Supporting Information

Proteoliposomes as energy transferring nanomaterials: enhancing the spectral range of light-harvesting proteins using lipid-linked chromophores

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Materials and Methods

LHCII protein purification

Trimeric LHCII complexes from spinach were biochemically purified as described previously (Adams et al., 2018). Briefly, spinach leaves (purchased from a local supermarket) were macerated in ice-cold buffer A (300 mM sucrose, 5 mM EDTA, 50 mM HEPES, pH 7.5), the liquid filtered through muslin cloth and chloroplasts collected by centrifugation, resuspended in buffer B (5 mM EDTA, 10 mM Tricine pH 7.4) and osmotically lyzed by adding an equal volume of buffer C (400 mM sucrose, 5 mM EDTA, 10 mM Tricine, pH 7.4). Isolated thylakoid membranes were adjusted to 0.5 mg Chl/mL and then solubilized with 0.5% (w/v) detergent *n*-dodecyl α-*D*-maltoside (α-DDM, Generon) in 20 mM HEPES buffer for 1 hr on ice. Thylakoid membrane proteins were then separated via ultracentrifugation on sucrose density gradients (8-13% w/w sucrose at 100,000 x g, 36 hr, 4 °C). The LHCII trimer band was then collected, concentrated using 30 kDa Amicon Ultra centrifugal filters (Merck Millipore, UK), and further purified using high-resolution size exclusion chromatography in 150 mM NaCl, 0.03% α-DDM, 20 mM HEPES (pH 7.5) using a 16/600 Superdex 200 prep grade column on an AKTA Prime FPLC system (GE Healthcare Life Sciences, PA, USA). After pooling the appropriate eluted fractions and concentrating using Amicon centrifugal filters once more, LHCII trimers were at a concentration of approx. 100 nM in buffer of 20 mM HEPES (pH 7.5) and estimated 0.3% α-DDM. SDS-PAGE and Native-PAGE was used to confirm protein purity and oligomerisation state, as previously. Purified LHCII trimers were characterised by absorption spectroscopy at each step and before every proteoliposome preparation. LHCII trimer molar concentration is estimated as [ChI mM concentration] x 42, where "chlorophyll mM concentration" is determined from absorbance measurements after methanol/acetone pigment extraction as in (Porra et al., 1989). This leads to an approximate conversion factor from optical density (at 675nm) to LHCII trimer mM concentration has a numerical value of 5.417 x 10⁻⁷ abs/mM.

Reconstitution of LHCII into proteoliposomes

Plant thylakoid lipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulphoquinovosyldiacylglycerol (SQDG) and L-α-phosphatidylglycerol (Soy PG), and the synthetic lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids as lyophilized solids. The fluorescently-tagged lipid Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE) was purchased as a solid

from Life Technologies (Invitrogen). Lipid mixtures were prepared by solubilising dry lipids with a 2:1 chloroform: methanol and mixing to obtain the desired ratios and final mass and subsequently dried under dry nitrogen gas flow for 40 minutes and then placed in vacuum desiccator for 10-16 hr to remove any residual trances of chloroform (room temperature, in the dark). Lipid aliquots were then either used immediately or stored under argon gas at -80 °C until use. Our standard thylakoid lipid mixture used for all samples contained 35% MGDG, 20% DGDG, 12% SQDG, 8% Soy PG and 25% DOPC (% wt/wt), adapted from Grab and co-authors (Grab 2016). TR-DHPE was added as required to aliquots containing the standard lipid mixture before drying. Glass vials were used throughout when working with lipids in organic solvents.

Aliquots of dry thylakoid lipid mixture (as prepared above) were solubilised with 0.5% α -DDM, 20 mM HEPES (pH 7.5) at room temperature for approx. 12-16 hr with agitation via a pinwheel rotator to generate a mixed micellar lipid-DDM solution (approx. 9:1 molar ratio of detergent-to-lipid). The starting protein-lipid-detergent suspension was prepared in plastic microfuge tubes by mixing calculated volumes of the following: lipid-DDM solution, aqueous buffers, and purified LHCII trimers to a final concentration of: 1 mM total lipid, 0.2% α-DDM, 20 mM HEPES (pH 7.5), 40 mM NaCl and the desired LHCII concentration. The desired LHCII concentration is achieved by calculating the volume of isolated LHCII trimers required to reach a defined lipid-to-protein (mol/mol) ratio for each sample (with molar concentration of lipids calculated from known masses and molecular weights and LHCII protein concentration determined from absorption as stated above). The lipid-DDM-protein mixture was then incubated with Bio-Beads (Bio-Rad) to gradually remove the detergent and allow proteoliposome formation via self-assembly, as follows: four incubation cycles with increasing quantities of fresh Bio-Beads (8 mg/mL, 20 mg/mL and 40 mg/mL and 100 mg/mL) for 90 min, 90 min, 90 min, and ~16 hr, respectively. Proteoliposomes were prepared in sets of 5 to 7, stored in the dark at 4 °C when not in use, and diluted samples from these immediately were characterised by ensemble spectroscopies (within 16 hr) and by microscopies (within 24-72 hr).

Ensemble absorption and fluorescence spectroscopy (cuvette-based)

Before spectroscopy measurements, proteoliposome samples were diluted in a buffer of 40 mM NaCl, 20 mM HEPES (pH 7.5), to obtain a large enough volume for use in a 10 x10 mm quartz cuvette (3 to 3.5mL) at a low enough absorbance of ≤0.1 at 675 nm to avoid inner filter effects (Yuan and Walt, 1957). Cuvette-based absorption spectroscopy was performed using an Agilent Technologies Cary 5000 UV-Vis-NIR absorption spectrophotometer equipped with an "integrating sphere" (also called a Diffuse Reflectance Accessory, Agilent) to remove any minor scattering effects.

Cuvette-based steady-state and time-resolved fluorescence spectroscopy was performed using an Edinburgh Instruments FLS980 fluorescence spectrophotometer equipped with dual excitation monochromators and dual emission monochromators. Samples were maintained at 20 °C and gently stirred during all measurements using a thermoelectrically-cooled cuvette-holder with magnetic stirring capabilities (Quantum Northwest TC 1 Temperature Controller). For steady-state emission spectra, a 450W Xenon arc lamp was used for excitation and a red-sensitive-PMT for detection (Hamamatsu R928 PMT). Emission scans with selective excitation of LHCII were acquired with excitation at 473 nm collecting emission from 500-800 nm (2 nm and 1 nm bandwidth excitation and emission slits, respectively). Emission scans with selective excitation of Texas Red were acquired with excitation at 540 nm collecting emission from 550-800 nm (1 nm bandwidth for both excitation and emission slits). Data acquisition parameters were 0.5 nm steps, integrating 0.1 s/ step and five scans averaged for all. LHCII fluorescence excitation range of 380-

380 nm (1 nm and 2 nm bandwidth excitation and emission slits, respectively. Data acquisition parameters were 1 nm steps, integrating 0.1 s/ step and a single scan for all samples.

