Eng. &Tech.Journal, Vol. 32,Part (B), No.4, 2014

The 4th International scientific Conference on Nanotechnology& Advanced Materials &Their Applications (ICNAMA 2013)3-4 Nov, 2013

Quantum Dots (QD-Dye Conjugate) are Ideal Candidates for FRET Investigations Using TIR Microscopy

Dr. Asma H. Mohammed
Center of Nanotechnology and advanced materials, University of Technology/Baghdad
Email:asmaahadimohammed@yahoo.com

ABSTRACT

Total Internal Reflection Microscopy has been employed to investigate Förster resonance energy transfer FRET for QD-dye conjugate system. QDs based donor systems are ideal for FRET for many reasons, one of them is that their broad excitation cross section which means that the direct excitation of the acceptor can be minimised via QD excitation at much shorter wavelengths, where little absorbance occurs in the acceptor. Förster resonance energy transfer (FRET) is a mechanism at which energy transfer between two fluorophores that occurs over a much shorter distance. A donor fluorophore in its excited state can transfer energy by a non-radiative, a long range dipole-dipole coupling mechanism to an acceptor fluorophore in close proximity (typically <10 nm). This is exactly the regime relevant to many biochemical processes and so significant effort has gone into developing FRET in recent years as a new tool for probing dynamics in condensed phase systems.

Keywords: QDs, FRET, Dipole-Dipole coupling

التكميم النقطي هي مرشحات مثالية لتحقيقات FRET باستخدام تقنية الانعكاس الكلي الداخلية (TIR)

الخلاصة

استخدم المجهر الانعكاس الكلي الداخلي الكلي لتحقيق انتقال الطاقة فورستر الرنينة FRET لنظام الصبغات المكممات النقطية QD . ألموسس على نظام المانح هو مثالي ل FRET لأسباب كثيرة، احد ها هو المقطع العرضي الواسع لتهيجها وهذا يعني أن التهيج المباشرة للمتقبل ممكن ان يصل الى قيمته الدنيا من خلال تهيج المكممات النقطية عند الاطوال الموجية القصيرة حيث يحدث امتصاص قليل للمتقبل. انتقال طاقة فوستر الرنينية هو مكانيكية بحيث الطاقة تنتقل بين صبغات متفلورة تفصل بينها مسافة قصيرة جدا. الصبغة المتفلورة المانحة في المستوي المتهيج ممكن ان تنقل طاقته وبطريقة غير مشعة وبنفس الوقت تحدث مكانيكية العزم المزدوج طويل المدى مع المستقبل فصلت بمسافة تقدر ب اقل من 10 نانومتر.

حديثا هذا النظام والمرتبط بعدة عمليات كيمياء الاحيائية وجهود حثيثة عاملة على تطوير FRET كاداة في نظام اعادة الطور.

https://doi.org/10.30684/etj.32.4B.4
2412-0758/University of Technology-Iraq, Baghdad, Iraq

INTRODUCTION

Fluorescence Resonance Energy Transfer (FRET) is an energy transfer mechanism named after the German scientist Theodor Förster .[1]. The conventional widefield fluorescence microscopy enables localisation of fluorescent molecules within the optical spatial resolution limits defined by the Rayleigh criterion, approximately 200 nanometres. FRET is an energy transfer process between two fluorophores that occurs over a much shorter distance. FRET is a method often utilised in folding and dynamic studies of proteins and nucleic material. Two fluorescent species are required with an overlap in the wavelengths of the emission of one species, the **donor**, with the absorbance of another, the **acceptor**. When the donor species is excited, it transfers energy non-radiatively to the acceptor species within 10-100 nm via a dipole interaction, exciting the acceptor. This is exactly the regime relevant to many biochemical processes and so significant effort has gone into developing FRET in recent years as a new tool for probing dynamics in condensed phase systems.(2,3). The fluorescence intensity of both species can be measured to calculate the FRET efficiency ***E***. This can be related to the distance between the two fluorophores by:

$$E = R_0^6 / R_0^6 + r^6 \qquad \ldots (1)$$

Where *r* is the distance between fluorophores and R_0 is the Förster distance, related to the spectral overlap integral between species by:

