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Nanometer-scale Fluorescence Resonance Optical Waveguides

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ABSTRACT

The telecommunications revolution has created a strong motivation to build photonic devices of ever smaller size and higher density. Using photosynthetic structures found in nature as an inspiration, we synthesized artificial structures that act like diffusive waveguides. These waveguides use FRET to transport energy, and we demonstrated the idea with 3- and 5-fluorophore structures which utilize DNA as a scaffold. A quantitative model that explains the results and provides the mechanism behind the energy transfer is also presented.

Most man-made photonic devices for optical wave guiding are based on the classical physics of bulk materials: Maxwell's equations allow propagating modes in the far field, and the wavelength of light imposes a fundamental lower limit on device size.[1−3] However, Nature has evolved several examples of photonic nanostructures to guide light over much smaller length scales for "light harvesting" in plants and photosynthetic bacteria.[4−5] This solution is fundamentally quantum mechanical and is related to fluorescence resonance energy transfer (FRET).[6−10] FRET is a near field dipole−dipole interaction that involves radiationless transfer of energy between two molecules in close proximity[11] and is commonly used to measure small distances.[12] Taking inspiration from photosynthetic light harvesting systems, we designed an artificial optical diffusive waveguide with a DNA backbone[13] of dimensions 14 nm by 3 nm. Optical energy is selectively injected into one end of the molecule and its exit is detected at the other; this represents the first step in building more complicated photonic devices using biological molecules and novel optical design principles.[14−17]

Previous work with multiple FRET interactions has focused on cascades of three different fluorophores,[18−19] in which the emission of each fluorophore in the cascade is matched with the absorption of the next. These have fundamental disadvantages in that they suffer significant energy loss with each transfer, since each heterogeneous FRET event creates a large red shift. This is difficult to generalize, and indeed prevents the design of waveguides. Here, we take advantage of the fact that some fluorophores can efficiently transfer energy nonradiatively among themselves. If the transfer efficiency (η) of these homogeneous FRET events is high enough, energy can be transported through many of these repeating units and will propagate diffusively along the waveguide. In this scheme energy is injected into the waveguide via an input fluorophore, is transported using a repeating fluorophore unit, and its propagation measured by an output fluorophore. Independently, Ohya et al.[20,21] have used similar ideas to investigate energy transfer in DNA molecules labeled with four fluorophores; they showed that energy from a donor to an acceptor could be passed through two mediator molecules of the same species in the case for which all molecules are arranged to be in phase with respect to the DNA helical period. Here we show that with a different choice of fluorophores it is possible to incorporate up to five fluorophores into nanoscale waveguides, have made the first measurement of transfer efficiency of the intermediary fluorophores in any waveguide, and have demonstrated a more general labeling geometry than used previously. Furthermore, we introduce a model that allows rigorous calculation of energy diffusion in these structures; earlier models considered only rectified energy transfer in cascades.

To inject light into the waveguide, we use FRET from the fluorophore 6-FAM [6-carboxyfluorescein] (F). TAMRA [6-tetramethylrhodamine-5(6)-carboxamide] (T) was used as the repeating element to transfer the energy within the waveguide, and Cy5 (C) for output energy detection (Figure 1). The fluorophores were spaced to achieve a compromise between optimum efficiency and minimizing FRET transfers that skip a fluorophore position,[22] and were placed both in phase and out of phase with respect to the helical periodicity of the DNA. The spacing between fluorophores is about 10 base pairs; the actual distance is also affected by possible location on opposing strands and by the length of linkers.

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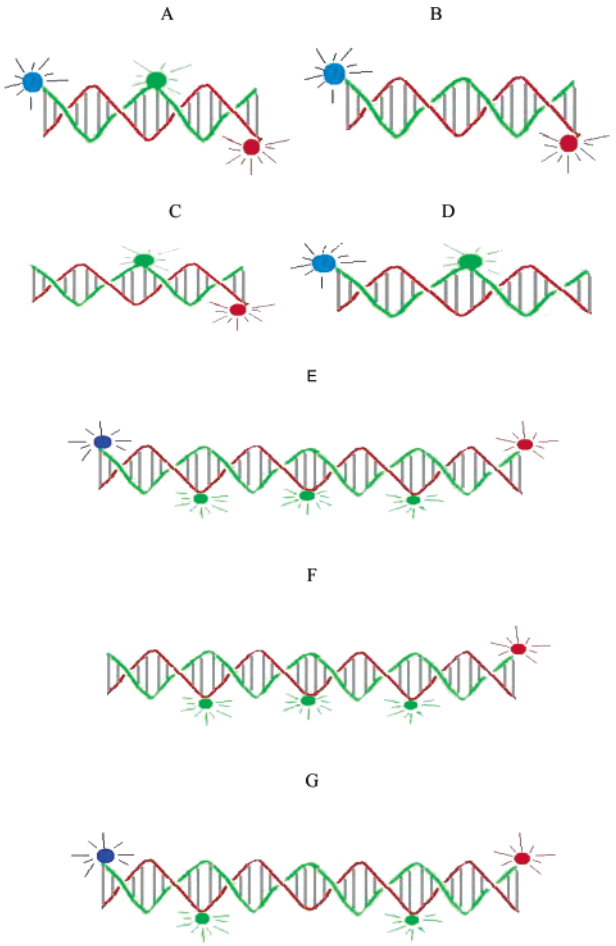

