughly 10^5) remained unchanged in both solvents (Figure 2c,f) and had values consistent with those of other bright fluorophores.[1a]

The fact that D_2O has no photoprotective effect on the dyes together with the lifetime and the fluorescence quantum yield measurements indicate that the speed (not the rate, see k_{rad} in Table 1) at which the photons are emitted is doubled in D_2O compared to that in H_2O under constant imaging conditions.[12] Simply put, the photon budget remains the same but is spent twice as fast in D_2O.

Finally we demonstrate that super-resolution imaging in D_2O can be used to resolve diffraction-limited subcellular structures. ATTO655-labeled primary antibodies were used to stain CCR5 in the membrane of stably transfected CHO cells where the high density of the receptor in the filopodia served as an excellent platform for structural super-resolution imaging (Figure 3). Under normal dSTORM conditions[5a] isolated emitters were localized (Figure 3c) which enabled a subdiffraction image to be reconstructed (Figure 3b).[13] In some instances both the random positioning of the receptor along the filopodia and the fact that the three-dimensional structure is imaged only in two dimensions allowed the distance between the edges of the filopodia to be measured (Figure 3b, inset), with peak-to-peak separations as small as 128 nm being clearly resolved with a localization precision of roughly 20 nm.

To summarize, we have demonstrated how to increase the brightness of a whole class of dim red-emitting fluorophores

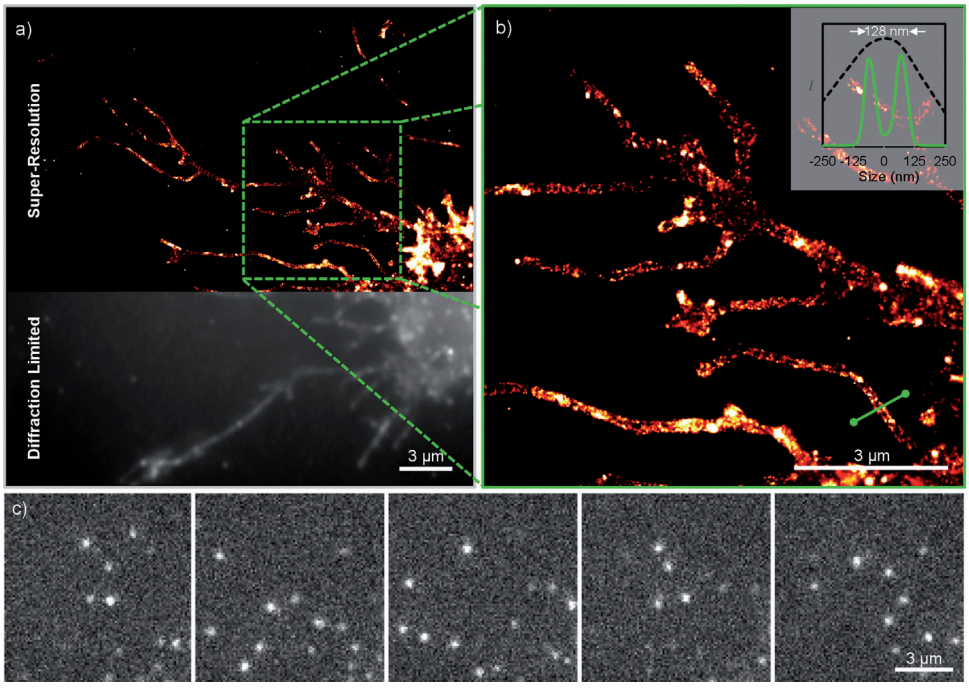

Figure 3. Super-resolution imaging in D_2O. a) Large field of view dSTORM image of the membrane-bound CCR5 receptor (top) compared to conventional diffraction-limited image (bottom). b) Enlarged area of the filopodia where well-defined structures are observed; inset: the filopodium has a peak-to-peak width of 128 nm with a localization precision of roughly 20 nm (green line). This is not resolvable in the diffraction-limited image (black dashed line). c) Imaging frames showing single isolated fluorescent molecules from the same field of view as in (b). Other than the increase in the fluorescence quantum yield, dSTORM imaging was unaffected by the presence of D_2O.

