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Understanding nonviral nucleic acid delivery with quantum dot-FRET nanosensors

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Abstract

Nonviral delivery of nucleic acids is a potentially safe and viable therapeutic modality for inherited and acquired diseases. However, current systems have proven too inefficient for widespread clinical translation. The rational design of improved carriers depends on a quantitative, mechanistic understanding of the rate-limiting barriers to efficient intracellular delivery. Separation of the nucleic acid from the carrier is one of the barriers, which may be analyzed by Förster resonance energy transfer (FRET), a mechanism used to detect interactions between fluorescently labeled molecules. When applied to the molecular components of polymer or lipid-based nanocomplexes, FRET provides information on their complexation status, uptake, release and degradation. Recently, the design of FRET systems incorporating quantum dots as energy donors has led to improved signal stability, allowing prolonged measurements, as well as increased sensitivity, enabling direct detection and the potential for multiplexing. The union of quantum dots and FRET is providing new insights into the mechanisms of nonviral nucleic acid delivery through convergent characterization of delivery barriers, and has the potential to accelerate the design of improved carriers to realize the potential of nucleic acid therapeutics and gene medicine.

Keywords

biophotonics; Förster resonance energy transfer; gene delivery; intracellular trafficking; nanomedicine; polyplex; quantum dots; siRNA delivery

Delivery of gene-loaded nanocomplexes into cells has emerged as an essential tool in modern therapeutics and biomedical research. Compared with viral vectors, the main advantages of nonviral gene carriers are diminished immunogenicity, ease of scale-up and tremendous potential for optimization. Their primary weakness, however, has been poor transfection efficiency. The low, transient transgene expression levels achieved with nonviral carriers have thus far precluded the widespread translation and clinical success of such delivery systems. The putative barriers associated with the low efficiency of nonviral vectors are illustrated in Figure 1. These include cellular binding and uptake, endosomal

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escape, cytosolic transit to the nucleus and unpacking, nuclear entry and transcriptional processing.

Quantitative intracellular trafficking studies enable the identification of these rate-limiting barriers, which can differ widely between delivery systems. Some carriers, especially those incorporating polyethylene glycol (PEG) for steric stabilization, are plagued by poor cellular uptake [1,2]. Others lack sufficient buffering capability to exploit the proton sponge effect for efficient endosomal escape [3]. Some types of nanoparticles reach the nucleus of target cells but still fail to mediate robust transgene expression [4]. With such disparate behavior among different types of nanocomplexes, the ability to track the delivery of nucleic acids within cells is crucial to understanding a carrier's unique strengths and limitations. However, measuring only the spatiotemporal trafficking activity of nanoparticles neglects other critical aspects of the complex process. To be effective, nucleic acids must be condensed and protected from enzymatic degradation *en route* to their destination, where they should be quickly released [5]. The dissociation status of the nanocomplex and payload integrity at each point are equally as important as their transport in ensuring that the therapeutic agent is delivered intact and available for processing. The ability to simultaneously detect subcellular location, nanocomplex stability and payload integrity with increased precision will promote the establishment of more robust structure–function relationships and accelerate the rational design of improved gene carriers.

Fluorescence-based methods have been the techniques of choice to study the physicochemical properties and intracellular trafficking of nanocomplexes [6–8]. Interactions between a nucleic acid payload and its polymer or lipid carrier can be probed using fluorescently labeled molecular components and followed from the extracellular domain to the cytoplasm or nucleus under physiological conditions [6,9–11]. Colocalization of their signals provides some indirect information about their interactions [12]. However, detection of dissociation and release is limited by the need to resolve distinct signals from components that may not have achieved sufficient separation. Therefore, such detection methods do not offer the requisite sensitivity to precisely detect the onset of dissociation, or to differentiate between molecules that are interacting or simply adjacent. Förster resonance energy transfer (FRET) is a technique that has advantages over colocalization due to its unique ability to resolve molecular interactions beyond the diffraction limit of conventional microscopy [13]. When FRET occurs, a donor fluorophore excites an acceptor via a nonradiative dipole–dipole interaction if they are sufficiently close (within ~10 nm). This so-called ‘molecular ruler’ can be used to determine distances between labeled molecules inside cells, including gene carriers and nucleic acids. The ability to detect nanometer-scale separations of nanocomplex components makes FRET a more powerful tool than fluorescence colocalization for studying nanocomplex stability and dissociation.

Recently, some researchers have chosen to design FRET pairs that employ luminescent semiconducting nanocrystals called quantum dots (QDs) as energy donors. Conventional fluorophores suffer from chemical and photo-degradation, photobleaching and broad spectra. Conversely, QDs possess resistance to bleaching and chemical degradation, broad absorption spectra, tunable narrow emission spectra and large energy separation between excitation and emission [14]. The increased stability of QDs makes the imaging of FRET signals over extended periods of time possible. Furthermore, the ability to tune emissions and excite multiple QDs at a single wavelength creates the potential for multiplexing [15]. The first reports proposing the use of QDs in biological sensing applications generated significant excitement [16,17]. In 2006, the specific benefits of FRET incorporating QDs (QD-FRET) for the study of nonviral gene delivery were published [18]. Since then, several groups have adapted and refined the technique to study many aspects of nonviral nucleic acid delivery. In this review, we focus on the use of QD-FRET as a tool to investigate the

intracellular fates of nanocomplexes used to deliver nucleic acids. We begin by detailing the rationale and appeal of the technique before exploring specific examples of its use and considerations when adopting QD-FRET to study nonviral nucleic acid delivery.

Why QD-FRET?

FRET is a process by which the energy of a donor chromophore is nonradiatively transferred to a nearby acceptor molecule via a dipole–dipole interaction, leading to a decrease in the donor emission and a concomitant increase in acceptor emission [13,19]. A general expression of energy transfer efficiency, E , defined in Equation 1, accounts for the fraction of excitons transferred from the donor to acceptor, where k_T is the rate of nonradiative energy transfer, τ_D is the excited-state radiative lifetime, and r is the distance between the donor and acceptor.

$$E = \frac{k_T}{\tau_D^{-1} + k_T} = \frac{R_0^6}{R_0^6 + r^6} \quad (1)$$

The Förster radius R_0 , defined as where 50% of energy transfer occurs, is a function of the refractive index of the medium (n , typically ranges from 1.3 to 1.5), the unperturbed donor photoluminescence quantum yield (Q_D), the spectral integral from the overlap of donor emission and acceptor absorption ($J[\lambda]$, in units of $M^{-1}cm^{-1}nm^4$) and the relative orientation of the donor and acceptor dipoles ($\kappa^2 \sim 2/3$), as shown in Equation 2.

$$R_0 = 0.211 [\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \text{ (in } \text{\AA}) \quad (2)$$

For most FRET pairs, R_0 is typically a few nanometers, enabling optical measurements of changes in donor–acceptor separation with angstrom resolution. Meanwhile, the sixth-power dependence of FRET efficiency on the donor–acceptor distance makes FRET well suited for probing conformational changes of molecules or detecting interactions between molecules.

