

**Ultrabright red to NIR emitting fluorescent organic nanoparticles made from quadrupolar dyes with giant two-photon absorption (2PA) in the NIR region. Confinement effect on fluorescence and 2PA and tuning of surface properties**

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## **Supplementary Information**

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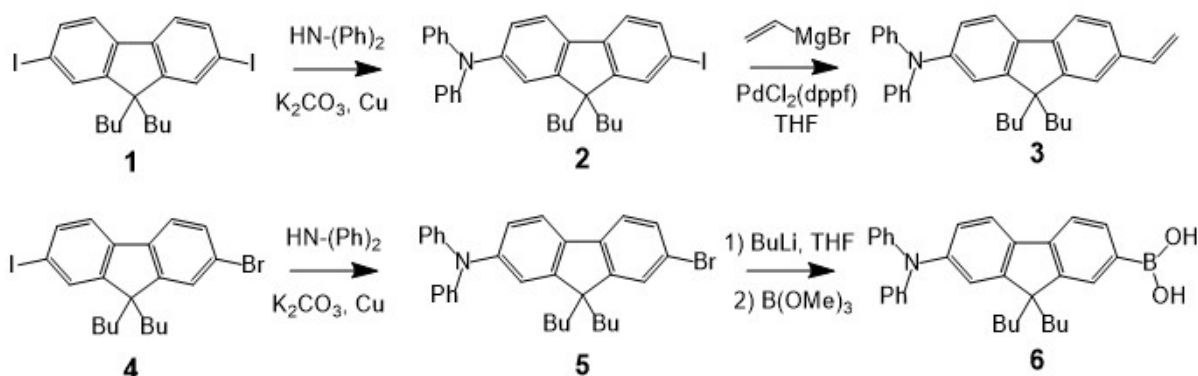
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## 1. EXTENDED MATERIALS AND METHODS

### 1.1. Dyes synthesis

QBDF, QBDeF, QBDF and QBDF chromophores were synthesized following three major steps: synthesis of the donors, synthesis of the cores and coupling between the two.

**Synthesis of donor moieties.** Dissymmetrical precursors diphenylamino-halo fluorene **2** and **5** were prepared by Ullmann coupling of diphenylamine with diiodo (**1**) or bromo-iodo (**4**) fluorene respectively in the presence of copper under basic conditions. The vinyl-conjugated donor **3** was then derived via a Corey-Kumada coupling while the boronic acid analog **6** was obtained after a halogen metal exchange and quenching with trimethylborate (**Scheme S1**).



**Scheme S1** Synthesis of donor precursors

Compound **1** has been previously described.<sup>1</sup>

Compound **2** was obtained as follows: A degassed solution of 2,7-diiodo-9,9'-dibutylfluorene (1.06 g, 2.0 mmol), diphenylamine (0.36 g, 2.1 mmol), K<sub>2</sub>CO<sub>3</sub> (1.90 g, 13.7 mmol), copper powder (0.09 g, 1.4 mmol) and 18-crown-6 ether (0.02 g, 70 μmol) in *o*-dichlorobenzene (7 mL)

was heated under reflux for 24 h. The suspension was then left to cool down at room temperature (RT), diluted in dichloromethane (50 mL) and filtered on Celite. Solvents were evaporated by vacuum distillation and the residuals chromatographed on silica gel using a petroleum ether/dichloromethane (90/10) mixture as eluant. Fractions containing the desired product were assembled and concentrated in a rotary evaporator. Precipitated compound **2** (diethyl oxide) was then filtered and dried under reduced pressure yielding a pale-grey solid (455 mg, 40 % yield). Mp = 129-130 °C. <sup>1</sup>H NMR (DMSO d<sub>6</sub>, 300 MHz): δ 7.79 (d, J = 1.3 Hz, 1H), 7.73 (d, J = 8.1 Hz, 1H), 7.66 (dd, J = 7.9; 1.4 Hz, 1H), 7.54 (d, J = 7.9 Hz, 1H), 7.29 (m, 4H), 7.08 (d, J = 2.1 Hz, 1H), 7.04 (m, 6H), 6.94 (dd, J = 8.3; 2.1 Hz, 1H), 1.86 (m, 4H), 1.03 (m, 4H), 0.66 (t, J = 7.5 Hz, 6H), 0.52 (m, 4H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ 153.26 151.73 148.05 147.91 140.80 135.99 135.23 132.04 129.39 124.16 123.49 122.89 121.02 120.68 119.10 91.69 55.39 40.07 26.17 23.16 14.09.

