S1. Broadband 2DES with ESA Subtraction

Figure S1. Real absorptive 2DES spectra with and without subtraction of excited state absorption. (A) Real absorptive 2DES spectra, plotted at various $t_2$ waiting times. Also shown is the 77 K linear absorption spectrum of the PSII-RC (right panel) with dashed lines showing the expected locations for the $Q_x$, $Q_y$ transitions for Chl vs Pheo as well as the anion band, (B) ESA-subtracted data.

Previous transient absorption studies of the PSII-RC have noted the dominant excited state absorption (ESA) signals that obscure weaker, oppositely-signed ground-state bleach (GSB) contributions that can provide insight into the excitonic structure and charge separation mechanisms (17, 30, 31). A method employed by Greenfield et al. is to subtract the ESA background at 557 nm (31) where there are no discernable features in the linear absorption spectrum. A similar ESA subtraction method has been used in a 2DES study of the bacterial reaction center (BRC) to reveal GSB features (68). Figure S1B shows the
2DES data following subtraction of the t2-dependent background signal at 557 nm, enabling clearer resolution of the weak GSB signatures of the Chl and Pheo Qx transitions. One can clearly observe absorption peaks corresponding to the pigment transitions indicated by dashed lines: Chl Qx 0-1 at 585 nm, Pheo Qx 0-0 at 545 nm, Pheo Qx 0-1 at 512 nm, CarD2 0-0 at 506 nm, CarD2 0-1 at 474 nm, and CarD1 0-0 at 489 nm. The spectral locations of these peaks are taken from the linear absorption analysis done by Shipman et al. (66) and Ratsep et al. (67). The Pheo anion band ESA at 455 nm, which signifies the reduction of Pheo, was reported by Hasting et al. (32).

Table S1. Mid-IR Peak Assignment Table

| Wavenumber (cm⁻¹) | Assignment                                             |
|-------------------|--------------------------------------------------------|
| 1657/1664 bandshift | Amide C=O response to CS (15, 34, 36)                  |
| 1670              | ChlD1 GSB (15, 35, 36)                                 |
| 1677              | Pheo GSB (15, 34, 36)                                  |
| 1704              | P GSB (35, 36)                                         |
| 1711              | P⁺ ESA (15, 35, 36, 43)                                |
| 1721              | Pheo GSB (minor contribution from P GSB) (27, 34, 36)  |
| 1724-1731         | P⁺ and Pheo⁻ ESA (15, 34-36, 43)                      |

S3. Lifetime Density Map Treatment

The lifetime density maps (LDMs) obtained from the OPTIMUS software package (37) underwent further treatment following reference (38) and personal communication with Mark Berg at U of SC. If the data (signal) is S(t), the inverse-Laplace transform φ(κ) is given by

\[
S(t) = \int_0^\infty d\kappa \phi(\kappa)e^{-\kappa t}
\]  

(1)

Note that t (time) and κ (rate) are conjugate variables, just as t and ω are in a Fourier transform. It is convenient to change the rate to a time constant T =1/κ (τ in reference (37)).
\[
S(t) = \int_0^\infty dT \Phi(T) e^{-t/T} \approx \sum_{i=1}^{100} \Phi_i \ e^{-t/T_i} (T_i - T_{i-1})
\]  

(see reference (37), Eq. 3), where \( \Phi(T) \) is called the spectral distribution function by Slavov et al. Again, time \( t \) and time constant \( T \) are distinct concepts, even though they have the same direction and units. Also assume we have 100 T points, which Slavov et al. say is standard.

The data is usually plotted on a log-time scale, but one of the important technical issues is that we need to perform the mathematical transformation. First, pick a time unit, e.g., \( T_0 = 1 \) ps. Then define new variables:

\[
y = \ln(t/T_0) \quad \text{and} \quad Y = \ln(T/T_0)
\]

and functions:

\[
\bar{S}(y) = S(t = T_0 e^y) \quad \text{and} \quad \bar{\Phi}(Y) = \Phi(T = T_0 e^Y) dT / d = \Phi(T_0 e^Y) T_0 e^{-Y}
\]

Then, on log scales,

\[
\bar{S}(y) = \int_{-\infty}^{\infty} dY \bar{\Phi}(Y) \exp(-e^{y-Y}) \approx \sum_{i=1}^{100} \Phi_i e^{-y/T_i} \ln\left(\frac{T_i}{T_{i-1}}\right)
\]