$$k_T(r) = 1 / \tau_D (R_0 / r)6 \qquad \ldots(2)$$

Where $k_T(r)$ is the rate of energy transfer and τ_D is the lifetime of the donor in the absence of energy transfer. To achieve accurate calculations of *r*, good spectral separation between the donor and acceptor excitation wavelengths is critical. CdSe QDs are ideal candidates for FRET based donor systems for many reasons, one is that their broad excitation cross section means that direct excitation of the acceptor can be minimised via QD excitation at much shorter wavelengths, where little absorbance occurs in the acceptor. The system examined here attempted to exploit FRET in the simplest of ways to demonstrate the potentially detrimental effect that QD bluing may have on the interpretation of energy transfer data. Here, a simple dye conjugated to a QD provided the acceptor. The imaging of the dispersed fluorescence allowed the direct monitoring of the FRET spectra and changes thereof due to the active photodynamics of the QD.

Although QDs as FRET donors have been demonstrated in a variety of ways

including other dye labelled protein acceptors,(4). FRET with Au nanoparticle

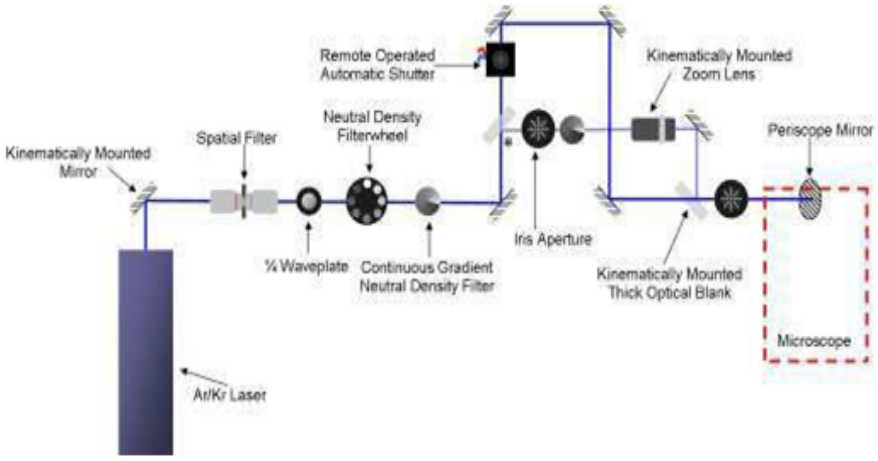

Figure 1 – The TIRFM optical track. Coherent light is produced by an Ar/Kr laser, spatially filtered to produce a clean and calumniated Gaussian beam, circularly polarised and sent to the microscope. A part of the beam is split from the main path* and tightly focused before recombining with the main beam for the Purposes of automatically focussing the microscope

quenchers in DNA sensing (5),and glucose sensors (6). Few examples appear to have been reported on the single molecule environment where knowledge of processes like PLA, PLE and bluing could all play a part in the FRET process, at least in the final interpretation of transfer efficiencies with respect to structural changes. Generally these studies are limited to diffusion through a confoncal volume and not single QD spectral dispersion.

Single QD–FRET based sensors have already been exploited by Zhang *et al.*(7), where they demonstrated FRET emission after DNA duplex hybridisation. Their system used commercially available streptavidin functionalised QDs as a 'capture probe' of a biotin functionalised short chain oligo and its conjugate pair that had a Cy5 acceptor attached. After hybridisation of the DNA and docking of the biotin, the QD FRET emission was observed. However these experiments were performed using confocal microscopy and looked at QD diffusion through an excitation volume, not single QD spectra.

Indeed another system examined by Zhou *et al.*(8)showed direct coupling of a dye-labelled DNA (acceptor) to QD donors, which significantly reduced the donor-acceptor distance and improved the FRET efficiency. They demonstrated a highly efficient FRET (88%) at a low acceptor-to-donor ratio of 2 at the single-molecule level. Again the single molecule measurements came from bursts through a confocal volume and spectral information could only be accessed via ensemble experiments

EXPERIMENTAL SET UP OF TIR SINGLE MOLECULE MICROSCOPY

The standard single molecule spectrometer setup was used. Here the resolution of the dispersion grating allowed adequate detection of single QD FRET spectral dynamics. Importantly the system intensity resolution is limited to the 8-bit resolution (256 levels) of the camera. Therefore in order to ensure that good single spectral FRET can be observed, intensity of both the donor and acceptor channels must approximate each other, as small FRET variations would be difficult to detect. Coherent laser light is produced by a Beamlok 2080 Argon/Krypton gas phase laser at a power of less than 40 mW. On leaving the laser, the beam is plane polarized and can feature unwanted high frequency noise, undesirable features for SM microscopy. Fluorophore absorption of photons is dependent on dipole orientation, meaning that only a small proportion of molecules correctly aligned would be excited by plane polarised light. To obtain high quality results, a degree of beam attenuation is required before the microscope. Fig 1 shows a schematic diagram of the optical track (path of laser beam through optical equipment designed to attenuate beam).

