Chapter 3

Photostabilizer-Rhodamine Conjugates for Biomolecule Labelling and STED Microscopy

3.1 Introduction

Small molecule fluorophores are widely used as versatile reagents and fluorescent molecular probes, which have enabled unprecedented insights into chemistry and biological research, and particularly, have emerged as key players for advanced fluorescence microscopy such as single-molecule fluorescence spectroscopy and super-resolution microscopy. Far-field optical microscopy and nanoscopy in its biological applications favor photostable fluorescent dyes with high absorption coefficient and large fluorescence quantum yield (greater than 0.5), low dark triplet state population, low background emission caused by unspecific labeling, good solubility in water, good biocompatibility and reactive group with a linker for conjugation to biological molecules such as proteins or nucleic acids. Meanwhile, red-emitting fluorescent dyes with absorption and emission in the far-red to near-infrared (IR) optical region (longer than 600 nm) have significant advantages for in vivo bioimaging. Comparing to ultraviolet-visible (UV-vis) light, red light can reduce scattering, drastically lower photo-damage to biological samples, provide good tissue penetration in depth and minimize background interference from cellular auto-fluorescence. One remarkable feature of small organic fluorescent probes is the capability of chemical manipulation to fulfill these desirable properties.

One such class of fluorophores is rhodamine dyes that have been widely used as fluorogenic markers and laser dyes in biological imaging and single-molecule based spectroscopy. In the practical implementation of some new physical concepts, rhodamines helped to overcome the diffraction limit in (far-field) optical microscopy. “Nanoscopic” techniques such as STED (stimulated emission depletion), STORM (stochastic reconstruction microscopy), PALM (photoactivation localization microscopy) and GSDIM (ground-state depletion with individual molecular return) allowed the optical resolution to be improved from about 200–350 nm to 20–35 nm by switching between the dark and the bright states of a fluorescent marker. The labeling specificity in bioconjugation reactions frequently depends on the polarity of the fluorophore and on related parameters, such as the presence of charged groups and solubility in aqueous buffer. To make them compatible with biomolecular applications, rhodamine family with low polarity and water solubility were further developed by the incorporation of various polar residues and adding functional groups for labeling (NHS, maleimide, hydrazide). Recently, Kolmakov and co-workers described the synthesis, properties and applications of larger bathochromic shifted rhodamine derivatives used for STED microscopy. The large red shift is achieved by introducing several fluorine atoms into the phenylring attached to C-9 (mesoposition in the xanthene scaffold),
which are quite reactive towards nucleophiles together with other modifications, such as additional double bonds and cycles, rigidized frame-works and donor substituents at nitrogen atoms. Among the rhodamine derivatives they prepared, the red-emitting rhodamine dye KK114, which turned out to be a bright and photostable and highly water-soluble fluorescent marker in various optical microscopy and nanoscopy techniques. Sulfonic acid groups (SO$_3$H) were introduced, because they are the least reactive and remain intact in many post-synthetic dye modifications.

However, these organic fluorophores still intrinsically suffer from transient excursions to dark states (blinking) as well as irreversible destruction (photobleaching). Intramolecular photostabilization was recently revived as a tool to impart organic fluorophores with “self-healing” or "self-protecting" properties by suppressing triplet-induced photo-damage. To date, biophysical applications of such fluorophore derivatives are rare due to their elaborate multiple-step synthesis, tremendously restricting the choice of fluorophore. In chapter 2 we described a general strategy to covalently link organic fluorophores to a photostabilizer in a simple and modular fashion. This approach uses amide-bond formation with commercially available starting materials. Unnatural amino acids were used as a flexible scaffold to bind organic fluorophores to a photostabilizer on a biomolecular target. Here, we use this strategy on rhodamine-fluorophores (RhodamineB and KK114). Single-molecule fluorescence microscopy showed a significantly increased photostability and suppression of triplet-induced blinking of fluorophore-photostabilizer conjugates compared to the non-stabilized parent fluorophores. Versatile use of RhodamineB- and KK114-photostabilizer derivatives in biomolecular research was demonstrated by labeling of antibodies and proteins in a single step, and by application in solution-based single-molecule Förster resonance energy transfer (smFRET) and super-resolution STED microscopy, where the improved characteristics of our conjugates optimize the sensitivity.

![Figure 3.1. Schematic overview of the synthesis of a photostabilizer-dye construct. In a next the photostabilizer-dye construct can be used to label a biomolecule as done conventionally with commercially available organic fluorophores.](image)
3.2 Results and Discussion

3.2.1 Synthesis and characterization of Photostabilizer-Dye Conjugates

Scheme 3.1. Synthesis of reactive photostabilizer-dye conjugates of RhodamineB for direct labeling of primary amines (compound 4) and thiols (compound 7). The strategy can be extended by a variation of the linker molecule 6.

The photophysical characterization of the photostabilizer-dye conjugates in the previous sections demonstrated the principle improvements in fluorescence emission properties brought by the UAAs scaffolding approach. Yet, our overall goal is to show the potential applicability of this new class of fluorophores in biological research. To achieve this, the most important step is the labeling of biomolecules such as proteins, nucleic acids or antibodies by our strategy. Direct labeling of biomolecules usually requires a complex (multi-step) synthesis process, demanding large amounts of substances (both fluorophore and biomolecular target). However, the amount of biomolecules is unfortunately often limited. To solve this problem, we reduced the synthetic steps needed for labeling to a minimum. We optimized the synthetic strategy by introducing a functional group (i.e. NHS-ester or maleimide) onto the photostabilizer-dye conjugates, rendering them readily available for
coupling with a biomolecular target (Figure 3.1). NPA was again used as a scaffold for this second generation of photostabilizer-dye conjugates.

As a first step, RhodamineB NHS-ester (1) was subjected to NPA (2) under basic conditions to yield 3 (Scheme 3.1). Subsequent activation with NHS/DCC in DMF afforded the NHS-ester derivative of the NPA-RhodamineB conjugate (4). With this compound in hand, we synthesized (5) by directly mixing (4) with ssDNA. The photophysical properties of (5) were characterized by immobilizing it on glass in deoxygenated buffer. In parallel, we intended to use NPA-RhodamineB for direct labeling of proteins. For this purpose, 4 was reacted with 2-maleimidoethylamine (6) to yield a maleimide-derivative of NPA-RhodamineB (7), which can react with the solvent-exposed cysteine residues of the recombinant proteins via Michael addition reactions. (Scheme 3.1, 8).

