Abstract: Reversibly photoswitchable 1,2-bis(2-ethyl-6-phenyl-1-benzothiophene-1,1-dioxide-3-yl)perfluorocyclopentenes (EBTs) having fluorescent “closed” forms were decorated with four or eight carboxylic groups and attached to antibodies. Low aggregation, efficient photoswitching in aqueous buffers, specific staining of cellular structures, and good photophysical properties were demonstrated. Alternating light pulses of UV and blue light induce numerous reversible photochemical transformations between two stable states with distinct structures. Using relatively low light intensities, EBTs were applied in biology-related super-resolution microscopy based on the reversible saturable (switchable) optical linear fluorescence transitions (RESOLFT) and demonstrated optical resolution of 75 nm.

The optical super-resolution techniques, as well as the fluorescent proteins and synthetic dyes required for them, revived fluorescence microscopy as one of the most powerful methods in the life sciences. The diffraction limit no longer restricts the spatial resolution in optical microscopy; it was overcome by using photoinduced switching of the fluorescent markers between dark and emitting states. For example, in biology-related optical microscopy based on the RESOLFT concept, an optical resolution of 30–40 nm has been achieved with reversibly photoswitchable fluorescent proteins (Dreiklang, rscEGFP) undergoing thousands of E/Z isomerizations. Owing to the thermal stabilities of the non-fluorescent and fluorescent states and adequate photoisomerization quantum yields, the switching between these states can be performed reversibly, with relatively low light intensities and comparatively low photobleaching. While fluorescent proteins demonstrate good performance in RESOLFT microscopy, synthetic dyes are far inferior in this respect. Man-made photochromic fluorescent dyes incorporated into silica gel nano-beads undergo tens of switching cycles and improve optical resolution. Recently, thiol-assisted photoswitching of a Cy3-Alexa647 conjugate has been implemented in the presence of potassium iodide (KI) under RESOLFT conditions and allowed, for example, to discern tubulin filaments approximately 130 nm apart. Still, RESOLFT microscopy of noteworthy subdiffraction resolution on biological samples has not yet been realized for synthetic organic fluorophores undergoing reversible photochemical transformation and switching between two stable states with distinct and stable structures.

The required properties of RESOLFT markers—sufficient photostability, high fluorescent modulation (> 90%), high brightness (product of the fluorescence quantum yield ($\Phi_f$) and extinction coefficient ($\varepsilon$)), thermal stability of the fluorescent and non-fluorescent forms, satisfactory switching kinetics, large separation between the absorption bands of the two stable states—have been achieved for synthetic photochromic dyes in organic solvents, but their performance in purely aqueous solutions remained poor (even in the presence of solubilizing residues and polar ionic groups). In most cases, these disappointing results were obtained for bi-chromophoric compounds with separate photochromic and fluorescent units, in which the fluorescence quenching was affected by the resonant energy transfer (RET) from the fluorescent unit (RET donor) to the colored form of the photochromic unit (RET acceptor). Alternatively, photo-switching of the emission signal without destructive readout caused by the excitation light was realized by using the electron-transfer process (from the excited state of fluorophore to the “closed form” of the photochromic unit). In any case, it is difficult to compensate the inherent hydrophobicity of the relatively large and complex molecular switch by adding polar or charged groups.

Great expectations in the field were associated with the new photochromic sulfones based on 1,2-bis(2-alkyl-6-aryl-1-benzothiophen-3-yl)perfluorocyclopentenes (see Scheme 1). By irradiation with UV light, the “open” form
Illumination with visible (blue) light restores the “open” isomer. Remarkably, the fluorescent “closed” forms emit green light with quantum efficiencies of up to 0.92,[11] and the photochromic (switching) and fluorescent (signal) functions are combined in one chemical entity. Using these sulfone derivatives as leads, we planned to prepare photochemically switchable fluorophores applicable in purely aqueous buffers that possess a reactive group and perform well (as bioconjugates) in commercially related RESOLFT microscopes using moderate light intensities of 1–10 kW cm−2 and switching times in the μs–ms range.

