SUPPLEMENTARY INFORMATION

Fluorogenic aptamers resolve the flexibility of RNA junctions using orientation-dependent FRET

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Key words: RNA, Fluorescent aptamer, FRET, riboswitch

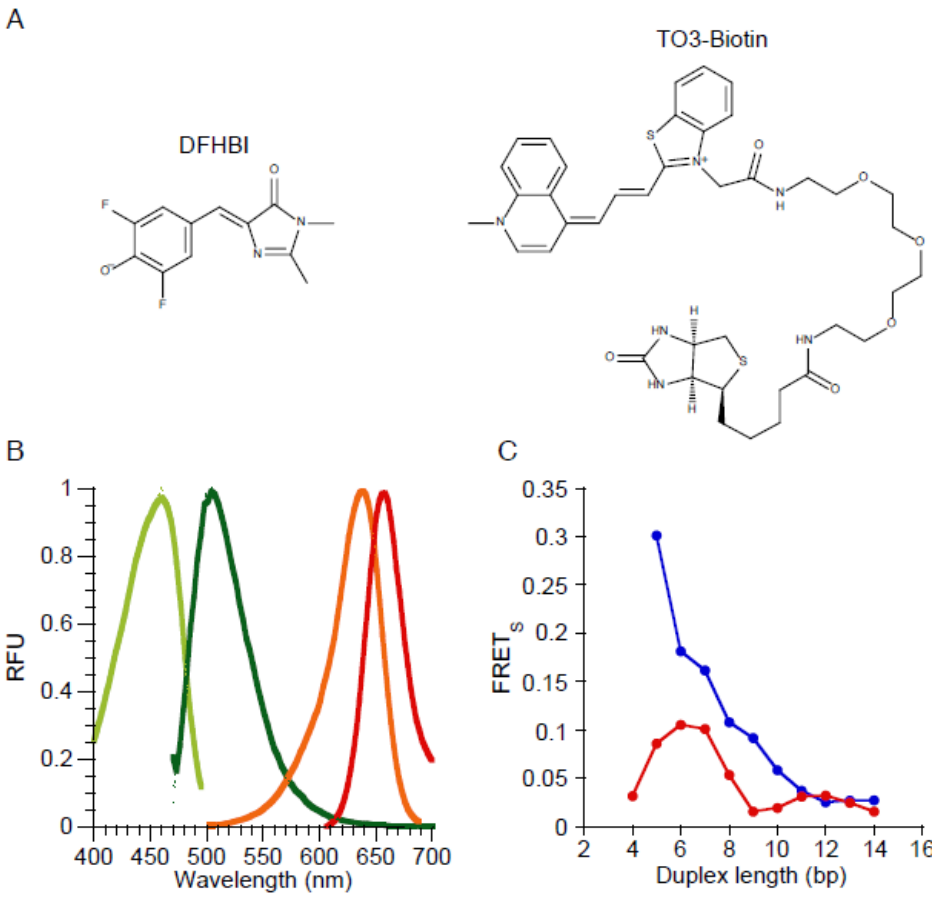


Fig. S1. Characterization of helical apta-FRET systems (A) Chemical structures of DFHBI and TO3-Biotin. **(B)** Excitation (light) and emission (dark) spectra of Broccoli/DFHBI (green) and Mango-III/TO3-biotin (red). The spectral overlap of these fluorophores are the area formed below the intersection between the DFHBI emission spectrum and the TO3-biotin excitation spectrum. **(C)** Comparison of duplex-length dependent apta-FRET from Mango-I (blue) and Mango-III (red). FRET as a function of duplex length for Broc-nbp-M1 (blue) and Broc-nbp-M3 constructs (red), both in the presence of TO3-Biotin & DFHBI.

