Non-competitive aptamer-based quenching resonance energy transfer assay for homogeneous growth factor quantification

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SUPPORTING INFORMATION

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Spectral characterization. The excitation and emission spectra of the 5'-FBA-Eu and cyanine dye type Quench V (Q5) were measured using Varian Cary Eclipse fluorescence spectrophotometer. Also luminescence lifetimes for 5'-FBA-Eu, bFGF-FBA-Eu complex with Q5, and FBA-Eu with Q5 were measured. All measurements were performed in assay buffer (0.11 mM KH_2PO_4, 0.56 mM Na_2HPO_4, 15.4 mM NaCl, 0.01% Triton-X, 1 mM $MgCl_2$, pH 7.4) with 50 nM 5'-FBA-Eu, 750 nM bFGF, and 2.6 µM Q5 in final volume of 500 µl. Luminescence maximums for Q5 and 5'-FBA-Eu emissions were 558 nm and 341 nm, and excitation maximums were 572 nm and 614 nm, respectively (Fig. S-1 A). Q5 has no effect to the shape of FBA-Eu emission spectra, but only for intensity (Fig. S-1 B).

The luminescence lifetime for Eu(III)-chelate conjugated to FBA in the presence of quencher was decreased significantly from 1.38 ms to 0.01 ms when fitted to single exponential decay, respectively (Fig. S-1 B). When bFGF-FBA-Eu complex was formed in the presence of Q5, two lifetimes for two luminescent populations were measured. In first population the unbound 5'-FBA-Eu was quenched, and in the other the FBA-Eu was protected from quenching due to binding to bFGF. Luminescence lifetimes were 0.01 ms for quenched and 0.58 ms for protected FBA-Eu when fitted to double exponential decay, respectively. Thus only protected FBA-Eu can produce TRL-signal in the used measurement window from 0.4 ms to 0.8 ms.

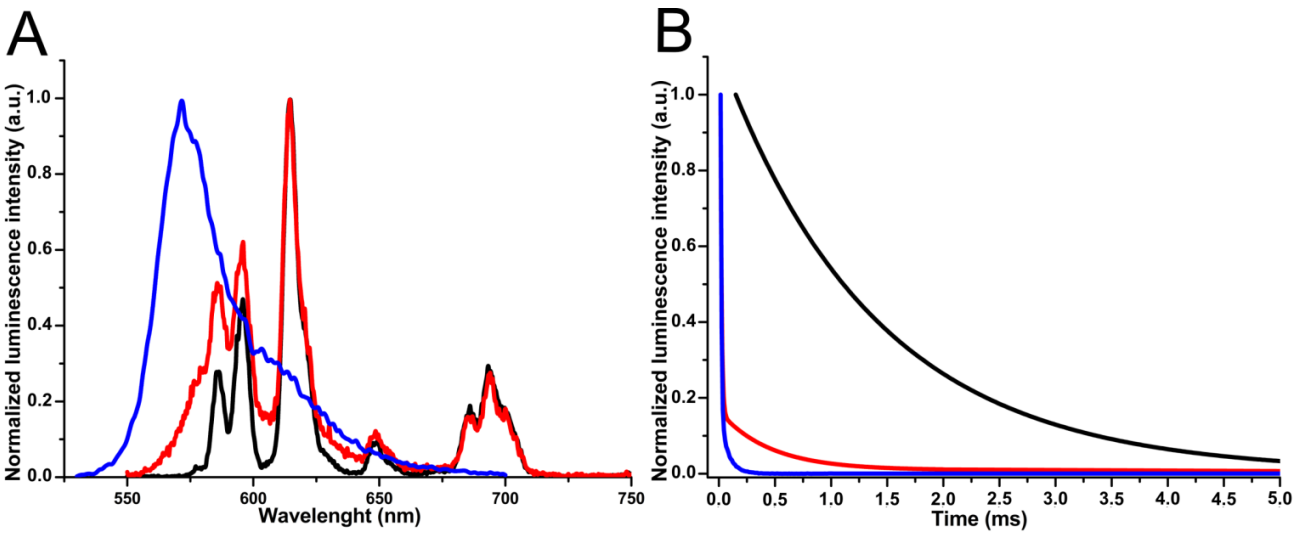

Figure S-1. Normalized emission spectra's and luminescence lifetimes. A) Emission spectra's for the Q5 (blue), protected bFGF-FBA-Eu complex in Q5 solution (red), and for bFGF-FBA-Eu complex without Q5 (black). B) Luminescence lifetimes for 5'-FBA-Eu with Q5 (blue), protected bFGF-FBA-Eu complex in Q5 solution (red), and for FBA-Eu without Q5 (black).

