Distance-dependent increase in quantum dot photoluminescence by molecular beacons containing dark quenchers

Förster resonance energy transfer is a fluorescence-based technique currently used for numerous biotechnological applications. Although organic fluorophores have traditionally been employed for this method, quantum dots have many unique optical characteristics that make them attractive candidates for this usage. As the present understanding of how quantum dots behave as energy donors is incomplete, it is necessary to conduct further studies towards the physicochemical nature of this phenomenon. Here, we attempt to assess the distance- dependency for quantum dot-based energy transfer by spacing dark quenchers at increasing distances from these nanoparticles' surfaces. We describe a surprising finding that quenchers can actually increase the average fluorescence intensity of quantum dot solutions, and we propose a theoretical explanation, which may allow others to more accurately conduct quantitative studies in the future.

Förster resonance energy transfer (FRET) describes a process of nonradiative energy transfer between a donor fluorophore in its excited state and an acceptor chromophore. This de-excitation pathway occurs instead of the donor emitting a photon, and thus the acceptor is said to have "quenched" the donor's fluorescence (for an excellent review, see reference 1). As this phenomenon is highly distance-dependent, typically said to only occur when the centre-to-centre distance between two molecules is <10 nm, the occurrence of FRET can be exploited for detecting interactions between labeled biomolecules[2]. Current applications include real-time PCR probes, biosensors, and more exploratory studies that aim to identify biomolecular interactions[1].

Although this technique has been around for many decades, until recently, its growth has been stunted by a number of disadvantages inherent to the traditional FRET setup. Initially, investigations using FRET were limited to using conventional fluorophores (e.g. organic dyes, fluorescent fusion proteins) as both donor and acceptor molecules. These fluorophores have many disadvantages, one of which is their broad absorption spectra and only small Stoke‟s shifts, which make direct excitation of the acceptor fluorophore a convoluting factor during data analysis[3]. Furthermore, they tend to have low quantum yields and to suffer from a high susceptibility to photobleaching, the latter of which means that donor fluorescence will be lost over the course of an experiment regardless of whether FRET occurs[1]. Not surprisingly, these obstacles have limited the accuracy and utility of the technique.

With the advent of nanotechnology, there have been marked improvements to many optical methods due to the availability of fluorescent nanoparticles. Quantum dots (QDs), for example, may serve as ideal FRET donors because of their many unique optical properties. QDs are nanoparticles composed of a semiconducting material that emits visible light following excitation. They can be coated with polymers and other materials to promote biocompatibility, solubility, and

conjugation to other biomolecules or surfaces. They are also well-suited as FRET donors because of their high surface area, as this allows them to be simultaneously linked to multiple quenchers, thus enhancing the energy transfer efficiency compared to a one-to-one donor-acceptor configuration[1]. Furthermore, they have a low susceptibility to photobleaching, their emission maximum can be tuned to maximize overlap with an acceptor's absorption spectrum, they have high quantum yields, and their broad absorption and narrow emission spectra make them suitable for multiplexed applications[1].

Nevertheless, even with the numerous advantages that QDs can offer, their use for FRET-based applications may be severely limited by their size. In contrast to organic dyes, QDs are colloidal in nature, with radii ranging from 5-50 nm depending on their surface functionalizations[1]. This necessarily blocks the close approach of a quenching molecule to the QD core. This may or may not affect the maximum distance that an acceptor could be located, depending on whether QDs behave like conventional fluorophores. For an organic dye, the energy transfer efficiency (*i.e.* the fraction of excitons that are transferred from D to A non-radiatively) is expressed as:

$$E = \frac{k_{\text{D-A}}}{k_{\text{D-A}} + \tau_{\text{D}}^{-1}} = \frac{R_0^6}{R_0^6 + r^6} \quad (1)$$

where $k_{\text{D-A}}$ designates the rate of energy transfer, τ_{D} designates the exciton radiative lifetime, r is the separation between donor and acceptor, and R_0 is the distance at which 50% FRET efficiency occurs[1]. This models a dipole-dipole interaction where r is calculated by assuming that the participating molecules can be represented by their centres *i.e.* that they can each be considered as single points. Most investigations using QD-based FRET in the literature make the assumption that it can be modeled similarly[1,2]. However, this may not be accurate. Whereas organic fluorophores are tiny molecules, nanocrystals have radii of several nanometers and the exciton that forms upon excitation is created at the surface of the crystal rather than at its centre. Thus, it is unclear whether the intermolecular distance between a QD donor and an acceptor should be measured from the QD centre or from the outer surface of its semiconductor core and whether there is still an r^6 distance dependency.