The ssDNA-photostabilizer-RhodamineB conjugates were isolated by HPLC. As shown in Figure 3.2, the major peak corresponding to ssDNA-NPA-RhodamineB conjugate had a longer retention time of 43 min compared to the pristine DNA, from which the efficiency of the coupling was calculated to be 94%, as determined by the relative integration of the peaks at 260 nm for labelled and unreacted oligonucleotides.

![Figure 3.2. HPLC-characterization and purification of functionalized oligonucleotides: ssDNA-fluorophore and ssDNA-NPA-fluorophore.](image)

After purification, ssDNA-NPA-RhodamineB conjugate was firstly characterized by UV-vis absorption spectroscopy. As shown in Figure 3.3a, two characteristic absorption peaks from DNA (~260 nm) and Rhodamine B (~555 nm) were
observed. Moreover, the UV spectrum of ssDNA-NPA-RhodamineB didn’t show an obvious change as compared to the corresponding ssDNA-RhodamineB conjugate.

Finally, the obtained conjugate was characterized by MALDI-TOF mass spectrometry which revealed certain mass increases compared to the non-modified DNA, further confirming the formation of the desired product. (Figure 3.3b)

**Figure 3.3. a)** UV-VIS absorption spectra of functionalized oligonucleotides: ssDNA-RhodamineB and ssDNA-NPA-RhodamineB showing the characteristic peak of DNA in the UV-range and the strong visible absorption of the dye. **b)** Maldi-TOF mass spectra of DNA-photostabilizer-dye conjugates. The comparison of the spectra of the non-modified DNA with that of the conjugates clearly indicates an increase of the molecular mass originating from coupling of dye (and stabilizer).

### 3.2.2 Photophysical characterization

Similar to the fluorophores studied before, RhodamineB showed strong blinking in deoxygenated PBS-buffer with typical observation times until photobleaching on the timescale of 10-20 seconds (Figure S3.1). Yet contrarily, RhodamineB showed strongly heterogeneous blinking characteristics with very short and longer off-times, even within one trace (Figure S3.1). Consequently, the autocorrelation function of this fluctuating signal could only be described by a biexponential decay with average off-times peaking at 7±4 ms and a significant fraction of values >20 ms, deviating from a normal distribution (Figure S3.1). This characteristic indicates the presence of multiple dark states or heterogeneous dye-environments. Still the blinking off-times were in the same range as determined before for the triplet state of the structurally-related rhodamine fluorophores, e.g., Alexa555 (22±6 ms) or
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ATT0565 (6±2 ms)\(^{12}\) and are hence considered to be triplet-related. Yet, the fluorescence lifetime of RhodamineB was found to be rather homogeneously distributed with an average of 3.1±0.4 ns (Figure S3.1). Strikingly, NPA-RhodamineB on ssDNA (5) showed strongly reduced blinking, an increased photostability resulting in a stable and non-blinking emission pattern with observation times of up to minutes (Figure S3.2), and an increased brightness (Figure S3.3).

### 3.2.3 Biomolecular FRET studies

Using this strategy, we aimed at improving our previous studies of the conformational states of the substrate-binding domain 2 (SBD2) of the ABC transporter GlnPQ.\(^{13}\) We monitored the conformational states of the protein (Figure 3.4a, open unliganded and closed liganded) via single-molecule Förster resonance energy transfer, smFRET, and alternating laser excitation (ALEX) using the green-emitting NPA-RhodamineB dye as the donor and the conventional red-emitting dye KK114\(^{7}\) as the acceptor (as described previously,\(^{13}\) SBD2 (mutant T369C/S451C) was labeled stochastically using a mixture of donor (RhodamineB or NPA-RhodamineB (7) and acceptor fluorophore KK114. The structural rearrangement in this protein upon ligand binding causes changes of the distance between these two labels of about 0.9 nm which is monitored via FRET. ALEX allows to distinguish the desired proteins labeled with both donor and acceptor (donor-acceptor) from those labeled with only donor (donor-only) or only acceptor (acceptor-only). Therefore, the fluorescence emission of the donor-only (F(DD)), that of the acceptor when excited via FRET (F(DA)) and directly via red excitation light (F(AA)) is determined (see Material and Methods). In our smFRET experiments, individual biomolecules were studied for short time periods of a few milliseconds while diffusing through the excitation volume of a confocal microscope. The challenge of such an experiment is to acquire intense fluorescent bursts during the short observation time under the required excitation intensity of 20-100 kW/cm\(^2\). Thereby, especially the donor fluorophore should show minimal blinking and photobleaching with maximized brightness, as achieved for the NPA-RhodamineB (compared to the parent fluorophore, previous chapter). Now, we assessed the brightness of the dyes when tagged to SBD2 and diffusing through our confocal observation spot using the well-established photon-counting histogram (PCH) technique. Figure 3.4b/c shows the histogram of brightness values (as obtained from >8500 single-molecule transits or bursts at 30 kW/cm\(^2\) intensity of the green laser) of the donor alone (F(DD)) and of the acceptor excited via FRET (F(DA)) when applying RhodamineB or its improved NPA-RhodamineB derivative as the donor. While the increase in brightness for NPA-RhodamineB was only subtle for F(DD), a more significant improvement in acceptor
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brightness (F(DA)) was found, which at the end is the essential read-out in smFRET experiments. A straightforward way to further maximize the donor brightness and thus the smFRET performance is to increase the excitation intensity. This, though, is usually limited by dark state populations (such as of the triplet state) that drives the achievable brightness into saturation, making a further increase in excitation intensity futile. However, Figure 3.4d shows that NPA-RhodamineB could be used at significantly higher excitation intensities, since triplet-state population was minimized.

