Supporting Information

A Quantum Dot-Based Concentric FRET Configuration for the Parallel Detection of Protease Activity and Concentration

Miao Wu, Eleonora Petryayeva, W. Russ Algar*

Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia, V6T 1Z1, Canada.

*Corresponding author: algar@chem.ubc.ca

Table of Contents

1. Detailed Experimental Methods S2

1.1 Enzyme specifications S2

1.2 Synthesis of DHLA-PEG S2

1.3 Ligand exchange with DHLA-PEG S3

1.4 Labeling peptide with Alexa Fluor 647 ..... S4

1.5 Preparation of aptamer-peptide chimeras S4

1.6 cFRET calibration curves S6

1.7 Correction factors for cFRET PL data analysis S6

1.8 Fitting aptamer progress curves at low THR concentrations and with LYZ S7

1.9 Two-probe non-cFRET system S7

1.10 Evaluating THR inhibition by HD1p S8

2. Additional Results and Discussion S9

2.1 Gel electrophoresis S9

2.2 FRET pairs S10

2.3 cFRET calibration curves S10

2.4 Additional cFRET data S11

2.5 Two-probe non-cFRET system data S14

3. References S18

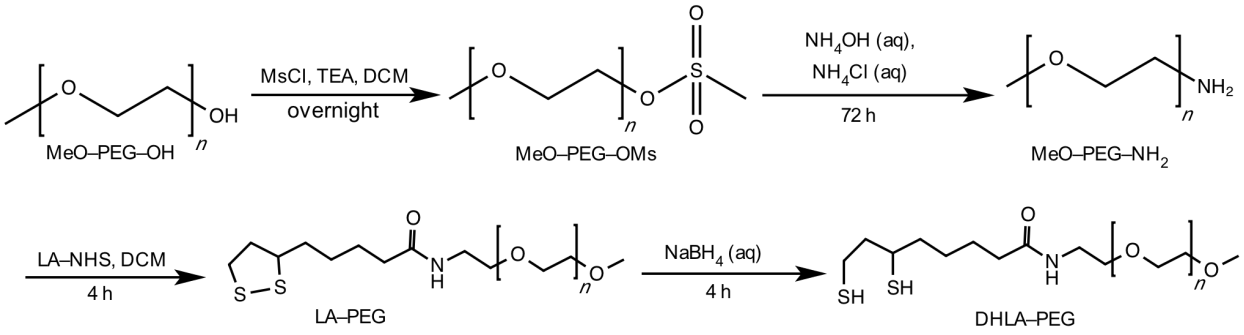
1. Detailed Experimental Methods

1.1 Enzyme specifications

Human α-thrombin (THR): Haematologic Technologies Inc. (Essex Junction, VT, USA); product no. HCT-0020, 3324 units/mg; EC Number 3.4.21.5. Trypsin (TRP): Sigma-Aldrich (Oakville, ON, Canada); product no. T1426, ≥ 10 000 BAEE units/mg protein, TPCK treated, from bovine pancreas; EC Number 3.4.21.4. Lysozyme (LYZ): Sigma-Aldrich; product no. L6876, ≥ 40 000 units/mg protein, from chicken egg white; EC Number 3.2.1.17.

1.2 Synthesis of DHLA-PEG

Scheme S1 shows the method for the synthesis of DHLA-PEG ligand.



Scheme S1. *Synthetic steps in the preparation of DHLA-PEG.*

Synthesis of MeO–PEG–OMs. 10 g of poly(ethylene glycol) methyl ether (MeO-PEG; average MW ~750 g/mol; ~13 mmol) was dried under vacuum at 50 °C overnight. The dried PEG was diluted with 30 mL of dichloromethane (DCM), 2.1 mL (16 mmol) of methanesulfonyl chloride (MsCl) was added, and the reaction was put under argon and cooled on ice. Next, 5.6 mL of triethylamine (TEA; 40 mmol) was added dropwise over 2 h. The reaction was brought to room temperature and stirred overnight. The reaction mixture was concentrated to dryness *in vacuo*, washed with 3×20 mL of ether, then dried *in vacuo*. Thin layer chromatography: 10:1 (v/v) $CHCl_3$:MeOH, R_f (MeO–PEG–OH) = 0.35, R_f (MeO–PEG–OMs) = 0.48. TLC plates were developed with iodine.

Synthesis of MeO–PEG–NH_2. MeO–PEG–OMs (entire yield from previous step) was dissolved in 20 mL of NH_4OH (30% w/w *aq*.) and 8 g of NH_4Cl was added. The reaction was stirred under

argon for 72 h. The mixture was diluted with ~20 mL water, washed with 3×20 mL portions of ether, and then extracted with 3×30 mL portions of DCM. The extract was dried over Na_2SO_4 and concentrated *in vacuo*. Thin layer chromatography: 10:1 (v/v) $CHCl_3$:MeOH, R_f (MeO–PEG–NH_2) = 0.02, R_f (MeO–PEG–OMs) = 0.48. TLC plates were developed with iodine and the presence of the primary amine was confirmed *via* the ninhydrin test. ESI^+ MS (MeOH): calcd. for $C_{35}H_{74}NO_{17}$ $[M+H^+]$ m/z = 781.0, found m/z = 780.8; calcd. for $C_{37}H_{78}NO_{18}$ $[M+H^+]$ m/z = 825.0, found m/z = 824.8. These peaks correspond to n = 17 and 18 in Scheme S1, respectively.

Synthesis of MeO–PEG–LA. The succinimidyl ester of lipoic acid (LA-NHS) was synthesized as described previously.[1] A mass of 0.6 g of LA-NHS was added to MeO–PEG–NH_2 (from previous step, ~9 g) in 50 mL DCM. The reaction was stirred under argon overnight. The product was concentrated *in vacuo* and purified by chromatography on silica gel with 20:1 v/v $CHCl_3$:MeOH as the eluent. Thin layer chromatography: 10:1 (v/v) $CHCl_3$:MeOH, R_f (MeO–PEG–NH_2) = 0.02, R_f (LA-NHS) = 0.92, R_f (MeO–PEG–LA) = 0.64. NMR ^1H ($CDCl_3$): 1.45 ppm (m, 2H), 1.65 ppm (m, 2H), 1.85 ppm (m, 2H), 2.15 ppm (t, 2H), 2.45 ppm (m, 2H), 3.15 ppm (m, 1H), 3.35 ppm (s, 3H), 3.42 ppm (m, 2H), 3.52 ppm (m, 4H), 3.62 ppm (s, 64H), 6.2 ppm (s, broad, 1H).

