Gamma Factor in sm-FRET

$$E = \frac{A}{A + D} = \frac{A}{A + \gamma \times D}$$

Optimizing Methods to Recover Absolute FRET Efficiency from Immobilized Single Molecules

James J. McCann,[†] Ucheor B. Choi,[‡§] Liqiang Zheng,[†] Keith Weninger,[§] and Mark E. Bowen[†*]
[†]Department of Pharmacology, and [‡]Department of Physiology and Biophysics, Stony Brook University, Stony Brook, New York; and [§]Department of Physics, North Carolina State University, Raleigh, North Carolina

ABSTRACT Microscopy-based fluorescence resonance energy transfer (FRET) experiments measure donor and acceptor intensities by isolating these signals with a series of optical elements. Because this filtering discards portions of the spectrum, the observed FRET efficiency is dependent on the set of filters in use. Similarly, observed FRET efficiency is also affected by differences in fluorophore quantum yield. Recovering the absolute FRET efficiency requires normalization for these effects to account for differences between the donor and acceptor fluorophores in their quantum yield and detection efficiency. Without this correction, FRET is consistent across multiple experiments only if the photophysical and instrument properties remain unchanged. Here we present what is, to our knowledge, the first systematic study of methods to recover the true FRET efficiency using DNA rulers with known fluorophore separations. We varied optical elements to purposefully alter observed FRET and examined protein samples to achieve quantum yields distinct from those in the DNA samples. Correction for calculated instrument transmission reduced FRET deviations, which can facilitate comparison of results from different instruments. Empirical normalization was more effective but required significant effort. Normalization based on single-molecule photobleaching was the most effective depending on how it is applied. Surprisingly, per-molecule γ-normalization reduced the peak width in the DNA FRET distribution because anomalous γ-values correspond to FRET outliers. Thus, molecule-to-molecule variation in gamma has an unrecognized effect on the FRET distribution that must be considered to extract information on sample dynamics from the distribution width.

INTRODUCTION

Fluorescence resonance energy transfer (FRET) is widely thought of as a spectroscopic ruler on the nanometer scale (1,2). Many biological phenomena occur on this scale, making FRET a popular tool in biology. The efficiency of energy transfer (E) between two fluorescent dyes is related to the fluorophore separation (r) by

is internally consistent as long as the photophysical and instrumental properties remain unchanged (4). E_{PR} is determined from the measured intensities (I) of the donor (D) and acceptor (A):

$$E_{PR} = \frac{I_A}{I_A + I_D}.$$ (2)

...ies with fluorophore separation, it is ...conclusions about the timescale and ...ural changes and molecular associations. ...n desirable to recover the true FRET ...ated from instrument and photophysical ...ed intensity values must be corrected as
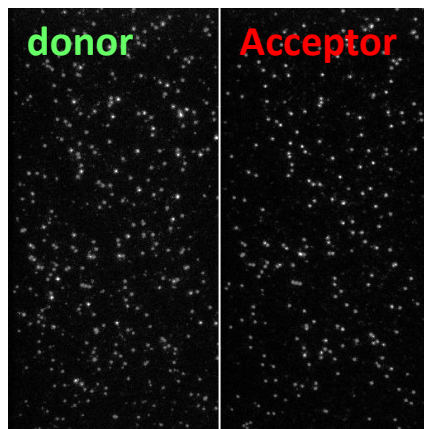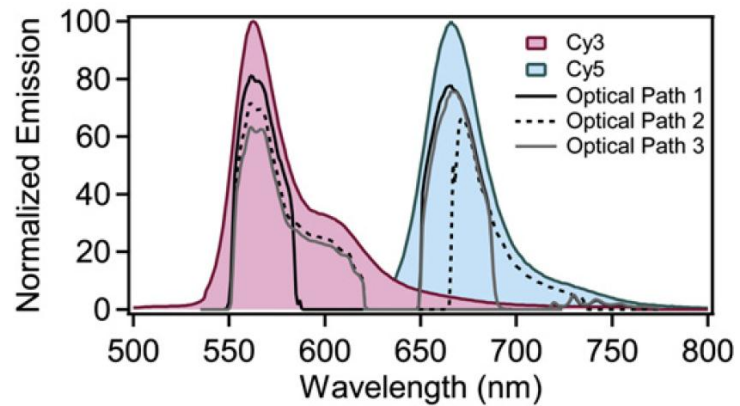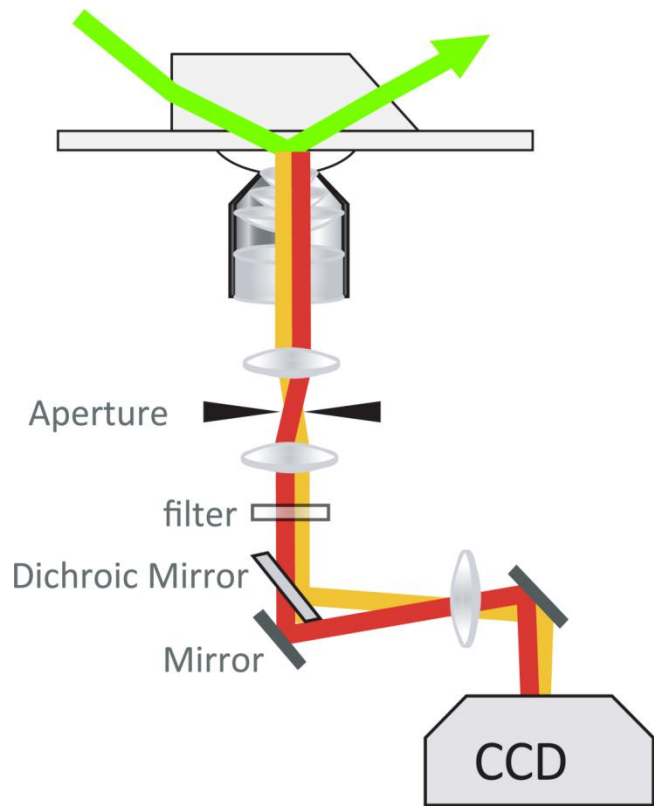
$$= \frac{I_A - \beta I_D}{((I_A - \beta I_D) + \gamma I_D)},$$ (3)

