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A dendritic single-molecule fluorescent probe that is monovalent, photostable, and minimally blinking

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Abstract

Single-molecule fluorescence techniques have emerged as a powerful approach to understand complex biological systems. However, a challenge researchers still face is the limited photostability of nearly all organic fluorophores, including the cyanine and Alexa dyes. We report a new, monovalent probe that emits in the far-red region of the visible spectrum with properties desirable for single-molecule optical imaging. This probe is based on a ring-fused boron-dipyrromethene (BODIPY) core that is conjugated to a polyglycerol dendrimer (PGD). The dendrimer makes the hydrophobic fluorophore water-soluble. This probe exhibits excellent brightness, with an emission maximum of 705 nm. We observed strikingly long and stable emission from individual PGD-BODIPY probes even in the absence of anti-fading agents such as Trolox, a combined oxidizing-reducing agent often used in single-molecule studies for improving the photostability of common imaging probes. These interesting properties greatly simplify use of the fluorophore.

Fluorophores, especially those that are bright, photostable, water-soluble, and biocompatible, are powerful tools for studying biological processes *in vivo* and *in vitro*[1-3].

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Author contributions

S.K.Y. and S.C.Z. conceived the study: S.K.Y. designed and synthesized the polyglycerol-dendronized fluorophores: X.S. performed the single-molecule experiments: X.S. and S.P. performed the microtubule labeling experiments: S.K.Y., X.S., T.H. and S.C.Z. wrote the manuscript.

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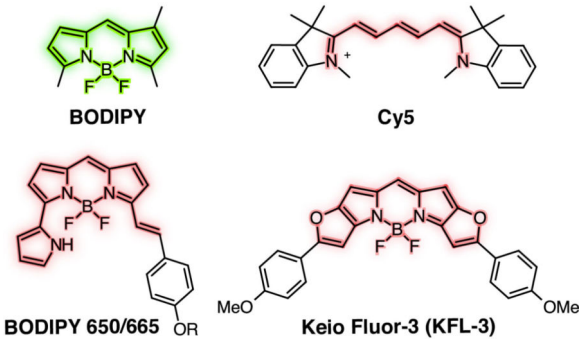
The most useful fluorophores also emit in the red or near-IR region and carry a single reactive functional group for conjugation. Through the efforts of many researchers a large number of organic fluorescent dyes are known and many are now commercially available. However, all fluorophores stop emitting light permanently as a result of photobleaching[4,5]. Temporary dark states are also suffered when irradiation induces transitions both to nonproductive chemical and excited states that do not fluoresce. These transient emission outages are known as blinking. Blinking and photobleaching are especially problematic when powerful lasers are used and with long-wavelength fluorophores such as Cy5[6-9], both commonly employed in single-molecule fluorescence studies[10-14].

Major advances in reducing the formation of dark states have been achieved by employing enzymatic deoxygenation systems and anti-fading agents, such as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), propyl gallate, β-mercaptoethanol, and cyclooctatetraene[15-20]. Beyond the effort required to find the optimal experimental combination of anti-fading agent and fluorophore, the agents themselves can present problems including cellular toxicity, lack of cell permeability, limited water-solubility, and potential interference with biological systems under investigation. Recently, Blanchard and colleagues reported an intriguing new strategy wherein a single anti-fading agent is covalently linked to a single fluorophore[20]. Herein we report an alternative approach; a near-IR emitting fluorophore with no anti-fading agent, that is brighter and more stable than Cy5, similar in its excitation and emission spectra, yet exhibits minimal blinking.

Results and discussion

Our approach focused on the boron-dipyrromethenes (BODIPYs) because of their high fluorescence quantum yields and narrow fluorescence bands[21,22]. Furthermore, the rate of intersystem crossing in simple BODIPY fluorophores is well known to be slow[23], although a very recent single-molecule fluorescence study showed a reduced degree of blinking upon the addition of oxidizing-reducing (ROXS) agents[24]. Although BODIPY absorbs at shorter wavelengths (λ_{max} = 505 nm)[2], has a low extinction coefficient and its photostability is not dramatically better than fluorescein[25,26], π-extended versions have improved each of these properties[27-31]. For example, Suzuki's recently described Keio Fluors (e.g., KFL-3) contain a furan-ring-fused BODIPY structure that emit in the red and near-IR region and exhibit remarkably high extinction coefficients and quantum yields in organic solvents[32,33].



