SUPPLEMENTAL TEXT

Supplement 1: Derivation of the relation between *D-A* stoichiometry and ratio *S*

The relation between *S* and stoichiometry can be extended to species with many n_D donor fluorophores and n_A acceptor fluorophores, and a common, average FRET efficiency $\langle E\rangle$ for each donor to the ensemble of acceptors. The photons counts for the 3 important emission streams become:

$$F_{D_{exc}}^{D_{em}} = n_D I_{D_{exc}} \sigma_{D_{exc}}^{D} \phi_D \eta_{D_{em}}^{D} \left(1-\langle E\rangle\right), \quad \text{(S1)}$$

$$F_{A_{exc}}^{A_{em}} = n_A I_{A_{exc}} \sigma_{A_{exc}}^{A} \phi_A \eta_{A_{em}}^{A}, \quad \text{(S2)}$$

and $F^{FRET} = n_D I_{D_{exc}} \sigma_{D_{exc}}^{D} \phi_A \eta_{A_{em}}^{A} \langle E\rangle$. (S3)

It follows easily that:

$$S_\gamma = \left(1+\frac{n_A}{n_D}\beta\right)^{-1}, \quad \text{(S4)}$$

showing that

$$\frac{n_A}{n_D} = \beta\left(S_\gamma^{-1}-1\right) \quad \text{(S5)}$$

which shows that S_γ reports on the *D-A* stoichiometry of a diffusing molecule.

Supplement 2: Relation of E_{PR}^{raw} to accurate-*E*: derivation

Crosstalk-uncorrected proximity ratio E_{PR}^{raw} is defined as $E_{PR}^{raw} = F_{D_{exc}}^{A_{em}} \Big/ \left(F_{D_{exc}}^{A_{em}} + F_{D_{exc}}^{D_{em}}\right)$. Using eqs. 5, 6, and 27, the numerator $F_{D_{exc}}^{A_{em}}$ can be rewritten as:

$$\begin{aligned} F_{D_{exc}}^{A_{em}} &= I_{D_{exc}} \sigma_{D_{exc}}^{D} \phi_A \eta_{A_{em}}^{A} E + l\left[I_{D_{exc}} \sigma_{D_{exc}}^{D} \phi_D \eta_{D_{em}}^{D} (1-E)\right] + d' I_{D_{exc}} \sigma_{D_{exc}}^{D} \phi_D \eta_{D_{em}}^{D} \\ &= I_{D_{exc}} \sigma_{D_{exc}}^{D} \left\{\phi_A \eta_{A_{em}}^{A} E + l\left[\phi_D \eta_{D_{em}}^{D} (1-E)\right] + d' \phi_D \eta_{D_{em}}^{D}\right\} \end{aligned} \quad \text{(S6)}$$

Using the definition of $\gamma = \phi_A \eta_{A_{em}}^{A} \big/ \phi_D \eta_{D_{em}}^{D}$, we reach a simplified expression for the numerator:

$$\begin{aligned} F_{D_{exc}}^{A_{em}} &= I_{D_{exc}} \sigma_{D_{exc}}^{D} \left\{\gamma \phi_D \eta_{D_{em}}^{D} E + l\left[\phi_D \eta_{D_{em}}^{D} (1-E)\right] + d' \phi_D \eta_{D_{em}}^{D}\right\} \\ &= I_{D_{exc}} \sigma_{D_{exc}}^{D} \phi_D \eta_{D_{em}}^{D} \left[\gamma E + l(1-E) + d'\right] \end{aligned} \quad \text{(S7)}$$

Using eq. S7 for the numerator, and considering that $F_{D_{exc}}^{D_{em}} = I_{D_{exc}} \sigma_{D_{exc}}^{D} \phi_D \ \eta_{D_{em}}^{D} \left(1-E\right)$, we obtain:

$$E_{PR}^{raw} = \gamma E + l\left(1-E\right) + d' \Big/ \left\{\left[\gamma E + l\left(1-E\right) + d'\right] + 1 - E\right\} =$$
$$E_{PR}^{raw} = \left[\left(\gamma - l\right)E + l + d'\right] \Big/ \left[\left(\gamma - l - 1\right)E + l + d' + 1\right] \qquad \text{(S8)}$$

Solving for E yields a simple expression that recovers E from E_{PR}^{raw} when correction factors γ, l, and d' are known:

$$E = \frac{1-\left(1+l+d'\right)\left(1-E_{PR}^{raw}\right)}{1-\left(1+l-\gamma\right)\left(1-E_{PR}^{raw}\right)} \qquad \text{(S9)}$$

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. DNA fragments used for accurate-*E* measurements using ALEX.