...for leakage of donor emission into the ...). The parameter γ accounts for differ-...onor and acceptor in detection efficiency ...ld (φ) (6,7):

$$\Big) \times \left(\frac{\varphi_A}{\varphi_D}\right) = \eta_{A/D} \times \varphi_{A/D}.$$ (4)

...on by γ adjusts for differences between ...otor dyes in their probability of photon ...tation and the probability that emitted ...cted. Because FRET is a ratio, the effect ...s not a constant but rather varies as a

hieve quantum yields distinct from those in the DNA samples. Correction for calculated FRET deviations, which can facilitate comparison of results from different instruments. effective but required significant effort. Normalization based on single-molecule photobleaching on how it is applied. Surprisingly, per-molecule γ-normalization reduced the peak width in se anomalous γ-values correspond to FRET outliers. Thus, molecule-to-molecule variation in t on the FRET distribution that must be considered to extract information on sample dynamics

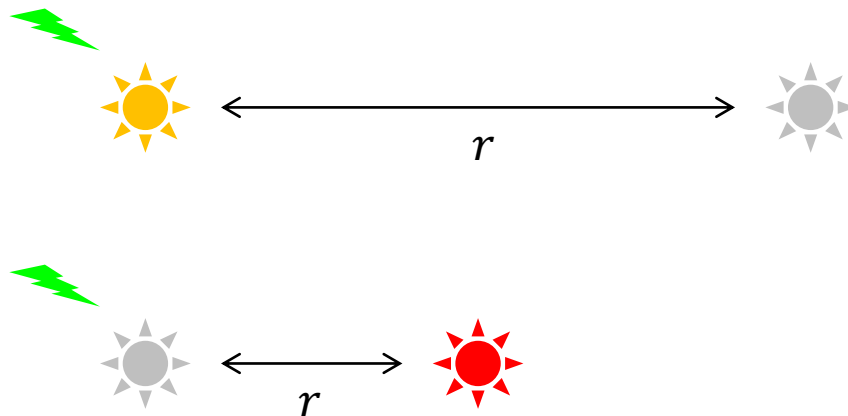# Experimental setup

FRET basic

FRET efficiency

$$E = \frac{1}{1 + (r/R_0)^6}$$

Relative proximity ratio

$$E = \frac{A}{A + D}$$

Gamma correction
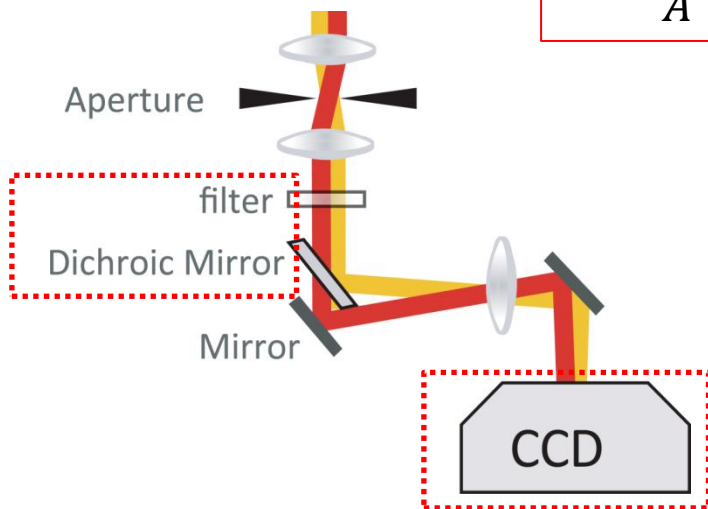
$$E = \frac{A}{A + \gamma \times D}$$

$$\gamma = \frac{\eta_A}{\eta_D} \times \frac{\phi_A}{\phi_D}$$

η : collection efficiency
ϕ : quantum yield

4 samples

3 optically different setups

$$E = \frac{A}{A + \gamma \times D}$$

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INTRODUCTION

Fluorescence resonance energy transfer (FRET) is widely thought of as a spectroscopic ruler on the nanometer scale (1,2). Many biological phenomena occur on this scale, making FRET a popular tool in biology. The efficiency of energy transfer (E) between two fluorescent dyes is related to the fluorophore separation (r) by

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}, \tag{1}$$

where R_0 is the Förster radius (3), which encompasses parameters such as spectral overlap, donor quantum yield, and the orientation of the transition dipoles.