Synthesis of MeO–PEG–DHLA. Approximately 1.4 g of MeO–PEG–LA was dissolved in 50 mL of water and cooled on ice. Next, 1.5 g of $NaBH_4$ in 100 mL of water was added portionwise to a solution of MeO–PEG–LA cooled in an ice bath. The reaction was stirred at room temperature for 4 h and then quenched with 12 M HCl (*aq*) until pH 1–2 was reached. The product was extracted with 3×30 mL portions of DCM, the extracts dried over sodium sulfate, solvent removed *in vacuo*, and the product stored under argon in the freezer. Thin layer chromatography: 10:1 (v/v) $CHCl_3$:MeOH, R_f (MeO–PEG–DHLA) = 0.62, R_f (MeO–PEG–LA) = 0.64.

1.3 Ligand exchange with DHLA-PEG

Hydrophobic QDs (200 µL, ~6 µM) were precipitated by the addition of 100 µL of methanol and centrifuged to a pellet (4800 rcf, 4 min). The pellet was redissolved in 200 µL chloroform and transferred to a 10 mL round bottom flask. The chloroform was evaporated under argon and

~0.3 g of DHLA-PEG was added. The mixture was brought to 50 °C under argon and left stirring for 6 h. The mixture was cooled to room temperature, then diluted with 2 mL of ethanol. Hexane was added until the solution became turbid, chloroform was then added dropwise until the turbidity disappeared, and finally hexane was added dropwise until turbid again. The QDs were centrifuged to a pellet (1900 rcf; 10 min) and the supernatant was discarded. The QD pellet was dissolved in borate buffer (50 mM, 250 mM NaCl, pH 9.5), passed through a 0.22 μm syringe filter, and washed using a 30 kDa centrifugal filter with 3×200 μL portions of borate buffer. The QDs were dissolved in borate buffered saline (50 mM, 13.7 mM NaCl, 0.27 mM KCl, 3 mM $MgCl_2$, pH 8.5) and quantified spectrophotometrically.

1.4 Labeling peptide with Alexa Fluor 647

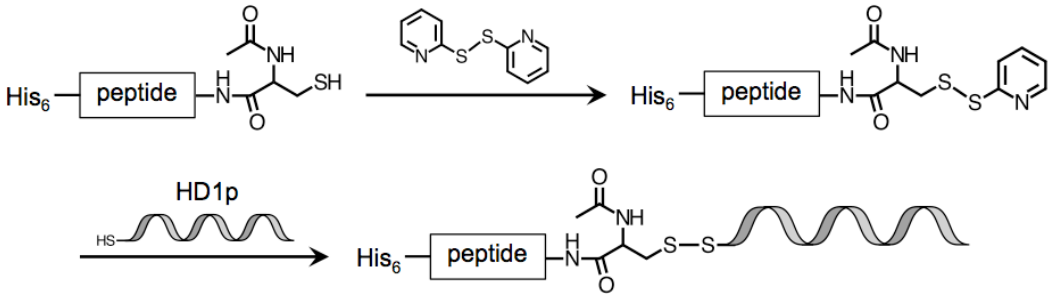
The terminal cysteine residue of the Sub(A647) peptide was labeled with Alexa Fluor 647 C_2 maleimide dye as described previously.[2,3] Briefly, ~1 mg (330 nmol) of peptide was dissolved in 50 μL of 50% v/v MeCN (aq) and diluted with 500 μL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (100 mM, 50 mM NaCl, pH 7.5). The peptide was mixed with 0.5 mg (384 nmol) of reactive dye dissolved in 25 μL DMSO and left overnight at room temperature, protected from light. The labeled peptide was purified over nickel (II) nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Toronto, ON, Canada). After binding to the Ni-NTA, the labeled peptide was washed with 2×10 mL of phosphate buffered saline (PBS), 5 mL of 1:1 v/v ethanol:PBS, and 2×10 mL of PBS, then eluted with 300 mM imidazole in PBS (the imidazole solution was filtered through activated carbon prior to use). The eluted peptide was desalted using Amberchrom CG–300 resin (Dow Chemical, distributed by Sigma-Aldrich). After adsorbing to the Amberchrom resin, the labeled peptide was washed with 4×5 mL of 0.2 M triethylamine acetate (TEAA) buffer and eluted with less than 1 mL of 70% v/v acetonitrile (aq). The purified peptide was quantified spectrophotometrically, split into 20 nmol portions, dried *in vacuo*, and stored at –20 °C. When needed, the peptide was first dissolved in 5 μL of 50% v/v acetonitrile (*aq*), and then diluted with buffer.

1.5 Preparation of aptamer-peptide chimeras

Pyridyl disulfide-activated Cys-His_6 (see Table 1) was prepared as described previously, see Scheme S2.[3] Briefly, 1 mg (550 nmol) of peptide (Cys-His_6) was dissolved in 10 μL 50% (v/v)

acetonitrile (*aq*) and then diluted with 290 µL ammonium acetate buffer (100 mM, pH 4.5). Approximately 2 mg (9 µmol) of 2,2′-dipyridyl disulfide (Aldrithiol-2; Sigma-Aldrich) was dissolved in 300 µL acetonitrile. These two solutions were mixed and allowed to react overnight. The reaction mixture was then diluted with two parts by volume of PBS and purified over Ni^{2+}–NTA–agarose and desalted over Amberchrom CG-300 resin. To quantify the amount of peptide, 50 µL of the eluent was reacted with a ~10^3-fold excess of tris-(2-carboxyethyl)phosphine (TCEP) to liberate 2-mercaptopyridine, which has an absorption peak at 343 nm with an extinction coefficient of 8080 $cm^{-1}\,M^{-1}$. The activated peptide (Py-S-Cys-His_6) was aliquoted into 20 nmol portions and dried *in vacuo*.

An oligonucleotide with the HD1 aptamer sequence (HD1p; Integrated DNA Technologies, Coralville, IA, USA) was received with the thiol linker protected as a disulfide. This disulfide was reduced with TCEP (20 nmol of aptamer and 100 µL of 40 mM TCEP in deionized water) for 4 h. Excess TCEP was removed using two successive Illustra NAP-10 columns (GE Healthcare) using PBS buffer as the eluent. A dried aliquot of Py-S-Cys-His_6 (20 nmol) was dissolved in 10 µL 50% (v/v) acetonitrile (*aq*) and mixed with the eluent (1.5 ml) containing reduced aptamer and let react overnight, see Scheme S2. The aptamer-peptide chimera, HD1p, was purified using Ni^{2+}–NTA–agarose, desalted with Amberchrom CG-300 resin, aliquoted into 20 nmol portions, then dried *in vacuo*. The amount of HD1p was quantified spectrophotometrically using the absorbance of the DNA at 260 nm (extinction coefficient ~212 600 $cm^{-1}\,M^{-1}$).