The Keio Fluors do not dissolve in water but linking hydrophilic moieties such as oligo(ethylene glycol), carboxylate, sulfonate, phosphonate, and ammonium groups has successfully conferred aqueous solubility on other BODIPYs[34-38]. In this study we solubilized a modified Keio Fluor with water-soluble and biocompatible polyglycerol dendrimers (PGDs), a strategy that was successful even with the very large aromatic surface of the perylenediimide (PDI) fluorophore[39,40]. However, the convenient point for attaching a reactive functional group that was built into the PDI unit, is absent in the Keio Fluor. Thus, as outlined in Figure 1, a universal PGD scaffold was developed that might be applied to any fluorophore and targeting (or stabilizing) group. In particular, a lysine-cored PGD with two orthogonal protecting groups increases the generality of the approach, allowing stepwise introduction of any two appropriately functionalized moieties (Fig. 1).

The synthesis of the biotinylated PGD-KFL used in single-molecule studies is outlined in Figure 2. Thus, 4*H*-furo[3,2-*b*]pyrrole **1**[32,33] was reacted with 4-formylbenzoic acid in the presence of trifluoroacetic acid (TFA) to obtain **2**. The carboxylic acid group in **2** was activated by conversion to the corresponding *N*-hydroxysuccinimide (NHS) ester and the product was treated with boron trifluoride diethyl etherate to produce BODIPY/KFL **3** in 30% yield for the three steps. The synthesis of the PGD with a bifunctional core began with Fmoc-Lys(Boc)-OH which was reacted with amine **4**[41] under standard peptide coupling conditions. The product trialkyne **5**, obtained in 92% yield was coupled with azide-cored PGD **6**[42] using copper-catalyzed 1,3-dipolar cycloaddition[43]. Bifunctional PGD **7** containing 24 allyl ether peripheral groups was obtained in 99% yield.

PGD **7** was readily purified by column chromatography and the extent of the click reaction was established by the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Thus, as seen in Figure 3, only a single main peak is observed (m/z = 3,370.3) that corresponds to the click reaction occurring at each of the three alkyne groups. After deprotection of the *tert*-butoxycarbonyl (Boc) group in **7** with TFA, the resulting PGD **8** was dihydroxylated using 2 mol% of $K_2OsO_4 \cdot 2H_2O$ per alkene to afford water-soluble PGD **9** with 48 hydroxyl end-groups. PGD **9** was purified by dialysis against water and complete Boc deprotection and dihydroxylation were observed in the ^{1}H nuclear magnetic resonance (NMR) and MALDI-TOF mass spectra of **8** and **9** (Fig. 3 and Supplementary Information).

As outlined above, the differentially protected lysine amino groups in **7** allow any two moieties with amine-reactive functionality to be covalently attached to the PGD core. For the immobilization needed in this study, the ε-amino group of the lysine core in **9** was biotinylated to afford **10** in 71% yield using biotin NHS ester in *N*,*N*-dimethylformamide (DMF) without any added reagents. The 9-fluorenylmethyloxycarbonyl (Fmoc) group was removed using piperidine to afford in 74% yield aminefunctionalized PGD **11**. Finally, conjugation of the BODIPY dye to PGD was performed by treating **11** with **3**, leading to the successful formation of biotinylated PGD-BODIPY **12**. All of the PGDs prepared (**7**-**12**), were characterized by ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry, which confirmed near quantitative conversions in each synthetic step (Fig. 3 and Supplementary Information). Although the preparation of **12** requires a number of steps, both the synthesis and purification are straightforward. In this study we chose to use polyglycerol dendrimers

to aid in characterization but the azide-cored PGD **6** could be replaced by an analogous clickable hyperbranched polyglycerol[44] thereby streamlining the synthesis considerably.

PGD-KFL **12** is highly water-soluble and exhibits absorption and emission maxima in water at λ_{abs} = 685 nm and λ_{em} = 705 nm, respectively (Fig. 4a). These spectroscopic characteristics are comparable to those of the nondendronized KFL **3** and other analogues[32,33] measured in organic solvents. The extinction coefficient and fluorescence quantum yield of **12** in water were determined to be ε = 190,500 $M^{-1}cm^{-1}$ and Φ = 0.57, respectively. Thus, the brightness of **12**, defined as the product of the extinction coefficient and the quantum yield ($\varepsilon \times \Phi$), is twice as high as that of Cy5 and Cy5.5 dyes which emit in a similar spectral window. Indeed, to the best of our knowledge, **12** is the brightest, water-soluble, monofunctional, far-red emitting organic fluorophore reported to date.

