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ORIGINAL PAPER

Time-Resolved FRET and FLIM of Four-way DNA Junctions

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Abstract Conformational transitions in a 4-way DNA junction when titrated with ionic solutions are studied using time-resolved fluorescence resonance energy transfer. Parameters characterising the transition in terms of critical ion concentration ($c_{1/2}$) and the Hill coefficient for ion binding are obtained by fitting a simple two-state model using steady-state spectra. Data obtained from a fluorescence lifetime plate reader and analysed by fitting a single exponential to donor fluorescence lifetime decays are shown to be in good agreement with the parameters obtained from steady-state measurements. Fluorescence lifetimes, however, offer advantages, particularly in being independent of fluorophore concentration, output intensity, inhomogeneity in the excitation source and output wavelength. We demonstrate preliminary FRET-FLIM images of DNA junction solutions obtained using a picosecond gated CCD which are in agreement with results from a fluorescence lifetime plate reader. The results suggest that time-resolved FRET-FLIM is sensitive to subtle structural changes and may be useful in assays based on 4-way DNA junctions.

Keywords Nucleic acids · Fluorescence · Energy transfer · Imaging

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Introduction

The DNA Holliday junction (HJ) is an essential element in processes of genetic recombination [1, 2]. It is a four-armed junction formed from the intersection of two DNA double helices, shown schematically in Fig 1(a). Recent studies have proposed the use of DNA junctions in nanotechnology devices [3, 4].

DNA junction structures show particular promise in nanoscale devices due to their branch point discontinuity which allows the adoption of two conformations, governed by electrostatic repulsion between negatively charged phosphate groups on the DNA backbone. In low salt solution conditions this repulsion causes the branch point to adopt a fully extended 'open' conformer. In high salt conditions, however, cations screen this repulsion and cause the branch point to adopt a more energetically favourable stacked, 'closed' conformer [5], as shown in Fig. 1(b).

Studies into the ion dependence of junction conformation have typically used gel electrophoresis [6], fluorescence resonance energy transfer (FRET) [7–9] and, more recently, molecular dynamics simulations [10] to probe conformations

