Supporting Information

Method for Accurate Determination of Dissociation Constants of Optical Ratiometric Systems: Chemical Probes, Genetically Encoded Sensors, and Interacting Molecules

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Materials. HEPES, TIS, Ac_2O, TPEN, CDTA, EDTA, HEDTA, DTT, TCEP (hydrochloride), dansyl chloride, PAR (4-(2-Pyridylazo)resorcinol), imidazole, Chelex 100, Tris base, SDS, Zincon were purchased from Sigma-Aldrich. Acetonitryle was obtained from Merck. NaCl, Et_2O, sodium glutamate, $CuSO_4 \cdot 5H_2O$ and analytical weights of $CaCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$ and were purchased from POCH (Gliwice, Poland). DMF, DCM, NMP, HBTU, TFA, DIEA, piperidine, TentaGel R Ram and Fmoc-proteced amino acids were obtained from Iris Biotech GmbH (Marktredwitz, Germany). The exact concentrations of metal salts was confirmed by representative series of ICP-MS measurements. All pH buffers used in this studies were treated with Chelex-100 resin to eliminate any metal ions contamination.

Peptide synthesis. Zinc ribbon peptide from TRAP protein (EVA peptide) was synthesized by solid phase synthesis using the Fmoc strategy on Tenta Gel R Ram (substitution 0.20 mmol/g) and Liberty 1 microwave-assisted synthesizer (CEM). The reagent excess, cleavage and purification were performed as previously published.[7] The purified peptide was identified by ESI mass spectrometry utilizing an API 2000 Applied Biosystems instrument. The mass values found and calculated were 3334.97 and 3334.95.

Equilibration of metal-dependent probes and sensor. Zincon in concentration of 50 µM was equilibrated in 1.0 mM chelator-Cu(II) metal buffers over 24 h. The set of CDTA, EDTA and HEDTA metal buffers was prepared in 50 mM HEPES buffer pH 7.4 with 100 mM NaCl and 0-1 mM $CuSO_4$ to obtain pCu ($-\log[Cu(II)_u]$) in the range 12.80-18.51. Samples were measured on a Jasco V-650 spectrophotometer. The EVA peptide (5 µM) was fractionally saturated in 1 mM EDTA, HEDTA and TPEN solutions in 50 mM HEPES pH 7.4 with various concentrations of $ZnSO_4$ over a period of 24 h. Transferred Zn(II) from metal buffer to peptide was considered during calculation of final pZn ($-\log[Zn(II)_u]$) values between 12.28 and 16.15. Samples were measured on a Hitachi F4500 fluorimeter. GEM-GECO1 sensor (0.5

μM) was equilibrated in conditions identical to those previously published: 10 mM EGTA in 30 mM MOPS pH 7.2 100 mM KCl with 0.1-10 mM Ca(II) in order to obtain pCa (-log[$Ca(II)_u$]) in the range 4.51-8.82. Samples were measured on a Hitachi F4500 fluorimeter. All metal buffers were prepared in chelexed buffers. Particular saturations of chelator solutions were obtained by mixing two solutions of chelator and fully saturated chelator (metal to ligand 1:1) to avoid pH shifting upon metal ions complexation. Accurate pCa, pCu and pZn values were obtained by correction of fractional transfer of metal ions from chelator complexed solutions to the sensor (Table S1-S3).

Characterization of FLIPE-1μ sensor with glutamate. Purified FLIPE-1μ sensor (0.25 μM) in phosphate buffer pH 7.0 was divided into aliquots and sodium glutamate was added to a final concentration ranging from 10 nM to 1 mM. The aliquots were incubated for 1 h at room temperature and measured on Hitachi F4500 fluorimeter according to the original article report by Frommer et al.[5] The concentration of unbound glutamate was calculated by subtracting the concentration of sensor-glutamate complex from total glutamate concentration (Table S4).

Detailed protocol for calculating the dissociation constant of GEM-GECO1.

1. Fluorescence intensity was measured at λ_1 = 464 nm (I_1) and λ_2 = 514 nm (I_2) for sensor in concentration of 0.5 μM in different Ca(II)-EGTA metal buffers (for details see Table S3). The corrected -log([$Ca(II)_u$]) values are taken from Table S3.

[$Ca(II)_u$] (M)	-log([$Ca(II)_u$])	I_1	I_2	$R_{1/2}$	$R_{2/1}$
1.52×10^{-9}	8.82	81.0	4000	0.02025	49.383
7.95×10^{-9}	8.10	81.9	3990	0.02032	49.205
1.68×10^{-8}	7.78	82.0	3990	0.02055	48.659
2.66×10^{-8}	7.57	83.16	3981	0.02089	47.872
3.77×10^{-8}	7.42	85.32	3972	0.02148	46.554
5.04×10^{-8}	7.30	89.85	3955	0.02272	44.018
6.47×10^{-8}	7.19	101.3	3920	0.02584	38.697
8.12×10^{-8}	7.09	127.4	3854	0.03306	30.251
1.01×10^{-7}	7.00	165.7	3738	0.04433	22.559
1.24×10^{-7}	6.91	228.1	3537	0.06449	15.506
1.51×10^{-7}	6.82	316.8	3259	0.09721	10.287
1.85×10^{-7}	6.73	407.2	2808	0.14501	6.896
2.26×10^{-7}	6.64	543.7	2458	0.22120	4.521
2.81×10^{-7}	6.55	717.0	1988	0.36066	2.773
3.52×10^{-7}	6.45	845.0	1643	0.51430	1.944
4.53×10^{-7}	6.34	922.6	1439	0.64114	1.560
6.01×10^{-7}	6.22	990.8	1294	0.76569	1.306
8.49×10^{-7}	6.07	1033	1213	0.85161	1.174
1.34×10^{-6}	5.87	1039	1125	0.92356	1.083
2.77×10^{-6}	5.56	1044	1110	0.94054	1.063
3.12×10^{-5}	4.51	1045	1100	0.95000	1.053

Measured borderline intensity values are:
I_{1u} = 81.0
I_{1b} = 1045
I_{2u} = 4000

I_{2b} = 1100

2. Convert the measured intensity data to ratio by dividing the intensity at λ_1 by intensity at λ_2 ($R_{1/2}$), or other way round ($R_{2/1}$) (see Table above).
3. Plot the ratio data versus concentration of unbound ligand. Alternatively, plot ratio data versus -log([L_u]).
4. Use the **Equation 8** (for $R_{1/2}$) or **Equation 9** (for $R_{2/1}$) to calculate the value of dissociation constant (K_d) and cooperativity factor (n). Use any software that allow to fit nonlinear curves. Set the end-point values (I_{1u}, I_{1b}, I_{2u}, I_{2b}) as fixed (constant).

In this case we used Origin 8.6 software. Below are listed ready to use formulas to copy-and-paste in Origin format (.fdf) for nonlinear curve fitting file.

