Resonance Energy Transfer-Based Nucleic Acid Hybridization Assays on Paper-Based Platforms Using Emissive Nanoparticles as Donors

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Abstract:

Quantum dots (QDs) and upconverting nanoparticles (UCNPs) are luminescent nanoparticles (NPs) commonly used in bioassays and biosensors as resonance energy transfer (RET) donors. The narrow and tunable emissions of both QDs and UCNPs make them versatile RET donors that can be paired with a wide range of acceptors. Ratiometric signal processing that compares donor and acceptor emission in RET-based transduction offers improved precision, as it accounts for fluctuations in the absolute photoluminescence (PL) intensities of the donor and acceptor that can result from experimental and instrumental variations. Immobilizing NPs on a solid support avoids problems such as those that can arise with their aggregation in solution, and allows for facile layer-by-layer assembly of the interfacial chemistry. Paper is an attractive solid support for the development of point-of-care diagnostic assays given its ubiquity, low-cost and intrinsic fluid transport by capillary action. Integration of nanomaterials with paper-based analytical devices (PADs) provides avenues to augment the analytical performance of PADs, given the unique optoelectronic properties of nanomaterials. Herein, we describe methodology for the development of PADs using QDs and UCNPs as RET donors for optical transduction of nucleic acid hybridization. Immobilization of green-emitting QDs (gQDs) on imidazole functionalized

cellulose paper is described for use as RET donors with Cy3 molecular dye as acceptors for the detection of SMN1 gene fragment. We also describe the covalent immobilization of blue-emitting UCNPs on aldehyde modified cellulose paper for use as RET donors with orange-emitting QDs (oQDs) as acceptors for the detection of HPRT1 gene fragment. The data described herein is acquired using an epifluorescence microscope, and can also be collected using technology such as a typical electronic camera.

Keywords: Quantum Dots, Upconverting Nanoparticles, Resonance Energy Transfer, Paper-Based Bioassays, Nucleic Acid Hybridization

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1 Introduction

1.1 Quantum Dots in Resonance Energy Transfer

Quantum dots (QDs) are colloidal semiconductor nanocrystals with diameter in the range of 2 to 10 nm.[1] The two commonly reported structural types of QDs are core QDs (e.g. CdSe, CdTe and CdS) and core/shell QDs (e.g. CdSe/ZnS, CdSe/CdS and $\text{CdSe}_x\text{S}_{1-x}/\text{ZnS}$).[1] The core/shell structural type is prevalent in bioassay development as the shell passivates the optical properties of QDs that originate from its core. QDs exhibit robust and unique optical properties that originate from quantum confinement effects. These properties include narrow (full-width-at-half-maximum in the range of 25-40 nm), symmetric and size tuneable emission spectra, greater photostability and brightness than organic fluorophores, high quantum yields and a large extinction coefficient over a broad range of wavelengths that stretches from the UV region to their first exciton peak in the visible region.[1] These properties are well-suited for the use of QDs in optical multiplexing for the development of bioassays. QDs are commercially available with numerous surface chemistries allowing for easy conjugation of biorecognition elements for use in bioassays.[1]

QDs are popular RET donors due to their high quantum yield and tuneable photoluminescence (PL). The surface area of QDs allows the immobilization of multiple recognition elements, where a single QD can participate in multiple RET events[1]. Our group has paired green-emitting QDs (gQDs) and red-emitting QDs (rQDs) with various molecular dyes as RET acceptors in nucleic acid hybridization assays[2, 3]. While QDs are popular RET donors, their use as RET acceptors has been limited to time gated measurements[4], chemiluminescence resonance energy transfer (CRET) [5] and bioluminescence resonance energy transfer (BRET) [6]. The broad absorbance band of QDs that stretches into the UV region of the spectrum makes them susceptible to direct excitation in the process of exciting the RET donor. We have recently paired QDs as RET acceptors with UCNPs[7, 8]. UCNPs are excited in the near-IR and IR region and emit in

the UV to near-IR region of the spectrum. This permits the excitation of the RET donor without the direct excitation of the QDs. We have previously shown the utility UCNP/QD RET pair for bioassay development using analytes such as proteins[7] and nucleic acids[8].

1.2 Upconverting Nanoparticles as Donors in Resonance Energy Transfer

UCNPs are luminescent nanocrystals that are typically tens of nanometers in size. They exhibit anti-Stokes emission based on the process of upconversion. Low-energy pump photons are accumulated in multiple long-lived excited-states of lanthanide ions that are supported in an inert crystal lattice. The subsequent relaxation to the ground electronic state results in the emission of radiation of higher energy than the excitation light[9]. A common class of UCNPs are lanthanide doped inorganic crystals, such as NaYF_4 and Y_2O_3 . The unique $4f^n 5d^{0-1}$ electronic orbitals of Ln^{3+} ions, which are shielded by the $5s^2$ and $5p^6$ sub-shell electrons, present energy states that can be long lived (up to 0.1 s) and are thus ideal for UC processes. There are five major upconversion processes observed in lanthanide doped UCNPs: excited state absorption (ESA), energy transfer upconversion (ETU), cooperative upconversion (CUC), photon avalanche (PA), and energy migration upconversion (EMU)[9]. Of these processes, ETU is the most efficient and is based on co-doping the inorganic lattice with two lanthanide species, a sensitizer (donor) and an activator (acceptor). For selection criteria of dopants, refer to reference [9]. The sensitizer ions are capable of absorbing photons in the NIR or IR region of the spectrum and effectively transfer the energy non-radiatively to an activator ion for UC luminescence. The sequential excitation of one activator and its subsequent relaxation to the ground electronic state results in an anti-Stokes emission[9]. Herein, we use Yb^{3+} and Tm^{3+} as sensitizer and activator, respectively, to obtain emission peaks in the UV and blue regions of the spectrum. By changing the identity and concentration of the

dopant ions, different emission profiles can be obtained. Lanthanide doped UCNPs are protected with an inert shell of the same material as the host lattice to prevent non-radiative relaxation pathways resulting from interactions with the high-energy vibrational modes of surface ligands and collisions with solvent molecules[9].

Our group has used UCNPs on paper as direct labels[10] and as RET donors with molecular dyes[11] and QDs [8]as acceptors in nucleic acid hybridization assays. Blue emitting NaYF₄: 0.5%Tm³⁺, 30% Yb³⁺/NaYF₄ core/shell UCNPs have been paired with orange emitting QD acceptors[8]; green and red emitting NaYF₄: 2%Er³⁺, 18% Yb³⁺/NaYF₄ core/shell UCNPs have been paired with Cy3 and Cy5.5 dyes[11]. The use of a near-IR excitation source minimizes background signals due to the suppression of light scatter and auto-fluorescence[9].

