**Figure 3 - Figure supplement 1A. Details for MFD Instruments Used in this Study.** Correction parameters for MFD that are specified for each experimental setup. The setup used to measure each variant is indicated in Supplementary File 3B. HHU stands for Heinrich Heine University.

|  |  |  |
| --- | --- | --- |
| Parameter | Setup 1 (Clemson) | Setup 2 (HHU) |
| Det. Efficiency Ratio (G/R) | 3.7 | 0.8 |
| Green Power (485 nm) | 80 µW | 60 µW |
| Red Power (640 nm) | 32 µW | 10 µW |
| PIE Repetition Rate | 20 MHz | 32 MHz |
| Repetition Time | 50.00 ns | 31.25 ns |
| Direct Acceptor Excitation, δ | 2.2 | 1.3 |
| Spectral Crosstalk, G→R, α | 1.7 | 1.7 |

**Figure 3 - Figure supplement 1B. Correction Parameters for Multiparameter Fluorescence Detection.** Correction parameters determined for each FRET variant based on buffer measurements for backgrounds, donor-only and directly-excited acceptor fluorescence for quantum yields (QYG, QYR), and standard stock fluorophore solutions for G-factors. Parameters constant for all samples measured on a given setup are summarized at the end. Details of variants can be found in Figure 1 and Table 1. HHU stands for Heinrich Heine University.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| FL Variant | QYG | QYR | BGG(D) (kHz) | BGR(D) (kHz) | BGR(A) (kHz) | G-factor | Setup |
| P2-G6 | 0.72 | 0.26 | 0.85 | 0.45 | 0.30 | 1.06 | HHU |
| P3-G4 | 0.75 | 0.53 | 0.54 | 0.21 | 0.38 | 1.06 | HHU |
| P2-G1 | 0.72 | 0.38 | 0.53 | 0.18 | 0.17 | 0.83 | Clemson |
| P3-G5 | 0.77 | 0.46 | 0.51 | 0.16 | 0.17 | 0.83 | Clemson |
| P1-G3 | 0.71 | 0.37 | 0.38 | 0.14 | 0.15 | 0.83 | Clemson |
| P1-G4 | 0.76 | 0.39 | 0.41 | 0.15 | 0.15 | 0.83 | Clemson |
| P1-G2 | 0.75 | 0.37 | 0.54 | 0.20 | 0.19 | 0.83 | Clemson |
| P3-S2 | 0.81 | 0.43 | 0.50 | 0.16 | 0.17 | 0.83 | Clemson |
| P2-S2 | 0.68 | 0.26 | 0.78 | 0.45 | 0.36 | 1.06 | HHU |
| S2-G6 | 0.70 | 0.26 | 0.74 | 0.39 | 0.29 | 1.06 | HHU |
| S3-G1 | 0.71 | 0.44 | 0.51 | 0.26 | 0.40 | 1.06 | HHU |
| S1-G1 | 0.73 | 0.49 | 0.51 | 0.26 | 0.40 | 1.06 | HHU |
| PSG Variant | QYG | QYR | BGG(D) (kHz) | BGR(D) (kHz) | BGR(A) (kHz) | G-factor | Setup |
| P2-G1 | 0.72 | 0.31 | 0.56 | 0.18 | 0.17 | 0.83 | Clemson |
| P3-G5 | 0.72 | 0.35 | 0.51 | 0.16 | 0.17 | 0.83 | Clemson |
| P1-G3 | 0.74 | 0.40 | 0.38 | 0.14 | 0.15 | 0.83 | Clemson |
| P1-G4 | 0.69 | 0.39 | 0.41 | 0.15 | 0.15 | 0.83 | Clemson |
| P1-G2 | 0.75 | 0.35 | 0.53 | 0.19 | 0.17 | 0.83 | Clemson |
| P3-S2 | 0.77 | 0.38 | 0.50 | 0.16 | 0.17 | 0.83 | Clemson |

