Supporting Information

A Novel Type of Quantum Dot – Transferrin Conjugate Using DNA Hybridization Mimics Intracellular Recycling of Endogenous Transferrin

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MATERIALS AND METHODS

Materials. QDs emitting at 650 nm were obtained from Nexdot in hexane and used as it is. Human holo Transferrin (Tf), Fluoroscein isothiocynate (FITC), tris(2-carboxyethyl)phosphine (TCEP), electrophoresis grade agarose, Ethidium Bromide (EtBr), Bovine Serum Albumin, Strepavidin, biotin maleimide, and streptavidin agarose beads were obtained from Sigma Aldrich. Alexa-647 NHS ester was obtained from FluoProbes, Interchim. Sulfo succinimidyl-4-(*N*-maleimidomethyl cylcohaxane-1-carboxylate) (sSMCC), Hams F-12 media, trypan blue, fetal bovine serum (FBS), trypsin, cell-culture grade Phosphate Buffered Saline (PBS) and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), Streptomycin, Penicillin, hygromycin and G418 antibiotics were obtained from Gibco, ThermoFischer Scientific. Polyacrylamide Gel was obtained from Biorad. InstantBlue

stain was obtained from Expedeon. Glass bottom LabTek-II imaging chambers and vivaspin centrifugal filters (100KDa, 30KDa, 3kDa) were purchased from VWR. HPLC purified, amine or fluorophore labelled DNA was purchased from IDT and used without further purification.

Methods.

A. Ligand exchange of quantum dots. QDs were obtained from Nexdot, received in hexane and used for ligand exchange as it is. For water dispersion of QDs, a classical two step ligand exchange procedure was followed. QDs were precipitated in ethanol and resuspended in MPA. After solubilization with MPA, the QDs were capped with the polymer comprising of 50% dithiol and 50% sulfobetaine zwitterions by protocols published previously.[1]

B. Bioconjugation reactions. All bioconjugates were prepared by following indicated procedures and purified using size exclusion chromatography or ultrafiltration as specified.

B1. *Conjugation of DNA on QDs.* DNA conjugation was carried according to our previous work.[2] Briefly, 300 nmol of sSMCC was added to a solution of 12.5 nmol DNA-NH_2 in 0.2M $NaHCO_3$, pH 8.3 (sSMCC/DNA=25/1). The reaction was kept for 45 min at room temperature under vigorous stirring to yield DNA-maleimide. Excess unreacted sSMCC was removed by ethanol precipitation. In parallel, 2.5 nmol QDs were reduced with 1000 nmol TCEP (TCEP/QD = 400) for 30 mins in PBS pH 7.4 under continuous stirring. Excess of TCEP was removed from the reaction using NAP-5 size exclusion columns. The reduced QD were mixed with DNA maleimide (DNA/QD=5) and left for reaction in presence of 1400mM NaCl overnight. The final products (QD-DNA) were first desalted by filtration (Vivaspin 10kDa) and then loaded on HPLC for size exclusion purification. For quantification of DNA/QD, detection of DNA-peak at 260 nm as described in our previous work was employed.[2]

B2. *Conjugation of DNA to Transferrin (Tf-cDNA).* DNA was conjugated to proteins by reaction of thiol-labelled complementary DNA (cDNA-SH) with Tf-maleimide. Briefly, 50 nmol of protein was reacted with 500 nmol of sSMCC (sSMCC/protein = 10) in 0.2M $NaHCO_3$, pH 8.3 for 1 hour under gentle agitation. The excess of sSMCC was removed by passing the reaction through vivaspin 30 kDa at least 5 times to obtain pure Tf-maleimide. In parallel, 1 µmol cDNA-SH was reduced with 400 µmol TCEP (TCEP/DNA = 50) in PBS pH 7.4 for at least 45 mins with constant stirring. Excess of TCEP was purified by passing the reaction through vivaspin 3 kDa at least 5 times. The cDNA-SH were mixed with the Tf-maleimide (DNA/protein = 20) and left for reaction in PBS pH 7.4 overnight under mild stirring. The conjugates (Tf-cDNA) were purified from excess DNA by SEC purification.

B3. *Functionalization of QD-DNA with Tf-cDNA.* QD-DNA (0.1 nmol) was incubated with Tf-cDNA (0.5 nmol) at 37°C and left for hybridization (Protein/QD = 5) in PBS supplemented with 10 mM $MgCl_2$ at pH 7.4 for an hour, followed by incubation at 4°C for >5 hours , till further use. Excess of unhybridized Tf-cDNA was purified by SEC as before. The conjugates ($QD^{DNA\text{-}Tf}$) were stored at 4°C in $NaHCO_3$ 0.2M until further use.

repeated washing on centrifugal membrane (vivaspin, 100 kDa) and purified by SEC.

