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Published in:
Nature Chemistry

DOI:
[10.1038/nchem.2525](https://doi.org/10.1038/nchem.2525)

2016

Document Version:
Peer reviewed version (aka post-print)

[Link to publication](#)

Citation for published version (APA):
Dostál, J., Pšenčík, J., & Zigmantas, D. (2016). In situ mapping of the energy flow through the entire photosynthetic apparatus. *Nature Chemistry*, 8(7), 705-710. <https://doi.org/10.1038/nchem.2525>

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***In situ* mapping of the energy flow through the entire photosynthetic apparatus**

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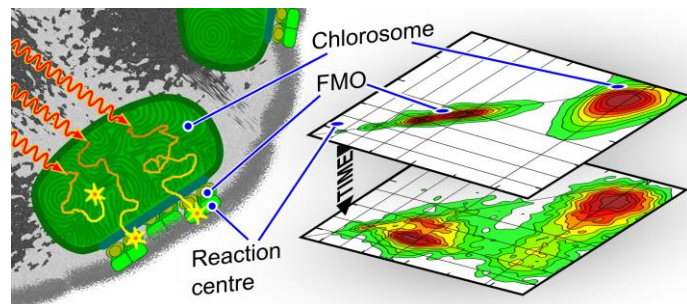
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Abstract

Absorption of sunlight is the first step in photosynthesis, which provides energy for the vast majority of organisms on Earth. The primary processes of photosynthesis have been extensively studied in isolated light-harvesting complexes and reaction centres. However, to fully understand biological light capturing it is crucial to reveal also the functional relations between the individual complexes. This information was scarce thereby preventing a full understanding of the light-capture functionality. Here we report direct tracking of the excitation energy flow through the entire photosynthetic system of green sulfur bacteria by means of two-dimensional electronic spectroscopy. We unravel functional organization of individual complexes in the photosynthetic unit and show that whereas energy is transferred within subunits on a sub- and few picoseconds timescale, energy flows at a timescale of tens of picoseconds between them. Thus, we demonstrate that the bottleneck of the energy transfer is between the constituents.

TOC Graphics



TOC Summary

The light capture in photosynthetic organisms depends on the efficiency of all energy transfer steps in the photosynthetic unit. Two-dimensional electronic spectroscopy was used on intact cells *in situ* to reveal and characterize functional connectivity between the individual complexes in the photosynthetic apparatus of green sulfur bacteria.

Green sulfur bacteria are photosynthetic organisms that are adapted to anaerobic environments with a limited access to solar energy. Representatives of these bacteria can be found at sea depths exceeding 100 m as well as in the proximity of black smokers at the ocean bottom,^{1,2} where the light levels are very low. Organisms inhabiting such environments had to develop highly efficient light-capturing mechanisms to survive. The photosynthetic apparatus of the green sulfur bacterium *Chlorobaculum (Cba.) tepidum* studied here consists of an assembly of light-harvesting complexes, a chlorosome and Fenna-Matthews-Olson (FMO) proteins, and reaction centres (RCs) (Fig. 1 inset). The chlorosome is mainly composed of tens to hundreds of thousands aggregated bacteriochlorophyll (BChl) *c* molecules, whereas the baseplate of the chlorosome consists of a pigment-protein complex containing BChl *a* and carotenoid molecules.³ It mediates the energy transfer from the BChl *c* aggregate to the rest of the photosynthetic apparatus. It has been suggested, but not confirmed experimentally, that several FMO trimers are in a close contact with the type I RC core complex and that they attach to the chlorosome baseplate.^{4,5} Most probably, four FMO trimers form a supercomplex with two reaction centres.⁶ While the photophysical processes in the isolated complexes have been extensively studied (for a review see e.g. ^{3,4,7}), little is known about the energy transfer between the complexes. For instance, energy transfer from the chlorosome to FMO and further to the RC has never been resolved. Time-resolved fluorescence measurements of cells provided some information about the energy transfer between spectrally well separated complexes in filamentous anoxygenic bacteria, which contain chlorosomes but not FMO complexes.^{8,9} For green sulfur bacteria only the energy transfer from the chlorosome to the BChl *a* containing complexes was observed.^{10,11} The lack of information on the energy transfer pathways between photosynthetic subunits prevents obtaining the full picture of light-harvesting.