FRET efficiency is used as a marker for colocalization and interaction, to study the magnitude of conformational changes and to calculate absolute distances. Measuring FRET using microscopy comes with a unique set of technical challenges to recover biologically relevant information. Microscopy experiments measure the donor and acceptor intensity by passing the emission through a series of optical elements to avalanche photodiode detectors or a sensitive digital camera (commonly an electron multiplied charge-coupled device (EMCCD)). The observed FRET efficiency has been called the relative proximity ratio (E_{PR}) because it

is internally consistent as long as the photophysical and instrumental properties remain unchanged (4). E_{PR} is determined from the measured intensities (I) of the donor (D) and acceptor (A):

$$E_{PR} = \frac{I_A}{I_A + I_D}. \tag{2}$$

Because E_{PR} varies with fluorophore separation, it is useful for drawing conclusions about the timescale and magnitude of structural changes and molecular associations. However, it is often desirable to recover the true FRET efficiency (E) separated from instrument and photophysical effects. The measured intensity values must be corrected as

$$E = \frac{I_A - \beta I_D}{((I_A - \beta I_D) + \gamma I_D)}, \tag{3}$$

where βI_D corrects for leakage of donor emission into the acceptor channel (5). The parameter γ accounts for differences between the donor and acceptor in detection efficiency (η) and quantum yield (φ) (6,7):

$$\gamma = \left(\frac{\eta_A}{\eta_D}\right) \times \left(\frac{\varphi_A}{\varphi_D}\right) = \eta_{A/D} \times \varphi_{A/D}. \tag{4}$$

Thus, normalization by γ adjusts for differences between the donor and acceptor dyes in their probability of photon emission upon excitation and the probability that emitted photons will be detected. Because FRET is a ratio, the effect of γ-normalization is not a constant but rather varies as a

3 setups
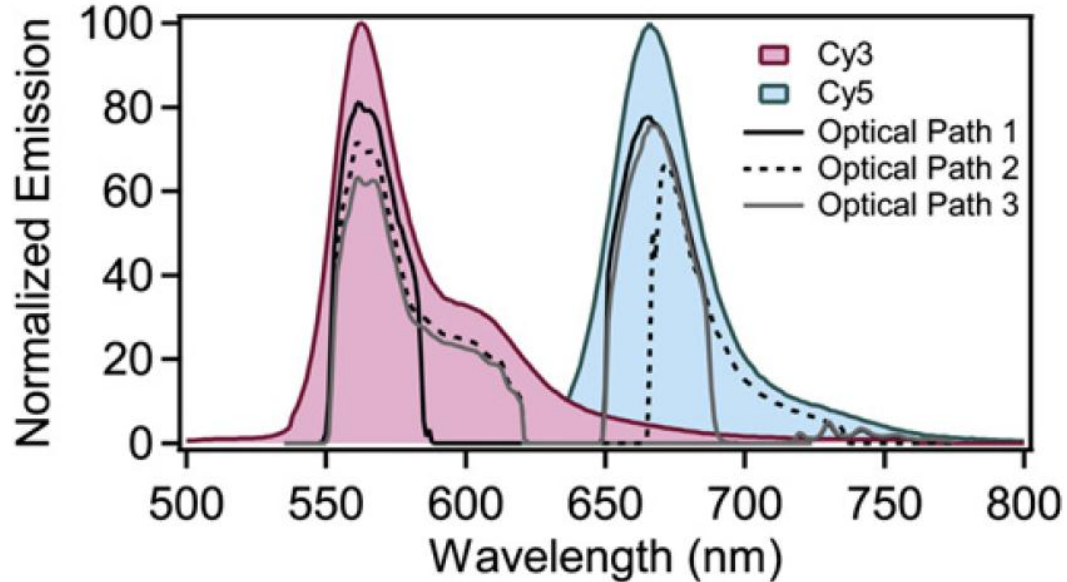
Optical Path 1 (Andor, ixon)
550 LP filter(chroma)
593 dichroic (semrock)
562/40 BP (semrock) / 670/30 BP (semrock)