B4. *Conjugation of biotin to QDs.* 2.5 nmol QDs were reduced with 1000 nmol TCEP (TCEP/QD = 400) for 30 mins in PBS pH 7.4 under continuous stirring. Excess of TCEP was removed from the reaction using NAP-5 size exclusion columns. The reduced QDs were reacted with 125 nmol Biotin-maleimide (Bt/QD=500) and left to react overnight. The subsequent day, conjugates were purified using SEC on PBS pH 7.4. These QDs (QD_{bt}) were subsequently used to synthesize QD_{bt}-DNA and $QD_{bt}{}^{DNA\text{-}Tf}$.

B5. *Quantification of DNA per QD and DNA per Tf.* In our previous work, we have demonstrated that for quantification of the number of DNA molecules conjugated per QD, two approaches can be taken. The primary approach involves the estimation of concentration of DNA by absorbance at 260 nm. First, blank subtracted absorbance spectra of QD and purified QD-DNA were normalized at 220 nm.

Then the spectrum of QD was subtracted from that of QD-DNA conjugate, to obtain the spectrum of conjugated DNA. The concentration of DNA was then estimated by the Beer-Lambert law using the extinction coefficient of DNA (provided by IDT). Several of these reactions were additionally quantified employing a more conventional approach of using fluorophore-labeled DNA. For these reactions, a dual labelled DNA with a NH2 at the 5′ end and Cy5.5 at the 3′ end was conjugated. After coupling and SEC purification of the conjugates, the quantification of DNA is performed using either the absorbance of DNA at 260 nm or the absorbance of Cy5.5 at 680 nm. Both approaches gave similar results. Having established that DNA quantification can be reliably carried out using absorption at 260nm, we used this method estimate the calibration curves in the present work. Similarly, for quantification of DNA/Tf, we obtained calibration curves to estimate the absorbance at 260nm corresponding to different conjugation efficiencies, by mixing different excess of DNA_{Cy5} with Tf_{FITC} (fixed concentration) for several concentrations of Tf."

C. Agarose beads assay. Streptavidin agarose beads were washed and equilibrated with 0.2M NaHCO3 pH 8.3 buffer before use. Biotinylated QDs samples (QD_{Bt}, QD_{Bt}-DNA or QD_{Bt}^{DNA-Tf}) were left to interact with the beads for 5 min under mild agitation. The beads were then washed 3 times by repeated centrifugation and re-suspension in 0.2M NaHCO3 to ensure removal of any non-specifically linked QDs on the streptavidin agarose beads. The samples were imaged using filter set with λexc= 450±25 and λems=610±20 for QDs and 485 ± 10 nm and emission filter 524 ± 12 nm for Tf-FITC. The nature of the assay allows only quantitative measurements.

D. Cell culture and experiments. IA2.2 cells (derivative of Chinese Hamster Ovary, CHO cells) were used for all these experiments. Cells were seeded (typically ~0.25 X 10^6) in Nunc Lab-Tek chambered slides (0.8 cm^2) and grown for 2 days in Ham's F12 complete media (Gibco, Life Technologies) supplemented with 10% heat inactivated FBS, 100µg/mL Streptomycin, 100µg/mL Penicillin, 100µg/mL hygromycin and 100µg/mL G418. For experiments, cells were washed in M1 buffer (140

mM NaCl, 20 mM HEPES, 1mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, pH 7.4)[3] thrice and incubated with 100nM QD^{DNA-Tf} at 37°C for 3 hours. After internalization of QD^{DNA-Tf}, surface bound QD^{DNA-Tf} was stripped by incubating in ascorbate buffer (160 mM sodium ascorbate, 40 mM ascorbic acid, 1 mM $MgCl_2$, pH 4.5) for 10 mins on ice followed by rigorous washing with ice cold M1 buffer on ice. The cells were fixed with ice cold methanol or imaged live.

D1. *Uptake experiments.* Specificity of uptake of QD^{DNA-Tf} was determined by incubating cells with 100nM QD or QD-DNA for 3 hours and compared with cells incubated with QD^{DNA-Tf}. After 3 hours, surface bound ligands was stripped by incubating cells in ascorbate buffer for 10 mins on ice followed by rigorous washing with ice cold M1 buffer on ice. The cells were fixed with ice cold methanol and imaged. Competitive experiments to compare selective uptake of QD^{DNA-Tf} were carried out in the presence of 1000X excess of Tf or 1000X ssDNA. Cell were incubated with 100 nM QD^{DNA-Tf} in presence of 100 µM unlabelled Tf or 100µM ss DNA and incubated for 3 hours. In parallel, cells were incubated with 100nM QD^{DNA-Tf} as a reference. After internalization of QD^{DNA-Tf}, surface bound QD^{DNA-Tf} was stripped by incubating in ascorbate buffer for 10 mins on ice, followed by rigorous washing with ice cold M1 buffer on ice. The cells were fixed with ice cold methanol and imaged.