The information on the functional connectivity between different subunits is also missing for other photosynthetic organisms, e.g. higher plants, and a method capable of directly unravelling connectivity between subunits is needed. Recently developed two-dimensional electronic spectroscopy (2DES) is a technique with high temporal and spectral resolution, which was successfully applied in studies of molecular couplings and energy transfer pathways in photosynthesis.¹²⁻¹⁴ The advantage of 2DES over transient absorption or emission spectroscopy techniques is that it provides full correlation maps between excitation and probing wavelengths. This allows obtaining the full connectivity network between electronic transitions. Until recently this technique could not be employed for studying intact cells, because of their intense light scattering. The latest 2DES developments enabled measurements even in highly scattering environments,¹⁵ including cells,¹⁶ however no information about energy transfer kinetics or connections between different subunits was revealed. Here we show that by employing scattering-resistant 2DES experimental setup,¹⁵ the complete absorptive 2D spectrum can be acquired and therefore the energy flow through the complete photosynthetic unit can be monitored. We resolve how the chlorosome, FMO and the RC are interconnected via the energy transfer channels in the cells of the green sulfur bacterium *Cba. tepidum*. Our study presents hitherto unavailable information about the functional connectivity and organization of the photosynthetic unit and opens new venues for modelling of intact photosynthetic systems.¹⁷

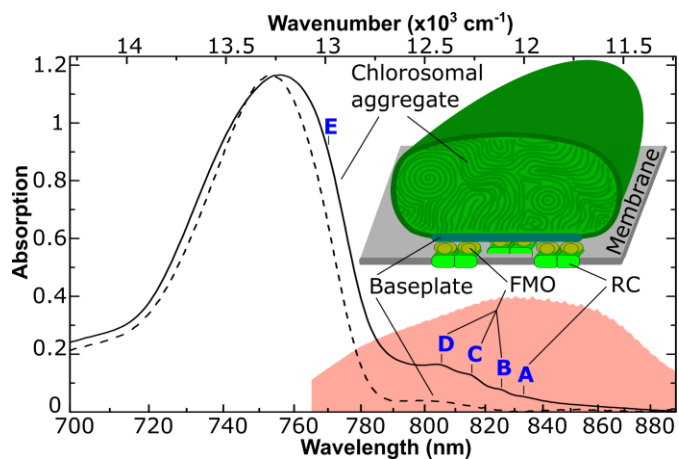


Fig. 1. The absorption spectrum of the intact *Cba. tepidum* cells (solid line) compared to the spectrum of isolated chlorosomes (dashed line) at 77 K. The laser spectrum used in the 2DES experiments is shown in light red. The letters A-E indicate positions of guiding lines used in 2D spectra (see Fig. 2). Inset: schematic representation of the photosynthetic apparatus of green sulfur bacteria.

Results and discussion

Absorption of photosynthetic complexes in cells. All experiments were conducted at anaerobic conditions that are essential for an efficient energy transfer to occur in the obligate anaerobe *Cba. tepidum*^{18,19} and at 77 K. At this temperature spectroscopic features of the individual complexes in the photosynthetic unit are well discernible in the absorption spectrum (Fig. 1): the prominent band corresponding to the chlorosomal aggregate with an absorption maximum at ~745 nm (13420 cm^{-1}), three absorption peaks associated with FMO at 806 nm (12410 cm^{-1}), 816 nm (12250 cm^{-1}) and 825 nm (12120 cm^{-1}), and a weak absorption of the RC with a peak at 833 nm (12000 cm^{-1}).²⁰ Most of the absorption corresponding to the baseplate at 785-825 nm ($12740\text{-}12120\text{ cm}^{-1}$) as seen in the spectrum of isolated chlorosomes in Fig. 1 and to the RC core complex at 750-840 nm ($13330\text{-}11900\text{ cm}^{-1}$)⁴ overlaps the FMO absorption, but is substantially weaker.²¹ Absorption of cells at the ambient temperature is similar, however, the weaker peaks are not resolved.²⁰ Distinguishable absorption of different constituent complexes of the photosynthetic apparatus enables us to observe features associated with the energy transfer within and between the individual light-harvesting complexes and RCs *in situ* by means of 2DES.

