Biosensors

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Quantum Dot Biosensors for Ultrasensitive Multiplexed Diagnostics**

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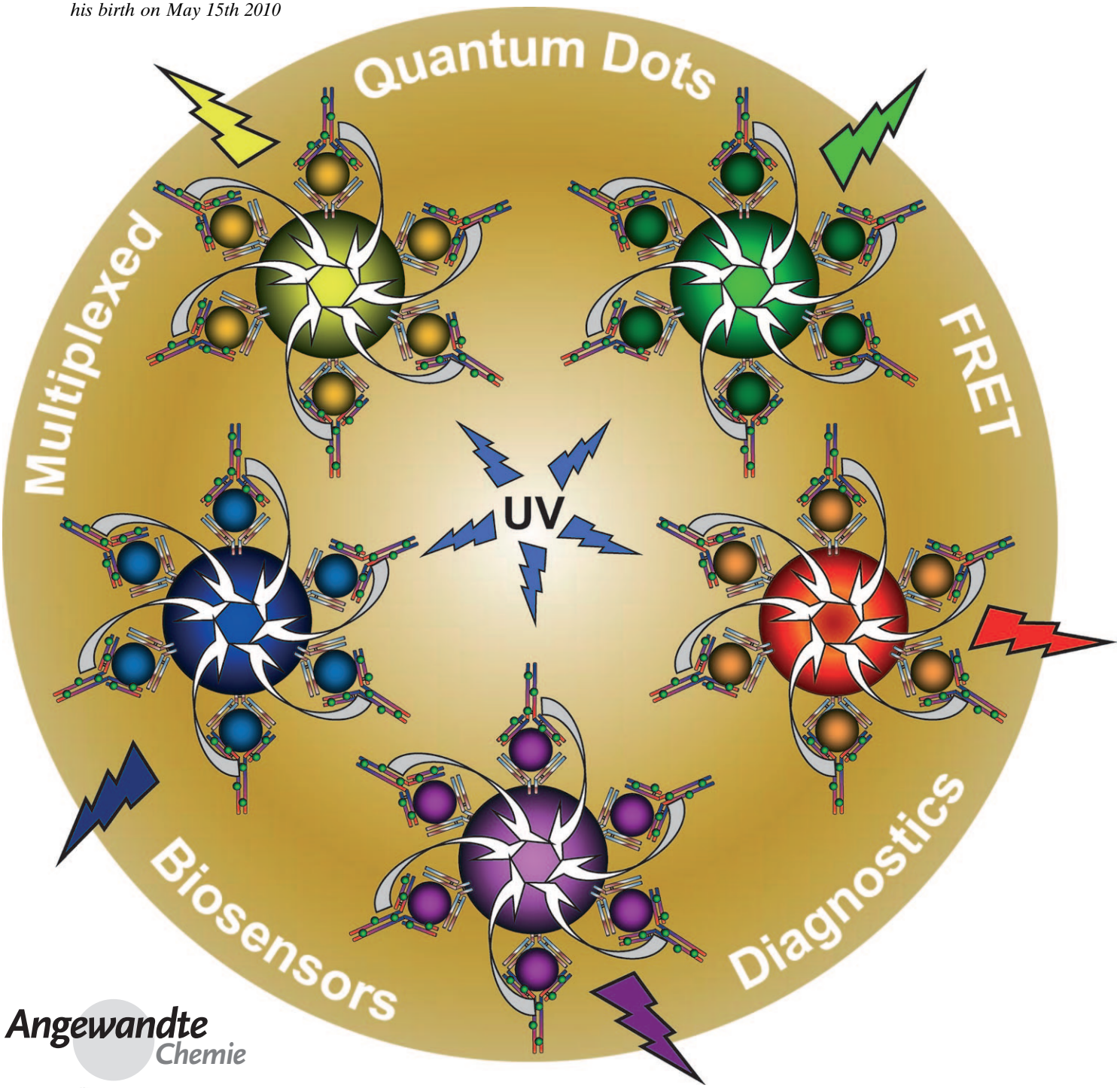
In memory of Theodor Förster on the centenary of his birth on May 15th 2010

