Double fluorescence resonance energy transfer to explore multicomponent binding interactions: a case study of DNA mismatches

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A first demonstration, using a four-component system of complementary and single base-mismatched oligonucleotides, that double fluorescence resonance energy transfer (FRET) can be used to interrogate multicomponent interactions in molecular complexes.

The study of intermolecular interactions is key to the understanding of fundamental biological processes. It is well established that many important biological complexes rely upon binding interactions between multiple components. A number of experimental approaches exist for the study of such non-covalent interactions, but few are capable of conveniently providing information about interactions between several components. Fluorescence resonance energy transfer (FRET)[1] between donor and acceptor fluorophores has been usefully employed in the study of molecular interactions in homogeneous solution.[2,3] Furthermore, the theory is sufficiently well understood to carry out inter- or intra-molecular distance mapping experiments,[4] typically in the 10–100 Å range. FRET experiments to study intermolecular interactions are usually carried out for two components (*i.e.* with a single donor and single acceptor). However, if a donor fluorophore is brought in proximity to more than one type of acceptor fluorophore, there exists the possibility of multiple FRET interactions resulting from irradiation at a single wavelength (Fig. 1). In principle it should be possible to simultaneously obtain information about multicomponent binding interactions. FRET has been used to study nucleic acid interactions in examples that include; duplex hybridisation,[5] triple helix[6] and DNA tetraplex formation,[7] and also for DNA diagnostic assays using molecular beacons to analyse point mutations.[8] We report a homogeneous system of oligonucleotides in which double FRET interactions have been used to study specific binding interactions between four components. The studies comprise of sequence-specific nucleic acid interactions and the localisation of single base mismatches, but the general principle will apply more broadly to other mutually binding systems.

The design of this four component system (Fig. 1) comprises a 27mer oligonucleotide (**M**) and three labelled 9mer oligonucleotide sequences (**R**, **F** and **T**), each of which is complementary to discrete, adjacent sections of the 27mer.[9] Each 9mer included a 5-propargylamino-modified thymidine base,[10] which was postsynthetically modified by reaction with the activated *N*-hydroxysuccinimide ester of either rhodamine-X (**R**OX), fluorescein (**F**AM) or carboxy-X-rhodamine (**T**MR) as indicated in Fig. 1. The excitation and emission maxima of these fluorophores are resolvable and the emission spectrum of FAM overlaps significantly with the excitation spectra of both TMR and ROX. On binding of all three 9mers to the target 27mer to give a fully assembled complex, the donor fluorophore (FAM) is *ca.* 20–25 Å from *both* acceptor fluorophores (TMR and ROX) such that excitation of FAM would lead to significant energy transfer to both TMR and ROX.[11] This was monitored by observing both the donor quenching effects and the sensitised emission of the two acceptor fluorophores.

The fluorescence spectrum for the matched four component system (**R**:**F**:**T**:**M**) is shown in Fig. 2(a) along with spectra of

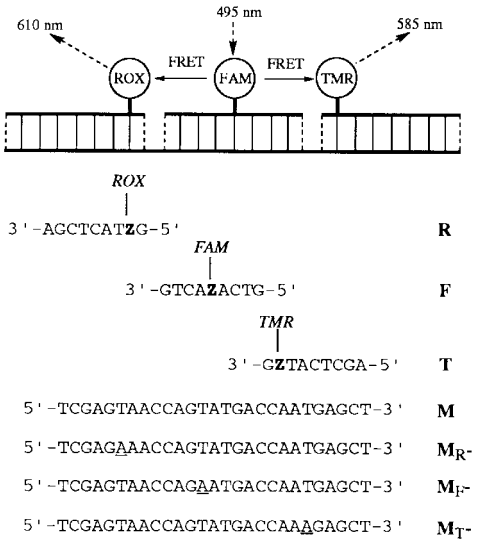

Fig. 1 Schematic of the four-component system, with the sequences used shown below. **Z** = 5-propargylamino dT, ROX = carboxy-X-rhodamine, FAM = carboxyfluorescein, TMR = carboxytetramethylrhodamine. The underlined bases represent T–A single base substitutions. The ROX (**R**), FAM (**F**) and TMR (**T**) labelled oligonucleotide probes bind contiguously on the target (**M**). Oligonucleotide targets $\mathbf{M_R}^-$, $\mathbf{M_F}^-$ and $\mathbf{M_T}^-$ incorporate a single internal A:A mismatch opposite probes **R**, **F** or **T**, respectively.

