Faraday Discussions
Cite this: *Faraday Discuss.*, 2015, **177**, 381

Local and Global Dynamics: general discussion

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DOI: 10.1039/C5FD90018B

Kenneth Ghiggino opened a general discussion of the paper by **Stephen Meech**: You refer to the origin of the non-exponential kinetics you observe in the BLUF proteins as arising from a distribution of ground state structures (or rotamers). Are you able to expand on what other evidence is available, or could be obtained, to confirm this hypothesis?

Stephen Meech responded: The evidence for multiple conformations of amino acids around the isoalloxazine ring was summarized by Udvarhelyi and Domratcheva.[1] There are experimental differences between NMR and X-ray structures, especially regarding Y21 and W104, and calculations show multiple conformers of Q63, separated by low barriers. It will be interesting to investigate the correlation of non-exponentiality and photoactivity as a function of temperature.

1 A. Udvarhelyi and T. Domratcheva, *J. Phys. Chem. B*, 2013, **117**, 2888–2897.

Neil Hunt asked: What was the rationale for the specific choice of the Y21 mutants used? What is the effect on the H-bonding structure observed in the wild-type protein upon mutation; is this conserved or broken?

Stephen Meech answered: The key point of these Y21 mutations is to remove the H-bond between Y21 and Q63. This was already known to be critical in photoactivity, and the effect is to trap the BLUF domain in its dark adapted state (as judged by the S0 to S1 absorption spectrum). All the Y21 mutants we have studied achieve this. The specific mutants were chosen to see the effect of polarity

(Y21I compared to Y21S) and electron donating ability (Y21C and Y21F (data not shown) compared to Y21I). The only Y21 mutant which behaved differently was Y21W, where the Trp to FAD electron transfer was clearly observed in TRIR and electronic spectroscopy, although the protein remained photoinactive.[1]

1 A. Lukacs *et al.*, *J. Am. Chem. Soc.*, 2014, **136**, 4605–4615.

John R. Helliwell remarked: On page 3 of your article you refer to a "disagreement among the published (protein) crystal structures with regard to the important (amino acid) residues Q63 and W104". This might be accounted for from possible differences between diffraction (and spectroscopic) measurements at 100 K and room temperature, *i.e.* for which structural differences in conformation and in structural dynamics do occur in proteins. See for example the references below (although unfortunately not for your studied proteins):

1 A. Deacon, T. Gleichmann, A. J. Kalb (Gilboa), H. Price, J. Raftery, G. Bradbrook, J. Yariv and J. R. Helliwell, The structure of concanavalin A and its bound solvent determined with small-molecule accuracy at 0.94 Å resolution, *Faraday Transactions*, 1997, **93**(24), 4305–4312.
2 Simon W. M. Tanley and John R. Helliwell, Structural dynamics of cisplatin binding to histidine in a protein, *Struct. Dyn.*, 2014, **1**, 034701.

E. D. Jemmis queried: Intramolecular ene-reactions with high specificity are known in organic chemistry, but achieved with difficulty. The specific template provided by the enzyme in your example makes this very easy. Any comments on this specific reaction to organic chemists, other than the evolutionary perfection that the enzyme has achieved?

Stephen Meech replied: Your question already hints at my answer. Proteins are typically exquisitely well adapted for their precise function, but their 'skills' are not always very transferable. I think that is the case here, where the mechanism we propose requires a difference in H-bonding ability between FAD ground and excited states, and a rather specific H-bonding arrangement among the key amino acids. It would be very challenging to reproduce this synthetically. Having said that, a synthetic system involving the isoalloxazine ring substituted with a moiety exhibiting intramolecular H-bonding might be an interesting one to study.

Himani Medhi asked: What do you mean by the Marker modes for the excited state decay and ground state recovery?

Stephen Meech responded: Through a study of model compounds we can identify signals at particular frequencies in the transient difference spectra where the dominant contribution is from one species. For example 1380 wavenumbers are dominated by the singlet excited state of the isoalloxazine ring, so this signal is the 'marker' for that state. If we follow its time dependence it tells us about the

decay (or formation) of that state. We find similar markers for the ground state recovery (1548 cm^{-1}) and the radical intermediate (around 1530 cm^{-1}).

Srihari Keshavamurthy opened the discussion of the paper by **Biman Bagchi**: In case of the reverse micelles, it seems like the frequency–frequency correlation functions already give a rather detailed insight regarding the heterogeneity inherent to the system. What additional/different insights does one obtain from the 2D-IR studies and results?

Biman Bagchi answered: I like the question and this was also my own initial response to 2D-IR and higher order non-linear techniques. I am surprised that more questions like this have not been asked. Yes, the core quantity remains the frequency–frequency time correlation function (FF-TCF). The strength of 2D-IR lies in its ability to use spectral differences among different "chromophores" in different locations to achieve the desired spatial resolution. Then one can associate a time constant to a given location. This is not possible easily by other methods. We can of course calculate FF-TCF but we need to measure it.

Arend G. Dijkstra remarked: Professor Bagchi, you are discussing the calculation of two-dimensional infrared spectra of water. Now, in water, all molecules will have similar vibrational frequencies. When two water molecules are close together, they will be coupled. This leads to the delocalization of the vibrational excitation, which should be observable in the 2D-IR spectrum. In you theoretical analysis, you use the line shape function, which assumes that the system Hamiltonian commutes with the system bath interaction. Therefore, you don't include the delocalization effect. Could you comment on this?