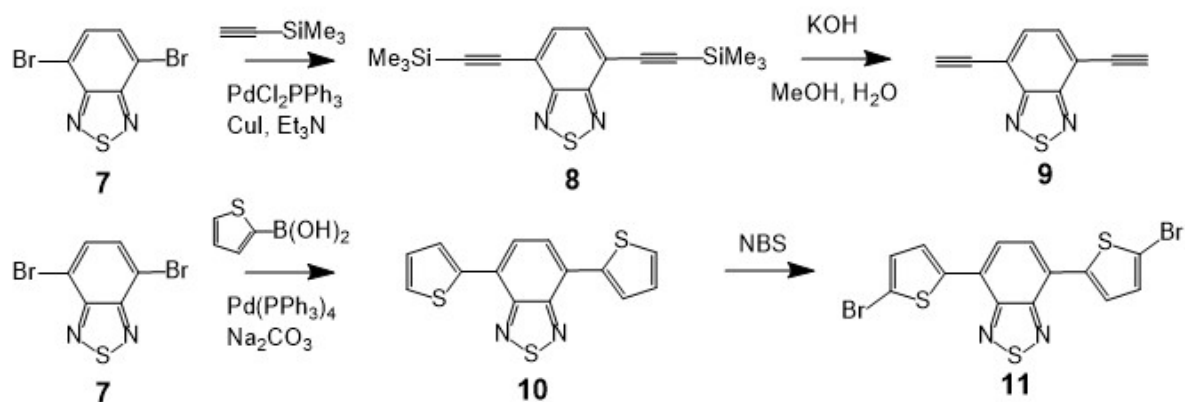
Compound **3** was obtained as follows: A solution of compound **2** (4 mL, 190 mM in THF) was degassed under argon for 10 min. PdCl<sub>2</sub>(dppf) (22 mg, 0.03 mmol) was added and the solution left to cool down to 0°C. A vinyl magnesium bromide solution (2.3 mL, 1 M in THF) was added dropwise and the reaction mixture agitated for 3h at RT. The reaction was quenched with water and the organic phase was extracted using diethyl ether, washed and dried on Na<sub>2</sub>SO<sub>4</sub>. The raw product was purified by silica gel chromatography using a petroleum ether/ethyl acetate (98/2) mixture as eluant. Fractions containing the desired product were assembled and concentrated under reduced pressure until crystallization of compound **3**. (185 mg, 75 % yield). Mp = 104-105°C. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 7.58 (d, J = 7.8 Hz, 1H); -7.56 (d, J = 8.2 Hz, 1H), 7.39 (dd, J = 8.2; 1.5 Hz, 1H), 7.36 (bs, 1H), 7.28 (m, 4H), 7.16 (m, 5H), 7.08 (m, 3H), 6.84 (dd, J = 17.6; 10.9 Hz, 1H), 5.83 (d, J = 17.6 Hz, 1H), 5.28 (d, J = 10.9Hz, 1H), 1.89 (m, 4H), 1.09 (m,

4H), 0.73 (t,  $J = 7.5$  Hz, 6H), 0.67 (m, 4H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz):  $\delta$  152.42 151.02 148.00 147.16 140.92 137.48 136.01 135.77 129.18 125.33 123.85 123.48 122.53 120.40 120.36 119.36 119.13 112.68 54.82 40.06 26.06 23.06 13.93.

Compound **5** has been previously described.<sup>2</sup>

Compound **6** has been previously described.<sup>3</sup>

**Synthesis of core moieties.** Conjugated cores were obtained by transforming the 4,7-dibromobenzothiadiazole **7** either into the bis ethynyl precursor **9** by Sonogashira coupling with trimethylsilyl ethylene (yielding intermediate **8**), followed by deprotection, or into the dibromothiophene precursor **11** by Suzuki coupling with 2-thienylboronic acid (yielding intermediate **10**) followed by bromination in the presence of NBS (**Scheme S2**).



**Scheme S2** Synthesis of core precursors

Compound **9** has been previously described.<sup>4</sup>

Compound **11** has been previously described.<sup>5</sup>

**Coupling donors and cores.** Suzuki coupling of compounds **6** and **7** yielded the  $\text{Q}_{\text{BDF}}$  dye. Sonogashira coupling of compounds **2** and **9** yielded the  $\text{Q}_{\text{BDeF}}$  dye. Heck coupling of

compounds **3** and **7** yielded the Q<sub>BDvF</sub> dye. Suzuki coupling of compounds **6** and **11** yielded the Q<sub>BDF</sub> dye (**Scheme S3**).

