Supporting Materials

Supplementary Methods.

Preparation of 96-well plate samples for LRET assays: Details of reagent addition for the preparation of 96-well plates are given for each type of LRET affinity and inhibition assay described in the main text.

Analysis of GFP-FKBP/rapamycin/FRB-eDHFR affinity using purified proteins: titration of GFP-FKBP.

- GFP-FKBP (6 μM in assay buffer) was serially diluted into 24 wells containing 50 μL assay buffer.
- Five rapamycin solutions were prepared (14, 8, 6, 2, 0.8 μM in Assay Buffer), and 25 μL of a given solution was added into wells containing 50 uL GFP-FKBP solutions such that [rapamycin] > [GFP-FKBP].
- A solution containing FRB-eDHFR (40 nM), TMP-TTHA-cs124(Tb^{3+}) (40 nM), and NADPH (4 μM) was prepared, and 25 μL of it was added into each well.
- The final concentrations of each component in sample wells were as follows:
  - GFP-FKBP, ranging from 3.0 μM to 0.4 pM
  - Rapamycin, ranging from 3.5 μM to 0.2 μM
  - FRB-eDHFR, constant in all wells at 10 nM
  - TMP-TTHA-cs124(Tb3+), constant in all wells at 10 nM
  - NADPH, constant in all wells at 1 μM
- A solution containing TMP-TTHA-cs124(Tb^{3+}) (20 nM), and NADPH (2 μM) was prepared for control wells, and 50 μL of it was added into each control well containing 50 uL GFP-FKBP dilutions.
- The final concentrations of each component in control wells were as follows:
  - GFP-FKBP, ranging from 3.0 μM to 0.4 pM
  - TMP-TTHA-cs124(Tb3+), constant in all wells at 10 nM
  - NADPH, constant in all wells at 1 μM

Analysis of GFP-FKBP/rapamycin/FRB-eDHFR affinity using purified proteins: titration of FRB-eDHFR.

- FRB-eDHFR (2 μM in assay buffer) was serially diluted into 16 wells containing 50 μL assay buffer.
- A solution containing GFP-FKBP (20 nM), TMP-TTHA-cs124 (2 μM), Tb^{3+} (0.2 μM), rapamycin (60 nM), and NADPH (4 μM) was prepared, and 50 μL of it was added into each sample well containing 50 μL FRB-eDHFR dilutions and into each control well containing 50 μL assay buffer.
- The final concentrations of each component in sample wells were as follows:
  - FRB-eDHFR, ranging from 1 μM to 30 pM
  - GFP-FKBP, constant in all wells at 10 nM
  - Rapamycin, constant in all wells at 30 nM

  - TMP-TTHA-cs124, constant in all wells at 1 μM
  - Tb^{3+}, constant in all wells at 0.1 μM
  - NADPH, constant in all wells at 2 μM
- The final concentrations of each component in control wells were as follows:
  - GFP-FKBP, constant in all wells at 10 nM
  - Rapamycin, constant in all wells at 30 nM
  - TMP-TTHA-cs124, constant in all wells at 1 μM
  - Tb^{3+}, constant in all wells at 0.1 μM
  - NADPH, constant in all wells at 2 μM

Analysis of GFP-FKBP/rapamycin/FRB-eDHFR affinity using proteins in cell lysate:

- Cell lysate (28 mg/ml (w/v)) was serially diluted into 24 wells containing 50 μL assay buffer.
- GFP-FKBP (6 μM) in cell lysate (28 mg/ml) was serially diluted into 24 wells containing 50 μL cell lysates, in reverse direction of cell lysate dilution.
- Five rapamycin solutions were prepared (14, 8, 6, 2, 0.8 μM in Assay Buffer), and 25 μL of a given solution was added into each sample well such that [rapamycin] > [GFP-FKBP].
- A solution containing FRB-eDHFR (40 nM), TMP-TTHA-cs124(Tb3+) (40 nM), and NADPH (4 μM) was prepared, and 25 μL of it was added into each sample well.
- The final concentrations of each component in sample wells were as follows:
  - GFP-FKBP, ranging from 3.0 μM to 0.4 pM
  - Rapamycin, ranging from 3.5 μM to 0.2 μM
  - FRB-eDHFR, constant in all wells at 10 nM
  - TMP-TTHA-cs124(Tb3+), constant in all wells at 10 nM
  - NADPH, constant in all wells at 1 μM
  - Cell lysate, constant in all wells at 14 mg/ml
- A solution containing TMP-TTHA-cs124(Tb3+) (20 nM), and NADPH (2 μM) was prepared for control wells, and 50 μL of it was added into each control well containing 50 μL GFP-FKBP dilutions in cell lysate.
- The final concentrations of each component in control wells were as follows:
  - GFP-FKBP, ranging from 3.0 μM to 0.4 pM
  - TMP-TTHA-cs124(Tb3+), constant in all wells at 10 nM
  - NADPH, constant in all wells at 1 μM
  - Cell lysate, constant in all wells at 14 mg/ml