D2. *Colocalization experiment.* For colocalization experiments of QD^{DNA-Tf} with Tf_{FITC}, QD^{DNA-Tf} was internalized and surface labelling was stripped by incubating in ascorbate buffer (160 mM sodium ascorbate, 40 mM ascorbic acid, 1 mM $MgCl_2$, pH 4.5) for 10 mins on ice, followed by rigorous washing with ice cold M1 buffer on ice. Then, the cells were pulsed with warmed 500 nM Tf_{FITC} for 7 minutes and chased for 10 mins at 37°C. At last, the cells were shifted to ice and surface were re-stripped as before. The cells were fixed with ice cold methanol.

D3. *Immunofluorescence experiment.* For colocalization experiments of QD^{DNA-Tf} with LAMP-1, QD^{DNA-Tf} was internalized and surface labelling was stripped by incubation in ascorbate buffer (160 mM sodium ascorbate, 40 mM ascorbic acid, 1 mM $MgCl_2$, pH 4.5) for 10 mins on ice followed by rigorous washing with ice cold M1 buffer on ice. The cells were fixed with ice cold methanol. Then the cells were washed with PBS (3X) and permeabilised with 1X saponin buffer for 30 minutes. Cells were incubated with anti-LAMP-1 (Mouse) in saponin buffer (1:100 dilution) for 1 hour followed by

3X washing in PBS. Anti-mouse-Alexa488 in saponin buffer was added (1:100 dilution) for 45 mins followed by washing with PBS.

D4. *Kinetics experiments.* Time for saturated endocytosis was estimated by incubating cells with 100nM $QD^{DNA\text{-}Tf}$ or 500nM $Tf_{\text{-}647}$ for indicated pulse duration followed by stripping of surface bound Tf-probes and fixation in methanol. For comparing the recycling experiments, cells labelled with 100nM $QD^{DNA\text{-}Tf}$ or 500nM $Tf_{\text{-}647}$ was additionally chased with Tf_{FITC} for indicated amounts of time, followed by surface stripping and fixation. Total cell intensity of each cell in both the channels (QD channel and FITC channel or 647 channel and FITC channel) was measured.

E.Imaging. All fluorescent imaging was carried out by using the following two set ups.

E1. *Imaging of beads and fixed cells.* All samples were imaged with Olympus IX81 microscope fitted with a 100 W Hg apo lamp housing and transformer with indicated objective and customizable filters. Images were acquired with EM CCD camera QuantEM:512SC Roper Scientific with >90% Quantum efficiency. The lamp was prestabilized before imaging and used with indicated magnification (10X or 60X). The QDs and conjugates were imaged using excitation filter 450 ± 25 nm and emission filter 655 ± 10 nm. The fluorescent proteins labelled with Alexa647 were imaged using excitation filter 560 ± 55 nm and emission filter 645 ± 75 nm and the proteins labelled with FITC were imaged using excitation filter 485 ± 10 nm and emission filter 524 ± 12 nm.

E2. *Live imaging.* For live imaging, inverted Spinning Disk Confocal Roper/Nikon Microscope embedded within a thermo-regulated enclosure and CO_2 from Life Imaging Services (LIS Cube Box). The spinning-disc system is set up on an inverted microscope Nikon TiE, equipped with a piezo stage MCL mounted on a XYZ encoding motorized scanning stage. This system also includes a Photo Ablation module in the form of the Roper iLAS2 system, images being recorded on a CoolSNAP HQ2 camera. For live imaging of QDs, excitation of mCherry with 50 ms exposure using 60x oil objective with NA 1.4 was used.