The second equation illustrates that optimal FRET occurs when there is appreciable spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, while the spectral overlap of the donor and acceptor emissions is minimized. Therefore, the broad absorption and emission spectra of organic fluorophores used in FRET present significant challenges for reducing direct acceptor excitation (simultaneous excitation of energy acceptor and donor) and crosstalk (overlap of donor and acceptor emission spectra), as depicted in Figure 2. To cope with these concerns, some researchers are turning to QDs as energy donors for FRET applications in an attempt to overcome some of the limitations associated with conventional organic FRET pairs. Their benefits include diminished spectral crosstalk and direct acceptor excitation due to the unique photophysical properties of QDs: narrow size-tunable emissions, extremely high extinction coefficients over a broad absorption range and enhanced photostability [20]. QDs have been preferentially adopted as energy donors rather than acceptors due to their relatively long radiative lifetime, broad excitation range and high extinction coefficients [21]. Readers seeking a deeper discussion of the photophysics of QD-FRET for biological applications are referred to the excellent review by Medintz and Mattoussi [20].

QD-FRET as a novel strategy to study nonviral delivery barriers

Many nonviral systems depend on interactions such as electrostatic attraction and chain entanglement to condense and protect nucleic acids during delivery. Such interactions have been studied using FRET pairs of organic fluorophores either doubly labeled on DNA [22]

or separately labeled on gene carriers and nucleic acids [23]. However, the former depends on stable coil-globule transition states of DNA and both require additional ratiometric analysis and disambiguation. Furthermore, the organic fluorophores used in conventional FRET are susceptible to photobleaching, limiting their utility in real-time studies of intracellular trafficking [13]. These weaknesses highlighted the need for an alternative.

The initial report of QD-FRET for the study of nonviral nucleic acid delivery described a sensitive and quantitative indicator of the stability and composition of chitosan–DNA nanocomplexes [18]. The authors labeled biotinylated plasmid DNA with streptavidin-decorated QDs, and conjugated the natural polysaccharide chitosan with Cy5 dye. The resulting high signal-to-noise ratio enabled digital detection of the formation and dissociation of single nanocomplexes using single molecule detection (Figure 3). They also used the frequencies of QD emission before and after nanocomplex disruption to estimate the number of plasmid copies contained within each particle (~30), which they validated with transmission electron microscopy. Cells readily internalized the QD-FRET nanocomplexes, and confocal microscopy revealed intact particles in both the cytoplasmic and nuclear domains. The intracellular dissociation rate of the complexes was quantified, and the disappearance of QD-FRET emission showed that most particles unpacked within 48 h.

Utilizing the ability to precisely detect DNA release, the same group developed a quantitative model to describe and predict distributions of free DNA throughout several subcellular compartments over time [24]. The average unpacking rate constants in the endosomal, cytoplasmic and nuclear domains were recorded for chitosan, polyethylenimine (PEI) and poly(phosphoramidate) (PPA) nanocomplexes. The results were then correlated with transfection efficiency to further elucidate the contributions of nanocomplex stability and intra-cellular trafficking during gene transfer. The data revealed a compartmental dependence of unpacking between carriers, with PEI unpacking at equal rates in both endosomal and cytosolic compartments and PPA unpacking preferentially in the cytosol. The results supported the conclusion that nanocomplexes must either escape the endosome quickly (PEI) or remain stable within them (PPA). If significant unpacking and degradation occurs within the endosome (chitosan), transfection efficiency may suffer. The calculated unpacking rate constants agreed with published computational models based on intracellular copy number, although release of DNA from PEI nanocomplexes occurred earlier than previously reported in studies using colocalization and conventional FRET [25–27].

The improved stability of the QD-FRET system allowed for direct measurement of nanocomplex stability over longer periods of time, and its inherent sensitivity led to the derivation of more robust quantitative models and the precise determination of the unpacking rate constants for three carriers. The technique has attracted the attention of delivery researchers as they seek high-quality quantitative data in understanding the mechanisms of nucleic acid delivery and the design of nonviral systems (Table 1). The field is gaining momentum as researchers continue to refine the technique, and reports of interesting results from QD-FRET studies emerge with increasing frequency.

Recent applications of QD-FRET to study nonviral delivery

QD-FRET in delivery of plasmid DNA

Plasmid DNA is the most commonly delivered nucleic acid, and it follows that QD-FRET has been most often applied in the study of its delivery. Ulasov *et al.* employed QD-FRET to study transfection by PEI–DNA nanocomplexes in multiple cell types, and used the results to derive new mathematical models to quantify intra-cellular trafficking [28]. They functionalized the particles with PEG and TAT peptide to modulate stability and cellular

uptake. PEG incorporation conceals the surface charge of nanoparticles and results in prolonged residence in circulation following systemic delivery, and the incorporation of TAT peptide enhances cellular penetration. The authors delivered complexes with different PEI:PEG and PEI:DNA ratios to cells and studied their trafficking via colocalization with fluorescently labeled plasma membranes, endolysosomal compartments and nuclei. Relying on QD-FRET to detect particle integrity, they used experimental data to calculate the rates of 15 different aspects of the transfection process including unpacking and accumulation of DNA in the cytosolic, endolysosomal and nuclear compartments. They observed that transfection efficiency was positively correlated with the rate of cellular uptake but negatively correlated with the rate of unpacking within the endosomes and lysosomes, and used these correlations to explain differences in transfection and establish the optimal component ratios in their carrier design to maximize efficiency.

For chitosan-based delivery systems, it is known that transfection depends heavily on polymer molecular weight, and overly tight binding is typically considered a critical barrier [29]. As chitosan possesses no stimulus-responsive degradative mechanism, DNA must be released in response to natural chemical changes in its surroundings. For instance, increasing ionic strength in the endosomal compartment may disrupt electrostatic interactions between DNA and the polycation. Another possibility is that hyperprotonation of the amine groups of chitosan may occur during endosomal acidification, leading to electrostatic repulsion, elongation and disentanglement of polymer chains from DNA. This effect would be more pronounced with higher-weight chitosan. Lee *et al.* observed this phenomenon using QD-FRET, and demonstrated that nanoparticles generated with higher-molecular-weight chitosan dissociate faster in acidic conditions similar to those found in the lysosome [30]. The result was reproduced in living cells, lending credence to the proposed mechanism by which high-molecular-weight chitosan mediates higher transfection levels than shorter polymer chains.

