Chemical approaches to the study of the ceramide synthase activity

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SUPPLEMENTARY MATERIAL I

Fluorescence spectroscopy studies

Table of contents

1. Absorption and emission spectra

In this section are shown the absorption and emission spectra of the synthesized fluorescent compounds at multiple concentrations in various solvents.
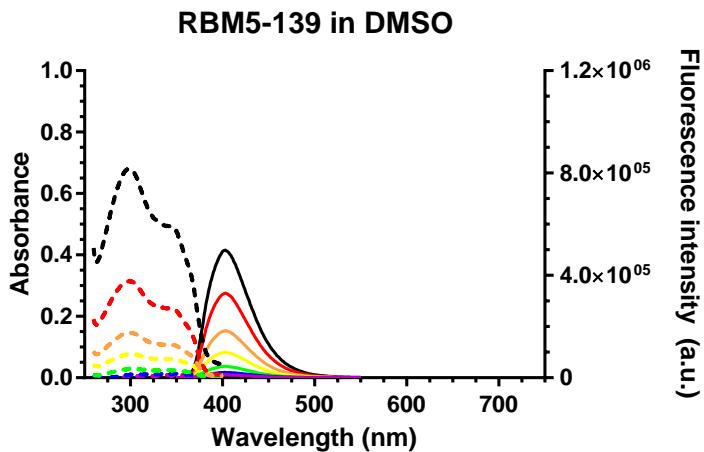
RBM5-139 in DMSO

Figure S1. Absorption (dotted line) and emission spectra (solid line) upon excitation at 340 nm of compound **RBM5-139** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black).
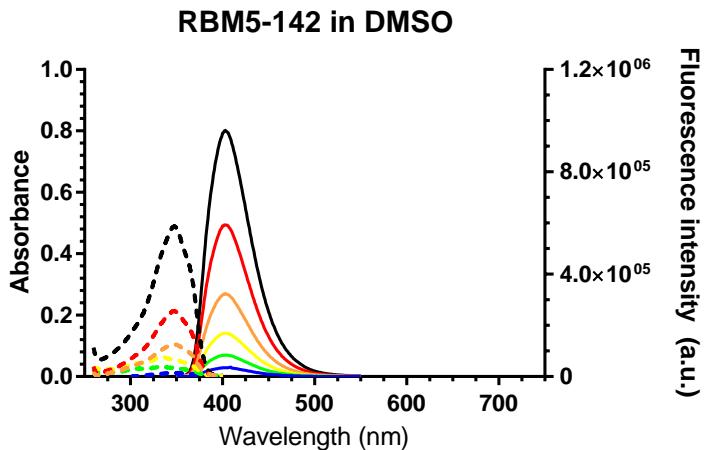
RBM5-142 in DMSO

Figure S2. Absorption (dotted line) and emission spectra (solid line) upon excitation at 340 nm of compound **RBM5-142** in DMSO at 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black).
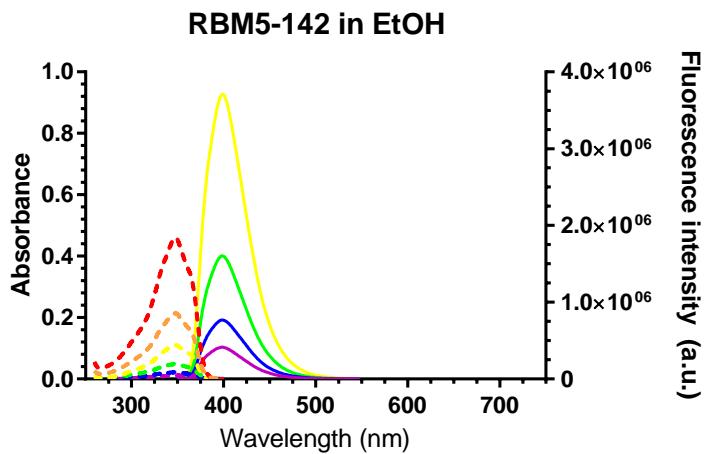
RBM5-142 in EtOH

Figure S3. Absorption (dotted line) and emission spectra (solid line) upon excitation at 340 nm of compound **RBM5-142** in EtOH at 0.25 µm (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange) and 10 µM (red). The solutions at concentrations greater than 2.5 µM could not be measured in the fluorimeter since they caused saturation of the detector.
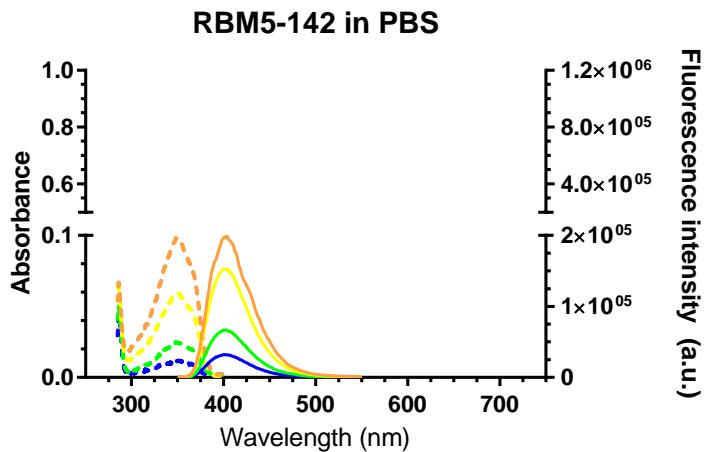
RBM5-142 in PBS

Figure S4. Absorption (dotted line) and emission spectra (solid line) upon excitation at 340 nm of compound **RBM5-142** in PBS containing 1 % DMSO and 0.1 % Triton X-100 at 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow) and 5 µM (orange). The solutions at concentrations greater than 5 µM were discarded since they showed a lack of linearity between concentration and absorbance.
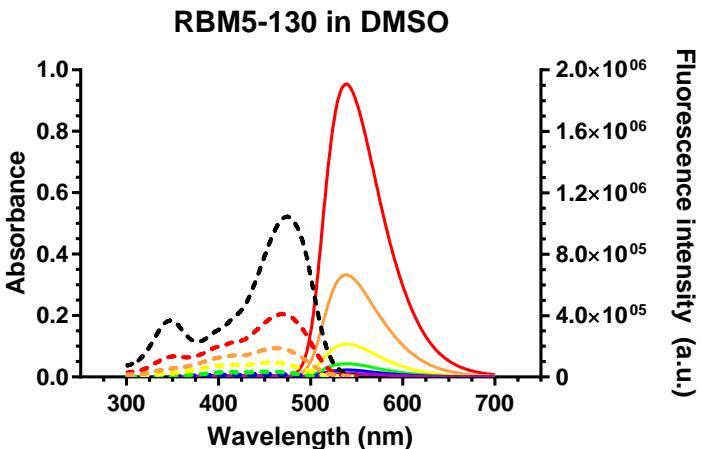
RBM5-130 in DMSO

Figure S5. Absorption (dotted line) and emission spectra (solid line) upon excitation at 470 nm of compound **RBM5-130** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). The solutions at concentrations greater than 10 µM could not be measured in the fluorimeter since they caused saturation of the detector.
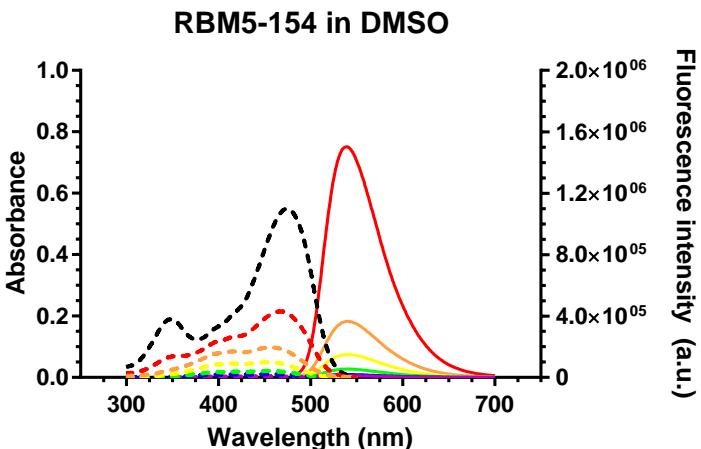
RBM5-154 in DMSO

Figure S6. Absorption (dotted line) and emission spectra (solid line) upon excitation at 470 nm of compound **RBM5-154** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). The solutions at concentrations greater than 10 µM could not be measured in the fluorimeter since they caused saturation of the detector.
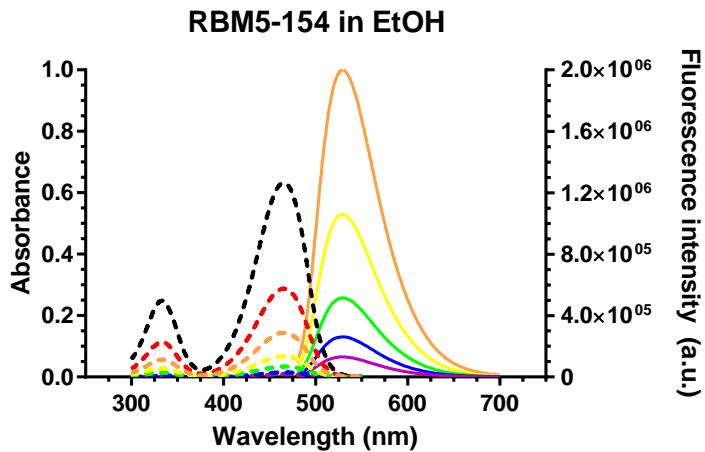
RBM5-154 in EtOH

Figure S7. Absorption (dotted line) and emission spectra (solid line) upon excitation at 470 nm of compound **RBM5-154** in EtOH at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). The solutions at concentrations greater than 5 µM could not be measured in the fluorimeter since they caused saturation of the detector.
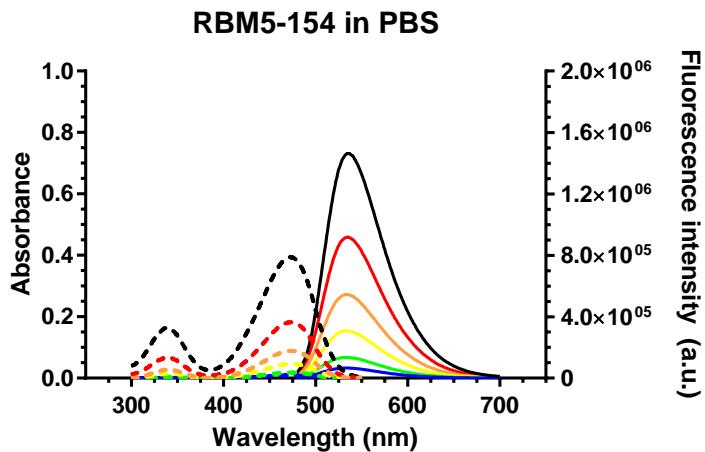
RBM5-154 in PBS

Figure S8. Absorption (dotted line) and emission spectra (solid line) upon excitation at 470 nm of compound **RBM5-154** in PBS containing 1 % DMSO and 0.1 % Triton X-100 at 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black).
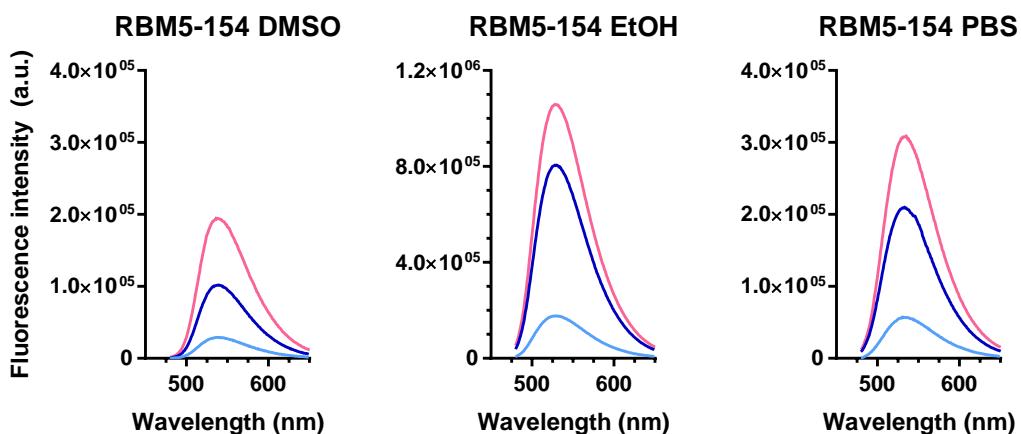
Figure S9. Emission spectra of **RBM5-154** at 2.5 µM in DMSO (left), EtOH (middle) and PBS (right) containing 1 % DMSO and 0.1 % Triton X-100 upon excitation at 340 nm (light blue), 455 nm (dark blue), 470 nm (light pink).
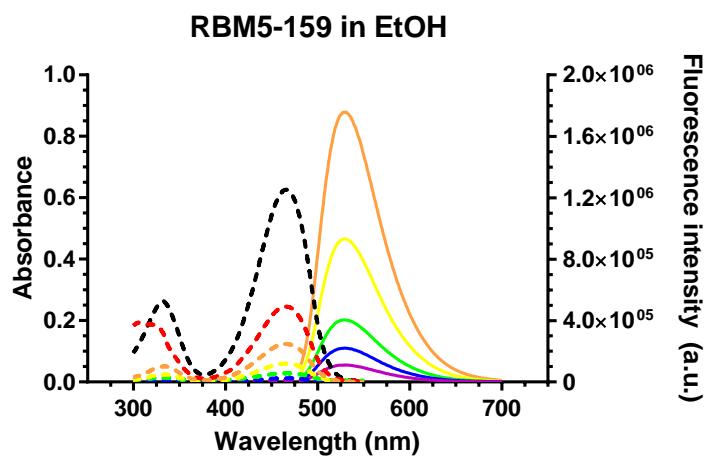
Figure S10. Absorption (dotted line) and emission spectra (solid line) upon excitation at 470 nm of compound **RBM5-154** in EtOH at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). Emission spectra are shown for concentrations up to 5 µM, since higher concentrations caused saturation of the detector.
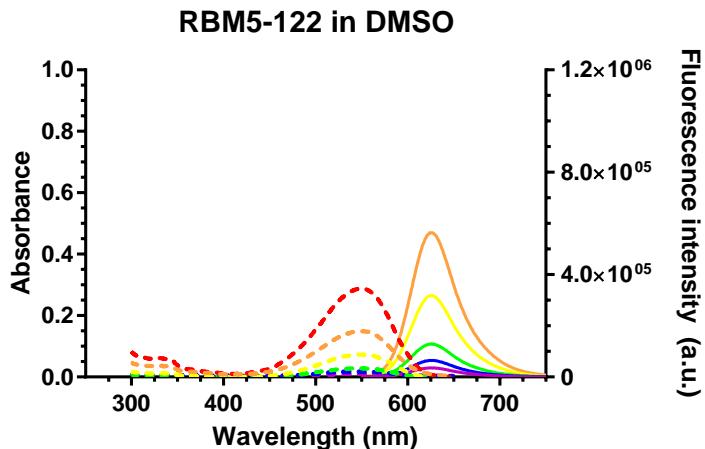
RBM5-122 in DMSO

**Figure S11**. Absorption (dotted line) and emission spectra (solid line) upon excitation at 510 nm of compound **RBM5-122** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange) and 10 µM (red). Emission spectra are shown for concentrations up to 5 µM, since a lack of proportionality between concentration and fluorescence intensity was detected at higher concentrations. This phenomenon was attributed to the poor solubility of the compound and the probable formation of aggregates.
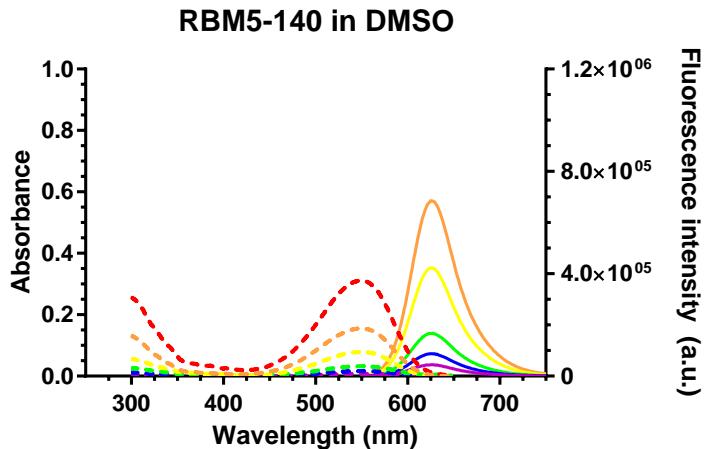
RBM5-140 in DMSO

Figure S12. Absorption (dotted line) and emission spectra (solid line) upon excitation at 510 nm of compound **RBM5-140** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange) and 10 µM (red). Emission spectra are shown for concentrations up to 5 µM, since a lack of proportionality between concentration and fluorescence intensity was detected at higher concentrations. This phenomenon was attributed to the poor solubility of the compound and the probable formation of aggregates.
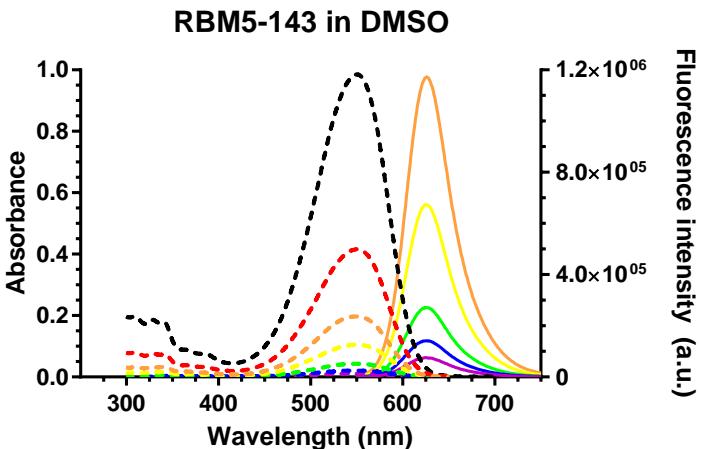
Figure S13. Absorption (dotted line) and emission spectra (solid line) upon excitation at 510 nm of compound **RBM5-143** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). Emission spectra are shown for concentrations up to 5 µM, since higher concentrations caused saturation of the detector.
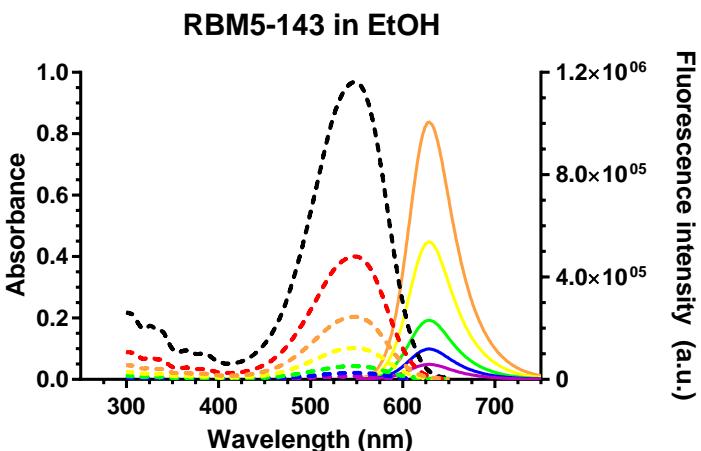

Figure S14. Absorption (dotted line) and emission spectra (solid line) upon excitation at 510 nm of compound **RBM5-143** in EtOH at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). Emission spectra are shown for concentrations up to 5 µM, since higher concentrations caused saturation of the detector.
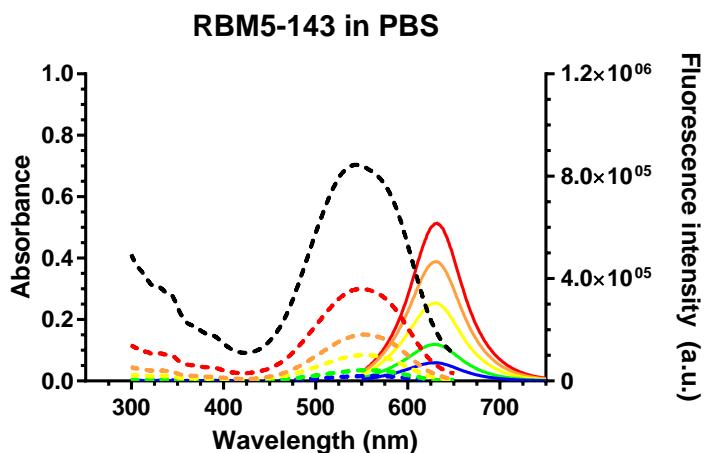
RBM5-143 in PBS

