Dynamics of the nucleosomal histone H3 N-terminal tail revealed by high precision single-molecule FRET

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Supplementary Methods

DNA sequence and labeling positions

The sequences of the used 170 bp and 210 bp DNA and acceptor positions (in red) are shown below. Dyad axis is marked with an asterisk (*). To account for the non-palindromic nature of the Widom 601 sequence, we call the left and the right side of the DNA α- and β-side, respectively. Base pairs on α-side are counted with negative numbers from the dyad axis; base pairs on β-side with positive numbers.

**Dyα**: Alexa 594 at - 9 bp, DNA length 170 bp

**Forward strand**:

\[
\text{5'}- \text{CATGCACAG GATGTATATA TCTGACACGT GCCTGGAGAC TAGGGAGTAA TCCCTTGGGC GGTTAAAACG}
\]

\[
\text{CTGGGAGAC GCGTACGTG CGTTTAAGCG GTGCTAGAGC TGTCTACGAC CAATTGAGCG GCCTCGGCAC CCGGATTCTC CAGGGCGGCC CGCATATCCC T-3'}
\]

**Dyβ**: Alexa 594/Cy5 at + 21 bp, DNA length 170 bp

**Reverse strand**:

\[
\text{3'}- \text{AGACTGTGCA GCAATTGCG CCGACCTCTG ATCCCTCATT ATCCCTCATT ACAGATGCTG TGTCTACGAC TACGATCTCG ACAGATGCTG GTTAACTCGC CGGAGCCGTG CCATTTTTGC GCCCCCTGTC GCTACGTGTCC GTACGTGTGC T-5'}
\]

**Eα**: Alexa 594/Cy5 at – 77 bp, DNA length 170 bp

**Forward strand**:

\[
\text{5'}- \text{CATGCACAG GATGTATATA TCTGACACGT GCCTGGAGAC TAGGGAGTAA TCCCTTGGGC GGTTAAAACG}
\]

\[
\text{CTGGGAGAC GCGTACGTG CGTTTAAGCG GTGCTAGAGC TGTCTACGAC CAATTGAGCG GCCTCGGCAC CCGGATTCTC CAGGGCGGCC CGCATATCCC T-3'}
\]

**Lα(170)**: Alexa 594/Cy5 at – 77 bp, DNA length 210 bp

**Forward strand**:

\[
\text{5'}- \text{CAAGGACGTT CTGATCTGATCTG TGTCTAGGAC TAGGGAGTAA TCGCTACGTGC GCCTGGAGAC TCCCTTGGGC GGTTAAAACG}
\]

\[
\text{TCCCCTTGGGC GCTTAAACGT CAGGGACAG TCGATTCTC CAGGGCGGCC CGCATATCCC TCCCTTGGGC GGTTAAAACG T-5'}
\]
Supplementary Figures