Some researchers choose to use intercalating DNA dyes as QD-FRET components. While the amount of dye must be tuned to optimize the signal-to-noise ratio, no chemical conjugation or separation steps are necessary. Intercalating dyes may also be a means to include more acceptors per donor than would otherwise be possible, increasing the overall FRET efficiency. In one such case, a lipid-based transfection reagent was used to deliver QD donors conjugated to DNA labeled with the BOBO-3 intercalating dye as the acceptor [31]. QD-FRET signal was detected throughout the cytoplasm and peri-nuclear region, indicating the presence of intact complexes in those regions 4 h after transfection. Although the QDs and DNA were stably conjugated, differences in QD-FRET signal were detected between condensed particles and those that were released. As the signal dissipated, the authors measured both trafficking and unpacking kinetics. The application of QD-FRET in a lipid-based delivery system and use of DNA-intercalating acceptors are the most novel and interesting features of these studies.

QD-FRET has also been incorporated into more complex delivery systems. Harashima's group has engineered a lipid-based tetra-lamellar multifunctional envelope-type nano device containing a polycation–DNA core encapsulated by inner nuclear membrane and outer endosome fusogenic envelopes that facilitate nuclear entry and endosomal escape, respectively [32]. Tetra-lamellar multifunctional envelope-type nano-device particles were formed with QD-labeled DNA and their synthetic polymer, a bio-cleavable polyrotaxane modified with dimethylaminoethyl-modified α -cyclodextrin and a disulfide-linked PEG chain (DMAE-ss-PRX), labeled with rhodamine energy acceptors. Using QD-FRET, they found that modulation of the supramolecular structure of DMAE-ss-PRX caused a shift in decondensation to regions of euchromatin in the nucleus. This was the first report of directed unpacking to a specific nuclear subdomain using a polymeric carrier. Using a low-

molecular-weight polymer resulted in equal delivery to regions of hetero- and euchromatin, but unpacking occurred preferentially in regions of heterochromatin. Particles containing a higher-molecular-weight polymer delivered more DNA to the nucleus, with more than 70% deposited to euchromatin regions. The authors hypothesized that the shorter polymer generates a weakly associated core that is easily disrupted by the high concentration of cationic histones contained in the heterochromatin adjacent to the nuclear membrane. More stable cores may reach the euchromatin regions intact, where activity of the nuclear transcriptional machinery may encourage their dissociation. Deposition of free DNA into transcriptionally active euchromatin regions increases transgene expression levels. Using QD-FRET, the authors quantified three states of complex dissociation and intranuclear disposition, elucidating a mechanism that accounts for the different efficiencies of two carriers.

Characterization and optimization of custom delivery systems have proven to be valuable applications for QD-FRET. Jiang *et al.* designed micellar DNA nanocomplexes with a PEG-*b*-PPA polymer for targeted delivery to the liver [33]. Micelles made with high-molecular-weight PPA blocks exhibited instability in physiological salt solutions [34]. Taking advantage of this rapid mode of unpacking, they added bioreducible crosslinkers to enhance stability prior to reaching the cytoplasm. The crosslinked micelles remained stable in serum and ionic solutions, but degraded in a similar manner to noncross-linked analogs in the presence of salt following crosslink reduction in the cytoplasm. This dual sensitivity provided stability in the extracellular and endocytic compartments, while enabling rapid cytosolic release of DNA. As expected, the custom carrier mediated higher and more prolonged transfection levels than noncrosslinked analogs. The authors used QD-FRET to study the intracellular behavior of crosslinked and non-crosslinked carriers by measuring distributions of intact and unpacked micelles in the cytosol, endolysosomal and nuclear compartments. The dual-sensitive micelles unpacked much more slowly, and tended to release their payload in the cytoplasm and nucleus. They yielded higher transfection levels, improved extracellular stability and more sustained gene expression. In this case, quantitative QD-FRET studies elucidated the mechanism by which enhanced extracellular micelle stability results in improved performance.

QD-FRET for alternative nucleic acid payloads

In addition to plasmid DNA, the delivery of other nucleic acids is also attractive for both clinical and research applications. siRNA delivered intracellularly triggers RNAi and leads to the specific knockdown of expression of the target gene. While efficient uptake, subcellular trafficking and cytoplasmic release remain necessary, delivery of siRNA does not require nuclear entry or transcription.

To study siRNA delivery and the effect of carrier functionalization with cell-penetrating peptides, Lee *et al.* designed a QD-FRET pair by attaching QDs to PEI and Cy5 to siRNA [35]. Instead of using image analysis to quantify nanocomplex disassembly, the authors opted for a high-throughput approach using flow cytometry. They measured the kinetics of endocytic and non-endocytic uptake, intracellular trafficking and dissociation of nanocomplexes with and without the cell-penetrating peptide Hph-1. Detection of QD-FRET intensity by flow cytometry showed that QD-PEI cell-penetrating peptide complexes were both internalized and unpacked faster than those composed of nonfunctionalized QD-PEI. Both types ultimately unpacked almost completely within 5 h. The difference in early-stage behavior was attributed to the need for QD-PEI complexes to escape endosomes before unpacking, and there was no difference in gene silencing after 24 h. Interestingly, QD-PEI complexes mediated more gene silencing than unconjugated PEI, which the authors attributed to altered physical properties that increased endocytic uptake. In this case, QD-FRET coupled with flow cytometry enabled high-throughput quantification of dissociation

kinetics. However, confocal fluorescence microscopy techniques and image analysis were needed to determine the subcellular compartment in which dissociation occurred.

Another group used QD-FRET to study complexes formed with QDs modified to bind and deliver siRNA. Qi *et al.* conjugated poly(maleic anhydride-*alt*-1-decene) modified with dimethyl-aminopropylamine to custom-synthesized QDs [36]. The resulting carriers were small (~12 nm), water-soluble particles with a positive ζ potential that could bind, protect and efficiently deliver siRNA into cells. Binding of fluorescein isothiocyanate (FITC)-labeled siRNA was detected by the quenching of the FITC signal by FRET following addition of sufficient red-emitting QDs serving as acceptors. The nanocomplexes remained colloidally stable and mediated robust gene silencing in both serum-free and complete culture media. QD emission, along with the absence of a FITC signal, allowed tracking of the binding and uptake of intact complexes. Appearance of a FITC signal after 1.5 h indicated the onset of siRNA release. After 5 h, FITC-labeled free siRNA was observed to have been distributed throughout the cytoplasm. By functionalizing QDs with an amphipathic brush, the authors generated a novel QD-based siRNA delivery system with integrated imaging capabilities. As QDs are further developed as carriers themselves, QD-FRET promises to play an even greater role in the design and use of such delivery systems.

