

Determination of the Associated Free Energy of the Unfolding of Cytochrome-*c*, the Intramolecular Distances of the Folded and Unfolded States, and the Effects of Förster Resonance Energy Transfer

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## ABSTRACT

In the field of biochemistry, the study of molecular structures of biological macromolecules is often carried out by use of spectroscopic techniques. The unfolding, or “denaturing” of proteins is carried out using a chemical denaturant, while the associated free energy of the unfolding process is determined using an unfolding curve, generated by spectroscopic measurements. The native structure of the protein cytochrome-*c* is denatured by urea, while UV-Vis absorption spectroscopy and fluorescence spectroscopy is used to determine the equilibrium of cytochrome-*c*’s native and denatured states, provided that each state exhibits distinct absorption and fluorescence signatures. Using unfolding curves and nonlinear regression analysis, we can determine the free energy associated with the unfolding process in the absence of a denaturant and how the use of a denaturant affects the associated free energy. A combination of Förster resonance energy transfer (FRET), and spectroscopic techniques, is utilized in the determination of intramolecular distances of the cytochrome-*c* protein and the fluorescence quenching heme cofactor. In studying the effects of FRET between the tryptophan-59 residue and heme, the Förster distance and the free energy determinations can be used to determine characteristics of the tertiary structure of cytochrome-*c*. Here we show that the Förster distance between Heme and tryptophan is  $\sim 31.79 \pm 0.5 \text{ \AA}$ , and in its native folded state, the heme cofactor and the tryotphan-59 residue cytochrome-*c* have an intramolecular distance of  $\sim 11.5 \pm 0.5 \text{ \AA}$ , which is lower than the Förster

distance, explaining the high efficiency of heme’s FRET quenching of the tryptophan-59 residue’s fluorescence. We were also able to extrapolate the free energy associated with the folding process of cytochrome-*c* in the absence of a denaturant as  $\sim 54.5 \pm 0.5 \text{ kJ} \cdot \text{mol}^{-1}$ .

## INTRODUCTION

The protein cytochrome-*c* is a natural fluorophore due to the presence of a tryptophan residue at position 59 in its peptide chain. The conjugated  $\pi$  bonds and the planar character (aromaticity) of the tryptophan residue allow it to re-emit electromagnetic radiation upon excitation. In its native tertiary structure, the presence of a covalently bound heme cofactor (necessary for the protein’s biological function) results in FRET between the two moieties, which quenches the tryptophan residue’s fluorescence. The energy absorbed by the tryptophan residue (FRET donor) is transferred to the heme (FRET acceptor) via quantum mechanical long-range dipole-dipole interactions, instead of being emitted by the tryptophan. This energy transfer is radiationless, thus no electromagnetic radiation is emitted during the transfer. We can compare the quenched fluorescence of the cytochrome-*c* (as it varies with urea concentration) to the fluorescence of a modified tryptophan residue in N-acetyltryptophanamide (NATA), in order to determine the Förster distance. Studying the FRET mechanism of cytochrome-*c* in conjunction with spectroscopic data allows us to measure intramolecular distances within the protein’s

structure and the thermodynamics associated with the structural changes that occur via the unfolding process.

This experiment was carried out in three parts, each with its own goal. Results of each part were then combined and analyzed together via the FRET mechanism in order to elucidate structural information of the cytochrome-*c* protein.

In the first part of this experiment, we sought to determine the free energy associated with the unfolding process of cytochrome-*c* and the efficiency of the heme's FRET quenching. In order to achieve this, we used a gradient of the chemical denaturant, urea, and generated absorption and emission spectra, allowing us to measure the relative concentrations of the native and denatured cytochrome-*c* as a function of the concentration of the urea. The resulting "unfolding curve" provides quantitative thermodynamic information regarding the stability of the protein. Exploiting the fact that the native tertiary structure and the denatured structure of cytochrome-*c* each possess unique absorption and emission spectra, we measured the shifts of absorption and emission spectra and changes in intensities that occurred with varying concentrations of urea to generate the aforementioned unfolding curve. The changes in relative concentrations between the native and denatured state allowed us to calculate the free energy associated with the unfolding of cytochrome-*c* in urea, which was then used to extrapolate the free energy of the equilibrium between these two states in the absence of urea, or any chemical denaturant using nonlinear regression analysis.

