Dynamics and Mechanism of Light Perception by UV Photoreceptor UVR8

DISSERTATION

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Abstract

UV RESISTANCE LOCUS 8 (UVR8) is a plant photoreceptor that senses ultraviolet-B (UV-B, 280 nm - 315 nm) region of the solar spectrum. UVR8 is a protein homodimer that dissociates into monomers in response to UV-B, triggering various UV protective mechanisms and photomorphogenesis in plants. Unlike other photoreceptors, UVR8 does not contain external chromophores and uses natural amino acid tryptophan (Trp or W) for light perception. Each UVR8 monomer has 14 tryptophan residues, among which 4 Trp residues (W285, W233, W337 in one monomer and W94 in the other monomer) are condensed to a pyramid cluster, serving as a reaction center. In the center, W285 and W233 are essential to the light-driven dimer dissociation. In this dissertation study, we revealed that Trp residues outside the reaction center can donate excitation energy to the center, enhancing the light harvesting efficiency of UVR8 by 100%. With extensive spectroscopy and mutagenesis studies, together with Förster Resonance Energy Transfer (FRET) theory, we determined rate constants of all possible elementary energy transfer steps, revealing a sophisticated Trp-to-Trp energy transfer network in UVR8. Time scales of these elementary resonance energy transfer reactions vary from tens of picoseconds to nanoseconds, depending on donor-acceptor distances, orientation factors and spectral overlap integrals. Furthermore, with femtosecond resolved spectroscopy, we discovered that UVR8 dimer dissociation is triggered by a series of electron transfer reactions at the Trp pyramid center. After excitation energy is funneled to the pyramid center, excitation
instantaneously localizes on W233, which serves as the energy sink. Subsequently, W233 excited state transfers an electron to W285 in 80 ps to form W285-W233+ charge separated intermediate. Competing with W285-W233 charge recombination (~4 ps), W285 anionic radical transfers an electron in ~ 17 ps to nearby arginine residues R286 or R338, which are involved in critical inter-subunit salt bridges. Microsecond molecular dynamics simulations revealed intermediate structures during UVR8 dissociation processes. Arginine neutralization breaks salt bridges and initiates a “Domino effect” to monomerize UVR8, which triggers downstream signaling steps.
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Major Field: Chemistry
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1.1 Ultraviolet-B Mediated Effects in Plants

Sunlight is of vital importance to sessile plants. Natural sunlight contains infrared (> ~760 nm), visible (~380 nm – 760 nm) and ultraviolet (UV, < ~380 nm) regions.\textsuperscript{1} By convention, the UV spectrum can further be divided into UV-A (315 nm - 400 nm), UV-B (280 nm – 315 nm) and UV-C (100 nm – 280 nm) regions.\textsuperscript{1} Since stratospheric ozone layer absorbs nearly all irradiation below 290 nm, UV-B is the most energetic part of solar spectrum on Earth’s surface.\textsuperscript{2-4} Ambient UV-B levels vary greatly depending on the time, season, latitude and altitude of measurements.\textsuperscript{2-4} Studies have shown that UV-B intensity maximize at noon with irradiance of a few hundred mW/m\textsuperscript{2}.\textsuperscript{5,6} With recent years’ depletion of ozone layer, UV-B irradiation that reaches the biosphere is increasing.\textsuperscript{2-4}

Although UV-B accounts for a small portion (less than 0.1% of total energy) of the sunlight, it causes various deleterious effects in plants.\textsuperscript{7-12} For example, UV-B results in the production of cyclobutane pyrimidine dimers (CPD)\textsuperscript{13} and pyrimidine-pyrimidone (6-4) photoproducts (6-4 PPs)\textsuperscript{14} in DNA, crosslinking adjacent bases on the same strand. Since DNA and RNA polymerases cannot read through such lesions, these photodamages can hinder normal DNA replication and transcription, impairing biological functions in cells.\textsuperscript{15,16} Moreover, high-energy photons of UV-B cause the formation of reactive oxygen species (ROS), including superoxide radicals, which could damage various biomolecules.\textsuperscript{17-20} Higher environmental UV-B also impairs photosynthesis in various
ways, including triggering degradation of protein D1 and D2 of Photosystem II (PS II), destruction of chlorophyll and carotenoids and reducing the activity of Rubisco. ¹⁰,2¹-2³

Plants have evolved various protective and repair mechanisms against UV-B damages.⁷-¹² Low doses of UV-B induce deposition of UV-absorbing flavonoids, a class of secondary metabolites, in epidermal tissues. By absorbing UV-B while allowing penetration of visible light, flavonoids function as “sunscreen” that reduces the penetration of UV-B into plant leaves.²⁴-²⁶ Plants have also evolved DNA repair mechanisms to repair damages in the genome. *Arabidopsis* has two distinct photolyases for the two types of photodamages: CPD photolyases and 6-4 photolyases.²⁷-³² Both photolyases contain flavin adenine dinucleotide (FAD) cofactors and utilize UV-A/blue light to repair photoproducts.¹⁵,³²,³³ In response to UV-B, transcription of the two photolyases is significantly promoted, reducing levels of CPD and 6-4 PP in plants.³⁴,³⁵ *Arabidopsis* mutants lacking photolyases are UV-B hypersensitive, with small and pale leaves when exposed to UV-B.²⁸,²⁹ Elevated UV-B also leads to increased somatic recombination frequency in plants, suggesting upregulation of homologous recombination repair in response to UV-B.³⁶ Stimulated by UV-induced ROS, plant cells show elevated activity of superoxide dismutase, glutathione reductase and peroxidases,³⁷-³⁹ which convert superoxide radicals to hydrogen peroxides and oxygen molecules, glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH) and hydrogen peroxides to water, respectively.⁴⁰-⁴³ Flavonoids also react with and therefore annihilates ROS.⁴⁴
UV-B is not only a damaging agent, but also an important environmental signal that triggers light-induced developmental changes, termed photomorphogenesis, in plants.\textsuperscript{12,45} Photomorphogenesis mediated by red light photoreceptors phytochromes and blue/UV-A light receptor cryptochrome has been extensively studied.\textsuperscript{46-50} Recent studies indicate that UV-B initiates similar photomorphogenesis responses, including suppression of hypocotyl elongation, reduction of leaf extension and promotion of apical hook opening in \textit{Arabidopsis} seedlings.\textsuperscript{11,12,45} Such finding suggest a cross-talk between UV-B signaling and visible light signaling pathways.\textsuperscript{51}

### 1.2 UV-B Signaling in Plant Cells

Plant cells perceive and respond to UV-B via various signaling mechanisms. High doses (typically $\geq 1 \text{ \mu mol}\cdot\text{s}^{-1}\cdot\text{m}^{2}$, depending on experiment conditions) and short wavelengths of UV-B induces genes related to stress responses, whereas low fluence rates and longer wavelengths of UV-B regulates genes involving in UV protective responses and photomorphogenesis.\textsuperscript{10,12,52} High dose UV-B signaling is initiated by damaging effects and is not UV-B specific. Plant ATM serine/threonine kinase (ATM) and serine/threonine-protein kinase ATR (ATR) can sense DNA double-stranded breaks and persistent single-stranded DNA, respectively, and mediate DNA damage signaling.\textsuperscript{53-55} \textit{Arabidopsis} mutants with impaired ATR are hypersensitive to UV-B.\textsuperscript{53} UV-B induced ROS activates different mitogen-activated protein kinase (MAPK) cascades, triggering ROS scavenging processes.\textsuperscript{17-20} High-dose UV-B signaling also cross-talk with other stress responses.\textsuperscript{12} For example, high levels of UV-B were found to upregulate expression
of pathogenesis-related protein 1 (PR1), which promote plant tolerance to pathogens and wound.\textsuperscript{56} On the other hand, some UV-B mediated responses, including flavonoid biosynthesis and photomorphogenesis, can be stimulated by much lower UV-B levels, when UV-induced damages are negligible.\textsuperscript{12,52,57} Since these responses are directly promoted by UV-B, not by cellular stress, they are referred to as UV-B specific signaling.\textsuperscript{9,12}

Screening of UV-B hypersensitive \textit{Arabidopsis} mutants has identified UV-B specific signaling components. \textit{Arabidopsis} mutants \textit{tt4} and \textit{tt5} lack functional chalcone synthase (CHS) and chalcone isomerase (CHI), respectively.\textsuperscript{26,58,59} CHS catalyzes the conversion of 4-coumaroyl-CoA to malonyl-CoA to naringenin chalcone, which is a key intermediate in flavonoid biosynthesis.\textsuperscript{60,61} CHI catalyzes another step of flavonoid formation: isomerization of chalcone to flavanone.\textsuperscript{61,62} CHS and CHI transcription is rapidly induced by low doses of UV-B, leading to deposition of UV-B absorbing flavonoids.\textsuperscript{11,63-66} Both \textit{tt4} and \textit{tt5} mutants show no flavonoid deposition and exhibit drastically lower survival rates than WT under elevated UV-B.\textsuperscript{26,58,59} \textit{Arabidopsis} variants \textit{uvr1} and \textit{uvr2-1} lack (6-4) photolyase and CPD photolyase, respectively, and therefore bear more DNA photodamages under UV-B irradiation.\textsuperscript{28,29} Later identified \textit{uvr8-1} mutant contains a 15-bp deletion in UV RESISTANCE LOCUS 8 (UVR8) gene,\textsuperscript{67} which encodes a β-propeller protein and has sequence similarity with Human Regulator of Chromatin Condensation 1 (RCC1), a Ran guanine nucleotide exchange factor.\textsuperscript{68} UVR8 shows little exchange activity but is capable of chromatin binding. UV-B induced CHS upregulation is abolished in \textit{uvr8-1} allele, indicating UVR8 acts upstream of flavonoid
synthesis in UV-B signaling. Subsequent studies using mature Arabidopsis leaves have shown that UVR8 regulates expression levels of over 70 genes, including genes encoding multiple enzymes involved in flavonoid biosynthesis, enzymes for alkaloid biosynthesis, chloroplast proteins, transcription factors, CPD photolyase and genes concerned with protection against oxidative stress, in response to UV-B.66 Importantly, under UV-B, UVR8 upregulates expression of bZIP transcription factor ELONGATED HYPOCOTYL 5 (HY5), an established key regulator in plant photomorphogenesis. UVR8 also induces expression of HY5 Homology, a functional redundant of HY5, in a UV-B dependent manner.66 Furthermore, UVR8 protein was shown to bind to the promoter region of HY5 gene, suggesting that HY5 is directly regulated by UVR8.66,69 A mutant uli3 shows impaired CHS induction and little hypocotyl elongation suppression in UV-B.70 The protein encoded by ULI3 gene exhibits 27% amino acid homology to a human 80-kDa diacylglycerol kinase, whose function is not directly related to UV-B.70 Later, constitutively photomorphogenic 1 (COP1) is found to be essential to UV-B responses.71 COP1 physically interacts with UVR8 in the presence of UV-B to promote HY5 expression.72 However, COP1 has been identified as a negative regulator of red/blue/UV-A light induced photomorphogenesis in plants.73-76 In dark, COP1 and SUPPRESSOR OF PHYA 1 (SPA1) form a protein complex that has E3 ligase activity towards photomorphogenesis promoting transcription factors including HY5.75 E3 ligase function of COP1/SPA1 is suppressed by red/blue/UV-A light.76

Since UVR8 is of vital importance of UV-B specific signaling, recent studies focused on UVR8 pathway. Expression of photolyases are induced by UV-B via UVR8
dependent and UVR8 independent pathways. COP1, HY5 and HYH facilitate, but is not essential to, UVR8 mediated photolyase light induction. UVR8 promotes the photosynthesis efficiency in the presence of UV-B. UVR8 is also required in UV-B mediated circadian clock entrainment. Circadian rhythm components CCA1, PRR9, GI and ELF4 are acutely induced by UV-B in a UVR8/COP1 dependent manner, but HY5/HYH are dispensable in such signaling.

From a pool of genes upregulated by UVR8 pathway, researchers identified negative regulators of UVR8 pathway: REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and REPRESSOR OF UV-B PHOTOMORPHOGENESIS 2 (RUP2). RUP1 and RUP2 expression is promoted by UV-B via a UVR8/COP1 dependent manner, and suppress UV-B signaling, providing a negative feedback loop. The current model of UVR8 signaling is shown in figure 1.1.

1.3 UVR8 is a UV-B Photoreceptor

It was not until recently that UVR8 was identified as a UV-B photoreceptor. UVR8 forms homodimers in dark and dissociate into monomers upon UV-B irradiation. UV-induced UVR8 monomers can interact with COP1 both in vivo and in vitro. Consistent to its gene regulation functions, UVR8 mainly exists in the cytosol in dark and accumulates in the nucleus in response to UV-B. As shown in figure 1.2, the structure of Arabidopsis UVR8 core domain has been solved by X-ray crystallography in the dimeric form, revealing a 7 bladed β-propeller architecture of each monomer. The two donut-shaped monomers are glued together by many electrostatic interactions.
Besides the core, UVR8 has N-terminal and C-terminal flexible regions, which are not shown in the crystal structure. Neither N-terminal part nor C-terminal part is necessary to UVR8 light-induced monomerization.\textsuperscript{82-84} However, UV-induced UVR8 nuclear accumulation requires the 23 N-terminal amino acids (N23), but the N23 region shows no similarity to any known subcellular localization signal sequences.\textsuperscript{81} The C-terminal is essential to UVR8-COP1 interaction.\textsuperscript{84} Importantly, UVR8-COP1 binding is critical to both the light mediated UVR8 nuclear accumulation and the subsequent signaling events.\textsuperscript{85}

Besides UVR8, \textit{Arabidopsis} contains at least three other types of photoreceptors: red/far red photoreceptors phytochromes (PHY A, PHY B, PHY C, PHY D and PHY E), blue/UV-A photoreceptors cryptochromes (CRY1 and CRY2) and blue/UV-A photoreceptors phototropins (PHOT1 and PHOT2).\textsuperscript{86-89} Each phytochrome protein has one Bilin chromophore that undergoes photon-induced isomerization to trigger protein conformational changes.\textsuperscript{87} CRYs and PHOTs contain flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), respectively.\textsuperscript{88,89} Uniquely, UVR8 doesn’t contain any external chromophores and uses natural amino acid tryptophan (Trp or W) for light perception.\textsuperscript{82,83} Each \textit{Arabidopsis} UVR8 monomer is 440 amino acid long and contains 14 Trp (W39, W92, W94, W144, W196, W198, W233, W250, W285, W300, W302, W337, W352 and W400), among which, W285 and W233 are critical to the light-induced dissociation function.\textsuperscript{80,82,83,90} Amino acid sequences with high similarity to \textit{Arabidopsis} UVR8 are found not only in angiosperms, but in the moss \textit{Physcomitrella patens}, the lycopod \textit{Selaginella moellendorffii}, and the green algae \textit{Chlamydomonas reinhardtii} and
Volvox carteri. Most of the Trp, including the critical W285 and W233, are conserved in these putative UVR8 orthologs, indicating that UVR8 appeared early in plant evolution.

In vivo and in vitro studies have shown that UVR8 dissociates into monomers in minutes when exposed to UV-B. The redimerization spends days in vitro, but takes minutes in vivo with the help of RUP1 and RUP2. Recent transient grating studies have shown that UVR8 monomerization occurs in 200 ms after excitation. However, lifetime of tryptophan singlet excited states is on picoseconds to nanoseconds time scale. Thus, photoreactions that triggers the dimer dissociation very likely occurs within nanosecond time scales, followed by millisecond conformational changes.

1.4 Femtosecond Resolved Spectroscopy

With the advancement of femtosecond lasers, time-resolved spectroscopy is a powerful tool for detecting ultrafast processes. In a typical femtosecond resolved pump-probe experiment, the laser system generates two pulsed laser beams: a pump beam and a probe beam. The pump beam initiates the reaction, whereas the probe beam hits the sample after an adjustable delay time to detect the responses of the sample. The detected signal, either absorption differences or fluorescence intensities, are plotted against delay times to obtain dynamic information of ultrafast processes initiated by the pump beam. The time resolution is mainly determined by pulse durations of the pump and probe laser pulses. With wavelength-tunable femtosecond laser systems, temporal resolution of femtoseconds and wavelength resolution of a few nanometers can be achieved.
simultaneously. Since femtosecond resolved spectroscopy was invented in the 1980s, it has been applied to many chemical and biological systems and provided novel insights into the dynamics.

In our lab, a comprehensive femtosecond laser system, which can perform both transient absorption and fluorescence up-conversion experiments, has been used to study ultrafast dynamics of proteins. The integrated experimental setup is schematically illustrated in figure 1.3. With this system, we can continuously tune wavelength from mid-infrared to deep UV region, with femtosecond resolution and an observation window of up to ~4 ns. The signal-to-noise ratio is gradually increased with repetition using fresh samples each time.
Figure 1.1 Signal transduction by UVR8

Acronyms: UVR8 (UV RESISTANCE LOCUS 8), COP1 (CONSTITUTIVELY PHOTOMORPHOGENESIS 1), RUP1 (REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1), RUP2 (REPRESSOR OF UV-B PHOTOMORPHOGENESIS 2), HY5 (ELONGATED HYPOCOTYL 5), HYH (HY5 HOMOLOG), PL (Photolyase), CHS (Chalcone Synthase), CHI (Chalcone Isomerase), CCA1 (Circadian Clock Associated 1), GI (GIGANTEA), PRR9 (Proline Rich 9) and ELF4 (EARLY FLOWERING 4).
Figure 1.2 X-ray structure of Arabidopsis UVR8 homodimer

The structure was solved by Wu et. al. (PDB bank: 4DNW). Each monomer has a 7-bladed β-propeller architecture. (A) A side view of UVR8 homodimer with charges residues involved intersubunit interactions highlighted (positive charged residues in blue and negatively charged residues in red). (B) A side view of UVR8 dimer with Trp residues shown in sticks. (C) A top view of UVR8 homodimer. Note the pseudo-C7 symmetry of monomers.
Figure 1.3 Schematic representation of the experimental setup with both the fluorescence up-conversion and the transient absorption configurations.\textsuperscript{96}

The dashed line is for the transient-absorption probe pathway and the solid line shows the fluorescence up-conversion beam pathway. Abbreviations: F-filter. MM-movable mirror. MP-movable parabolic mirror. $\lambda/2$-halfwave plate. PD-photodiode. Milenium, Tusnami, Spitfire, Evolution 30, and SSA are the pump laser, femtosecond oscillator, two-stage amplifier, amplifier’s pump laser, and single-shot autocorrelator, respectively.
2.1 Introduction

Each UVR8 monomer contains 14 tryptophan residues, which, except for the C-terminal W400, are shown in the X-ray structure. The remaining 13 Trp, based on their positions, can be classified into 3 groups (figure 2.1A): distal Trp ($W_d$: W39, W92, W144, W196, W300 and W352), peripheral Trp ($W_p$: W198, W250 and W302) and pyramid center Trp ($W_c$: W94, W233, W285 and W337). The 6 distal tryptophan residues and a tyrosine Y248 occupy 7 equivalent positions on the 7 β-blades and form a highly symmetrical ring (figure 2.1B). W233, W285, W337 and W94 from the other monomer form a pyramid cluster at the dimer interface. W198, W250 and W302 lie on the periphery of the pyramid cluster (figure 2.1C). Previous studies have shown that mutation of either W285 or W233 to phenylalanine/alanine abolishes light-induced dissociation of UVR8. A recent complete in vivo screening demonstrates that the other 12 Trp are not essential to UVR8 function, but W337F/A partially impaired UV response, highlighting the importance of the pyramid cluster. Notably, W285F mutant has nearly identical structure to UVR8 WT, suggesting that W285 plays photochemical role rather than a structural role in UVR8 light perception. Our ultrafast spectroscopy studies on W285F shows W285 participates in a ~150 ps photoreaction that greatly quenches the fluorescence in WT. Significantly, W285F exhibits a red shifted fluorescence spectrum with an intensity 6 times of WT, implying that other Trp transfer excitation energy to the pyramid center. If assuming no energy transfer and W285 only quenches excitation
within the 4 $W_c$, the quantum yield of $W_c$ would exceed unity. Theoretical studies also support Trp-to-Trp energy transfer scenario and suggest W233 is the final energy acceptor.\textsuperscript{98,99} Like photosynthesis systems, it is very likely that distal Trp and peripheral Trp can serve as antenna and transfer energy to the pyramid center, increasing light perception efficiency of UVR8. However, there is still no solid experimental evidence that a light harvesting network exists in UVR8, not to mention the time scales and detailed scheme of the excitation energy transfer processes. In this chapter, we aim to solve the complete Trp-to-Trp energy transfer dynamics in UVR8.

2.2 Materials and Methods

2.2.1 Expression and Purification of Arabidopsis UVR8

*Arabidopsis* UVR8 protein samples for this study were prepared following the protocol reported by Wu et. al. with minor modifications.\textsuperscript{82} pET 29 (b) plasmid expressing full-length wild type *Arabidopsis* UVR8 was kindly provided by Prof. Yigong Shi at Tsinghua University. Plasmids expressing UVR8 mutants were prepared with Quickchange site-directed mutagenesis kits (Qiagen) following the manufacturer’s protocol. For protein expression, pET 29 (b) plasmids containing wild type or mutant UVR8 nucleotide sequences were transformed into *E. coli* BL21-DE3 cells. After the cells were grown to an OD of 1.0-1.2 at 37 °C, protein expression was induced by adding IPTG (200 μM/L final concentration in medium). *E. coli* cells were grown in 18 °C for another 18-22 hours before harvesting with centrifuge. For protein purification, *E. coli* cells containing desired UVR8 proteins were suspended and kept stirring overnight in
UVR8 lysis buffer (150 mM NaCl, 25 mM Tris and pH=8.0) supplemented with lysozyme (1 mg/mL). The suspended cells were then sonicated and centrifuged at 20K revolutions per minute (RPM) speed. The supernatant was applied to Ni-Sepharose resin (GE) and then washed with 20 column volumes of wash buffer (150 mM NaCl, 25 mM Tris, 15 mM Imidazole and pH=8.0). The desired protein was then eluted with elution buffer (150 mM NaCl, 25 mM Tris, 250 mM Imidazole and pH=8.0). The eluted sample was subjected to an anion exchange column (Source 15Q, GE). Gradient elution (120 mM to 300 mM NaCl) was used to obtain final purified UVR8 protein samples. For all experiments, protein samples were kept in the UVR8 lysis buffer (150 mM NaCl, 25 mM Tris and pH=8.0) unless stated otherwise. Dimer/monomer state of proteins were determined by size exclusion chromatography (SEC) as previously reported. UV-induced dissociation function of protein samples were tested either by SEC or by fluorescence intensity change under continuous UV illumination.

2.2.2 Determination of Dimer/monomer State and Dissociation Function

UVR8 dimer and monomer have distinct molecular weights and show different elution volumes on size exclusion chromatography (SEC). Dimer/monomer state of proteins were determined by SEC as previously reported. Briefly, 100 μL of UVR8 protein sample (10 μM) were injected to a Superdex 200 column (25 mL column volume, GE) for molecular weight determination. To test UV-induced dissociation function, UVR8 mutant samples were placed in a 5-mm cuvette and treated with a broadband UV lamp for 10 min. The irradiated samples were tested by SEC. Functional mutants show
monomer peaks in chromatography profile. Dissociation functions were also investigated by fluorescence intensity change under continuous UV illumination. Fluorescence intensity of functional mutants gradually increase under UV illumination.

2.2.3 Steady-state Absorption and Emission

The absorption spectra were measured using Cary 50 UV-Vis Spectrophotometer (Agilent). The OD at absorption peak (282 nm) was adjusted to around 1.0 in 5-mm quartz cuvettes for every sample, which corresponds to protein concentrations from ~20 μM to ~50 μM depending on the number of tryptophan residues in the mutants. Absorption spectra of the 3 Trp groups were obtained with the normalized absorption spectra of UVR8 WT, 4Wc/400F and mono-6Wd using the following equations:

\[
A_d(\lambda) = A_{\text{mono-6W}_d}(\lambda)
\]  
(2.1)

\[
A_p(\lambda) = \frac{9 \times A_{4W_c/400F}(\lambda) - 6 \times A_{\text{mono-6W}_d}(\lambda)}{3}
\]  
(2.2)

\[
A_c(\lambda) = \frac{13 \times A_{\text{WT}}(\lambda) - 9 \times A_{4W_c/400F}(\lambda)}{4}
\]  
(2.3)

where \(A_{\text{mono-6W}_d}, A_{4W_c/400F}\) and \(A_{\text{WT}}\) are normalized absorption spectra of UVR8 mono-6Wd, 4Wc/400F and WT (as shown in figure 2.1D), respectively. \(A_d, A_p\) and \(A_c\) are the normalized absorption spectra of distal, peripheral and pyramid center Trp (as shown in figure 2.1E), respectively. By applying the above equations, we are assuming that the tryptophan residues in different Trp groups have the same molar extinction coefficient at the absorption peaks at ~282 nm.

