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Authors
Ashfold, Michael
Bai, Shuming
Bradforth, Stephen
et al.

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DISCUSSIONS

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Photo-protection/photo-damage in natural systems: general discussion

Michael Ashfold, Shuming Bai, Stephen Bradforth, Pavel Chabera, Jeffrey Cina, Carlos E. Crespo-Hernández, Natércia das Neves Rodrigues, Marta Duchi, Graham Fleming, Christopher Grieco, Scott Habershon, Michael Haggmark, Sharon Hammes-Schiffer, Shou-Ting Hsieh, Bern Kohler, Heiko Lokstein, Andrew Marcus, Todd Martinez, Spiridoula Matsika, Thomas A. A. Oliver, Luis Ortiz-Rodríguez, Tomas Polivka, Minjung Son, Vasilios Stavros, Collin Steen, Matthew Turner, Peter Jomo Walla and Jack Woolley

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Collin Steen opened discussion of Tomas Polivka's paper: It would be very interesting to study the excited state dynamics of this E-FCP protein in the context of the whole organism. Dynamics and behavior are frequently altered in an *in vivo* sample compared to an isolated one. As an example, I'd like to present some recent results from Graham Fleming's group at UC Berkeley in which we apply *in vivo* spectroscopy to study the photoprotective quenching mechanisms in intact cells of the heterokont algae *Nannochloropsis oceanica*.[1] *N. oceanica* is a small marine algae, 2–3 μM in size, which results in minimized light scattering compared to larger organisms such as *E. huxleyi*. It also possesses a relatively simple membrane structure and pigment composition including the higher plant xanthophyll cycle carotenoids (violaxanthin-antheraxanthin-zeaxanthin), one type of chlorophyll, and Chl *a*-binding LHCX proteins, which are closely related to the FCP proteins discussed in the present contribution (DOI: 10.1039/c8fd00193f). We have also made use of two knock-out mutants of *N. oceanica*: *vde*, which is unable to synthesize Zea in high light, and *lhcx1*, which lacks the LHCX1 protein.

Fluorescence lifetime snapshots revealed that wild type cells undergo a significant decrease in the average chlorophyll fluorescence lifetime (t_{avg}) during 10 min of high-light exposure, which is indicative of the onset of quenching. However, both mutants lack any substantial decrease in t_{avg} (Fig. 1B in Park *et al.*[1]), indicating that VDE and LHCX1 are both required for photo-protection in this species of algae.

In order to gain insight into the mechanism behind the dissipation of excess excitation energy, we employed *in vivo* Transient Absorption (TA) spectroscopy on *N. oceanica* cells that had been dark acclimated (unquenched) and after exposure

to high light for 30 min (quenched). The decay of the signal at 980 nm following excitation of Chl *a* was clearly different in the unquenched and quenched states (Fig. 2C in Park *et al.*[1]). Given that this spectral region corresponds to $Car^{\bullet+}$ ESA, this observed difference provides evidence for charge transfer (CT) quenching in which energy is transferred from the Q_y state of Chl to a heterodimer CT state consisting of $Car^{\bullet+}$ and $Chl^{\bullet-}$. Probing at 540 nm also revealed differences between the unquenched and quenched conditions (Fig. 2A in Park *et al.*[1]), thereby confirming the presence of excitation energy transfer (EET) quenching from the Chl Q_y to the Car S_1 state. Interestingly, the *vde* and *lhcx1* mutants exhibited no difference between unquenched and quenched samples probed at either 980 nm or 540 nm (Fig. 3 in Park *et al.*[1]). This strongly suggests that VDE (and thus Zea) as well as LHCX1 are required for the CT and EET photoprotective quenching mechanisms in intact *N. oceanica*.

So in summary: (1) We observe Chl-Car energy transfer processes in living cells of the algae *Nannochloropsis oceanica*; (2) these energy transfer processes involve the $Car^{\bullet+}$ (CT) and Car S_1 (EET) states; (3) this energy transfer specifically requires VDE (Zea) as well as the LHCX1 protein, which is closely related to FCP. I understand that it is not possible to directly compare energy transfer dynamics in intact *N. oceanica* and isolated E-FCP proteins, but what do you make of the differences between your results and ours? Additionally, do you have plans to study energy transfer dynamics of the FCP protein *in vivo*? What differences would you anticipate between FCP and LHCX1 in the context of a fully intact system?

1 S. Park, C. J. Steen, D. Lyska, A. L. Fischer, B. Endelman, M. Iwai, K. K. Niyogi and G. R. Fleming, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 3385–3390.

Tomas Polivka responded: We do not fully understand the energy transfer pathways in isolated E-FCP, especially their response to high- or low-light conditions. We know that the carotenoid composition (Fx to hFx ratio) varies with light conditions, but we do not know how this may affect the energy transfer pathways. So we first plan to explore the isolated protein in detail, later we may focus on an *in vivo* system containing E-FCP. Concerning the differences between E-FCP and an intact system from *N. oceanica*, the key difference is the carotenoid composition. The direct Chl-Car quenching observed in *N. oceanica* requires carotenoids with low S_1 energy, such as zeaxanthin. Since such carotenoids are not present in E-FCP, the absence of quenching is rather expected. Fucoxanthin is obviously unable to quench the singlet states of Chl-a, because its S_1 energy is too high to do it.

Tomas Polivka addressed Collin Steen: The kinetics you showed in your presentation certainly suffers from annihilation. Then, when normalizing to the long-time plateau of your kinetics, if the amount of annihilation is different in the quenched and unquenched sample, such a difference must affect your results. How do you deal with this?

Collin Steen replied: Thanks for bringing up the topic of Chl*–Chl* annihilation as it does influence the observed kinetics. The signals measured at 540 nm are a combination of Car S_1 ESA and Chl ESA. High-light exposure activates

a variety of de-excitation pathways, resulting in a reduced overall amplitude of Chl ESA in the quenched sample. However, the kinetics of the ESA signals were observed to be kinetically equivalent at time delays $\geq$40 ps where Car S_1 states are nearly fully de-populated. In order to extract the contribution of Car S_1 ESA from the overall Chl ESA, we scale the kinetic profile measured in the dark (mostly Chl ESA) to match that in high light based on signals at long time delays and subtract the two.

This normalization procedure of the EET kinetic traces at long time delays is based on the assumption that the amount of annihilation is consistent during the transition from dark to high light. If the amount of annihilation were different under the quenched and unquenched conditions, we would expect to see a significant change in the Chl ESA and GSB dynamics. However, in a previous paper, we found that the decay of the Chl ESA and GSB signals probed at 620 and 680 nm respectively were identical for dark- and high-light-exposed spinach thylakoid membranes.[1] Therefore, it is reasonable to assume that Chl*–Chl* annihilation is consistent during the transition from dark to high light and thus the decay of Chl ESA is kinetically equivalent. This is further supported by the *N. oceanica vde* mutant that lacks Zea, which upon scaling exhibits identical decays at 540 nm under unquenched and quenched conditions.[2]

1 S. Park, A. L. Fischer, C. J. Steen, M. Iwai, J. M. Morris, P. J. Walla, K. K. Niyogi and G. R. Fleming, *J. Am. Chem. Soc.*, 2018, **140**, 11965–11973.
2 S. Park, C. J. Steen, D. Lyska, A. L. Fischer, B. Endelman, M. Iwai, K. K. Niyogi and G. R. Fleming, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 3385–3390.

Natércia das Neves Rodrigues addressed Collin Steen: This is a question regarding your TA experiments on living cells, which are of course rather complex samples. I am curious to know how much this was a problem experimentally, *i.e.* how difficult it was to generate signal, if there were problems with excessive scatter or any other experimental challenges that arose from using a complex sample like this. Moreover, I am interested in knowing how you addressed these, both experimentally – *e.g.* did you have to avoid translating the sample, or were there other things you had to adjust experimentally – and in terms of analysis, *e.g.* did you have problems with over-complicated spectra, how you addressed these and how you isolated the spectral features of interest?

Collin Steen answered: Experimentally, it is important that the sample is continuously translocated during the experiment and bubbled regularly between measurements to prevent sample damage. To minimize scattering of the pump we use a polarization filter set to the probe polarization. To ensure a clean probe signal we also add a notch filter specific to the pump wavelength prior to our detector. In terms of analysis, we are able to identify the molecular species involved in the quenching of excitation energy based on the features of the difference kinetic decay profile, which is taken as the difference between the sample in its quenched (high-light) state and its unquenched (dark) state. By fitting this difference profile, we obtain rise and decay time constants for the species of interest, which can be compared to literature values for the lifetimes for various pigments. Verification of the molecular species of interest is also based on comparing the observed excited-state absorption spectrum with the literature. Additionally, the use of mutants or chemical treatments that target specific

molecules in the organism can provide further insight into assignment of the spectra and decay profiles.

Pavel Chabera asked Tomas Polivka: It would seem that the synthetic pathway for producing carotenoid with an extra hexanol group is rather complicated. Why does the organism (plankton) undertake this challenge? You indicate in the paper (DOI: 10.1039/c8fd00193f) that this is not happening for protection against excess light – why? Is there any better plausible explanation?

Tomas Polivka replied: Our results show that the overall efficiency of carotenoid-chlorophyll energy transfer is not affected by fucoxanthin (Fx) - hexanoylfucoxanthin (hFx) exchange, though the energy transfer is slower if hFx is the donor. Similarly, there is no additional quenching of Chl singlet excited states if hFx is present. Yet, the triplet quenching, which has not been studied in detail in our paper, could still be affected by Fx-hFx exchange. The enhanced synthesis of hFx under high-light conditions is clearly proven, so there should be some advantage of having hFx in the antenna under high-light conditions. But the data we have collected so far do not give any clear answers to what this advantage actually is.