In the second part of this experiment, we studied the mechanism of the heme's quenching of the tryptophan residue's fluorescence to determine the Förster. In order to achieve this, we measured the absorption and fluorescence of NATA. As a modified tryptophan residue, NATA has similar spectroscopic properties as the tryptophan-59 residue in cytochrome-*c* without the quenching effects of the heme. By comparing the fluorescence

data of the NATA to the fluorescence data of the cytochrome-*c* denatured in varying concentrations of urea, we were able to determine the efficiency of the tryptophan-59/heme FRET quenching as a function of distance. The efficiency of FRET at 50% then allowed us to find the Förster distance.

In the third part of this experiment, we combine the information from the first two parts, specifically the determined Förster distance and the fluorescence efficiency (as a function of distance, which varied with the concentration of urea), for structural analysis of the cytochrome-*c* protein. This allows us to find the actual distance between the tryptophan-59 residue and the heme cofactor in cytochrome-*c*'s native folded (globular) tertiary structure.

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## METHODOLOGY

As previously mentioned, this experiment was carried out in three parts.

In order to generate the unfolding curve of the first part, fluorescence and absorption spectra were measured for cytochrome-*c* in varying concentrations of urea. A set of samples (1.5 mL each) of ~10  $\mu$ M cytochrome-*c* with urea in a 20 mM pH 7.4 potassium phosphate ( $KP_i$ ) buffer were prepared, varying the urea concentration from 3.0 M to 10.0 M in 0.5 M increments. An additional sample without any urea was also prepared. Absorption and fluorescence spectra were recorded for each sample. The absorption spectra were recorded with a Evolution-201 UV-Vis spectrophotometer, from 650 to 300 nm (absorption data points recorded at 0.5 nm increments) with a high sensitivity setting and a 5 nm bandpass. The fluorescence spectra were recorded using a F-2710 FL spectrophotometer, from 300 to 650 nm (emission data points recorded at 0.5 nm increments) with an excitation wavelength of 290.0 nm, a scan speed of 300 nm/min, a PMT voltage of 400 V, and a response time of 0.08 s. All spectra were recorded using glass cuvettes with 1 cm path lengths.

For the second part of the experiment, fluorescence and absorption spectra were measured for NATA. Three samples (1.5 mL each) of  $\sim 10 \mu\text{M}$  NATA in the same 20 mM pH 7.4 potassium phosphate ( $\text{KP}_i$ ) buffer that was used in part one. Fluorescence and absorption spectra were recorded using the same parameters in part one, with the exception of changing the excitation wavelength to 280.0 nm, to account for the difference between NATA and the tryptophan-59 residue.

Calculations for each sample can be found in the appendix. The samples were prepared using stock solutions prepared by the stock room from the Department of Chemistry, Hunter College CUNY. All data obtained was corrected, normalized, and analyzed using Microsoft Excel along with the solver add-on. Calculations were performed using matlab software. Refer to the following section for details.

## RESULTS AND DISCUSSION

For the first part of this experiment, both the absorption and fluorescence data were analyzed using Microsoft Excel. Spectra were recorded for blanks (without the cytochrome-*c*) and subtracted from the corresponding absorption and fluorescence spectra. The concentrations of the remaining cytochrome-*c* in the each sample were calculated using the Beer-Lambert Law (eq. 1 – refer to appendix) in order to normalize the fluorescence spectrum, accounting for any fluorescence measured from the remaining cytochrome-*c*. Normalization of the each fluorescence spectrum was achieved by dividing the spectrum by the remaining cytochrome-*c* concentration. The normalized fluorescence data at 350 nm (the emission wavelength) was then used to find the experimental fraction of unfolded cytochrome-*c* in each concentration of urea using eq. 2. The theoretical fraction for each concentration of urea was found using a separate a (eq. 3). Exploiting the fact that the folded and unfolded states are in equilibrium, eq. 3 describes

the fraction of cytochrome-*c* in the unfolded state using the Boltzmann distribution. Using the solver add-on in Microsoft Excel, we were able to fit the theoretical fraction to the experimental values, and using nonlinear regression analysis, extrapolate values for  $m$  ( $\text{kJ} \cdot \text{M}^{-1} \cdot \text{mol}^{-1}$ ), the rate of change of the free energy of denaturant with respect to denaturant concentration and  $C_m$  (M), the midpoint concentration, which results in equal amounts of the cytochrome-*c* in the folded and unfolded states. By minimizing the sum of squared errors (with the solver add-on) to match the theoretical and experimental data points, we determined the value of  $m$  as  $6106.30 \text{ J} \cdot \text{M}^{-1} \cdot \text{mol}^{-1}$  and the value of  $C_m$  as  $8.91163 \text{ M}$ . Figure 1 shows the result of this curve fitting.