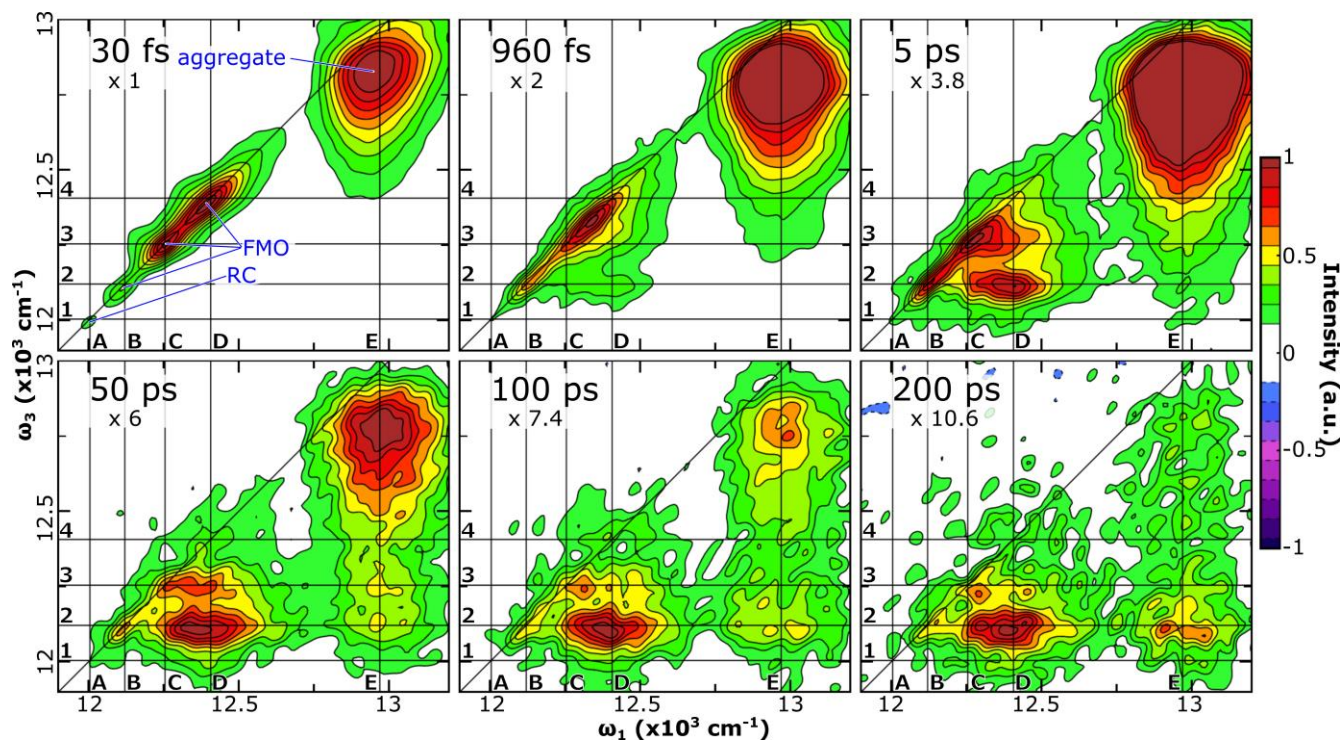


Fig. 2. The selected 2D spectra (absorptive part) of the intact *Cba. tepidum* cells measured between 11900 and 13200 cm^{-1} (760-840 nm) at indicated population times at 77 K. The spectra are normalized to the maximum of the FMO signal (the relative amplitude multiplier is shown in the top left corner). The vertical and horizontal lines show positions of the distinct peaks (from left to right and from bottom to top: reaction centre - 12000 cm^{-1} ; FMO - 12120 cm^{-1} , 12250 cm^{-1} and 12410 cm^{-1} ; an extra line in the chlorosome region at 12940 cm^{-1}). The letters and numbers associated with the lines are there to facilitate identification of individual peaks. The distinct transitions from the chlorosome aggregate, FMO and reaction centre can be identified as diagonal peaks in the 2D spectrum acquired at the early population time (30 fs). The excitation energy transfer within and between the individual complexes is observed at later population times as decay of the diagonal peaks and formation of cross-peaks below the diagonal.