Figure 1. Location of fluorophores. Cartoon of Bi−FRET molecules (A) FTC, (B) F_C, (C) _TC, (D) FT_, and Cartoon of Quad-FRET molecules (D) FTTTC, (E) _TTTC, and (F) FT_TC where circles colored blue = F (6-FAM), green = T (TAMRA), red = C (Cy5). The complimentary strands of DNA are shown in green and red. The figures are not to scale.

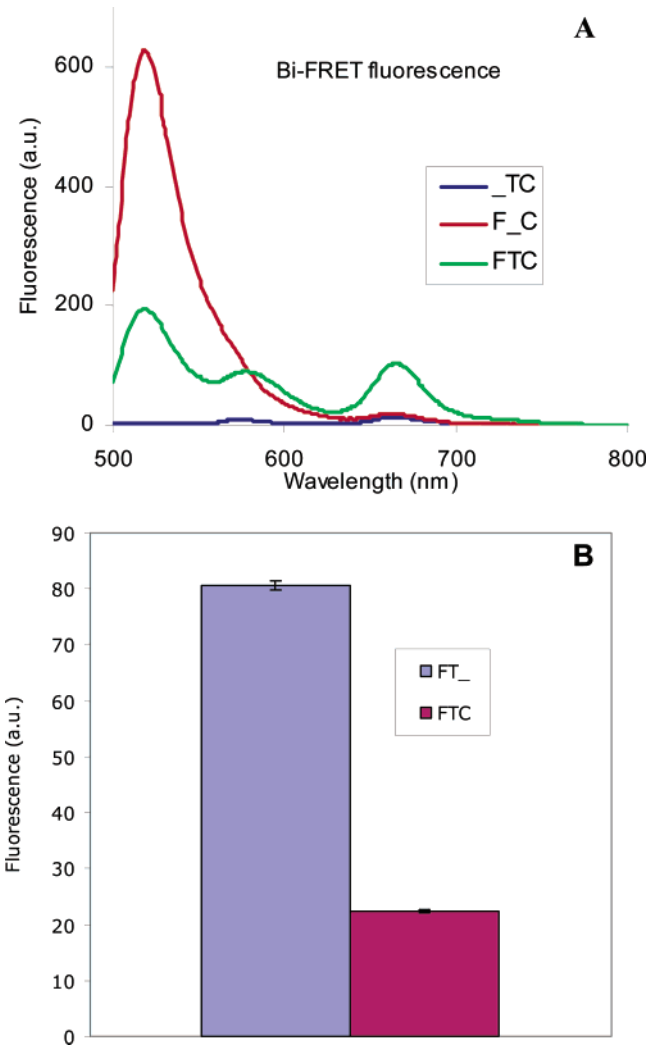

Figure 2. Bi-FRET results. (A) Bi−FRET fluorescence emission spectra at 480 nm excitation. There are peaks at three wavelengths which represent the emission maxima for the three fluorophores in the order FAM, TAMRA, and Cy5, respectively (from left to right). There is an increase in the fluorescence of FAM when the TAMRA is absent. The graph shows a prominent peak in FTC spectra from the Cy5 fluorescence that is absent in F_C and _TC. (B) TAMRA fluorescence from FTC and FT_. Each bar in the graph was made using eight trial measurements. A curve fitting procedure was used to extract the TAMRA fluorescence from emission spectra after normalizing for molecular concentration. There is a considerable drop in TAMRA fluorescence when the Cy5 is present, indicating efficient energy transfer.

Under optimal conditions the FRET process can be nearly 100% efficient. The DNA sequences, double stranded for stiffness, were chosen to minimize hairpins. The excitation wavelength (480 nm) is a compromise between efficient excitation of FAM and minimizing direct excitation of TAMRA and Cy5.