Supplementary Figure 1

Supplementary Figure 1. MFD-PIE analysis of Donor only (nucleosomes with unlabeled DNA) and double labeled mutDyβ nucleosomes. Stoichiometry, $S_{\text{PIE}}$, versus FRET efficiency, $E_{\text{FRET}}$, of data obtained from (A) 40 pM $\alpha_n$ nucleosomes plus 260 pM unlabeled nucleosomes at 5 mM NaCl and (B) 40 pM mutDyβ nucleosomes plus 1960 pM unlabeled nucleosomes at 100 mM NaCl. (C) Histogram of the donor fluorescence intensity decay from selected D1A bursts (red line) and best fit model curve (black line, lower left panel). (D) Histogram of the donor fluorescence intensity decay from selected D2A bursts (green line) and best fit
model curve (black line, lower left panel). (E) Histogram of the donor fluorescence intensity decay from selected D1 D2A bursts (blue line) and best fit model curve (black line, lower left panel). The decay histograms are analyzed by the M2 model. The weighted residuals shown on top (gray line) with the reduced sum of the weighted squared deviation between the model and the data of the decay histogram, $\chi^2_\nu$. Model distance distributions are shown on right panels. Subspecies (D1A, D2A and D1D2A) positions indicated with circles are kept as in Fig. 2A. FRET bursts selection is indicated by wine colored rectangle.
Supplementary Figure 2. All potential quenchers in the nucleosome crystal structure (PDB ID: 1KX5) with full histone H3NtT tails. Fluorescence of Alexa dyes can be quenched by interactions with Trp, Tyr, Met, and His residues through a combination of static and dynamic quenching mechanisms (1). Donor dyes attachment amino acids C/9 (A) and G/9 (B) on the H3NtT are shown with magenta and blue color, respectively. Acceptor dye attachment nucleotide B/21 on DNA is shown in red color.
Supplementary Figure 3. seTCSPC decay histograms generated from selected bursts of molecules between D-only (gray region) and FRET (wine rectangle) selections on Fig. 2A (dashed blue rectangle, 0.8 < $S_{PE}$ < 0.9). Decay histograms for photons detected in the donor detection channels are shown as dark and bright green lines and for photons detected in the acceptor channels are shown as red and orange lines (parallel and perpendicular channels, respectively). The existence of a second delayed decay in the acceptor detection channels proves that the selected molecules carry both donor and acceptor bright dyes (on the acceptor decay histograms acceptor emission after direct excitation by the delayed red laser pulse around 17 ns is visible).
Supplementary Figure 4

Independent fits of all samples. (A) Ensemble FRET analysis of distance changes between wild type H3NtT and various positions on the DNA. For visualization data were normalized to the maximal and minimal amplitude of the sigmoidal fit. (B) Ensemble FRET analysis of distance changes between H3NtT and the DNA in nucleosomes bearing two point mutations in the $\alpha 3$ domain of H2A, H2A R81E/R88E. Inflection points of the proximity curves are significantly left-shifted in mutE$\alpha$ and mutD$\alpha$ nucleosomes; curve progression is changed to a two-step behavior in mutE$\alpha$ and mutL(170), indicating allosteric effects induced by the mutation.

|                  | $E_\alpha$ | $L_\alpha$(170) | Dy$\alpha$ | Dy$\beta$ | mutE$\alpha$ | mut$L_\alpha$(170) | mutD$\alpha$ | mutD$\beta$ |
|------------------|------------|-----------------|------------|-----------|--------------|-------------------|--------------|-------------|
| $c_{1/2}$[1] [mM] | -          | -               | -          | -         | 299 ± 6      | 322 ± 17          | -            | -           |
| $c_{1/2}$[2] [mM] | .590 ± 12  | .607 ± 26       | -          | -         | -            | 697 ± 352         | -            | -           |
| $c_{1/2}$[3] [mM] | -          | -               | 819 ± 3    | 806 ± 3   | 723 ± 23     | 766 ± 69          | 744 ± 9      | 708 ± 9     |
| $b_1$ [mM]       | -          | -               | -          | -         | 28 ± 7       | 30 ± 16           | -            | -           |
| $b_2$ [mM]       | 103 ± 11   | 125 ± 25        | -          | -         | -            | 66 ± 63           | -            | -           |
| $\Delta P_1$*    | 0$^*$      | 0$^*$           | 0$^*$      | 0$^*$     | -0.72 ± 0.03 | -0.74 ± 0.13      | 0$^*$        | 0$^*$       |
| $\Delta P_2$*    | -1.00 ± 0.03 | -1.00 ± 0.07    | 0$^*$      | 0$^*$     | -0.72 ± 0.03 | -0.74 ± 0.13      | 0$^*$        | 0$^*$       |
| $\Delta P_3$*    | 0$^*$      | 0$^*$           | -1.00 ± 0.02 | -1.00 ± 0.02 | -0.28 ± 0.03 | -1.01 ± 4.3     | -1.00 ± 0.04 | -1.00 ± 0.04 |
| $P(0)^*$         | 1.00 ± 0.03 | 1.00 ± 0.05     | 1.00 ± 0.01 | 1.00 ± 0.01 | 1.00 ± 0.04 | 1.00 ± 0.04       | 1.00 ± 0.02  | 1.00 ± 0.02  |