Fluorescence lifetime measurements used either (i) a 473 nm pulsed diode laser (EPL-475, Edinburgh Instruments) for selective excitation of LHCII, collecting emission at 681 nm with 10 nm bandwidth emission slits, or (ii) a 566 nm pulsed LED (EPLED-560, Edinburgh Instruments) for selective excitation of Texas Red, collecting emission at 610 nm with 10 nm bandwidth emission slits. A laser/LED repetition rate of 0.5 MHz was always used. A dedicated high-speed red-sensitive PMT was used for detection (Hamamatsu H10720-20 PMT). A built-in neutral density (ND) filter wheel was applied to the pulsed laser for LHCII lifetime measurements to set excitation power as desired, an average power of approximately 1.5µW for LHCII (pulse energy of 3.0pJ) and 90 nW for Texas Red (pulse energy of 0.18fJ). Control measurements for excitation power versus fluorescence lifetime showed that singlet-singlet annihilation effects are likely to be avoided using these settings (see **supplementary Figure S13**). Decay curves from the Edinburgh FLS980 system were fitting using the manufacturer's supplied software. All ensemble spectroscopy data was further analysed in Origin Pro (v.9) graphing software.

Our initial time-resolved measurements of Texas Red on the most quenched samples detected very low signal in when using the Edinburgh FLS980 spectrometer. Therefore, additional decay curves were acquired for Texas Red using an alternative system, the Horiba Quantamaster fluorescence spectrometer equipped with a higher power supercontinuum excitation laser. Here the acquisition parameters were: 0.5 MHz laser repetition rate, excitation set to 540 nm (1 nm slit) and emission collected at 610 nm (5 nm slit). The Quantamaster system produced data with a higher signal-to-noise and improved resolution and we confirmed that the trends were consistent between the Edinburgh and Quantamaster systems by measuring the majority of samples using <u>both</u> instruments, discussed in the **supplementary Figure S14**.

Epifluorescence microscopy analysis and photo-bleaching experiments

Substrates were glass coverslips 50 x 25 mm (#1.5 thickness), prepared by piranha cleaning for 40 min and used within 48 hr. Hydrophobic ultrathin adhesive imaging spacers (0.12-mm depth, 9-mm diameter) were then attached to substrates to create small wells to confine a droplet of buffer (Electron Microscopy Sciences, Hatfield, PA), for an open sample setup to allow multiple buffer exchanges. Proteoliposome samples were diluted 1/50 in a buffer of 10 mM MES (pH 6.0) 150 mM NaCl buffer and incubated with clean glass for 30 minutes in the dark (note, a lower pH buffer was used here during membrane adsorption in an attempt to promote interactions with the highly electronegative glass). Samples were then washed with seven changes of 10 mM MES (pH 6.0) 150 mM NaCl buffer in order to remove any loosely associated membranes, before being washed a final time with three changes of 20 mM HEPES (pH 7.5) 20mM NaCl to keep imaging conditions consistent with spectroscopy, returning the pH to our standard 7.5.

Epifluorescence microscopy was performed using a Nikon E600 microscope equipped with a Andor Zyla 4.2 sCMOS detector and appropriate filter cubes (LHCII cube: excitation 450-475 nm, dichroic 500 nm, emission 650-800 nm; Texas Red cube: excitation 540-580 nm, dichroic 595, emission 600-660 nm). Images were taken with a using an x40 air objective (N.A. 0.6), 1s exposure and appropriate ND filters inserted to maintain the maximum number of counts at a level for good detector signal-to-noise and linearity (10-75% of detector saturation). Two-channel imaging (Texas Red + LHCII) of a field of view was performed sequentially by switching between cubes and ND filters as appropriate.

For deliberate photo-bleaching of LHCII, an aperture was inserted to expose an approx. 30 µm diameter region of the sample for a continuously period of 120 s through the LHCII filter

cube at full power (i.e. no ND filters). During the bleaching, the aperture diameter was increased incrementally by approx. 10 μ m every 30 s to bleach multiple regions by different amounts (i.e., regions 1 to 4 in main paper **Figure 3B**). Subsequently, full-field images were acquired sequentially with LHCII and Texas Red filter cubes, to visualize the effect of photobleaching.

Fluorescence Lifetime Imaging Microscopy (FLIM) and single-proteoliposome analysis

FLIM measurements were performed on a Microtime 200 time-resolved confocal fluorescence microscope (PicoQuant GmbH). This system uses an Olympus IX73 inverted optical microscope as a sample holder with light passing into and exiting various filter units for laser scanning, emission detection and timing electronics. Excitation lasers were reflected toward the sample by a 488/561 (dual band) dichroic mirror. Prior to the dichroic mirror, a small portion of the beam is deflected towards a photodiode that provides an average power readout for the excitation source before the objective lens. The excitation beam is focused through a 100X oil objective (N.A. 1.4) (UPlanSApo, Olympus). For calculating excitation power, we estimate that there is ~85% transmission efficiency at the excitation wavelengths of 485 nm and 561nm.

The excitation sources, an LDH 485 nm and an LDH 561 nm laser heads (PicoQuant), were driven in Pulsed Interleaved Excitation (PIE) mode by a PDL 828 Sepia II burst generator module (PicoQuant) at a pulse rate of 10 MHz per laser (i.e. 20 MHz overall). The pulse width for the LDH 485 nm and LDH 561 nm lasers were 90 ps and 70 ps respectively. The voltage supplied to each laser was set at the minimum required to allow lasing and kept constant for all measurements (this maintains the shortest possible pulse FWHM and provides the best temporal resolution). Laser power was set to the desired output using a combination of neutral density filters and a micro blade cut-off that partially blocks the laser beam. An excitation fluence of 0.026 mJ/cm² was used in order to limit any damage to the samples, this value was selected after trailing a series of excitation powers on control samples as shown in **Figure S15**).

Emission from the sample was passed through the same objective lens and dichroic mirror, towards a detection arm of the optical path. LHCII emission and Texas Red emission were separated by a 635LP filter in a beamsplitter tower that directed the emission towards two detectors. Emission wavelengths shorter than 635 nm, were directed though a 620/60 emission filter before being detected by a hybrid Photomultiplier Tube (PMT) detector (PicoQuant). Emission wavelengths longer than 635 nm, were directed though a 690/70 emission filter before being detected by a Single Photon Avalanche Diode (SPAD) detector (PicoQuant). With this arrangement of detectors, the PMT was optimised to detect Texas Red emission, and SPAD detected emission from LHCII. Timing electronics were a time-correlated single photon counting (TCSPC) TimeHarp 260 module (PicoQuant).

The PIE beam was directed across the sample using a "FLIMBee" mirror-based galvanometer scanner (PicoQuant). FLIM measurements were generally taken for 256 x 256 pixels across a 25 x 25 μ m field of view. The dwell time for each pixel was set to 25 μ s, such that an entire frame was captured in 1.64 s, and 500 frames were accumulated for each field of view. Collecting the data in this manner, allowed for the quantification of fluorophore bleaching, and the separation of frames into subgroups such that the lifetimes can be analysed at different times during the measurement. Analysis of fluorescence decay curves were performed using inbuilt fitting functions in the SymPhoTime software (PicoQuant GmbH) to fit a bi-exponential decay that was reconvoluted with measured Instrument Response Functions (IRFs). A good fit was confirmed when residuals were minimized, and Chi² was <1.1.

Supplementary results (explanations and figures)

Protein biochemistry: obtaining a high purity sample of trimeric LHCII

Denaturing and native gel electrophoresis was performed on all LHCII preparations. Representative gels are shown below.



Figure S1 SDS-PAGE gel with either Coomassie (left panel) or SYPRO-RUBY stain (middle panel). Gel lanes show, in order: protein standard, LHCII "before FPLC" (containing impurities, i.e., before size exclusion chromatography), purified trimeric LHCII (i.e., trimer fractions after size exclusion chromatography), purified monomeric LHCII (monomer fractions after size exclusion chromatography). Native-PAGE gel (right panel) was ran at 4°C and then stained with Coomassie. Native gel lanes show, in order: LHCII "before FPLC", purified trimeric LHCII, and purified monomeric LHCII, as above.

