Activated Phosphonated Trifunctional Chelates for Highly Sensitive Lanthanide-based FRET Immunoassays applied to Total Prostate Specific Antigen Detection

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Supplementary Information (8 pages including this one)

Figure S1. ^{1}H-NMR spectrum of **L*** (25°C, 200 MHz, D_2O)

Figure S2. UV-Vis absorption spectrum of L* labelled streptavidin in TRIS/HCl 0.01 M, pH 7.4 buffer.

Figure S3. Excitation spectrum (λ_{em} = 545 nm) of TbL* labelled streptavidine in TRIS/HCl 0.01 M, pH 7.4 buffer.

Figure S4. Time-resolved (delay = 10 µs) emission spectrum of the TbL* labelled streptavidine in TRIS/HCl 0.01 M, pH 7.4 buffer (λ_{exc} = 330 nm).

Figure S5. Tb based emission decay profile of the TbL* labelled streptavidine in TRIS/HCl 0.01 M, pH 7.4 buffer (λ_{exc} = 330 nm and λ_{em} = 545 nm).

Figure S6. MALDI-TOF mass spectra of AB1 (A) and AB1 labelled with L* (B). A shift of *ca* 2500 Da is observed for labelled antibody in the expanded region.

Figure S7. UV-Vis absorption spectra of **L3** (blue) and Tb**L3** (violet) and luminescence spectra of Tb**L3** (green, λ_{exc} = 328 nm) in TRIS/HCl 0.01M at pH 7.4

Scheme S1. Full ^{1}H-NMR assignment for **L3** and **L***.

PL decay time analysis

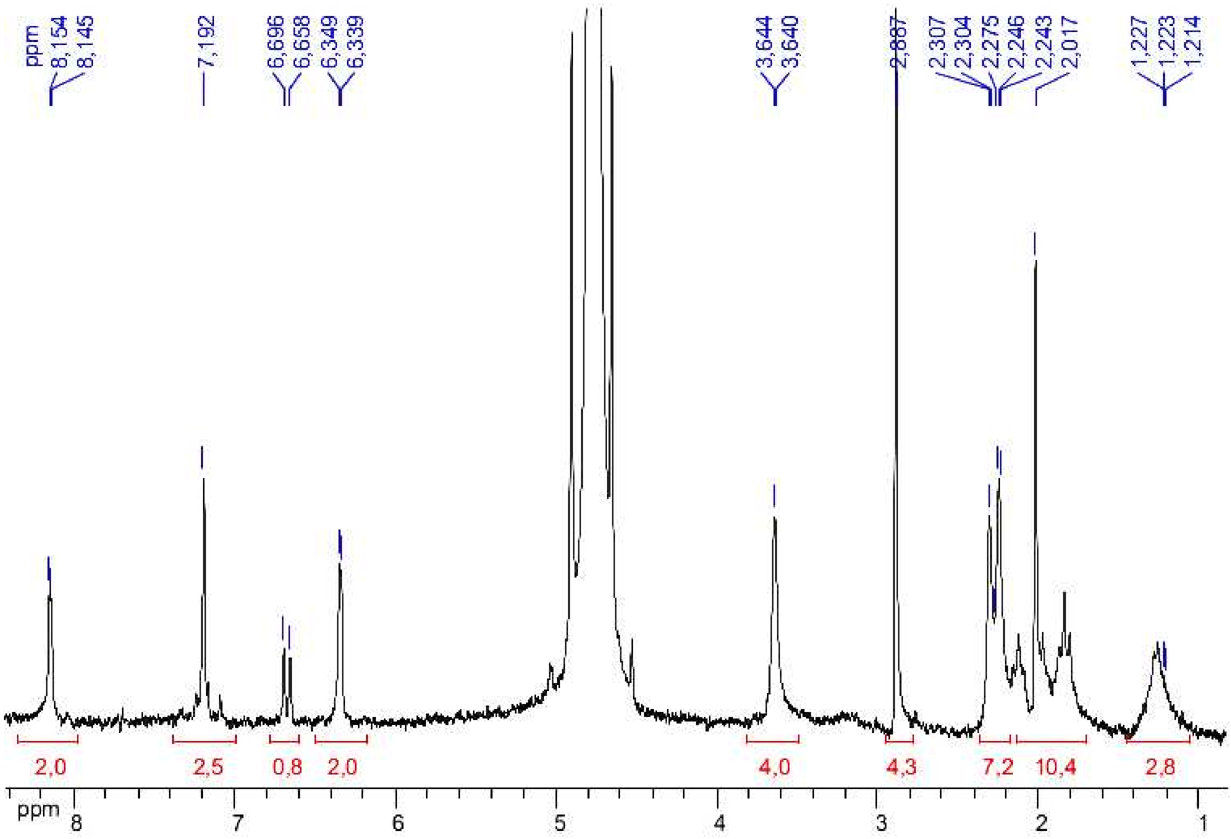

Figure S1. ^{1}H-NMR spectrum of **L*** (25°C, 200 MHz, D_2O)