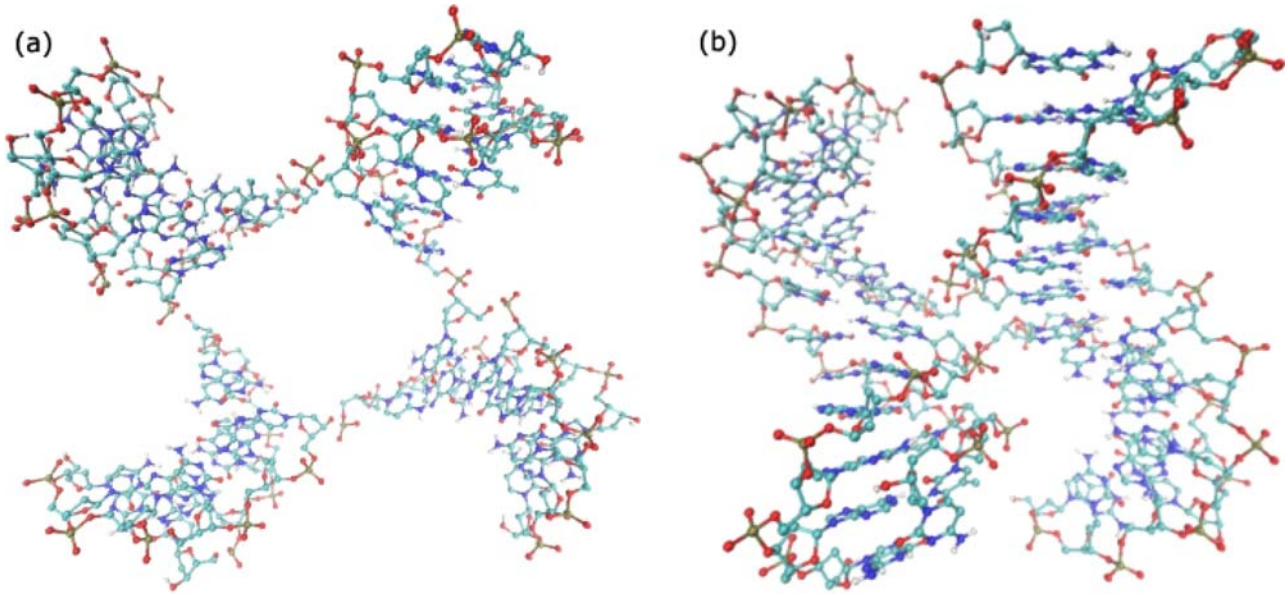

Fig. 1 Schematic representation of open (a) and closed (b) DNA junction conformers. In the absence of metal cations, the open conformer is adopted due to electrostatic repulsion between negatively charged phosphates on the DNA backbone. Under the influence of sufficient cations the junction branch point can collapse, allowing the adoption of the stacked, closed conformer

adopted by DNA junction structures. FRET studies typically involve labelling one junction arm with a donor and one with an acceptor chromophore and using the efficiency of energy transfer to infer information about fluorophore separation.

Donor fluorescnce lifetime studies have been used to investigate DNA junction conformation previously [11]. Fluorescence lifetime measurement is likely to be particularly useful in identifying the state of junctions in any nanotechnology application, as this is a property of the fluorophore and is therefore largely independent of local sample concentration, output intensity, inhomogeneity in the excitation source and output wavelength [12].

In this work, information about the conformation of DNA junctions in solution was obtained from FRET spectroscopy. Initially, the DNA junction system used here was characterised in solutions of differing cation type and concentration using steady state fluorescence spectroscopy. The results were used to fit a simple two-state model, to which further results could be compared.

Donor fluorescence lifetime data were measured in two different solution conditions, which were indicated by steady-state spectra to correspond to the open and closed HJ conformations, with the aim of showing that significant donor lifetime changes result from increased FRET efficiency through a conformational change.

Several measurements of donor lifetime data were then taken for DNA junctions in differing ionic solutions. These results were then compared to those obtained from steady-state spectra. Fluorescence lifetime imaging microscopy (FLIM) data for droplets of DNA junction samples in the open and closed conformations were then collected, with the aim of showing that FLIM could also rapidly determine the spatial variation of junction conformation and give results comparable to the steady-state and time-resolved measurements.

Methods and analysis

Sample preparation

DNA junctions were prepared from a mixture of pure oligonucleotides (Eurogentec) in overall 20 mM Tris/TrisH^+ Cl^- (pH 7.5) with 5 mM added $MgCl_2$. These were, in the indicated proportions: 1-FTGCAATCCTGAGCACA (5 μM), 2-TGTGCTCACCGAATCGGA (10 μM), 3-TCCGATT CGGACTATGCA (10 μM), 4-RTGCATAGTGATTGCA (10 μM). F is Carboxyfluorescein (commercially known as FAM) bound at the 5′ terminus of oligonucleotide 1 and R is Carboxytetramethylrhodamine (known as TAMRA) bound at the 5′ terminus of oligonucleotide 4. These proportions were used in order to maximise the incorporation of donor into complete junctions and ensuring that complete junctions dominate when the solution is excited at the donor excitation wavelength. Assembly involved heating the mixture to 80°C in a water bath for a period of 30 min and slow cooling in the water bath to favour the most thermodynamically stable product. Finally, samples were buffer exchanged in Amersham Biosciences Microspin G25 ion exchange columns, removing Mg^{2+} ions and producing DNA junction solutions of concentration 1 μM after dilution, as required for this work.

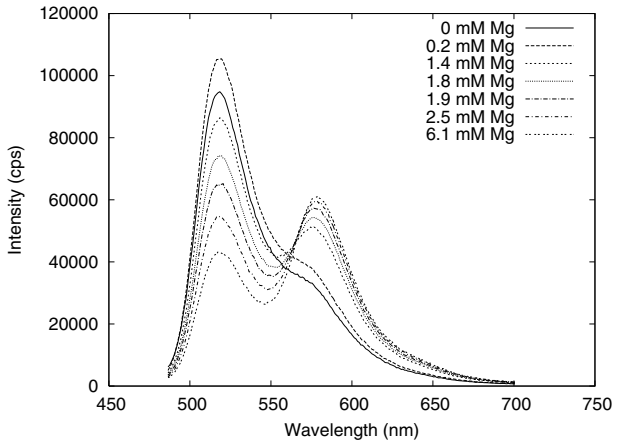

Fig. 2 Typical steady-state fluorescence emission spectra obtained from a 1 μM sample of HJ solution with the indicated concentration of Mg^{2+}