$^*$ fixed parameter, * data was normalized to $P(0) = 1$ and $P(0) + \Sigma(\Delta P) = 0$ and refitted.
Supplementary Figure 5

Dynamic PDA

A  wt Dyβ 100mM NaCl

B  wt Dyβ 400mM NaCl

C  wt Dyβ 600mM NaCl
D mutDyβ 100mM NaCl

E mutDyβ 400mM NaCl

F mutDyβ 600mM NaCl
Supplementary Figure 5. Dynamic Photon Distribution Analysis (dynPDA) of Dyβ and mutDyβ nucleosomes reveals frequency of conversion between H3NtT conformations and allosteric effects. Bursts were divided into 1, 2 and 3 ms (presented here) time windows. Donor-acceptor distances (x-axis) were calculated from integer photon counts and are affected by photon shot noise, leading to not entirely smooth distributions. The resulting three $\langle R_{DA}\rangle_E$ distance distribution histograms for each salt concentration were used for global fitting. Their distribution function (left panel, black line) was approximated by seven interconverting dynamic species ($dyn$HF-MF, $dyn$MF-LF, $dyn$HF-LF, $dyn$MF-VLF, $dyn$LF-VLF, $dyn$MF-NoFRET and $dyn$LF-NoFRET) and five static states (HF, MF, LF, VLF and NoFRET). Partial histograms for each contribution are also presented on the left panel (colors are defined in the plot legend). The shot noise free distance distributions calculated as a sum of five Gaussian-distributed probabilities of donor-acceptor distances and seven allowed dynamic transitions between pairs of those static species (2) are shown on the right panel. Best fit quality is shown as weighted residuals (w. res.) in the upper panel (black line).

(A-C) dynPDA of 40 pM Dyβ plus 1960 pM unlabeled nucleosomes at 100 (A, the corresponding MFD-PIE plot is shown in Fig. 2A), 400 (B) and 600 (C) mM NaCl.

(D-F) dynPDA of 40 pM mutDyβ plus 1960 pM unlabeled nucleosomes at 100 (D, the corresponding MFD-PIE plot is shown in Supplementary Fig. 2B), 400 (E) and 600 (F) mM NaCl.
Supplementary Tables

Supplementary Table 1: Complete table of global fit results from proximity curves in Fig. 1B, C. Empty fields: parameter not fitted, jointed fields: global parameter.

|                  | $E_a$ | $L_{a(170)}$ | $Dy_\beta$ | $Dy_a$ | mut$E_a$ | mut$L_{a(170)}$ | mut$Dy_\beta$ | mut$Dy_a$ |
|------------------|-------|---------------|-------------|--------|----------|---------------|--------------|----------|
| $c_{1/2}(1)$ [mM] | -     | -             | -           | -      | -        | 310 ± 6       | -            | -        |
| $c_{1/2}(2)$ [mM] | 596 ± 12 | -            | -           | -      | -        | 674 ± 60      | -            | -        |
| $c_{1/2}(3)$ [mM] | -     | 819 ± 3       | 806 ± 3     |        | 743 ± 9  | 708 ± 9       | -            | -        |
| $b_1$ [mM]       | -     | -             | -           |        | 27 ± 7   | -            | -            | -        |
| $b_2$ [mM]       | 112 ± 12 | -             | -           | -      | -        | 39 ± 21       | -            | -        |
| $b_3$ [mM]       | -     | 20 ± 2        | 34 ± 3      |        | 42 ± 6   | 47 ± 7        | -            | -        |
| $\Delta P_i^*$   | 0#    | 0#            | 0#          |        | 0#       | -0.73 ± 0.03  | -0.68 ± 0.04 | 0#        |
| $\Delta P_i^*$   | -1.00 ± 0.05 | 0#          | 0#          |        | 0#       | 0.7 ± 0.8     | 0#           | 0#        |
| $P(0)^*$         | 1.00 ± 0.03 | 1.00 ± 0.01  | 1.00 ± 0.01 | 1.00 ± 0.02 | 1.00 ± 0.04 | 1.00 ± 0.04 |