After leaving the laser the beam is first redirected by reflection from a kinematically mounted mirror. Kinematic mounting is advantageous as it allows fine adjustment to the direction of the beam/position of the footprint. Once redirected, the beam passes through Figure (1) The **Spatial Filter** – consisting of two matched objective type lenses separated by a fully articulate pinhole, the spatial filter removes aberrations from the laser beam, providing a smooth Gaussian beam cross section. The beam enters the rear of the first lens and is tightly focussed onto the pinhole. Diffraction limitation leads to the formation of an “Airy disk” or Fourier transform of the beam. As shown in Figure (2), only the centre of the focal spot is perfect; only this portion is passed by the pinhole. The second lens re-collimates the now-perfect beam

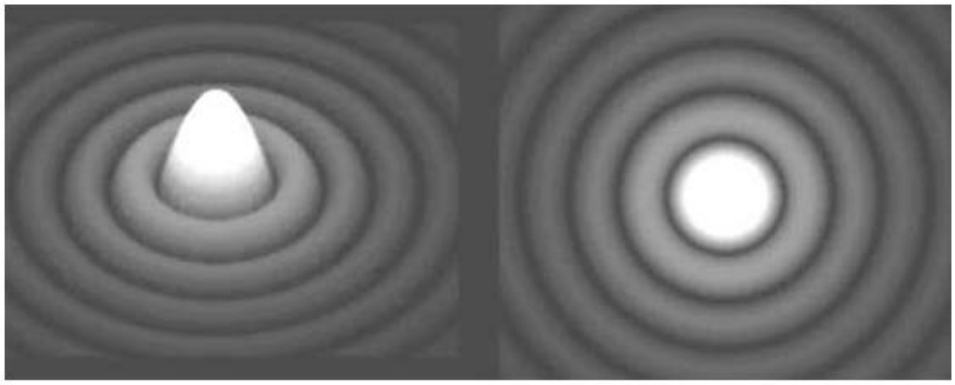

Figure 2: 3D representation of Airy disk showing side and plan view. Beam is only perfect at the centre of the focal spot. Image obtained from www.gla.freniere.com/sa_Huygens.htm

The **Quarter Waveplate** – consisting of a birefringent crystal mounted in a free rotating mount for easy adjustment, the ¼ waveplate causes a phase-shift of ¼ of a wavelength between the two perpendicular axes of a polarised light beam, resulting in plane-polarised light becoming circularly polarised (and vice versa).

Neutral Density (ND) Filters – solid ND filters mounted in a rotating wheel or a rotating disk with continuously graduated density, neutral density filters attenuate the laser beam across all wavelengths. These allow the laser to be run within optimal parameters while attenuating the beam strength for the current experiment.

Optical Blank – plain glass disks allow most of the beam to pass freely while reflecting some light from the front and rear surfaces, allowing a weak beam to be split from the main beam and utilised separately. Thicker blanks allow greater spatial separation of the front and rear reflections, making it easier to use one while filtering out the other using an iris aperture.

Remotely Operated Shutter –controlled from the computer, this prevents damage being caused to the CCD camera by intense laser radiation.

- **Power density of the laser source**

Power densities can vary markedly with beam conditions such as diameter and attenuation from adsorptive components in the optical path. Throughout this work bespoke modification of the beam was required for different experiments; for example in the dual channel imaging the beam is apertured to ensure both long and short wavelength images of the footprint can be imaged on a single CCD chip. For generalisation, all power intensities henceforth will be quoted as that emergent from the special filter and before being directed into the microscope. Typically, powers measured after the spatial filter correlate linearly with the intensity at the sample as shown in Figure 3.