Optical Path 2 (Roper, Cascade)
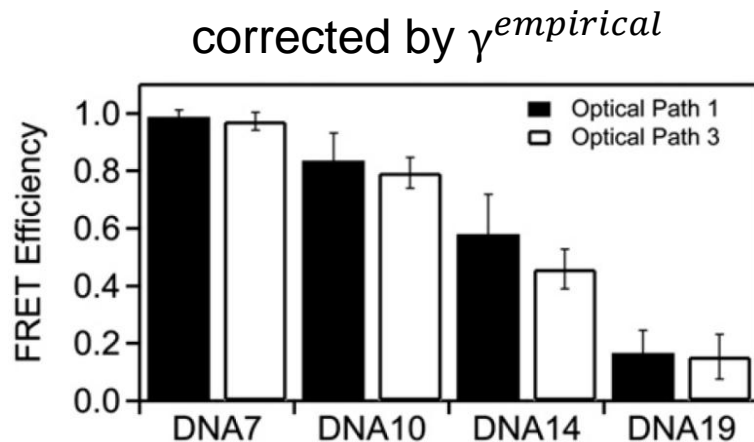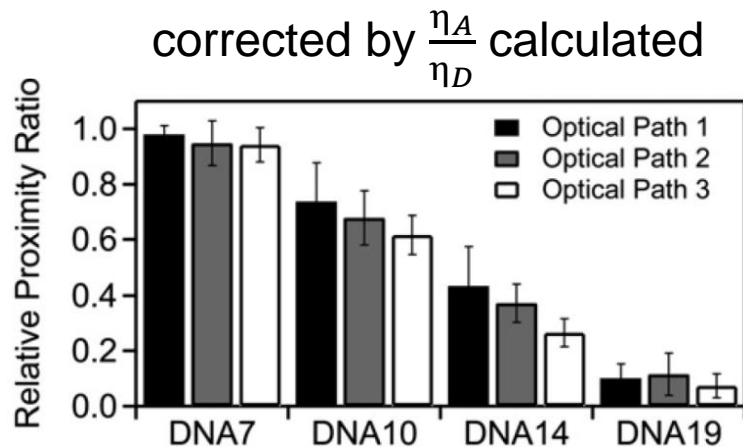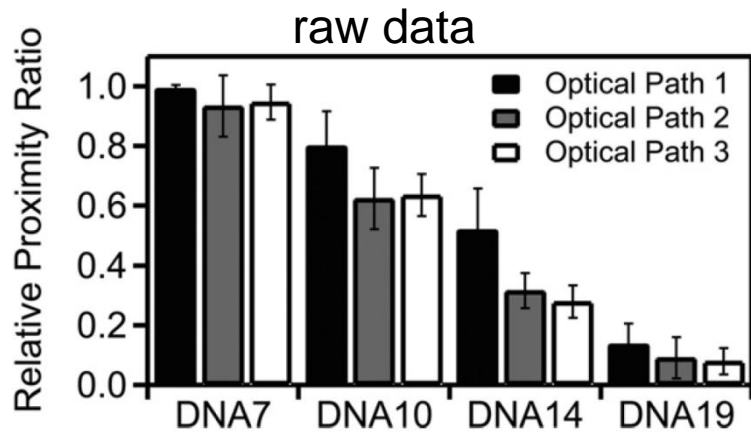645 dichroic (chroma)
585/70 BP (chroma) / 700/75 BP (chroma)