Figure S15. Absorption (dotted line) and emission spectra (solid line) upon excitation at 510 nm of compound **RBM5-143** in PBS containing 1 % DMSO and 0.1 % Triton X-100 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). Emission spectra at concentrations higher than 2.5 µM were not used for calculations due to the lack of linearity between concentration and fluorescence intensity. This phenomenon was attributed to the poor solubility of the compound and the probable formation of aggregates.
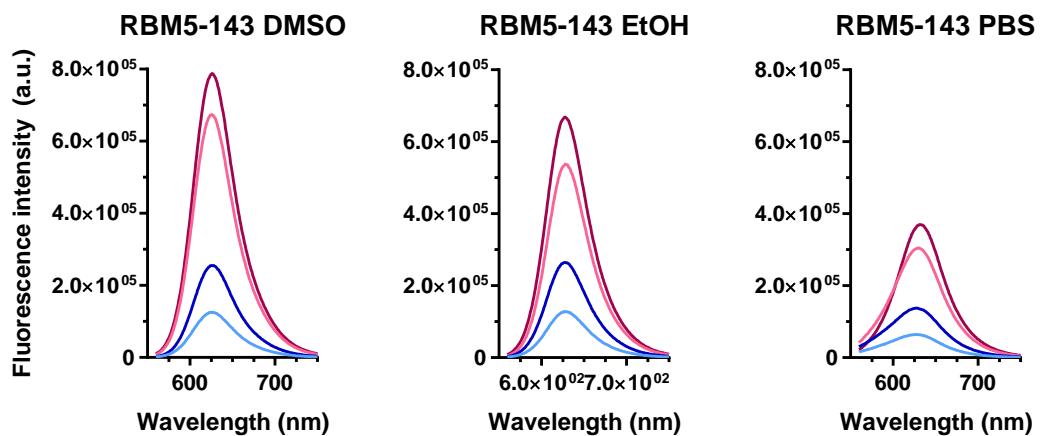
Figure S16. Emission spectra of **RBM5-143** at 2.5 µM in DMSO (left), EtOH (middle) and PBS (right) upon excitation at 455 nm (light blue), 470 nm (dark blue), 510 nm (light pink) and 550 nm (dark pink).
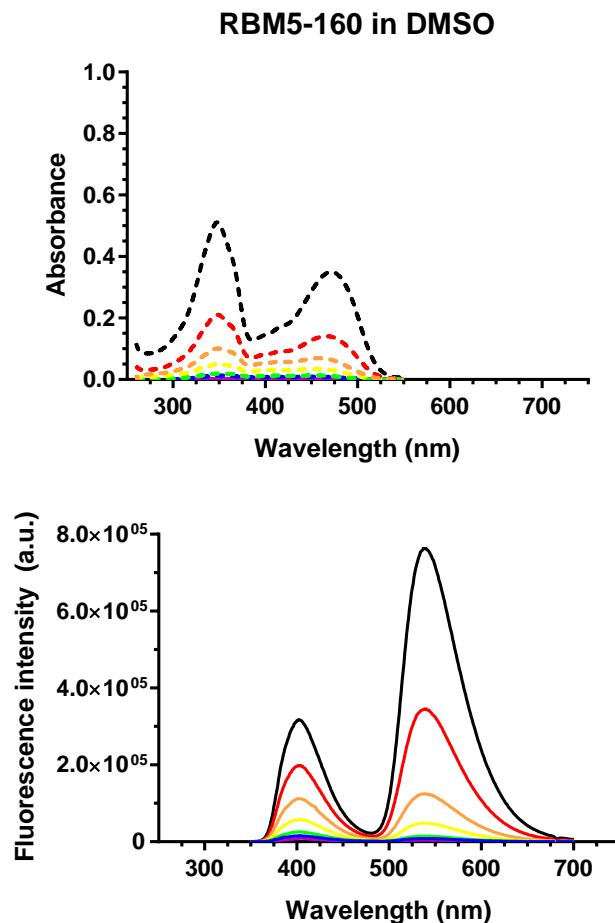
RBM5-160 in DMSO

Figure S17. Absorption (top) and emission spectra (bottom) upon excitation at 340 nm of compound **RBM5-160** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black).
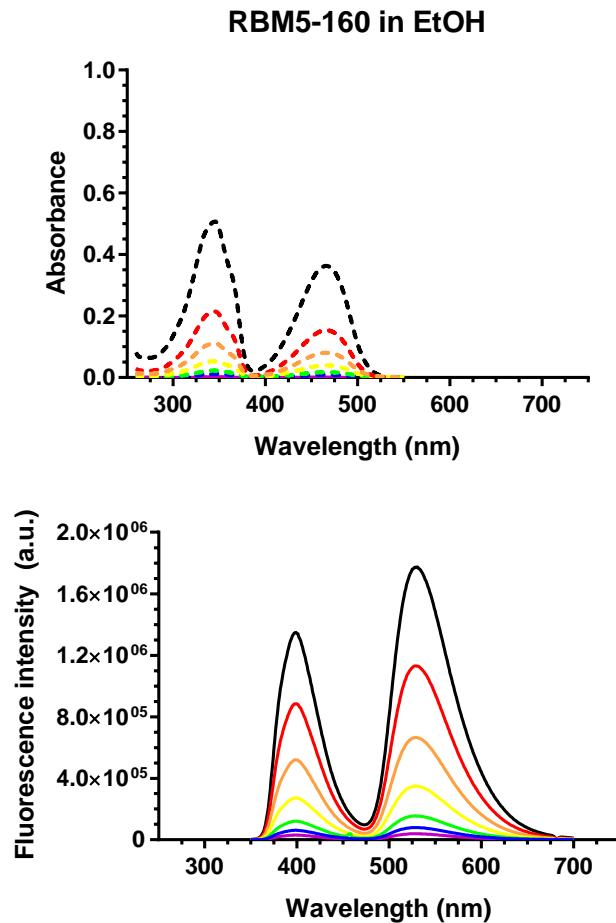
RBM5-160 in EtOH

**Figure S18**. Absorption (top) and emission spectra (bottom) upon excitation at 340 nm of compound **RBM5-160** in EtOH at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black).
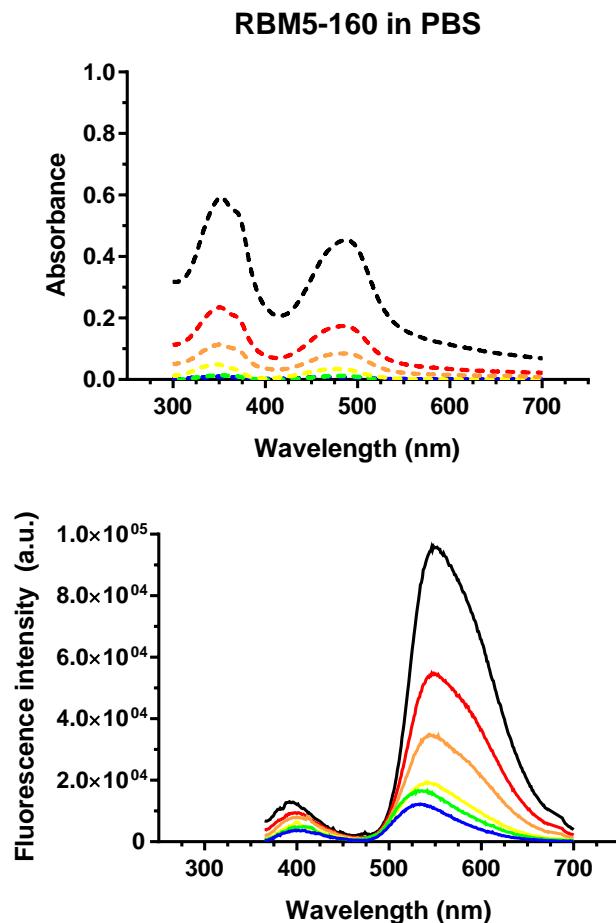
RBM5-160 in PBS

Figure S19. Absorption (top) and emission spectra (bottom) upon excitation at 340 nm of compound **RBM5-160** in PBS containing 1 % DMSO and 0.1 % Triton X-100 at 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). The poor solubility of **RBM5-160** in PBS probably caused its aggregation, resulting in lower fluorescence emission, together with a lack of linearity between absorbance and fluorescence.
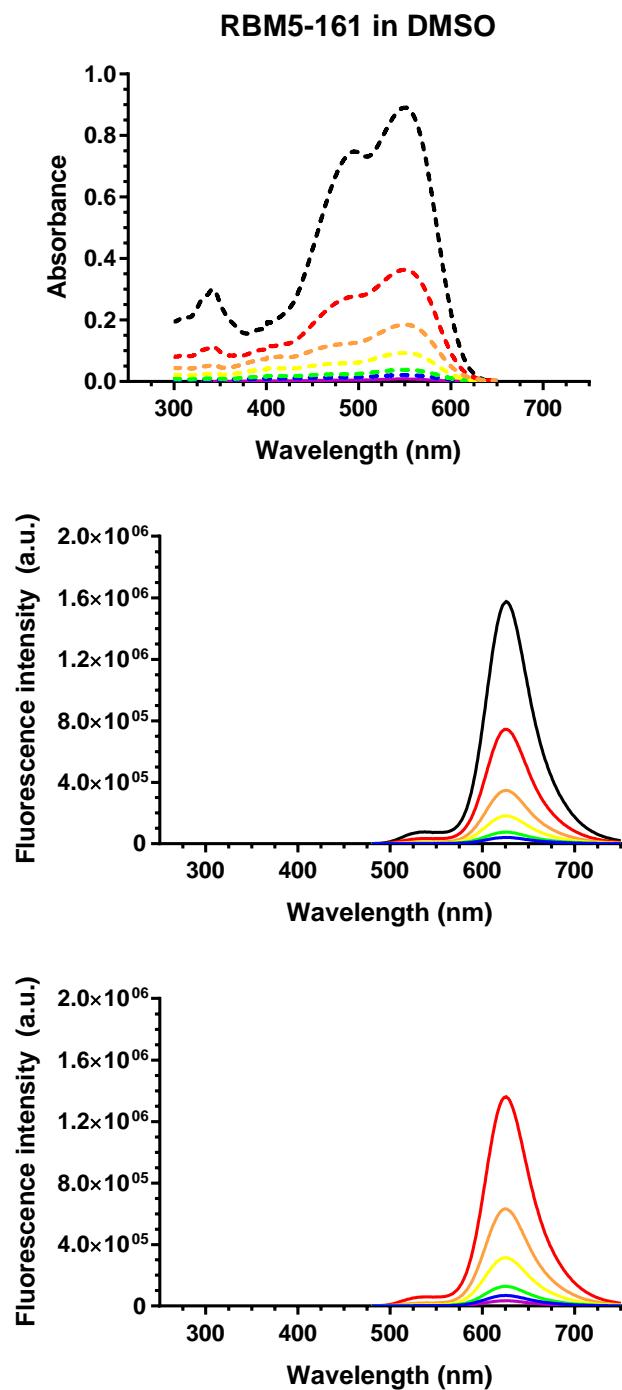
RBM5-161 in DMSO

Figure S20. Absorption spectra (top) and emission spectra upon excitation at 455 (middle) or 470 nm (bottom) of compound **RBM5-161** in DMSO at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black).
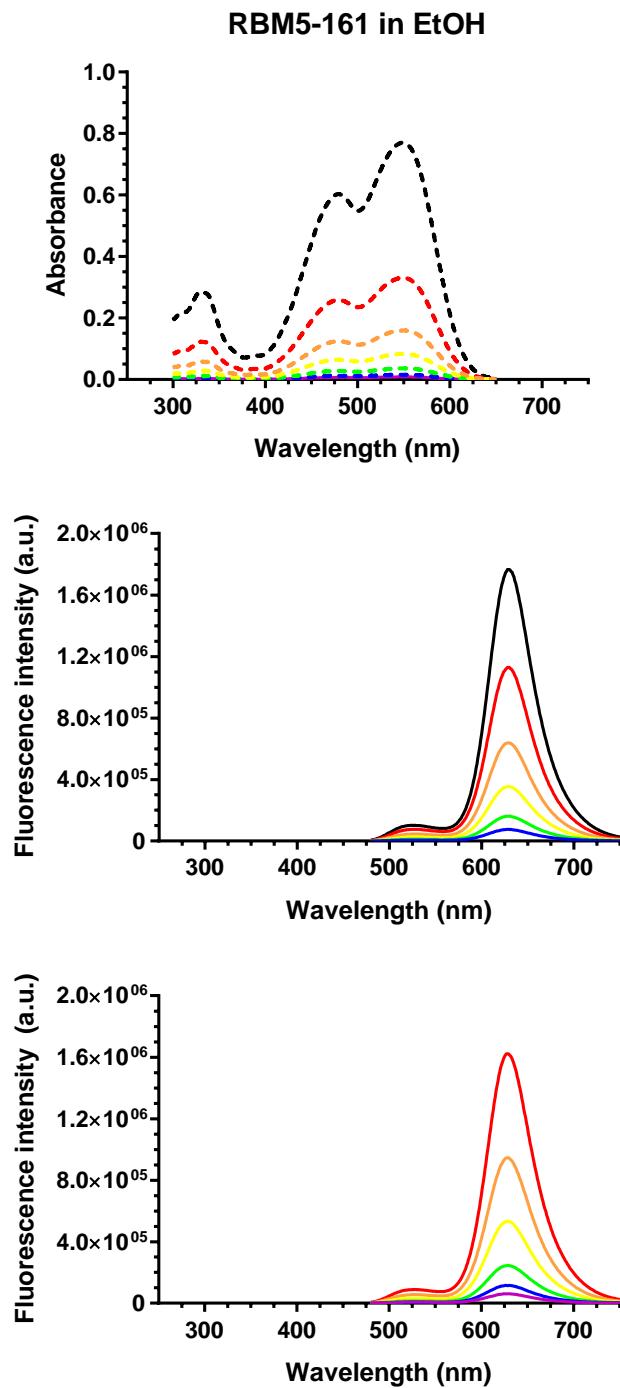
RBM5-161 in EtOH

Figure S21. Absorption spectra (top) and emission spectra upon excitation at 455 (middle) or 470 nm (bottom) of compound **RBM5-161** in EtOH at 0.25 µM (purple), 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black).
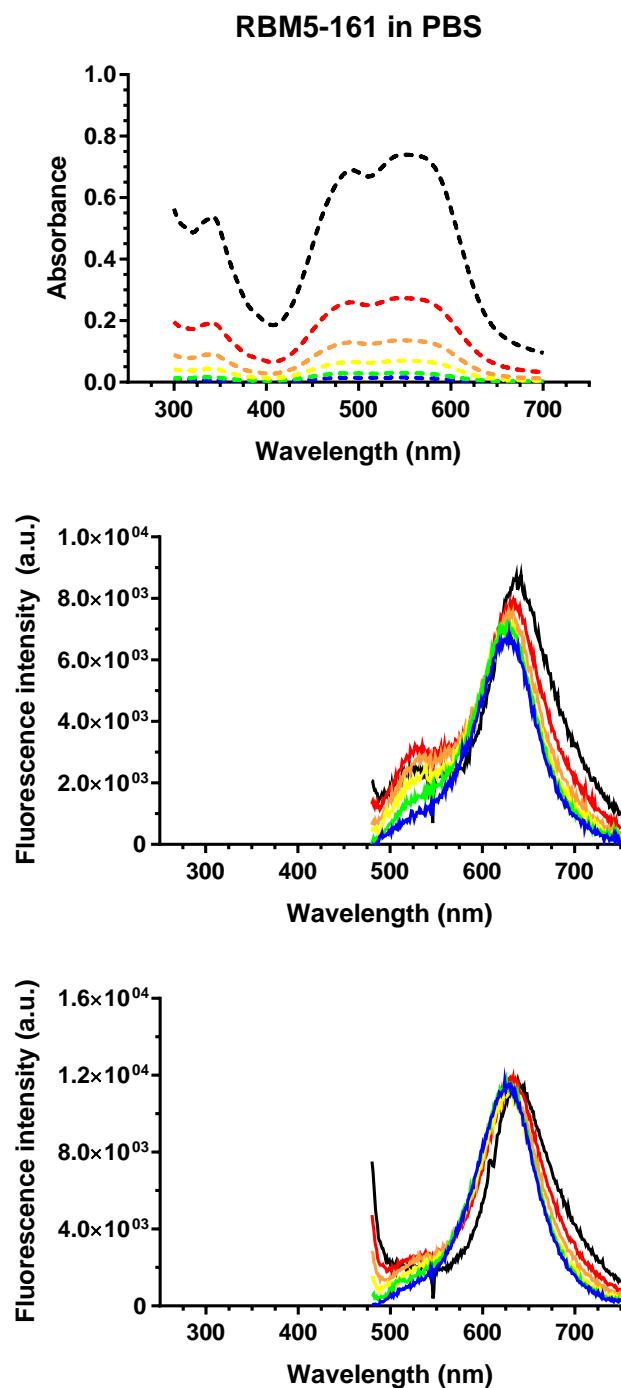
Figure S22. Absorption spectra (top) and emission spectra upon excitation at 455 (middle) or 470 nm (bottom) of compound **RBM5-161** in PBS containing 1 % DMSO and 0.1 % Triton X-100 at 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange), 10 µM (red) and 25 µM (black). The poor solubility of **RBM5-161** in PBS probably caused its aggregation, resulting in lower fluorescence emission, together with a lack of linearity between absorbance and fluorescence.

2. Linear plots to calculate the molar extinction coefficients

In this section are presented the absorbance at the maximum absorption wavelength (λ_{max}^{Abs}) *versus* concentration plots that were used to calculate the molar extinction coefficient (ε). The regressions were forced to intercept the origin.
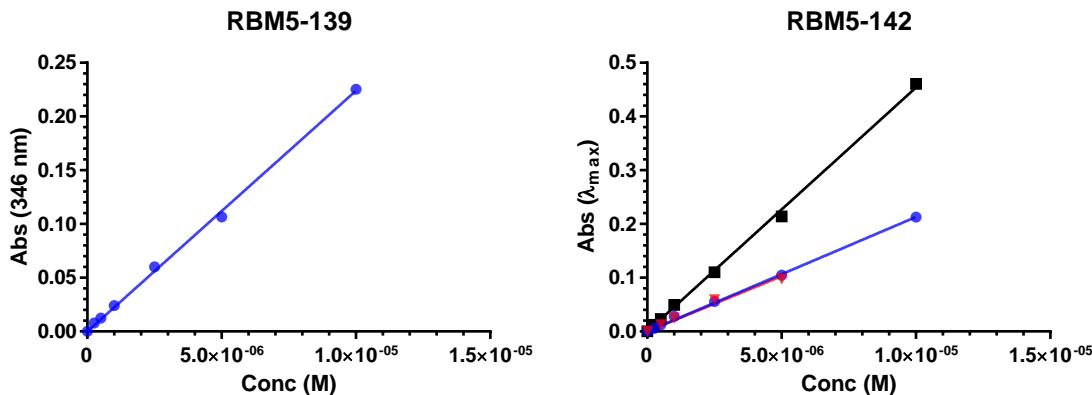
Compound	Solvent	λ_{max} (nm)	Slope (ε) ± SE	R^2	Legend
RBM5-139	DMSO	346	22,389 ± 263.5	0.9986	-●-
RBM5-142	DMSO	346	21,296 ± 270.9	0.9983	-●-
	EtOH	348	45,370 ± 545.6	0.9986	-■-
	PBS	350	20,558 ± 897.9	0.9834	-▼-

Figure S23. Linear plots of absorbance (at λ_{max}) *vs* concentration for compounds **RBM5-139** and **RBM5-142** in solution. Solvent color code: DMSO (blue), EtOH (black), PBS containing 1% DMSO and 0.1 % Triton X-100 (red).

RBM5-130
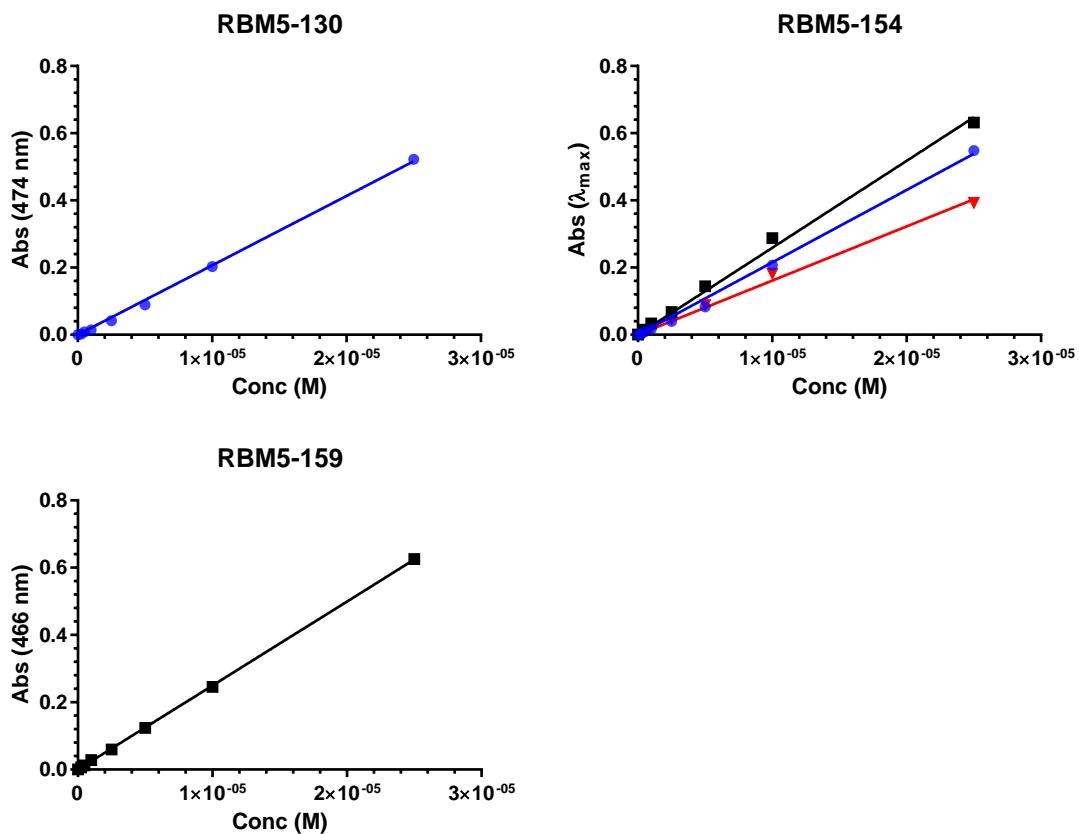
RBM5-154

RBM5-159

Compound	Solvent	λ_{max} (nm)	Slope (ε) $\pm$ SE	R^2	Legend
RBM5-130	DMSO	474	20,654 $\pm$ 278.1	0.9982	-●-
RBM5-154	DMSO	476	21,543 $\pm$ 436.6	0.996	-●-
	EtOH	465	25,882 $\pm$ 544.4	0.9957	-■-
	PBS	475	16,121 $\pm$ 373.9	0.9947	-▼-
RBM5-159	EtOH	466	24,959 $\pm$ 87.49	0.9999	-■-

Figure S24. Linear plots of absorbance (at λ_{max}) *vs* concentration for compounds **RBM5-130**, **RBM5-154** and **RBM5-159** in solution. Solvent color code: DMSO (blue), EtOH (black), PBS containing 1% DMSO and 0.1 % Triton X-100 (red).