Scheme S2. *Steps in the preparation of HD1p-Cys-His_6 chimeras.*

1.6 cFRET calibration curves

To calibrate the cFRET configuration, $[HD1p/cHD1(Cy3)]_M$-QD-$[Sub(A647)]_N$ conjugates were prepared in two steps. First, a stock solution of HD1p/cHD1(Cy3) hybrids was prepared by mixing HD1p and cHD1(Cy3) in a 1:1 ratio at M μM (each) and incubating for 2 h. Second, M×10 pmol of HD1p/cHD1(Cy3) hybrids were mixed with N×10 pmol of Sub(A647) from N μM stock solutions and diluted to a total volume of 45 μL with BBS. Next, 10 μL of 1.0 μM QDs (10 pmol) were added to the mixture and let stand at room temperature for 4 h. Control experiments tracking PL intensities over time after mixing QD and peptides confirmed that 4 h was sufficient to obtain a relatively stable PL ratio. Different (M, N) combinations were prepared, spanning the ranges M = 0, 3, 6, 9, 12 and N = 0, 2, 4, 6, 8 as a 5×5 matrix of samples (data not shown), and M = 0, 4, 8, 12 and N = 0, 3, 6, 9 (see Figure S3). Prior to PL measurements, 50 μL of the conjugate solution was diluted with 50 μL of BBS buffer and transferred to a 96-well plate.

1.7 Correction factors for cFRET PL data analysis

The correction factors used in eqns. 2–3 eliminated crosstalk between the QD, Cy3, and A647 emission with PL intensity measurements at 524, 564, and 668 nm. These correction factors are defined by eqns. S1–S3, where $I_x(\lambda)$ is the intensity from emitter x measured at wavelength λ.

$$_{QD}\sigma_{564} = \frac{I_{QD}(564)}{I_{QD}(524)} \tag{S1}$$

$$_{QD}\sigma_{668} = \frac{I_{QD}(668)}{I_{QD}(524)} \tag{S2}$$

$$_{Cy3}\sigma_{668} = \frac{I_{Cy3}(668)}{I_{Cy3}(564)} \tag{S3}$$

The kinetic PL ratio data (used to calculate progress curves) was further corrected for small non-proteolytic changes in PL and instrumental drift by normalization to control data (no protease added). For the calculated PL ratio, (t, [E]), as a function of time, t, and enzyme concentration, [E], the data was normalized according to Eqn. S4. This equation applies to all FRET ratios. Although the normalization of the FRET ratios in Eqns. 6–7 is not noted explicitly, normalized data was always used for calculation of progress curves of (M, N) *versus* time.

$$\rho(t,[E])_{\text{Normalized}} = \frac{\rho(t,[E])}{\rho(t,0)}\rho(0,0) \tag{S4}$$

1.8 Fitting aptamer progress curves at low THR concentrations and with LYZ

Eqn. 8 in the main text reduces to Eqn. S5 *via* a Taylor series expansion and truncation prior to the quadratic term. Progress curves with little or no change in signal (*e.g.*, no discernable curvature at low THR concentrations or with LYZ) were fit with this equation—a line for which we approximated $\Delta = c/d$.

$$M(t) = -Amt + (M_\infty + A) = -ct + d \tag{S5}$$

1.9 Two-probe non-cFRET system

1.9.1 Calibration curves

For the two-probe non-cFRET configuration, 50 μL of QD524-[HD1p/cHD1(Cy3)]$_M$ and QD624-[Sub(THR)A647]$_N$ were prepared by mixing 10 pmol of QD624-[Sub(THR)A647]$_N$ conjugate (in 25 μL) and 5 pmol QD524-[HD1p/cHD1(Cy3)]$_M$ conjugate (in 25 μL). These two conjugate solutions were prepared analogous to those in the cFRET configuration.

1.9.2 Assays

With the conventional FRET configuration and n samples, QD624-[Sub(A647)]$_8$ (10n pmol, 25n μL) and QD524-[HD1p/cHD1(Cy3)]$_{12}$ (5n pmol, 25n μL) were prepared separately, following steps analogous to the preparation of the cFRET probes, then mixed prior to assays. The assays were then executed analogous to the cFRET configuration assays.

1.9.3 Data analysis

While the emission peaks for the QD524, Cy3, QD624, and A647 were resolved from one another, the longer wavelength tails of the emission spectra had some small overlap with other red-shifted dyes. Starting from the QD524 PL peak, it was possible to account for these small amounts of crosstalk using eqns. S6–S9, where $_x\sigma_\lambda$ is a correction factor for the PL intensity, I, of emitter x at wavelength λ, which is the ratio of emission from x at wavelength λ over that at its

emission peak. Correction factors were determined from spectra measured for the emitters individually.

$$I_{\mathrm{QD524}} = I(524) \tag{S6}$$

$$I_{\mathrm{Cy3}} = I(564) - {}_{\mathrm{QD524}}\sigma_{564} I_{\mathrm{QD524}} \tag{S7}$$

$$I_{\mathrm{QD624}} = I(624) - {}_{\mathrm{Cy3}}\sigma_{624} I_{\mathrm{Cy3}} \tag{S8}$$

$$I_{\mathrm{A647}} = I(668) - {}_{\mathrm{QD624}}\sigma_{668} I_{\mathrm{QD624}} - {}_{\mathrm{Cy3}}\sigma_{668} I_{\mathrm{Cy3}} \tag{S9}$$

The corrected intensity values were then used to calculate PL ratios, ρ, according to Eqns. S10–S11.

$$\rho_{\mathrm{Cy3/QD524}} = \frac{I_{\mathrm{Cy3}}}{I_{\mathrm{QD524}}} \tag{S10}$$

$$\rho_{\mathrm{A647/QD624}} = \frac{I_{\mathrm{A647}}}{I_{\mathrm{QD624}}} \tag{S11}$$

Conversion of PL ratios to the number of cHD1(Cy3) and Sub(A647) per QD were done using Eqns. S12–S13 obtained from calibration curves (see Section 2.5.1).

$$\rho_{\mathrm{Cy3/QD524}} = 0.0127M - 0.0045 \tag{S12}$$

$$\rho_{\mathrm{A647/QD624}} = 0.2194N + 0.0866 \tag{S13}$$

1.10 Evaluating THR inhibition by HD1p

To measure the inhibition of THR activity by HD1p, two sets of samples were prepared: one set utilized [Sub(A647)]$_8$-QD524-[HD1p]$_M$ conjugates (where $M = 0$, 5, 10, 15, 20, 30), and the other set utilized [Sub(A647)]$_8$-QD524 mixed with M×[HD1p], where the HD1p was not ligated to Cys-His$_6$. For each M, THR was added to the conjugate or mixture with dilution to equal final volumes. The final concentration of THR was 60 nM. The A647/QD524 PL ratio was measured every 90 s for 4 h. Since calibration curves for these systems were routinely found to be linear, the PL ratios were converted to approximate progress curves by extrapolating the relative change in the PL ratio to the relative change in the number of peptides per QD.