Biman Bagchi replied: Of course. We are effectively considering a dilute solution of H–O–D and –O–D stretch. We neglect the difference between –O–H and –O–D which could be significant. If I have –O–D stretch in a dilute solution, that will weakly couple with the surrounding –O–H stretch. So, by neglecting the coupling and consequent delocalization among the –O–H stretch, we are essentially mimicking the –O–D stretch. One can of course do –O–D stretch.

R. J. Dwayne Miller commented: Your calculations of the 2D-IR spectra of liquid water were for pure H_2O; yet the comparison is being made to H–O–D experimental studies in terms of probing the hydrogen bond dynamics. However, for pure H_2O, the O–H stretch is resonantly coupled to adjacent waters and this resonant coupling greatly enhances the overall coupling to all the bath modes, collective modes of the liquid water state. We developed nanofluidic cells to enable the study of pure H_2O and the spectral diffusion is much faster than what you are calculating (Cowan *et al.*, *Nature*, 2005).[1] Generally, it was thought that the energy transfer for the purely resonant case for neat H_2O would dominate the spectral diffusion dynamics, *i.e.* the spatial propagation of the O–H vibrational

excitation would average out structural variations in the water structure in relation to hydrogen bond dynamics. We did a temperature dependence in which the energy transfer rate is unaffected and found that the spectral diffusion dramatically slowed down at lower temperatures within changes as small as 10 degrees (Kraemer *et al.*, *PNAS*, 2008).[2] This study demonstrated that the spectral diffusion and relation to the hydrogen bond dynamics, under the full resonant conditions of neat water, is extremely sensitive to the details of the degree of hydrogen bonding. We ascribed the fast spectral diffusion in H_2O to the higher frequency collective librational modes of liquid water, consistent with the observed times scales, and the degree of hydrogen bonding with respect to the rms motion of such modes. The bottom line is that the vibrational modes of pure water, under fully resonant conditions, are extremely sensitive to relatively small changes in the correlation lengths within the hydrogen bond network. It would be great if just the temperature dependence for the 2D spectra of liquid H_2O could be recovered. The problem is in treating the excitonic coupling and the accuracy of the potentials used. We developed a split operator approach (Paarmann *et al.*, *J. Chem. Phys.*, 2009)[3] that handles this aspect of the problem. This aspect of the problem can be treated fairly well with this approach. My understanding (personal discussions with James Skinner, University of Wisconsin) is that it is very difficult to treat the temperature dependence of neat water as the potentials are highly tweaked for the room temperature properties of liquid water. For example, the melting point found by MD for ice is well below the thermodynamic value. It is difficult to give a corrected temperature for the MD to correlate to the experiment.

The above discussion is to put in context how sensitive the O–H vibrational stretch is to the details of the hydrogen bond network and that the fully resonant terms need to be included to properly treat pure H_2O. I realize that the main intent of these calculations is to theoretically treat the time dependent frequency correlations, *i.e.* the observable in 2D-IR, to infer structural correlations of water within biologically relevant environments. Given the relatively high sensitivity of the water potential and the importance of resonant interaction terms within the hydrogen bond network, these factors may be important in how water interacts with membranes and biomolecule surfaces. The O–D stretch of H–O–D is generally used to probe these issues, given the much longer lifetime and much slower spectral diffusion, which gives a larger dynamics range for separating different contributions to the water dynamics. However, the resonant terms in neat H_2O give rise to enhanced coupling to intermolecular modes, as in the case for librations, that may even affect structural correlations. The intermolecular potential is different from H–O–D and these would not be taken into account. Can you comment on what needs to be done theoretically to treat water in its full splendour? As a first starting point, would it be possible to treat the extremely sensitive temperature dependence for spectral diffusion? This high sensitivity may help both refine the potentials used for water and for probing the effects of biological interfaces on the dynamic structure of water.

1 M. L. Cowan, B. D. Bruner, N. Huse, J. R. Dwyer, B. Chugh, E. T. J. Nibbering, T. Elsaesser and R. J. D. Miller, *Nature*, 2005, **434**, 199–202.
2 D. Kraemer, M. L. Cowan, A. Paarmann, N. Huse, E. T. J. Nibbering, T. Elsaesser and R. J. Dwayne Miller, *PNAS*, 2008, **105**(2), 437–442.
3 A. Paarmann, T. Hayashi, S. Mukamel and R. J. D. Miller, *J. Chem. Phys.*, 2009, **130**, 204110.

Biman Bagchi answered: As I stated before, we are effectively considering a dilute solution of H–O–D and –O–D stretch. We neglect the difference between –O–H and –O–D which could be significant. If I have –O–D stretch in a dilute solution, that will weakly couple with the surrounding –O–H stretch. So, by neglecting the coupling and consequent delocalization among –O–H stretch, we are essentially mimicking –O–D stretch. One can of course do –O–D stretch. I agree that one should directly treat H–O–D or D_2O in dilute solutions. That should not be difficult – maybe the statistics will be an issue. As to the sensitivity of water potential to charged surfaces, there is a real problem. Our force-fields are not good enough (as far I know) to pick-up small differences between various surfaces and molecules at various locations.

Himangshu Prabal Goswami asked:

(i) Do 2D-IR response functions need to always decay to an equilibrium value?

(ii) If answer to question 1 is yes, then for the exponential in eqn 2 of your paper to be negative, such that the 2D-IR response function decays to an equilibrium value, the following mathematical inequality must hold good among the line-shape functions $g(t)$:

$$g(t_1) + g(t_2) + g(t_3) + g(t_1 + t_2 + t_3) > g(t_1 + t_2) + g\,(t_2 + t_3). \quad (1)$$

What underlying physics takes care of this inequality?