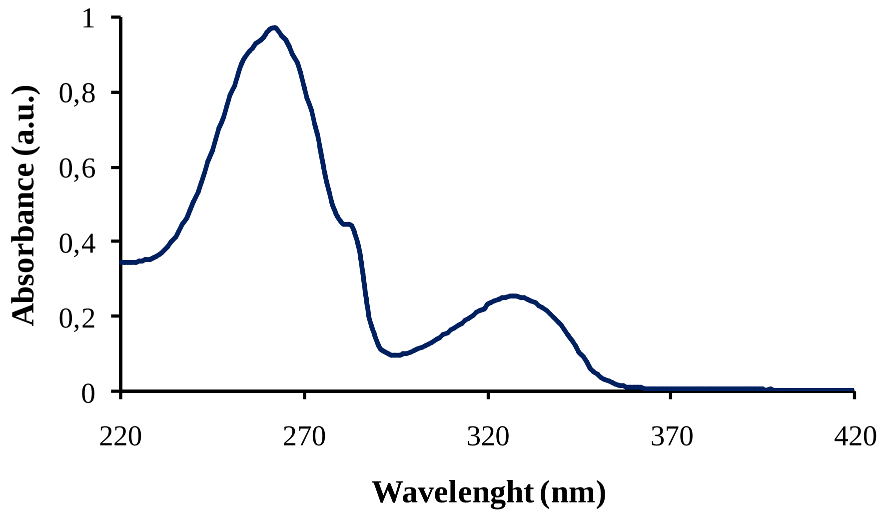

Figure S2. UV-Vis absorption spectrum of TbL* labelled streptavidin in TRIS/HCl 0.01 M, pH 7.4 buffer.

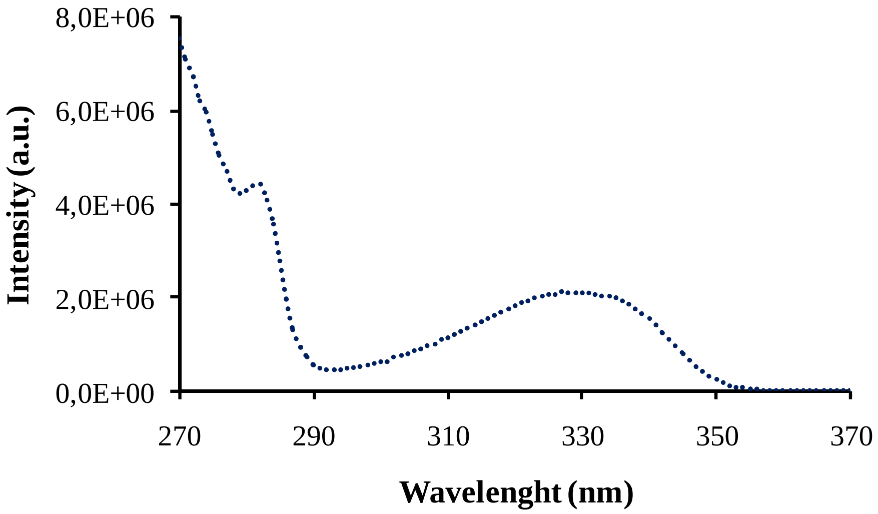

Figure S3. Excitation spectrum (λ_{em} = 545 nm) of TbL* labelled streptavidine in TRIS/HCl 0.01 M, pH 7.4 buffer.

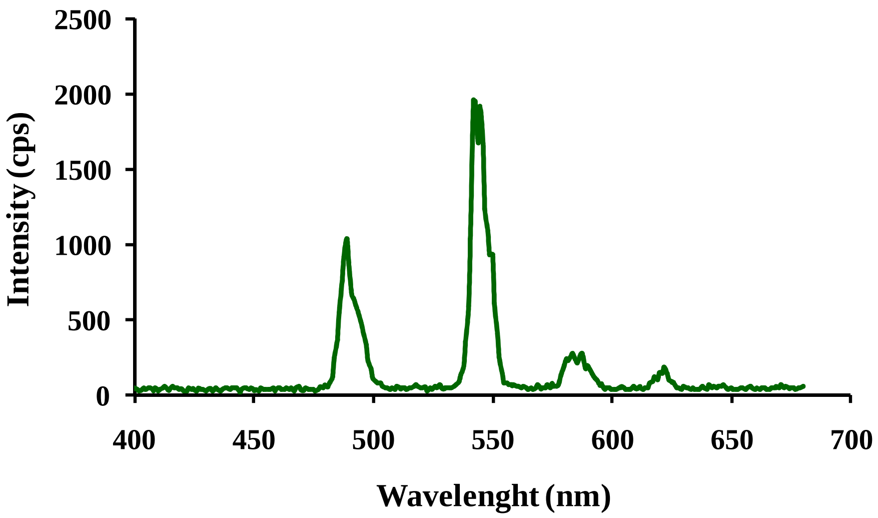

Figure S4. Time-resolved (delay = 10 µs) emission spectrum of the TbL* labeled streptavidine in TRIS/HCl 0.01 M, pH 7.4 buffer.