Analytical Ficoll gradient analysis: evidence for lipid and protein colocalisation

Analytical ultracentrifugation of selected proteoliposomes and control samples on Ficoll density gradients was performed to observe the differential sedimentation of any sub-populations. A standard quantity of each samples (150 μ L undiluted sample) was loaded onto 5-20% continuous Ficoll gradients in SW55 tubes (in 20 mM HEPES, 40 mM NaCl buffer). Note, Ficoll has a similar density to sucrose but aqueous solutions of Ficoll have a much lower osmolality, making it a gentler medium for isolation of vesicles whilst avoiuding osmotic lysis. Gradients were centrifuged at 226,000 xg for 10 hr at 4 °C (with no brake) and then immediately photographed.



Figure S2 Analytical Ficoll gradients between 5-20% (Left) Representative proteoliposomes sample (0.7 μ M LHCII, 8 μ M Texas Red, 1 mM total lipids) showing a clear single population and no aggregates of LHCII; (**Right**) LHCII in detergent at a 0.7 μ M concentration showing clear sedimentation of LHCII not reconstituted into proteoliposomes.

Fluorescence emission spectra of LHCII within proteoliposomes reveals only minimal peak shifts and broadening

Shown below are fluorescence emission spectra from proteoliposome Series 2, all normalized to an emission of 1.0 at the peak maximum ~681 nm. Shifts of wavelength (in the x-axis) are no more than 1 nm for any sample. Broadening of the peaks increases with decreasing LHCII concentration to a maximum of 19% of the peak area compared to isolated LHCII, suggest that up to 19% of chlorophylls may have an altered environment. Whilst not ideal this is entirely in line with multiple other proteoliposome studies (Adams et al., 2018; Natali et al., 2016; Pandit et al., 2011) where LHCII appears to be destabilized when the protein-to-lipid ratio is very low. We speculate that LHCII-LHCII interactions may be stabilizing. This is outside of the scope of the current work. For the current study, whilst subtle changes to spectra are detected it is important to note that the vast majority of LHCII appears undamaged and that we can still calculate accurate FRET and enhancement of LHCII emission.



Figure S3 Normalised steady-state emission spectra of sample Series 2 with selective LHCII excitation at 473nm. Note that the spectra are all relatively similar with minimal emission peak shift and broadening with different LHCII concentrations. All measurements were taken in a buffer of 20mM HEPES (pH 7.5), 40mM NaCI.

Fluorescence excitation and linear absorption analysis

Shown below are LHCII fluoresce excitation spectra for proteoliposome samples with constant Texas Red concentration **supplementary Figure S4A** and constant LHCII concentration **supplementary Figure S4B**. LHCII fluoresce excitation spectra scans over a range of excitation wavelengths while collecting selective LHCII emission at 686nm, this value was selected to allow the excitation peak maximum of LHCII to be collected (~675nm). Linear absorption is calculated by 1 – the sample transmission. Sample transmission can be obtained from the optical density of the sample using the equation: $T = 10^{-0D}$ (T = Transmission) (OD = optical density). Overlapping excitation spectra and linear absorption (normalised to maximum fluorescence excitation and absorption -675nm for LHCII) suggests a well-connected system where energy absorbed at any wavelength is transferred to the same final fluorescent component. The close overlap of excitation and linear absorption in an LHCII only sample (**supplementary Figure S4B** *solid and dashed black lines respectively*) is indicative of intact and fluorescently active LHCII.

Comparison between acceptor fluoresce excitation and linear absorption over the region of donor absorption (with acceptor only excitation and linear absorption subtracted) can be used to estimate donor to acceptor energy transfer efficiency using the equation:

 $ETE = 1 - \frac{(1-T) - ex}{2}$ (1-T) where ((1-T) = linear absorption) (ex – fluorescence excitation). If the excitation spectra is lower than the linear absorption there is less than 100% energy transfer from donor to acceptor at that wavelength, the ratio of excitation to linear absorption over the donor region is therefore directly related to ETE. As LHCII to TR ratio increases (supplementary Figure S4A) these is a significant increase in ratio of TR excitation to linear absorption, this is consistent with steady state fluorescence emission and time resolved fluorescence data which also shows increasing FRET efficiency with LHCII concentration. Samples with a constant LHCII concentration (supplementary Figure S4B) do not show significant variations in TR excitation to linear absorption ratio suggesting consistent TR to LHCII ETE. Fluorescence explation and linear absorption were not used as a primary method of calculating ETE as the de-composition of LHCII and TR fluorescence emission spectra is more accurate than the de-composition of fluorescence excitation and linear absorption for samples with a high LHCII:TR ratio. However, the calculated ETE values for samples with low LCHII:TR ratios are consistent with what is reported by TR fluorescence emission and lifetime analysis. Full analysis of TR excitation to linear absorption ratio and ETE is reported in Supplementary Table S4.



Figure S4 Representative LHCII fluorescence excitation (ex) and linear absorption (1-T) spectra for proteoliposomes (individual sample concentrations in legend, all at 1mM total lipid). (A) Constant Texas Red concentration – sample series 2 (B) Constant LHCII concentration – sample series 1. LHCII fluoresce excitation measured at 686nm emission and normalised to 1.0 at chl a absorption peak (~675nm). Linear absorption calculated from 1 – sample transmission as described in above text and normalised to 1.0 at chl a absorption peak (~675nm).

Quantification of the LHCII and Texas Red concentration in proteoliposomes by "spectral decomposition" analysis of the absorption spectra

All analysis, below, was performed using Origin Pro (v.9) graphing software. All absorption spectra were "baselined" to give an absorbance of zero at 800 nm as expected in these samples, to remove any differences in noise (e.g. detectors, slight differences in cuvettes, etc.). All absorption spectra were then corrected for dilution by multiplying by dilution factor.

LHCII content was estimated before any spectral decomposition because there is no significant absorption from Texas Red >630 nm. LHCII content assessed from the integrated area from 635-700 nm, with LHCII concentration then calculated as:

AREA / 67.3x10⁶/M/cm (value from absorption of a known concentration of LHCII in detergent).

Area was used to ensure that any slight peak broadening did not lead to a loss of relative absorption.

Before quantification of concentration of Texas Red, the component peaks within absorption spectra were decomposed because LHCII overlaps throughout the TR absorption range. To obtain the TR-only component, a representative LHCII absorption spectrum (originally collected from LHCII in detergent or in liposomes) was normalised to the test sample's absorbance at 675 nm (LHCII ChI *a* Q_y peak) and then subtracted, to give a result as in **supplementary Figure S5**, below.



Figure S5 Example of the de-composition of absorption spectra for a representative proteoliposome sample (0.52μ M LHCII, 5.0μ M TR, 1mM total lipids). Absorption spectra from a LHCII-only control sample (Red) is fitted to the 675nm absorption peak of the raw absorption data (Black) and subtracted. The resulting spectra is the absorption from only the Texas Red component in each sample (Blue).