The 3'-VBA-Eu dissociation constant. The dissociation constant (K_d) for VBA was determined performing a panel of VEGF titrations with different 3'-VBA-Eu concentrations (Figure S-2). The IC_{50} values were determined from different titrations and blotted against 3'-VBA-Eu concentration. The K_d value was calculated using linearized Cheng-Prusoff equation, $IC_{50} = (([K_i]/K_d)*[C]) + K_i$.[1] The y-axis cutting point for the linear representation was the K_d value for the 3'-VBA-Eu. The K_d value calculated for 3'-VBA-Eu was 0.44 ± 0.4 nM, which was in accordance with the corresponding literature value (0.5 nM).[2] This indicates that the Eu(III)-chelate does not interfere aptamer binding to VEGF target.

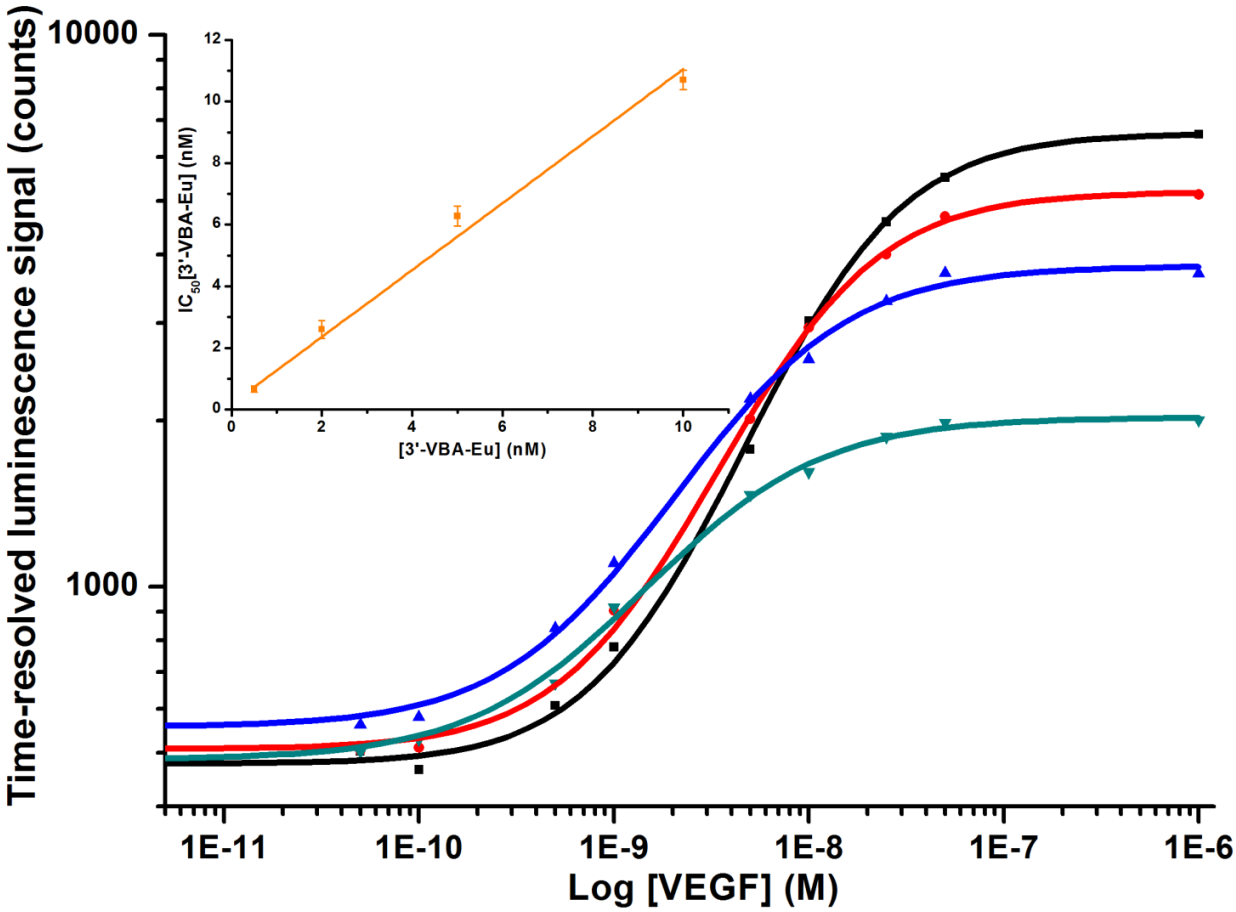

Figure S-2. The 3'-VBA-Eu dissociation constant (K_d) determination. The outset represent VEGF titration curves in the QRET assay with 0.5 nM (green), 2 nM (blue), 5 nM (red), and 10 nM (black) 3'-VBA-Eu concentration, respectively. In the inset, IC_{50} versus concentration of the aptamer have been plotted, which was then used to determine K_d value for 3'-VBA-Eu.

FBA-Eu binding kinetics. The aptamer binding kinetics was measured using 10 nM, 25 nM, and 2.3 µM concentrations for 5'-FBA-Eu, bFGF, and Q5, respectively. Unlike in other assays where 5'-FBA-Eu and bFGF were preincubated before Q5 addition, now the Q5 and 5'-FBA-Eu were mixed at the beginning to perform quenching before target addition. The total quenching with Q5 and the Eu(III)-chelates TRL-signal stabilization takes around 2 min, and the aptamer binding kinetics was measured after bFGF addition (Fig. S-3). Assay was performed in four separate reactions due to fast kinetics of aptamer binding.[3] Kinetics were monitored using same measurement protocol and equipment than in other assays. Due to equipment limitations, the first measurement point was not until 18 s seconds after bFGF addition, and after that the measurement was repeated in every nine seconds. Due to these limitations, over 95 % of maximal TRL-signal was monitored already in first measuring point. The k_{obs} for Eu-FBA binding was 0.135 ± 0.015 s^{-1}, signifying k_{on} rate of $1.35*10^{-3}$ M^{-1} s^{-1}.The signal-to-background ratio (S/B) in kinetic measurement was 20.7, which was in same range than with preincubation. This proves that preincubation was not essential requirement, and all components could be added in one step if needed. With these modifications the total assay time can be reduced, and the non-competitive QRET assay can be accomplished in less than 3 min without losing any assay performance. For the first time the QRET assay shows the indications to real-time reaction monitoring.

