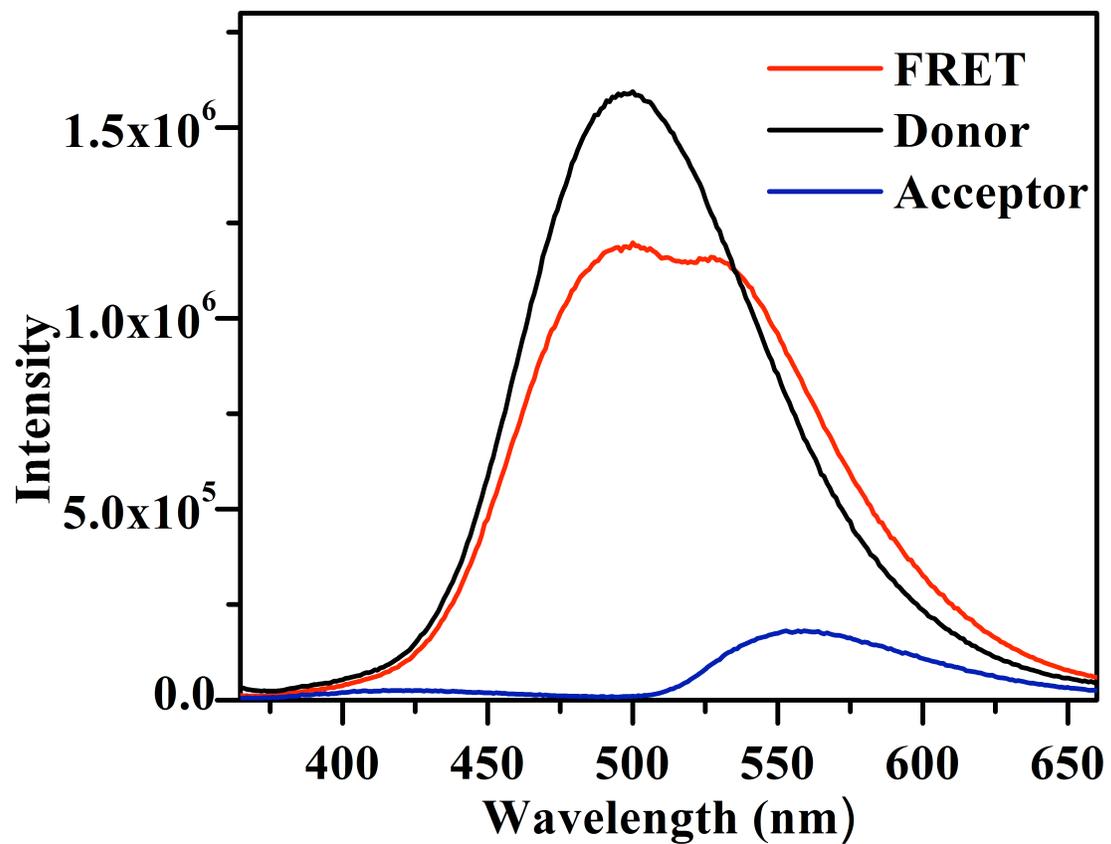


Supporting Information:

Supporting Information Figure S1. SecA donor quenching and signal peptide acceptor enhancement. Fluorescence spectra of IAEDANS-labeled SecA-Cys-827 with unlabeled SP22 (—), IAEDANS-labeled SecA-Cys-827 and IANBD-labeled SP22 (—) and IANBD-labeled SP22 and SecA-Cys-827 (—). The SecA donor was at 7 μM and the SP22 acceptor was at 18 μM with excitation at 336 nm in all cases.



Supporting Information Figure S2. Binding affinity of unlabeled SP2 and IANBD-labeled-SP2 with IAEDANS-labeled SecA-Cys-256. (A) Binding of SP2 to SecA-256C-IAEDANS measured by change in fluorescence intensity upon signal peptide binding. The dissociation constant was determined to be $1.2 \pm 0.1 \mu\text{M}$. (B) Binding of SP2-IANBD to SecA-256C-IAEDANS measured by energy transfer. The dissociation constant was found to be $8.1 \pm 2.2 \mu\text{M}$. SecA 256C-IAEDANS was maintained at a constant concentration of $1 \mu\text{M}$ and SP2 (IANBD) was titrated into the system from 0-30 μM . All experiments were performed in TKE buffer at 20°C .