\( \bar{\Phi}(Y) \) is what we call the rate spectrum. The decay spectrum \( S(y) \) is defined by:

\[
\hat{S}(y) = -\frac{d\bar{S}}{dy} = \int_{-\infty}^{\infty} dY \bar{\Phi}(Y) e^{y-Y} \exp(-e^{y-Y})
\]

If we define a response function
\[ R(Y) = e^Y \exp(-e^Y) \]  

(7)

it has the form of a convolution of the rate spectrum:

\[ \hat{S}(y) = \int_{-\infty}^{\infty} dY \Phi(Y) R(y - Y) \]  

(8)

In principle, the decay spectrum can be calculated directly as a derivative of the decay data (Eq. (6)). However, a point-to-point derivative would be very sensitive to noise. One needs to smooth the data first, then take the derivative of the smoothed function. The LDA analysis from Slavov et al. (37) is a perfectly fine choice for a smoothing function. Equation (8) then provides a simple way to calculate the derivative. Varying the regularizing parameter or even a GLA provide alternative choices for a smoothing function. However, so long as the final fit goes through the data with a small \( \chi^2 \), the resulting decay spectrum should be pretty much the same.

Assume that we are happy with these aspects of the analysis and have a rate spectrum vector \( \Phi = [\Phi_1(\lambda), ..., \Phi_{100}(\lambda)] \) and a corresponding vector of time constants \( T = [T_1, ..., T_{100}] \) that produce a satisfactory fit to the data. We need to discretize Eq. (8):

\[ \hat{S}_i(\lambda) = \sum_j \Phi_j(\lambda) R_{i-j} \delta Y = [\Phi * R] \delta Y \]  

(9)

The point spacing, \( \delta Y = \ln(T_i/T_{i-1}) \), should be constant. It is not an essential factor, but it is useful for keeping absolute normalizations. Any standard analysis package will have a built-in function for taking the convolution of vectors, as on the right of Eq. (9). The tricky part is understanding the ranges for \( i \) and \( j \) that are built into the function. The vector convolution itself is independent of the \( x \)-axis values, but it is useful to generate them to keep track of what is going on.

The procedure consists of the following steps. First, convert the time constants to a log-time scale (Eq. (3)), \( Y = [\ln(T_1/T_0, ..., \ln(T_{100}/T_0))] \). Choose the time unit so zero is near the center of the range, e.g., \( Y = [Y_{-39}, ..., 0, ..., Y_{60}] \). Calculate the response function on the same range using Eq. (7), \( R = [R(Y_{-39}), ..., R(Y_{60})] \). Most vector convolution routines will produce a result that is 201 points long, \( \hat{S}(\lambda) = [S_{-99}(\lambda), ..., S_{0}(\lambda), ..., S_{99}(\lambda)] \) with implied log times \( y = \)
\[ y_{-99}, \ldots, 0, \ldots, y_{99} \] and times \( t = [T_0 \exp(y_{99}), \ldots, T_0, \ldots, T_0 \exp(y_{99})]. \) Finally, truncate the vectors so that only the times within the range of the original data are kept. Supplementary Figure S2 demonstrates the effect of this treatment on the LDM, showing how it nicely removes unphysical oscillatory components from the LDM.

**Figure S2.** Lifetime Density Maps (LDMs) before and after treatment described in S3. (A) (LDM) output from OPTIMUS software (37) upon 662-668 nm excitation, (B) LDM after treatment described in S3. The artificially induced oscillations along the time axis are removed.

**S4. Additional Lifetime Density Maps**

In the main text we reported lifetime density maps (LDMs) for two excitation wavelength regions that exhibited the clearest excitation-frequency dependence. In Supplementary Figure S3 we show analogous LDMs in which we use a narrower window of integration along the excitation frequency axis to more completely explore the excitation frequency dependence across the Qy region.
Figure S3. LDMs of broadband-2DES (left) and 2DEV (right) at different excitation frequency regions spanning the entire \( Q_y \). Blue features indicate a rise of GSB or decay of ESA, while conversely, Red indicates decay of GSB or rise of ESA.

S5. Additional Global Target Analysis of the 2-pathway Model

We first try to fit the broadband-2DES with the 2-pathway model from Romero et al(17). This model does not fit well with our data (Figure S4). Opposite-sign features are observed on the 2D SAS of the compartments. There are also Pheo bleach features in compartments S4 and S6, which represent the \( P_{01} \) pathway and should therefore not contain such features.
Result from fitting broadband-2DES data: **A.** 2-pathway model from Romero et al. **B.** 2D SAS of each compartment.