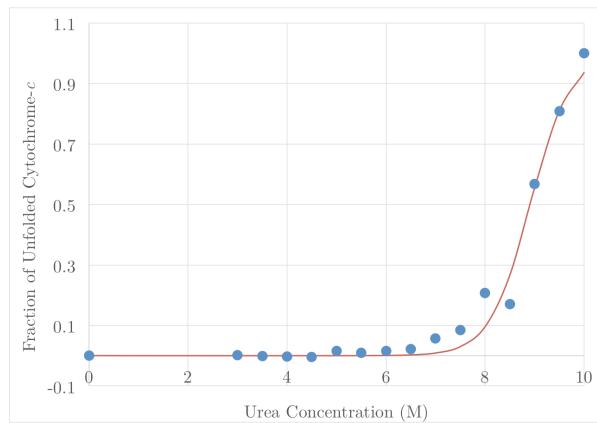


FIG 1. Unfolding Curve of Cytochrome-*c* in Urea. The blue circles represent the experimental fraction of unfolded cytochrome-*c* while the red line represents the theoretical fraction of unfolded cytochrome-*c*.

Using these values of  $m$ ,  $C_m$ , and eq. 4, we were able to find the free energy associated with the cytochrome-*c* folding process in the absence of urea (0 M), which is  $54.5 \text{ kJ} \cdot \text{mol}^{-1}$ , or  $\sim 13.0 \text{ kcal} \cdot \text{mol}^{-1}$ . This value matches closely to the free energy (for pH 7 and temp. 298 K) found in a similar study performed by Travaglini-Allocatellim, et. al..

A possible source of error in this part of the experiment can be seen in the anomalous point for the experimental fraction at a urea concentration of 8.5 M. Although the concentration of denaturant was greater than the sample for 8.0 M urea, the fraction of unfolded

cytochrome-*c* was less. This was likely due to experimental error, such as improper measurement of the protein or the denaturant. This anomalous data point would result in a slightly different value of  $m$  and  $C_m$ , as the solver add-on would try and account for this point. By removing this data point and performing the nonlinear regression analysis with solver again yields  $m$  and  $C_m$  values of  $6309.87 \text{ J} \cdot \text{M}^{-1} \cdot \text{mol}^{-1}$  and  $8.76260 \text{ M}$  respectively. This corresponds to a 3.23% and 1.70% deviation respectively from the original values.

In the second part of this experiment, the spectroscopic data measured for the  $\sim 10 \mu\text{M}$  NATA samples were analyzed using Microsoft Excel. Absorption and fluorescence spectra were measured for three samples of  $\sim 10 \mu\text{M}$  NATA and averaged. Since we consider the cytochrome-*c* to be fully denatured in 10.0 M urea, the FRET efficiency is near zero, and thus the absorbance in the visible region is solely due to the heme cofactor. Therefore, the absorbance data of the cytochrome-*c* in 10.0 M urea could be used to determine the absorbance of the FRET acceptor, the tryptophan-59 residue. The absorbance data of the cytochrome-*c* in 10.0 M urea was converted to the extinction coefficient by dividing the absorbance by the absorbance at 410 nm and then multiplying by the extinction coefficient at 410 nm,  $105,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$  (since the path length of the cuvette was 1 cm). The fluorescence data for NATA was then combined with the extinction coefficient (as a function of wavelength) of the cytochrome-*c* in 10.0 M urea. By doing this, we model the modified tryptophan NATA as tryptophan-59, the FRET donor. The combined data was then used with eq. 5 to find the Riemann sum approximation of  $J_{DA}$ , the overlap integral of the donor fluorescence spectrum (in this case, the NATA), and the acceptor absorption spectrum (in this case, the heme cofactor). Figure 2 shows the overlap of the fluorescence spectra of NATA and the absorption spectrum of cytochrome-*c* in 10.0 M urea, essentially the absorption spectrum of the heme

cofactor. Before graphing this spectroscopic data in figure 2, both spectrums were normalized by dividing by their largest values in order to have comparable heights.