**Figure 3.4.** Improving smFRET-ALEX measurements by using NPA-RhodamineB as a donor and KK114 as acceptor: representative study on the protein GlnPQ-SBD2. **a)** Crystal structures of the SBD2 (T369C/S451C) open (upper panel) and closed state (lower panel, after binding of the ligand glutamine shown in red) with label positions of donor (D) and acceptor (A). **b/c)** Histogram of fluorophore brightness values as determined from PCH on single-molecule transits of labeled SBD2 diffusing through the observation volume of our confocal microscope: comparison of donor-brightness F(DD) (**b**) and acceptor-brightness when excited via FRET, F(DA) (**c**), when instituting RhodamineB (RhoB, grey) and its NPA-derivative (NPA-RhoB, green) as donor (>8500 transits). Excitation intensities of 30 kW/cm² at 532 nm and 20 kW/cm² at 640 nm. **d)** Dependence of the brightness of F(DD) for increasing green excitation intensity: Comparison of NPA-RhodamineB (NPA-RhoB, green) and RhodamineB (RhoB, black) as a donor on SBD2 (> 1500 transits). **e)** Two-dimensional histogram (2D) of joint pair values of S (labeling stoichiometry, dashed lines 0.43 < S < 0.6 donor-acceptor population DA) and E* (FRET-efficiency, i.e. interprobe distance) determined from 24180 transits of SBD2 without ligand. Excitation intensities of 30 kW/cm² at 532 nm and 20 kW/cm² at 640 nm. **f)** Corresponding one-dimensional histograms of E* values for increasing amounts of ligand (from upper to lower panels as indicated). An increased population of SBD2 molecules with increased E* = 0.72 values
(dashed line, closed conformation) becomes obvious (as opposed to the open conformation, \( E^* = 0.59 \), dashed line).

With these optimized properties in mind, Figure 3.4e/f shows the results of our smFRET-ALEX experiments on SBD2 labeled with NPA-RhodamineB (donor) and KK114 as acceptor. Figure 3.4e shows a two-dimensional (2D) histogram of joint values of S and \( E^* \) as determined from >24000 single-molecule transits of unliganded SBD2. While S represents the relative labeling stoichiometry of the protein (donor-only population with \( S > 0.8 \), donor-acceptor population \( DA \) with \( 0.3 < S < 0.8 \), and the acceptor only population with \( S < 0.3 \)), \( E^* \) indicates the FRET efficiency and thus distance between the donor and acceptor (with larger values indicating decreased donor-acceptor distances). Without ligand, we observed a single donor-acceptor population with an average \( E^* \)-value of 0.59 (Figure 3.4e/f, upper panel). This value is in agreement with the \( E^* \) value determined in previous work\(^{13}\) and is indicative of the open protein conformation with 4.9 nm interprobe distance (Figure 3.4a). Absolute differences originated from the varying Förster radius \( R_0 \) of the fluorophore pairs used here compared to those used previously (Cy3B/ATTO647N and Alexa555/Alexa647).\(^{14}\) Upon addition of the ligand (1.1 µM and 200 µM, Figure 3.4f, middle and lower panels) the ALEX-smFRET experiments indicated increasing amounts of a species characterized by \( E^* = 0.72 \), which indicates the “closed” state (with a decreased interprobe distance of \(~4.0 \text{ nm}\)). While a ligand concentration of 1.1 µM is close to the \( K_d \)-value of the protein,\(^{14}\) and consequently results in a mix of open and closed states, a concentration of 200 µM saturates ligand binding and therefore results in a 100% population of the closed state. Our current results are in good agreement with biochemical data from isothermal calorimetry and with our previous smFRET data, confirming our recent hypothesis of an induced-fit type mechanism in GlnPQ.\(^{13}\) Additionally, it confirms successful labeling of the protein with the custom-made NPA derivative and that the results from the FRET-assay are indeed independent of the fluorophore pair that is used.\(^{13}\)

3.2.4 Synthesis of KK114 photostabilizer-dye conjugates

A common tool in cellular far-field fluorescence microscopy is the use of immunofluorescence, where the majority of cells are fixed and individual structures or proteins are tagged using specific primary antibodies and fluorophore-labeled secondary antibodies. In recent years, pioneering developments in far-field fluorescence microscopy, so called super-resolution microscopy have revolutionized cellular imaging, allowing the visualization of biological structures with nanometer-resolution, i.e., beyond the physical diffraction limit that thus far prevented to resolve structures with a precision better than approximately ~250
nm. Given the importance of photostability and brightness in such techniques, we investigated the potential of fluorophores with intramolecular photostabilization for immunofluorescence and specifically super-resolution imaging. Therefore, we have used the NHS-ester of the dye KK114\textsuperscript{9} and its derivative β-Ala-NPA-KK114-NHS (Scheme 3.2) to tag secondary antibodies using standard procedures.

**Scheme 3.2. Synthesis of reactive photostabilizer-dye conjugates of KK114 used for direct labeling of antibodies.**

The synthesis shown in Scheme 3.2 was conducted starting from KK114, which was coupled with commercially available (S)-nitrophenylalanine using conventional peptide coupling reagent HATU to yield 10. The dye conjugate 10 was elongated by one β-alanine moiety, yielding 12. The elongation of the linking site was performed as described for a sulfocysteine-decorated carbopyronine dye.\textsuperscript{9} Such an elongation provided a high hydrolytic stability of the corresponding NHS ester (obtained from the resulting dye conjugate) with 85% of yield. That proved to be true for the NHS ester of NPA-KK114 15 as well. The preliminary experiments showed that the unmodified NHS ester has an insufficient stability and is therefore unsuitable for antibody conjugation.

The NPA-KK114 and NHS ester of NPA-KK114 were isolated by HPLC (see Figure S3.4), and characterized by UV/Vis-absorption spectroscopy (Figure S3.5). After
purification, all compounds run as single peaks in the HPLC-chromatogram (Figure S3.4) and show UV/Vis-absorption peaks characteristic for the respective chromophore (~637 nm) (Figure S3.5). (See the Materials and Methods section and the Supplementary Information for further details on chemical synthesis and characterization of functionalized oligonucleotides and reactive precursor molecules.)