Mapping energy transfer with 2DES. Laser spectrum used in the 2DES measurements covered all the near infrared absorption bands of the photosynthetic apparatus (Fig. 1). To avoid the dominance of the signal from the chlorosome over other spectral features, the laser spectrum was adjusted to excite only the red edge (765-790 nm) of the chlorosomal aggregate band centred at 745 nm. The absorptive (real) part of the 2D spectra, corresponding to the absorption signal, acquired at various delay times after excitation (population times) is shown in Fig. 2. The spectra can be intuitively interpreted as a set of transient absorption spectra stacked next to each other along the excitation frequency (horizontal) axis. The absorptive 2D spectrum acquired at the 30 fs population time consists of a series of diagonal peaks that can be attributed to different transitions in the linear absorption spectrum (Fig. 2). Stimulated emission (SE) and ground-state-bleach (GSB) signals contribute equally to each diagonal peak. The red slope of the chlorosome band, the three main FMO peaks and a weak RC peak are clearly discernible. The elongated featureless shape of the chlorosome band extending below the diagonal peak is a part of the very intense chlorosome peak lineshape.

The time evolution of the 2D spectrum (Fig. 2), seen as a formation and decay of the cross-peaks below the diagonal (i.e. SE signal red-shifted with respect to the excitation frequency), reveals how energy flows within and between the individual complexes in the photosynthetic unit. Individual points in the 2D spectrum evolve in a complex manner with exponential rates ranging from $(100 \text{ fs})^{-1}$ to $(200 \text{ ps})^{-1}$. To facilitate a quantitative analysis of the energy transfer rates in the photosynthetic unit of green sulfur bacterium *Chlorobaculum tepidum* we split the 2D spectra into two parts corresponding to the excitation of FMO together with the RC, and to the excitation of the chlorosome, respectively. Each of these regions was fitted by four exponentials. Amplitudes of all fitted components are plotted in Figs. 3 and 4 as the two-dimensional decay associated spectra (DAS).²² DAS analysis is the most suitable way of global analysis in this case, since it does not require any additional assumptions to be applied to the model. The robustness of the extracted timescales was confirmed by repeatedly performed fitting using different initial conditions which all led to the same results. Note that positive signals, corresponding to the ground state bleach (GSB) and stimulated emission (SE), dominate the 2D spectra (Fig. 2) and the negatively signed excited state absorption plays only a minor role. Consequently, the positive (negative) peaks in the DAS (Figs. 3 and 4) primarily indicate energy leaving from (arriving to) different parts of the photosynthetic unit.²²