commonly used for biological imaging by replacing H_2O in the imaging buffer by D_2O. This increase is caused by a reduced efficiency of hydrogen-bond-assisted nonradiative deactivation in the deuterated solvent. This effect is in principle applicable to any type of fluorescence imaging involving oxazines, and we show that it can be used in super-resolution imaging to increase the precision with which single molecules are localized. Our newly introduced method is very simple and cost-effective, since D_2O is compatible with biological buffers and only needed at the time of imaging. Experimenting in heavy water would be advantageous to time-sensitive measurements because the rate at which photons are detected is twice as high, allowing for faster imaging. Furthermore in contrast to other recently introduced methods[14] our approach is live-cell compatible.[15] Our results indicate that understanding and modulating the photophysics of fluorophores broadly used in the life sciences is a worthwhile strategy to significantly improve super-resolution imaging techniques.

Received: March 19, 2013
Published online: July 4, 2013

Keywords: dyes/pigments · fluorescence · oxazines · super-resolution microscopy

[1] a) S. J. Lord, N. R. Conley, H.-L. D. Lee, S. Y. Nishimura, A. K. Pomerantz, K. A. Willets, Z. Lu, H. Wang, N. Liu, R. Samuel, R. Weber, A. Semyonov, M. He, R. J. Twieg, W. E. Moerner, *ChemPhysChem* **2009**, *10*, 55 – 65; b) G. Lukinavičius, K. Umezawa, N. Olivier, A. Honigmann, G. Yang, T. Plass, V. Mueller, L. Reymond, I. R. Corrêa, Jr., Z.-G. Luo, C. Schultz, E. A. Lemke, P. Heppenstall, C. Eggeling, S. Manley, K. Johnsson, *Nat. Chem.* **2013**, *5*, 132 – 139; c) C. Wurm, K. Kolmakov, F. Gottfert, H. Ta, M. Bossi, H. Schill, S. Berning, S. Jakobs, G. Donnert, V. Belov, S. Hell, *Opt. Nanosc.* **2012**, *1*, 7; d) K. Kolmakov, V. N. Belov, J. Bierwagen, C. Ringemann, V. Mueller, C. Eggeling, S. W. Hell, *Chem. Eur. J.* **2010**, *16*, 158 – 166; e) K. Kolmakov, V. N. Belov, C. A. Wurm, B. Harke, M. Leutenegger, C. Eggeling, S. W. Hell, *Eur. J. Org. Chem.* **2010**, 3593 – 3610.

[2] a) S. T. Hess, T. P. K. Girirajan, M. D. Mason, *Biophys. J.* **2006**, *91*, 4258 – 4272; b) E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, H. F. Hess, *Science* **2006**, *313*, 1642 – 1645; c) M. J. Rust, M. Bates, X. W. Zhuang, *Nat. Methods* **2006**, *3*, 793 – 795; d) J. Fölling, M. Bossi, H. Bock, R. Medda, C. A. Wurm, B. Hein, S. Jakobs, C. Eggeling, S. W. Hell, *Nat. Methods* **2008**, *5*, 943 – 945.

[3] W. E. Moerner, *J. Microsc.* **2012**, *246*, 213 – 220.

[4] a) L. Stryer, *J. Am. Chem. Soc.* **1966**, *88*, 5708 – 5712; b) R. Sens, K. H. Drexhage, *J. Lumin.* **1981**, *24 – 25*, 709 – 712; c) H. Inoue, M. Hida, N. Nakashima, K. Yoshihara, *J. Phys. Chem.* **1982**, *86*, 3184 – 3188; d) A. Fürstenberg, E. Vauthey, *Photochem. Photobiol. Sci.* **2005**, *4*, 260 – 267.