The emission spectra of UVR8 samples were measured in 5-mm quartz cuvettes
using Fluoromax-3 spectrofluorimeter (Horiba) at various protein concentrations. Protein concentrations that were used for emission spectra measurements are ~2 μM, ~10 μM, ~50 μM and ~50 μM for excitation wavelengths 290 nm, 300 nm, 310 nm and 315 nm, respectively. For fluorescence quantum yield (QY) determination, fluorescence spectra of samples with a series of concentrations were measured with 290 nm excitation and then integrated, and the integrals were then plotted versus absorbance at 290 nm. Tryptophan in water was used as a standard. For each sample, 3 independent measurements were conducted and the error bars were determined using the standard deviation of the 3 measurements.

2.2.4 Femtosecond Fluorescence Up-conversion Method

The experimental layout has been detailed elsewhere. Briefly, the pump wavelength was set at 290 nm, generated by OPA-800 (1 kHz, Spectra-Physics), and its pulse energy was attenuated to ~100 nJ. The instrument response time under the current noncollinear geometry is about 400 fs, and all data were taken at a magic angle (54.7°). The samples (~150 μM) were kept in spinning quartz cells during irradiation to avoid heating and photobleaching. Samples in the cells were replaced with fresh samples about every 1 million excitation pulses.

2.2.5 Sub-Nanosecond Resolved Time Correlated Single Photon Counting (TCSPC) and Spectra of Exponential Components

Sub-nanosecond resolved TCSPC data were acquired using the commercially
available FluoTime 200 system (Picoquant) with PLS-290 pulsed LED (8 mHz, 290 nm, ~1 µW, Picoquant) as the excitation source. The FWHM of instrument response function (IRF) was 700-800 ps. All samples (10 µM) were kept in 5 mm quartz cuvettes during measurements. Fluorescence decay transients at 305 nm, 310 nm, 315 nm, 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm, 350 nm, 355 nm, 360 nm, 365 nm, 370 nm, 375 nm and 380 nm (16 wavelengths) were taken for all samples (data shown in figure 2.4) at a magic angle (54.7°). For WT and W198/250/302/400F samples, no observable dissociation was detected after the measurements, as confirmed using Superdex200 size column.

\[
S(t) = \int_{-\infty}^{\infty} \text{IRF}(\tau)(\sum_i A_i e^{-(t-\tau)/\tau_i} + A_{\text{scat}}) d\tau
\]  

(2.4)

All sub-nanosecond resolved TCSPC data were then fitted using FluoFit software (Picoquant) with convoluted multiple-exponential decay models described in Equation 2.4, where \( S(t) \) is the measured signal; \( \text{IRF}(t) \) is the instrument response function as measured by buffer scattering signal; \( A_i \) and \( \tau_i \) are the amplitude and time constant of the \( i^{\text{th}} \) exponential component; and \( A_{\text{scat}} \) is the contribution of scattering to total signal. For each sample, transients of 16 different wavelengths were globally fitted with a same set of time constants. The time constants of the fastest component were fixed in all samples since it is below the IRF. For WT and W198/250/302/400F only, constraints were applied to \( \tau_{\text{DA2}} \) amplitudes at different wavelengths to allow the \( \tau_{\text{DA2}} \) emission spectrum to be similar to \( W_d \). Otherwise, \( \tau_{\text{DA2}} \) will mix with \( \tau_3 \), rendering both components not being accurately resolved. After the fitting parameters were obtained, the spectrum of the \( m^{\text{th}} \)
exponential component (as shown in figure 2.5C) was given by the following equation:

\[ I_m(\lambda) = I(\lambda) \frac{A_m \tau_m}{\sum_i A_i \tau_i} \]  

(S5)

where \( I_m(\lambda) \) is the intensity of the emission spectrum (\( \lambda_{ex}=290 \text{ nm} \)) associated with the \( m^{th} \) component; \( A_i \) and \( \tau_i \) are the amplitude and time constant of the \( i^{th} \) exponential component from the TCSPC transient at wavelength \( \lambda \) (\( \lambda=305, 310, 315\ldots \) 380 nm); \( I(\lambda) \) is the intensity of the steady-state emission spectrum (\( \lambda_{ex}=290 \text{ nm} \)) of the same sample at wavelength \( \lambda \).

2.2.6 Picosecond Resolved Time Correlated Single Photon Counting (TCSPC)

The desired fundamental wavelengths (870 nm, 930 nm, 945 nm and 960 nm, 0.4-0.6 W, \(~100 \text{ fs pulse duration}) were generated with a tunable (700 nm to 980 nm) Tsunami Ti-Sapphire oscillator (80 MHz, Spectra-physics), and were then subject to third harmonic generation with a commercial tripler (TPL fs tripler, mini optic) to obtain the corresponding UV excitation light (290 nm, 310 nm, 315 nm and 320 nm). The power of the excitation beam (80 MHz) was attenuated to \(~0.4 \text{ µW} \) before being directed to the sample chamber of a commercially available FluoTime 200 system (Picoquant), where the protein samples (~50 µM) were placed in a 5-mm cuvette for measuring. The instrument response function (FWHM 40 ps) was determined by measuring the scattering signal of UVR8 lysis buffer (150 mM NaCl, 25 mM Tris and pH=8.0). For functional mutants, no observable dimer dissociation was detected after the measurements, as confirmed using Superdex200 size column.
2.2.7 Energy Transfer Rate and Time Constant Calculations for Individual Donor-Acceptor Pairs with FRET Theory

Förster (FRET) and Dexter (DET) energy transfer rates can be deducted from fundamental quantum mechanical theories (See Appendix A). Details about FRET theory can also be found elsewhere.\textsuperscript{103} Briefly, energy transfer rates $k_{\text{FRET}}$ (in ns\textsuperscript{-1}) were calculated as follows:

$$ k_{\text{FRET}} = \frac{1}{\tau_D} \left( \frac{R_0}{R} \right)^6 = 8.79 \times 10^{23} \frac{\kappa^2 Q_D J}{R^6 n^4 \tau_D} \quad (2.6) $$

where $J$ is the spectral overlap integral (in cm\textsuperscript{3}M\textsuperscript{-1}) between donor emission spectrum and acceptor absorption spectrum (shown in figure 2.1F), which was obtained using the following equation:

$$ J = \frac{\int_{\lambda}^{\infty} F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int_{0}^{\infty} F_D(\lambda) d\lambda} \quad (2.7) $$

where $F_D$ is the normalized emission spectrum of the donor ($W_d$ or $W_p$). $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor (in M\textsuperscript{-1}cm\textsuperscript{-1}). $\varepsilon_A(\lambda)$ has the same shape as $W_p$ or $W_c$ absorption spectra with the maxima adjusted to 5500 M\textsuperscript{-1}cm\textsuperscript{-1}, which is the literature molar extinction coefficient of tryptophan in proteins at the absorption peak.\textsuperscript{104} The unit of wavelength $\lambda$ was converted to centimeter to give $J$ values in cm\textsuperscript{3}M\textsuperscript{-1}.

$R$ (in Å) is the center-to-center distance between the donor and acceptor (data in table 2.2, 2.3 and 2.7) based on X-ray structure reported by Wu et al.\textsuperscript{82} We used the
midpoint of the C₃a-C₇a bond of indole ring as the center of the indole chromophores for
distance calculations.

κ² is the orientation factor defined as follows:

$$\kappa^2 = (\mu_\Lambda \cdot \mu_D - 3(\mu_\Lambda \cdot R)(\mu_D \cdot R))^2$$  \hspace{1cm} (2.8)

where $\mu_\Lambda$ and $\mu_D$ are the normalized transition dipole moments of the acceptor and the
donor respectively, which were determined using X-ray structure and previously reported
transition dipole moment of indole 1Lₐ state. $R$ is the normalized vector connecting
centers of donor and acceptor tryptophan residues. The calculated κ² values are shown in
table 2.4.

n is the refractive index within protein and the value of 1.33 was used. $Q_D$ is the
fluorescence quantum yield (QY) of donor without acceptors. $\tau_D$ is the donor lifetime in
the units of nanosecond.

For distal tryptophan donors (W_d), the fluorescence QY of mono-6W_d (0.15) was
used in calculations. Since UVR8 distal Trp have two lifetimes, the amplitude weighted
average lifetime was used here.

$$\tau_D = \frac{A_1\tau_{D1} + A_2\tau_{D2}}{A_1 + A_2}$$  \hspace{1cm} (2.9)

In equation 2.9, $\tau_{D1}$ and $\tau_{D2}$ are 0.5 ns and 2.7 ns, as determined by experiments.
$A_1$ and $A_2$ are the corresponding amplitudes, which are 0.29 and 0.71 respectively.

For peripheral tryptophan donors (W_d), the fluorescence quantum yield couldn’t
be measured directly. We applied the following relations for the calculation:
\[
\tau_{\text{radiative}} = \frac{\tau_d}{Q_d} \tag{2.10}
\]

\[
k_{\text{FRET}} = \frac{1}{\tau_D} \left( \frac{R_0}{R} \right)^6 = 8.79 \times 10^{23} \frac{k^2 J}{R_0^6 n^4 \tau_{\text{radiative}}} \tag{2.11}
\]

In equation 2.10 and 2.11, \(\tau_{\text{radiative}}\) is the radiative lifetime of the donor tryptophan. The typical value of radiative lifetime of tryptophan is approximately 20 ns in polar environment. Thus, \(\tau_{\text{radiative}}\) of 20 ns was used in equation 2.11 for calculating energy transfer rates from peripheral tryptophan donors.

After obtaining the FRET rates, the corresponding time scales \(\tau_{\text{FRET}}\) (in ns) for each donor-acceptor pair (table 2.6 and 2.9) were calculated using equation 2.12:

\[
\tau_{\text{FRET}} = \frac{1}{k_{\text{FRET}}} \tag{2.12}
\]

2.2.8 Total FRET Rates and Model Simulations for Distal Tryptophan FRET

Every distal tryptophan transfers energy to all 14 interfacial Trp on both UVR8 subunits. This process was treated as parallel reactions in our model. Considering a distal tryptophan residue \(W_m\) (\(m=39, 92, 144, 196, 300\) and 352), the total FRET rate of all parallel channels was given using the following equation, using theoretical \(k_{\text{FRET}}\) obtained from the previous section.

\[
k_{\text{total}, W_m} = \frac{1}{\tau_{\text{FRET}, W_m W_i}} = \sum_i \frac{1}{\tau_{\text{FRET}, W_m W_i}} \tag{2.13}
\]

where \(k_{\text{FRET}, W_m W_i}\) is the calculated FRET rate from one distal tryptophan \(m\) (\(W_m\)) to one
interfacial tryptophan $i$ (Wi). $\tau_{FRET,Wm,Wi}$ is the corresponding time constant shown in table 2.6. $N$ is the number of interfacial Trp in the protein. For WT, $N$ is 14 (7 tryptophans on each subunit). For mutants, $N$ is less than 14 because certain interfacial Trp were knocked out. The corresponding time constant was obtained via the following equation:

$$\tau_{\text{total},Wm} = \frac{1}{k_{\text{total},Wm}} \tag{2.14}$$

Similarly, the $k_{\text{total}}$ and $\tau_{\text{total}}$ were obtained for energy transfer from W$_p$ to W$_c$ for each of the three W$_p$, using equations 2.13 and 2.14. However, energy transfer to the cluster that is further away was not considered ($N=4$ in equation 13 for WT) due to negligible contribution to the total transfer rates (see table 2.8).

Furthermore, the average FRET rate and time constant (theoretical) of the 6 distal Trp were given in the following equation (theoretical $\tau_{\text{Ave}}$ in table 2.10 Column 3).

$$\text{Theoretical} \frac{1}{\tau_{\text{Ave}}} = k_{\text{Ave}} = \frac{\sum_{m} k_{\text{total},Wm}}{6} \tag{2.15}$$

The 2$^\text{nd}$ lifetime of distal tryptophan $m$ ($m=39, 92, 144, 196, 300$ or 352) in the presence of acceptors was obtained as follows.

$$\tau_{\text{DA2,Wm}} = 1 / (1 / \tau_{D2} + 1 / \tau_{\text{total},Wm}) \tag{2.16}$$

By summing up the decay dynamics of all the 6 distal Trp (colored dashed lines in figure 2.6), we simulated the total fluorescence dynamics (black solid lines in figure 2.6) based on theoretical FRET calculations:

$$\text{Simul.}(t) = \sum_{m} W_{m} \exp(-t / \tau_{\text{DA2,Wm}}) \tag{2.17}$$
We found the simulated decay curve can be fitted with single exponential decays using one effective $\tau_{DA2}$ (see figure 2.6). Furthermore, by comparison, we found the following relation (table 2.10 Column 4 and 5):

$$\text{Effective } \tau_{DA2} \approx \frac{1}{\tau_{D2}^{-1} + \tau_{Ave}^{-1}} \quad (2.18)$$

Thus, from experimentally measured average $\tau_{DA2}$, experimental $\tau_{Ave}$ (table 2.11 Column 5) was obtained:

$$\text{Experimental } \tau_{Ave} = \frac{1}{\tau_{DA2}^{-1}(\text{Exp}) - \tau_{D2}^{-1}} \quad (2.19)$$

$\tau_{D2}$ is 2.7 ns. The experimental $\tau_{Ave}$ was then compared with the theoretical value obtained from equation 2.15 (table 2.11 Column 4). Errors of experimental $\tau_{Ave}$ were estimated by assuming 0.1 ns fitting errors in $\tau_{DA2}$. Thus, experimental $\tau_{Ave}$ errors are relatively large when $\tau_{DA2}$ is close to 2.7 ns.

### 2.2.8 Model Simulation of Picosecond Resolved TCSPC Data

Procedure for model simulations are detailed in Appendix B. Briefly, we derived the excited state kinetics for different Trp using initial populations, calculated FRET rates and experimentally measured lifetimes. TCSPC transients were then simulated with temporal evolutions of Trp excited states and emission spectra of 3 Trp groups.

### 2.2.9 Energy Transfer Efficiency and Light Perception Calculations in UVR8

Details about FRET efficiency calculations can be found elsewhere.$^{103}$ Briefly,
FRET efficiency (E) is defined as follows:

\[ E = 1 - \frac{\tau_{DA}}{\tau_D} \]  

(2.20)

where \( \tau_{DA} \) and \( \tau_D \) are donor fluorescence decay time constants with and without the acceptors respectively. In our study, \( \tau_D \) was experimentally measured when the acceptors were removed by site-directed mutagenesis. \( \tau_{DA} \) was calculated using \( \tau_D \) and theoretically calculated total FRET rates \( \tau_{total} \) (defined in equation 2.13 and 2.14):

\[ \tau_{DA} = \frac{1}{\tau_D^{-1} + \tau_{total}^{-1}} \]  

(2.21)

As above mentioned, distal tryptophan residues have two lifetimes: \( \tau_{D1} \) and \( \tau_{D2} \), which are 0.5 ns and 2.7 ns. To consider the overall FRET efficiency of the two different subpopulations, we used the population weighted average efficiency for each distal Trp Wm (m=39, 92, 144, 196, 300 or 352):

\[ E_{Wm} = (1 - R_{D2})E_1 + R_{D2}E_2 \]  

(2.22)

in which, \( R_{D2} \) is the amplitude ratio (0.71) of the 2.7 ns lifetime; \( E_1 \) and \( E_2 \) are the FRET efficiencies of the two components and were calculated as follows:

\[ E_1 = 1 - \frac{(\tau_{D1}^{-1} + \tau_{total,Wm}^{-1})^{-1}}{\tau_{D1}} \]  

(2.23)

\[ E_2 = 1 - \frac{(\tau_{D2}^{-1} + \tau_{total,Wm}^{-1})^{-1}}{\tau_{D2}} \]  

(2.24)

In each UVR8 protein sample, the overall FRET efficiency for all 6 \( W_d \) is the arithmetic average of the FRET efficiencies of 6 individual \( W_d \) (from equation 2.22).
\[ E = \frac{\sum E_{Wm}}{6} \]  

(2.25)

Alternatively, the overall energy transfer efficiency was calculated with experimental average FRET time (Exp. \( \tau_{ave} \)) as defined in equation 2.19 (shown in table 2.11):

\[ E = (1 - R_{D2})(1 - \frac{(\tau_{D1}^{-1} + \tau_{ave}^{-1})^{-1}}{\tau_{D1}}) + R_{D2}(1 - \frac{(\tau_{D2}^{-1} + \tau_{ave}^{-1})^{-1}}{\tau_{D2}}) \]  

(2.26)

Energy transfer efficiencies from W198, W250 and W302 to the pyramid center were calculated based on our models, in which each \( W_p \) has two lifetimes and 15% (\( R_{nt} \)) with no energy transfer. Similarly, the population weighted average efficiency for each \( W_p \) is:

\[ E_{Wi} = (1 - R_{nt})(1 - \frac{(\tau_{1, Wi}^{-1} + \tau_{total,Wi}^{-1})^{-1}}{\tau_{1, Wi}}) + R_{2, Wi}(1 - \frac{(\tau_{2, Wi}^{-1} + \tau_{total,Wi}^{-1})^{-1}}{\tau_{2, Wi}}) \]  

(2.27)

where \( R_{2, Wi} \) is the amplitude ratio of the slower lifetime of Wi (i=198, 250 or 302); \( \tau_{1, Wi} \) and \( \tau_{2, Wi} \) are the faster (~1 ns) and slower (6-8 ns) lifetimes of Wi, respectively. \( \tau_{total,Wi} \) is the total FRET rate of Wi as defined in equations 2.13 and 2.14. Values are shown in table 2.15.

For WT/W400F, the energy transfer efficiency from \( W_d \) directly to the pyramid center (4\( W_c \)) was calculated for each \( W_d \).

\[ E_{direct,Wm4Wc} = E_{Wm} \frac{R_{Wm}}{R_{Wm} + 1} \]  

(2.28)

\( E_{Wm} \) is the FRET efficiency for \( W_m \) (m=39, 92, 144, 196, 300 or 352) in WT/W400F as calculated with equation 2.22. \( R_{Wm} \) is the FRET branching ratio for \( W_m \) shown in table 2.6.
The energy transfer efficiency from each \( W_d \) to certain \( W_p \) was calculated as follows:

\[
E_{WmW_i} = E_{Wm} \frac{\tau_{FRET,WmW_i}^{-1} + \tau_{FRET,WmW_i(b)}^{-1}}{\tau_{total,Wm}^{-1}}
\]  

(2.29)

where \( \tau_{total,Wm} \) is the total FRET time constant defined in equation 2.13 (shown in figure 2.3A) for \( Wm \) (\( m = 39, 92, 144, 196, 300 \) or 352). \( \tau_{FRET,WmW_i} \) and \( \tau_{FRET,WmW_i(b)} \) are the FRET time constants for D-A pairs \( Wm-Wi \) and \( Wm-Wib \) (\( i = 198, 250 \) or 302) respectively (data shown in table 2.6).

The energy transfer efficiency from each \( W_d \) to the pyramid center via two-step transfer through \( W_p \) was calculated using the following equation:

\[
E_{twostep,WmWc} = E_{W198}^{WmW198} + E_{W250}^{WmW250} + E_{W302}^{WmW302}
\]

(2.30)

\( E_{W198}, E_{W250} \) and \( E_{W302} \) are the energy transfer efficiencies to the pyramid center given by equation 2.27. \( E_{WmW198}, E_{WmW250} \) and \( E_{WmW302} \) are the energy transfer efficiencies from \( Wm \) (\( m = 39, 92, 144, 196, 300 \) or 352) to \( W198, W250 \) and \( W302 \) respectively, calculated using equation 2.29.

Total efficiencies for \( W_d \) energy transfer to the pyramid center is the sum of efficiencies of the direct and indirect transfer.

\[
E_{Wm4W_c} = E_{direct,Wm4W_c} + E_{twostep,Wm4W_c}
\]

(2.31)

All results are shown in table 2.15 and 2.16.

Finally, the fraction of absorbed photons (\( \lambda_{ex}=290 \) nm) that is ultimately utilized to excite the pyramid center in UVR8 WT, the light perception efficiency, was given by
the following equation:

\[
E_{290nm} = \frac{A_d(290nm) \sum W_i E_{W_{400w}} + A_p(290nm) \sum W_i E_{W_i} + 4A_e(290nm)}{6A_d(290nm) + 4A_p(290nm) + 4A_e(290nm)}
\]  

(2.32)

In equation 2.32, we assumed that W400 has the same extinction coefficient as Wp (A_{W400}=A_p) and doesn’t involve the energy transfer network of UVR8. With the efficiency values in table 2.15 and 2.16, the light harvesting efficiency was calculated to be 0.65, meaning that for every 100 photons absorbed by UVR8 WT, about 65 of them finally reach the pyramid center. If all energy transfer efficiencies (E) are set to zero, equation 2.32 gives the probability of direct excitation of the pyramid center, which is 0.32.

The total efficiency of the 80 ps photoreaction channel (λ_{ex}=290 nm) is:

\[
E_Q = R_{c1}E_{290nm}
\]  

(2.33)

With R_{c1}=0.75, E_Q was calculated to be 0.49, meaning that for every 100 photons absorbed by UVR8 WT, about 49 of them were quenched via the 80 ps channel.

2.3 Results and Discussion

2.3.1 Absorption and Emission Spectra of 3 Trp Groups

We first seek to obtain the absorption and emission spectra of the 3 Trp groups by designing Trp mutations. Knockout of all the 3 peripheral and 4 pyramid center Trp causes the protein (W285/233/94/337/198/250/302F) does not yield well-folded UVR8 protein. However, well-folded protein can be obtained when an additional residue R286
is mutated (W285/233/94/337/198/250/400F/W302H/R286A or mono-6W₉), which makes the mutant monomeric without UV light. Since previous FTIR experiments have suggested that UVR8 dimer and monomer have similar overall tertiary structure, the local environments of buried distal Trp in the mono-6W₉ mutant should be the same as those in dimers.¹⁰⁰ By site-directed mutagenesis, we can knockout the whole pyramid center Trp group (mutant W285/233/94/337/400F or 4W₉/400F), or the peripheral Trp group (mutant W198/250/302/400F) or both Trp groups (mono-6W₉).

For absorption spectra, significant differences were observed at the red-side region beyond 300 nm (figure 2.1D inset). For WT and W198/250/302/400F, in which the Trp pyramid center is intact, the absorption spectra extend to above 320 nm, which may come from the exciton couplings among the 4 closely packed pyramid center Trp.⁸³,⁹³ However, the 2 mutants with pyramid center Trp completely knocked out (4W₉/400F, mono-6W₉) have negligible absorbance at wavelengths beyond 314 nm, suggesting that pyramid Trp, not distal Trp or peripheral Trp, contribute to UVR8 absorption in such region (above 314 nm). Similarly, by comparing the absorption spectra for 4W₉/400F and mono-6W₉, we found that distal Trp (W₉) show negligible absorbance beyond 310 nm, whereas peripheral Trp (Wₙ) absorb UV light with wavelengths up to 314 nm. From the absorption spectra of WT and mutants, absorption spectra of 3 groups of Trp were readily obtained by spectra subtraction (equations 2.1-2.3, figure 2.1E). Importantly, the absorption data indicates that 310 nm pump light selectively excites the interfacial Trp (Wₙ and Wₙ) without exciting distal Trp (W₉), and that 315 nm pump light only excites Wₙ.
Using different pump wavelengths and proteins samples (290 nm: mono-6W\textsubscript{d}, 310 nm: 4W\textsubscript{d}400F, 315 nm: WT), we obtained the steady-state emission spectra of 3 Trp groups (figure 2.1E), which have distinct emission peaks: 320 nm for W\textsubscript{d}, 340 nm for W\textsubscript{p} and 350 nm for W\textsubscript{c}. The mutant mono-6W\textsubscript{d} (distal Trp only) has a structured emission spectrum with the peak at 320 nm, which is characteristic of tryptophan residues buried in proteins\textsuperscript{107}, consistent with X-ray structure. Unlike the 6 buried distal tryptophan residues, the 3 peripheral tryptophan residues show typical fluorescence spectra for tryptophan residues on protein surface, with peaks at 340 nm. To further test W\textsubscript{p} emission, we measured the emission spectra at 3 different excitation wavelengths (290 nm, 300 nm and 310 nm) for mutants containing distal Trp and peripheral Trp. As shown in figure 2.2, the emission spectra gradually shift to the red side as the excitation wavelength was tuning to longer wavelengths, owing to the different absorption ratios of W\textsubscript{p} to W\textsubscript{d} at various excitation wavelengths. Importantly, all these mutants have nearly identical emission spectra with 310 nm excitation, suggesting that the 3 peripheral tryptophan residues have very similar emissions (figure 2.2H). The pyramid tryptophan group has the reddest emission (peak 350 nm) among the 3 groups, which as pointed out by previous computational studies, is due to the nearby electrostatic environment that stabilizes W233 La excited state\textsuperscript{99}. The steady-state spectrum data reveal a striking hierarchical arrangement, in which tryptophan chromophores that are further from the reaction center have bluer absorption and emission, favoring energy donating, whereas tryptophan chromophores that are nearer to the reaction center have redder absorption and emission, favoring energy accepting.
The direction of energy flow was quantitatively evaluated by calculating FRET spectral overlap integrals (equation 2.7) for forward and backward energy transfer among 3 Trp groups (Fig. 2.1F). As shown in figure 2.1F, the overlap integrals for $W_d$-to-$W_p$, $W_d$-to-$W_c$ and $W_p$-to-$W_c$ are $1.38 \times 10^{-16}$ cm$^3$M$^{-1}$, $2.67 \times 10^{-16}$ cm$^3$M$^{-1}$ and $7.21 \times 10^{-17}$ cm$^3$M$^{-1}$, respectively, whereas the overlap integrals for the corresponding backward transfer are $1.01 \times 10^{-17}$ cm$^3$M$^{-1}$, $4.08 \times 10^{-18}$ cm$^3$M$^{-1}$ and $6.91 \times 10^{-18}$ cm$^3$M$^{-1}$, respectively, which are at least one order of magnitude smaller than those for the forward transfer. Thus, the backward energy transfer could be neglected and the excitation energy primarily flows in one direction from $W_d$ to $W_p$, from $W_d$ to $W_c$ and from $W_p$ to $W_c$.