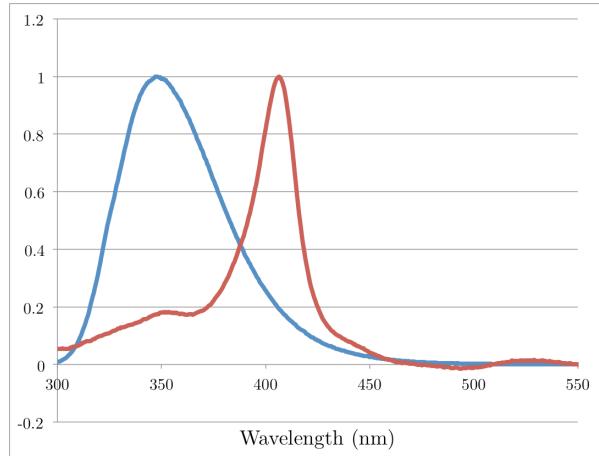


FIG 2. Spectrum of NATA Fluorescence and Cyt-*c* in 10.0 M Urea Absorbance.

The blue line corresponds to the fluorescence spectra of  $10 \mu\text{M}$  NATA while the red line corresponds to the absorbance of the  $10 \mu\text{M}$  cyt-*c* in 10.0 M urea.

The value of  $J_{DA}$  was found to be  $5.2026 \times 10^{14}$ . Using this value, the orientation factor  $\kappa^2$ , the refractive index  $n$ , and the quantum yield of the donor in the absence of acceptor  $\Phi_D$ , with eq. 6, we calculated  $R_0$ , the distance between the heme cofactor and the tryptophan-59 residue at which the FRET efficiency is 50% (the Förster distance).  $R_0$  was determined to be  $31.79 \pm 0.5 \text{ \AA}$ . This value matches closely to the Förster distance between a heme and a tryptophan residue measured by Sanchez, et. al., 34 Å.

A possible source of error in this part of the experiment could be the calculation of the  $J_{DA}$  value. The  $J_{DA}$  value was approximated as a sum of Riemann squares with width  $d\lambda = 0.5 \text{ nm}$ . If we were able to measure the spectroscopic data in smaller intervals, it might yield a more accurate  $J_{DA}$  value, and thus a more accurate Förster distance. The Förster distance measure in this experiment deviates 6.5% from the one measured by Sanchez, et. al..

In the third part of this experiment, the data from the previous two parts were combined to determine the efficiency of the FRET between

the heme and the tryptophan-59 residue as a function of intramolecular distance. Using the Förster distance determined in the previous part and eq. 7, a range of FRET efficiencies were generated for acceptor-donor distances between 1 and 100 Angstroms. The FRET efficiency was then determined as a function of urea concentration using the normalized fluorescence data from the first part (representing the FRET efficiency of the donor (tryptophan-59 residue) with acceptor (the heme),  $F_{DA}$ ) and the calculated value for the FRET efficiency of the donor without the acceptor,  $F_D$ . The value of  $F_D$  was found by dividing the fluorescence intensity of the 10  $\mu\text{M}$  NATA solution at 350 nm by its concentration (as the NATA mimics the properties of the tryptophan-59 residue). We then derived an equation for intramolecular distance  $r$  as a function of FRET efficiency  $E$ , eq. 8, allowing us to plot the distance dependence of FRET efficiency as shown in figure 3.

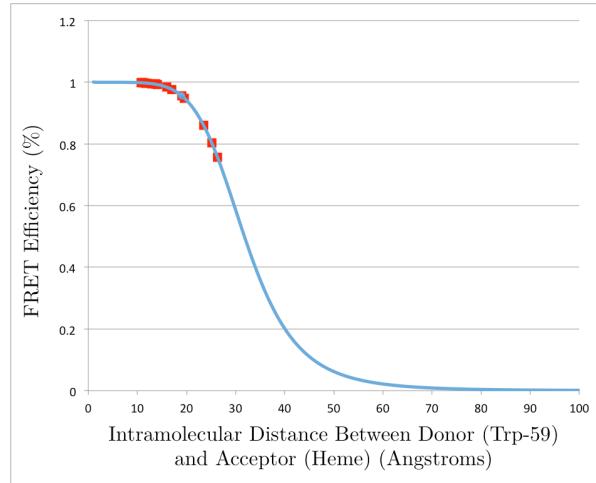


FIG 3. FRET Efficiency vs. Distance Between FRET Donor and FRET Acceptor.

The blue line corresponds to the theoretically calculated distance dependence of FRET efficiency and the red circles correspond to the experimental FRET efficiency as a function of distance.