After screening many substituents attached to positions C2(2′) and C6(6′) in 1,2-bis(1-benzothiophene-1,1-dioxide-3-yl)perfluorocyclopentene, we have chosen a symmetrical core structure with 2(2′)-ethyl and 6(6′)-phenyl groups (Scheme 1, dyes 1 and 2).[11] These groups, and the absence of strong electron-donor residues at C6,6′ provide a relatively low (but not too low) quantum efficiency of the ring-opening reaction (Table 1). It was sufficient for a high number of excitation–emission cycles of the closed isomer in a read-out process, before its back-isomerization to the open form takes place (erase or refresh step). In contrast, strong electron donors at C6,6′ slow down the ring-opening reaction drastically[11a,12] and might promote photodegradation of the closed form. Ethyl groups at C2(2′) (not methyl groups) are essential for increasing the fluorescence quantum yields in highly polar solvents.[11b] Photophysical properties of newly synthesized dyes 1 and 2 in aqueous buffers are summarized in Table 1 (the absorption and emission spectra are presented in Figure S6). The differences in properties measured in aqueous solutions and in methanol (Table S2) are not large. For instance, the emission efficiencies and fluorescence lifetimes are reduced by only 20–30% in PBS at pH 7.4, with slightly larger variations observed for compound 1.

A difficult task was to find and attach to the dye core the most appropriate hydrophilic residues, to prevent aggregation of the free dyes and their bioconjugates in aqueous solutions. Numerous approaches to hydrophilic, water soluble diarylethenes with sulfonic[13] carboxylic acid[11,14] hydroxy groups, or inositol residues,[14] as well as poly(ethylene glycol) chains,[14a,15] have been already reported. These polar groups are necessary because the cores of the best synthetic switchers are inherently hydrophobic, and these compounds are poorly soluble in water,[14c] form nanoggregates[13b] or need cyclodextrins as additives for solubilization.[13a] Moreover, they often lack a reactive group for bioconjugation. We have found that the presence of several carboxylic acid groups conferred the required properties to dyes 1 and 2, despite the common notion that the carboxylic acid is inferior to the sulfonic or phosphoric (phosphonic) residue in terms of solubilization.

Indeed, the presence of four or eight carboxylates provided a fairly good solubility at pH > 5 and inhibited aggregation (in the micromolar range) even after photocyclization (the closed isomer aggregates more readily).[11d] Unlike most of the other solubilizing groups, one of the carboxylates can be conveniently transformed into any reactive group. Using mono N-hydroxysuccinimidy esters and standard labeling methods, we have successfully conjugated these two new hydrophilic diarylethenes with secondary antibodies. Remarkably, all the bioconjugates retain the photochromic and fluorescent properties of free dyes 1 and 2. They were used in conventional (confocal) microscopy and in super-resolution RESOLFT microscopy.

Diarylethenes 1 and 2 were obtained via three different routes: using a Suzuki–Miyaura cross coupling of C6(6′)-diodio derivative[11a] (Scheme S2/S3) with either 3,5-diformylphenyl boronic (route A; 23% yield over 2 steps), or 5-boronoisophthalic acid (route B; 46% yield), or a pinacol ester of 3,5-di(tert-butoxycarbonyl)phenylboronic acid (route C; 13% over 2 steps).[11a] An additional Jones oxidation step in route A or an acidic treatment (with CF3COOH in CH2Cl2) in route C was applied. To improve the hydrophilic properties and suppress the aggregation even further, we prepared diarylethene 2 with eight carboxylic groups. This was achieved in 41% yield by a similar strategy using a Suzuki–Miyaura cross-coupling followed by cleavage of tert-butyl esters under acidic conditions.[11a] The amino-reactive mono NHS esters of these dyes were prepared by the gradual and controlled addition of the measured quantities of N,N-dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to solutions of 1 or 2 and N-hydroxsuccinimide in DMF with catalytic amounts of