(iii) If the answer to question number 1 is no, the above inequality may sometimes hold and sometimes not. Then what physical processes are involved in having control over the value of the line shape functions so that the response increases or decreases? How do we control these parameters or processes so that response function reaches a saturation value or indefinitely increases?

Biman Bagchi responded: The force–force time correlation function (FF-RCF) always decays to zero. At long times, the 2D-IR spectra become symmetric and spherical. I do not see any issue here.

Sanghamitra Mukhopadhyay addressed **Biman Bagchi** and **Martin Zanni**: Using 2D-IR spectroscopy to find spatio-temporal correlations in aqueous systems is very promising. My question is whether neutron spectroscopy can be used in the same manner to find the same correlations? In this context I would like to mention that there is the VESUVIO instrument at ISIS (http://www.isis.stfc.ac.uk/instruments/vesuvio/) which is used for neutron Compton scattering and femtosecond dynamics. Since the neutron is very much sensitive to hydrogen, do you think this instrument has potential for this type of spectroscopy?

Martin Zanni replied: I believe that the neutron source would need to be coherent, although there may be incoherent 2D analogs.

Biman Bagchi commented: The uniqueness of the 2D-IR method lies in its ability to probe the response of a chemical bond (in our case –O–H stretch), and also our ability to interpret the results. I am not too aware of the abilities of neutron Compton scattering. But it seems it will provide different kinds of information, like spatial structure and translational dynamics. It will certainly be worthwhile to do neutron Compton experiments near an interface to learn more. This can nicely complement 2D-IR studies.

Debabrata Goswami asked: The basic premise of the presented work on 2D-IR calculation is the dephasing across the entire system. This knowledge could give you better insight into the different structures, the theory might be very complex and it might be hard to use all that information. Please comment.

Stephen Meech responded: This is an interesting question. My first thought is that the large number of protein modes contributing to the spectral region of interest here (which encompasses the amide backbone) is too large to be addressed by 2D-IR. It is however true that NMR methods routinely tackle small proteins where all the very numerous nuclei in question contribute. 2D-NMR had a considerable head start on 2D-IR, so it is possible that there is further technical, experimental and theoretical progress to be made which might clean up or simplify the 2D-IR spectra. Meanwhile, one approach is to incorporate unnatural amino acids at key locations which have intense IR transitions in regions of the spectrum where the protein does not absorb. A second way forward, specific to photoactive proteins, is to perform pump–2D-IR probe measurements. We have shown for flavoproteins that the fact of electronic excitation perturbing the protein structure[1] and the subsequent relaxation could make them probes for 2D-IR.

1 A. Lukacs *et al.*, *J. Am. Chem. Soc.*, 2011, **133**, 16893–16900.

Biman Bagchi also commented: I should clarify that we tried to correlate our results with experiments that treat dilute H–O–D or D_2O in water. That was done in our paper in a crude way by turning off the interactions among –O–H vibrations that can lead to the delocalization of the vibration. In experiments such a delocalization is not usually probed for H–O–D or D_2O. The question of coupling between –O–H modes in neat water is certainly relevant. In the old days this was called "exchange dephasing" and has been considered. In experiments one always uses a "chromophore" (like H–O–D) to avoid such problems.

Martin Zanni remarked: It seems that a lot of these questions hinge around what 2D-IR is good for. Experimental methodology of 2D-IR is very advanced now. Theory is also very good. What can it do and what are the future directions? As Stephen Meech just said, for biology we have coupled 2D-IR with sophisticated labeling methods, cell free protein expression and isotope labels. There is a whole

community that can put non-natural amino acids into proteins. The future is very bright for interrogating important questions in biology, as well as other topics.

Stephen Meech answered: I agree that progress in 2D-IR has been spectacular, and that its combination with unnatural amino acid (UAA) substitution presents a means to escape from the spectral congestion we typically encounter in proteins. As you suggest, in the longer term UAA substitution offers far more than the simple placement of convenient IR probes. For example, the possibility of placing photoactive molecules at critical points in the protein structure suggests a large number of exciting experiments.

R. J. Dwayne Miller opened the discussion of **Jonathan D. Hirst**'s paper: Your group has worked on fully accounting for all the interactions of a system with the surrounding bath for theoretically modelling 2D spectra. The first input is to get the 1D absorption spectra right and to do that it is essential to use an accurate density of states for the bath–solvent interactions. This approach works very well for absorption spectra and one can get extremely good agreements. However, CD spectra are much more sensitive to anisotropic interactions locally to the chromophore, which gives rise to the unique ability to probe chiral structures or excitonic features involving coupling between chromophores. I would think that fitting CD spectra is significantly more involved in properly accounting for these interactions. Can anyone comment on the challenges to properly treat CD spectra theoretically to put this present work in the proper context?

Shaul Mukamel replied: The numerical effort involved in calculating non-chiral or chiral tensor components of the linear response is comparable. Circular dichroism (CD) signals are related to the chiral response and usually carry more information since they involve an interference between contributions of different parts of the molecule due to the field phase variation across the molecule. This gives the CD signals an extra sensitivity to the local structure compared to non-chiral absorption spectra. This is why the technique is widely used for determining the secondary structure of proteins. Averaging over random orientations can be performed analytically using tensor calculus, yielding universal compact expressions. My group has modeled CD and chiral third order signals in chromophore aggregates and proteins in the visible and infrared respectively.[1,2]

1 D. Abramavicius, B. Palmieri, D. Voronine, F. Sanda and S. Mukamel, Coherent Multidimensional Optical Spectroscopy Excitons in Molecular Aggregates; Quasiparticle *vs.* Supermolecule Perspectives, *Chem. Rev.*, 2009, **109**, 2350–2408.
2 W. Zhuang, T. Hayashi and S. Mukamel, Coherent Multidimensional Vibrational Spectroscopy of Biomolecules; Concepts, Simulations and Challenges, *Angew. Chem., Int. Ed.*, 2009, **48**, 3750–3781.