2.3.2 Energy Transfer Rate Calculations with FRET Theory

We next applied FRET ( Förster Resonance Energy Transfer) theory, using X-ray structure and spectral overlap integrals, to calculate energy transfer time constants ($\tau_{\text{FRET}}$) for all possible Trp donor-acceptor pairs (shown in figure 2.3 and tables 2.2-2.9). As shown in figure 2.3A, the calculated time constants from $W_d$ to $W_p/W_c$ vary significantly, depending on the distances and orientations, from a few ns to more than 1000 ns. Notably, the distal tryptophan residues from one subunit can donate excitation energy to interfacial Trp on both subunits, which allows higher energy transfer efficiency. By comparing sums of FRET rates to the Trp on 2 subunits, we found that $W92$ and $W144$ exhibit comparable strength of energy transfer to interfacial Trp on both monomers, whereas the rest 4 distal Trp prefer FRET to interfacial Trp within the same subunit, due to shorter distances (table 2.6). Rates of 14 parallel FRET channels were
added to obtain the total FRET time scales ($\tau_{total}$ in figure 2.3) for the 6 $W_d$ (3.6 ns, 2.3 ns, 2.0 ns, 2.1 ns, 1.8 ns and 1.6 ns for W39, W92, W144, W196, W300 and W352 respectively), which are comparable to typical tryptophan lifetimes, suggesting significant energy transfer efficiency. In addition, total FRET time scales to pyramid tryptophan residues and those to peripheral tryptophan residues were calculated, which indicates that energy transfer to pyramid center Trp group is faster than that to peripheral Trp group for all 6 distal Trp. This feature, which is due to more favorable transition dipole coupling and greater spectral overlap between distal Trp and pyramid Trp, increases the portion of energy that is directly transferred to the reaction center.

As shown in figure 2.3B, as a result of shorter donor-acceptor distances, theoretical energy transfer time constants from $W_p$ to $W_c$ fall in the range of picoseconds to nanoseconds. By comparing $\kappa^2/R^6$, we found that $W_p$ nearly exclusively transfers energy to the nearer pyramid center (table 2.8). In the $W_p$-to-$W_c$ FRET pathways (figure 2.3B), we found that W250 and W302 mainly transfer energy to W233 and W285, respectively, with the rate constants of 120 ps and 50 ps, respectively. Surprisingly, compared with W250 and W302, which have $\tau_{total}$ of 100 ps and 50 ps, respectively, owing to unfavorable orientations and longer distances, W198 has a dramatically slower calculated total FRET rate, with a $\tau_{total}$ of 2.0 ns. Such an uneven energy transfer pattern from 3$W_p$ to 4$W_c$ is consistent with recent computational results.98
2.3.3 Experimental Energy Transfer Rates

We then used time resolved fluorescence spectroscopy to test the accuracy of the calculated time constants for \( W_d \)-to-\( W_p/W_c \) energy transfer. As shown by mono-6\( W_d \) transients (figure 2.5A-B), \( W_d \) exhibits two lifetimes (\( \tau_{D1} \): 500 ps, 29%; \( \tau_{D2} \): 2.7 ps, 71%) without the acceptors. Fluorescence decay transients gated at 16 wavelengths were measured for various UVR8 protein samples containing 6\( W_d \) and certain interfacial Trp (shown in fig. 2.4) to measure the lifetime change of 6\( W_d \) in the presence of acceptors. From global fitting, we constructed the time constant associated spectra for all observed decay time constants, as shown in figure 2.5C. For the 7 mutants that consist of \( W_d \) and \( W_p \) only (figure 2.5C1-7), we observed three time scales: 0.5 ns, 1.9-2.4 ns and 6-8 ns. The 6-8 ns component must come from the lifetime \( W_p \), since it was not seen in mono-6\( W_d \). Further verifying such assignment, the spectra associated with the 6-8 ns time constants (yellow square, figure 2.5C1-7) have nearly the same shape as \( W_p \) spectra (green solid lines, figure 2.5C1-7) for all 7 mutants, as measured with 310 nm pump. Similarly, judging by spectrum shapes, the 0.5 ns and 1.9-2.4 ns time scales correspond to the first and second lifetimes of \( W_d \) in the presence of acceptors (\( \tau_{DA1} \), \( \tau_{DA2} \)). Supporting such argument, in all mutants, \( \tau_{DA2} \) is smaller than \( \tau_{D2} \) (2.7 ns) due to energy transfer to \( W_p \), whereas \( \tau_{DA1} \) is fixed to 0.5 ns because it is expected to be almost unchanged in the presence of nanosecond energy transfer channels. For the mutant W198/250/302/400F (figure 2.5C8) and WT (figure 2.5C9), 4 time constants are needed to globally fit the transients. Since faster \( W_d \) FRET rates were expected with the pyramid center, \( \tau_{DA1} \) (in purple circles) was fixed to 0.45 ns instead of the previous 0.50 ns in our
fitting based on the theoretical estimates. \( \tau_{DA2} \) were found to be 1.5 ns and 1.2 ns for W198/250/302/400F and WT, respectively. The two additional time scales, which are 1.4 ns and 5.4 ns in the mutant; 1.6 ns and 6.9 ns in WT, must be assigned to \( W_p \) or \( W_c \). In W198/250/302/400F mutant, where all 3 \( W_p \) are knocked out, the total emission spectrum of 1.4 ns and 5.4 ns component (cyan hexagons) is nearly identical to that of WT pumped at 315 nm (red solid line), validating our assignment. On the other hand, the total spectrum of the 1.6 ns and 6.9 ns components for WT is blue shifted compared to \( W_c \) emission, indicating \( W_p \) contributions. For comparison, we next simulated the overall decay dynamics of \( W_d \) 2.7 ns lifetime component with calculated FRET time constants (figure 2.6). Significantly, even though the 6 \( W_d \) donors have distinct decay dynamics (dashed lines in figure 2.6) due to different FRET rates, the simulated total dynamics (black solid lines in figure 2.6) can be perfectly described with a single exponential decay, whose time constant is in good agreement with experimental \( \tau_{DA2} \) in all mutants, showing that our proposed scheme shown in figure 2.3 is accurate for energy transfer from \( W_d \) to \( W_p/W_c \). Collectively, our model suggests that 6 \( W_d \) donate excitation energy to the 14 interfacial Trp (7 from each monomer) with an average time constant of 2.2 ns and with an efficiency of 0.45 in WT.

To investigate the energy transfer dynamics from \( W_p \) to \( W_c \), ps resolved time correlated single photon counting (TCSPC) experiments were conducted with 310 nm and 315 nm pump wavelengths to avoid \( W_d \) signal. Unlike 6 \( W_d \), the positions of 3 \( W_p \) are not symmetrical, which may lead to different lifetimes. Thus, as shown in figure 2.7, we determined lifetimes of W302, W250 and W198 one by one with 310 nm excitation,
using mutants containing 6 W<sub>d</sub> and the desired W<sub>p</sub> (6W<sub>d</sub>+1W<sub>p</sub> mutants). Furthermore, the fluorescence decay transients (pump 310 nm) of “6W<sub>d</sub>+2W<sub>p</sub>” and “6W<sub>d</sub>+3W<sub>p</sub>” mutants agree well with sum of individual W<sub>p</sub> lifetime decay dynamics measured in “6W<sub>d</sub>+1W<sub>p</sub>” mutants (figure 2.8), which validates the measured values and suggests no interaction among the 3 W<sub>p</sub>. Notably, fluorescence decay dynamics of the pyramid center (figure 2.9A), as measured with W400F, has two time scales: 80 ps (75%) and 1.4 ns (25%). The 80 ps is consistent with the previously reported quenching times, which has been suggested to be W285/W233 charge separation time, whereas the latter time scale was not discovered before due to a lack of clean 4W<sub>c</sub> data. Since the mutants W94F and W337F show similar W<sub>c</sub> dynamics (figure 2.9D) to W400F, such double exponential dynamics is an intrinsic property of the pyramid, and may derive from the structural fluctuations of the pyramid center. Contradictory to W198/250/302/400F data, we did not observe a 5.4 ns component in W400F pump 315 nm transients. To reconcile such discrepancy, pump 315 nm experiments were conducted with certain W<sub>p</sub> mutants (figure 2.9C). The data suggest that mutation of W302, but not W198 or W250, slows down pyramid center dynamics, likely due to structural perturbations, giving the 5.7 ns slow time scale as observed in W198/250/302/400F mutant. Therefore, W302 was kept in the following mutant studies. Transients measured at various fluorescence wavelengths are shown in figure 2.10-2.14. Consistent to previous observations, all transients display a small percentage 6-8 ns component, which is close to W<sub>p</sub> lifetimes. Such finding indicates that a small population of W<sub>p</sub> exhibit extremely slow energy transfer, probably due to fluctuations of the flexible loop structures at the dimer interface, allowing the Trp
to sample conformations that are unfavorable to energy transfer. With 15% of such non-energy-transfer \( W_p \) population and the calculated FRET rates, we simulated pump 310 nm transients, which agree well with experimental data for all different fluorescence wavelengths in all 4 mutants (figure 2.10-2.13). These results suggest that energy transfer dynamics from \( W_p \) to \( W_c \) also follows FRET scheme, but with about 15% non-energy-transfer population. Because both \( W_p \) to \( W_c \) are located at flexible loop region, UVR8 protein may sample conformations that are extremely unfavorable to \( W_p \) to \( W_c \) energy transfer, leading to the non-energy-transfer population.

Besides FRET mechanism, another widely observed resonance energy transfer mechanism is Dexter resonance energy transfer. Unlike FRET, which is mediated by transition dipole coupling between the donor and the acceptor, Dexter energy transfer occurs via an electron exchange between the donor and the acceptor at short distances. In our case, due to large Trp transition dipole moment, coulomb coupling is dominant over exchange coupling, suggesting that Dexter resonance energy transfer mechanism is not important in UVR8, comparing to Förster mechanism.\(^{105,108}\) We will not discuss the possibility of Dexter energy transfer mechanism here.

### 2.3.4 Energy Transfer Efficiencies and Overall Efficiency of UVR8

Finally, combining all our data, we simulated pump 290 nm fluorescence transients for W400F, (figure 2.14), where all 3 Trp groups were included, and we found simulated curves are in good agreement with experimental data. From time integrals of
simulation curves for different Trp groups, we constructed emission spectra for the Trp groups (figure 2.15), which have similar shapes to those measured directly (solid lines in figure 2.15). Based on our model, assuming W400 does not participate in the network, we calculated percentage of excitation energy transferred to the pyramid center. For \(W_d\), the overall energy transfer efficiency to \(W_c\) (\(E_{4Wc}\)) is 0.42, lower than total FRET efficiency (0.45) due to loss in two-step transfer via \(W_p\). With 15% non-transfer population, W198, W250 and W302 have energy transfer efficiency of 0.59, 0.83 and 0.84, respectively. Given the extinction coefficients of 3 Trp groups, for every 100 photons (290 nm) absorbed by UVR8 WT, 32 of them directly excite the pyramid center, 33 of them reach the pyramid center through energy transfer. Compared to 32% direct absorption by the 4 \(W_c\), the energy transfer processes increase light perception efficiency of UVR8 by two-fold, from 32% to 65%. Among the 65 photons at the pyramid center, 49 (75% of 65) get quenched via the 80 ps channel.

2.4 Conclusion

With extensive mutagenesis and spectroscopy studies, we revealed a remarkable Trp-to-Trp energy transfer network, which enhances UVR8 light perception efficiency. Owing to solvation and electrostatic environments, UVR8 distal Trp (\(W_d\): W39 W92 W144 W196 W300 W352), peripheral Trp (\(W_p\): W198 W250 W302) and pyramid center Trp (\(W_c\): W94 W233 W285 W337, reaction center) have distinct emission spectra with peaks at about 320 nm, 340 nm and 350 nm, respectively. The absorption spectra of the 3 Trp groups show significant difference in the red side region (above 300 nm): the distal
Trp have negligible absorbance beyond 310 nm; the peripheral Trp absorption spectrum extends to ∼314 nm; the pyramid center Trp absorption red side tail reaches beyond 320 nm. This hierarchical arrangement causes the overlap integrals of forward energy transfers to be more than 10-fold greater than those of backward transfers, which enables excitation energy to flow in one direction from distal Trp to peripheral Trp and pyramid center Trp, or from peripheral Trp to pyramid center Trp. The time scales of various energy transfer pathways were determined with FRET theory and time-resolved spectroscopy. By studying mutant model systems, we found that Förster theory works well in calculating rate constants for energy transfer from distal Trp to interfacial Trp, which is due to relatively long distances between donors and acceptors. Energy transfer from peripheral Trp to pyramid center Trp also follow FRET dynamics, with picosecond to nanosecond time scales, but with a 15% non-transfer population, probably owing to structural flexibility at interfacial loop region. Like the photosynthetic systems, energy transfer processes in UVR8 greatly enhance its light harvesting efficiency by 100%, making it an effective UV-B sensor in plants. After the UV-B excitation energy are funneled to the reaction center, 75% excited population is quenched on a ∼80 ps time scale, which causes UVR8 dimer to dissociate, while a portion of excitation energy dissipates on 1.4 ns time scale.
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Table 2.1 Mutant summary

*Mutation of all peripheral Trp without knocking out R286 leads to no protein yield.
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<td>8.0</td>
<td>21.9</td>
<td></td>
</tr>
<tr>
<td>W94b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 Distance matrix for tryptophan residues in UVR8*

*Results were based on X-ray structure reported by Wu et al. We used the midpoint of the C<sub>3a</sub>-C<sub>7a</sub> bond of indole ring as the center of the indole chromophores for distance calculations.
Table 2.3 Distances between distal and interfacial tryptophan residues*

<table>
<thead>
<tr>
<th>R (Å)</th>
<th>W39</th>
<th>W92</th>
<th>W144</th>
<th>W196</th>
<th>W300</th>
<th>W352</th>
</tr>
</thead>
<tbody>
<tr>
<td>W94</td>
<td>20.5</td>
<td>14.1</td>
<td>14.9</td>
<td>21.8</td>
<td>28.3</td>
<td>26.0</td>
</tr>
<tr>
<td>W198</td>
<td>29.7</td>
<td>26.8</td>
<td>20.1</td>
<td>13.6</td>
<td>20.7</td>
<td>27.0</td>
</tr>
<tr>
<td>W233</td>
<td>21.7</td>
<td>21.1</td>
<td>18.9</td>
<td>17.2</td>
<td>17.1</td>
<td>19.7</td>
</tr>
<tr>
<td>W250</td>
<td>27.4</td>
<td>28.8</td>
<td>25.6</td>
<td>19.8</td>
<td>14.3</td>
<td>21.3</td>
</tr>
<tr>
<td>W285</td>
<td>19.4</td>
<td>21.6</td>
<td>22.3</td>
<td>21.7</td>
<td>16.0</td>
<td>16.4</td>
</tr>
<tr>
<td>W302</td>
<td>21.1</td>
<td>26.8</td>
<td>28.8</td>
<td>27.1</td>
<td>14.0</td>
<td>14.1</td>
</tr>
<tr>
<td>W337</td>
<td>16.7</td>
<td>17.4</td>
<td>18.6</td>
<td>20.0</td>
<td>17.3</td>
<td>16.2</td>
</tr>
<tr>
<td>W94b</td>
<td>23.3</td>
<td>23.8</td>
<td>23.9</td>
<td>23.8</td>
<td>21.5</td>
<td>21.9</td>
</tr>
<tr>
<td>W198b</td>
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<td>25.2</td>
<td>18.7</td>
<td>20.6</td>
<td>33.9</td>
<td>35.9</td>
</tr>
<tr>
<td>W233b</td>
<td>27.0</td>
<td>21.0</td>
<td>19.6</td>
<td>24.2</td>
<td>32.0</td>
<td>31.1</td>
</tr>
<tr>
<td>W250b</td>
<td>30.8</td>
<td>21.6</td>
<td>18.8</td>
<td>25.9</td>
<td>38.1</td>
<td>37.0</td>
</tr>
<tr>
<td>W285b</td>
<td>24.3</td>
<td>18.4</td>
<td>20.1</td>
<td>27.0</td>
<td>33.0</td>
<td>30.1</td>
</tr>
<tr>
<td>W302b</td>
<td>25.2</td>
<td>18.0</td>
<td>22.1</td>
<td>31.5</td>
<td>38.2</td>
<td>33.6</td>
</tr>
<tr>
<td>W337b</td>
<td>24.7</td>
<td>21.0</td>
<td>22.6</td>
<td>27.6</td>
<td>31.4</td>
<td>28.9</td>
</tr>
</tbody>
</table>

*Results were based on X-ray structure reported by Wu et al.\textsuperscript{82} We used the midpoint of the C\textsubscript{3a}-C\textsubscript{7a} bond of indole ring as the center of the indole chromophores for distance calculations.
<table>
<thead>
<tr>
<th>$\kappa^2$</th>
<th>W39</th>
<th>W92</th>
<th>W144</th>
<th>W196</th>
<th>W300</th>
<th>W352</th>
</tr>
</thead>
<tbody>
<tr>
<td>W94</td>
<td>0.46</td>
<td>0.27</td>
<td>0.00</td>
<td>0.01</td>
<td>0.58</td>
<td>0.57</td>
</tr>
<tr>
<td>W198</td>
<td>0.16</td>
<td>0.01</td>
<td>0.20</td>
<td>0.25</td>
<td>2.71</td>
<td>1.01</td>
</tr>
<tr>
<td>W233</td>
<td>0.00</td>
<td>0.20</td>
<td>1.42</td>
<td>0.73</td>
<td>0.98</td>
<td>0.42</td>
</tr>
<tr>
<td>W250</td>
<td>1.09</td>
<td>0.56</td>
<td>0.61</td>
<td>0.14</td>
<td>0.22</td>
<td>1.17</td>
</tr>
<tr>
<td>W285</td>
<td>1.58</td>
<td>0.34</td>
<td>0.22</td>
<td>0.55</td>
<td>0.20</td>
<td>1.15</td>
</tr>
<tr>
<td>W302</td>
<td>1.25</td>
<td>1.20</td>
<td>1.20</td>
<td>0.24</td>
<td>0.02</td>
<td>0.40</td>
</tr>
<tr>
<td>W337</td>
<td>0.02</td>
<td>0.00</td>
<td>0.37</td>
<td>0.14</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>W94b</td>
<td>0.17</td>
<td>0.34</td>
<td>0.99</td>
<td>0.53</td>
<td>1.70</td>
<td>1.69</td>
</tr>
<tr>
<td>W198b</td>
<td>0.02</td>
<td>0.14</td>
<td>0.05</td>
<td>2.80</td>
<td>1.67</td>
<td>0.53</td>
</tr>
<tr>
<td>W233b</td>
<td>1.03</td>
<td>0.18</td>
<td>1.00</td>
<td>0.63</td>
<td>0.66</td>
<td>1.03</td>
</tr>
<tr>
<td>W250b</td>
<td>0.61</td>
<td>0.94</td>
<td>0.02</td>
<td>0.37</td>
<td>0.26</td>
<td>0.44</td>
</tr>
<tr>
<td>W285b</td>
<td>0.04</td>
<td>0.57</td>
<td>0.12</td>
<td>1.64</td>
<td>1.30</td>
<td>0.59</td>
</tr>
<tr>
<td>W302b</td>
<td>0.92</td>
<td>0.13</td>
<td>0.06</td>
<td>0.00</td>
<td>0.59</td>
<td>0.70</td>
</tr>
<tr>
<td>W337b</td>
<td>0.02</td>
<td>0.21</td>
<td>2.64</td>
<td>0.56</td>
<td>0.02</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 2.4 $\kappa^2$ between distal and interfacial tryptophan residues for all 84 D-A pairs

*All values were calculated using equation 2.8.
Table 2.5 $\kappa^2/R^6$ between distal (W_d) and interfacial tryptophan residues (W_p and W_c) for all 84 D-A pairs

<table>
<thead>
<tr>
<th>$\kappa^2/R^6$ (10^{-9}\text{Å}^6)</th>
<th>W39</th>
<th>W92</th>
<th>W144</th>
<th>W196</th>
<th>W300</th>
<th>W352</th>
</tr>
</thead>
<tbody>
<tr>
<td>W94</td>
<td>6.28</td>
<td>34.50</td>
<td>0.09</td>
<td>0.05</td>
<td>1.13</td>
<td>1.87</td>
</tr>
<tr>
<td>W198</td>
<td>0.23</td>
<td>0.02</td>
<td>3.01</td>
<td>38.90</td>
<td>34.70</td>
<td>2.60</td>
</tr>
<tr>
<td>W233</td>
<td>0.03</td>
<td>2.26</td>
<td>31.20</td>
<td>28.40</td>
<td>39.60</td>
<td>7.19</td>
</tr>
<tr>
<td>W250</td>
<td>2.58</td>
<td>0.99</td>
<td>2.14</td>
<td>2.26</td>
<td>25.80</td>
<td>12.70</td>
</tr>
<tr>
<td>W285</td>
<td>29.50</td>
<td>3.35</td>
<td>1.77</td>
<td>5.21</td>
<td>12.00</td>
<td>59.80</td>
</tr>
<tr>
<td>W302</td>
<td>14.00</td>
<td>3.22</td>
<td>2.13</td>
<td>0.61</td>
<td>2.62</td>
<td>49.40</td>
</tr>
<tr>
<td>W337</td>
<td>1.16</td>
<td>0.11</td>
<td>9.12</td>
<td>2.22</td>
<td>0.52</td>
<td>0.64</td>
</tr>
<tr>
<td>To A*</td>
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<td>44.40</td>
<td>49.50</td>
<td>77.70</td>
<td>116.00</td>
<td>134.00</td>
</tr>
<tr>
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<td>1.03</td>
<td>1.87</td>
<td>5.36</td>
<td>2.88</td>
<td>17.00</td>
<td>15.30</td>
</tr>
<tr>
<td>W198b</td>
<td>0.01</td>
<td>0.55</td>
<td>1.15</td>
<td>36.80</td>
<td>1.10</td>
<td>0.25</td>
</tr>
<tr>
<td>W233b</td>
<td>2.62</td>
<td>2.09</td>
<td>17.90</td>
<td>3.10</td>
<td>0.62</td>
<td>1.13</td>
</tr>
<tr>
<td>W250b</td>
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<td>9.18</td>
<td>0.36</td>
<td>1.22</td>
<td>0.09</td>
<td>0.17</td>
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<tr>
<td>W285b</td>
<td>0.19</td>
<td>14.60</td>
<td>1.86</td>
<td>4.27</td>
<td>1.00</td>
<td>0.79</td>
</tr>
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<td>3.56</td>
<td>3.73</td>
<td>0.56</td>
<td>0.00</td>
<td>0.19</td>
<td>0.49</td>
</tr>
<tr>
<td>W337b</td>
<td>0.00</td>
<td>14.20</td>
<td>20.00</td>
<td>1.27</td>
<td>0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>To B**</td>
<td>8.14</td>
<td>46.20</td>
<td>47.20</td>
<td>49.60</td>
<td>20.10</td>
<td>18.20</td>
</tr>
</tbody>
</table>

*Sum of the 7 $\kappa^2/R^6$ values to the interfacial Trp on the same subunit as the distal Trp donor.