the individual two component systems (**R**:**M**, **F**:**M**, **T**:**M**) that contribute to the former. Three additional target 27mers ($\mathbf{M_R}^-$, $\mathbf{M_F}^-$ and $\mathbf{M_T}^-$) were designed each with a single central A:A base mismatch to perturb binding of oligonucleotide component **R**, **F** or **T** respectively and examine the effect on energy transfer between the donor and both acceptors (Fig. 1).[12] The fluorescence spectra of these mismatched systems (**R**:**F**:**T**:$\mathbf{M_R}^-$, **R**:**F**:**T**:$\mathbf{M_F}^-$, **R**:**F**:**T**:$\mathbf{M_T}^-$) are presented in Fig. 2(b) where it is clear that mismatches are distinguished by distinct differences

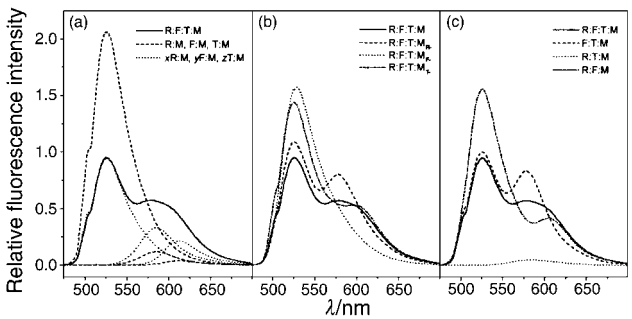

Fig. 2 Relative fluorescence profiles (arbitrary units) for; (a) the basis two-component systems **R**:**M**, **F**:**M**, **T**:**M** (- - - -) and the matched four-component system **R**:**F**:**T**:**M** (——). Also shown is the scaled contributions of **R**:**M**, **F**:**M** and **T**:**M** (· · · · ·) to **R**:**F**:**T**:**M** where $x = 5.33$, $y = 0.46$, $z = 2.75$; (b) mismatched systems **R**:**F**:**T**:$\mathbf{M_R}^-$ (- - -) **R**:**F**:**T**:$\mathbf{M_F}^-$ (· · · · ·) and **R**:**F**:**T**:$\mathbf{M_T}^-$ (··——··——); (c) control systems **F**:**T**:**M** (- - -), **R**:**T**:**M** (· · · · ·) and **R**:**F**:**M** (··—··—).

Table 1 Fluorimeter Assay. Relative fluorescence intensities (arbitrary units) and the contributions from ROX, FAM and TMR to the four component matched and mismatched systems and the three component controls. The relative contributions *x*, *y*, *z* are solved by simultaneous equations[13]

	I_{525}	I_{585}	I_{610}	*x*	*y*	*z*
R:**M**	0	0.01	0.04	1	0	0
F:**M**	2.06	0.36	0.12	0	1	0
T:**M**	0	0.12	0.06	0	0	1
R:**F**:**T**:**M**	0.95	0.56	0.44	5.33	0.46	2.75
R:**F**:**T**:$\mathbf{M_R}^-$	1.08	0.76	0.39	0.96	0.53	4.65
R:**F**:**T**:$\mathbf{M_F}^-$	1.55	0.43	0.19	0.47	0.75	1.26
R:**F**:**T**:$\mathbf{M_T}^-$	1.43	0.52	0.48	7.37	0.69	1.51
F:**T**:**M**	0.99	0.79	0.33	−1.20	0.48	5.26
R:**T**:**M**	0	0.05	0.03	0.23	0	0.38
R:**F**:**M**	1.55	0.37	0.40	7.40	0.75	0.08

in the fluorescence profiles. Melting temperature studies confirmed that the unlabelled (non-fluorescent) 9mers exhibited good discrimination between matched (80–87% bound at 25 °C) and mismatched (< 20% bound at 25 °C) targets.

Control experiments [Fig. 2(c)] were performed on three-component matched systems containing two of the three fluorescent 9mer oligonucleotides bound to the 27mer target. These were the ROX–FAM FRET couple (**R**:**F**:**M**), the TMR–FAM FRET couple (**F**:**T**:**M**) and the ROX–TMR non-FRET pair (**R**:**T**:**M**).

In order to determine the variations in the relative fluorescence contribution of individual fluorophores to each system, the fluorescence spectra were deconvoluted using a set of simultaneous equations.[13] Using this method, each of the three hybridised matched systems **R**:**M**, **F**:**M** and **T**:**M** was assigned a standard value of 1 for the individual contribution to the fluorescence signal of ROX, FAM and TMR, respectively at their respective maximum emission intensities (610, 525, 585, nm). The contributions to the fluorescence resulting from FRET were then determined relative to these values. The relative fluorescence intensities and the calculated contributions from ROX, FAM and TMR to the single four-component matched system, the four-component mismatched systems and controls are summarised in Table 1. The matched system (**R**:**F**:**T**:**M**) exhibited FRET between the donor and *both* acceptors, as indicated by a decrease in the relative fluroescence of FAM (1.0 to 0.46) and an increase in the relative fluroescence of ROX (1.0 to 5.33) and TMR (1.0 to 2.75).