RBM5-122
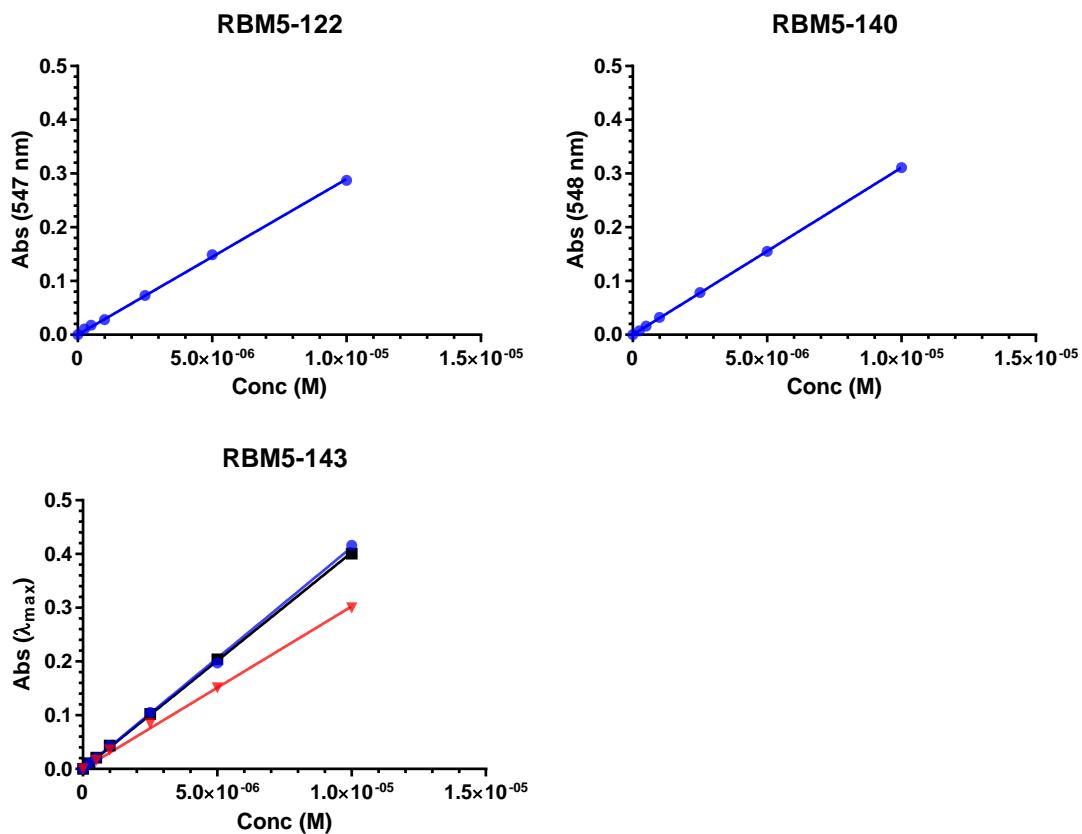
RBM5-140

RBM5-143

Compound	Solvent	λ_{max} (nm)	Slope (ε) ± SE	R^2	Legend
RBM5-122	DMSO	547	28,966 ± 238.1	0.9993	-●-
RBM5-140	DMSO	548	31,095 ± 53.41	1	-●-
RBM5-143	DMSO	550	41,242 ± 352.5	0.9993	-●-
	EtOH	548	40,269 ± 170.7	0.9998	-■-
	PBS	550	30,244 ± 407.6	0.9983	-▼-

Figure S25. Linear plots of absorbance (at λ_{max}) vs concentration for compounds **RBM5-122**, **RBM5-140** and **RBM5-143** in solution. Solvent color code: DMSO (blue), EtOH (black), PBS containing 1% DMSO and 0.1 % Triton X-100 (red).
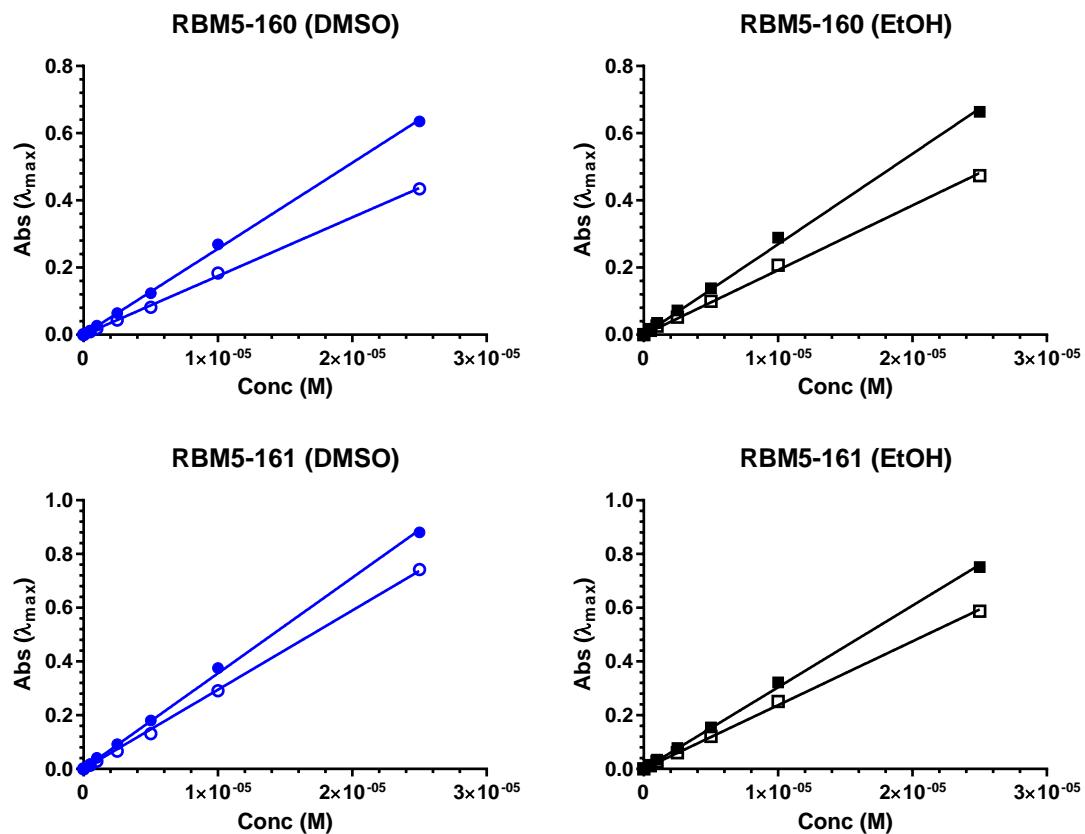
Compound	Solvent	λ_{max} (nm)	Slope (ε) ± SE	R^2	Legend
RBM5-160	DMSO	348	25,594 ± 218.3	0.9993	-●-
		469	17,466 ± 160.3	0.9992	-○-
	EtOH	345	26,928 ± 368.7	0.9982	-■-
		466	19,244 ± 279.9	0.9979	-□-
RBM5-161	DMSO	495	29,482 ± 277.5	0.9992	-○-
		551	35,577 ± 336.9	0.9991	-●-
	EtOH	479	23,736 ± 233	0.9991	-□-
		549	30,389 ± 311	0.999	-■-

Figure S26. Linear plots of absorbance (at λ_{max}) *vs* concentration for compounds **RBM5-160** and **RBM5-161** in DMSO (blue) and EtOH (black).

3. Linear plots to calculate the fluorescence quantum yields

In this section are displayed the absorbance at the excitation wavelength (λ_{Ex}) *versus* integrated fluorescence intensity plots that were used to calculate the quantum yields (Φ) of the different fluorescent compounds. The regressions were forced to intercept the origin.
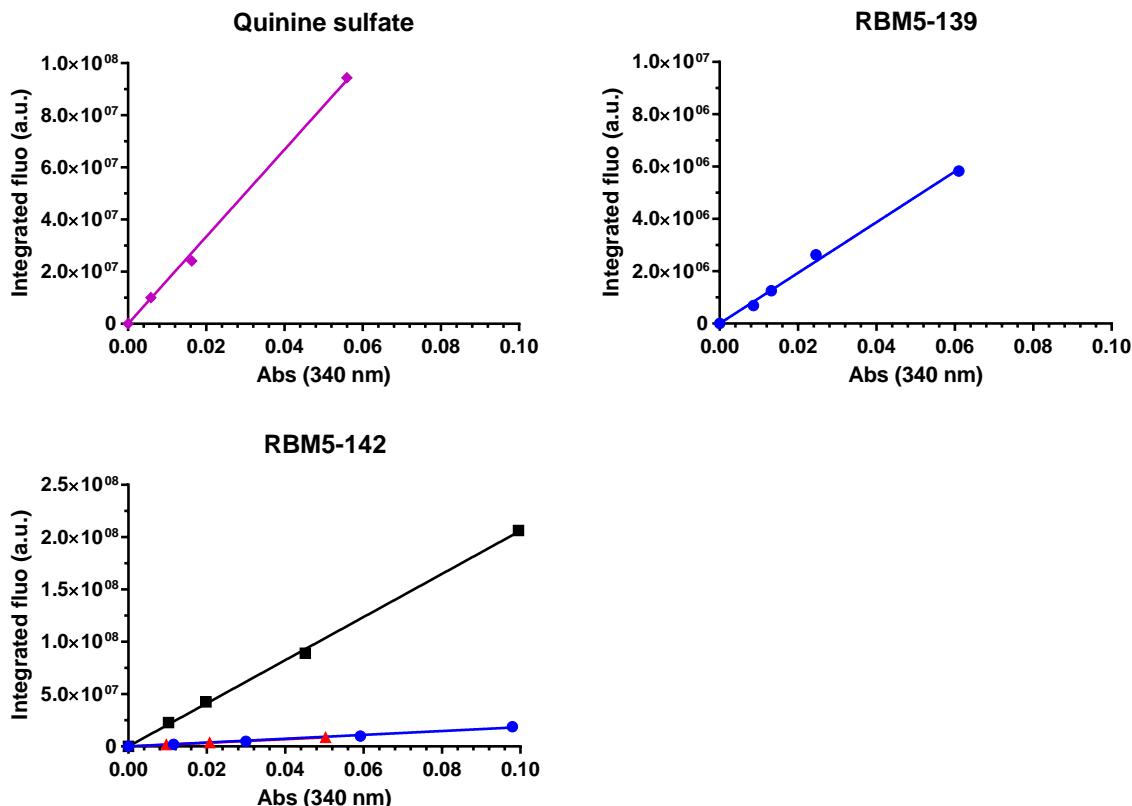
Compound	Solvent	Slope	SE	R²	Legend
Quinine sulfate	1.0 M aq. H₂SO₄	1.67E+09	3.16E+07	0.9981	-♦-
RBM5-139	DMSO	9.68E+07	2.19E+06	0.9959	-●-
RBM5-142	DMSO	1.84E+08	5.81E+06	0.9915	-●-
	EtOH	2.06E+09	2.19E+07	0.9991	-■-
	PBS	1.72E+08	2.36E+06	0.9987	-▼-

Figure S27. Linear plots of integrated fluorescence intensity (AUC[350-600 nm]) *vs* absorbance (at λ_{Ex} = 340 nm) for fluorescence standard quinine sulfate in 1.0 M aq. H₂SO₄ (purple), and MCC-labelled compounds **RBM5-139** and **RBM5-142** in different solvents: DMSO (blue), EtOH (black) and PBS containing 1 % DMSO and 0.1 % Triton X-100 (red).
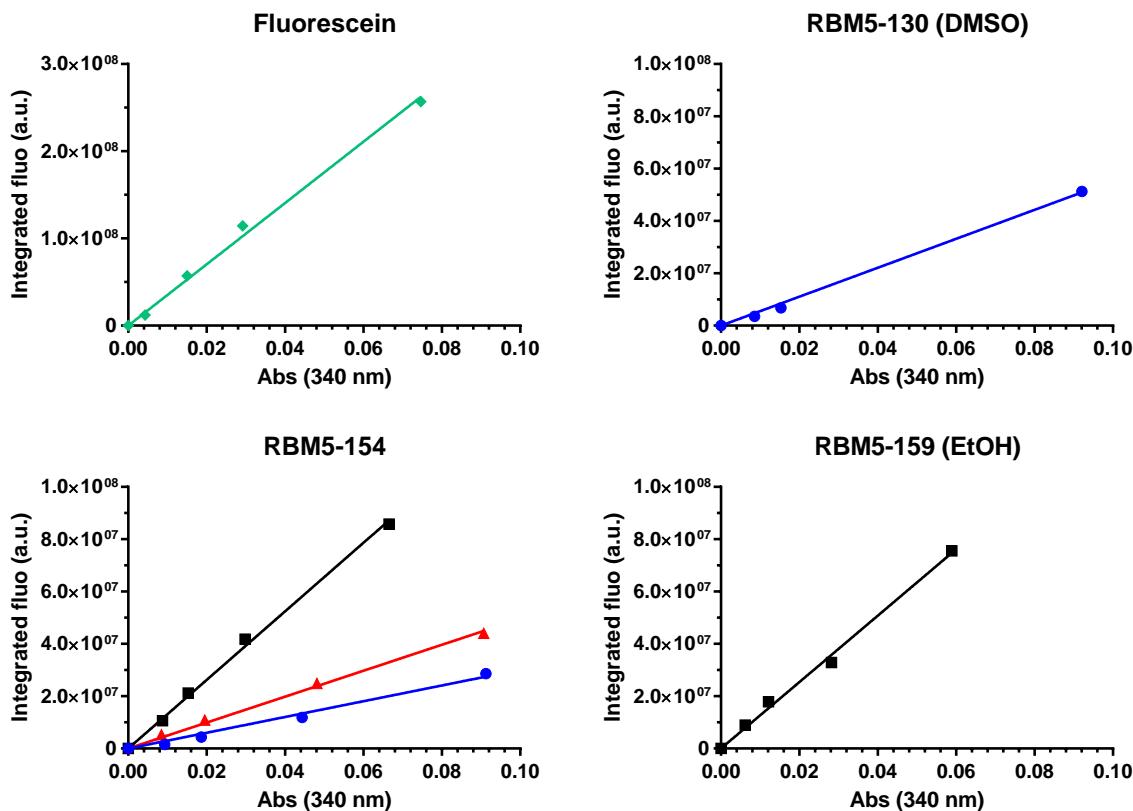
Compound	Solvent	Slope	SE	R²	Legend
Fluorescein	0.1 M aq. NaOH	3.51E+09	8.64E+07	0.9954	-◆-
RBM5-130	DMSO	5.53E+08	1.31E+07	0.9974	-●-
RBM5-154	DMSO	3.01E+08	1.26E+07	0.9877	-●-
	EtOH	1.31E+09	2.27E+07	0.9975	-■-
	PBS	4.96E+08	1.04E+07	0.9962	-▼-
RBM5-159	EtOH	1.27E+09	3.05E+07	0.9953	-■-

Figure S28. Linear plots of integrated fluorescence intensity (AUC[480-700 nm]) *vs* absorbance (at λ_{Ex} = 470 nm) for fluorescence standard fluorescein in 0.1 M aq. NaOH (green), and NBD-labelled compounds **RBM5-130**, **RBM5-154** and **RBM5-159** in different solvents: DMSO (blue), EtOH (black) and PBS containing 1 % DMSO and 0.1 % Triton X-100 (red).
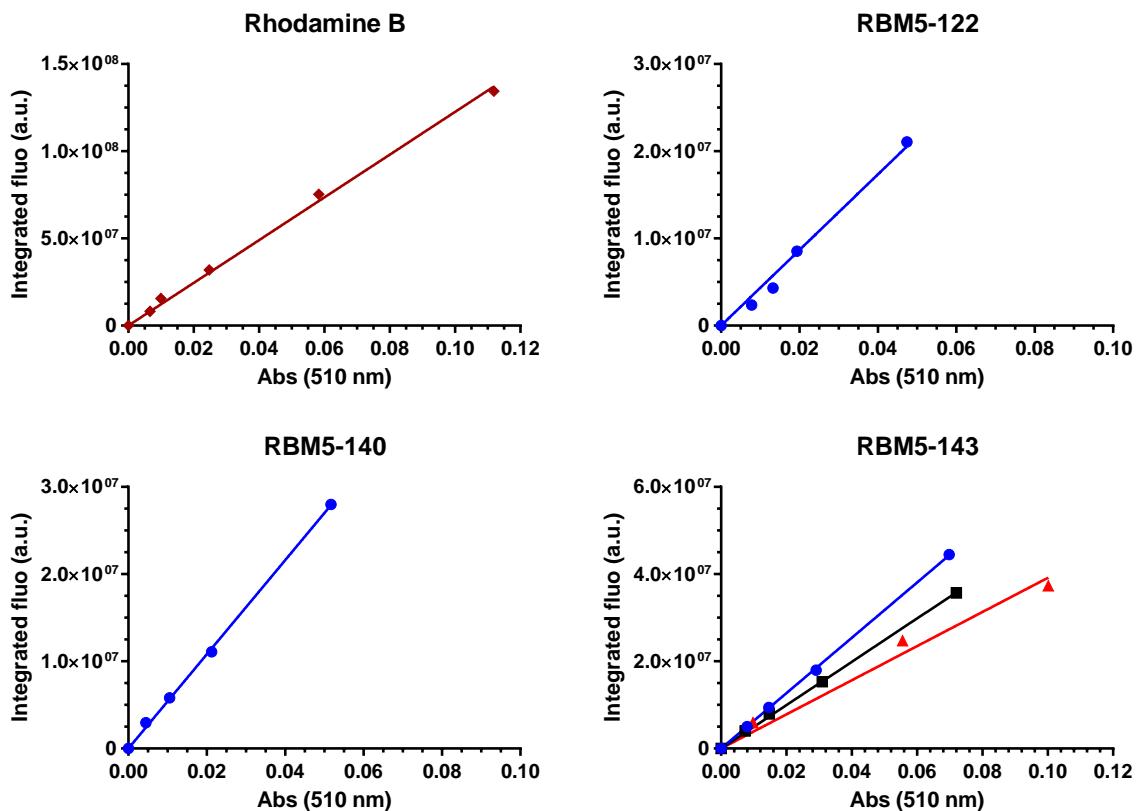
Compound	Solvent	Slope	SE	R^2	Legend
Rhodamine B	EtOH	1.23E+09	2.04E+07	0.9974	-♦-
RBM5-122	DMSO	4.34E+08	1.72E+07	0.9878	-●-
RBM5-140	DMSO	5.40E+08	6.06E+06	0.999	-●-
RBM5-143	DMSO	6.34E+08	3.39E+06	0.9998	-●-
	EtOH	4.97E+08	4.41E+06	0.9994	-■-
	PBS	3.91E+08	2.06E+07	0.9811	-▼-

Figure S29. Linear plots of integrated fluorescence intensity (AUC[520-800 nm]) *vs* absorbance (at λ_{Ex} = 510 nm) for fluorescence standard rhodamine B in EtOH (dark red), and NR-labelled compounds **RBM5-122**, **RBM5-140** and **RBM5-143** in different solvents: DMSO (blue), EtOH (black) and PBS containing 1 % DMSO and 0.1 % Triton X-100 (red).

4. Calculation of the spectral overlap integrals

In this section are shown the donor emission and acceptor absorption spectra used to calculate the spectral overlap integrals.

DMSO

EtOH

PBS
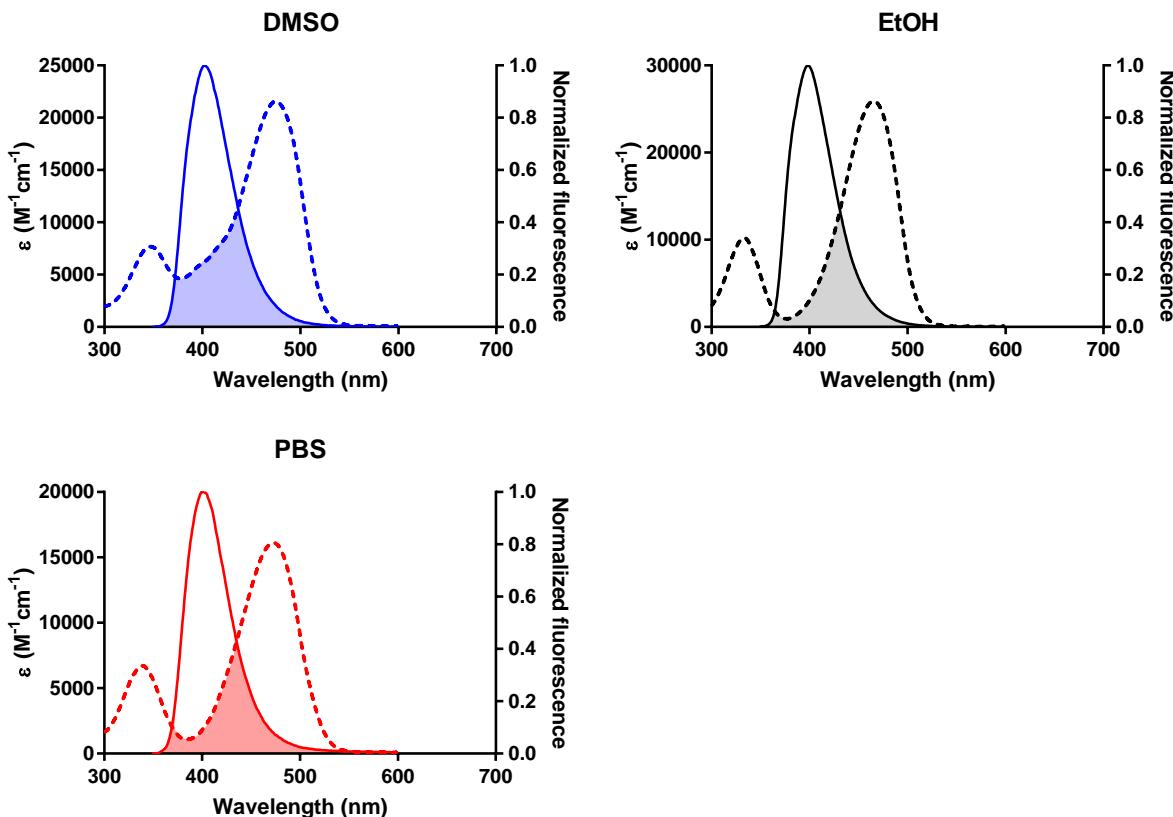
FRET pair	Solvent	Spectral overlap integral (J) ($nm^4 \cdot M^{-1} \cdot cm^{-1}$)	R_0 (Å)
Donor: MCC (**RBM5-142**) Acceptor: NBD (**RBM5-154**)	DMSO	2.79E+14	23.5
	EtOH	2.34E+14	35.1
	PBS	1.61E+14	22.0