Recently, the effect of distance on QD FRET efficiency was studied using a system in which a QD donor was connected to a Cy3 acceptor via different lengths of an oligopeptide linker[4]. After the results were normalized for a one to one donor-acceptor configuration, it was found that the efficiency of energy transfer was related to r^6, making the traditional energy transfer approximation seemingly reliable. If this is, indeed, an appropriate way to model how QDs behave, then such results should be reproducible using different kinds of linking and quenching molecules.

For this report, we attempted to investigate the effect of quencher distance on QD FRET efficiency, using molecular beacons to place dark quenchers at increasing distances from quantum dot surfaces. Although we are unable to comment on the r^6 dependency for FRET, our results hint that the heterogeneity of QD solutions may have been previously overlooked, and additional considerations must be made when using QDs for FRET experiments.

Assessing Quantum Dot Size

Molecular beacons (MBs) have generally proven to be a facile method for investigating the FRET efficiencies of different

fluorophore-quencher pairs. We thus sought to use this method to place quenchers at various distances from the surface of a QD (~0 nm, 1 nm & 2 nm), with a representative MB shown in Figure 1 (for the DNA sequences see Methods). Given that distance measurements were the focus of this study, it was deemed necessary to first establish the size of the CdSe/ZnS quantum dots being used for these MBs.

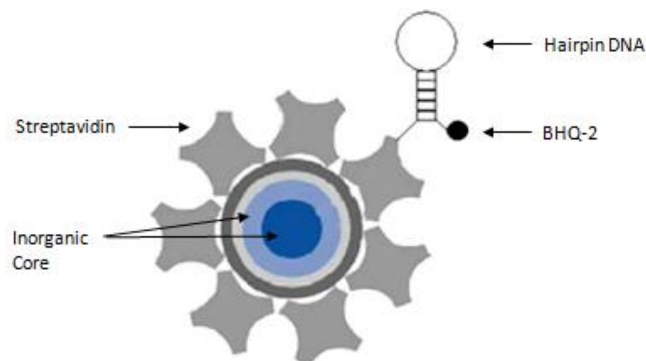

Figure 1 | Schematic diagram of a molecular beacon. A streptavidin coated quantum dot is linked to biotinylated hairpin DNA containing a black hole quencher at its terminus. This quencher will be closest to the surface for MB1 and furthest for MB3. Adapted from Cady *et al.*[5].

Transmission Electron Microscopy (TEM) was initially used to provide insight towards the size and shape of the QD cores. As shown in Figure 2, the QDs are rod shaped, with the dark regions indicative of the high-density inorganic semiconducting cores. Due to the polymer and streptavidin coats being both less dense and more susceptible to damage by the high-energy electron beam, they are not part of these dark regions and seem to have actually melted off (seen as the "film"). This was ideal, as measurements of specifically the QD core could then be made, with the average length being 11.3 ± 2.0 nm and average width being 4.5 ± 0.7 nm (n=14).

To then establish the radius of the fully intact QDs (*i.e.* the centre to surface distance) Dynamic Light Scattering (DLS) was used. In order to give the most accurate reflection of the functional QD size, the QDs were dispersed in the same PBS buffer for these measurements that was later used for the FRET experiments[6]. The QDs were found to have an average hydrodynamic radius of 11.4 ± 0.4 nm. This was actually larger than anticipated, making it less likely that the longer MB DNA strands (MB2 and MB3) would be close enough to the QD for quenching to occur.

Establishing QD-DNA binding

Having gained an appreciation for the size of QDs used, the next step was to create the proposed MBs. To avoid the inconsistencies and difficulties affiliated with covalent coupling methods, a biotin-streptavidin approach was used to link the DNA to the QDs. The success of this strategy was demonstrated by running the attempted QD- DNA conjugates on a gel alongside isolated QDs. As shown in Figure 3a, the attempted QD-DNA conjugates migrated more quickly, presumably because the negative charges associated with the backbone of attached DNA attracted them to the cathode. That there is a smear in these lanes (3 & 4), vs. a sharp band, indicates that the MBs made are heterogeneous in the number of DNA molecules

attached. Nevertheless, such results provide evidence that a streptavidin-biotin approach is a convenient and reliable means of assembling MBs.