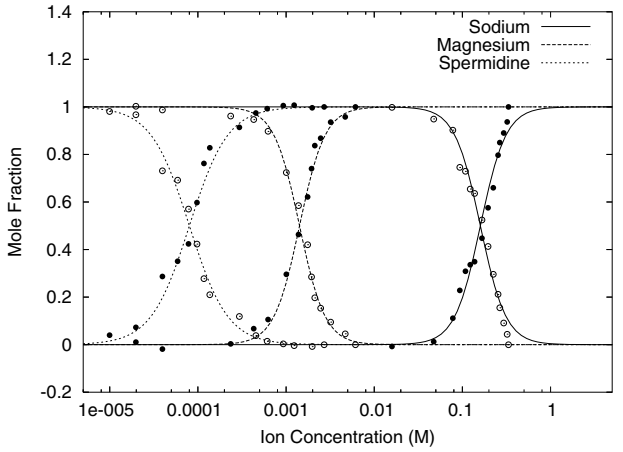

Fig. 3 Mole fractions of the open and closed conformers obtained from steady-state spectroscopic data, indicated by ○ and ● respectively. The solid lines indicate fits obtained by varying $c_{1/2}$ and n. Data is shown for DNA junctions with Na^+, Mg^{2+} and Spermidine (3+) as the titrated ion

Steady-state characterisation of DNA junction system

Changes in junction conformation in solution were detected optically using steady-state fluorescence emission spectroscopy to monitor the efficiency of FRET between F and R.

Fluorescence emission spectra were recorded from samples in a Starna 26.5-F Far UV quartz fluorescent cell (10 mm path length) using a Jobin Yvon Spex Fluoromax spectrofluorimeter (Instruments S.A.). Excitation was at a fixed wavelength of 476.5 nm. Emission was collected at 90° to the excitation beam. The emission intensity was normalised to the intensity of the excitation beam to correct for variations in the intensity output of the excitation lamp.

To allow changes in junction conformation to be characterised, steady-state fluorescence emission spectra were collected for samples in differing solutions. This was achieved by titration of a number of different ionic solutions. For each solution studied, a 50 μl sample of 1 μM junction solution prepared as above was placed in the cuvette. An emission spectrum was then collected from this sample before a series of additions of small volumes of concentrated cation solution, again prepared in a overall 20 mM, pH 7.5 Tris/TrisH^+Cl^- buffer solution.

Care was taken not to change the total sample volume by more than 10% in an attempt to keep the sample concentration roughly constant, with spectra corrected for small changes in overall volume. After each addition, the sample was allowed 2 min to equilibrate before an emission spectrum was collected. Repeated collection of steady-state spectra indicated that 2 min is a sufficient time delay to allow any conformational change to complete.

Simple two-state analysis was applied to this system, assuming the equilibrium:

$$\mathrm{HJ}_{\mathrm{open}} + nM^{z+} \leftrightharpoons \mathrm{HJ}_{\mathrm{closed}} \tag{1}$$

where $\mathrm{HJ}_{\mathrm{open}}$ is the HJ system before titration of ionic solution, $\mathrm{HJ}_{\mathrm{closed}}$ is the HJ system after titration and M^{z+} is a cation of charge z.

As such, the fluorescence spectra can be described by:

$$I(\lambda) = I_{\mathrm{open}}(\lambda)x_{\mathrm{open}} + I_{\mathrm{closed}}(\lambda)x_{\mathrm{closed}} \tag{2}$$

with

$$x_{\mathrm{open}} = 1 - x_{\mathrm{closed}} \tag{3}$$

where $I_{\mathrm{open/closed}}(\lambda)$ is the emission spectrum of the HJ solution in the fully open/closed conformation and $x_{\mathrm{open/closed}}$ is the mole fraction of the HJ population in the open/closed conformation.