2. Additional Results and Discussion

2.1 Gel electrophoresis

Figure S1 shows gel electrophoresis results that demonstrate the self-assembly of the Sub(A647) peptide, HD1p aptamer probe, and HD1p/cHD1(Cy3) aptamer hybrid *via* their polyhistidine tags. The PEG-coated QDs have no charge and thus negligible electrophoretic mobility. At pH 8.3, Sub(A647) will carry a net negative charge (from the aspartic acid and glutamic acid residues, and the A647) and thus impart some limited mobility to QD-Sub(A647) conjugates. Although larger, oligonucleotides have a greater net negative charge (from the phosphate backbone) and conjugation of HD1p and HD1p/cHD1(Cy3) hybrid impart even greater mobility to the QD, albeit still limited. With the addition of THR to the QD-HD1p/cHD1(Cy3) conjugates, a relative decrease in mobility is observed from the added size of the bound THR.

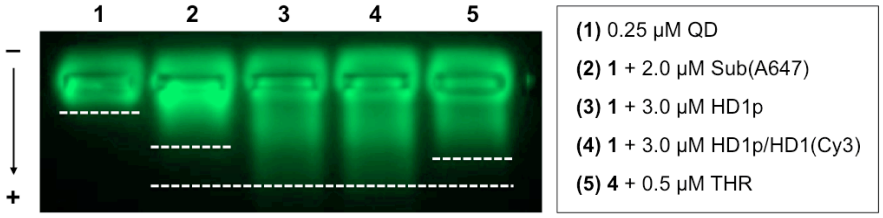


Figure S1. Pseudocolor PL image of an agarose gel (1.0%) showing the polyhistidine-mediated assembly of the Sub(A647) peptides and HD1p or HD1p/cHD1(Cy3) aptamers to the QD, as well as binding of THR to the aptamer. The arrow at the left indicates the polarity of the electric field (7.3 V/cm). The dashed lines indicate the approximate leading edge of the bands.

2.2 FRET pairs

Figure S2 shows the absorption and emission spectra for the QD524, QD624, cHD1(Cy3), and Sub(A647). The spectral overlap integrals, J, and Förster distances, R_0, for the relevant FRET pairs are listed in Table S1.

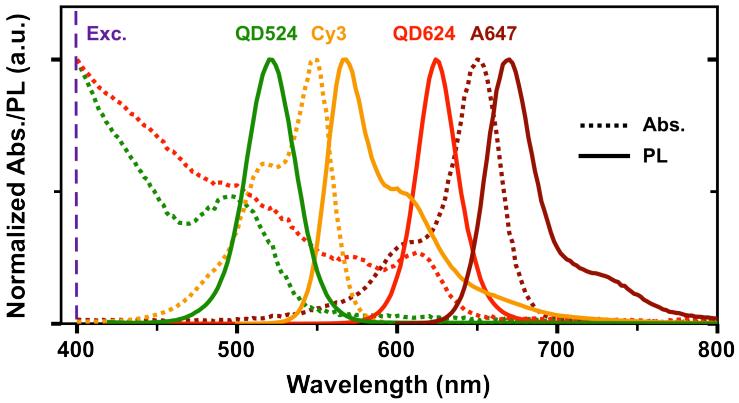


Figure S2. Normalized absorption and emission spectra for the QD524 and QD624 donors and Cy3 and A647 dyes acceptor used in this work.

Table S1. FRET pair characteristics.

FRET pair	J **(cm^6 mol^{-1})**	R_0 **(nm)**
QD524-Cy3	2.3×10^{-10}	4.7
QD524-A647	5.6×10^{-11}	3.7
Cy3-A647	6.9×10^{-10}	5.9
QD624-A647	2.0×10^{-9}	6.0

2.3 cFRET calibration curves

Figure S3 shows two-dimensional cFRET calibration plots of the Cy3/QD and A647/QD624 PL ratios as a function of the number of cHD1(Cy3) per QD, M, and the number of Sub(A647) per QD, N. These plots are for a 4×4 matrix of calibration samples and were used to process the assay data. A larger 5×5 matrix of calibration samples was measured on another occasion to confirm the same general trends in the two-dimensional data. Figure S4 shows representative PL spectra in full. PL ratios may vary between batches of QDs; however, the linear trends permit rescaling of calibration data obtained with one batch of QDs for use with another batch.