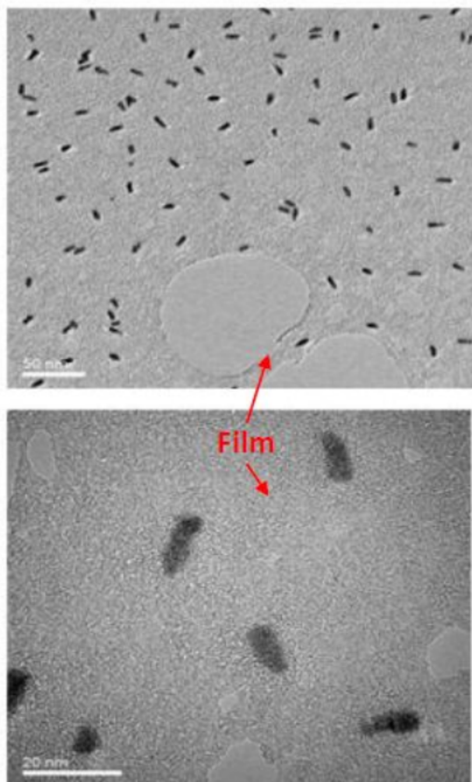

Figure 2 | TEM images of 605 nm emitting streptavidin coated QDs at two different resolutions. A film (indicated) can be seen, which consists of the streptavidin and polyethylene glycol coat.

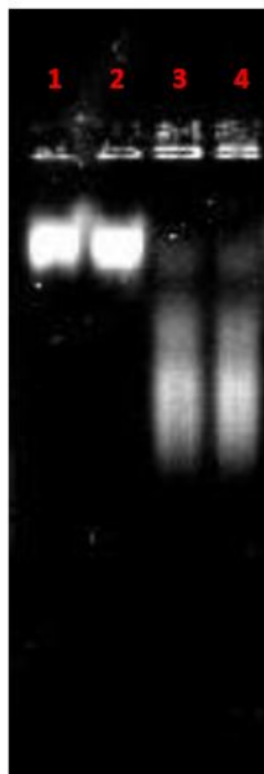

Figure 3| Electrophoretic characterization of MB1 complexes. 0.7% agarose gel with independent 605 nm QDs in lanes 1&2 and attempted MB1 conjugates in lanes 3&4 (50:1 DNA to QD ratio used).

Evaluating Fluorescence Quenching

Before comparing the FRET efficiencies of the three different MBs, it seemed appropriate to determine the optimal DNA to QD ratio for quenching. MB1 DNA was used for these experiments as it was the most likely to permit FRET (since it places the quencher closest to the QD surface). To take into consideration both the nanoparticle concentration as well as the fluorescence intensity for this type of comparison, the average brightness per nanoparticle was measured. This was achieved by using Fluorescence Correlation Spectroscopy (FCS), as the G(0)s of the autocorrelation decays are inversely related to particle concentration, and the mean fluorescence intensities can be calculated from the count rate trajectories. Surprisingly, FCS analysis determined that as the quencher ratio increased, the QDs were, on average, brighter (see Table 1a).

Table 1| Brightness per nanoparticle measurements for 605 nm streptavidin-coated QDs a. Incubated with different ratios of MB1 DNA **b.** Incubated with a 50:1 ratio of MB1 or MB3 DNA.

a	Avg. η (kHz)	Std. Dev.
Control	4.94	0.15
10:1	4.84	0.12
25:1	5.56	0.43
50:1	6.58	0.31

b	Avg. η (kHz)	Std. Dev.
Control	5.16	0.23
MB1	5.89	0.21
MB3	4.98	0.06

Given the unexpected nature of such results, it was hypothesized that perhaps the presence of a quencher reduced the degree of QD blinking (*i.e.* intermittent "on & off" states, see reference 7). If the presence of a quencher could reduce the occurrence of QDs switching into "dark" states, then this could potentially lead to QDs with greater average brightness. To investigate this possibility, FCS autocorrelation decays for the 50:1 MB1 sample and an isolated QD sample were collected across a series of laser powers (see Figure 4). Autocorrelation decays that slant downwards at short lag times (vs. a plateau) are indicative of blinking, and this phenomenon should increase with laser power[7]. Comparing the two power series, it does not appear that the presence of a quencher reduces the occurrence of blinking. Thus, this