Copy-and-paste formulas

1. Formulas for nonlogarithmic curves fitting.
   a) $R_{1/2}$ = y = *f* (x):

      y = ((I1b*x^n)+(I1u*K^n))/((I2b*x^n)+(I2u*K^n));

      Fitted or fixed parameters: I1u,I1b,I2u,I2b,K,n

   b) $R_{2/1}$ = y = *f* (x):

      y = ((I2b*x^n)+(I2u*K^n))/((I1b*x^n)+(I1u*K^n));

      Fitted or fixed parameters: I1u,I1b,I2u,I2b,K,n
2. Formulas for logarithmic curves fitting.
   a) $R_{1/2}$ = y = *f* (-log(x)):

      K = 10^-(pK);

      Z = 10^(-x);

      y = ((I1b*Z^n)+(I1u*K^n))/((I2b*Z^n)+(I2u*K^n));

      Fitted or fixed parameters: I1u,I1b,I2u,I2b,pK,n

b) $R_{2/1}$ = y = *f* (-log(x)):

K = 10^(-pK);

Z = 10^(-x);

y = ((I2b*Z^n)+(I2u*K^n))/((I1b*Z^n)+(I1u*K^n));

Fitted or fixed parameters: I1u,I1b,I2u,I2b,pK,n

Dissociation constant and cooperativity factor values of Ca(II)-GEM-GECO1 complex calculated based on above formulas in Origin 8.6 software are:

a) For $R_{1/2}$ data processing: K_d = 221 ± 4 nM, n = 2.74 ± 0.06

b) For $R_{2/1}$ data processing: K_d = 215 ± 2 nM, n = 3.09 ± 0.05.

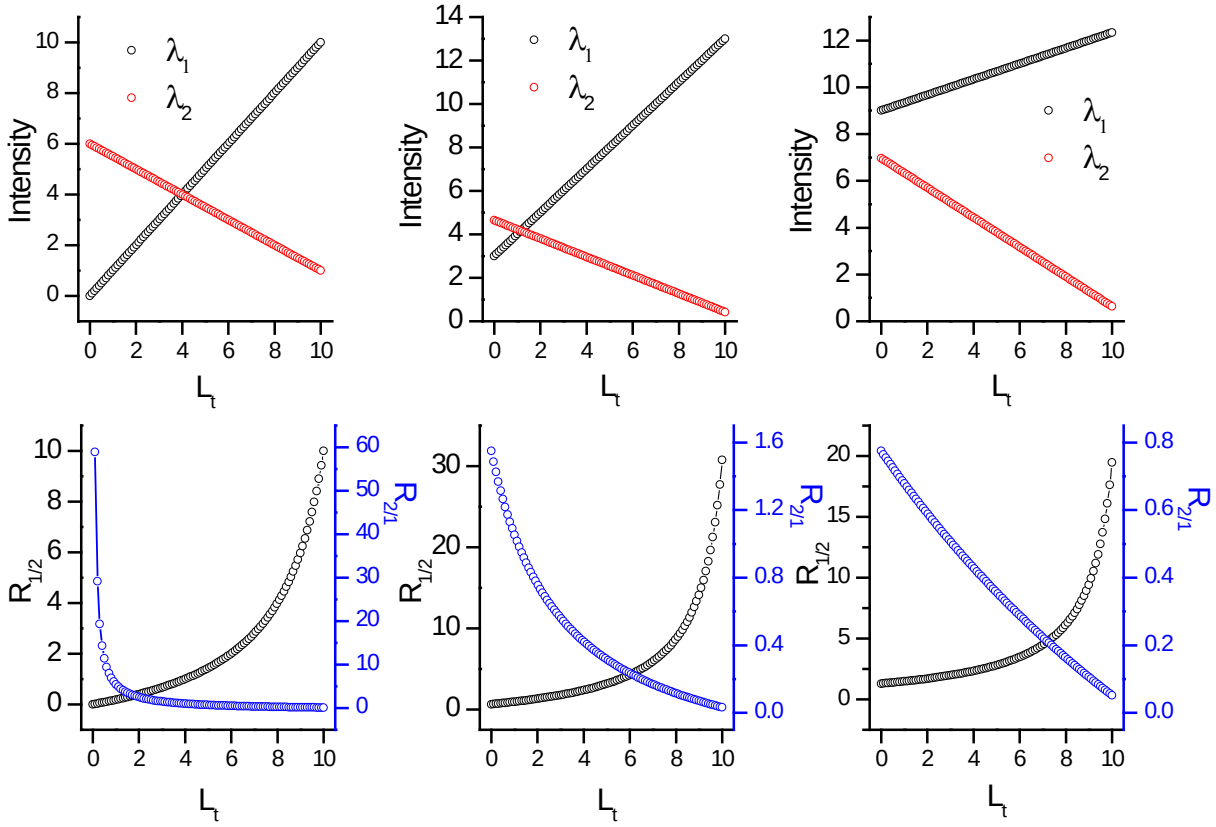


Figure S1. Nonlinearity of ratio intensities versus total concentration of ligand in the case of ratiometric probes with high affinity up to the stoichiometric point. Top row demonstrates an examples of linear response between both wavelengths and their intensities. Bottom row presents ratios of intensities, $R_{1/2}$ and $R_{2/1}$. Simulation was generated in Origin 8.6 software. L_t refers to ligand total concentration.