Another payload of interest is oligodeoxynucleotides (ODN). Antisense ODN delivered to the cytoplasm of cells can inhibit expression of a specific gene by binding its complementary mRNA transcript to block ribosomal access and hasten its degradation. QD-FRET has been used to study and compare the uptake and intracellular fate of ODN delivered by either lipoplex or polyplex [37]. The incorporation of QD-FRET pairs did not affect the performance of the ODN in either case. PEI-ODN nanocomplexes were internalized more readily during the first hour of transfection, probably due to a more positive ζ potential than the lipoplexes. The intracellular QD-FRET signals from both carrier types equilibrated by the end of the 4-h transfection period, and some unpacking was observed as early as 4 h post-transfection. Polymer-ODN nanocomplexes demonstrated a much higher rate of dissociation than lipoplexes, and unpacking of nearly all complexes of both types was observed by 48 h post-transfection. Furthermore, the presence of an intracellular QD-FRET signal in cells incubated with liposomes indicates that the complexes are internalized via endocytosis rather than membrane fusion, which would result in immediate dissociation and elimination of FRET. In this case, despite the differences in uptake and unpacking rates, ODN-mediated inhibition of gene expression was similar for both delivery systems.

QD-FRET for mechanistic studies

In addition to its utility in the study of nanoparticle trafficking and identification of intracellular barriers to delivery, QD-FRET is also applicable to the more fundamental, mechanistic studies associated with the design phase of nonviral carrier systems. Specifically, QD-FRET has been used in cell-free systems to scrutinize nanocomplex self-assembly, composition and stability. A thorough understanding of these processes and properties combined with precise quantitative data will accelerate the evolution of improved carriers.

An interesting quenching phenomenon discovered between QDs and the pentablock copolymer poly(diethylaminoethylmethacrylate)/Pluronic® F127 allowed Zhang *et al.* to study the effects of chloroquine on complex stability and DNA release in a cell-free assay [38]. Chloroquine is a lysosomotropic agent known to enhance transfection in many systems, and may participate in the dissociation of DNA from polymer carriers. The emission of cysteine-coated QDs was quenched via FRET by both plasmid DNA and pentablock copolymers, but not by preformed polymer-DNA nanocomplexes. Reductions in QD emission in the presence of complexes thus indicated dissociation and the release of

freed components. The addition of chloroquine resulted in quenching that varied linearly with the concentration of chloroquine added. This result revealed the contribution of chloroquine to complex destabilization and demonstrated the feasibility of using QD-FRET to measure induced dissociation in a well-defined, cell-free setting.

In an attempt to understand the poorly understood process of nanocomplex self-assembly, Ho *et al.* combined QD-FRET with microfluidic technology to monitor polymer-DNA complexation with millisecond resolution [39]. By generating complexes on a microfluidic chip, they were able to eliminate many of the forces involved with typical bulk mixing to isolate the assembly process. Solutions of Cy5-labeled chitosan and QD-DNA were introduced into micro-channels under laminar flow conditions, and self-assembly occurred at the channel interface (Figure 4). The band of QD-FRET-mediated Cy5 signal grew wider and brighter with distance as the complexes nucleated, matured and diffused outward, creating a spatial signal pattern that was subsequently transformed to determine the kinetic parameters of self-assembly. The authors described two distinct stages of the assembly process: a primary stage where assembly was determined to be diffusion-limited, and a secondary diffusion reaction-limited stage. QD-FRET provided a sensitive and quantitative indication of the onset and progression of molecular interactions throughout the self-assembly process. Such a system could be used to screen the chemical properties of carriers that affect DNA condensation, nanocomplex size and structure, and ultimately their efficiency. The same group also used single-molecule detection to compare the rates of QD emission before and after nanocomplex disruption to determine the number of plasmid copies within each nanoparticle [18]. The single particle analysis of the nanocomplex facilitates understanding of the physicochemical basis for its biological performance.

Two-step QD-FRET

The narrow emission spectra and broad absorption windows of QDs make them a natural choice for multiplexed applications, allowing the simultaneous detection of two or more processes. Instead of investigating each delivery barrier independently, Chen *et al.* recently proposed an integrated approach to monitor DNA release and degradation simultaneously with two-step QD-FRET [34]. Their system is based on three fluorophores and two parallel energy transfers (Figure 5). Three distinct states of DNA could be distinguished through quantitative ratiometric analysis of FRET efficiencies:

- When double-labeled plasmid DNA (QD/nuclear dye [ND]-DNA) is condensed in stable nanocomplexes, the QD donor drives energy transfer through the ND (E_{12}), which acts as a relay to Cy5 (E_{23}) conjugated on the polymeric gene carrier. For stable nanocomplexes, both the ND and Cy5 are 'on' or actively exhibiting FRET-mediated emission;
- When the nanocomplex begins to unpack and release intact DNA, only the ND is 'on' through E_{12} while Cy5 is 'off' due to the loss of E_{23} ;
- When free DNA is degraded, both ND and Cy5 are 'off' due to the loss of both E_{12} and E_{23} .

The technique was used to resolve mechanistic differences between two different carrier systems: quickly-dissociating PEG-*b*-PPA micelles and more stable chitosan nanocomplexes. Chitosan protects DNA more effectively, but its slow release ultimately limits its efficacy relative to PEG-*b*-PPA. This novel two-step QD-FRET method allows for detailed assessment of the onset of DNA release and degradation simultaneously, and is only possible due to the narrow emission spectra of the QD donor. An organic donor fluorophore would probably have resulted in significant levels of direct second acceptor excitation, which might then bias the analysis. Given the heterogeneity of nanocomplexes and

intracellular microenvironments, the single-particle resolution makes two-step QD-FRET particularly powerful in understanding the critical barriers to gene delivery.