The first series of experiments involved molecules having up to 3 fluorophores. These molecules are 19 base pairs long and called Bi−FRET molecules since they were intended to test double energy transfer events. We synthesized the molecule FTC, which has FAM and TAMRA on one strand and Cy5 on the other (Figure 1A). Other molecules synthesized for control experiments were F_C, _TC and FT_, where a blank indicates no fluorophore at that position (Figure 1B, 1C, 1D). When FTC is illuminated with a wavelength that primarily excites FAM, the fluorescence shows a pronounced peak in the emission spectrum of Cy5, as one would expect from two successive FRET events. To eliminate the possibility that TAMRA or Cy5 are excited directly, control experiments with _TC were performed, and in order to show that the energy did not transfer directly from FAM to Cy5, control experiments with F_C were performed. The spectra from these experiments can be seen in Figure 2A. Further experiments were performed with FTC and FT_. These experiments involved annealing single-stranded DNA having FAM and TAMRA attached with an excess of the complementary strand (having no labels or Cy5, respectively). TAMRA fluorescence was normalized using the fluorescence from FAM. We used a least-squares curve fitting procedure to extract the contribution of TAMRA to the fluorescence spectra (see Methods); the results are summarized in Figure 2B. The drop in fluorescence of TAMRA is clearly observed when Cy5 is located near it. We did not find significant difference in the FAM fluorescence for the two molecules. This experiment enables us to calculate the TAMRA-to-Cy5

efficiency (η^{bf}_{TC}) and provides more evidence that the energy transfer occurs from FAM to Cy5 via the TAMRA intermediary.

The Bi−FRET experiments demonstrate the feasibility of injecting and detecting light using FRET. To create an actual waveguide, one needs to repeat the intermediary waveguide fluorophore. We worked next with molecules having as many as 5 fluorophores (Quad-FRET).[23] These molecules were designed such that one of the DNA strands had the injector and detector fluorophores, while the complementary strand had the repeating waveguide fluorophores (Figure 1E, 1F, 1G). For this molecule, we expect that the energy can move both forward and backward on the T fluorophores. Excitation of the FTTTC molecule at 480 nm showed energy transfer from FAM to Cy5. As a control we used the molecule _TTTC, where we saw significantly lower fluorescence from the Cy5. We also did experiments with FT_TC, which revealed spectra similar to that of FTTTC but with a drop in the fluorescence of the Cy5 (Figure 3A). We find that in all these molecules there is a small shift of <5 nm in the emission spectra of FAM, which has also been observed by others.[24]

To more carefully analyze this system we quantitated the fluorescence of Cy5 while exciting Quad-FRET molecules at 480 nm. Figure 3B shows the collected results from 49 experiments. The error bars in the graph are mostly due to uncertainty in molecular concentrations. Cy5 is not significantly directly excited at 480 nm (confirmed by absorption spectra), and so the fluorescence must come from either FRET transfers from FAM to Cy5 via the three TAMRA molecules or to a smaller extent from the directly excited TAMRA to Cy5. The fluorescence from _TTTC is due to a small amount of direct excitation of TAMRA at the 480 nm excitation used. A drop in the fluorescence of the Cy5 is clearly seen when one of the TAMRA molecules is missing. Moreover, in molecules without the injection fluorophore there is again a reduction in the fluorescence of Cy5− agreeing with our model of the process.

The data are consistent with quantitative calculations using a simple model[24] based on energy balance. We may write for each excited fluorophore species X_n:

$$\frac{dX_n}{dt} = \epsilon_n \Gamma - k_n X_n + \sum_{m=\text{donor}} k_{mn} X_m - \sum_{o=\text{acceptor}} k_{no} X_n$$

where ϵ_n = the extinction coefficient at the excitation wavelength, Γ = constant factor depending on excitation light and the geometry used, k_n = radiative and all other non-FRET decay rates (intersystem crossing, quenching, internal conversion) and k_{mn} = FRET transfer from m (donor) to n (acceptor). $\Gamma\epsilon_n$ gives the number of excited fluorophore species created per unit time by excitation light. The model takes into account the possibility of forward and backward energy transfer and can be used to determine steady-state emission rates and transfer efficiencies for arbitrary combinations of fluorophores without resorting to time dependent measurements (details in Supporting Information).