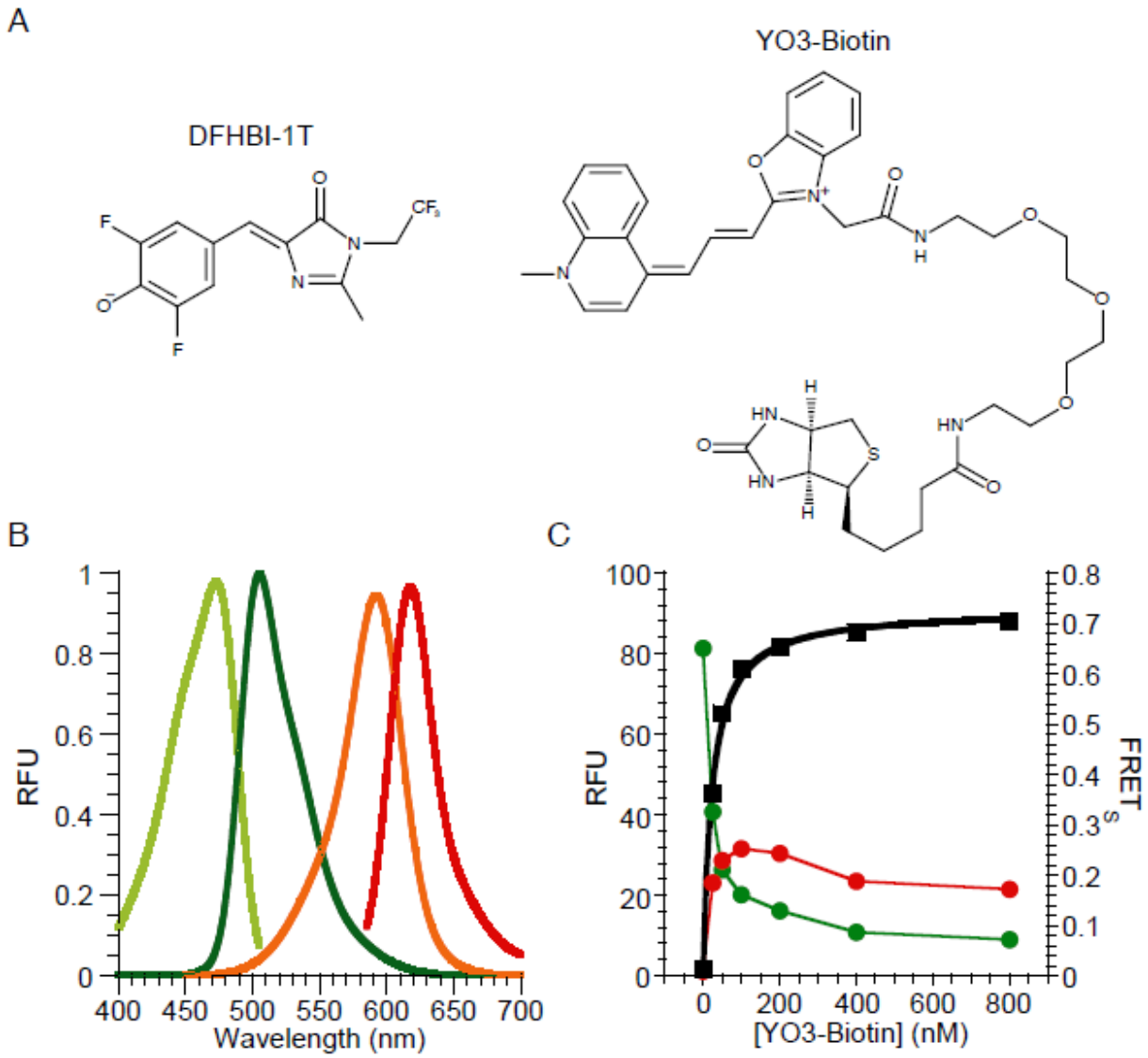
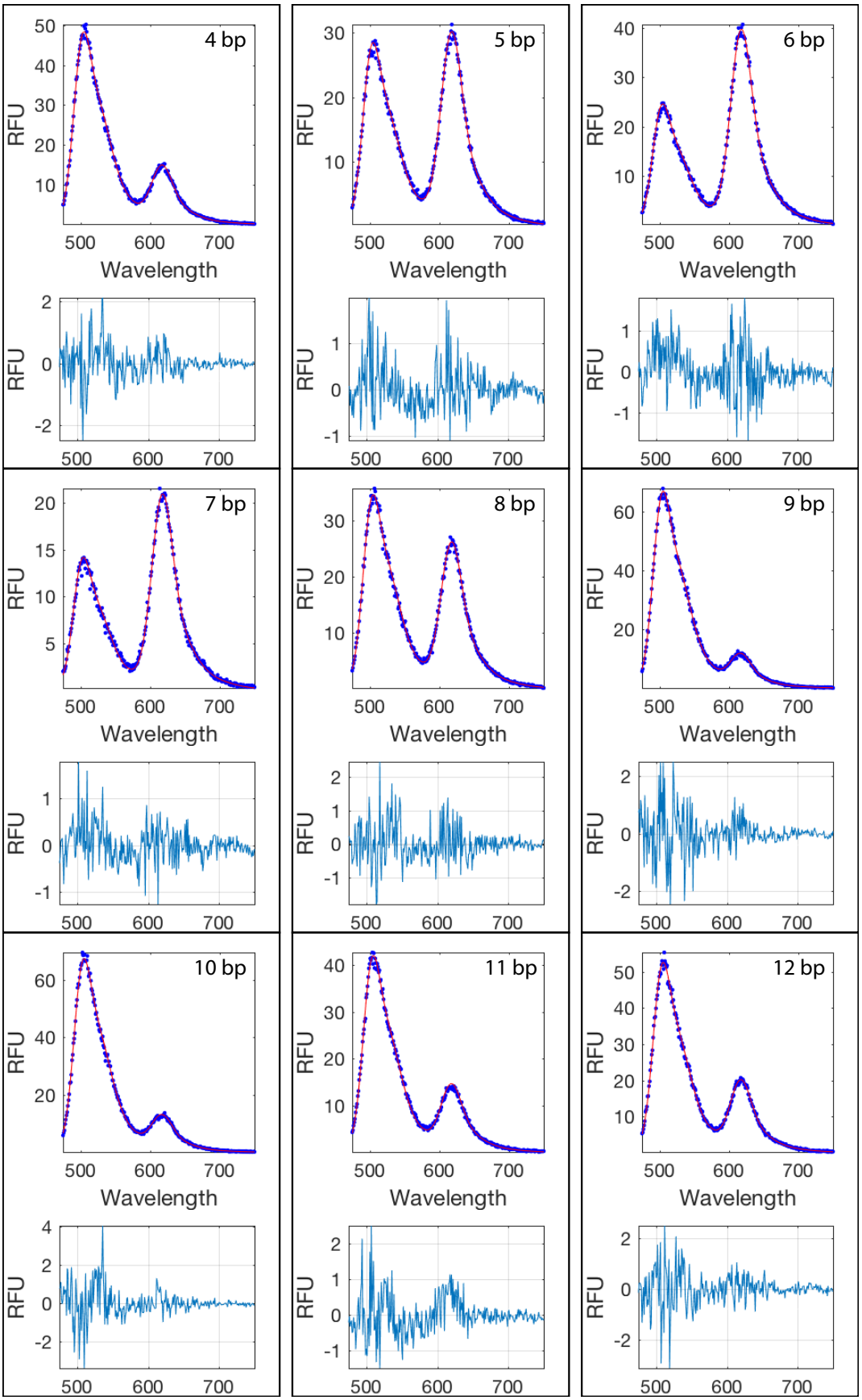


