

Communication

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# A Highly Fluorescent Nucleobase Molecular Rotor

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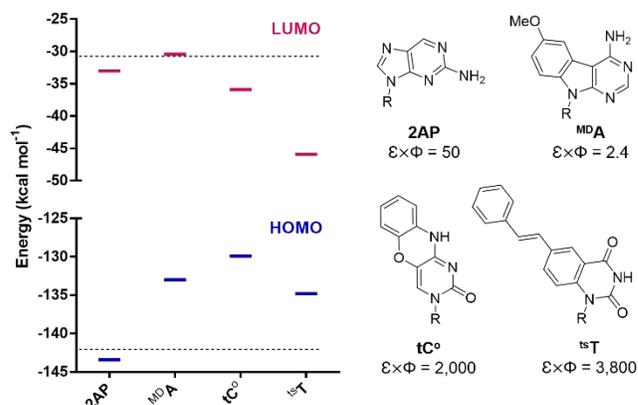
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## Supporting Information Placeholder

**ABSTRACT:** Fluorescent base analogs (FBAs) are powerful probes of nucleic acids' structures and dynamics. However, previously reported FBAs exhibit relatively low brightness and therefore limited sensitivity of detection. Here we report the hitherto brightest FBA that has ideal molecular rotor properties for detecting local dynamic motions associated with base pair mismatches. The new *trans*-stilbene annulated uracil derivative "tsT" exhibits bright fluorescence emissions in various solvents ( $\epsilon \times \Phi = 3,400 - 29,700 \text{ cm}^{-1} \text{ M}^{-1}$ ) and is highly sensitive to mechanical motions in duplex DNA ( $\epsilon \times \Phi = 150 - 4,250 \text{ cm}^{-1} \text{ M}^{-1}$ ). tsT is thereby a "smart" thymidine analog, exhibiting a 28-fold brighter fluorescence intensity when base paired with A as compared to T or C. Time-correlated single photon counting revealed that the fluorescence lifetime of tsT ( $\tau = 4 - 11 \text{ ns}$ ) was shorter than its anisotropy decay in well-matched duplex DNA ( $\theta = 20 \text{ ns}$ ), yet longer than the dynamic motions of base pair mismatches (0.1 - 10 ns). These properties enable unprecedented sensitivity in detecting local dynamics of nucleic acids.

Fluorescent nucleobase analogs (FBAs) provide powerful tools for probing nucleic acids' structures, dynamics and binding interactions.<sup>1,2</sup> Compared to conjugated fluorophores and intercalating dyes, FBAs have the advantage of highly precise positioning within RNA and DNA structures. However, fluorescence quenching of FBAs by neighboring residues via photo-induced electron transfer (PET) can dramatically limit their brightness.<sup>3-6</sup> For example, small electron-poor systems such as 2-aminopurine (2AP) are reductively quenched by purines,<sup>7</sup> and electron-rich systems such as <sup>MDA</sup> are oxidatively quenched by pyrimidines.<sup>8</sup> The brightest, previously reported fluorescent nucleobase analogs, such as tC<sup>o</sup> ( $\epsilon \times \Phi \approx 2,000 \text{ cm}^{-1} \text{ M}^{-1}$ ),<sup>9,10</sup> avoid PET quenching by having HOMO-LUMO energy levels that are intermediate between the HOMO of guanine and the LUMO of thymidine (Figure 1 and Figure S1). However, the fluorescence intensity of tC<sup>o</sup> and related analogs exhibit little-to-no environmental sensitivity and therefore have limited utility as reporters of base pair mismatches.<sup>11,12</sup> Given the growing clinical interest in point-of-care detection of single nucleotide polymorphisms (SNPs),<sup>13</sup> there have been numerous efforts towards the development of SNP sensors based on FBA-mismatch detection.<sup>8,14-27</sup> However, these