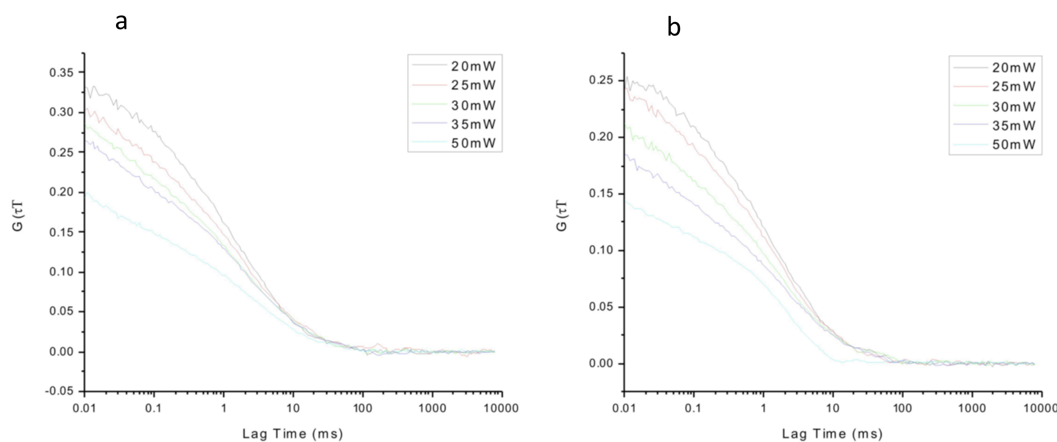

Figure 4| Autocorrelation decays at variable two-photon laser powers for 10nM samples of a. 605 nm streptavidin- coated QDs exposed to a 50:1 ratio of MB1 DNA **b.** isolated

An alternative explanation for the increased MB brightness could be that DNA-conjugated QDs have a greater two-photon cross-section. This seems plausible given the network of π-electrons along the DNA backbone, which could absorb photons and transmit this energy to the QD, acting essentially like an antenna. Such a phenomenon would counteract and could easily disguise the effects of quenching. Thus, to establish whether there was any masked quenching, FCS was used to derive particle brightness for QDs attached to MB1 (which was expected to show quenching), MB3 (which was likely too far away for quenching), as well as quencher-free controls. The results are summarized in Table 1b. It is notable that the MB3 sample was not brighter than the quencher-free control. Considering that this sample was likely too far for quenching, this finding seems to eliminate the hypothesis that the presence of DNA, itself, will increase particle brightness. It is interesting, however, that the MB1 sample was brighter than the other two samples. This is consistent with the previous findings shown in Table 1a, and suggests that only QDs with quenchers close enough for energy transfer become brighter.

Towards a Better Understanding of QD-based FRET

Close inspection of the MB1 FCS autocorrelation decays reveals that the particle concentration was lower than in the MB3 and control samples, despite the samples being prepared identically. A similar situation is shown in Fig. 4, where the MB1 G(0) is larger than the control with no quencher. In Fig. 4a and b, the black traces show this at short lagtime. A lower concentration of brighter particles is usually characteristic of aggregation; however, there was no evidence of aggregates in the count rate trajectories. Ultimately, there is only one explanation that we believe can account for both the required proximity of the quencher and the particle concentration and fluorescence trends that lead to brighter MBs. This explanation is based upon the notion that QD samples are not homogenous. As outlined by Yao *et al.*, some of the QDs in a solution will be dimmer than the average fluorescence intensity and some will be brighter[8]. It seems possible that the dimmer dots in the MB1 sample might be more readily quenched than the brighter QDs, perhaps due to surface differences such as a thinner organic polymer coat or more streptavidin proteins. Such a quenching behaviour would leave behind an apparently smaller concentration of brighter QDs, which is consistent with all of the fluorescence data collected. There is little mention of selective quenching in the literature, and thus this finding might add new insight towards how QD FRET applications should be approached. Clearly, if there is preferential quenching of certain QDs in a sample (rather than all equally) this will complicate the mathematical modeling used for quantitative studies. Further investigations that may challenge or support this hypothesis could involve dosing in the MB1 DNA to a QD sample being analyzed by FCS. A subsequent decrease in particle concentration but increase in average brightness would be in support of our hypothesis. This should also be repeated using longer MB DNA strands to ensure that increases in particle brightness only occur when the quencher is close enough for FRET to occur.