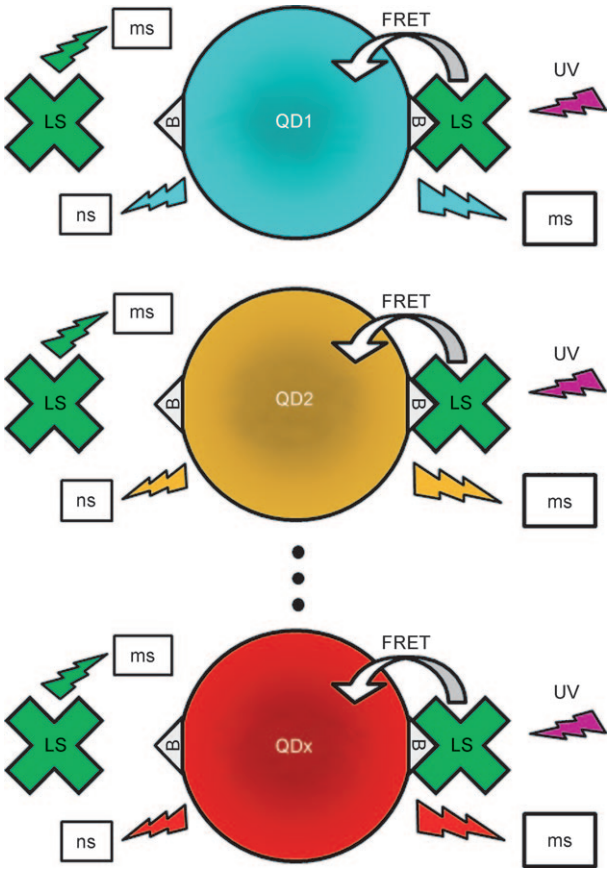


Nanobiosensors have become an emerging field within diagnostic applications.[1–8] Although the in vitro diagnostic market is one of the largest segments for the health care industry, with annual growth rates of approximately 10%,[9] specialized technologies such as point-of-care testing and multiplexing continue to generate exceptional interest.[10] The annual growth rate of in vitro diagnostic tests based on multiplexed reporter technology has been estimated to reach 26% within the next few years.[11] Multiplexed diagnostics allows the simultaneous measurement of multiple clinical parameters from the same sample volume, thereby decreasing the time and cost required for recording each data point. The desire for multiplexed diagnostics is also driven by the appearance of many new biomarkers (e.g., for Alzheimer's disease)[12] with higher selectivity and sensitivity.

Herein we present a homogeneous assay format that combines both very high sensitivity and multiplexed detection, and provides large benefits compared to conventional diagnostic assays. Our system (Scheme 1) is based on FRET (Förster resonance energy transfer) from luminescent terbium complexes (LTCs) to several different semiconductor quantum dots (QDs) established by molecular recognition between streptavidin (sAv) and biotin (Biot). This biological binding system was chosen to demonstrate a proof-of-concept for the multiplexed FRET assay because it is transferable to practical diagnostic assays that use for example, RNA-, DNA-, aptamer-, peptide-, or protein-based recognition. A competitive immunoassay using Eu–QD FRET for the single analyte estradiol has already been demonstrated.[13] In order to evaluate the limits of detection (LODs) concerning in vitro diagnostics, we compared our system to the well-established and highly sensitive immunoassay FRET pair of europium-(III) tris(bipyridine) (EuTBP) and allophycocyanin (APC),[14,15] which is applied in many ultrasensitive diagnostic immunoassays and drug screening kits for single analytes.[16,17]

The special chemical and physical properties of QDs are well-known,[18,19] and QDs are commonly used in various life-science applications.[1–8,20–24] Their size-tunable absorption and emission wavelengths, and their narrow and symmetric emission bands make QDs ideal candidates for optical multiplexing. Although QDs have been used as FRET donors in bioassays with various organic dye acceptors,[25–29] it was shown that QDs are inefficient FRET acceptors when common organic fluorophores are used as donors.[30] Use of QDs as efficient FRET acceptors requires lanthanide-based donors with long (up to milliseconds) excited-state lifetimes;[31–34] the only other alternatives are bioluminescence (BRET) or chemiluminescence (CRET) resonance energy transfer without external light excitation.[35–37]

The combination of QD acceptors with LTC donors in time-resolved FRET results in significant sensitivity, distance, and multiplexed detection advantages compared to other donor–acceptor pairs.[31] The long-lived (milliseconds) terbium luminescence of the LTCs allows time-gated detec-