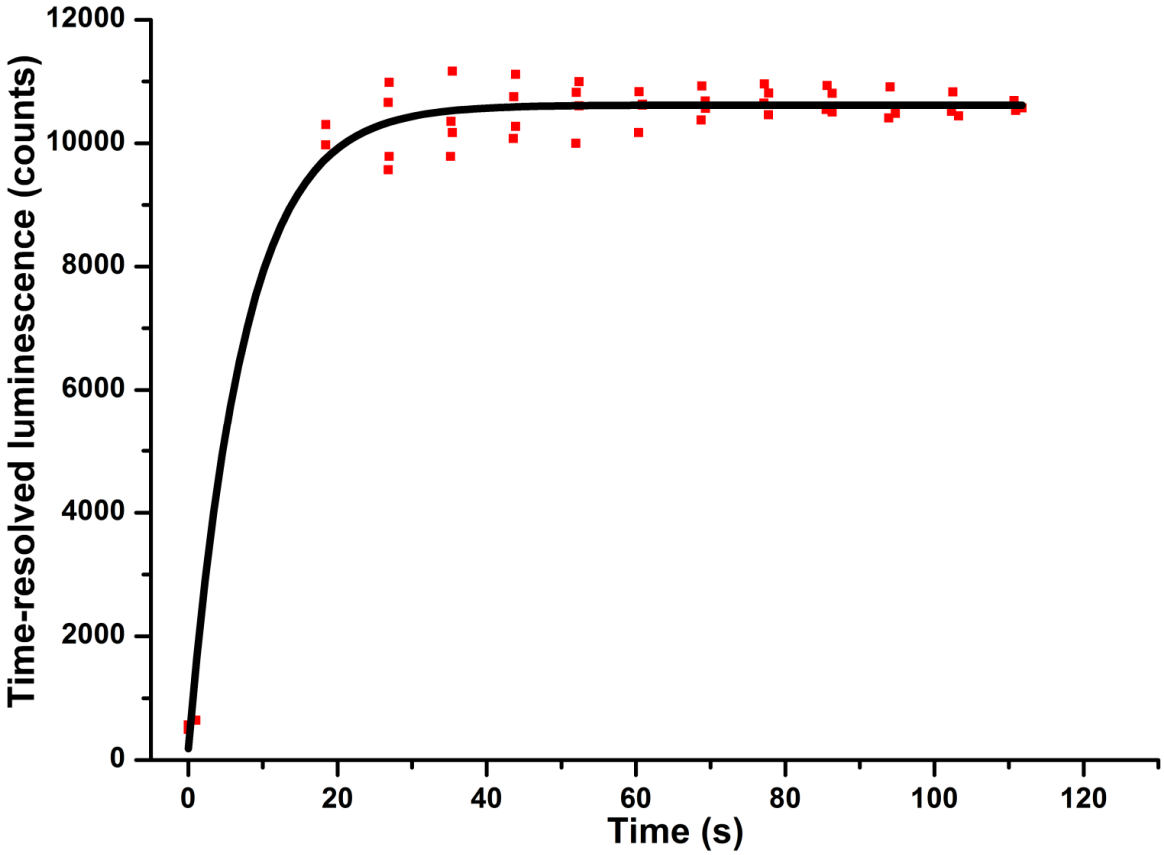

Figure S-3. Eu-FBA binding kinetics to bFGF in the non-competitive QRET assay. Aptamer binding kinetics was monitored using 10 nM Eu-FBA, 25 nM bFGF, and 2.3 µM Q5 concentrations. The results are shown as signals from four individual measurements using nine second measurement interval.

VEGF titration in serum. The suitability of the QRET assay in more complex matrix was tested using diluted serum, which is one of the most complicated but also relevant assay matrixes. The influence of serum was tested using VEGF spiked pooled human serum. Serum dilution 1:100 and 1:50 was tested (1% or 2% serum in final concentration). Compared to VEGF assay in buffer, the S/B was compromised with increasing serum concentration. The limit-of-detection (LOD) in 1:100 and 1:50 diluted serum were 1.0 ± 0.06 nM, and 3.8 ± 0.16 nM, respectively (Figure S-4). The average CV% in 1:100 and 1:50 diluted serum were 8.6% and 11.1%, respectively. The highest S/B ratios monitored were 4.3 and 2.6 in 1:100 and 1:50 diluted serum, respectively. In 1:100 and 1:50 diluted serum, 3.8 µM and 5.8 µM Q5 concentrations were used, respectively. Higher quencher concentration was needed due to high nonspecific protein concentration, thus some quencher was lost due to serum proteins. Higher quencher concentration also affects the S/B ratio, thus also specific signal was partly quenched. In buffer the equilibrium forms in minutes, but in serum the equilibrium needs more time to form. In 1:100 and 1:50 diluted serum the optimal incubation time was 60 min. One's equilibrium was formed; the signal stays stable throughout the whole tested 240 min of incubation. However, also 10 min incubation time can be used with serum dilution without much loss on overall performance, but only the CV% was affected. These assays in serum proves that VEGF-VBA-Eu complex formation was not only protecting the Eu(III)-chelate from quenching but also aptamer from degradation.