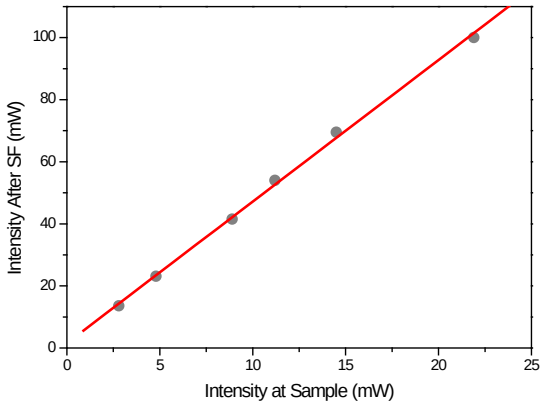

Figure (3) Laser intensity power calibration: Laser intensity values were taken after spatial filter in the experimental setup and compared to the intensity at the sample plane resulting in a strongly linear correlation.

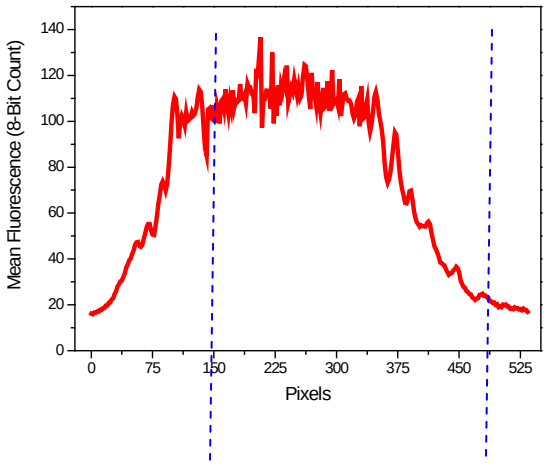

Figure (4) The near 'top-hat' TIR beam intensity profile obtained from the fluorescence of a high concentration of physisorbed tetramethylrhodamine.

- **The TIR Footprint**

(TMR) dye. The profile is used to limit biasing in the data analysis by selecting a region of near-uniform intensity. The profile confirms that an image of approximately 275 pixel squared is irradiated with a mean intensity (106 ± 11) varying by only ~ 10 % across the field.

Uniformity of the laser intensity across the visual field is an important consideration in the analysis of images and an obvious source of data biasing where large variations are observed. Here we demonstrate the approximate uniformity of the power intensity of our optical system by using a high concentration of tetramethylrhodamine (TMR) dye (~ 100 nM) dispersed on a coverslip to image the cross-section of the circular TIR footprint Figure(4). Evidently, from the characteristic 'top-hat' illumination profile, sample areas of $>275^2$ pixels will yield near-uniform excitation such that data analysed within a region of interest (ROI) equal or smaller than this area will be subject to minimal biasing. Deviation between the maximum at the centre of the beam and minimum at the periphery equated to a mean of 106 ± 11 with 10% variation across a 275 square pixel area.

The Imperfect System. The spectral specifics of the system under examination were chosen to be intentionally flawed. This was to highlight the importance of bluing in relation to FRET. Under normal conditions FRET occurs when a donor absorbs a shorter wavelength photon, undergoes non-radiative

resonance transfer of its excitation energy to excite an acceptor molecule and then reradiates this energy as Stokes shifted light.

From a radiative transition in the acceptor molecule. However in this case, the dye emission was chosen such that no observable FRET should occur initially. Indeed the donor molecule (QD) was chosen to be red shifted to the acceptor (dye); exactly the wrong way round for FRET to take place. However during the course of irradiation, in the intensity regime that is conducive to bluing, the system should evolve from a situation of no FRET (via irreversible spectral shifting) to one in which spectrally resolved FRET would be observed Figure (5). In fact it was envisaged that temporal mapping of the overlap integral may be resolvable as the QD undergoes irreversible oxidation through the absorption profile of the acceptor molecule. This process would typify the need to understand correct power regimes when employing QDs in a FRET reporter capacity as a life science tool and reiterate the warningfrom the nanoworld.