The performance of PGD–KFL **12** in single-molecule applications was assessed using the immobilization and imaging methods described previously (Fig. 4b)[39,45]. The single-molecule experiments were carried out with total-internal-reflection fluorescence microscopy, using a 633 nm laser as the excitation source. Laser light was focused onto the sample slide within an area of ~100 by 50 μm^2 and the power measured directly before the objective was ~6.7 mW (~130 W cm^{-2} at the sample plane). Under conditions typically used for imaging individual Cy5-labeled molecules where Trolox[17] and an oxygen scavenging system[45] were used, bright fluorescence of single, immobilized PGD–KFL molecules were detected and readily distinguishable from the background signal (see Supplementary Fig. S1). This result indicates that the PGD–KFL fluorophore is well suited for single-molecule imaging applications. We then obtained the intensity trajectories of individual **12** molecules and observed that many of them were very photostable and survived 60 s of excitation without photobleaching, under conditions typically used in our laboratory for single-molecule fluorescence resonance energy transfer (FRET) imaging[45]. As in the imaging experiments above, these conditions employed both Trolox, an agent that has been shown to effectively suppress blinking and extend the lifetime of Cy5, and an oxygen scavenging system consisting of glucose, glucose oxidase and catalase.

The long-lasting emission observed for **12** may be attributed to one or more of the following factors: Trolox-mediated enhancement of photostability[17], suppression of photobleaching as a result of deoxygenation[45], an intrinsic property of the BODIPY core (i.e., KFL unit) in **12**, or a type of protective effect from the dendrimer encapsulation. To understand the mechanism, we imaged molecules of **12** in T50 buffers containing no additives (Fig. 4c), Trolox only (Fig. 4d), the oxygen scavenging system only (Fig. 4e), and both Trolox and the oxygen scavenging system (Fig. 4f). Under these conditions, the single-molecule photobleaching time, $\tau_{photobleaching}$ [39,45], of **12** was determined to be 3.3 ± 0.2 s, 3.4 ± 0.8 s, > 60 s, and > 60 s, respectively. These data suggest that the deoxygenation alone can produce the long-lasting emission of **12** and Trolox is likely dispensable.

Not only does **12** exhibit long-lasting fluorescence emission, it displays striking photostability within individual intensity trajectories (Fig. 5). By imaging immobilized molecules over a course of 300 s without Trolox, but with the oxygen scavenging system, $\tau_{photobleaching}$ was determined as 67 ± 7 s and 45 ± 13 s for **12** and Cy5, respectively. This

same experiment confirmed that most of the immobilized molecules exhibited a single photobleaching step and are, thus, individual fluorophores rather than dye aggregates. Prior to photobleaching the majority of the **12** molecules exhibited highly stable emission at 100 ms time resolution. As seen in Fig. 5a, the total-internal-reflection fluorescence images of **12** exhibited many bright spots, whereas the commonly used single-molecule probe Cy5 showed far fewer bright spots (Fig. 5b). The extent of surface immobilization was similar for both dyes so the difference can be attributed to the well-documented blinking behavior of Cy5. Consistent with this result, the time-dependent decay in average Cy5 intensity exhibited a significantly higher level of noise than did **12** (Fig. 5c,d). The clearest difference between **12** and Cy5 is seen in individual time trajectories where both show long-lasting fluorescence but **12** alone exhibits a dramatically more stable, non-blinking emission (Fig. 5e), in contrast to the behavior of Cy5 (Fig. 5f). The difference can be seen for many more molecules in a comparative movie and additional intensity traces provided in the Supplementary Information. For a better comparison of the two probes, we also obtained the total number of photons detected (320,000 ± 40,000 for **12** and 140,000 ± 30,000 for Cy5), and found it to be as 2.3 times larger for **12** than for Cy5.

It is worth noting that the blinking of Cy5 can be effectively suppressed by the addition of Trolox[17], but this additive does not always work[4]. Furthermore, Trolox and all additives must be tested experimentally to ensure that they do not perturb the biological system under investigation. For other fluorophores the specific additive that will be most useful must be determined on a case-by-case basis and beyond this complication, additives have a number of limitations, including toxicity and poor membrane permeability (*vide supra*). The results with PGD–KFL **12** suggest that it may be possible to find fluorophores that are inherently photostable and non-blinking.

