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Toward Two-Photon Absorbing Dyes with Unusually Potentiated Nonlinear Fluorescence Response

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ABSTRACT: The combination of two two-photon-induced processes in a Förster resonance energy transfer (FRET)-operated photochromic fluorene-dithienylethene dyad lays the foundation for the observation of a quartic dependence of the fluorescence signal on the excitation light intensity. While this photophysical behavior is predicted for a four-photon absorbing dye, the herein proposed approach opens the way to use two-photon absorbing dyes, reaching the same performance. Hence, the spatial resolution limit, being a critical parameter for applications in fluorescence imaging or data storage with common two-photon absorbing dyes, is dramatically improved.

Multiphoton processes find extensive use in imaging applications^{1–3} and data storage⁴ with specifically designed organic chromophores. This preference is tightly related to the nonlinear dependence of the absorption probability on the excitation light intensity. For two-photon absorption (2PA) a quadratic dependence applies,^{5–8} and for higher-order multiphoton absorption cubic (3PA)⁹ or quartic (4PA),¹⁰ dependencies are expected. The practical consequence of this photophysical phenomenon translates into a much higher density of excited chromophores in the focal point of the exciting laser beam as compared to areas that are out-of-focus. If fluorescence emission is used to monitor the excited state, this translates into superior 3D spatial resolution, for example in fluorescence microscopy. Noteworthy, there is a dramatic increase in the spatial resolution with an increasing number of simultaneous excitations; i.e., 3PA and 4PA are superior to 2PA. However, on the downside the absorption probability of 3PA and 4PA is dramatically decreased, requiring very high laser intensities, and the excitation wavelength would be shifted to longer than 1200 nm for typical dyes with one-photon absorption at around 400 nm. Few reports on 3PA and 4PA dyes^{9–16} and their occasional use in bioimaging^{17–20} can be found in the literature. Even more rarely, organic dyes²¹ or materials²² that feature SPA are described. However, the mentioned drawbacks widely limit their generalized application from a technological point of view, making imaging with 2PA dyes the most frequently chosen approach.^{1,3}

In this work we introduce a molecular design that builds on 2PA, but presenting the above-described advantages of a nonlinear dependence beyond a 2PA system. The basic approach relies on the structural and photophysical integration^{23,24} of a 2PA dye (a fluorene derivative, FL) with a dithienylethene photoswitch (DTE) in a dyad; see Figure 1.^{25–27} Opposed to multiphotonically addressable DTE switches that are electronically integrated in π -extended architectures,^{28–31} the two chromophores are electronically

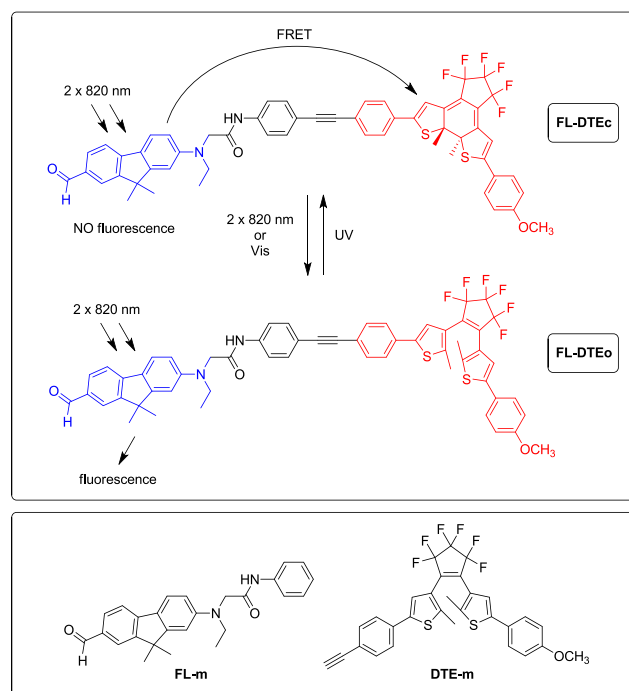


Figure 1. Top: working principle of a photochromic dyad having a DTE appended to a 2PA dye. Bottom: Structures of model compounds.