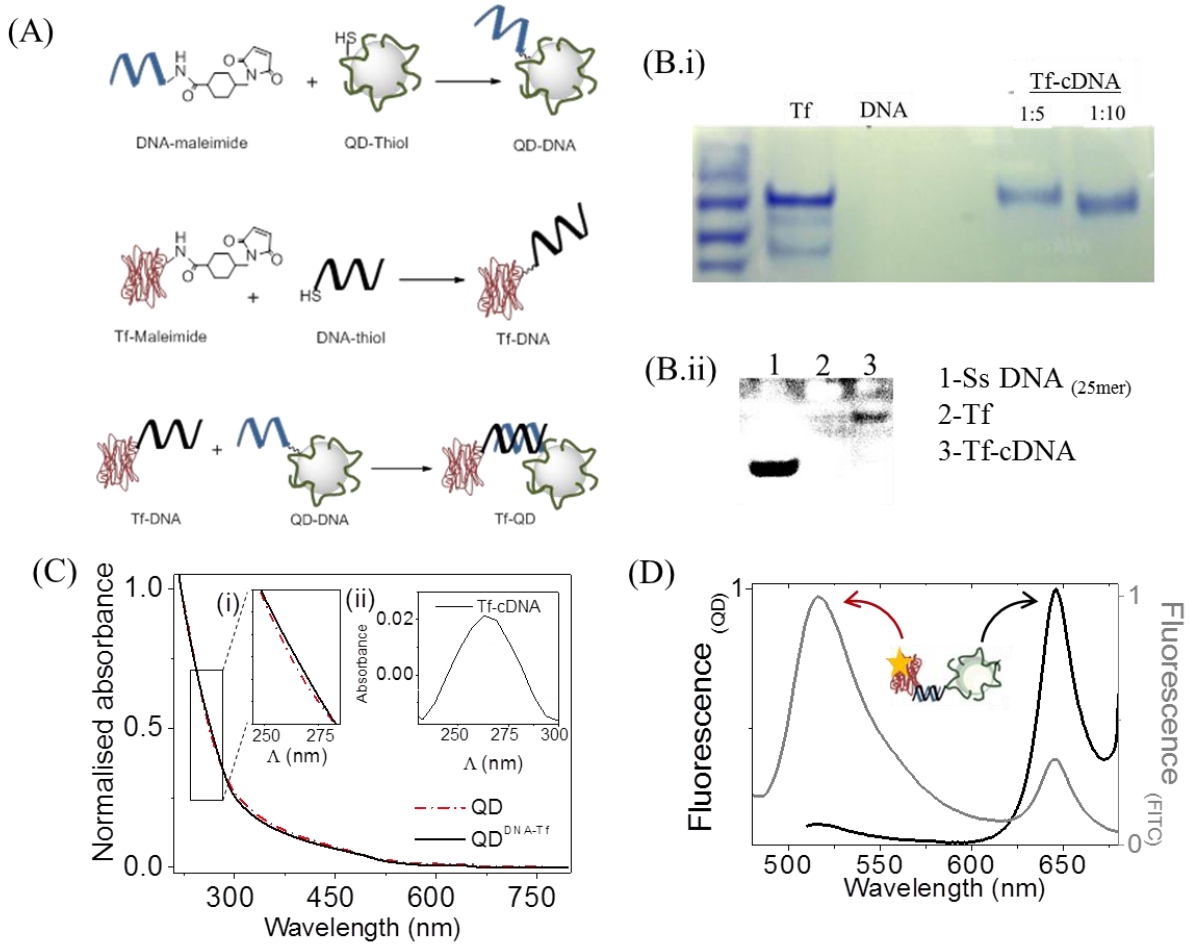

Figure S1. Stepwise assembly and characterization of $QD^{DNA\text{-}Tf}$. (A) conjugation of DNA maleimide to thiols on QD (top) Conjugation of maleimide-functionalized Transferrin (Tf) to thiol- labelled DNA (middle) Assembly of $QD^{DNA\text{-}Tf}$ *via* DNA hybridization (bottom). (B) Electrophoretic characterization of Tf-cDNA conjugates (i) PAGE cell stained with InstantBlue™ protein staining reagent showing change in electrophoretic migration of Tf upon conjugation to DNA. Unconjugated DNA is not visible (ii) 3% agarose gel stained with EtBr showing change in electrophoretic migration of DNA upon conjugation with Tf. Unconjugated protein is not stained with EtBr. **(C)** Absorbance spectra of $QD^{DNA\text{-}Tf}$ (black) post purification superimposed on equal concentration of QD-DNA (grey) to show increase in absorbance within 250-285 nm. Inset shows difference spectra to quantify number of Tf per QD. **(D)** Dual mode of detection of $QD^{DNA\text{-}Tf}{}_{FITC}$ shown by fluorescence spectroscopy. Nanobioconjugate is assembled by hybridization of Tf_{FITC}-cDNA (Tf labelled with FITC conjugated to DNA) to QD-DNA and purified by SEC Emission spectra corresponding to FITC on Tf (λ_{ex}=480, λ_{em}=490-690; grey) and QD_{650} (λ_{ex}=350, λ_{em}=575-690; black) is shown, confirming that the nanobioconjugate has both the QD and the Tf.