Texas Red content was assessed on these decomposed spectra from the peak height at 591nm with TR-DHPE concentration then calculated as:

PEAK HEIGHT / 85,000/M/cm¹ (value is the extinction coefficient of TR-DHPE in a 1 cm pathlength cuvette provide by the supplier in agreement with other published works (Titus, Haugland et al. 1982)

Time resolved fluorescence data of control samples: isolated non-quenched Texas Red and LHCII

Fluorescence decay curves of isolated (not quenched) Texas Red (**Figure S6A**) and LHCII (**Figure S6B**) are fitted to bi-exponential, leading to a mean amplitude-weighted lifetime of 4.4 ns of 3.9 ns, respectively. These values were used to calculate the relative fluorescence lifetime quenching of components when reconstituted into proteoliposomes, where quenching occurs due to energy transfer in the case of Texas Red and self-quenching in the case of LHCII. The instrument response function (IRF) measures the scattering of laser excitation from non-fluorescent control samples to determine the fastest possible response of the detectors (the IRF is used for re-convolution fitting of decay curve data).



Figure S6 Time resolved fluorescence data from (A) solution of liposomes comprised of 2.1 μ M TR-DHPE and 1.0 mM total lipids in a buffer 10 mM HEPES, pH 7.5 (absorbance ~0.1 at 561 nm). Taken using QuantaMaster fluorescence spectrometer with supercontinuum pulsed laser at a 0.5 MHz repetition rate, excitation set to 540 nm (1 nm slit) and 610 nm emission (5 nm slit). Instrument response function (IRF) taken using colloidal silica beads. (B) LHCII trimers in a buffer of 0.03% w/v α -DDM, 10 mM HEPES, pH 7.5 (absorbance ~0.1 at 675 nm). Taken using Edinburgh Instruments FLS980 fluorescence spectrometer with excitation from a 475 nm pulsed laser at a 0.5 MHz repetition rate emission collected at 610 nm emission (10 nm slits). Instrument response function (IRF) taken using colloidal silica beads.

Disruption of proteoliposomes with detergent as a secondary proof of FRET

Shown below is the fluorescence emission spectra of a proteoliposome sample under selective Texas Red excitation (540 nm) before and after solubilisation in detergent. The detergent (DDM) is known to disrupt lipid bilayers and will, after several minutes of mixing, isolate the majority of lipids and LHCII trimers into detergent micelles. The isolation of lipids and proteins is expected to limit any TR-to-LHCII energy transfer, as they will be too spatially distant for efficient FRET to occur and also limit any self-quenching of LHCII due to protein-protein interactions. As expected, the detergent treatment did indeed appear to greatly diminish TR-LHCII and LHCII-LHCII interactions, resulting in the regeneration of fluorescence of both components, as shown in **Figure S7**. Texas Red fluorecence emission intensity of detergent-solubilized proteoliposomes increased from 3% to 87%, as compared to a control sample of TR-DHPE prepared from the start in solubilizing detergent. This regeneration confirms that both LHCII and Texas Red quenching occur due to the membrane archetecure and nanoscale interactions.



Figure S7 Fluroescence emission data from 2.84 μ M LHCII, 8.0 μ M Texas Red, 1mM total lipid proteoliposomes diluted x60 in 20 mM HEPES, 40 mM NaCl. Post DDM spectra taken after 0.1% w/w α -DDM added to cuvette and stirred for 5 minutes in order to solubilise lipids and proteins. Red and green arrows highlight the fluorescnece emission recovery ("de-quenching") of TR and LHCII respectively.

Quantification of the "Relative fluorescence" of LHCII and Texas Red by "spectral decomposition" analysis of fluorescence spectra

All analysis, below, was performed using Origin Pro (v.9) graphing software. Before guantification of relative fluorescence intensity data, the LHCII and Texas Red component peaks within fluorescence emission spectra were "decomposed", as described below. To obtain the LHCII-only component, a representative Texas Red emission spectrum (originally collected from Texas Red only liposomes) was normalised to the measured sample's emission at 610nm (Texas Red emission peak) and then subtracted (result as in supplementary Figure S8A-C). To obtain the Texas Red-only component, a representative LHCII emission spectrum was normalised to roughly 80% of the measured sample's emission at 681nm (result as in supplementary Figure S8D-F). This was % value was found empirically (via multiple iterations) to be the optimal value which produces a decomposed spectrum for TR where the value at 681nm is 17.7% of its peak maximum at 610nm, the expected value for pure isolated Texas Red. All emission spectra were then corrected for dilution by multiplying by dilution factor. It was critical that our values for emission intensity were accurate for all sample sets acquired over many weeks apart, therefore, we performed control measurements each day to check the absolute consistency of the FLS980 fluorescence spectrophotometer, relative to a known isolated LHCII in α-DDM sample. Values were always within 3% of previously acquired data. Positive residuals in the 650-700nm region of all decomposition attributed to the slight broadening of the LHCII emission peak at different concentrations in proteoliposomes as outlined in supplementary Figure S3.



Figure S8 Example of de-composition of emission spectra for a representative proteoliposome sample (0LHCII and TR concentrations stated in legend, all with 1 mM total lipids). (A-C) Removal of TR emission: a TR-only control emission spectrum (Red) is normalised to the 610 nm peak in spectra measured for TR+LHCII proteoliposome samples (Black) and subtracted, leaving only the LHCII emission peak (Blue) (D-F) Removal of LHCII emission: an LHCII-only control emission spectrum (Red) is normalised to 681 nm peak in spectra measured for TR+LHCII proteoliposome samples (Black) and subtracted, leaving only the TR emission peak (Blue), which is expected to have a magnitude at 681 nm that is 17.7% of the magnitude at 610 nm.

The "relative fluorescence intensity" per molecule of interest was defined as the value for emission intensity divided by the molar concentration (concentration calculated as in the ESI

section *Quantification of the LHCII and Texas Red concentration in proteoliposomes by "spectral decomposition" analysis of the absorption spectra*). Thus, "relative LHCII fluorescence intensity" (i.e., the LHCII emission per mole of LHCII) was calculated by integrating the fluorescence spectrum between 500-800 nm (after decomposition to remove any TR contribution) and dividing this value by LHCII concentration (in mM). And, "relative TR fluorescence emission peak height at 610 nm (after decomposition to remove any LHCII contribution) and dividing this value by TR-DHPE concentration (in mM).

Observation of significant "self-quenching" of LHCII within proteoliposomes due to its clustering in agreement with previous studies

The phenomenon of LHCII self-guenching which is known to occur in proteoliposomes was guantified. Taking this into account allows us to accurately determine the enhancement of LHCII fluorescence which occurs due to energy transfer from Texas Red, considering a specific known level of LHCII self-quenching. Steady-state and time-resolved fluorescence spectra were measured on samples of LHCII isolated in *α*-DDM detergent micelles and samples of LHCII reconstituted into proteoliposomes at a comparable concentration to the main text sample set "proteoliposome Series 1" (0.7 µM LHCII). Steady-state fluorescence emission data (Figure S9A) shows a 59% decrease in LHCII emission when reconstituted into proteoliposomes compared to isolated LHCII (from 2.51x10⁹ counts/mol to 1.06x10⁹ counts/mol). A similar decrease of 49% is observed for the mean fluorescence lifetime of LHCII (from 3.96 ns for isolated LHCII compared to 2.02 ns for LHCII in proteoliposomes) (Figure S9B). "Enhancement" of LHCII fluorescence emission due to increasing concentration of Texas Red was assessed on the sample set "proteoliposome Series 2" (i.e., main text Figure 2D, green plot) was calculated as relative to this expected value for LHCII emission in proteoliposomes containing 0.7 µM LHCII, in order to take guenching due to LHCII-LHCII interactions into account.