Analysis of competitive inhibition of GFP-FKBP/rapamycin/FRB-eDHFR interaction using ascomycin.

- Ascomycin (0.4 μM in assay buffer) was serially diluted into 12 wells containing 50 μL assay buffer.
- A solution containing FRB-eDHFR (60 nM), GFP-FKBP (60 nM), TMP-TTHA-cs124(Tb3+) (60 nM), and NADPH (6 μM) was prepared, and 25 μL of it was added into each sample well and into each control well containing 75 μL assay buffer.
- 25 μL rapamycin solution (60 nM in assay buffer) was added into each sample well.
- The final concentrations of each component in sample wells were as follows:
  - Ascomycin, ranging from 200 nM to 98 pM
  - GFP-FKBP, constant in all wells at 15 nM
  - FRB-eDHFR, constant in all wells at 15 nM
  - Rapamycin, constant in all wells at 15 nM
  - TMP-TTHA-cs124(Tb3+), constant in all wells at 15 nM
  - NADPH, constant in all wells at 1.5 μM
- The final concentrations of each component in control wells were as follows:
  - GFP-FKBP, constant in all wells at 15 nM
  - FRB-eDHFR, constant in all wells at 15 nM
  - Rapamycin, constant in all wells at 15 nM
  - TMP-TTHA-cs124(Tb3+), constant in all wells at 15 nM
  - NADPH, constant in all wells at 1.5 μM

Supplementary Figures.