**Scheme S3** Final couplings



Q<sub>BDF</sub> has been previously described.<sup>5</sup>

Q<sub>BDeF</sub> was obtained as follows: A degassed toluene solution (5 mL) containing compound **2** (342 mg, 0.60 mmol), compound **9** (50 mg, 0.27 mmol), PdCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub> (10 mg, 14 μmol), CuI (6 mg, 31 μmol) and triethylamine (0.5 ml, 3.7 mmol) was heated to 40 °C for 48 h. Solvents were then evaporated by distillation under reduced pressure. The obtained residue was chromatographed on silica gel using a petroleum ether/dichloromethane (70/30) mixture as eluent. Fractions containing the desired product were assembled prior to the addition of pentane in order to let Q<sub>BDeF</sub> compound precipitate as orange powder (191 mg, 66 % yield). Mp = 264 °C. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 9.88 (s, 2H), 7.89 (s, 2H) 7.73 (d, J = 8.7 Hz, 4H), 7.70 (m, 10H), 7.39 (m, 4H), 7.22 (m, 10H), 7.15(d, J = 8.7 Hz, 4H), 1.97 (m, 8H), 1.13 (m, 8H), 0.75 (t, J = 7.1 Hz, 12H), 0.68 (m, 8H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz): δ 154.47 152.65 150.75 147.92 147.87 142.21 135.21 132.43 131.35 129.25 126.16 124.12 123.24 122.82 120.89 119.73 119.12 118.83 117.21 99.13 85.42 55.17 40.03 26.04 23.04 13.95. HRMS (FD) calcd. for C<sub>76</sub>H<sub>70</sub>N<sub>4</sub>S: 1070.5321, found: 1070.5332.

Q<sub>BDvF</sub> was obtained as follows: A degassed distilled DMF solution (10 mL) containing compound **3** (381 mg, 0.81 mmol), compound **7** (100 mg, 0.34 mmol), Pd(OAc)<sub>2</sub> (38 mg, 0.17 mmol), triphenylphosphine (40 mg, 0.15 mmol), K<sub>2</sub>CO<sub>3</sub> (200 mg, 1.45 mmol) and tetrabutylammonium bromide (218 mg, 0.68 mmol) was heated to 110 °C for 18 h. Solvents were then evaporated by distillation under reduced pressure. The obtained residue was chromatographed on silica gel using a petroleum ether/dichloromethane gradient (30% to 100% dichloromethane) as eluent. Fractions containing the desired product were assembled prior to the addition of pentane in order to let Q<sub>BDvF</sub> compound precipitate as red powder (164 mg, 45 % yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz): δ 8.12 (d, J = 16.2 Hz, 2H), 7.77 (s, 2H), 7.73 (d, J = 16.2 Hz, 2H), 7.66 (s, 4H), 7.60 (m, 4H), 7.29 (m, 8H), 7.16 (m, 10H), 7.05 (m, 6H), 1.95 (m, 8H), 1.13 (m, 8H), 0.74 (t, J = 7.4 Hz, 12H), 0.70 (m, 6H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 150 MHz): δ 154.07 152.56 151.27 147.96 147.37 141.44 135.89 135.73 133.90 129.40 129.20 126.85 126.20 123.94 123.43 123.41 122.62 121.11 120.49 119.40 119.21 55.03 40.14 26.10 23.07 13.96. HRMS (FD) calcd. for C<sub>76</sub>H<sub>74</sub>N<sub>4</sub>S: 1074.5634, found: 1074.5644.

Q<sub>BDiF</sub> has been previously described.<sup>5</sup>

## 1.2. FONs preparation

FONs were prepared by nanoprecipitation of dyes in water as previously described. In short, 200 μL of dye solution (1 mM in THF (spectroscopic grade, Thermo Fisher)) were rapidly added to 19.8 ml of freshly distilled water at room temperature (RT) under continuous sonication (10 mW) for 3 min. Coating with PAH was performed by dropwise addition of 50 μL PAH solution (1 mM in water) to 4.95 mL of freshly prepared FONs solution at RT under magnetic agitation (1200 rpm) for 30 min or more. FONs were stored at RT when not used on the day of preparation (stability measurements, **Figure S4** and **Figure S5**).

### 1.3. Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy was performed on a Hitachi H7650 electron microscope at the Bordeaux Imaging Centre core facility as previously described. Carbon-membrane coated copper grids were ionized using the Glow discharge technique prior to FONs deposition. Bare FONs were imaged on positively charged grids. After FONs deposition and drying, grids were contrasted with Nano-W® (methylamine tungstate) or uranyl-acetate and dried prior to imaging. Images were analyzed using a custom-written Matlab script to extract the median nanoparticle diameters shown in Figure 1 and reported in Table 1. The average number of dye molecules per NP, denoted  $N$ , was determined from the median nanoparticle diameter  $\varnothing_{\text{TEM}}$  using the formula:

$$N = \frac{4}{3} * \pi * \left(\frac{\varnothing_{\text{TEM}}}{2}\right)^3 * \frac{\rho}{M} * N_A$$
 where the density of the NP  $\rho$  is assumed to be equal to 1 g.cm<sup>-3</sup>,  $M = 1023.44$  g.mol<sup>-1</sup> for Q<sub>BDF</sub>,  $M = 1071.48$  g.mol<sup>-1</sup> for Q<sub>BDeF</sub>,  $M = 1075.52$  g.mol<sup>-1</sup> for Q<sub>BDeVF</sub>, and  $M = 1187.68$  g.mol<sup>-1</sup> for Q<sub>BDeVF</sub> is the molecular weight of the dyes and  $N_A = 6.022.1023$  mol<sup>-1</sup> is the Avogadro constant.