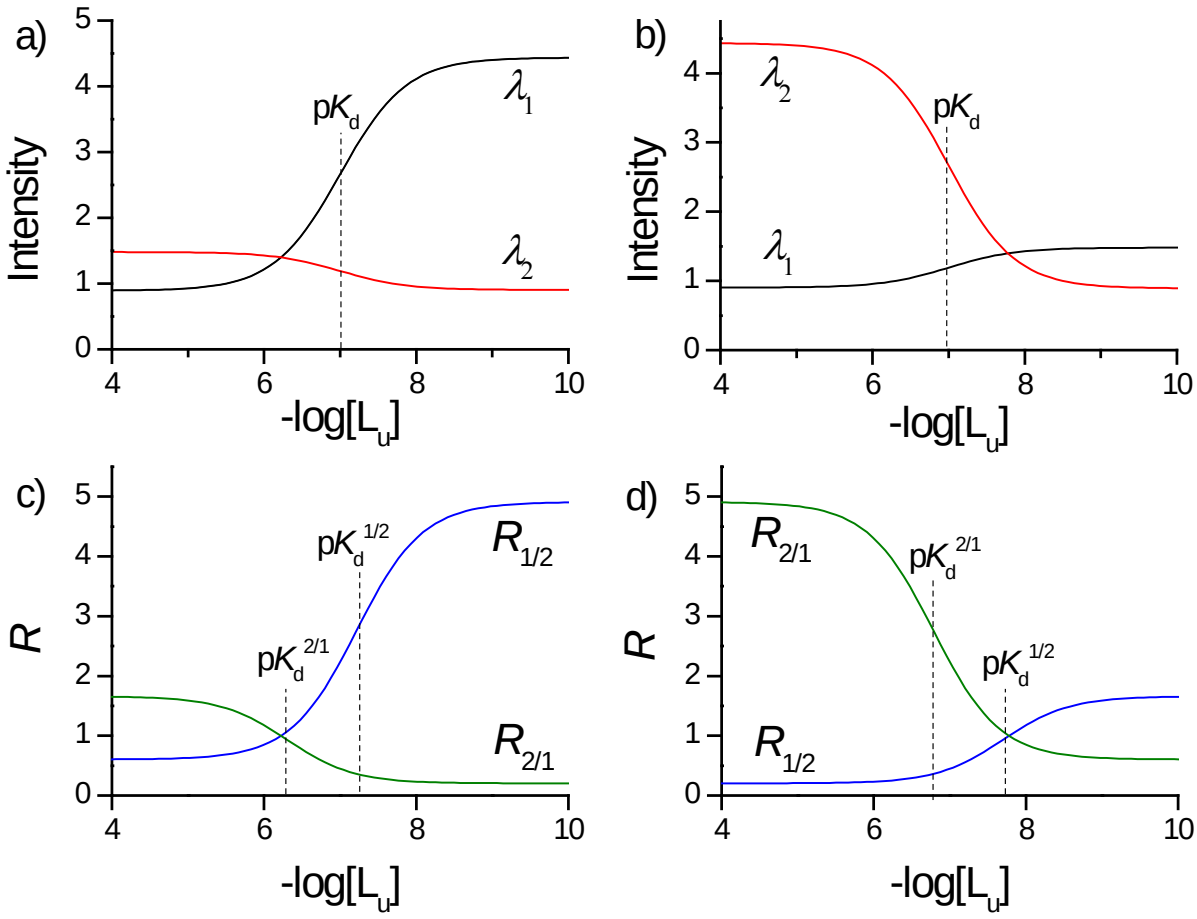


Figure S2. a) - d) Logarithmic plots of intensities of λ_1 and λ_2 and their ratios for simulated examples presented in the Figure 1 (a and b), showing the difference of between single wave and ratio-based calculated K_d. [L_u] denotes unbound (free) ligand. $pK_d^{1/2}$ and $pK_d^{2/1}$ refer to -logK_d values determined directly from $R_{1/2}$ and $R_{2/1}$ ratios, respectively.

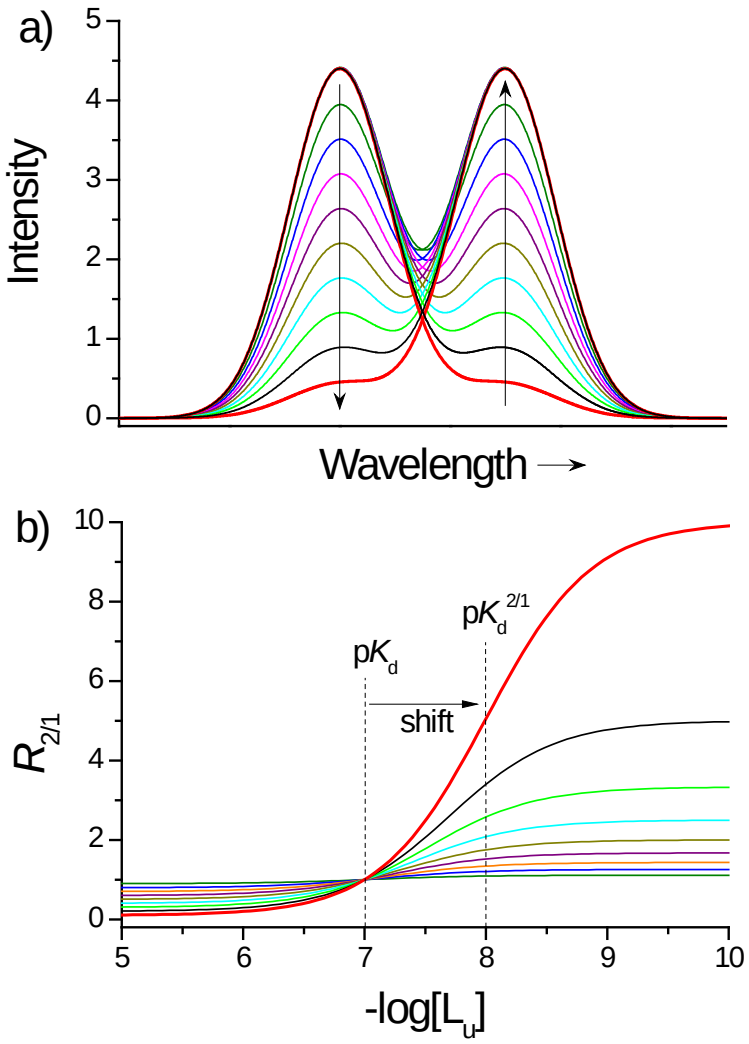
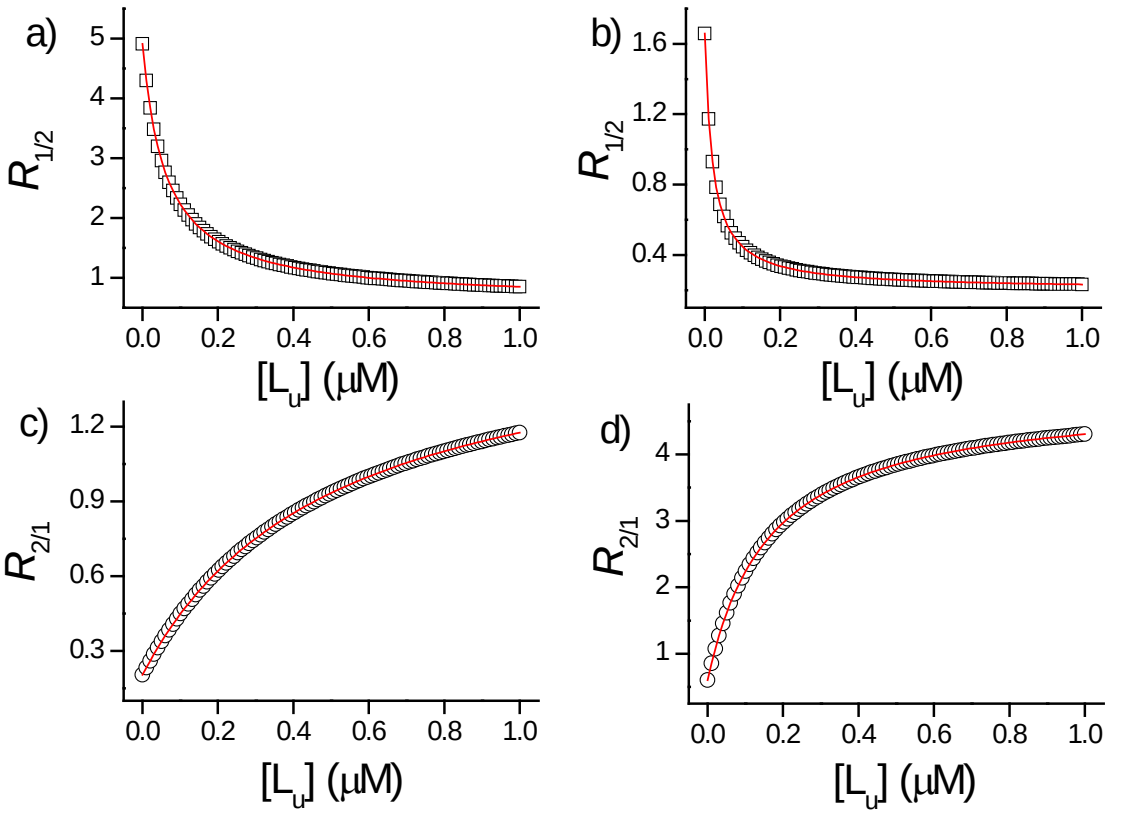