John R. Helliwell said: The understanding of the CD solution spectra of proteins is an important object of study as we cannot crystallize every protein. You remark that "No obvious improvement is observed in the calculated spectra of

beta sheet proteins" when considering the "influence of vibrational structure". Beta sheets are relatively rigid objects in protein fold examples and so this is presumably to be expected. Could you please clarify the choice of protein classes, *i.e.* where I would assume that those that are intrinsically dynamic would be optimal?

Jonathan D. Hirst responded: We have studied four broad classes of protein: α-helical, mixed α–β, and two classes of β-sheet protein: β-I and β-II proteins. The β-I and β-II proteins show different circular dichroism spectra (Manavalan and Johnson, 1983).[1] This may in part be explained by the greater conformational flexibility that β-II proteins exhibit compared to β-I proteins (Hirst *et al.*, 2003).[2] The rigidity or conformational dynamics referred to here is in the context of roughly nanosecond timescales. Such motion is of a much lower frequency than the frequency of the C–N bond stretch. Its influence in our calculations would be directly manifested in the off-diagonal elements of the exciton Hamiltonian matrix. In contrast, the dynamics associated with the vibronic structure of the ππ* transition is on a sub-picosecond scale. Our consideration of the vibronic structure is directly manifested in both the diagonal and the off-diagonal terms of the Hamiltonian matrix. Thus, *a priori*, one would not particularly expect the influence of the vibronic structure to be more important in the circular dichroism spectroscopy of more intrinsically (conformationally) dynamic proteins.

1 P. Manavalan and W. C. Johnson, Jr., *Nature*, 1983, **305**, 831.
2 J. D. Hirst, S. Bhattacharjee and A. V. Onufriev, *Faraday Discuss.*, 2003, **122**, 253.

Mike Ashfold remarked: At what energies do we need to worry about contributions from higher excited states to the electronic absorption spectra of proteins? What are the main contributors to the 'other broadening effects' that you accommodate by modelling each vibronic transition within the ππ* absorption band with a 15 nm Gaussian function?

Jonathan D. Hirst answered: There are, of course, higher excited states of interest. In other work, we have characterised charge transfer states (Gilbert and Hirst, 2004; Oakley and Hirst, 2006)[1,2] in dipeptides and in proteins, which have helped assign peaks observed between 160 nm and 180 nm in synchrotron radiation circular dichroism spectra of proteins (Bulheller *et al.*, 2008).[3] At yet shorter wavelengths, we have calculated the $\pi_b\pi^*$ and $n'\pi^*$ excitations to occur at around 130 nm (Besley and Hirst, 1998).[4] The coupling of these higher energy transitions to those at 190 nm and 220 nm has been explored (Bulheller *et al.*, 2008),[3] but does not lead to a quantitative improvement in the accuracy of the computed protein circular dichroism spectra. An important contributor to broadening will come from the thermal effects, which lead to a conformational ensemble of ground state geometries. We have established this in an earlier study (Besley *et al.*, 2004)[6] for small amides using molecular dynamics (MD) simulations at various temperatures to sample the conformational ensemble, followed

by time-dependent density functional theory calculations on a series of snapshots from the MD trajectory.

1 A. T. B. Gilbert and J. D. Hirst, Charge-Transfer Transitions in Protein Circular Dichroism Spectra, *J. Mol. Struct.: THEOCHEM*, 2004, **675**, 53–60.
2 M. T. Oakley and J. D. Hirst, Charge-Transfer Transitions in Polypeptides, *J. Am. Chem. Soc.*, 2006, **128**, 12414–12415.
3 B. M. Bulheller, A. J. Miles, B. A. Wallace and J. D. Hirst, Charge-Transfer Transitions in the Vacuum-Ultraviolet of Protein Circular Dichroism Spectra, *J. Phys. Chem. B*, 2008, **112**, 1866–1874.
4 N. A. Besley and J. D. Hirst, *Ab Initio* Study of the Effect of Solvation on the Electronic Spectra of Formamide and *N*-Methylacetamide, *J. Phys. Chem. A*, 1998, **102**, 10791–10797.
5 N. A Besley, M. T. Oakley, A. J. Cowan and J. D. Hirst, A Sequential Molecular Mechanics/Quantum Mechanics Study of the Electronic Spectra of Amides, *J. Am. Chem. Soc.*, 2004, **126**, 13502–13511.

Siva Umapathy enquired: how would you consider the electric transition moments of transitions which are close to each other where one transition is less chiral and the other is more chiral? Would this not affect the line shape? The absorption coefficient of one transition could be very large, so if there is overlap of the two transitions the line shape will be influenced by the non chiral residue. Is it possible to use the same approach for drug protein interactions? And if the drug itself is highly chiral? Can they be measured with a similar technique?