Fig. S2. apta-FRET compatibility of Broccoli/DFHBI-1T and Mango III/YO3-Biotin. (**A)** Chemical structures of DFHBI-1T and YO3-Biotin. (**B)** Excitation (light) and emission (dark) spectra of Broccoli/DFHBI (green) and Mango-III/YO3-biotin (red). The spectral overlap of these fluorophores are the area formed below the intersection between the DFHBI emission spectrum and the YO3-biotin excitation spectrum. (**C)** Titration of YO3-Biotin with DFHBI-1T and Broc-6bp-M3 RNA held constant. Fluorescence emission of DFHBI-1T (green), YO3-Biotin (red), and FRET (black).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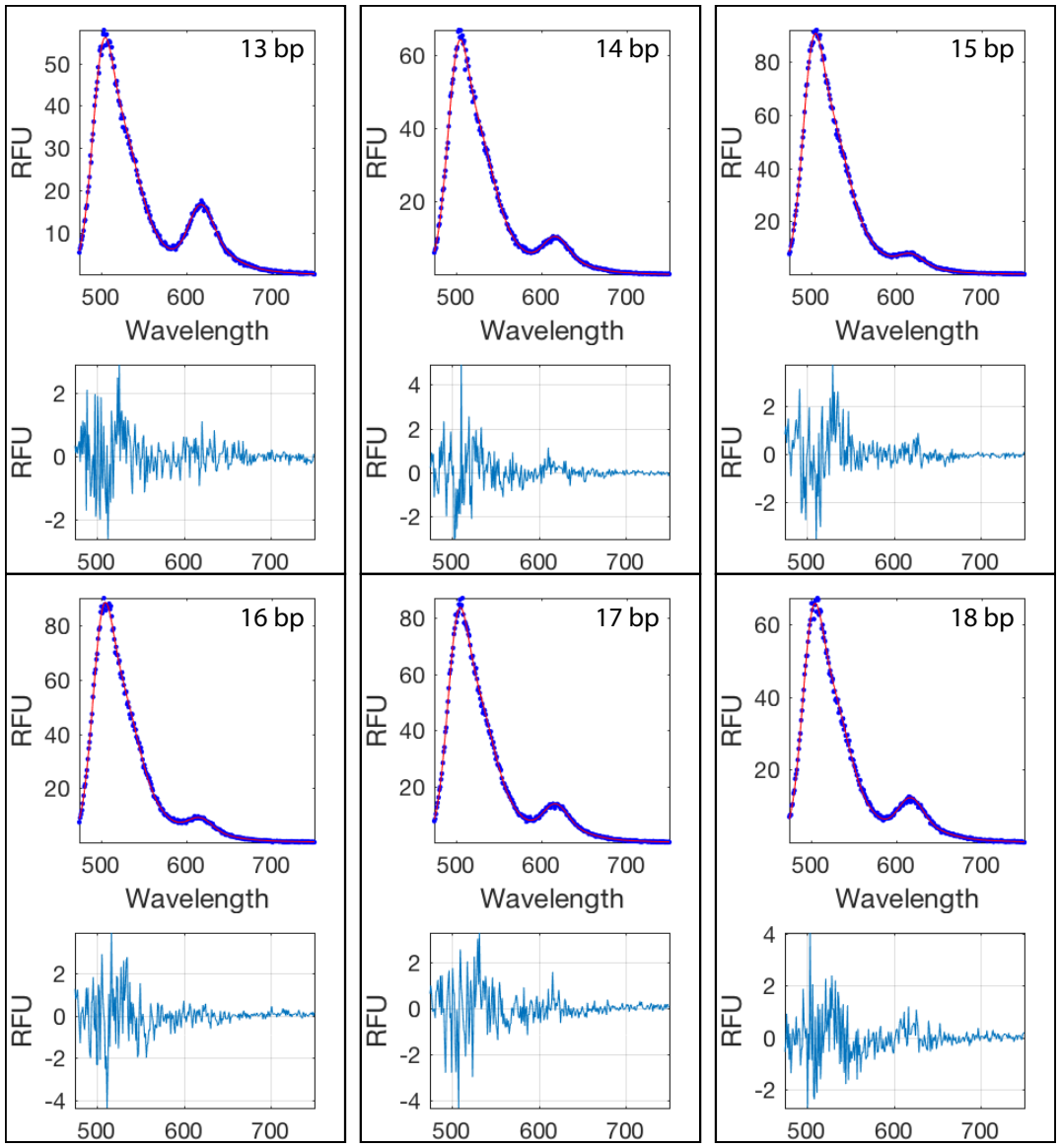


Fig. S3. Gaussian fits for Broc-nbp-M3 apta-FRET constructs. For each construct, one replicate of raw FRET emission data is shown. Constructs are labelled in the top right corner of the graph by the length of the duplex (e.g. '4 bp' is M3-4bp-Broc). Data points (blue) are fit to a triple Gaussian (red curve; Eq. 6). Residuals are shown below each graph.

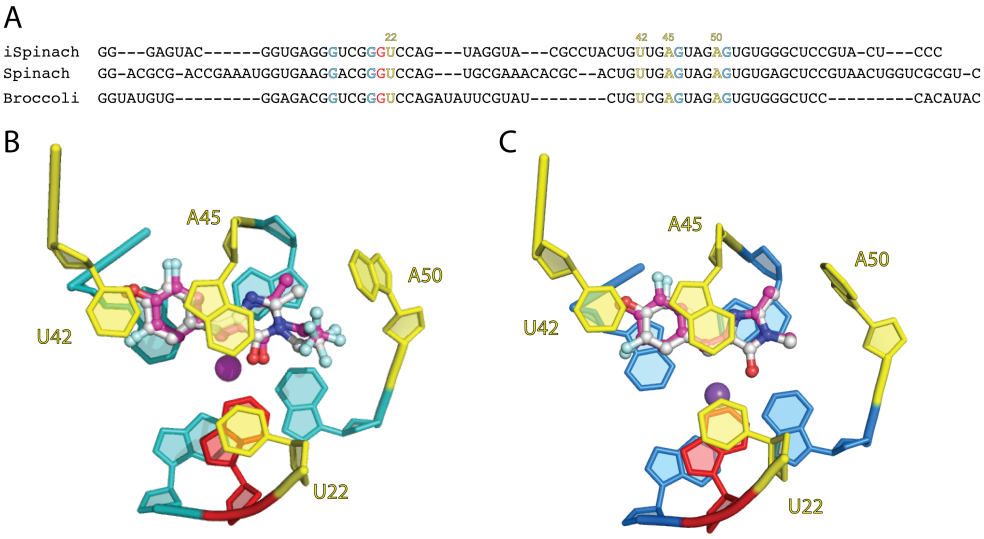


Fig. S4. Sequence and structural comparison of DFHBI/DFHBI-1T aptamers.