Pavel Chabera followed: In a "standard carotenoid model", it is believed that only groups attached to the conjugated backbone of a carotenoid can influence its excited state properties. How do you explain that the hexanol attached to the molecule but *not* conjugated to carotenoid's conjugated system can influence its properties? In particular, that it can switch off the ICT state?

Tomas Polivka answered: Yes, it is correct that we have always considered the groups that are not in conjugation to be spectroscopically silent. On the other hand, we know that spectroscopic properties of keto-carotenoids are strongly affected by solvent polarity. Thus, there is clearly a way to modify spectroscopic properties by something that is not a part of the conjugated system. Here, the attached hexanoyloxy tail obviously affects the electron distribution along the conjugated backbone, preventing stabilization of the ICT state.

Graham Fleming asked: Can you add a bit more detail on why you say charge transfer is necessary for rapid energy transfer?

Tomas Polivka responded: The coupling of the ICT state to the S_1 state modifies the transition dipole moment of the donor transition. It presumably enhances the transition dipole moment (as evidenced by *e.g.* increased S_1 fluorescence quantum yield of keto-carotenoids), making the donor–acceptor coupling stronger, resulting in more efficient energy transfer.

Heiko Lokstein remarked: There has been a lot of discussion recently about the so-called S* states of carotenoids. I wonder whether you have observed an S* state in either one of your investigated carotenoids, or, more specifically, whether there was any difference in S* transient absorption spectra between them.

Tomas Polivka answered: No, we have not observed any spectral features attributable to S* signal in these carotenoids. It is not so surprising as the S* signal is typically reported for carotenoids with long conjugation length, let's say about N=11 or longer. Fucoxanthin and its derivatives are much shorter.

Vasilios Stavros said: Tomas, you present some really interesting data. I was wondering whether you had considered looking at the influence of temperature on the excited state dynamics of hFx, as conformational flexibility may influence the role of S_1/ICT on excited state energy transfer.

Tomas Polivka responded: No, we have not studied temperature dependence so far though I agree it could provide valuable information as it indeed can reveal some extra information about the role of the hexanoyloxy tail in hFx. In the future, we plan to run experiments at both low temperature (down to about 200 K to keep the solution liquid) and high (up to about 325 K to prevent degradation).

Shuming Bai opened discussion of the paper by Scott Habershon: As I understand, you apply the machine learning diabatization to obtain an "on-the-fly" PES for the MCTDH method. My first question: In principle, other effective diabatization methods should be applicable in your approach, right? My second question: The universal diabatization method is still quite challenging for the systems with more than several atoms. For "on-the-fly" applications it can be more difficult because different configurations of state can join in along the dynamics, as you have found. Is it possible to monitor situations like this, and provide warning information during the calculation?

Scott Habershon replied: For the first question, the answer is yes; in principle, our approach is compatible with any diabatization scheme.

For the second part of the question, we agree that some sort of diabatization monitoring is essential; we are already thinking about this somewhat. In the projection diabatization scheme which we employ, for example, one can keep track of the energies of the different adiabatic states throughout the sampled configurations in our on-the-fly scheme; if adiabatic states change in energy such that they become relevant to the diabatization scheme (as in the example considered in our paper), then we can of course go back and re-run the dynamics with these additional states included. This procedure could be repeated until no further states are required. As an alternative, we are also investigating how one might include more than one set of reference electronic states in our projection diabatization scheme; this is ongoing work, however.

Bern Kohler asked: Have you considered applying your on-the-fly simulations to uracil, a nucleobase with well-understood internal conversion pathways?

Scott Habershon answered: Yes - that is exactly the sort of molecular system we'd like to be in a position to directly simulate using these on-the-fly methods. With 12 atoms, so a maximum of 30 active degrees-of-freedom (3N-6), this is at the upper-end of the system sizes we can currently model; however, the development of more efficient potential energy surface sampling schemes and improved software parallelisation should help us push towards this limit.

Natércia das Neves Rodrigues said: In the model you describe, do you have to restrict and select which coordinates to include or are you able to use the full 3N dimensional space?

Scott Habershon responded: Although the methods we describe can in principle be used to describe the full 3N-6-dimensional space of an N-atom molecule, the expense of *ab initio* calculations and propagation of the MCTDH equations-of-motion mean that one usually has to make a selection of the "active" vibrational coordinates. Various approaches towards this have been suggested previously, including the one in our contribution (DOI: 10.1039/c8fd00228b), but this aspect remains something of an open problem.

Natércia das Neves Rodrigues remarked: In my gas-phase experiments, after I pump my system with one photon, I then ionize the system in order to probe it. This ionization is effectively a second transition from the excited state to a given cationic state. I have found that the Franck–Condon factor for this transition—which changes in time as the wavepacket samples the excited state surface and is projected onto different parts of the cationic state surface—affects my experimental observable. In particular, the auto-correlation function which describes the time-dependent wavepacket behavior alone does not successfully model my experimental observations, as it does not account for the changing probe step Franck–Condon factor. Is it realistic that your models may be expanded to model this time-dependent projection of the excited state population onto the cationic state? Alternatively, do you know of any other methods which may model this behavior?

Scott Habershon answered: Yes, we believe that wavefunction methods like MCTDH could be used to model processes such as that described, although we expect it would be very challenging. For example, one could imagine introducing a time-dependent term to the Hamiltonian to simulate excitation to the ionic states at different stages during the MCTDH evolution. However, accounting for the configuration-dependent Franck–Condon factors seems like it would require further work; performing these "jumps" for trajectory-based methods might be more straightforward using Franck–Condon factors calculated at each configuration for each trajectory, but performing a similar simulation for a grid-based method would be much more demanding.

It is already possible within the MCTDH framework, using pre-fitted potential energy surfaces, to model time-resolved photoelectron spectroscopy experiments, so such an extension to the on-the-fly method should be possible. The cation potential energy surfaces can be fitted as for neutral states, and time-dependent fields can be applied to model laser pulses. So, assuming that the transition dipole (and polarisability, *etc.*, if necessary) matrix elements are also fitted, then excitations to the cation states my also be modelled. It is also necessary to account for the energy of the ejected electron; this can be achieved by introducing an extra discrete variable representation-type grid into which the electron wavefunction is excited, a so-called discretised continuum.

Jeffrey Cina asked: Could you please comment on the relative computational overhead of the MCTDH and electronic structure portions of these calculations?

Scott Habershon replied: In the initial calculation, where one is running a MCTDH simulation while simultaneously sampling configurations at which to perform *ab initio* electronic structure calculations, the computational burden lies towards the electronic structure calculations. In follow-up calculations, where one can perform an MCTDH simulation on the constructed potential energy surface, the computational expense lies firmly with the propagation of the MCTDH equations-of-motion.

It is also worth noting that the distribution of computational expense will be highly problem-dependent. For example, problems with many strongly-coupled degrees-of-freedom might require more *ab initio* electronic structure calculations in order to generate an accurate representation of the potential energy surface, such that this stage may be extremely costly. In addition, the exponential scaling of the MCTDH algorithm itself will eventually become the bottleneck in the simulations as the system-size increases. In the initial dynamics calculations reported here, using the normal modes chosen using forces and couplings, the overwhelming computational cost was in the electronic structure calculations. For each of the molecules studied in this paper (DOI: 10.1039/c8fd00228b), the percentage CPU times for the dynamics were: 0.35% (1a), 4.6% (2a), 0.11% (3a) and 0.5% (4a).

For the simulations of 4a using the 5 "steepest" normal modes, as well as the "false" normal mode directed towards the conical intersection, MCTDH accounted for about 40% of the total computational cost. This much larger proportion was due to the much larger DVR grids being used.

In addition to the point about highly-correlated potential energy surfaces being more difficult to fit, we also note that such systems also require more single-particle functions in the MCTDH calculation to account for the motion of the coupled modes.

Sharon Hammes-Schiffer questioned: How do you generate the initial conditions, and how sensitive are the results to them? Does MCTDH properly describe zero-point energy and the transfer of energy between vibrational modes?

Scott Habershon answered: The initial wavepacket is a Hartree product of ground-state harmonic oscillator eigenfunctions, centered at the minimum-energy geometry of the electronic ground-state. As such, harmonic zero-point energy is included in the initial wavefunction.

The MCTDH equations-of-motion are derived using the time-dependent variational principle. As such, they correctly describe zero-point energy conservation and other quantum-mechanical effects such as tunnelling. In addition, energy transfer between different vibrational modes is also captured (as long as this coupling is also described by the underlying potential energy surface, of course).

We have not investigated the effects of alternative initial conditions in this paper, although this is possible in the MCTDH method. For example, an initial imaginary-time relaxation of the wavepacket on the ground-state would account for any effects of anharmonicity on the shape of the initial wavepacket. This wavepacket can then be vertically promoted to the excited electronic state or, pushing towards higher accuracy, a laser pulse can be modelled as a time-dependent field which, allied with a representation of the transition-dipole

moment surface, allows a more realistic representation of the excitation. To adopt this more accurate representation of the excitation process in our on-the-fly method requires more work to model the transition dipoles, but this should be feasible.