* fixed parameter, # data was normalized to $P(0) = 1$ and $P(0) + \sum \Delta P_i = 0$ and refitted

$$P(X) = P(0) + \frac{\Delta P_i}{1 + \exp\left(c_{(1/2)} - X \right) / b_i}$$

Supplementary Table 2: Equilibrium constants and relaxation times for $Dy_\beta$ nucleosomes at different salt concentrations as calculated from dynPDA fit results.

| NaCl [mM] | Rate constant, [ms⁻¹] | Dynamic (A-B) | HF-MF | MF-LF | HF-LF | MF-VLF | LF-VLF | HF-NoFRET | MF-NoFRET | LF-NoFRET |
|-----------|------------------------|---------------|-------|-------|-------|--------|--------|-----------|-----------|-----------|
| 100       | $k_{12}$ | 0.77 | 1.28 | 4.72 | 4.24 | 2.26 | 4.99 | 1.39 | 0.88 | 2.33 | 19.36 | 0.69 | 0.45 | 4.37 | 1.15 |
| 400       | $k_{21}$ | 0.87 | 1.22 | 10.25 | 7.75 | 1.84 | 1.90 | 1.58 | 0.82 | 9.13 | 25.80 | 0.97 | 0.52 | 6.07 | 1.11 |
| 600       | $k_{31}$ | 0.80 | 1.18 | 17.19 | 15.70 | 1.61 | 1.83 | 1.73 | 0.78 | 22.69 | 31.25 | 1.23 | 0.58 | 7.56 | 1.09 |
| NaCl [mM] | Equilibrium constant | | | | | | | | | | | | | | |
| 100       | $K_{MF-MF}$ | 0.60 | 1.76 | 1.12 | 1.58 | 0.12 | 1.54 | 3.80 | | | | | | | |
| 400       | $K_{MF-LF}$ | 0.71 | 1.32 | 0.97 | 1.94 | 0.35 | 1.86 | 5.46 | | | | | | | |
| 600       | $K_{MF-VLF}$ | 0.80 | 1.09 | 0.88 | 2.22 | 0.73 | 2.11 | 6.96 | | | | | | | |
| NaCl [mM] | Relaxation time, [ms] | | | | | | | | | | | | | | |
| 100       | $\tau_{MF-MF}$ | 0.490 | 0.135 | 0.236 | 0.441 | 0.046 | 0.882 | 0.181 | | | | | | | |
| 400       | $\tau_{MF-LF}$ | 0.479 | 0.056 | 0.268 | 0.416 | 0.029 | 0.668 | 0.139 | | | | | | | |
| 600       | $\tau_{MF-VLF}$ | 0.470 | 0.030 | 0.291 | 0.399 | 0.019 | 0.553 | 0.116 | | | | | | | |
**Supplementary Table 3:** Equilibrium constants and relaxation times for mutDyβ nucleosomes at different salt concentrations as calculated from dynPDA fit results.