attempts have thus far resulted in either bright FBAs with low sensitivity towards SNPs,<sup>15-19</sup> or highly sensitive FBAs with low brightness.<sup>8,20,21</sup> Here we present the brightest and most sensitive FBA-mismatch reporter to date. The *trans*-stilbene-containing thymidine analog "tsT" acts as an ideal molecular rotor for SNP detection,<sup>22-27</sup> with a fluorescent lifetime ( $\tau = 4 - 11 \text{ ns}$ ) that is slightly longer as compared to timescale of local dynamic motions of base pair mismatches (0.1 - 10 ns).<sup>28-30</sup> This provides unprecedented sensitivity and specificity for detecting a single adenine residue in the opposing strand, with a ~20-fold brighter fluorescence intensity for matched "A" ( $\epsilon \times \Phi = 3,000 - 4,250 \text{ cm}^{-1} \text{ M}^{-1}$ ) versus mismatched "C, T, and G" bases ( $\epsilon \times \Phi = 150 - 520 \text{ cm}^{-1} \text{ M}^{-1}$ ).

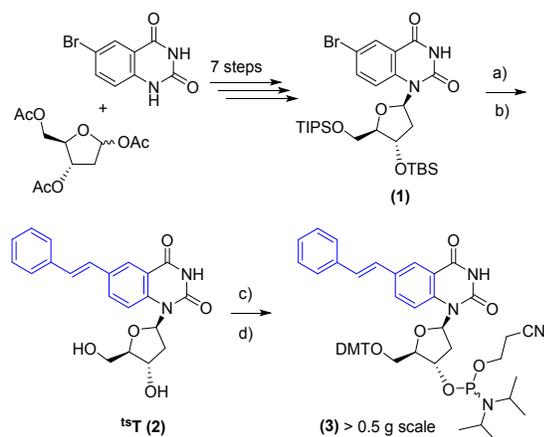


**Figure 1.** Average brightness of selected FBAs in well-matched duplex DNA ( $\text{cm}^{-1} \text{ M}^{-1}$ ) and their calculated HOMO-LUMO energy levels ( $\text{kcal mol}^{-1}$ ). DFT calculations (where R = Me) were performed using B3LYP/6-31+G(d) in water (see Table S1 and Figure S1 for details). The dashed lines represent the LUMO level of the most easily reduced nucleobase (T), and the HOMO level of the most easily oxidized base (G).<sup>31</sup>

Design of the new fluorescent thymidine analog "tsT" was inspired by the excellent base pairing specificities of 6-substituted quinazolines,<sup>19,32-34</sup> together with the desirable photophysical properties of *trans*-stilbene that include viscosity-sensitive emissions.<sup>35</sup> Density Functional Theory (DFT) calculations predicted that a ring-fused, uracil-*trans*-stilbene derivative "tsT" should not undergo fluorescence

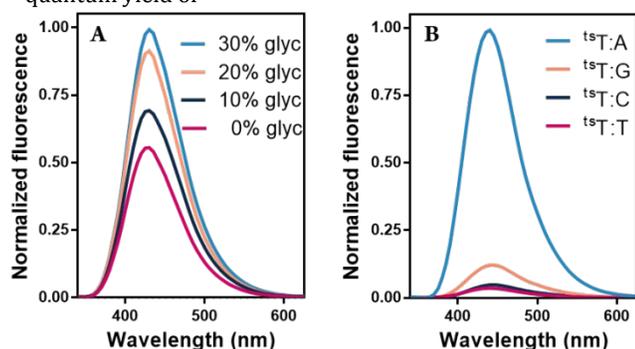
quenching via PET (Figure 1). We therefore commenced synthesis of <sup>ts</sup>T by preparing 6-bromo-quinazoline-2,4-(3H)-dione nucleoside **1** as a single diastereoisomer on a multi-gram scale in a 46% overall yield (7 steps) according to our previously established protocol.<sup>36</sup> Suzuki-Miyaura coupling, followed by silyl deprotection furnished <sup>ts</sup>T nucleoside **2** in a 92% isolated yield over 2 steps (Scheme 1 and Scheme S1).