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decoupled by implication of an insulating spacer. However, in such architectures photophysical communication via electron or energy transfer is possible.^{32–35} In our specific case Förster resonance energy transfer (FRET), implying the colored closed form DTEc but not the colorless open form DTEo, was envisioned.^{36–38} As illustrated in Figure 1, on two-photon excitation the emission of the FL chromophore is quenched by a FRET process, which ultimately sensitizes the isomerization of DTEc to its colorless form. In the latter the unquenched fluorescence of the FL chromophore is observed on two-photon excitation.

As follows from the nonlinear (quadratic) dependence of the 2PA probability on the excitation light intensity, the combination³⁹ of two two-photon-induced processes, i.e., (a) FRET sensitized DTEc \rightarrow DTEo isomerization and thereby the generation of the emissive form of the dyad (FL-DTEo) and (b) two-photon-excited fluorescence, should lead to a quartic dependence for the first instances of the photochromic conversion. On the other extreme, once all DTEc is converted into DTEo, the “normal” quadratic dependence, arising from two-photon-excited fluorescence, would apply.

The choice of the FL chromophore was motivated by its favorable photophysical properties (see Table 1 and spectra in

Table 1. Photophysical Key Data of FL-DTE, DTE-m, and FL-m in Methanol

	$\lambda_{\text{abs,max}}^a$ (nm)	ϵ ($\text{M}^{-1}\text{cm}^{-1}$) ^b	$\lambda_{\text{f,max}}^c$ (nm)	Φ_{f}^d	τ_{f}^e (ns)
FL-DTEo	336	34 900	555	0.36	2.4
FL-DTEc	600	10 900	— ^f	— ^f	— ^f
DTEo-m	302	42 600	— ^g	— ^g	— ^g
DTEc-m	592	18 700	— ^g	— ^g	— ^g
FL-m	378	20 400	555	0.36	2.4

^aLongest-wavelength absorption maximum. ^bMolar absorption coefficient at $\lambda_{\text{abs,max}}$. ^cFluorescence emission maximum. ^dFluorescence quantum yield; 15% error. ^eFluorescence lifetime, measured by time-correlated single-photon counting; 5% error. ^fFL-DTEc is nonfluorescent. Some residual fluorescence is observed due to the presence of 6% FL-DTEo in the photostationary state. ^gNot measured; essentially not fluorescent.

the Supporting Information).⁴⁰ In methanol the separate FL model chromophore (FL-m, see structure in Figure 1) shows a broad intramolecular charge-transfer fluorescence with a maximum at 555 nm ($\Phi_{\text{f}} = 0.36$, $\tau_{\text{f}} = 2.4$ ns). The 2PA spectrum features a maximum at 770 nm with a significant 2PA cross section ($\sigma_{2\text{PA}}$) of 156 GM. The 2PA spectrum essentially coincides with the charge-transfer (one-photon) absorption band of the chromophore, when the wavelength scale of the former is divided by 2, i.e., $\lambda_{2\text{PA}} \approx 2\lambda_{1\text{PA}}$. Importantly, the two-photon-excited fluorescence spectrum is the same as observed by one-photon excitation into the absorption band of the chromophore ($\lambda_{\text{abs,max}} = 378$ nm), confirming that the same excited state is involved in either process.

When integrated into the FL-DTE dyad (see Supporting Information for details on the synthesis of the ring-open form; the ring-closed form is generated *in situ* by UV-light irradiation) the fluorene shows its fluorescence only when the DTE is encountered in its open form; see Figure 2 and Table 1. However, for the closed form a significant spectral overlap ($J = 1.54 \times 10^{15} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$) between the FL emission and the DTEc absorption (see data in Table 1) yields a practically quantitative FRET process ($\Phi_{\text{FRET}} \approx 1$); see

