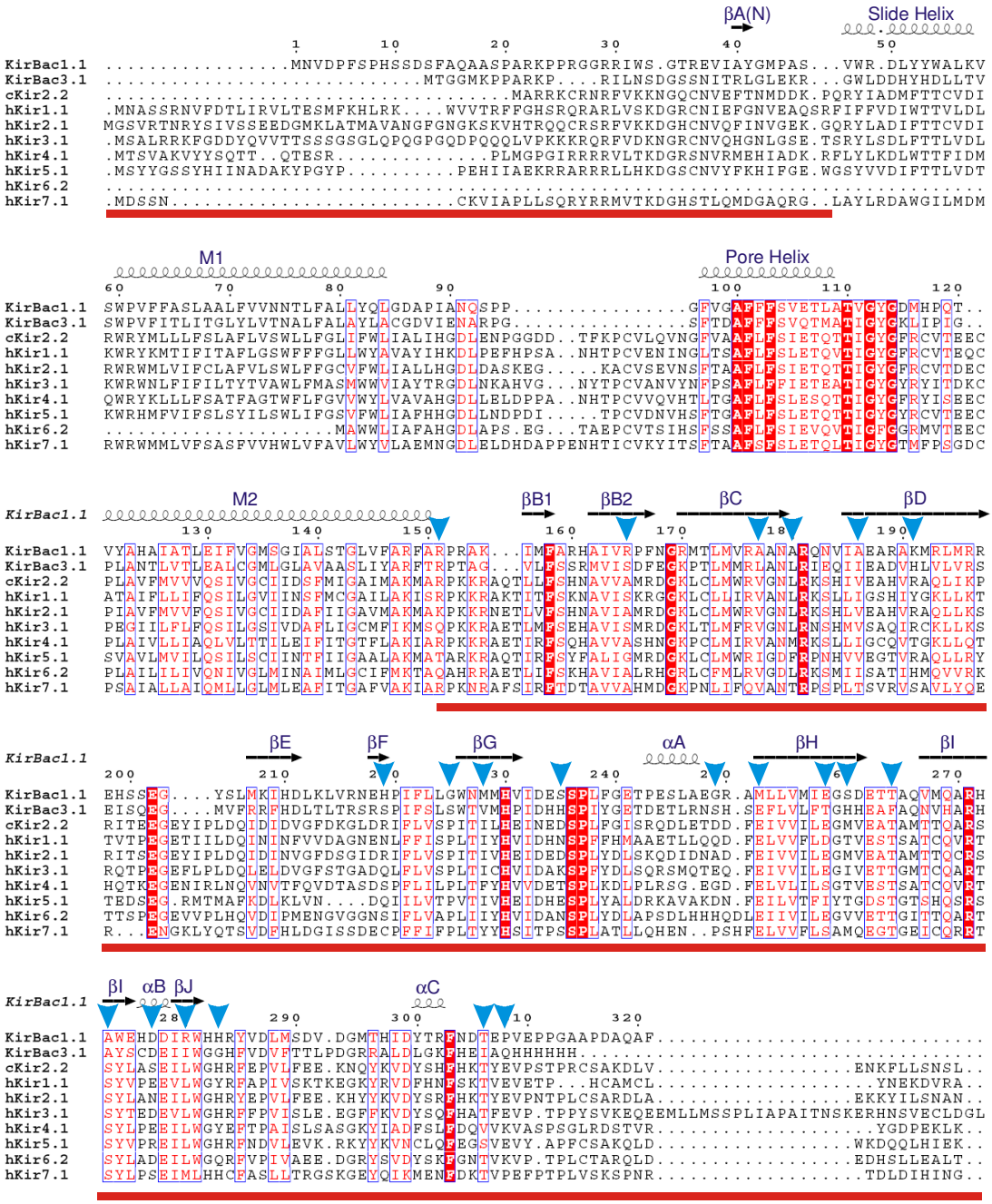