Optical Path 3 (Andor, ixon)
550 LP (chroma)
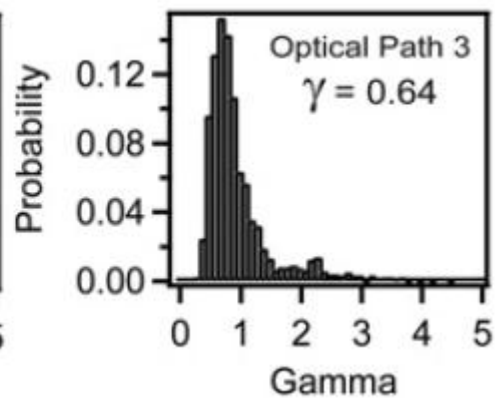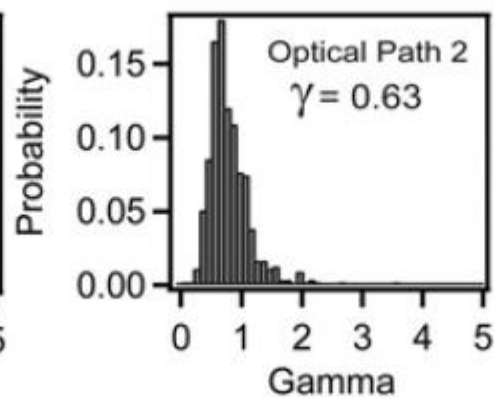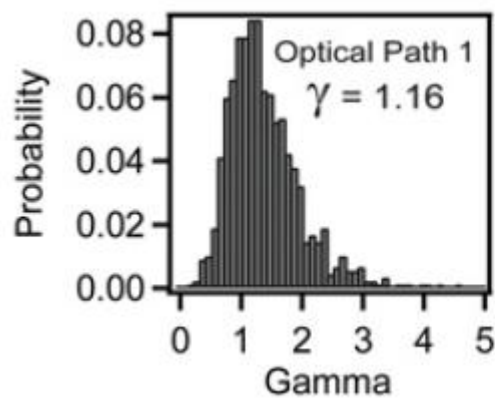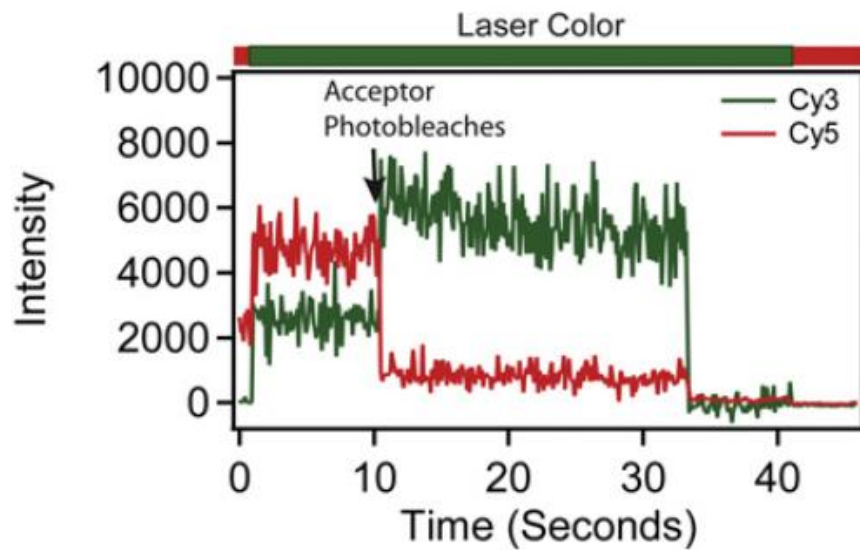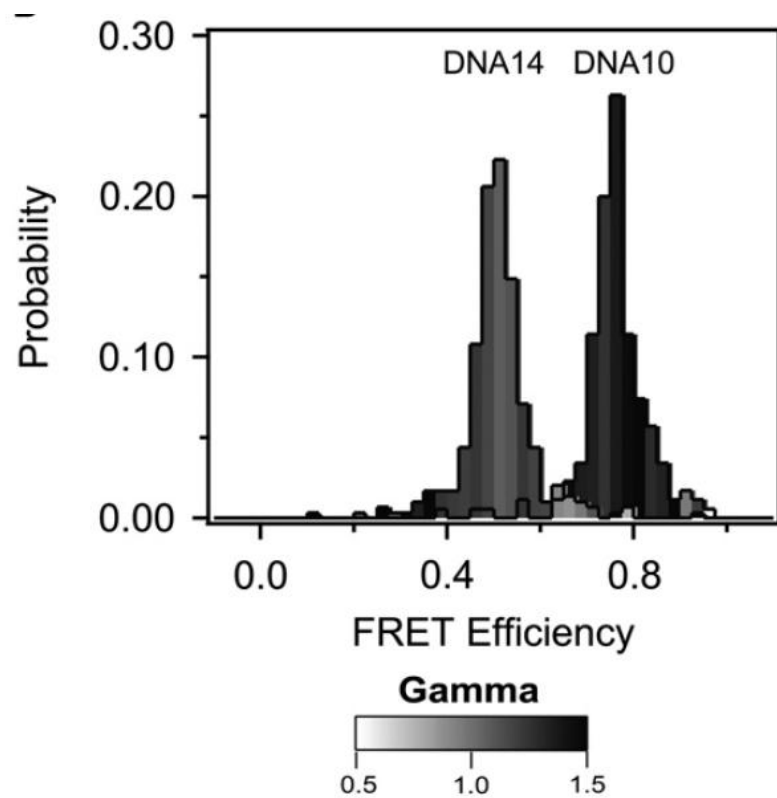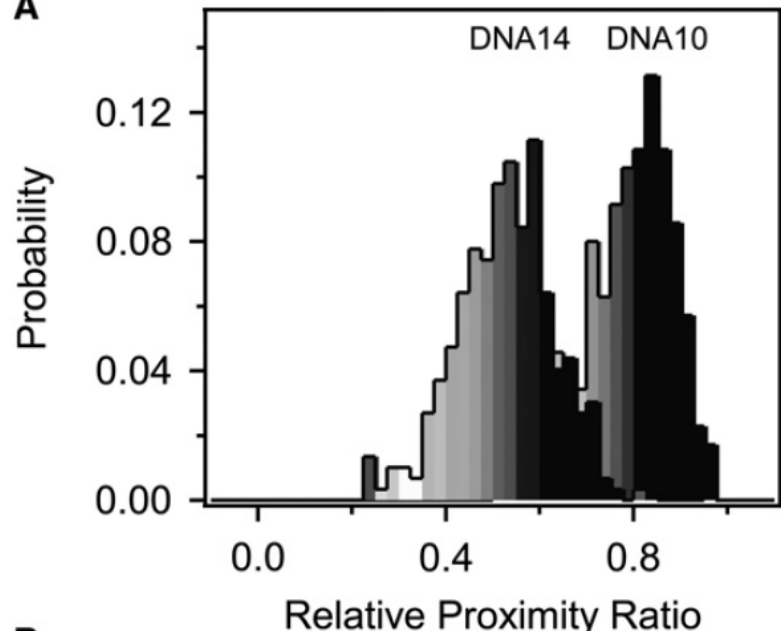645 dichroic (chroma)
585/70 BP (chroma) / 670/30 BP (semrock)