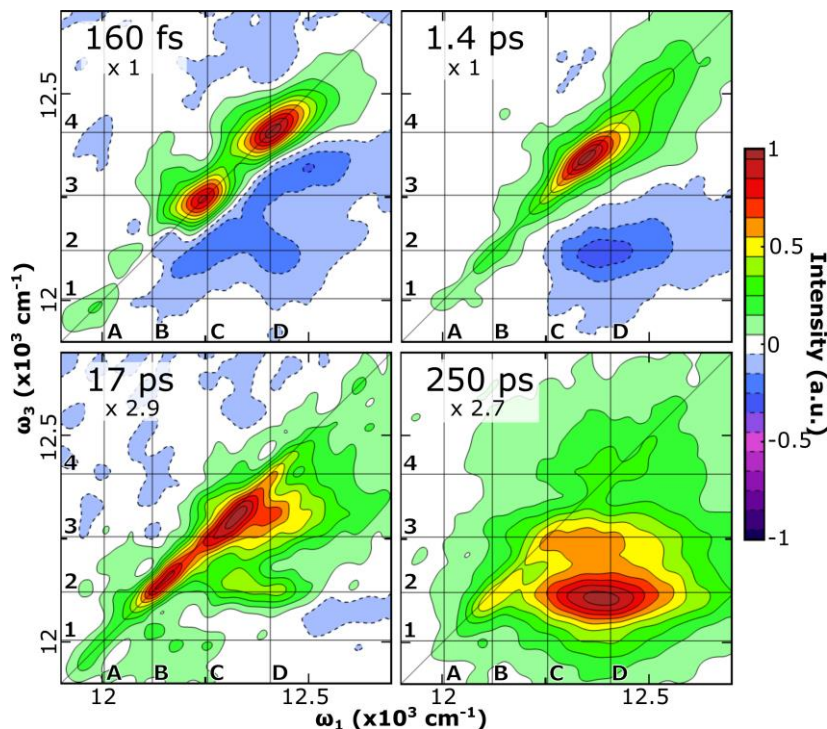


Fig. 3. Decay associated spectra of the FMO-RC spectral region. The spectra characterize the energy relaxation within FMO and energy transfer from FMO to the RC on the indicated timescales. All spectra are normalized (the relative amplitude multiplier is shown in the top left corner). The 160 fs and 1.4 ps components mainly depict the energy transfer between the individual energy states of the FMO complex. The 17 ps component is attributed to slow relaxation processes within the FMO protein and to the excitation energy transfer from FMO to RC. The 250 ps component reflects the slow decay of the FMO population trapped at the lowest excited state.

Analysing the energy transfer network. First, we analyze relaxation within the FMO complex. The two prominent diagonal FMO peaks at 12410 cm^{-1} and 12250 cm^{-1} decay on a timescale of 160 fs and indicate the energy transfer to the lower exciton states in FMO (Fig. 3), in agreement with previous experiments on the isolated FMO complexes.^{12,23} However, the exciton relaxation from the states located between these two strongest peaks ($\sim 12330\text{ cm}^{-1}$) is ten times slower (Fig. 3; 1.4 ps component). All internal energy transfer processes in FMO are completed within ~ 50 ps. The rather slow processes are reflected by the 17 ps component (Fig. 3) that reveals mixture of energy transfer steps through intermediate states to the lowest level in FMO. Such residual processes on the ~ 20 ps time scale are observed only at cryogenic temperatures and their assignment is still debated.^{19,23,24} Additionally, the 17 ps component reflects the lowest FMO state decay, which is assigned to the energy transfer from the lowest FMO state to the RC as will be discussed below. After completion of all internal energy transfer processes in FMO, the FMO spectrum (Fig. 2, $T > 50$ ps) features fully relaxed SE and GSB signal localized at the lowest FMO state (12120 cm^{-1} , line 2) accompanied by GSB signal clearly visible at 12250 and 12410 cm^{-1} (lines 3 and 4). Such equilibrated 2D spectrum uniformly decays with 250 ps component (Fig. 3). This observation confirms that the lowest energy state is excitonically delocalized over a few sites within an FMO monomer.^{25,26}

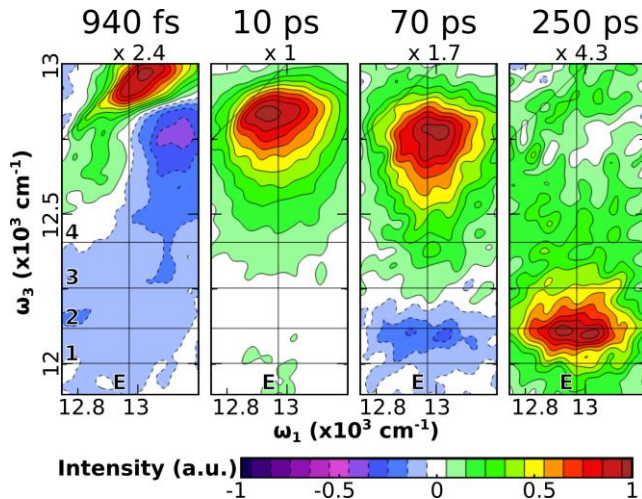


Fig. 4. Decay associated spectra of the chlorosome excitation region. The spectra characterize the energy transfer through the whole photosynthetic unit after the chlorosome excitation. All spectra are normalized and the relative amplitude multiplier is shown below the lifetimes characterizing each component). The 940 fs component describes the exciton diffusion within the chlorosome band. The decay of the signal on the 10 ps timescale is attributed to exciton-exciton annihilation in the chlorosomal aggregate. The energy transfer from chlorosome to FMO is described by the 70 ps component. The slowest 250 ps component reflects the decay of the excitation energy trapped at the lowest FMO state.