### 3.2.5 Photostabilizer-dye conjugates in confocal and super-resolution STED microscopy

These antibodies were used to specifically immunolabel nuclear pore complexes (NPC) in fixed mammalian PtK2 cells. Figure 3.5a and Figure S3.6 depict that we could successfully apply NPA-KK114 to visualize the spatial distribution of these NPCs and is shown in the representative confocal scanning images. However, it also becomes obvious that these complexes appear as rather large (>250 nm) blurred spots due to the diffraction limit. To increase resolution, we employed super-resolution STED microscopy, which in its most common application adds a STED laser to the confocal microscope that features a focal intensity distribution with a central zero to allow imaging with sub-diffraction spatial resolution. Figure 3.5b and Figure S3.6 clearly show the successful implementation of NPA-KK114 in STED microscopy. Now the NPCs were much better resolved and appeared as much smaller spots (85-90 nm, which is a reasonable value considering the use of primary and secondary antibodies). When compared to conventional KK114, the NPA-KK114 showed an increased photostability under STED conditions. Repeated scanning of the same area of the cell revealed that fading of fluorescence was reduced for NPA-KK114, while brightness was only subtly increased (Figure 3.5d-f, Figure S3.6). This improvement in photobleaching resistance under STED conditions via an intramolecular mechanism highlights future directions towards improved dynamic STED imaging without the need of adding (potential toxic) chemical compounds.
Figure 3.5. Use of photostabilizer-dye conjugates in confocal and super-resolution STED microscopy of immunolabeled cells – data from nuclear pore complexes in fixed mammalian PtK2 cells immunolabeled with KK114 and NPA-KK114. (a/b) Confocal (a) and STED (b) image of a representative cell stained with NPA-KK114 (scale bars 5 µm). (c) Normalized intensity profile along the dashed line in the white boxes marked in the images of (a) and (b), exemplifying how neighboring NPCs can be much better resolved in the STED (red) compared to the confocal (black) recordings. (d-f) Repeated scanning of the same area of the cells in the STED mode indicates reduced photobleaching in the case of NPA-KK114 compared to KK114: images 1, 4 and 8 for KK114 (d) and NPA-KK114 (e) (scale bars 1 µm), and (f) bleaching constant from an exponential fit in terms of number of images for KK114 and NPA-KK114 (Total number of subsequent images recorded = 30, n=4).

3.3 Conclusion

In summary, we introduced a versatile and simple design concept to synthesize photostabilizer-dye conjugates on a specific target using unnatural amino acids. The strategy is based on a straightforward and modular synthesis route using amide-
bond formation with commercially available starting materials. The unnatural amino acids p-nitrophenylalanine was used as a scaffold to link Rhodamine B and KK114 fluorophores covalently to biomolecular targets, i.e., DNA, antibodies and proteins in our case study. We are convinced, however, that other targets (RNA, affinity-tags, etc.) can also be labeled via similar means while maintaining the positive effects of photostabilization on these targets. Both of the RhodamineB- and KK114-photostabilizer derivatives show a considerable increase in photostability, brightness as well as a suppression of triplet-based blinking compared to the non-stabilized parent fluorophore, which are important parameters for single-molecule fluorescence microscopy applications. The approach allows labeling of biomolecules in a single step.

Finally, photostabilizer-dye conjugates were used in two state-of-the-art applications, i.e., the study of conformational changes in proteins via smFRET and super-resolution imaging using STED microscopy. Our results show that NPA-RhodamineB could be used at significantly higher excitation intensities comparing to RhodamineB, since triplet-state population was minimized and would allow for faster and better data acquisition. By labelling antibodies with KK114-photostabilizer conjugates to visualize nuclear pore complexes through stimulated emission depletion (STED) microscopy, we showed that intramolecular photostabilization could also be used for immunofluorescence combined with significantly increased number of possible successive STED images that can be acquired. The intramolecular photostabilized organic fluorophores demonstrated effectively enhanced photophysical properities and excellent imaging performance in STED microscopy. Hence, we are convinced that the presented strategy will stimulate broader use of intramolecular photostabilization and help to emerge this strategy to the new gold-standard for photostabilization.

3.4 Experimental Section

3.4.1 Synthesis of photostabilized-Rhodamine dyes

Synthesis of ("RhodamineB-Maleimide")
(S)-Rhodamine B 4-(4-(2-(maleimide)
ethylamino)-4-oxobutryl) piperazine
amide: Rhodamine B 4-(3-Carboxy-
propionyl) piperazine amide was
prepared as described previously in
literature. To rhodamine B 4-(3-
Carboxypropionyl)piperazine amide (200
mg, 0.3 mmol) and N-hydroxysuccinimide (45.2 mg, 0.4mmol), in 20mL anhydrous
DMF, N,N'-dicyclohexylcarbodiimide (79.1 mg, 0.4 mmol) was added. After stirring
for 12 h at room temperature under inert conditions, the precipitated
dicyclohexylurea (DCU) was removed by filtration. RhodamineB-NHS (1) was used
without further purification. To the crude rhodamineB-NHS (1) and N-(2-
Aminoethyl) maleimide trifluoroacetate salt (76.2 mg, 0.3 mmol), in 10mL DMF,
50µL triethylamine was added. After stirring for 12 h at room temperature under
inert conditions the solvent was removed by evaporation in vacuo. The product
(RhodamineB- maleimide) was purified by column chromatography (SiO2,
DCM/Methanol: 20/1) and isolated as pink solid. Yield: 154.9 mg (0.21 mmol, 70%).
^1^H NMR (400 MHz, CDCl3) δ 7.74 – 7.60 (m, 2H), 7.53 (d, J = 6.9 Hz, 1H), 7.34 – 7.27
(m, 1H), 7.22 (t, J = 10.4 Hz, 2H), 7.03 (d, J = 7.5 Hz, 1H), 6.93 – 6.81 (m, 1H), 6.75 (d,
J = 6.3 Hz, 1H), 6.70 (s, 1H), 6.66 (s, 1H), 6.54 (s, 1H), 3.60 (tq, J = 14.8, 7.6 Hz, 10H),
3.43 (d, J = 24.8 Hz, 10H), 2.66 –2.60 (m, 2H), 2.42 (t, J = 6.8 Hz, 2H), 1.31 (t, J = 6.9
Hz, 12H). ^13^C NMR (101 MHz, CDCl3) δ 172.90 , 171.02 , 157.69 , 155.67 , 135.13 ,
134.13 , 132.14 , 130.15 , 130.01 , 127.57 , 113.83 , 95.95 , 77.21 , 46.06 , 45.39 ,
41.71 , 41.12 , 38.47 , 37.50 , 28.69 , 12.55. HRMS: (C_{42}H_{49}N_{6}O_{6}) 733.41 (found),
733.37 (calc).