### Scheme 1. Synthesis of <sup>ts</sup>T nucleoside (**2**) and <sup>ts</sup>T phosphoramidite (**3**)<sup>a,b</sup>.



<sup>a</sup> Reagents and conditions: (a) *trans*-2-phenylvinyl boronic acid pinacol ester (2.1 equiv.), Cs<sub>2</sub>CO<sub>3</sub> (3.2 equiv.), Pd(dppf)Cl<sub>2</sub>, 1,4-dioxane, Ar, 100 °C, 24 h, (98%). (b) TBAF (4.9 equiv.), THF, 25 °C, 22 h, (94%). (c) DMTCl (1.4 equiv.), pyridine, Ar, 25 °C, 165 min, (68%). (d) 2-cyanoethyl N, N-diisopropyl chlorophosphoramidite (4.0 equiv.), DIPEA (5.0 equiv.), CH<sub>2</sub>Cl<sub>2</sub>, Ar, 0 to 25 °C, 150 min, (90%). TIPS = triisopropylsilyl, TBS = *tert*-butyldimethylsilyl, dppf = 1,1'-Bis(diphenylphosphino)ferrocene, TBAF = tetra-*n*-butylammoniumfluoride, THF = tetrahydrofuran, DMTCl = 4,4'-dimethoxytriphenylmethyl chloride, DIPEA = *N,N*-diisopropylethylamine. <sup>b</sup> See the SI for synthetic details and

As compared to *trans*-stilbene itself ( $\Phi = 0.02 - 0.04$ ), the <sup>ts</sup>T nucleoside **2** exhibits a similar extinction coefficient ( $\epsilon_{310\text{nm}} = 36,500 \pm 5,600 \text{ cm}^{-1} \text{ M}^{-1}$ ), yet much larger quantum yield ( $\Phi = 0.11 - 0.75$ ) in various solvents (Table S2). Decreasing quantum yields and increasing Stokes' shifts of nucleoside **2** were observed with increasing solvent polarity (Figure S2). These results are consistent with formation of a twisted intramolecular charge transfer (TICT) excited state leading to non-radiative decay.<sup>37,38</sup> To further evaluate the potential of <sup>ts</sup>T as a fluorescent molecular rotor, the absorption and fluorescence emission of nucleoside **2** was measured in various mixtures of methanol and glycerol. These solvents have very similar polarities yet drastically different viscosities. The quantum yield of



**Figure 2.** (A) Fluorescence of <sup>ts</sup>T nucleoside **2** in mixtures of methanol and glycerol; and (B) Fluorescence of <sup>ts</sup>T functionalized duplex DNA (ODN1) containing matched or mismatched base pairs in PBS buffer (pH = 7.4). All emission spectra were collected using an excitation wavelength of 310

nm. <sup>ts</sup>T increased with increasing viscosity (Figure 2A and Figure S3), consistent with rotation-induced fluorescent quenching. Encouraged by these results, we synthesized <sup>ts</sup>T phosphoramidite **3** for its incorporation into DNA. Nucleoside **2** was treated with DMTCl and chlorophosphoramidite to obtain >500 mg of pure <sup>ts</sup>T phosphoramidite **3**. The entire 11-step sequence required only 7 chromatographic separations and provided **3** in a 26% overall isolated yield.

Using standard, solid-phase supported synthesis,<sup>39</sup> <sup>ts</sup>T was incorporated into three different oligonucleotide DNAs (ODN1 – ODN3). Following their purification and annealing to complementary strands, canonical B-form secondary structures were observed using circular dichroism (Figure S6). The maximum absorbance of <sup>ts</sup>T remains very strong in DNA ( $\epsilon_{310\text{nm}} = 30,600 \pm 700 \text{ cm}^{-1} \text{ M}^{-1}$ ) and sufficiently red shifted to have no overlap with the natural bases (Fig S7A). The brightness of the three duplexes containing a <sup>ts</sup>T:A base pair ( $\epsilon \times \Phi = 3,000 - 4,250 \text{ cm}^{-1} \text{ M}^{-1}$ ) were about the same as the free nucleoside **2** in PBS buffer ( $3,420 \text{ cm}^{-1} \text{ M}^{-1}$ ), consistent with a lack of <sup>ts</sup>T fluorescence quenching by neighboring residues via PET. Thermal denaturation experiments revealed that <sup>ts</sup>T exhibits similar base pairing specificity as thymidine,<sup>40</sup> with the temperature of ODN1 duplex melting ( $T_m$ ) being <sup>ts</sup>T:C/T < <sup>ts</sup>T:G < <sup>ts</sup>T:A (Table 1 and Figure S8). The brightness of <sup>ts</sup>T was much more sensitive to this same trend, with  $\epsilon \times \Phi$  values ranging from 150 – 4,250  $\text{cm}^{-1} \text{ M}^{-1}$  for <sup>ts</sup>T:C/T < <sup>ts</sup>T:G << <sup>ts</sup>T:A (Table 1, Figure 2B and Figure S7B). Similar trends were also observed for the relative broadness of thymidine imino proton resonances in <sup>1</sup>H NMR spectra of T:G and T:T base pairs in duplex DNA.<sup>41</sup> Together these results suggested that fluorescence quenching of <sup>ts</sup>T is related to increased molecular motions of mismatched <sup>ts</sup>T:C/T base pairs over both <sup>ts</sup>T:G (wobble) and <sup>ts</sup>T:A base pairs.