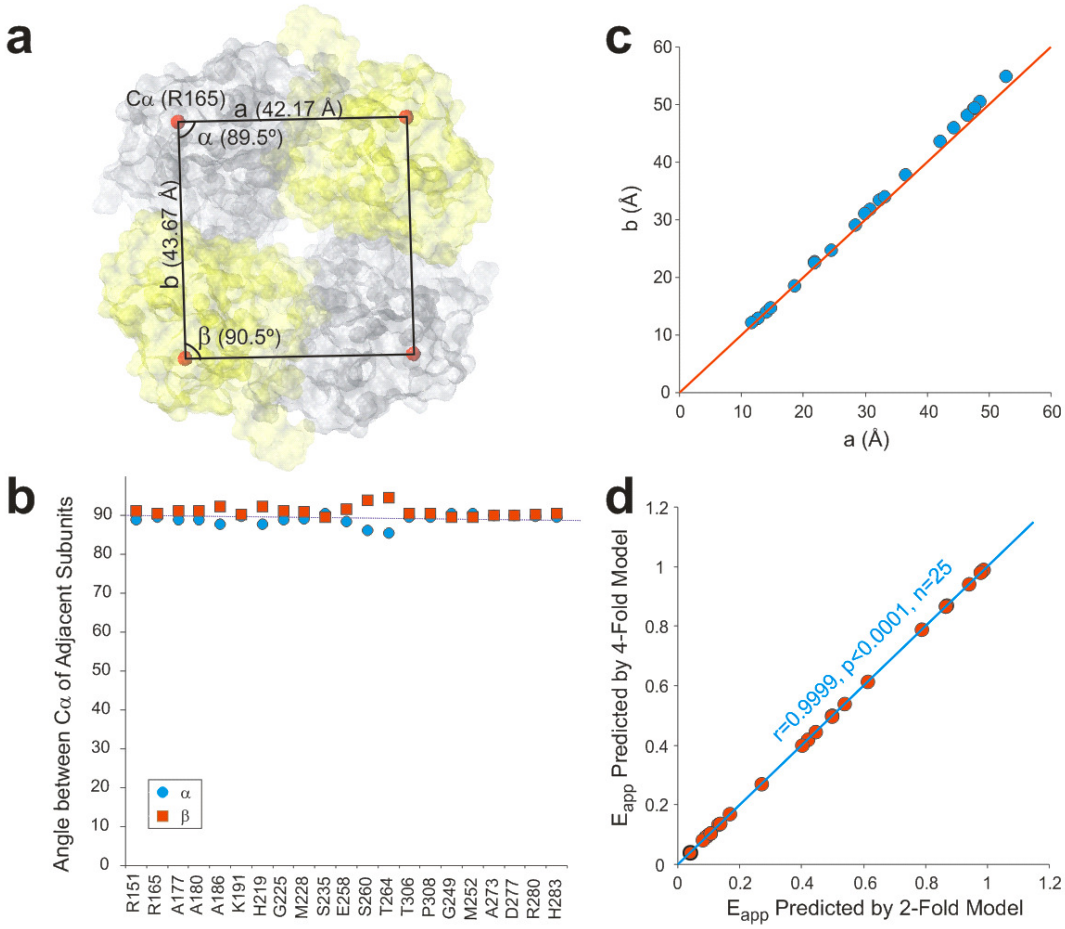