![Scheme 1. Photoswitchable EBTs 1 and 2 decorated with four or eight carboxylic acid groups; see Table 1 for spectral properties of the "open" (OF, non-fluorescent) and the "closed" (CF, fluorescent) forms.][2] For compounds with R = H and R = C6H11, see Refs. [9a, b, c] and [9d, e], respectively.

| Table 1: Photophysical properties of free dyes 1 and 2 in PBS (pH 7.4). |
|-----------------|-------|-------|
| Parameter       | State 1 | State 2 |
| λmax,abs [nm]   | CF 450/35 000 | 448/45 000 |
| ε [M−1 cm−1]    | CF 534, 558 | 522, 550 |
| λmax,flu [nm]   | CF 354, 558 | 354, 558 |
| φf [flu]        | CF 0.48 | 0.57 |
| τ [ns]          | CF 1.72 ± 0.05 | 2.04 ± 0.05 |
| φ0−φc [flu]     | 0.19 ± 0.04 | 0.23 ± 0.04 |
| φ1−φc [flu]     | 1.2 × 10−4 ± 3 × 10−4 | 2.0 × 10−4 ± 3 × 10−4 |

[a] lowest energy absorption peak. [b] fluorescence quantum yield; fluorescein in 0.1 M NaOH was used as standard. [c] At 365 nm. [d] At 470 nm (see Supporting Information for details).
4-(N,N-dimethyl)aminopyridine (DMAP).\[16\] The stable mono-NHS ester of dye 1 was isolated by preparative RP-HPLC, while mono-NHS ester of dye 2 could be only generated in solution without isolation. Then, the secondary antibodies were labeled with these mono-NHS esters and purified by gel-filtration.\[16\] MALDI-TOF mass spectrometry and UV/Vis spectroscopy were used to evaluate the degrees of labeling (DOL; see Table S1 in the Supporting Information). We varied the DOL values to achieve the best immunofluorescence staining and acquire the best-possible images under confocal and RESOLFT conditions.

The conjugates obtained with secondary antibodies were highly fluorescent and photochromic; we used them for immunostaining of tubulin filaments and nucleopore complexes in Vero cells. Confocal measurements (Figure S1) showed their selective binding with primary antibodies and tubular network in the whole fixed cells. Cells immunolabeled with dyes 1 and 2 were used for pump–probe fluorescence measurements in a modified confocal microscope using a wide-field illumination (Figure S2). For that, the stained cells were mounted onto an object slide with a cavity filled with PBS (pH 7.4). Switching between the open and closed forms was performed by laser light of 375 nm and 491 nm wavelength, respectively. The measurement was initialized by a 10 ms pulse of 491 nm light (17 kW cm\(^{-2}\)) to switch all the fluorophores in the observation area to the “dark” state. Then a pulse sequence was applied consisting of a 20 μs pulse of 375 nm light (0.8 kW cm\(^{-2}\)), a 500 μs delay, a 10 ms pulse of 491 nm light (17 kW cm\(^{-2}\)) and another 2 ms delay. This pulse sequence was repeated 200 times (in Figure 1 and Figure S3, 100 and 200 cycles are shown, respectively). These measurements showed that half of the initial fluorescence is bleached after performing about 11 switching cycles for dye 1 and 21 switching cycles for dye 2, respectively. A bi-exponential fit of the obtained decay curve of the ring-opening (“off”-switching) reaction gave the time constants of \(t_1 = 241\) µs and \(t_2 = 1.31\) ms for dye 1 (79% of the amplitude belongs to \(t_1\) and 21% to \(t_2\)). Under these irradiation conditions, the corresponding decay times for dye 2 are \(t_1 = 126\) µs (77%) and \(t_2 = 674\) µs (23%). The residual fluorescence per cycle was evaluated by comparison of the maximal fluorescence in a cycle and the average value of the residual fluorescence recorded during the last 100 μs illumination with 491 nm light (as a mean value of 100 cycles). The residual fluorescence was found to be 4.3% for dye 1 and 1.7% for dye 2, respectively.