### 1.4. Photophysical measurements

All photophysical studies were performed on freshly prepared, air-equilibrated solutions at RT.

#### 1.4.1. Quantum Yield

One-photon absorption spectra were recorded on a Jasco V-670 UV/Vis spectrophotometer and used as raw data (no baseline correction for diffusion). Emission spectra, corrected for the wavelength-sensitivity of the detection unit, were recorded in 4-faced 1 cm fluorescence quartz cuvettes on a Horiba Fluoromax-4 spectrofluorometer. Samples were excited around the absorbance maximum after adjusting the optical density below 0.1. Fluorescence quantum yields



were calculated using the following formula:  $\Phi_f = \Phi_{ref} * \frac{E}{E_{ref}} * \left( \frac{1-10^{-A_{ref}}}{1-10^{-A}} \right) * \left( \frac{n}{n_{ref}} \right)^2$  with  $\Phi_f$  the fluorescence quantum yield of the product,  $\Phi_{ref}$  the fluorescence quantum yield of the reference (either 4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran (DCM) in ethanol:  $\Phi_{ref} = 0.437$ , Nile Blue in ethanol:  $\Phi_{ref} = 0.27$  or Nile Red in DMSO:  $\Phi_{ref} = 0.79$ ),  $E$  the area under the curve of the emission spectrum of the sample excited at  $\lambda_{ex}$ ,  $E_{ref}$  the area under the curve of the emission spectrum of the reference excited at  $\lambda_{exRef}$ ,  $A$  the absorbance of the sample at  $\lambda_{ex}$ ,  $A_{ref}$  the absorbance of the reference at  $\lambda_{exRef}$ ,  $n$  the refractive index of the sample solvent ( $n_{THF} = 1.407$ ,  $n_{H2O} = 1.333$ ) and  $n_{ref}$  the refractive index of the reference solvent ( $n_{EtOH} = 1.36$ ,  $n_{DMSO} = 1.4785$ ).

#### 1.4.2. 2PA Cross-sections

The two-photon absorption (2PA) cross section ( $\sigma_2$ ) was determined from two-photon excited fluorescence (TPEF) measurements. TPEF brightness curves  $\sigma_2 \cdot \Phi_f = f(\lambda)$ , where  $\Phi_f$  is the fluorescence quantum yield determined as described above (See 1.4.1), were obtained by integrating the corrected emission of a sample excited in 10 nm increments at various wavelengths  $\lambda$  following the method described in Xu et al.<sup>6</sup> and corrected as in Katan et al.<sup>7</sup> Emission was read on a compact CCD spectrometer module (BWTEK BTC112E). Excitation was achieved using a Ti:Sapphire oscillator (Coherent Chameleon, Nd:YVO4, 140 fs, 80 MHz) supplemented with an optical parametric oscillator (OPO, PP-BBO) for wavelengths longer than 1000 nm. The quadratic dependence of the fluorescence intensity on the excitation power was checked at all wavelengths. TPEF values were measured against Fluorescein in 0.01 M aqueous NaOH for the 680-1000 nm spectral range and against Nile Red in DMSO in the 1000-1160 nm spectral range, applying the appropriate solvent-related refractive index corrections. The

excitation beam was focused onto the sample placed in a 2-faced 1 cm absorbance quartz cuvette through a microscope objective (10X, NA 0.25). Emission light was redirected towards the spectrometer using a dichroic mirror (Chroma 675dcxru) followed by an emission filter (Chroma e650sp-2p).

#### 1.4.3. Brightness

The brightness of dyes and nanoparticles were determined as  $\epsilon \cdot \Phi_f$  or  $\sigma_2 \cdot \Phi_f$  where  $\epsilon$  is the molar attenuation coefficient determined using Beer-Lambert Law:  $A(\lambda) = \epsilon(\lambda) \cdot l \cdot C$ ,  $A$  being the absorbance value at the wavelength  $\lambda$ ,  $l$  the length of the optical path and  $C$  the sample concentration. Molar attenuation coefficient values and cross section values provided in Table 2, denoted  $\epsilon$  and  $\sigma_2$ , were determined using the dye concentration  $C_{\text{dye}}$  whereas molar attenuation coefficient values and cross-section values provided in Table 3, denoted  $\epsilon_{\text{FONs}}$  and  $\sigma_{2,\text{FONs}}$  were determined using the nanoparticle concentration  $C_{\text{NP}}$ .  $C_{\text{dye}}$  was determined by lyophilizing an aliquot of FONs suspension, dissolving it in an equal volume of  $\text{CHCl}_3$  or THF, measuring its absorbance and applying Beer-Lambert's law.  $C_{\text{NP}}$  was calculated as  $C_{\text{dye}} \cdot N^{-1}$  where  $N$  is the number of dye molecules per NP determined as described above (See 1.3).