Figure S9 Quantification of the self-quenching of LHCII when it is reconstituted into proteoliposomes (0.7 μ M LHCII and 1 mM total lipids, in a buffer of 20 mM HEPES 40 mM NaCI) versus isolated LHCII in detergent micelles (buffer of 20 mM HEPES, 40 mM NaCI, 0.03% α -DDM) (A) Steady-state fluorescence emission spectra taken with selective LHCII excitation (473 nm, parameters as previously) (B) Time-resolved fluorescence emission data taken using Edinburgh instruments FLS980 fluorescence spectrometer with 475 nm pulsed laser at 0.5 MHz repetition rate.

Observation that "self-quenching" of Texas Red is minimal in our liposomes

Steady-state and time-resolved fluorescence emission spectra of Texas Red were measured for control samples omitting LHCII in order to determine a value for fully emissive TR (no quenching). These values for "relative TR fluorescence" were used as a baseline to calculate the TR quenching due to FRET in the energy transfer efficiency (ETE) equation. TR-DHPE was measured in two "isolated" forms of (i) in chloroform, and (ii) in DDM detergent micelles, and compared to (iii) TR-DHPE reconstituted into liposomes at a comparable concentration to "proteoliposome Series 2". Each sample of isolated TR-DHPE was measured at different dilutions and the final results averaged (n= 3). Three individually prepared samples of TR-DHPE in liposomes were measured and the results averaged. Slight differences in the magnitude of TR fluorescence emission 'per mol' and amplitude-weighted lifetime were measured (Figure S9A and S9B, respectively): in detergent 8.95x10¹¹ counts/mol and 5.09 ns, in chloroform 8.79x10¹¹ counts/mol and 4.14 ns, in liposomes 8.55 x10¹¹ counts/mol and 4.21 ns. Differences in fluorescence may be attributed to different local solvent environments, supported by the shifts to emission peaks in the x-direction. Self-quenching of TR-DHPE in liposomes cannot be ruled out, but is not considered further in this study. These values for steady-state and time-resolved fluorescence of Texas Red in liposomes were used as the non-quenched value for energy transfer efficiency calculations as it is most representative of the local environment of Texas Red in samples, i.e., $^{F_{D}}$ and $^{\tau_{D}}$ in eqn. 1 and 2 for main text Figure 2D.



Figure S10 Quantification of TR-DHPE fluorescence when in chloroform, detergent micelles and incorporated into liposomes, all samples omitting LHCII. Chloroform used was analytical grade and 99.9% pure, detergent samples used a buffer of 20 mM HEPES 40 mM NaCl 0.1% α -DDM, all liposomes samples were at a total lipid concentration of 1mM and TR-DHPE between 10-15 μ M in a buffer of 20 mM HEPES 40 mM. (A) Steady-state "relative fluorescence emission" of Texas Red 'per mol' in different solvent conditions (each spectra is an average of three samples). Acquired with selective Texas Red excitation at 540 nm, all other parameters as described previously. (B) Time-resolved fluorescence emission of Texas Red in different solvent conditions. Representative decay curves from one sample per condition. Taken using QuantaMaster fluorescence spectrometer with supercontinuum pulsed laser at 0.5 MHz repetition rate with excitation set to 540 nm, all other parameters as described previously.

Analysis of particle widths

An analysis of the full-width at half-maximum (FWHM) was performed using ImageJ by manually drawing intensity profiles across the width of single proteoliposomes. The width at half of the maximum was measured from these intensity plots and tabulated along with the maximum intensity. This results below show a mean FWHM in the LHCII and TR channels of 310±30 nm and 305±40 nm (for measuring n=20 representative proteoliposomes), with maximum intensity in each channel ranging from 18-250 (mean= 79) in the LHCII channel and ranging from 66-2610 (mean= 1010) in the TR channel. Therefore, we conclude that the majority of proteoliposomes in FLIM images are diffraction-limited spots, as our FLIM resolution is ~300nm, suggesting that their true diameter is <300 nm. Figure S11(A+B) show representative FLIM images of proteoliposomes on a surface displayed with different intensity ranges. Figure S11D displays a summary of individual proteoliposomes FWHM analysis.



Figure S11 (A)and (B) show display the same area FLIM images with the intensity ranges set to LHCII:2-25, TR:0-225 and LHCII:2-75, TR:0-600 respectively. (C) Shows labelled proteoliposomes used in single proteoliposomes FWHM analysis which is summarised in (D).

Gallery of FLIM image data: multiple fields of view from the tested three proteoliposomes (or liposome) samples

In **supplementary figure S12**, we present representative (25 X 25 μ m) fields of view for proteoliposomes containing a "high" concentration of LHCII (2.8 μ M LHCII, 12 μ M TR, 1 mM total lipids), a "low" concentration of LHCII (1.2 μ M LHCII, 12 μ M TR, 1 mM total lipids), and a control "TR-only" liposome sample containing TR-DHPE without any proteins (12 μ M TR, 1 mM total lipids).

In the TR-only liposome sample, there is very little signal in the LHCII channel. This minimal signal observed can be attributed to the spectral overlap of the emission filters and detectors (quantifed overlap of <1% of the TR signal). In the TR channel, the donor lifetime is usually high (3-4 ns) as expected for non-quenched TR, represented by a "more red" colouration in the false-colour mapping of the FLIM images.

In the proteoliposome samples, we observe a clear LHCII signal for both samples (99% confidence that these signals are above the TR overlap and detector noise thresholds), with a high degree of colocalisation with the corresponding TR signals (as discussed in the main text). The presence of LHCII in both of these samples was found to correspond to the quenching of the TR, which can be observed qualitatively as the preponderance of shorter lifetimes, appearing "bluer" in the FLIM false-colour mapping (as compared to TR-only liposomes).

The sample with high LHCII concentration on average appears to have shorter "bluer" TR lifetimes and lower TR intensities than the low LHCII concentration sample, when comparing across multiple fields of view. This provided an initial indication of the higher extent of donor quenching in the prescence of a higher acceptor concentration. However, the variety of proteoliposomes and corresponding lifetimes within one field of view presents the need for a comprehensive single-particle anaylsis.



Figure S12 Gallery of FLIM images.

Optimization of excitation power parameters for ensemble (cuvette-based) time-resolved fluorescence measurements: avoiding annihilation effects

A series of excitation powers were trialled on control samples of isolated LHCII, to analyze how fluorescence lifetime varies with laser excitation power and determine a "safe" low power which will not adversely damage the sample. For example, it is known that high excitation fluences can cause singlet-singlet annihilation events in LHCII aggregates which reduce the LHCII lifetime (Barzda et al., 2001). In **supplementary Figure S13**, we observed that LHCII mean fluorescence lifetime was relatively constant at 3.75 to 3.85 ns for low powers of $0.1 - 1 \mu$ W, but started to decrease at power higher than 1μ W. To avoid such possible annihilation effects (i.e., a decrease in the lifetime due to use of high laser power), all subsequent LHCII lifetimes were measured using laser power at under 5% of this threshold. For Texas Red lifetimes, see the following page.



Figure S13. Measured fluorescent lifetime of isolated LHCII (20 mM HEPES, pH 7.5, 0.03% α -DDM) taken using Edinburgh Instruments FLS980 fluorescence spectrometer at a range of laser powers. Left and right panels display (i) the full y-range of 0-4 ns or (ii) a zoomed in range, shown for clarity.