By rearranging Eqs. 2 and 3 it is possible to obtain expressions for x_{open} and x_{closed} in terms of fluorescence emission intensities:

$$x_{\mathrm{open}} = \frac{I(\lambda_D) - I_{\mathrm{closed}}(\lambda_D)}{I_{\mathrm{open}}(\lambda_D) - I_{\mathrm{closed}}(\lambda_D)} \tag{4}$$

$$x_{\mathrm{closed}} = \frac{I(\lambda_A) - I_{\mathrm{open}}(\lambda_A)}{I_{\mathrm{closed}}(\lambda_A) - I_{\mathrm{open}}(\lambda_A)} \tag{5}$$

where these are expressed at the peak donor and acceptor emission wavelengths, λ_D and λ_A, in order to optimise the signal to noise ratio and hence reduce the errors in x.

The equilibrium is controlled by the dissociation constant in terms of concentration, K_C, which can be written

Table 1 Parameters obtained from fits of Eqs. 8 and 9 to experimentally determined values of x_{open} and x_{closed}, with associated errors. Values quoted for R^2 are those returned by the fitting algorithm

M^{z+}	$c_{1/2}$ (mM)	n	R^2
Li^+	125 ± 2	3.5 ± 0.2	0.99
Na^+	160 ± 3	3.0 ± 0.2	0.98
K^+	143 ± 5	2.6 ± 0.2	0.97
Ca^{2+}	1.42 ± 0.04	2.9 ± 0.2	0.99
Mg^{2+}	1.42 ± 0.02	3.0 ± 0.2	0.99
Spermidine (3+)	0.078 ± 0.002	2.01 ± 0.12	0.99

as:

$$K_C = \frac{[\mathrm{HJ}_{\mathrm{open}}]c^n}{[\mathrm{HJ}_{\mathrm{closed}}]} = \frac{x_{\mathrm{open}}c^n}{x_{\mathrm{closed}}} \quad (6)$$

where c is the concentration of titrated cation and n, which is analagous to a Hill coefficient and is the observed stoichiometry of ion binding in Eq. 1, determines the steepness of transition.

If $c_{1/2}$ is defined as the switching ion concentration at which $x_{\mathrm{open}} = x_{\mathrm{closed}} = 1/2$ then Eq. 6 can further be written as:

$$K_C = c_{1/2}^n \quad (7)$$

By combining Eqs. 3 and 7, we obtain:

$$x_{\mathrm{open}} = \frac{c_{1/2}^n}{c^n + c_{1/2}^n} \quad (8)$$

$$x_{\mathrm{closed}} = \frac{c^n}{c^n + c_{1/2}^n} \quad (9)$$

Equations 8 and 9 can then be fitted to the experimental data by varying $c_{1/2}$ and n.

Time-resolved FRET of open and closed junction conformations

Samples were prepared as described previously.

Donor excitation was achieved using laser pulses of wavelength 468 nm, at a rate of 10 MHz, from a Picoquant picosecond diode laser head (model no. LDH-P-C-470). The beam polarisation was aligned vertically using an Edinburgh Instruments SB 900 Soleil-Babinet compensator. Fluorescence was collected at 90° to the excitation beam through a monochoromator and polariser set to the 'magic angle', 54.7°, to avoid anisotropy effects. Data was collected by the principle of time correlated single photon counting (TCSPC) using an Edinburgh Instruments FL 920 spectrometer.

Time-resolved fluorescenece emmission data for HJ in the open and closed conformations was collected with the emission monochromator set to 518 nm, corresponding to the peak emission of the donor fluorophore. Closed samples were prepared in 10 mM $MgCl_2$ (known, from steady state results, to be a regime in which the DNA junctions adopt the fully closed conformation).

For each measurement an appropriate instrument response function was recorded using a colloidal scattering sample with the emission monochromator set to the excitation laser wavelength. All data were collected to a peak of 10, 000 counts to ensure similar signal to noise on each measurement.

The data collected obey Eq. 10:

$$J(t) = i(t) \otimes I(t) \quad (10)$$

where $J(t)$ is the observed decay, $I(t)$ is the true decay and $i(t)$ is the appropriate instrument response function.