Spiridoula Matsika asked: In this study you discovered that there was a problem with the chosen diabatization scheme because the DD-MCTDH predictions did not agree with the known ultrafast dynamics. Is there a way to test/know if your calculations predict the correct behavior if this behavior is not known *a priori*?

Scott Habershon responded: Yes - one can, in principle, compare the features of the diabatic and adiabatic states as an indicator of potential problems in diabatization. In addition, in one of the diabatization schemes used in this paper (projection diabatization) (DOI: 10.1039/c8fd00228b), one can monitor the adiabatic states as they change in energy across the range of sampled configuration space, allowing one to self-consistently update the manifold of states used in the diabatization scheme. Finally, although we have not investigated fully, we believe that the projection and propagation diabatization schemes certainly have room for improvement, for example by combining the two different approaches to seek a "best-of-both-worlds" approach.

Jack Woolley commented: Given that certain vibrational modes are required to traverse conical intersections, could this method, knowing the correct active space, be used as a black box software to explore how different chemical functionalities associated with the two nitrogen atoms influence the excited state dynamics? Recent experimental data on a model MAA and the MAA Shinorine shows subtle differences (see Fig. 1 below) suggesting that careful thought behind choice of functionality will be crucial in developing next generation, nature inspired sunscreens, and hence theory will be very important, to aid with functionalisation of core structures, like those discussed in the contribution.

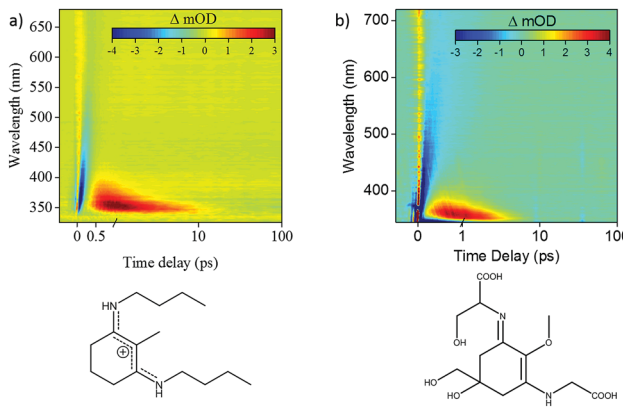

Fig. 1 (a) False colour heat map of transient absorption data, reported by Woolley *et al.*[1] for a similar system to those studied by Habershon and co-workers. (b) False colour heat map of transient absorption date of the MAA Shinorine.

1 J. M. Woolley, M. Staniforth, M. D. Horbury, G. W. Richings, M. Wills and V. G. Stavros, *J. Phys. Chem. Lett.*, 2018, **9**, 3043–3048.

Scott Habershon replied: Yes, the ultimate (if ambitious) aim of this line of research is to try to move towards a more "black box" method for modelling quantum chemical dynamics as a route to exploring the impact of different chemical functionality on molecular photochemistry. Such a method would enable design of photo-functional molecules such as sensors or switches. However, as noted in the question, barriers remain to this, such as the accuracy and expense of *ab initio* electronic structure calculations and methodological aspects such as the appropriate choice of active vibrational modes. Knowing which vibrational modes are essential to capture the experimentally-observed dynamics is very challenging, and may require a multi-method approach, such as using trajectory-surface hopping to inform the set-up of MCTDH simulations.

Todd Martinez asked: Is the diabatization process that you are using dependent on the path taken? If so, how do you deal with the resulting ambiguity?

Scott Habershon answered: In the propagation diabatization scheme, we propagate the diabatization matrix outwards from some initial geometry (usually the minimum-energy geometry on the lowest electronic state). In this scheme, the diabatization matrix is propagated using line integrals along the shortest-path between configurations (defined using simple Euclidean distance). As a result, there is some ambiguity here; if one changes the set of sampled configurations, then the propagation of the diabatization matrix will also change, and so the diabatic states will also change.

To date, we have not noticed this becoming a problem in our simulations. Our impression is that the density of configurations which must be sampled to generate an accurate potential energy surface implicitly means that there is little variation between the propagation of the diabatization matrix between different simulation runs. In addition, we have recently begun using Sobol grid sampling to generate configurations which are subsequently used to construct the diabatic potential energy surfaces and inter-state couplings; this means that the MCTDH dynamics is less susceptible to "jitter" in the constructed potential energy surfaces which might arise from differences in sampled configurations. This point is discussed more in a previous publication.[1]

1 G. W. Richings, C. Robertson and S. Habershon, *J. Chem. Theory Comput.*, 2019, 15, 857–870.

Vasilios Stavros said: You discuss possible factors that could influence the agreement between your calculations and those of Losantos and coworkers. One is the inclusion of more diabatic states in the projection scheme, which would lower the barrier along a relaxation coordinate. Do your calculations include solvation effects and if not, is it possible that the disagreement may also be due to the absence of these effects?

Scott Habershon replied: Our calculations do not include solvation effects, and are performed in vacuum. It is possible that solvent effects can change the character of the electronic states, and that could, of course, influence the accuracy of comparison with experiment.

Including solvation effects directly into our on-the-fly scheme is the next frontier here. We can see some ways forward, such as using an Ehrenfest-type scheme for mixed quantum/classical dynamics whereby the solvent molecules are modelled using classical molecular dynamics, but the on-the-fly nature of our approach might enable alternatives; we are actively investigating these aspects.

Peter Jomo Walla opened discussion of the paper by Heiko Lokstein: I would like to make a comment about the relative contributions of chlorophyll two-photon excitation and carotenoid two-photon excitation to the two-photon excitation spectra of LHC II and other pigment-protein complexes. In 2017 we addressed this question in a paper in which we explored relative cross-sections in two-photon excitation spectra of chlorophylls (Chls) as well as carotenoids.[1] After recognizing Dr Lokstein's contribution (DOI: 10.1039/c8fd00198g) I contacted him because, based on his reported results, he concluded that there is almost no contribution from carotenoid two-photon excitation to the two-photon spectra of LHC II. However, these results did not consider the two-photon excitations spectra of carotenoids as observed by us.[1] We therefore agreed to join forces and directly compare all two-photon results that we both have obtained in our previous studies. I have prepared a slide that summarizes the major conclusions from these comparisons (shown in Fig. 2).

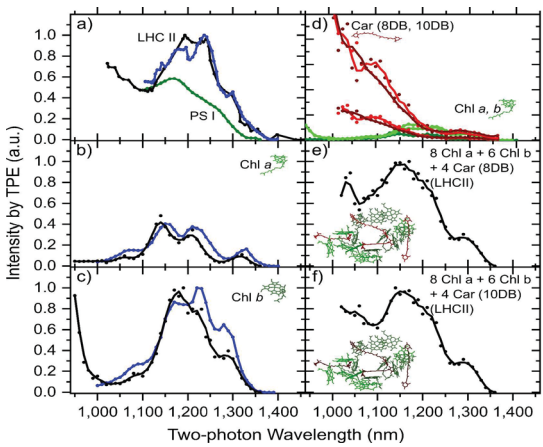

Fig. 2 Comparison of experimental two-photon excitation spectra of LHC II, PS I and isolated pigments. (a) Two-photon excitation spectra of LHC II by Walla *et al.*[2] (black curves) and Betke and Lokstein (blue) (DOI: 10.1039/c8fd00198g), as well as, PS I by Wehling *et al.*[3] (green). (b) and (c) Two-photon excitation spectra of isolated Chl a and b from Gacek *et al.*[1] and Betke and Lokstein (blue) (DOI: 10.1039/c8fd00198g). (d) Two-photon excitation spectra of carotenoids with 8 and 10 conjugated double bonds from Gacek *et al.*[1] (red) in comparison to two-photon excitation spectra of Chls *a* and *b*. (green). The relative magnitudes of the spectra in (d) reflect the relative contributions of the individual pigments to two-photon spectra in ref. 1. (e) and (f) LHC II two-photon excitation spectra reconstructed from summing experimental two-photon excitation spectra of 8 Chls *a*, 6 Chls *b* and 4 carotenoids (xanthophylls) from the individual pigment two-photon excitation spectra shown in (d). The protein and pigment structures were created with VMD: Visual molecular dynamics,[4] from a data set of Su *et al.*[5]

In general, the two-photon spectra observed in our work are in good agreement with those reported by Dr Lokstein. Fig. 2a link accordingly demonstrates that the LHC II spectrum reported in Betke and Lokstein (blue) agrees quite well with the one we reported in 2000 in Walla *et al.*,[2] (black in Fig. 2a) only that the spectral range reported in our publication is larger. For comparison, Fig. 2a also shows the two-photon excitation spectrum of PS I that we reported in 2005, for example.[3] Also the agreement of the two-photon spectra of Chl *a* reported in Gacek *et al.*[1] as well as Betke Lokstein (DOI: 10.1039/c8fd00198g) in solution (Fig. 2b, black and blue, respectively) and of Chl *b* in solution (Fig. 2c) is quite reasonable. Minor differences are likely due to different solvents that were used in both studies. A surprising result observed in Gacek *et al.* was that Chl *b* has, in general, a significantly larger two-photon absorption cross-section than Chl *a*.[1]

However, the larger spectral range reported for LHC II in Walla *et al.*[1] (Fig. 2a, black) demonstrates that there are more contributions from pigments other than Chls since the Chls two-photon absorption cross section drastically drops in the spectral range below 1100 nm (Fig. 2b and c). These additional signals from other pigments to the two-photon excitation spectra of LHC II can be readily explained by significant contributions from carotenoid dark states since the two-photon excitation spectra of carotenoids reported in Gacek *et al.*[1] are quite large in this spectral range (Fig. 2d).