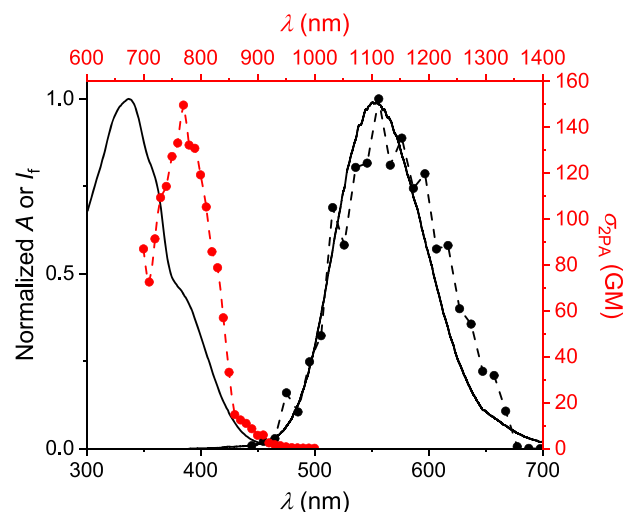


Figure 2. One-photon absorption (left) and fluorescence (right) spectra of FL-DTEo (black solid lines); 10 μM in methanol. Note that the absorption spectrum of FL-DTEo shows the FL band as a shoulder at ca. 384 nm and the accordingly adapted 2PA spectrum (red dashed line and red points) coincides spectrally with that feature. The two-photon-excited fluorescence spectrum (black dashed line and black points) resembles the conventional fluorescence spectrum (black solid line).

Supporting Information for details. The critical FRET radius R_0 was determined as 53 Å (see Supporting Information), far larger than the modeled distance between the centers of the two chromophores ($R = 22$ Å). For the colorless FL-DTEo the experimental fluorescence quantum yield and lifetime are identical to those of the model FL-m (Table 1), being reasoned with the zero spectral overlap integral for this form. This confirms the absence of electronic communication between the FL excited state and the DTEo ground state. After formation of FL-DTEc on irradiation with UV light (302 nm; $\Phi_{\text{oc}} = 0.50$), a residual fluorescence (ca. 6% of that of FL-DTEo) is observed, which, however, is attributed to the minor content of the open form in the photostationary state (DTEc-m/DTEo-m = 94/6; determined by ^1H NMR spectroscopy for the model DTE). Hence, in practical terms quantitative FRET is operative for FL-DTEc, while no such pathway is observed for FL-DTEo. Although photoinduced electron transfer from FL to DTEc cannot be excluded, it is very unlikely that that this process contributes significantly to the quenching (see Supporting Information). The switching is reversed by shining red light on the dyad ($\Phi_{\text{co}} = 0.008$), and 100% of the open form is observed in the corresponding photostationary state.

Having established the FRET behavior of the dyad we proceeded to obtain experimental proof for the two-photon-excited processes in the FL-DTE dyad, i.e., FRET-induced ring opening of FL-DTEc and fluorescence from FL-DTEo. The 2PA spectrum of FL-DTEo (Figure 2) coincides in shape and cross section ($\lambda_{2\text{PA,max}} = 770$ nm, $\sigma_{2\text{PA}} = 150$ GM) with the one of FL-m (see Supporting Information). The operation in the two-photon regime is confirmed by the double logarithmic plot of the fluorescence versus the laser intensity, yielding the expected slope of 2 (see Supporting Information). As for FL-m, the two-photon-excited fluorescence of FL-DTEo spectrally coincides with the emission resulting from conventional one-photon excitation (Figure 2). Importantly, starting with the dyad in its closed nonfluorescent form (FL-DTEc) the irradiation with 820 nm laser light yields the fast increase of

the FL fluorescence emission (see below). Noteworthy, at half of this wavelength, i.e., 410 nm, the DTE photochrome shows negligible absorption (zero for DTEo and $<1000 \text{ M}^{-1} \text{ cm}^{-1}$ for DTEc). Hence, it can be safely assumed that 2PA of the DTE at the chosen excitation wavelength is at most minor, while the FL features a significant cross section (86 GM at 820 nm). Thus, the formation of the fluorescent FL-DTEo form by direct two-photon-induced ring opening is unlikely and instead the above proposed FRET-sensitized ring-opening isomerization is operative.

The beforehand made observations lay the foundation for the proof-of-principle experiment, which we expected to confirm our initial assumption that the combination of two-photon-induced processes yields a dependence of the fluorescence intensity versus laser intensity that goes beyond the “normal” quadratic relationship and which in theory should approximate a quartic function. In Figure 3 the kinetics of the