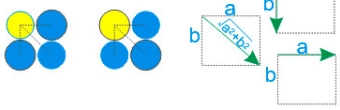
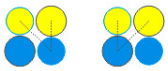
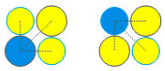
Supplementary Figure S1: Sequence alignment of Kir channels

Sequence alignments were performed using ClustalW2.0 and formatted by online server, ESPript2.2. Secondary structure elements were assigned based on KirBac1.1 crystal structure (2WLL). Residues constitute the 'Kir domain' were marked by thick red solid lines, residues in the KirBac1.1 cytoplasmic domain that were examined by FRET measurements in the present study are marked by blue arrows.



Supplementary Figure S2: Correlation analysis on measured apparent FRET efficiencies with these predicted by KirBac1.1 crystal structure (2WLL) with 2-fold or four fold symmetry. (a) Assymmetry of KirBac1.1 crystal structure. Surface map of the sytoplasmic domain of each subunit, and the four a-carbon of residue R165 are highlighted as brown spheres. The angle between Ca of adjacent subunits is referred to as a and b, and the distance between Ca of adjacent subunits is referred to as a or b. (b) The angle between Ca of adjacent subunits from KirBac1.1 crystal structure (2WLL). Most of the FRET measured residues show essentially rectangular distribution within tetramers, and most have considerably less than 5º of deviation. (c) Comparison of Ca distances between adjacent subunits from crystal structure of KirBac1.1 (2WLL). Difference in a versus b Ca distance between adjacent subunits are smaller than 2 Å. (d) Correlation analysis between apparent FRET efficiencies predicted by KirBac1.1 crystal structure (2WLL) with 2-fold and 4-fold symmetric FRET model. The distances between □-carbons of FRET measured residues at two adjacent subunits from crystal structure of KirBac1.1 (2WLL, referred to as a or b) were used for calculating the apparent FRET efficiencies. In the 2-fold symmetric FRET model, both a and b distances were used for calculations of apparent FRET efficiencies using the equations and parameters as described in supplementary Table S1; in the 4-fold symmetric FRET model, the average values of corresponding distance a and b were used to replace the distance parameters a or b. The correlation coefficient r=0.9999, p<0.0001 and results in essentially no difference in correlation between apparent FRET efficiencies measured experimentally and these predicted by either 2-fold or 4-fold FRET model.

Supplementary Table S1 Fluorophore labeling stoichiometry and FRET model parameters

Name	Stoichiometry	Energy transfer pathway	$p_{D_nA_{4-n}}$ *	n	$E^{D}_{D_nA_{4-n}}$ **
DDDD			$\frac{r^4}{(1+r)^4}$	4	0
DDDA			$\frac{4r^3}{(1+r)^4}$	3	$E_1 = \frac{1}{3}\left(\frac{R_o^6(\frac{1}{a})^6}{1+R_o^6(\frac{1}{a})^6} + \frac{R_o^6(\frac{1}{b})^6}{1+R_o^6(\frac{1}{b})^6} + \frac{R_o^6\left(\frac{1}{\sqrt{a^2+b^2}}\right)^6}{1+R_o^6\left(\frac{1}{\sqrt{a^2+b^2}}\right)^6}\right)$
DDAA			$\frac{2r^2}{(1+r)^4}$	2	$E_2 = \frac{R_o^6\left((\frac{1}{a})^6 + \left(\frac{1}{\sqrt{a^2+b^2}}\right)^6\right)}{1+R_o^6\left((\frac{1}{a})^6 + (\frac{1}{\sqrt{a^2+b^2}})^6\right)}$
DDAA			$\frac{2r^2}{(1+r)^4}$	2	$E_3 = \frac{R_o^6\left((\frac{1}{b})^6 + \left(\frac{1}{\sqrt{a^2+b^2}}\right)^6\right)}{1+R_o^6\left((\frac{1}{b})^6 + (\frac{1}{\sqrt{a^2+b^2}})^6\right)}$
DADA			$\frac{2r^2}{(1+r)^4}$	2	$E_4 = \frac{R_o^6\left((\frac{1}{a})^6 + (\frac{1}{b})^6\right)}{1+R_o^6\left((\frac{1}{a})^6 + (\frac{1}{b})^6\right)}$
DAAA			$\frac{4r}{(1+r)^4}$	1	$E_5 = \frac{R_o^6\left((\frac{1}{a})^6 + (\frac{1}{b})^6 + \left(\frac{1}{\sqrt{a^2+b^2}}\right)^6\right)}{1+R_o^6\left((\frac{1}{a})^6 + (\frac{1}{b})^6 + \left(\frac{1}{\sqrt{a^2+b^2}}\right)^6\right)}$
AAAA			$\frac{1}{(1+r)^4}$	0	0

D: Donor fluorophores, blue circles;
A: Acceptor fluorophores, yellow circles;
r: Ratio of donor to acceptor in labeling mixtures.
DnA4-n: The specific configuration of labeling proteins containing n donor and 4-n acceptor fluorophores;
R0: Förster distance of FRET pairs, which are 29 Å for A/D pair and 33 Å for E/D pair with an orientation factor as 2/3;
*The probability of labeling proteins forming configuration that contain n donor and 4-n acceptor fluorophores;
**The effective FRET efficiencies of labeling proteins containing n donor and 4-n acceptor, determined by the decrease in donor emissions.