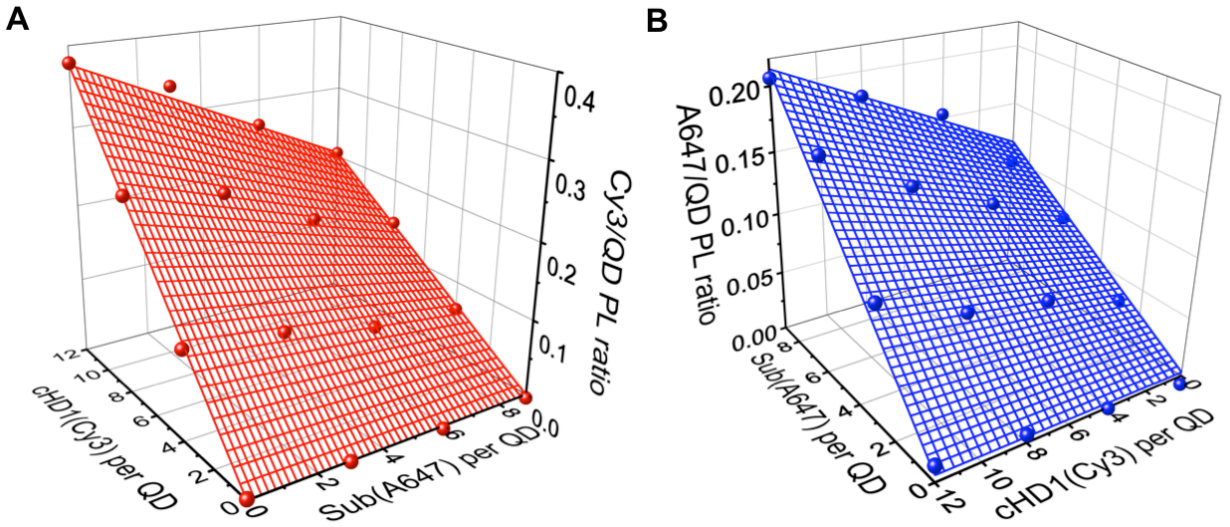


Figure S3. Changes in (A) the Cy3/QD PL ratio and (B) the A647/QD PL ratio as a function of the average numbers of HD1p/cHD1(Cy3) per QD, *M*, and Sub(THR)A647 per QD, *N*. Note the change in xy axes between (A) and (B).

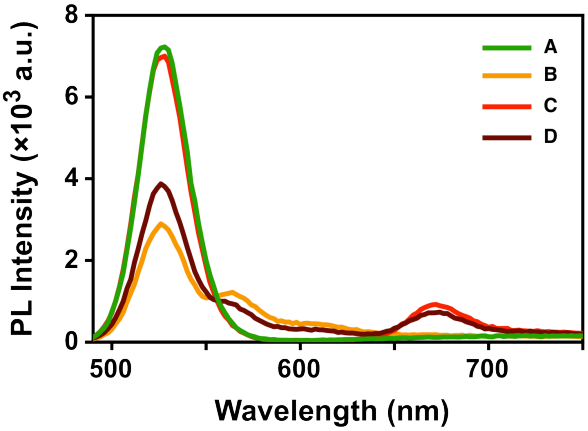


Figure S4. Representative PL emission spectra for the concentric FRET system: (A) QD524; (B) $[HD1p/cHD1(Cy3)]_{12}$-QD524 (C) QD524-$[Sub(A647)]_8$; (D) $HD1p/cHD1(Cy3)]_{12}$-QD524-$[Sub(A647)]_8$. These spectra are not from the same batch of materials as the data in Fig. S3, but show the same general trend. Note that binding of polyhistidine-terminated peptides to these QDs can increase their brightness.

2.4 Additional cFRET data

Figure S5 shows the PL ratio data for the cFRET probe in response to TRP and LYZ (see Fig. 2 for processed data). Figure S6 shows progress curves for various concentrations of TRP (0.16–5.0 μM, scaling by factors of two).

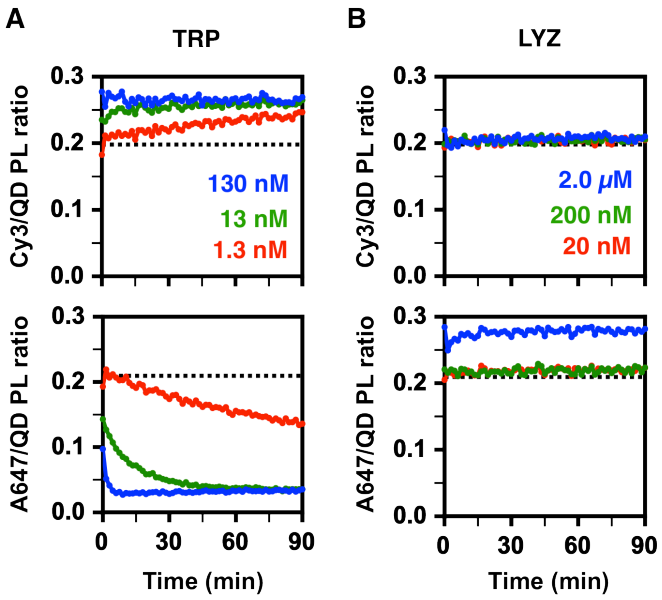


Figure S5. Normalized PL ratio data for the (A) TRP and (B) LYZ progresses curves shown in Fig. 2 (main text). The dashed line is normalized data for a control sample with no enzyme.

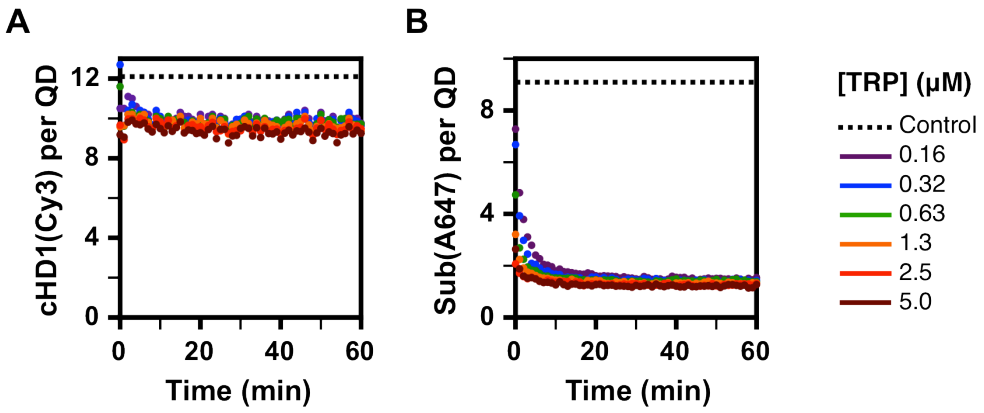


Figure S6. Calculated progress curves for the cFRET probes in response to various concentrations of TRP: (A) displacement of cHD1(Cy3) from HD1p; (B) hydrolysis of Sub(A647). Complete proteolysis of Sub(A647) is nearly instantaneous for micromolar concentrations of TRP.

Figure S7 shows the PL ratio data for the cFRET probe in response to various concentrations of THR and argatroban. Figure S8 shows the processed data, a summary of which is presented in Fig. 4A.