(A) Sequence alignment of the iSpinach, Spinach, and Broccoli aptamers. Sequences used in alignment derive from the crystalizing constructs for iSpinach and Spinach[22,25]. **(B)** Structural superposition of iSpinach-DFHBI-1T and Spinach-DFHBI-1T (PDB ID: 4Q9R) binding pockets. Spinach/DFHBI-1T (cartoon and grey ball-stick) overlaid with iSpinach/DFHBI (purple ball-stick). **(C)** Structural superposition of the iSpinach-DFHBI complex (PDB ID: 5OB3) with the Spinach-DFHBI complex (PDB ID: 4TS0). iSpinach-DFHBI (cartoon and purple ball-stick) overlaid with Spinach-DFHBI (grey ball-stick).

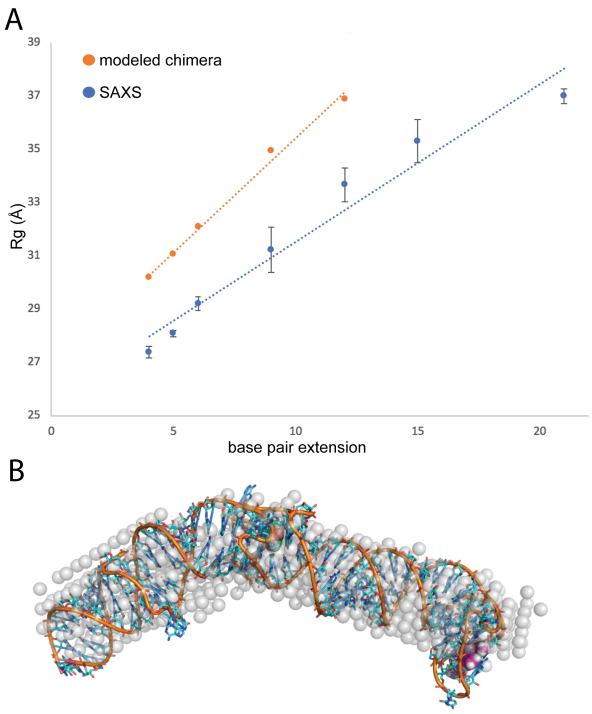


Fig. S5. Structural characterization of Broc-nbp-M3 apta-FRET chimeras. (A) Comparison of calculated R_gs from modeled chimeras (orange) with R_g measurements from the Guinier region of solution scattering profiles (blue). **(B)** Molecular envelope from SAXS for the Broc-12-M3 chimera overlaid with the Broc-12-M3 model.

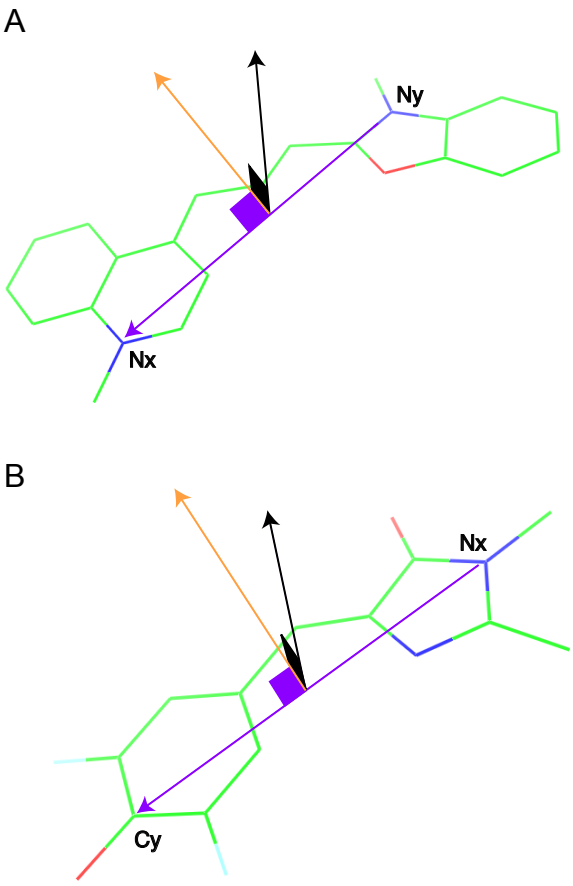
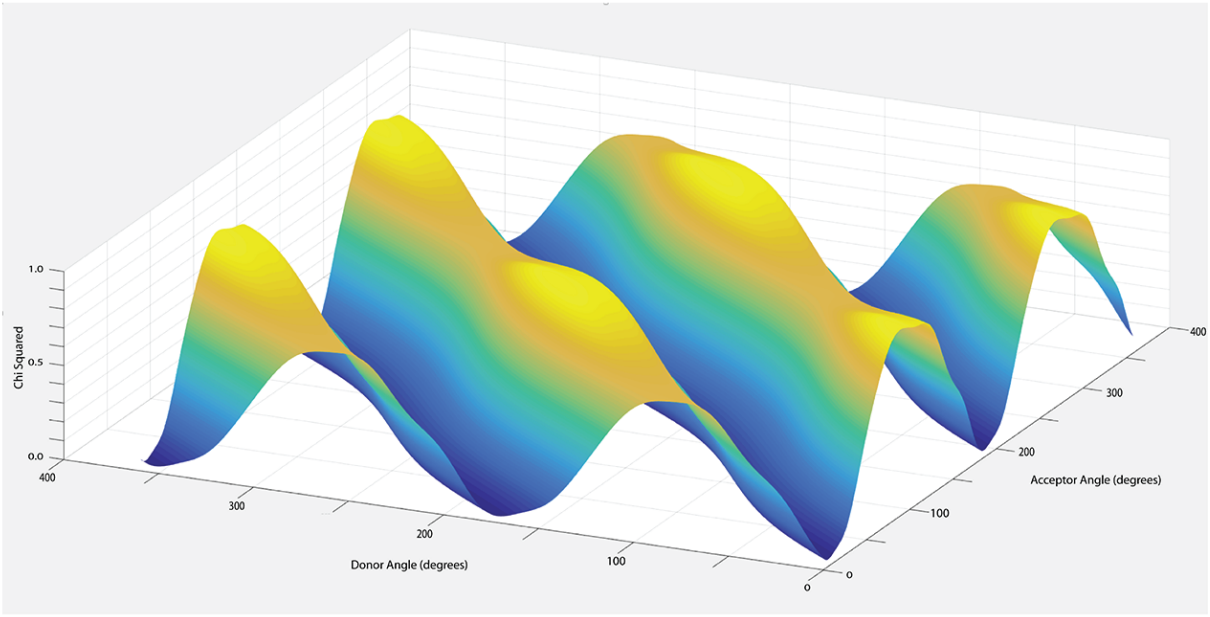