Relative magnitudes of the two-photon excitation spectra of individual carotenoids and Chls were determined in Gacek *et al.*[1] Thus, it appears to be reasonable to reconstruct a LHC II two-photon excitation spectrum by using the sum of 8 Chls *a*, 6 Chls *b* and 4 carotenoids corresponding to the stoichiometric ratio of these pigments in LHC II. In fact, the agreement of these reconstructed spectra (Fig. 2e and f) with that of the experimental two-photon spectrum of LHC II (Fig. 2a) is quite reasonable.

The spectrum of PS I (green curve in Fig. 2a) demonstrates that the relative contribution of carotenoids may be even larger here. PS I contains exclusively Chl *a* (besides β-carotene), hence, this is in good agreement with the observation that Chl *a* has a smaller two-photon cross-section than Chl *b*.

The observations made by Dr Lokstein (DOI: 10.1039/c8fd00198g) that LHC II, containing different complements of carotenoids having also different numbers of conjugated double bonds, have very similar two-photon excitation spectra agrees well with our observation (Gacek *et al.*[1]), that carotenoids with different numbers of conjugated double bonds have surprisingly similar two-photon excitation spectra (Fig. 2d).

In summary, carotenoid two-photon excitation contributes significantly to the two-photon excitation spectra of PS I and LHC II below a spectral range of around 1100 -1200 nm. This observation also provides evidence that in both PS I and LHC II, excitation energy transfer occurs from carotenoid dark states to Chls; otherwise no Chl fluorescence would be observed after carotenoid two-photon excitation. Dr Lokstein and we agree to join forces and further explore our observations, in order to provide more quantitative information about what the contributions of individual pigments, Chls and carotenoids (xanthophylls) at various excitation wavelengths in two-photon excitation and absorption spectra of photosynthetic pigment-protein complexes are.

Dr Lokstein might also elucidate further, to what extend these insights as well as further observations obtained by his group support current suggestions about

the molecular mechanisms relevant in the regulation of photosynthetic light-harvesting.

1 D. A. Gacek, A. L. Moore, T. A. Moore and P. J. Walla, *J. Phys. Chem. B*, 2017, **121**, 10055.
2 P. J. Walla, J. Yom, B. P. Krueger and G. R. Fleming, *J. Phys. Chem. B*, 2000, **104**, 4799.
3 A. Wehling and P. J. Walla, *J. Phys. Chem. B*, 2005, **109**, 24510.
4 W. Humphrey, A. Dalke and K. Schulten, *J. Mol. Graph.*, 1996, **14**, 33.
5 X. Su, J. Ma, X. Wei, P. Cao, D. Zhu, W. Chang, Z. Liu, X. Zhang and M. Li, *Science*, 2017, **357**, 815.

Heiko Lokstein responded: I became aware of Dr Walla's 2017 paper[1] when I was contacted by him following the online publication of our current Faraday Discussions paper (DOI: 10.1039/c8fd00198g). We both do agree that our experimental data are rather similar, but our interpretations of these were considerably differing, namely regarding the relative contributions of carotenoids and chlorophylls (Chls) to the two-photon excitation (TPE) spectra of various photosynthetic pigment-protein complexes, and, in particular those of plant major light-harvesting complex II (LHC II). In fact, we concluded that there is (almost) no contribution from carotenoids to two-photon excitation spectra of LHC II. Moreover, we pointed out that direct TPE of (Bacterio)Chls in the presumed spectral region of the forbidden carotenoid S_1 ($2^1A_g^-$) state(s) was largely neglected in previous publications.

However, after discussions with Dr Walla and careful re-examination of our own data we both came to the conclusion that our previous bold statements have to be reconsidered. We therefore agreed to join forces and directly compare all the two-photon excitation results that we had obtained.

Indeed, Walla and co-workers had previously reported TPE data for a more extended spectral range, extending below 1100 (550 nm) for LHC II. These data may indicate that there may be contributions from pigments other than Chls (namely from dark states of carotenoids), since the Chls two-photon absorption cross section apparently drops in the TPE range below 1100 (550 nm) nm, possibly carotenoid contributions may even be apparent already at 1200 (600) nm.

Summarizing, we do agree that, apparently, carotenoid two-photon excitation can significantly contribute to the two-photon excitation spectra of photosynthetic pigment protein complexes, including PS I and LHC II, in particular in the spectral range below 1100 (550 nm), possibly even already at 1200 (600) nm, however this would need to be further quantified.

Dr Walla and we agreed to further examine our observations, in order to provide a more quantitative view of the contributions of individual pigments, Chls and carotenoids (xanthophylls), to two-photon excitation at various excitation wavelengths in photosynthetic pigment-protein complexes.

Moreover, we would like to stress that the mechanism of photoprotective non-photochemical Chl-fluorescence quenching (NPQ) proposed by Dr Wallas group, based on a combination of TPE and pulse-amplitude modulated fluorescence (PAM) experiments[2] would be consistent with our own observations: Non-linear polarization spectroscopy in the frequency domain (NLPF) experiments with LHC II in different states of aggregation (commonly serving as a model system for NPQ) indicated altered xanthophyll-Chl interactions: Upon pumping in the Chl *a*+*b* Qy spectral region (640–680 nm) and probing at selected wavelengths in the energetically much higher allowed xanthophyll absorption

region (490–510 nm) it was found that a xanthophyll molecule absorbing at ~510 nm strongly interacts with a Chl *b* absorbing at about 650 nm.[3] Upon aggregation of LHC II the picture changes completely: now the same xanthophyll molecule, probed at 510 nm, interacts with Chl(s) *a* absorbing at ~682 nm, comprising the terminal emitter in LHC II.[4]

1 D. A. Gacek, A. L. Moore, T. A. Moore and P. J. Walla, *J. Phys. Chem. B*, 2017, **121**, 10055.
2 S. Bode, C. C. Quentmeier, P. N. Liao, N. Hafi, T. Barros, L. Wilk, F. Bittner and P. J. Walla, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 12311.
3 B. Voigt, M. Krikunova and H. Lokstein, *Photosynth. Res.*, 2008, **95**, 317.
4 H. Rogl, R. Schödel, H. Lokstein, W. Kühlbrand and A. Schubert, *Biochemistry*, 2002, **41**, 2281.

Tomas Polivka remarked: Your detection of two-photon absorption relies on carotenoid-chlorophyll energy transfer. Can the absence of any carotenoid contribution to the two-photon signal in LHCII simply mean there is no carotenoid-chlorophyll energy transfer *via* the S_1 state in LHCII?

Heiko Lokstein responded: A very good question. Indeed, it seems that excitation energy transfer onto chlorophyll S_1 following supposed two-photon excitation of carotenoid S_1 states is somewhat inefficient (or even negligible) as also suggested by previous studies with purple bacterial LH2 complexes (*e.g.*, Krikunova *et al.*[1]) – but further experimental work is clearly required.

1 M. Krikunova, A. Kummrow, B. Voigt, M. Rini, H. Lokstein, A. Moskalenko, H. Scheer, A. Razjivin and D. Leupold, *FEBS Lett.*, 2002, **528**, 227–229.

Tomas Polivka addressed Peter Jomo Walla and Heiko Lokstein: From the data presented by Peter Walla it seems that there is onset of carotenoid contribution to the two-photon absorption in LHCII at significantly higher energies than expected. Do you think this imply that the energy threshold for carotenoid-chlorophyll S_1-mediated energy transfer channel is much higher than expected? Only hot S_1 contributes to energy transfer? Or even some other state?

Peter Jomo Walla responded: We were also surprised by this observation and it might indeed indicate energy transfer from hot carotenoid S_1 states. We have already speculated about this possibility in a paper in 2002 (Walla *et al.*[1], see also A. Wehling and P. J. Walla[2]). When the 0-0-transition of the carotenoid S_1 state is significantly higher than that of the tetrapyrrole acceptor, the contributions of the carotenoid S_1 0-0-transtion relative to hot states might be larger in two-photon excitation spectra. On the other hand, the reported two-photon excitation spectra (Gacek *et al.*[3]) might also indicate the presence of further dark states between the carotenoid S_1 and carotenoid S_2 state such as the putative carotenoid S_x or carotenoid S* states proposed by others. We currently cannot disentangle the exact nature of these states but the results provide evidence that there are dark carotenoid states, either hot S_1, S_x or S* states, that can efficiently transfer energy to tetrapyrroles and contribute to two-photon excitation in dyads and pigment-protein complexes.

1 P. J. Walla, P. A. Linden, K. Otha and G. R. Fleming, *J. Phys. Chem. B*, 2002, **106**, 1909.
2 A. Wehling and P. J. Walla, *J. Phys. Chem. B*, 2005, **109**, 24510.
3 D. A. Gacek, A. L. Moore, T. A. Moore and P. J. Walla, *J. Phys. Chem. B*, 2017, **121**, 10055.

Heiko Lokstein replied: It seems, taking also the data of Dr Walla and co-workers into account, that obviously two-photon absorption in LHC II starts at significantly higher energies than previously expected, (*i.e.*, for S_1 of the xanthophylls). In fact, we cannot exclude that the energy threshold for carotenoid-to-chlorophyll excitation energy transfer is much higher, so that possibly hot S_1 states or, even, some other, yet unassigned, state(s) contribute… Clearly, further experimental work is required.