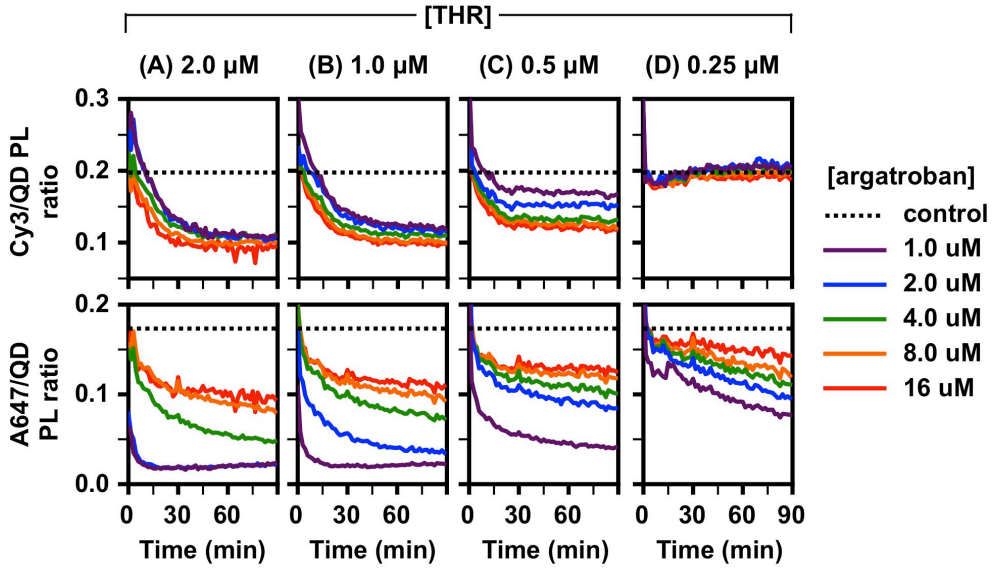


Figure S7. Normalized PL ratio data showing the response of the cFRET probe to various concentrations of THR (A-D) and argatroban. See Fig. S6 for the progress curves. The dashed line is normalized data for a control sample with no enzyme.

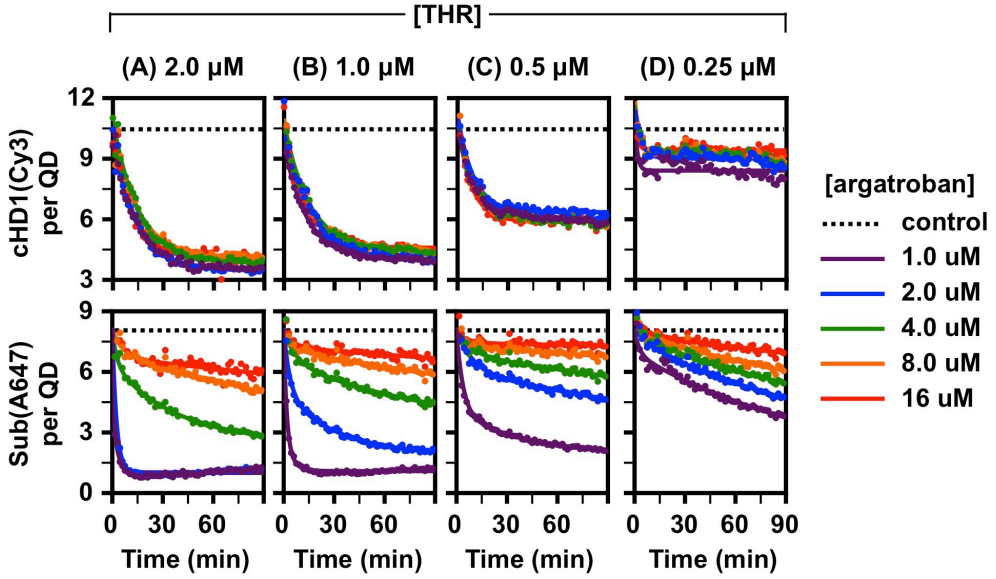


Figure S8. Progress curve showing the response of the cFRET probe to various concentrations of THR (A-D) and argatroban. This data is summarized in Fig. 4A (main text). The dashed line is normalized data for a control sample with no enzyme.

Figure S9 shows the PL ratio data for the cFRET probe in response to TLCK-treated THR (see Fig. 4B for processed data).

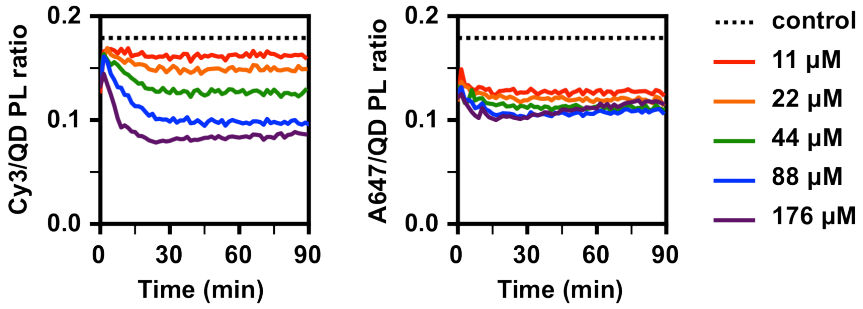


Figure S9. Normalized PL ratio data showing the response of the cFRET probe to various concentrations of TLCK-treated THR. This data is summarized in Fig. 4B (main text). The dashed line is normalized data for a control sample with no enzyme.

2.5 Two-probe non-cFRET system data

2.5.1 Calibration data

Figure S10 shows PL ratio calibration data for the QD524-$[HD1p/cHD1(Cy3)]_M$ and QD624-$[Sub(THR)A647]_N$ conjugates.

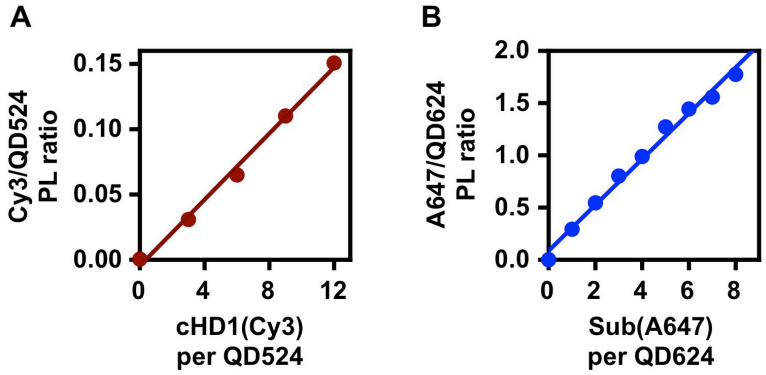


Figure S10. Calibration curves for QD524-$[HD1p/cHD1(Cy3)]_M$ and QD624-$[Sub(THR)A647]_N$ FRET probes as discrete entities.