Jonathan D. Hirst replied: The circular dichroism of a protein in the far-ultraviolet is dominated by the local, regular repeating secondary structure. Helices and beta-strands are chiral, *e.g.* helices are typically right-handed and strands also have a distinctive twist. This "macromolecular" chirality is much more important than the chirality of individual amino acids. If a ligand binding to a protein has chromophoric groups with electronic transitions in the far- or near-ultraviolet, then these, in principle, regardless of whether the ligand itself is chiral or achiral, could be affected by binding and differences in circular dichroism could (and have been) used to study protein–ligand interactions.

Elangannan Arunan asked: You mentioned the Rydberg state being pushed away due to intermolecular interactions and hence the two bands observed are clearly from $n\pi^*$ and $\pi\pi^*$. In general, when one orbital is perturbed by another, the interaction could lead to two new orbitals one getting stabilized and another de-stabilized. Would this not affect the nature of the two states observed?

Jonathan D. Hirst responded: The Rydberg states present in the gas phase are destabilised by Pauli repulsion interactions with the solvent (Besley and Hirst, 1998).[1] This destabilisation is sufficiently large that the interaction between the Rydberg states and the low-lying valence states is small. For the (planar) *N*-methylacetamide molecule in isolation, the $n\pi^*$ and $\pi\pi^*$ states have different symmetries and do not interact. In the asymmetric environment of the (rest of the) protein, the two states will (as you suggest) mix, and this interaction is

explicitly present in the corresponding off-diagonal term of the exciton Hamiltonian matrix.

1 N. A. Besley and J. D. Hirst, *Ab Initio* Study of the Effect of Solvation on the Electronic Spectra of Formamide and *N*-Methylacetamide, *J. Phys. Chem. A*, 1998, **102**, 10791–10797.

Artur Nenov remarked: In the construction of the Hamiltonian matrix (eqn 9 of your paper) the off-diagonal elements (chromophore–chromophore couplings) are assumed to be purely electrostatic. Could you comment on the role of other interactions like induction, dispersion and quantum-mechanical exchange?

Jonathan D. Hirst answered: The magnitude of non-electrostatic interactions in the calculation of the off-diagonal elements of the exciton Hamiltonian is a potentially important question. For various conformations of a model dipeptide, the *ab initio* transition energies at the CASSCF level (Oakley and Hirst, 2006)[1] cannot be reproduced by a simple splitting of states due to coupling *via* electrostatic interactions. In several other molecular systems, including DNA (Nachtigallova *et al.*, 2008)[2] and naphthalene (Scholes and Ghiggino, 1994),[3] non-classical short range exchange interactions have been considered in the illustrative cases of dimers. Thus, the question does warrant further investigation.

1 M. T. Oakley and J. D. Hirst, *J. Am. Chem. Soc.*, 2006, **128**, 12414–12415.
2 D. Nachtigallova *et al.*, *Phys. Chem. Chem. Phys.*, 2008, **10**, 5689–5697.
3 G. D. Scholes and K. P. Ghiggino, *J. Phys. Chem.*, 1994, **98**, 4580–4590.

Artur Nenov asked: Was the origin of the $0 \leftarrow 0$ transition (190 nm) computed (adiabatic S0-S1 energy gap) or taken from experimental data?

Jonathan D. Hirst replied: The vertical transition energy computed at the CASPT2 level with a large active space, a large basis set and a continuum model of solvent, is 193 nm (Besley and Hirst, 1998),[1] which is close to the value of 190 nm that we have taken from the experiment.

1 N. A. Besley and J. D. Hirst, *Ab Initio* Study of the Effect of Solvation on the Electronic Spectra of Formamide and *N*-Methylacetamide, *J. Phys. Chem. A*, 1998, **102**, 10791–10797.

Kiran Moirangthem remarked: The paper discusses the computational study of interpreting the CD spectra of proteins, with the incorporation of vibronic coupling where the present approach well explains even the negative bands in the CD spectra of alpha-helical proteins (which occur around 208 nm and 222nm). But I am curious to know why we don't see this absorption at 208 nm (ignoring the forbidden $n\pi$ absorption at 222 nm) in the absorption spectrum shown in Fig. 3 of your paper? Why do we not see even the slightest hump corresponding to the absorption at 208 nm in the spectrum shown in Fig. 3?

Jonathan D. Hirst responded: The figure in our paper shows the absorption spectrum of *N*-methylacetamide, which only has one peptide chromophore. Thus, there is only a single peak at ~190 nm. For polypeptides, where there are several peptide chromophores, then the band or shoulder at ~208 nm, arising from the coupling of $\pi\pi^*$ transitions on different peptide groups, is evident, *e.g.* early experiments showed this for poly-L-lysine (Rosenheck and Doty, 1961).[1]

1 K. Rosenheck and P. Doty, The far ultraviolet absorption spectra of polypeptide and protein solutions and their dependence on conformation, *Proc. Natl. Acad. Sci. U. S. A.*, 1961, **47**, 1775–1778.