Figure S30. Absorption spectra (dotted line) of compound **RBM5-154** (acceptor) expressed in molar extinction coefficient units ($M^{-1}cm^{-1}$) and normalized emission spectra (solid line) upon excitation at 340 nm of compound **RBM5-142** (donor) in various solvents: DMSO (blue), EtOH (black) and PBS containing 1 % DMSO and 0.1 % Triton X-100 (red). The absorption spectra were scaled so that the molar extinction coefficients at the maximum absorption wavelength would match the corresponding values reported in **Figure S23**.
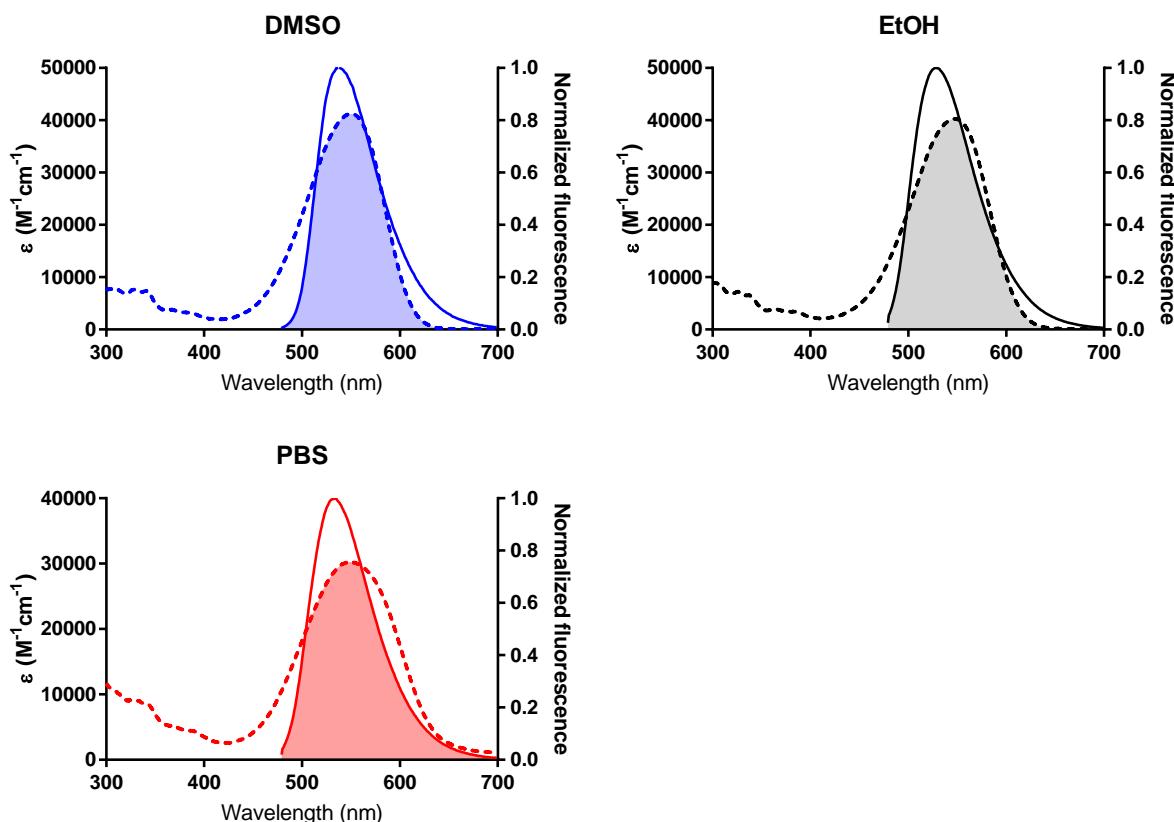
FRET pair	Solvent	Spectral overlap integral (J) ($nm^4 \cdot M^{-1} \cdot cm^{-1}$)	R_0 (Å)
Donor: NBD (**RBM5-154**) Acceptor: NR (**RBM5-143**)	DMSO	2.77E+15	36.0
	EtOH	2.60E+15	46.8
	PBS	2.23E+15	39.1

Figure S31. Absorption spectra (dotted line) of compound **RBM5-143** (acceptor) expressed in molar extinction coefficient units ($M^{-1}cm^{-1}$) and normalized emission spectra (solid line) upon excitation at 470 nm of compound **RBM5-154** (donor) in various solvents: DMSO (blue), EtOH (black) and PBS containing 1 % DMSO and 0.1 % Triton X-100 (red). The absorption spectra were scaled so that the molar extinction coefficients at the maximum absorption wavelength would match the corresponding values reported in **Figure S24**.

5. Deconvolution of the composite spectra

In this section are shown the deconvolutions of the composite absorption and emission spectra of compounds **RBM5-160** and **RBM5-161** used to obtain the corresponding isolated spectra of the individual donor and acceptor components.
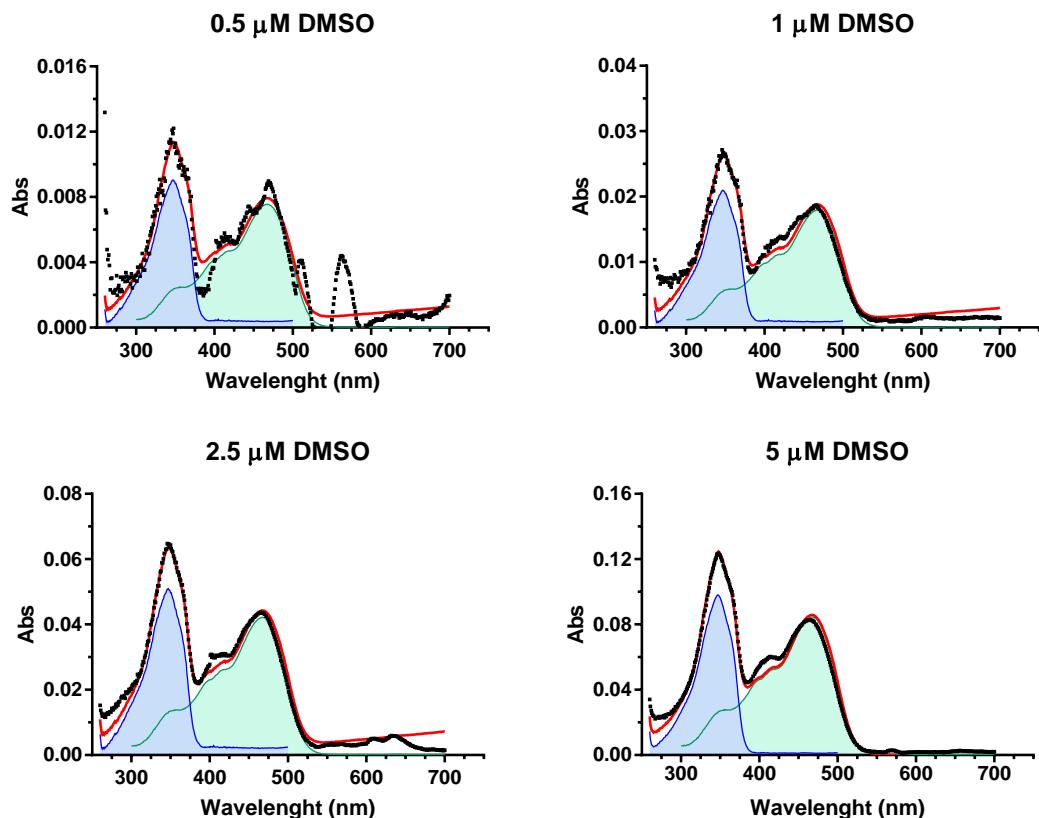
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R²
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-142 5 µM	0.08601 ± 0.00225	RBM5-154 10 µM	0.0351 ± 8.59E-4	0.85508
1	RBM5-142 5 µM	0.19947 ± 0.00264	RBM5-154 10 µM	0.08329 ± 0.00101	0.96333
2.5	RBM5-142 5 µM	0.48471 ± 0.00479	RBM5-154 10 µM	0.19583 ± 0.00183	0.97907
5	RBM5-142 10 µM	0.45945 ± 0.00343	RBM5-154 10 µM	0.39284 ± 0.00289	0.98683

Figure S32. Deconvoluted absorption spectra of compound **RBM5-160** in DMSO at concentrations ranging from 0.5 µM to 5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).

0.5 μM EtOH

1 μM EtOH

2.5 μM EtOH
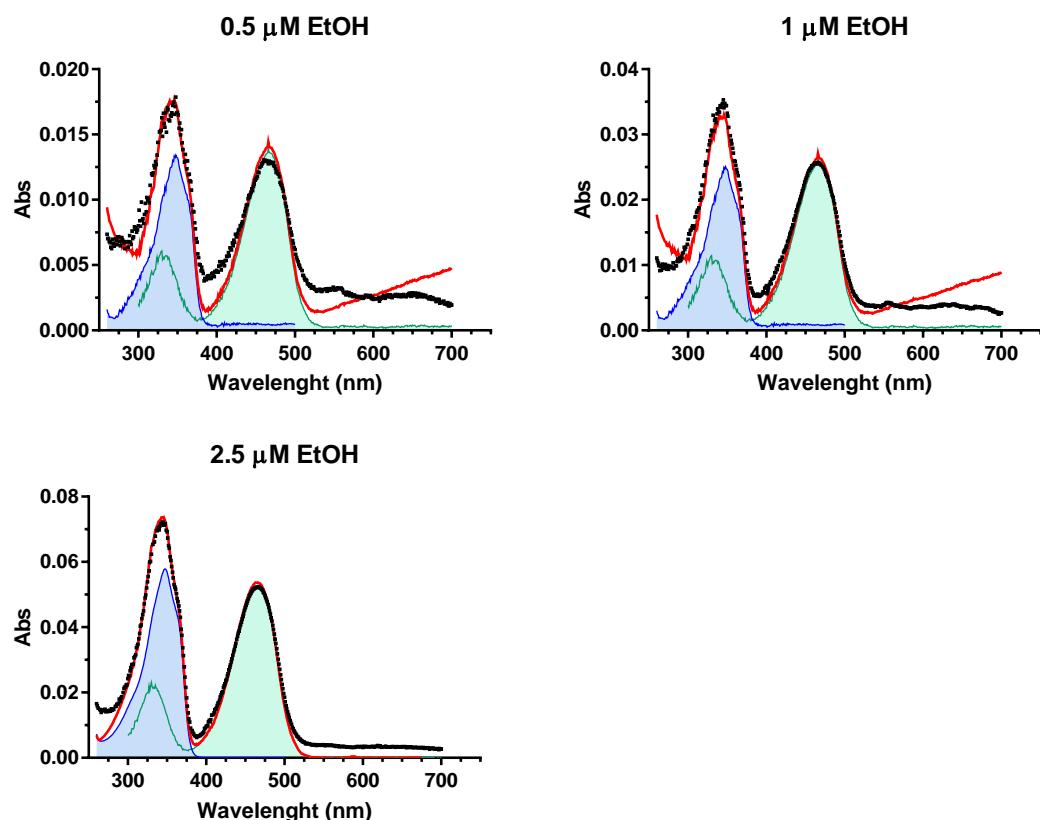
Conc (μM)	Component 1 (Donor)		Component 2 (Acceptor)		R^2
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-142 1 μM	0.27148 ± 0.00560	RBM5-154 1 μM	0.40059 ± 0.00685	0.90269
1	RBM5-142 1 μM	0.50687 ± 0.01007	RBM5-154 1 μM	0.75288 ± 0.01232	0.9307
2.5	RBM5-142 25 μM	0.05527 ± 5.50E-04	RBM5-154 2.5 μM	0.79542 ± 0.0078	0.97596

Figure S33. Deconvoluted absorption spectra of compound **RBM5-160** in EtOH at concentrations ranging from 0.5 µM to 2.5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).

0.5 µM DMSO

1 µM DMSO

2.5 µM DMSO
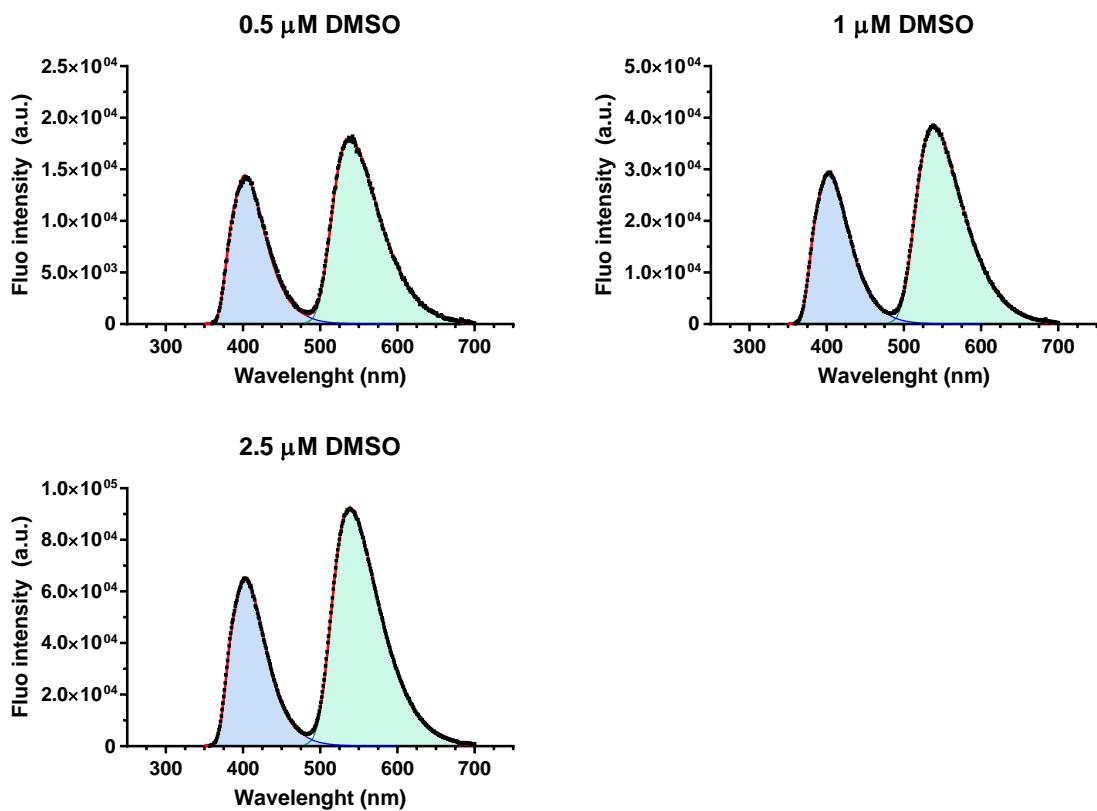
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R^2
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-142 10 µM	0.02417 ± 6.90E-05	RBM5-154 5 µM	0.25339 ± 5.34E-04	0.99863
1	RBM5-142 10 µM	0.04948 ± 8.70E-05	RBM5-154 5 µM	0.54089 ± 6.74E-04	0.99951
2.5	RBM5-142 10 µM	0.10964 ± 1.81E-04	RBM5-154 5 µM	1.29846 ± 0.0014	0.99962

Figure S34. Deconvoluted emission spectra upon excitation at 340 nm of compound **RBM5-160** in DMSO at concentrations ranging from 0.5 µM to 2.5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).

0.5 µM EtOH

1 µM EtOH

2.5 µM EtOH
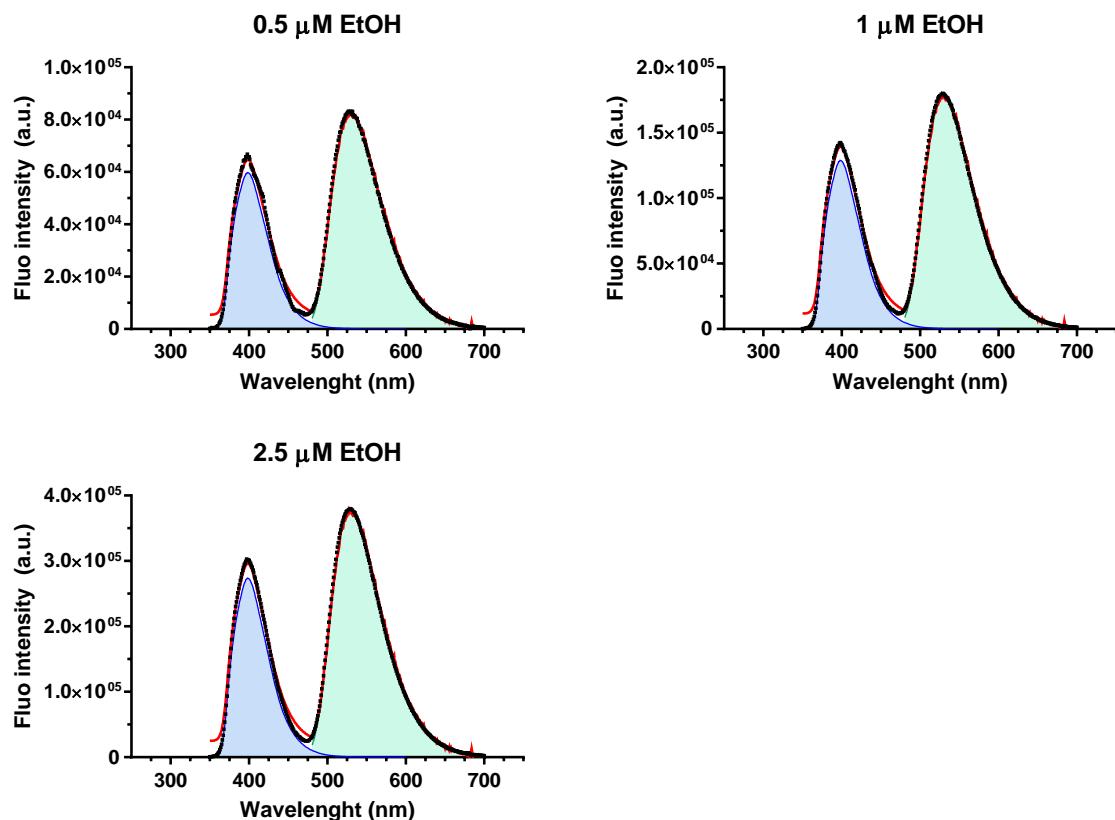
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R²
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-142 1 µM	0.07776 ± 4.91E-04	RBM5-154 0.25 µM	4.24179 ± 0.01815	0.99467
1	RBM5-142 1 µM	0.16768 ± 9.93E-04	RBM5-154 0.25 µM	9.18401 ± 0.03671	0.99533
2.5	RBM5-142 1 µM	0.35616 ± 0.00214	RBM5-154 0.25 µM	19.40169 ± 0.07912	0.99516

Figure S35. Deconvoluted emission spectra upon excitation at 340 nm of compound **RBM5-160** in EtOH at concentrations ranging from 0.5 µM to 2.5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).
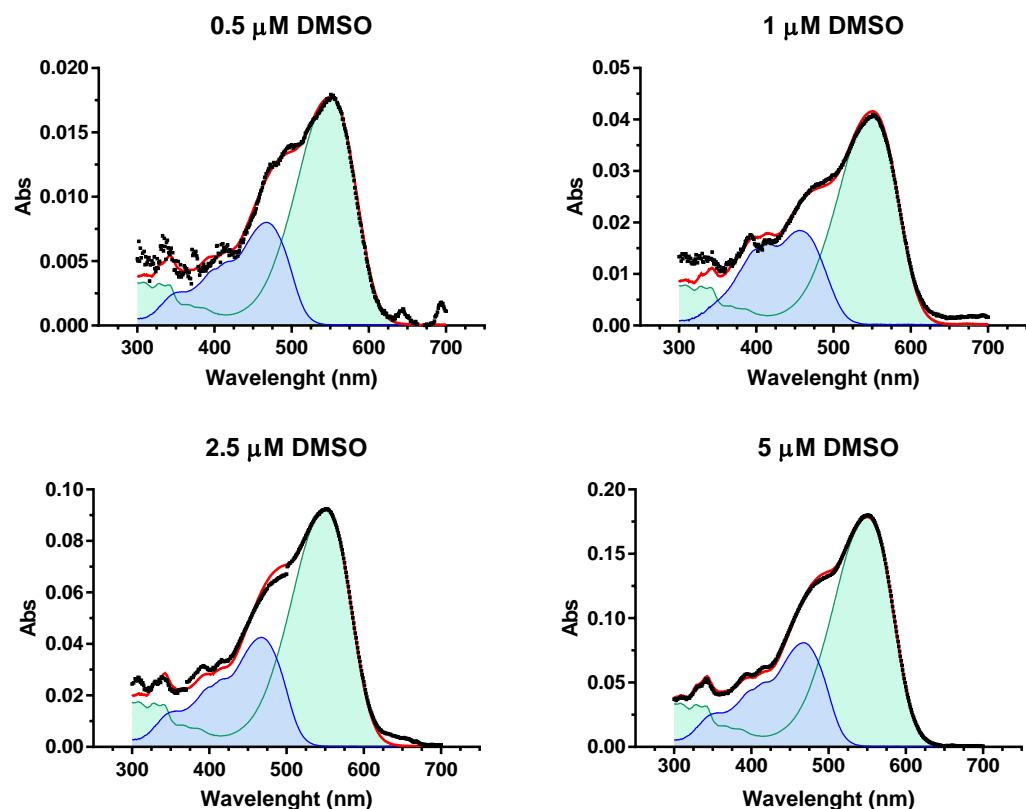
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R^2
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-154 10 µM	0.03725 ± 4.49E-04	RBM5-143 10 µM	0.04255 ± 2.37E-04	0.98756
1	RBM5-154 5 µM	0.18834 ± 0.00241	RBM5-143 10 µM	0.09941 ± 6.42E-04	0.98171
2.5	RBM5-154 10 µM	0.19765 ± 0.00162	RBM5-143 10 µM	0.22143 ± 8.59E-04	0.9939
5	RBM5-154 10 µM	0.37605 ± 0.00131	RBM5-143 10 µM	0.42829 ± 6.94E-04	0.999

Figure S36. Deconvoluted absorption spectra of compound **RBM5-161** in DMSO at concentrations ranging from 0.5 µM to 5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).