**Sum of the 7 $\kappa^2/R^6$ values to the interfacial Trp on the other subunit.
<table>
<thead>
<tr>
<th>$\tau_{\text{FRET}}$ (ns)*</th>
<th>W39</th>
<th>W92</th>
<th>W144</th>
<th>W196</th>
<th>W300</th>
<th>W352</th>
</tr>
</thead>
<tbody>
<tr>
<td>W94</td>
<td>29.7</td>
<td>5.4</td>
<td>2051.1</td>
<td>3508.5</td>
<td>165.2</td>
<td>99.8</td>
</tr>
<tr>
<td>W233</td>
<td>5394.6</td>
<td>82.6</td>
<td>6.0</td>
<td>6.6</td>
<td>4.7</td>
<td>26.0</td>
</tr>
<tr>
<td>W285</td>
<td>6.3</td>
<td>55.7</td>
<td>105.5</td>
<td>35.8</td>
<td>15.6</td>
<td>3.1</td>
</tr>
<tr>
<td>W337</td>
<td>160.9</td>
<td>1681.6</td>
<td>20.5</td>
<td>84.1</td>
<td>357.6</td>
<td>293.5</td>
</tr>
<tr>
<td>W94b</td>
<td>181.2</td>
<td>99.8</td>
<td>34.8</td>
<td>64.8</td>
<td>11.0</td>
<td>12.2</td>
</tr>
<tr>
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<td>10.4</td>
<td>60.2</td>
<td>302.0</td>
<td>165.2</td>
</tr>
<tr>
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<td>100.4</td>
<td>43.7</td>
<td>186.7</td>
<td>236.0</td>
</tr>
<tr>
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<td>9.3</td>
<td>147.0</td>
<td>7744.9</td>
<td>2399.1</td>
</tr>
<tr>
<td>To Pyramid**</td>
<td>4.57</td>
<td>2.56</td>
<td>2.14</td>
<td>3.94</td>
<td>2.60</td>
<td>2.15</td>
</tr>
<tr>
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<td>1549.9</td>
<td>16122.0</td>
<td>120.0</td>
<td>9.3</td>
<td>10.4</td>
<td>138.9</td>
</tr>
<tr>
<td>W250</td>
<td>140.0</td>
<td>365.9</td>
<td>168.8</td>
<td>159.8</td>
<td>14.0</td>
<td>28.4</td>
</tr>
<tr>
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<td>169.5</td>
<td>588.2</td>
<td>137.8</td>
<td>7.3</td>
</tr>
<tr>
<td>W198b</td>
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<td>314.0</td>
<td>9.8</td>
<td>328.3</td>
<td>1444.5</td>
</tr>
<tr>
<td>W250b</td>
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<td>39.3</td>
<td>997.6</td>
<td>296.0</td>
<td>4170.1</td>
<td>2099.6</td>
</tr>
<tr>
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<td>101.4</td>
<td>96.8</td>
<td>647.2</td>
<td>108774.9</td>
<td>1910.8</td>
<td>744.6</td>
</tr>
<tr>
<td>To peripheral***</td>
<td>17.1</td>
<td>20.4</td>
<td>38.6</td>
<td>4.53</td>
<td>5.60</td>
<td>5.50</td>
</tr>
</tbody>
</table>

| FRET Branching Ratios**** (Pyramid/Peripheral) | 3.7  | 8.0  | 18   | 1.1  | 2.2  | 2.6  |

Table 2.6 Calculated FRET time scales from distal Trp to interfacial Trp for all 84 D-A pairs

*Values were calculated with equation 2.6.

**Total FRET time constants of the parallel 8 energy transfers to the 8 pyramid center Trp, calculated using equation 2.13 and 2.14

***Total FRET time constants of the parallel 6 energy transfers to the 6 peripheral center Trp, calculated using equation 2.13 and 2.14

****Obtained by dividing total peripheral FRET time constants by total pyramid FRET time constants
<table>
<thead>
<tr>
<th>R (Å)</th>
<th>W198</th>
<th>W250</th>
<th>W302</th>
</tr>
</thead>
<tbody>
<tr>
<td>W94b</td>
<td>13.1</td>
<td>11.2</td>
<td>10.7</td>
</tr>
<tr>
<td>W233</td>
<td>8.6</td>
<td>8.2</td>
<td>11.5</td>
</tr>
<tr>
<td>W285</td>
<td>13.2</td>
<td>9.2</td>
<td>7.1</td>
</tr>
<tr>
<td>W337</td>
<td>13.9</td>
<td>12.3</td>
<td>10.7</td>
</tr>
<tr>
<td>W94</td>
<td>17.8</td>
<td>21.2</td>
<td>21.2</td>
</tr>
<tr>
<td>W233b</td>
<td>17.5</td>
<td>21.8</td>
<td>23.4</td>
</tr>
<tr>
<td>W285b</td>
<td>21.3</td>
<td>24.4</td>
<td>23.9</td>
</tr>
<tr>
<td>W337b</td>
<td>19.9</td>
<td>22.0</td>
<td>21.1</td>
</tr>
</tbody>
</table>

Table 2.7 Distances between peripheral and pyramid center tryptophan residues

*Results were based on X-ray structure reported by Wu et al. We used the midpoint of the C<sub>3a</sub>-C<sub>7a</sub> bond of indole ring as the center of the indole chromophores for distance calculations.
<table>
<thead>
<tr>
<th>$\kappa^2/R^6$ (10^{-9}\AA^6)</th>
<th>W198</th>
<th>W250</th>
<th>W302</th>
</tr>
</thead>
<tbody>
<tr>
<td>W94b</td>
<td>70.5</td>
<td>478</td>
<td>172</td>
</tr>
<tr>
<td>W233</td>
<td>310</td>
<td>8250</td>
<td>22.2</td>
</tr>
<tr>
<td>W285</td>
<td>21.6</td>
<td>1560</td>
<td>19000</td>
</tr>
<tr>
<td>W337</td>
<td>82.3</td>
<td>19.1</td>
<td>421</td>
</tr>
<tr>
<td>To the closer pyramid*</td>
<td>485</td>
<td>10000</td>
<td>20000</td>
</tr>
<tr>
<td>W94</td>
<td>2.0</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>W233b</td>
<td>15.1</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>W285b</td>
<td>18.7</td>
<td>6.5</td>
<td>10.9</td>
</tr>
<tr>
<td>W337b</td>
<td>5.6</td>
<td>30</td>
<td>33.7</td>
</tr>
<tr>
<td>To the further pyramid**</td>
<td>41</td>
<td>39</td>
<td>46</td>
</tr>
</tbody>
</table>

Table 2.8 $\kappa^2/R^6$ between peripheral and pyramid center tryptophan residues

*Sum of the 4 $\kappa^2/R^6$ values to the closer 4Wc

**Sum of the 4 $\kappa^2/R^6$ values to the further 4Wc
<table>
<thead>
<tr>
<th>$\tau_{\text{FRET (ns)}}^*$</th>
<th>W198</th>
<th>W250</th>
<th>W302</th>
</tr>
</thead>
<tbody>
<tr>
<td>W94b</td>
<td>14.0</td>
<td>2.1</td>
<td>5.7</td>
</tr>
<tr>
<td>W233</td>
<td>3.2</td>
<td>0.12</td>
<td>44.4</td>
</tr>
<tr>
<td>W285</td>
<td>45.7</td>
<td>0.63</td>
<td>0.05</td>
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<tr>
<td>W337</td>
<td>12.0</td>
<td>518</td>
<td>2.3</td>
</tr>
<tr>
<td>$\tau_{\text{total}}^{**}$</td>
<td>2.0</td>
<td>0.1</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 2.9 Calculated FRET time scales from peripheral Trp to pyramid center Trp

*Calculated using equation 2.11

**Calculated using equation 2.13 and 2.14 with $\tau_{\text{FRET}}$
<table>
<thead>
<tr>
<th>Mutant Type</th>
<th>Mutant</th>
<th>$\tau_{\text{Ave}}$ (ns) (Theor.)*</th>
<th>$\tau_{\text{DA2}}$ (ns) calculated from $\tau_{\text{Ave}}$ and $\tau_{\text{D2}}$ (Theor.)**</th>
<th>Effective $\tau_{\text{DA2}}$ (ns) from fitting of simulation curves (Theor.)***</th>
<th>$\tau_{\text{DA2}}$ (ns) (Exp.)****</th>
</tr>
</thead>
<tbody>
<tr>
<td>6W$<em>{d}$+1W$</em>{p}$</td>
<td>4W$_{c}$/400/198/250F</td>
<td>26.9</td>
<td>2.43</td>
<td>2.47</td>
<td>2.42±0.1</td>
</tr>
<tr>
<td>6W$<em>{d}$+1W$</em>{p}$</td>
<td>4W$_{c}$/400/198/302F</td>
<td>37.2</td>
<td>2.49</td>
<td>2.52</td>
<td>2.37±0.1</td>
</tr>
<tr>
<td>6W$<em>{d}$+1W$</em>{p}$</td>
<td>4W$_{c}$/400/250/302F</td>
<td>18.2</td>
<td>2.33</td>
<td>2.39</td>
<td>2.29±0.1</td>
</tr>
<tr>
<td>6W$<em>{d}$+2W$</em>{p}$</td>
<td>4W$_{c}$/400/198F</td>
<td>15.6</td>
<td>2.28</td>
<td>2.31</td>
<td>2.28±0.1</td>
</tr>
<tr>
<td>6W$<em>{d}$+2W$</em>{p}$</td>
<td>4W$_{c}$/400/250F</td>
<td>10.8</td>
<td>2.14</td>
<td>2.19</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>6W$<em>{d}$+2W$</em>{p}$</td>
<td>4W$_{c}$/400/302F</td>
<td>15.6</td>
<td>2.19</td>
<td>2.26</td>
<td>2.15±0.1</td>
</tr>
<tr>
<td>6W$<em>{d}$+3W$</em>{p}$</td>
<td>4W$_{c}$/400F</td>
<td>8.4</td>
<td>2.03</td>
<td>2.04</td>
<td>1.94±0.1</td>
</tr>
<tr>
<td>6W$<em>{d}$+4W$</em>{c}$</td>
<td>W198/250/302/400F</td>
<td>2.8</td>
<td>1.36</td>
<td>1.39</td>
<td>1.50±0.1</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>2.1</td>
<td>1.17</td>
<td>1.19</td>
<td>1.20±0.1</td>
</tr>
</tbody>
</table>

Table 2.10 Summary of distal Trp FRET rates

*Time constant of the average FRET rate of the 6 W$_{d}$ in each mutant. Defined in equation 2.15.

**Theoretical donor lifetime in the presence of the acceptor. Defined in 2.16.

***Decay time constant of simulated curve in equation 2.17.

**** $\tau_{\text{DA2}}$ measured with nanosecond resolved TCSPC (figure 2.5C).
<table>
<thead>
<tr>
<th>Mutant Type</th>
<th>Mutant</th>
<th>FRET Scheme</th>
<th>$\tau_{\text{Ave}}$ (ns) (Theor.)*</th>
<th>$\tau_{\text{Ave}}$ (ns) (Exp.)**</th>
<th>E (Theor.)***</th>
<th>E (Exp.)****</th>
</tr>
</thead>
<tbody>
<tr>
<td>6W$_d$+1W$_p$</td>
<td>4W$_d$/400/198/250F</td>
<td>6W$_d$ to W302</td>
<td>26.9</td>
<td>23±10</td>
<td>0.062</td>
<td>0.08±0.03</td>
</tr>
<tr>
<td></td>
<td>4W$_d$/400/198/302F</td>
<td>6W$_d$ to W250</td>
<td>37.2</td>
<td>19±6</td>
<td>0.050</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td></td>
<td>4W$_d$/400/250/302F</td>
<td>6W$_d$ to W198</td>
<td>18.2</td>
<td>15±4</td>
<td>0.082</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td>6W$_d$+2W$_p$</td>
<td>4W$_d$/400/198F</td>
<td>6W$_d$ to W250/302</td>
<td>15.6</td>
<td>15±4</td>
<td>0.11</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td></td>
<td>4W$_d$/400/250F</td>
<td>6W$_d$ to W198/302</td>
<td>10.8</td>
<td>9.5±2</td>
<td>0.14</td>
<td>0.17±0.03</td>
</tr>
<tr>
<td></td>
<td>4W$_d$/400/302F</td>
<td>6W$_d$ to W198/250</td>
<td>12.2</td>
<td>10.6±2</td>
<td>0.12</td>
<td>0.16±0.04</td>
</tr>
<tr>
<td>6W$_d$+3W$_p$</td>
<td>4W$_d$/400F</td>
<td>6W$_d$ to 3W$_p$</td>
<td>8.4</td>
<td>6.9±1.5</td>
<td>0.18</td>
<td>0.22±0.04</td>
</tr>
<tr>
<td>6W$_d$+4W$_c$</td>
<td>198/250/302/400F</td>
<td>6W$_d$ to 4W$_c$</td>
<td>2.8</td>
<td>3.4±0.5</td>
<td>0.39</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>6W$_d$ to 3W$_p$+4W$_c$</td>
<td>2.1</td>
<td>2.2±0.3****</td>
<td>0.45</td>
<td>0.44±0.03</td>
</tr>
</tbody>
</table>

Table 2.11 Overall FRET efficiencies of 6 distal Trp in various UVR8 samples

*Time constant of the average FRET rate of the 6 W$_d$ in each mutant. Defined in equation 2.15.

**Obtained using experimental $\tau_{\text{DA}2}$ and $\tau_{\text{D}2}$ with equation 2.19.

*** Overall FRET efficiency from 6 W$_d$ calculated using equation 2.25 for each mutant.

****Overall FRET efficiency from 6 W$_d$ calculated with equation 2.26, using the experimental $\tau_{\text{Ave}}$.

*****FRET rate constant in WT matches the value (2.3 ns) calculated from $1/(1/6.9+1/3.4)$. 

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### Table 2.12 A<sub>d</sub>, A<sub>p</sub> and A<sub>c</sub> values used in model simulations

<table>
<thead>
<tr>
<th>A&lt;sub&gt;d&lt;/sub&gt;(310 nm)</th>
<th>A&lt;sub&gt;p&lt;/sub&gt;(310 nm)</th>
<th>A&lt;sub&gt;c&lt;/sub&gt;(310 nm)</th>
<th>A&lt;sub&gt;d&lt;/sub&gt;(290 nm)</th>
<th>A&lt;sub&gt;p&lt;/sub&gt;(290 nm)</th>
<th>A&lt;sub&gt;c&lt;/sub&gt;(290 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.044</td>
<td>0.109</td>
<td>0.668</td>
<td>0.846</td>
<td>0.888</td>
</tr>
</tbody>
</table>

*Direct readings of absorption curves in figure 2.1E. The same A<sub>d</sub> value was used for all the 6 different W<sub>d</sub>. The same A<sub>p</sub> value was used for W198, W250 and W302. In mutants where certain W<sub>p</sub> were knocked out, the A<sub>p</sub> value for the corresponding W<sub>p</sub> was set to 0 in our simulations.
<table>
<thead>
<tr>
<th>Description</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>The first lifetime of W\textsubscript{d}</td>
<td>$\tau_{D1}$</td>
<td>0.5 ns</td>
</tr>
<tr>
<td>The second lifetime of W\textsubscript{d}</td>
<td>$\tau_{D2}$</td>
<td>2.7 ns</td>
</tr>
<tr>
<td>Amplitude ratio of the second lifetime of W\textsubscript{d}</td>
<td>$R_{D2}$</td>
<td>0.71</td>
</tr>
<tr>
<td>The first lifetime of W\textsubscript{198}</td>
<td>$\tau_{1,\text{W198}}$</td>
<td>1.2 ns</td>
</tr>
<tr>
<td>The second lifetime of W\textsubscript{198}</td>
<td>$\tau_{2,\text{W198}}$</td>
<td>6.6 ns</td>
</tr>
<tr>
<td>Amplitude ratio of the second lifetime of W\textsubscript{198}</td>
<td>$R_{2,\text{W198}}$</td>
<td>0.82</td>
</tr>
<tr>
<td>The first lifetime of W\textsubscript{250}</td>
<td>$\tau_{1,\text{W250}}$</td>
<td>1.2 ns</td>
</tr>
<tr>
<td>The second lifetime of W\textsubscript{250}</td>
<td>$\tau_{2,\text{W250}}$</td>
<td>6.2 ns</td>
</tr>
<tr>
<td>Amplitude ratio of the second lifetime of W\textsubscript{250}</td>
<td>$R_{2,\text{W250}}$</td>
<td>0.85</td>
</tr>
<tr>
<td>The first lifetime of W\textsubscript{302}</td>
<td>$\tau_{1,\text{W302}}$</td>
<td>1.1 ns</td>
</tr>
<tr>
<td>The second lifetime of W\textsubscript{302}</td>
<td>$\tau_{2,\text{W302}}$</td>
<td>8.2 ns</td>
</tr>
<tr>
<td>Amplitude ratio of the second lifetime of W\textsubscript{302}</td>
<td>$R_{2,\text{W302}}$</td>
<td>0.87</td>
</tr>
<tr>
<td>Population ratio of W\textsubscript{p} with no energy transfer to the pyramid center*</td>
<td>$R_{nt}$</td>
<td>0.15</td>
</tr>
<tr>
<td>The first fluorescence decay time of the pyramid center</td>
<td>$\tau_{c1}$</td>
<td>0.08 ns</td>
</tr>
<tr>
<td>The second fluorescence decay time of the pyramid center</td>
<td>$\tau_{c2}$</td>
<td>1.4 ns</td>
</tr>
<tr>
<td>Amplitude ratio of the 0.08 ns component</td>
<td>$R_{c1}$</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Table 2.13 Lifetimes and amplitude ratios used in model simulations and energy transfer efficiency calculations

*R\textsubscript{nt} value was determined based on the agreement between the simulated and experimental transients. All other values were determined by experiments.
<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>FL\textsubscript{d} (a.u.)</th>
<th>FL\textsubscript{p} (a.u.)</th>
<th>FL\textsubscript{c} (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>310</td>
<td>1.46</td>
<td>0.247</td>
<td>0.068</td>
</tr>
<tr>
<td>315</td>
<td>1.84</td>
<td>0.421</td>
<td>0.150</td>
</tr>
<tr>
<td>320</td>
<td>1.89</td>
<td>0.612</td>
<td>0.268</td>
</tr>
<tr>
<td>325</td>
<td>1.83</td>
<td>0.786</td>
<td>0.433</td>
</tr>
<tr>
<td>330</td>
<td>1.79</td>
<td>0.916</td>
<td>0.541</td>
</tr>
<tr>
<td>335</td>
<td>1.60</td>
<td>0.987</td>
<td>0.636</td>
</tr>
<tr>
<td>340</td>
<td>1.35</td>
<td>1</td>
<td>0.72</td>
</tr>
<tr>
<td>345</td>
<td>1.17</td>
<td>0.963</td>
<td>0.772</td>
</tr>
<tr>
<td>350</td>
<td>0.969</td>
<td>0.891</td>
<td>0.790</td>
</tr>
<tr>
<td>355</td>
<td>0.768</td>
<td>0.798</td>
<td>0.755</td>
</tr>
<tr>
<td>360</td>
<td>0.601</td>
<td>0.695</td>
<td>0.711</td>
</tr>
<tr>
<td>365</td>
<td>0.477</td>
<td>0.593</td>
<td>0.650</td>
</tr>
<tr>
<td>370</td>
<td>0.372</td>
<td>0.497</td>
<td>0.580</td>
</tr>
<tr>
<td>375</td>
<td>0.279</td>
<td>0.411</td>
<td>0.518</td>
</tr>
<tr>
<td>380</td>
<td>0.216</td>
<td>0.336</td>
<td>0.469</td>
</tr>
</tbody>
</table>

Table 2.14 Values of FL\textsubscript{d}, FL\textsubscript{p} and FL\textsubscript{c} at various wavelengths for model simulations

*FL\textsubscript{d}, FL\textsubscript{p} and FL\textsubscript{c} are the fluorescence intensity per unit population per unit time for excited W\textsubscript{d}, W\textsubscript{p} and W\textsubscript{c}, respectively. They have same shapes as the emission spectra of the 3 Trp groups as shown in figure 2.1E. Their absolute values were optimized in model simulations, based on the agreement between the simulated and experimental transients.
<table>
<thead>
<tr>
<th></th>
<th>W198</th>
<th>W250</th>
<th>W302</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy transfer</td>
<td>2 ns</td>
<td>0.1 ns</td>
<td>0.05 ns</td>
</tr>
<tr>
<td>time constant (τ\text{total})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First lifetime (τ₁)</td>
<td>1.2 ns (18%)</td>
<td>1.2 ns (15%)</td>
<td>1.1 ns (13%)</td>
</tr>
<tr>
<td>Second lifetime (τ₂)</td>
<td>6.6 ns (82%)</td>
<td>6.2 ns (85%)</td>
<td>8.2 ns (87%)</td>
</tr>
<tr>
<td>Population ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>without energy transfer (R_{nt})</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Energy transfer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>efficiency to 4W_c *</td>
<td>0.59</td>
<td>0.83</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Table 2.15 Energy transfer efficiency from W_p to the pyramid center

*Efficiency was calculated with equation 2.27.
<table>
<thead>
<tr>
<th></th>
<th>W39</th>
<th>W92</th>
<th>W144</th>
<th>W196</th>
<th>W300</th>
<th>W352</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total FRET efficiency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((E)^*)</td>
<td>0.34</td>
<td>0.44</td>
<td>0.47</td>
<td>0.45</td>
<td>0.49</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>Efficiency of direct</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>transfer to (4W_c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((E_{direct,4Wc})^{**})</td>
<td>0.27</td>
<td>0.39</td>
<td>0.44</td>
<td>0.24</td>
<td>0.33</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Efficiency to W198</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((E_{W198})^{***})</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.20</td>
<td>0.09</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Efficiency to W250</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((E_{W250})^{***})</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Efficiency to W302</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>((E_{W302})^{***})</td>
<td>0.06</td>
<td>0.02</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Efficiency to (4W_c) via</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(W_p) ((E_{twostep})^{****})</td>
<td>0.06</td>
<td>0.04</td>
<td>0.02</td>
<td>0.13</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Total FRET efficiency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>to the pyramid center ((E_{4Wc})^{*****})</td>
<td>0.33</td>
<td>0.43</td>
<td>0.46</td>
<td>0.37</td>
<td>0.44</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Table 2.16 Energy transfer efficiency from \(W_d\) to the pyramid center in WT/W400F

*The fraction of \(W_d\) excited population that is transferred to interfacial Trp. Calculated with equation 2.22.

** The fraction of \(W_d\) excited population that is directly transferred to the pyramid center. Calculated with equation 2.28.

*** The fraction of \(W_d\) excited population that is transferred to each of the 3 peripheral Trp. Calculated with equation 2.29.

**** The fraction of \(W_d\) excited population that is transferred to the pyramid center via \(3W_p\). Calculated with equation 2.30.

***** The fraction of \(W_d\) excited population that is transferred to the pyramid center, both directly and indirectly. Calculated with equation S52. The values are smaller than the total efficiency, meaning that a small fraction of energy dissipates in non-FRET channels in \(W_p\), during the two-step energy transfer.
Figure 2.1 UVR8 structure and steady-state spectra.

(A) A side view of UVR8 dimer with 3 groups of Trp highlighted in different colors: W_d-purple, W_p-cyan and W_c-orange. W_p and W_c lie at the dimer interface whereas W_d are buried in the middle of β sheets. (B) A top view of one subunit from the dimer interface shows the highly symmetrical locations of 6 W_d (W39, W92, W144, W196, W300 and W352). (C) A top view from the dimer interface demonstrates the relative positions of the pyramid center (W285, W233, W337 and W94b) and the 3 W_p (W198, W250 and W302). (D) Normalized absorption and emission spectra for UVR8 WT (red), W198/250/302/400F (green), 4W_c/400F (dark yellow) and Mono-6W_d (purple). The inset highlights absorption difference at the red side. (E) Normalized absorption and emission spectra for W_d (purple), W_p (purple) and W_c (red). The inset provides a zoom-in view of the absorption-emission overlap region. (F) Spectral overlap integrals for W_d-to-W_p (panel 1 top), W_p-to-W_d (panel 1 bottom), W_d-to-W_c (panel 1 top), W_c-to-W_d (panel 1 bottom), W_p-to-W_c (panel 1 top) and W_c-to-W_p (panel 1 bottom) energy transfer. The overlap regions are shaded using light blue color.
Figure 2.2 Steady-state emission spectra measured with various excitation wavelengths for UVR8 mutants containing the 6 distal tryptophan residues and combinations of the 3 peripheral tryptophan residues.

(A) 4Wc/400/198/250F. (B) 4Wc/400/198/302F. (C) 4Wc/400/250/302F. (D) 4Wc/400/198F. (E) 4Wc/400/250F. (F) 4Wc/400/302F. (G) 4Wc/400F. (H) Steady-state emission spectra ($\lambda_{ex}=310$ nm) for all the mutants are nearly identical, indicating that the 3 peripheral Trp (Wp) have similar emissions with peaks at around 340 nm.
Figure 2.3 Schematic view of Trp-to-Trp energy transfer network in UVR8.

Each line represents one energy transfer pathway. As shown by the legend, colors of the lines are based on calculated FRET time constants and $\kappa^2/R^6$. The most dormant paths are shown in red. (A) The 84 possible energy transfer pathways from $6W_d$ to 14 interfacial Trp ($W_p+W_c$ on both subunits). The summed $\kappa^2/R^6$ and the total FRET time constants (calculated with equations S13 and S14) are shown below the residue labels. (B) The 12 possible energy transfer pathways from $3W_p$ to $4W_c$. The summed $\kappa^2/R^6$ and the total FRET time constants are shown above the residue names.
Figure 2.4 Fluorescence decay transients ($\lambda_{ex}=290$ nm) from 305 nm to 380 nm for UVR8 WT and 8 mutants measured with sub-nanosecond resolved time correlated single photon counting (TCSPC).