Agreement of time-resolved fluorescence data from our Edinburgh FL-980 fluorescence spectrometer and the improved Agilent Quantamaster system

Due to the low Texas Red signal observed in time-resolved measurements on the most quenched samples when using the Edinburgh FLS980 spectrometer, the data for TR decay curves (as in main text Figure 2C) was acquired using a Horiba Quantamaster fluorescence spectrometer equipped with a higher power excitation laser. Decay curves from the Quantamaster system were fitting using DecayFit Fluorescence Decay Analysis software v1.3. The Texas Red lifetime values obtained from both spectrometers were shown to be consistent between the Edinburgh and Quantamaster instruments (**supplementary Figure S14**) over the series of proteoliposome samples, however, the Quantamaster providing decay curves data with an improved higher signal to noise. Therefore, data from the Quantamaster system is used for main text Figure 2C and 2D. Furthermore, the agreement between the magnitude of TR fluorescence lifetime where laser power is very low (LED in our standard FL-980 system) or moderate (supercontinuum laser in the Quantamaster system) gives us confidence that are no significant annihilation effects with Texas Red. This is agreement with the finding that Texas Red lifetime does not change with laser fluence in FLIM (see next page).



Figure S14 Measured TR fluorescncent lifetimes from proteolipsome Series 1 (constant TR concentration, varying LHCII concentration) taken using Edinburgh Instruments FLS980 spectrometer (Black datapoints) and Horiba Quantamaser spectrometer (Red datapoints). The lifetimes recorded on both systems are very similar suggesting that no high excitation power annihilation is occuring when using the higher power Quantamaster system.

Optimization of excitation power parameters for FLIM measurements: avoiding annihilation effects

A series of excitation powers on the FLIM were trialled on control samples of either isolated LHCII (in detergent solution, with the excitation laser focussed at a plane within the droplet) and Texas Red at low concentration in liposomes (absorbed to the glass substrate as usual). Excitation fluence (power per unit area) at the sample surface were calculated to be from 0.002 mJ/cm² to 0.388 mJ/cm². As for the cuvette-based measurements (above), control measurements were performed for FLIM to analyze how fluorescence lifetime varies with laser excitation power and determine a "safe" low power which will not adversely damage the sample.

In **supplementary Figure S15**, we observed that LHCII mean lifetime was 3.5 to 4.0 ns for low fluences of 0.002 to 0.026 mJ/cm² but started to decrease at fluence higher than 0.026 mJ/cm² and was <1 ns at 0.388 mJ/cm². In contrast, TR mean lifetime was very similar at ~3 ns for all tested laser powers, so one assumes that fluence was well below the threshold for annihilation effects.

Therefore, in all FLIM acquisitions reported the current study, an excitation fluence of 0.026 mJ/cm² was used for both lasers, because it provided a balance between a high enough power to produce reasonable signal but low enough that neither LHCII nor TR undergo significant annihilation events.



Figure S15 Mean fluorescence lifetimes of isolated LHCII and TR liposomes measured using the FLIM system at a range of laser fluences. Green datapoints: emission from isolated LHCII in α -DDM solution with variation of the 488 nm laser power. Red datapoints: emission from Texas Red in liposomes with variation of the 561 nm laser power.

Tabulated data for ensemble spectroscopy (i.e., data for main text figures 1-2)

Table S1 below shows the numerical analysis of the ensemble spectroscopy data used to estimate the LHCII and TR content and then calculate "LHCII enhancement" (for proteoliposome Series 1) or the energy transfer efficiency (for proteoliposome Series 2). "Measured LHCII Absorbance" is recorded here as the integrated area between 635-800 nm measured using Origin software in absorption spectra and then used to calculate the concentration of LHCII (in μ M), as explained in **Figure S5.** The LHCII concentration is also calculated as a percentage of the total mass (lipid and protein total), as is common in proteoliposome studies (wt/wt%). "Measured TR Absorbance" is recorded here as the height of the Texas Red peak from absorption spectra and used to calculate its concentration (in μ M) in liposomes, as stated in **Figure S5.** The concentration of TR-DHPE is also given as a percentage relative to the total lipid (mole/mole), because this provides a good approximation of the effective density of the tagged lipid within the overall lipid bilayer, as commonly stated when studying supported lipid bilayers by fluorescence microscopy.

"Relative TR Emission" was calculated from steady-state fluorescence spectra analysed as in **Figure S8** and used to estimate the ETE (energy transfer efficiency) via equation 1 given in main text **Figure 2.** "Calculated TR $<\tau>$ " (i.e., mean fluorescence lifetime) is recorded from the analysis of time-resolved fluorescence spectra and used to estimate ETE (energy transfer efficiency) via equation 2 given in main text **Figure 2.** "Relative LHCII Emission" was calculated from steady-state fluorescence spectra analysed as in **Figure S8**, with selective excitation of Texas Red, in order to determine the enhancement of LHCII due to TR-to-LHCII energy transfer. Following on from this, "LHCII Emission Enhancement" was calculated as the percentage increase of the Relative LHCII Emission of a given sample compared to that recorded for the control sample of LHCII liposomes containing a 0.7 μ M LHCII (comparable LHCII concentration to the whole proteoliposomes series 1, as described in **Figure S9**).