We then try to simplify the target model by removing the contribution of Chlz. The target model thus becomes a 6-compartment model (Figure S5). The 2D SAS show quite convincing evidence for the 2-pathway model. There is excitation frequency dependence between the 2D SAS of the 2 pathways. Also, there is no Pheo features in compartments S2 and S4 which represent the P_{D1} pathway. However, when we try to fit this model to the 2DEV data, we were not able to obtain a good fit, as the artificial opposite-sign features between the 2D SAS appear once again (Figure S5B, lower panel).
Figure S5 Result from fitting broadband-2DES and 2DEV data: (A) 2-pathway model with Chlz contributions removed, (B) 2D SAS of each compartment.
We also try a slightly modified model compared to the main text, in which the population of Trap1* is transferred directly to RP2 (Figure S6). The GTA fit produces slightly different time constants.

Figure S6. Result from simultaneously fitting broadband-2DES and 2DEV data: (A) Slightly modified 5-compartment model with 2 trap states, (B) 2D SAS of each compartment.
Figure S7. Linear absorption and exciton states from the global target model of Figure S6. Linear absorption of the D1D2 RC at 77 k (green solid curve) fit with three Gaussian distributions that represent the three distinct initial exciton states RC* (red solid curve, centered at 679.5 nm, FWHM 4.1 nm), Trap1* (blue solid curve, centered at 666.6 nm, FWHM 6.0 nm), and Trap2* (yellow solid curve, centered at 671.1 nm, FWHM 6.2 nm). The black dashed line is the sum of the three Gaussian distributions.
Figure S8. 1D SAS of the compartments in the visible (right) and mid-IR (left). The amplitudes are normalized with respect to the final charge separated state RP2 ($P_{D1}^{+}\text{Pheo}_{D1}^{-}$).
Figure S9 Result from simultaneously fitting broadband-2DES and 2DEV data to a 5-compartment model: (A) 5-compartment model with 2 trap states, (B) 2D SAS of each compartment.

![Diagram showing 2D SAS of each compartment](image)

Figure S10. 1D SAS of the compartments in the visible (right) and mid-IR (left). The amplitudes are normalized with respect to the final charge separated state RP2 (P_D1^+PheoD1^-).

S6. Reduction of 2D SAS to 1D SAS

We fit two Gaussian distributions to the linear absorption of the sample (Figure 5, main text). The variables in the fits are the amplitudes, center wavelength, and variances of the 2 Gaussian distributions. The values of the means are initially set based on the excitation frequency dependence of the 2D SAS, and only allowed to vary very slightly (± 2 nm). We then use these Gaussian distributions as window along the excitation axis on the 2D SAS to obtain the 1D SAS of each species. That can be achieved by doing a simple matrix multiplication:

\[
\text{SAS}_{1D}(\omega_3) = \text{SAS}_{2D}(\omega_3, \omega_1) \times \text{Gaussian}(\omega_1)
\]

To further validate our model, we reconstruct the 2D spectra using the 1D SAS. First, we reconstruct the 2D SAS from the 1D SAS by using the following equation:
$$\text{SAS}_{2D}(\omega_3, \omega_1) = \text{SAS}_{1D}(\omega_3) \otimes \text{Gaussian}(\omega_1)$$

Where $\otimes$ is the outer product. We then use the reconstructed 2D-SAS with their appropriate time-dependent concentration to reconstruct the 2D data. The 2D SAS of the final charge separated state RP2 is taken directly from the output of the GTA (Figure 4 in the main text) instead of reconstructed. The result of this reconstruction of 2D maps is shown in Figure S6. The reconstructed 2D spectra capture the overall dynamics of the raw 2D spectra. An analogous procedure can be readily employed should the target model include more than two exciton states by including additional Gaussians in the linear absorption fit as has been done in Figure S6 where three exciton states were proposed in the target model.
Figure S11. Reconstructed 2D spectra compared with raw 2D spectra. Comparisons for waiting times $t_2 = 0.2, 5, 20.3,$ and 392.6 ps.
S7. Comparison of 2DEV Data with the Visible Pump Mid-IR Probe Studies of Groot et al. (15)