Figure S3. Simulation of theoretical sensor responses with pK_d = 7 and Hill's coefficient n = 1; a) Colors of spectra demonstrate several examples of probes with various intensities of bound and unbound states. Arrow indicates direction of intensity changes; b) Intensities ratio of particular probe example in the function of unbound ligand concentration [L_u].



a)	Value	SE
I_{1b}	0.9037	Fixed
I_{1u}	1.4801	Fixed
I_{2b}	4.4361	Fixed
I_{2u}	0.8922	Fixed
K_d	1E-7	3.25607E-24
n	1	2.79907E-17
Adj. R-Square = 1		

b)	Value	SE
I_{1b}	0.9037	Fixed
I_{1u}	1.4801	Fixed
I_{2b}	4.4361	Fixed
I_{2u}	0.8922	Fixed
K_d	1E-7	3.25607E-24
n	1	2.79907E-17
Adj. R-Square = 1		

c)	Value	SE
I_{1b}	0.8922	Fixed
I_{1u}	4.4361	Fixed
I_{2b}	1.4801	Fixed
I_{2u}	0.9037	Fixed
K_d	1E-7	8.33864E-24
n	1	4.90969E-17
Adj. R-Square = 1		

d)	Value	SE
I_{1b}	0.9037	Fixed
I_{1u}	1.4801	Fixed
I_{2b}	4.4361	Fixed
I_{2u}	0.8922	Fixed
K_d	1E-7	7.89529E-24
n	1	5.69835E-17
Adj. R-Square = 1		

Figure S4. Precise calculation of dissociation constant from the model spectra presented in Figure 2 using our method of data processing. K_d is the same as one set to create model data (100 nM) in case of both ratios. [L_u] is unbound ligand concentration.

Table S1. The chemical components of Cu(II) buffers used in this study and related unbound Cu(II) concentration values. Metal buffers were prepared in 50 mM HEPES buffer pH 7.4 with 100 mM NaCl. Measurements were done at 25°C.

Competitor 1 mM	total Cu(II) (mM)	$[Cu(II)_u]$ (M)	$-\log([Cu(II)_u])$	$[Cu(II)_u]$ corrected (M)[d]	$-\log([Cu(II)_u])$ corrected[d]
CDTA[a]	0.03	3.11×10^{-19}	18.51	3.11×10^{-19}	18.51
	0.05	5.29×10^{-19}	18.28	5.29×10^{-19}	18.28
	0.1	1.12×10^{-18}	17.95	1.12×10^{-18}	17.95
	0.125	1.44×10^{-18}	17.84	1.44×10^{-18}	17.84
	0.175	2.13×10^{-18}	17.67	2.13×10^{-18}	17.67
	0.25	3.35×10^{-18}	17.47	3.31×10^{-18}	17.48
EDTA[b]	0.05	6.52×10^{-18}	17.19	6.17×10^{-18}	17.21
	0.1	1.37×10^{-17}	16.86	1.23×10^{-17}	16.91
	0.2	3.09×10^{-17}	16.51	2.75×10^{-17}	16.56
	0.3	5.31×10^{-17}	16.28	4.68×10^{-17}	16.33
	0.4	8.25×10^{-17}	16.08	7.24×10^{-17}	16.14
	0.5	1.24×10^{-16}	15.91	1.07×10^{-16}	15.97
	0.6	1.86×10^{-16}	15.73	1.58×10^{-16}	15.8
	0.7	2.90×10^{-16}	15.54	2.40×10^{-16}	15.62
	0.8	4.97×10^{-16}	15.30	3.80×10^{-16}	15.42
	0.9	1.13×10^{-15}	14.95	7.24×10^{-16}	15.14
	0.95	2.41×10^{-15}	14.62	1.17×10^{-15}	14.93
HEDTA[c]	1.0	3.31×10^{-15}	14.48	2.95×10^{-15}	14.53
	1.0	1.12×10^{-12}	11.95	1.58×10^{-13}	12.80

[a]Protonation and stability constants of CDTA: $\beta_{HL} = 12.30$, $\beta_{H_2L} = 18.42$, $\beta_{CuL} = 21.92$.[1]

[b]Protonation and stability constants of EDTA: $\beta_{HL} = 10.17$, $\beta_{H_2L} = 16.28$, $\beta_{H_3L} = 18.96$, $\beta_{H_4L} = 20.96$, $\beta_{CuHL} = 21.70$, $\beta_{CuL} = 18.70$.[1]

[c]Protonation and stability constants of HEDTA: $\beta_{HL} = 9.81$, $\beta_{H_2L} = 15.18$, $\beta_{H_3L} = 17.78$, $\beta_{CuHL} = 19.92$, $\beta_{CuL} = 17.50$.[1]

[d]Correction was made to account transfer of metal from metal buffer component to Zincon.

Table S2. The chemical components of Zn(II) buffers used in this study and related unbound Zn(II) concentration values. Metal buffers were prepared in 50 mM HEPES buffer pH 7.4. Measurements were done at 25°C.

Competitor 1 mM	total Zn(II) (mM)	$[Zn(II)_u]$ (M)	$-\log([Zn(II)_u])$	$[Zn(II)_u]$ corrected (M)[d]	$-\log([Zn(II)_u])$ corrected[d]
TPEN[a]	0.1	7.16×10^{-17}	16.15	7.16×10^{-17}	16.15
	0.2	2.76×10^{-16}	15.56	2.74×10^{-16}	15.56
EDTA[b]	0.05	1.30×10^{-15}	14.89	1.25×10^{-15}	14.89
	0.1	2.74×10^{-15}	14.56	2.68×10^{-15}	14.57
	0.15	4.36×10^{-15}	14.36	4.27×10^{-15}	14.37
	0.2	6.18×10^{-15}	14.21	6.04×10^{-15}	14.22
	0.3	1.06×10^{-14}	13.98	1.04×10^{-14}	13.98
	0.4	1.65×10^{-14}	13.78	1.61×10^{-14}	13.79
	0.5	2.47×10^{-14}	13.61	2.41×10^{-14}	13.62
	0.6	3.71×10^{-14}	13.43	3.59×10^{-14}	13.44
	0.7	5.78×10^{-14}	13.24	5.55×10^{-14}	13.26
HEDTA[c]	0.1	9.15×10^{-14}	13.04	8.25×10^{-14}	13.08
	0.15	1.45×10^{-13}	12.84	1.35×10^{-13}	12.87
	0.2	2.06×10^{-13}	12.69	1.94×10^{-13}	12.71
	0.3	3.53×10^{-13}	12.45	3.37×10^{-13}	12.47
	0.4	5.49×10^{-13}	12.26	5.27×10^{-13}	12.28

[a]Protonation and stability constants of TPEN: β_{HL} = 7.19, β_{H_2L} = 12.04, β_{H_3L} = 15.36, β_{H_4L} = 18.31, β_{ZnL} = 15.40.[1]

[b]Protonation and stability constants of EDTA: β_{HL} = 10.17, β_{H_2L} = 16.28, β_{H_3L} = 18.96, β_{H_4L} = 20.96, β_{ZnHL} = 19.44, β_{ZnL} = 16.40.[1]

[c]Protonation and stability constants of HEDTA: β_{HL} = 9.81, β_{H_2L} = 15.18, β_{H_3L} = 17.78, β_{ZnL} = 14.60.[1]

[d]Correction was made to account transfer of metal from metal buffer component to EVA peptide.