**Figure 3 - Figure supplement 1C. Fit Parameters from seTCSPC using a Two State Model.** Species fluorescent lifetimes were used to estimate effective fluorescence-averaged lifetimes accounting for fluorophore linker dynamics. Details on model function and definition of parameters can be found in *Materials and Methods*. Information about variants is found in Figure 1 and Table 1.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| FL Variant | Donor Fraction | Scattering Amplitude | τD(0) |  | rD|D,∞ | rA|A,∞ | rA|D,∞ | Species Fraction xI | Species Fraction xII | τD(A),xI | τD(A),xII | τD(A),fI | τD(A),fII | χ,seTCSPC2 |
| P2-G6 | 0.02 | 0.07 | 3.60 | 0.72 | .09 | .14 | .07 | 0.46 | 0.54 | 2.73 | 0.39 | 2.78 | 0.65 | 2.54 |
| P3-G4 | 0.00 | 0.17 | 3.76 | 0.82 | .18 | .14 | .08 | 0.46 | 0.54 | 2.67 | 0.53 | 2.75 | 0.83 | 1.49 |
| P2-G1 | 0.01 | 0.04 | 3.59 | 0.85 | .14 | .19 | <.01 | 0.46 | 0.54 | 3.41 | 0.46 | 3.41 | 0.74 | 1.22 |
| P3-G5 | 0.15 | 0.12 | 3.86 | 0.85 | .14 | .21 | .02 | 0.46 | 0.54 | 2.45 | 0.33 | 2.57 | 0.59 | 1.13 |
| P1-G3 | 0.39 | 0.09 | 3.56 | 0.83 | .19 | .13 | .01 | 0.46 | 0.54 | 3.31 | 0.97 | 3.31 | 1.25 | 2.12 |
| P1-G4 | 0.11 | 0.05 | 3.79 | 0.89 | .15 | .22 | .08 | 0.46 | 0.54 | 3.58 | 1.13 | 3.58 | 1.41 | 2.11 |
| P1-G2 | 0.00 | 0.05 | 3.77 | 0.83 | .12 | .20 | .04 | 0.46 | 0.54 | 3.44 | 0.15 | 3.45 | 0.34 | 1.15 |
| P3-S2 | 0.02 | 0.0 | 4.04 | 0.88 | .19 | .25 | .02 | 0.46 | 0.54 | 0.28 | 2.09 | 0.37 | 2.27 | 0.45 |
| P2-S2 | 0.00 | 0.20 | 3.38 | 0.71 | .04 | .20 | .04 | 0.46 | 0.54 | 0.41 | 2.03 | 0.67 | 2.14 | 1.96 |
| S2-G6 | 0.00 | 0.16 | 3.50 | 0.72 | .11 | .13 | <.01 | 0.46 | 0.54 | 0.33 | 1.80 | 0.58 | 1.97 | 0.92 |
| S3-G1 | 0.00 | 0.20 | 3.56 | 0.77 | .14 | .05 | .01 | 0.46 | 0.54 | 0.80 | 2.95 | 1.09 | 2.98 | 1.84 |
| S1-G1 | 0.00 | 0.12 | 3.64 | 0.79 | .18 | .14 | .09 | 0.46 | 0.54 | 2.96 | 0.76 | 2.99 | 1.06 | 1.61 |
| PSG  Variant | Donor Fraction | Scattering Amplitude | τD(0) | κ2 | rD|D,∞ | rA|A,∞ | rA|D,∞ | Species Fraction xI | Species Fraction xII | τD(A),xI | τD(A),xII | τD(A),fI | τD(A),fII | χr,seTCSPC2 |
| P2-G1 | 0.00 | 0.09 | 3.60 | 0.79 | .14 | .08 | .07 | 0.52 | 0.49 | 3.01 | 0.65 | 3.03 | 0.94 | 0.94 |
| P3-G5 | 0.04 | 0.10 | 3.59 | 0.85 | .14 | .25 | .02 | 0.52 | 0.49 | 1.93 | 0.36 | 2.09 | 0.62 | 0.85 |
| P1-G3 | 0.11 | 0.06 | 3.72 | 0.84 | .19 | .16 | .01 | 0.52 | 0.49 | 3.57 | 0.92 | 3.57 | 1.22 | 2.14 |
| P1-G4 | 0.05 | 0.06 | 3.46 | 0.87 | .14 | .25 | .07 | 0.52 | 0.49 | 3.38 | 0.66 | 3.38 | 0.94 | 1.37 |
| P1-G2 | 0.00 | 0.06 | 3.77 | 0.83 | .14 | .18 | .04 | 0.52 | 0.49 | 3.12 | 0.23 | 3.14 | 0.45 | 1.64 |
| P3-S2 | 0.05 | 0.07 | 3.87 | 0.86 | .14 | .25 | .04 | 0.52 | 0.49 | 0.47 | 2.52 | 0.77 | 2.62 | 0.72 |

