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Supplemental Information

LRET Determination of Molecular Distances during pH Gating of the Mammalian Inward Rectifier Kir1.1b

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Supplementary Materials

Förster energy transfer data analysis

Luminescence decay was recorded as a function of time and bi-exponential lifetime decay functions were fit to the data according to:

$$y = A_f \exp(t/\tau_f) + A_s \exp(t/\tau_s) + baseline$$

where A are the amplitude factors and tau (τ) are the decay time constants. Since excitation of the Tb maleimide chelate produced both fast (*f*) and slow (*s*) donor states (1), 2 sensitized acceptor time constants (τ_{SE}) were determined for each Tb donor state. The resulting donor only (τ_{DO}) and sensitized emission (τ_{SE}) time constants were used to calculate intramolecular *Cys-Cys* distances for both closed (pH 6) and open (pH 8) conditions, according to:

$$E = 1 - \left(\tau_{SE} / \tau_{DO} \right)$$

The distance (r) between donor and acceptor is related to the Förster energy transfer according to:

$$r = R_o \left(1/E - 1 \right)^{1/6}$$

where R_0 is the distance at which there is 50% Förster energy transfer. The above equations were modified by Selvin for lanthanide resonance (2) from the original Förster Energy transfer theory as described in Chapter 13.2 of (3).

Determination of R_0

For each donor acceptor pair, R_0 was calculated from first principles using either the Tb-Fluorescein spectra appropriate for the C189-Kir1.1b, C289-Kir1.1b dimers or the Tb-Atto465 spectra for the A161C-Kir1.1b dimers.

$$R_0(\text{Å}) = 0.211(\kappa^2 n^{-4} Q_D J(\lambda))^{1/6}$$

$$\kappa^2 = 2/3 = relative\ orientation\ of\ transition\ dipoles\ (donor/acceptor)$$

$$n = 1.4 = refractive\ index\ of\ medium$$

$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda = spectral\ overlap\ integral$$

The overlap integral, $J(\lambda)$ is the numerically computed area encompassed by both the terbium donor emission and the acceptor absorbance spectrum.

$$F_D(\lambda) = corrected\ fluorescence\ intensity\ of\ donor$$

$$\varepsilon_A(\lambda) = extinction\ coefficient\ of\ acceptor$$

$$Q_D = quantum\ yield\ of\ donor\ in\ absence\ of\ acceptor = \tau_{DO}/4.75ms$$

Spectra used to determine the C189 R_0 for Terbium-BODIPY-FL pair

Using the above equations, together with the numerically calculated spectral overlap integral resulted in $R_0 = 42.7$ Å.

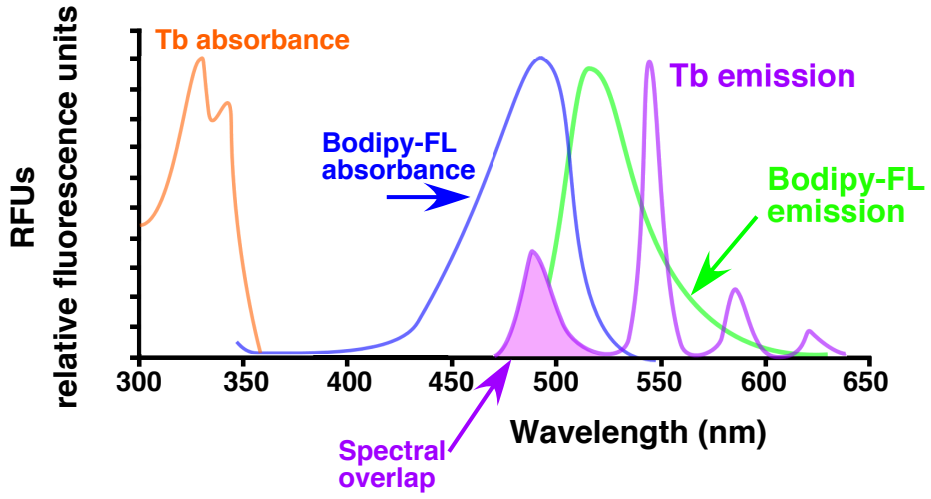

Fig S.1 Absorbance and emission spectra for the Terbium-Bodipy-FL donor-acceptor pair. The shaded area represents the spectral overlap common to both the Tb donor emission and Bodipy-FL acceptor absorbance.