Fig. S6. Coordinate system definitions using YO3 **(A)** and DFHBI-1T structures **(B)**. A reference vector is taken from Nx to Ny (purple vector). The normal of the plane is shown by the black arrow. The 12'O clock vector (orange) is calculated as the cross product of the black and purple unit vectors so as to enforce perpendicularity with the clockface normal. It is in-plane with the fluorophore as denoted by right angles to the purple and black unit vectors.

A



B

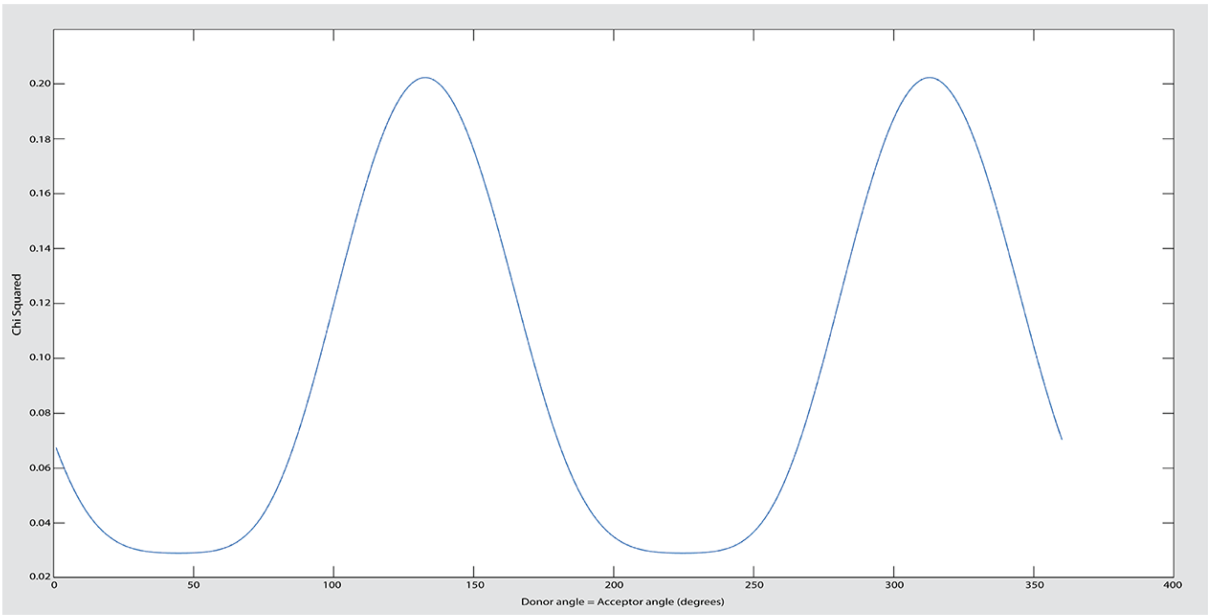


Fig. S7. **(A)** Chi square analysis, high Chi squared values are colored in yellow low chi squared values in dark blue. **(B)** Chi Squared values along the acceptor donor $\theta = \gamma$ valley shown in panel **(A)**.

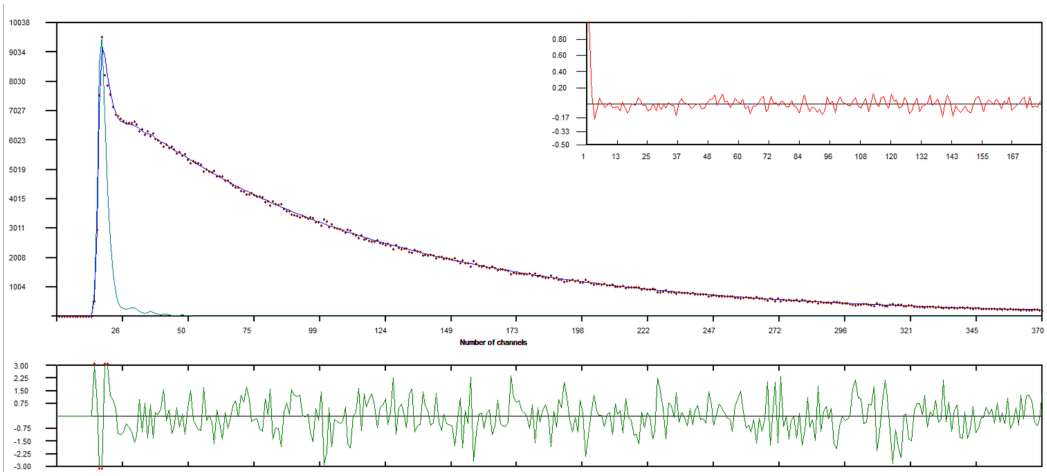


Fig. S8. FRET lifetime data. Fluorescence lifetime trace of Broc-9-M3 bound to DFHBI-1T and YO3-Biotin. The fluorescence lifetime decay (red points) with fit (dark blue) and lamp (light blue). Below in green is the weighted residuals and in the top right is the autocorrelation (red). The weighted residuals and autocorrelation were used with χ^2 to assess the quality of the fit. χ^2 for the fit is 1.36.

Table S1. RNA constructs used in this study. Red indicates an iMango-III U10A core.

Broccoli is colored green.