Sample desc	ription	Dilution Factor	LHCII Absorbance	Calcul Conce	ated LHCII Intration	TR Absorbance	Calcul Conce	ated TR ntraion	Relative TR Emission per mol TR	Calculate d TR <t></t>	ETE (steady- state)	ETE (time resolved)	Relative - LHCII Emission per mol LHCII	LHCII Emission Enhancement
Initial concentration			Integranted area	1					Peak height				Integrated	
(calculated final			(635-800nm)			OD at 591nm			at 610nm				area 625-	Enhancemnt relative
concentrations	sin	Pre	dilution		(wt/wt%	dilution		(mol/mol	(540nm				800nm (540nm	to 0.52µM LHCII
subsiquent col	umns)	Measurment	corrected	(µM)	LHCII/total)	corrected	(µM)	lipid%)	Excitation)	(ns)	(%)	(%)	Excitation)	sample with no TR (%)
LHCII-0.70µM/	No TR	x15	34.91	0.52	2. 7.4	0.000	0.0	0.00		-	•	-	 1.03E+11 	
LHCII-0.70µM/	TR-1.4µM	x15	35.45	0.53	3 7.5	0.079	0.9	0.09	-	-	-	-	 1.33E+11 	25
LHCII-0.70µM/	TR-2.8µM	x15	38.35	0.57	/ 8.1	0.186	2.2	0.22	-	-	-	-	 1.57E+11 	50
LHCII-0.70µM/	TR-4.2µM	x15	36.97	0.55	5 7.8	0.313	3.7	0.37	-	-	-	-	 1.76E+11 	83
LHCII-0.70µM/	TR-5.6µM	x15	37.53	0.56	5 7.9	0.399	4.7	0.47	-	-	-	-	 1.92E+11 	106
LHCII-0.70µM/	TR-7.1µM	x15	36.34	0.54	1 7.7	0.514	6.0	0.60	-	-	-	-	 2.10E+11 	129
LHCII-0.70µM/	TR-8.5µM	x15	37.48	0.56	5 7.9	0.599	7.0	0.70	-	-	-	-	 2.34E+11 	153
LHCII-0.70µM/	TR-9.9µM	x15	38.50	0.57	/ 8.1	0.721	8.5	0.85	-	-	-	-	 2.47E+11 	181
LHCII-0.70µM/	TR-11.3µM	x15	37.98	0.56	6 8.0	0.807	9.5	0.95	-	-	-	-	 2.67E+11 	171
LHCII-0.70µM/	TR-12.7µM	x15	36.68	0.54	1 7.7	0.954	11.2	1.12	-	-	-	-	 2.93E+11 	221
LHCII-0.70µM/	TR-14.1µM	x15	40.99	0.61	8.6	1.047	12.3	1.23	-	-	-	-	 2.96E+11 	214
No LHCII/	TR-6.8µM	x15	i 0.00	0.00) 0.0	0.364	4.3	0.43	1.88E+12	4.43	BN/A	N/A		
LHCII-0.20µM/	TR-6.8µM	x15	i 10.93	0.16	5 2.4	0.710	8.4	0.84	6.68E+11	2.98	3 <mark>64</mark>	.5 32.	7.	
LHCII-0.35µM/	TR-6.8µM	x15	i 19.19	0.28	3 4.2	0.656	7.7	0.77	4.08E+11	2.2	1 78	.3 50.	1 ·	
LHCII-0.50µM/	TR-6.8µM	x15	i 23.10	0.34	l 5.0	0.453	5.3	0.53	4.45E+11	2.34	4 76	.3 47.	2.	
LHCII-0.60µM/	TR-6.8µM	x15	33.09	0.49	7.0	0.618	7.3	0.73	2.53E+11	1.65	5 86	.6 62.	8.	
LHCII-0.70µM/	TR-6.8µM	x15	35.50	0.53	5 7.5	0.424	5.0	0.50	2.72E+11	1.70) 85	.5 61.	6.	
LHCII-0.90µM/	TR-6.8µM	x15	50.94	0.76	5 10.4	0.429	5.0	0.50	2.00E+11	1.40) 89	.4 68.	4.	
LHCII-1.40µM/	TR-6.8µM	x30	79.74	1.18	3 15.4	0.672	7.9	0.79	8.76E+10	1.05	5 95	.3 76.	3.	
LHCII-2.75µM/	TR-6.8µM	x30	102.01	1.51	18.9	0.466	5.5	0.55	9.76E+10	1.02	2 94	.8 77.	0.	
LHCII-2.80µM/	TR-6.8µM	x45	166.12	2.47	27.5	0.448	5.3	0.53	6.52E+10	1.15	5 96	.5 74.	0.	
LHCII-3.50µM/	TR-6.8µM	x60	191.46	2.84	l 30.5	0.682	8.0	0.80	3.67E+10	0.72	2 98	.0 83.	7.	

Table S1 Display of data shown in main text Figures 1 and 2 for proteoliposome series 1 (upper half of table) and proteoliposome series 2 (lower half of table). Note that all samples were prepared with the same total lipid concentration of the thylakoid lipid mixture at 1 mM. Note that the concentrations given in this table (and referred to in the main text) represent the concentration of LHCII and TR-DHPE in samples prior to any dilution for measurement, and should be used for comparison to each other and to the total lipid concentration. Proteoliposome samples were diluted as stated in "dilution factor" column prior to measurements. Raw data from ensemble spectroscopy was then corrected for this dilution factor.

Single particle FLIM tabulated data (i.e., data for main text figure 4C)

In order to calculate the fluorescence lifetimes on a per-proteoliposome basis, manual analysis was performed by fitting fluorescence decay curves extracted from a region of interest, with each region defined to represent an individual proteoliposome. Only well-resolved proteoliposomes with sufficient signal to produce a good fit were selected (criteria of counts >500 and fit chi² <1.2). This selection process may produce inherent bias as we do not assess the weakly emitting particles, but nevertheless the trends are clear.

Table S2 below shows representative data from our single-proteoliposome analysis of FLIM images which is subsequently used to estimate TR and LHCII co-localization (comparison of their intensities) and also to produce the population distributions (frequency histograms) in main text Figure 4C. Individual particles were selected and numbered for consistency and to avoid repeat measurements. A perimeter was then drawn around each particle in order to select a defined number of pixels considered to make up that individual particle. The intensities of LHCII and Texas Red signal from each particle were measured and known detector "bleed-through" was subtracted. "Corrected signal" is given after subtraction for spectral overlap in the opposite channel (Raw LHCII signal contains significant bleedthrough of TR fluorescence, so this was quantified with control measurements and removed; in contrast there is minimal bleedthrough of LHCII fluorescence into the TR channel). The amplitude-weighted mean fluorescence lifetime of Texas Red ($T_{average}$) was then calculated from a bi-exponential decay function fitted to the produced fluorescence decay curves. A_1 , T_1 and A_2 , T_2 represent the amplitude (A) and lifetime (T) of the two exponential components. The chi² fit quality parameter is shown (chi² <1.2 represent a good fit). The lifetime of LHCII is not assessed in the current study.

Partic numb	le er	Pixels in partice	LHCII Raw Signal	LHCII corrected Signal	TR Raw Signal	TR Corrected Signal	TR A ₁	TR A ₂	TR T ₁	TR T ₂	TR T _{average}	TR χ²
#		#	Counts	Counts	Counts	Counts	Counts	Counts	ns	ns	ns	
	1	42	204	102	10572	10561	560	355	2.17	0.00	1.33	0.96
	2	32	88	47	3903	3897	61	20	0.76	2.70	1.22	0.96
	3	21	284	259	2364	2351	24	-87	2.30	0.03	0.84	1.01
	4	39	40	-10	4760	4756	894	243	2.44	0.00	1.90	0.97
	5	30	111	78	3047	3041	102	96	2.70	0.74	1.76	1.04
	6	20	115	100	1287	1281	180	17	0.93	3.00	1.20	0.71
	7	23	67	43	2255	2251	155	345	3.20	1.30	1.91	1.17
	8	34	40	15	2053	2049	74	6	1.40	4.70	1.60	0.76
	9	63	563	430	13605	13578	570	1000	2.50	0.77	1.40	1.01
	10	57	1557	1484	7054	6986	288	1200	2.40	0.67	1.00	1.06

Table S2 Representative single-particle analysis of FLIM images of LHCII-TR proteoliposomes. This type of data was used to calculate the histograms shown in Figure 4C-D of the main text.

Estimation of energy transfer efficiency in single proteoliposomes via FLIM

Our photobleaching experiments determined that a quenching of LHCII led to an increase in Texas Red fluorescence (so-called "fluorescence recovery" or "de-quenching" of TR) and we know that this occurred during FLIM acquisitions. We attempted to correct for this effect to provide a more reliable estimate for TR lifetime for the calculation of FRET efficiency (our main interest). Therefore, single-proteoliposome data of TR mean lifetime averaged over the entire field of proteoliposomes within an image was assessed for a period of the initial 170 s of an acquisition (frames 1 - 500). From this, the *TR lifetime change due to LHCII photobleaching* over the entire image was determined to be: a 53% increase for the high-LHCII proteoliposomes and a 52% increase for low-LHCII proteoliposomes, shown as the "Ratio" in **supplementary Table S3**, below.