For analysis purposes, it is necessary to de-convolute $I(t)$ from $i(t)$ before fitting via Eq. 11:

$$I(t) = \sum_i \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \quad (11)$$

where α_i are fractional amplitudes of each lifetime and τ_i are donor lifetimes [13]. From these parameters an average lifetime, $\bar{\tau}$ can be defined by Eq. 12 [11]:

$$\bar{\tau} = \sum_i \alpha_i \tau_i \quad (12)$$

Time-resolved FRET characterisation of conformational changes

Time-resolved data were collected using an Edinburgh Instruments Fluorescence Lifetime Plate Reader for a number

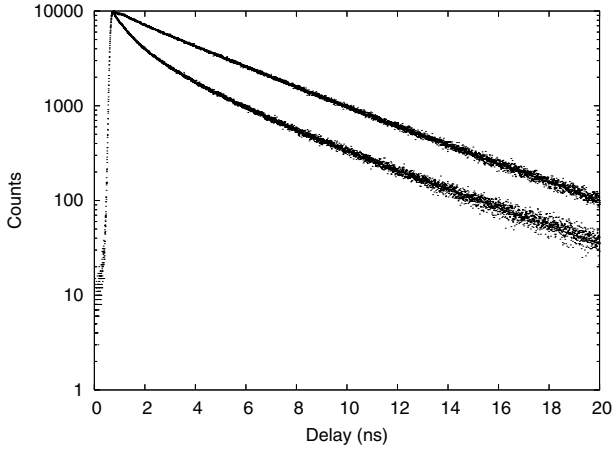

Fig. 4 Time-resolved fluorescence data for 'open' (top) and 'closed' HJ samples. The open sample has a clearly longer average lifetime than the closed. Re-convoluted fits to this data are shown by the solid lines, with details of the required parameters given in Table 2

Table 2 Parameters obtained from reconvolution fits to time-resolved fluorescence emission data obtained from 'open' and 'closed' HJ samples. Data for a donor-only molecule is also included

	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	$\bar{\tau}$ (ns)
Donor Only	0.667 ± 0.140	4.7	0.231 ± 0.105	2.8	0.103 ± 0.016	0.3	3.84 ± 0.73
Open	0.58 ± 0.09	4.5	0.31 ± 0.07	3.0	0.111 ± 0.013	0.4	3.6 ± 0.4
Closed	0.275 ± 0.006	4.0	0.325 ± 0.006	1.3	0.400 ± 0.011	0.3	1.66 ± 0.03

of DNA junction samples at varying cation concentration, with a number of different cations. 100 μl samples were dispensed in a BMG Labtech 96 well plate. Donor excitation was performed at 470 nm and time-resolved emission data were collected at 520 nm. Data were collected for 60 s per well, providing a sufficient number of counts to identify changes in donor lifetime between the open and closed conformations.

This data was fitted via a single exponential of the form of Eq. 11, with $i = 1$:

$$I(t) = \alpha \ \exp\left(\frac{-t}{\tau_{\text{app}}}\right) \tag{13}$$

where t_{app} is the apparent donor lifetime obtained from this fit. All fits are carried out from the peak of the donor fluorescence decay and can now be used to monitor changes in junction conformation, analagously to donor intensities in Eq. 4 for steady-state spectra.

FRET-FLIM of DNA junction conformation

A 5 μl droplet of HJ solution on a glass coverslip was excited at the donor wavelength as described previously. FLIM data was then collected using a LaVision PicoStar HR picosecond gated image intensifier gated at 600 ps intervals. Five images were averaged per interval with an exposure time of 500 ms per image.

FLIM data was collected for an HJ droplet in the open conformation and in the closed conformation after the addition of $MgCl_2$ solution to bring the Mg^{2+} ion concentration to 10 mM, the regime of the closed conformation. Each pixel was fitted to Eq. 13, where the intensity is now also a function of position on the image, $I(x, y, t)$ and the donor lifetime is now also a function of position, $\tau_{\text{app}}(x, y)$.

Results and discussion

Steady-state characterisation of DNA junction system

Figure 2 shows two typical spectra obtained under titration of an ionic solution. They show an increase in acceptor peak intensity at 580 nm at the expense of a reduction in donor peak intensity at 518 nm.

