Electronic Supplementary Information

PET and FRET Utility of an Amino Acid Pair: Tryptophan and 4-Cyanotryptophan

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Experimental Methods

**Förster Distance Calculation**

The Förster distance ($R_0$) of the Trp (donor) and 4CN-Trp$^1$ (acceptor) FRET pair was calculated using the following equation:

$$R_0^6 = \left( \frac{9000 ( \ln 10 ) \kappa^2 Q_D}{128 \pi^5 N \eta^4} \right) J(\lambda), \quad (S1)$$

where $\kappa^2$ is the orientation factor, assumed to be 2/3, $Q_D$ is the fluorescence quantum yield of the donor in the absence of the acceptor which is 0.14 for Trp in water,$^3,4$ $N$ is the Avogadro’s number, $\eta$ is the refractive index of the medium (1.33 for water), and $J(\lambda)$ is the overlap integral, determined by the following equation:

$$J(\lambda) = \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda, \quad (S2)$$

where $F_D(\lambda)$ is the area-normalized emission spectrum of the donor and $\varepsilon_A(\lambda)$ is the wavelength-dependent molar absorption coefficient of the acceptor.

**Stern-Volmer Titration and Numerical Fitting**

Static fluorescence quenching experiments ($\lambda_{ex} = 330$ nm) was carried out using free 4CNI-3AA and NATA. To determine the underlying quenching rate$^5$, we employed a diffusion model as described in detail elsewhere$^6,7$ to numerically fit the experimental Stern-Volmer curve (Figure 4 in the main text). Briefly, the decaying fluorescence signal $I(t)$ is described by:

$$I(t) = I_0 \exp \left( - \frac{t}{\tau_0} - C_0^0 \int_0^t k(t') dt' \right), \quad (S3)$$

where $\tau_0$ is the fluorescence lifetime of the fluorophore, $C_0^0$ is the quencher bulk concentration, and $k(t)$ is the time-dependent quenching rate which is calculated using:

$$k(t) = 4\pi \int_{C_0^Q a_0}^\infty r^2 k_0(r) C_Q(r,t) dr, \quad (S4)$$

In the above equation, $C_Q(r,t)$ is the concentration of quencher at distance $r$ from the fluorophore at time $t$, and $k_0(r)$ is the distance-dependent quenching rate, defined as:

$$k_0(r) = k_0 e^{-\beta(r-a_0)}, \quad (S5)$$

where $k_0$ is the quenching rate when the pair are in van der Waals contact (when $r = a_0$), and $\beta$ is a constant. For this study, the value of $a_0$ was set to 7.0 Å, which corresponds to the sum of the
van der Waals radii of two tryptophan sidechains. By defining a normalized concentration of the quencher as:

\[ y(r,t) = \frac{C_Q(r,t)}{C_Q^0} \tag{S6} \]

we can model the time- and distance-dependent quenching using the following equation:

\[ \frac{\partial}{\partial t}y(r,t) = -D\nabla^2y(r,t) - k_Q(r)y(r,t). \tag{S7} \]

We numerically solved for \( C_Q(r,t) \) using the following initial conditions and boundary conditions:

\[ y(r,t = 0) = 1, \tag{S8} \]
\[ \left( \frac{\partial}{\partial t}y(r,t) \right)_{r = a_0} = 0, \tag{S9} \]
\[ \lim_{r \to \infty} y(r,t) = 1, \tag{S10} \]

and subsequently obtained \( k(t) \) and \( I(t) \) for each bulk quencher concentration \( C_Q^0 \). For the Stern-Volmer data presented in Figure 4, the best fit yielded the following parameters: \( k_0 = 6.8 \text{ ns}^{-1} \), \( \beta = 1.3 \text{ Å}^{-1} \), and \( a_0 = 7.0 \text{ Å} \).

**Molecular Dynamics Simulations**

Molecular dynamics (MD) simulations on the 4CN-Trp-Trp peptide were carried out to characterize its conformational distribution in aqueous solution. First, the force field parameters for the 4-cyanoindole moiety were determined using the Force Field Toolkit Plugin\(^8\) v1.1 for VMD\(^9\) v1.9.4 with the initial charge, bond, and angle parameters from CHARMM36\(^10\) and CGenFF v4.1 for Small Molecule Drug Design.\(^11\) The peptide model was built using Vega ZZ\(^12\) v3.1.1 with an initial structure of a fully extended conformation (\( \phi = \varphi = 180^\circ \)) and then solvated using the Automatic PSF Generation Plugin v.1.3 in VMD. Simulations were carried out using the NAMD\(^13\) v.2.12 software package. Following a 1 ns equilibration run at 298 K and 1 atm in the NPT ensemble, subsequent production runs totaling 150 ns were performed on the equilibrated system at 298 K in the NVT ensemble. A trajectory was built by saving a snapshot every 500 fs, resulting in 300,000 total frames. Analysis of the MD trajectory was accomplished using VMD; for each frame, the minimum distance between any atoms in the 4-cyanoindole ring and the indole ring in the peptide was determined and used for constructing the distance distribution plot.
**Table S1.** Fluorescence lifetime (τ) and relative amplitude (A) determined from fitting the fluorescence decay of each peptide in Figure S1 to a single or bi-exponential function. The data for 4CN-Trp-Met were taken from Ref. 1.

<table>
<thead>
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<th>Peptide Name</th>
<th>Peptide Sequence</th>
<th>τ (ns)</th>
<th>A (%)</th>
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**Figure S1.** Absorption spectra of Gly-Trp-Gly (33 µM) and 4CN-Trp-Gly (33 µM) in water, as indicated.
Figure S2. Fluorescence decay kinetics of 4CN-Trp-X peptides, as indicated. In each case, the smooth black line corresponds to the best fit of the respective data to a single-exponential or a bi-exponential function and the resultant lifetime(s) is given in Table S1. Residuals of the fits are on top of each respective panel.
Figure S3. Fluorescence decay kinetics of 4CN-Trp-Gly, 4CN-Trp-Pro-Pro-Trp, 4CN-Trp-Pro-Trp, and 4CN-Trp-Trp, as indicated. The smooth line in each case correspond to the best fit of the data to either a single-exponential function (4CN-Trp-Gly and 4CN-Trp-Pro-Pro-Trp) or a bi-exponential function (4CN-Trp-Pro-Trp and 4CN-Trp-Trp) and the resultant lifetime(s) are listed in Table 1 of the main text.

Figure S4. The trajectory of the separation distance between the two fluorophores in the 4CN-Trp-Trp peptide obtained from the MD simulations.
**Figure S5.** Representative structures of the two conformational populations of the 4CN-Trp-Trp peptide. The distances between the 4-cyanoindole and indole moieties are 2.96 Å (A) and 6.62 Å (B), respectively.

**References**