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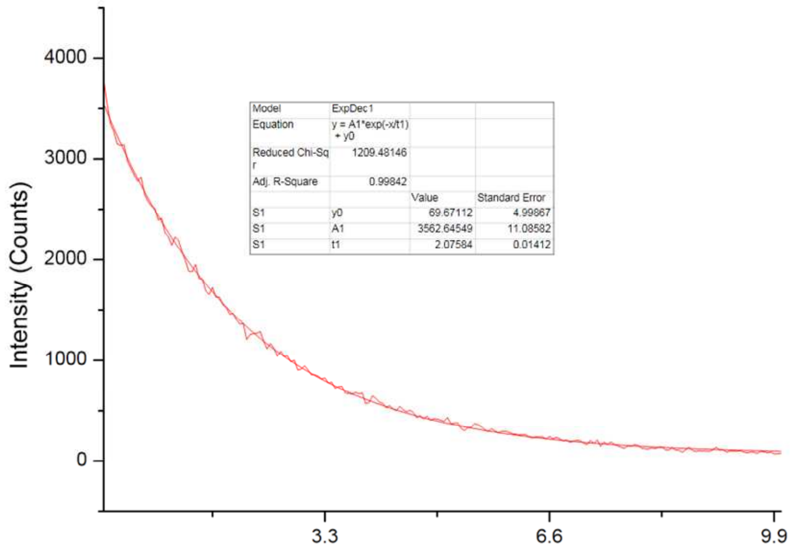
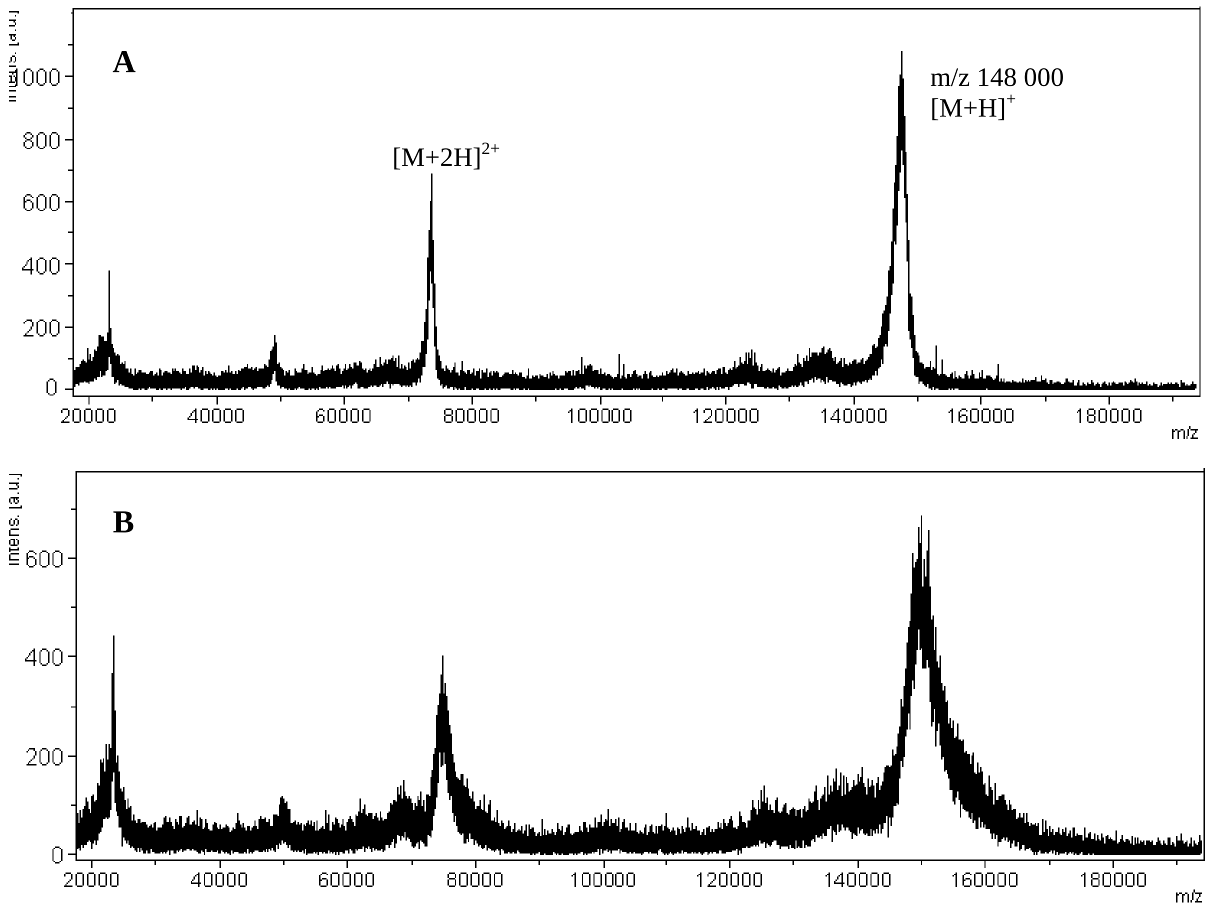


Figure S5. Tb based emission decay profile of the TbL* labelled streptavidine in TRIS/HCl 0.01 M, pH 7.4 buffer (λ_{exc} = 330 nm and λ_{em} = 545 nm).