Table S3. The chemical components of Ca(II) buffers used in this study and related unbound Ca(II) concentration values. Metal buffers were prepared in 30 mM MOPS pH 7.2 100 mM KCl. Measurements were done at 25°C.

Competitor 10 mM	total Ca(II) (mM)	$Ca(II)_u$ (M)	$-\log([Ca(II)_u])$	$Ca(II)_u$ corrected (M)[b]	$-\log([Ca(II)_u])$ corrected[b]
EGTA[a]	0.1	1.52×10^{-9}	8.82	1.52×10^{-9}	8.82
	0.5	7.95×10^{-9}	8.10	7.95×10^{-9}	8.10
	1.0	1.68×10^{-8}	7.78	1.68×10^{-8}	7.78
	1.5	2.66×10^{-8}	7.57	2.66×10^{-8}	7.57
	2.0	3.77×10^{-8}	7.42	3.77×10^{-8}	7.42
	2.5	5.04×10^{-8}	7.30	5.04×10^{-8}	7.30
	3.0	6.47×10^{-8}	7.19	6.47×10^{-8}	7.19
	3.5	8.12×10^{-8}	7.09	8.12×10^{-8}	7.09
	4.0	1.01×10^{-7}	7.00	1.01×10^{-7}	7.00
	4.5	1.24×10^{-7}	6.91	1.24×10^{-7}	6.91
	5.0	1.51×10^{-7}	6.82	1.51×10^{-7}	6.82
	5.5	1.85×10^{-7}	6.73	1.85×10^{-7}	6.73
	6.0	2.26×10^{-7}	6.64	2.26×10^{-7}	6.64
	6.5	2.81×10^{-7}	6.55	2.81×10^{-7}	6.55
	7.0	3.52×10^{-7}	6.45	3.52×10^{-7}	6.45
	7.5	4.53×10^{-7}	6.34	4.53×10^{-7}	6.34
	8.0	6.02×10^{-7}	6.22	6.01×10^{-7}	6.22
	8.5	8.51×10^{-7}	6.07	8.49×10^{-7}	6.07
	9.0	1.34×10^{-6}	5.87	1.34×10^{-6}	5.87
	9.5	2.79×10^{-6}	5.56	2.77×10^{-6}	5.56
	10.0	3.18×10^{-5}	4.50	3.12×10^{-5}	4.51

[a]Unbound Ca(II) was calculated based on data from Grynkiewicz, Poenie and Tsien[2], which were used to calculate the Ca(II)dissociation constant of GEM-GECO1[3].

[b]Correction was made to account transfer of metal from metal buffer component to GEM-GECO1 protein.

Table S4. Total and unbound concentrations of glutamate (Glu) used for calibration of FLIPE-1μ sensor. Measurements were performed in 20 mM phosphate buffer pH 7.0 at 25°C.

Total Glu (M)	-log(Glu_{total})	[Glu_u][a]	-log[Glu_u][a]
1×10^{-8}	8.00	8.40×10^{-9}	8.08
1×10^{-7}	7.00	9.23×10^{-8}	7.03
2×10^{-7}	6.70	1.85×10^{-7}	6.73
3×10^{-7}	6.52	2.78×10^{-7}	6.56
4×10^{-7}	6.40	3.70×10^{-7}	6.43
5×10^{-7}	6.30	4.63×10^{-7}	6.33
6×10^{-7}	6.22	5.57×10^{-7}	6.25
7×10^{-7}	6.15	6.50×10^{-7}	6.19
8×10^{-7}	6.10	7.44×10^{-7}	6.13
9×10^{-7}	6.05	8.38×10^{-7}	6.08
1×10^{-6}	6.00	9.32×10^{-7}	6.03
1.5×10^{-6}	5.82	1.41×10^{-6}	5.85
2×10^{-6}	5.70	1.89×10^{-6}	5.72
2.5×10^{-6}	5.60	2.37×10^{-6}	5.63
3×10^{-6}	5.52	2.86×10^{-6}	5.54
3.5×10^{-6}	5.46	3.35×10^{-6}	5.47
4×10^{-6}	5.40	3.85×10^{-6}	5.41
5×10^{-6}	5.30	4.84×10^{-6}	5.32
6×10^{-6}	5.22	5.83×10^{-6}	5.23
7×10^{-6}	5.15	6.83×10^{-6}	5.17
8×10^{-6}	5.10	7.83×10^{-6}	5.11
9×10^{-6}	5.05	8.83×10^{-6}	5.05
1×10^{-5}	5.00	9.82×10^{-6}	5.01
1.2×10^{-5}	4.92	1.18×10^{-5}	4.93
1.4×10^{-5}	4.85	1.38×10^{-5}	4.86
1.6×10^{-5}	4.79	1.58×10^{-5}	4.80
1.8×10^{-5}	4.74	1.78×10^{-5}	4.75
2×10^{-5}	4.70	1.98×10^{-5}	4.70
4×10^{-5}	4.40	3.98×10^{-5}	4.40
6×10^{-5}	4.22	5.98×10^{-5}	4.22
8×10^{-5}	4.10	7.98×10^{-5}	4.10
1×10^{-4}	4.00	9.98×10^{-5}	4.00
2.5×10^{-4}	3.60	2.50×10^{-4}	3.60
5×10^{-4}	3.30	5.00×10^{-4}	3.30
1×10^{-3}	3.00	1.00×10^{-3}	3.00

[a]Concentrations of unbound Glu were achieved by subtraction of FLIPE-1μ-Glu complex concentration from total pool of Glu.

Table S5. Comparison of the results obtained using different fitting methods for Zincon-Cu(II) complex. Hill's equation was used for both single wavelength and ration calculations and one binding site equation was used to process the ratio data. There is a significant difference in the ratio based results, especially between $R_{1/2}$ and $R_{2/1}$. However the results obtained by processing the single wavelength in good agreement with the ones obtained by our calculation method.