$\frac{\eta_A}{\eta_D}$ can be calculated based on this calculated spectrum

raw data
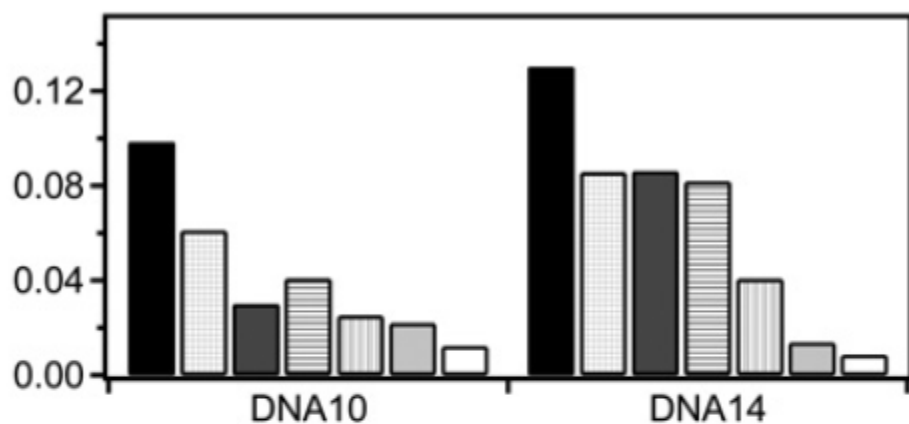
Relative Proximity Ratio

- ■ Optical Path 1
- ▨ Optical Path 2
- ☐ Optical Path 3

DNA7   DNA10   DNA14   DNA19

Cy5

5′ GGACTGCCGCCTGGGGAGCCGCACGACGACACGACAAAG 3′

|||||||||||||||||||||||||||||||||||||||

3′ CCTGACGGCGGACCCCTCGGCGTGCTGCTGTGC 5′

Cy3 Cy3 Cy3   Cy3
7   10 14   19

corrected by $\frac{\eta_A}{\eta_D}$ calculated

Relative Proximity Ratio

- ■ Optical Path 1
- ▨ Optical Path 2
- ☐ Optical Path 3

DNA7   DNA10   DNA14   DNA19

corrected by $\gamma^{empirical}$

FRET Efficiency

- ■ Optical Path 1
- ☐ Optical Path 3