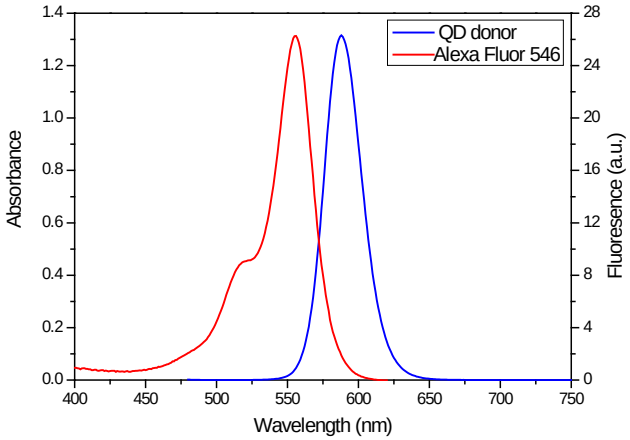

Figure (5) FRET system setup: The donor molecule starts 'downfield' of the acceptor, where no FRET should occur. As the sample is irradiated the QD peak emission blue shifts to a point where the emission of the QD superimposes on the absorption of the acceptor dye and FRET should occur.

Qdot 585 ITK Streptavidin conjugate kit was commercially sourced (Invitrogen UK, Q10011MP, Lot # 454208, 2 µM stock solution). Unlike the standard QD streptavidin conjugates, (used in all previous experiments) the QD ITK™ streptavidin conjugates have the streptavidin covalently attached to the inner amphiphilic coating without a PEG linker. It was hypothesised that this would decrease the donor acceptor distance, which is critical in achieving effective FRET.

Sample Preparation. The acceptor probes chosen were both 5(-and-6-)TMR biocytin (Molecular Probes UK, T12921 lot2801-5) and Alexa Fluor 547 biocytin (Molecular probes A-12923, lot#4281-4). While both were examined the

Alexa Fluor 547 yielded much clearer images during the processing stages, hence all data presented here originates from that system. A systematic molar dilution ratio experiment yielded an optimised fraction of 2:1 for the QD and dye dilution respectively. Samples were diluted in PBS buffer, then combined, followed by vortex mixing, then finally left to incubate for 20 minutes prior to image capture.

Due to initial efficient bleaching of the dye molecule at higher powers, experiments were conducted to at lower excitation powers which yielded optimised images. The capture of images in this power regime required a higher gain on the intensifier to observe any dispersed fluorescence and hence were much more susceptible to increased noise levels. Nonetheless useful spectrally resolved images were obtained under the conditions defined in Table (1).

Table (1) Experimental conditions in the initial PLA experiment.

Excitation Intensity	10mW	**Camera settings**	Voltage 8.0, Black 0.55, Gain 1.65
Excitation Wavelength	514nm (SpectaPhysics Argon Ion 165)	**Concentration Sample**	dil, 5×10^3 from stock (2uM)
Emission Filter	Semrock, FF562-Em02 (FF01-593/40)	**Scan**	(175 pixel^2) 3×3: 3000 frames@ 10fps
Sample Prep	Ozonated Coverslip (20minutes)	**Microscope mode**	TIR

RESULTS & DISCUSSION

Initially, higher Dye:QD ratios yielded low single QD fluorescence. With the ratio as high as 10 almost no single QD fluorescence was detectable at all, it is currently unclear as to whether this is due to either efficient quenching which has been demonstrated in similar systems,(11,12) or whether electrostatic interactions at the surface modify the QD's ability to physisorb onto the coverslip. In order to alleviate fast photobleaching of the dye, experiments were conducted at a much lower excitation regime. At this new laser power, although infrequent, it was possible by visual inspection to manually extract single instances of what appeared to be single QD FRET. Samples were then temporally projected through the image stack using ImageJ demonstrating two spectrally separated peaks, examples of which are shown in Figure (6).

The slight process of bluing before the QD-Dye conjugate is rendered FRET active (highlighted in red). **(c)** Single QD from the same sample that displays no obvious QD-Dye FRET emission, sample undergoes photo- oxidation under similar power conditions, which may be evidence in support of photoionisation suppression via

electron transfer. (White line is included as a reference to guide the eye)These characteristic 'double peak' spectra, although infrequent in occurrence, are believed to originate from the single molecule QD-Dye FRET interaction. The first thing to note is that the double peak examples extracted here tended to be of lower overall integrated intensity compared to other QDs in the same sample that displayed normal behaviour, this may be the result of electron transfer and hence inefficiency in the radiative fluorescence process. Secondly, the distance between the two peaks typically equated to 5-20 nm and would be consistent with a FRET system. Instances of this type, in similar systems, have already been demonstrated in the bulk.[13,14] In control experiments direct excitation of dye at 457 nm resulted in negligible dye emission, at least with the same intensifier settings used for QD imaging.