R. J. Dwayne Miller opened discussion of the paper by **Artur Nenov**: To help the discussion and to illustrate the intellectual challenges of this topic, it is important to realize that DNA needs to have some degree of what is called "radiation hardening" to minimize UV induced damage. DNA is the "hard drive backup" for the cell. The genetic code gives the instruction set for replication that would be lost with UV induced damage to DNA, *e.g.* in the formation of T-T dimers. The cell has developed an elaborate correction mechanism based on DNA photolyase but there has also been early evidence that DNA is intrinsically immune to UV induced damage or is inherently radiation hardened. Early studies based simply on fluorescence quantum yields on individual bases showed there was essentially no fluorescence (10^{-4} or less)[1] and by extension even smaller fluorescence quantum yields would be expected for DNA. The typical radiative rate for molecules representative of nucleotides would put the excited state lifetime on the picosecond timescale. At this time, the energy gap law for nonradiative relaxation was formulated. The extremely fast relaxation of the excited states of DNA was thought to proceed through the very high density of vibrational states within the low frequency modes of the DNA strand that would enhance the Franck–Condon factors (lead to resonant terms) connecting the S0 and S1 ground and excited states. The additional low frequency modes effectively create new vibrational relaxation pathways from that of the isolated nucleotide bases and this would reduce the excited state lifetimes further from estimates based on just the nucleotide bases. The excited state lifetimes of DNA strands would be expected to be subpicosecond based on the fluorescent quantum yields of the individual bases, with accurate fluorescence quantum yields under ambient conditions beyond the detection capabilities at the time. This was the picture for many years. There was always the doubt (RJDM) that there were dark states that had gone undetected in the fluorescence studies. Then came the work of Kohler's group and others that did the first femtosecond studies of the isolated DNA bases to fully characterize the excited state dynamics (see reference 1 in Artur Nenov's paper). The individual nucleotide bases showed extremely fast excited state relaxation processes on the picosecond to sub-picosecond time scale, corroborating the early fluorescence quantum yield studies. For a rigid molecular system, this observation is in violation of the classical energy gap law as formulated based on measurements of cyclic aromatic systems. (It is interesting to speculate how the theoretical developments would have advanced with this glaring exception to the breakdown in the formation of the Franck–Condon overlap or nuclear wavefunction overlap between coupled electronic states.) These observations have led

to a much improved understanding of the electronic structure of the DNA bases in which conical intersections provide the very fast nonradiative relaxation.

At this point, the state of affairs in understanding DNA photophysics seemed to be secure. The DNA bases themselves exhibited extremely fast nonradiative relaxation to provide the inherent radiation hardening against UV induced damage. The full DNA single and/or double strand structure was not essential to provide new nonradiative relaxation channels to quickly dissipate the UV generated excited states. This present paper represents yet another new twist in the DNA story. The high level time dependent *ab initio* calculations find evidence for excimer and charge transfer (CT) states that necessarily involve very reactive photointermediates. Experimentally there is recent evidence for long lived excited states on the 10–100 ps timescale based on ground state recovery studies of model oligomers of DNA (see for example cited references 1, 6, 60 and 77 of Artur Nenov's paper). These excited state dynamics are 1–2 orders of magnitude slower than observed for individual bases. On a preliminary basis, my group has recently completely a series of 2D-UV studies of DNA with sufficient bandwidth in the UV range. The advantage of 2D spectroscopy is that the spectra remove inhomogeneous broadening and it is possible to directly observe coupled states that would be hidden in these previous studies. We find evidence for the very CT states predicted by this work. Such states would be much more reactive than other possible excited state intermediates. One can understand this based on the close proximity of the nucleotides within the DNA chain that open up electronic coupling between the excited state of adjacent bases both for excimer formation and for electron hopping and the ground valence states for hole hopping. This interaction will lead to a very rapid charge separation and the creation of very reactive intermediates – the very thing DNA needs to avoid. It appears DNA still holds some surprises. With this above narrative on the key issues in understanding DNA photophysics, what do you think are the key questions regarding the excited state dynamics of DNA that may be probed by 2D methods or others? Are the initial ultrafast processes the relevant photophysics to understand the presumably inherent UV protection of DNA (now in question)?

1 M. Daniels and W. Hauswirth, *Science*, 1971, **171**(3972), 675–677.

Sankarampadi Aravamudhan answered: In the variety of pulse experiments in the UV, Vis, and IR region of the electromagnetic spectrum, what was obviously a prime concern was the time scales (slow or fast) of the relaxation processes. The spontaneous emission is a radiative relaxation and with the internal conversion and inter system crossings preceding the spontaneous emissions, the terms fluorescence, delayed fluorescence and phosphorescence are applicable. All these are explainable when the excited state (population) occupation number has been changed due to the stimulated absorption. All these processes thus characterize the change in population differences between the lower ground state and the higher excited state. If the population differences have to be altered, then the stimulated absorption must result in a transition to account for the complete transition of the electron. The induced (stimulated) transition probabilities depend on the radiation power experimentally applied. The reciprocal of the transition probabilities are the time of transit of the electron under perturbation.

If the transition times corresponding to the induced transitions are larger than the pulse widths, then the perturbation lasts less than the time required for the electron to make a transition to the other level and thus non-stationary states are created which radiate with characteristic decoherent times. This decoherent process is not appropriately described by population difference changes. Maybe these decoherence times characterize the homogeneous line broadening or what is referred to as the natural line width spectroscopically. And this lifetime and the decoherence times are related by the uncertainty relation to the line widths. Whereas the relaxation time which governs changes in population differences are to be reckoned among the processes which couple the molecule to the medium degrees of freedom and the fluctuations induce these relaxations. These two kinds of relaxation times are well distinguished in the time scales of magnetic resonance experiments. This difference between the magnetic resonance descriptions under pulsed excitations and the optical transients were highlighted in the early papers by A. H. Zewail[1] and R. G. Brewer.[2] When ultra fast events under high power laser pulses are concerned, it is yet to be reconciled as to the distinction between the decoherence and consequent radiation transients and the transients of the type which arise by spontaneous emissions.