When the spectra are analysed according to the theory described above, good fits to the data (Fig. 3) are obtained, shown by both the quoted R^2 and visual inspection. The parameters of these fits, and those obtained similarly for other cations, are included in Table 1.

It has been reported that the junctions used here are prone to dissociation in the low salt conditions above [14], an observation confirmed on these samples using gel electrophoresis [15]. Similarly, it can also be shown that this is not an issue at the high salt conditions corresponding to the closed conformation. This is not a problem for demonstrating these solution based analysis techniques, although the use of these junctions in devices would require the structural integrity to be enhanced by, for example, increasing the junction arm length.

Time-resolved FRET of open and closed junction conformations

Figure 4 shows time-resolved fluorescence data and fits obtained for HJ samples in the open and closed

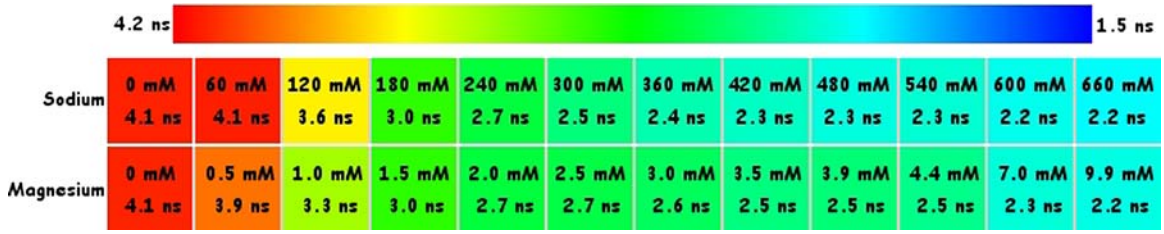

Fig. 5 Donor lifetimes observed for 1 μM HJ under the influence of Sodium and Magnesium. The concentration of each ion is indicated for each sample

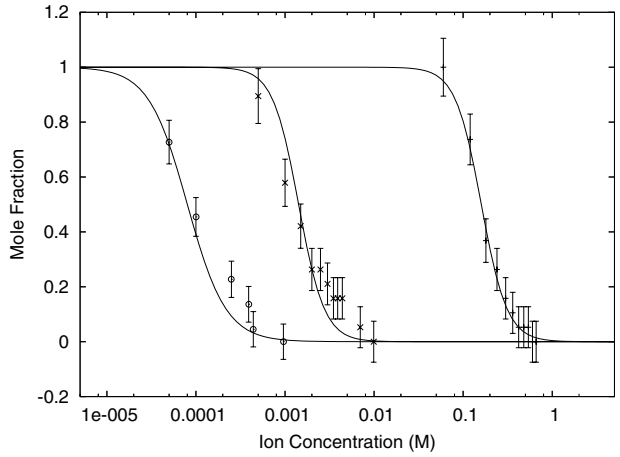

Fig. 6 Normalised open HJ concentration calculated from donor lifetimes of DNA junctions under the influence of (+) Na^+, (×) Mg^{2+} and (○) Spermidine (3+). These show agreement with steady state results indicated by the solid lines

conformations. The shapes of these data suggest that, in the open conformation, the donor spectrum is close to a single exponential. However, the spectrum from a closed HJ sample suggests significant contributions from shorter lifetimes. The re-convoluted fits to this data, shown in Table 2 support these observations; a triple exponential form is revealed with a reduction in all three lifetimes in the closed conformer compared to the open. This structure is also shown in results from a donor-only molecule. It has been observed previously [11] that the best fit to the fluorescence decay of the donor-only junction was obtained with multiple fluorescence lifetimes. As in this work, the major and longest lifetime was similar to the lifetime of free donor under the same conditions, and can therefore be attributed to dye in a solvent-rich environment. The two minor lifetimes were shorter, which indicates quenching in a small population of the donors due to dye-DNA and dye-linker interactions facilitated by free rotation about the flexible linker. The donor average lifetime ($\bar{\tau}$) in the closed conformation is reduced by 2 ns.