A. DNA fragments used for analysis of detection correction factor γ. Acceptor (*A*): Alexa 647); donor (*D*): TMR. In the *A*-containing DNA fragments, the acceptor was incorporated on an amino-modifier C6 dT residue of an invariant 3-bp sequence (in bold), in order to eliminate any differences in the fluorophore properties due to differences in the local DNA environment.

B. Additional DNA fragments used for comparison of experimental accurate-*E* values with values predicted with cylindrical models of DNA. The 2-bp insertion used to generate the new fragments without changing the local environment of the fluorophores is shown in grey boxes.

Fig. S2. Dependence of γ and β on laser power ratio.

A. The dependence of E_{PR}-1/S plot on excitation power ratio $I_{A_{exc}} / I_{D_{exc}}$. The applied power ratios are 0.3, 0.2, and 0.1.

B. Values of γ and β derived from Fig. S2A. β is proportional to the power ratio, while γ is unaffected.

FIG. S1

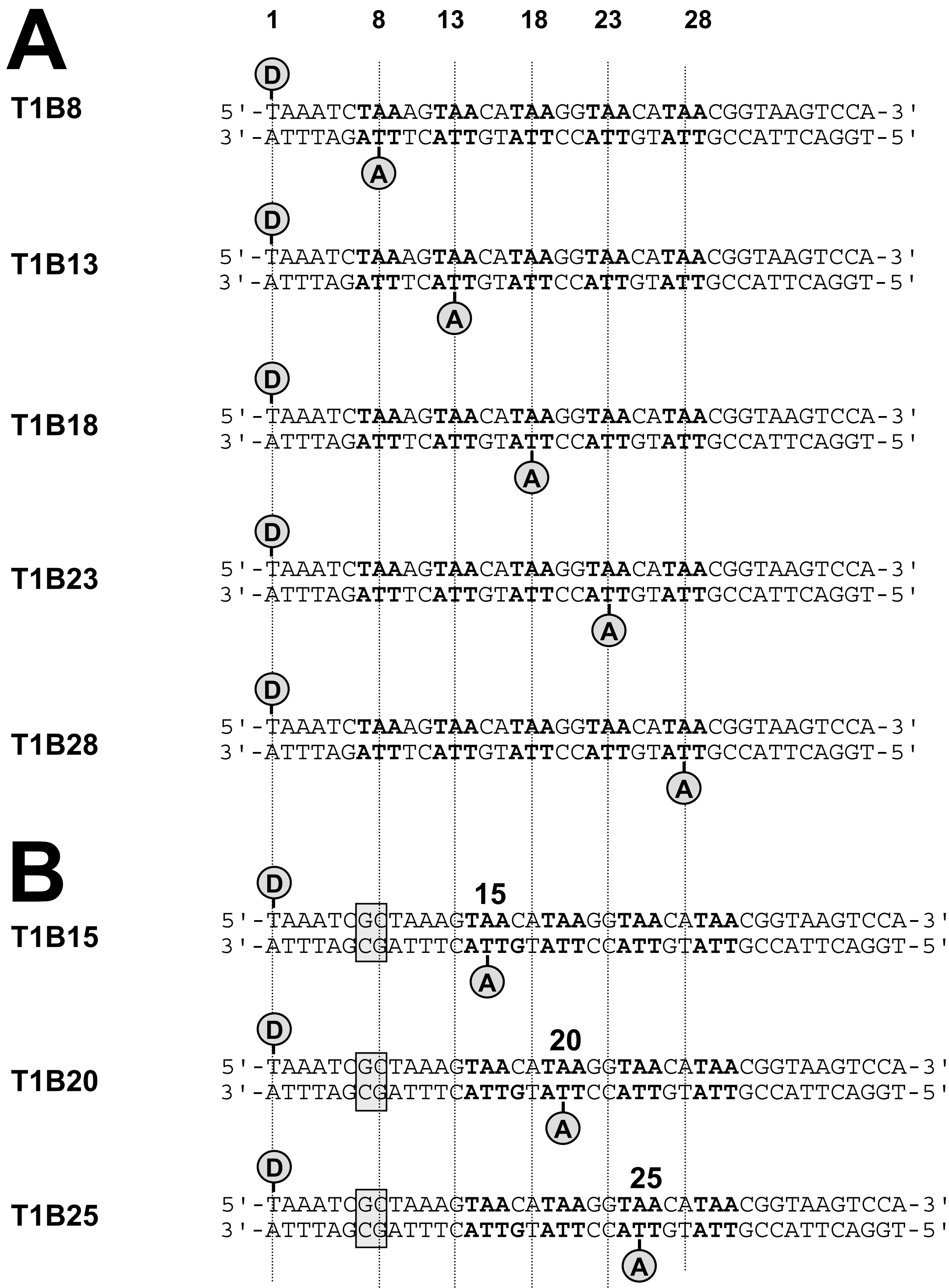
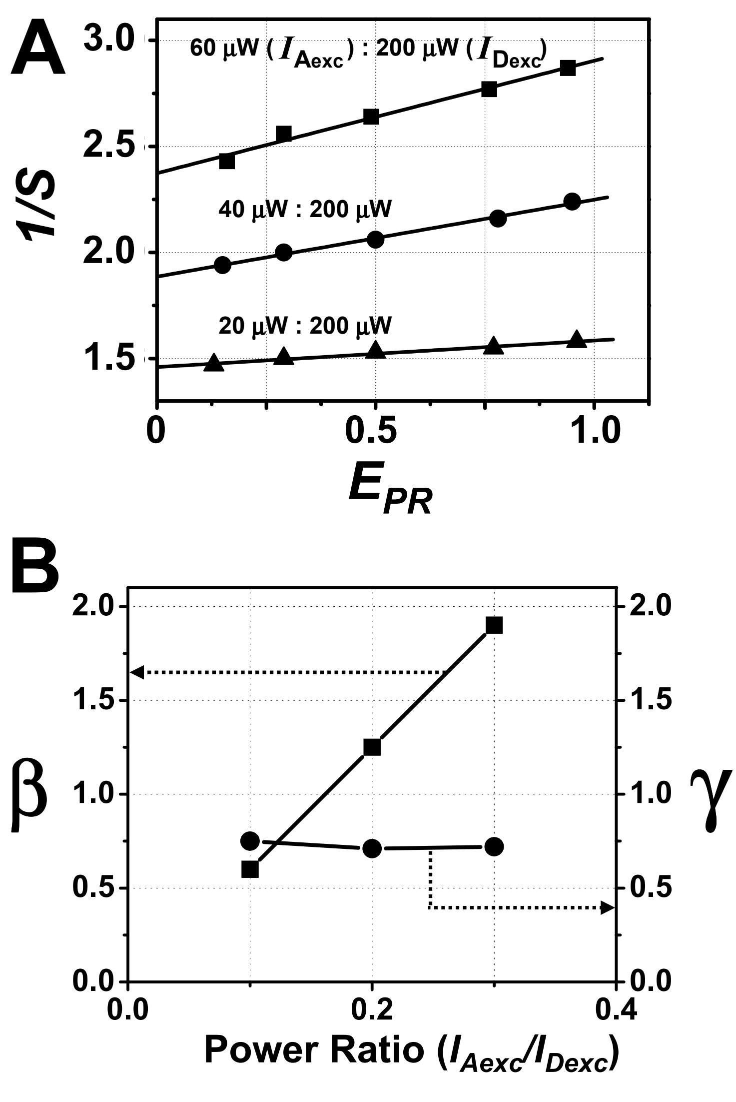

A
1
8
13
18
23
28
D
T1B8
5'-TAAATCTAAAGTAACATAAGGTAACATAACGGTAAGTCCA-3'
3'-ATTTAGATTTCATTGTATTCCATTGTATTGCCATTCAGGT-5'
A
T1B13
T1B18
T1B23
T1B28
B
15
T1B15
5'-TAAATCGCTAAAGTAACATAAGGTAACATAACGGTAAGTCCA-3'
3'-ATTTAGCGATTTCATTGTATTCCATTGTATTGCCATTCAGGT-5'
20
T1B20
25
T1B25

FIG. S2



A
60 μW (I_{Aexc}) : 200 μW (I_{Dexc})
40 μW : 200 μW
20 μW : 200 μW
1/S
3.0
2.5
2.0
1.5
0
0.5
1.0
E_{PR}
B
β
γ
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
Power Ratio (I_{Aexc}/I_{Dexc})