Minjung Son addressed Heiko Lokstein and Peter Jomo Walla: Could you make some general comments on the sensitivity of this experiment as to whether it can distinguish between contributions from different carotenoid molecules, *i.e.*, whether the carotenoid is a β-carotene or a lutein, or whether the carotenoid has eight conjugated bonds or nine, for example?

Peter Jomo Walla replied: The two-photon excitation spectra of carotenoid-tetrapyrrole dyads containing carotenoids with different numbers of conjugated double bonds looked surprisingly similar (Gacek *et al.*[1]). Again, dominant contributions from hot carotenoid S_1 energy transfer might be an explanation in this case. Then, the cut off-wavelength in two-photon excitation spectra and energy transfer for each vibrational donor level would rather depend on the limiting energy level of the acceptor and not so much on varying 0-0-transtion energies of the carotenoid donors themselves. However, our studies also indicate that the overall efficiency of energy transfer depends on the number of conjugated double bonds or the carotenoid used, *i.e.* the relative contribution of the carotenoid donors to two-photon spectra varies compared to that of the tetrapyrrole acceptor. Two-photon excitation spectra of pigment-protein complexes reconstituted with different carotenoids that include more of the spectral range dominated by carotenoid dark states might provide further insights into that.

1 D. A. Gacek, A. L. Moore, T. A. Moore and P. J. Walla, *J. Phys. Chem. B*, 2017, **121**, 10055.

Heiko Lokstein answered: It appears that – regarding basically our data (see also Fig. 5 of the current paper (DOI: 10.1039/c8fd00198g)) – we cannot distinguish contributions from different carotenoids, or, whether the carotenoid has eight conjugated bonds or nine, or even more… This may, at least in part, also be related to inefficient excitation energy transfer to chlorophylls.

Stephen Bradforth asked: As your experiment uses two photons from the same beam, this limits somewhat the polarization information available. However, in your presentation you showed polarization ratio data comparing two circular polarized photons to two linearly polarized photons. Can you interpret what that ratio information is revealing?

Heiko Lokstein answered: The polarization-dependent experiment does not aim at obtaining information about (relative) orientations of the molecules. However, unlike in linear absorption, for two-photon absorption the polarization of the exciting light matters, even for randomly oriented molecules.[1] Thus, two-photon excited fluorescence was recorded for linear and circular excitation with otherwise identical measuring conditions.

The two-photon polarization ratio can be an indicator for the symmetry of the molecules investigated.[1] For the $S_0 \rightarrow S_1$ transition of a carotenoid (or linear polyene), a value of ~ 0.8 would be expected.[2] In our polarization measurements with LHCII, however, values between 1 and 1.2 were obtained for the spectral range of 610–690 nm and close to 1 below 600 nm. This would suggest that the excited molecules have, unlike carotenoids, a molecular symmetry different from the C_{2h} point group. Furthermore, the polarization ratio has been measured for pure chlorophyll *a* in the range between 660 to 680 nm. It was found to be comparable to the values for LHCII. These observations, in turn, can be taken as further indication that the two-photon-excited molecules in this spectral region are not carotenoids, but chlorophylls in LHCII.

1 W. M. McClain, *J. Chem. Phys.*, 1971, **55**, 2789–2796.
2 R. R. Birge and B. M. Pierce, *J. Chem. Phys.*, 1979, **70**, 168–178.

Vasilios Stavros addressed Heiko Lokstein and Peter Jomo Walla: In terms of the (simplified) energy level scheme presented in Fig. 2 (DOI: 10.1039/c8fd00198g), I would anticipate large spectral changes in the excited state absorption (ESA) following excited state energy transfer from the carotenoid to the chlorophyll. From the previous studies that you cite in your paper,[1–4] is there any evidence that such large spectral shifts are observed in, say, transient absorption studies? If so, I would expect this to be indicative of initial TPE of the carotenoid.

1 B. P. Krueger, J. Yom, P. J. Walla and G. R. Fleming, *Chem. Phys. Lett.*, 1998, **310**, 57–64.
2 P. J. Walla, P. A. Linden, C. P Hsu, G. D. Scholes and G. R. Fleming, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 10808–10813.
3 P. J. Walla, J. Yom, B. P. Krueger and G. R. Fleming, *J. Phys. Chem. B*, 2000, **104**, 4799–4806.
4 P. J. Walla, P. A. Linden, K. Ohta and G. R. Fleming, *J. Phys. Chem. A*, 2002, **106**, 1909–1916.

Peter Jomo Walla responded: Actually, there is also some evidence for carotenoid two-photon excitation from two-photon ESA studies. When exciting pure carotenoids at two-photon wavelengths corresponding to energies significantly below the allowed carotenoid S_2 absorption ($\lambda_{TPE}\sim$1200–1310 nm), carotenoid dark state ESA decay kinetics ($\lambda_{det}\sim$550 nm) are observed corresponding to the lifetime of the dark carotenoid S_1 state in the absence of any energy transfer (~9–15 ps). When repeating the same experiment with the carotenoids in bacterial light-harvesting complexes the carotenoid dark state ESA decay shifts to 2 ps in *Rb. sphaeroides*,[1,2] confirming the time constant for carotenoid dark state to bacteriochlorophyll energy transfer previously reported by Zhang *et al.*[3] In complexes with less overall carotenoid-chlorophyll energy transfer (*Rps. acidophila*) two-photon ESA constants are observed that are closer to the carotenoid S_1 lifetime in the absence of energy transfer ($\sim$ 7 ps). Also, different ESA kinetics are observed when exciting the same state energy by two- as well as one-photon excitation in green plants or algae. For example, the fast decay kinetics ($\sim$ 0.8 ps) observed in the spectral range of the carotenoid dark state ESA ($\lambda_{det}\sim$550 nm) with a two-photon excitation wavelength of $\lambda_{TPE}\sim$1150 nm is not observed in the same complex with one-photon excitation at $\lambda_{OPE}\sim$575 nm.[4] In both cases identical state energies are excited with both types of excitation but different kinetics are observed. This excludes that the same pigment, *i.e.* only Chlorophylls, is excited in both cases as exciting the same pigment at the same state energy should result in the same ESA kinetics. Again, the faster kinetics observed after two-

photon excitation in the spectral range of the carotenoid dark state ESA that is not observed after chlorophyll one-photon excitation of the same state energies corresponds to the time scales for energy transfer from forbidden carotenoid states to chlorophylls.

1 P. J. Walla, P. A. Linden, C. P. Hsu, G. D. Scholes and G. R. Fleming, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 10808–10813.
2 Walla, Fleming *et al.*, Ultrafast Phenomeny XII, 671–673, (2000).
3 J.-P. Zhang, R. Fujii, P. Qian, T. Inaba, T. Mizoguchi, Y. Koyama, K. Onaka and Y. Watanabe, *J. Phys. Chem. B*, 2000, **104**, 3683.
4 A. Wehling and P. J. Walla, *J. Phys. Chem. B*, 2005, **109**, 24510.

Heiko Lokstein answered: A very good question, indeed. I have to admit that we have not performed ourselves excited state absorption (ESA) studies following two-photon excitation of carotenoids, except for β-carotene in solution. However, as apparent from the discussion, it seems that excitation energy transfer to (bacterio)chlorophylls following two-photon excitation of carotenoids, at least in the spectral region above 1200 (600) nm, is inefficient (or even negligible). Thus, the origin of the previously observed ESA signals following (supposed) two-photon excitation of carotenoids in pigment-protein complexes may well have to be reconsidered, as also suggested by our current paper.

Graham Fleming asked: The potential surfaces of carotenoids are very complex and there may be multiple minima on a single electronic state surface such as S_1. In fact, in experiments we did some time ago we found that the transient infrared spectra of β-apo carotenal obtained by either exciting S_2 or two photon excitation of S_1 were not the same and the lifetime was different also.[1] Thus representing the energy levels by a single line can lead to significant ambiguity in my view.

1 Y. Pang, M. A. Prantil, A. J. Van Tassle, G. A. Jones and G. R. Fleming, *J. Phys. Chem. B*, 2009, **113**, 13086–13095.

Heiko Lokstein replied: Indeed, we have to admit that the (generally) used energy level schemes for carotenoids are somewhat over-simplified. There is also theoretical evidence that the states reached by different excitation schemes are not the same. However, the allotted presentation time precluded introduction of any more sophisticated representations!

Andrew Marcus opened a discussion of the paper by Spiridoula Matsika: You found that in general the distance between adjacent pyrimidine bases is one of the key factors that can affect the energies of charge transfer states and the rate of cyclobutane pyrimidine dimer (CPD) formation. Is CPD formation similarly sensitive to the local conformation (or relative orientation) of adjacent pyrimidine bases?

Spiridoula Matsika answered: This question has been addressed before by many studies. Studies by Schatz and co-workers examined that questions several years ago[1,2] and found that while the orientation plays some role, the most important factor is the distance between the two thymine double bonds. More recent studies have looked at many geometrical parameters in more detail,[3] and

they find other geometrical parameters affecting CPD formation but the distance is still the most significant.

1 M. McCullagh, M. Hariharan, F. D. Lewis, D. Markovitsi, T. Douki and G. C. Schatz, *J. Phys. Chem. B*, 2010, **114**, 5215–5221.
2 Z. Z. Pan, M. McCullagh, G. C. Schatz and F. D. Lewis, *J. Phys. Chem. Lett.*, 2011, **2**, 1432–1438.
3 I. Conti, L. C. Martinez Fernandez, L. Esposito, S. Hofinger, A. Nenov, M. Garavelli and R. Improta, *Chem.–Eur. J.*, 2017, **23**, 15177–15188.