**Synthesis of 3 (“NPA-RhodamineB”)**

(S)-Rhodamine B 4-(4-((1-Carboxy -2-(4-
nitrophenyl)ethyl)amino)-4-oxobutryl)
piperazine amide: Rhodamine B 4-(3-Carboxy-
propionyl) piperazine amide was
prepared as described previously in
literature. To rhodamine B 4-(3-Carboxy-
propionyl) piperazine amide (200 mg, 0.3 mmol) and N-hydroxysuccinimide (45 mg,
0.4 mmol), in 20 mL anhydrous DMF, N,N'-dicyclohexylcarbodiimide (79 mg, 0.4 mmol) was added. After stirring for 12 h under inert conditions at room
temperature, the precipitated dicyclohexylurea (DCU) was removed by filtration. RhodamineB-NHS (1) was used for coupling without further purification. To
RhodamineB-NHS (1) (212 mg, 0.3 mmol) in 30 mL DMF, (S) nitrophenylalanin (NPA) (344 mg, 1.6 mmol) was added, followed by 50 µL triethylamine. After stirring for 12 h at room temperature under inert conditions the solvent was evaporated in vacuo. The product (NPA-RhodamineB (3)) was purified by column chromatography (SiO₂, DCM/Methanol: 10/1) and isolated as pink solid. Yield: 210.2 mg (0.262 mmol, 80%). 1H NMR (400 MHz, CD₃OD) δ 1.28-1.32 (t, 12H, J=6.8), 2.41 (br s, 2H), 2.47 (br s, 2H), 3.01 (dd, 1H, J=14.1, 5.1),3.34 (m, 10H), 3.66-3.71 (q, 8H, J=7.2), 4.48(dd, J=11.0, 6.2, 1H), 6.96-6.97 (br s, 2H), 7.06 (d, 2H, J=2.0), 7.24-7.27 (d, 2H, J=9.6), 7.41 (d, 2H, J=7.3), 7.49-7.50 (m, 1H), 7.68-7.69 (m, 1H), 7.74-7.75 (m, 2H), 8.02 (d, J=8.2, 2H). 13C NMR (101 MHz, CD₃OD) δ = 175.9, 172.4, 171.2, 168.1, 157.8, 155.8, 155.5, 146.8, 146.5, 135.1, 131.7, 131.0, 129.9, 127.5, 122.7, 114.0, 113.4, 96.0, 55.4, 48.3, 48.0, 47.8, 47.6, 47.4, 47.2, 47.0, 45.5, 38.0, 30.4, 27.9, 11.5. HRMS: (C₄₅H₅₁N₆O₈ +) 803.3987 (found) 803,3763 (calc.).

4 and 7 (“NPA-RhodamineB-NHS” and “NPA-RhodamineB-Maleimide”)

(S)-Rhodamine B 4-(4-((1-oxo-1-(2-(maleimide)ethylamino)-3-(4-nitro-phenyl)) isopropylamino) -4-oxobutyryl) piperazine amide: To (S)-rhodamine B 4-(4-((1-Carboxy-2-(4- nitrophenyl)ethyl) amino) -4-oxobutyryl) piperazine amide (3) (80.4 mg, 0.1 mmol) and N-hydroxysuccinimide (13.6 mg, 0.12mmol), in 20mL anhydrous DMF, N,N'-dicyclohexylcarbodi-imide (23.7 mg, 0.12 mmol) was added. After stirring for 12 h under inert conditions at room temperature, the precipitated dicyclohexylurea (DCU) was removed by filtration. The resulting RhoB-NPA-NHS was used further without purification. To the crude RhoB-NPA-NHS mixture and N-(2-aminoethyl)maleimide trifluoroacetate salt (25.4 mg, 0.1 mmol), in 5 mL DMF, 17 µL triethylamine was added. After stirring for 12 h at room temperature under inert conditions the solvent was removed by evaporation in vacuo. The product (RhoB-NPA-maleimide) was purified by column chromatography (SiO₂, DCM/Methanol: 15/1) and isolated as pink solid. Yield: 55.5 mg (0.06 mmol, 60%). HRMS: (C₅₁H₅₇N₆O₉ +) 925.53 (found), 925.42 (calc.).
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10 (“NPA-KK114”)

The dye KK114 (9) (10 mg, 0.011 mmol), 2 N-hydroxysuccinimide (13 mg, 0.11 mmol), HATU reagent (12 mg, 0.032 mmol), and Et3N (25 μL, 0.17 mmol) were combined in anhydrous DMF (0.5 mL). The solution was stirred at room temperature overnight under an argon atmosphere. L-4-Nitrophenylalanine (2) (20 mg, 0.09 mmol) in DMF (1 mL) and more Et3N (25 μL, 0.17 mmol) was introduced, and stirred at room temperature overnight under an argon atmosphere. The solvent was evaporated in vacuo. The crude product was dissolved in water (few mL) and filtered (Rotilabo syringe filters, 0.22 μm). The solution was evaporated in vacuo. The crude product was dissolved in water (few mL) and filtered (Rotilabo syringe filters, 0.22 μm). The solution was concentrated to 1 mL and purified by preparative HPLC. Yield: 4.5 mg (4.2 μmol, 37%). 1H-NMR (400 MHz, Acetone-d6; mixture of diasterioisomers: only broad unresolved signals were recorded) δ = 1.31/1.35/1.37 (s×3, 12 H, CH3), 1.54 (m, 2 H, CH2), 2.04 (m, 4 H, CH2×2), 2.80 (m, 2 H, CH2CO), 3.00 (s, 3 H, NCH3), 3.18 (m, 2 H, CH2), 3.30 (m, 2 H, CH2), 3.52 – 3.64 (m, 2 H, CH2Ph, m, 4 H, CH2SO3), 3.70 (m, 4 H, CH2N), 4.50 (m, 1H, CHCO2H), 5.85 (m, 2 H), 7.30 – 7.50 (m, 4 H), 8.00 – 8.20 (m, 2 H) ppm. MS (ESI) m/z (positive): found 1080 (100%) [M+H]. HRMS (negative): (C51H49F4N5O13S2): 1078.2612 (found M–H), 1078.2626 (calc.). HPLC was performed with Shimadzu LC-10AD VP over Agilent SB C18 column (4.6 x 75mm, particle size 3.5 μm) with gradient A/B: MeCN/H2O+50 mM TEAB; 0:100→60:40 in 20 min, to 100:0 at 25 min.; tR = 13.8 min.