(A) 4Wc/400/198/250F. (B) 4Wc/400/198/302F. (C) 4Wc/400/250/302F. (D) 4Wc/400/198F. (E) 4Wc/400/250F. (F) 4Wc/400/302F. (G) 4Wc/400F. (H) W198/250/302/400F. (I) WT.
Figure 2.5 W_d-to-W_p/W_c energy transfer investigated by time-resolved fluorescence pumped at 290 nm.

(A) Time correlated single photon counting (TCSPC) transients for mono-6W_d gated at 320 nm, 325 nm and 330 nm. (B) Femtosecond resolved fluorescence up conversion transients for mono-6W_d gated at 320 nm, 325 nm and 330 nm. Together with TCSPC data, the lifetimes of W_d were determined to be \( \tau_{D1} \approx 0.5 \text{ ns (29\%)} \) and \( \tau_{D2} \approx 2.7 \text{ ns (71\%)} \). (C) Time scales from global fitting and time constant associated spectra for 4W_d/400/198/250F (panel 1), 4W_d/400/198/302F (panel 2), 4W_d/400/250/302F (panel 3), 4W_d/400/198F (panel 4), 4W_d/400/250F (panel 5), 4W_d/400/302F (panel 6), 4W_d/400F (panel 7), W198/250/302/400F (panel 8) and WT (panel 9). For each panel, the energy transfer scheme for the corresponding mutant is shown on the up-right corner. The black solid lines are the steady-state emission spectra of the protein samples. The time constant associated spectra are shown using discrete symbols in different shapes and colors as labeled in the legend and the time constant assigned to \( \tau_{DA2} \) are underscored in each panel. Please note that panel 8 and 9 show the total spectra of the last two time scales, which is the sum of the two time constant associated spectra. To explain the origin of the observed time scales, emission spectra of W_d (purple solid), W_p (green solid) and W_c (red solid) are shown in the panels and compared with the time constant associated spectra.
Figure 2.6 Model simulations demonstrate that the total fluorescence decay dynamics of the 6 distal Trp in UVR8 can be described with single exponential decays in the following UVR8 protein samples.

(A) 4Wc/400/198/250F, (B) 4Wc/400/198/302F, (C) 4Wc/400/250/302F, (D) 4Wc/400/198F, (E) 4Wc/400/250F, (F) 4Wc/400/302F, (G) 4Wc/400F, (H) W198/250/302/400F, (I) WT. In each panel, the 6 dashed lines correspond to the simulated single exponential decays of $\tau_{DA2}$ based on experimental $\tau_{D2}$ (2.7 ns) and theoretical total FRET times ($\tau_{\text{total}}$) for W39 (dark red), W92 (dark yellow), W144 (green), W196 (dark cyan), W300 (dark blue) and W352 (dark purple) respectively. The simulated total decay dynamics (equation 2.17) of the 6 distal Trp (black solid lines, sum of the 6 dashed lines) can be fitted with single decay time constant (shown in black). The simulated curves from experimental average $\tau_{DA2}$ (orange solid lines) agrees well with theoretical curves (black solid lines), indicating that FRET theory provides accurate description of the energy transfer processes from W_d in UVR8.
Figure 2.7 Fluorescence decay transients ($\lambda_{ex}=310$ nm) from 330 nm to 380 nm for 8 mutants measured with picosecond resolved time correlated single photon counting (TCSPC).

(A) 4W/400/198/250F. (B) 4W/400/198/302F. (C) 4W/400/250/302F. (D) 4W/400/198F. (E) 4W/400/250F. (F) 4W/400/302F. (G) 4W/400F. For all mutants, transients at different wavelengths are very similar. Fitting results are shown for each mutant.
Figure 2.8 Numerical simulations show that the Wₚ lifetimes observed in “6W_d+2Wₚ” mutants and “6W_d+3Wₚ” mutants match the sum of decay transients of their containing peripheral Trp.

The mutants: (A) 4Wₖ/400/198F. (B) 4Wₖ/400/250F. (C) 4Wₖ/400/302F. (D) 4Wₖ/400F. For each mutant, decay transients of individual Wₚ (colored dashed lines, red-W198; green-W250; blue-W302) were simulated using double exponential decay model with the time constants and amplitude ratios (shown at the right side of the legend) measured from “6W_d+1Wₚ” mutants, which add up a total decay transient (black solid line). The total transients agree with the simulated curves (orange solid lines) using time constants and ratios (shown in figure 2.7) directly measured in “6W_d+2Wₚ” and “6W_d+3Wₚ” mutants.
Figure 2.9 Fluorescence decay transients measured with picosecond resolved TCSPC.

(A) Decay transients at various fluorescence wavelengths for W400F ($\lambda_{ex}=315$ nm). (B) Decay transients at 350 nm with various excitation wavelengths ($\lambda_{ex}=290$ nm, 310 nm, 315 nm and 320 nm) for W400F. Fluorescence decay signals with 315 nm and 320 nm excitation wavelengths gives pure pyramid center ($W_c$) dynamics. In addition to $W_c$ dynamics, 310 nm excitation transients also have contributions from $W_p$; 290 nm excitation transients contain dynamics of all 3 Trp groups. (C) Fluorescence dynamics of the pyramid center measured by 315 nm excitation, for W400F (dark yellow), W198/400F (red), W250/400F (blue), W198/250/400F (green) and W198/250/302/400F (cyan). All transients are similar except that for W198/250/302/400F, indicating that mutation of W302 affects pyramid center fluorescence dynamics. (D) Fluorescence dynamics of the pyramid center measured by 315 nm excitation for W400F, W94F and W337F. All transients are similar.
Figure 2.10 Fluorescence decay transients ($\lambda_{ex}$=310 nm) for W198/250/400F measured with picosecond resolved TCSPC.

Transients were taken at various fluorescence wavelengths: (A) 330 nm, (B) 335 nm, (C) 340 nm, (D) 345 nm, (E) 350 nm, (F) 355 nm, (G) 360 nm, (H) 365 nm, (I) 370 nm, (J) 375 nm and (K) 380 nm. For each wavelength, the simulated curve (green) and contributions from different Trp groups ($W_c$: red, $W_p$: dark yellow) are shown.
Figure 2.11 Fluorescence decay transients ($\lambda_{ex}=310$ nm) for W198/400F measured with picosecond resolved TCSPC.

Transients were taken at various fluorescence wavelengths: (A) 330 nm, (B) 335 nm, (C) 340 nm, (D) 345 nm, (E) 350 nm, (F) 355 nm, (G) 360 nm, (H) 365 nm, (I) 370 nm, (J) 375 nm and (K) 380 nm. For each wavelength, the simulated curve (green) and contributions from different Trp groups ($W_c$: red, $W_p$: dark yellow) are shown.
Figure 2.12 Fluorescence decay transients (λ<sub>ex</sub>=310 nm) for W250/400F measured with picosecond resolved TCSPC.

Transients were taken at various fluorescence wavelengths: (A) 330 nm, (B) 335 nm, (C) 340 nm, (D) 345 nm, (E) 350 nm, (F) 355 nm, (G) 360 nm, (H) 365 nm, (I) 370 nm, (J) 375 nm and (K) 380 nm. For each wavelength, the simulated curve (green) and contributions from different Trp groups (W<sub>C</sub>: red, W<sub>F</sub>: dark yellow) are shown.
Figure 2.13 Fluorescence decay transients ($\lambda_{ex}=310$ nm) for W400F measured with picosecond resolved TCSPC.

Transients were taken at various fluorescence wavelengths: (A) 330 nm, (B) 335 nm, (C) 340 nm, (D) 345 nm, (E) 350 nm, (F) 355 nm, (G) 360 nm, (H) 365 nm, (I) 370 nm, (J) 375 nm and (K) 380 nm. For each wavelength, the simulated curve (green) and contributions from different Trp groups ($W_c$: red, $W_p$: dark yellow) are shown.
Figure 2.14 Fluorescence decay transients (λ<sub>ex</sub>=290 nm) for W400F measured with picosecond resolved TCSPC.

Transients were taken at various fluorescence wavelengths: (A) 310 nm, (B) 315 nm, (C) 320 nm, (D) 325 nm, (E) 330 nm, (F) 335 nm, (G) 340 nm, (H) 345 nm, (I) 350 nm, (J) 355 nm, (K) 360 nm, (L) 365 nm, (M) 370 nm, (N) 375 nm and (O) 380 nm. For each wavelength, the simulated curve (green) and contributions from different Trp groups (W<sub>c</sub>: red, W<sub>p</sub>: dark yellow, W<sub>d</sub>: blue) are shown.
Figure 2.15 Comparison between directly measured and simulation constructed spectra.

(A) W198/250/400F. (B) W198/400F. (C) W250/400F. (D) W400F. In each panel, the black line is the steady-state emission with the excitation labeled. Discrete colored labels represent spectra of Trp groups constructed from time integrals of simulation curves: purple triangles-$W_d$, dark yellow squares-$W_p$ and cyan hexagons-$W_c$. Solid lines are the measured emission spectra of 3 Trp groups.
Chapter 3: Dynamics and Mechanism of UV-Induced Dissociation of UVR8

3.1 Introduction

Based on our studies in the previous chapter, after UV irradiation, a tryptophan-to-tryptophan energy transfer network funnels excitation energy to the pyramid center, where a photoreaction occurs in 80 ps to trigger dimer dissociation. In chapter 3, we seek to answer questions (1) what is the nature of the photoreaction; (2) What is the mechanism of UVR8 photo-induced dissociation?

Mutagenesis studies have been conducted by other researchers to identify functionally important Trp residues. UVR8 WT appears as a dimer on SDS-PAGE as long as the sample is not boiled. But weak dimers can appear as monomers in SDS-PAGE, and SEC is proven to be a more accurate method to test dimer/monomer state. Upon identification of UVR8 as a photoreceptor, researchers found W285 is critical to UV induced monomerization. W285F appears as a dimer in non-boiled SDS-PAGE and SEC, but is not UV responsive. W285A is a monomer in non-boiled SDS-PAGE but elutes as a dimer in SEC. Interestingly, although W285A is not UV responsive, it interacts with COP1 in dimeric form and is constitutively active in vivo. W233F/A mutants are also constitutive dimers. The other 2 pyramidal Trp (W94 and W337) are not essential to dissociation function, with W94A and W337F/A as functional dimers. A complete in vivo mutant screening of all 14 Trp suggests that only W285 and W233 are essential to UV induced dimer dissociation of UVR8, whereas W337 mutation partially impairs UVR8 function. Results from Wu et. al. also support the critical role of W285
and W233. Furthermore, researchers found that the pyramid Trp are excitonically coupled and W233 is the most important in the exciton.

Two UVR8 monomers are glued together by various electrostatic interactions and hydrogen-bonds: R286 interacts with D96 and D107, R338 form salt-bridges with E43 and D44, R146 with E182, R200 with E158 and R354 with E53. These interactions must break to unzip the UVR8 dimer (figure 1.1 and 3.1A). Studies have shown that single alanine mutation of either R286 or R338 abrogates dimer formation, whereas R146A, R200A and R354A are functional dimers. D96N and D107N are UV-responsive dimers but the double mutant D96N/D107N is a constitutive monomer. Notably, R286 and R338 are near the critical W285.

Although there is consensus that W285, W233 and R286/338 play crucial roles in UVR8 monomerization (shown in figure 3.1B), the exact dissociation mechanism has been controversial. Christie et. al. proposed charge transfer from excited Trp pyramid to R286/338, leading to charge neutralization of the salt-bridge(s). Wu et. al. suggested excited state proton transfer of Trp, disrupting cation-π interactions at the dimer interface. A computational study by Voityuk et. al. suggests that charge separation between W285 and W233 produces W285 plus radical/W233 minus radical intermediate (W285+W233⁻), leading to proton transfer from Asp/Glu to R286/R338, which breaks the dimer interactions. Another theoretical study by Li et. al. proposed 3 possible mechanisms: 1) electrostatic repulsion of Trp excited breaks nearby salt-bridges formed by R286 or R338; 2) neutralization of R286/338 by two step election hopping from W233 to W285 to Arg; 3) neutralization of R286/338 by multiple proton transfer reactions.
Mathes et al. proposed proton-coupled electron transfer (PCET) from W285 triplet state to nearby salt-bridges formed by R286/338, neutralizing both Arg and Asp/Glu. Here, we seek to use extensive mutagenesis studies, femtosecond resolved spectroscopy and computational methods to unveil the mechanism of light perception by UVR8.

3.2 Materials and Methods

3.2.1 UVR8 Protein Sample Preparation and Steady-State Measurements

The purification of full-length A. thaliana UVR8 has been detailed in section 2.2.1. The UVR8 ΔC plasmids were built by cloning truncated A. thaliana UVR8 (1-380 amino acids) genes into pET-28 (a) vectors. Cell growth and protein purification of UVR8 ΔC follow the same protocol as the full-length UVR8. We sequenced the mutated DNA to ensure that the correct mutation(s) were introduced. For 290 nm excitation, we used protein samples with an optical density about 0.43 at 280 nm (~4.7 μM dimer in 5-mm cuvette) to measure the steady-state absorption spectrum and an optical density about 0.086 at 282 nm (~1 μM dimer in 5-mm cuvette) to obtain the steady-state fluorescence spectrum. For 310 nm and 315 nm excitations, samples with much higher protein concentrations (~50 μM dimer in 5-mm cuvette) were used for measurements. For experiments at different buffers (low pH, D2O), protein was exchanged to the desired buffer immediately before experiments with a pre-equilibrated desalting spin column. The ingredients of various buffers are: (1) low pH (pH 4.5): 150 mM NaCl, 23 mM Na2HPO4, 13.5 mM citric acid and (2) D2O: 150 mM NaCl, 25 mM Tris-HCl at pH 8.0 exchanged to deuterium oxide. Dimer/monomer state of proteins were determined as
described in section 2.2.2. UV-induced dissociation function of protein samples were tested either by SEC or by fluorescence intensity change under continuous UV illumination.83,100

3.2.2 Dissociation Quantum Yield Measurements

The quantum yield of UVR8 dimer dissociation is measured as follows. We prepared a 125-μL sample containing ~10 μM UVR8 dimer in a 5 mm × 5 mm square cuvette. The protein solution is then irradiated by a UV-B lamp (UVP, 6W, ~1.4 mW/cm²) at a distance of 14 cm with at 300-nm longpass filter (UV-B, figure 3.14 solid line) or without the filter (UV-B+UV-C, figure 3.14 dashed line). The irradiated sample was then centrifuged at 14000 rpm for 5 minutes and loaded on a 10/30 superdex-200 column (GE Healthcare). The percentage of dimer dissociated was analyzed by fitting the ratio of areas of the dimer peak to the monomer peak with Gaussian function.

3.2.3 Femtosecond Resolved Broadband Transient Absorption Spectroscopy

For all measurements, 290 nm or 315 nm pump pulses (1 kHz) were generated by a commercial optical parametric amplifier (TOPAS, Spectra-Physics). The pump pulse energy was attenuated to 80-100 nJ/pulse before being focused into UVR8 samples (4 mg/mL), which is kept in a 5-mm quartz cell with constant magnetic stirring. We generated white-light continuum (WLC) probe pulses by focusing the 800-nm pulses on a sapphire crystal with 2-mm thickness and the resulting broadband pulses were split into a probe beam and a reference beam. The probe beam was focused and overlapped with the
pump beam at the sample cell. After that, both probe and reference beams were focused into the optical fibers before entering the entrance slit of the imaging spectrometer (IHR320, Horiba). The incoming pulses were dispersed by a 300 grooves/mm grating and then imaged onto a 1024×256 pixel CCD detector (Synapse, Horiba) for data acquisition and analysis. The experiments were done at the magic angle (54.7°) with ~250 fs instrument response. To further minimize the local photobleaching and monomer accumulation, the data were only averaged for 50 laser shots (50 ms) at each time delay and the pump beam was blocked for 1 second in between data acquisition to allow sufficient equilibrium. For UV responsive proteins, samples were replaced with fresh samples before any observable dimer dissociation occurs, as checked by the superdex-200 size-exclusion column. For pump 315 spectra, Savitzky-Golay smoothing (100 points of window) was conducted, and then the smoothed curves were averaged with raw data to give the data shown in figure 3.7 and figure 3.3D.

3.2.4 Femtosecond Resolved Single Wavelength Transient Absorption Spectroscopy

The experimental layout has been detailed elsewhere.\textsuperscript{96,114} Briefly, for all measurements, pump and probe beams (1 kHz) were generated by two optical parametric amplifiers (TOPAS, Spectra-Physics). UVR8 protein samples (4 mg/mL) were kept in a 5-mm quartz cell with constant magnetic stirring. The pump pulse energy was attenuated to 80-100 nJ/pulse before being focused into the sample cell. The instrument response time is about 250 fs and all experiments were done at the magic angle (54.7°). The data were only averaged for 50 laser shots (50 ms) at each time delay and the pump beam was
blocked for 1 second in between data acquisition to allow sufficient equilibrium. For functional UVR8 proteins, samples were frequently replaced with fresh samples to ensure negligible monomer formation during experiments.

3.2.5 Kinetic Model Fitting for Pump 315 nm Transient Absorption Data

The complete reaction scheme is shown in figure 3.9. 315 nm light selectively excite the pyramid Trp (Wc), which have reactive (75% 80 ps) and nonreactive (25% 1.4 ns) subpopulations. Assuming the initial Trp excited state concentration is n0, and the reactive fraction is Rc (0.75), the temporal evolution of various intermediates can be solved by the following equations:

Reactive W*: $[W_{c1}]_t = n_0 R_c e^{-\frac{t}{\tau_{cs}}}$ (3.1)

Nonreactive W*: $[W_{c2}]_t = n_0 (1 - R_c) e^{-\frac{t}{\tau_{c2}}}$ (3.2)

Charge separated intermediate state:

$$\frac{d[W_{285^-W233^+}]}{dt} = \tau_{cs}^{-1} [W_{c1}]_t - (\tau_{CR}^{-1} + \tau_{ET}^{-1} + \tau_{DP}^{-1}) [W_{285^-W233^+}]_t$$ (3.3)

W285 neutral W233 plus:

$$\frac{d[W_{285W233^+}]}{dt} = \tau_{ET}^{-1} [W_{285^-W233^+}]_t - \tau_{DP}^{-1} [W_{285W233^+}]_t$$ (3.4)

W285 minus W233 neutral radical:

$$\frac{d[W_{285^-W233^+}]}{dt} = \tau_{DP}^{-1} [W_{285^-W233^+}]_t - \tau_{ET}^{-1} [W_{285^-W233^+}]_t$$ (3.5)
where $\tau_{CS}$, $\tau_{c2}$, $\tau_{CR}$, $\tau_{ET}$ and $\tau_{DP}$ are time scales of charge separation, nonreactive decay of pyramid center, charge recombination, electron transfer to Arg from W285 minus radicals and deprotonation of W233$^+$, respectively. With 630 nm probe wavelengths, mainly Trp excited state and Trp cationic radical are detected.

Total $W^*$ population:

$$[W^*]_t = [W_{c1}]_t + [W_{c2}]_t \quad (3.6)$$

Total $W^+$ population:

$$[W^+]_t = [W285^{-}W233^+]_t + [W285W233^+]_t \quad (3.7)$$

According to the Beer-Lambert law, the signal of absorption transient at 630 nm probe wavelength can be acquired as:

$$\Delta A \propto \varepsilon_{W^*} [W^*]_t + \varepsilon_{W^+} [W^+]_t \quad (3.8)$$

Based on studies by Kohler et. al.,$^{115}$ the extinction coefficients of $W^*$ ($\varepsilon_{W^*}$) and $W^+$ ($\varepsilon_{W^+}$) were set to 4000 M$^{-1}$cm$^{-1}$ and 3000 M$^{-1}$cm$^{-1}$ respectively. As determined by fluorescence dynamics (figure 3.2B), $R_{c1}$, $\tau_{CS}$ and $\tau_{c2}$ are 0.75, 80 ps and 1.4 ns. Applying equation 3.8 to fit our experimental WT transient (figure 3.3E), $\tau_{CR}$ and $\tau_{ET}$ were determined to be 4 ps and 17 ps, respectively. $\tau_{DP}$ is several nanoseconds and cannot be fully resolved in our 3 ns time window.

3.2.6 Numerical Simulation of Pump 290 nm Absorption Transients

290 nm light can pump all 3 Trp groups: distal Trp ($W_d$), peripheral Trp ($W_p$) and pyramid center Trp ($W_c$). We first simulated Trp excited state temporal evolution for 3
Trp groups with our proposed energy transfer model (detailed in appendix B part 1). With $[W_{c1}]_t$, population evolution of electron transfer intermediates was simulated using equations 3.3, 3.4, 3.5 and 3.7.

Total $W^*$ population:

$$[W^*]_t = [W_{c1}]_t + [W_{c2}]_t + [W_p]_t + [W_d]_t \quad (3.9)$$

The signal of absorption transient at 630 nm probe wavelength, and signal contributions from $W^*$ and $W^+$ can be acquired as:

$$\Delta \varepsilon \propto \varepsilon_{W^*}[W^*]_t + \varepsilon_{W^+}[W^+]_t = S(W^*) + S(W^+) \quad (3.10)$$

In our simulations, the extinction coefficients of $W^*$ ($\varepsilon_{W^*}$) and $W^+$ ($\varepsilon_{W^+}$) were set to 4000 M$^{-1}$cm$^{-1}$ and 3000 M$^{-1}$cm$^{-1}$ respectively.\(^{115}\) $R_{c1}$, $\tau_{CS}$ and $\tau_{c2}$ were 0.75, 80 ps and 1.4 ns respectively. $\tau_{CR}$, $\tau_{ET}$ and $\tau_{DP}$ values were 4 ps, 17 ps and 7.5 ns respectively. The simulated results are shown in figure 3.6.

3.2.7 MD Simulations

This part was done in collaboration with Prof. Jiali Gao’s research group at University of Minnesota. The X-ray structure of UVR8 (PDB entry: 4D9s) was solvated in a cubic box of 104×104×104 Å$^3$ using TIP3P water model with a minimum distance of 10 Å between the protein and the edges of the periodic box. Counter ions were added to neutralize charge and produce an ion concentration of approximately 0.15 M. The simulations of ground state, electron excited and transfer state were set up using CHARMM c38a2.\(^{116}\) CHARMM27 force field with CMAP correction was used for ground state calculation.\(^{117,118}\) The system was first subjected to energy minimization,
followed by gradually heating from 10 K to 300 K every 10K using 100 ps NPT simulations at each temperature. During energy minimization and heating, harmonic restraints with the force constant of 5.0 kcal/(molÅ²) for backbone and 4.0 kcal/(molÅ²) for heavy atoms of sidechain were kept on the non-water and non-counter ions. For simulations of the electron excited and transfer states, the corresponding patch residues were used, which the charges in the force field were modified from time dependent density functional theory (TDDFT) and multistate density functional theory (MSDFT). All simulations were first carried out by using CHARMM during initial 20 ns equilibrations and 20 ns production for further combined quantum mechanical and molecular mechanical (QM/MM) studies. Then the ground state, R338 neutralized, R286 neutralized and R234 neutralized state were selected to do long time simulations up to 2 μs using GROMACS-4.6.5 molecular dynamics code to study the mechanisms of dimer dissociations. The non-pair list was updated every 10 steps. The grid neighbor searching method was applied in the simulation with a 10 Å cutoff distance for the short-range neighbor list. Electrostatic interactions were treated by using the Partial-Mesh Ewald (PME) summation method with a 14 Å for long range and 10 Å for short-range electrostatic cutoff, respectively. The short-range cutoff for van der Waals interactions during the simulation was 12 Å. The isotropic pressure coupling was achieved by Parrinello-Rahman method with a compressibility of 4.5x10⁻⁵ bar⁻¹. All simulations were carried out at 300 K in NPT ensemble and 1 atm pressure with a time step of 2 fs.
3.2.8 Count Water Number at the Interface

This part was done by Prof. Jiali Gao’s research group. Three carbon α (CA) atoms of D129, W285 and A24 for each monomer were selected to make two planes. The interfacial area is between the planes. The geometric center of the interface may approximately equal to the center of mass for those six CA atoms. Then we counted the number of oxygen atoms of water both in between the 2 planes and within 18 Å distance of the center of mass of the 6 CA. Using this method, the 2 μs trajectories of MD simulation for ground state, R338 neutralized, R286 neutralized and R234 neutralized were analyzed to reveal the key dimer dissociation features.

3.2.9 QM/MM Calculation

This part was done by Prof. Jiali Gao’s research group. We extracted 100 snapshots (one snapshot every 200 ps) from the 20 ns production simulations of ground state, electron excited and transfer states to statistically investigate the electron transfer (ET) rate based on the QM/MM calculations. The single excited state of residue was acquired by time dependent range-separated hybrid functional TD-CAM-B3LYP.\textsuperscript{123} MSDFT calculations were performed by PBE0 functional\textsuperscript{124} with HF correction for the off-diagonal Hamiltonian matrix element. 6-31+G(d) basis set was used for all the calculation. All the QM/MM calculations were performed with a locally modified version of GAMSS code\textsuperscript{125} in CHARMM quantum part.