Although a range of behaviours were again observed some useful features should be noted and are shown in Figure 6. If indeed the FRET system is occurring, blinking is clearly evident from the removal of intensity from both acceptor and donor channels (shown in green). Other features that may be indicative of FRET include: complete FRET transfer in the form of suppression of the QD peak (cyan), through a situation where fluorescence is retained in both acceptor and donor spectral space (pink), to a situation where no FRET is apparent and all of the fluorescence is retained in the QD emission (yellow). In addition to these events, on the same sample under identical power conditions the observation of bluing was observed in QDs that did not exhibit the double-peak spectra. Although not displayed by all QDs on the surface, the lack of photobluing in the doublepeak spectra may be indicative of a bluing suppression in QD-FRET conjugates, and this is highlighted in Figure (6).

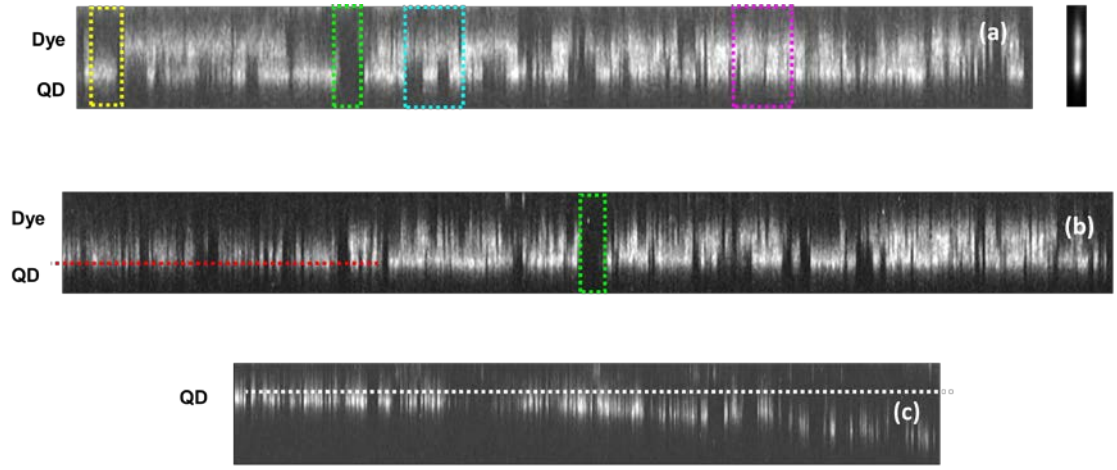

Figure (6a & b) Single QD dye conjugation: Shown here are temporal 3D projections.

The single conjugate dispersion demonstrating various behaviours including: FI (Green) minimal FRET emission (Yellow) complete FRET transfer (Cyan) and comparable emission (Pink). Evident from the QD emission is the slight process of bluing before the QD-Dye conjugate is rendered FRET active (highlighted in red). **(c)** Single QD from the same sample that displays no obvious QD-Dye FRET emission, sample undergoes photo-oxidation under similar power conditions, the may be evidence in support of photoionisation suppression via electron transfer. (White line is included as a reference to guide the eye)Possible explanations for the low incidents of positive examples include a sub dye-to-QD dilution ratio and also the fact that a low concentration was required to view dispersed spectra. Also the low power regime limits the QDs ability to undergo photobluing. However smaller QDs may still exist from either; unusually blue QDs in the size distribution or a high susceptibility to the bluing process. There is of course the possibility that two spatially colocalised QDs may be under interrogation rather than any dye emission. However evidence in support of the existence of the QD-FRET system includes: (1) the distance between the two peaks is of the correct order of magnitude for single QD transfer. However it is still unclear whether electronic transfer itself provides a non-radiative route for the donor electron to relax to the valence state and avoid ionisation by Auger excitation. Although this would be consistent with the ionisation model proposed as it may suppress ionisation and therefore oxidation, depending on relative rates of the competing processes. (2) Peaks are collinear and so reduce the likelihood of the double peak originating from two separate QDs (3) Samples exhibit all single QD phenomena including, blinking, bluing and (to some extent) PLE and PLA. In the case of double peaked spectra many of these observables are correlated in both peaks.