13 (“β-Ala-NPA-KK114-OH”, the modified dye conjugate with the linking site elongated by one β-alanine moiety)

The modification was performed as follows: compound NPA-KK114 (10) (2.2 mg, 2 μmol) was reacted with β-alanine methyl ester hydrochloride (11) (2 mg, 13 μmol) in the presence of HATU reagent (4 mg, 11 μmol) and Et3N (4 μL, 28 μmol) in DMF (0.5 mL) at room temperature under an argon atmosphere. As the reaction was complete (in ca. 2 h, as established by HPLC), the solution was quenched with water (1 mL) and loaded onto a column with reverse-phase silica gel (Macherey-Nagel, Polygoprep 60-50 C18, 2 g) and water containing 0.1 % v/v TFA as mobile phase. In
the course of the elution, MeCN was gradually added to the mobile phase up to the ratio of 1:1. The pure fractions were pooled, filtered (Rotilabo® syringe filters, 0.22 µm), concentrated, and freeze-dried to afford ca. 2 mg of a crude methyl ester β-AlaNPA-KK114-OMe (12). Without further treatment, the intermediate was saponificated by stirring overnight at room temperature in a dilute alkaline solution made of water (3 mL) and 1 M aq. NaOH (0.15 mL). The solution was acidified with an excess of TFA (10 µL) and the product isolated by column chromatography (over 2 g of RP-SiO2) exactly as described above for the starting methyl ester. Filtration and evaporation of the pure fractions of the acid β-AlaNPA-KK114-OH (13) afforded a crystalline blue solid, well soluble in basified water or DMF, sparingly soluble in acetone. Yield: 1.4 mg (1.2 µmol, 60% over two steps). MS (ESI) m/z (negative): found 1149 (100%) [M–H]. HRMS: (C_{54}H_{54}F_{4}N_{6}O_{14}S_{2}): 1149.2989 (found M–H), 1149.2996 (calc.). The HPLC analyses were performed by means of Knauer Smartline semi-preparative high pressure gradient system with two pumps, mixing chamber, column thermostat 4000 (25 °C), and an UV detector 2550 (gradient A/B: MeCN/H2O+0.1 % v/v TFA; 10:90→100:0 in 20 min): tR = 9.4 min.

15 (β-Ala-NPA-KK114-NHS, The active ester of the modified dye conjugate)

The NHS ester was obtained as follows: N,N’-disuccinimidyl carbonate (14) (DSC reagent, 1.5 mg, 6 µmol) was added to a solution of acid β-Ala-NPA-KK114-OH (13) (1.2 mg, 1 µmol) in DMF (0.30 mL) containing Et3N (3 µL, 20 µmol) in a 2.5 mL vial with a magnetic stirring bar. The vial was flushed with argon, sealed, and the solution stirred until the reaction was complete (ca. 30 min, as established by HPLC). The solution was thoroughly evaporated to dryness straight from the vial in a high vacuum (for evaporation a small hole in the cap was pierced and the vial was placed inside a flask). The residue was sonificated for 3 min with ethyl acetate (2 mL) and the suspension centrifuged. The mother liquor, which contained DSC and NHS, was separated. The precipitate was dried from the residual solvent and re-dissolved in DMF (0.3 mL). The solution was divided in to 6 aliquots (50 µL, each containing ca. 200 µg of the active ester) that were carefully evaporated in vacuo, as described above, flushed with argon, and stored at −20°C. The dried aliquots proved sufficiently pure (98%, HPLC). MS (ESI) m/z (negative, %) = 1246 (100%) [M–H]; HRMS (negative): (C_{58}H_{57}F_{4}N_{7}O_{16}S_{2}): 1246.3175 (found M–H), 1246.3167 (calc.); HRMS (positive): (C_{58}H_{57}F_{4}N_{7}O_{16}S_{2}):
1286.2848 (found M+K), 1286.2871 (calc.). HPLC (A/B: MeCN/H₂O + 0.1% v/v TFA; 10:90 → 100:0 in 20 min): tᵣ = 10.2 min.

### 3.4.2 Synthesis of photostabilizer- Rhodamine B conjugates

All reagents were purchased from commercial suppliers and used without further purification unless stated otherwise. Synthetic oligomers (NH₂-C6-5’-TAA TAT TCG ATT CCT ACT TAT ATT GCA TAG CTA TAC G-3’) were received in HPLC-purified quality from IBA or Eurofins (Germany). A Varian 400 (400 and 100 MHz) was used to record ¹H-NMR and ¹³C-NMR spectra. Chemical shifts (δ) are denoted in ppm using residual solvent peaks as internal standard (δ_H=7.26 and δ_C=77.0 for CDCl₃, δ_H=3.31, 4.78 and δ_C=49.15 for CD₃OD). High-resolution mass spectra (HRMS) were recorded on an Orbitrap XL (Thermo Fisher Scientific; ESI pos. or neg. mode). Flash chromatography was performed using a Grace Reveleris® Flash System (40 µm silica column).

**Isolation and characterization of functionalized oligonucleotides:** Reversed phase HPLC (rp-HPLC) analysis and preparative purifications (isolation) was performed on a Shimadzu LC-10AD VP machine equipped with Waters Xterra MS C18 column (3.0 x 150 mm, particle size 3.5 µm) and Waters Xterra MS C18 prep column (7.8 x 150mm, particle size 10 µm) using a gradient of acetonitrile / triethylammonium acetate buffer (50 mM, pH 7.0). Gradient 1: 05/95 0 to 10 min, to 65/35 at 60 min, to 75/25 at 65 min, to 05/95 at 75 min for 15 min. Flow 0.5 ml/min analytical run or 1.0 ml/min preparative run. The DNA was isolated by collecting the major peak of interest (see Figure 3.2). The resulting compounds were characterized by UV/Vis-absorption spectroscopy (Figure 3.3a) and Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Figure 3.3b). Spectra were recorded on an ABI Voyager DE-PRO MALDI TOF (delayed extraction reflector) Biospectrometry Workstation mass spectrometer.