Similarly, another group used two-step QD-FRET to monitor the delivery of the chemotherapeutic drug doxorubicin by a QD-RNA carrier [40]. They conjugated QDs to RNA aptamers, loaded with intercalated doxorubicin and targeting the prostate-specific membrane antigen. In the intact complex, both QD donor and doxorubicin acceptor fluorescence were quenched, the QD being quenched by doxorubicin and doxorubicin in turn being quenched by intercalation with the aptamer. Drug release resulted in recovery of both QD and doxorubicin fluorescence, which were then used to detect cancer cells that had internalized the targeted nanocomplexes. The complexes used in this study killed antigen-expressing cancer cells with high selectivity, demonstrating that QD-FRET has potential in combined therapeutic and diagnostic applications.

Selection of QD-FRET pairs & practical concerns

To maximize the benefits of QD-FRET in the study of nonviral nucleic acid delivery, a researcher planning to adopt the technique should give some thought to the relevant practical considerations when designing the system. Adoption of QD-FRET has probably been slowed by the perceived complexity of the chemical modification process and potential effect on the bioactivity of nucleic acids that arises from the size and surface properties of conjugated QDs. One group reported a decrease in DNA bioactivity after conjugation, probably due to a combination of random incorporation of biotin sites into coding regions and steric hindrance of the transcriptional machinery [18]. However, other groups have demonstrated high expression levels with QD-labeled DNA [41], and total retention of nucleic acid bioactivity is not crucial in mechanistic studies.

Determination of the ideal labeling frequency requires some optimization. Less than one QD per plasmid DNA molecule on average would provide sufficiently high sensitivity, given that each complex typically consists of multiple plasmids. Such a configuration would also guarantee minimum interference with complexation as well as false positives from excess free QDs. Similar principles apply to the labeling of gene carriers, although multiple organic fluorophore acceptors are typically required. The optimization involves the number of fluorophores per molecule, acceptor-to-donor ratio (which could also be tuned by adjusting the carrier-to-payload ratio), and balance between reasonable energy transfer efficiency and unaltered expression. When reduced bioactivity is a problem, one alternative is to use mixtures of labeled and unlabeled reagents. An additional consideration unique to the use of intercalating dyes for intracellular studies is that nuclear staining may occur, reducing the detectable fluorescence from nanocomplexes or released nucleic acids. Some QDs are large, so the physical properties of labeled and naive particles should be compared before moving forward. Cell-free characterization of the QD-FRET signal and nanocomplex formation is strongly recommended before moving to intracellular studies. QDs are poorly suited as acceptors in QD-FRET, due to their broad excitation windows and long exciton lifetimes. The cytotoxicity of labeled complexes should also be evaluated to avoid unexpected complications.

To date, most published QD-FRET pairs have been composed of commercially available fluorophores. The optical characteristics of the pairs described in this review have been summarized in Table 2. Theoretically speaking, QD-FRET pairs are chosen for their substantial spectral overlap $J(\lambda)$. Practically, however, detection sensitivity is also determined by the optical capabilities available to the user, including the excitation source, filter combinations and spectral response of the detector. While cellular imaging reveals the spatial distribution of complexes at given time points, flow cytometry-based detection

enables the rapid screening of a range of experimental conditions. A combination of the two techniques would supply a more complete picture of where and when nanocomplexes unpack. While a more exhaustive discussion of detection techniques, modes of analysis and methods of determining QD-FRET are beyond the scope of this review, we refer interested readers to a comprehensive review on FRET imaging [42].

Future perspective

Nonviral delivery of nucleic acids represents a potentially safe and effective treatment option for many diseases. However, success to date has been hindered by poor transfection efficiency. Elucidation of the rate-limiting barriers in non-viral gene transfer will help the engineering of more efficient carriers. QD-FRET has been a valuable addition to the arsenal of tools used to quantify the formation, composition, unpacking and degradation of nucleic acid nanocomplexes. The unique optical properties of QDs result in improved sensitivity, increased precision and ultimately the ability to derive more robust structure–function relationships. The versatility of QD-FRET enables not only studies of specific subcellular behavior with confocal microscopy, but also multiplexing and high-throughput analysis using flow cytometry.

QDs encounter some criticism for potentially interfering with biological entities due to their larger size compared with organic fluorophores [43]. The challenges to QD-based assays are retention of bioactivity and functionality, although QD conjugation has been shown to have minimal effects in many cases. Furthermore, the additional mass of larger QDs may alter the diffusivity of biomolecules, or disrupt the movement of bio-molecules. However, the ongoing improvement of QD synthesis yields fluorophores with reduced size but undiminished brightness, emission and photostability. These QDs promise to facilitate future QD-FRET studies with reduced concerns about interference.

The utility of QD-FRET in understanding nucleic acid delivery will continue to expand. QDs are already used in a number of *in vivo* applications [44], and can be tuned to operate in the near infrared range required for tissue penetration. This would open the door for the development of QD-FRET theranostics [45], as the field transitions from the molecular to the integrative level. As QD synthesis is further refined so that multiple QDs can be excited at a single wavelength and emit in distinct spectral ranges, the frequency of multicolor applications and multiplexing should increase. This will lead to even more detailed information, as multiple processes are monitored simultaneously. By enabling prolonged, sensitive, and real-time imaging of nonviral nucleic acid delivery, QD-FRET will help the field move away from trial-and-error approaches and promote the rational design of more effective systems.

References

Papers of special note have been highlighted as:

- of interest
- ■ of considerable interest

1. Verbaan FJ, Oussoren C, Snel CJ, Crommelin DJ, Hennink WE, Storm G. Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice. *J Gene Med.* 2004; 6(1):64–75. [PubMed: 14716678]
2. Schaffer DV, Lauffenburger DA. Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. *J Biol Chem.* 1998; 273(43):28004–28009. [PubMed: 9774415]

