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Two-photon FRET pairs based on coumarin and DBD dyes†

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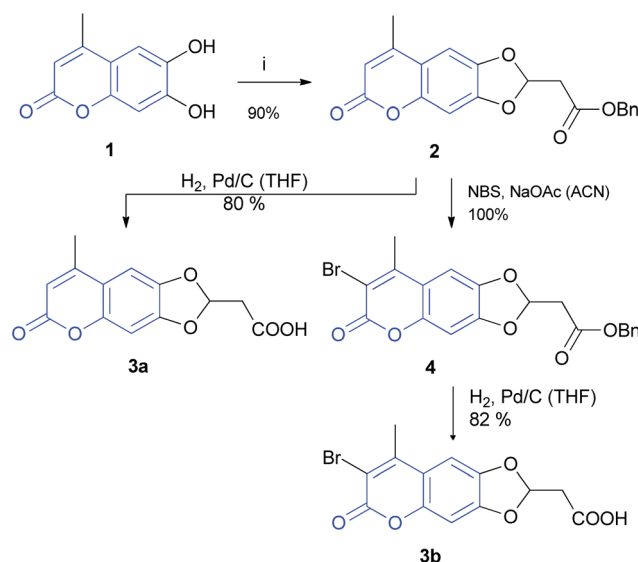
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The synthesis and photophysical properties of two new FRET pairs based on coumarin as a donor and DBD dye as an acceptor are described. The introduction of a bromo atom dramatically increases the two-photon excitation (2PE) cross section providing a 2PE-FRET system, which is also suitable for 2PE-FLIM.

Amongst various analytical techniques, the evaluation of fluorescence signals is one of the most sensitive methods.¹ Therefore, fluorescence spectroscopy has found a wide range of applications in biology, biochemistry, and clinical diagnostics. Besides classic steady state measurements that evaluate the fluorescence intensity, methods utilizing the fluorescence lifetime have gained growing interest in recent years. Both parameters are successfully applied in fluorescence imaging. The pivotal element of these methods is the fluorescent dye and its properties. Small fluorescent organic molecules play, beside quantum dots (QD)² and luminescent lanthanide complexes,³ the central role in current fluorescence spectroscopy. The potential of a fluorescent dye is determined by the combination of its photophysical parameters, such as fluorescence quantum yield (Φ_F), molar extinction coefficient (ϵ), fluorescence lifetime (τ_F), absorption and emission wavelength (λ_{ABS} , λ_{EM}), and stability against photobleaching. Because the penetration depth of light in biological tissue is increased with increasing wavelength, high λ_{ABS} and λ_{EM} are desirable. However, a long-wavelength absorption maximum is usually connected with low thermal and photochemical stability of the dyes. This problem can be circumvented by two-photon excitation (2PE),⁴ which is based on the simultaneous absorption of two photons with lower energy instead of one photon with high energy. Consequently, the excitation wavelength λ_{EXC} is almost doubled in 2PE. The probability of the 2PE is described by its 2PE cross

section σ_2 . Unfortunately, σ_2 is low for most organic molecules.⁵ On the other hand the applicability of many fluorescence dyes suitable for 2PE is limited when considering the other photophysical properties. The combination of the outstanding properties of two fluorescent dyes within one molecule can be achieved by a pair of dyes capable for Förster Resonance Energy Transfer (FRET).⁶ This phenomenon, in which one dye (the donor D) is excited and the energy is transferred to the other dye (the acceptor A), is observed if the emission spectrum of D and the absorption spectrum of A sufficiently overlap. Furthermore, the FRET efficiency is strongly distance-dependent (the distance with 50% FRET efficiency is called Förster distance R_0) and is influenced by the relative arrangement of the transition dipole moments of D and A. A special type of FRET exists if the donor D is capable of 2PE. In this case, the fluorescence of the donor D as well as the acceptor A can be observed at shorter wavelength than the excitation ("Anti-Stokes shift"). Although some