Luis Ortiz-Rodríguez commented: It is known that DNA can adopt different conformations such as A-, B-, or Z-forms. In your work, it is mentioned that conformational restrictions exerted by the helical structure of DNA are involved in the stabilization of the charge transfer (CT) states. One parameter highlighted in the paper (DOI: 10.1039/c8fd00184g) is the distance between the stacking bases, which is very sensitive to the conformation of DNA. Could you comment on the DNA conformation selected in the investigation? Do other parameters such as the sugar pucker, intrastrand phosphate-phosphate distance and twist angle play an important role in the stabilization of the CT-states?

Spiridoula Matsika answered: The DNA conformation that we adopted in this study is a regular B DNA form. Other parameters may play some role in the stabilization of the CT states, but we did not examine them in detail. The distance however seems to explain the trends we observe, and there is a direct correlation between the distance and energy of the CT states, so our results indicate that distance is the most important parameter.

Marta Duchi remarked: McCamant's group have reported that the AMBER ff14SB and ff14 force fields designed for double stranded DNA do not accurately predict the conformations of single-stranded thymine due to the over-stabilisation of 5′-thymine in a *syn* conformation.[1] Do you observe similar effects in your molecular dynamics simulations?

1 C. Nganou, S. D. Kennedy and D. W. McCamant, *J. Phys. Chem. B*, 2016, **120**, 1250–1258.

Spiridoula Matsika responded: The study by McCamant focused on single stranded thymine strands and dinucleotides, and it was concluded that the current AMBER force field does not accurately predict the conformation of single-stranded thymine. Our work considers double stranded oligonucleotides which is expected to be much better represented by AMBER force fields. That said, we do not have a direct way to compare our results to experiments to verify the validity of the simulations. Our results, however, indirectly agree with experiment, and we believe this is a form of validation.

Bern Kohler said: You argue persuasively that a low-lying CT state can quench CPD formation by depopulating a dimer precursor (DP) state that would otherwise form the photoproduct. My question has to do with whether the probability that an initial excited state can reach the DP state in the first place could also be an important factor in determining sequence-dependent CPD quantum yields. In your earlier study,[1] you argue that the energetic proximity of the bright exciton state formed from the two $\pi\pi^*$ states of the two T bases to the DP state will

promote internal conversion to the latter state. However, it seems that the initial excited state could also decay to a non-DP state that is lower in energy such as an $n\pi^*$ state localized on either T. In addition, there is a bright $n\pi^*$ state localized on G that could decay to the DP state, possibly even resulting in some CPD formation. In a sequence like GTTA, one could also ask whether excitons associated with GT or TA mediate dimerization by the central thymines. Can you say anything about whether the many internal conversion pathways that seem to exist for the various kinds of initial excitations make it more or less likely to reach the DP state that is subject to quenching by the CT mechanism?

1 W. Lee and S. Matsika, *Phys. Chem. Chem. Phys.*, 2015, **17**, 9927–9935.

Spiridoula Matsika responded: It is absolutely true that there are many pathways present, and initial excitation can decay in many different ways instead of reaching the DP state. That is a reason that CPD formation in general has a very low quantum yield. In our studies we assume that these other pathways will be similar in the different sequences we studied, and in that case, what differentiates the sequences is the probability to decay from the DP state to the CT state. This seems like a reasonable assumption since the most important difference between the sequences is the energy of the CT states. A more complete study would have to examine all the different pathways and their competition, but that is a formidable task.

Vasilios Stavros stated: I have two questions/comments:

1. Am I correct in assuming that the role of the cation is simply as a spectator, or does it influence the energy of the charge transfer state?
2. Can you say a little more about solvation, specifically the size of the octahedral box used in your simulations, and what guided your choice of box size?

Spiridoula Matsika responded: 1. The cations can influence the energy of the CT state depending on their positions. However, they are expected to be found around negatively charged backbone in general (because they are positively charged), so their positions are very unlikely to be sequence dependent. Therefore, it is not likely that the cations make any significant contribution to the CT energy differences observed in our comparisons. In this sense, I think that, in our comparisons, it is safe to consider the role of the cation as a spectator.

2. For solvation, water molecules were added to make the minimum distance between any atom of DNA and the edge of the octahedral box be 8 ångstroms.

The number of water molecues added is different depending on the sequences, but they are usually around 8000–9000 molecules. For example, a total 8732 of water molecules were added for the TmCG sequence.

Stephen Bradforth commented: Your calculations show very subtle differences in the CT state energies. Have you performed a sensitivity analysis with respect to the solvation model on your results?

Spiridoula Matsika answered: That is a very good point. We have not performed a sensitivity analysis with respect to the solvation model. It is correct however that

the solvation model can affect the exact energies we see. We do not expect this to alter the trends we see and the conclusions we have made, but if we are interested in very accurate energetics we should explore the solvation model further.

Tom Oliver commented: Echoing the earlier comment made by Bern Kohler, the nature of the initially photoexcited state can greatly alter the yield of charge-transfer in DNA strands.[1] In our recent study of 2'-deoxyguanosine 3'-monophosphate 5'-thymidine, d(GpT) we observed the relative product branching into $d(G^{+}pT^{-})$ products was wavelength independent between 273–257 nm, and determined that the initial Franck–Condon excited states of the dinucleotide are significantly delocalised across both nucleobases.[2] Could you comment on the nature of the initially excited states predicted by your ADC(2) calculations, and any whether $^{1}\pi\pi^{*}$ or $^{1}n\pi^{*}$ states preferentially couple to charge-transfer states.

1 Y. Zhang, J. Dood, A. A. Beckstead, X.-B. Li, K. V. Nguyen, C. J. Burrows, R. Improta and B. Kohler, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 11612–11617.
2 M. Duchi, M. P. O'Hagan, R. Kumar, S. J. Bennie, M. Carmen Galan, B. F. E. Curchod and T. A. A. Oliver, *Phys. Chem. Chem. Phys.*, 2019, DOI: 10.1039/C8CP07864E.

Spiridoula Matsika replied: The initially excited states in the Franck–Condon region in our calculations had various degrees of delocalization along two bases. Some of them were localized on one base, and some were delocalized over two bases, either the two pyrimidine bases, or in some cases between thymine and the adjacent guanine base. In agreement with the above ref. 2, we also see that the nature of the vertical excited states is very dependent on the specific geometry and relative orientation of the bases. The charge transfer states we see involve π orbitals, so they are mainly coupled to the $\pi\pi$ states.

Carlos E. Crespo-Hernández remarked: According to the results presented in Fig. 3 in your paper (DOI: 10.1039/c8fd00184g), the energy of the charge transfer state of a given sequence varies by more than 1 eV depending on the specific molecular dynamics snapshot used in the calculation. Can reliable inferences be made using an average charge transfer energy that is based on 20 snapshots given that the standard deviation is 0.2 to 0.4 eV for each of the sequences studied in this work? In other words, is the averaging of 20 snapshots accurate enough to represent the average charge transfer energy of a given sequence relative to the average energy of the other sequences? How sensitive are the average charge transfer energies, and the standard deviations, to the number of snapshots used in the calculation?

Spiridoula Matsika replied: The strong variation of the energy of the CT states with the snapshot is physical and insightful since it shows how sensitive the CT states are to the environment. We believe the average to be qualitatively correct, especially for this type of study, which compares different systems. So we expect the trends observed in this study to be preserved even if we do larger simulations with more than 20 snapshots. The average is probably not statistically converged, but given the expense of the calculations this is the best that can be done at the present time.

Carlos E. Crespo-Hernández followed: The reported one-electron reduction potentials for cytosine and thymine shown in Table 1 are practically identical taking into consideration the theoretical and experimental accuracy of the methods used

in ref. 48 and 49 in your paper (DOI: 10.1039/c8fd00184g), respectively. Then, the significantly different average charge transfer energy predicted for the GTC/GCT and GTT/GCC pair of sequences seems surprising, given that the average distance between the two bases involved in charge transfer does not vary by more than 0.1 ångstroms according to Fig. 4 in your paper. Are other base stacking geometric parameters (such as tilt, twist, shift, slide, *etc.*) important in the determination of the average charge transfer energies?

Spiridoula Matsika responded: The difference in the experimental reduction potentials between thymine and cytosine is actually substantial. And it is well established that thymine is reduced more readily than cytosine. This we believe is one component leading to the difference in the energies of CT states. The distance seems to be another parameter since shorter distances are observed between GT compared to GC. So, these two effects contribute in the same direction of affecting the energy of CT states. We think the additive effect makes the shift on the CT states stronger. Other geometrical parameters may play some small role, but we have not explored that in this work. It is worth examining in more detail in the future.

Shou-Ting Hsieh communicated: The research is interesting, and sorry for such a naive question. I just think that our DNA sequence was determined when we were born (neglecting the probability of mutation). Therefore, even though we know some people have a sequence that may cause CPD easily, it is hard to do any "modification". I would like to know what kinds of method may be developed based on this research. I guess that one day in the future, maybe we can predict the probability of CPD formation based on the DNA sequence. As precision medicine becomes more important, this may allow people to know with how much care they need to prevent UV light.

Spiridoula Matsika communicated in reply: We are still far away from being able to predict the probability of CPD formation based on someone's DNA sequence and offer solutions. But progress is made in small steps, and we hope that the current fundamental research such as ours and that of others can help in making progress towards this direction. By the way, there are experimental groups that try to determine experimentally the effect of sequence on CPD formation, such as the group of Prof. John-Stephen Taylor at Washington University.