Figure S2 (A)

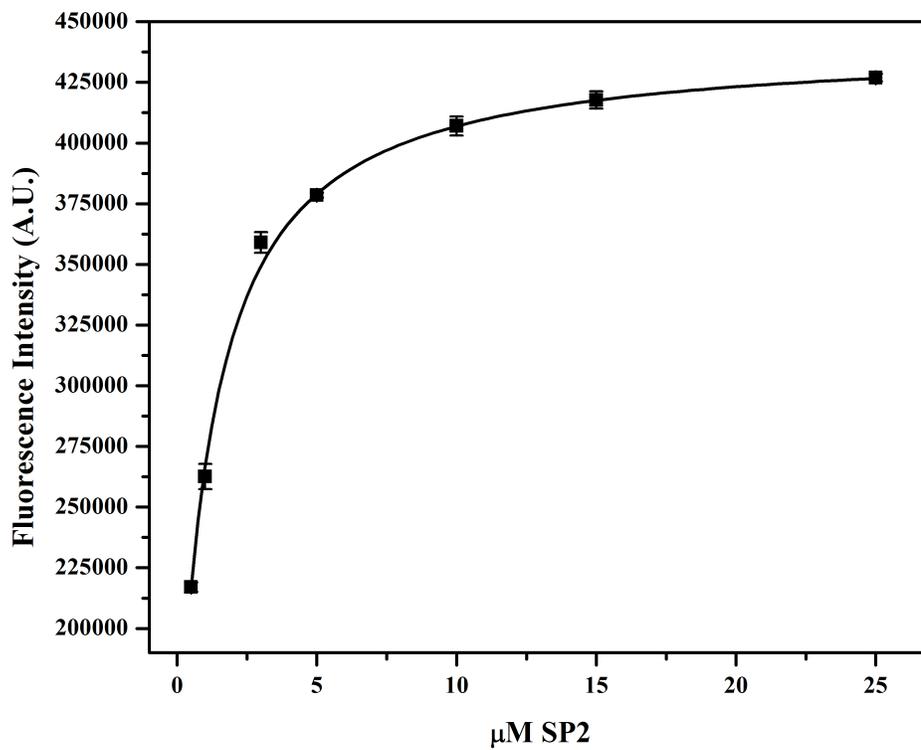
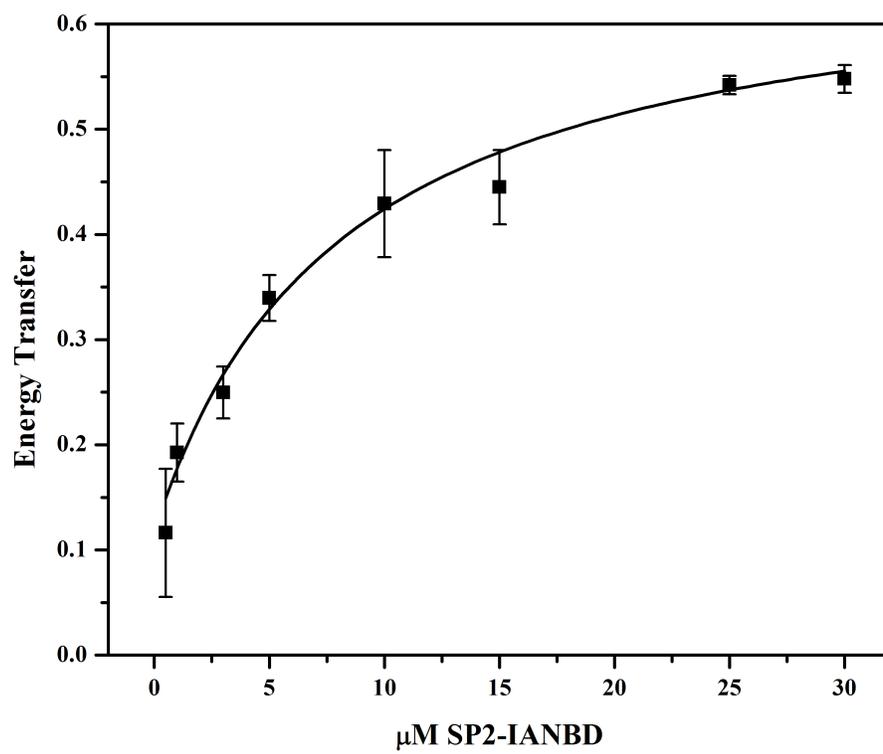
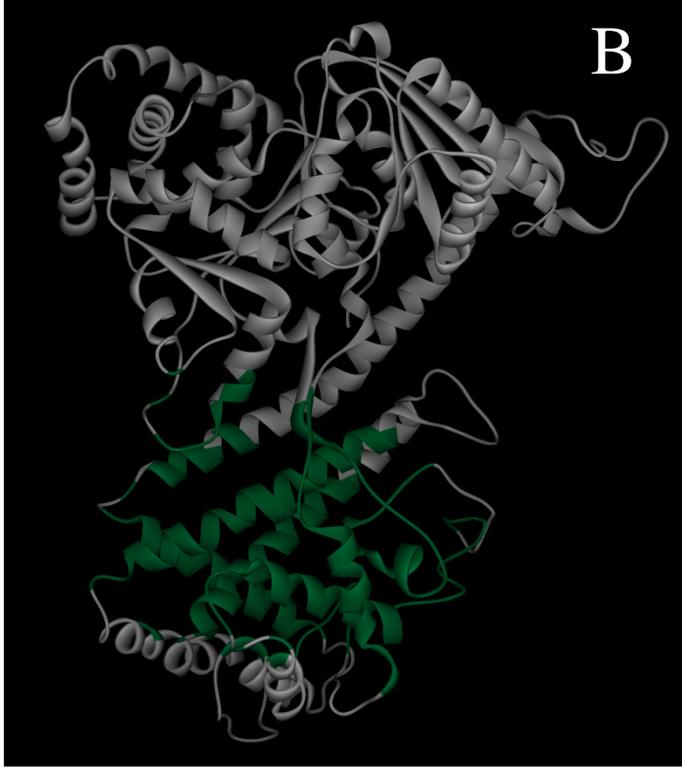