1.3 Solid-Phase Assays

Solid-phase resonance energy transfer (RET)-based bioassays are characterized by immobilization of a donor nanoparticle (e.g. QDs or UCNPs) on the surface of a solid support that is modified with a suitable surface chemistry to facilitate its immobilization[1]. The immobilized donor nanoparticle surface is then conjugated to a biorecognition element to allow selective interaction with a solution-phase analyte. The selective binding event modulates the luminescence of donor nanoparticle, which is achieved by pairing an appropriate acceptor (a fluorescent dye or another nanoparticle) with the donor nanoparticle. The selective binding interaction modulates the efficiency of energy transfer between the donor and the acceptor, where the resulting emission from an acceptor serves as an analytical signal[1].

Solid-phase RET-based bioassays that make use of nanoparticles as an active component of a transduction interface offer various advantages in terms of assay development and analytical performance when compared with the corresponding solution-phase assay. Immobilization of nanoparticles on a solid support eliminates the need to maintain their colloidal suspension, which allows implementation of solution conditions (e.g. solvent composition and reaction conditions) that may be beneficial to the analytical performance of the assay[1, 12]. On the other hand, exposure of such solution-phase conditions to a colloidal suspension of nanoparticles can potentially compromise their colloidal stability, the latter being an integral requirement for the performance of solution-phase nanoparticle assays. Immobilization of a biorecognition element is greatly simplified in the case of solid-phase assays, where the immobilized nanoparticles can be exposed to a high concentration of reagents to facilitate bioconjugation. Excess reagents can be rinsed from the surface without the need for time-consuming purification steps that are typically encountered in a multi-step synthetic protocol. As a result, bioconjugation can be readily achieved[1, 12]. A solid support that is modified with a selective chemistry can be used to capture targets of interest from a complex matrix. The selectivity of biomolecular interaction can be improved by exposing the substrate to washes in order to suppress the contribution of interferents prior to measurement[1, 12]. Solid-phase assays are also useful for the development of reusable assays. The biomolecule-target complex can be dissociated by changing the environment (e.g. by means of manipulation of temperature, ionic strength or introduction of denaturing agents), while retaining the immobilized selective chemistry on the surface of a solid support for subsequent interrogation of another sample solution for the analyte of interest[1, 12]. It should be noted that reusability is an important feature of biosensors. Solid-phase nanoparticle assays are also amenable to integration with a number of near-field optical transduction techniques, which include

evanescent wave excitation[13, 14] and plasmonics[15]. These techniques not only improve the versatility of nanoparticle-based solid-phase assays, but they can also have a pronounced effect on the analytical performance of the assays.

Another significant advantage of solid-phase assays employing nanoparticle mediated RET for signal transduction is the improvement in assay sensitivity that arises from enhancement of energy efficiency at an interface. When donors and acceptors are immobilized at sufficiently high density at an interface there are additional energy transfer pathways between donors and acceptors, where multiple donors can interact with a single acceptor in addition to the interaction of one donor with multiple acceptors[1]. At an interface, there are no discrete donor-acceptor pairs. Instead, there is a two-dimensional plane of donors and acceptors. As a result, the probability of energy transfer to a given acceptor increases for solid-phase RET-based assays. This enhancement is sufficient to compensate for the loss of available surface area of a nanoparticle for selective interaction, which is experienced when one face of a nanoparticle is blocked by immobilization to an interface[1].

1.4 Paper as a Support for Development of Diagnostic Assays

There is a growing interest in the development of decentralized diagnostic assays that can be applied at the point-of-care and point-of-need settings in order to improve patient care and to make health care more accessible. In this regard, paper-based assays have attracted considerable attention in recent years given the advantageous attributes of paper substrates. These attributes include: (1) low-cost and widespread commercial availability of paper substrates with different pore sizes and flow rates[16]; (2) cost-effective, high-throughput and ease of patterning of paper-based analytical devices (PADs) using methods such as wax printing, stamping and drawing[17, 18]; (3) PADs can be operated independent of any supporting equipment given the autonomous

fluid flow offered by the hydrophilic cellulosic fibers of paper substrates; (4) well established methods for surface modification of cellulose for biomolecule immobilization; (5) an elegant approach to eradicate biohazard by means of incineration of paper; (6) requirement of a small reagent/sample volume and (7) compatibility with biological samples[19]. A number of these attributes are in congruence with the ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end-users) guidelines set by the World Health Organization in order to develop diagnostics for the developing world[19]. Examples of PADs include dipsticks assays, lateral flow devices, paper-based 96-zone and 384-zone plates, microfluidic paper-based analytical devices (μ PAD), three-dimensional PADs and origami PADs[20].

1.5 Paper-Based Solid-Phase Nucleic Acid Hybridization Assays Using the gQD/Cy3 and UCNP/QD RET pairs

In this chapter, we describe methods for solid-phase nucleic acid hybridization assays on a paper-based platform using QDs and UCNPs as donors in RET-based transduction scheme. The paper substrates were patterned using a wax printing method and subsequently chemically derivatized with functional groups that were suitable for the immobilization of the donor nanoparticles. In case of the gQD/Cy3 (donor /acceptor) RET pair (Figure 1a), the paper substrates were chemically modified with imidazole groups to allow immobilization of gQDs that were pre-modified with oligonucleotide probes. Subsequent hybridization of the target strand and the Cy3-labeled reporter strand brought the Cy3 acceptor dye in close proximity to the surface of immobilized gQDs to allow RET-sensitized emission from the Cy3 acceptor dye, which served as an analytical signal upon excitation of gQDs. In case of the UCNP/oQD RET pair (Figure 1b), the paper substrates were modified with aldehyde groups to allow covalent immobilization of blue-emitting UCNPs.

The immobilized UCNPs were subsequently bioconjugated to an oligonucleotide probe. The hybridization of the target strand and the reporter strand that was bioconjugated to an oQD resulted in a close proximity of the UCNP and the oQD, where the resulting RET-sensitized emission from the oQD served as an analytical signal upon excitation of UCNPs. This chapter also entails experimental and data analysis methods for a ratiometric transduction of nucleic acid hybridization using the aforementioned RET pairs. Readout of the donor and acceptor emissions is described using both an epifluorescence microscope and a low-cost electronic camera employing colored digital imaging. Given the advantageous attributes of paper substrates for the development of low-cost diagnostic assays, the nucleic acid hybridization assays described herein can potentially find applications in field-portable and remote diagnostic applications.