The striking difference in photophysical properties for PGD-KFL **12** and Cy5 may be attributed to certain intrinsic differences between the two fluorophores, or the PGD that surrounds the Keio Fluor. We first tested the hypothesis that the ring-fused structure in **12** contributes to its photostability, by using a related BODIPY probe named BODIPY 650/665X. This probe is closely related to **12** in structure and has similar spectroscopic parameters; however, it does not possess the same ring-fused structure as found in **12**. Under the same imaging condition as shown in Figure 5, we found that the majority of the **12** molecules exhibited non-blinking fluorescence at 100 ms time resolution (Supplementary Fig. S2), whereas most of the BODIPY 650/665X molecules, although significantly less blinking in comparison to Cy5, nonetheless showed detectable blinking behavior (Supplementary Fig. S3). This result is consistent with the transition to the dark state involving the alkene group in BODIPY 650/665X and the polyene unit in Cy5, both absent in **12**, underscoring the importance of the rigid, fused-aromatic structure in Keio Fluor.

To address the second possibility, that the PGD surrounding the KFL unit in **12** plays a role in its special properties, we examined KFL **3** without the PGD shell by attaching it directly to DNA. This probe exhibited a significantly higher level of blinking under the same imaging conditions (Supplementary Fig. S5). Thus the polyglycerol dendrimers also plays a role in suppressing the blinking of the KFL core in **12**. This stabilization may be considered analogous to the green fluorescent protein (GFP) wherein the protein matrix surrounding the

chromophore protects it from quenching by bulk solvent. To examine whether the effect of the PGD might be generalized to other fluorophores, commercially available Cy5 and BODIPY 650/665X NHS-esters were covalently linked to amine **11**. PGD-BODIPY 650/665X and PGD-Cy5 were immobilized and imaged under the identical conditions used previously. By comparing the fraction of blinking molecules, we found no marked difference with or without attachment of PGD for both of the fluorophores above (Supplementary Fig. S6,7). Overall, these results indicate that the non-blinking of PGD-KFL **12** originates through a combination of the ring-fused structure of the KFL unit and its PG dendronization, with the former predominating. However, the affect of the PGD is only observed with KFL **3**; it is not observed with Cy5 or BODIPY 650/665X. The non-blinking properties of **12** could be observed even with a significantly higher laser intensity. Thus, another single-molecule experiment was carried out using a Cobolt MLD™ 640 nm diode laser module with 50 mW power laser (1,000 W cm^{-2}) where the non-blinking emission was successfully retained as seen in individual time trajectories shown in Supplementary Fig. S8.

Although the biological applicability of **12** is beyond the scope of this study, the fluorescent labeling of microtubule network in mouse embryonic fibroblast (MEF) cells were explored to illustrate the usefulness of **12** in targeted biological imaging. Fixed cells were labeled with primary antibody against β-tubulin and biotinylated secondary antibody against the primary, followed by incubation with neutravidin. Finally, cells were labeled with **12**, showing clear microtubule network (Supplementary Fig. S9a). In contrast, when neutravidin was excluded from the procedure, only basal level of staining was observed with **12** (Supplementary Fig. S9b), clearly illustrating specific binding of **12** to microtubules through neutravidin.

Conclusions

We have developed a new far-red fluorescent probe, PGD-KFL **12**, that is exceptionally bright and of comparable photostability to a commonly used fluorophore Cy5. The most notable feature of **12** is its long-lasting fluorescence emission with a strikingly low level of blinking in single-molecule imaging experiments even in the absence of additives such as Trolox. This overcomes a significant obstacle in single-molecule fluorescence imaging. The design is based on the known Keio Fluor (KFL-3), which was rendered water-soluble by covalent linkage to a biocompatible, polyglycerol dendrimer (PGD). A series of comparative studies with Cy5 and BODIPY 650/665X, both within a PGD and attached only to DNA showed that the remarkably stable fluorescence output from **12** originates both in the KFL fused ring structure and, to a lesser extent, the polyglycerol dendrimeric shell. Although the latter requires a multistep preparation, it may be possible to streamline the synthesis by using a hyperbranched polyglycerol.

The new approach developed here uses an orthogonally protected lysine unit that allows any fluorophore to be linked to the dendrimer core along with a second moiety. In the current study the second group was biotin, which was used for surface immobilization. However, the second moiety could just as easily be a second fluorophore, a different targeting agent, or a stabilizing group. Each of these possibilities is under active investigation. More broadly, the findings here suggest the possible development of a series of next-generation

fluorophores with different excitation and emission profiles that are photostable, monovalent, and non-blinking in the absence of additives. A set of single-molecule optical probes with such properties would likely find widespread applicability as tools in any biological study that uses fluorescence microscopy.