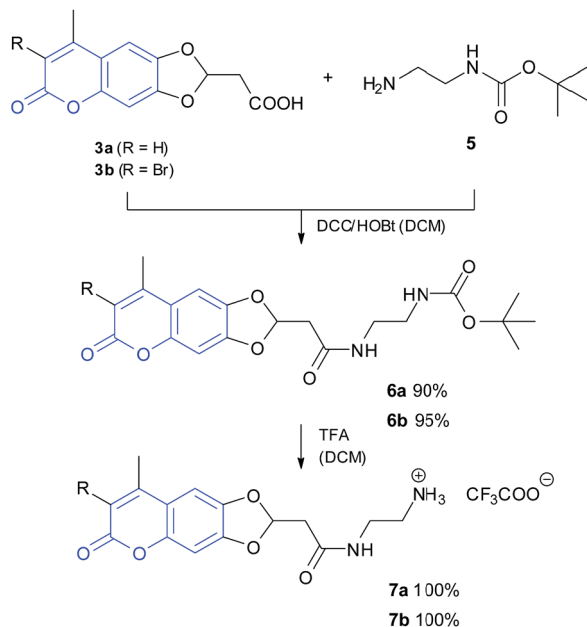


Scheme 1 Synthesis of coumarins 3a,b (i benzyl prop-2-ynoate, DMAP).

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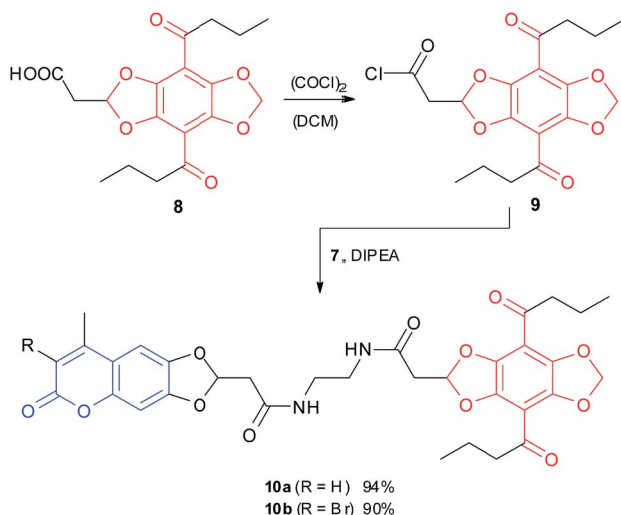




Scheme 2 Synthesis of compounds 7a,b.

examples of 2PE-FRET pairs were reported in the literature,⁷ the number of 2PE-FRET systems is rather limited.

In the last few years, we have developed a new class of fluorescent dyes, whose structure is based on [1,3]dioxolo[4,5-*f*][1,3]benzo-dioxole (DBD).^{8–14} These dyes are characterised by large Stokes shifts ($\Delta\lambda = \lambda_{EM} - \lambda_{ABS} > 100$ nm), combined with long fluorescent lifetimes ($\tau_F > 20$ ns) and exceptional bleaching stability. The 2PE cross-sections σ_2 of DBD dyes are, however, rather low (*vide infra*). Recently, we reported on a first FRET pair with DBD dyes as acceptor and 2,5-diphenyloxazol (PPO) as donor.¹³ Herein we wish to report on the synthesis and properties of new FRET pairs with coumarin derivatives as donor and DBD dyes as acceptor for 2PE-FRET application.



Scheme 3 Synthesis of FRET pairs 10a,b.

Table 1 Summary of spectroscopic data of compounds 2, 4, 8, 10a, and 10b (DMSO)

| Compound | λ_{ABS}/nm | λ_{EM}/nm | τ_F/ns | $\epsilon/\text{M}^{-1} \text{cm}^{-1}$ | Φ_F | σ_2/GM^a |
|----------|---------------------------|--------------------------|--------------------|---|----------|------------------------|
| 2 | 343 | 413 | 0.8 ^b | 10 878 | 0.23 | 0.01 ± 17% |
| 4 | 353 | 436 | 1.3 ^b | 15 269 | 0.17 | 13.86 ± 4% |
| 8 | 437 | 570 | 24.8 ^c | 3094 | 0.68 | 0.04 ± 3% |
| 10a | 344, 433 | 570 | 24.0 ^d | — | — | — |
| 10b | 355, 434 | 570 | 23.5 ^d | — | — | — |

^a Excited at 780 nm (2, 8, 10a,b) or 720 nm (4), GM = 10⁻⁵⁰ cm⁴ per s per photon (threefold measure). ^b Fluorescence lifetime was detected at 420 nm ($\lambda_{EXC} = 375$ nm). ^c Fluorescence lifetime was detected at 570 nm ($\lambda_{EXC} = 440$ nm). ^d Fluorescence lifetime was detected at 570 nm ($\lambda_{EXC} = 372$ nm).