**0.5 µM EtOH**

1 µM EtOH

2.5 µM EtOH

5 µM EtOH
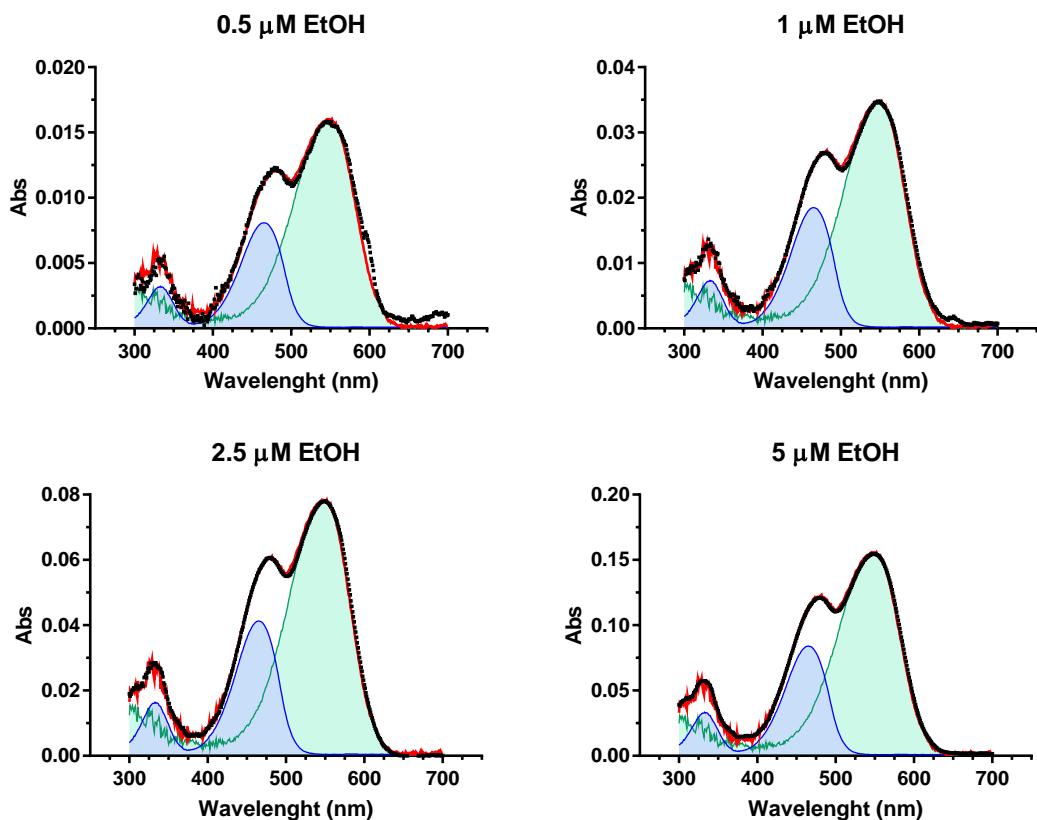
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R^2
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-154 5 µM	$0.05614 \pm 7.55E\text{-}04$	RBM5-143 0.5 µM	0.75626 ± 0.00469	0.98533
1	RBM5-154 5 µM	$0.12838 \pm 9.25E\text{-}04$	RBM5-143 0.5 µM	1.63968 ± 0.00575	0.99532
2.5	RBM5-154 5 µM	0.28649 ± 0.00164	RBM5-143 0.5 µM	3.70021 ± 0.01022	0.99723
5	RBM5-154 5 µM	0.58311 ± 0.00331	RBM5-143 0.5 µM	7.35667 ± 0.02059	0.99708

Figure S37. Deconvoluted absorption spectra of compound **RBM5-161** in EtOH at concentrations ranging from 0.5 µM to 5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).

0.5 µM DMSO


1 µM DMSO

2.5 µM DMSO

5 µM DMSO
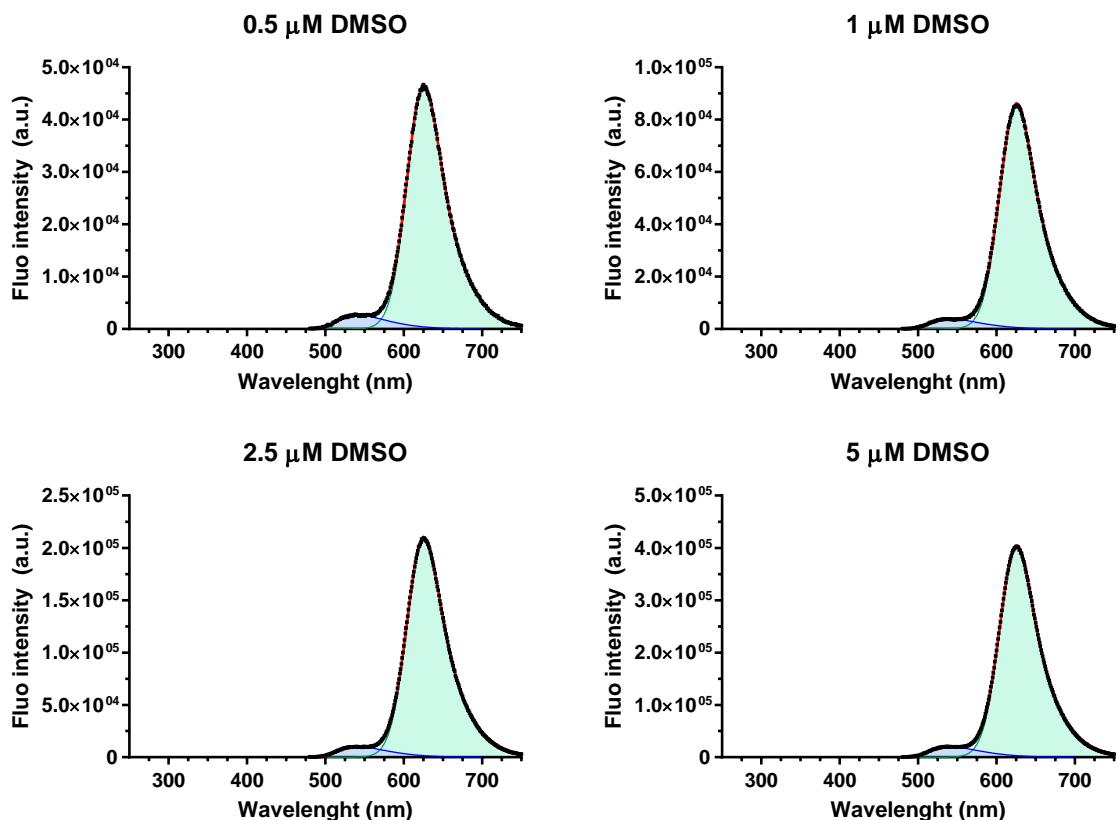
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R^2
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-154 2.5 µM	0.01816 ± 2.36E-04	RBM5-143 5 µM	0.09554 ± 7.77E-05	0.99979
1	RBM5-154 2.5 µM	0.02643 ± 4.13E-04	RBM5-143 5 µM	0.17701 ± 1.36E-04	0.99982
2.5	RBM5-154 2.5 µM	0.06856 ± 8.15E-04	RBM5-143 5 µM	0.43157 ± 2.68E-04	0.99988
5	RBM5-154 2.5 µM	0.13542 ± 0.00121	RBM5-143 5 µM	0.83084 ± 3.96E-04	0.99993

Figure S38. Deconvoluted emission spectra upon excitation at 455 nm of compound **RBM5-161** in DMSO at concentrations ranging from 0.5 µM to 5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).
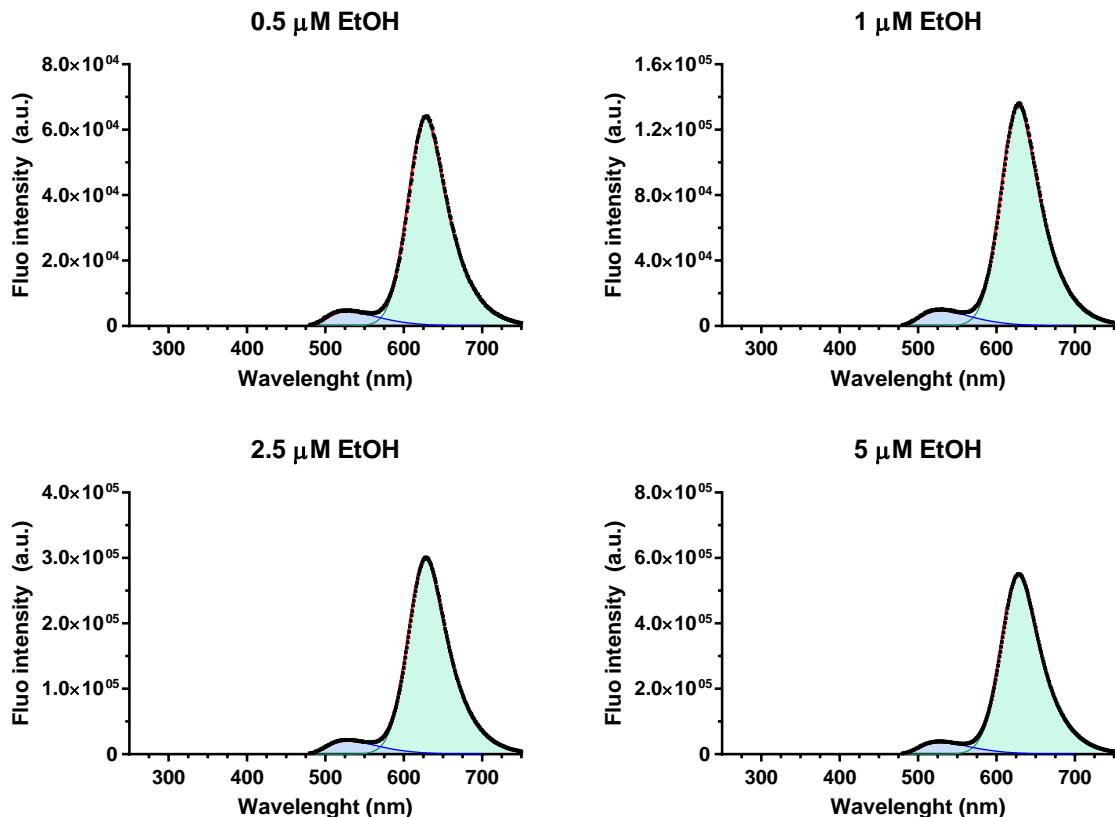
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R²
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-154 5 µM	0.00226 ± 4.59E-05	RBM5-143 2.5 µM	0.23964 ± 3.79E-04	0.99924
1	RBM5-154 5 µM	0.00479 ± 9.18E-05	RBM5-143 2.5 µM	0.5079 ± 7.57E-04	0.99932
2.5	RBM5-154 5 µM	0.01037 ± 2.00E-04	RBM5-143 2.5 µM	1.12472 ± 0.00165	0.99934
5	RBM5-154 5 µM	0.01842 ± 3.91E-04	RBM5-143 2.5 µM	2.06403 ± 0.00323	0.99925

Figure S39. Deconvoluted emission spectra upon excitation at 455 nm of compound **RBM5-161** in EtOH at concentrations ranging from 0.5 µM to 5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).

0.5 µM DMSO

1 µM DMSO

2.5 µM DMSO

5 µM DMSO
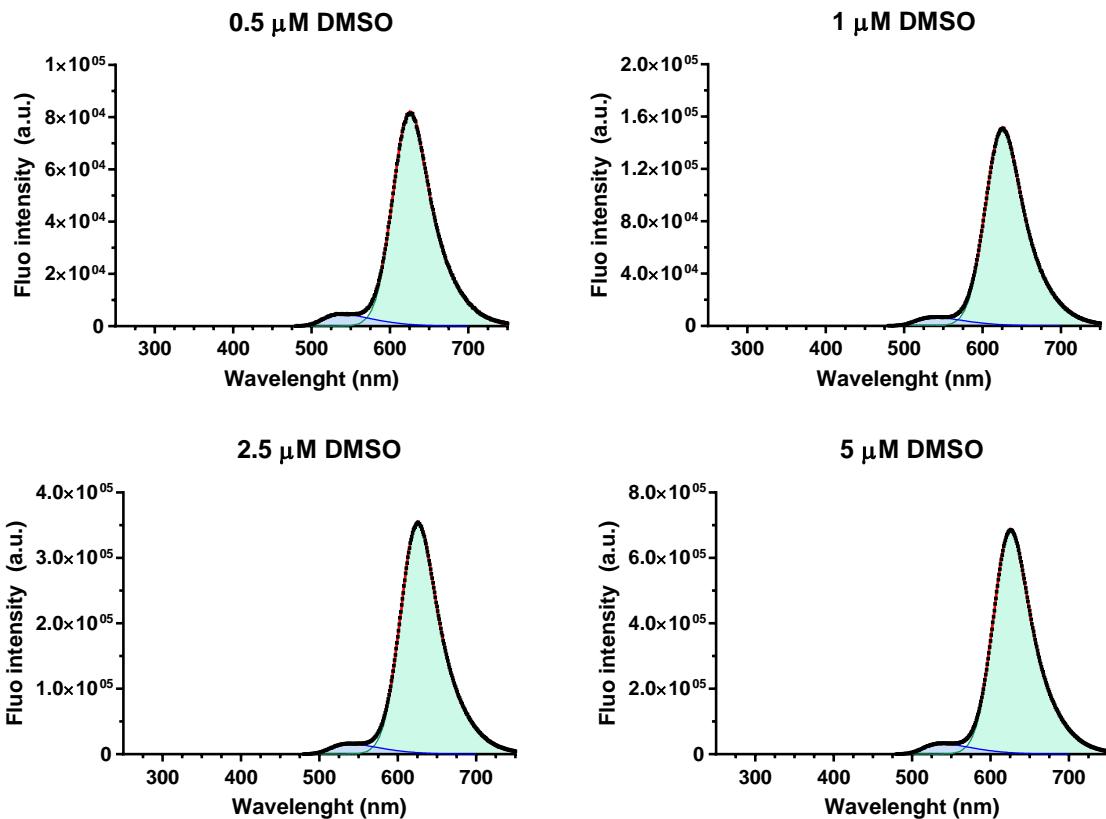
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R^2
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-154 1 µM	0.08346 ± 0.00111	RBM5-143 1 µM	0.74621 ± 6.38E-04	0.99976
1	RBM5-154 1 µM	0.1197 ± 0.00169	RBM5-143 1 µM	1.38051 ± 9.72E-04	0.99984
2.5	RBM5-154 1 µM	0.2892 ± 0.00368	RBM5-143 1 µM	3.23379 ± 0.00212	0.99986
5	RBM5-154 1 µM	0.57693 ± 0.00768	RBM5-143 1 µM	6.26587 ± 0.00443	0.99984

Figure S40. Deconvoluted emission spectra upon excitation at 470 nm of compound **RBM5-161** in DMSO at concentrations ranging from 0.5 µM to 5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).

Figure S41. Deconvoluted emission spectra upon excitation at 470 nm of compound **RBM5-161** in EtOH at concentrations ranging from 0.5 µM to 2.5 µM. Legend: original data (■), fitted curve (–), donor contribution (–), acceptor contribution (–).
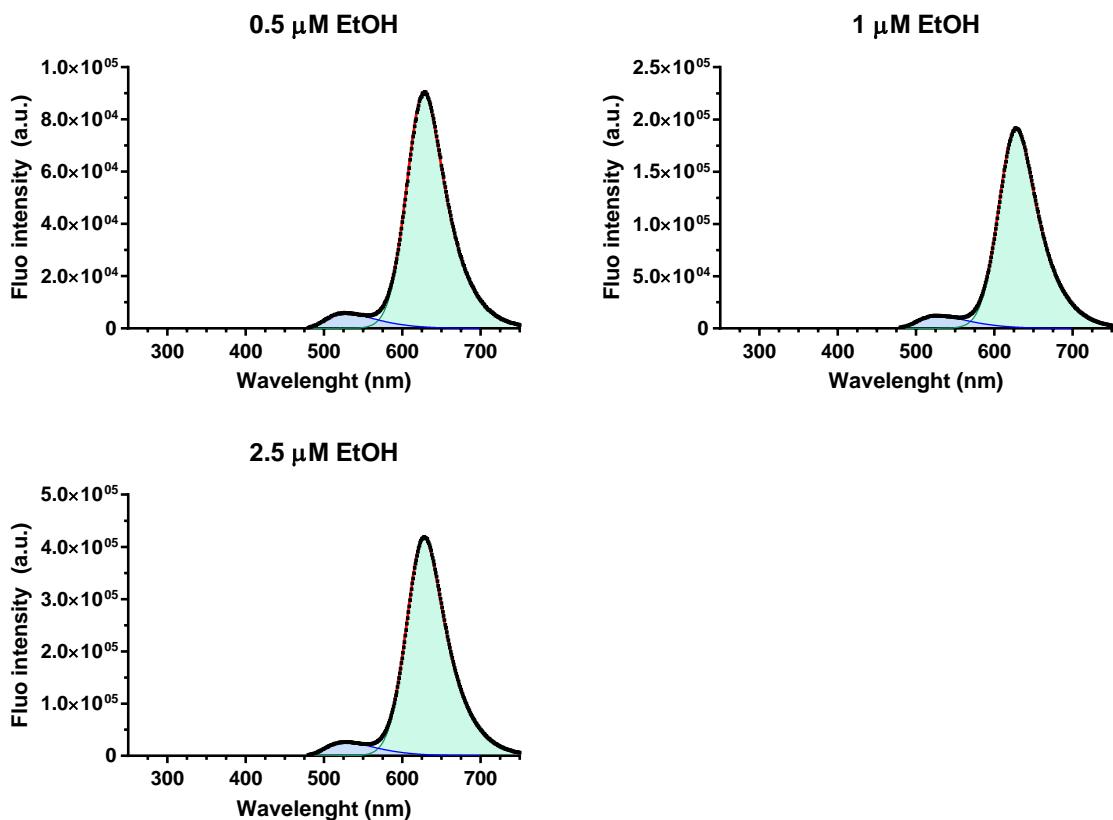
Conc (µM)	Component 1 (Donor)		Component 2 (Acceptor)		R^2
	Ref. spectrum	Coefficient ± SE	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-154 2.5 µM	0.00537 ± 6.94E-05	RBM5-143 5 µM	0.17544 ± 1.56E-04	0.99976
1	RBM5-154 2.5 µM	0.01121 ± 1.26E-04	RBM5-143 5 µM	0.37236 ± 2.84E-04	0.99982
2.5	RBM5-154 2.5 µM	0.02397 ± 3.01E-04	RBM5-143 5 µM	0.81496 ± 6.78E-04	0.99979

6. Linear plots to calculate the FRET efficiency

In this section are displayed the absorbance at the excitation wavelength (λ_{Ex}) *versus* integrated fluorescence intensity plots used to calculate the FRET efficiencies from the attenuation of the donor emission. To this end, we compared the slopes of the plots corresponding to the donor in the absence and the presence of the acceptor. The regressions were forced to intercept the origin.
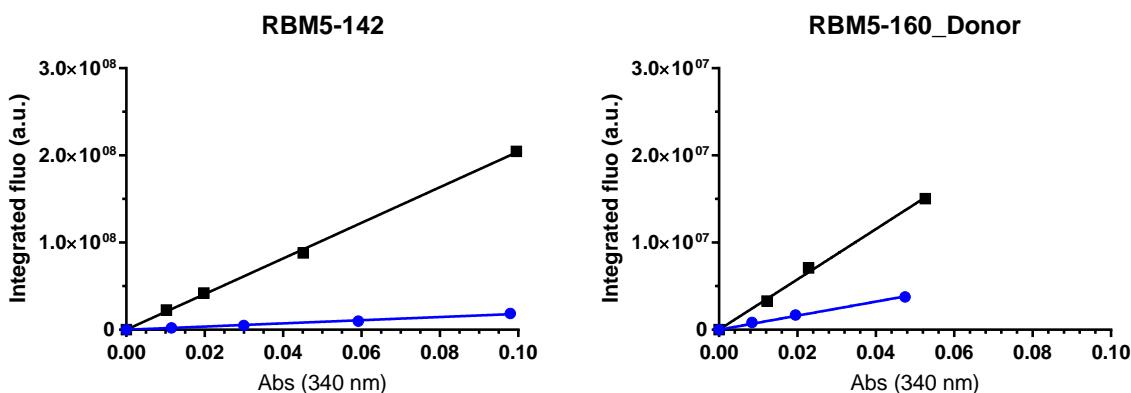
Compound	Solvent	Slope	SE	R^2	Legend
RBM5-142	DMSO	1.82E+08	5.79E+06	0.9914	-●-
	EtOH	2.04E+09	2.16E+07	0.9991	-■-
RBM5-160_Donor	DMSO	8.06E+07	2.26E+06	0.9947	-●-
	EtOH	2.88E+08	5.54E+06	0.9975	-■-

Figure S42. Linear plots of integrated fluorescence intensity (AUC$_{DMSO}$[365-495 nm] or AUC$_{EtOH}$[360-490 nm]) *vs* absorbance (at λ_{Ex} = 340 nm) for compounds **RBM5-142** (Donor in the absence of the acceptor) and **RBM5-160_Donor component** (Donor in the presence of the acceptor) in DMSO (blue) and EtOH (black).
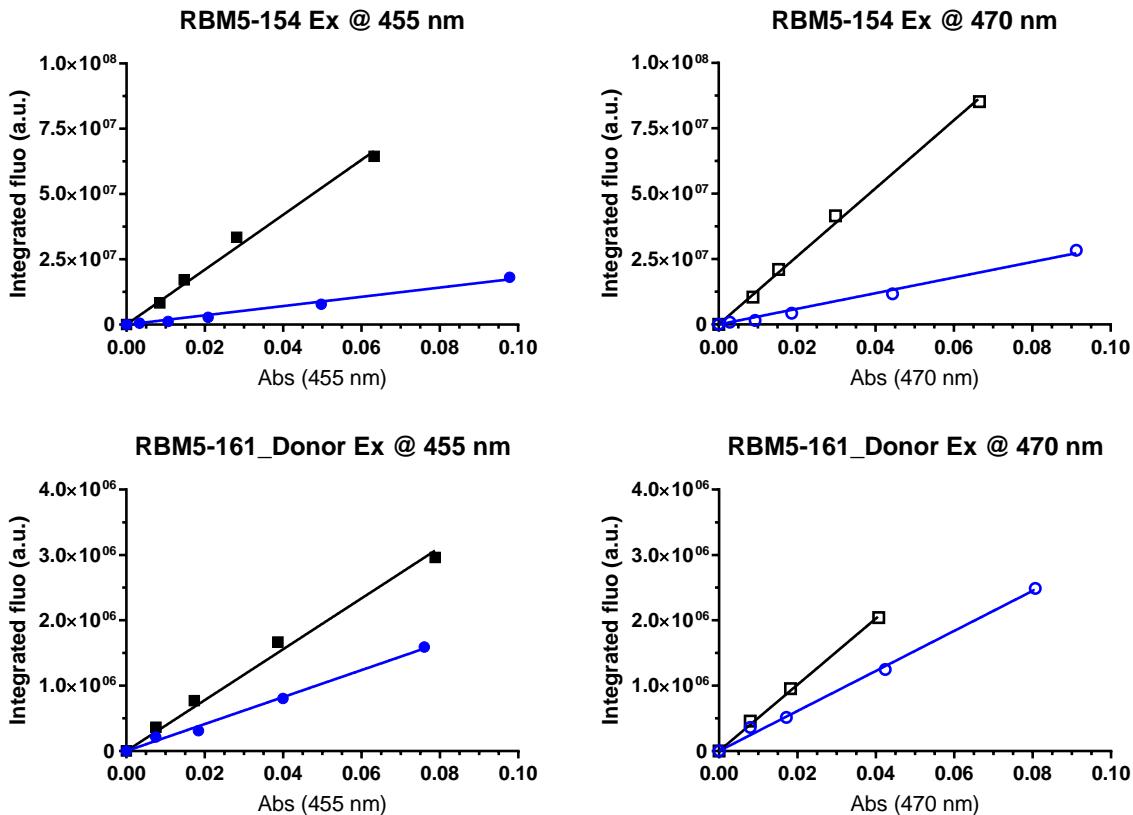
Compound	Solvent	λ_{ex} (nm)	Slope	SE	R^2	Legend
RBM5-154	DMSO	455	1.77E+08	6.78E+06	0.9881	-●-
		470	2.99E+08	1.12E+07	0.9888	-○-
	EtOH	455	1.05E+09	3.34E+07	0.9913	-■-
		470	1.30E+09	2.26E+07	0.9975	-□-
RBM5-161_Donor	DMSO	455	2.06E+07	5.40E+05	0.9944	-●-
		470	3.06E+07	6.86E+05	0.9958	-○-
	EtOH	455	3.89E+07	1.25E+06	0.991	-■-
		470	5.07E+07	8.24E+05	0.9982	-□-

Figure S43. Linear plots of integrated fluorescence intensity (AUC$_{DMSO}$[490-670 nm] or AUC$_{EtOH}$[480-660 nm]) *vs* absorbance (at λ_{Ex} = 455 or 470 nm) for compounds **RBM5-154** (Donor in the absence of the acceptor) and **RBM5-161_Donor component** (Donor in the presence of the acceptor) in DMSO (blue) and EtOH (black).