3. Akita H, Ito R, Khalil IA, Futaki S, Harashima H. Quantitative three-dimensional analysis of the intracellular trafficking of plasmid DNA transfected by a nonviral gene delivery system using confocal laser scanning microscopy. *Mol Ther.* 2004; 9(3):443–451. [PubMed: 15006612]
4. Hama S, Akita H, Ito R, Mizuguchi H, Hayakawa T, Harashima H. Quantitative comparison of intracellular trafficking and nuclear transcription between adenoviral and lipoplex systems. *Mol Ther.* 2006; 13(4):786–794. [PubMed: 16364692]
5. Grigsby CL, Leong KW. Balancing protection and release of DNA: tools to address a bottleneck of non-viral gene delivery. *J R Soc Interface.* 2010; 7(Suppl 1):S67–S82. [PubMed: 19734186]
6. Kiang T, Bright C, Cheung CY, Stayton PS, Hoffman AS, Leong KW. Formulation of chitosan-DNA nanoparticles with poly(propyl acrylic acid) enhances gene expression. *J Biomater Sci Polym Ed.* 2004; 15(11):1405–1421. [PubMed: 15648571]
7. Suh J, Wirtz D, Hanes J. Efficient active transport of gene nanocarriers to the cell nucleus. *Proc Natl Acad Sci USA.* 2003; 100(7):3878–3882. [PubMed: 12644705]
8. Panyam J, Labhasetwar V. Dynamics of endocytosis and exocytosis of poly(D,L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells. *Pharm Res.* 2003; 20(2):212–220. [PubMed: 12636159]
9. Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc Natl Acad Sci USA.* 1999; 96(9):5177–5181. [PubMed: 10220439]
10. Lucas B, Remaut K, Sanders NN, Braeckmans K, De Smedt SC, Demeester J. Studying the intracellular dissociation of polymer-oligonucleotide complexes by dual color fluorescence fluctuation spectroscopy and confocal imaging. *Biochemistry.* 2005; 44(29):9905–9912. [PubMed: 16026163]
11. Lucas B, Remaut K, Sanders NN, Braeckmans K, De Smedt SC, Demeester J. Towards a better understanding of the dissociation behavior of liposome-oligonucleotide complexes in the cytosol of cells. *J Control Release.* 2005; 103(2):435–450. [PubMed: 15763625]
12. Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc Natl Acad Sci USA.* 1999; 96(9):5177–5181. [PubMed: 10220439]
13. Lakowicz, JR. Principles of Fluorescence Spectroscopy. 4. Kluwer Academic/Plenum; NY, USA: 2006. p. 443–472.
14. Clapp AR, Medintz IL, Mattoussi H. Forster resonance energy transfer investigations using quantum-dot fluorophores. *Chemphyschem.* 2006; 7(1):47–57. [PubMed: 16370019]
15. Ho YP, Kung MC, Yang S, Wang TH. Multiplexed hybridization detection with multicolor colocalization of quantum dot nanoprobe. *Nano Lett.* 2005; 5(9):1693–1697. [PubMed: 16159207]
16. Bruchez M, Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. *Science.* 1998; 281(5385):2013–2016. [PubMed: 9748157]
17. Chan WCW, Nie SM. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science.* 1998; 281(5385):2016–2018. [PubMed: 9748158]
18. Ho YP, Chen HH, Leong KW, Wang TH. Evaluating the intracellular stability and unpacking of DNA nanocomplexes by quantum dots-FRET. *J Control Release.* 2006; 116(1):83–89. Specific benefits of quantum dot (QD)-Förster resonance energy transfer (FRET) for the study of nonviral gene delivery first reported. [PubMed: 17081642]
19. Förster T. Intermolecular energy transference and fluorescence. *Ann Phys.* 1948; 2:55–75.
20. Medintz IL, Mattoussi H. Quantum dot-based resonance energy transfer and its growing application in biology. *Phys Chem Chem Phys.* 2009; 11(1):17–45. Fundamental photophysics of QD-FRET and its use in biological applications. [PubMed: 19081907]
21. Clapp AR, Medintz IL, Fisher BR, Anderson GP, Mattoussi H. Can luminescent quantum dots be efficient energy acceptors with organic dye donors? *J Am Chem Soc.* 2005; 127(4):1242–1250. [PubMed: 15669863]
22. Itaka K, Harada A, Nakamura K, Kawaguchi H, Kataoka K. Evaluation by fluorescence resonance energy transfer of the stability of nonviral gene delivery vectors under physiological conditions. *Biomacromolecules.* 2002; 3(4):841–845. [PubMed: 12099831]

23. Kong HJ, Liu J, Riddle K, Matsumoto T, Leach K, Mooney DJ. Non-viral gene delivery regulated by stiffness of cell adhesion substrates. *Nat Mater.* 2005; 4(6):460–464. [PubMed: 15895097]
24. Chen HH, Ho YP, Jiang X, Mao HQ, Wang TH, Leong KW. Quantitative comparison of intracellular unpacking kinetics of polyplexes by a model constructed from quantum dot-FRET. *Mol Ther.* 2008; 16(2):324–332. Used data from QD-FRET experiments to derive quantitative model of transport and unpacking. [PubMed: 18180773]
25. Lauffenburger DA, Varga CM, Hong K. Quantitative analysis of synthetic gene delivery vector design properties. *Mol Ther.* 2001; 4(5):438–446. [PubMed: 11708880]
26. Lauffenburger DA, Varga CM, Tedford NC, Thomas M, Klibanov AM, Griffith LG. Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. *Gene Ther.* 2005; 12(13):1023–1032. [PubMed: 15815703]
27. Mitragotri S, Dinh AT, Pangarkar C, Theofanous T. Understanding intracellular transport processes pertinent to synthetic gene delivery via stochastic simulations and sensitivity analyses. *Biophys J.* 2007; 92(3):831–846. [PubMed: 17085500]
28. Ulasov AV, Khramtsov YV, Trusov GA, Rosenkranz AA, Sverdllov ED, Sobolev AS. Properties of PEI-based polyplex nanoparticles that correlate with their transfection efficacy. *Mol Ther.* 2011; 19(1):103–112. Used QD-FRET to derive a detailed quantitative model for carrier optimization. [PubMed: 21045811]
29. Lavertu M, Methot S, Tran-Khanh N, Buschmann MD. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials.* 2006; 27(27):4815–4824. [PubMed: 16725196]
30. Lee JI, Ha KS, Yoo HS. Quantum-dot-assisted fluorescence resonance energy transfer approach for intracellular trafficking of chitosan/DNA complex. *Acta Biomater.* 2008; 4(4):791–798. [PubMed: 18326480]
31. Lim TC, Bailey VJ, Ho YP, Wang TH. Intercalating dye as an acceptor in quantum-dot-mediated FRET. *Nanotechnology.* 2008; 19(7):075701. [PubMed: 21817649]
32. Shaheen SM, Akita H, Yamashita A, et al. Quantitative analysis of condensation/decondensation status of pDNA in the nuclear sub-domains by QD-FRET. *Nucleic Acids Res.* 2011; 39(7):e48. [PubMed: 21288880]
33. Jiang X, Zheng Y, Chen HH, Leong KW, Wang TH, Mao HQ. Dual-sensitive micellar nanoparticles regulate DNA unpacking and enhance gene-delivery efficiency. *Adv Mater Weinheim.* 2010; 22(23):2556–2560. [PubMed: 20440698]
34. Chen HH, Ho YP, Jiang X, Mao HQ, Wang TH, Leong KW. Simultaneous non-invasive analysis of DNA condensation and stability by two-step QD-FRET. *Nano Today.* 2009; 4(2):125–134. Demonstrated that two-step QD-FRET could measure stability and degradation simultaneously. [PubMed: 20161048]
35. Lee H, Kim IK, Park TG. Intracellular trafficking and unpacking of siRNA/quantum dot-PEI complexes modified with and without cell penetrating peptide: confocal and flow cytometric FRET analysis. *Bioconjug Chem.* 2010; 21(2):289–295. Applied QD-FRET to siRNA delivery and developed high-throughput measurement with flow cytometry. [PubMed: 20078095]
36. Qi L, Gao X. Quantum dot-amphipol nanocomplex for intracellular delivery and real-time imaging of siRNA. *ACS Nano.* 2008; 2(7):1403–1410. [PubMed: 19206308]
37. Wu Y, Ho YP, Mao Y, et al. Uptake and intracellular fate of multifunctional nanoparticles: a comparison between lipoplexes and polyplexes via quantum dot mediated Förster resonance energy transfer. *Mol Pharm.* 2011; 8(5):1662–1668. [PubMed: 21740056]
38. Zhang B, Zhang Y, Mallapragada SK, Clapp AR. Sensing polymer/DNA polyplex dissociation using quantum dot fluorophores. *ACS Nano.* 2011; 5(1):129–138. [PubMed: 21190373]
39. Ho YP, Chen HH, Leong KW, Wang TH. The convergence of quantum-dot-mediated fluorescence resonance energy transfer and microfluidics for monitoring DNA polyplex self-assembly in real time. *Nanotechnology.* 2009; 20(9):095103. [PubMed: 19417478]
40. Bagalkot V, Zhang L, Levy-Nissenbaum E, et al. Quantum dot-aptamer conjugates for synchronous cancer imaging, therapy, and sensing of drug delivery based on bi-fluorescence resonance energy transfer. *Nano Lett.* 2007; 7(10):3065–3070. [PubMed: 17854227]