#### 1.4.4. Lifetime

Fluorescence decays were measured on a Horiba Fluorolog spectrofluorometer in the time-correlated single photon counting (TCSPC) configuration. Samples were excited using a 455 nm or 570 nm NanoLED. The instrument response was determined by measuring the light scattered by a Ludox<sup>®</sup> suspension. Lifetime values were obtained from the deconvolution fit analysis of the decay profiles.

### 1.5. Zeta Potential

Zeta potentials were measured from undiluted FONs samples on a Nano Particle Analyser SZ-100 (Horiba).

### 1.6. Cell culture

Cos7 cells were cultured in DMEM supplemented with 1% Glutamax and 10 % Fetal Bovine Serum (FBS) and were maintained in an incubator at 37°C, 5% CO<sub>2</sub>. 48h prior to an imaging experiment, 80.000 cells were seeded in 3 cm culture grade petri dishes. 24h later, 1% (v/v) FONs were added to the dishes and incubated on the cells at 37°C for 24 h. On the day of imaging, the FONs containing medium was removed and the dishes washed twice with pre-warmed PBS.

### 1.7. Imaging

Cellular imaging was performed on an upright confocal microscope (TCS SP5, Leica Microsystems) equipped with an HCX IR-APO L 25X water immersion objective (NA 0.95). One-photon excitation was achieved by an Argon laser (458 nm, 488 nm and 514 nm) or a Helium-Neon laser (561 nm). Two-photon excitation was achieved by a tunable pulsed Mai-Tai HP laser (Spectraphysics). The system was equipped with a 400 Hz scanner and emission was detected on conventional photomultiplier tubes (PMTs).

## 2. SUPPLEMENTARY TABLES

**Table S1.** Radiative and non-radiative decay rates for dyes in THF and as FONs subunits

Dye		$\lambda_{em}^a$ [nm]	$\Phi_f^b$	$\tau^c$ [ns] (%)	$\tau_f^d$ [ns]	$k_r^e$ [ $10^9 s^{-1}$ ]	$k_{nr}^f$ [ $10^9 s^{-1}$ ]
<b>Q<sub>BDF</sub></b>	in THF	628	0.66	5.5	5.5	0.12	0.06
	in FONs	591	0.30	2.1 (40) 5.0 (60)	3.8	0.08	0.18
<b>Q<sub>BDeF</sub></b>	in THF	688	0.14	2.2	2.2	0.06	0.39
	in FONs	623	0.04	4.6 (40) 1.0 (30) 17.5 (30)	2.6	0.02	0.37
<b>Q<sub>BDvF</sub></b>	in THF	646	0.55	3.6	3.6	0.15	0.13
	in FONs	646	0.02	4.5 (10) 1.6 (70) 0.58 (20)	1.8	0.01	0.55
<b>Q<sub>BDFtF</sub></b>	in THF	647	0.43	3.9	3.6	0.12	0.16
	in FONs	689	0.08	1.1 (50) 2.4 (50)	1.8	0.05	0.53