1	Broc-4bp-M1	GGAUCA-GAGACGGUCGGGUC-CA----------ACGAA-GGGACGGUGCGGAGAGGAG- AGU-----------UG-UCGAGUAGAGUGUGGGCUC-UGAUCC
2	Broc-5bp-M1	GGAUCA-GAGACGGUCGGGUC-CAC---------ACGAA-GGGACGGUGCGGAGAGGAG- AGU----------GUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
3	Broc-6bp-M1	GGAUCA-GAGACGGUCGGGUC-CACG--------ACGAA-GGGACGGUGCGGAGAGGAG- AGU---------CGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
4	Broc-7bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGU-------ACGAA-GGGACGGUGCGGAGAGGAG- AGU--------ACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
5	Broc-8bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGUC------ACGAA-GGGACGGUGCGGAGAGGAG- AGU-------GACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
6	Broc-9bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGUCG-----ACGAA-GGGACGGUGCGGAGAGGAG- AGU------CGACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
7	Broc-10bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGUCGA----ACGAA-GGGACGGUGCGGAGAGGAG- AGU-----UCGACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
8	Broc-11bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGUCGAG---ACGAA-GGGACGGUGCGGAGAGGAG- AGU----CUCGACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
9	Broc-12bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGC--ACGAA-GGGACGGUGCGGAGAGGAG- AGU---GCUCGACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
10	Broc-13bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCA-ACGAA-GGGACGGUGCGGAGAGGAG- AGU--UGCUCGACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
11	Broc-14bp-M1	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCACACGAA-GGGACGGUGCGGAGAGGAG- AGU-GUGCUCGACGUG-UCGAGUAGAGUGUGGGCUC-UGAUCC
12	Broc-4bp-M3	GGAUCA-GAGACGGUCGGGUC-CA--------------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU--------------------UG- UCGAGUAGAGUGUGGGCUC-UGAUCC
13	Broc-5bp-M3	GGAUCA-GAGACGGUCGGGUC-CAC-------------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU-------------------GUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
14	Broc-6bp-M3	GGAUCA-GAGACGGUCGGGUC-CACG------------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU------------------CGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
15	Broc-7bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGU-----------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU-----------------ACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
16	Broc-8bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUC----------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU----------------GACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
17	Broc-9bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCG---------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU---------------CGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
18	Broc-10bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGA--------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU--------------UCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
19	Broc-11bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAG-------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU-------------CUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
20	Broc-12bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGC------------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU------------GCUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC

21	Broc-13bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCA----------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU----------UGCUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
22	Broc-14bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCAC----------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU----------GUGCUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
23	Broc-15bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCACA---------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU---------UGUGCUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
24	Broc-16bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCACAG--------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU--------CUGUGCUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
25	Broc-17bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCACAGC-------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU-------GCUGUGCUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
26	Broc-18bp-M3	GGAUCA-GAGACGGUCGGGUC-CACGUCGAGCACAGCU------ACGAA- GGAAGGAUUGGUAUGUGGUAUA-UUCGU------AGCUGUGCUCGACGUG- UCGAGUAGAGUGUGGGCUC-UGAUCC
27	Broc-iM3-U10A (from Fig. 5B)	GGAACUG-AGCAGGCA-AUGAUCCAA-GGAAGGAUUGGUAUGGGGUAG-UUGUGGAUCAU- GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAU-GAGACGGUCGGGUC- CAGAUAUUCGUAUCUG-UCGAGUAGAGUGUGGGCUCAUCUGU-GGGA-CAGUUCC
28	NiCo(M,B: 0,0)	GGAACUG-AGCAGGCA-AUGACC------GAA-GGAAGGAUUGGUAUGUGGUAUA-UUC-- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGU---UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA---ACAUCUGU-GGGA- CAGUUCC
29	NiCo(M,B: 1,0)	GGAACUG-AGCAGGCA-AUGACC-----CGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCG- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGU---UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA---ACAUCUGU-GGGA- CAGUUCC
30	NiCo(M,B: 2,0)	GGAACUG-AGCAGGCA-AUGACC----GCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGC----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGU---UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA---ACAUCUGU-GGGA- CAGUUCC
31	NiCo(M,B: 3,0)	GGAACUG-AGCAGGCA-AUGACC---UGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCA---GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGU---UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA---ACAUCUGU-GGGA- CAGUUCC
32	NiCo(M,B: 4,0)	GGAACUG-AGCAGGCA-AUGACC--CUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAG--GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGU---UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA---ACAUCUGU-GGGA- CAGUUCC
33	NiCo(M,B: 5,0)	GGAACUG-AGCAGGCA-AUGACC-GCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAGC-GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGU---UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA---ACAUCUGU-GGGA- CAGUUCC
34	NiCo(M,B: 6,0)	GGAACUG-AGCAGGCA-AUGACCUGCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAGCAGGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGU---UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA---ACAUCUGU-GGGA- CAGUUCC
35	NiCo(M,B: 0,1)	GGAACUG-AGCAGGCA-AUGACC------GAA-GGAAGGAUUGGUAUGUGGUAUA-UUC-- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUG--UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA--CACAUCUGU-GGGA- CAGUUCC
36	NiCo(M,B: 1,1)	GGAACUG-AGCAGGCA-AUGACC-----CGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCG- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUG--UG-

		UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA--CACAUCUGU-GGGA- CAGUUCC
37	NiCo(M,B: 2,1)	GGAACUG-AGCAGGCA-AUGACC----GCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGC----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUG--UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA--CACAUCUGU-GGGA- CAGUUCC
38	NiCo(M,B: 3,1)	GGAACUG-AGCAGGCA-AUGACC---UGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCA---GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUG--UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA--CACAUCUGU-GGGA- CAGUUCC
39	NiCo(M,B: 4,1)	GGAACUG-AGCAGGCA-AUGACC--CUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAG--GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUG--UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA--CACAUCUGU-GGGA- CAGUUCC
40	NiCo(M,B: 5,1)	GGAACUG-AGCAGGCA-AUGACC-GCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAGC-GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUG--UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA--CACAUCUGU-GGGA- CAGUUCC
41	NiCo(M,B: 6,1)	GGAACUG-AGCAGGCA-AUGACCUGCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAGCAGGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUG--UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA--CACAUCUGU-GGGA- CAGUUCC
42	NiCo(M,B: 0,2)	GGAACUG-AGCAGGCA-AUGACC------GAA-GGAAGGAUUGGUAUGUGGUAUA-UUC-- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGC-UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA-GCACAUCUGU-GGGA- CAGUUCC
43	NiCo(M,B: 1,2)	GGAACUG-AGCAGGCA-AUGACC-----CGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCG- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGC-UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA-GCACAUCUGU-GGGA- CAGUUCC
44	NiCo(M,B: 2,2)	GGAACUG-AGCAGGCA-AUGACC----GCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGC----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGC-UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA-GCACAUCUGU-GGGA- CAGUUCC
45	NiCo(M,B: 3,2)	GGAACUG-AGCAGGCA-AUGACC---UGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCA---GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGC-UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA-GCACAUCUGU-GGGA- CAGUUCC
46	NiCo(M,B: 4,2)	GGAACUG-AGCAGGCA-AUGACC--CUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAG--GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGC-UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA-GCACAUCUGU-GGGA- CAGUUCC
47	NiCo(M,B: 5,2)	GGAACUG-AGCAGGCA-AUGACC-GCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAGC-GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGC-UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA-GCACAUCUGU-GGGA- CAGUUCC
48	NiCo(M,B: 6,2)	GGAACUG-AGCAGGCA-AUGACCUGCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA- UUCGCAGCAGGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGC-UG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CA-GCACAUCUGU-GGGA- CAGUUCC
49	NiCo(M,B: 0,3)	GGAACUG-AGCAGGCA-AUGACC------GAA-GGAAGGAUUGGUAUGUGGUAUA-UUC-- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGCAUG- UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CAUGCACAUCUGU-GGGA- CAGUUCC
50	NiCo(M,B: 1,3)	GGAACUG-AGCAGGCA-AUGACC-----CGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCG- ----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGCAUG-

		UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CAUGCACAUCUGU-GGGA-CAGUUCC
51	NiCo(M,B: 2,3)	GGAACUG-AGCAGGCA-AUGACC----GCGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCGC----GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGCAUG-UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CAUGCACAUCUGU-GGGA-CAGUUCC
52	NiCo(M,B: 3,3)	GGAACUG-AGCAGGCA-AUGACC---UGCGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCGCA---GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGCAUG-UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CAUGCACAUCUGU-GGGA-CAGUUCC
53	NiCo(M,B: 4,3)	GGAACUG-AGCAGGCA-AUGACC--CUGCGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCGCAG--GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGCAUG-UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CAUGCACAUCUGU-GGGA-CAGUUCC
54	NiCo(M,B: 5,3)	GGAACUG-AGCAGGCA-AUGACC-GCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCGCAGC-GGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGCAUG-UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CAUGCACAUCUGU-GGGA-CAGUUCC
55	NiCo(M,B: 6,3)	GGAACUG-AGCAGGCA-AUGACCUGCUGCGAA-GGAAGGAUUGGUAUGUGGUAUA-UUCGCAGCAGGUCAU-GCAGCCGG-GCUGCGAAAGCGGC-A-ACAGAUGUGCAUG-UCGAGUAGAGUGUGGGCUC-GAAA-GAGACGGUCGGGUC-CAUGCACAUCUGU-GGGA-CAGUUCC
56	Mango-III	GCUACGAAGGAAGGAUUGGUAUGUGGUAUAUUCGUAGC
57	iSpinach	GGGAGUACGGUGAGGGUCGGGUCCAGUAGGUACGCCUACUGUUGAGUAGAGUGUGGGCUCC GUACUCCC

Table S2. Representative fitted emission peak shapes in Gaussian analysis of Broc-nbp-M3 constructs (Table S1.B).

Duplex length (bp)	Broccoli/DFHBI-1T em peaks (nm)					Mango/YO3-Biotin em peaks (nm)				
	Fitted	Peak	Loc	m_1	n_1	Fitted	Peak	Loc	m_2	n_2
4	499.8	519.5	543.3	-0.7	26.1	616.0	634.2	663.4	-0.1	10.3
5	500.1	519.8	543.6	-0.4	16.1	615.8	634.0	663.2	-0.3	22.7
6	500.2	519.9	543.7	-0.3	13.0	615.7	633.9	663.1	-0.4	28.5
7	500.5	520.2	544.0	0.0	8.9	615.7	633.9	663.1	-0.4	18.7
8	499.9	519.6	543.4	-0.6	18.9	615.8	634.0	663.2	-0.3	18.5
9	499.6	519.3	543.1	-0.9	35.2	616.0	634.2	663.4	-0.1	7.7
10	499.6	519.3	543.1	-0.9	39.8	616.1	634.3	663.5	0.0	10.0
11	499.7	519.4	543.2	-0.8	32.1	616.0	634.2	663.4	-0.1	13.8
12	499.8	519.5	543.3	-0.7	31.6	616.1	634.3	663.5	0.0	15.5
13	499.7	519.4	543.2	-0.8	36.8	615.9	634.1	663.3	-0.2	13.0
14	499.7	519.4	543.2	-0.8	45.2	616.1	634.3	663.5	0.0	8.4
15	499.4	519.1	542.9	-1.1	55.4	615.9	634.1	663.3	-0.2	5.4
16	499.5	519.2	543.0	-1.0	56.4	616.5	634.7	663.9	0.4	6.5
17	499.5	519.2	543.0	-1.0	49.9	616.1	634.3	663.5	0.0	9.8
18	499.6	519.3	543.1	-0.9	42.2	615.9	634.1	663.3	-0.2	9.5
Standard deviation	0.3	0.3	0.3			0.2	0.2	0.2		

See Eq. 6 in the main text for details of the exact functional representation used. Briefly, m1 and m2 are deviations in the peak wavelength position expected for the fluorophore aptamers in isolation. n1 and n2 are the amplitude terms for each peak shape respectively.

Table S3. Observed E'_FRET values

Helix_Len	E'_FRET	ERR
4	0.215	0.011
5	0.498	0.017
6	0.610	0.020
7	0.596	0.031
8	0.406	0.006
9	0.128	0.003
10	0.146	0.011
11	0.229	0.006
12	0.255	0.013
13	0.198	0.007
14	0.112	0.005
15	0.062	0.002
16	0.073	0.000
17	0.119	0.001
18	0.133	0.003

Helix Length - construct number, average E'_{FRET} value and E'_{FRET} error determined from analysis of the constructs shown in **Table S1.B** and **Table S2.** E'_FRET is determined from the average of three independent replicates and the error the corresponding standard deviation.