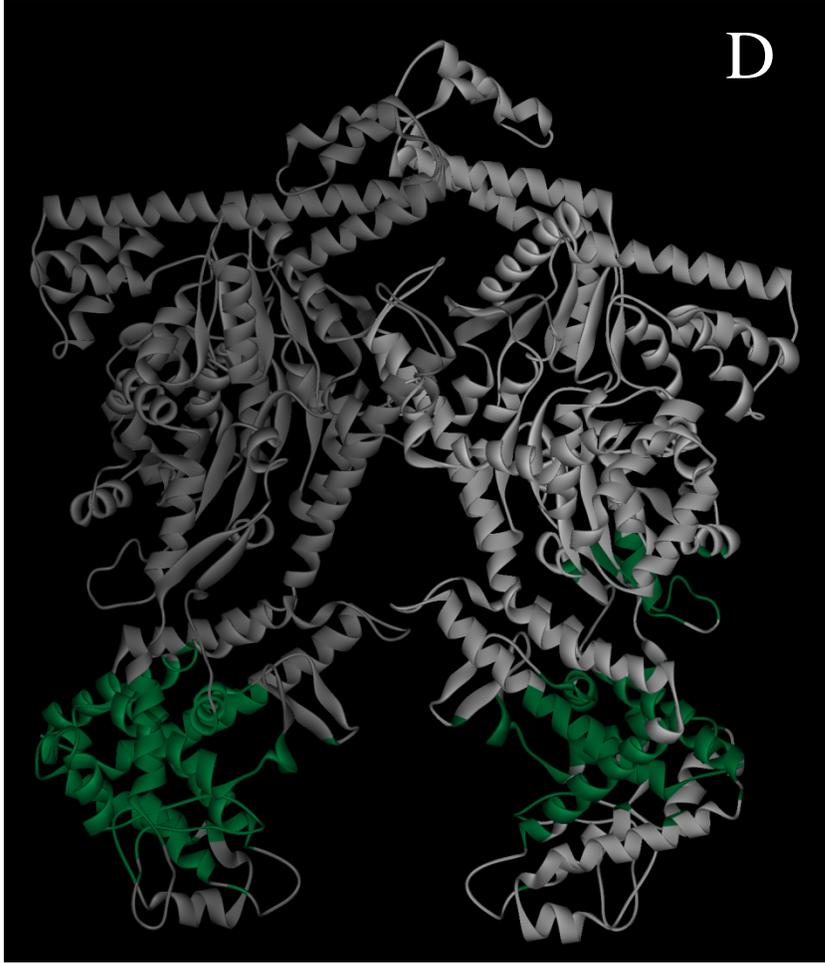
Figure S2 (B)



Supporting Information Figure S3. Mapped signal peptide binding site on various SecA crystal structures. The FRET-mapped binding site is colored in dark green on the SecA structure from (A) *Bacillus subtilis* (pdb code 1m6n) (1), (B) *Bacillus subtilis* (pdb code 2ibm) (2), (C) *Escherichia coli* (pdb code 2fsf) (3), (D) *Thermus thermophilus* (pdb code 2ipc) (4), and (E) *Mycobacterium tuberculosis* (pdb code inl3) (5). The *T. thermophilus* SecA structure is shown as a parallel dimer, all other structures are shown as monomers.









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