In **R**:**F**:**T**:$\mathbf{M_R}^-$, the mismatch will significantly reduce the proportion of **R** binding. In this case, the system should be very similar to the **F**:**T**:**M** control system. The relative emissions of FAM (0.53) and TMR (4.65) compare well with the **F**:**T**:**M** system (relative emissions of 0.48 and 5.26 respectively). The second mismatch system (**R**:**F**:**T**:$\mathbf{M_F}^-$) will reduce the binding of **F**, which results in no observed FRET between the donor and either acceptor. This resembles the control system **R**:**T**:**M**, in which **F** does not bind, and ROX or TMR produces no significant FRET emission signal. The final system (**R**:**F**:**T**:$\mathbf{M_T}^-$) positions a mismatch that prevents binding of **T**. The result is FRET between FAM and ROX only, as in the control system **R**:**F**:**M**. The two systems compare well, with the relative emissions of FAM (0.69) and ROX (7.37) being very similar to the **R**:**F**:**M** system (relative emissions of 0.75 and 7.40, respectively).

An alternative format for analysis involved fluorescence imaging of samples in a multiwell plate. Samples containing each of the three 9mers (**R**, **F**, **T**) and one of the four matched (**M**) or mismatched ($\mathbf{M_R}^-$, $\mathbf{M_F}^-$, $\mathbf{M_T}^-$) targets were irradiated at 488 nm and emission measured through a 530(±15) nm (FAM), 570(±15) nm (TMR) or 610 nm (ROX) longpass filter. Data were obtained for multiple repeats of each four-component system and analysed using the simultaneous equation method.[13] A plot of relative ROX *vs.* relative TMR emission for each sample from both the earlier fluorimeter studies and fluorimager microtitre plate assays is shown in Fig. 3. Data points were

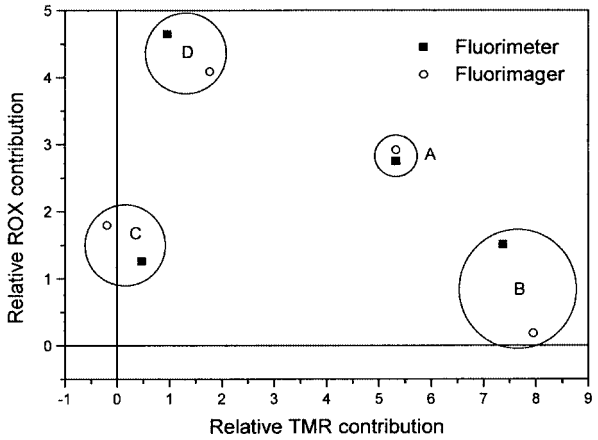

Fig. 3 Relative contributions of both TMR and ROX to fluorescence on binding 9mers to each of the four 27mer targets. Region A corresponds to the matched four component system **R**:**F**:**T**:**M** and regions B, C, D to the mismatched systems **R**:**F**:**T**:$\mathbf{M_R}^-$, **R**:**F**:**T**:$\mathbf{M_F}^-$ and **R**:**F**:**T**:$\mathbf{M_T}^-$ respectively. Fluorimager data (○) have been normalised to the TMR contribution in the matched four-component system of the fluorimeter data (■).

observed to fall into four distinct regions corresponding to the four target types. Region A represents the situation in which both TMR and ROX emission are observed (*i.e.* the matched system, **R**:**F**:**T**:**M**). Region B defines the **R**:**F**:**T**:$\mathbf{M_R}^-$ mismatched system, in which there is TMR emission but no ROX emission. Region C represents the **R**:**F**:**T**:$\mathbf{M_F}^-$ mismatch, which does not exhibit FRET because the donor (FAM) oligonucleotide does not bind. Finally, region D defines the **R**:**F**:**T**:$\mathbf{M_T}^-$ mismatch, in which there is only ROX, but no TMR emission.

These studies provide an insight into the fluorescence behaviour of a double FRET system. The results have shown that a single base mismatch can be detected and mapped in a four component system. Careful design and judicious selection and placement of fluorophores, will enable the general principle to be applied to the study of other multimolecular complexes, and address whether specific combinations of molecules complex under a given set of conditions. The microplate studies also suggest that double FRET can be utilised in a multiplexed format for a more routine analysis of multicomponent interactions.

Notes and references

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