For QM/MM calculation, the diabatic states of electronic localized excitation and electron transfer can be written as:\textsuperscript{126}
\begin{align}
\Phi_{ab}(S_t) &= \Psi_1 \cdots \hat{A}\{\Psi_a^S \Psi_b^S\} \cdots \Psi_N \quad \text{for } N-1 \text{ fragments} \\
\Phi_{ab}(CT) &= \Psi_1 \cdots \hat{A}\{\Psi_a^{\ast\ast} \Psi_b^{\ast\ast}\} \cdots \Psi_N \quad \text{for } N-1 \text{ fragments}
\end{align}

where \( \hat{A}\{\Psi_a^S \Psi_b^S\} \) specifies an antisymmetric wave function for the locally excited residue \( a \), coupled with monomer \( b \) in the ground state, while \( \hat{A}\{\Psi_a^{\ast\ast} \Psi_b^{\ast\ast}\} \) is an antisymmetric wave function for electron transfer from residue \( a \) to residue \( b \). They can be constructed using the corresponding fragment block-localized KS orbitals. The other \( N-1 \) fragments are treated as classically molecular mechanics (MM).

Using Marcus-Hush theory under the harmonic approximation, two parabolas along a reaction coordinate can be used to represent the potential energy curves of reaction and product states.\textsuperscript{127,128} In the diabatic representation, the two curves cross and the crossing point is the transition state. With the assumption that both parabolas have the same curvature, the activation energy \( \Delta G^+ \) is given by

\[ \Delta G^+ = \frac{(\lambda + \Delta G^0)^2}{4\lambda} \quad (3.13) \]

here \( \Delta G^0 \) is the driving force and \( \lambda \) is the reorganization energy owing to the geometric relaxation accompanying. For the nonadiabatic ET, the rate constant \( k_{ET} \) is calculated by taking golden rule-based expression in the high temperature limit, i.e.,\textsuperscript{129,130}

\[ k_{ET} = \frac{4\pi}{h} V^{\frac{1}{2}} \left( \frac{1}{4\pi \lambda k_B T} \right)^{\frac{1}{2}} \exp \left[ -\left( \frac{\lambda + \Delta G^0}{4\lambda k_B T} \right) \right] \quad (3.14) \]
where $h$ and $k_B$ represent Plank and Boltzmann constant, respectively, and $T$ stands for the temperature. $V_{rp}$ is the electronic coupling matrix element, which is half of energy gap formed an upper and a lower curve at the crossing point. In the adiabatic representation that the two curves avoid crossing. The reorganization energy $\lambda$ and driving force $\Delta G^0$ can be obtained using linear response method:\textsuperscript{131}

$$\lambda = \frac{1}{2} \left[ \left( G(P)_r - G(P)_p \right) + \left( G(R)_p - G(R)_r \right) \right]$$

$$= \frac{1}{2} \left[ \left( G(P) - G(R)_r \right) + \left( G(R) - G(P)_p \right) \right]$$

$$\Delta G^0 = \frac{1}{2} \left[ \left( G(P)_r - G(R)_p \right) + \left( G(P)_p - G(R)_r \right) \right]$$

$$= \frac{1}{2} \left[ \left( G(P) - G(R)_r \right) - \left( G(R) - G(P)_p \right) \right]$$

(3.15)

(3.16)

here capital P and R stand for product and reactant electronic states while lowercase p and r means the equilibrium structures of product and reactant states. The free energy of these four points can be statistically calculated from 100 selected snapshots using QM/MM methods based on block localized density functional theory (BLDFT).

The coupling energies are calculated as $V_{rp} = \langle \Phi_{ab}(r) | H | \Phi_{ab}(p) > - S_{rp} \epsilon_g$ where $S_{rp}$ is the overlap integral between the two nonorthogonal Kohn-Sham determinants for state r and p, and $\epsilon_g$ is the adiabatic ground state energy obtained as lowest root by diagonalizing the $2 \times 2$ Hamiltonian matrix. The first term is off-diagonal Hamiltonian matrix element: $H_{rp} = \langle \Phi_{ab}(r) | H | \Phi_{ab}(p) >$ which has both the implicit functional of the diabatic state density and the transition density. It can be calculated by:\textsuperscript{132,133}
\[ H_{rp} = H_{rp}^{\text{BLKS}} + S_{rp} \frac{\Delta E_c^r + \Delta E_c^p}{2} \]  

(3.17)

\[ \Delta E_c^r \text{ and } \Delta E_c^p \] are correlation energy for two diabatic states, which can be approximated by the energy difference between BLDFT and HF theory using BLKS orbitals. Results are shown in table 3.3.

3.3 Results and Discussion

3.3.1 W233 is the Energy Sink and W285 is a Quencher

Here, except for the conserved W352, we have systematically mutated thirteen other tryptophan residues. All single-point tryptophan mutants, except for W285F and W233F, exhibit similar emission spectra (figure 3.1C) and fluorescence decay dynamics (figure 3.2) to wild type (WT), and maintain the normal function of UV-induced dimer dissociation (figure 3.2), indicating that only W285 and W233 are critical to UVR8 monomerization. Mutation of W285 or W233 leads to higher fluorescence quantum yield (Q_F) than WT (figure 3.1C inset). Knockout of W233 gives a moderate Q_F increase from 0.07 (WT) to 0.19 (W233F) and a peak shift from 332 nm (WT) to 335 nm (W233F). Strikingly, single mutation of W285 shifts protein emission peak to 355 nm and changes the overall Q_F of UVR8 Trp from 0.07 (WT) to 0.42 (W285F). From the two values, we can deduce that the other tryptophan residues must donate excitation energy to pyramidal cluster, otherwise the average fluorescence quantum yield of the pyramidal Trp would exceed unity. Notably, the double mutation W285F/D129N shows a much blue-shifted (peak ~340 nm) fluorescence spectrum and decreased peak-intensity compared with
W285F single mutant (figure 3.1C). Since the red shifted emission in W285F mainly originates from the pyramid center,97 this finding clearly indicates that, D129, which is hydrogen-bonded to W233, plays the major role in stabilizing the pyramid center excited state. As shown in figure 3.1D, UVR8 dissociation follows the rate law of first-order reaction with a dissociation quantum yield (QY) of 0.1 (dimer dissociated per photon absorbed) for WT, which is consistent with the value determined by other methods.100 D129N has only 30-40 percent while D129K has about 10 percent of dissociation QY of WT, due to inefficient energy transfer and possibly lower yields in later reaction steps. In addition, the WT in deuterium oxide (D\(_2\)O) has a similar quantum yield of wild type (figure 3.1D), suggesting that proton transfer does not involve in critical branching steps.

To elucidate the roles of the 4 pyramid Trp, especially W285 and W233, we made all mutation combinations in the cluster. By scrutinizing the fluorescence spectra of pyramid center mutants, we found that the 15 mutants can be categorized into three major classes (figure 3.3A): (1) functional proteins with both W285 and W233 intact; (2) nonfunctional mutants without W285 but with W233; (3) other nonfunctional mutants with W233 knocked out. All functional mutants (the first class: W94F, W337F, W94/337F) have similar steady-state fluorescence spectra and fluorescence decay dynamics to WT (figure 3.4). The second class of mutants show features of pyramid center emission, emission peaks of 350-355 nm and a significant (>40 %) ~12 ns lifetime component in fluorescence decay transients (figure 3.4). On the other hand, W233 knockout mutants (the third class), whether W285 is kept or not, exhibit peaks at ~337 nm with the ~12 ns component disappearing in TCSPC transients (figure 3.4B). Such
findings unambiguously demonstrate that the > 350 nm pyramid center emission mainly derives from W233. Previous circular dichroism (CD) studies pointed out that four pyramid center tryptophan residues are excitonically coupled. Based on our results, we conclude that any excitation within the pyramid center, including excitation energy transferred to the pyramid center, will instantaneously relax to the lowest energy W233 excited state via the strong exciton coupling. With the selective 315 nm pump, Trp cluster fluorescence dynamics of WT and 4 pyramid center Trp single mutants are measured and shown in figure 3.3B. Excluding the role of W94 and W337 in the photoreaction, W94F and W337F show the same fluorescence decay dynamics as WT, with a 75% 80 ps photoreaction dynamics and a 25% 1.4 ns nonreactive dynamics, which may derive from structural fluctuations of the cluster. Significantly, mutation of either W285 or W233 cause the 80 ps photoreaction to vanish, confirming that the critical reaction occurs between W285 and W233. W285F data mainly reflect the W233 fluorescence lifetime with a 94% 12.6 ns component; W233F transient shows pyramid center fluorescence dynamics without W233.

3.3.2 Charge Separation Between W285 and W233

A number of researchers have proposed light-induced charge separation (CS) between W285 and W233, but no solid experimental evidence has been provided. To test the CS hypothesis, we performed femtosecond resolved transient absorption experiments to determine the reaction intermediates. We first performed the broadband transient absorption spectra (figure 3.3C) for WT and W285/233F with 290 nm pump. At
3 ps, the absorption spectra of WT and W285/233F are quite similar, representing the featureless Trp excited state (W*) absorption. At later delay times (300 ps and 1 ns), we observed a Trp cationic radical (W+) band around 620-630 nm in WT, but not in the control sample W285/233F or WT monomer (figure 3.5), revealing electron transfer (ET) reaction. Due to complicated energy transfer in WT, W+ formation has multiple time scales. As demonstrated by numerical simulations (figure 3.6), although ET is fast (80 ps), W+ gradually accumulates over a period of nanoseconds, causing W+ to be more prominent at 1 ns than 300 ps. Furthermore, the simulation results show that W* species still dominates at 1 ns, with W+ signal ratio of ~13%, consistent to the relatively weak W+ feature observed in the 1 ns spectrum. To exclude W* signal from distal Trp and peripheral Trp, we selectively pumped the pyramid center with 315 nm laser and measured the broadband transient absorption spectra at various delay times (figure 3.3D and 3.7). WT and W285F have similar W* absorption at 3 ps. Unlike 290 nm excitation data, significant W+ absorption signal, with the maximum at 620-630 nm, was observed at 300 ps, 1 ns and 3 ns. At 3 ns, deprotonated tryptophan neutral radical at ~500 nm was detected, indicating nanosecond deprotonation of W+ following initial charge transfer. The W+ accumulation observed here rules out the recently proposed PCET scenario. Notably, no W+ was observed in the structurally similar mutant W285F, implying that direct ET from W233 excited state to arginine is not likely. Thus, the 80 ps ET reaction must be a charge separation (CS) between W285 and W233. W285, flanking by positively charged R286 and R338, is more likely to be the electron acceptor than W233, which is hydrogen bonded to negatively charged D129. Supporting such
assignment, our calculations predict that W233 excited state transfers an electron to W285 in 67 ps, which is very close to the experimentally determined 80 ps time scale, followed by another ET from W285 minus radical to the arginine residues R286 or R338 to break the critical salt-bridge(s) (table 3.3). Others proposed that the electrostatic perturbations by W285-W233 CS is strong enough to break salt-bridges and lead to dimer dissociation.\textsuperscript{98} We tested such scenario with two mutants W285K/W233D and W285D/W233K and found both are dimeric (figure 3.8), suggesting that charge separation alone is not sufficient to drive monomerization. Based on these findings, we conclude that W285/W233\textsuperscript{+} is formed via the 80 ps CS reaction, and then W285 anionic radical state donates an electron to neutralize the neighboring R286 or R338.

3.3.3 R286/338 Charge Neutralization and Dissociation Quantum Yields

After the photoinduced W285-W233 charge separation, the reaction could evolve along two pathways: charge recombination or electron transfer to arginine residues (figure 3.9). Knowing the W\textsuperscript{*} dynamics in the pyramid center, we can map out W\textsuperscript{+} temporal evolution by single wavelength transient absorption with probe wavelengths around 630 nm. Unlike the mutant W285F, WT transient probed at 630 nm (figure 3.3E blue curve) is drastically different from the W\textsuperscript{*} decay dynamics (figure 3.3E dark yellow curve) determined by time-resolved fluorescence, due to W\textsuperscript{+} contribution. We observed a ~4 ps rise (initial bump of figure 3.3E inset), representing the total rate of 3 dumping channels of [W285-W233\textsuperscript{*}]: charge recombination, ET to Arg and deprotonation of W\textsuperscript{*}. The much slower charge separation (80 ps) causes reverse kinetics and tiny [W285-
W233$^+$ accumulation, resulting in a small amplitude of the ~4 ps rise. Among the 3 pathways, only ET to Arg leads to W$^+$ accumulation in the form of [W285W233$^+$]. Thus, the amplitude of W$^+$ reflects the branching ratio of the competing charge recombination and ET to Arg reactions. By model fitting (section 3.2.5), we measured the BET to be ~ 4 ps, ET to Arg as ~17 ps and a productive (ET to Arg) branching ratio ($E_{ET}$) of 0.20. By multiplying the light perception efficiency ($E_{QY}$ 0.65), charge separation efficiency ($E_{cs}$ 0.75) and $E_{ET}$ together, a branching constant of 0.098 was obtained, which is in excellent agreement with steady-state dissociation quantum yield of 0.10, suggesting that after Arg charge neutralization, all subsequent steps proceed to final UVR8 monomers.

3.3.4 Further Mutagenesis Studies

More mutagenesis studies were carried out to test our proposed reaction mechanism. To further confirm the charge separation direction, we mutated the W285 and/or W233 to tyrosine (Y), which has less electron affinity than W, to modulate electron transfer direction. All 3 mutants show faster fluorescence decay dynamics than the corresponding redox-inactive phenylalanine mutants (figure 3.10A), implying charge separation reactions in Y mutants, but with much slower rates than WT. Among the 3 mutants, W285Y, with a reversed CS direction, exhibits negligible response to extended UV-B+UV-C light while W233Y, with the same CS direction as the proposed mechanism, retains the highest dissociation efficiency (figure 3.10B), indicating that the charge needs to move from residue 233 to 285 to disrupt the inter-subunit interactions. Consistent with earlier findings, when R286 or R338 was changed to neutrally
charged alanine (A) or glutamine (Q), both mutants fail to form dimer after purification and show slower fluorescence decay dynamics than WT, but the R286K or R338K mutants behave same as wild type (figure 3.10C and 3.10D). However, the mutation of other interface salt-bridge arginine residues (R146, R200, R354) does not have obvious effect on dimerization state or dissociation function, highlighting the key function of R286 and R338 as a switch that turns on UVR8 monomerization. Mutation of charged residues R234 or D129 near W233 gives functional, but less efficient UVR8 variants, with slower fluorescence decay dynamics (figure 3.10E and 3.10F), suggesting that electron transfer reaction is facilitated by the local environment, which is optimized by evolution. Hydrogen-bonded to W233 indole ring, D129 may also play the role of anchoring W233 to the conformation that favors electron transfer. We also removed the whole hydrophobic cluster with the mutant W285/233/94/337G, and found that the mutant is a constitutive dimer (figure 3.8), suggesting that the cation-π interactions are not necessary for dimer formation.

3.3.5 MD Simulations of UVR8 Dimer Dissociation

To follow later dissociation steps, we conducted molecular dynamics (MD) simulations for Arg charge neutralized states and the ground state as a control. Although complete monomerization takes milliseconds, as determined by previous transient grating experiments, an apparent tendency to dissociation was observed in the 2 μs R286 and R338 neutralized trajectories, but not in ground state or R234 neutralized simulations. After R286 or R338 becomes neutral, water molecules flush into the dimer interface.
(figure 3.16A), weakening electrostatic interactions, causing a few Å increase in inter-subunit distance (figure 3.16B). Underneath the pyramid cluster, a central water channel is formed by the seven blades of the β-propeller monomer (figure 3.11 and 3.12). When monomerization is initiated by R286 or R338 neutralization, the hydrophobic gate formed by pyramidal Trp on top of the water channel can open, allowing channel water molecules to exchange with interfacial waters (figure 3.16C and figure 3.16D). By examining MD snapshots, we found that ground state and neutralized R234 are structurally stable with closed hydrophobic gate, while neutralized R286 and R338 make large conformational changes (figure 3.13). The most prominent structural changes occur in blades 5 and 6, where W285 and W233 are located (figure 3.13). Importantly, we observed unwinding of the β-propeller architecture in both R286 and R338 neutralized trajectories (figure 3.16E and figure 3.13), consistent to earlier dynamic X-ray studies.\textsuperscript{137} Furthermore, for neutralized R338, bending of the dimer, a state preceding dimer dissociation (figure 3.16E and figure 3.13), was observed.

3.4 Conclusions

Based on all these findings and previous data, including the crystal structure of Arabidopsis UVR8, we propose a reaction scheme for UVR8 photo-induced dissociation (figure 3.9). After an energy transfer network funnels excitation energy to the pyramid center, ~75% of excitation energy centered on W233 is used for charge separation. A second ET from W285\textsuperscript{-} to R286/R338 competes with charge recombination, resulting in 0.20 efficiency, which is reasonably high given the challenging chemistry in this step.
The relative long distance between Arg and W233, together with the W233\(^+\) deprotonation, “locks” the electron on Arg by preventing back electron transfer from R286/R338 to W233, leading to the near unity yield in successive elementary steps. With the neutralized Arg, the protein and water molecules orchestrate dimer dissociation, triggering UV-protective mechanisms in cells.
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Table 3.1 UVR8 Full Length Mutant Summary

*Emission peaks have ±1 nm experimental errors.
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Table 3.2 UVR8 ΔC Mutant Summary
The reorganization energies and driving forces are computed on the basis of linear response theory from molecular dynamics simulations of the initial and final diabatic states for each electron transfer reaction, and the electronic coupling matrix elements are averaged over 100 configurations during 20 ns molecular dynamics simulations. The free energy barriers are determined according to Marcus-Hush theory for electron transfer.

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<th>$\Delta G^0$ (kcal/mol)</th>
<th>$\lambda$ (kcal/mol)</th>
<th>$\Delta G^+$ (kcal/mol)</th>
<th>$k_{ET}$ (s$^{-1}$)</th>
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Table 3.3 Computed rate constants for the initial electron transfer reactions
Figure 3.1 UVR8 structure and steady-state data.

(A) Overall structure of UVR8 homodimer with tryptophan pyramid center (orange), interfacial acidic residues (pink), interfacial basic residues (blue) and water molecules (light blue) highlighted. (B) Local structure around the tryptophan pyramid center. Two critical salt-bridges, R286-D96-D107 and R338-E43-D44, are near W285. D129 and R234 are close to W233. D129 is hydrogen-bonded to W233 indole ring. (C) Steady-state emission spectra of WT, Trp single mutants and W285F/D129N are shown in various colored lines. The inset shows fluorescence quantum yields of selected UVR8 proteins. (D) Dissociation kinetics and quantum yields of various UVR8 samples.
Figure 3.2 UV response test and time-resolved fluorescence of single-point tryptophan mutants.

(A) Steady-state fluorescence peak intensity ($\lambda_{pu}=290$ nm) changes under continuous UV illumination for WT, 13 single-point tryptophan-to-phenylalanine mutants (except W352) and W285F/D129N. An increasing fluorescence intensity indicates normal dissociation function, whereas decreasing peak intensity is due to photobleaching of the nonfunctional mutants. (B) Time-resolved fluorescence transients of WT, 13 single-point tryptophan mutants and W285F/D129N are determined by sub-nanosecond TCSPC. Note that all mutants except W233F, W285F and W285F/D129N have similar TCSPC transients.
Figure 3.3 Steady-state and time resolved spectra for WT and pyramid center Trp ($W_c$) mutants.

Continued
Figure 3.3 Steady-state and time resolved spectra for WT and pyramid center Trp ($W_c$) mutants.

(A) Steady-state emission spectra ($\lambda_{pu}=290$ nm) of WT and all 15 $W_c$ mutants are shown in different colors. Legends from a same class are listed in a same column. (B) Fluorescence decay transients of WT and $W_c$ single mutants, as measured with picosecond resolved TCSPC ($\lambda_{pu}=315$ nm). The fitting results are labeled near the transients. (C) Broad band transient absorption spectra ($\lambda_{pu}=290$ nm) of WT (blue lines), W285/233F (orange lines) and indole/CCl$_4$ model system (grey lines) at various delay times. For shape comparison, absolute intensities of the spectra were adjusted by multiplying by different factors, as labeled near the left side of each spectrum. The spectrum change caused by tryptophan cation radicals ($W^+$) was shaded in light green. (D) Broad band transient absorption spectra ($\lambda_{pu}=315$ nm) of WT (blue lines), W285F (red lines) at various delay times. For shape comparison, absolute intensities of the spectra were adjusted by multiplying by certain factors, as labeled near the left side of each spectrum. The spectrum change caused by Trp cation radicals ($W^+$) and by Trp neutral radicals was shaded in light green and light blue, respectively. (E) Single wavelength transient absorption transients ($\lambda_{pu}=315$ nm, $\lambda_{pr}=630$ nm) for WT (raw data: circles; fitting line: blue line) and W285F (raw data: quares; fitting line: red line). For WT, the transient was deconvoluted into Trp excited state ($W^*$ dark yellow dashed line) and Trp cation radicals ($W^+$ green dashed line) contributions.
Figure 3.4 UV response test and time-resolved fluorescence of tryptophan pyramidal-cluster mutants.

(A) Steady-state fluorescence peak intensity ($\lambda_{pu}=290$ nm) changes under continuous UV illumination for WT and 15 combinations of tryptophan-to-phenylalanine mutants. An increasing fluorescence intensity indicates normal dissociation function. (B) Time-resolved fluorescence transients of WT and 15 combinations of tryptophan-to-phenylalanine mutants are determined by sub-nanosecond TCSPC. According to their emission spectra and function, we categorized these mutants into three classes.
Figure 3.5 Pump 290 nm femtosecond-resolved broadband absorption spectra of various samples.

(A) WT dimer, (B) W285/233F, and (C) WT after UV-B irradiation (monomer). Note that 300-ps and 1-ns spectra of W285/233F and WT+UV samples are very close (dashed orange and cyan lines). However, a significant difference was observed in WT dimer which is due to the observation of W⁺ intermediate.
The initial excited populations and energy transfer rates were based on our proposed energy transfer models. In the simulations, charge separation time is 80 ps, charge recombination time is 4 ps, ET to Arg is 17 ps and deprotonation time is 7.5 ns. Extinction coefficients of W* and W+ were set to 4000 M⁻¹cm⁻¹ and 3000 M⁻¹cm⁻¹ respectively, according to Kohler et. al. (14)
Figure 3.7 Pump 315 nm femtosecond-resolved broadband absorption spectra of (A) WT dimer and (B) W285F.

Raw data were shown in grey lines and smoothed data were shown in color lines. Note that, for W285F, only 3-ps raw data were shown. The spectra of W285F at different time delays are close. However, significant differences were observed in WT dimer, owing to the capture of Trp cationic radicals (~630 nm) and Trp neutral radicals (~500 nm).
Figure 3.8 Size exclusion chromatography results for C-terminal truncated (ΔC) samples.

Figure 3.9 Reaction scheme at the pyramid reaction center.

CS, CR, ET and DP are W285-W233 charge separation, W285-W233$^+$ charge recombination, electron transfer to Arg from W285 minus radicals and deprotonation of W233$^+$ reaction steps, respectively. $\tau_{CS}, \tau_{CR}, \tau_{ET}$ and $\tau_{DP}$ are time scales of corresponding reactions.
Figure 3.10 Steady-state emission ($\lambda_{pu}=290$ nm), time resolved fluorescence spectra ($\lambda_{pu}=290$ nm) and size exclusion column (SEC) results before or after UV irradiation for certain key mutants.

(A) Sub-nanosecond resolved TCSPC transients of W285 and/or W233 phenylalanine/tyrosine mutants. The inset shows steady-state emission spectra of tyrosine mutants. (B) SEC profiles before (red) or after (blue) 30 minutes of UV-B+UV-C irradiation for W285Y, W285/233Y and W233Y. (C) Sub-nanosecond resolved TCSPC transients and steady-state emission spectra (inset) of R286 and R338 mutants. (D) SEC results before (red) or after (blue) 10 minutes of UV-B irradiation for R286 and R338 mutants. (E) Sub-nanosecond resolved TCSPC transients and steady-state emission spectra (inset) of WT, D129N, D129K and R234Q. (F) SEC results before (red) or after (blue) 10 minutes of UV-B irradiation for WT, D129N, D129K and R234Q.
Figure 3.11 The gate connecting the dimer interface and the interior water channel of each UVR8 monomer is gated by W233, W285 and W337, which form the tryptophan pyramid with W94 of the other subunit.

(A) A top view of the water gate in the closed state. (B) A top view of the water gate in the open state. A separation of 5.4 Å between W285 and W337 was observed in the open state. (C) The channel water and interfacial water molecules are in direct contact and exchangeable when the gate is open. (D) A histogram of the inter-trypthophan distance along the molecular dynamics trajectory. The R286-neutralized and the R338-neutralized states were observed to have open state (large distances). For ground state and R234-neutralized state, the gate remains closed during the 2 μs simulation.
Figure 3.12 A bottom view of the water gate and water exchange between central channel and interface.