## 7. Linear plots to calculate the DEB

In this section are found the absorbance at the excitation wavelength (λ_{Ex}) versus integrated fluorescence intensity plots that were used to calculate the bleed-through related to the emission of the donor component (DEB). To this end, we compared the slopes of the plots corresponding to the total fluorescence of the click adducts **RBM5-160** and **RBM5-161** and those of the fluorescence of the isolated donor component after spectral deconvolution. The regressions were forced to intercept the origin.
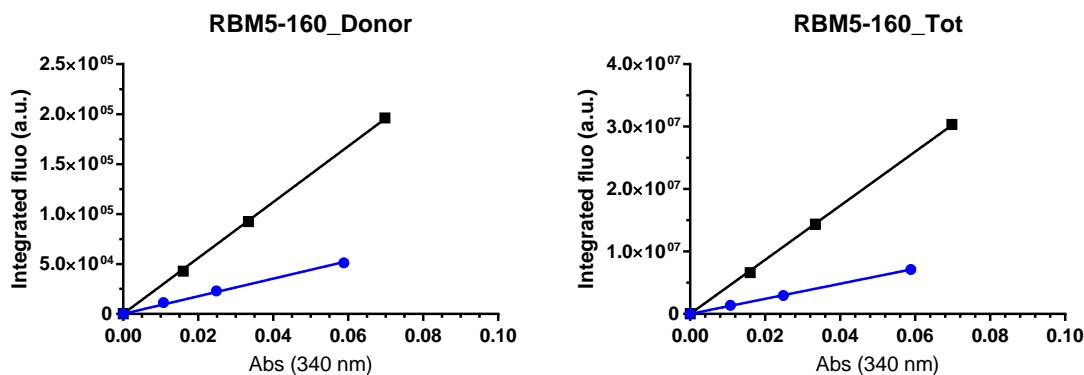
Compound	Solvent	Slope	SE	R^2	Legend
RBM5-160_Donor	DMSO	8.83E+05	2.04E+04	0.9964	-●-
	EtOH	2.80E+06	1.59E+04	0.9998	-■-
RBM5-160_Total	DMSO	1.21E+08	8.90E+05	0.9996	-●-
	EtOH	4.33E+08	2.56E+06	0.9998	-■-

Figure S44. Linear plots of integrated fluorescence intensity (AUC$_{DMSO}$[490-665 nm] or AUC$_{EtOH}$[480-655 nm]) *vs* absorbance (at λ_{Ex} = 340 nm) for compound **RBM5-160_Donor component** (only the emission of the donor is considered) and **RBM5-160_Total spectrum** (both the emission of the donor and the acceptor moieties are considered) in DMSO (blue) and EtOH (black).
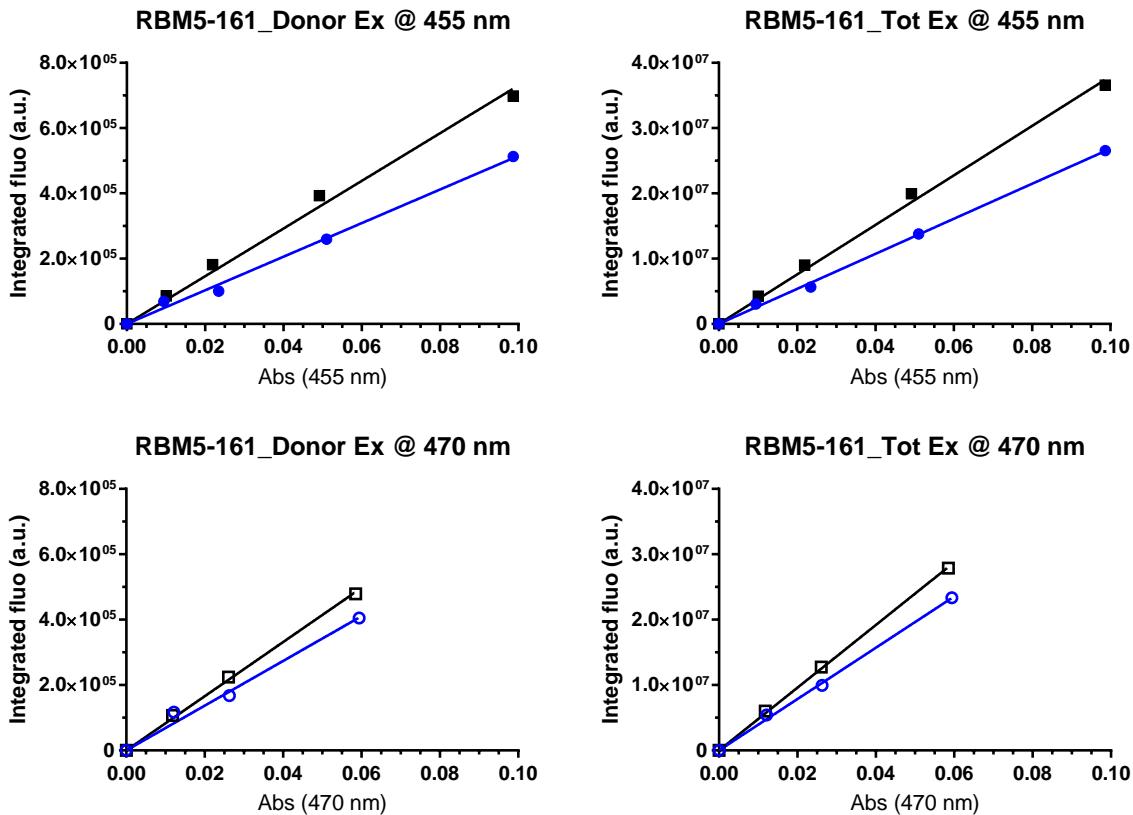
Compound	Solvent	λ_{ex} (nm)	Slope	SE	R^2	Legend
RBM5-161_Donor	DMSO	455	5.15E+06	1.29E+05	0.9949	-●-
		470	6.84E+06	3.14E+05	0.9852	-○-
	EtOH	455	7.30E+06	2.10E+05	0.9928	-■-
		470	8.28E+06	1.21E+05	0.9985	-□-
RBM5-161_Total	DMSO	455	2.68E+08	3.68E+06	0.9985	-●-
		470	3.92E+08	6.41E+06	0.9982	-○-
	EtOH	455	3.79E+08	7.76E+06	0.9964	-■-
		470	4.79E+08	4.06E+06	0.9995	-□-

Figure S45. Linear plots of integrated fluorescence intensity (AUC[570-730 nm]) *vs* absorbance (at λ_{Ex} = 455 or 470 nm) for compound **RBM5-161_Donor component** (only the emission of the donor is considered) and **RBM5-160_Total spectrum** (both the emission of the donor and the acceptor moieties are considered) in DMSO (blue) and EtOH (black).

8. Calculation of the coefficients to generate the AEB spectra

In this section are shown the correction of the experimental emission spectra of acceptor components under different excitation conditions. The obtained coefficients were used to generate the "calculated" acceptor emission bleed-through spectra.
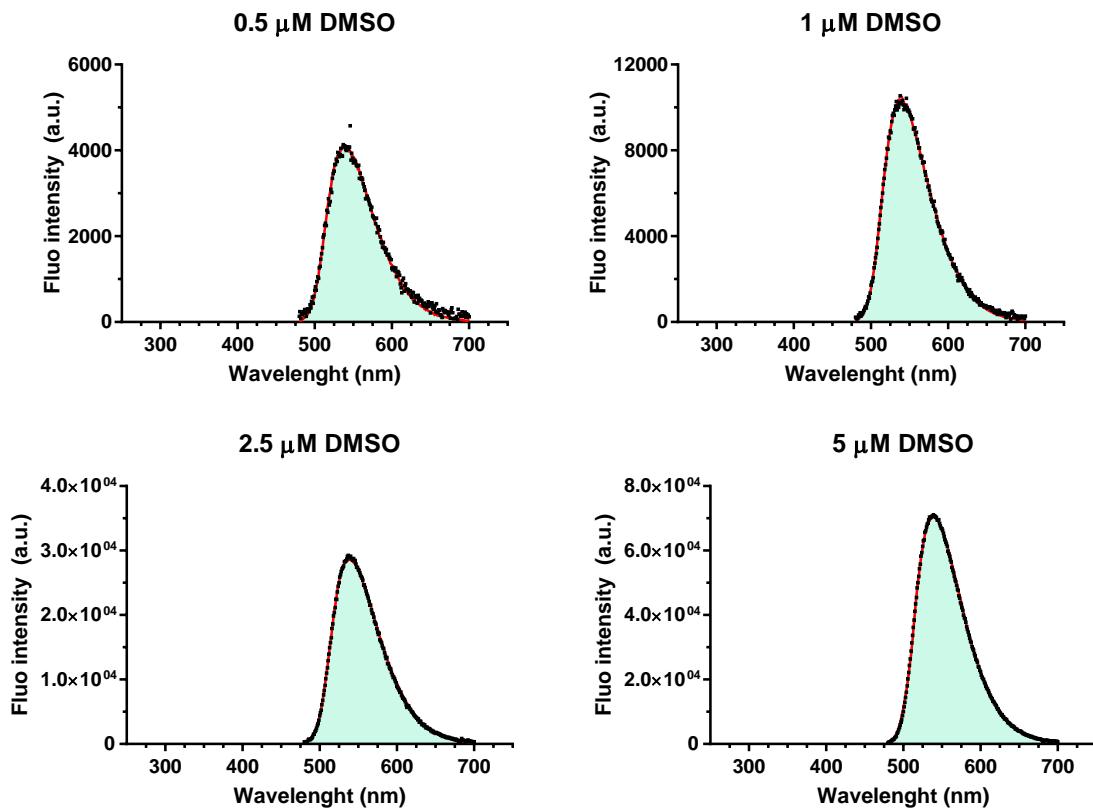
Conc (µM)	Component 1		R^2
	Ref. spectrum	Coefficient ± SE	
0.5	RBM5-154 0.5 µM DMSO Fluo@470 nm	$0.12267 \pm 6.70\text{E-}04$	0.99298
1	RBM5-154 1 µM DMSO Fluo@470 nm	$0.11954 \pm 2.94\text{E-}04$	0.99864
2.5	RBM5-154 2.5 µM DMSO Fluo@470 nm	$0.14723 \pm 2.23\text{E-}04$	0.9995
5	RBM5-154 5 µM DMSO Fluo@470 nm	$0.13796 \pm 1.57\text{E-}04$	0.99972

Figure S46. Deconvoluted emission spectra upon excitation at 340 nm of compound **RBM5-154** in DMSO at concentrations ranging from 0.5 µM to 2.5 µM. Legend: original data (■), fitted curve (–), acceptor contribution (–).
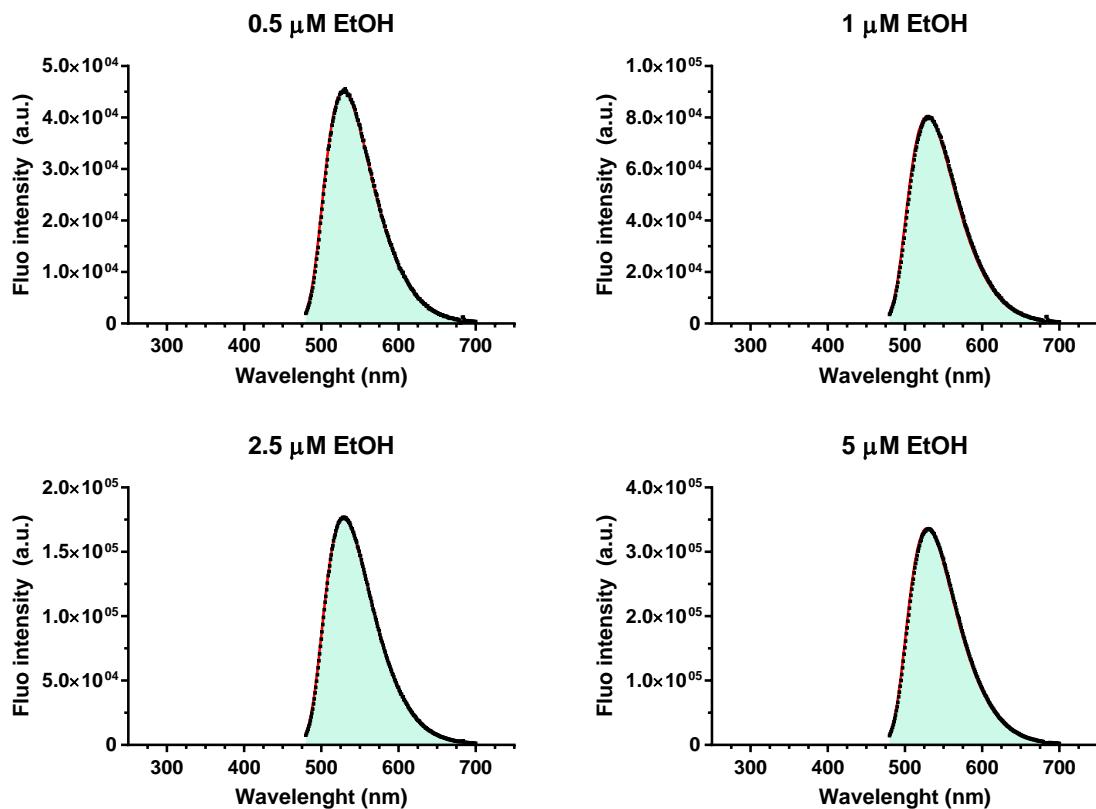
Conc (µM)	Component 1		R²
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-154 0.5 µM EtOH Fluo@470 nm	0.17282 ± 3.18E-04	0.99925
1	RBM5-154 1 µM EtOH Fluo@470 nm	0.15615 ± 4.70E-04	0.99799
2.5	RBM5-154 2.5 µM EtOH Fluo@470 nm	0.16631 ± 1.81E-04	0.99974
5	RBM5-154 5 µM EtOH Fluo@470 nm	0.16833 ± 4.14E-04	0.99866

Figure S47. Deconvoluted emission spectra upon excitation at 340 nm of compound **RBM5-154** in EtOH at concentrations ranging from 0.5 µM to 2.5 µM. Legend: original data (■), fitted curve (–), acceptor contribution (–).

0.5 μM DMSO

1 μM DMSO
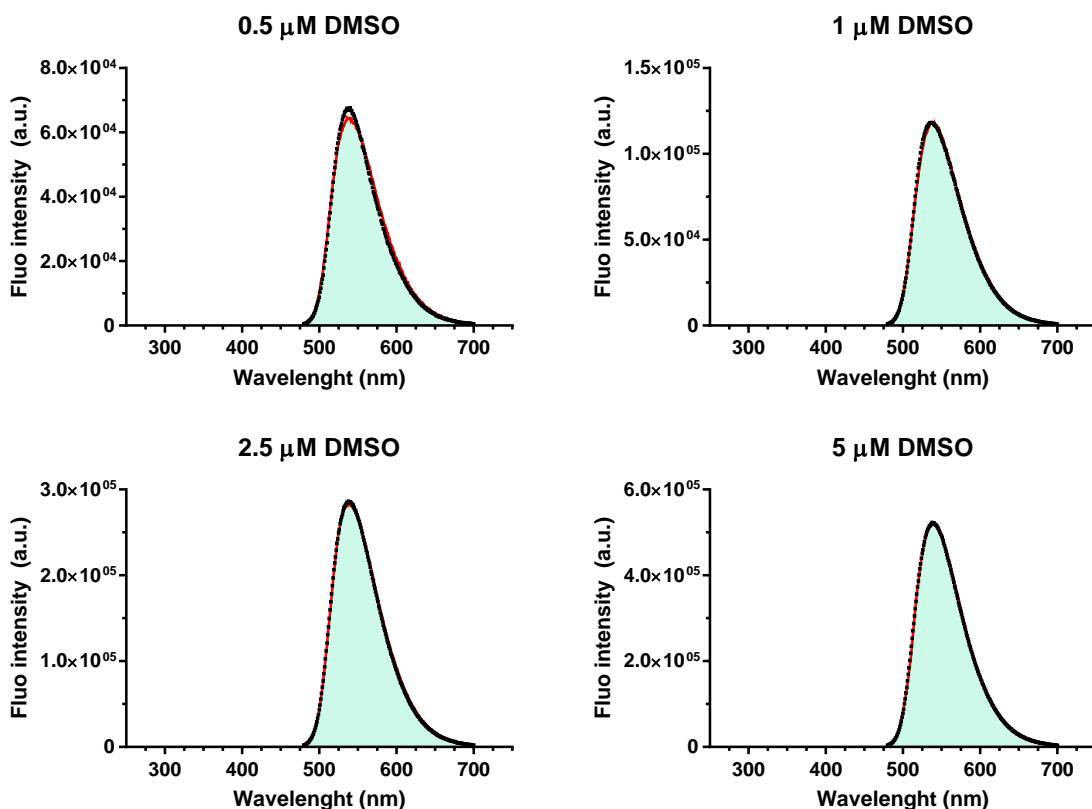
2.5 μM DMSO

5 μM DMSO

Conc (μM)	Component 1		R^2
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-154 0.5 μM DMSO Fluo@470 nm	1.93543 ± 0.00881	0.99563
1	RBM5-154 1 μM DMSO Fluo@470 nm	1.36264 ± 0.00308	0.99889
2.5	RBM5-154 2.5 μM DMSO Fluo@470 nm	1.45386 ± 0.00222	0.99949
5	RBM5-154 5 μM DMSO Fluo@470 nm	1.01559 ± 0.00134	0.99962

Figure S48. Deconvoluted emission spectra upon excitation at 470 nm of compound **RBM5-160** in DMSO at concentrations ranging from 0.5 μM to 2.5 μM. Legend: original data (■), fitted curve (–), acceptor contribution (–).
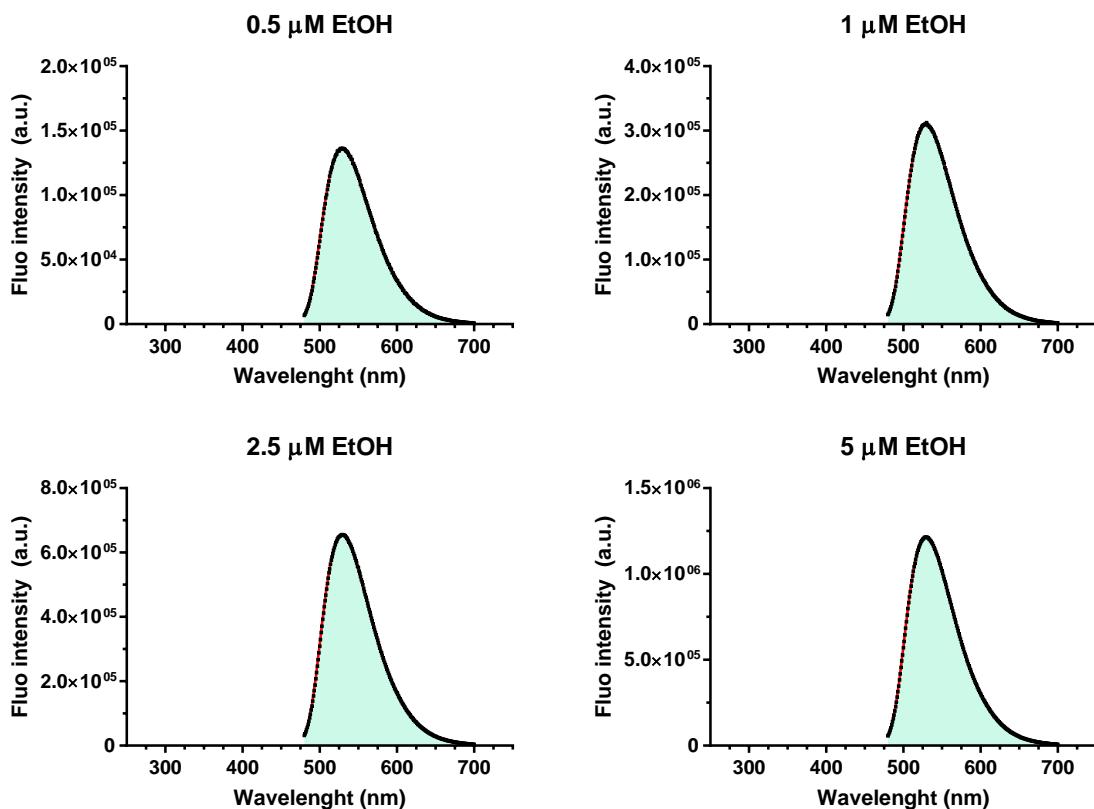
Conc (μM)	Component 1		R²
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-154 0.5 μM EtOH Fluo@470 nm	0.52058 ± 3.71618E-4	0.99989
1	RBM5-154 1 μM EtOH Fluo@470 nm	0.59993 ± 3.39746E-4	0.99993
2.5	RBM5-154 2.5 μM EtOH Fluo@470 nm	0.6181 ± 4.16827E-4	0.9999
5	RBM5-154 5 μM EtOH Fluo@470 nm	0.60572 ± 2.77769E-4	0.99995

Figure S49. Deconvoluted emission spectra upon excitation at 470 nm of compound **RBM5-160** in EtOH at concentrations ranging from 0.5 μM to 2.5 μM. Legend: original data (■), fitted curve (–), acceptor contribution (–).