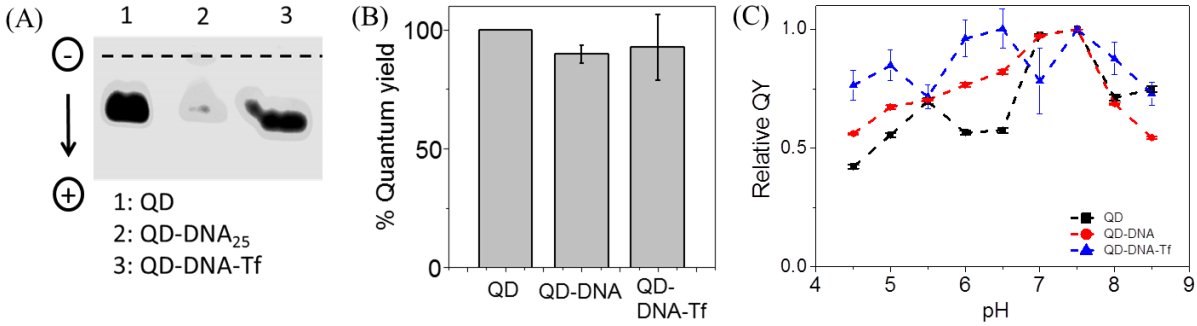

Figure S2. Biochemical properties of QD^{DNA-Tf} compared to QD-DNA and unconjugated QD (A) Electrophoresis of nanobioconjugates on an 0.8% agarose gel. Unconjugated QD (1), QD-DNA (2) or QD^{DNA-Tf} (3) have similar migration towards positive terminal. (B) QD-DNA and QD^{DNA-Tf} retain high QY as QD after multiple conjugation and reactions at physiological pH (7.4). Each point represents mean and SEM of 3 independent samples of 30nM concentration. (C) pH-dependent fluorescence QY of QD (black, square), QD-DNA (red, circles) and QD^{DNA-Tf} (blue, triangles). All nanobioconjugates retain high QY in a broad range of pH. Mean and SEM of 3 independent samples of 25nM concentration is shown.

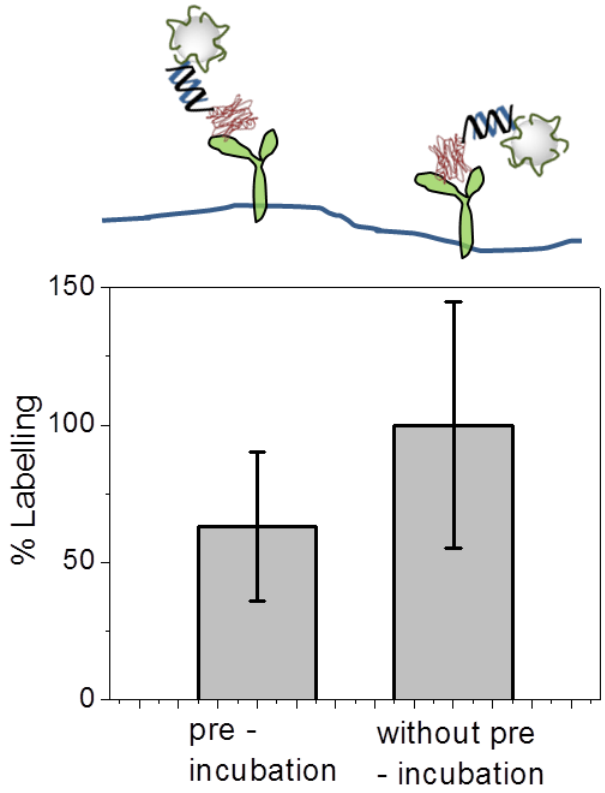

Figure S3. Effect of pre-incubation of cells with $QD^{DNA\text{-}Tf}$ at 4°C on intracellular labelling. Ia2.2 cells are incubated with 100 nM $QD^{DNA\text{-}Tf}$ for 2 hours with (left) or without (right) a pre-incubation step for 30 minutes at 4°C. No substantial increase in intracellular fluorescence is seen upon facilitated ligand binding. Total cell intensity of 50 cells is estimated. Bar graphs represent normalized mean and SEM of two independent experiments.