DNA7   DNA10   DNA14   DNA19

$\gamma^{photobleach}$

A

A — Standard Deviation of Mean FRET Efficiency for DNA10 and DNA14

B — Mean Peak Width for DNA10 and DNA14

Normalization Method

- ■ Raw
- ⊞ Filter Trans. $\eta_{A/D}$
- ■ Empical $\eta_{A/D}$
- ⊟ $\gamma^{Empirical}$
- ⊡ $\gamma^{Universal}$ (Photobleach)
- ▨ γ^{Global} (Photobleach)
- ☐ $\gamma^{Individual}$ (Photobleach)
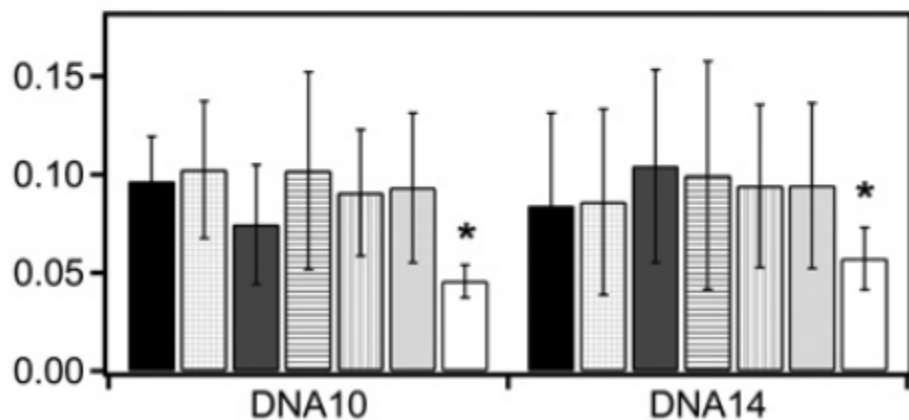
mean and std from three optical paths
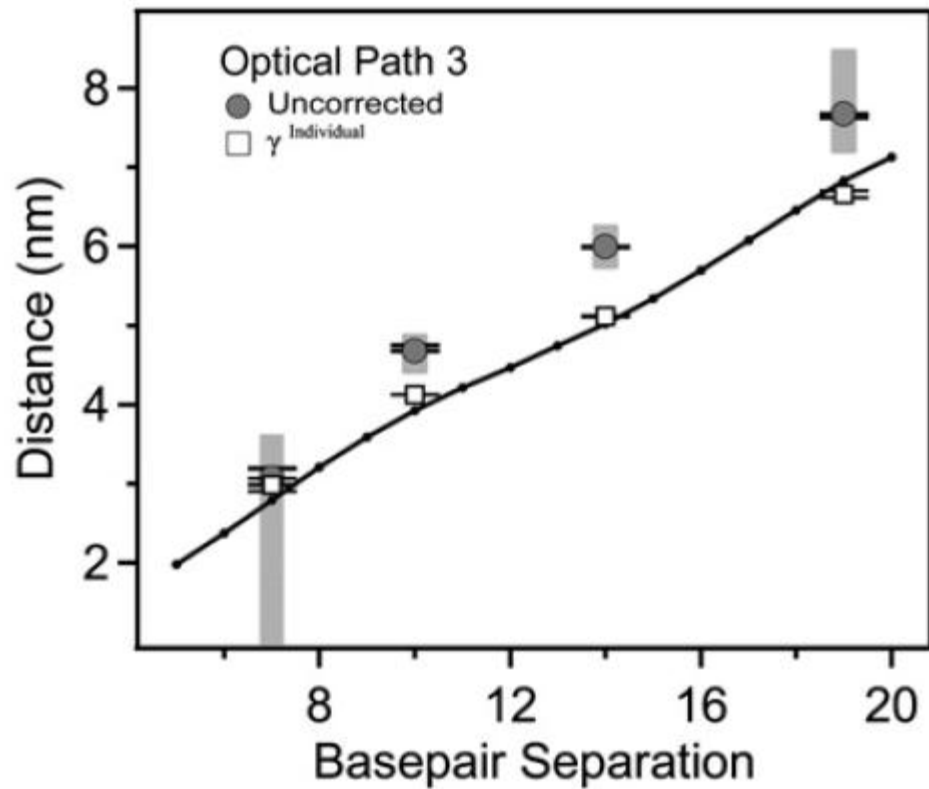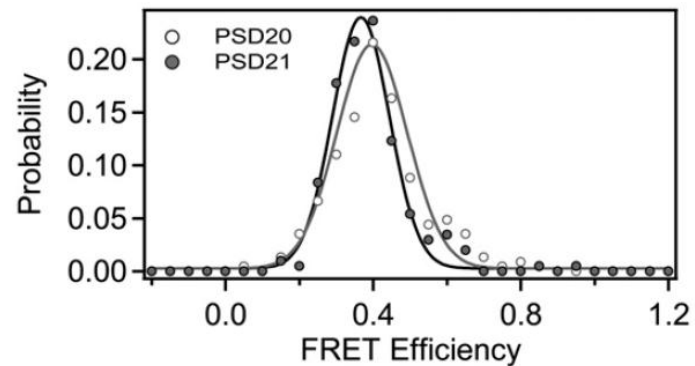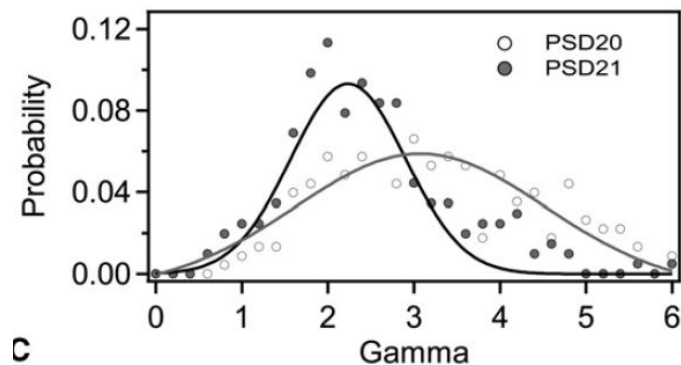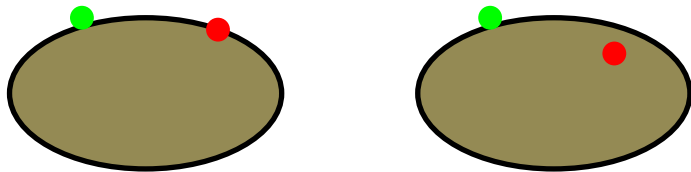
Recovering the absolute FRET