Now we examine the connectivity between different complexes of the photosynthetic unit, which is clearly observed in the sequence of the 2D spectra presented in Fig. 2. An appearance of the cross-peaks below the chlorosome diagonal peak, connecting chlorosome and FMO absorption bands (positions E2, E3 in Fig. 2) implies that energy is transferred from the chlorosome to FMO. However, an intermediate energy transfer step through the baseplate remains obscured, because absorption of the baseplate is too weak and is not observed. The cross-peaks appearing at the D1 and E1 positions reveal the energy transfer from FMO to the RC. The 2DES data thus unambiguously show that the FMO complex serves as a mediator of the energy transfer between the chlorosome and RC. Although FMO was placed between the chlorosome and cytoplasmic membrane containing RCs in all structural models, an energy transfer from the chlorosome to FMO has never been observed experimentally. Here we provide the clear experimental evidence of the FMO energy transfer function, which was sought after for decades.

The quantitative insight into these processes is provided by analysis of DAS components of the chlorosome part of the 2D spectra (Fig. 4). The fastest process (940 fs component in Fig. 4) can be attributed to relaxation within the chlorosome band.²⁷ The shape of the 940 fs DAS component clearly shows the decay of the higher-energy states of the chlorosome (positive DAS amplitude) and build-up of the population at the red-edge of the chlorosome absorption band, corresponding to the lowest states of the chlorosome (negative DAS amplitude). Due to the extremely high density of pigments and the consequent presence of multiple excitations, an exciton annihilation occur in the chlorosome, which is resolved in our experiments as a decaying 10 ps component without any corresponding rise. Annihilation results in the non-exponential dynamics governed by exciton diffusion in chlorosomes, however, here we approximate it by an additional exponential decay in the DAS analysis, which is justified by the adequacy of the fit. The majority of the chlorosome to FMO energy transfer proceeds with the 70 ps time constant. The energy arriving to FMO can be observed as the rising signal of the lowest FMO state (appearance of the cross-peak E2 in Fig. 2 and the negative 70 ps component in Fig. 4).

Note that the excitation energy transfer from the chlorosome to the upper FMO states is much slower than the subsequent relaxation to the lowest FMO state, which was discussed above. As a result, the upper states of FMO exhibit only a small transient population, which explains why the corresponding cross-peaks in the 2D spectrum (E3, E4 in Fig. 2) are quite weak. When the population rate of some state is slower than the depopulation rate, the solution of linear rate equations changes its sign compared to the case when population is faster than depopulation. Thus, the positive cross-peaks at the positions E3, E4 in the 70 ps DAS (Fig. 4) indicate the population transfer from the chlorosome to FMO rather than the relaxation within FMO. Lastly, the remaining population leaves the lowest FMO state with the 250 ps time constant, which was also resolved in the case of direct FMO excitation.

The intrinsic RC dynamics can be traced by observing the RC diagonal peak A1 in Fig. 2. This peak decays mainly on the 160 fs and 1.4 ps timescales (Fig. 3). These time constants correspond to the energy relaxation and charge separation dynamics within the RC. The energy transfer from FMO to the RC can be observed as a decay of FMO peaks and the cross-peaks dynamics on line 1, which are associated with excitation arrival to the RC. We assign substantial part of the strong 17 ps decay component of the FMO states in Fig. 3 to the energy transfer from FMO to the RC. Similarly to the energy transfer from the chlorosome to FMO, the transfer between FMO and RC is slower than the subsequent processes occurring within the RC. As described above, this leads to the weak signals of the RC cross-peaks (B1, C1, and D1 in Fig. 2) and the sign inversion of the DAS components in the same region. In agreement with this interpretation, the 160 fs and 1.4 ps DAS (Fig. 3) exhibit negative cross-peaks in the spectral region corresponding to the RC. Positive small-amplitude peaks on the line 1 (B1, C1) of the 17 ps DAS provide further support that the energy transfer from FMO to the RC occurs with the 17 ps transfer time

However, not all excitations reaches the RC on this timescale – it is clear from the significant amplitude of the cross-peaks D2 and E2 in the 200 ps 2D spectrum in Fig. 2 that some fraction of the excitation energy stays in the lowest FMO state for a much longer time. The DAS support this observation (Fig. 3) by showing that population leaves the lowest FMO state with 17 ps and 250 ps components. Based on the analysis of the peak amplitudes in 2D spectra we estimate that ~25% of the energy originally absorbed by FMO is still present in the lowest state after 200 ps.