| NaCl [mM] | dynamic (A-B) | HF-MF | MF-LF | HF-LF | MF-VLF | LF-VLF | MF-NoFRET | LF-NoFRET |
|-----------|---------------|-------|-------|-------|--------|--------|------------|------------|
|           | Rate constant, [ms⁻¹] | k₁₂  | k₂₁  | k₃₂  | k₁₃  | k₂₄  | k₄₂  | k₃₄  | k₄₃  | k₂₅  | k₃₅  | k₄₅  | k₂₆  | k₃₆  | k₄₆  | k₂₇  | k₃₇  | k₄₇  |
| 100       | 9.50          | 21.08 | 15.08 | 6.16  | 2.38  | 0.49  | 2.37  | 0.40  | 1.79  | 22.30 | 0.57  | 0.39  | 5.62  | 0.78  |
| 400       | 12.41         | 23.82 | 29.94 | 19.18 | 2.51  | 0.57  | 1.92  | 0.44  | 6.55  | 31.25 | 0.80  | 0.44  | 5.49  | 0.63  |
| 600       | 14.02         | 25.84 | 47.31 | 40.89 | 2.61  | 0.63  | 1.67  | 0.47  | 15.57 | 39.13 | 1.01  | 0.41  | 5.40  | 0.54  |

| NaCl [mM] | Equilibrium constant | K_{HF-MF} | K_{MF-LF} | K_{HF-LF} | K_{MF-VLF} | K_{LF-VLF} | K_{MF-NoFRET} | K_{LF-NoFRET} |
|-----------|----------------------|------------|------------|------------|------------|------------|----------------|----------------|
| 100       | 0.45                 | 2.45       | 4.83       | 5.89       | 0.08       | 1.45       | 7.18           |
| 400       | 0.52                 | 1.56       | 4.42       | 4.34       | 0.21       | 1.83       | 8.74           |
| 600       | 0.57                 | 1.16       | 4.17       | 3.55       | 0.40       | 2.46       | 9.97           |

| NaCl [mM] | Relaxation time, [ms] | t_{HF-MF} | t_{MF-LF} | t_{HF-LF} | t_{MF-VLF} | t_{LF-VLF} | t_{MF-NoFRET} | t_{LF-NoFRET} |
|-----------|-----------------------|------------|------------|------------|------------|------------|----------------|----------------|
| 100       | 0.033                 | 0.047      | 0.348      | 0.361      | 0.042      | 1.045      | 0.156          |
| 400       | 0.028                 | 0.020      | 0.325      | 0.423      | 0.026      | 0.808      | 0.164          |
| 600       | 0.025                 | 0.011      | 0.309      | 0.467      | 0.018      | 0.706      | 0.168          |
Supplementary Notes

Supplementary Note 1: Static and dynamic FRET lines

All MFD plots (Fig. 3A, B, D, E) for Dyβ and mutDyβ (Alexa488 and Cy5) are presented with static and dynamic FRET lines, to demonstrate the presence of static and dynamic fractions in the data. Each population exhibits kinetic exchange faster than the molecular dwell time (< 10 ms) within the bursts. The theoretical dependence between FRET efficiency and species weighted average donor fluorescence lifetime in presence of acceptor dye is described as

$$E_{\text{static}} = 1 - \frac{\langle \tau \rangle_x}{\tau_{D(0)}}$$  \hspace{1cm} (1.1)

Here we use an empirical dependence of species weighted average donor lifetime $\langle \tau \rangle_x$ on fluorescence weighted average donor lifetime $\langle \tau \rangle_F$ as a polynomial with $c_i$ coefficients obtained by numerical simulations (3)

$$\langle \tau \rangle_x = \sum_{i=0}^{n} c_i \left( \langle \tau \rangle_F \right)^i$$  \hspace{1cm} (1.2)

In this work we used the following joint parameters, which are common for the two FRET pairs Alexa488/Cy5:

**Alexa488/Cy5, static FRET line (Fig. 3A, B, D, E, orange):**

$c_0=-0.0225$, $c_1=0.3806$, $c_2=0.4007$, $c_3=-0.0838$, $c_4=0.0056$ with $\tau_{D(0)}=4.1$ ns.

The dynamic FRET line is described as

$$E_{\text{dyn}} = 1 - \frac{\tau_{F1} \cdot \tau_{F2}}{\tau_{D(0)} \left( \tau_{F1} + \tau_{F2} - \langle \tau \rangle_x \right)}$$  \hspace{1cm} (1.3)

where $\tau_1$ and $\tau_2$ are the donor fluorescence lifetimes defining the limiting FRET levels of the respective line. We have assumed that the limiting states of each DA sample remain the same for all NaCl concentrations.