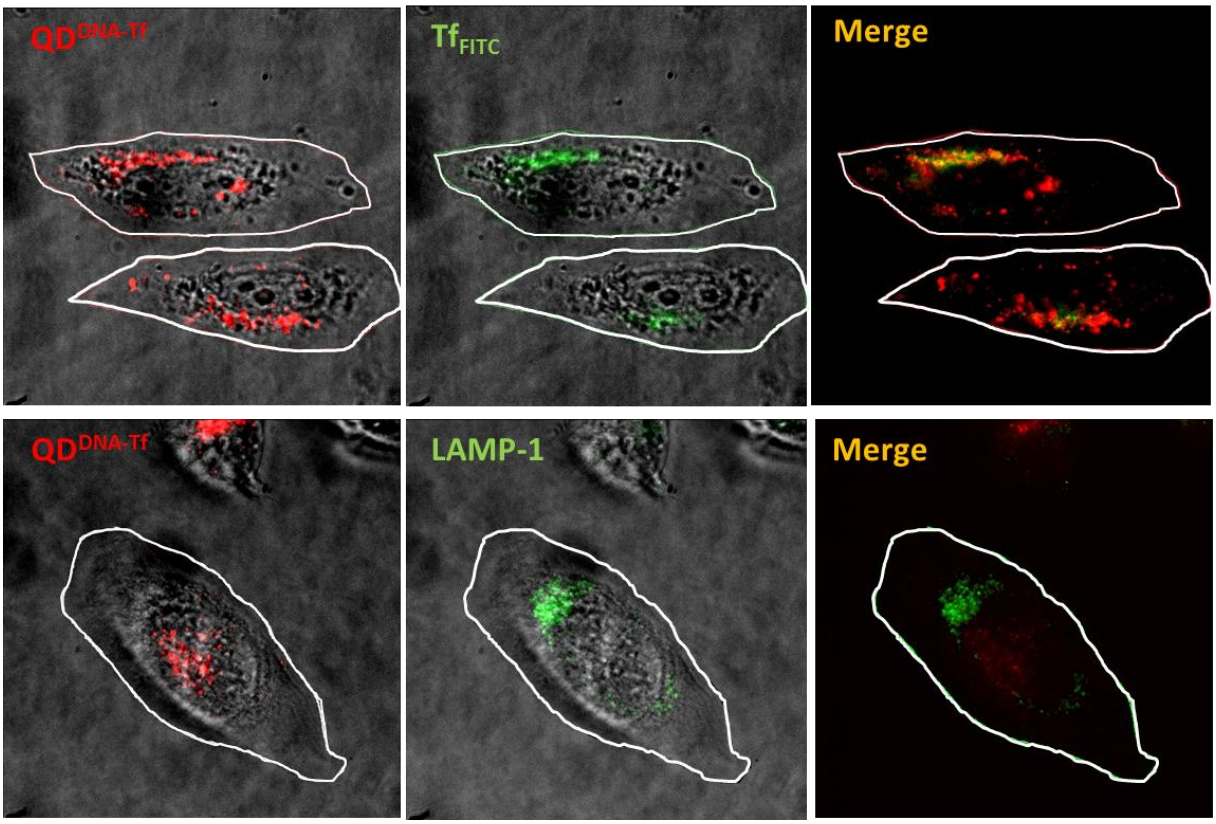

Figure S4. Phase contrast images of cells labelled with $QD^{DNA\text{-}Tf}$ nanobioconjugate along with Tf_{FITC} (top) and LAMP-1 (bottom).

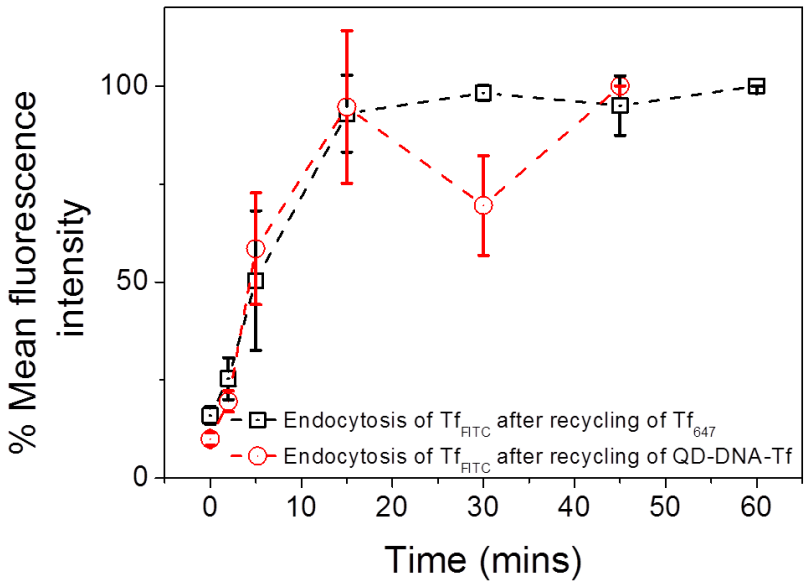

Figure S5. Impact of endocytosis of QD^{DNA-Tf} on subsequent endocytosis of Tf-TfR . Cells are pulsed with 500 nM Tf_{647} for 1 hour (Black) or 100 nM QD^{DNA-Tf} for 3 hours (Red) and chased with 500nM Tf_{FITC} for indicated period of time and fixed. Intracellular intensity of FITC channel show similar profiles in both cases. Total cell intensity of 60 cells is quantified for every time point and mean and SEM of two independent experiments is plotted. (Tf_{FITC}: λ_{exc}: 485±10 nm, λ_F : 524±12 nm).