The coumarin chromophore was chosen because it is already known to be suitable for 2PE.¹⁵ The synthesis starts with the commercially available 6,7-dihydroxy-coumarin 1. The reaction with benzyl prop-2-ynoate¹⁶ in the presence of catalytical amounts of DMAP afforded coumarin 2 with very good yield.

This type of cyclisation has already been described for other catechols^{8,10,17} but never applied to coumarins. Because the introduction of halogen atoms regularly increases the 2PE cross-section, 2 was brominated with NBS to give 3-bromo-coumarin in quantitative yield. Subsequently, esters 2 and 4 were deprotected by catalytic hydrogenation to the carboxylic acids 3a,b (Scheme 1). The connection between coumarin and DBD chromophore was accomplished by a 1,2-diaminoethane linker. For this purpose, esters 3a,b were converted into amides 6a,b by reaction with commercially available *N*-Boc-1,2-diaminoethane 5, which were subsequently deprotected to give primary amines 7 (Scheme 2).

Finally, the FRET pairs 10a,b were prepared from amines 7a,b and acyl chloride 9, which is easily accessible from the known DBD acid 8 (Scheme 3).¹⁰

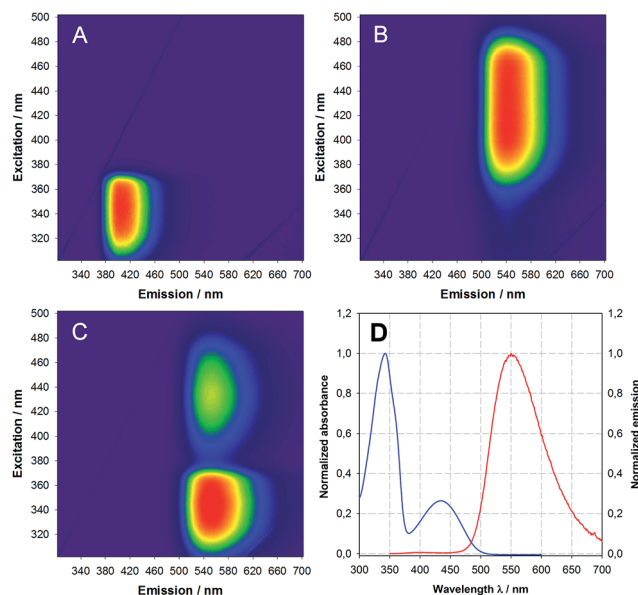


Fig. 1 3D excitation emission matrix (EEM) of (A) coumarin 2, (B) DBD 8, (C) FRET pair 10a and (D) absorption (blue) and emission (red) spectra of FRET pair 10a in DCM (excitation at 340 nm).