**Alexa488/Cy5, dynamic FRET line between HF and LF states with $\tau_{D(0)}=4.1$ ns (Fig. 3A, B, D, E, magenta):**

$\tau_1=1.4$ ns, $\tau_2=3.8$ ns, $c_0=-1.7081$, $c_1=1.4489$, $c_2=0$.

Supplementary Note 2: Accessible volume simulations

We model dye distributions by the AV approach (4, 5) according to (2, 3). The dyes are approximated by a sphere with an empirical radius of $R_{\text{dye}}$, where the central atom of the fluorophore is connected by a flexible linkage of a certain effective length $L_{\text{link}}$ and width $w_{\text{link}}$ to the nucleobases of the DNA (see figure below).

The overall length of the linkage is given by the actual length of the linker and the internal chemical structure of the dye. A geometric search algorithm finds all dye positions within the linkage length from the attachment point which do not cause steric clashes with the macromolecular surface. All allowed positions are considered as equally probable which allows one to define an accessible volume for the dye (AV). We
have used typical parameters for the simulations: Alexa488: $L_{\text{link}} = 20 \, \text{Å}$, $w_{\text{link}} = 4.5 \, \text{Å}$, $R_{\text{dye}} = 1.5 \, \text{Å}$, Cy5: $L_{\text{link}} = 22 \, \text{Å}$, $w_{\text{link}} = 4.5 \, \text{Å}$, $R_{\text{dye}} = 3.5 \, \text{Å}$.

Molecular drawing (A) and sketch (B) for the AV parameters. $R_{\text{dye}(i)}$, $L_{\text{link}}$ and $w_{\text{link}}$ indicated by arrows.

**Supplementary Note 3: Time-resolved fluorescence decay analysis**

*Sub-ensemble TCSPC*

The model function was fit to the experimental fluorescence intensity decays using the iterative re-convolution approach. Here, the model-decay curves were convoluted with the experimental instrument response function (IRF). Furthermore a constant offset $c$ of the fluorescence intensity was considered. The experimental time-resolved fluorescence intensities of the FRET-sample and the donor reference sample are presented as:

$$
F_{\text{FRET}}(t) = N_0 \cdot \left[ (1 - x_{\text{NoFRET}}) \cdot F_D(t) + x_{\text{NoFRET}} \cdot F_D(0) \right] \otimes \text{IRF} + s \cdot \text{IRF} + c
$$

$$
F_{\text{Ref}}(t) = N_0 \cdot F_D(0) \otimes \text{IRF} + s \cdot \text{IRF} + c
$$

Here, $s$ takes into account scattered light from the sample and $x_{\text{NoFRET}}$ represents fraction of the NoFRET species. The normalized to unit area model functions were scaled by the experimentally measured photon number $N_0$. This reduces the number of free fitting parameters by 1.

**Model functions**

The fluorescence decay of the donor in the absence of acceptor can be multi-exponential due to local quenching. To account for these effects the donor only reference samples were fitted by a multi-exponential relaxation model.

$$
F_D(0)(t) = \sum_i x_D^{(i)}(0) \cdot \exp(-t/\tau_D^{(i)}(0))
$$

Here $\tau_D^{(i)}(0)$ are the donor fluorescence lifetime components and $x_D^{(i)}(0)$ the pre-exponential factors.