Methods

Synthesis and characterization details for all new compounds, additional data for single-molecule imaging, and movies showing the single-molecule imaging of **12** and Cy5 can be found in the Supplementary Information. Supplementary Movies were recorded at 10 frames per second over a period of 300 s are played at 10 times the actual imaging speed.

DNA labeling

DNA D27-5Am (/5Biosg/ACAAGTATAGGATCCCCGAGAA/iAmMC6T/CGAG)[46] containing an amino-C6-dT modification at the fifth base from the 3′-end and a biotin moiety at the 5′-end was purchased from Integrated DNA Technologies. This DNA was dissolved in water at 1 mM concentration. BODIPY 650/665X succinimidyl ester (5 mg) purchased from Molecular Probes was dissolved in 280 μL of dimethyl sulfoxide (DMSO) immediately before use. KFL **3** (1 mg) was dissolved in 56 μL DMSO immediately before use.

The D27-5Am solution described above (1 μL) was mixed with 17 μL of sodium tetraborate buffer at 100 mM concentration (pH 8.5) and 3 μL BODIPY 650/665X succinimidyl ester solution in DMSO. For the labeling with **3**, 1 μL DNA, 9 μL buffer and 10 μL dye were used instead. The mixtures were incubated at room temperature overnight. After incubation, free dye was removed by using two Micro Bio-Spin columns. The DNA labeling efficiency was determined to be 35% for BODIPY 650/665X and 8% for **3**. The low labeling efficiency observed in both cases was likely due to limited solubility of both dyes in aqueous solutions

Single-molecule imaging

Molecules of PGD-BODIPY **12**, or Cy5- or BODIPY 650/665X-labeled DNA at a concentration of 125 pM were immobilized on a PEG-passivated surface through biotin-NeutrAvidin linkage as described[39,45]. Prior to imaging, the sample was rinsed extensively with an imaging buffer at pH 8.0, containing 10 mM Tris and 50 mM NaCl with no other additives (T50), with 2 mM Trolox, with the oxygen scavenging system - 0.8% (w/v) dextrose, 1.0 mg/mL glucose oxidase, and 0.04 mg/mL catalase, or with both 2 mM Trolox and the oxygen scavenging system[45].

Single-molecule imaging was performed with prism-type total internal reflection fluorescence microscopy as described previously[39,45]. In brief, a 633 nm HeNe laser was used to excite the fluorophore of immobilized molecules through total internal reflection and scattered excitation light was removed by a notch filter. Fluorescence emission was collected using a 60×, 1.2 NA, water immersion objective lens (Olympus America) and detected at 100 ms time resolution with an EMCCD camera (Andor). Using a custom program written in IDL, individual molecules were identified and their intensity time traces were extracted. The total intensity of all the molecules identified within each imaging area

was then calculated using a custom MATLAB program. The decay in the average intensity as a function of time was fitted to a single exponential, with the lifetime obtained as the characteristic single-molecule photobleaching time[39,45]. This measurement was repeated four to six times in different imaging areas for each sample for a statistical analysis of the results obtained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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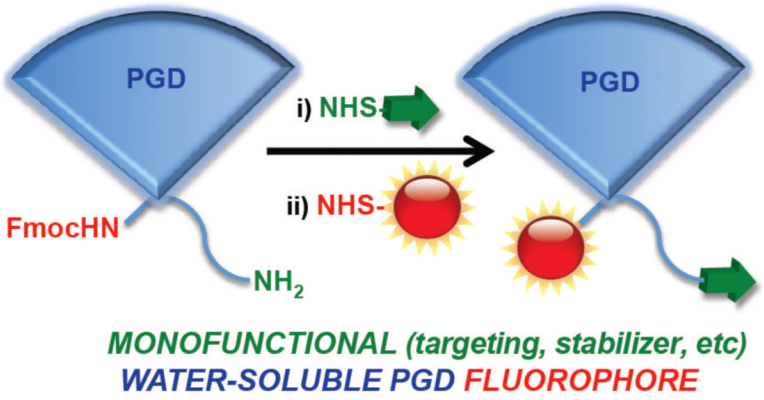

Figure 1. Schematic representation of the synthesis of water-soluble and monofunctional polyglycerol-dendronized fluorophores

Water-soluble polyglycerol dendrimers (PGDs) with two reactive groups lead to the stepwise incorporation of a targeting moiety and a fluorophore. In this approach, a free amine in the PGD reacts with a *N*-hydroxysuccinimide (NHS) ester-functionalized targeting group. After deprotection of 9-fluorenylmethyloxycarbonyl (Fmoc) group, amide coupling between the resulting amine and a NHS-fluorophore produces a water-soluble and monofunctional PGD-fluorophore conjugate.