To demonstrate the applicability of dyes 1 and 2 for RESOLFT microscopy, fixed immunolabeled Vero cells were imaged using a modified 1C RESOLFT QUAD Scanning microscope (Abberior Instruments, Göttingen). The imaging was performed by applying the following pulse sequence: switching the diarylethenes to their fluorescent “closed” isomers with 355 nm light (130 W cm\(^{-2}\)) for 50 μs, 200 μs delay, application of the doughnut-shaped 488 nm beam (36 kW cm\(^{-2}\)) for 1.2 ms to switch the diarylethenes in the periphery of the focal spot to their non-fluorescent “open” isomers. Finally, the remaining area with the fluorescent isomers was probed for 80 μs with a Gaussian shaped 488 nm light beam (9.7 kW cm\(^{-2}\)). The same pulse sequence was applied to record the confocal images except that the off-switching was omitted. Using this imaging scheme, it was possible to acquire RESOLFT images with an optical resolution of 74 nm FWHM\[17\] and confocal images with a resolution of 175 nm FWHM.\[17\] The confocal and RESOLFT images are given in Figure 2, Figure S4 (tubulin filaments), and Figure S5 (nucleopore complexes). The relatively high confocal resolution (Figure 2 and Figure S4) results from the 355 nm activation spot that is significantly smaller than the excitation spot at 488 nm wavelength. This two-step process of activation and excitation yields an effective confocal point spread function (PSF) which can be described by the product of the activation, excitation, and detection PSFs.\[18\]

Both diarylethenes show a high switching contrast in the pump–probe measurements. Although the switching-off rate of the free dye 2 is only about twice the rate of dye 1 (Table 1), it turned out that during RESOLFT measurements, dye 2 (attached to a protein) switches off much faster than dye 1 (1.5 ms for dye 2 versus 7 ms for dye 1); this is probably the reason why dye 1 bleached faster and provided poorer resolution (85 nm). The resolution achieved in the RESOLFT measurements was limited mainly by photobleaching and not by the residual fluorescence after off-switching.

To date, reversibly photoswitchable fluorescent proteins still offer a significantly lower switching fatigue (about 100 \(\times\) more cycles),\[19\] yet we have shown the possibility to prepare reversibly photoswitchable organic dyes that perform in purely aqueous solutions and are suitable for biology-related RESOLFT microscopy. Importantly, the simple EBT scaffold presented a viable core structure and was decorated with four
or eight carboxylic acid groups. Compounds 1 and 2 underwent reversible photochemical transformations and switched between two stable states with distinct structures in aqueous PBS buffer and without stabilizing reagents and additives. The results of the present study allow for the first time manmade photoswitchable fluorophores to be compared with photoswitchable fluorescent proteins. Though the performance of synthetic dyes in RESOLFT microscopy is still inferior to that of fluorescent proteins (in respect of bleaching, number of switching cycles, and phototoxic effects of UV light), the simplicity of EBT structures is encouraging. Indeed, low aggregation, efficient photoswitching in aqueous buffers, and chemical reactivity were gained straightforwardly, by introducing numerous freely rotating COOH groups to a highly symmetrical core structure. All these results can be very helpful for future work. It will focus on the design and characterization of reversibly photoswitchable and fatigue-resistant dyes that isomerize into a stable fluorescent form by irradiation with light of 405 nm and longer wavelengths. Moreover, their ability for labeling living cells (using SNAP-Tag or HaloTag® technologies) will be evaluated.

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[10] The “closed” form is yellow and emits green light.


[16] For synthetic procedures, details and analytical data, see the Supporting Information. Dyes 1 and 2 are base-sensitive; under alkaline conditions they react with water giving products with a -CF₂CF₂C(O)-fragment instead of initial perfluorocyclopentene ring. The same transformation occurred in wet DMSO.

[17] Full width at half minimum (FWHM) of the Lorentzian fit.

[18] The point spread function (PSF) is the spatial intensity distribution of a point-like source imaged by an optical system.


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