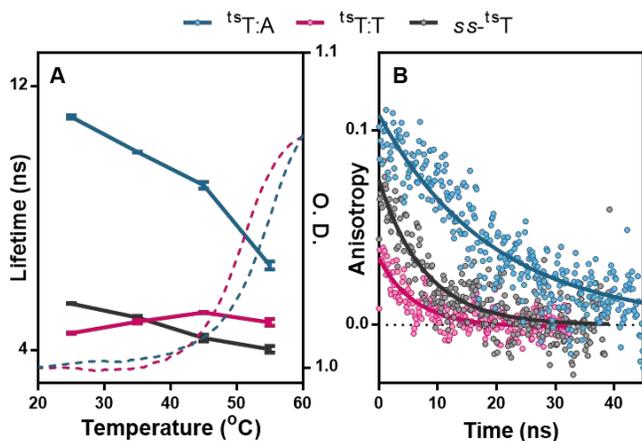
**Table 1: Fluorescence quantum yield ( $\Phi$ ), brightness ( $\epsilon \times \Phi$ ) and melting temperature ( $T_m$ ) of double-stranded or single-stranded (ss) DNA.<sup>a</sup>**

Oligonucleotide	$\Phi$	$\epsilon \times \Phi$ ( $\text{cm}^{-1} \text{ M}^{-1}$ )	$T_m$ (°C)
<sup>ts</sup> T:A	0.139	4,250	55.0 <sup>c</sup>
<sup>ts</sup> T:G	0.017	520	53.0
<b>ODN1<sup>b</sup></b>			
<sup>ts</sup> T:C	0.005	150	51.0
<sup>ts</sup> T:T	0.005	150	50.8
ss- <sup>ts</sup> T	0.028	860	-
<sup>ts</sup> T:A	0.098	3,000	60.2 <sup>d</sup>
<b>ODN2<sup>b</sup></b>			
<sup>ts</sup> T:T	0.012	370	56.1
ss- <sup>ts</sup> T	0.020	610	-
<sup>ts</sup> T:A	0.134	4,100	54.1 <sup>e</sup>
<b>ODN3<sup>b</sup></b>			
ss- <sup>ts</sup> T	0.026	800	-

<sup>a</sup> All measurements were performed at 25 °C in PBS buffer (pH = 7.4), with DNA concentrations = 2.0  $\mu\text{M}$ . <sup>b</sup> ODN1 sequence: 5'-GCCGTA<sup>ts</sup>TCGTATACAC-3'. ODN2 sequence: 5'-GCCGTA<sup>ts</sup>TGCGTATACAC-3'. ODN3 sequence: 5'-GCCGTA<sup>ts</sup>TATGTATACAC-3'. <sup>c</sup>  $T_m$  of the corresponding unmodified duplex = 49.7 °C. <sup>d</sup>  $T_m$  of the unmodified duplex DNA = 56.3 °C. <sup>e</sup>  $T_m$  of the unmodified duplex DNA = 49.3 °C.