Calculation of distances between paired C189 residues in the CTD

As discussed in the text, dimers of Kir1.1b were constructed with a single Cys on one subunit and no Cys on the other subunit. Dimers were translated in a cell-free (CF) wheat germ system for mammalian integral membrane proteins. LRET was used to measured Förster energy transfer between donor and sensitized acceptor labeled C189 residues on diagonally opposite subunits of tetrameric Kir1.1b that had been incorporated into anionic liposomes. Donor only (τ_{DO}) and sensitized emission (τ) time constants were determined for both fast and slow Tb donor states. The quantities required for the distance calculation are summarized in the table below for the C189 expts. Yellow denotes assumed constants for the Tb-BODIPY-FL donor / acceptor pair. Pink denotes measured or fitted quantities derived from the spectral overlap integral (J) or the time constants (τ_{DO} and τ) determined from the LRET lifetime decays, and Green denotes values calculated from the energy transfer equations of the previous section. Average C189-C189 distances at pH 8 and pH 6 and Δ = pH 8 - pH 6 are summarized in the cyan box.

Table S.1 C189 dimer LRET data

Fast Tb3+ donor											
κ^2	J ($M^{-1}cm^{-1}nm^4$)	n	$\tau_{donor\text{-}free}$ (ms)	τ_{DO} (ms)	Q_D	R_0 (Å)	$\tau_{pH\ 8}$ (ms)	$\tau_{pH\ 6}$ (ms)	$r_{pH\ 8}$ (Å)	$r_{pH\ 6}$ (Å)	Δr_{pH} (Å)
2/3	9.42E+14	1.4	4.75	0.7108	0.15	36.0		0.1180		27.5	
2/3	9.42E+14	1.4	4.75	0.755	0.16	36.3	0.1440		28.3		
											0.8
Slow Tb3+ donor											
κ^2	J ($M^{-1}cm^{-1}nm^4$)	n	$\tau_{donor\text{-}free}$ (ms)	τ_{DO} (ms)	Q_D	R_0 (Å)	$\tau_{pH\ 8}$ (ms)	$\tau_{pH\ 6}$ (ms)	$r_{pH\ 8}$ (Å)	$r_{pH\ 6}$ (Å)	Δr_{pH} (Å)
2/3	9.42E+14	1.4	4.75	1.948	0.41	42.5		0.7190		38.9	
2/3	9.42E+14	1.4	4.75	1.924	0.41	42.4	0.8020		40.1		
											1.2
Yellow are assumed constants								AVG	34.2	33.2	1.0
Pink are measured/fitted constants								SE	5.9	5.7	0.2
Green are calculated values								SD	8.36	8.08	0.28

Calculation of distances between paired C289 residues in the CTD

Similar to C189 (Table S.1 above), LRET was used to measure Förster energy transfer between donor and sensitized acceptor labeled C289 residues on diagonally opposite subunits of tetrameric Kir1.1b that had been incorporated into anionic liposomes. Donor only (τ_{DO}) and sensitized emission (τ) time constants were determined for both fast and slow Tb donor states. The quantities required for the distance calculation are summarized in the table below for the C289 expts. Yellow denotes assumed constants for the Tb-BODIPY-FL donor / acceptor pair. Pink denotes measured or fitted quantities derived from the spectral overlap integral (J) or the time constants (τ_{DO} and τ) determined from the LRET lifetime decays, and Green denotes values calculated from the energy transfer equations of the previous section. Average C289-C289 distances at pH 8 and pH 6 and Δ = pH 8 - pH 6 are summarized in the cyan box.