Endocytosis of NP-Tf conjugates involve binding of the protein to its receptor followed by engagement of additional receptors. This eventually results in membrane invagination around the conjugates followed by internalization. The large size (and thus higher surface area) of the NP-Tf conjugate can potentially engage several receptors on the surface and within the endosomes upon internalization. This can cause an imminent deficit of the receptor at the membrane. Along with this, additionally delayed intracellular dynamics of conjugates can potentially perpetuate the deficit at the cell membrane, affecting uptake of 'fresh' ligands. To test if endocytosis of the surface pool of TfR was significantly depleted, post endocytosis of the first pulse with QD^{DNA-Tf}, cells were pulsed immediately after with Tf_{FITC} at designated time intervals. This second round of endocytosis of Tf_{FTIC} had similar kinetics with $t_{1/2}$ = 4.6±1.7 and 6.0±0.5 min when cells were initially pulsed with QD^{DNA-Tf} or Tf_{A647} (Figure SI4). This indicates that internalization of synthetic QD^{DNA-Tf} conjugates does not affect normal trafficking and recycling of TfR in the cell.

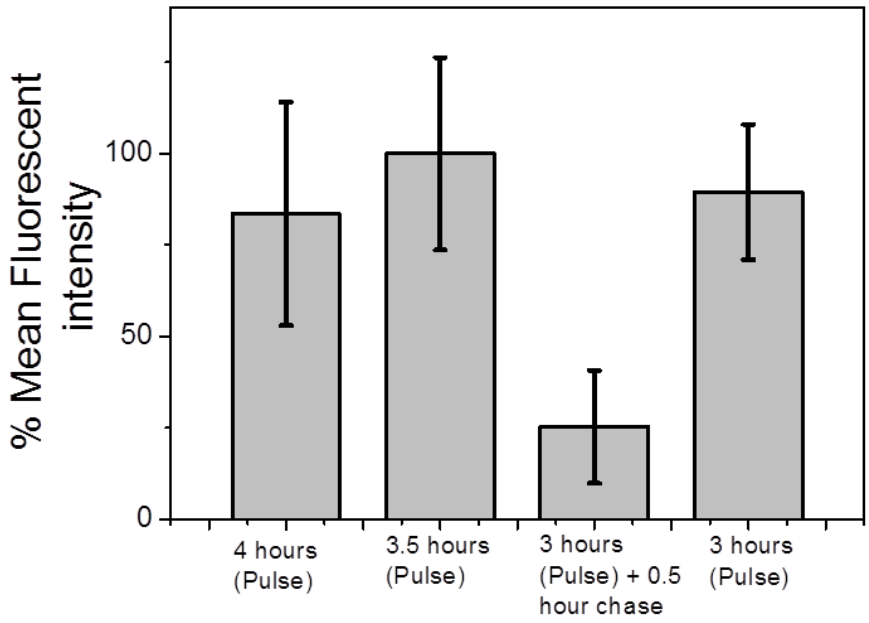

Figure S6: Intracellular photostability of $QD^{DNA\text{-}Tf}$. Cells are pulsed with 100nM $QD^{DNA\text{-}Tf}$ and chased with unlabeled Tf for indicated periods of time, surface stripped and fixed. Mean and SEM of 2 independent experiments with 80 cells/dish each are plotted. Substantial decrease in intracellular labelling is seen exclusively in case of chase with unlabeled Tf causing $QD^{DNA\text{-}Tf}$ conjugates to recycle.

Table SI1: Intracellular fluorescence intensity in indicated experimental conditions

Experiment	% intensity[a]
4 hours pulse + 0 hr Chase	83.5±35.8
3.5 hours pulse + 0 hr Chase	100.0±26.3
3 hours pulse + 0.5 hr Chase	25.2±15.4
3 hours pulse + 0 hr Chase	89.5±18.5

[a] for estimation of the intracellular intensity, mean fluorescence intensity of 80 cells/dish were individually measured and expressed as a percentage of intensity of dish pulsed for 3.5 hours.

To further confirm that the recycling of $QD^{DNA\text{-}Tf}$ with time is a real biological affect, we tested the photostability of $QD^{DNA\text{-}Tf}$. Cells were incubated with $QD^{DNA\text{-}Tf}$ for 3, 3.5 and 4 hours the intracellular intensity was compared to cells which have been additionally chased for 30 mins post 3 hours of pulse. The intracellular intensity remained consistent (80-100%) in the 'only pulsed' cells. Strikingly, in the control cells with additional chase period of 30 mins, the intracellular intensity had reduced by 75-90%. This suggests that prolonged incubation of $QD^{DNA\text{-}Tf}$ in cells does not change the photophysical stability of the conjugates (Figure SI5, Table SI 1). This further supports that recycling of conjugates by 'reduction in fluorescence over time' probed herein is devoid of any artifacts from photophysical instability of the QD.