41. Srinivasan C, Lee J, Papadimitrakopoulos F, Silbart LK, Zhao M, Burgess DJ. Labeling and intracellular tracking of functionally active plasmid DNA with semiconductor quantum dots. *Mol Ther.* 2006; 14(2):192–201. [PubMed: 16698322]
42. Jares-Erijman EA, Jovin TM. FRET imaging. *Nat Biotechnol.* 2003; 21(11):1387–1395. [PubMed: 14595367]
43. Smith AM, Duan H, Mohs AM, Nie S. Bioconjugated quantum dots for *in vivo* molecular and cellular imaging. *Adv Drug Delivery Rev.* 2008; 60(11):1226–1240.
44. Michalet X, Pinaud FF, Bentolila LA, et al. Quantum dots for live cells, *in vivo* imaging, and diagnostics. *Science.* 2005; 307(5709):538–544. [PubMed: 15681376]
45. Ho YP, Leong KW. Quantum dot-based theranostics. *Nanoscale.* 2010; 2(1):60–68. [PubMed: 20648364]

Websites

101. Invitrogen. www.invitrogen.com
102. Fluorophores.org. Database of fluorescent dyes, properties and applications. <http://fluorophores.mgfb.at>
103. PubSpectra. <http://home.earthlink.net/~pubspectra>

Executive summary

Background

- Clinical success of nucleic acid therapeutics is limited by the lack of safe and effective delivery systems.
- Rational design of improved nonviral carriers requires a quantitative understanding of the mechanisms and rate-limiting barriers to efficient delivery.
- Quantum dot (QD)-Förster resonance energy transfer (FRET) provides a stable and sensitive metric of intermolecular interactions and nanocomplex localization.

Why QD-FRET?

- The benefits of QDs include a broad absorption range, narrow size-tunable emission and enhanced chemical and photostability.
- QDs are used preferentially as energy donors due to their long radiative lifetimes, broad excitations and high extinction coefficients.

QD-FRET as a novel strategy to study delivery barriers

- QD-FRET was first used in nucleic acid delivery to study nanocomplex condensation, composition and dissociation.
- Results were used to derive a quantitative model used to explain the strengths and limitations of polymer-based carrier systems.

Recent applications of QD-FRET to study nonviral delivery

- QD-FRET has enabled the measurement of plasmid DNA condensation, uptake, release, degradation and subnuclear disposition.
- The technique is also used to study delivery of alternative nucleic acid payloads including siRNA and antisense oligonucleotides.
- Cell-free mechanistic studies have also benefited from the increased stability and sensitivity of QD-FRET.

Two-step QD-FRET

- The unique optical properties of QDs allow for the simultaneous measurement of two or more interactions with a series of FRET pairs.

Selection of QD-FRET pairs

- Optimization of the QD-FRET design can maximize sensitivity and minimize unwanted changes, such as altered nanocomplex physicochemical properties and diminished bioactivity.

Future perspective

- QD-FRET is a powerful and versatile technique used to understand the mechanisms of nucleic acid delivery, and will play an increasingly important role in the design of improved nonviral carrier systems.

Figure 1. Putative barriers contributing to the low transfection efficiency typically achieved by nonviral delivery systems

Additional processes such as exocytosis and endolysosomal degradation may also contribute to diminished transfection. Unpacking may occur in any of the subcellular compartments, while it is also possible for intact complexes to enter the nucleus.

Figure 2. Absorption and emission spectra of conventional and quantum dot-based systems
(A) A conventional organic fluorophore-based Förster resonance energy transfer pair and
(B) a quantum dot-Förster resonance energy transfer pair with a quantum dot energy donor. Direct acceptor excitation and donor-acceptor crosstalk are common problems with traditional organic fluorophores. Substituting quantum dot donors avoids these concerns, while improving other properties such as a larger spectral overlap between donor emission and acceptor absorption.

Figure 3. Detection of intracellular unpacking

When labeled molecular components are condensed into a nanocomplex, quantum dot-Förster resonance energy transfer results in acceptor emission upon excitation of the donor. Once the complex unpacks within the cell, quantum dot-Förster resonance energy transfer is abolished and donor emission is regained.

QD: Quantum dot.

Figure 4. Quantum dot-Förster resonance energy transfer has been used to quantify the kinetics of complexation using microfluidics

605 QD-labeled pDNA and Cy5-labeled polymers were loaded at equal volume flow rates. QD-FRET signal was observed along the interface of the streams, demonstrating complex formation. Monochrome fluorescence images ($285 \times 200 \mu\text{m}$) of FRET-mediated Cy5 signal (670 nm) indicating the degree of complexation were obtained at different time intervals, calculated from their axial distance from the junction.