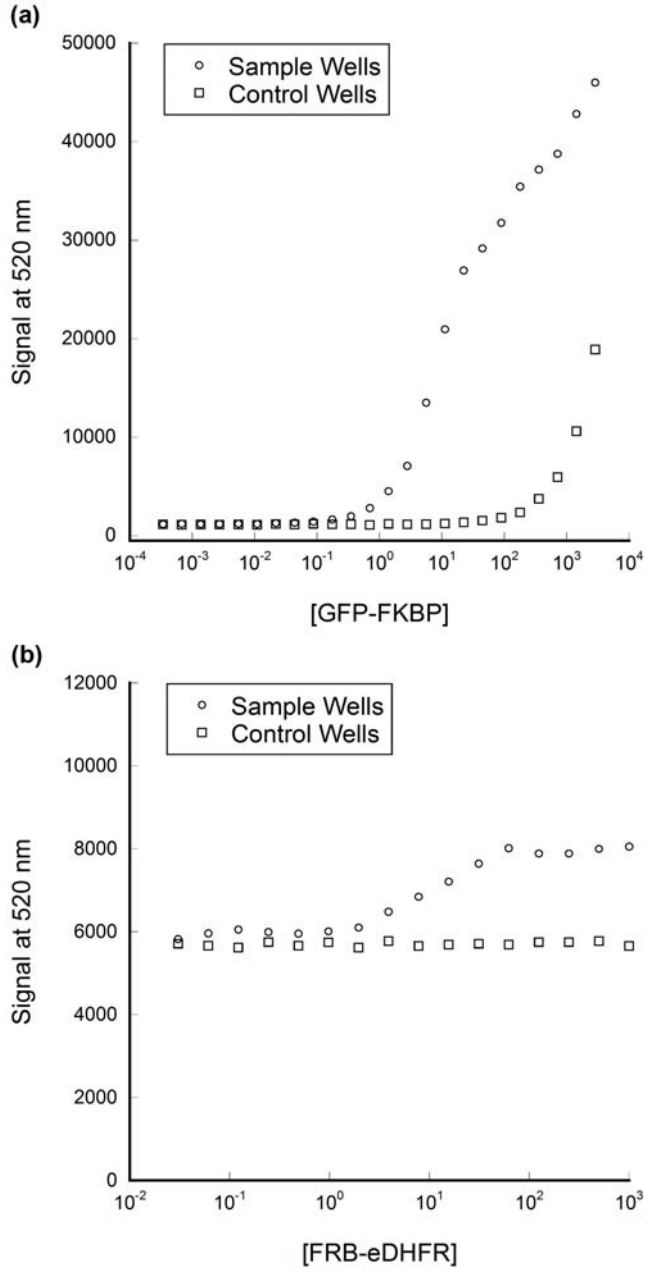
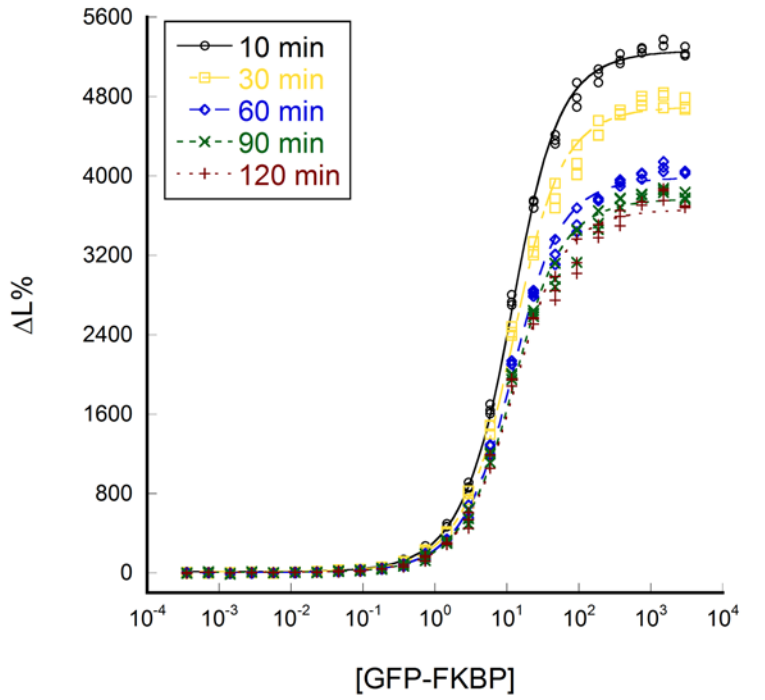


Figure S1. Raw LRET signal (λ_{em} = 520 nm) observed for representative binding affinity measurements. (a) Signal observed in sample and negative control wells when purified GFP-FKBP was titrated against constant levels of FRB-eDHFR (10 nM) and other assay components. The signal observed in negative controls increases with GFP-FKBP concentration because of non-specific, diffusion-mediated LRET between luminescent terbium complex and GFP. The increased background prevents saturation of the uncorrected LRET sample signal. (b) Signal observed in sample and negative control wells when purified FRB-eDHFR was titrated against constant levels of GFP-FKBP (10 nM) and other assay components. The signal observed in negative controls is persistently high (~5800 counts) across the titration range because there is a high level (100 nM) of luminescent terbium complex present in all wells.



y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	12.949	9.4125
m2	5261.3	15.159
m3	6.6236	0.16426
R^2	0.99933	NA

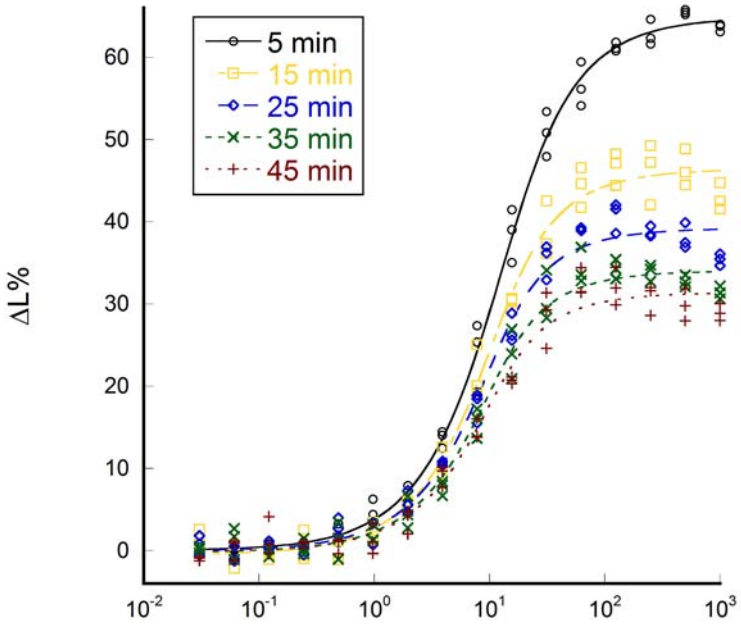
y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	13.605	13.443
m2	4696	21.846
m3	7.222	0.28106
R^2	0.99828	NA