**Synthesis of ssDNA-RhodamineB:** Step 1: Rhodamine B 4-(3-Carboxypropionyl) piperazine amide was prepared according to literature.¹ In a 5 ml round bottom flask, Rhodamine B 4-(3-carboxypropionyl)piperazine amide (200 mg, 0.3 mmol) and N-hydroxysuccinimide (45.2 mg, 0.4mmol) were dissolved in 20 mL dry DMF, after the addition of N,N'-dicyclohexylcarbodiimide (79.1 mg, 0.4 mmol), the reaction was carried out for 12 h under inert atmosphere at room temperature. Precipitated dicyclohexylurea (DCU) was removed by filtration. RhodamineB-NHS was used for coupling without further purification. The lyophilized ssDNA-NH₂ was resuspended in MilliQ water and adjusted to 20 µM in 0.2 M NaHCO₃ buffer (pH 8.35). To this solution, RhodamineB-NHS crude mixture in 100 µl of DMF was added and
the mixture was vortexed thoroughly. After incubation overnight the oligonucleotide was purified with an illustra NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra), see Figure 3.2.

**Synthesis of ssDNA-NPA-RhodamineB:** Step 1: The lyophilized ssDNA-NH₂ was resuspended in MilliQ water and adjusted to 20 µM in 0.2 M NaHCO₃ buffer (pH 8.35). To this solution, RhoB-NPA-NHS (4) in 100 µl of DMF was added and the mixture was vortexed thoroughly. After incubation overnight, the oligonucleotide was purified on illustra NAP 5 column (vide supra) and isolated by preparative rp-HPLC (gradient 1, vide supra) to yield 5, see Scheme 1. The yield was found to be 25% for coupling NPA-RhodamineB-NHS to ssDNA.

### 3.4.3 Single-molecule FRET and ALEX spectroscopy

For data acquisition we used the same home-built confocal microscope as described above. Fluorescence photons arriving at the two detection channels (donor detection channel D or acceptor detection channel A) were assigned to either donor- or acceptor-based excitation based on their photon arrival time as described previously. Three relevant photon streams were extracted from the data corresponding to donor-based donor emission F(DD), donor-based acceptor emission F(DA) and acceptor-based acceptor emission F(AA). During diffusion, fluorophore stoichiometry's S and apparent FRET efficiencies E* were calculated for each fluorescent burst above a certain threshold yielding a two-dimensional histogram.(11, 12) Uncorrected FRET efficiency E* monitors the proximity between the two fluorophores and is calculated according to E* = F(DA)/(F(DD)+F(DA)). Stoichiometry S is defined as the ratio between the overall fluorescence intensity during the green excitation period over the total fluorescence intensity during both green and red periods and describes the ratio of donor-to-acceptor fluorophores in the sample: S = (F(DA)+F(DD))/(F(DD)+F(DA)+F(AA)). Using published procedures to identify bursts corresponding to single molecules, we obtained bursts characterized by three parameters (M, T, and L). A fluorescent signal is considered a burst provided it meets the following criteria: a total of L photons are collected, having M neighboring photons within a time interval of T microseconds. We applied a burst search on the sum of all three detection channels (all photons) using parameters M = 15, T = 500 µs and L = 50; additional per-bin thresholding removed spurious changes in fluorescence intensity and selected for intense single-molecule bursts (all photons > 150 photons unless otherwise mentioned). Binning of the detected bursts into a 2D E*/S histogram yielded sub-populations that can be separated according to their S-values.
ALEX-experiments were carried out at room temperature using 25-50 pM of double-labeled protein in imaging buffer. The imaging buffer consisted of 50 mM KPi (pH 7.4), 150 mM KCl and 1 mM Trolox when determining the differences in brightness of the donor alone (F(DD)) and that of the acceptor excited via FRET (F(DA)), when either the NPA-RhodamineB derivative or RhodamineB fluorophore is used. For observing the conformational dynamics of the SBD2 protein the buffer consisted of 50 mM KPi (pH 7.4) and 150 mM KCl. ALEX titration experiments with adding increasing concentrations of ligand to the imaging buffer (0 µM, 1.1 µM and 200 µM of glutamine) were recorded in one continues experiment.

3.4.4 Count rate versus excitation intensity

The count rate per molecule dependence on the excitation intensity was recorded using 20-100 nM of SBD2 protein labelled only with NPA-RhodamineB or RhodamineB in imaging buffer (10 mM KPi, 2.7 mM KCl, 137 mM NaCl (pH 7.4), 10% w/v glucose, 50 µg/ml glucose-oxidase, 100-200 µg/ml catalase, and 0.1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The count rate per molecule was acquired via excitation from the 543 nm laser on a LSM 710 confocal laser scanning microscope (CLSM, Carl Zeiss, Jena, Germany) through a C-apochromat 40×/1.20 w Korr M27 water immersion objective (NA = 1.2). Excitation from the 543 nm Laser (at different intensities, 0.0 to 47.4 kW/cm²) was coupled into the objective by a dichroic beam splitter (MBS 458/543). Fluorescence was collected through the same objective with appropriate spectral filtering (NFT 635 VIS and LP 580).

3.4.5 STED and confocal – microscopy

STED microscopy and the corresponding confocal microscopy was performed on an Abberior Instruments Resolft microscope (Abberior Instruments, Goettingen, Germany) to which a Ti:Sa STED laser was added (MaïTai, Newport-Spectra Physics) (as outlined in detail in 16) Excitation was performed at 640nm (LDH-D-C-640P laser diode, Picoquant) and STED at 780 nm at 80 MHz. Pulsing of the excitation laser was triggered by the Ti:Sa laser using a photodiode (APS-100-01), an amplifier (CON-TTL, both Becker & Hickl, Berlin, Germany) along with a ps-delay unit (either Abberior Instruments or LAS-015617, MPD/Laser2000 UK). A donut-shaped intensity distribution of the STED laser focus was realized by the incorporation of a vortex phase plate (VPP-1a, RPC Photonics, Rochester, NY) into the collimated STED beam. Laser focusing and fluorescence collection was done by an oil-immersion microscope objective (UPlanSapo 100x/1.4oil, Olympus, Japan). The collected fluorescence was detected by an avalanche-photodiode (SPCM-AQRH-13, Excelitas)
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Technologies) with a 650-690 nm bandpass filter (670/40, AHF Analysentechnik, Tuebingen Germany).