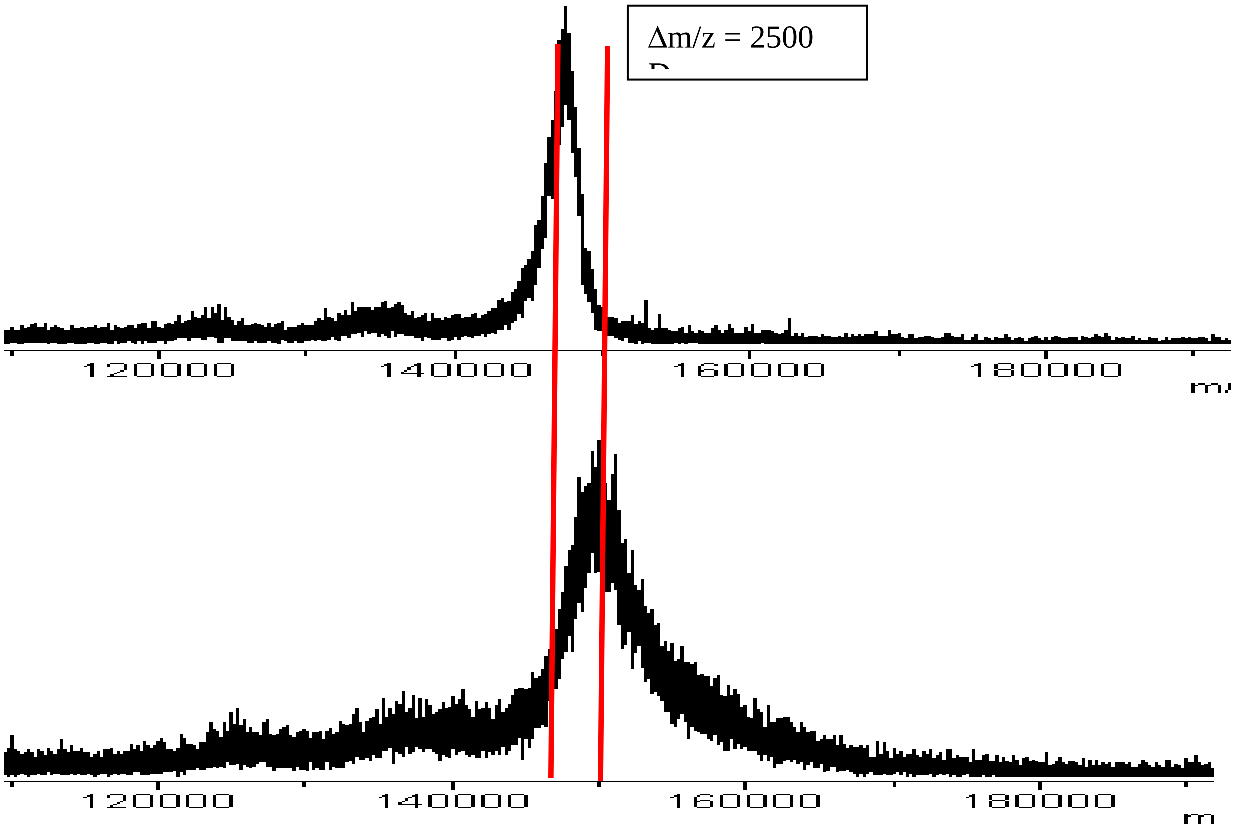


Figure S6. MALDI-TOF mass spectra of AB1 (A) and AB1 labelled with L* (B). A shift of *ca* 2500 Da is observed for labelled antibody in the expanded region.

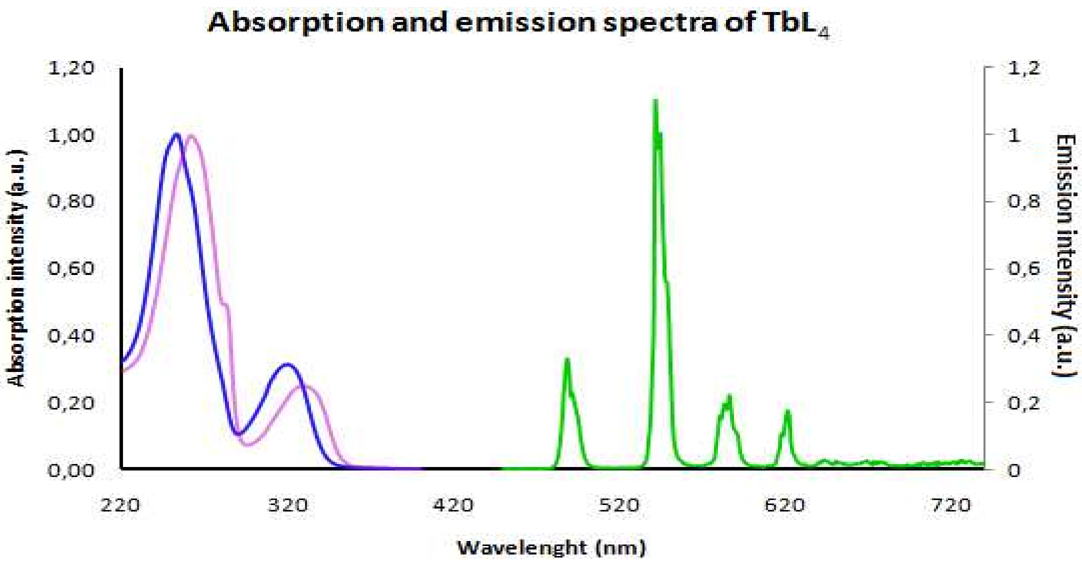
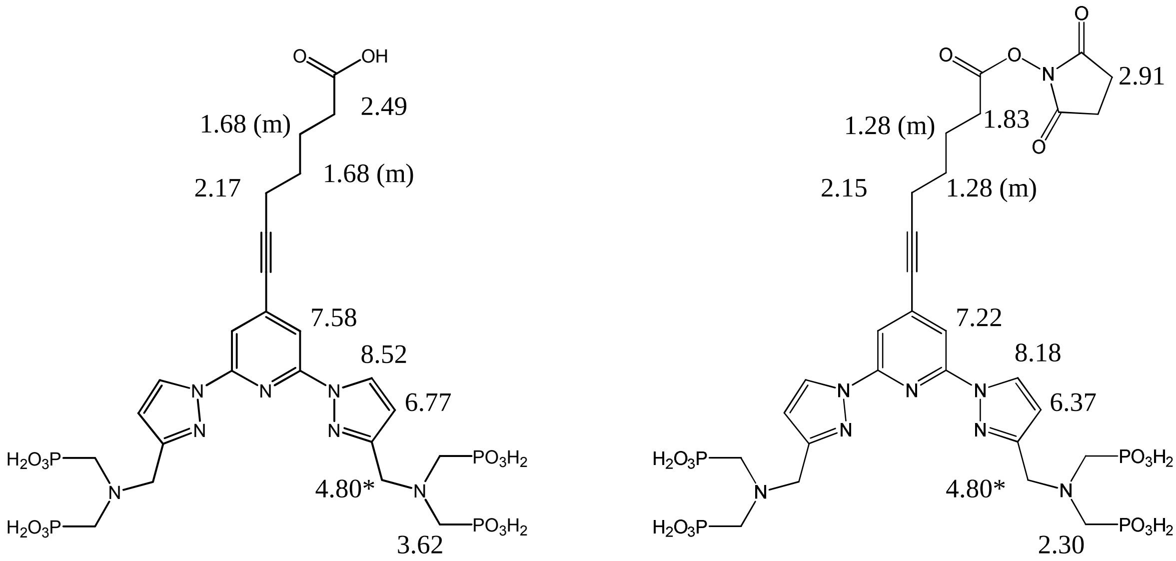