Single-proteoliposome analysis was performed on the full 1-500 frames of data, necessary to provide a large enough photon count level for an adequate decay curve for a good fit (typically 3000 counts with a range from 1000-10000). The data without any correction applied is shown in main text **Figure 4C**. These raw values for TR mean lifetime were then adjusted by dividing by the appropriate value for *TR lifetime change due to LHCII photobleaching* as above (0.52 and 0.53), to give a representation of the mean TR lifetime unbiased by photobleaching effects. Energy transfer efficiency (ETE) was then calculated

 $ETE_{LT} = 1 - \frac{\tau_{DA}}{\tau_D}$

from the corrected TR lifetime data, using the conventional relationship, for the high-LHCII proteoliposomes and the low-LHCII proteoliposomes (as *DA*) using the TR-only liposome sample FLIM data as the donor-only sample (as *D*). This FRET efficiency estimation is used to produce the histogram in **Figure 4D** in the main text.

	High L	HCII	Low LHCII				
	Caclulated		Caclulated				
Frames	TR <t></t>	Error	TR <t></t>	Error			
#	ns	ns	ns	ns			
1-50	0.713	0.018	0.712	0.026			
1-500	1.335	0.008	1.360	0.007			
Ratio	0.534	-	0.524	-			

Supplementary Table S3. The difference in measured TR lifetime over frames 1-50 vs 1-500.

LHCII fluorescence excitation and linear absorption tabulated data Table SX

shows the numerical analysis of LHCII fluorescence excitation and linear absorption used as a secondary method of estimating energy transfer efficiency from TR to LHCII. "Value at 591nm in linear absorption spectrum (1-T%)" is taken from linear absorption normalised to 1.0 at LHCII excitation peak (~675 nm). Linear absorption is calculated from 1 – transmission spectra. Transmission (T) is calculated from measured optical density (OD) using the equation: $T = 10^{-0D}$. The linear absorption is normalised to 1.0 at LHCII absorption peak (~675 nm). "Value at 591nm in fluorescence excitation spectrum" is taken from LHCII fluorescence excitation spectra normalised to 1.0 at LHCII excitation peak (~675 nm). "591 nm(1-T%) – Excitation" is the difference in linear absorption and LHCII when values of linear absorption and LHCII excitation for LHCII only control samples are removed from all linear absorption and LHCII excitation values respectively. "FRET efficiency" is then calculated

$$E = 1 - \frac{(1-T) - ex}{2}$$

 $ETE = 1 - \frac{1}{(1-T)}$ ((1-T) = linear absorption) (ex – fluorescence excitation). For samples with a high LHCII: TR concentration ratio the 1-T% and Excitation values (with control LHCII removed) can be very close, small fluctuations in spectra due to noise can result in under/overestimated calculations of ETE for these samples. However, for samples with a low LHCII to TR ratio and a difference in (1-T%) and Excitation well above the noise calculated FRET efficiencies follow the same trend as when calculated from TR emission quenching.

Sample description		Value at 591nm in linear absorption spectrum (1-T%)	Value at 591nm in fluorescence excitation spectrum	591nm (1-T%) - Excitation	FRET Efficiency (%)		
Calcualted		Normalised to 1.0	Normalised to 1.0	After LHCII only	Calculated	Calculat	ed from
concentraions		at Chl a peak	at Chl a peak	removed	from ex and 1-T	relative	emission
LHCII-0.52µM/	No TR	0.285	0.28	0.000	-	-	
LHCII-0.53µM/	TR-0.9µM	0.378	0.375	-0.002	102.6	-	
LHCII-0.57µM/	TR-2.2µM	0.489	0.479	0.005	97.8	-	
LHCII-0.55µM/	TR-3.7µM	0.647	0.609	0.033	91.0	-	
LHCII-0.56µM/	TR-4.7μΜ	0.725	0.721	-0.001	100.2	-	
LHCII-0.54µM/	TR-6.0µM	0.883	0.829	0.049	91.8	-	
LHCII-0.56µM/	TR-7.0μΜ	0.936	0.909	0.022	96.7	-	
LHCII-0.57µM/	TR-8.5µM	1.074	0.988	0.081	89.7	-	
LHCII-0.56µM/	TR-9.5µM	1.115	1.046	0.065	92.2	-	
LHCII-0.54µM/	TR-11.2µM	1.295	1.160	0.130	87.1	-	
LHCII-0.61µM/	TR-12.3µM	1.280	1.215	0.060	94.0	-	
No LHCII/	TR-4.3µM			-	-	-	
LHCII-0.16µM/	TR-8.4µM	2.756	1.866	0.885	64.2		64.5
LHCII-0.28µM/	TR-7.7μΜ	1.626	1.353	0.268	80.0		78.3
LHCII-0.34µM/	TR-5.3µM	1.137	1.023	0.109	87.2		76.3
LHCII-0.49µM/	TR-7.3µM	1.036	0.947	0.085	88.7		86.6
LHCII-0.53µM/	TR-5.0μΜ	0.771	0.738	0.029	94.1		85.5
LHCII-0.76µM/	TR-5.0µM	0.638	0.643	-0.009	102.5		89.4
LHCII-1.18µM/	TR-7.9µM	0.608	0.594	0.009	97.2		95.3
LHCII-1.51µM/	TR-5.5µM	0.455	0.460	-0.009	105.4		94 .8
LHCII-2.47µM/	TR-5.3µM	0.405	0.393	0.007	93.8		96.5
LHCII-2.84µM/	TR-8.0µM	0.456	0.422	0.030	82.6		98.0

Supplementary Table S4 Analysis of linear absorption and LHCII excitation data displayed in figure SX, proteoliposome series 1 (upper half of table) and proteoliposome series 2 (lower half of table).

References

Adams, P.G., Vasilev, C., Hunter, C.N., and Johnson, M.P. (2018). Correlated fluorescence quenching and topographic mapping of Light-Harvesting Complex II within surface-assembled aggregates and lipid bilayers. Biochimica et Biophysica Acta, Bioenergetics *1859*, 1075-1085.

Barzda, V., Gulbinas, V., Kananavicius, R., Cervinskas, V., van Amerongen, H., van Grondelle, R., and Valkunas, L. (2001). Singlet–Singlet Annihilation Kinetics in Aggregates and Trimers of LHCII. Biophysical Journal *80*, 2409-2421.

Grab, O., Abacilar, M., Daus, F., Geyer, A., and Steinem, C. (2016). 3D-Membrane Stacks on Supported Membranes Composed of Diatom Lipids Induced by Long-Chain Polyamines. Langmuir *32*, 10144-10152.

Natali, A., Gruber, J.M., Dietzel, L., Stuart, M.C., van Grondelle, R., and Croce, R. (2016). Light-harvesting Complexes (LHCs) Cluster Spontaneously in Membrane Environment Leading to Shortening of Their Excited State Lifetimes. Journal of Biological Chemistry *291*, 16730-16739.

Pandit, A., Shirzad-Wasei, N., Wlodarczyk, L.M., van Roon, H., Boekema, E.J., Dekker, J.P., and de Grip, W.J. (2011). Assembly of the major light-harvesting complex II in lipid nanodiscs. Biophysical Journal *101*, 2507-2515.

Porra, R.J., Thompson, W.A., and Kriedemann, P.E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta, Bioenergetics *975*, 384-394.

Titus, J. A.; Haugland, R.; Sharrow, S. O.; Segal, D. M., Texas red, a hydrophilic, red-emitting flourophore for use with flourescein in dual parameter flow microfluorometric and fluorescence microscopic studies, Journal of Immunological Methods 1982, 50, (2), 193-204.

Yuan, P., and Walt, D. (1957). Calculation for Fluorescence Modulation by Absorbing Species and Its Application to Measurements Using Optical Fibers. Analytical Chemistry *59*, 2391-2394.