FRET: Förster resonance energy transfer; QD: Quantum dot.

Adapted with permission from [39].

Figure 5. Simultaneous detection of complex stability and nucleic acid degradation

Two-step QD-Förster resonance energy transfer enables the simultaneous detection of nanocomplex unpacking and nucleic acid degradation using a three-fluorophore system (QD, ND and Cy5) through two parallel energy transfer processes (E_{12} and E_{23}). Under excitation of the QD donor, three distinct states of DNA could be distinguished: when condensed within intact nanocomplexes, the QD donor drives energy transfer through the ND (E_{12}), which acts as a relay to the carrier-bound acceptor (E_{23}), resulting in emission from Cy5; when the nanocomplex is unpacked only the ND is 'on' through E_{12} , while Cy5 is 'off' due to the loss of E_{23} . Emission from the ND, through E_{12} , suggests that DNA remains intact; when free DNA is degraded, both ND and Cy5 are 'off' due to the loss of both E_{12} and E_{23} , leaving only emission from the QD donor.
 ND: Nuclear dye; QD: Quantum dot.

Table 1

Reports of quantum dot-Förster resonance energy transfer used to study nonviral delivery of nucleicacids.

Payload and carrier	Mechanism detected	Detection approach	Significance of findings	Ref.
pDNA, chitosan	Unpacking	Confocal microscopy, single molecule detection	First report of QD-FRET used to study unpacking of polymer-DNA nanocomplexes	[18]
pDNA, PEI, PPA, chitosan	Unpacking	Confocal microscopy	Kinetics of trafficking and unpacking correlated with transfection efficiency	[24]
pDNA, PEI-PEG-TAT	Uptake, trafficking, unpacking	Confocal microscopy	Correlated transfection with faster uptake and diminished endosomal unpacking in multiple cell lines; used results to optimize carrier composition	[28]
pDNA, chitosan	Unpacking	Confocal microscopy	Higher MW chitosan unpacks faster at low pH and results in higher transfection efficiency	[30]
pDNA, liposome	Uptake, integrity	Confocal microscopy	Demonstrated intact QD-DNA complexes delivered to cytoplasm and perinuclear region by liposomes	[31]
pDNA, DMAE-SS-PRX	Unpacking	Confocal microscopy	Showed that different carriers deliver DNA to different nuclear subdomains	[32]
pDNA, PEG-PPA	Unpacking	Confocal microscopy	Validated increased extracellular stability of crosslinked micelles, leading to increased transfection efficiency	[33]
pDNA, PEG- <i>b</i> -PPA, chitosan	Unpacking, degradation	Confocal microscopy, single molecule detection	Three distinct states of DNA condensation and integrity distinguished extra- and intracellularly	[34]
siRNA, PEI	Uptake, unpacking	Confocal microscopy, flow cytometry	Adding Hph-1 to QD-PEI results in faster unpacking; QD-PEI silences better than PEI alone	[35]
siRNA, QD-PMAL	Uptake, unpacking	Confocal microscopy	QDs coated with amphipol delivered siRNA efficiently while allowing intracellular tracking of integrity	[36]
ODN, liposome, PEI	Uptake, unpacking	Confocal microscopy, flow cytometry	ODN complexes formed with a polycation are internalized and unpacked more quickly than ODN liposomes	[37]
pDNA, PDEAEM, copolymer	Unpacking	Spectrofluorometry	Demonstrated role of chloroquine in nanocomplex dissociation	[38]
pDNA, chitosan	Complexation	Fluorescence microscopy	Determined kinetics of nanocomplex self-assembly with millisecond resolution	[39]
Doxorubicin DNA aptamer, QD	Drug release	Confocal microscopy	Simultaneous detection of drug release, identification of cancer cells and targeted cell killing; demonstrated potential of QD-FRET in theranostics	[40]

DMAE: Dimethylaminoethyl-modified α -cyclodextrin; FRET: Förster resonance energy transfer; MW: Molecular weight; ODN: Oligodeoxynucleotide; PDEAEM: Poly(di ethylaminoethylmethacrylate); PEG: Polyethylene glycol; PEI: Polyethylenimine; PMAL: Poly(maleic anhydride-*alt*-1-decene) modified with dimethylaminopropylamine; PPA: Polyphosphoramidate; PRX: Polyrotaxane; QD: Quantum dot; ss: Disulfide link.

Table 2
Optical parameters of quantum dot-Förster resonance energy transfer pairs chosen to study nucleic acid delivery.

Fluorophores/QD-FRET pair	Emission (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Quantum yield	Spectral overlap, $J(\lambda)$ ($M^{-1}cm^{-1}nm^4$)	Förster radius, $R_0(\text{\AA})$	Ref.
<i>Individual fluorophores</i>						
Cy3	570	1.5×10^5 (Ex: 552 nm)	0.15			
Cy5	670	2.5×10^5 (Ex: 650 nm)	0.28			
Texas red	614	8.5×10^4 (Ex: 596 nm)	NA			
Rhodamine	573	1.29×10^5 (Ex: 560 nm)	NA			
AlexaFluor647	668	2.37×10^5 (Ex: 633 nm)	0.33			
BOBO-3	605	1.48×10^5 (Ex: 570 nm)	0.39			
Adirondack green QD	525	1.3×10^5 (Ex: 488 nm)	0.4			
Qdot545	545	2.9×10^5 (Ex: 488 nm)	0.4			
Qdot605	603	11×10^5 (Ex: 488 nm)	0.4			
Qdot625	621	27×10^5 (Ex: 488 nm)	0.4			
<i>QD-FRET pairs</i>						
Qdot525-Cy3				7.02×10^{15}	59.19	[34]
Qdot525-BOBO-3				4.44×10^{15}	52.26	[31,34]
Adirondack green QD-Texas red				1.17×10^{15}	43.92	[30]
Qdot545-rhodamine				8.07×10^{15}	60.58	[32]
Qdot605-Cy5				1.24×10^{16}	65.09	[18,24,33, 37,39]
Qdot605-AlexaFluor647				1.11×10^{16}	63.89	[28]
Qdot625-Cy5				2.13×10^{16}	71.24	[35]

Values were estimated according to Equation 2 for the FRET pairs used in the studies reviewed here.

Orientation factor (κ^2) and refraction index (n) were assumed to be 2/3 and 1.4, respectively.

Other parameters were obtained from the manufacturers when possible, or adapted from [101–103].

Ex: Excitation; FRET: Förster resonance energy transfer; NA: Not available; QD: Quantum dot.