Figure S7. UV-Vis absorption spectra of **L3** (blue) and Tb**L3** (violet) and luminescence spectra of Tb**L3** (green, λ_{exc} = 328 nm) in TRIS/HCl 0.01M at pH 7.4



Scheme S1. Full [1]H-NMR assignment for **L3** and **L***. The asterisks denote the fact that the signals can be hidden by the peak of the water, m = multiplet.

PL decay time analysis. The decay time analysis in the donor and acceptor channel was performed as described in reference 41 (from the manuscript). All decay curves were fit using a multi-exponential PL intensity decay function:

$$I = \Sigma A_i \exp(-t/\tau_i) = A \Sigma \alpha_i \exp(-t/\tau_i) \qquad (1)$$

where A is the total amplitude and α_i are the amplitude fractions ($\Sigma\alpha_i = 1$). All PL lifetime averaging for the dynamic FRET quenching process was performed using amplitude weighted average lifetimes:

$$<\tau> = \Sigma\alpha_i\tau_i \qquad (2)$$

First the decay curve of the pure Tb donor was fit using a double-exponential decay function, which led to the amplitude fractions α_{D1} and α_{D2}, the PL decay times τ_{D1} and τ_{D2} (with $\tau_{D2} > \tau_{D1}$) and the average PL decay time of the pure donor (in the absence of the acceptor) $<\tau_D>$. The FRET-quenched decay curves in the donor detection channel were fit using a triple-exponential decay function, leading to the amplitude fractions α_{DA*1}, α_{DA*2} and α_{DA*3} and the PL decay times τ_{DA1}, τ_{DA2} and τ_{DA3}, for which the third decay time component was fixed to $\tau_{DA3} = \tau_{D2}$ in order to take into account the emission of unquenched donors. For the calculation of the average donor decay time in the presence of the acceptor $<\tau_{DA}>$ only the first two amplitudes and decay times were used (as the third component represents unquenched donors). Therefore the amplitude fractions must be redefined for these two decay times τ_{DA1} and τ_{DA2}:

$$\alpha_{DA1} = \frac{\alpha_{DA*1}}{\alpha_{DA*1} + \alpha_{DA*2}} \quad \text{and} \quad \alpha_{DA2} = \frac{\alpha_{DA*2}}{\alpha_{DA*1} + \alpha_{DA*2}} \qquad (3)$$

As the unquenched donor possesses two decay time components (τ_{D1} and τ_{D2}), $<\tau_{DA}>$ must be corrected for the shorter time component (τ_{D1}) by a factor z_D (the fraction of unquenched donors in the short time components), which is determined by comparing the amplitude fractions of τ_{D2} and τ_{DA3} ($\tau_{DA3} = \tau_{D2}$) multiplied by the amplitude fraction α_{D1}:

$$z_D = \alpha_{D1}(\alpha_{DA*3}/\ \alpha_{D2}) \qquad (4)$$

The average FRET-quenched decay time is then:

$$\langle \tau_{DA} \rangle = \frac{\alpha_{DA1}\tau_{DA1} + \alpha_{DA2}\tau_{DA2} - z_D\tau_{D1}}{1 - z_D} \qquad (5)$$

and the average FRET-efficiency is:

$$\langle \eta_{FRET} \rangle = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle} \qquad (6)$$