y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	4.7676	12.066
m2	3984.3	19.494
m3	6.8322	0.28436
R^2	0.99809	NA

y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	6.6977	13.208
m2	3768	21.48
m3	7.2741	0.34568
R^2	0.99742	NA

y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	7.0238	15.774
m2	3661.6	25.736
m3	7.4957	0.43498
R^2	0.9961	NA

Figure S2. System reaches equilibrium after ~80 min. when purified GFP-FKBP is titrated against purified FRB-eDHFR to measure dissociation constant, as revealed by representative data plots and associated curve fitting results. The curve fit result were obtained by non-linearly fitting the data to Equation 2 (main text), with m1, m2 and m3 representing L_{min}, L_{max} and K_D, respectively.



y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	0.056611	0.38076
m2	64.865	0.52594
m3	6.831	0.43119
R^2	0.99631	NA

y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	-0.49062	0.48575
m2	46.396	0.63019
m3	4.2178	0.5267
R^2	0.98918	NA

y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	-0.015767	0.42812
m2	39.224	0.54253
m3	3.3667	0.47369
R^2	0.98823	NA

y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	-0.18774	0.41884
m2	34.045	0.52988
m3	3.3086	0.52497
R^2	0.98526	NA

y = m1-(m1-m2)*((m3+10+m0)-(...		
	Value	Error
m1	-0.15597	0.44914
m2	31.401	0.56883
m3	3.3462	0.61538
R^2	0.98014	NA

Figure S3. System reaches equilibrium after ~30 min. when purified FRB-eDHFR is titrated against purified GFP-FKBP to measure dissociation constant, as revealed by representative data plots and associated curve fitting results. The curve fit result were obtained by non-linearly fitting the data to Equation 2 (main text), with m1, m2 and m3 representing L_{min}, L_{max} and K_D, respectively.