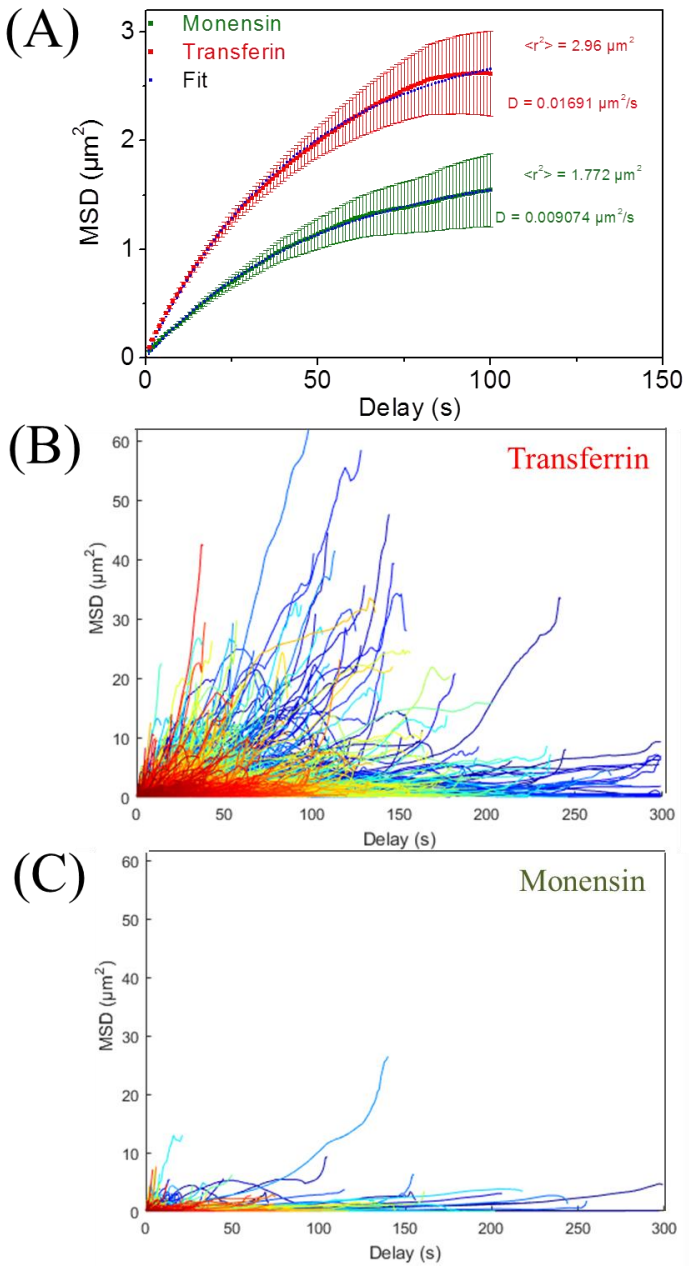

Figure S7. Comparative endosomal dynamics of $QD^{DNA\text{-}Tf}$ in normal and Monensin treated cells. Quantification of endosomal dynamics was carried out using Wave Tracer and Trackmate. (A) The weighted mean MSD for > 2000 tracks per condition and average motion characterized using Confinement or corralled diffusion model is shown. Monensin treated cells show greater confinement in comparison to untreated cells. **(B)** Representative MSD of individual tracks in a typical cells and (C) Representative MSD of individual tracks in an untreated cell is shown. Untreated cells show more frequent and longer tracks than Monensin treated cells.

The live cell imaging was performed using spinning disk confocal microscope, SP7. CHO cells pulsed with Tf-QDs for 3 hours were chased with unlabelled Tf and continuously imaged for 30 mins with the 50 msec acquisition time and 21 confocal planes frames time point. Post-acquisition, the background was siphoned off and the cell area was analyzed for single particle dynamics. The 3D frames were stacked and these 2D stacks were analyzed over time as follows: tracks of the particles were calculated using Wave Tracer Using this analysis, the motion was characterized for >2 000 tracks segments per condition by the value of diffusion coefficient D, in the equation of the Mean Square displacement (MSD) described as cited.[4] For each MSD, the instantaneous diffusion coefficient, D, was calculated from linear fits of the first 4 points of MSD. Histograms of this MSD for (treated and untreated cells) are shown in Figure (4C). In addition, confinement studies were performed. For this analysis, images were analyzed using Fiji and particle tracks were estimated using Trackmate plugin post suitable thresholding. Tracks were analyzed using Matlab (MathWorks) scripts. MSD was estimated using MSD analyzer Matlab class 2.[5] Then weighted mean MSD was estimated for > 2000 tracks segments per condition. Average motion was characterized using confinement or corralled diffusion model (Figure **S6 A**) [1]. Confinement geometry was not considered in this model for simplicity. Approximation of the confinement size and diffusion coefficient (D) were calculated from weighted mean MSD for each condition using 30% of the maximum lag time. Representative MSD of individual tracks in a typical cells (Figure S6**,** B) and untreated cell (Figure S6, C) is shown. The untreated cells show frequent and longer tracks than the Monensin treated cells.

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