(A) Water channel in closed state. (B) Water channel is partially open in an R286-neutralized snapshot. (C) Water channel open in an R286-neutralized snapshot. (D) Channel water molecules (in spheres) and interfacial water molecules (yellow sticks) can exchange via the opened gate.
Figure 3.13 Typical MD snapshot structures from neutralized R234 (yellow), neutralized R286 (blue) and neutralized R338 (cyan), comparing with a ground state snapshot structure (grey).

(A) Side view. (B) Top view. The ground and neutralized R234 are very stable, while neutralized R286 and R338 make large conformational changes. The most prominent structural changes occur in blade 5 and 6, where W285 and W233 are located, respectively. An unwinding of the dimer was seen in neutralized R286 and R338 structures.
Figure 3.14 Spectrum of UV light (with filter, black line; without filter, grey dashed line) in units of photons and ratio of photons absorbed by UVR8 sample.
Figure 3.15 Snapshot configurations from molecular dynamics simulations illustrating interfacial salt-bridge interactions for the dark state of UVR8 dimer (A), and the activated states with Arg286 neutralization (B) and with Arg338 neutralization (C).

For clarity, only key salt bridges are shown from one side of the interface. Key salt bridges across the dimer interface found in the X-ray crystal structures are mostly maintained during molecular dynamics simulation of the dark state. For both activated states, the salt bridge between Arg286 and Asp96 breaks apart first (within one nanosecond), reorienting Asp96 towards Arg234. Subsequently, several interfacial salt bridges are interrupted, forming solvent-separated ion pairs or fully dissociated ions. Major changes in ion-pair interactions in the arginine-neutralized configurations are indicated by light-blue arrows.
Figure 3.16 Summary of MD simulations

(A) Number of water molecules at the dimer interface changes with time for ground state, R286 neutralized, R338 neutralized and R234 neutralized simulations. (B) Inter-subunit distance (center of mass distance) changes with time for ground state, R286 neutralized, R338 neutralized and R234 neutralized simulations. (C) An MD snapshot showing the channel waters (blue spheres) and interfacial waters (red spheres). (D) A few Channel water molecules (blue spheres) enter dimer interface via the opened gate 5 ns after the hydrophobic cluster opens. (E) A typical snapshot from R338 neutralized simulation trajectory (cyan) after 1 μs is compared with a ground state MD snapshot (grey). For comparison, two structures were aligned using one monomer (left monomer).
Chapter 4: Summary and Perspective

In this dissertation study, we systematically studied the UV-B (280-315 nm) photoreceptor UVR8 from *Arabidopsis thaliana* and revealed the complete light perception mechanism of this molecular machinery. In chapter 1 of this dissertation, we reviewed previous studies on plant responses to solar UV-B irradiation, UV-B mediated signaling events and biological functions of UVR8. In chapter 2, we reported a striking light harvesting network in UVR8 and solved all energy transfer rates among the tryptophan chromophores in UVR8. In chapter 3, we revealed reaction dynamics at UVR8 reaction center and the mechanism of UV-induced dimer dissociation. Here, in chapter 4, we will summarize all our findings and point out unsolved questions about UVR8.

Based on data from more than 100 mutants in this dissertation research and results from previous studies, including the crystal structure of *Arabidopsis thaliana* UVR8, we solved the complete mechanism of UV-B light perception of UVR8 (shown in figure 4.1). The proposed mechanism has two major parts: a tryptophan-to-tryptophan energy transfer network (shown in figure 4.1A) that funnels excitation energy to the reaction center (central Trp pyramid); a series of electron transfer reactions at the reaction center that triggers UVR8 dimer dissociation (shown in figure 4.1 B-D). Upon UV-B irradiation, all 3 Trp groups can directly absorb photons: distal Trp (W_d ~37%), peripheral Trp (W_p ~23%) and pyramid center Trp (W_c ~32%). After excitation, distal Trp can transfer 34% of excitation energy directly to the reaction center and 11% to the peripheral Trp group. 75% of excitation energy at the peripheral Trp, including energy
that is donated from W₅, is transferred to the reaction center. The overall light perception efficiency of the reaction center is 65%, compared with the 32% direct excitation, the energy transfer network increases the light harvesting efficiency of UVR8 by 100%. After the energy is funneled to the pyramid center, 75% of excited state population undergoes an 80 ps (τₜₜ) charge separation reaction to form W285-W233⁺ intermediate (figure 4.1B). Subsequently, the extra electron on W285 can either move forward to neutralize the critical arginine residues R286 or R338 (τₑₑ ~17 ps) to dissociate the dimer, or move back to W233 through a futile charge recombination (τₑₑ ~4 ps) channel, giving a 20% branching ratio for the productive pathway (figure 4.1C). By multiplying the light perception efficiency (65%), the charge separation efficiency (75%) and the branching coefficient for arginine neutralization pathway (20%) together, we obtain a quantum yield of 9.8%, in excellent agreement with the experimentally measured overall dissociation quantum yield of UVR8 (10%). As shown by MD simulation, after photo-induced arginine neutralization, the critical salt-bridges formed by R286/R338 break and water molecules flood into the dimer interface, further shielding and weakening the electrostatic interactions. The whole dissociation process takes milliseconds, as determined by other methods.⁹³

Similar energy transfer networks, with the same biological function of increasing light harvesting efficiency, have been discovered in the photosynthesis systems in various organisms, including angiosperms, gymnosperms, green algae, purple bacteria and cyanobacteria.¹³⁸ However, there are differences between the light harvesting processes in photosynthesis systems and those in UVR8. Photosynthesis systems utilize visible-
light-absorbing pigments (including chlorophylls, carotenoids and phycobilins) as antenna to harvest solar energy. UVR8 uses natural amino acid tryptophan as chromophore to perceive UV light to trigger cell signaling. Photosynthetic light harvesting uses different types of chromophores with distinct absorption and emission spectra to control energy transfer direction. UVR8 only contain tryptophan chromophores and the unidirectional energy flow is achieved by dramatically different environment of 3 Trp groups. Photosynthetic light harvesting networks can involve multiple protein complexes (e.g., energy transfers from LH1 and LH2 to the photosynthetic reaction center in purple bacteria), whereas UVR8 energy transfer occurs within the protein homodimer. After the energy is funneled to the reaction center, both photosynthesis systems and UVR8 use the excitation energy to initiate electron transfer reactions. Like the bacteriochlorophyll (Bchl) “special pair” in purple bacteria photosynthetic reaction center, UVR8 has W285-W233 tryptophan pairs in the pyramid center, which undergo charge separation reaction.

UVR8 is the only identified UV photoreceptor. Whether there are other UV photoreceptors is an important research question. UVR8 in different plant species show high sequence similarity (figure 4.2). The critical tryptophan and arginine residues (Arabidopsis UVR8 W285 W233 R286 and R338) are highly conserved among various species, suggesting all UVR8 orthologs may share a same light perception mechanism. However, it is still worthwhile to solve the structure and examine the mechanism of other UVR8 orthologs. Furthermore, due to limited time window in our experiments, we only solved the early processes within nanosecond time scales. Efforts should be made to
elucidate dynamics at later stages: When will arginine neutral radical lose the electron to restore to the positively charged state? What are the conformational changes on microsecond to millisecond time scales? Also, it’ll be interesting to use 2-dimentional spectroscopy to investigate the exciton coupling among the pyramid center Trp.
Figure 4.1 Summary of UV Light Perception Mechanism of UVR8

(A) Efficiency of light harvesting network in UVR8. Note that 3 excitation percentages only add up to 92%, since W400 absorbs about 8% of UV-B light. (B) Initial charge separation between two critical tryptophan residues W285 and W233. (C) Two competing steps: futile charge recombination and electron transfer to arginine residues. (D) Water molecules flood into the dimer interface and the dimer dissociates into monomers.
Figure 4.2 Sequence alignment of UVR8 from 10 species.

Abbreviations: ARATH- Arabidopsis thaliana, RICCO- Ricinus communis (Castor bean), VITVI- Vitis vinifera (Grape), POPTR- Populus trichocarpa (Western balsam poplar), ORYSI- Oryza sativa subsp. indica (Rice), SELML- Selaginella moellendorfii (Spikemoss), PHYPA- Physcomitrella patens subsp. patens (Moss), CHLVA- Chlorella variabilis (Green alga), VOLCA- Volvox carteri (Green alga), CHLRE- Chlamydomonas reinhardtii (Chlamydomonas smithii). The critical residues W285, W233, R286 and R338 (highlighted in red) in Arabidopsis UVR8 are highly conserved among different plant species.
References


chromophore amino acids Journal of Chemical Information and Modeling, 51(6), 1287-1295.


electron transfer constitutes the photoactivation mechanism of the plant photoreceptor UVR8 Journal of the American Chemical Society, 137(25), 8113-8120.


Appendix A: Energy Transfer Theory

1. Simple 2-State System

Total Hamiltonian:

\[ \hat{H} = \hat{H}_0 + V \]  \hspace{1cm} (a.1)

\( H_0 \) is Hamiltonian of donor and acceptor without interactions. \( V \) is the interaction Hamiltonian.

For the simplest case of energy transfer from one donor state to one acceptor state, assuming initial state (donor excited state) is \( \psi_1 \), final state (acceptor excited state) is \( \psi_2 \), and they are eigenstates of \( H_0 \):

\[ \hat{H}_0 \psi_1 = E_1 \psi_1 \]
\[ \hat{H}_0 \psi_2 = E_2 \psi_2 \] \hspace{1cm} (a.2)

Where \( E_1 \) and \( E_2 \) are the energies of \( \psi_1 \) and \( \psi_2 \) respectively.

Total wavefunction of the system is:

\[ \psi = c_1 \psi_1 e^{-\frac{E_1 t}{\hbar}} + c_2 \psi_2 e^{-\frac{E_2 t}{\hbar}} \] \hspace{1cm} (a.3)

Applying Time dependent Schrödinger equation:

\[ \hat{H} \psi = i\hbar \frac{\partial \psi}{\partial t} \]

\[ (\hat{H}_0 + V)(c_1 \psi_1 e^{-\frac{E_1 t}{\hbar}} + c_2 \psi_2 e^{-\frac{E_2 t}{\hbar}}) = i\hbar (c_1 \dot{\psi}_1 e^{-\frac{E_1 t}{\hbar}} + c_2 \dot{\psi}_2 e^{-\frac{E_2 t}{\hbar}} - \frac{i}{\hbar} E_1 c_1 \psi_1 e^{-\frac{E_1 t}{\hbar}} - \frac{i}{\hbar} E_2 c_2 \psi_2 e^{-\frac{E_2 t}{\hbar}} ) \]

\[ V(c_1 \psi_1 e^{-\frac{E_1 t}{\hbar}} + c_2 \psi_2 e^{-\frac{E_2 t}{\hbar}}) = i\hbar (c_1 \dot{\psi}_1 e^{-\frac{E_1 t}{\hbar}} + c_2 \dot{\psi}_2 e^{-\frac{E_2 t}{\hbar}} ) \] \hspace{1cm} (a.4)
Multiply $\langle \Psi_2 | e^{\frac{E_{21}}{\hbar}} | \psi \rangle$ on both sides:

$$
c_1 \langle \Psi_2 | V | \Psi_1 \rangle e^{-\frac{E_{21}}{\hbar}} + c_2 \langle \Psi_2 | V | \Psi_2 \rangle = i\hbar \dot{c}_2 \quad (a.5)
$$

Assuming state $\psi_2$ population is negligible. This is true if there is fast relaxation in the acceptor (reversed kinetics, no accumulation):

$$
c_1 \approx 1 \quad c_2 \approx 0 \quad (a.6)
$$

Coupling between initial and final states:

$$
V_{21} = \langle \Psi_2 | V | \Psi_1 \rangle \quad (a.7)
$$

$$
\dot{c}_2 = -\frac{i}{\hbar} V_{21} e^{-\frac{E_{21}}{\hbar}} \quad (a.8)
$$

Integrate to obtain $c_2$ at a small time $\tau$:

$$
c_2(\tau) = \int_0^\tau -\frac{i}{\hbar} V_{21} e^{-\frac{E_{21}}{\hbar} \tau} dt = \frac{e^{-\frac{E_{21}}{\hbar} \tau}}{E_2 - E_1} - 1 V_{21} \quad (a.9)
$$

Probability on $\psi_2$ state at time $\tau$:

$$
|c_2(\tau)|^2 = |V_{21}|^2 \frac{2 - e^{-\frac{E_{21}}{\hbar} \tau} - e^{-\frac{E_{21}}{\hbar} \tau}}{(E_2 - E_1)^2} = |V_{21}|^2 \frac{4\sin^2 \left(\frac{E_2 - E_1}{2 \hbar} \tau\right)^2}{(E_2 - E_1)^2} = |V_{21}|^2 \frac{\sin^2 \left(\frac{E_2 - E_1}{2 \hbar} \tau\right)^2}{\left(\frac{E_2 - E_1}{2 \hbar}\right)^2} \quad (a.10)
$$

Energy transfer rate $k_{12}$:
\[ \kappa_{12} = \left| c_2(\tau) \right|^2 = \frac{2\pi}{\hbar} \left| V_{21} \right|^2 \frac{\sin\left(\frac{E_2 - E_1}{\hbar} \tau \right)}{2\pi \tau^{-1} \left(\frac{E_2 - E_1}{2\hbar} \right)^2} \approx \frac{2\pi}{\hbar} \left| V_{21} \right|^2 \delta\left(\frac{E_2 - E_1}{\hbar} \right) = \frac{2\pi}{\hbar} \left| V_{21} \right|^2 \delta(E_2 - E_1) \]

(a.11)

When time scale of energy transfer is long, energy uncertainty is minimal, the following line-shape function is delta function:

\[ \lim_{\tau \to \infty} \frac{\sin\left(\frac{E_2 - E_1}{\hbar} \tau \right)}{2\pi \tau^{-1} \left(\frac{E_2 - E_1}{2\hbar} \right)^2} = \delta\left(\frac{E_2 - E_1}{\hbar} \right) \]  

(a.12)

Expression of \( \kappa_{12} \) in different domains:

\[ \kappa_{12} = \frac{2\pi}{\hbar} \left| V_{21} \right|^2 \delta(E_2 - E_1) = \frac{2\pi}{\hbar^2} \left| V_{21} \right|^2 \delta(\omega_2 - \omega_1) = \frac{1}{\hbar^2} \left| V_{21} \right|^2 \delta(\nu_2 - \nu_1) \]  

(a.13)

Units: \( V_{21} \) in erg (cm\(^2\)gs\(^{-2}\)), \( \hbar \) in cm\(^2\)gs\(^{-1}\), delta function in erg\(^{-1}\).

2. General Resonance Energy Transfer

For real molecules, both the donor and acceptor have numerous states. Total energy transfer rate (\( \kappa_{ET} \)) is a state density weighted integration of all energy transfer rates for any initial-final state pair:

\[ \kappa_{ET} = \frac{2\pi}{\hbar} \int_{-\infty}^{\infty} \rho_1(E_1) \rho_2(E_2) \delta(E_2 - E_1) dE_1 dE_2 \]  

(a.14)

Antisymmetric wavefunction for initial state \( \psi_1 \) and final state \( \psi_2 \):

\[ \Psi_1 = \frac{1}{\sqrt{2}} (\varphi_{D_1}(r_1) \varphi_{A}(r_2) - \varphi_{D_2}(r_2) \varphi_{A}(r_1)) \chi_{A_i,n} \chi_{A_j} \]  

(a.15)
\[ \Psi_2 = \frac{1}{\sqrt{2}}(\varphi_D(r_1)\varphi_A^*(r_2) - \varphi_D^*(r_2)\varphi_A(r_1)) \chi_{D,m}\chi_{A,k}^* \]  

(a.16)

For the initial state \( \psi_1 \), the donor is at the \( n \)th vibrational state of the electronic excited state, while the acceptor is at the 1st vibrational state of the electronic ground state. For the final state \( \psi_2 \), the donor is at the \( m \)th vibrational state of the electronic ground state, while the acceptor is at the \( k \)th vibrational state of the electronic excited state. \( \varphi_{D*} \) and \( \varphi_D \) are electronic wavefunctions of excited state and ground state molecular orbitals respectively on the donor molecule. \( \varphi_{A*} \) and \( \varphi_A \) are electronic wavefunctions of excited state and ground state molecular orbitals respectively on the acceptor molecule. \( \chi \) are the nuclear wavefunctions of corresponding states.

Electrostatic interactions between the donor and the acceptor in CGS-Gaussian units:

\[ V = \frac{e^2}{\varepsilon |r_1 - r_2|} \]  

(a.17)

Plug in \( \psi_1 \) and \( \psi_2 \) to calculated interaction term \( V_{21} \):

\[ V_{21} = \frac{e^2}{\varepsilon} \left\langle \varphi_D(r_1)\varphi_A^*(r_2) \left| \frac{1}{|r_1 - r_2|} \right| \varphi_D^*(r_2)\varphi_A(r_1) \right\rangle - \left\langle \varphi_D(r_1)\varphi_A^*(r_2) \left| \frac{1}{|r_1 - r_2|} \right| \varphi_A(r_1)\varphi_D(r_2) \right\rangle \chi_{D,m} \chi_{D',k}^* \chi_{A,k} \chi_{A,l} \]

(a.18)

The coupling is the product of an electronic coupling \( V_{el} \) and a nuclear part. We can notice that the nuclear part is the product of two Frank-Condon factors.

\[ V_{21} = V_{el} \left\langle \chi_{D,m} \chi_{D',k}^* \chi_{A,k} \chi_{A,l} \right\rangle \]  

(a.19)

The electronic coupling \( V_{el} \) has two parts: a coulomb term \( V_{COU} \) and an exchange term \( V_{EX} \).
\[ V_{el} = \frac{e^2}{\varepsilon^2} \left( \langle \varphi_D(r_1) \varphi_A(r_2) \rangle \frac{1}{|r_1 - r_2|} \langle \varphi_D(r_2) \varphi_A(r_1) \rangle - \langle \varphi_D(r_1) \varphi_A(r_2) \rangle \frac{1}{|r_1 - r_2|} \langle \varphi_D(r_2) \varphi_A(r_1) \rangle \right) = V_{CO2} + V_{EX} \]  

(a.20)

Assuming fast equilibrium \((k_{relax} >> k_{ET})\):

\[ k_{12} = \frac{2\pi}{\hbar} |V_{el}|^2 \left( \sum_n \sum_m \frac{e^{-E_n/k_{relax}}}{Z_D} \right)^2 \left( \sum_k \sum_l \frac{e^{-E_l/k_{relax}}}{Z_A} \right)^2 \delta(E_2 - E_1) \]  

(a.21)

\( Z \) is the partition function.

Sum of all possible transfer rates:

\[ k_{ET} = \frac{2\pi}{\hbar} |V_{el}|^2 \left( \sum_n \sum_m \frac{e^{-E_n/k_{relax}}}{Z_D} \right)^2 \left( \sum_k \sum_l \frac{e^{-E_l/k_{relax}}}{Z_A} \right)^2 \delta(E_n - E_m - E_k + E_l) \]  

(a.22)

\[ k_{ET} = \frac{2\pi}{\hbar} |V_{el}|^2 \int_0^\infty \left( \sum_{E \leq E_D < E + dE} \frac{e^{-E/k_{relax}}}{Z_D} \right)^2 \left( \sum_{E \leq E_A < E + dE} \frac{e^{-E/k_{relax}}}{Z_A} \right)^2 \delta(E_n - E_m - E_k + E_l) dE \]  

(a.23)

where \( \Delta E_D = E_n - E_m \quad \Delta E_A = E_k - E_l \)

\[ k_{ET} = \frac{1}{\hbar^2} |V_{el}|^2 \int_0^\infty \left( \sum_{E \leq E_D < E + dE} \frac{\ln \nu}{Z_D} \right)^2 \left( \sum_{E \leq E_A < E + dE} \frac{\ln \nu}{Z_A} \right)^2 \delta(E_n - E_m - E_k + E_l) dV \]  

(a.24)

We next will deduct relationship between the thermally weighted Frank-Condon factor and experimentally measured spectra.

Einstein A coefficient in CGS-Gaussian unit system:

\[ \frac{32n\pi^3 \nu^3}{3\hbar c^3} \]

An arbitrarily measured fluorescence spectrum is proportional to the radiative rate:

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\[ f(\nu) \propto \left( \frac{32\pi^3 \nu^3}{3\hbar c^3} \right) |\mu_{DD'}|^2 \left( \sum_{\nu < \nu_D \nu + \nu_D} \frac{e^{-\frac{\hbar \nu u}{k_B T}}}{Z_{D'}^2} \left| \left( \chi_{D,m} \right| \chi_{D',n} \right|^2 \right) \]  

(a.25)

\[ N \text{ is the arbitrary normalizing factor} \]

\[ N f(\nu) = \left( \frac{32\pi^3 \nu^3}{3\hbar c^3} \right) |\mu_{DD'}|^2 \left( \sum_{\nu < \nu_D \nu + \nu_D} \frac{e^{-\frac{\hbar \nu u}{k_B T}}}{Z_{D'}^2} \left| \left( \chi_{D,m} \right| \chi_{D',n} \right|^2 \right) \]  

(a.26)

Integration over frequency:

\[ N \int f(\nu) d\nu = \int \left( \frac{32\pi^3 \nu^3}{3\hbar c^3} \right) |\mu_{DD'}|^2 \left( \sum_{\nu < \nu_D \nu + \nu_D} \frac{e^{-\frac{\hbar \nu u}{k_B T}}}{Z_{D'}^2} \left| \left( \chi_{D,m} \right| \chi_{D',n} \right|^2 \right) d\nu \]  

(a.27)

\[ N \int f(\nu) d\nu = k_{\text{rad}} = \frac{\phi}{\tau_D} \]

\[ N = \frac{\phi}{\tau_D \int f(\nu) d\nu} \]  

(a.28)

\[ \sum_{\nu < \nu_D \nu + \nu_D} \frac{e^{-\frac{\hbar \nu u}{k_B T}}}{Z_{D'}^2} \left| \left( \chi_{D,m} \right| \chi_{D',n} \right|^2 \right) = \frac{3\hbar c^3 \phi f(\nu) \nu^{-3}}{32\tau_D n \pi^3 |\mu_{DD'}|^2 \int f(\nu) d\nu} \]  

(a.29)

For acceptor absorption:

Eternal electrical field \( E = E_0 (e^{-2\pi\nu t} + e^{2\pi\nu t}) = 2E_0 \cos(2\pi\nu t) \)

Energy density \( \rho(\nu) = \varepsilon E_0^2 / 2\pi = n^2 E_0^2 / 2\pi \) (erg cm\(^{-3}\))

Irradiance \( I(\nu) = \frac{C}{n} \rho(\nu) = ncE_0^2 / 2\pi \) (erg cm\(^{-2}\) s\(^{-1}\))
Absorption rate
\[ k_{abs} = \frac{1}{3\hbar^2} |\mu|^2 |E_0|^2 \delta(\nu - \nu_0) \] (a.30)

Absorption by sample:
\[ \frac{2\pi}{3\hbar^2} |\mu_{AA'}|^2 \frac{\rho(\nu)}{n^2} \sum_{\nu - \Delta \nu < \nu + \Delta \nu} \frac{e^{\frac{\nu - \nu_0}{k_B T}}}{Z_{AA'}} \left| \chi_{AA',\delta} \left| \chi_{AA} \right| \right|^2 2\pi\hbar \nu (N_A C \times 10^3) = -\frac{d\rho(\nu)}{dt} \] (a.31)

On the other hand, Beer’s law gives
\[ \frac{I}{I_0} = 10^{-A} = 10^{-\varepsilon CI} \] (a.32)

\[ \ln I - \ln I_0 = -\varepsilon CI \ln 10 \] (a.33)

\[ \frac{dI(\nu)}{dl} = -I(\nu)\varepsilon C \ln 10 = -\frac{n}{c} \rho(\nu)\varepsilon C \ln 10 \] (a.34)

\[ \frac{d\rho(\nu)}{dt} = \frac{c}{n} \frac{dI(\nu)}{dl} = \frac{c^2}{n^2} \frac{dI(\nu)}{dl} \] (a.35)

So
\[ \frac{2\pi}{3\hbar^2} |\mu_{AA'}|^2 \frac{\rho(\nu)}{n^2} \sum_{\nu - \Delta \nu < \nu + \Delta \nu} \frac{e^{\frac{\nu - \nu_0}{k_B T}}}{Z_{AA'}} \left| \chi_{AA',\delta} \left| \chi_{AA} \right| \right|^2 2\pi\hbar \nu (N_A C \times 10^3) = -\frac{c^2}{n^2} \left( -\frac{n}{c} \rho(\nu)\varepsilon C \ln 10 \right) \] (a.36)

\[ \sum_{\nu - \Delta \nu < \nu + \Delta \nu} \frac{e^{\frac{\nu - \nu_0}{k_B T}}}{Z_{AA'}} \left| \chi_{AA',\delta} \left| \chi_{AA} \right| \right|^2 = \frac{3000\hbar c n \ln 10 \varepsilon_A}{4\pi^2 N_A |\mu_{AA'}|^2} \nu \] (a.37)