Efficiency of the energy transfer from the FMO to the RC is a long-standing question. It has been demonstrated that the overall efficiency of this energy transfer step is less or equal to 40% in isolated FMO-RC complexes or membrane preparations,^{28–32} for which isolation procedures were held responsible. Our estimate points to 75% efficiency; however, two additional factors should be considered. First, the estimate can be affected by the annihilation processes in FMO, which would lead to an overestimation of the efficiency. On the other hand the energy transfer efficiency could be underestimated, because the RC can process only one excitation at a time and excitation congestion in the photosynthetic apparatus is likely to occur due to multiple excitations competing for the entry into RC. Consequently, excitation energy gets accumulated at the lowest state of FMO from where it is dissipated with a 250 ps time constant. Energy transfer to the RC on the long timescale cannot be excluded, although rather unlikely, because it would require that one quarter of the FMO complexes are connected to the RCs in a different, but well defined way.

All energy transfer pathways with transfer rates obtained in this study are summarized in Fig. 5. It becomes apparent that the energy transfer rates between the chlorosome and FMO, and between FMO and the RC are slower than the relaxation within the chlorosome and FMO. Thus, we establish that the inter-complex energy transfer presents the slowest link in the overall energy transfer through photosynthetic unit. These results set the baseline for the future discussion on the possible coherent or incoherent energy transfer in the whole photosynthetic unit of green sulfur bacteria.

Because of the narrowing of absorption and emission bands the energy transfer may proceed with slightly different transfer rates at physiological temperatures as compared to 77 K. Nevertheless, the functional connectivity between the individual complexes should not be affected at 77 K. This is supported by the previously reported experiments, showing that upon

freezing and thawing the changes of the energy-transfer efficiency in whole cells of another chlorosome containing bacterium, *Chloroflexus aurantiacus*, were fully reversible.³³ To confirm that freezing to 77 K does not compromise viability of the *Chlorobaculum tepidum* cells we performed freezing/thawing experiments with the positive outcome (see Supplementary information).

Conclusions

We demonstrated that 2DES enables step by step tracking of the energy flow through the entire photosynthetic unit in a bacterial cell. This allowed us to determine how different parts of photosynthetic apparatus are connected to each other and to demonstrate for the first time that FMO serves as an energy conduit between chlorosome and RC. Our findings open opportunities for achieving the full understanding of functional organization of photosynthetic systems. Scattering-resistant 2DES method of measuring energy transfer pathways *in situ* is not limited to photosynthetic bacteria and will be certainly applied to other photosynthetic organisms.

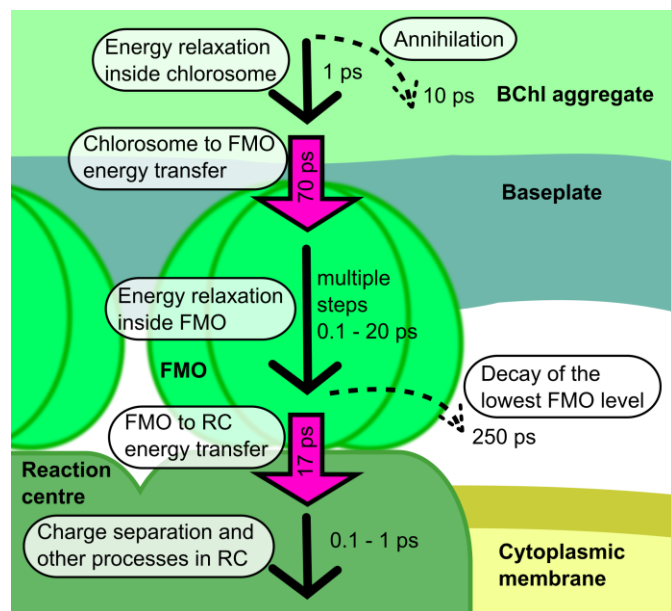


Fig. 5. A scheme of the excitation energy flow through the entire photosynthetic apparatus of *Cba. tepidum*. The main energy transfer processes within and between photosynthetic complexes are shown with characteristic time scales.

Methods

Experimental setup. The detailed description of the experimental setup used to obtain 2DES spectra can be found in ¹⁵. During experiments the coherence time was scanned from -170 fs to 450 fs with the 2 fs step, which ensured the resolution of 40 cm⁻¹ along the excitation (coherence) frequency axis. The same resolution was achieved along the detection frequency axis, determined by the time domain window used in the Fourier analysis. The duration of the laser pulses was ~15 fs. The laser repetition rate was 20 kHz and the excitation energy – 1 nJ per pulse, the beams were focused to the 0.1 mm diameter spot.