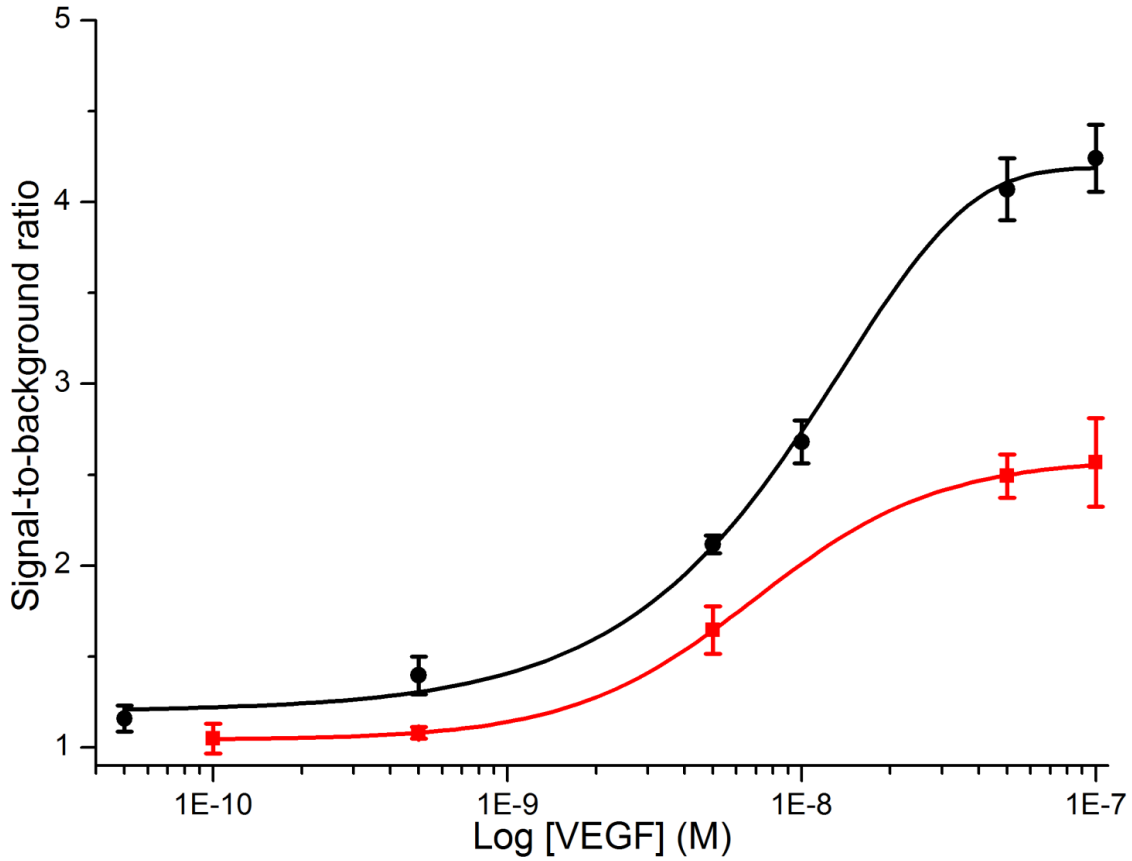

Figure S-4. The influence of diluted human serum in VEGF assay done with 3'-VBA-Eu. S/B ratios in quenching resonance energy transfer (QRET) assay using 1% serum (black) or 2% serum (red) were 4.3 and 2.6, respectively. The results are shown as means ± SD of four replicates.