Scheme 1. Principle of the multiplexed FRET assay. LTC-labeled streptavidin (LS) binds to biotinylated (B) QDs to result in a proximity between LTCs and QDs, thus allowing FRET to occur. Before binding (left-hand side) UV excitation leads to LTC and QD luminescence with millisecond and nanosecond decay times, respectively. After binding (right-hand side) energy is transferred from LTCs to QDs, thus resulting in luminescence quenching of LTCs and the appearance of a long-lived QD luminescence with millisecond decay times. Use of time-gated detection leads to suppression of the nanosecond luminescence, and the millisecond luminescence intensities at the varying emission wavelengths can be directly used to measure the concentrations of the different FRET complexes (LS–QD1 to LS–QD*x*).

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tion that leads to an almost complete suppression of short-lived sample autofluorescence, and fluorescence from directly excited QDs. Exceptionally large Förster radii (R_0, the donor–acceptor distance where FRET is 50% efficient) up to 11 nm can be achieved because of the large overlap integral values, whereas conventional donor–acceptor pairs exhibit significantly smaller R_0 values that rarely exceed 6 nm.[38] By choosing QDs with emission wavelengths in between or beyond the well-separated LTC luminescence bands allows multiplexed FRET from one LTC to many QDs from the green to the NIR spectral range.

As a comparative approach, two different LTCs were used as energy donors. TbL is based on a glutamate skeleton that is N-functionalized with two anionic bipyridyl chromophoric units,[39] and was synthesized in our laboratories. TbL was compared to Lumi4Tb, which is based on an isophthalamide structure,[40,41] and is commercially available (Lumiphore Inc., USA). The LTCs were conjugated to sAv (LTC–sAv) with labeling ratios of 11.5 TbL per sAv and 4.4 Lumi4Tb per sAv unit. Luminescence decay times were (1.6 ± 0.2) ms for TbL–sAv and (2.3 ± 0.1) ms for Lumi4Tb–sAv. The LTC absorption and emission spectra (Figure 1 in the Supporting Information) are separated by approximately 100 nm, thus leading to a clear distinction between excitation and emission radiation.

The five different QDs used in our study were commercially available QDots™ (Invitrogen, USA) with emission maxima at 529, 565, 604, 653, and 712 nm. The QDs were selected for their high extinction coefficients over a broad wavelength range, which leads to good spectral overlap with the LTCs (Figure 2 in the Supporting Information) and for their emission wavelengths to fit within the gaps of the LTC emission spectra (Figure 3 in the Supporting Information). Moreover, the narrow and symmetrical emission bands of the QDs and the choice of suitable bandpass filters result in a multiplexed detection system with low spectroscopic cross-talk between independent detection channels. The CdSe/ZnS (CdSeTe/ZnS in the case of QD712) core–shell QDs range from spheres with 3 nm diameter (QD529) to ellipsoid with 6 and 12 nm axes (QD712). Each QD is polymer-coated and has 5–7 Biot units attached to its surface. The Biot–QDs range from 12 nm (Biot–QD529) to 18.5 nm (Biot–QD712) hydrodynamic diameters (Table 1 in the Supporting Information).

For visualization of the multiplexed detection of the five different LTC–QD FRET systems, a Biot–QD mixture was added in small aliquots to a stock solution of LTC–sAv and emission spectra were recorded with time-gated detection. The appearance of long-lived QD emission bands is very strong evidence for simultaneous multiplexed FRET from the LTC donors to all five different QD acceptors. Figure 1 shows the QD FRET emission bands that grow out of the Lumi4Tb emission spectrum as the Biot–QD/Lumi4Tb–sAv concentration ratio is increased. As indicated in the enlarged region of interest, the increasing signals arise from long-lived FRET-sensitized QD emission, thus verifying the simultaneous fivefold multiplexed FRET from Lumi4Tb to QDs in the system.

In order to perform highly sensitive multiplexed detection of all five FRET systems within one sample, the time-delayed luminescence intensities of the LTC donors I(D) and the QD acceptors I(A) were measured for increasing Biot–QD/LTC–sAv concentration ratios. Figure 2 shows the luminescence intensity ratios ($R = I(A)/I(D)$) as a function of Biot–QD concentration for Lumi4Tb–sAv and unconjugated (free) Lumi4Tb, as a nonbinding control. After addition of minute amounts of Biot–QD mixtures to the LTC–sAv solution, the FRET-sensitized QD luminescence intensity significantly increased whereas the LTC luminescence intensity decreased (see Figure 5 in the Supporting Information for luminescence decay curves), thus resulting in the increase of the R value for all five FRET systems (Figure 2).The ultrahigh sensitivity of the multiplexed detection is underlined by the sub-picomolar LODs for all five different FRET systems (Table 1, first row).