The FRET-sensitized decay curves in the acceptor detection channel were fit using a quadruple-exponential decay function, leading to the amplitude fractions α_{AD*0}, α_{AD*1}, α_{AD*2} and α_{AD*3} and the PL decay times τ_{AD0}, τ_{AD1}, τ_{AD2} and τ_{AD3}, for which the fourth decay time component was fixed to $\tau_{AD3} = \tau_{D2}$ in order to take into account the emission of unquenched donors, which is much less intense compared to the donor channel but still present due to spectral crosstalk of the Tb emission in the dye acceptor detection channel. The correction factor z_A (the fraction of unquenched donors in the short time components) is almost negligible but is still taken into account for a correct treatment:

$$z_A = \alpha_{D1}(\alpha_{AD*3}/\ \alpha_{D2}) \qquad (7)$$

In order to calculate the average FRET decay time $<\tau_{AD}>$ only the amplitudes and lifetimes with $i = 0$ to 2 are taken into account ($i = 3$ represents the unquenched donor emission). Moreover, the amplitudes α_{AD*i} must be corrected by the FRET rates $k_i = 1/\tau_{ADi} - 1/<\tau_D>$, to take into account the dependence of the excitation of the acceptors (and therefore the amplitude fractions) on the different FRET efficiencies for the different distances (corresponding to the decay times τ_{ADi}). The corrected amplitude fractions are (for $i = 0$ to 2):

$$\alpha_{\mathrm{ADi}} = \frac{\alpha_{\mathrm{Ai}} / k_{\mathrm{i}}}{\sum \alpha_{\mathrm{Ai}} / k_{\mathrm{i}}} \tag{8}$$

The average FRET decay time is then calculated by:

$$\langle \tau_{\mathrm{AD}} \rangle = \frac{\alpha_{\mathrm{AD1}}\tau_{\mathrm{AD1}} + \alpha_{\mathrm{AD2}}\tau_{\mathrm{AD2}} + \alpha_{\mathrm{AD3}}\tau_{\mathrm{AD3}} - z_{\mathrm{A}}\tau_{\mathrm{D1}}}{1 - z_{\mathrm{A}}} \tag{9}$$

and the average FRET-efficiency is:

$$\langle \eta_{\mathrm{FRET}} \rangle = 1 - \frac{\langle \tau_{\mathrm{AD}} \rangle}{\langle \tau_{\mathrm{D}} \rangle} \tag{10}$$

For each FRET decay time the donor-acceptor distance r_x can be calculated by:

$$r_{\mathrm{x}} = \left(\frac{\tau_{\mathrm{x}} R_0^6}{\tau_{\mathrm{D}} - \tau_{\mathrm{x}}} \right)^{1/6} \tag{11}$$

where τ_x represents the different lifetimes τ_{DAi}, τ_{ADi}, $<\tau_{DA}>$ or $<\tau_{AD}>$. The fractions of FRET-pairs found at the different distances corresponding to τ_{DAi} and τ_{ADi} are given by the amplitude fractions of these decay times.

Complete decay time fit data and resulting FRET data an distances

Decay time fit results and calculated FRET parameters for AF647 as acceptor

Donor Channel R_0(nm) 5.3

Channel 25-3999 fixed decay background

D or DA	TPSA [nM]	τ_1	A_1	α_{DA*1}	α_{DA1}	τ_2	A_2	α_{DA*2}	α_{DA2}	$\langle\tau_{DA}\rangle$	τ_3 (fixed)	A_3	α_{DA*3}	$\langle\tau_D\rangle$	z(D)	$\langle\tau_{DA}\rangle$ corr	η_{FRET}	averaging
D	0.00					568	268	0.09			2955	2679	0.91	2738				
DA	2.00	289	155	0.06	0.37	541	261	0.10	0.63	447	2950	2302	0.85	2567	0.08	436	0.84	
DA	4.00	263	336	0.13	0.44	924	421	0.16	0.56	631	2950	1929	0.72	2296	0.07	635	0.77	
DA	6.00	278	500	0.19	0.47	1107	566	0.22	0.53	718	2950	1516	0.59	2029	0.06	728	0.73	
DA	8.00	251	648	0.25	0.47	1110	726	0.28	0.53	704	2950	1232	0.47	1766	0.05	711	0.74	
DA	10.00	263	738	0.29	0.48	1165	797	0.31	0.52	731	2950	1004	0.40	1608	0.04	738	0.73	
DA	12.00	253	688	0.28	0.48	1115	736	0.30	0.52	698	2950	1054	0.43	1656	0.04	704	0.74	
DA	14.00	265	663	0.26	0.48	1096	705	0.28	0.52	693	2950	1142	0.46	1720	0.05	699	0.74	
DA	16.00	255	657	0.26	0.47	1097	733	0.29	0.53	699	2950	1164	0.46	1725	0.05	705	0.74	
DA	18.00	238	631	0.25	0.47	1056	709	0.28	0.53	671	2950	1220	0.48	1757	0.05	676	0.75	
DA	20.00	236	552	0.22	0.45	1003	680	0.27	0.55	660	2950	1299	0.51	1835	0.05	665	0.76	
		259	557	0.22	0.46	1021	633	0.25	0.54	665	2950	1386	0.53		0.05	670	0.76	
		255	635	0.25	0.47	1094	707	0.28	0.53	697	2950	1204	0.47	1762	0.05	703	0.74	
distance *r*		**3.6**				**5.0**										**4.4**		

intensity information		
% $\tau_1*\alpha_1$	% $\tau_2*\alpha_2$	% $\tau_3*\alpha_3$
0.000	0.019	0.98
0.006	0.020	0.97
0.014	0.063	0.92
0.027	0.120	0.85
0.035	0.175	0.79
0.048	0.227	0.73
0.042	0.200	0.76
0.041	0.179	0.78
0.038	0.183	0.78
0.033	0.166	0.80
0.028	0.147	0.82
0.0	0.148	0.82
0.0	0.162	0.79