Fig. 1 Illustration of interfacial chemistry for paper-based solid-phase transduction of nucleic acid hybridization using the (a) gQD/Cy3 RET pair and the (b) UCNP/QD RET pair. The paper substrates was patterned using wax printing and the circular hydrophilic paper zones were derivatized with (a) imidazole groups for the immobilization of QD-probe oligonucleotide conjugates and (b) aldehyde moieties for immobilization of UCNPs. Sequential addition and hybridization of target and the (a) Cy3-labeled or the (b) QD-labeled reporter oligonucleotides provided the necessary proximity for RET sensitized emission from the acceptor (a) Cy3 dye or

(b) oQD upon excitation of donor (a) gQDs(emission maximum at 525 nm) or (b) UCNP with (a) a UV or (b) IR excitation source. Upon a selective hybridization event, the emission PL shifts from the donor to the acceptor and the modulation of the donor and the acceptor PL serve as an analytical signal.

2 Materials

2.1 Reagents

- 1 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)(HEPES) buffer: 100 mM HEPES
(pH 7.2)
- 2 1-(3-Aminopropyl)imidazole (API, $\geq 97\%$)
- 3 1.7 mL microcentrifuge tubes
- 4 2.5 M NaCl solution prepared using cartridge-purified water (the Milli-Q water)
- 5 Anhydrous ethanol.
- 6 Avidin from egg white
- 7 Borate buffer (BB1): 50 mM borate (pH 9.2)
- 8 Borate buffered saline (BB2): 50 mM borate (pH 9.2, 100 mM NaCl)
- 9 Chloroform, reagent grade
- 1 Dithiothreitol($>99\%$)
- 0
- 1 Ethyl acetate, reagent grade
- 1
- 1 Hydrophobic green-emitting QDs (gQDs) and orange emitting QDs (oQDs). We use
2 commercially available alkyl-ligand coated ternary alloyed $\text{CdSe}_x\text{S}_{1-x}/\text{ZnS}$ QDs (gQDs with
peak PL at 525 nm and oQDs with peak PL at 575 nm) from Cytodiagnostics Inc.
(Burlington, ON, Canada)
- 1 Hexanes, reagent grade
- 3
- 1 L-Glutathione, reduced (GSH)
- 4
- 1 Lithium chloride (LiCl, anhydrous, ACS reagent, $\geq 99\%$)
- 5
- 1 Maleimidohehexanoic acid – G(Aib)GHHHHHH from CanPeptide (Pointe-Claire, QC)
- 6

1 Methanol, reagent grade
7
1 NHS-PEG₄-biotin from Thermo Scientific (Rockford, IL USA)
8
1 Octadecene, reagent grade
9
2 Oleic acid, reagent grade, 90 %
0
2 Phosphate-buffered saline (PBS)
1
2 Phosphorylethanolamine (PEA)
2
2 Sodium (meta)periodate(NaIO₄, ≥ 99%)
3
2 Sodium cyanoborohydride (NaCNBH₃, reagent grade, 95%)
4
2 Sodium hydroxide
5
2 Sterile ultrapure Milli-Q water (specific resistance ≥18 MΩ.cm)
6
2 Tetramethylammonium hydroxide (TMAH): 25 % w/w solution in methanol
7
2 Thulium (III) acetate hydrate(99.9%)
8
2 Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) powder (≥ 98%)
9
3 Whatman® cellulose chromatography papers (Grade 1, 20 cm × 20 cm)
0
3 Ytterbium(III) acetate hydrate(99.9%)
1
3 Yttrium(III) acetate hydrate (99.9%)
2
3 Oligonucleotide sequences listed in Table 1 (see Note 1). The sequences were from
3 Integrated DNA Technologies (Coralville, IA, USA)

Oligonucleotide Sequences for the Hybridization Assays

Table 1. Probe, target and reporter oligonucleotide sequences used in the hybridization assays with the gQD/Cy3 and UCNP/QD RET pairs.

Name	Sequence (5' to 3' direction)
gQD/Cy3 RET	

SMN1 probe	DTPA-5'-ATT TTG TCT GAA ACC CTG T-3'
SMN1 FC target	5'-TCC TTT ATT TTC CTT ACA GGG TTT CAG ACA AAA T-3'
SMN1 Cy3 reporter	Cy3-5'-AAGGAAAATAAAGGA-3'
UCNP/QD RET pair	
HPRT1 probe	5'-Biotin-CAA AAT AAA TCA AGG TCA-3'
HPRT1 FC target	5'-GAT GAT GAA CCA GGT TAT GAC CTT GAT TTA TTT TG-3'
HPRT1 reporter	5'-TAACCTGGTTCATCATC-Thiol-3'

Abbreviations: FC = fully-complementary, DTPA = dithiol phosphoramidite, Cy3 = cyanine 3.

2.2 Instrumentation and Equipment

1	Nikon Eclipse L150 epifluorescence microscope (Nikon, Mississauga, ON, Canada) custom fitted with the following components: 4× Nikon WD Plan Fluor objective lens (NA = 0.13); a filter cube comprising ZET405/20× as an excitation filter, Z405rdc as a dichroic mirror and a HQ430lp as an emission filter (Chroma Technologies Corp., Bellows Falls, VT, USA); a 25 mW diode laser excitation source with an output of 402 nm (Radius 402, Coherent Inc. Santa Clara, CA, USA) and a diode array spectrometer (QE65000, Ocean Optics Inc. Dunedin, FL, USA) as a detector.
2	Nikon Eclipse L150 epifluorescence microscope (Nikon) custom fitted with the following components: 40× Nikon WD Plan Fluor objective lens (NA = 0.60); a filter cube comprising a ZET964/86bp excitation filter (Chroma), a ZT1064rdc-sp dichroic mirror (Chroma) and a 455/35 (Nikon) or 570/20 (Chroma) emission filters; a 2.5 W tunable 980 nm collimated diode laser (Laserglow Technologies, Toronto, ON, CAN) and a H5784-20 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA)
3	Handheld ultraviolet (UV) lamp (UVGL-58, LW/SW, 6W, The Science Company®, Denver, Co, USA)
4	HP8452A Diode-Array Spectrophotometer (HP Corporation, Palo Alto, CA, USA)
5	iPad mini (Apple, Cupertino, CA, USA)
6	Low-binding polypropylene microcentrifuge tubes, 1.7 mL capacity
7	Micropipettes and tips