Tom Oliver opened discussion of Christopher Grieco's paper: What is the mechanism for the very fast intersystem crossing you observe in the quinone moiety?

Christopher Grieco replied: The very fast intersystem crossing process appears to occur from the $n\pi^*$ singlet state of the quinone to the triplet manifold. Although we are directly exciting to the $\pi\pi^*$ singlet state in our experiments, the observation of the two excited state absorption bands described in the paper at the earliest resolvable time delay is evidence that internal conversion from the $\pi\pi^*$ singlet state to the $n\pi^*$ singlet state occurs very rapidly. In accordance to the El-Sayed rules, the intersystem crossing process likely occurs from this state to a $\pi\pi^*$ triplet state. Because of the rapid time constant for this process, we consider that the energy levels of these two states may also be close. However, we currently do

not have data to support this. We believe quantum mechanical calculations would provide much further insight into the intersystem crossing mechanism.

Matthew Turner said: In a previous paper[1] you suggested that, when in cyclohexane, 4-*t*-butylcatechol could aggregate through two possible pathways; a "linear" and a "bifurcated" hydrogen bonding structure. In contrast, in this current paper (DOI: 10.1039/c8fd00231b) you discuss the dimer of 3,5-di-*t*-butylcatechol (C) + 3,5-di-*t*-butyl-*o*-quinone (Q) but only consider the "linear" structure. Is it possible the "bifurcated" dimer could form and if so how would this effect the interpretation of your results?

1 C. Grieco, F. R. Kohl, Y. Zhang, S. Natarajan, L. Blancafort and B. Kohler, *Photochem. Photobiol.*, 2019, **95**, 163–175.

Christopher Grieco responded: In the paper, we studied intermolecular hydrogen bonding interactions that occur within 4-*tert*-butylcatechol aggregates in cyclohexane solutions. The "bifurcation" refers to an intermolecularly hydrogen-bonded O-H group in a catechol molecule that can simultaneously donate an intermolecular hydrogen bond to another neighboring catechol molecule (see Fig. 3a). In this *Faraday Discussions* paper (DOI: 10.1039/c8fd00231b), we considered a linearly hydrogen-bonded structure between Q and C. In this case, we argue (see the text of the paper) that a single O-H group is unlikely to hydrogen bond to both of the carbonyl groups in Q simultaneously (see Fig. 3b). We consider that a bifurcated structure in which the intramolecularly hydrogen-bonded O-H group in C can also donate a hydrogen bond to a carbonyl group in Q is also unlikely (see Fig. 3c). This is because forming a heterodimer of C and C causes the free O-H band in the FTIR spectrum to decrease in intensity, which signifies that the free O-H group must be donating a hydrogen bond.

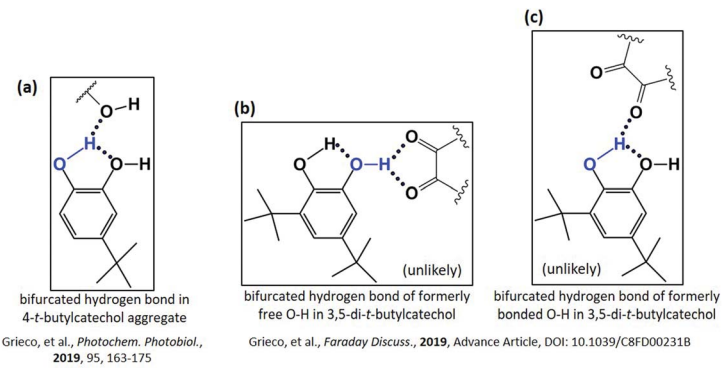

Fig. 3 (a) Possible bifurcated hydrogen bond structure in a 4-*t*-butylcatechol aggregate. The O–H group that intramolecularly hydrogen bonds to the other O–H group in the catechol also intermolecularly hydrogen bonds to an O–H group of a neighboring molecule. (b) Bifurcation of the intermolecular hydrogen bond between an O–H group of 3,5-di-*t*-butylcatechol and two carbonyl groups in a neighboring 3,5-di-*t*-butyl-*o*-quinone molecule. (c) Bifurcated hydrogen bond structure for the O–H group in a 3,5-di-*t*-butylcatechol molecule that retains its intramolecular hydrogen bond while simultaneously donating an intermolecular hydrogen bond to one of the carbonyl groups of a 3,5-di-*t*-butyl-*o*-quinone molecule.

1 C. Grieco, F. R. Kohl, Y. Zhang, S. Natarajan, L. Blancafort and B. Kohler, *Photochem. Photobiol.*, 2019, **95**, 163–175.

Carlos E. Crespo-Hernández said: Fig. 2 in your paper (DOI: 10.1039/c8fd00231b) shows that the Q : C heterodimer can be excited selectively from the Q and C monomer components in the Q + C mixture at wavelengths longer than 450 nm. Even though the two-state model that you describe for discriminating the transient absorption spectra produced by the monomers and heterodimers is reasonable, I wonder if you had considered exciting the mixture at 450 nm or longer wavelengths in order to measure directly the spectral and excited-state dynamics of the Q : C heterodimer independently of those of the Q monomer?

Christopher Grieco answered: The process of discriminating the transient absorption spectra produced by the monomers (Q+C) and heterodimers Q : C is an effective, but undesirable method for studying the excited state processes in the Q : C heterodimer. This is because it requires multiple transient absorption measurements to be made, which can intrinsically carry error into the final spectrum determined for Q : C due to the spectral subtraction process involved. A more desirable measurement is as you describe, and it appears possible if exciting with 450 nm. This experiment is worth trying. However, there is a small amount of Q monomer absorption present at 450 nm, and it may still contribute to the transient absorption signal. In particular, the absorption % by Q monomers in the Q+C mixture at 450 nm is ~17%. Using a longer wavelength, such as 500 nm, would be challenging because of possible resonance with the n→pi* transition. Measuring a higher concentration of Q+C would shift the solution equilibrium to Q : C and favorably change the relative absorption of Q and Q : C at 450 nm (*i.e.* > 30 mM Q + 30 mM C). Provided that other experimental conditions can be accommodated, such as a smaller sample path length, the experiment using 450 nm could then be ideal.

Carlos E. Crespo-Hernández asked: In the introductory remarks of your presentation, you described that eumelanin exhibits a complex chemical heterogeneity in which stacking and hydrogen-bonding interactions are important. Could you comment on how stacking and other intermolecular interactions in eumelanin may modulate the excited-state dynamics and the formation of neutral semiquinone radicals you elucidated in the Q : C heterodimer model system?

Christopher Grieco answered: Intermolecular interactions, such as π-stacking, that are present in eumelanin are expected to modulate the excited state dynamics and the formation of neutral semiquinone radicals. For example, alternative, competitive excited state pathways may turn on due to the presence of these different intermolecular interactions. We are actively studying these processes, and believe that the rapid (~10 ps) decay of the excited states in eumelanin materials may involve deactivation pathways that are distinct from what we observe in the Q+C model system studied here (photophysical relaxation *versus* photochemical change). Regardless of the differences between eumelanin and our model system, we believe that it is possible that some of the excited states in eumelanin follow a similar hydrogen atom (net) transfer reaction as in the model

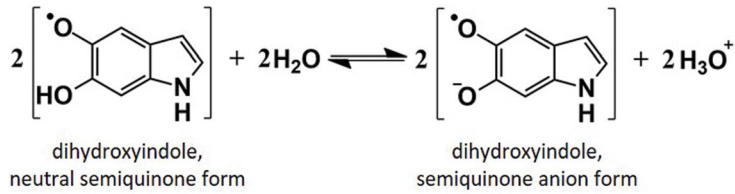

Fig. 4 Redox equilibrium of the neutral and anionic semiquinone forms of 5,6-dihydroxyindole in aqueous solution.

system. To add to this discussion, the neutral semiquinone of the eumelanin precursor molecules are known to undergo a subsequent proton transfer event that could lead to a metastable semiquinone radical ion, which may play an additional role (see Fig. 4). This can occur in eumelanin because its structure is likely to be more polar than the Q and C compounds, and is suspended or dissolved in a polar solvent (water).

Michael Ashfold commented: The photochemistry reported for the benzoquinone:catechol (Q : C) heterodimer is reminiscent of that displayed by other hydrogen-bonded organic chromophores, where Q is the hydrogen-bond acceptor and C the hydrogen-bond donor. [1-3] UV excitation typically populates a $\pi\pi^*$ state, in which the π and π^* orbitals are both localized on either Q or C –forming a configuration that is commonly termed a locally excited (LE) state. The UV absorption data shows that the orbitals involved in the $\pi^* \leftarrow \pi$ transition in the Q : C heterodimer are localized on Q (DOI: 10.1039/c8fd00231b). Analogy with the benzoquinone-water (Q:H_2O) complex, for example, suggests that this $\pi\pi^*$ LE state will evolve to a $\pi\pi^*$ charge-transfer (CT) state with the π-hole localized on C and a π^* electron on Q.[4]

This photoinduced charge separation affects the subsequent hydrogen migration from C to Q.[1] But, as noted elsewhere in this Discussion (DOI: 10.1039/c8fd00240a), the nature of the migrating species (*i.e.* proton *vs.* hydrogen atom) is sensitive to the non-adiabatic coupling between the LE and CT states and thus to the topography of the adiabatic excited state potential energy surface (PES). If the diabatic PESs of the LE and CT states associated with the minimum energy reaction path (MERP) are within the Marcus normal region, then the state reached by $\pi^* \leftarrow \pi$ excitation at the Franck–Condon (FC) geometry has dominant LE character and non-adiabatic coupling between the LE and CT states is weak. The migrating species is likely to be hydrogen atom-like: the H atom 'carries' the electron from C towards Q, and the excited adiabatic PES only gains appreciable CT character after the O-H bond has started extending. In contrast, if the diabatic PESs of the LE and CT states associated with the MERP are barrierless or within the Marcus-inverted region, excitation at the FC geometry samples a region of strong non-adiabatic coupling between the LE and CT states and the migrating species will tend to be proton-like. In this case, an intermolecular Q^-–C^+ charge separation is established at very early time, which is then neutralized by proton-transfer from the O-H group of C to the O atom of Q.