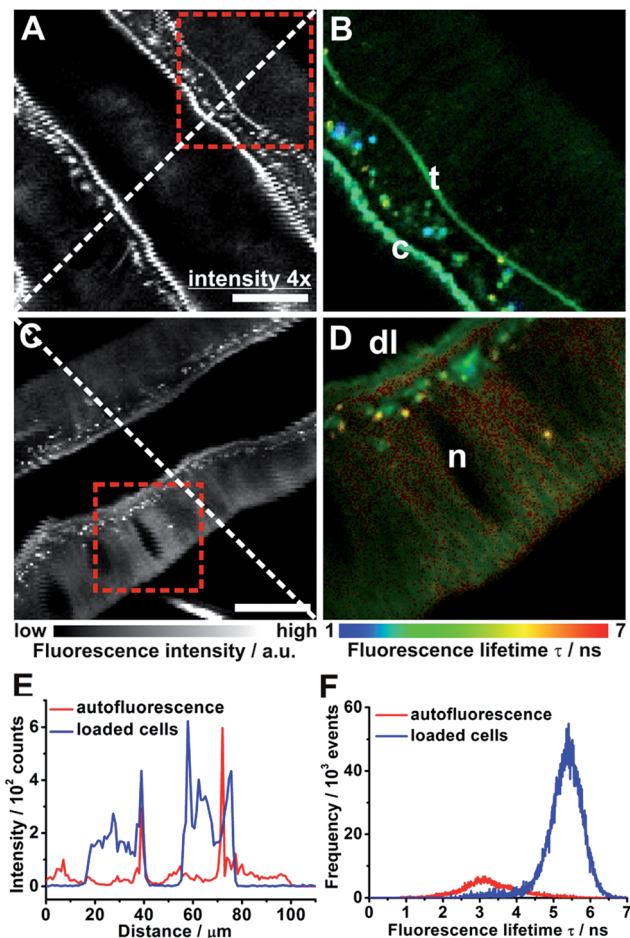


Fig. 2 Autofluorescence intensity image of a salivary duct $\times 4$ boosted for visibility (A) and corresponding 2PE-FLIM image of the red rectangle (B). Fluorescence intensity image of **10b**-loaded salivary duct (C) and corresponding 2PE-FLIM image of the red rectangle (D). Scale bar = 20 μm ; c = luminal cuticule; t = trachea; dl = duct lumen; n = nucleus. (E) Intensity profiles along the dashed lines in (A) and (C). (F) Fluorescence lifetime histograms for the 2PE-FLIM images shown in (B) and (D).

Next, we investigated the spectroscopic properties of compounds **10a,b** in comparison with the starting compounds **2**, **4**, **8**. The results in DMSO are summarized in Table 1 (for other solvents see ESI[†]). Of particular note are the large Stokes shift (133 nm) and the long fluorescence lifetime (24.8 ns) of DBD dye **8** compared with coumarins **2** and **4**. The successful FRET in compound **10a,b** was shown by 3D-fluorescence experiments, which are exemplarily outlined for **10a** in Fig. 1.

The highly efficient FRET in compound **10a** is stressed in Fig. 1C by (i) the strong quenching of the donor emission (compare to Fig. 1A) and (ii) the strong emission of the DBD acceptor under indirect excitation *via* the coumarin donor (compare to Fig. 1B). This result was to be expected, because the maximum possible distance between the chromophores (1.5–1.6 nm) is markedly below the Förster radius R_0 for this FRET pair (2.56 nm, for details see the ESI[†]).

Whereas the 2PE cross section σ_2 is very low for DBD compound **8** and coumarin **2**, the introduction of bromine in

coumarin **4** significantly enhances the σ_2 value to 0.74 GM (see Table 1).

In order to explore potential applications of the 2PE-FRET pairs-sensor **10b** in living cells, its uptake into such was studied in 2PE-fluorescence lifetime imaging (2PE-FLIM) experiments. Insect salivary gland lobes were incubated for 15 min with 2 μM **10b** and then, the tubular-like salivary ducts were imaged. Salivary ducts without **10b**-loading displayed a comparatively low autofluorescence when excited at 780 nm as expected for 2PE (Fig. 2A and E).¹⁸ Mainly the luminal cuticule and tracheae contributed to the autofluorescence (Fig. 2B) and the corresponding fluorescence lifetime distribution fluctuated around 3 ns (Fig. 2F). In contrast, the fluorescence intensity of **10b**-loaded salivary ducts was up to one order of magnitude higher than that of unloaded ducts (Fig. 2C and E). Such a loading efficiency has previously been reported for other ion-sensitive fluorescent dyes.¹⁹ Here, the duct cells were stained strongly and **10b** did not accumulate in specific cellular compartments. However, almost no fluorescence could be observed in the nuclei and the duct lumen (Fig. 2D). This result is a prerequisite for successful intracellular recordings using the novel 2PE-FRET pairs presented. The sufficient **10b**-loading into living cells could also be observed in the fluorescence lifetime distribution, which was now shifted to longer lifetimes around 5.5 ns (Fig. 2F).

Conclusions

Both single chromophores exhibit specific pros and cons. The 3-bromocoumarin shows a sufficiently large 2PE cross section (0.74 GM) but has a low fluorescence lifetime (1.3 ns) and a relatively short emission wavelength (436 nm).

By contrast, the DBD chromophore shows long emission wavelength (570 nm), large fluorescence lifetime (23–25 ns) but very low 2PE cross section. The FRET pair **10b** perfectly combines the advantages of these dyes. After long-wavelength two-photon excitation at 780 nm an efficient FRET takes place resulting in a long-lived emission at 570 nm. The applicability of **10b** for 2PE-FLIM was demonstrated with the aid of living cells of insect salivary gland lobes. In this application the large contrast range of 1–7 ns is noteworthy. Currently, we are investigating synthetic routes to derivatives of **10b**, which are suitable for coupling with various biomolecules.

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