Acceptor Channel

Channel 25-2500 fixed decay background

τ_{DA}	TPSA [nM]	τ_0	A_0	α_{AD*0}	k_{FRET0}	α_{AD0}	τ_1	A_1	α_{AD*1}	k_{FRET1}	α_{AD1}	τ_2	A_2	α_{AD*2}	k_{FRET2}	α_{AD2}	$\langle\tau_{DA}\rangle$	τ_3 (fixed)	A_3	α_{AD*3}	z(A)	$\langle\tau_{DA}\rangle$ corr	η_{FRET}	averaging
2738	2.00	49	267	0.36	0.0202	0.058	232	274	0.37	0.00394	0.30	858	118	0.16	0.00080	0.64	622	2950	75	0.103	0.0103	623	0.77	
2738	4.00	57	574	0.39	0.0172	0.070	234	590	0.40	0.00392	0.32	858	233	0.16	0.00080	0.61	603	2950	74	0.050	0.0050	604	0.78	
2738	6.00	54	867	0.37	0.0180	0.066	223	1015	0.44	0.00411	0.34	833	359	0.16	0.00083	0.59	574	2950	73	0.032	0.0032	574	0.79	
2738	8.00	66	1264	0.43	0.0148	0.091	253	1204	0.41	0.00358	0.36	869	403	0.14	0.00079	0.55	575	2950	67	0.023	0.0023	575	0.79	
2738	10.00	60	1346	0.40	0.0162	0.078	244	1495	0.44	0.00374	0.38	858	466	0.14	0.00080	0.55	565	2950	64	0.019	0.0019	565	0.79	
2738	12.00	59	1229	0.40	0.0167	0.077	236	1327	0.43	0.00387	0.36	846	443	0.14	0.00082	0.57	568	2950	61	0.020	0.0020	568	0.79	
2738	14.00	67	1275	0.43	0.0146	0.093	259	1201	0.41	0.00349	0.37	873	394	0.13	0.00078	0.54	572	2950	62	0.021	0.0021	572	0.79	
2738	16.00	58	1175	0.39	0.0168	0.072	236	1341	0.44	0.00387	0.36	851	445	0.15	0.00081	0.57	573	2950	65	0.021	0.0021	573	0.79	
2738	18.00	65	1179	0.42	0.0150	0.086	260	1173	0.42	0.00349	0.37	904	370	0.13	0.00074	0.55	595	2950	61	0.022	0.0022	595	0.78	
2738	20.00	61	1020	0.39	0.0161	0.076	238	1128	0.43	0.00384	0.35	837	395	0.15	0.00083	0.57	566	2950	66	0.025	0.0025	566	0.79	
		60	1020	0.40	0.0166	0.077	242	1075	0.42	0.00378	0.35	859	363	0.15	0.00080	0.57	581	2950	67	0.034	0.0034	581	0.79	
		61	1170	0.41	0.02	0.08	244	1235	0.43	0.00	0.36	859	409	0.14	0.00	0.56	573	2950	65	0.02	0.00	573	0.79	
distance *r*		**2.8**					**3.6**					**4.7**										**4.2**		

intensity information			
% $\tau_1*\alpha_1$	% $\tau_2*\alpha_2$	% $\tau_3*\alpha_3$	% $\tau_4*\alpha_4$
0.033	0.16	0.25	0.56
0.056	0.23	0.34	0.37
0.060	0.29	0.38	0.27
0.089	0.33	0.37	0.21
0.079	0.35	0.39	0.18
0.077	0.33	0.40	0.19
0.092	0.34	0.37	0.20
0.072	0.33	0.40	0.20
0.085	0.34	0.37	0.20
0.072	0.31	0.39	0.23
0.071	0.30	0.37	0.26
0.076	0.32	0.38	0.23