Fitting method	pK_d	n	I_{1b}	I_{1u}	I_{2b}	I_{2u}
λ_1 – Hill's equation	16.18(1)	1.12(2)	0.123(5)	1.158(3)	-	-
λ_2 – Hill's equation	16.07(1)	1.02(2)	-	-	0.887(5)	0.044(3)
$R_{1/2}$ – one binding site	17.35(2)	-	-	-	-	-
$R_{1/2}$ – Hill's equation	17.33(2)	1.12(5)	-	-	-	-
$R_{2/1}$ – one binding site	15.28(3)	-	-	-	-	-
$R_{2/1}$ – Hill's equation	15.32(1)	1.23(2)	-	-	-	-
$R_{1/2}$ – Our method	16.15(4)	1.10(3)	0.123	1.158	0.887	0.044
$R_{2/1}$ – Our method	16.13(2)	1.15(3)	0.123	1.158	0.887	0.044

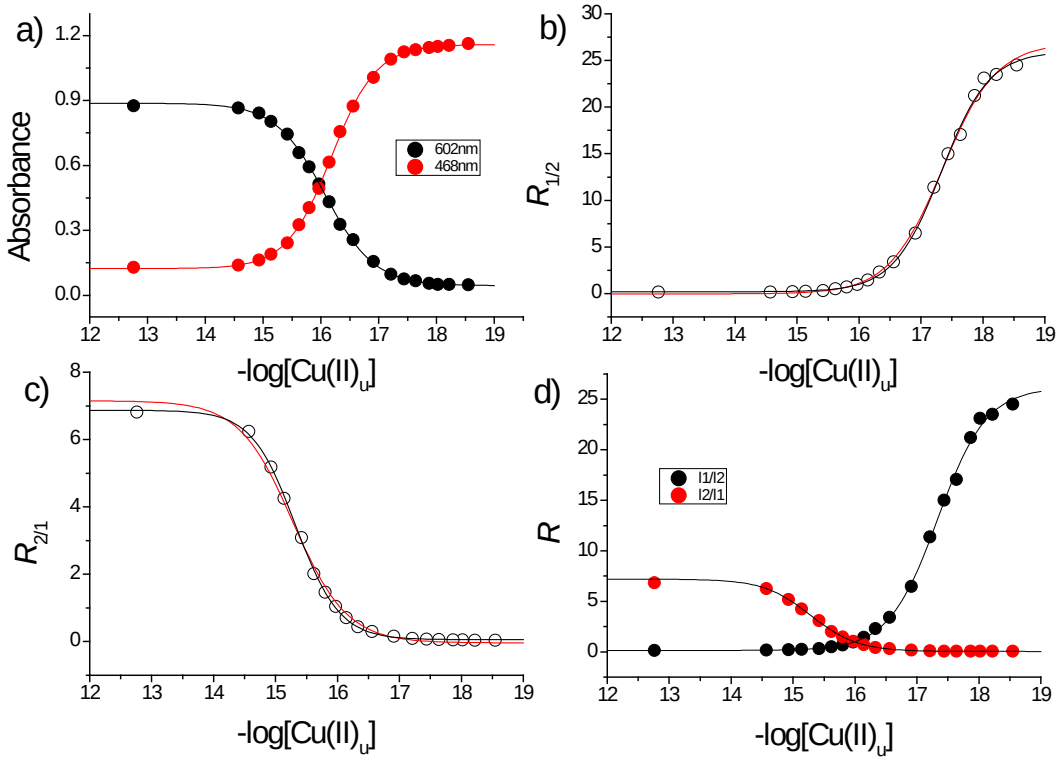


Figure S5. Characterization of Zincon-Cu(II) complex. (a) Spectral response to changes it the concentration of unbound Cu(II). The plot illustrates the absorbance changes at 468 and 602 nm, which are the maxima of Cu(II) unbound and bound Zincon, respectively. (b) changes in the ratio, calculated based on $I_{1/2}$ or $I_{2/1}$ as response to different concentration of unbound Cu(II). (c) and (d) Plots from (b) were separated and used for calculation of the dissociation constant with Hill's equation (black line) and one site binding equation, where Hill's factor is essentially fixed at 1, which shows the importance of presence of this factor in the equation.

Table S6. Comparison of the results obtained using different fitting methods for EVA-Zn(II) complex. Hill's equation was used for both single wavelength and ration calculations and one binding site equation was used to process the ratio data. There is a significant difference in the ratio based results, especially between $R_{1/2}$ and $R_{2/1}$. However the results obtained by processing the single wavelength in good agreement with the ones obtained by our calculation method.

Fitting method	pK_d	n	I_{1b}	I_{1u}	I_{2b}	I_{2u}
λ_1 (donor) – Hill's equation	13.87(3)	1.07(6)	1124(6)	1490(6)	-	-
λ_2 (acceptor) – Hill's equation	13.90(2)	1.02(5)	-	-	307(2)	146(2)
$R_{1/2}$ – one binding site	14.22(2)	-	-	-	-	-
$R_{1/2}$ – Hill's equation	14.22(2)	0.98(5)	-	-	-	-
$R_{2/1}$ – one binding site	13.77(2)	-	-	-	-	-
$R_{2/1}$ – Hill's equation	13.78(2)	1.05(4)	-	-	-	-
$R_{1/2}$ – Our method	13.89(1)	1.01(3)	1124	1490	307	146
$R_{2/1}$ – Our method	13.89	1.04(2)	1124	1490	307	146

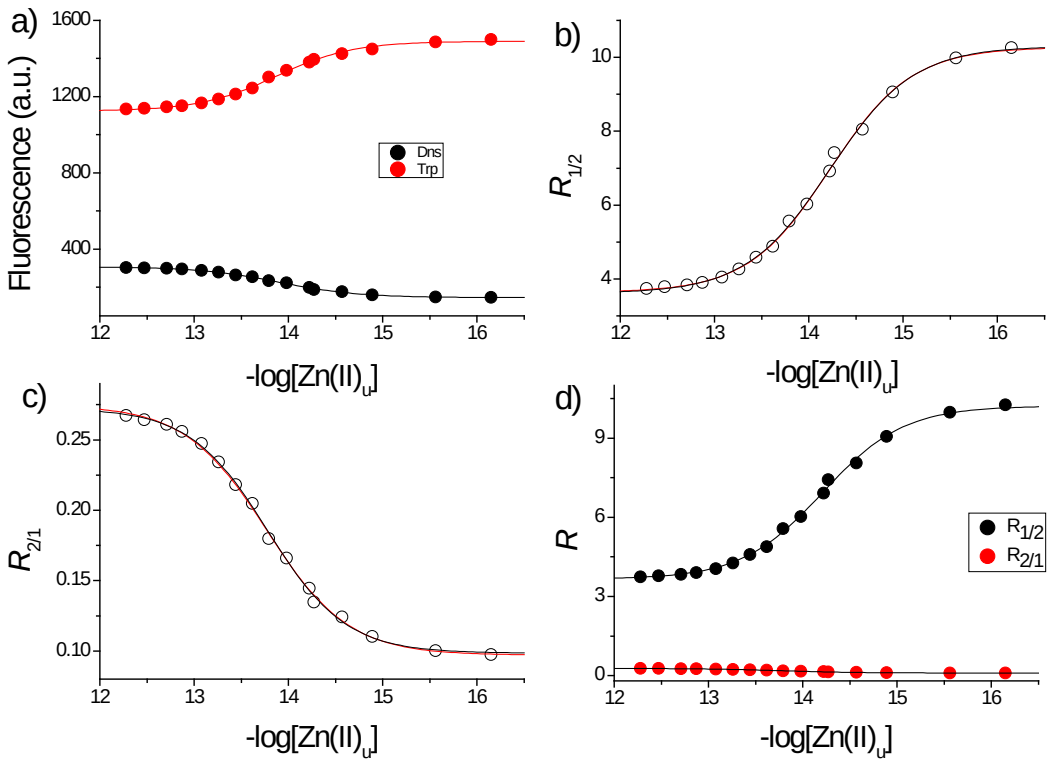


Figure S6. Characterization of EVA-Zn(II) complex. (a) Spectral response to changes in the concentration of unbound Zn(II). The plot illustrates the fluorescence changes at 355 and 545 nm, which are the maxima of Zn(II) unbound (low FRET) and bound (high FRET) EVA, respectively. (b and c) changes in the ratio, calculated based on $I_{1/2}$ or $I_{2/1}$ as response to different concentration of unbound Zn(II). (d) plots b and c were put together and used for calculation of the dissociation constant with Hill's equation (black line) and one site binding equation, where Hill's factor is essentially fixed at 1.