Measurement of the single LTC–sAv–Biot–QD assays under the same experimental conditions led to similar LODs

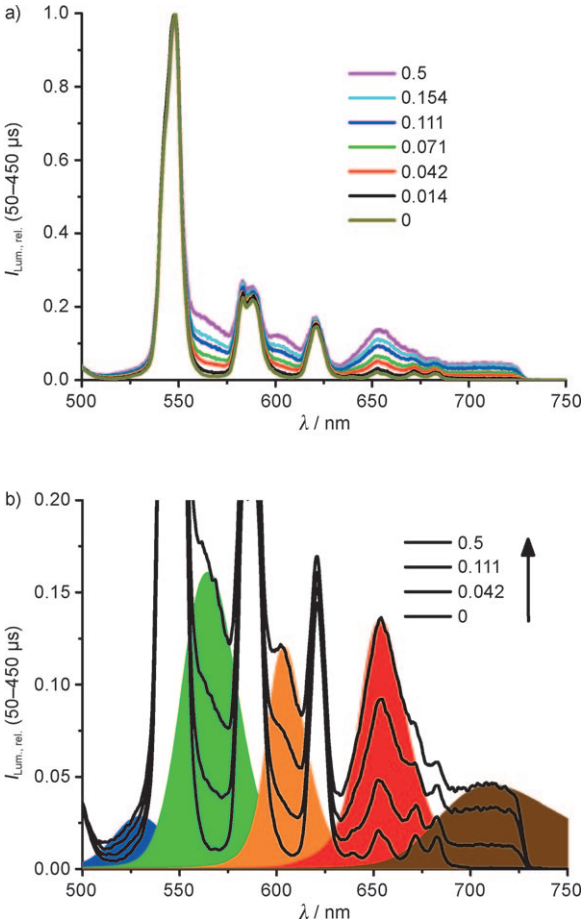

Figure 1. a) Time-resolved emission spectra of increasing concentration ratios of Biot–QD per Lumi4Tb–sAv unit. Background autofluorescence of the biological samples and QD emission from direct laser excitation were efficiently suppressed by time-gated detection (50–450 µs). Spectra are normalized to the 545 nm peak of Lumi4Tb. b) Close-up view on the area of interest with a superimposition of the steady-state emission spectra of QD529 (blue), QD565 (green), QD604 (orange), QD653 (red), and QD712 (brown). The increasing intensities of the QD emission bands in the time-gated spectra with increasing Biot-QD to Lumi4Tb-sAv ratio arise from simultaneous FRET from Lumi4Tb to all five different QDs. The results for TbL are similar to those of Lumi4Tb (see Figure 4 in the Supporting Information).