**Figure 3 - Figure supplement 1D. Interdye Distances from the Global Fit of seTCSPC Decays.** Distances resulting from seTCSPC fits. Model details are in *Materials and Methods*. All R0 for distance calculations taken as 52 Å, state widths set to 6 Å. Model fit parameters can be found in Supplementary File 3C. Details of variants can be found in Figure 1and Table 1. Uncertainties corresponding to 95% confidence intervals were estimated using the F-test for the ratio of χr,seTCSPC2of the final fit to the χr,seTCSPC2 under variation of each parameter independently. The number of degrees of freedom was 2663 for all curves (number of data points-number of parameters).

|  |  |  |
| --- | --- | --- |
| **FL Variant** | **(****Å)** | **(Å)** |
| P2-G6 | 64.0 ± 2.0 | 36.6 ± 4.4 |
| P3-G4 | 60.4 ± 2.9 | 38.5 ± 1.1 |
| P2-G1 | 84.7 ± 7.0 | 37.8 ± 3.8 |
| P3-G5 | 57.1 ± 3.2 | 35.0 ± 6.0 |
| P1-G3 | 79.8 ± 9.8 | 44.2 ± 8.3 |
| P1-G4 | 83.3 ± 5.2 | 45.1 ± 5.8 |
| P1-G2 | 76.9 ± 7.4 | 30.6 ± 4.5 |
| P3-S2 | 33.8 ± 6.0 | 52.6 ± 2.1 |
| P2-S2 | 37.4 ± 3.5 | 55.6 ± 1.6 |
| S2-G6 | 35.6 ± 3.4 | 52.5 ± 1.4 |
| S3-G1 | 42.3 ± 2.8 | 67.8 ± 4.2 |
| S1-G1 | 66.5 ± 2.9 | 41.7 ± 2.1 |
| FL Fraction | 46.1% | 53.9% |
| **PSG Variant** | **〈RDA,A〉(Å)** | **〈RDA,B〉(Å)** |
| P2-G1 | 68.2 ± 6.2 | 40.4 ± 7.6 |
| P3-G5 | 53.3 ± 3.0 | 36.0 ± 5.5 |
| P1-G3 | 89.0 ± 10.5 | 43.2 ± 4.3 |
| P1-G4 | 96.5 ± 12.0 | 40.8 ± 2.0 |
| P1-G2 | 67.5 ± 2.1 | 32.9 ± 3.5 |
| P3-S2 | 37.4 ± 3.9 | 57.7 ± 5.7 |
| PSG Fraction | 51.8% | 48.2% |

**Figure 3 - Figure supplement 1E. Parameters for Calculation of Static FRET-Lines in MFD Plots.** The static FRET-lines are calculated using these parameters and shown for visual reference in MFD plots in Figure 3 and Figure 4–figure supplement 2. FRET-lines are corrected for dynamic averaging due to fluorophore linker dynamics via polynomials relating the species fluorescence lifetimes, τD(A),x, from seTCSPC fits and linker-movement corrected apparent fluorescence-averaged fluorescence decay lifetimes, τD(A),f. These polynomials take the form: . Polynomial coefficients are determined via simulations of state widths in the Margarita software package. Static FRET-lines describe the expected relationship between and FRET efficiency for non-dynamic populations as is varied from 0 to the donor-only lifetime. Values for are listed in Supplementary File 3C. Details of variants can be found in Figure 1 and Table 1.

The static FRET-lines take the form: .