8	Neutral density (ND) filters (ND 4, ND 8 and ND 16)
9	Orbital shaker
10	0.2 μ m Polyethersulfone (PES) syringe filters
11	Xerox ColorQube 8570DN solid ink wax printer (Xerox Canada, Toronto, ON, Canada)
12	Amicon Ultra-0.5 mL 100 kDa centrifugal filters (Millipore Corporation, Billerica, MA, USA)

3 Methods

3.1 UCNP synthesis

3.1.1 Synthesis of Core NaYF₄: 0.5%Tm³⁺, 30% Yb³⁺ UCNPs

1. Add 0.4562, 0.2534 and 0.0042 g of Y(CH₃CO₂)₃.xH₂O, Yb(CH₃CO₂)₃.4H₂O, Tm(CH₃CO₂)₃.xH₂O and a stir bar into a 250 mL three-neck round bottom flask.
2. Add 30mL of octadecene and 12 mL of oleic acid into the round bottom flask.
3. Place the round bottom flask on a heating mantle controlled by a temperature precision controller.
4. Insert the precision controller thermometer in the central neck of the three-neck round bottom flask through a septum. Attach a two-neck collection round bottom flask via a bent adaptor to one of the side necks of the reaction flask. Seal the remaining necks of the reaction and the collection flasks with septa. (see Note 2)
5. Stir the mixture gently under vacuum at 115 °C for 30 min. The solution should turn clear and colourless at this point.

6. Cool the mixture to 50 °C under a gentle stream of argon. Insert the in-line via a needle in the unused neck of the three-neck round bottom flask. Insert the out-line via a needle in the unused neck of the collection round bottom flask. The argon flow should be maintained for the remainder of the synthesis.
7. Prepare a 20 mL methanol solution containing 0.20g NaOH and 0.30 g NH₄F via sonication. The solution will turn cloudy. Shake the solution occasionally to help dissolve the solids (see Note 3). Add the methanol solution to the reaction mixture.
8. Allow the reaction to stir for 30 min at 50 °C before heating it to 75 °C to evaporate the methanol. The solution should become clear at this point. (see Note 4)
9. Increase the temperature to 300 °C rapidly and maintain it for 1 hour. Wrap the reaction flask with glass fibre to help keep the solution warm. (see Note 5)
10. Allow the reaction mixture to cool to room temperature under argon.
11. Add an equal volume of absolute ethanol to the reaction mixture and centrifuge the solution at 4500 rpm to collect the UCNPs.
12. Resuspend the UCNPs in hexanes and repeat step 8. Repeat two more times.
13. Store the washed core UCNPs in hexanes in a glass vial at 4 °C for subsequent growth of shell.

3.1.2 Synthesis of NaYF₄: 0.5%Tm³⁺, 30% Yb³⁺/NaYF₄ core/shell UCNPs

14. Add 0.5738 g of Y(CH₃CO₂)₃.xH₂O into a 250 mL three-neck round bottom flask.
15. Follow steps 2 to 5.
16. Cool the mixture to 80 °C under argon and add the core UCNPs from step 13.
17. Cool the reaction temperature to 50 °C after all the hexane has evaporated.
18. Prepare a 20 mL methanol solution containing 0.14g NaOH and 0.26 g NH₄F via sonication. Add the mixture to the reaction vessel. The reaction mixture will turn cloudy. (see Note 3)

19. Allow the reaction to stir for 30 min before heating the mixture to 75 °C to evaporate the methanol. The solution should be clear at this point. (see Note 4)
20. Increase the temperature to 300 °C rapidly and maintain it for 1 hour. (see Note 5)
21. Allow the reaction mixture to cool to room temperature under argon.
22. Add an equal volume of absolute ethanol and centrifuge at 4500 rpm to collect the UCNPs.
23. Resuspend the UCNPs in hexanes and repeat step 8. Repeat twice.
24. Store the oleic acid capped core/shell UCNPs in hexanes or toluene in a glass vial at 4 °C for subsequent modification.

3.2 Preparation of water soluble QDs and UCNPs

3.2.1 Preparation of water soluble UCNPs

1. Mix 2 mL of hexanes containing 100 mg of oleic acid capped core/shell UCNPs from step 24, 400 mg of phosphorylethanolamine(PEA) and 1 mL of tetramethylammonium hydroxide (TMAH) in 10 mL of absolute ethanol in a capped glass vial.
2. Stir the reaction vigorously overnight at 70 °C in an oil bath.
3. Allow the reaction to cool to room temperature and recover PEA capped UCNPs by centrifugation at 3500 rpm.
4. Resuspend the UCNPs in ethanol via sonication and mix the solution with an equal volume of hexanes before centrifugation at 4500 rpm.
5. Repeat step 4 two times.
6. Resuspend the washed PEA capped UCNPs in 10 mL of water and pass the solution through a 0.2 µm polyether sulfone (PES) syringe filter to remove aggregates. If desired, evaporate water using a rotary evaporator to concentrate UCNPs.

7. Store the PEA capped UCNPs in excess PEA at 4 °C.

3.2.2 Preparation of water soluble QDs

1. Add 75 μL of 10 μM alkyl QDs into 2 mL of chloroform in a glass vial.
2. Dissolve 0.2 g of reduced L-Glutathione (GSH) in 600 μL of TMAH.
3. Add the solution of QDs drop-wise to the GSH solution while swirling.
4. Allow the cloudy mixture to sit overnight in the dark.
5. Extract water soluble GSH coated QDs using BB2 in 100 μL fractions. Place the collected fractions in a 1.7 mL microcentrifuge tube.
6. Add an equal volume of ethanol and collect the QDs by centrifugation at 8000 rpm for 5 min.
7. Resuspend the QDs in 200 μL of BB2.
8. Repeat steps 6 and 7 two more times.
9. Resuspend the GSH-QDs in BB1.
10. Measure the absorbance spectrum of the QDs and use their first exciton peak and known molar extinction coefficient to determine the NP concentration.
11. Store the GSH-QD at 4 °C for subsequent use.

3.3 Bioconjugation of Oligonucleotides to QDs

3.3.1 Bioconjugation of hexahistidine-terminated oligonucleotides to GSH-QDs

1. Incubate thiol-terminated oligonucleotides (27 nmol in 100 μL) with 500 equivalents of dithiothreitol (DTT) (27 μL of 0.5 M DTT solution in 1 x PBS) and 100 μL of 1 x PBS buffer for 1 h at room temperature to reduce disulfides moieties into sulfhydryl groups.
2. Extract excess DTT with 600 μL of anhydrous ethyl acetate four times.