Two mutant protein with different labeling.
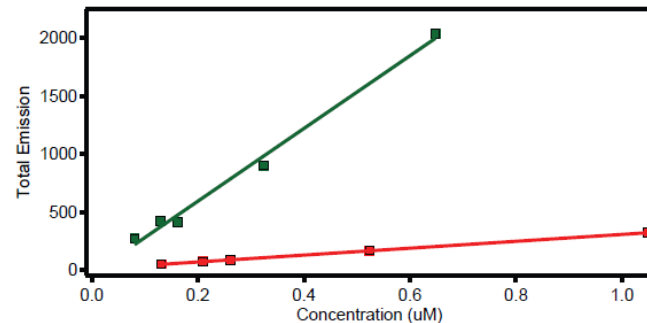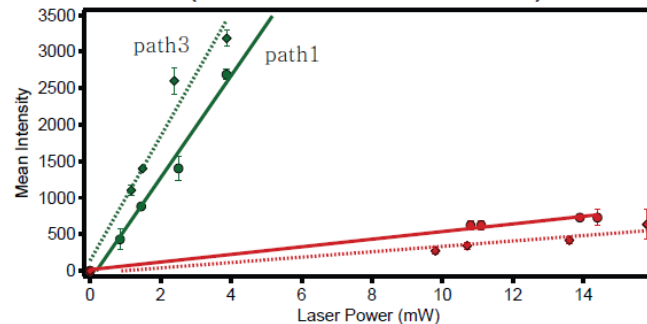Dye separation for each mutant is similar.
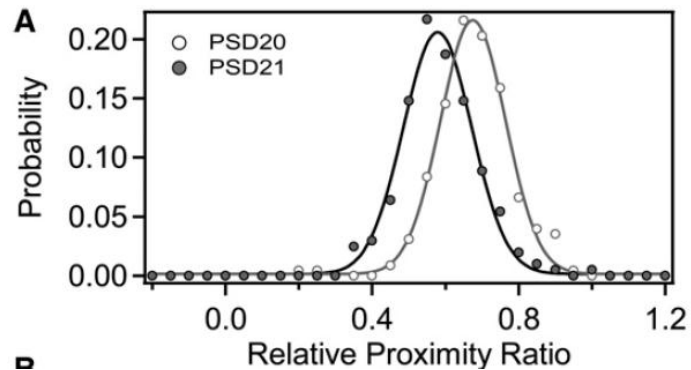
Supplements

$\gamma^{empirical}$

$$\gamma = \frac{\eta_A}{\eta_D} \times \frac{\phi_A}{\phi_D}$$

$$\frac{\dfrac{m_A{}^{SM}}{m_A{}^{Ensemble}}}{\dfrac{m_D{}^{SM}}{m_D{}^{Ensemble}}} = \frac{\dfrac{\eta_A \times \phi_A}{\eta^{Ensemble} \times \phi_A \times I_{ex}}}{\dfrac{\eta_D \times \phi_D}{\eta^{Ensemble} \times \phi_D \times I_{ex}}} = \frac{\eta_A}{\eta_D}$$
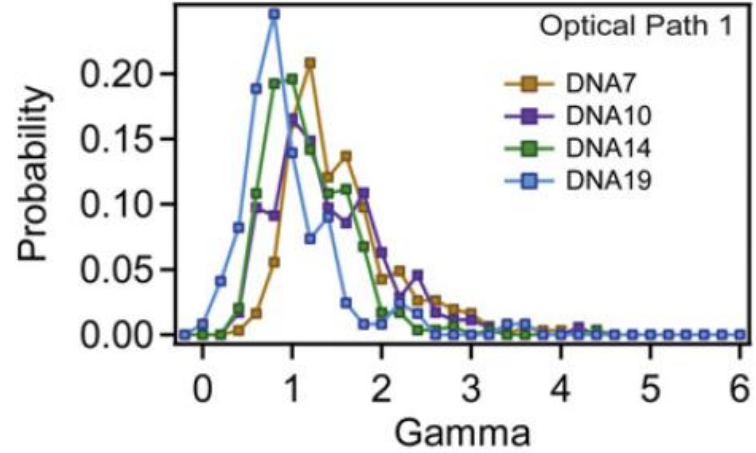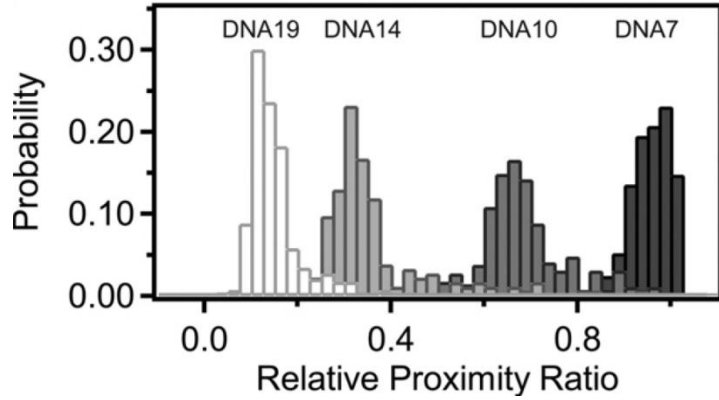
$$F = \eta \times \phi_A \times I_{ex} \times C$$

$$m^{Ensemble} = \eta^{Ensemble} \times \phi \times I_{ex}$$

$$m^{SM} = \eta \times \phi$$

A — Cy Dyes on DNA (532 nm Excitation)

ϕ_A and ϕ_D can be measured with reference sample Rhodamine etc.

optical path 3

Leakage correction

$$E = \frac{(A - L \times D)}{(A - L \times D) + (D + L \times D)} = \frac{(A - L \times D)}{A + D}$$

$$E = \frac{(A - L \times D)}{(A - L \times D) + \gamma \times (D + L \times D)}$$