bFGF titration in serum. The influence of serum was also tested using bFGF spiked pooled human serum. Serum dilution 1:100, 1:50, and 1:10 were tested (1%, 2%, and 10% serum in final assay volume). Compared to VEGF assay, one higher serum concentration was tested because of higher S/B ratios monitored in buffer with Eu-FBA. Like in VEGF assay, also in the bFGF assay the S/B ratio was compromised with increasing serum concentration. The LOD in 1:100 and 1:50 diluted serum were 6.1 ± 0.17 nM and 11.5 ± 0.56 nM, respectively (Figure S-5). In 1:10 diluted serum the LOD was 102.3 ± 2.9 nM, which was in line with other results. The average CV% in 1:100, 1:50, and 1:10 diluted serum were 5.6%, 7.4%, and 6.6%, respectively. The good CV% values are due to still high TRL-signal monitored even in 10% serum concentration. The S/B ratios monitored were 42.5, 26.7, and 6.6 with 1:100, 1:50, and 1:10 diluted serum, respectively. In 1:100, 1:50, and 1:10 diluted serums, 3.8 µM, 5.8 µM, and 6.0 µM Q5 concentrations were used, respectively. In 10% serum, the 6.0 µM Q5 concentration could not quench the TRL-signal as low than with less serum (lowest signal 4000 counts compared to normal 600 counts). This was due to high matrix interference when 10% serum used, and thus as low background as with 2% serum cannot be achieved with 10% serum until with very high (over 20 µM) quencher concentration, which was otherwise problematic. Like in VEGF assay, 60 min incubation time was sufficient with 1:100 and 1:50 diluted serum. With 1:10 dilution the maximal S/B ratio was recorded not until after 180 min of incubation. However, like in VEGF assays also 10 min incubation can be used with only minor loss on overall performance.

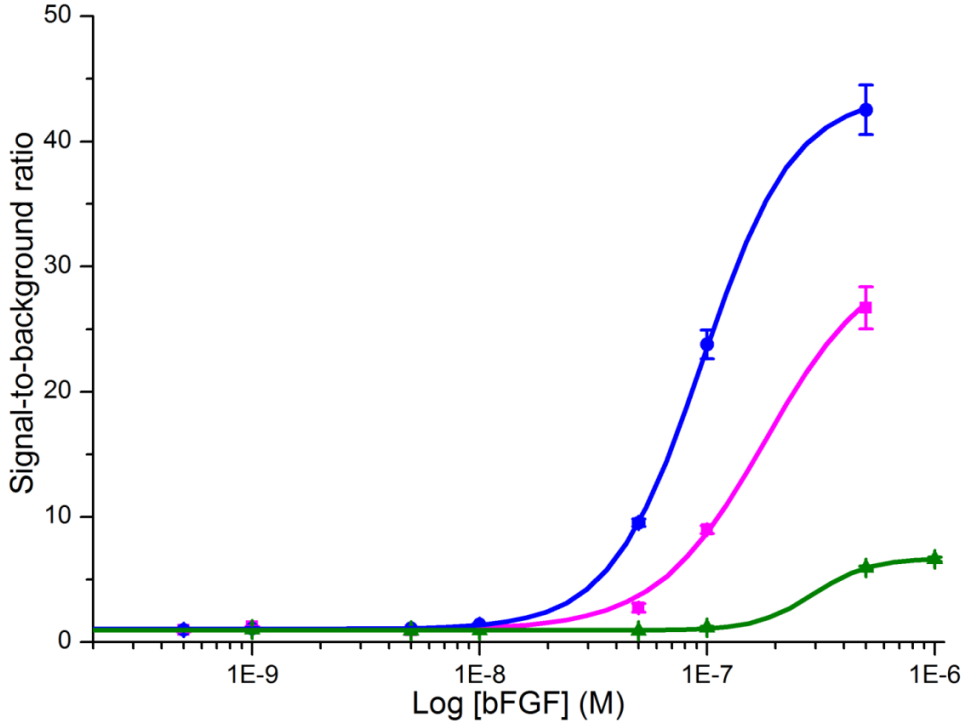

Figure S-5 The influence of diluted human serum in bFGF assay done with 5'-FBA-Eu. S/B ratios in quenching resonance energy transfer (QRET) assay using 1% serum (blue), 2% serum (pink), and 10% serum (green) were 42.5, 26.7, and 6.6, respectively. The results are shown as means ± SD of four replicates.

References

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2. H. Kaur, L. Y. Yung, PLOS ONE, 2012, 7, e31196.
3. B. Strehlitz, N. Nikolaus, R. Stoltenburg, Sensors, 2008, 8, 4296-4307.