3. Add 6-maleimidohexanoic acid – G(Aib)GHHHHHH (0.7 mg in 30 μ L of DMSO) to the oligonucleotide solution in 20 times molar excess and allow the reaction mixture to shake for 12 h at room temperature.(see Note 6)
4. Purify the modified oligonucleotides using a NAP-5 desalting column as per the manufacturer's instructions.
5. Quantify the purified hexahistide-modified oligonucleotides by UV-vis spectroscopy (λ_{max} = 260nm).
6. Store the hexahistidine-modified oligonucleotides at -20 °C.
7. Incubate hexahistidine-modified oligonucleotides (HPRT1 reporter) with GSH-QDs at the desired QD:DNA ratio for 1 hour in BB2 in a 1.7 mL microcentrifuge tube on an orbital shaker. (see Note 7)
8. Purify the hexahistidine-modified oligonucleotides by centrifugation three times using an Amicon Ultra-0.5 mL 100kDa centrifugal filter as per the manufacturer's instructions to remove excess nucleic acids.
9. Store the washed nucleic acid conjugated QDs in BB2 at 4 °C.

1.3.2 Bio-conjugation of DTPA-terminated oligonucleotides to GSH-QDs

1. Prepare 50 mM solution of TCEP by dissolving 7 mg of TCEP in 500 μ L of borate buffer (BB1).
2. To a 1.7 mL microcentrifuge tube, pipette 169 μ L of borate buffered saline (BBS).
3. To the same tube, pipette 82 μ L of 50 mM TCEP solution that was prepared in step 1.
4. Add 8.2 nmol of DTPA terminated oligonucleotide probe (SMN1 probe) to a solution prepared in step 3 and place the tube on an orbital shaker for 15 min (see Note 8).

5. Pipette 200 pmol of GSH-gQDs to the solution in step 4 (see Note 9).
6. Pipette additional volume of borate buffered saline (BB2) to the solution in step 5 such that the final solution volume is 493 μL . For the example of QD-probe conjugates preparation provided in this section, an additional 169 μL of borate buffered saline (BB2) will need to be added.
7. Agitate the contents of the tube overnight on an orbital shaker.
8. Prepare a fresh 50 mM TCEP solution using step 1.
9. Pipette a 40 μL aliquot of 50 mM TCEP solution from step 8 into the contents of the tube in step 7.
10. Incrementally pipette 100 μL of 2.5 M NaCl solution to the tube in step 9. We use an interval time of 10 min for each sequential addition of 10 μL of 2.5 M NaCl (see Note 10).
11. Place the tube in step 10 on an orbital shaker for an overnight incubation and subsequently store the contents of the tube (QD-probe conjugates) inside a fridge at 4 $^{\circ}\text{C}$ until further use. The concentration of QD-probe conjugates in this solution is *ca.* 312 nM (total solution volume is 633 μL).

3.4 Wax Printing and Chemical Modification of Paper

3.4.1 Wax printing of paper substrates

1. Using an appropriate software, draw a pattern of the paper device that is to be printed on the Whatman® cellulose chromatography paper substrates. We use AutoCAD 2012 software (see Note 11). For the purpose of the work described in this chapter, the dimensions of each paper device

is 25 mm × 60 mm and a single sheet of 20 cm × 20 cm Whatman® chromatography paper substrate comprised 18 paper devices (arranged in a 6 × 3 array format). We use paper devices that contain 32 circular zones with a diameter 5 mm (see Note 12), arranged in a 4 × 8 array format. We print on bare paper, and the zones are surrounded by a filling with black ink.

2. Print the paper substrates using a Xerox ColorQube 8570DN solid ink wax printer (see Note 13).
3. Preheat an oven or a hot plate to 120 °C.
4. Place the paper sheet (printed side up) inside the oven or on top of the hotplate for 2.5 min to confine the hydrophilic paper zones across the thickness of the paper substrate (see Note 14).
5. Allow the paper sheet to cool to room temperature.
6. Using scissors, trim the patterned paper sheet to individual paper devices.

3.4.2 Chemical derivatization of paper zones with aldehyde functionality

1. Attach a binder clip to the end of a wax-printed paper substrate in order to suspend the paper device in air. We do so by inserting a micropipette tip in the arms of the binder clip such that the bottom of the micropipette tip rests inside one of the holders of the microcentrifuge tray rack (see Note 15).
2. Prepare 1.4 M solution of LiCl by dissolving 30 mg of LiCl in 500 µL of Milli-Q water.
3. Prepare 94 mM solution of NaIO₄ by dissolving 10 mg of NaIO₄ in 500 µL of Milli-Q water.
4. Mix the two solutions prepared in steps 2 and 3 in equal volumes.

5. Spot a 5 μL aliquot of the solution prepared in step 4 onto each paper zone and incubate the spotted paper devices inside an oven set at 50 $^{\circ}\text{C}$ for 30 min.
6. Repeat step 5.
7. Wash the paper devices three times with Milli-Q water (see Note 16).
8. Place the washed paper devices on any form of absorbent blotting paper in order to wick off the excess water.
9. Dry the paper devices inside a vacuum desiccator.

3.4.3 Chemical derivatization of aldehyde modified paper zones with imidazole functionality

1. Suspend aldehyde modified paper substrates in air using a binder clip as mentioned previously.
2. Prepare 320 mM solution of NaCNBH_3 by dissolving 10 mg of NaCNBH_3 in 500 μL of 100 mM HEPES buffer (pH 8.0).
3. To 400 μL of 320 mM solution of NaCNBH_3 prepared in step 2, pipette 12 μL of API. The concentration of API in the resulting solution is 0.24 M.
4. Pipette a 5 μL aliquot of the solution prepared in Step 3 onto each paper zone that was modified with the aldehyde functionality.
5. Incubate the spotted paper substrates under ambient conditions for 1 hour.

6. Submerge each paper device inside a 50 mL conical centrifuge tube filled with borate buffer (BB1).
7. Place the conical centrifuge tubes on an orbital shaker for 15 min in order to wash the paper devices.
8. Remove the imidazole modified paper substrates from the conical tubes and place them on any form of an absorbent blotting paper in order to wick off the excess solution.
9. Dry the paper substrates inside a vacuum desiccator.

3.5 Immobilization of QDs and UCNPs on Paper

3.5.1 Immobilization of QD-probe oligonucleotide conjugates on imidazole modified paper substrates

1. Suspend imidazole modified paper substrates in air using a binder clip as mentioned previously.
2. Spot a 3 μ L aliquot of 312 nM solution of QD-probe conjugates solution onto each paper zone that was modified with imidazole functionality and incubate for 1 hour at room temperature in dark (see Note 17).
3. Wash the paper devices for 15 min with borate buffer (BB1) using an orbital shaker by submerging each paper device inside a 50 mL conical centrifuge tube containing BB1.
4. Wick off the excess solution from the paper substrates using any form of absorbent blotting paper and dry the QD-probe conjugates modified paper substrates inside a vacuum desiccator.