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| FL Variant | p0 | p1 | p2 | p3 | p4 |
| P2-G6 | -0.020 | 0.362 | 0.465 | -0.109 | 0.008 |
| P3-G4 | -0.020 | 0.368 | 0.442 | -0.099 | 0.007 |
| P2-G1 | -0.020 | 0.362 | 0.466 | -0.109 | 0.008 |
| P3-G5 | -0.021 | 0.372 | 0.430 | -0.094 | 0.006 |
| P1-G3 | -0.019 | 0.361 | 0.471 | -0.111 | 0.008 |
| P1-G4 | -0.021 | 0.369 | 0.438 | -0.098 | 0.007 |
| P1-G2 | -0.020 | 0.369 | 0.441 | -0.099 | 0.007 |
| P3-S2 | -0.022 | 0.378 | 0.407 | -0.086 | 0.005 |
| P2-S2 | -0.018 | 0.353 | 0.500 | -0.124 | 0.009 |
| S2-G6 | -0.019 | 0.358 | 0.481 | -0.115 | 0.008 |
| S3-G1 | -0.019 | 0.361 | 0.471 | -0.111 | 0.008 |
| S1-G1 | -0.020 | 0.364 | 0.459 | -0.106 | 0.008 |
| PSG Variant | p0 | p1 | p2 | p3 | p4 |
| P2-G1 | -0.020 | 0.362 | 0.465 | -0.109 | 0.008 |
| P3-G5 | -0.020 | 0.362 | 0.466 | -0.109 | 0.008 |
| P1-G3 | -0.020 | 0.367 | 0.448 | -0.102 | 0.007 |
| P1-G4 | -0.019 | 0.357 | 0.486 | -0.118 | 0.009 |
| P1-G2 | -0.020 | 0.369 | 0.441 | -0.099 | 0.007 |
| P3-S2 | -0.021 | 0.372 | 0.427 | -0.093 | 0.006 |

**Figure 3 - Figure supplement 1F. Parameters for Calculation of Dynamic FRET-Lines in MFD Plots.** The dynamic FRET-lines are calculated using these parameters and shown for visual reference in MFD plots in Figure 3 and Figure 4–figure supplement 2. Two-State dynamic FRET-lines describe the path in FRET efficiency vs plots on which populations exhibiting fractional mixing between two limiting, Gaussian-distributed states would fall. Dynamic lines are corrected like static lines for linker dynamics. Lifetimes for limiting states are taken from seTCSPC analysis (Figure 3A and Supplementary File 3C). Details of variants can be found in Figure 1 and Table 1.

Dynamic FRET-lines take the form: .

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Variant | p0 | p1 | p2 | p3 |
| P2-G6 | -2.499 | 1.894 | 0.000 | 0.000 |
| P3-G4 | -2.134 | 1.768 | 0.000 | 0.000 |
| P2-G1 | -2.608 | 1.767 | 0.000 | 0.000 |
| P3-G5 | -2.653 | 2.023 | 0.000 | 0.000 |
| P1-G3 | -1.355 | 1.400 | 0.000 | 0.000 |
| P1-G4 | -1.482 | 1.414 | 0.000 | 0.000 |
| P1-G2 | -4.946 | 2.435 | 0.000 | 0.000 |
| P3-S2 | -3.337 | 2.453 | 0.000 | 0.000 |
| P2-S2 | -1.984 | 1.907 | 0.000 | 0.000 |
| S2-G6 | -2.118 | 2.049 | 0.000 | 0.000 |
| S3-G1 | -1.675 | 1.560 | 0.000 | 0.000 |
| S1-G1 | -1.763 | 1.586 | 0.000 | 0.000 |
| Variant | p0 | p1 | p2 | p3 |
| P2-G1 | -1.955 | 1.641 | 0.000 | 0.000 |
| P3-G5 | -2.146 | 2.003 | 0.000 | 0.000 |
| P1-G3 | -1.713 | 1.479 | 0.000 | 0.000 |
| P1-G4 | -2.014 | 1.597 | 0.000 | 0.000 |
| P1-G2 | -3.712 | 2.179 | 0.000 | 0.000 |
| P3-S2 | -2.247 | 1.844 | 0.000 | 0.000 |