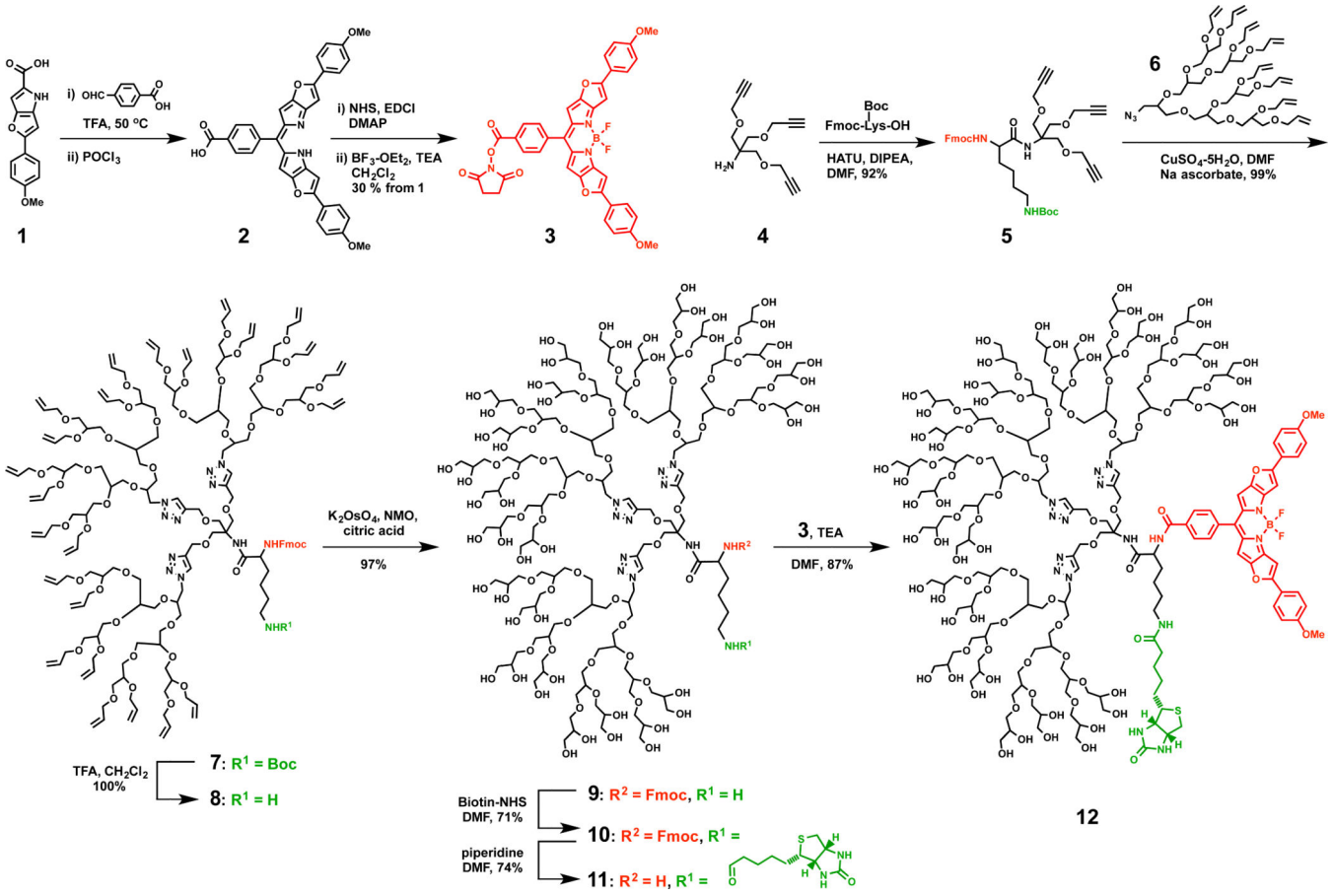

Figure 2. Synthesis of biotinylated PGD-BODIPY 12

TFA, trifluoroacetic acid; NHS, *N*-hydroxysuccinimide; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; TEA, triethylamine; Fmoc, 9-fluorenylmethyloxycarbonyl; Boc, *tert*-butoxycarbonyl; HATU, 2-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA, *N*,*N*-diisopropylethylamine; NMO, *N*-methylmorpholine *N*-oxide. The dihydroxylation stereochemistry was not controlled in the PGD synthesis so it contains a mixture of diastereomers.