3.5.2 Immobilization of UCNPs on aldehyde modified paper substrates

1. Mix 200 μL of PEA-UCNPs (3 mg mL^{-1}) with 200 μL of a 0.1 mM sodium cyanoborohydride solution in HEPES buffer (100mM, pH 7.2).
2. Suspend aldehyde modified paper substrates in air using a binder clip as mentioned previously.
3. Pipet 5 μL of the solution in Step 1 to each aldehyde modified paper zone.
4. After incubating for 10 min., wash the paper substrate in 0.1% v/v aqueous Tween® 20 solution for 5 min in a conical centrifuge tube.
5. Wash the paper substrate with purified water for 2 min
6. Place the washed paper substrate on any form of absorbent blotting paper to wick off the excess water.
7. Dry the paper substrate inside a vacuum desiccator.

3.5.3 Layer-by-layer assembly of the assay using immobilized UCNPs on paper substrates

1. Suspend paper substrates with immobilized UCNPs in air using a binder clip as mentioned previously.
2. Pipet 3 μL of a freshly prepared 2 mM aqueous NHS-PEG-biotin solution to each spot of the paper substrate containing UCNP and allow to air dry.
2. Wash the paper for 2 min with purified water in a conical centrifuge tube.

3. Place the washed paper substrate on an absorbent blotting paper to wick off the excess water before placing it inside a vacuum desiccator to dry.
4. Pipet 5 μL of a 20 μM avidin solution in HEPES buffer (100 mM, pH 7.2) to each paper zone. Allow the zones to air dry.
5. Wash the paper for 2 min with BB1 in a conical centrifuge.
6. Place the washed paper substrate on an absorbent blotting paper to wick off the excess water before placing it inside a vacuum desiccator to dry.
6. Pipet 5 μL of 10 μM biotinylated oligonucleotide probe (HPRT1 probe) solution in BB1 to each paper zone. Allow the paper to air dry.
7. Repeat steps 5 and 6.

3.6 Calibration Curve of Target DNA

1. Prepare dilutions of the SMN1 FC or HPRT1 FC target in the concentration range of 20 nM to 15 μM using 50 mM borate buffered saline (pH 9.2, 100 mM NaCl) (see Note 18).
2. Prepare a dilution of SMN1 Cy3 reporter at 10 μM to 15 μM concentration or HPRT1 oQD reporter at 2 μM using borate buffered saline (BB2).
3. Spot 3 μL of borate buffered saline (BB2) onto the paper zones that belong to the top row of the paper device (see Note 19).
4. Spot a 3 μL aliquot of SMN1 FC or HPRT1 FC target at various concentrations onto each of the different rows of the paper device (excluding the first row, see point 3). In order to collect replicate

measurements, the same concentration of the target can be spotted onto the paper zones that belong to the same row of the paper device (see Note 20).

5. Allow the targets/sample solutions to incubate on the paper device for 1 hour (see Note 21).

6. Spot a 3 μ L aliquot of 10 μ M to 15 μ M solution of SMN1 Cy3 reporter or of 2 μ M HPRT1 oQD reporter onto each of the paper zones that were subjected to the target/sample hybridization and allow the reporter solution to incubate on the paper device for 30 min (see Note 22).

7. Wash the paper substrates for 5 min with borate buffered saline (BB2) using an orbital shaker by submerging each paper device inside a 50 mL conical centrifuge tube containing BB2. Add 1% Tween ® 20 by volume to the wash solution when QDs are used as acceptors.

8. Wick off the excess solution from the paper substrates using an absorbent blotting paper and dry the paper substrates inside a vacuum desiccator prior to the data collection (see Note 23).

3.7 Data Acquisition

3.7.1 gQD/Cy3 RET Pair

1. Using the epifluorescence microscope platform (402 nm laser excitation source and diode array spectrometer as a detector), measure the PL spectrum from each paper zone that was modified with the selective chemistry (immobilized QD-probe conjugates) (see Note 24). Also, collect a background spectrum from imidazole modified paper zone (without immobilized QD-probe conjugates).

2. Representative data acquired using the epifluorescence microscope platform is shown in Figure 2b and c.

3. For the hybridization assays using the gQD/Cy3 RET pair, the red-green-blue (RGB) color palette of a digital camera can also be used to acquire quantitative information. Using the long wavelength (365 nm) setting of a handheld UV lamp (UVGL-58, LW/SW, 6W, The Science Company®), illuminate the paper devices for the excitation of immobilized QDs on paper substrates and collect a colored digital image of the paper device under the default settings of the built-in Camera application of an iPad mini in a dark environment (see Note 25). Proceed to section 3.8.2 for data analysis.

2. Representative data from the iPad detection platform is shown in Figure 2a and c.

Fig.2 Solid-phase QD-RET transduction of nucleic acid hybridization on paper substrates using the gQD/Cy3 RET pair. (a) Colored digital image and the corresponding pseudo-colored PL images of gQDs (G channel) and Cy3 (R channel) after R-G-B splitting of the colored digital image with increasing amount of SMN1 FC TGT. The amounts of SMN1 FC TGT in (i) to (viii) were 0, 0.94, 1.9, 3.8, 7.5, 15, 30 and 45 pmol, respectively. (b) Normalized PL spectra acquired using the

epifluorescence microscope platform showing the assay response with increasing amounts of SMN1 FC TGT. The amounts of SMN1 FC TGT in (i) to (xii) were 0, 0.057, 0.12, 0.23, 0.47, 0.94, 1.9, 3.8, 7.5, 15, 30 and 45 pmol, respectively. (c) Calibration curves showing the RET ratio response (red) and the R/G ratio response (black) with increasing amount of SMN1 FC TGT. Figure adapted with permission from reference [21]. Copyright 2014 American Chemical Society.

3.7.2 UCNP/QD RET Pair

1. Using the epifluorescence microscope platform with a 980nm laser excitation source and the PMT detector, measure the PL from each paper zone. Appropriate optical emission filters are used to collect the UCNP and QD signals independently. Herein, we use 455/35 nm and 570/20 nm band-pass filters to collect UCNP and QD luminescence, respectively. The paper is scanned on the microscope stage and an image is obtained using LabView. (see Note 26)
2. Representative data from the epifluorescence detection platform is shown in Figure 3.