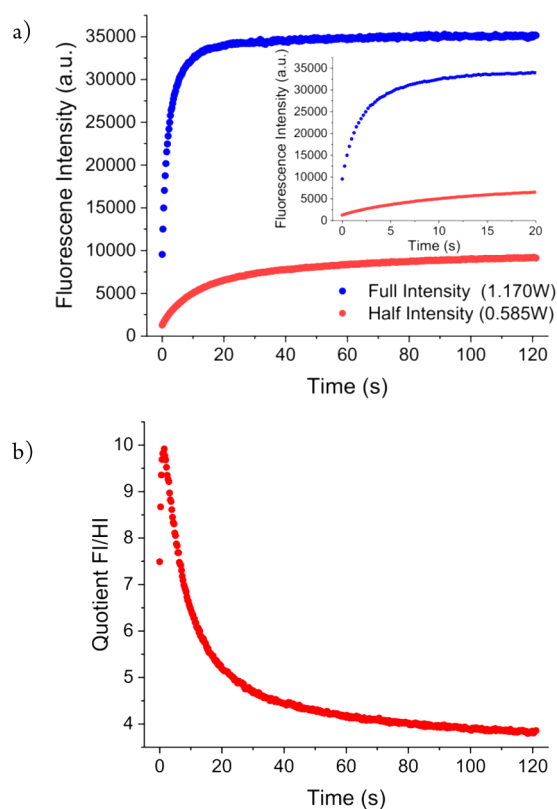


Figure 3. (a) Kinetics of fluorescence buildup on irradiation of FL-DTEc ($10 \mu\text{M}$ in methanol) with 820 nm laser light at full intensity (FI, blue points) and at half intensity (HI, red points). See Supporting Information for the associated rate constants. Inset: Zoom in on the first 20 s . (b) Ratio of time-dependent fluorescence intensities shown in (a).

buildup of the FL fluorescence on 820 nm light irradiation of FL-DTEc at full and half laser intensity are shown. The time-dependent ratio of the kinetics shows a quotient of 10 in the first instances of the irradiation (ca. 1.5 s under the chosen irradiation conditions). For a “pure” quartic dependence a value of 16 (2^4) would be expected. The deviation from this number can be modeled and led to the presence of already 6% FL-DTEo in the initial solution, corresponding to the experimental value observed for the photostationary state of the open-to-closed photoisomerization (see Supporting

Information). At longer irradiation times, and with the build-up of successively higher concentrations of the open fluorescent form FL-DTEo, this quotient decays and approaches a value of 4. Expectedly, this corresponds to the sole two-photon-excited fluorescence of FL-DTEo. Noteworthy, the fitting of the build-up kinetics (at full and half laser intensity) yielded two rate components: a fast one, assigned to the FRET-induced isomerization in the focal volume, and a second slower one ascribed to diffusion of molecules out of the focal volume of the excitation light beam. Interestingly, the ratio of the rate constants of the fast components at different laser intensities shows a value of 4.2, clearly corroborating the two-photon nature of the isomerization (see Supporting Information).

In summary, we propose an innovative approach toward dye systems that enables the harnessing of a dramatically improved dependence of the fluorescence signal on the excitation light intensity. Conventional two-photon absorbing fluorophores, in concordance with photophysical theory, yield a quadratic dependence. By combining a 2PA dye with a photochromic system that can be switched by two-photon-initiated FRET and the resulting form of the dyad showing two-photon-excited fluorescence, two 2PA processes are entangled. This would lead effectively to a quartic dependence for a photochromic system with quantitative (100%) conversion between the isomeric forms. We have been able to approach this theoretical limit, reaching a factor of 10 for comparing excitation with full and half laser intensity (as opposed to solely 4 for exciting the 2PA dye alone). Noteworthy, in practical terms this still outperforms a 3PA dye, for which a factor of 8 (2^3) is expected. Hence, the presented approach enables success in overcoming the photophysically imposed limitations of 2PA dyes, while maintaining the technological benefits of working in the two-photon excitation regime. Our structurally modular approach (nonconjugative combination of the two functional units) allows matching literally any 2PA dye with a FRET-efficient photochromic system to achieve the herein described phenomenon.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c07377>.

Details on the synthesis of new compounds (FL-m and FL-DTE), NMR spectroscopic data and copies of ^1H and ^{13}C NMR spectra, HRMS data, details on DFT calculations (including atomic coordinates of the optimized FL-DTEc structure), description of photo-physical/photochemical characterization procedures, modeling of fluorescence response, considerations on photoinduced electron transfer, and additional spectral data for FL-m and DTE-m (PDF)

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Notes

The authors declare no competing financial interest.

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