As an additional comment, the paper by Artur Nenov deals with "deactivation pathways". In this context it is my current understanding that unless the processes responsible for the occurrence of transients in the ultra fast regimes is well specified and the respective characteristic times (time scales) are known, interpretations on the basis of such transients at the inference for the chemical dynamics and biological functions would entail many ambiguities. I quote the statement on page 3 of this paper: "But this was exactly what Ahmed Zewail set out to do. He had realised, from an experiment in the 1970s on anthracene molecules at low temperature, that molecules could be brought to vibrate in pace. 'Coherent preparation' of a sample system is thus a key point in all his experiments. We shall return to this concept". For most of the contexts it should be quite clear whether the relevant transient time constant is due to the decoherence phenomenon or the spontaneous emission. Probably the two processes are distinctly different.

1 http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1999/press.html
2 R. G. Brewer, *Coherence in Spectroscopy and Modern Physics, NATO Advanced Study Institutes Series*, Springer, 1978, vol. 37, pp. 41–84.

Artur Nenov answered: The mechanism explaining the existence of the long-lived signal in DNA polynucleotides and the nature of the excited state associated with it has been eagerly debated in the recent years. Both theoretical and experimental studies favor different hypotheses. A summary of the latest results can be found in the paper submitted by us for this Faraday meeting. We believe that 2D electronic spectroscopy (2D-ES) has the best prospects to link the long lifetime to a particular mechanism. The main goal of our paper is to demonstrate that every deactivation channel in DNA has its characteristic spectroscopic fingerprints in a 2D spectrum as a direct consequence of the different electronic nature of the excited states and the ability of 2D-ES to disentangle the population transfer between excited states populated upon UV radiation. These fingerprints relate to

the excited state absorption (ESA) and stimulated emission (SE) bands. *Ab initio* theoretical simulations, as the ones performed in our group,[1,2] will help to identify these fingerprints, which has a twofold benefit: firstly, they can serve as a guide for setting up 2D-ES experiments through identifying spectral windows of interest; and secondly, they will aid the interpretation of the 2D spectra, with the ultimate goal of deciphering the nature of the electronic states involved in the formation and recovery of the long lived state. I would like to note that a sufficient bandwidth in the visible and UV is required in order to resolve the above-mentioned fingerprint bands. As demonstrated in our paper there exist bands which exhibit geometry dependent spectral shifts of several thousand cm^{-1}, which qualifies them as excellent fingerprints, but poses a challenge for their experimental detection. It is, therefore, all the more pleasing to hear that broad bandwidths are already feasible in the UV region as well. Related to this I would also like to draw your attention to our computational studies on aromatic compounds (adenine, indole, phenol, *etc.*),[3,4] which reveal a number of characteristic ESA bands that can be detected by probing in the visible region. Therefore, I would encourage the realization of "two-color" experiments using narrowband UV-pump pulses and supercontinuum Vis-probes, as recently reported for example by the group of Riedle.[5]

1 I. Rivalta, A. Nenov, O. Weingart, G. Cerullo, M. Garavelli and S. Mukamel, *J. Phys. Chem. B*, 2014, **118**(28), 8396–8405.
2 A. Nenov, S. a Beccara, I. Rivalta, G. Cerullo, S. Mukamel and M. Garavelli, *ChemPhysChem*, 2014, **15**(15), 3282–3290.
3 A. Nenov, I. Rivalta, S. Mukamel and M. GAravelli, *Comput. Theor. Chem.*, 2014, **1040–1041**, 295–303.
4 A. Nenov, I. Rivalta, G. Cerullo, S. Mukamel and M. Garavelli, *J. Phys. Chem. Lett.*, 2014, **5**(4), 767–771.
5 N. Krebs, I. Pugliesi, J. Hauer and E. Riedle, *New J. Phys.*, 2013, **15**, 085016.

Sankarampadi Aravamudhan commented: Those experiments which are carried out in such a way that the spectrum is obtained by slow scanning through the two level resonance are the continuous radiation or continuous wave regime with low power electromagnetic radiation and when only incoherent light source is used. Coherent sources are capable of delivering high powers. At high power radiation the induced transition rates of the stimulated transitions would be high and a continuous application of high power radiation would not result in a steady state population difference to be maintained and the observed absorption and emissions would be time dependent observable parameters, and hence not conducive for further inferences. Hence, when high powers are available the electromagnetic radiation (the light irradiation) is in the form of a pulse, the pulse widths are significantly less than the relaxation times and the induced transition times. If the induced transition rates are fast at high power levels, the reciprocal of the rate, which is the transition time, would be small for one molecule (particle) to undergo the transition from a lower energy level to a higher level (presuming the lower energy level is more populated under thermal equilibrium). If the pulse widths are small compared to the induced transition times ($1/W_{01} = 1/W_{10}$), then at the end of the pulse the particle would not have transited fully from say, the lower level to the higher level. Thus the particle would be left in a non stationary state since only the lower energy level or the higher energy level is an Eigen state.