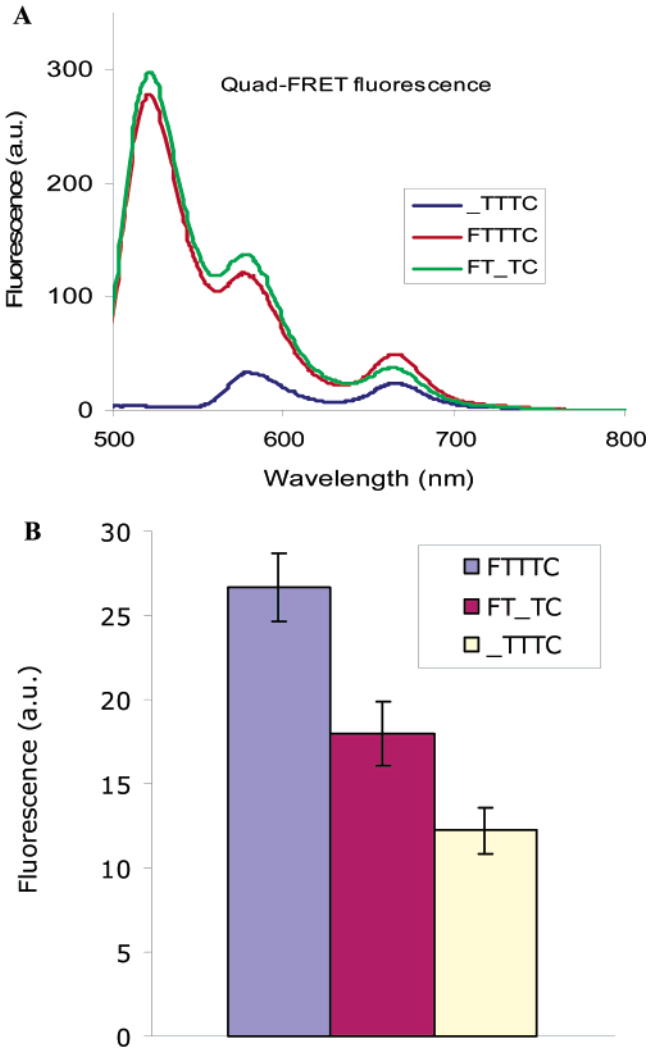

Figure 3. Quad-FRET results. (A) The fluorescence from 40-mer DNA molecules at 480 nm excitation. The peaks in fluorescence at three wavelengths are due the emission maxima of the three fluorophores used (FAM, TAMRA, and Cy5, respectively, from left to right). B) The fluorescence from Cy5 in Quad-FRET molecules. The graph was made from 49 separate trials (FTTTC, 19; FT_TC, 18; _TTTC, 12). The fluorescence of the Cy5 was extracted from emission spectra by curve fitting. The fluorescence of molecules with one TAMRA missing or FAM missing is smaller than the case where all the five fluorophores are present on the molecule.

Assuming steady state and using the model (see Supporting Information) in the Bi−FRET case, we calculate the TAMRA to Cy5 efficiency $\eta^{bf}_{TC} = 0.72 \pm 0.03$ and FAM to TAMRA efficiency $\eta^{bf}_{FT} = 0.69 \pm 0.07$. This means in the Bi−FRET about 40% of the excitation that the FAM absorbs ultimately reaches the Cy5. Multiplying this by the quantum yield of Cy5 will tell how many photons are emitted. In the more complicated Quad-FRET case we find the TAMRA to Cy5 efficiency $\eta^{qf}_{TC} = 0.74 \pm 0.05$ and FAM to TAMRA efficiency $\eta^{qf}_{FT} = 0.35 \pm 0.09$. The TAMRA to TAMRA efficiency was found to be $\eta^{qf}_{TT} > 0.95$. For the Quad-FRET molecule the FAM and TAMRA fluorophores are on different strands and are more widely spaced, so the FAM to TAMRA efficiency is reduced compared to the Bi−FRET case. We found that the fluorescence of Cy5 in FT_TC

is greater than in _TTTC. This suggests that there is significant energy transfer in the T_T part of FT_TC, consistent with the high TAMRA to TAMRA efficiency. In Quad-FRET molecules we took into account the possibility of transfers that skip a fluorophore to obtain the efficiencies. This is not an important consideration in the Bi−FRET case due to the spectral separation of FAM and Cy5. To the first order about 20% of excitations that FAM absorbs reach Cy5 in Quad-FRET molecules with all 5 fluorophores.