0.5 μM DMSO

1 μM DMSO
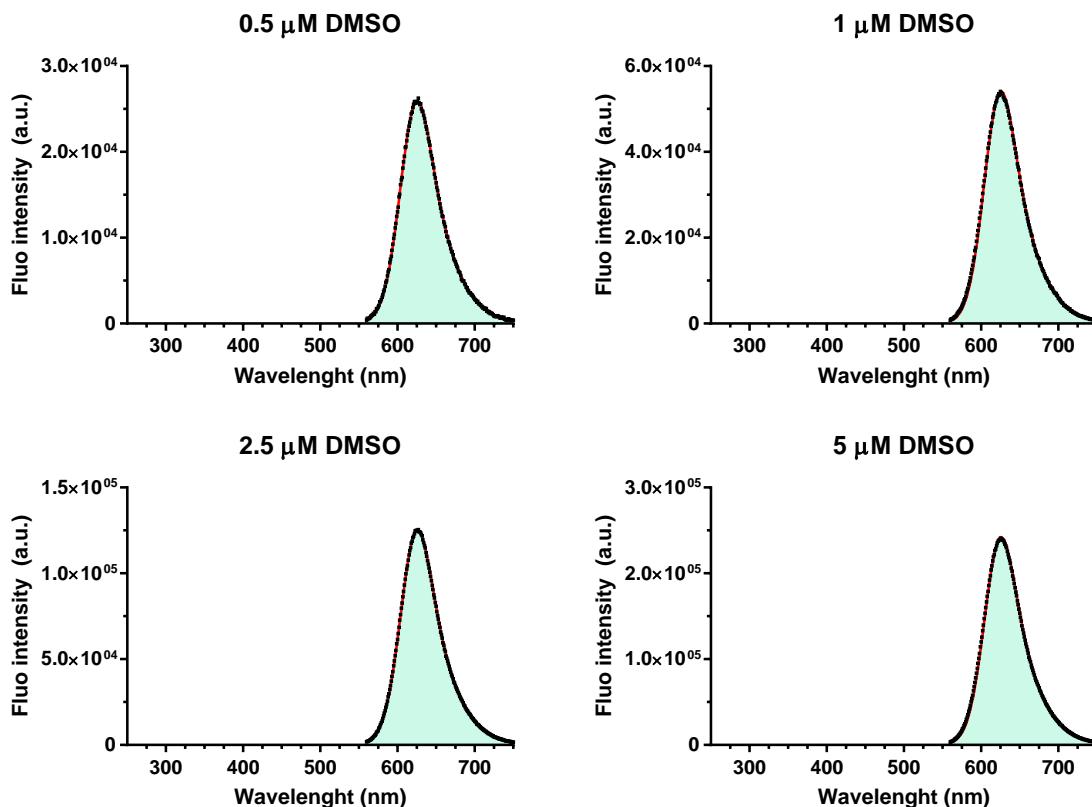
2.5 μM DMSO

5 μM DMSO

Conc (μM)	Component 1		R^2
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-143 0.5 μM DMSO Fluo@550 nm	0.14677 ± 1.44E-04	0.99977
1	RBM5-143 1 μM DMSO Fluo@550 nm	0.15008 ± 2.54E-04	0.99931
2.5	RBM5-143 2.5 μM DMSO Fluo@550 nm	0.15892 ± 9.34E-05	0.99992
5	RBM5-143 5 μM DMSO Fluo@550 nm	0.1805 ± 2.69137E-4	0.99946

Figure S50. Deconvoluted emission spectra upon excitation at 455 nm of compound **RBM5-143** in DMSO at concentrations ranging from 0.5 μM to 2.5 μM. Legend: original data (■), fitted curve (–), acceptor contribution (–).

0.5 μM EtOH
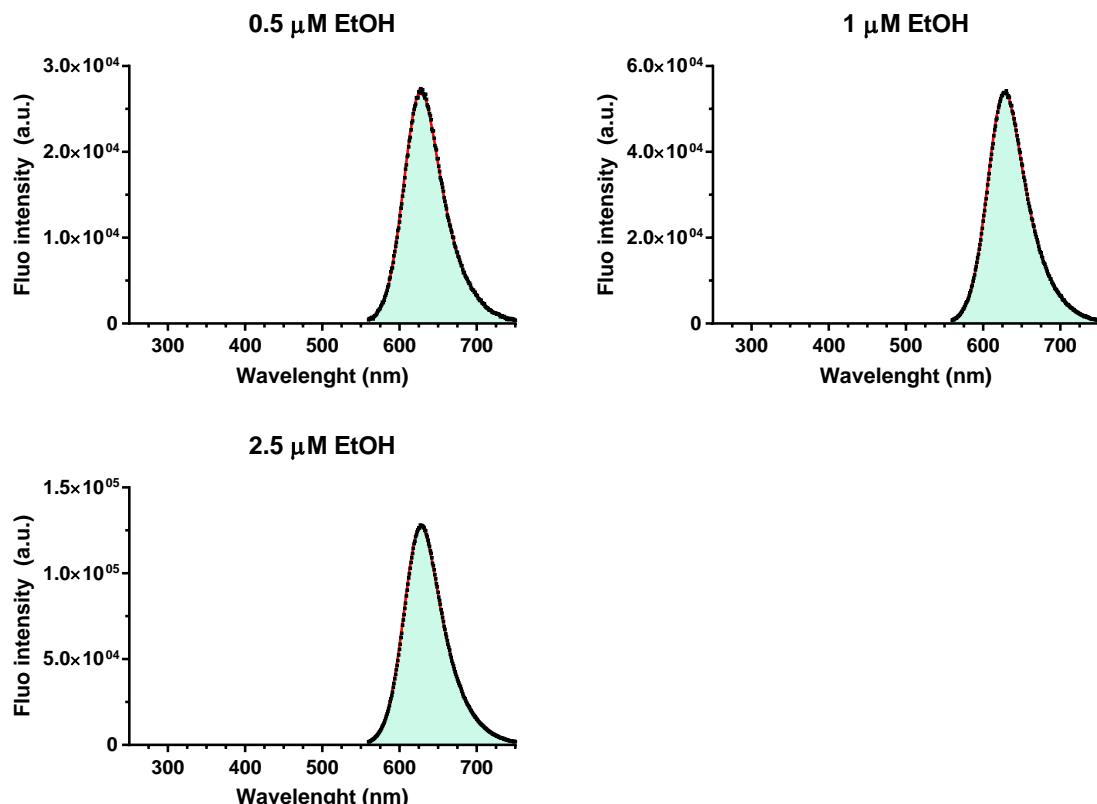
1 μM EtOH

2.5 μM EtOH

Conc (μM)	Component 1		R^2
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-143 0.5 μM EtOH Fluo@550 nm	0.17534 ± 2.27E-04	0.9996
1	RBM5-143 1 μM EtOH Fluo@550 nm	0.17667 ± 1.72E-04	0.99977
2.5	RBM5-143 2.5 μM EtOH Fluo@550 nm	0.1908 ± 2.25E-04	0.99967

Figure S51. Deconvoluted emission spectra upon excitation at 455 nm of compound **RBM5-143** in EtOH at concentrations ranging from 0.5 μM to 2.5 μM. Legend: original data (■), fitted curve (–), acceptor contribution (–).

0.5 μM DMSO
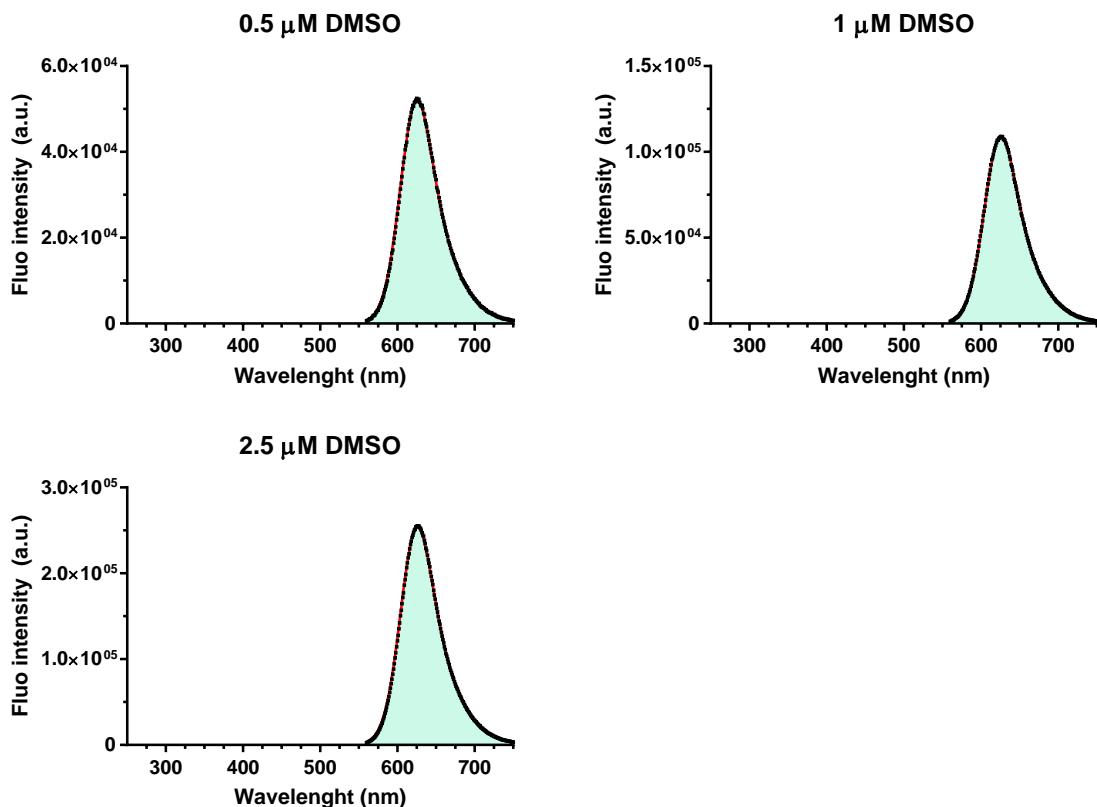
1 μM DMSO

2.5 μM DMSO

Conc (μM)	Component 1		R^2
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-143 0.5 μM DMSO Fluo@550 nm	0.29458 ± 3.33E-04	0.9997
1	RBM5-143 1 μM DMSO Fluo@550 nm	0.30279 ± 1.95E-04	0.9999
2.5	RBM5-143 2.5 μM DMSO Fluo@550 nm	0.32259 ± 3.46E-04	0.99973

Figure S52. Deconvoluted emission spectra upon excitation at 470 nm of compound **RBM5-143** in DMSO at concentrations ranging from 0.5 μM to 2.5 μM. Legend: original data (■), fitted curve (–), acceptor contribution (–).

0.5 μM EtOH
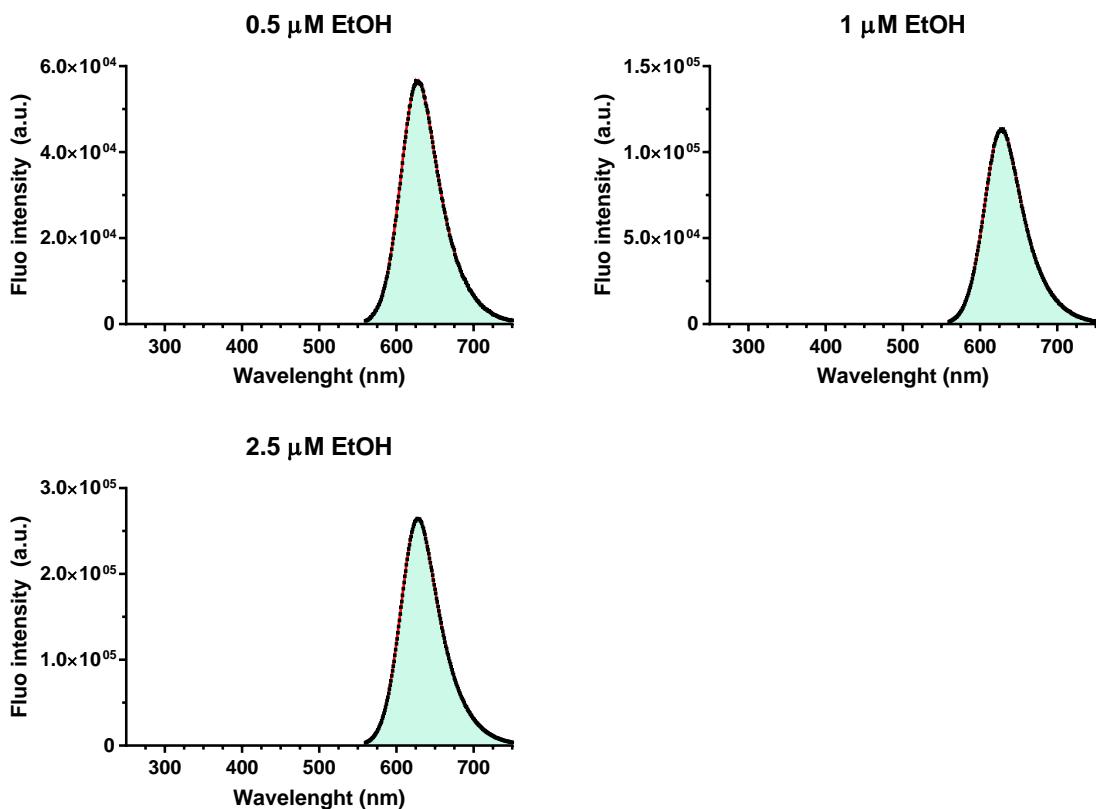
1 μM EtOH

2.5 μM EtOH

Conc (μM)	Component 1		R^2
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-143 0.5 μM EtOH Fluo@550 nm	0.3659 ± 2.31E-04	0.99991
1	RBM5-143 1 μM EtOH Fluo@550 nm	0.37194 ± 2.04E-04	0.99993
2.5	RBM5-143 2.5 μM EtOH Fluo@550 nm	0.39546 ± 1.54E-04	0.99996

Figure S53. Deconvoluted emission spectra upon excitation at 470 nm of compound **RBM5-143** in EtOH at concentrations ranging from 0.5 μM to 2.5 μM. Legend: original data (■), fitted curve (–), acceptor contribution (–).

0.5 μM DMSO

1 μM DMSO
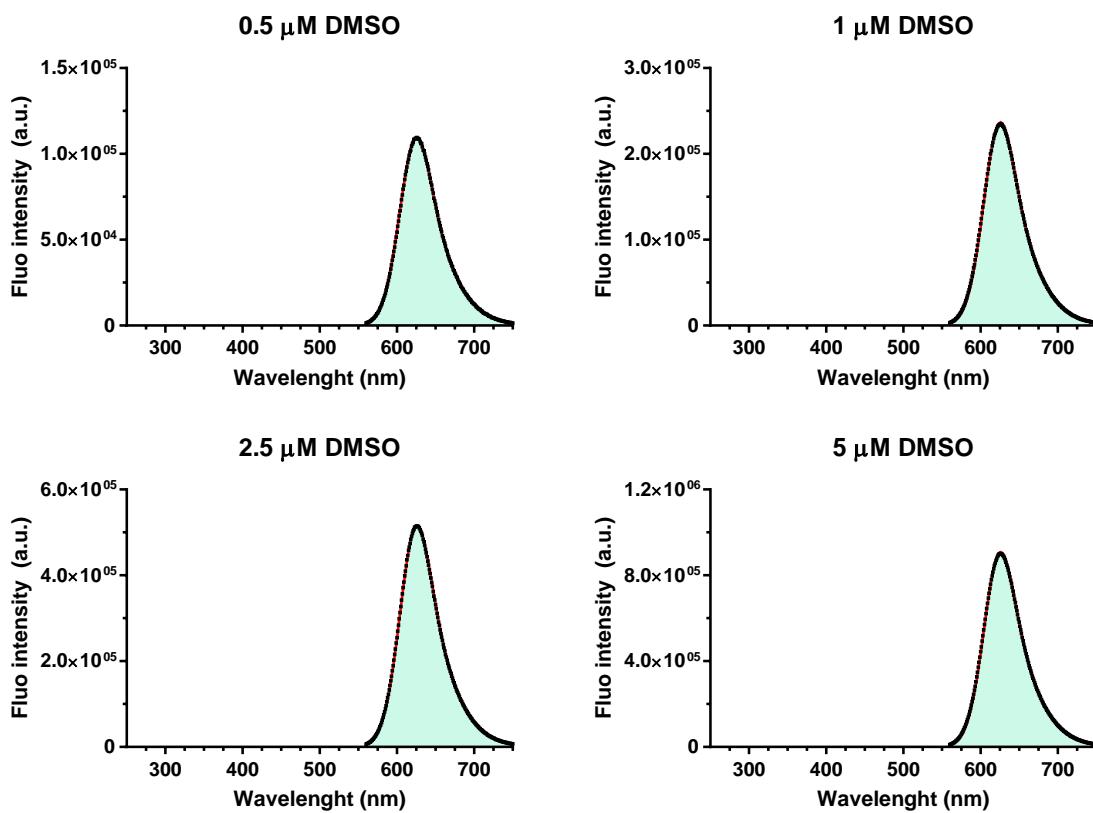
2.5 μM DMSO

5 μM DMSO

Conc (μM)	Component 1		R²
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-143 0.5 μM DMSO Fluo@550 nm	0.62139 ± 4.04E-04	0.9999
1	RBM5-143 1 μM DMSO Fluo@550 nm	0.65685 ± 7.13E-04	0.99971
2.5	RBM5-143 2.5 μM DMSO Fluo@550 nm	0.65501 ± 3.44E-04	0.99993
5	RBM5-143 5 μM DMSO Fluo@550 nm	0.67721 ± 5.93E-04	0.99981

Figure S54. Deconvoluted emission spectra upon excitation at 550 nm of compound **RBM5-161** in DMSO at concentrations ranging from 0.5 μM to 2.5 μM. Legend: original data (■), fitted curve (–), acceptor contribution (–).
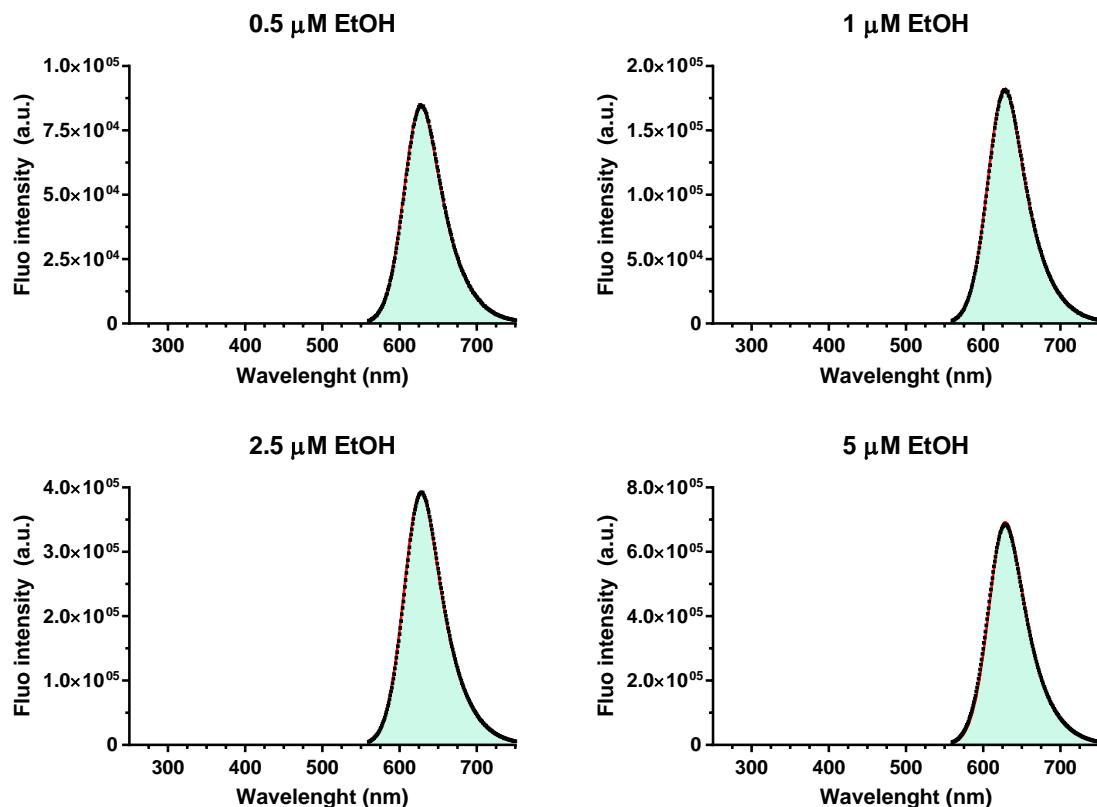
Conc (μM)	Component 1		R²
	Reference spectrum	Coefficient ± SE	
0.5	RBM5-143 0.5 µM EtOH Fluo@550 nm	0.5481 ± 6.80E-04	0.99963
1	RBM5-143 1 µM EtOH Fluo@550 nm	0.59619 ± 5.70E-04	0.99978
2.5	RBM5-143 2.5 µM EtOH Fluo@550 nm	0.5869 ± 7.60E-04	0.9996
5	RBM5-143 5 µM EtOH Fluo@550 nm	0.56779 ± 9.77E-04	0.99928

Figure S55. Deconvoluted emission spectra upon excitation at 550 nm of compound **RBM5-161** in EtOH at concentrations ranging from 0.5 µM to 2.5 µM. Legend: original data (■), fitted curve (–), acceptor contribution (–).