Sample preparation. The *Cba. tepidum* cells were grown as described in ³⁰. Prior the measurements the cell suspension was concentrated by centrifugation and diluted in 1:2 (v/v) ratio with glycerol to obtain absorbance of about 0.2 at 800 nm in the 0.2 mm thick sample cell. The sample was reduced by sodium dithionite (final concentration of 20 mM) and incubated for at least 2 hours in air-tight vessel at room temperature. All experiments were carried at 77 K in the optical cryostat (Oxford Optistat DN2).

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Acknowledgments

We thank the group of František Vácha for cell cultivation and for the isolation of the chlorosomes. We are grateful to David Bína and Heiko Lokstein for their help with the viability experiments described in the supplementary information. The work in Lund was supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. The work in Prague was supported by the Czech Science Foundation (project P501/12/G055).

Author Contributions

J.D. designed and performed the experiments, analyzed the data and wrote the manuscript, J.P. designed and performed the experiments and wrote the manuscript, D.Z. conceived the idea, designed and performed the experiments and wrote the manuscript.

Additional information

Supplementary information is available in the online version of the paper. Correspondence and requests for materials should be addressed to František Vácha (vacha@jcu.cz).

Competing Financial Interests

Authors declare no competing financial interests.

Supplementary information

***In situ* mapping of the energy flow through the entire photosynthetic apparatus**

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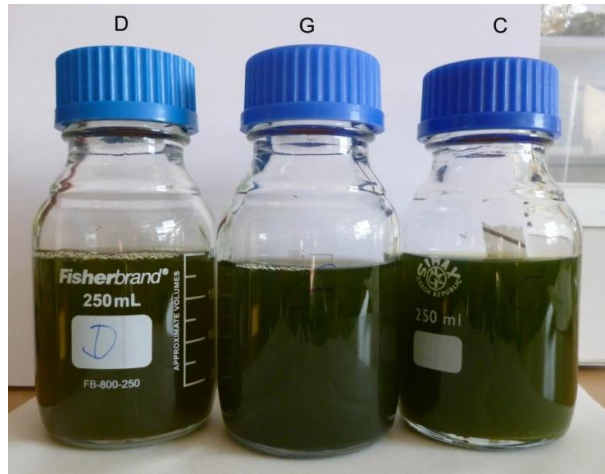
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Cells viability experiment

To test how the cryogenic conditions affect *Chlorobaculum tepidum* bacterial cells, we have prepared the cell sample in the same way as we did for the two-dimensional electronic spectroscopy measurements. The cell suspension was concentrated by centrifugation and diluted in 1:2 (v/v) ratio with glycerol. The glycerol-containing mixture was then split in two parts: one part (sample labelled D) was reduced by sodium dithionite (final concentration of 20 mM). The second part was not treated with dithionite (sample G). Both samples were slowly frozen in liquid nitrogen vapours to 77 K to mimic the freezing procedure in the optical cryostat. We note, that the available protocols recommend flash-freezing to 77 K, but we intentionally treated the cells in the same – likely more harsh – way as in the optical experiments. After keeping them at 77 K for some time, the two samples were thawed and injected into two flasks with the growth medium. In addition, a control sample (labelled as C) of bacteria, which was not mixed with glycerol and not frozen and thawed was injected at the same time. After standard growing time of three days at 48 °C we did not observe any differences between the three cultures, which would exceed the usual batch-to-batch variations. This experiment shows that the viability of cells was not compromised by the freezing and thawing in a mixture with glycerol. The visual comparison of the three samples after the final growth period is provided in Supplementary Figure 1.

It is also worth noting that storing bacteria at 77 K in 15-80% glycerol solutions is a standard procedure for the long-term storage of bacterial cultures.¹



Supplementary Figure 1. The three different samples of *Chlorobaculum tepidum* after induced growing. The samples D and G were mixed with the glycerol and frozen at 77 K, later to be thawed for growing. The sample G in addition was treated with dithionite before freezing. The control sample C was not frozen before growing.

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