[5] a) M. Heilemann, S. van de Linde, A. Mukherjee, M. Sauer, *Angew. Chem.* **2009**, *121*, 7036 – 7041; *Angew. Chem. Int. Ed.* **2009**, *48*, 6903 – 6908; b) W. C. Duim, B. Chen, J. Frydman, W. E. Moerner, *ChemPhysChem* **2011**, *12*, 2387 – 2390; c) J. Vogelsang, T. Cordes, C. Forthmann, C. Steinhauer, P. Tinnefeld, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8107 – 8112.

[6] a) S. van de Linde, U. Endesfelder, A. Mukherjee, M. Schuettpelz, G. Wiebusch, S. Wolter, M. Heilemann, M. Sauer, *Photochem. Photobiol. Sci.* **2009**, *8*, 465 – 469; b) T. Kottke, S. van de Linde, M. Sauer, S. Kakorin, M. Heilemann, *J. Phys. Chem. Lett.* **2010**, *1*, 3156 – 3159; c) T. Cordes, M. Strackharn, S. W. Stahl, W. Summerer, C. Steinhauer, C. Forthmann, E. M. Puchner, J. Vogelsang, H. E. Gaub, P. Tinnefeld, *Nano Lett.* **2010**, *10*, 645 – 651.

[7] a) A. Grofcsik, M. Kubinyi, W. J. Jones, *Chem. Phys. Lett.* **1996**, *250*, 261 – 265; b) P. Fita, M. Fedoseeva, E. Vauthey, *J. Phys. Chem. A* **2011**, *115*, 2465 – 2470.

[8] S. Fleming, A. Mills, T. Tuttle, *Beilstein J. Org. Chem.* **2011**, *7*, 432 – 441.

[9] J. Arden-Jacob, K.-H. Drexhage, M. Hammers-Schneider, N. U. Kemnitzer, A. Zilles (ATTO-Tec GmbH), EP2374795A1, 2011.

[10] O. Hartley, H. Gaertner, J. Wilken, D. Thompson, R. Fish, A. Ramos, C. Pastore, B. Dufour, F. Cerini, A. Melotti, N. Heveker, L. Picard, M. Alizon, D. Mosier, S. Kent, R. Offord, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16460 – 16465.

[11] G. T. Dempsey, J. C. Vaughan, K. H. Chen, M. Bates, X. Zhuang, *Nat. Methods* **2011**, *8*, 1027 – 1036.

[12] a) T. Hirschfeld, *Appl. Opt.* **1976**, *15*, 3135 – 3139; b) C. Eggeling, J. Widengren, R. Rigler, C. A. M. Seidel in *Applied Fluorescence in Chemistry, Biology and Medicine*, Springer, Berlin, **1999**, pp. 193 – 240.

[13] a) J. S. Biteen, M. A. Thompson, N. K. Tselentis, G. R. Bowman, L. Shapiro, W. E. Moerner, *Nat. Methods* **2008**, *5*, 947 – 949; b) J. L. Ptacin, S. F. Lee, E. C. Garner, E. Toro, M. Eckart, L. R. Comolli, W. E. Moerner, L. Shapiro, *Nat. Cell Biol.* **2010**, *12*, 791 – 798; c) S. F. Lee, M. A. Thompson, M. A. Schwartz, L. Shapiro, W. E. Moerner, *Biophys. J.* **2011**, *100*, L31 – L33.

[14] a) J. C. Vaughan, S. Jia, X. W. Zhuang, *Nat. Methods* **2012**, *9*, 1181 – 1184; b) J. C. Vaughan, G. T. Dempsey, E. Sun, X. Zhuang, *J. Am. Chem. Soc.* **2013**, *135*, 1197 – 1200.

[15] D. M. Czajka, C. S. Fischer, A. J. Finkel, J. J. Katz, *Am. J. Physiol.* **1961**, *201*, 357 – 362.