Table S.2 C289 dimer LRET data

Fast Tb3+ donor											
κ^2	J ($M^{-1}cm^{-1}nm^4$)	n	$\tau_{donor\text{-}free}$ (ms)	τ_{DO} (ms)	Q_D	R_0 (Å)	$\tau_{pH\ 8}$ (ms)	$\tau_{pH\ 6}$ (ms)	$r_{pH\ 8}$ (Å)	$r_{pH\ 6}$ (Å)	Δr_{pH} (Å)
2/3	9.42E+14	1.4	4.75	0.9	0.19	37.4	0.0500	0.0540	23.3	23.6	-0.3
Slow Tb3+ donor											
κ^2	J ($M^{-1}cm^{-1}nm^4$)	n	$\tau_{donor\text{-}free}$ (ms)	τ_{DO} (ms)	Q_D	R_0 (Å)	$\tau_{pH\ 8}$ (ms)	$\tau_{pH\ 6}$ (ms)	$r_{pH\ 8}$ (Å)	$r_{pH\ 6}$ (Å)	Δr_{pH} (Å)
2/3	9.42E+14	1.4	4.75	1.98	0.42	42.6	0.2400	0.2100	30.7	29.9	0.8
Yellow are assumed constants								AVG	27.0	26.8	0.2
Pink are measured/fitted constants								SE	3.7	3.1	0.5
Green are calculated values								SD	5.19	4.42	0.76

Spectra used to determine the A161C R_0 for the Terbium-Atto465 pair

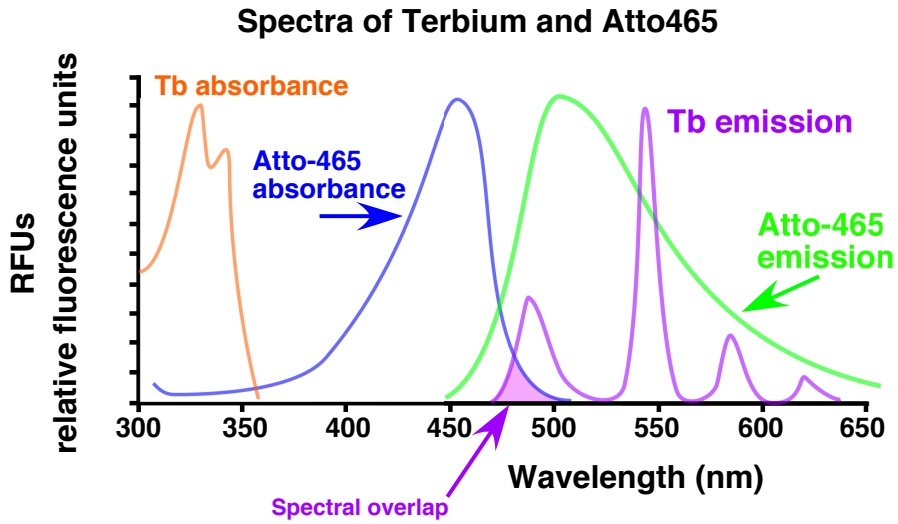

Fig S.2 Absorbance and emission spectra for the Terbium-Atto465 donor-acceptor pair. The shaded area represents the spectral overlap common to both the Tb donor emission and the Atto465 acceptor absorbance.

Calculation of distances between paired C161 residues in the CTD

Similar to C189 and C289 (Table S.1,2 above), LRET was used to measure Förster energy transfer between donor and sensitized acceptor labeled C161 residues on diagonally opposite subunits of tetrameric Kir1.1b that had been incorporated into anionic liposomes. C161 was formed by replacing a native *Ala* at this position by a *Cys* residue. C161 is adjacent to the hydrophobic HBC gate at L160-Kir1.1b.