Table S7. Comparison of the results obtained using different fitting methods for FLIPE-1μ-glutamate complex. Hill's equation was used for both single wavelength and ration calculations and one binding site equation was used to process the ratio data. There is a significant difference in the ratio based results, especially between $R_{1/2}$ and $R_{2/1}$. However the results obtained by processing the single wavelength in good agreement with the ones obtained by our calculation method.

Fitting method	pK_d	n	I_{1b}	I_{1u}	I_{2b}	I_{2u}
λ_1 – Hill's equation	5.86(1)	1.03(1)	440(1)	257(1)	-	-
λ_2 – Hill's equation	5.85(1)	1.03(1)	-	-	457(2)	886(1)
$R_{1/2}$ – one binding site	5.58(1)	-	-	-	-	-
$R_{1/2}$ – Hill's equation	5.57(1)	1.02(1)	-	-	-	-
$R_{2/1}$ – one binding site	6.09(1)	-	-	-	-	-
$R_{2/1}$ – Hill's equation	6.08(1)	1.03(1)	-	-	-	-
$R_{1/2}$ – Our method	5.86(1)	1.02(1)	440	257	457	886
$R_{2/1}$ – Our method	5.86(1)	1.03(1)	440	257	457	886

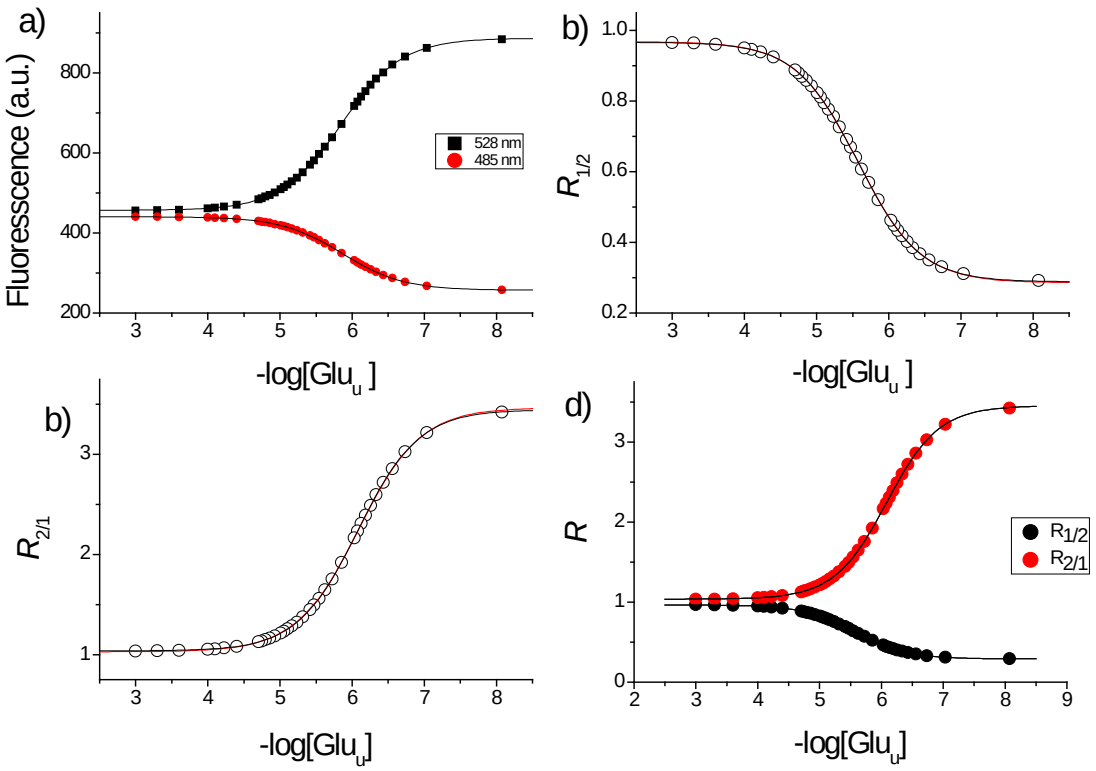


Figure S7. Characterization of FLIPE-1μ-glutamate complex. (a) Spectral response to changes it the concentration of unbound glutamate (see table footnote). The plot illustrates the fluorescence changes at 485 and 528 nm, which are the maxima of glutamate unbound (low FRET) and bound (high FRET) FLIPE, respectively. (b) changes in the ratio, calculated based on $I_{1/2}$ or $I_{2/1}$ as response to different concentration of unbound Glutamate. (c) and (d) Plots from (b) were separated and used for calculation of the dissociation constant with Hill's equation (black line) and one site binding equation, where Hill's factor is essentially fixed at 1.

Table S8. Comparison of the results obtained using different fitting methods for Ca(II)-GEM-GECO1 complex. Hill's equation was used for both single wavelength and ration calculations and one binding site equation was used to process the ratio data. There is a significant difference in the ratio based results, especially between $R_{1/2}$ and $R_{2/1}$. However the results obtained by processing the single wavelength in good agreement with the ones obtained by our calculation method.

Fitting method	pK_d	n	I_{1b}	I_{1u}	I_{2b}	I_{2u}
λ_1 – Hill's equation	6.64(1)	2.89(7)	1048(5)	81(3)	-	-
λ_2 – Hill's equation	6.67(1)	2.97(6)	-	-	1140(7)	3996(10)
$R_{1/2}$ – one binding site	6.23(11)	-	-	-	-	-
$R_{1/2}$ – Hill's equation	6.46(5)	2.81(8)	-	-	-	-
$R_{2/1}$ – one binding site	7.03(12)	-	-	-	-	-
$R_{2/1}$ – Hill's equation	7.03(3)	3.15(6)	-	-	-	-
$R_{1/2}$ – Our method	6.66(1)	2.74(6)	1045	81	1100	4000
$R_{2/1}$ – Our method	6.67(1)	3.09(5)	1045	81	1100	4000