For the purposes of this work, the change in donor lifetime shown here is sufficient to identify changes in junction conformation, with a fractional change of ~ 0.5.

Time-resolved FRET characterisation of conformational changes

Figure 5 shows examples of results obtained using Edinburgh Instruments Fluorescence Lifetime Plate Reader. Examples are provided for coordinating ions of valency 1 and 2. The fractional change in apparent lifetime is similar to that for $\bar{\tau}$, allowing this quantity to be used to monitor changes in junction conformation.

The values of x_{open} obtained from these results are shown in Fig. 6, plotted alongside the results obtained from steady-state measurements. These show satisfactory agreement.

FRET-FLIM of DNA junction conformation

Figure 7 shows FLIM images of a 5 μl droplet of junction solution, originally in the open conformation and 'switched' with magnesium to the closed conformation. Donor lifetimes were calculated using single exponential fits to FLIM data, and as such should be compared to the lifetimes obtained for magnesium using a plate reader, which suggested a change in lifetime from 4.1 to 2.2 ns. The images presented show a lifetime magnitude and change which are in agreement.

Conclusions and comments

The use of time-resolved FRET emission data to monitor changes in the conformation of DNA junctions in solution has been investigated. Data collected for such samples showed clear contrast in donor emission lifetimes between the open and closed conformers, with an average lifetime lower in the closed conformer than in the open conformer due to increased FRET.

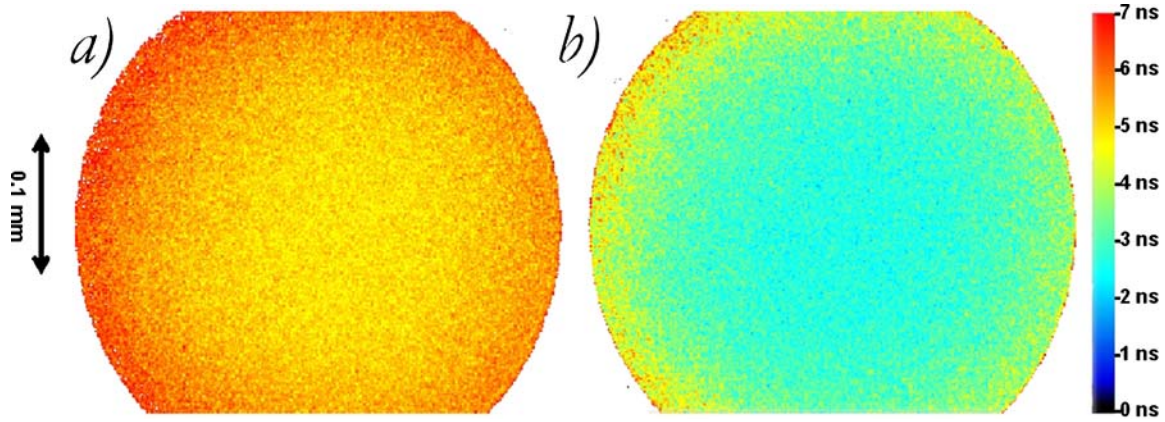

Fig. 7 FLIM images of HJ droplets in both the open configuration (a) and closed using Mg^{2+} ions (b), where the lifetimes indicated on the scale are in ns

Time-resolved donor emission data obtained for HJ samples under the influence of a number of different cations showed agreement with results obtained from steady-state measurements when using a simple single-exponential fit to the time-resolved data. A fractional change in apparent donor emission lifetime between the open and closed conformations of $\sim 50\%$ was observed.

FRET-FLIM data from droplets of open and closed DNA junction samples showed significant contrast between the two states, with an observed lifetime change of similar magnitude to those obtained using time-resolved fluorescence spectroscopy.

This work validates the use of fluorescence emission lifetime spectroscopy techniques to monitor changes in conformation of nanoscale DNA junctions in solution. The use of FLIM in the analysis of protein solutions spotted on surfaces has been reported [16] and this is complemented by the results presented here using a lifetime plate reader and FLIM-FRET. These promising results present the feasibility of using time-resolved fluorescence spectroscopy as a tool for high throughput analysis of DNA assays based on similar DNA junction structures, for which FLIM-FRET is likely to be particularly useful.

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