The energy transfer occurring in these molecules is probably not coherent, due to thermalization in the vibration energy levels of the molecules. However, these waveguides are not expected to be used in long-haul applications; rather, they could serve as short-range interconnects in dense optical circuits. One can estimate the propagation time from random walk statistics: the time for energy to propagate through a waveguide of length L is $t_{\text{prop}} \sim (L/d)^2\tau$, where τ is the excited-state lifetime of the fluorophores and d is the average spacing between fluorophores. In the case of the Quad-FRET molecule, we estimate that $t_{\text{prop}} \sim 10-20$ nanoseconds. The main source of losses in the waveguides is the energy efficiency of each transfer, which may be optimized further by using other fluorophores and changing the distance d between them. However, there is a clear tradeoff between minimizing propagation time and minimizing energy loss, both of which are governed by the choice of d.

In summary, we have demonstrated a general method to use FRET to transport energy along a DNA backbone. Using a repeating fluorophore unit in a molecule with 5 fluorophores, we showed how nanoscale optical waveguides could be constructed using DNA and commonly available fluorophores. A model that explains the mechanism behind the transfers and allows quantitative calculations was presented. These molecules are among the smallest photonic devices that can be built today and may form the basis of more complicated active devices. Future work in this area can be pursued in a number of directions, including using intercalating fluorophores, creating self-assembling photonic circuits, and using Dexter transfer[26] to build electronic devices. These molecules can also be used to overcome a long standing limitation of FRET: although FRET acts as a "spectroscopic ruler", in practice it is a short ruler with limited dynamic range. By arranging multiple transfers, the useful distances that can be measured with FRET increase linearly with the number of fluorophores in the chain.

Methods. *Sample Preparation.* The oligo sequences used were 5′ AAGGGAACCACTCAATGTC 3′ (strand X) and 5′ TAGACAAGAAAGAGGACTGAGTA ACTGATAGGGACAACAT 3′ (strand Y) and their complements (X* and Y*). Fluorophores were attached as follows: in Bi−FRET, Cy5 to the 5′ end of X, FAM to the 5′ end of X* and TAMRA to the 10th base of X*; and in Quad-FRET, Cy5 to the 1st base of X, TAMRA to 10th, 20th, and 30th bases of X*, and FAM to the 3′ end of X. These oligos with fluorophores attached were obtained from TriLink Biotechnologies, CA 92121 (www.trilinkbiotech.com). The strands were mixed in equimolar amounts with STE NaCl buffer (0.1 M NaCl, 10 mM Tris.Cl, 1 mM EDTA, pH 8.0). Annealing to form double-stranded DNA was done by heating to 74 °C, keeping the temperature constant for 10 min, and then cooling to room temperature for 2−3 h (PTC-150 Minicycler, MJ Research). In the case of fluorescence we used 800 μL of sample with about 20 nM of the molecule and measured the fluorescence. The concentration used is too small to allow significant intermolecular FRET when compared to intramolecular FRET. Emission curves were obtained using a fluorimeter (Shimadzu RS 5301PC) and absorption curves using a UV spectrophotometer (Shimadzu UV-1601). Due to the presence of fluorophores, it is not possible to use conventional UV absorption methods to quantify DNA and alternative approaches were used.

Data Analysis. From the spectra, the following method was then used to obtain the contribution of a fluorophore. First we obtained emission/excitation curves for the three fluorophores: F(λ), T(λ), and C(λ). These three curves were used to fit the emission/excitation curves S(λ) obtained from experiments. The three constants f, t, and c were determined using a least-squares fit in the regions the spectra were measured.

$$S(\lambda) = fF(\lambda) + tT(\lambda) + cC(\lambda) \tag{1}$$

This way we can separate out the contributions of the fluorophores at any wavelength. Noise due to scattering was subtracted before the fit.

Error Analysis. Concentrations of DNA molecules have an error associated with them due to imperfect mixing, annealing, and pipetting calibration; we estimate the total of these errors to be less than 15%, and they can be reduced in some cases by direct measurement and normalization of concentrations. For instance 530 nm excitation (FAM not significantly excited) may be used to normalize and compare FTC and _TC or FTTTC and _TTTC. Other possible sources of error include Raman and Rayleigh scattering, instrument errors, fluorescence leakage − fluorescence of a fluorophore leaking into the spectra of another fluorophore and excitation of acceptor fluorophores at donor excitation wavelength.[7] The quantum yield and absorption spectra of fluorophores depends in an essentially unknown way on a host of other factors including pH, temperature, length, and sequence of DNA strand[27] and whether the strand is single or duplex DNA.[27−28] Since the Förster radius depends on the 1/6 power of the quantum yield, absorption maximum and the spectral overlap integral, consequently the Förster radius is not very sensitive to changes in those parameters.

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Supporting Information Available: Fluorescence measurements in multi-FRET systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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