Multi-exponential donor decays were accounted for in the analysis of the FRET samples by global fitting. We assumed that all donor species are quenched by the same FRET rate constant $k_{\text{RET}}$. This is true if quenching does not change the donor radiative lifetime and when FRET is uncorrelated with the donor quenching. Based on these assumptions, the donor fluorescence intensity decay in the presence of acceptor dye $F_{D(A)}(t)$
can be factorized into the donor fluorescence decay in absence of FRET and the time-resolved FRET-induced donor quenching $\varepsilon_{D(A)}(t)$:

$$F_{D(A)}(t) = F_{D(0)}(t) \cdot \varepsilon_{D(A)}(t)$$ \hspace{1cm} (3.3)

We relate the FRET-induced donor decay to the distribution of distances by the rate-constant of energy transfer as defined by Förster:

$$k_{\text{RET}} = k_F \cdot \kappa^2 \cdot \left(\frac{R_{\text{Or}}}{R_{\text{DA}}}\right)^6$$

Here, $R_{\text{Or}}$ is a reduced Förster-radius, $k_F$ - the radiative rate constant of fluorescence and $\kappa^2$ is the orientation-factor. The reduced Förster-radius is given by:

$$R_{\text{Or}} = \left[\frac{9(n(10))}{128\pi^5N_A} \cdot \frac{j}{n^4}\right]^{\frac{1}{6}} = 0.2108 \, \text{Å} \cdot \left[\frac{j(\lambda)}{\text{mol}^{-1} \cdot \text{dm}^3 \cdot \text{cm}^{-1} \cdot \text{nm}^{-1}}\right]^{\frac{1}{6}},$$

where $N_A$ is Avogadro’s constant, $n$ is the refractive index of the medium and $j = \int f_\lambda(\lambda) \cdot \varepsilon_\lambda(\lambda) \cdot \lambda^4 \cdot d\lambda$ is the overlap integral between $f_\lambda(\lambda)$, the donor emission spectrum and $\varepsilon_\lambda(\lambda)$, the acceptor absorption spectrum. This reduced Förster-radius stresses that the FRET-rate constant is independent of quenching and specific for the dye-pair under the condition that the spectral overlap is independent of dynamic quenching.

With these assumptions, the FRET-induced donor decay relates to the distance distribution $p(R_{\text{DA}})$ by:

$$\varepsilon_{D(A)}(t) = \int p(R_{\text{DA}}) \cdot \exp(-t \cdot \langle\kappa^2\rangle \cdot k_F \cdot [1 + (R_{\text{Or}}/R_{\text{DA}})^6]) dR_{\text{DA}}$$ \hspace{1cm} (3.4)

Usually the orientation factor can be approximated by an average $\langle\kappa^2\rangle \approx 2/3$. We used a reduced Förster-radius of $R_{\text{Or}}=52$ Å which was determined for the donor Alexa488 with a radiative rate constant $k_F=0.2451$ ns$^{-1}$ and acceptor Cy5.

In the fit we have used continuous distance distributions which are described by a superposition of normal distributions:

$$p(R_{\text{DA}}) = \sum_{l=1}^{N} x_{\text{DA}}^{(i)} \cdot \frac{1}{2 \sigma_{\text{DA}} \sqrt{2\pi}} \cdot \exp \left(-2 \cdot \left[\frac{R_{\text{DA}} - \langle R_{\text{DA}}^{(i)} \rangle}{2 \sigma_{\text{DA}}}\right]^2\right)$$ \hspace{1cm} (3.5)

Here, $\langle R_{\text{DA}}^{(i)} \rangle$ is the mean of the state $(i)$ distance distribution with species fraction $x_{\text{DA}}^{(i)}$ and a halfwidth $\sigma_{\text{DA}}$ set to a physical meaningful value of 6 Å (flexible dye-linkers) estimated from dye clouds AV simulations. Number of Gaussian distributed distances $N$ was equal to 1 in model M1 and 2 in model M2.

The final analysis model is obtained by substituting eq. 3.5 into eq. 3.4 and eq. 3.4 into eq. 3.3. Finally, the fluorescence intensity decay of the donor in presence and absence of FRET are globally fitted. By the global (joint) analysis of the reference sample and the FRET-sample the photo-physical properties (dynamic quenching) of the donor dye are taken into account.
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