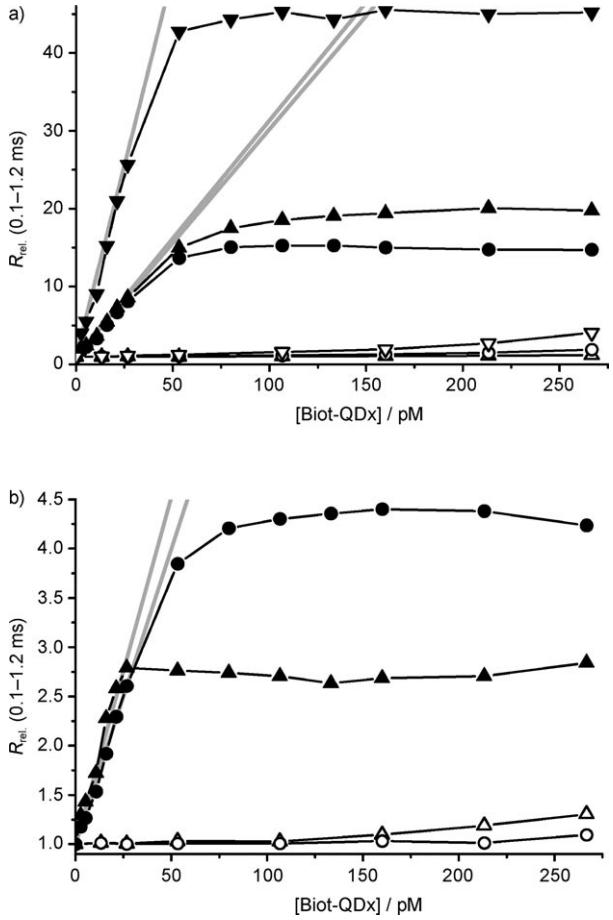

Figure 2. Relative luminescence intensity ratios (normalized to unity at [Biot–QDx] = 0 for better comparison) of QD (FRET acceptor) and Lumi4Tb (FRET donor) luminescence over Biot–QDx concentration for a) $x = 712$ (▼), $x = 565$ (▲), and $x = 653$ (●); and b) $x = 604$ (●) and $x = 529$ (▲). The filled symbols show FRET experiments where the QDs are FRET-sensitized by Lumi4Tb by Lumi4Tb–sAv–Biot–QD binding (increasing ratio *R* over concentration up to a certain ratio level where the binding is saturated). The open symbols show control experiments with Biot–QDx and unconjugated Lumi4Tb, where no binding and hence no FRET occurs (the small increase at higher QD concentrations results from extremely strong short-lived luminescence from directly excited QDs that is still measurable after 0.1 ms). The increase of *R* reaches a maximal limit around 60 pM of Biot–QDx (except Biot–QD529 because of weak luminescence signals). The overall Biot–QD concentration is then 300 pM (because of five different Biot–QDx) and the Lumi4Tb–sAv concentration is ca. 1800 pM, which results in bioconjugates with 6 Lumi4Tb–sAv per Biot–QDx unit. This value is similar to the labeling ratio of 5–7 Biot per QD (as specified by Invitrogen) and it shows the saturation of Biot–QDs with Lumi4Tb–sAv. Results for TbL can be found in Figure 6 in the Supporting Information.

(Table 1, second row) compared to the multiplexed measurement, thus demonstrating the low spectroscopic cross-talk of our technique. This major advantage results from the narrow and symmetric wavelength bandwidths of the QDs, the well-separated emission bands of the LTCs, and the choice of adequate filters. For all measurements, control experiments were performed using LTCs without sAv, thus excluding potential signals that arise from dynamic FRET or luminescence from directly excited QDs.

In order to evaluate the LODs of our high affinity sAv–Biot system in practical diagnostic applications such as immunoassays (using antigen–antibody binding with much lower affinity) it is necessary to use a well-established reference for comparison. For this reason, we performed the same experiments of sAv–Biot binding with the EuTBP–APC FRET pair (see above), which enables extremely high sensitivity for many scientific and commercial diagnostic assays.[14–17] Even the geometrical parameters of the FRET pairs are quite similar: the EuTBP donor is also a lanthanide complex (the EuTBP/sAv labeling ratio was 4.1:1) and the APC acceptor (10–15 Biot units per APC) is also relatively large (disc-shaped 105 kDa phycobiliprotein with ca. 3 nm thickness and 10–13 nm diameter).[42,43] The sAv–Biot assay of this single FRET pair resulted in an LOD of (24 ± 12) pM.[32] The sub-picomolar LODs of all different FRET pairs within the multiplexing experiment (and the single assays) are significantly lower (Table 1, third row). Hence, our LTC–QD multiplexed assay not only provides simultaneous detection of up to five different bioanalytes, but it also offers a sensitivity that is approximately 40–240-fold higher than a well-established homogeneous immunoassay FRET pair, and therefore has a great potential for practical diagnostic applications.

In conclusion, we have demonstrated a novel and fast technique that is suitable for multiplexed ultrasensitive detection. The multiplexed technology offers the significant benefits of simultaneously measuring different biocomplexes with extremely high sensitivity, reproducibility, and speed. One of the primary goals for future QD-based technology should be the development of stable and reproducible QDs with an efficient, robust, and stable antibody labeling chemistry, in order to achieve real immunoassays. The development of efficient QD-based sensors plays an important role for bio-nanotechnology and we believe that our high-sensitivity multiplexed FRET tool will bring a significant contribution for RNA-, DNA-, aptamer-, peptide-, and protein-based sensing applications in the fields of biology, chemistry, medicine, and physics.