To gain insight into the relationships between  ${}^{\text{ts}}\text{T}$  dynamic motions and brightness in DNA, time-correlated single photon counting (TCSPC) experiments were conducted using  $ds\text{-}{}^{\text{ts}}\text{T:A}$ .<sup>a</sup> All measurements were performed using 2.0  $\mu\text{M}$  of DNA in PBS buffer (pH = 7.4). All values reflect averages and standard deviations of three measurements.<sup>b</sup> See Table S5 for details. <sup>c</sup> Calculated from  $E\theta$  (Eq. S5). <sup>d</sup> Value reflects local dynamics, not  $R_{\text{H}}$ .

${}^{\text{ts}}\text{T:T}$  (4.5 ns), reflecting the same trends as their steady-state brightness. Upon heating each sample over a “pre-melting” temperature range of 25  $^{\circ}\text{C}$   $\rightarrow$  45  $^{\circ}\text{C}$  where no global melting of the duplexes was observed, the  $\tau$  values of  ${}^{\text{ts}}\text{T}$  decreased in a linear fashion for both  $ds\text{-}{}^{\text{ts}}\text{T:A}$  (-0.11 ns per  $^{\circ}\text{C}$ ) and  $ss\text{-}{}^{\text{ts}}\text{T}$  (-0.05 ns per  $^{\circ}\text{C}$ , Figure 3A). These results are consistent with increased “breathing” motions of nucleobases with increasing temperatures.<sup>42</sup> Surprisingly, the  $\tau$  values of  $ds\text{-}{}^{\text{ts}}\text{T:T}$  increased over this same temperature range (+0.03 ns per  $^{\circ}\text{C}$ ), suggesting increasing rigidity of the mismatch with increasing temperature. This result is consistent with the higher affinity and positive entropy change exhibited by T:T mismatches for Hg<sup>II</sup> binding over these temperatures.<sup>43,44</sup> At 55  $^{\circ}\text{C}$  the  $\tau$  values for all three samples neared convergence due to thermal melting of the duplexes (Figure 3A).



**Figure 3.** (A) Temperature-dependent fluorescence lifetime values (averaged values of three measurements with standard errors connected by solid lines) compared to global thermal melting according to absorbance changes (O.D., dashed lines) at 260 nm. (B) Time-resolved fluorescence anisotropy of  $ss\text{-}{}^{\text{ts}}\text{T}$ ,  $ds\text{-}{}^{\text{ts}}\text{T:T}$  and  $ds\text{-}{}^{\text{ts}}\text{T:A}$  (ODN1) at 25  $^{\circ}\text{C}$ . All measurements were conducted in PBS buffer (pH = 7.4).

**Table 2. Fluorescence lifetime ( $\tau$ ), anisotropy decay ( $\theta$ ), and hydrodynamic radius ( $R_{\text{H}}$ ) of ODN1 double-stranded or single-stranded (ss).<sup>a, b</sup>**

	T ( $^{\circ}\text{C}$ )	$\tau$ (ns)	$\theta$ (ns)	$R_{\text{H}}$ (nm) <sup>c</sup>
${}^{\text{ts}}\text{T:A}$	25	$11.1 \pm 0.1$	$20 \pm 4.2$	$2.70 \pm 0.18$
	35	$10.0 \pm 0.1$	$19 \pm 3.8$	$2.68 \pm 0.18$
	55	$6.6 \pm 0.1$	$8.5 \pm 2.8$	$2.09 \pm 0.22$
${}^{\text{ts}}\text{T:T}$	25	$4.5 \pm 0.1$	$5.3 \pm 1.6$	$1.74 \pm 0.17^{\text{d}}$