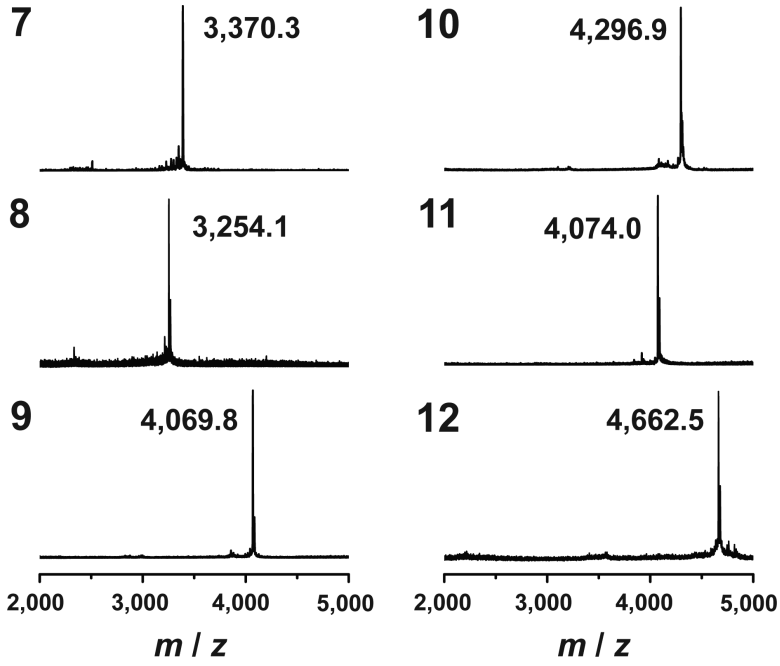

Figure 3. MALDI-TOF mass spectra of 7-12

Quantitative conversions in all chemical reactions employed for the synthesis of polyglycerol dendrimers **7-12** are shown by complete shifts of molecular ion peaks in each synthetic step.