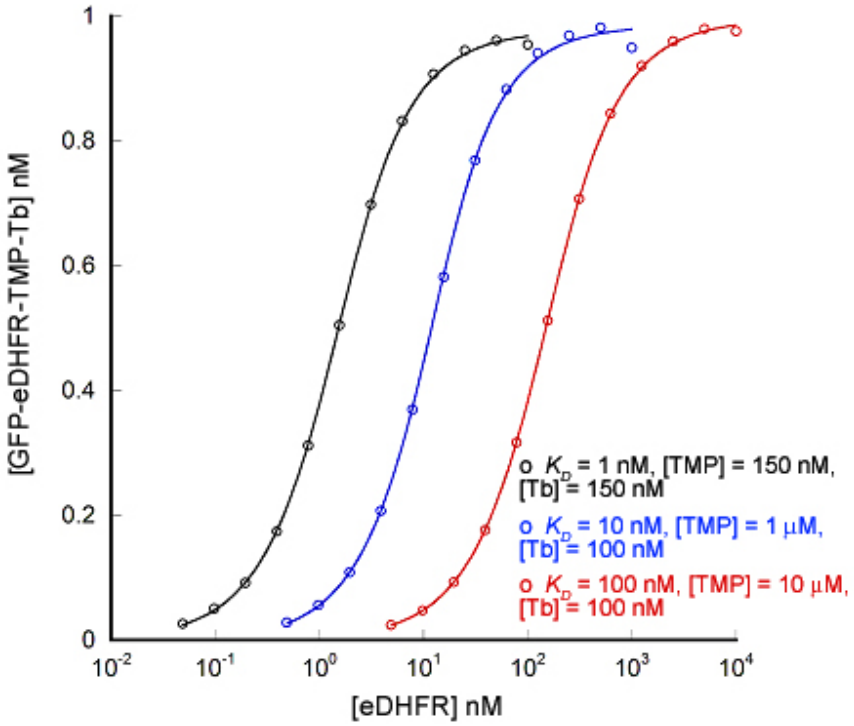


Figure S4. A broad range of affinities between eDHFR and GFP fusion proteins can be measured by varying the amount of TMP conjugate and/or the amount of terbium in the assay. The plot shows that the expected equilibrium concentration of a luminescent, GFP-eDHFR-TMP-Tb complex (y-axis) can be made to vary over a similar range under different titration conditions. Calculations were made such that for a given assumed K_D value, [GFP] = K_D , $0.01K_D$ < [eDHFR] < $100K_D$, and [TMP] and [Tb] were as shown in the plot legend.

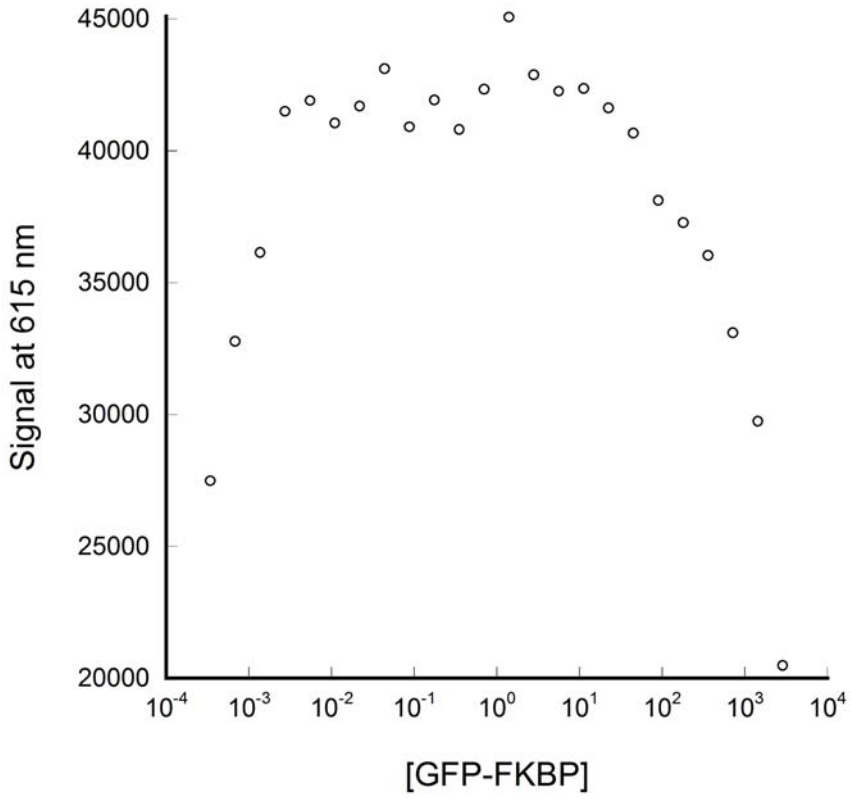


Figure S5. Raw Tb^{3+} signal (λ_{em} = 615 nm) observed for a representative data set obtained when bacterial lysates containing GFP-FKBP were titrated against a fixed concentration of FRB-eDHFR. The Tb^{3+} luminescence is substantially reduced at the extreme ends of the titration range because these sample wells contain less assay buffer than wells in the mid-range of the titration. The assay buffer is formulated to prevent non-specific binding of TMP-TTHA-cs124(Tb^{3+}) to sample wells and resultant sequestration out of the excitation light path. Four data point from either end were omitted for non-linear curve fit calculations (see main text for details).

References.

1. Rajapakse, H. E., Reddy, D. R., Mohandessi, S., Butlin, N. G., and Miller, L. W. (2009) Luminescent terbium protein labels for time-resolved microscopy and screening, *Angew Chem Int Ed Engl 48*, 4990-4992.
2. Reddy, D. R., Pedro Rosa, L. E., and Miller, L. W. (2011) Luminescent Trimethoprim-Polyaminocarboxylate Lanthanide Complex Conjugates for Selective Protein Labeling and Time-Resolved Bioassays, *Bioconjug Chem 22*, 1402-1409.
3. Lovrien, R., and Matulis, D. (2005) Assays for total protein, *Curr Protoc Microbiol Appendix 3*, Appendix 3A.