We compare our 2DEV data to the pump mid-IR probe experiments of Groot et al. (15) in Supplementary Fig. S12. For the comparison, we first reduce our 2DEV data to approximate their experiments by integrating the 2DEV data at the appropriate center wavelength and bandwidth along the excitation axis. To mimic their experiments at 669 nm excitation we integrated over a 9 nm window, while we used an 8 nm window at 681 nm excitation, consistent with their experimental conditions. Since they published evolution associated difference spectra instead of raw data, we applied the same global fits to our data using the time constants reported in their work (15). For 669 nm excitation we fit our data with time constants of 0.2, 3, and 32 ps, 2 ns, and a non-decaying component. For 681-nm excitation the time constants are 0.6, 21, and 500 ps and a non-decaying component. Results from our data are generally consistent with that of Groot et al. with some minor differences.
Figure S12. EADS upon 669-nm (black lines) and 681-nm excitation (red lines) from reference (15) (top) and our data (bottom, smaller plots). The lifetimes of the spectra are 3 and 0.6 ps (a); 32 and 21 ps (b), 2 ns and 500 ps (c); infinite and infinite (d), for the 669- and 681-nm data sets, respectively. In d, the blue line is P01′Pheo01′ spectrum from steady-state Fourier transform IR experiments (34, 43). Note that the sign convention here is opposite to our 2DEV spectra, i.e., GSB is negative.
S8. Effect of $t_1$ Scan Limit on 2DEV Spectra and comparison with Yoneda et al. (27)

2DEV signals are weak due to the small transition dipole strength of the mid-IR transitions. Therefore scanning unnecessarily long $t_1$ delays after the signal has already decayed will add noise to the spectra. However, an unreasonably short maximum $t_1$ delay will cause premature truncation of the signal, broadening the spectrum along the excitation frequency axis and sacrificing the distinct advantage of 2D spectroscopy to resolve the excitation-frequency dependence. Ideally, the $t_1$ delay should be chosen to allow resolution dictated by the sample itself (32). Supplementary Figure S13a and b show 2DEV spectra taken with maximum $t_1$ delays of 100 fs and 210 fs respectively, clearly demonstrating that excitation frequency dependent features are considerably better resolved with the longer $t_1$ delay, especially at early $t_2$ times.

Our 2DEV data show features that are distinct from those reported by Yoneda et al. (27). Several differences between our experiments likely contribute, including the increased spectral resolution of our excitation frequency axis (resolution of ~159 cm$^{-1}$ compared to ~333 cm$^{-1}$ of Yoneda et al. (27)). Our experiment used 500 Hz excitation compared to 1 kHz to reduce contributions from long-lived triplet states. Other differences between our 2DEV spectra and those of Yoneda et al. (27) are likely a result of variation in our sample preparation of the isolated D1D2-cyt b 559 complex. Since the original isolation method of Nanba and Satoh (33), considerable work has been devoted to preserving the RC pigment content and integrity of the D1D2-cyt b 559 complex. Our isolation approach is based on the work of Berthold et al. (56) and van Leeuwen et al. (57). We report a ratio of 1.2 of the linear absorption recorded at 417:435 nm to verify removal of the CP43 and CP47 antenna complexes, following the work of Eijckelhoff et al. (58). Yoneda et al. (27) report a ratio of 1.16. As discussed in Supplementary Information S7, our 2DEV data is consistent with the pump-mid IR probe experiments of Groot et al.(15) who used the same sample preparation protocol.
Figure S13. Effect of $t_1$ scan limit on 2DEV data. (A) 2DEV spectra obtained when limit the coherent time $t_1$ is limited to 100 fs; (B) 2DEV spectra obtained when utilizing the full $t_1$ range of 210 fs. A half-Tukey window was applied to the time-domain data, then the $t_1$ axis was zero-padded to $2^{10}$ points before Fourier transformation in both cases.
S9. Representative fits to the global target model

**Figure S14. Representative fits to the global target model.** Blue excitation (665 nm, panels A and B) and red excitation (680 nm, panels C and D) at various probe wavelengths. Probe wavelengths in the visible are shown in panels A and C, at 455 nm (anion band), 545 nm (Pheo Qx) and 604 nm (Chl ESA). Probe wavelengths in the mid-IR are shown in panels B and D at 1670 cm$^{-1}$ (ChlD1 GSB), at 1704 cm$^{-1}$ (P GSB), at 1721 cm$^{-1}$ (Pheo GSB).
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