Figure 3 shows that the distance at which FRET efficiency is approximately 50% is  $\sim 32 \text{ \AA}$ , within a 0.66% deviation with the Förster distance of  $31.79 \pm 0.5 \text{ \AA}$  found in the second part of this experiment. Figure 3 also shows how the FRET efficiency decreases with increasing concentration

of urea. This is due to urea denaturing the protein. The further cytochrome-*c* unfolds, the greater the distance between the tryptophan-59 residue and the heme cofactor becomes, resulting in a decrease in FRET efficiency. The denaturing urea is used to show the distance dependence of FRET. In the absence of urea (0 M), cytochrome-*c* is in its native folded state, with a FRET efficiency of nearly 100%. In contrast, when the protein is saturated with urea (10.0 M), it becomes almost completely unfolded, reducing the FRET efficiency to  $\sim 75\%$ . The urea concentration at which the FRET efficiency is between these two values ( $\sim 87.5\%$ ) is 8.5 M, which matches the unfolding curve from part A, the point at which the protein is unfolding.

Using this data, we can also show the effects of urea denaturing on the distance between the tryptophan-59 residue and the heme cofactor in cytochrome-*c*, as shown in figure 4.

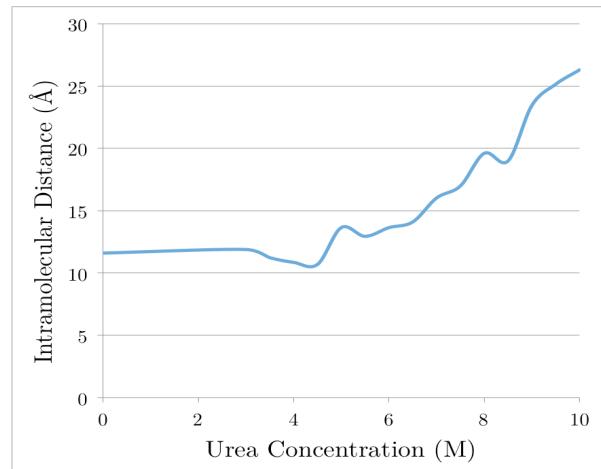


FIG 4. Distance Between FRET Donor and FRET Acceptor vs. Urea Concentration.

The blue line corresponds to effect of varying urea concentrations on the distance between the tryptophan-59 residue and the heme cofactor in cytochrome-*c*.

The distance between tryptophan-59 residue and the heme cofactor in cytochrome-*c*'s native state (corresponding to the sample with 0 M urea) was found to be  $11.60 \text{ \AA}$ . In contrast, the distance in the completely unfolded/denatured state (corresponding to the sample with 10 M urea) was found to be  $26.30 \text{ \AA}$ . Even in a nearly completely denatured state, the distance between the

tryptophan-59 residue and the heme cofactor is less than the Förster distance, thus FRET still dominates. These distances for folded and unfolded cytochrome-*c* match the values measured by Sanchez, et. al.. In addition, these values are also within range Förster distance, allowing the Förster distance calculated in the second part to be used as a reference measurement.

Any possible source of error in this part of the experiment would be purely computational, as it only involved analyzing the data from the previous parts. The curve of figure 4 shows a slight dip at 8.5 M urea, further indicating error for this sample as previously discussed. As previously mentioned, the extrapolated Förster distance calculated in this part falls within a 0.66% deviation of the Förster distance calculated in the second part of this experiment.

A major difference between the first part and the latter parts of this experiment is how we treated the folding and unfolding of cytochrome-*c*. In the first part of this experiment, in determining the fraction of unfolded protein and the associated free energy, we treated cytochrome-*c* molecules existing in equilibrium between the folded and unfolded state, as shown in eq. 2 and eq. 3, in which the system was modeled using a Boltzmann distribution of the two states. In contrast, in the latter parts of this experiment, we examined the kinetics and mechanism of the unfolding process using the FRET quenching efficiency, measured with the spectroscopic data. The assumption of considering the folded and unfolded states of the protein being in equilibrium is not entirely consistent with the intramolecular distances found in the last part of this experiment, specifically because we were able to show using the FRET efficiencies, that there are states in which cytochrome-*c* was only partially unfolded.