Donor only (τ_{DO}) and sensitized emission (τ) time constants were determined for both fast and slow Tb donor states. The quantities required for the distance calculation are summarized in the table below for the C161 expts. Yellow denotes assumed constants for the Tb-Atto465 donor / acceptor pair. Pink denotes measured or fitted quantities derived from the spectral overlap integral (J) or the time constants (τ_{DO} and τ) determined from the LRET lifetime decays, and Green denotes values calculated from the energy transfer equations of the previous section. Average C161-C161 distances at pH 8 and pH 6 and Δ= pH 8 - pH 6 are summarized in the cyan box.

Table S.3 C161 dimer LRET data

Fast Tb3+ donor											
κ^2	J ($M^{-1}cm^{-1}nm^4$)	n	$\tau_{donor\text{-}free}$ (ms)	τ_{DO} (ms)	Q_D	R_0 (Å)	$\tau_{pH\ 8}$ (ms)	$\tau_{pH\ 6}$ (ms)	$r_{pH\ 8}$ (Å)	$r_{pH\ 6}$ (Å)	Δr_{pH} (Å)
2/3	6.18E+13	1.4	4.75	0.4412	0.09	21.1	0.0818	0.0159	16.5	12.2	4.3
Slow Tb3+ donor											
κ^2	J ($M^{-1}cm^{-1}nm^4$)	n	$\tau_{donor\text{-}free}$ (ms)	τ_{DO} (ms)	Q_D	R_0 (Å)	$\tau_{pH\ 8}$ (ms)	$\tau_{pH\ 6}$ (ms)	$r_{pH\ 8}$ (Å)	$r_{pH\ 6}$ (Å)	Δr_{pH} (Å)
2/3	6.18E+13	1.4	4.75	1.748	0.37	26.5	1.080	0.0667	28.7	15.5	13.2
Yellow are assumed constants								AVG	22.6	13.8	8.8
Pink are measured/fitted constants								SE	6.1	1.6	4.5
Green are calculated values								SD	8.67	2.33	6.34

Labeling protocol for single-*Cys* dimers

To minimize ambiguity in Förster energy transfer, single-Cys dimers of Kir1.1b (Fig 1 of the text) were constructed as originally described in (4). The dimers were produced in a cell-free (CF) wheat-germ system (CFS-WGE-2240, Cambridge Isotope Labs, MA), supplemented with anionic (POPE+POPG) extruded liposomes (5).

The entire cell-free (CF) reaction product was centrifuged for 3m at 18,000g. The resulting pellet was washed in 158mM KMOPS, suspended, and partially solubilized in the same volume of 35mM CHAPS plus 158mM KMOPS. Following an additional centrifugation (3m at 18,000g), the supernatant (containing CHAPS) was used for donor / acceptor labeling. To estimate the amount of soluble protein, fluorescent lysine residues were incorporated into our dimers during CF translation (FluoroTect GreenLys in vitro translation labeling system, L5001 Promega). A fluorescent scan (Typhoon 9400, GE Healthcare) of the resulting electrophoresis indicated that only 5% (0.025ug/uL or 0.3uM) of the original CF translated protein (0.5ug/uL) was solubilized.

In the donor-only condition, 110uM Tb-chelate (LanthaScreen PV3580, Life Technologies) was incubated at 23° C for 90 min in the dark with 0.3uM of CHAPS-solubilized, single-Cys dimer. For the donor-acceptor condition, 110uM of both the Tb-chelate donor and acceptor (BODIPY FL-N, B-10250, Life technologies; or Atto465M, ATTO-TEC, D-57076 Siegen, Germany) were incubated at 23° C for 90 min in the dark with 0.3uM of CHAPS-solubilized single-Cys dimer.

Despite an elevated label/protein molar ratio almost all of the free label could be removed using multiple washes with protein desalting spin columns (Thermo Scientific, cat# 89849). Free label that could not be removed did not interfere with our donor or acceptor lifetime measurements. In some experiments (e.g. C289), we observed better energy transfer when the concentration of acceptor label was 100x less than the concentration of Tb donor label, probably because CTD cysteines have a higher affinity for BODIPY-FL-N acceptor than for Tb maleimide.