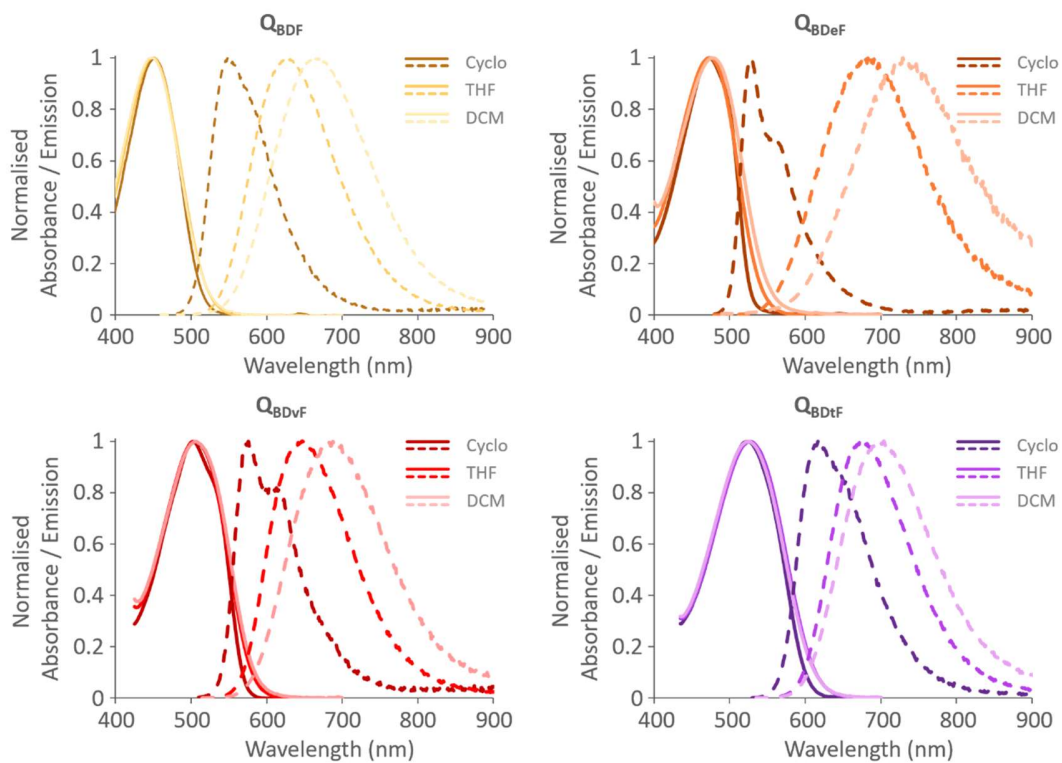
<sup>a)</sup> Emission maximum wavelength (same values as in Table 1); <sup>b)</sup> Fluorescence quantum yield (same values as in Table 1); <sup>c)</sup> Fluorescence lifetime. When the lifetime is multi-exponential, the contribution of each lifetime value to the fit is indicated between brackets; <sup>d)</sup> average lifetime defined as the weighed sum of lifetimes indicated in b). Note that  $\tau_f = 1/(k_r + k_{nr})$ ; <sup>e)</sup> Radiative decay constant calculated as  $k_r = \tau_f/\Phi_f$ ; <sup>f)</sup> Non-radiative decay rate calculated as  $k_{nr} = (1 - \Phi_f)/\tau_f$ .

**Table S2.** Potential reversal upon coating with PAH

<b>Dye</b>	$\zeta_{\text{bare}}^{\text{a)}}$ [mV]	$\zeta_{\text{+PAH}}^{\text{b)}}$ [mV]
<b>QBDF</b>	-65	+73
<b>QBDeF</b>	-67	+65
<b>QBDvF</b>	-62	+66
<b>QBDF</b>	-63	+65

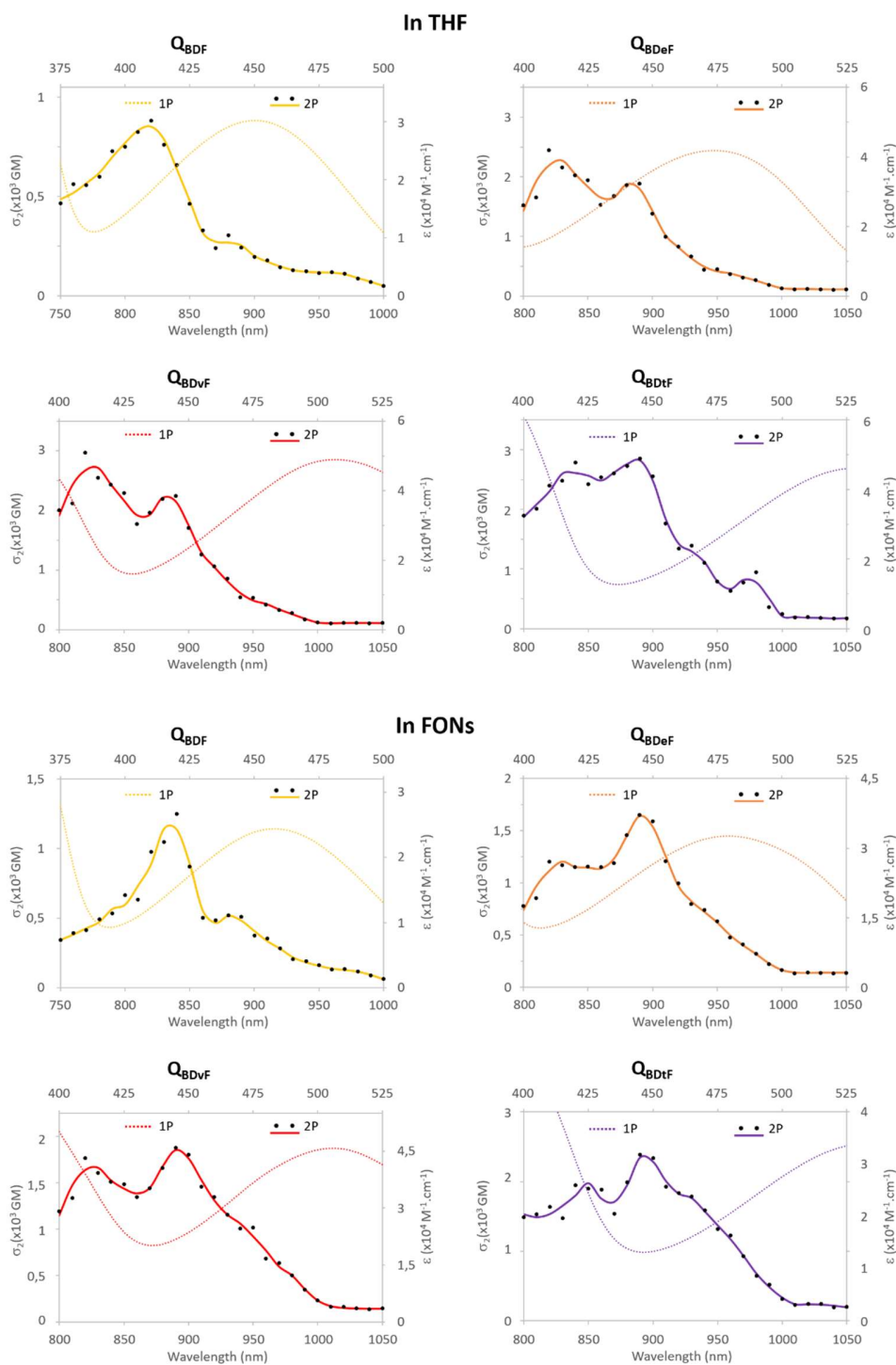
<sup>a)</sup> Zeta potential of bare FONs (same values as in Table 1); <sup>b)</sup> Zeta potential of PAH coated FONs