In summary, this study sought to explore the physicochemical nature of QD FRET by assessing the effect of QD-quencher distanceon FRET efficiency. The motivation was that by better understanding the nature of QD FRET, the technique could be used more accurately. Interestingly, a decrease in fluorescence intensity was never clearly observed for the quenchers placed at different lengths from the QD surface. Given the larger-than-expected size of the QDs, lack of quenching from the more distant quenchers was not surprising. It was, however, surprising that the MBs containing surface quenchers were actually brighter than the others. Our data suggest that these quenchers may be selectively eliminating fluorescence from the

dimmer particles in a naturally heterogeneous QD solution, leaving behind the brighter particles. Although further experiments will have to be conducted to more rigorously test this explanation, if supported, this finding will contribute to our overall goal of better understanding QD FRET.

Methods

Molecular beacon design and preparation

Oligonucleotides encoding three MB sequences were purchased from Biosearch Technologies. The sequences were as follows: MB1: Biotin-GCA GCA CGT CCT ACC CCA AGG CTG C BHQ-2, MB2: Biotin-TAA GCA GCA CGT CCT ACC CCA AGG CTG C BHQ-2 and MB3: Biotin-ACA TAA GCA GCA CGT CCT ACC CCA AGG CTG C BHQ-2 (underlined regions form stem of hairpin). The DNA was reconstituted in ultrapure water and stored at -20 °C as 100 µM stock solutions.

Size characterization of CdSe QDs

Streptavidin-coated QDs that emit maximally at 605 nm were purchased from Invitrogen. For characterization, 10 nM QD solutions were prepared using PBS as a dispersant. DLS was performed using a Malvern Nano ZS, with which ≥15 data sets were averaged for each run. TEM images of the quantum dots were obtained using a high resolution FEI Tecnai F20 TEM with an acceleration voltage of 200 kV.

Gel Electrophoresis of QD-DNA conjugates

Gel electrophoresis was used to assess whether the biotinylated DNA became conjugated to the QDs. 10 µL of 50 nM quantum dot samples containing either a 50:1 DNA to QD ratio or no DNA (but the equivalent volume of water) were mixed with 5 µL loading buffer. The samples were loaded into a 0.7% agarose gel, and electrophoresis was carried out at 100 V for 25 min. Gels were imaged with a BioRad Gel DocTM XR, using UV light to excite the gel-embedded QDs.

Assessment of Fluorescence Quenching

For all quenching experiments, 605 nm streptavidin-coated QDs were diluted in 1x PBS buffer (pH 7.4) with MB DNA added to make either 10:1, 25:1, or 50:1 DNA to QD ratios. In all instances, the final QD concentration was 10 nM. Sample fluorescence was analyzed using a previously described FCS setup8. Briefly, sample solutions were placed in custom quartz chambers on a Zeiss Axiovert 200 microscope. Excitation occurred via two-photon excitation with a 780 nm Ti:Sapphire laser (100fs, 82MHz). A dichroic filter directed the resulting sample fluorescence towards an avalanche photodiode, via bandpass filters. Acquired FCS autocorrelation decays were then plotted and fitted using OriginPro (OriginLab Software). Brightness per particle calculations were made by multiplying the mean fluorescence intensity by fitted G(0) values, where G(0) is represented by the equation[9]:

$$G(0) = \frac{\sum_i \eta_i^2 N_i}{\left(\sum_i \eta_i N_i\right)^2} \qquad (2)$$

in which η and N are the brightness and number, respectively, of particle i. This equation represents a sample with particles of differing brightnesses. In the instance where there is only one type of particle, this equation reduces to $G(0) = 1/N$. One can also use the previous approximation if the numbers and brightnesses of the particles are not known.

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