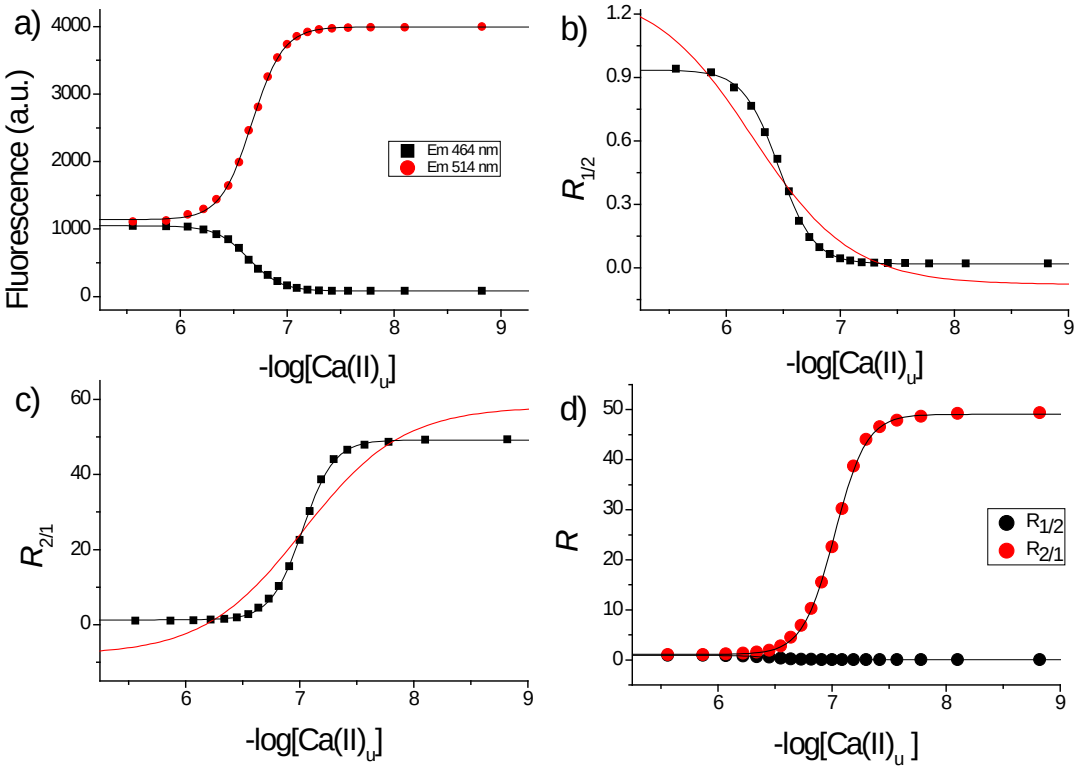


Figure S8. Characterization of Ca(II)-GEM-GECO1 complex. (a) Spectral response to changes it the concentration of unbound Ca(II). The plot illustrates the fluorescence changes at 464 and 514 nm, which are the maxima of Ca(II) bound and unbound GEM-GECO1, respectively. (b) changes in the ratio, calculated based on $I_{1/2}$ or $I_{2/1}$ as response to different concentration of unbound Ca(II). (c) and (d) Plots from (b) were separated and used for calculation of the dissociation constant with Hill's equation (black line) and one site binding equation, where Hill's factor is essentially fixed at 1. There is a striking difference, but this is not surprising as GEM-GECO1 binds four Ca(II) ions.

Table S9. Comparison of the results obtained using different fitting methods for $(hook)_2$-Zn(II) complex. Hill's equation was used for both single wavelength and ration calculations and one binding site equation was used to process the ratio data. There is a significant difference in the ratio based results, especially between $R_{1/2}$ and $R_{2/1}$. However the results obtained by processing the single wavelength in good agreement with the ones obtained by our calculation method. Note that two hook peptides are involved in binding one Zn(II).[4]

Fitting method	pK_d	n	I_{1b}	I_{1u}	I_{2b}	I_{2u}
λ_1 (donor) – Hill's equation	13.85(3)	0.79(3)	135(3)	457(6)	-	-
λ_2 (acceptor) – Hill's equation	13.82(1)	0.77(2)	-	-	143.9(2)	87.9(5)
$R_{1/2}$ – one binding site	14.06(3)	-	-	-	-	-
$R_{1/2}$ – Hill's equation	14.10(2)	0.83(3)	-	-	-	-
$R_{2/1}$ – one binding site	13.15(4)	-	-	-	-	-
$R_{2/1}$ – Hill's equation	13.13(2)	0.72(2)	-	-	-	-
$R_{1/2}$ – Our method	13.84(2)	0.77(2)	135	457	143.9	87.9
$R_{2/1}$ – Our method	13.84(1)	0.80(2)	135	457	143.9	87.9

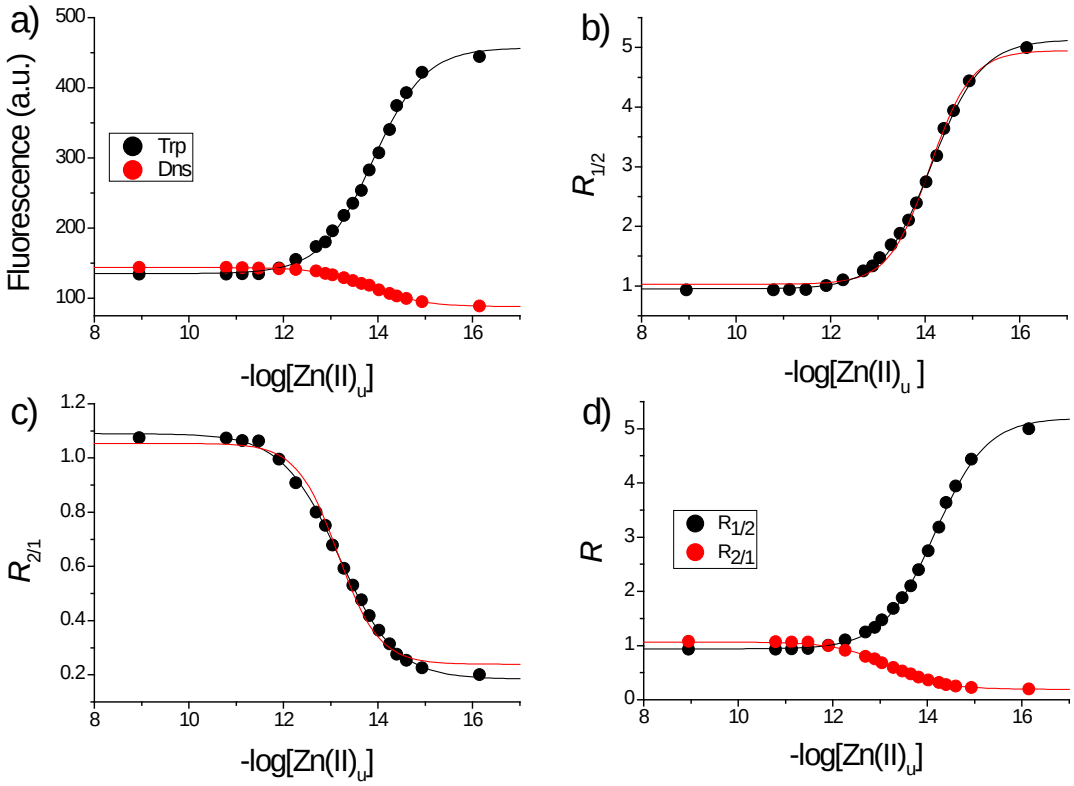


Figure S9. Characterization $(hook)_2$-Zn(II) complex. (a) Spectral response to changes it the concentration of unbound Zn(II). The plot illustrates the fluorescence changes at 355 and 545 nm, which are the maxima of Zn(II) unbound and bound hook, respectively. (b) changes in the ratio, calculated based on $I_{1/2}$ or $I_{2/1}$ as response to different concentration of unbound Zn(II). (c) and (d) Plots from (B) were separated and used for calculation of the dissociation constant with Hill's equation (black line) and one site binding equation, where Hill's factor is essentially fixed at 1.

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