Fig.3 Solid-phase UCNP/QD (donor/acceptor) RET transduction of nucleic acid hybridization on paper substrates. (a) Pseudo-colored microscope images of UCNPs (blue channel), oQDs (orange channel) and an overlay of both images with increasing amount of HPRT1 FC target. The amounts of HPRT1 FC target ranged from 15 fmol to 6 pmol. (b) Calibration curve showing the RET ratio with increasing amount of HPRT1 FC target.

4 Data Analysis

4.1 Analysis for gQD/Cy3 RET Pair

4.1.1 Analysis of PL Spectra

1. Subtract background spectrum from each PL spectrum that was collected from the paper zones with immobilized QD-probe conjugates.
2. Normalize each background corrected PL spectrum to the maximum PL intensity of gQDs, i.e., divide all the PL intensities by the maximum PL intensity of gQDs (the λ_{max} for gQDs is in the range of 520 nm to 530 nm). The resulting spectra will depict profiles as shown in Figure 2b.
3. Calculate RET Ratio for each background subtracted and normalized PL spectrum using Equation 1. In Equation 1, the wavelength range of 560 nm to 590 nm in the numerator of each term is used to integrate the Cy3 (acceptor) PL intensity, while the wavelength range of 510 nm to 540 nm in the denominator of each term is used to integrate the gQD (donor) PL intensity. The subscripts DA and D denote measurements made in the presence and absence of the acceptor, respectively (see Note 27).

$$FRET\ ratio = \frac{\int_{560}^{590} \frac{dI}{I} PL(I) \div \int_{510}^{540} \frac{dI}{I} PL(I) \div \theta_{DA}}{\int_{560}^{590} \frac{dI}{I} PL(I) \div \int_{510}^{540} \frac{dI}{I} PL(I) \div \theta_D} \quad (1)$$

4. Plot the calculated RET ratios in step 3 as a function of the amount of target DNA that was spotted onto each paper zone. An example of the resulting data set is shown in Figure 2c.

4.1.2 Analysis of Digital Images

1. Open the acquired colored digital images using the ImageJ software (National Institute of Health, Bethesda, MB, USA).

2. Click on the image tab and sequentially select the color and then split channels option from the dropdown menu in order to split each colored digital image into the corresponding red, green and blue color channels. The channels of interest are the red and green color channels, which respectively interrogate the acceptor (Cy3) and the donor (gQDs) PL intensities as shown in Figure 2a (see Note 28).

3. Draw a circle around a paper zone in the split red and green color channels images by selecting the oval drawing tool (see Note 29).

4. Click on the Analyze tab and select the measure option from the dropdown menu. This will open a new window displaying the area, mean, min and max values corresponding to the paper zone that was selected. The value of interest is the mean value.

5. Determine the mean value from each of the paper zones by dragging the same drawn circle from one paper zone to another and subsequently repeating step 4 for different paper zones.

6. Calculate the R/G ratio corresponding to each of the paper zones using Equation 2. In equation 2, I_R corresponds to the mean intensity of the paper zone in the red channel, while I_G corresponds

to the mean intensity of the same paper zone in the green channel. The subscripts DA and D denote measurements made in the presence and absence of the acceptor, respectively (see Note 30).

$$R / G \text{ ratio} = \frac{\bar{I}_R}{\bar{I}_G} \bigg|_{DA} - \frac{\bar{I}_R}{\bar{I}_G} \bigg|_D \quad (2)$$

7. Plot R/G ratios as a function of the amount of target DNA that was spotted onto each paper zone. An example of the resulting data set is shown in Figure 2c.

4.2 Analysis of Microscope Images for the UCNP/QD RET Pair

1. Using the ImageJ software (National Institute of Health, Bethesda, MB, USA), open the images that were acquired by scanning the paper using an epifluorescence microscope.
2. Click on Plugins, Macros, Record. (see Note 31)
3. Draw circles around each paper zone for the resultant image using 420-490nm optical filter using the oval drawing tool. The circle drawn for one zone can be dragged over to other zones to ensure that the mean intensity determined from each zone represents the same surface area.
4. After each circle is drawn, press the “M” key on the keyboard to measure an integrated intensity in the paper zone. This will display the area, and the mean, minimum and maximum intensity values of the paper zone that was selected. Use the mean value as the average signal from each zone.
5. In the Macros window, save the resultant code.
6. Open the image the was scanned using the 460-480 nm filter
7. Go to Plugins, Macro, Run and select the code that was saved. This will automatically give results from each zone on this image in the same order as the zones were created in the previous image.

8. Calculate a RET Ratio (Equation 3) for each reaction zone by taking the ratio of the signal in the QD (acceptor) channel and UCNP (donor) channel.

$$RET Ratio = \frac{I_{QD}}{I_{UCNP}} - \frac{I_{QD}}{I_{UCNP}} \quad (3)$$

9. Plot the calculated RET ratios as a function of amount of DNA target that was spotted onto each paper zone. Take an average of the three paper zones that were subjected to the same target concentration. An example of the resulting data set is shown in Figure 3.

5 Notes

Note 1: SMN1 sequence is a genetic marker that is diagnostic of the neuromuscular disorder known as spinal muscular atrophy [22]. We have used the SMN1 sequences as model sequences to demonstrate the principles of solid-phase QD-RET nucleic acid hybridization assays. HPRT1 is a housekeeping gene found in mammalian cells. It was selected as a generic target to demonstrate the principles of solid-phase RET between UCNPs and QDs. Housekeeping genes are routinely used for control and calibration in biotechnological applications and genomic studies[8].

Note 2: A small incision is made in the septum for the thermometer.

Note 3: This solution can be prepared after step 5 has been set up and will be ready to use in this step.

Note 4: Mark the solvent level on the reaction flask before the addition of the methanol and wait for the solvent level to return to the same level. The methanol should evaporate and condense in the collection flask. Check the side flask to determine the end of condensation.

Note 5: Be careful not to get any material trapped between the heating mantle and the reaction flask as it is a fire hazard.

Note 6: Ensure that the 6-maleimidohexanoic acid solution is prepared before extracting the excess DTT. Add the 6-maleimidohexanoic acid solution to the reduced thiol DNA immediately after extraction to ensure that the sulfhydryl functionality is fully available.

Note 7: A maximum of approximately 20 nucleic acids can be loaded on one QD using this method.

Note 8: As an example, if the concentration of SMN1 probe was 239.8 μM , then 34 μL of the probe solution will need to be pipetted. TCEP is used as a reducing agent to reduce the disulfide (DTPA) moiety associated with each oligonucleotide probe to a dithiol[23]. TCEP is added at 500 times molar excess of the DTPA probe concentration. Therefore, if a different amount of DTPA probe is used, then the volume of 50 mM TCEP solution should be adjusted accordingly.