2.5.2 Assay data

Figure S11 shows the raw PL ratio data and progress curves for the two-probe non-cFRET system in response to various concentrations of THR, TRP, and LYZ. Figure S12 shows the raw PL ratio data and progress curves in response to various concentrations of THR and argatroban. Figure S13 shows the raw PL ratio data and progress curves in response to various concentrations of TLCK-THR. Figure S14 summarizes the data in Figs. S11–S13 in terms of k and Δ.

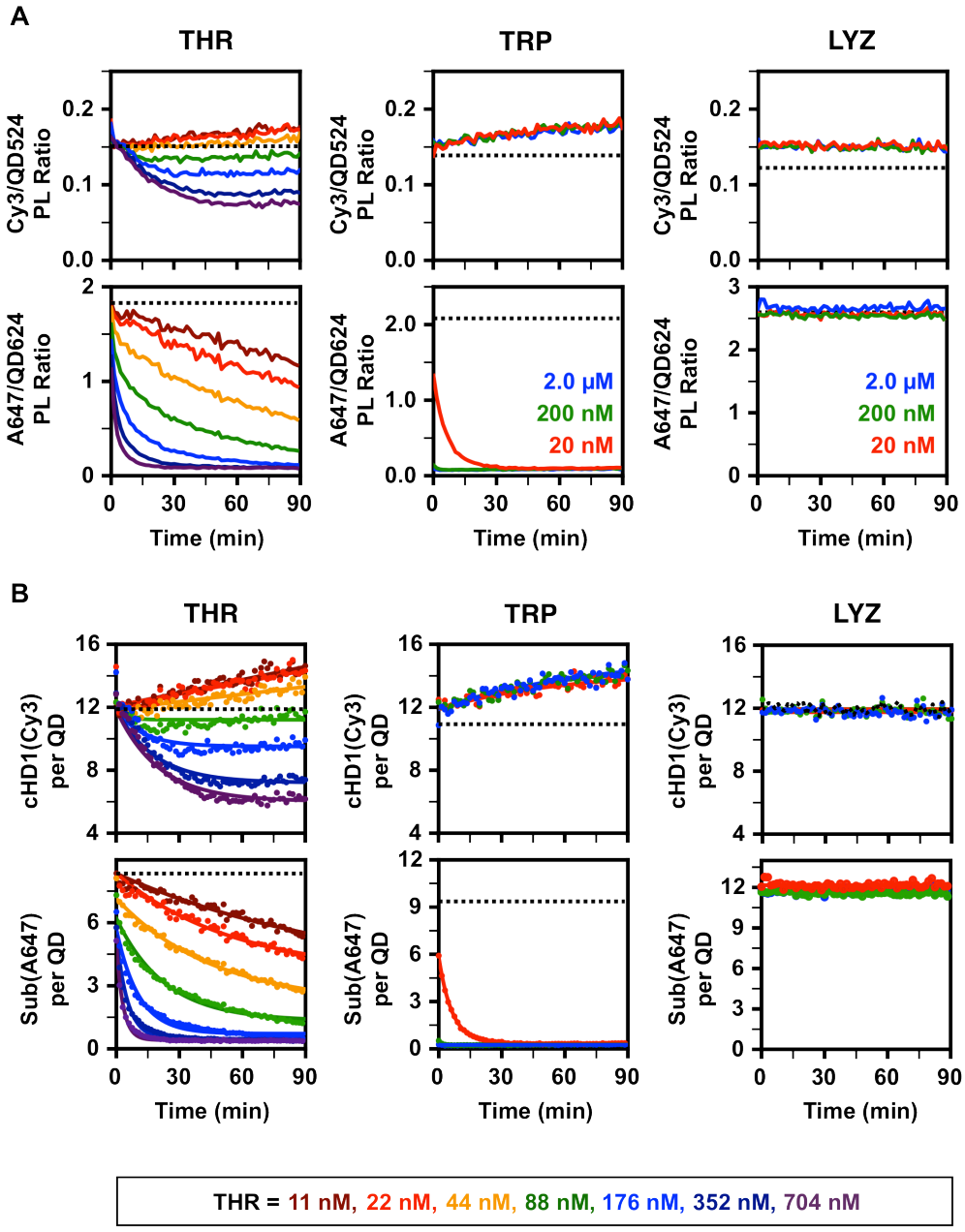


Figure S11. (A) Normalized PL ratio data and (B) progress curves for the response of the two-probe non-cFRET system, QD524-[HD1p/cHD1(Cy3)]$_{12}$ and QD624-[Sub(THR)A647]$_8$, to different concentrations of THR, TRP, and LYZ. For TRP and LYZ, the legend in panel A also applies to panel B. The dashed line is normalized data for a control sample with no enzyme.