Table S4. Summary of crystallographic statistics

	Mango-III-YO3-Biotin	iSpinach-DFHBI-1T
Data collection		
Space group	$P2_1\ 2_1\ 2_1$	C2
Cell dimensions		
a, *b*, *c* (Å)	62.38, 67.13, 76.77	150.56, 48.69, 30.61
α, β, γ (°)	90, 90, 90	90, 91.4, 90
Resolution (Å)	48.41 – 2.72 (2.80 – 2.72)[a]	75.26 – 2.01 (2.10-2.01)[a]
R_{merge}	0.035 (>1.0)	0.05 (1.00)
$CC_{1/2}$	1.00 (0.34)	0.999 (0.627)
$\langle I \rangle / \langle \sigma(I) \rangle$	27.6 (1.0)	13.5 (1.1)
Completeness (%)	99.9 (100)	98.7 (92.1)
Redundancy	6.3 (6.4)	5.3 (5.0)
Refinement		
Resolution (Å)	48.40 – 2.80 (2.90 – 2.80)	75.3 – 2.10 (2.18 – 2.1)
No. reflections	13695 (1351)	11686 (1167)
R_{work} / R_{free}	17.36 / 21.46	19.59 / 23.24
No. atoms	1688	1612
RNA	1632	1493
Ligands	48	22
Ions	8	43
Water	0	54
B-factors (Å^2)	80.47	66.10
RNA	80.68	58.12
Ligand	74.44	40.06
Ions	70.8	54.88
R.m.s deviations		
Bond lengths (Å)	0.006	0.006
Bond angles (°)	1.20	1.12
Coord. Precision (Å)	0.56	0.25

[a]Values in parentheses are for highest-resolution shell. One crystal was used for each data set.

Table S5. Helical Model parameters used in fitting figure 2B

Helix Le	R_x	R_y	R_z	a1_x	a1_y	a1_z	a2_x	a2_y	2_z	d1_x	d1_y	d1_z	d2_x	d2_y	d2_z
4	-15.291	-8.440	29.273	0.489	-0.033	-0.872	0.348	0.924	0.160	0.797	0.483	-0.363	0.572	-0.409	0.711
5	-23.984	-7.928	25.935	0.750	-0.111	-0.652	0.005	0.987	-0.163	0.797	0.483	-0.363	0.572	-0.409	0.711
6	-30.340	-9.662	20.375	0.912	-0.096	-0.398	-0.168	0.798	-0.579	0.797	0.483	-0.363	0.572	-0.409	0.711
7	-34.247	-15.318	15.174	0.986	0.065	-0.154	-0.167	0.422	-0.891	0.797	0.483	-0.363	0.572	-0.409	0.711
8	-35.100	-23.448	12.431	0.947	0.321	0.003	0.010	-0.021	-1.000	0.797	0.483	-0.363	0.572	-0.409	0.711
9	-33.274	-31.812	13.468	0.808	0.588	0.021	0.305	-0.389	-0.869	0.797	0.483	-0.363	0.572	-0.409	0.711
10	-30.015	-38.088	18.394	0.615	0.782	-0.105	0.624	-0.563	-0.542	0.797	0.483	-0.363	0.572	-0.409	0.711
11	-27.011	-40.638	26.059	0.427	0.840	-0.334	0.864	-0.488	-0.123	0.797	0.483	-0.363	0.572	-0.409	0.711
12	-25.878	-39.010	34.446	0.307	0.744	-0.594	0.949	-0.188	0.255	0.797	0.483	-0.363	0.572	-0.409	0.711
13	-27.627	-34.091	41.305	0.292	0.524	-0.800	0.850	0.241	0.468	0.797	0.483	-0.363	0.572	-0.409	0.711
14	-32.351	-27.825	44.873	0.387	0.251	-0.887	0.601	0.661	0.450	0.797	0.483	-0.363	0.572	-0.409	0.711
15	-39.187	-22.591	44.442	0.562	0.013	-0.827	0.280	0.938	0.205	0.797	0.483	-0.363	0.572	-0.409	0.711
16	-46.591	-20.432	40.588	0.761	-0.115	-0.638	-0.009	0.982	-0.187	0.797	0.483	-0.363	0.572	-0.409	0.711
17	-52.839	-22.414	34.987	0.920	-0.090	-0.383	-0.173	0.780	-0.601	0.797	0.483	-0.363	0.572	-0.409	0.711
18	-56.576	-28.260	29.876	0.987	0.078	-0.142	-0.161	0.397	-0.904	0.797	0.483	-0.363	0.572	-0.409	0.711

Helix Length - construct number, R- displacement from center of donor to center of acceptor fluorophores in Å. a_1 and a_2 unit vectors defining the normal to the YO3 fluorophore clockface and the 12 O Clock position in the YO3 plane respectively. d_1 and d_2 define the same coordinates for DFHBI-1T.

Table S6. Fluorescent lifetime data

	6 bp		8 bp		9 bp	
	α	τ	α	τ	α	τ
Donor Only	4.23	4.69 ± 0.07	3.47	4.75 ± 0.05	2.41	4.80 +0.07 -0.06
Acceptor Only	3.10	5.2 +0.9 -0.2	2.96	6.1 ± +1.5 -0.3	3.30	5.72 +0.05 -0.06
Donor/Acceptor Lifetime 1	1.26	0.8 +0.2 -0.1	2.67	1.5 +0.1 -0.2	2.38	3.9 +1.8 -1.2
Donor/Acceptor Lifetime 2	0.43	4.7 +0.4 -0.3	0.53	3.5 +1 -0.6	0.34	1.2 +1.8 -0.8

Lifetime contributions in Table SX were determined with a nonlinear least squares fit. Errors were determined using 90% confidence limits.

Compare with Lewis, Zhang and Zuo et al. JACS 2005