## CONCLUSION

Using a combination of FRET and spectroscopic techniques, we were able to determine the free energy associated with the

unfolding process of cytochrome-*c*. Although quite trivial, by determining the free energy for this process as positive, we are able to show that this unfolding process is non-spontaneous in water at pH 7, close to the physiological pH in which this protein exists (in the mitochondria of the cell). We were also able to determine the midpoint concentration of urea denaturation of cytochrome-*c* as 8.5. The Förster distance was found to be  $\sim 31.79 \pm 0.5 \text{ \AA}$ , which we were able to use as a relative reference for the measurement of the intramolecular distances between the tryptophan-59 residue and the heme cofactor as a function of urea concentration, as well as the distance dependence of the FRET efficiency between heme and tryptophan. Specifically, we found that even in 10.0 M urea concentration, the FRET quenching of the tryptophan-59 residue still dominates. This is either the result of 10.0 M urea not completely denaturing the cytochrome-*c*, or that the maximum distance between the heme cofactor and the tryptophan-59 residue in the unfolded state is less than the Förster distance. To determine which, further experimentation can be carried out, possibly increasing the concentration of urea past 10.0 M and observing if the FRET efficiency further decreases, indicating further unfolding.

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## APPENDIX

All calculated values from the discussion and result section were determined using the following equations and matlab software.

$$A = \varepsilon l C \quad \text{eq. 1}$$

Where  $A$  is the absorptivity of the sample,  $\varepsilon$  ( $\text{cm}^{-1} \cdot \text{M}^{-1}$ ) is the absorption coefficient of the sample at a given wavelength,  $l$  (cm) is the path length of the sample (cuvette width), and  $C$  (M) is the concentration of the sample.

$$f = \frac{F(C) - F_0}{F_\infty - F_0} \quad \text{eq. 2}$$

Where  $f$  is the fraction of unfolded protein,  $F(C)$  is the fluorescence of the sample at a given concentration  $C$ ,  $F_0$  is the fluorescence of the protein in its native state (in the absence of denaturant), and  $F_\infty$  is the fluorescence of the protein in its completely denatured state (saturated with denaturant).

$$f = \frac{e^{-m(\frac{C_m - C}{RT})}}{1 + e^{-m(\frac{C_m - C}{RT})}} \quad \text{eq. 3}$$

Where  $m$  ( $\text{kJ} \cdot \text{M}^{-1} \cdot \text{mol}^{-1}$ ) is the rate of change of the free energy of denaturant with respect to denaturant concentration,  $C_m$  (M) is the midpoint concentration,  $R$  ( $\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ), and  $T$  (K) the temperature of the system.

$$\Delta G_U^\circ = \Delta G_{H_2O}^\circ - mC \quad \text{eq. 4}$$

Where  $\Delta G_U^\circ$  ( $\text{kJ} \cdot \text{mol}^{-1}$ ) is the free energy associated with the unfolding process of the protein as a function of denaturant concentration and  $\Delta G_{H_2O}^\circ$  ( $\text{kJ} \cdot \text{mol}^{-1}$ ) is the free energy associated with the unfolding process in the absence of denaturant.

$$\Delta G_{H_2O}^\circ = mC_m \quad \text{eq. 4.5}$$

When the concentration of the denaturant is 0, eq. 4 can be used to determine the  $\Delta G_{H_2O}^\circ$ .

$$J_{DA} = \frac{\int F_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 \cdot d\lambda}{\int F_D(\lambda) \cdot d\lambda} \quad \text{eq. 5}$$

Where  $J_{DA}$  is the overlap integral of the donor fluorescence spectrum and the acceptor absorption spectrum,  $F_D$  is the fluorescence of the donor molecule by itself,  $\varepsilon_A(\lambda)$  is the molar extinction coefficient of the acceptor by itself,  $\lambda$  (nm) is the wavelength, and  $d\lambda$  (nm) is the interval length at which the fluorescence and absorption data is measured at.

$$R_0 = \sqrt[6]{(8.79 \times 10^{-5}) \left( \frac{\kappa^2 \Phi_D}{n^4} \right) J_{DA}} \quad \text{eq. 6}$$

Where  $R_0$  (Å) is the Förster distance,  $\kappa^2$  is the orientation factor between the transition dipoles of the donor and acceptor,  $\Phi_D$  is the quantum yield of the donor in the absence of an acceptor, and  $n$  is the refractive index.

$$E = 1 - \frac{F_{DA}}{F_D} \quad \text{eq. 7}$$

Where  $E$  is the FRET energy transfer efficiency and  $F_{DA}$  is the fluorescence intensity of the donor in the presence of the acceptor.

$$r = \sqrt[6]{\frac{R_0^6}{E} - R_0^6} \quad \text{eq. 8}$$

This is the derived equation for intramolecular distance  $r$  (Å) between FRET donor and acceptor.

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