9. Calculated AEB spectra

In this section are displayed the acceptor emission bleed-through (AEB) spectra calculated using the previous coefficients as described in the Experimental Section 6.2.9.
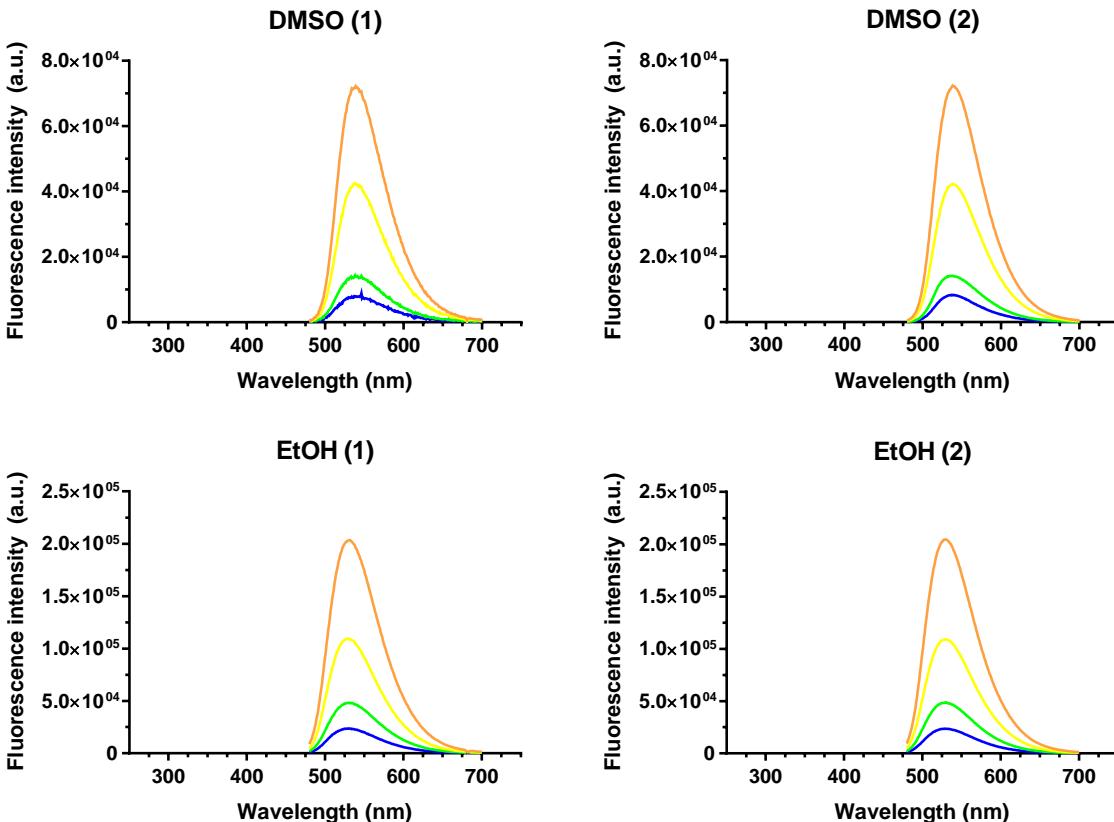
Figure S56. Calculated emission spectra of compound **RBM5-160** owing to the direct excitation of the acceptor component upon irradiation at 340 nm (calculated acceptor bleed-through) at 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow), 5 µM (orange) in DMSO (top) and EtOH (bottom). Left panels: spectra calculated from "RBM5-154_Ex@340nm"; right panels: spectra calculated from "RBM5-160_Ex@470nm".
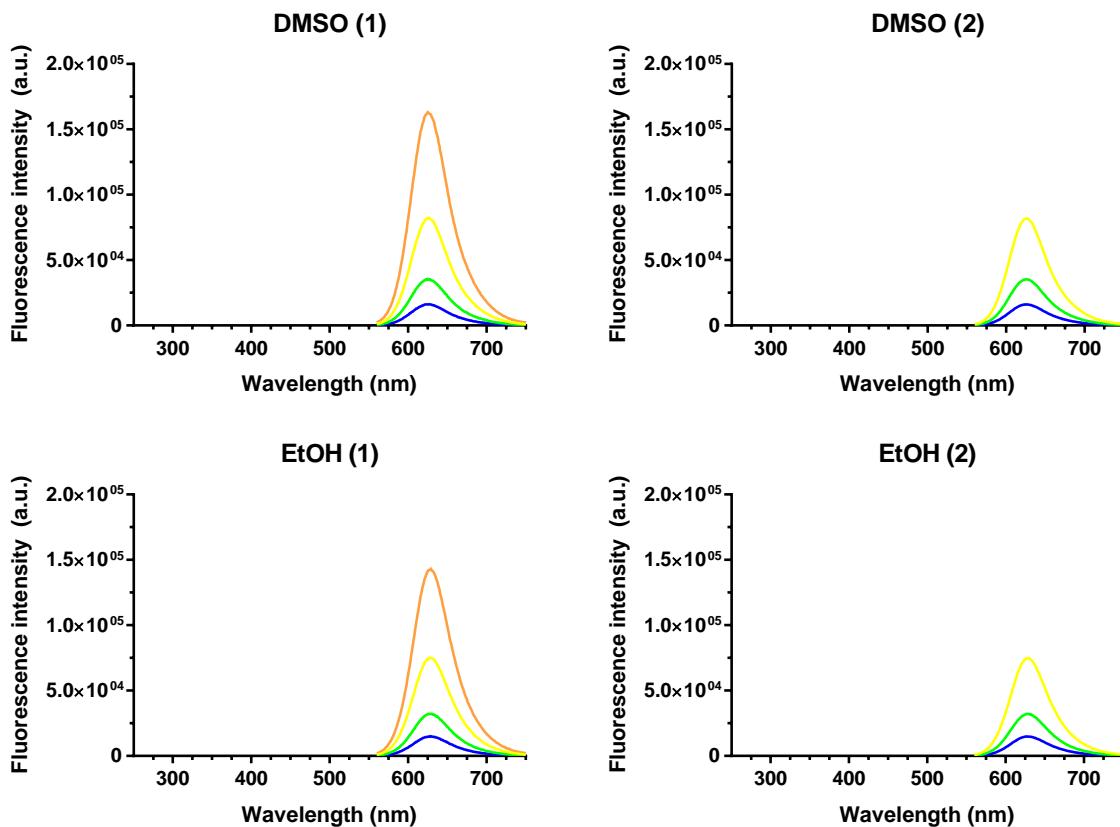
Figure S57. Calculated emission spectra of compound **RBM5-161** owing to the direct excitation of the acceptor component upon irradiation at 455 nm (calculated acceptor bleed-through) at 0.5 µM (blue), 1 µM (green), 2.5 µM (yellow) and 5 µM (orange) in DMSO (top) and EtOH (bottom). Left panels: spectra calculated from "RBM5-143_Ex@455nm"; right panels: spectra calculated from "RBM5-161_Ex@550nm".
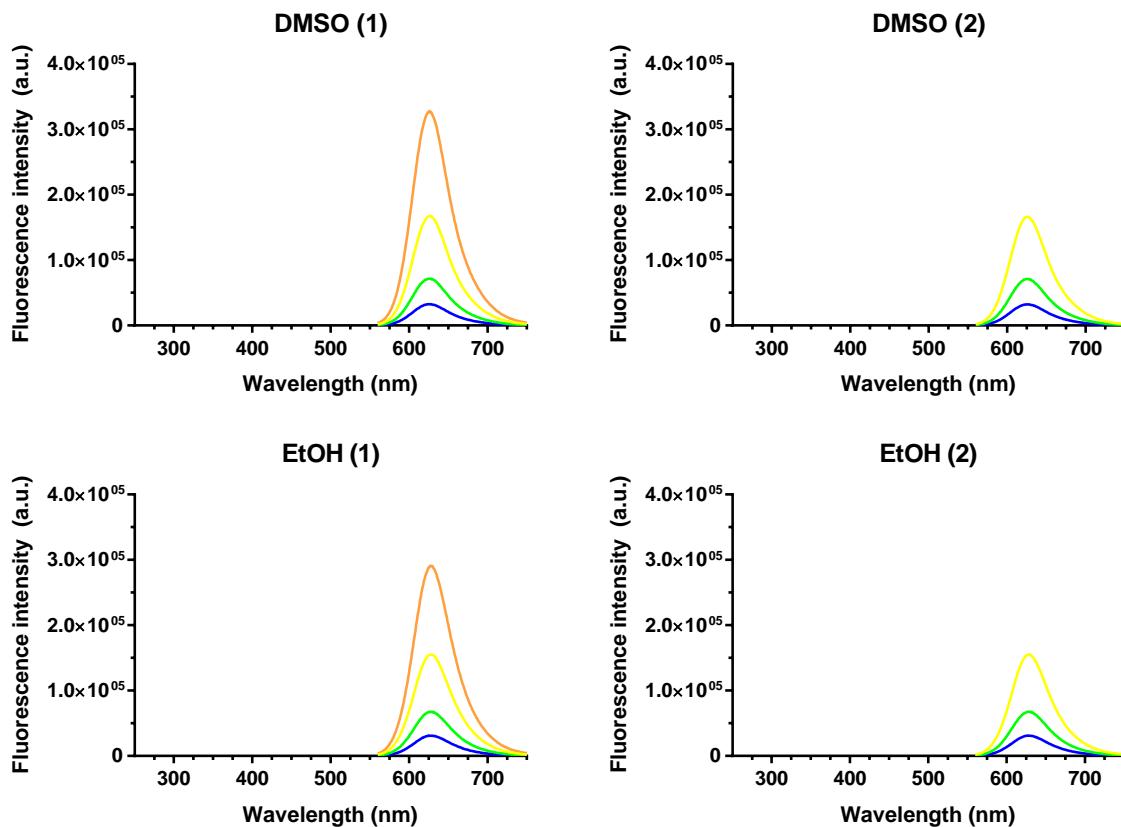
Figure S58. Calculated emission spectra of compound **RBM5-161** owing to the direct excitation of the acceptor component upon irradiation at 470 nm (calculated acceptor bleed-through) at 0.5 µM (blue), 1 µM (green) and 2.5 µM (yellow) in DMSO (top) and EtOH (bottom). Left panels: spectra calculated from "RBM5-143_Ex@470nm"; right panels: spectra calculated from "RBM5-161_Ex@550nm".

10.Linear plots to calculate the AEB

In this section are depicted the absorbance at the excitation wavelength (λ_{Ex}) *versus* integrated fluorescence intensity plots that were generated to calculate the bleed-through resulting from the direct excitation of the acceptor at the λ_{Ex} (AEB). The regressions were forced to intercept the origin.
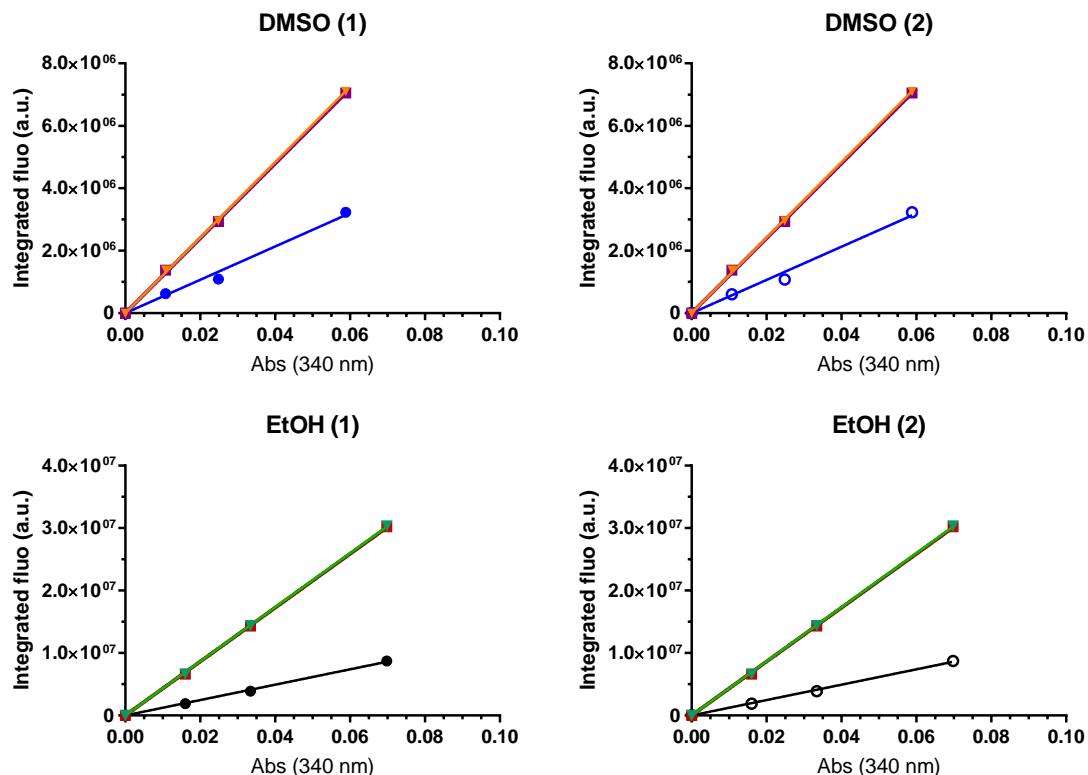

Compound	Solvent	Calc. method	Slope	SE	R^2	Legend
RBM5-160_Total	DMSO	–	1.21E+08	8.85E+05	0.9997	-▼-
	EtOH	–	4.33E+08	2.57E+06	0.9998	-▼-
RBM5-160_Acceptor component	DMSO	–	1.20E+08	8.81E+05	0.9997	-■-
	EtOH	–	4.31E+08	2.56E+06	0.9998	-■-
RBM5-160_Acceptor bleed-through	DMSO	1	5.34E+07	2.32E+06	0.9885	-●-
		2	5.32E+07	2.36E+06	0.9882	-○-
	EtOH	1	1.23E+08	1.94E+06	0.9983	-●-
		2	1.23E+08	2.13E+06	0.998	-○-

Figure S59. Linear plots of integrated fluorescence intensity (AUC$_{DMSO}$[490-665 nm] or AUC$_{EtOH}$[480-655 nm]) *vs* Abs (at λ_{Ex} = 340 nm) for **RBM5-160_Total** (emission both from the donor and the acceptor); **RBM5-160_Acceptor** (emission of the acceptor both resulting from FRET and direct excitation at 340 nm) and **RBM5-160_Acceptor bleed-through** (emission from the direct excitation of the acceptor) in DMSO and EtOH. Calculation methods: 1, from "RBM5-154_Ex@340"; 2, from "RBM5-160_Ex@470".
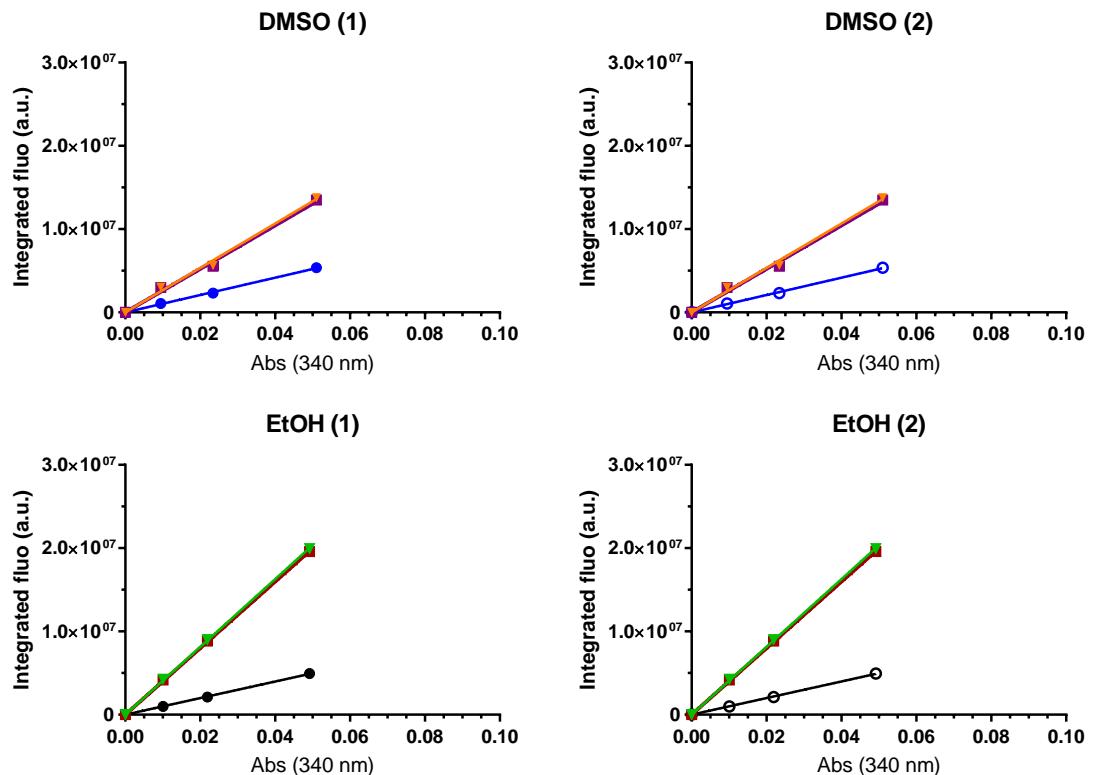
Compound	Solvent	Calc. method	Slope	SE	R^2	Legend
RBM5-161_Total	DMSO	–	2.67E+08	8.41E+06	0.9934	-▼-
	EtOH	–	4.07E+08	2.01E+06	0.9998	-▼-
RBM5-161_Acceptor component	DMSO	–	2.61E+08	8.11E+06	0.9936	-■-
	EtOH	–	3.99E+08	1.94E+06	0.9998	-■-
RBM5-161_Acceptor bleed-through	DMSO	1	1.04E+08	1.52E+06	0.9986	-●-
		2	1.04E+08	1.88E+06	0.9977	-○-
	EtOH	1	9.95E+07	8.33E+05	0.9995	-●-
		2	9.96E+07	8.55E+05	0.9995	-○-

Figure S60. Linear plots of integrated fluorescence intensity (AUC[570-730 nm]) *vs* Abs (at λ_{Ex} = 455 nm) for **RBM5-161_Total** (emission both from the donor and the acceptor); **RBM5-161_Acceptor** (emission of the acceptor resulting both from FRET and direct excitation at 455 nm) and **RBM5-161_Acceptor bleed-through** (emission from the direct excitation of the acceptor at 455 nm) in DMSO and EtOH. Calculation method 1: from "RBM5-143_Ex@455"; calculation method 2: from "RBM5-161_Ex@550".
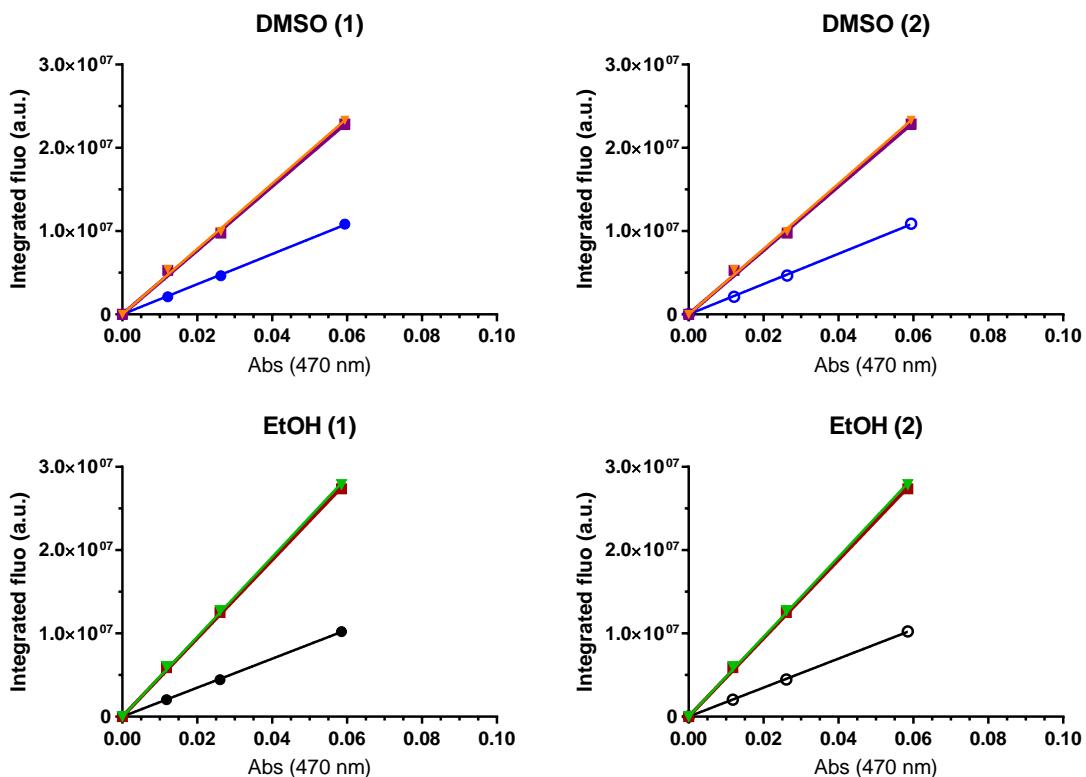
Compound	Solvent	Calc. method	Slope	SE	R^2	Legend
RBM5-161_Total	DMSO	–	3.92E+08	6.42E+06	0.9982	-▼-
	EtOH	–	4.79E+08	4.05E+06	0.9995	-▼-
RBM5-161_Acceptor component	DMSO	–	3.84E+08	6.09E+06	0.9983	-■-
	EtOH	–	4.71E+08	3.91E+06	0.9995	-■-
RBM5-161_Acceptor bleed-through	DMSO	1	1.81E+08	1.46E+06	0.9996	-●-
		2	1.82E+08	1.44E+06	0.9996	-○-
	EtOH	1	1.74E+08	8.54E+05	0.9998	-●-
		2	1.74E+08	8.87E+05	0.9998	-○-

Figure S61. Linear plots of integrated fluorescence intensity (AUC[570-730 nm]) *vs* Abs (at λ_{Ex} = 470 nm) for **RBM5-161_Total** (emission both from the donor and the acceptor); **RBM5-161_Acceptor** (emission of the acceptor resulting both from FRET and direct excitation at 470 nm) and **RBM5-161_Acceptor bleed-through** (emission from the direct excitation of the acceptor at 470 nm) in DMSO and EtOH. Calculation method 1: from "RBM5-143_Ex@470"; calculation method 2: from "RBM5-161_Ex@550".