Although, as intended, mapping of the FRET overlap integral was not observed, it is clear that the degree of bluing at these lower powers (required to stop dye bleaching) does not result in a large enough spectral shift to facilitate FRET in a large population. However there remains the possibility that the FRET process itself may be limiting the rate of single QD photobluing if charge transfer kinetics are much faster than that of photo-oxidation. Indeed if this is the case this may be a process that can be exploited to retard photo- oxidation, an inbuilt antioxidant for a future generation of QD perhaps? What is evident from the demonstration in Figure c, is that a period of induction can be seen prior to the FRET conjugate being rendered active. During this period, the peak of the QD is seen to blue slightly, suggesting that this is the source of its FRET activation as the photo-oxidation of the QD surface renders its emission blue-shifted to a wavelength at which electron transfer can occur (highlighted in red). Work is ongoing to elucidate the origins of this phenomenon.

Finally and as a summery the QD-FRET conjugate work could be extensively developed, most obviously by choosing an acceptor dye that should undergo FRET during normal conditions rather than the intentionally ‘imperfect system’ examined. The work conducted here suggests that single QD spectral FRET dynamics should be experimentally achievable under this system.

CONCLUSIONS

QD-dye conjugates system. QDs based donor systems are ideal for FRET. Förster resonance energy transfer (FRET) is a mechanism at which energy transfer between two fluorophores that occurs over a much shorter distance. A donor fluorophore in its excited state can transfer energy by a non- radiative, a long range dipole-dipole coupling mechanism to an acceptor fluorophore in close proximity (typically <10 nm). it was envisaged that temporal mapping of the overlap integral may be resolvable as the QD undergoes irreversible oxidation through the absorption profile of the acceptor molecule. This process would typify the need to understand correct power regimes when employing QDs in a FRET reporter capacity as a life science tool and reiterate the warningfrom the nanoworld.

REFERENCES

[1].Förster, Theodor. Naturwissenschaften., 1946, 6, 166-175.
[2].Ha, T.; Enderle, Th.; Ogletree, D.F.; Chemla, D.S.; Selvin, P.R.; Weiss, S. Proc. Natl.Acad. Sci. U.S.A.1996, 199693, 6264-6268
[3].Jares-Erijman, E.A.; Jovin, T.M. Nat. Biotechnol. 2003, 21, 1387-1395
[4].Clapp, A.R.; Medintz, I.L.; Mauro, J.M.; Fisher, B.R.; Bawendi, M.G.; Mattoussi, H. J.Am. Chem.Soc. 2004, 126, 301-310
[5].Xu, S.; Zhang, J.; Dong, Q.; Sun, B. Proceedings of SPIE, 2008, 6829, 68291F
[6].6Pickup, J.C.; Hussain, F.; Evans, N.D.; Rolinski, O.J.; Birch, D.J.S. Biosens. And Bioelectr. 2005, 20, 2555-2565
[7].Zhang, C.-Y.; Yeh, H.-C.; Kuroki, M.T.; Wang, T.-H. Nat. Mater. 2005, 4, 826-831
[8].Algar, W.R.; Krull, U.J. Langmuir 2006, 22, 11346-11352
[9].Weber, P.C.; Ohlendorf, D.H.; Wendoloski, J.J.; Salemme, F.R. Science 1989, 243, 85-88
[10].Olmberg, A.lomstergren, A. ord, ukacs, undeberg, hl n, Electrophoresis, 2005, 26, 501-51
[11].Yildiz, A.; Forkey, J.N.; McKinney, S.A.; Ha, T.J.; Goldman, Y.E.; Selvin, P.R. Science2003, 300, 2061-2065
[12].Gordon, M.P.; Ha, T.; Selvin, P.R. Proc. Natl. Acad. Sci. U.S.A. 2004, 17, 101, 6462-6465
[13].Toprak E.; Enderlein J.; Syed S.; McKinney S.A.; Petschek R.G.; Ha T.; Goldman Y.E.; Selvin P.R. Proc. Natl. Acad. Sci. U.S.A. 2006, 103, 6495-6495
[14].Huang, B.;Wang, W.; Bates, M.; Zhuang X. Science 2008, 319, 810-813