$ss\text{-}{}^{\text{ts}}\text{T}$	25	$5.4 \pm 0.1$	$7.9 \pm 1.7$	$1.98 \pm 0.14$
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To evaluate local and global dynamic motions, TCSPC experiments were used to measure time-resolved fluorescence anisotropy decay ( $\theta$ , Figure 3B, Eq. S4). At 25  $^{\circ}\text{C}$ , the  $\theta$  values of  ${}^{\text{ts}}\text{T}$  in DNA followed the same trends as steady-state quantum yields and fluorescence lifetime measurements, where  $ds\text{-}{}^{\text{ts}}\text{T:A}$  ( $\theta = 20$  ns)  $\gg$   $ss\text{-}{}^{\text{ts}}\text{T}$  ( $\theta = 7.9$  ns)  $>$   $ds\text{-}{}^{\text{ts}}\text{T:T}$  ( $\theta = 5.3$  ns). The  $\theta$  values of  $ds\text{-}{}^{\text{ts}}\text{T:A}$  at 25  $^{\circ}\text{C}$  and 35  $^{\circ}\text{C}$  were essentially identical ( $\theta = 19 - 20$  ns, Table 2 and Figure S10), suggesting that the  $\theta$  values are reflecting global motions of the duplex at these temperatures. We therefore used the  $\theta$  value at 25  $^{\circ}\text{C}$  to calculate the hydrodynamic radius of the duplex ( $R_{\text{H}}$ , Eq. S5), giving  $R_{\text{H}} = 2.70 \pm 0.18$  nm (Table 2). This value is in full agreement with duplex DNA  $R_{\text{H}}$  values previously measured using fluorescence anisotropy, sedimentation velocity and Monte Carlo simulations.<sup>9,45,46</sup> At 55  $^{\circ}\text{C}$  the  $\theta$  of  $ds\text{-}{}^{\text{ts}}\text{T:A}$  (8.5 ns) approached that of  $ss\text{-}{}^{\text{ts}}\text{T}$  at 25  $^{\circ}\text{C}$  (7.9 ns). These values are consistent with the conversion of a “rod-like” duplex ( $R_{\text{H}} = 2.70$  nm) into a “worm-like” chain of ssDNA ( $R_{\text{H}} = 2.09$  nm, Table 2).<sup>47,48</sup> The close agreements between our results obtained using  ${}^{\text{ts}}\text{T}$  with the global physical properties of ssDNA,<sup>49,50</sup> suggest that ssDNA contains partially-ordered nucleobases with correlation times dominated by concerted, global motions of the entire molecule. In contrast, the  $\theta$  for mismatched, duplex  $ds\text{-}{}^{\text{ts}}\text{T:T}$  (5.3 ns) primarily reflects rapid, local dynamics, since this value is much lower than that of  $ds\text{-}{}^{\text{ts}}\text{T:A}$  (20 ns), and it is even lower than  $ss\text{-}{}^{\text{ts}}\text{T}$  at 25  $^{\circ}\text{C}$  (7.9 ns). These results are consistent with previous NMR measurements and molecular dynamics simulations suggesting the presence of rapid, local motions of base pair mismatches in otherwise canonical duplexes.<sup>28</sup> To our knowledge this is the first time that an FBA has been used to determine the hydrodynamic radii of single-stranded DNA, and our results suggest that the dynamic motions of a base pair mismatch in duplex DNA are even greater than those present in single-stranded DNA.

In summary,  ${}^{\text{ts}}\text{T}$  is the brightest and most environment sensitive FBA reported to date ( $\epsilon \times \Phi = 150 - 29,700$   $\text{cm}^{-1} \text{M}^{-1}$ ). The fluorescence lifetime of  ${}^{\text{ts}}\text{T}$  ( $\tau = 4 - 11$  ns) is longer than the time scale of local dynamic motions of base pair mismatches (0.1 - 10 ns),<sup>28-30</sup> yet faster than the global anisotropy decay of  ${}^{\text{ts}}\text{T}$  in duplex DNA ( $\theta = 20$  ns). These properties together enable unprecedented sensitivity and specificity in the detection of an adenine residue in the complement strand. In addition to highly sensitive base pair match/mismatch discrimination,  ${}^{\text{ts}}\text{T}$  has provided insights into the fundamental dynamic behavior of duplex and single-stranded DNA using TCSPC experiments. The exceptional brightness and sensitivity of  ${}^{\text{ts}}\text{T}$  may enable other types of demanding applications, such as tracking of single-molecule dynamics.<sup>51</sup>

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Description of materials and methods, computational details, synthetic procedures, compounds characterization and NMR spectra, circular

dichroism (CD), thermal denaturation (T<sub>m</sub>) and fluorescence spectra, Scheme S1, Figures S1-S10 and Tables S1-S5.

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### Notes

The authors declare no competing financial interests.

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