The final proteoliposomes used in the LRET measurements were prepared by removal of CHAPS from a mix of labeled protein and CHAPS-solubilized POPE, POPG, and PIP_2, using partially dehydrated G50 KMOPS columns at either pH 6 or pH 8 (6). The resulting proteoliposomes, containing labeled tetrameric Kir1.1b channels and anionic lipid, were maintained at either pH 6 or pH 8, inside and out.

Similar amounts of protein in the Cys-free and single-Cys A161C dimer samples

Background contamination in the LRET measurements was assessed by comparing the donor signal from a *Cys*-free Kir1.1b sample to the donor signal from one of our single-*Cys* dimer proteins (A161C-Kir1.1b). Both the *Cys*-free and *Cys*-containing proteoliposome samples were prepared by the same CF translation and labeling protocols, and the amount of A161C dimer protein (80kD) was comparable to the amount of Cys-free monomer (40kD) on SDS electrophoresis (Fig S.3). In the recording shown in Fig 9 of the text the *Cys*-free sample had a significant amount of a known wheat-germ heat-shock protein, Hsp70 at 70kD (7), which was not present in the A161C dimer sample (Fig S.3). In general, the amount of Hsp70 in any CF translation sample was quite variable.

Consequently, the large difference in Tb signal intensity between single-Cys A161C dimer liposomes and the Cys-free liposomes (Fig 9 of text) implies that extraneous material from the CF system was not being significantly labeled and would not interfere with our LRET measurements even though there were other proteins present, including a wheat-germ Hsp 70.

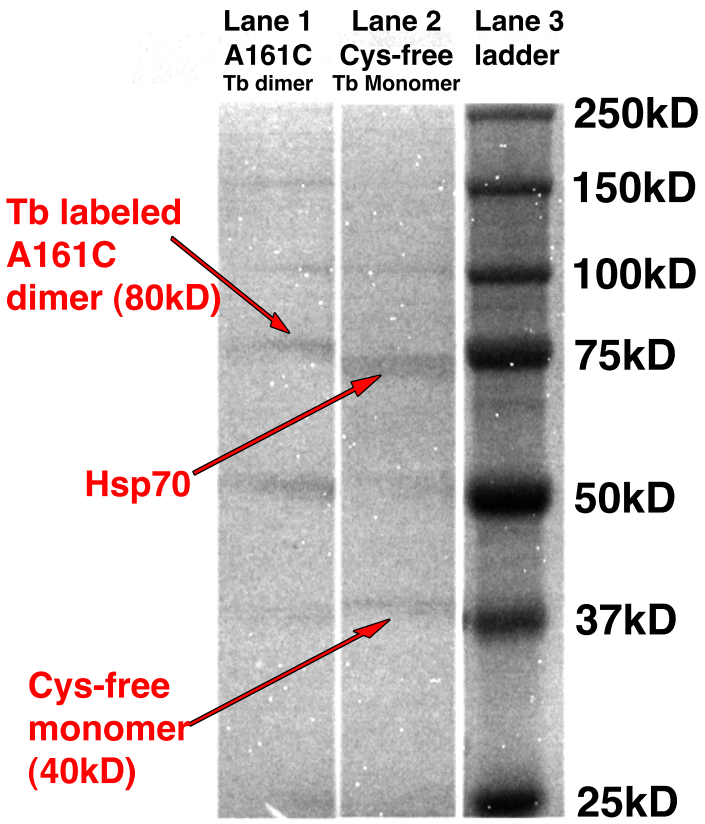

Fig S.3. SDS gel (10%) stained with Pierce Zinc reversible stain kit (Thermo Scientific Cat# 24582), showing similar amounts of single-Cys A161C dimer and Cys-less monomer in the final LRET proteoliposomes. Both proteins were produced by our CF wheat-germ system and labeled with a similar protocol. Lane 1: A161C single-Cys dimer (80kD) LRET proteoliposomes, plus other unidentified bands from the cell-free translation. Lane 2: Cys-less 40kD monomer of Kir1.1b, plus Hsp70, together with several other unidentified CF bands. Lane 3: ladder

Supporting References

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