Decay time fit results and calculated FRET parameters for XL665 as acceptor

Donor Channel R_0(nm) 7.1

Channel 25-3999 fixed decay background

D or DA	TPSA [nM]	τ_1	A_1	α_{DA*1}	α_{DA1}	τ_2	A_2	α_{DA*2}	α_{DA2}	$\langle\tau_{DA}\rangle$	τ_3 (fixed)	A_3	α_{DA*3}	$\langle\tau_D\rangle$	z(D)	$\langle\tau_{DA}\rangle$ corr	η_{FRET}	averaging
D	0.00					197	285	0.19			3164	1251	0.81	2614				
DA	2.00	134	308	0.20	0.62	921	188	0.12	0.38	433	3160	1079	0.69	2301	0.16	476	0.82	
DA	4.00	149	353	0.22	0.53	1166	315	0.20	0.47	628	3160	937	0.58	2106	0.13	694	0.73	
DA	6.00	174	363	0.23	0.46	1343	432	0.28	0.54	810	3160	758	0.49	1957	0.11	886	0.66	
DA	8.00	193	371	0.24	0.40	1424	546	0.36	0.60	926	3160	597	0.39	1807	0.09	998	0.62	
DA	10.00	197	431	0.28	0.40	1476	643	0.43	0.60	963	3160	439	0.29	1600	0.07	1017	0.61	
DA	12.00	197	426	0.28	0.38	1499	698	0.46	0.62	1006	3160	394	0.26	1565	0.06	1056	0.60	
DA	14.00	195	414	0.29	0.38	1506	678	0.47	0.62	1010	3160	357	0.25	1539	0.06	1058	0.60	
DA	16.00	198	412	0.28	0.38	1483	678	0.47	0.62	997	3160	361	0.25	1535	0.06	1045	0.60	
DA	18.00	202	423	0.28	0.38	1486	695	0.46	0.62	1001	3160	380	0.25	1548	0.06	1050	0.60	
DA	20.00	188	429	0.29	0.39	1477	662	0.45	0.61	971	3160	375	0.26	1530	0.06	1018	0.61	
		183	393	0.26	0.43	1378	554	0.37	0.57	874	3160	568	0.37		0.08	930	0.64	
		196	422	0.28	0.38	1488	676	0.46	0.62	991	3160	384	0.26	1553	0.06	1041	0.60	
distance *r*		**4.7**				**7.4**										**6.6**		

intensity information		
% $\tau_1*\alpha_1$	% $\tau_2*\alpha_2$	% $\tau_3*\alpha_3$
0.000	0.014	0.99
0.011	0.048	0.94
0.016	0.109	0.88
0.021	0.191	0.79
0.026	0.284	0.69
0.035	0.392	0.57
0.035	0.441	0.52
0.036	0.458	0.51
0.037	0.451	0.51
0.037	0.445	0.52
0.036	0.436	0.53
0.0	0.326	0.65
0.04	0.44	0.53

Acceptor Channel

Channel 25-2000 fixed decay background

τ_{DA}	TPSA [nM]	τ_0	A_0	α_{AD*0}	k_{FRET0}	α_{AD0}	τ_1	A_1	α_{AD*1}	k_{FRET1}	α_{AD1}	τ_2	A_2	α_{AD*2}	k_{FRET2}	α_{AD2}	$\langle\tau_{DA}\rangle$	τ_3 (fixed)	A_3	α_{AD*3}	z(A)	$\langle\tau_{DA}\rangle$ corr	η_{FRET}	averaging
2614	2.00	17	210	0.39	0.0571	0.026	159	178	0.33	0.00591	0.21	746	105	0.19	0.00096	0.76	604	3160	46	0.086	0.0195	612	0.77	
2614	4.00	29	241	0.33	0.0344	0.025	200	256	0.36	0.00462	0.20	857	170	0.24	0.00078	0.78	706	3160	54	0.074	0.0169	715	0.73	
2614	6.00	39	282	0.34	0.0252	0.028	247	300	0.36	0.00367	0.20	959	206	0.24	0.00066	0.77	790	3160	54	0.064	0.0145	798	0.69	
2614	8.00	35	283	0.29	0.0283	0.020	226	372	0.38	0.00405	0.19	944	264	0.27	0.00068	0.79	791	3160	60	0.061	0.0139	800	0.69	
2614	10.00	35	318	0.30	0.0279	0.020	241	395	0.37	0.00377	0.18	988	289	0.27	0.00063	0.80	833	3160	60	0.056	0.0128	841	0.68	
2614	12.00	46	300	0.28	0.0215	0.024	230	389	0.36	0.00396	0.17	945	320	0.30	0.00068	0.81	803	3160	66	0.061	0.0139	812	0.69	
2614	14.00	43	311	0.30	0.0227	0.024	252	372	0.36	0.00358	0.18	991	280	0.27	0.00063	0.79	832	3160	59	0.058	0.0132	840	0.68	
2614	16.00	40	284	0.28	0.0243	0.021	238	378	0.37	0.00381	0.18	965	297	0.29	0.00065	0.80	818	3160	61	0.060	0.0136	827	0.68	
2614	18.00	37	263	0.26	0.0270	0.017	230	404	0.39	0.00397	0.18	975	300	0.29	0.00064	0.81	828	3160	60	0.059	0.0134	836	0.68	
2614	20.00	39	304	0.30	0.0251	0.021	250	382	0.38	0.00362	0.18	1034	272	0.27	0.00058	0.80	872	3160	54	0.053	0.0121	880	0.66	
		36	280	0.31	0.0293	0.023	227	343	0.37	0.00410	0.19	940	250	0.26	0.00069	0.79	788	3160	57	0.063	0.0144	796	0.70	
		39	295	0.29	0.03	0.02	238	385	0.37	0.00	0.18	977	289	0.28	0.00	0.80	825	3160	60	0.06	0.01	834	0.68	
distance *r*		**3.5**					**4.8**					**6.5**										**6.3**		

intensity information			
% $\tau_1*\alpha_1$	% $\tau_2*\alpha_2$	% $\tau_3*\alpha_3$	% $\tau_4*\alpha_4$
0.014	0.11	0.31	0.57
0.019	0.14	0.39	0.45
0.024	0.16	0.44	0.38
0.019	0.16	0.47	0.35
0.019	0.16	0.49	0.33
0.022	0.15	0.49	0.34
0.024	0.16	0.49	0.33
0.020	0.16	0.49	0.33
0.016	0.16	0.50	0.33
0.021	0.17	0.50	0.31
0.020	0.15	0.46	0.37
0.02	0.16	0.49	0.33