### 3. SUPPLEMENTARY FIGURES



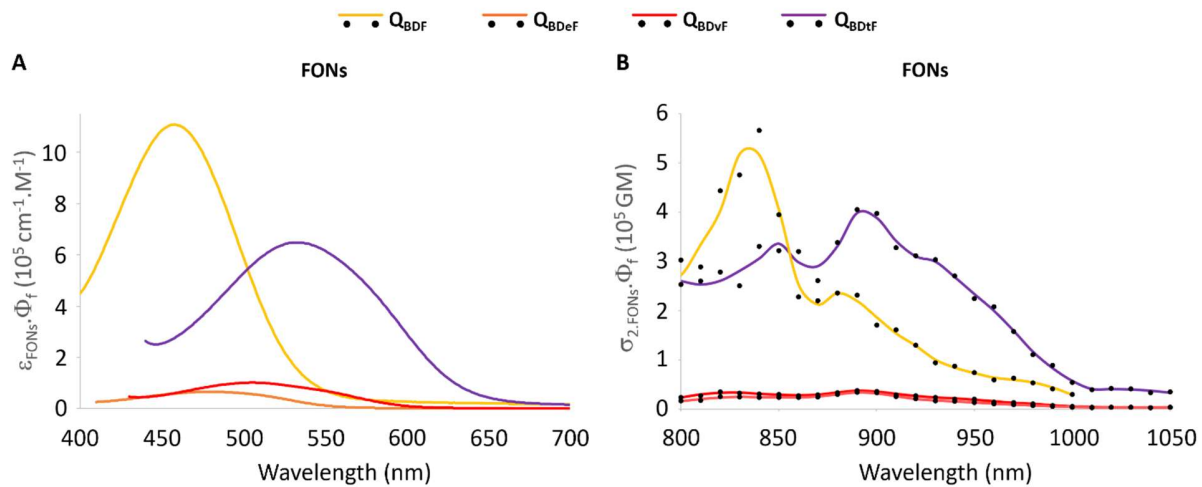
**Figure S1.** Solvatochromic behavior of the dyes.

Absorbance (solid lines) and emission (dashed lines) spectra were measured in Cyclohexane (Cyclo, dark hues), Tetrahydrofuran (THF, medium hues) or Dichloromethane (DCM, light hues). Solvent polarity increases in the order Cyclo > THF > DCM.