Plug in to the energy transfer rate equation:
\[ k_{ER} = \frac{1}{\hbar^2} |V_{el}|^2 \frac{1}{|\mu_{AA'}|^2 |\mu_{DD'}|^2} \int_{0}^{\infty} \left( \frac{3000\hbar c n \ln 10 \varepsilon_A}{4\pi^2 N_A} \right) \left( \frac{3\hbar c^3 \phi}{32\tau_D n \pi^3} \right) f_D(\nu) \varepsilon_A \nu^{-4} d\nu \] (a.38)
\[ k_{ET} = |V_d|^2 \frac{1}{|\mu_{AA}|^2 |\mu_{D0}|^2} \int_0^{\infty} \left( \frac{9000 \ln 10 \phi e^{4}}{128 \pi^2 N_A r_D} \right) f_D(v) \varepsilon_A(v) v^{-4} dv \]  

(a.39)

3. Förster Theory

For Förster theory, when donor and acceptor are far apart (edge-to-edge distance over 5-6 angstroms), exchange coupling is zero:

\[ V_{EX} = -\left< \varphi_D(r_1) \varphi_A(r_2) \right| \frac{e^2}{\varepsilon |r_1 - r_2|} \left| \varphi_A(r_1) \varphi_D(r_2) \right> \approx 0 \]  

(a.40)

\[ V_{el} = V_{COU} = \frac{e^2}{\varepsilon} \left< \varphi_D(r_1) \varphi_A(r_2) \right| \frac{1}{|r_1 - r_2|} \left| \varphi_D(r_1) \varphi_A(r_2) \right> \approx \frac{e^2}{n^2} \left< \varphi_D(r_1) \varphi_A(r_2) \right| \frac{1}{|r_1 - r_2|} \left| \varphi_D(r_1) \varphi_A(r_2) \right> \]  

(a.41)

Fast oscillating field:

\[ n = \sqrt{\varepsilon \mu} \approx \sqrt{\varepsilon} \]  

(a.42)

Multi-pole Expansion

\[ V = \frac{e^2}{n^2 |r_1 - r_2|} = e^2 \left( \frac{1}{|r_D - r_A|} - \frac{\Delta r_1 |R|}{|r_D - r_A|^3} + \frac{\Delta r_2 |R|}{|r_D - r_A|^3} + \frac{|R|^3 (\Delta r_1 \Delta r_2) - 3 (\Delta r_1 |R|)(\Delta r_2 |R|)}{|r_D - r_A|^5} + \ldots \right) \]  

(a.43)

\[ \Delta r_1 = r_1 - r_D \]

\[ \Delta r_2 = r_2 - r_A \]  

\[ R = r_2 - r_1 \approx r_A - r_D \]  

(a.44)

\[ V_{el} = \left< \varphi_D(r_1) \varphi_A(r_2) \right| e^2 \frac{1}{n^2 |r_1 - r_2|} \left| \varphi_D(r_1) \varphi_A(r_2) \right> \]  

(a.45)
Dipole-dipole interaction is dominant when donor-acceptor distance is much larger than the molecular sizes

\[ V \approx \left| R \right|^2 \left( \Delta r_1 \cdot \Delta r_2 \right) - 3(\Delta r_1 \cdot R)(\Delta r_2 \cdot R) \]
\[ n^2 |R|^5 \]  

(a.46)

Transition dipole moment:

\[ \left\langle \varphi_D \left| e \Delta r_1 \right| \varphi_{D'} \right\rangle = -\mu_{DD'} \]
\[ \left\langle \varphi_A \left| e \Delta r_2 \right| \varphi_{A'} \right\rangle = -\mu_{AA'} \]  

(a.47)

\[ V_{el} = \left\langle \varphi_{D'}(r_1)\varphi_{A'}(r_2) \right| \frac{\left| R \right|^2 \left( \Delta r_1 \cdot \Delta r_2 \right) - 3(\Delta r_1 \cdot R)(\Delta r_2 \cdot R)}{n^2 |R|^5} \left| \frac{\left( \mu_{DD'} \cdot \mu_{AA'} \right) - 3(\mu_{DD'} \cdot R)(\mu_{AA'} \cdot R)}{n^2 |R|} \right) \]

(a.48)

where

Define 3 angles:

\[ \cos \theta = \frac{\mu_{DD'} \cdot \mu_{AA'}}{|\mu_{DD'}||\mu_{AA'}|} \]
\[ \cos \alpha = \frac{\mu_{DD'} \cdot R}{|\mu_{DD'}||R|} \]  

(a.49)

\[ \cos \beta = \frac{\mu_{AA'} \cdot R}{|\mu_{AA'}||R|} \]

\[ V_{el} = \frac{\cos \theta - \cos \alpha \cos \beta}{n^2 R^3} |\mu_{DD'}||\mu_{AA'}| \]  

(a.50)

Then

\[ k_{ET} = \left( \frac{\cos \theta - \cos \alpha \cos \beta}{n^2 R^3} \right)^2 \int_0^{\infty} \left( \frac{9000 \ln 10 \phi e^4}{128 \pi^2 N_A \tau_D} \right) f_D(v) e_A(v)v^{-4} f_D(v)dv \]  

(a.51)
Orientation factor:

\[ \cos \theta - \cos \alpha \cos \beta = \kappa \quad (a.52) \]

\[
k_{FRET} = \frac{9000 \ln(10)}{128\pi^2 N_A} \frac{\kappa^2 \phi}{n^4 R^6 \tau_D} \int_0^\infty \frac{f_D(v) \varepsilon_A(v) e^4 v^{-4}}{f_D(v)} dv \quad (a.53)
\]

Convert to wavelength domain:

\[ f_D(\lambda) \lambda^2 / c = f_D(v) \]

\[ dv = c\lambda^{-2} d\lambda \quad (a.54) \]

\[
k_{FRET} = \frac{9000 \ln(10)}{128\pi^2 N_A} \frac{\kappa^2 \phi}{n^4 R^6 \tau_D} \int_0^\infty \frac{f_D(\lambda) \varepsilon_A(\lambda) \lambda^4}{f_D(\lambda)} d\lambda = \frac{9000 \ln(10)}{128\pi^2 N_A} \frac{\kappa^2 \phi J}{n^4 R^6 \tau_D} \quad (a.55)
\]

Spectral Overlap:

\[ J = \int_0^\infty \frac{f_D(\lambda) \varepsilon_A(\lambda) \lambda^4}{f_D(\lambda)} d\lambda \quad (a.56) \]

\[
k_{FRET} = 8.79 \times 10^{-25} \frac{\kappa^2 \phi J}{n^4 R^6 \tau_D} \quad (a.57)
\]

If \( J \) in \( \text{cm}^3 \text{M}^{-1} \), \( R \) in cm.

\[
k_{FRET} = 8.79 \times 10^{23} \frac{\kappa^2 \phi J}{n^4 R^6 \tau_D} \quad (a.58)
\]

If \( J \) in \( \text{cm}^3 \text{M}^{-1} \), \( R \) in angstroms.

Efficiency:

\[ E = \frac{k_{FRET}}{k_{FRET} + \tau_D^{-1}} \quad (a.59) \]
\[ k_{\text{FRET}} = \frac{1}{\tau_D} \]
\[ E = 0.5 \] \hspace{1cm} (a.60)
\[ R_0^6 = 8.79 \times 10^{21} \frac{k^2 \phi J}{n^4} \]
\[ E = \frac{R_0^6}{R_0^6 + R^6} \] \hspace{1cm} (a.61)

4. Dexter Theory

For small transition dipole moments, triplet to triplet energy transfer or forbidden excitation, the exchange interaction dominates (Dexter theory):

\[ V_{et} = V_{EX} = -\langle \varphi_D (r_1) \varphi_A^* (r_2) \rangle \frac{e^2}{n^2 |r_1 - r_2|} \langle \varphi_A (r_1) \varphi_D^* (r_2) \rangle \] \hspace{1cm} (a.62)

\[ k_{DEX} = \frac{|V_{DEX}|^2}{|\mu_{AA}|^2 |\mu_{DD}|^2} \frac{9000 \ln(10) c^4 \phi}{128 \pi^4 \nu_A^2 \tau_D} \int f_D (v) e^{-V^4} dV = \frac{|V_{DEX}|^2}{|\mu_{AA}|^2 |\mu_{DD}|^2} \frac{9000 \ln(10) \phi}{128 \pi^4 \nu_A^2 \tau_D} \int f_D (\lambda) e^{-\lambda^4} d\lambda \] \hspace{1cm} (a.63)

\[ k_{DEX} = 8.79 \times 10^{-25} \frac{|V_{DEX}|^2}{|\mu_{AA}|^2 |\mu_{DD}|^2} \frac{\phi}{\tau_D} J \] \hspace{1cm} (a.64)

Dipole moment in esu cm (1E18 Debye), If J in cm$^3$M$^{-1}$, R in cm. To calculate V, charge in esu (e=4.8032E-10 esu), distance in cm.

\[ \frac{k_{DEX}}{k_{\text{FRET}}} = |V_{DEX}|^2 / \left( \frac{k^2}{n^4 R^6} |\mu_{AA}|^2 |\mu_{DD}|^2 \right) \] \hspace{1cm} (a.65)

Alternatively,
\[ f_D(v) = \frac{\tau_D}{\phi} \int f_D(v)dv \left( \frac{32n\pi^3 v^3}{3h^3} \right) \mu_{Dv'} \left( \sum_{h_v \leq \Delta E_{Dv'}<h_v+\mu_{Dv'}} \frac{e^{\frac{-h_v}{kT}}}{Z_{D'v'}} \left| \langle \chi_{D,m} | \chi_{D',n} \rangle \right|^2 \right) \]  
(a.66)

\[ \int f_D(v)dv = \frac{\tau_D}{\phi} \int f_D(v)dv \left( \frac{32n\pi^3 v^3}{3h^3} \right) \mu_{Dv'} \left( \sum_{h_v \leq \Delta E_{Dv'}<h_v+\mu_{Dv'}} \frac{e^{\frac{-h_v}{kT}}}{Z_{D'v'}} \left| \langle \chi_{D,m} | \chi_{D',n} \rangle \right|^2 \right) d\nu = \sum_{n} \sum_{m} \frac{e^{\frac{-h_v}{kT}}}{Z_{D'v'}} \left| \langle \chi_{D,m} | \chi_{D',n} \rangle \right|^2 = \sum_{n} \frac{E_n}{Z_{D'}} = 1 \]  
(a.67)

Unit of RET rate: s

\[ \epsilon_A(v) = \frac{4\pi^2 N_A |\mu_{A\lambda}|^2}{3000\hbar c \ln 10} \left( \sum_{v \leq \Delta v} e^{\frac{hv}{kT}} \left| \langle \chi_{A',\lambda} | \chi_{A,\lambda} \rangle \right|^2 \right) \]  
(a.69)

\[ A(v) = \frac{4\pi^2 N_A |\mu_{A\lambda}|^2}{3000\hbar c \ln 10} \left( \sum_{v \leq \Delta v} e^{\frac{hv}{kT}} \left| \langle \chi_{A',\lambda} | \chi_{A,\lambda} \rangle \right|^2 \right) \text{Cl} \]  
(a.70)

\[ \bar{A}(v) = \frac{A(v)\nu^{-1}}{\int A(v)\nu^{-1}d\nu} = \sum_{v \leq \Delta v} e^{\frac{hv}{kT}} \left| \langle \chi_{A',\lambda} | \chi_{A,\lambda} \rangle \right|^2 \]  
(a.71)

\[ k_{DEX} = \frac{1}{h^2} |V_{EX}|^2 \int \bar{f}_D(v)\bar{A}(v)dv \]  
(a.72)

Units: V in erg (cm²gs⁻²), h bar in cm²gs⁻¹, \( \int f_D(v)\bar{A}(v)dv \) in s. Calculated rate in s⁻¹.

Assuming H atom s orbital in one dimension:

\[ |V_{EX}|^2 = \frac{e^4}{\hbar^2} \int \int Ne^{-\eta_1/a_0}e^{-\eta_1/a_0} \frac{1}{|r_1-r_2|}e^{-(\eta_1+R)/a_0}e^{-(\eta_1+R)/a_0}dr_1dr_2 \]  
(a.73)
\[ |V_{ex}|^2 = \frac{e^4}{n^4} \left( \int \int Ne^{-\eta_1/\lambda_0} e^{-\eta_1/\lambda_0} \frac{1}{r_1 - r_2} e^{-\eta_2/\lambda_0} e^{-\eta_2/\lambda_0} d_1 d_2 \right) \left( e^{-2R/(\lambda_0 + \lambda_1)} \right) \]  

(a.74)

\[ |V_{ex}|^2 = K \exp(-2R/L) \]  

(a.75)

L is related to ground state and excited state orbital radius (some call it averaged van der waals radius for initial and final states), R is the donor acceptor distance, K is the coupling if donor and acceptor orbital overlap.

For more complicated orbital, orientation must be considered.

\[ k_{dex} = \frac{1}{\hbar^2} K \exp(-2R/L) \int \tilde{f}_D(\nu) \tilde{A}(\nu) d\nu \]  

(a.76)

Practically, it’s very hard to obtain \( \tilde{A}(\nu) \) (blue side has contributions of higher bright states)
Appendix B: Simulation of TCSPC Transients

1. Simulation of Kinetics of 3 Trp Groups
The excited populations of UVR8 tryptophan residues at different times (from 0 to 30 ns, 4 ps interval) were numerically simulated based on kinetic models. Parameters are shown in table 2.12, 2.13 and 2.14. The calculated energy transfer time constants for individual D-A pairs in table 2.6 and 2.9 were also needed.

Since distal tryptophan residues have two lifetimes, for each W_d, the excited population was treated as the sum of two subpopulations with the same energy transfer rate but with distinct lifetimes (equation b.1). The differential equations and corresponding boundary conditions describing the population evolution of a distal tryptophan W_m (m=39, 92, 144, 196, 300 or 352) are shown as below:

\[
[W_m] = [W_{m1}] + [W_{m2}],
\]

\[
\frac{d[W_{m1}]}{dt} = -\left(\tau_{\text{total,} W_m}^{-1} + \tau_D^{-1}\right)[W_{m1}]_0 \quad [W_{m1}]_{t=0} = A_d(\lambda)(1-R_{D2})
\]

(b.1)

\[
\frac{d[W_{m2}]}{dt} = -\left(k_{\text{total,} W_m} + \tau_D^{-1}\right)[W_{m2}]_0 \quad [W_{m2}]_{t=0} = A_d(\lambda)R_{D2}
\]

(b.2)

Equations b.2 and b.3 define the decay dynamics of the 0.5 ns and 2.7 ns components, respectively. In the equations, \(\tau_{\text{total,} W_m}\) is the theoretical total FRET time constant (figure 2.3A) calculated using equation 2.13 and 2.14 for W_m (m=39, 92, 144, 196, 300 or 352). \(\tau_D\) are the two lifetimes of W_d, which are 0.5 ns and 2.7 ns, respectively. R_{D2} is
the fraction of amplitude for the 2.7 ns lifetime, which is 0.71 as determined by femtosecond fluorescence up-conversion (Fig. 3B). $A_d(\lambda)$ is the value of the normalized $W_d$ absorption spectrum (given by equation 2.1, shown in figure 2.1E) at the excitation wavelength $\lambda$ ($\lambda=290 \text{ nm or 310 nm}$). The decay transients of all $W_d$ were then added together to obtain the total dynamics of distal tryptophan group:

$$[W_d]_t = [W39]_t + [W92]_t + [W144]_t + [W196]_t + [W300]_t + [W352]_t \quad (b.4)$$

For each peripheral tryptophan residues $W_i$ ($i=198, 250 \text{ or 302}$), depending on the lifetime and whether or not transfer energy to $W_c$, the total excited population was divided into 4 subpopulations: (1) short lifetime and energy transfer to $W_c$, (2) long lifetime and energy transfer to $W_c$, (3) short lifetime and no energy transfer to $W_c$, (4) long lifetime and no energy transfer to $W_c$.

$$[W_i]_t = [W_{i1}]_t + [W_{i2}]_t + [W_{i3}]_t + [W_{i4}]_t \quad (b.5)$$

Each subpopulation has a distinct differential equation and boundary conditions for numerical simulation:

$$\frac{d[W_{i1}]_t}{dt} = -\left(\tau_{\text{total,}W_i}^{-1} + \tau_{1,W_i}^{-1}\right)[W_{i1}]_t + (1 - R_m)R_{2,W_i}\sum_{m}^{W_i}\left(\tau_{FRET,WmW_i}^{-1} + \tau_{FRET,WmW_i(b)}^{-1}\right)[Wm]_t$$

$$[W_{i1}]_{t=0} = A_p(\lambda)(1 - R_m)(1 - R_{2,W_i}) \quad (b.6)$$

$$\frac{d[W_{i2}]_t}{dt} = -\left(\tau_{\text{total,}W_i}^{-1} + \tau_{2,W_i}^{-1}\right)[W_{i2}]_t + (1 - R_m)R_{2,W_i}\sum_{m}^{W_i}\left(\tau_{FRET,WmW_i}^{-1} + \tau_{FRET,WmW_i(b)}^{-1}\right)[Wm]_t$$
Equations b.6, b.7, b.8 and b.9 describe the kinetics of the 4 subpopulations. On the right side of each equation, the first term gives the decay kinetics due to lifetime and energy transfer to W_c; the second term defines the rise kinetics due to energy transfer from W_d.

In the above equations, \( \tau_{\text{total},Wi} \) is the theoretical total FRET time constant from peripheral Trp Wi (i=198, 250 or 302) to the 4 pyramid center tryptophan residues calculated using equation 2.13 and 2.14 (shown in figure 3.3B). \( \tau_{1, Wi} \) and \( \tau_{2, Wi} \) are the two lifetimes of Wi. \( \tau_{\text{FRET},WmWi} \) is the FRET time constant from one distal Trp Wm to the peripheral Trp Wi and \( \tau_{\text{FRET},WmWi(b)} \) is the FRET time constant from the same W_d to the W_p on the other subunit (Table S6), which takes the intersubunit energy transfer into account. \([Wm]_t\) is the kinetics of excited distal tryptophan m (m=39, 92, 144, 196, 300 or 352) simulated as above mentioned. \( R_{2, Wi} \) is the fraction of amplitude for the slower lifetime (6-8 ns) and \( R_{nt} \) stands for the population ratio of W_p that does not transfer energy to W_c. \( A_p(\lambda) \) is the value of the normalized W_p absorption spectrum (given by equation S2, shown in figure 3.1E) at the excitation wavelength \( \lambda \) (\( \lambda = 290 \) nm or 310 nm). For peripheral Trp that were knocked out in the mutant, the \( A_p(\lambda) \) value was set to 0. For each mutant, the simulated
transients for peripheral Trp present in the mutant were added together to give the total dynamics of peripheral tryptophan group:

\[ [W_p]_t = \sum_i [W_i]_t \]  

(b.10)

Our model treated the 4 pyramid center tryptophan residues \((4W_c)\) as a whole. Since 2 decay time constants were observed (0.08 ns and 1.4 ns) for the pyramid center, we divide \(W_c\) into two subpopulations:

\[ [W_c]_t = [W_{c1}]_t + [W_{c2}]_t \]  

(b.11)

\[
\frac{d[W_{c1}]_t}{dt} = -\tau_{c1}^{-1}[W_{c1}]_t + R_{c1} \sum_m \sum_n (\tau_{FRET,WmWn}^{-1} + \tau_{FRET,WmWn(b)}^{-1})[W_m]_t + R_{c1} \sum_i \tau_{total, Wi}^{-1}([W_i]_t + [W_i^2]_t)
\]

(b.12)

\[
[W_{c1}]_{t=0} = 4A(\lambda)R_{c1}
\]

\[
\frac{d[W_{c2}]_t}{dt} = -\tau_{c2}^{-1}[W_{c2}]_t + (1 - R_{c1}) \sum_m \sum_n (\tau_{FRET,WmWn}^{-1} + \tau_{FRET,WmWn(b)}^{-1})[W_m]_t + R_{c1} \sum_i \tau_{total, Wi}^{-1}([W_i]_t + [W_i^2]_t)
\]

\[
[W_{c2}]_{t=0} = 4A(\lambda)(1 - R_{c1})
\]

(b.13)

In equations b.12 and b.13, \(\tau_{c1}\) and \(\tau_{c2}\) are the two fluorescence decay times of the pyramid center, which are 0.08 ns and 1.4 ns, respectively. \(\tau_{FRET,WmWn}\) and \(\tau_{FRET,WmWn(b)}\) are the FRET time constants from one distal Trp Wm (m=39, 92, 144, 196, 300 or 352) to the pyramid center Trp Wn on the same and on the other subunit (table 2.6), respectively. \([W_m]_t\) is the kinetics of excited distal tryptophan m (m=39, 92, 144, 196, 300 or 352)
simulated as above mentioned. \( \tau_{\text{total,}\text{Wi}} \) is the theoretical total FRET time constant from peripheral Trp Wi (i=198, 250 or 302) to the 4 pyramid center tryptophan residues calculated using equation 2.13 and 2.14 (figure 3.3B). [Wi\(_i\)] and [Wi\(_j\)] are the kinetics of the two subpopulations (as above mentioned) of peripheral Trp Wi, that transfer energy to \( W_c \). \( R_{c1} \) is the amplitude ratio of the 80 ps component, which is 0.75. \( A_c(\lambda) \) is the value of the normalized \( W_c \) absorption spectrum (given by equation S3, shown in figure 3.1E) at the excitation wavelength \( \lambda \) (\( \lambda = 290 \) nm or 310 nm).

2. Simulation of Fluorescence Transients and Contributions from 3 Trp Groups
The simulated total signal (Simul.) was given by the sum of contributions from \( W_d \), \( W_p \) and \( W_c \):

\[
\text{Simul.}(t) = S(W_d) + S(W_p) + S(W_c)
\]  
(b.14)

Which were calculated using the following equations:

\[
S(W_d) = A \cdot FL_d(\lambda) \int_{-\infty}^{+\infty} IRF(\tau) [W_d]_{\tau-\tau} d\tau 
\]  
(b.15)

\[
S(W_p) = A \cdot FL_p(\lambda) \int_{-\infty}^{+\infty} IRF(\tau) [W_p]_{\tau-\tau} d\tau 
\]  
(b.16)

\[
S(W_c) = A \cdot FL_c(\lambda) \int_{-\infty}^{+\infty} IRF(\tau) [W_c]_{\tau-\tau} d\tau 
\]  
(b.17)

\([W_d], [W_p] \text{ and } [W_c] \) are the kinetics of distal, peripheral and pyramid center Trp as defined in equations b.4, b.10 and b.11, respectively. IRF is the instrument response function as recorded using buffer scattering. \( FL_d, FL_p \) and \( FL_c \) are the fluorescence intensity of \( W_d, W_p \) and \( W_c \) per unit population per unit time, respectively. \( FL_d, FL_p \) and
FLc have the same spectra shapes as fluorescence spectra shown in figure 3.1E, but with arbitrary absolute intensities (table 2.14). For comparison with experimental data, a same normalizing factor (A) was applied to equations b.15, b.16 and b.17 to give a normalized total simulation curve.

For a certain mutant with at one excitation wavelength, picosecond resolved TCSPC data at multiple fluorescence wavelengths were simulated using our model. Given the fact that the steady-state emission is the sum of time integrals of time-resolved fluorescence from 3 Trp groups (equation b.18), with the steady-state emission spectra of the mutant and simulation data at various fluorescence wavelengths, the spectra of W_d, W_p and W_c can be determined in a way similar to that described in equation S5.

\[ I(\lambda) = I_d(\lambda) + I_p(\lambda) + I_c(\lambda) \]  
(b.18)

\[ I_d(\lambda) = I(\lambda) \frac{\int_{0}^{+\infty} FL_d(\lambda)[W_d],dt}{\int_{0}^{+\infty} FL_d(\lambda)[W_d],dt + \int_{0}^{+\infty} FL_p(\lambda)[W_p],dt + \int_{0}^{+\infty} FL_c(\lambda)[W_c],dt} \]  
(b.19)

\[ I_p(\lambda) = I(\lambda) \frac{\int_{0}^{+\infty} FL_p(\lambda)[W_p],dt}{\int_{0}^{+\infty} FL_d(\lambda)[W_d],dt + \int_{0}^{+\infty} FL_p(\lambda)[W_p],dt + \int_{0}^{+\infty} FL_c(\lambda)[W_c],dt} \]  
(b.20)

\[ I_c(\lambda) = I(\lambda) \frac{\int_{0}^{+\infty} FL_c(\lambda)[W_c],dt}{\int_{0}^{+\infty} FL_d(\lambda)[W_d],dt + \int_{0}^{+\infty} FL_p(\lambda)[W_p],dt + \int_{0}^{+\infty} FL_c(\lambda)[W_c],dt} \]  
(b.21)

Where \( I(\lambda) \) is the steady-state emission spectrum. The shapes of resulted \( I_d, I_p \) and \( I_c \)
(figure 2.15, colored symbols) agree well with directly measured emission spectra of $W_a$, $W_p$ and $W_c$ Trp groups, which further validates our energy transfer model.