3.4.6 Immunolabeling

Gm5756T human fibroblast were fixed with 3% paraformaldehyde for 10 min, rinsed several times in PBS, permeabilized in 0.1% Triton x100 for 10 min, rinsed again several times in PBS, blocked in 2% BSA/5%FCS in PBS for 1 hour. Samples were incubated with primary mouse antibodies to stain nuclear pore diluted 1:500 in blocking buffer for 1 hour at room temperature. Coverslips were washed five times in 1% BSA in PBS and then incubated for 30 min with goat anti-mouse antibodies (Abberior GmbH, Gottingen, Germany) diluted 1:250 in blocking buffer. Samples were washed five times in 1% BSA in PBS and mounted in the buffer system utilizing an oxygen-scavenging system as used before in the single-molecule experiments.

Author Contribution

Thorben Cordes conceived the study. Jasper H. M. van der Velde, Andreas Herrmann and Thorben Cordes designed experiments. Jingyi Huang, Jasper H. M. van der Velde and Atieh Aminian Jazi performed single-molecule imaging and ALEX experiments. Jingyi Huang, Jochem H. Smit, Kirill Kolmakov and Giorgos Guoridis provided new reagents including chemical characterization. Jingyi Huang performed the synthesis, purification and characterization of NPA-RhodamineB, DNA-NPA-RhodamineB and NPA-KK114. Silvia Galiani and Christian Eggeling performed STED microscopy. Gerard Roelfes provided proteins for FRET studies. Jasper H. M. van der Velde and Thorben Cordes analysed data and all authors contributed to writing the paper.
Chapter 3

Reference


Appendix

Supplementary Figure 3.1. Details of photophysical characterization of RhodamineB. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized RhodamineB molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_f$. (d) Representative fluorescence time traces of the selected single emitters. (e) Autocorrelation decay of a single emitter (black, with the fit in red) (f) Histogram of off-state lifetimes, with Gaussian fit (red line) and mean plus
standard deviation value. Here all individual fluctuating fluorescence time traces were autocorrelated, and the autocorrelation decay was fitted to obtain values of the lifetime of the dark off-state. (g) Representative TIRF images of immobilized RhodamineB molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (h) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 4 kW cm-2 for confocal measurement and 50 W cm-2 for TIRF-experiments (excitation at 532 nm, detection with HC582/75). Brightness and contrast settings 2300 (low) to 6800 (high) and 3 (low) to 50 (high) for TIRF and confocal images, respectively.

Supplementary Figure 3.2. Photophysical characterization of NPA-RhodamineB. (a) Schematic view of the photostabilizer-dye conjugate on DNA and the experimental strategy for surface immobilization. (b) Confocal scanning image of immobilized NPA-RhodamineB molecules, including the identification of positions of single emitters for further analysis (right panel) (scale bar 5 µm). (c) Histogram of fluorescence lifetimes determined for each of these single emitters, including Gaussian fit (black line) and average lifetime value $\tau_{fl}$. (d) Representative TIRF images of immobilized NPA-RhodamineB molecules at two different time points (0 and 2 min, as given) after starting illumination and recording (scale bar 2 µm). (e) Representative fluorescence time traces of single emitters from TIRF recordings, indicating long-term blinking and photobleaching characteristics. Excitation intensities are 0.66 kW·cm-2 for
confocal and 50 W·cm⁻² for TIRF-experiments (excitation at 532 nm, detection with HC582/75). Brightness and contrast settings 2300 (low) to 6800 (high) and 3 (low) to 50 (high) for TIRF and confocal images, respectively.

**Supplementary Figure 3.3.** Average and standard deviation of the mean (error bars) of photophysical parameters of RhodamineB without and with Trolox (2 mM TX) and of NPA-RhodamineB, as determined from the TIRF data of Supplementary Figures 10 and 11: photobleaching time $\tau_{bleach}$ (left panel), total number of detected photons $N_{total}$ (middle left), brightness (middle right), and signal-to-noise ratio (SNR, right panel). Recorded in aqueous PBS buffer at pH 7.4 in the absence of oxygen (continuous 532 nm excitation at $\approx$50 W·cm⁻²). Values in bar graphs were derived from $N > 1000$ molecules.
Supplementary Figure 3.4. HPLC-characterization and purification of KK114 derivatives: a) HPLC analysis of NPA-KK114 (10), b) β-Ala-NPA-KK114-OH (13) and c) β-Ala-NPA-KK114-NHS (15).

Supplementary Figure 3.5. UV-VIS absorption spectra of β-Ala-NPA-KK114-NHS (15) showing the characteristic absorption of the KK114 dye.
Supplementary Figure 3.6. Comparison of performance of KK114 and NPA-KK114 fluorophores for super-resolution STED microscopy of immunolabeled nuclear pore complexes in fixed fibroblast cells. Confocal (a,c) and STED (b,d) images of a representative cell stained with KK114 (a,b) and NPA-KK114 (c,d). (scale bars 5 µm). (e) Total image intensity with number of repeated scan of the same area of the cells in the STED mode for KK114 (black) and NPA-KK114 (red), indicating reduced photobleaching in the case of NPA-KK114. Data from ≥ 3 measurement series, with error bars depicting the standard deviation. (f) Average brightness and standard deviation (error bars) for KK114 and NPA-KK114 labeling. (g) Size (full-width-at-half-maximum (FWHM)) of the emission peaks for KK114 and NPA-KK114.
half-maximum FWHM) of nuclear pore complexes in the initial STED images (n > 300 nuclear pore complexes) for KK114 and NPA-KK114 labeling.