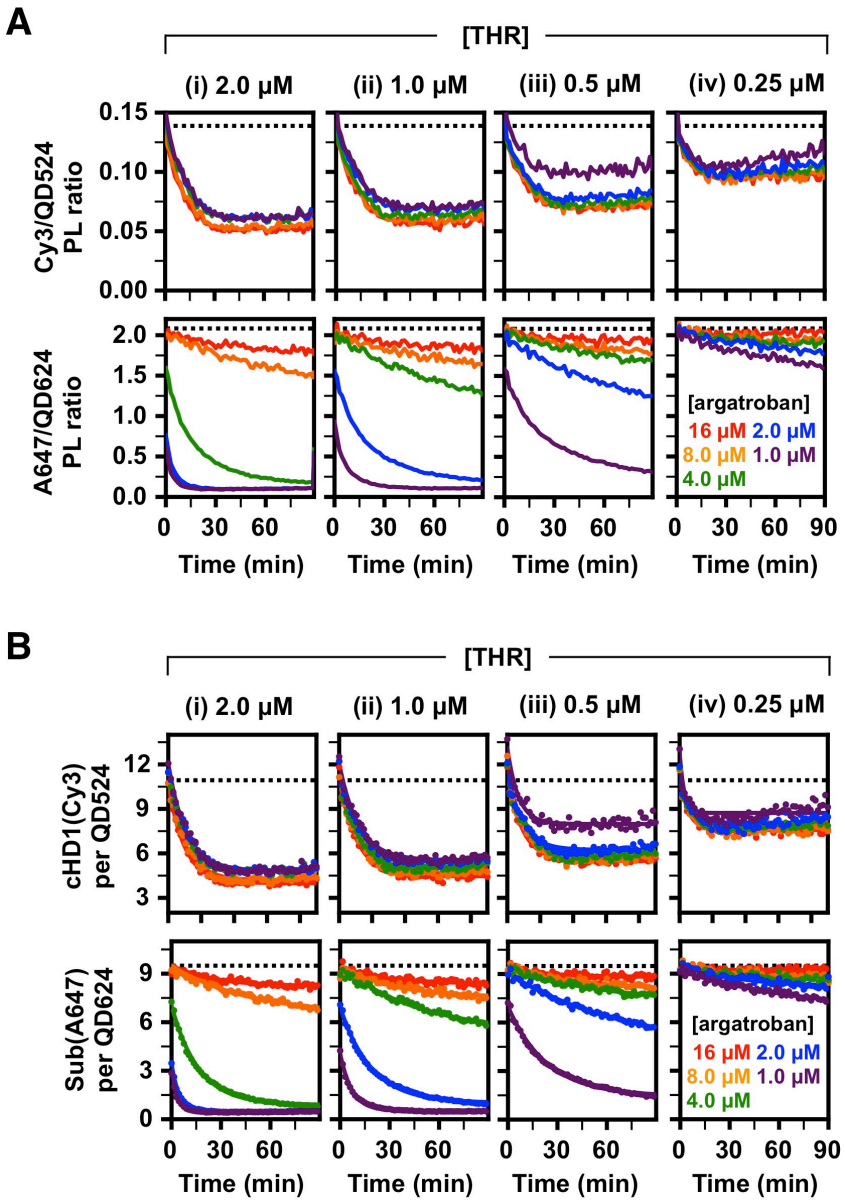


Figure S12. (A) Normalized PL ratio data and (B) progress curves for the response of the two-probe, non-cFRET system, QD524-[HD1p/cHD1(Cy3)]$_{12}$ and QD624-[Sub(THR)A647]$_8$, to different concentrations of THR and argatroban. The dashed line is normalized data for a control sample with no enzyme.

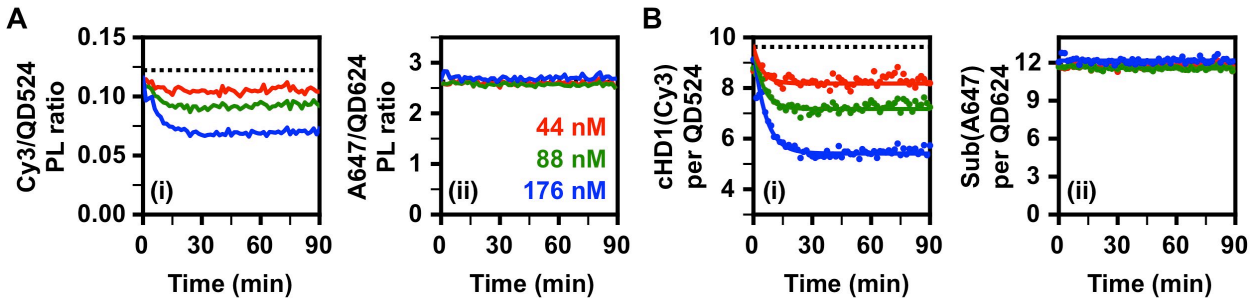


Figure S13. (A) Normalized PL ratio data and (B) progress curves showing the response of the (i) QD524-[HD1p/cHD1(Cy3)]$_{12}$ and (ii) QD624-[Sub(THR)A647]$_8$ FRET probes to different concentrations of TLCK-THR. The dashed line is normalized data for a control sample with no enzyme.

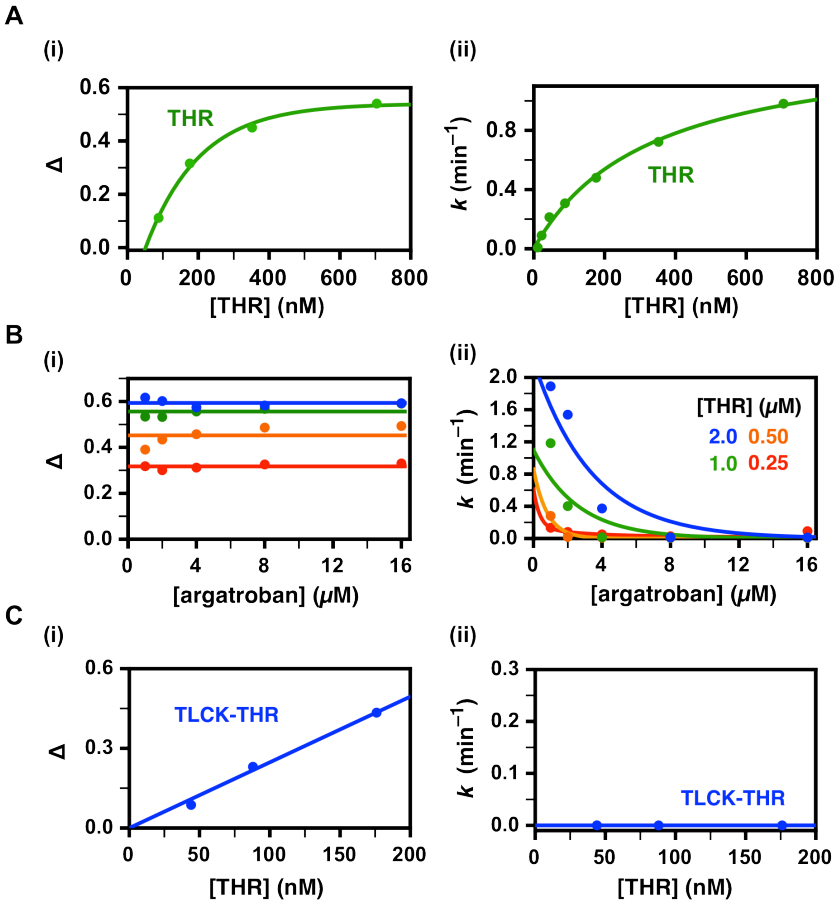


Figure S14. Summary of (i) Δ and (ii) *k* values for the two-probe non-cFRET system, calculated from the following the experiments: (A) THR assays in Fig. S10; (B) THR and argatroban assays in Fig. S11; and (C) TLCK-THR assays in Fig. S12.

3. References

(1) Algar, W. R.; Krull, U. J. *Sensors* **2011**, *11*, 6214-6236.
(2) Petryayeva, E.; Algar, W. R.; Medintz, I. L. *Appl. Spectrosc.* **2013**, *67*, 215-252.
(3) Algar, W. R.; Blanco-Canosa, J. B.; Manthe, R. L.; Susumu, K.; Stewart, M. H.; Dawson, P. E.; Medintz, I. L. *Meth. Mol. Biol.* **2013**, *1025*, 47-73.