Note 9: As an example, if the concentration of GSH-QDs was 5.1 μM , then *ca.* 39 μL of the QD solution will need to be pipetted. The amount of QDs added in this step is such that the stoichiometric ratio between the QDs and DTPA probe is 1 to 40, respectively. The bioconjugation of DTPA probe to CdSeS/ZnS (core/shell) QDs is driven by self-assembly *via* the coordination linkage of dithiol moiety to the Zn^{2+} atoms on ZnS shell of the QDs[24-26].

Note 10: This step is referred to as the salt aging of QD-probe conjugates. Salt aging is used to increase the loading capacity of oligonucleotide probes to the surface of QDs by screening the electrostatic repulsion associated with the negative charge of the sugar-phosphate backbone of oligonucleotides[21]. We have previously reported that without the salt-aging step, an average of 17 oligonucleotide probes can be self-assembled to the surface of similar GSH-QDs when incubated with 40 times molar excess of the oligonucleotide probes[21]. However, with the salt-

aging step, an average of 35-40 oligonucleotide probes can be self-assembled to the surface of the GSH-QDs for the aforementioned preparation[21].

Note 11: Ensure that the pattern of the paper device fits within the 20 cm × 20 cm dimensionality of the Whatman® cellulose chromatography paper sheet.

Note 12: After the wax melting step, the actual diameter of each paper zone will be smaller than what was originally designed, i.e., less than 5 mm. An estimate of the size of the features that were wax printed on the paper substrates after the melting step can be made as described by Carrilho *et al.*[17].

Note 13: A custom setting of the printer may be required in order to print the paper substrate.

Note 14: This step melts and spreads the wax laterally and vertically, resulting in a formation of hydrophobic wax barriers across the thickness of the paper. Owing to the lateral spreading of the wax, the resulting diameter of each hydrophilic paper zone on the paper device will be approximately 3 mm[17].

Note 15: This ensures that any solution that is spotted onto the paper zones does not come in contact with any other surface, which can otherwise result in reagent loss.

Note 16: We wash paper devices by submerging each paper device inside a 50 mL conical centrifuge tube that is filled with Milli-Q water.

Note 17: Dilutions of the stock QD-probe conjugates solution can also be prepared and spotted onto the imidazole modified paper zones. Quantitative information can also be acquired using various concentrations of the QD-probe conjugates solution(e.g. 8.7, 52 or 167 nM) provided that calibration data exist for these concentrations of immobilized QD-probe conjugates.

Note 18: It is preferred that a serial dilution method is used in order to prepare various dilutions of SMN1 FC TGT. Also, ensure that the NaCl concentration in each of the target solutions is adjusted to 100 mM. We use 2.5 M NaCl solution in order to adjust NaCl concentration to 100 mM.

Note 19: This allows for the collection of PL spectra and images from paper zones that were modified with immobilized QD-probe conjugates but not exposed to the target sample.

Note 20: There are four paper zones along each row of the paper device. This allows four replicate measurements to be collected by spotting the same target concentration along a particular row of the paper device. To evaluate non-specific adsorption, a row of paper zones can also be spotted with SMN1 NC target.

Note 21: During this incubation step, the target/sample solutions may dry on the paper device. However, this does not affect the experimental outcome.

Note 22: We typically apply reporter that is in 2-3 fold molar excess of the target concentration. For experiments involving single nucleotide polymorphism discrimination, the paper substrates are first washed with 15% (v/v) formamide solution in borate buffer (BB1) for 15 min and then with borate buffered saline (BB2) for 1 min prior to the reporter hybridization step.

Note 23: We have previously reported that data collection from dry paper substrates offers at least an order of magnitude higher assay sensitivity and at least an order of magnitude lower limit of detection as compared to the corresponding hydrated paper substrates[21].

Note 24: We use a 4× objective lens (NA = 0.13) for the collection of PL spectra from paper substrates. Depending on the concentration of QD-probe conjugates that was spotted onto the

paper zones and the power of the laser excitation source, a neutral density (ND) filter may be required in order to attenuate the laser excitation intensity to prevent detector saturation. We use a ND 8 filter for experiments that rely on excitation with the 25 mW diode laser and QD-probe conjugates spotted onto the paper substrates at 312 nM (936 fmol) concentration. Due to ratiometric signal processing, fluctuations in the absolute PL intensities of donor and acceptor that are caused by factors such as variations in detector sensitivity, excitation source intensity and sample dilutions do not significantly affect the experimental outcome[27]. While QDs exhibit a strong and broad absorption band[1], which is a useful attribute to efficiently excite QDs with a broad range of excitation wavelengths, the selection of excitation wavelength close to 400 nm is done in order to minimize direct excitation of the Cy3 acceptor[1].

Note 25: For the collection of PL images, place the paper substrates and the UV lamp on a horizontal surface and hold the iPad mini orthogonal to the horizontal surface. Depending on the concentration of QD-probe conjugates that was spotted onto the paper zones, a placement of ND filter in front the iPad mini camera may be required to prevent pixel saturation. For QD-probe conjugates spotted onto the paper devices at 312 nM (936 fmol) concentration, we place paper devices 10 cm away from the UV lamp and use ND 16 filter. The selection of ND filter (ND 4, ND 8 or ND 16) will also be dependent on the separation distance between the lamp and the paper device.

Note 26: We use a 40x objective lens ($NA = 0.60$) to collect the photoluminescence from the paper. The tunable power of the IR laser is set between 0.4 and 1 W depending on the concentration and brightness of immobilized UCNPs.

Note 27: The second term in Equation 1 is a correction factor and accounts for the crosstalk of the gQDs donor PL with the Cy3 acceptor PL.

Note 28: After splitting the color channels, the images will be in the grey color scheme. Pseudo-coloring of the images can be done by clicking on the image tab and then selecting the Lookup Tables option from the dropdown menu, followed by selecting the desired color scheme. We use red color for displaying the red color channel and green color for displaying the green color channel.

Note 29: Make sure the drawn circle fits within the dimensions of the paper zone. The same drawn circle can be dragged from one paper zone to another in order to analyze the mean PL intensity from each paper zone. This also ensures that the mean intensity is determined from each of the paper zones that represent the same unit area.

Note 30: The second term in Equation 2 is a correction factor and accounts for the crosstalk of the gQDs donor intensity in the red channel.

Note 31: This allows the software to track the locations on the paper from where the signals are collected as an integrated area for the image collected with the 420-490 nm filter. This can then be applied to the image collected with the 460-480 nm filter.

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