Experimental Section

TbL was synthetically coupled to the free lysine residues of sAv[33] with a TbL/sAv labeling ratio of 11.5:1. Lumi4Tb was supplied by Lumiphore Inc. in the streptavidin-labeled form with a Lumi4Tb/sAv labeling ratio of 4.4:1. Luminescence decay times were measured in borate buffer (50 mM, pH 8.3) containing bovine serum albumin (BSA; 2%) and KF (0.5 M). KF improves the performance of TbL as it replaces coordinated water that effectively quenches luminescence. Although KF is not necessary for Lumi4Tb luminescence, it was included to maintain experimental consistency. The sizes of the CdSe/ZnS and CdSeTe/ZnS core–shell quantum dots were analyzed with TEM and the hydrodynamic diameters of the Biot–QDs were analyzed with exclusion chromatography on HPLC. Both methods were performed by Invitrogen. All samples were excited with a pulsed Nd:YAG-OPO laser system (OPO: GWU, Germany; laser: Spectra Physics, USA) with 20 Hz repetition rate and a pulse energy of 10 μJ at 320 nm. This excitation wavelength was selected to yield similar extinction coefficients for both TbL–sAv and Lumi4Tb–sAv ($16\,500\ M^{-1}cm^{-1}$).

Table 1: LODs determined from the linear part of the assay calibration curves (grey lines in Figure 2) and calculated as the zero standard value (R at [Biot–QDx] = 0) plus three times the zero standard deviation from 40 different measurements.

Biot–QD	529	565	604	653	712
LOD_M [pM][a]	0.63 ± 0.15	0.12 ± 0.03	0.37 ± 0.16	0.10 ± 0.02	0.12 ± 0.04
LOD_S [pM][b]	0.95 ± 0.3	0.17 ± 0.03	0.32 ± 0.12	0.05 ± 0.02	0.08 ± 0.02
SIF[c]	38 ± 28	200 ± 150	65 ± 60	240 ± 168	200 ± 167

[a] Multiplexed. [b] Single assay. [c] SIF (sensitivity improvement factor) = LOD(st)/LOD_M, where LOD(st) is the LOD of EuTBP–sAv–Biot–APC ((24 ± 12) pM) as standard reference. High error values are due to summation of both relative LOD errors.

Fivefold multiplexing was measured on a spectrograph with intensified CCD camera detection (LOT-Oriel, Germany). Measurements were performed after 1 h incubation at room temperature. Time-gated (50–450 μs) luminescence spectra (500–725 nm) of the LTC donor and the five different QD acceptors were collected simultaneously. A Biot–QD mixture (containing Biot–QD529 (3 nM), Biot–QD565 (2 nM), Biot–QD604 (1 nM), Biot–QD653 (1 nM), and Biot–QD712 (3 nM) in borate buffer) was added in small aliquots to a solution of LTC–sAv (10 nM). QD concentrations were varied to normalize differences in spectral overlap and luminescence quantum yields, thus yielding similar luminescence intensities for all five QDs. A modified KRYPTOR immunoanalysis platereader (Cezanne, France) with two photomultiplier detection channels and changeable bandpass filters (see Figure 7 in the Supporting Information for the detection setup) was used for the ultrasensitive detection measurements. The sample volume was 150 μL. A Biot–QD mixture (0.4 nM of each Biot–QD) was added in small aliquots to a stock solution of LTC–sAv (2 nM). For the single LTC–sAv–Biot–QD assays stock solutions of Biot–QD (1 nM) were added in small aliquots to a stock solution of LTC–sAv (1 nM). After a minimum of 30 min incubation, the luminescence intensities of the LTC donors I(D) and the QD acceptors I(A) were measured in a time-resolved window ranging from 0.1 to 1.2 ms. Spectral resolution was achieved with bandpass filters (ca. 10 nm FWHM) with maximum transmission close to the emission maxima of the QD acceptors (between the emission bands of the LTCs) and at the 545 nm emission maximum of the LTC donors (see Figure 3 in the Supporting Information). For suppression of sample or measurement fluctuations and for correction of the decreasing LTC concentration with addition of the QD mixture, the luminescence intensity ratio $R = I(A)/I(D)$ was employed.

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