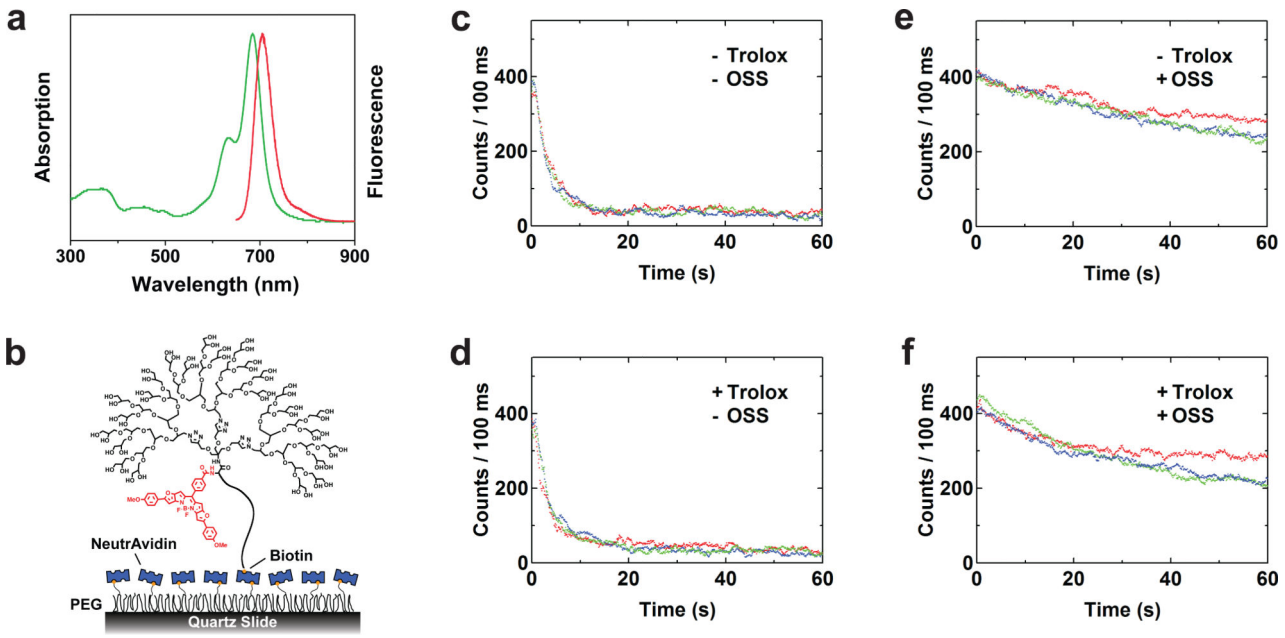

Figure 4. Photophysical properties of 12

a, Normalized absorption (green) and emission (red) spectra of **12** in water. **b**-**f**, Single-molecule analysis of **12**: experimental scheme, in which **12** was immobilized on a PEG-coated surface through biotin-neutravidin linkage (**b**), averaged fluorescence intensity as a function of time measured in three different imaging areas (red, green and blue) in T50 buffers containing no additives (**c**), Trolox only (**d**), oxygen scavenging system (OSS) only (**e**), and both Trolox and OSS (**f**). See Methods for details of the imaging experiments.

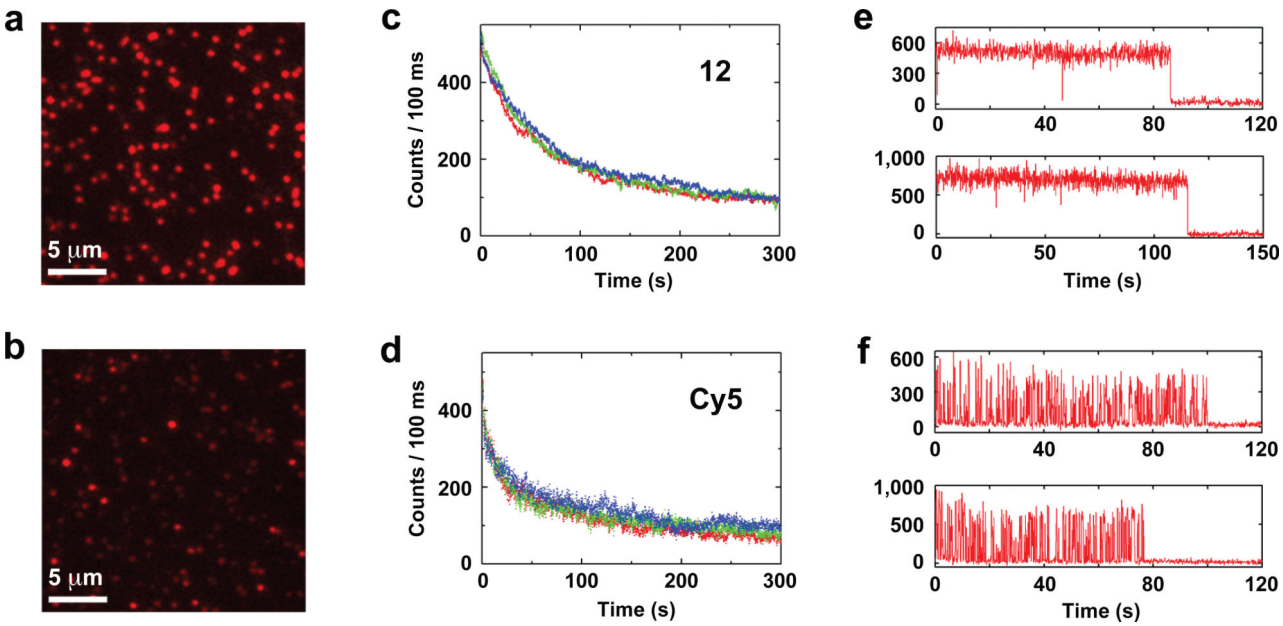

Figure 5. Photostability comparison of 12 with Cy5

Single-molecule imaging was performed in the presence of oxygen scavenging system without Trolox. **a**,**b**, Fluorescence images of immobilized **12** (**a**) and Cy5 (**b**) with pseudo colors. **c**,**d**, Averaged fluorescence intensity of **12** (**c**) and Cy5 (**d**) as a function of time measured in three different imaging areas (red, green and blue). **e**,**f**, Typical fluorescence intensity traces of individual **12** (**e**) and Cy5 (**f**) molecules. **12** shows non-blinking fluorescence in contrast to the blinking behavior of Cy5. 10× accelerated movies of **12** and Cy5 are provided in Supplementary Movie.