In the case of Q:H_2O, the calculated LE and CT $^1\pi\pi^*$ states show a Marcus inverted evolution along the Q….H–OH…. coordinate.[2] The analogous singlet

reaction path in Q : C can be expected to be Marcus inverted also, since catechol is more acidic than water and thus a stronger proton-donor. The calculated $^3\pi\pi^*$ reaction path in Q:H_2O is more reminiscent of normal Marcus behavior.[2] The barriered nature of this process is likely to extend to Q : C also and offers a possible explanation for the deduced ns timescale of the hydrogen migration on the triplet PES. The present data for Q : C heterodimer can be expected to inspire future computational studies of this and related systems.

1 T. Schultz, E. Samovlova, W. Radloff, I.V. Hertel, A.L. Sobolewski and W. Domcke, *Science*, 2004, **306**, 1765–1768.
2 M.Barbatti, *J. Am. Chem. Soc.*, 2014, **136**, 10246–10249.
3 T. Kumpulainen, B. Lang, A. Rosspeintner and E. Vauthey, *Chem. Rev.*, 2017, **117**, 10826–10939, and references therein.
4 T.N.V. Karsili, D. Tuna, J. Ehrmaier and W. Domcke, *Phys. Chem. Chem. Phys.*, 2015, **17**, 32183–32193.

Christopher Grieco responded: We thank you for your in-depth, insightful analysis. We look forward to future computational studies of the processes you describe. Your discussion also inspires future experimental studies in different solvent environments that may perturb the CT state energy in a predictable way, forming an additional way to test the models you discuss.

Some additional responses: does the charge separation process compete with internal conversion from $\pi\pi^* \rightarrow n\pi^*$ in Q? The $n\pi^*$ state is possibly lower in energy than the $\pi\pi^*$ state like in xanthone (for example, see Satzger *et al.*[1]). Our transient absorption spectra of Q monomers in different solvents show peaks that match optical transitions expected for the $n\pi^*$ state of Q. Future computational studies may be useful for determining whether these spectral assignments are correct and to help determine how the $n\pi^*$ state plays a role in the excited state pathways in Q.

1 H. Satzger, B. Schmidt, C. Root, W. Zinth, B. Fierz, F. Krieger, T. Kiefhaber and P. Gilch, *J. Phys. Chem. A*, 2004, **108**, 10072–10079.

Todd Martinez asked: Is there any experimental information about these processes in protic solvents?

Christopher Grieco replied: Such experimental information would be very useful, but no experiments on Q : C heterodimers have been made in polar solvents yet. This appears difficult to study because the dimerization constant is expected to depend on solubility, which changes with solvent polarity. For example, our unpublished results show that the Q : C heterodimers form in much smaller yields in a polar solvent, chloroform, compared to in cyclohexane. For other polar, protic solvents, it is difficult to establish whether a significant population of heterodimers form because such solvents typically contain O–H or N–H groups whose IR spectrum interferes with that of the solute. These groups also compete with hydrogen bonding to the Q molecule and can also lead to lower Q : C heterodimer yields. Increasing the Q : C formation yield may require exceptionally high concentrations of solute (*e.g.* $\gg$ 100 mM) that may not be feasible in experiments. We note that changes in the UV-vis spectrum may be used to quantify Q : C heterodimer formation yields in protic solvents because the Q spectrum is sensitive to changes in solvent polarity, but this is a less direct method.

Alternatively, the problem of reduced Q : C heterodimer formation yield in polar solvent environments may be solved by future studies of covalently linked Q and C dimer compounds, such as the ones studied by Van Ahn and Williams.[1] This is because spatially constraining the Q and C pair may favor intermolecular hydrogen bonding between them.

1 N. Van Anh and R. M. Williams, *Photochem. Photobiol.*, 2012, **11**, 957–961.

Andrew Marcus remarked: Your model for the photo protection and radical scavenging mechanisms of eumelanin focus on the chemical interactions between DHI and DHICA. In a living organism, could these molecular interactions be mediated in a significant way by the presence of proteins or other biochemical species?

Christopher Grieco answered: While the presence of proteins may affect the molecular interactions in eumelanin in living organisms, we believe they may not generally be significant. The eumelanin structure is believed to consist of large agglomerates of nanoparticles that are hundreds of nanometers in size. These nanoparticles are believed to consist of aggregated structures containing the DHI and DHICA moieties. For example, see Fig. 4 in Watt *et al.*[1] These structures are expected to predominantly include intermolecular interactions between eumelanin chromophores that likely give rise to many of its photoproperties. However, it is possible that the photochemistry at the surface of the particles, which may experience interactions with the aqueous solvent environment and surrounding proteins, could be modulated and would be interesting to study. Indeed, proteins have been shown to bind to eumelanin.[2]

1 A. A. R. Watt, J. P. Bothma and P. Meredith, *Soft Matter*, 2009, **5**, 3754–3760.
2 S. Sharma, S. Wagh and R. Govindarajan, *Pigment Cell Res.*, 2002, **15**, 127–133.

Michael Haggmark commented: In the last scheme of the paper, there is a lifetime associated with the complex separating. I was wondering if the melanin polymer is confined and as such unable to separate, what dynamics would follow. That is to say, would it be a more faithful model system due to the confinement? Have you considered extending this experiment to more confined conditions, perhaps in a glassy matrix? It would make a very interesting follow up project.

Christopher Grieco responded: The likelihood for solvent-separation of the semiquinone (SQ) radical pair is certainly a process that can change the rate of decay of the radicals to restore Q and C. We agree that a model in which the SQ radicals cannot spatially separate would be a more faithful system for studying the characteristics of the SQ radical pair. However, in our paper we are primarily interested in the dynamics that precede formation of the SQ radical pair, and believe that the Q+C system is a faithful model system for these processes. We agree that it would very interesting to follow up on the work in this paper by studying Q+C in a more confined condition. Two approaches come to mind: (1) study the complex in a glassy matrix (as you've suggested), such as a glass-forming solvent; (2) study a covalently tethered Q+C system, such as those studied by Van Ahn and Williams.[1] Each system has its limitations: in the first, one would need to ensure that heterodimers form, and may be limited to experiments using lower

temperatures; in the second one, the molecules may still be able to spatially separate (become solvent separated). Regardless, we believe both systems would likely provide valuable information about the reactivity of the SQ radical pair.

1 N. Van Anh and R. M. Williams, *Photochem. Photobiol.*, 2012, **11**, 957–961.

Vasilios Stavros remarked: Chris, you show some really beautiful data. My question follows from the previous comment regarding restricting the position of the C/Q pair. Have you considered increasing the viscosity of your solvent? I would expect the time for separation of your contact pair to increase from 14 ps due to the imposed friction.

Christopher Grieco answered: We have considered increasing the viscosity of the solvent to see if we observe an increase of the separation time of the contact semiquinone (SQ) radical pair, but we have not yet designed or attempted any experiments. The chosen solvent would not only need higher viscosity, but must also support the formation of Q : C heterodimers in large enough quantities to measure. Such an experiment would be very insightful and help provide better evidence for spatial separation of the SQ radical pair. Your question reminds me of an alternative model we previously considered (not discussed in the paper) that may explain the spectral narrowing of the SQ absorption band: intersystem crossing. For example, after the net hydrogen transfer event has occurred, the total multiplicity of the contact SQ pair may be singlet. It is possible for a subsequent spin-flip to occur to form a triplet SQ radical pair state. As such, this process may be confused with spatial separation of the SQ radical pair. We currently favor the latter process, but are interested in future experiments that can help support our interpretation of the spectral narrowing.

Vasilios Stavros followed: Am I correct in saying that you do not see any sharpening of the radical feature in Fig. 4d in the paper (due to vibrational cooling) (DOI: 10.1039/c8fd00240a) because of the much slower formation of a neutral radical from the triplet-mediated HAT channel (>1 ns)?

Christopher Grieco answered: What you are saying is expected because the sharpening of the radical feature at 370 nm would occur with a much faster rate than the formation of the neutral radicals from the triplet-mediated hydrogen atom transfer channel. In other words, our resolution of spectral sharpening would be poor. However, it would be interesting if we could observe spectral sharpening of the radical feature after it forms from the singlet-mediated hydrogen atom transfer channel, which occurs much more quickly. Unfortunately, strong signal overlap from excited singlet and triplet states of Q also absorb in the same spectral region that make it difficult to discern the peak at 370 nm. We envision that a time-resolved vibrational spectroscopy experiment could be used to resolve vibrational cooling of the neutral radical by monitoring dynamic shifts in the vibrational frequency of a marker band (if one could be found).

Conflicts of interest

There are no conflicts to declare.