**Figure S2.** One- vs two-photon absorbance.

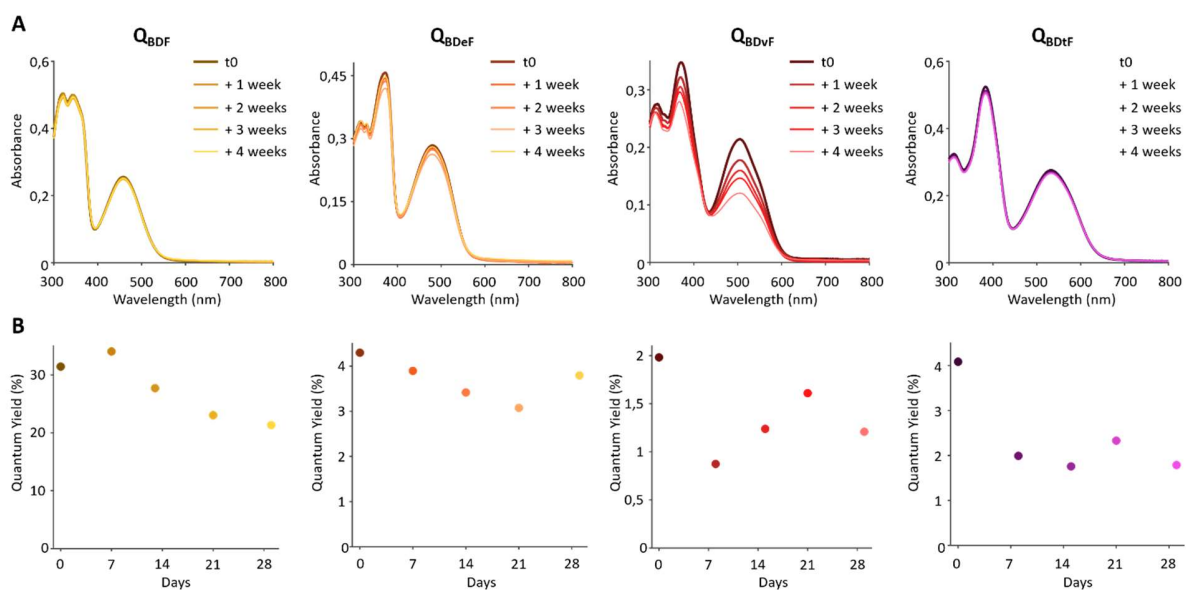
Overlapped one-photon (dashed lines, secondary axes) and two-photon (smoothed solid lines, primary axes) absorbance data of dyes in THF (Top) or of FONs (Bottom).



**Figure S3.** FONSs one- and two-photon brightness.

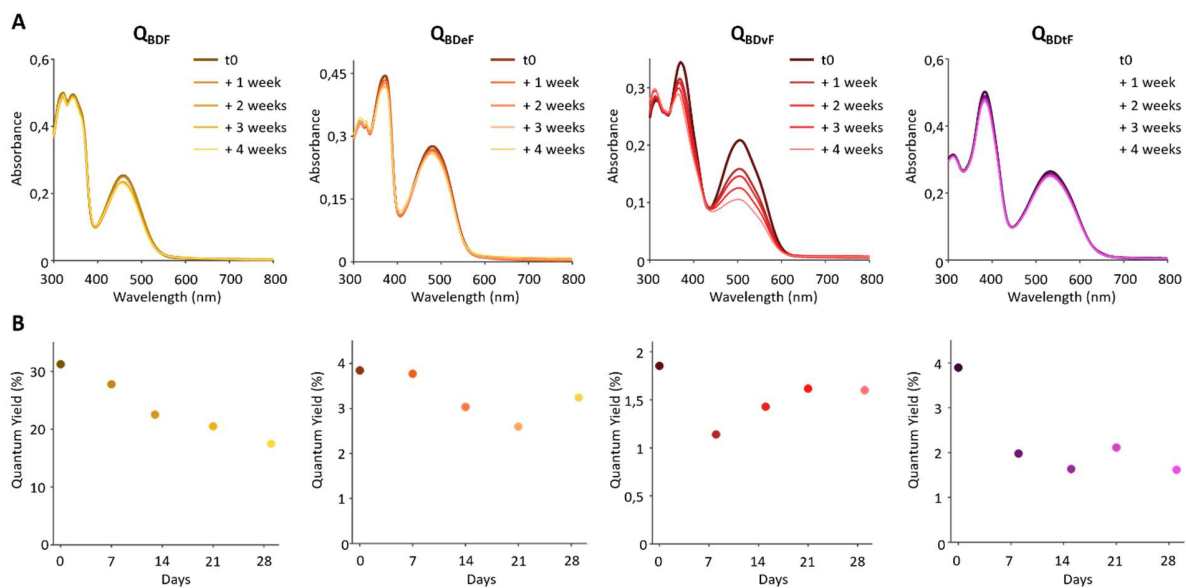
Overlapped one-photon (A) and two-photon (B) brightness of FONSs. Same color code in both panels.





**Figure S4.** Bare FONs stability in time.

**A** Overlapped FONs absorbance spectra measured once a week for a month after FONs preparation. **B** Normalized FONs quantum yields measured once a week for a month after FONs preparation. Color coding defined in panel A is maintained in panel B.



**Figure S5.** PAH coated FONs stability in time.

**A** Overlapped PAH coated FONs absorbance spectra measured once a week for a month after FONs preparation. **B** Normalized PAH coated FONs quantum yields measured once a week for a month after FONs preparation. Color coding defined in panel A is maintained in panel B.

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