Such a non stationary state would be a linear combination of the lower level ψ_l and the upper level ψ_u. Since such linear combinations of ψ_u and ψ_l are generated by a coherent radiation which corresponds to the description as time dependent perturbation (corresponding to the electromagnetic radiation pulse), it is a time dependent non-stationary state at the end of the pulse, and matter in such a state would radiate energy. This radiation with an initial amplitude would soon tend towards the thermal equilibrium distributing the molecules (particles) with certain time independent population differences characteristic of a thermal equilibrium population distribution among the two levels. Thus these experiments should always be reckoned with the appropriate transition times for the induced transitions and relaxation transitions. Only then it would be convincingly set out for a specified coherent superposition of the two levels, the superposition arising due to the time dependent perturbation. Under time independent

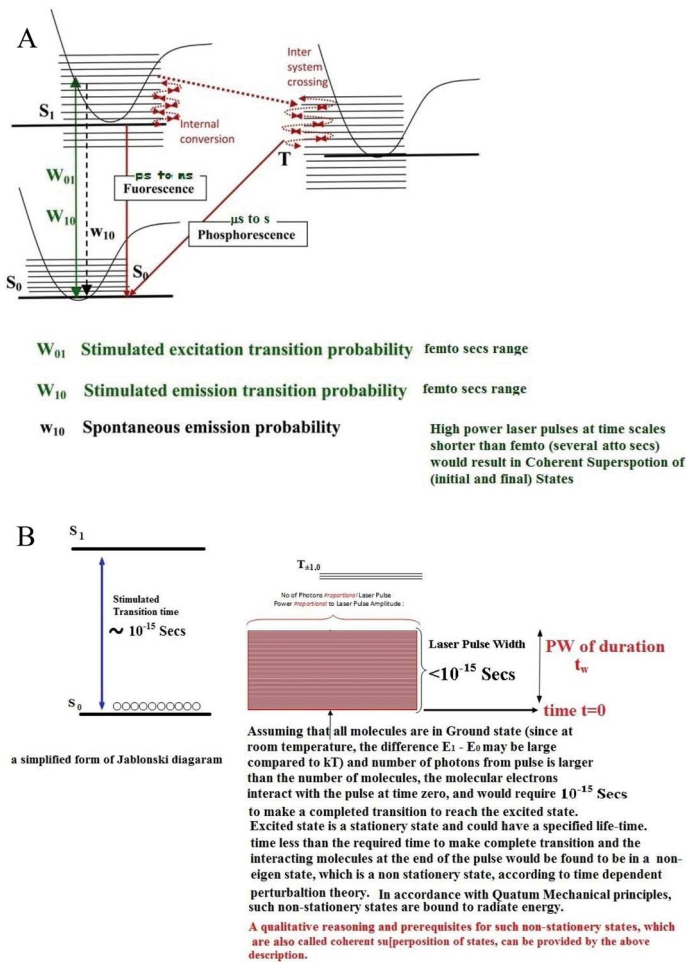

Fig. 1 (A) Jablonsky Diagram: description of related processes with typical time scales. (B) A simplified form of Jablonsky Diagram: a consideration of laser pulse characteristics with reference to transition time scales.

perturbations such states are time independent mixed states, but not non stationary coherent superpositions (Fig. 1).

These W values are expressed as the number of molecules per second that make the transitions. The reciprocal of the W values would then correspond to the time in secs required for one molecule to undergo a transition. Corresponding to every pair of two levels, there could be radiationless relaxation transitions. The reciprocal of the relaxation probabilities (different upward and downward rates) would correspond to the relaxation time which is defined conveniently as the time in terms of equilibrium population difference between the relevant 2-level system.

As an additional remark to the previous question by Dwayne Miller, I have mentioned the natural line width and indicated the inhomogeneous width due to overlap of lines. However, having to content myself with the qualitative assertion of the instantaneity of the event without proving that this "instantaneity" means a time scale several orders of magnitude less than the ultra fast pico/femto/atto seconds regime, may not be intellectually scientific, even if it sounds as unknown as origin of the universe or of life on planet earth.

Shaul Mukamel replied: This question should only be asked in the context of a specific experimental observable. Otherwise it is a philosophical issue. The absorption line shape involves two interactions with the radiation field and their separation is controlled by the dephasing time (the inverse line width). This can be interpreted as the time it takes to absorb the photon. Other measurements can be controlled by different processes.

R. J. Dwayne Miller also responded: The above discussion is all perfectly fine. The only comment I can make is that the use of lasers, and therein coherent radiation, does not imply that one is not in the perturbative limit as you seem to imply. With lasers it is possible to go to very high peak powers and couple many electronic states together in a nonstationary superposition that creates interferences that do not reflect the intrinsic dynamics of the individual states (in terms of nonradiative transitions). This feature of laser radiation is explicitly used in the Coherent Control (see the work of Brumer and Shapiro).[1] However, it is rather straight forward to control the intensity to be in the weak field limit. In this perturbative limit, one can use very short laser pulses with an appropriate bandwidth to probe the states of interest. For transform limited pulses, the different Fourier components of the pulse all have the same phase relationship at the carrier central wavelength to give the minimum pulse duration for a given bandwidth. If the laser bandwidth is larger than the spectral width of the transitions of interest, the pulse will be shorter than the intrinsic molecular dynamics of interest. One does not change the light-matter interaction. The Hamiltonian describing the perturbation by the field is exactly the same for the weak CW light condition, with the exception that the frequencies now have a common phase factor. This allows one to prepare the initial state as you describe and watch in the time domain the very processes you show in your figure. The information content is the same in both cases. Please note that the dynamics you depict in the figure contribute to the spectral linewidth in the frequency domain spectrum collected using CW excitation, which can be thought of as uncertainty broadening or more

microscopically as relaxation induced Fourier components to the induced polarization in the medium. In this regard, the equivalence of frequency domain spectroscopy and time domain spectroscopy has been well established. The two methods of probing the system response are Fourier transforms of one another. (See the work by Loring and Mukamel on a similar issue raised in comparing time domain and frequency domain Coherent Anti-Stokes Raman.[2] Here the issue was whether the nonlinear aspects of the excitation could yield more information on homogeneous line widths. The equivalence of time domain and frequency domain for the same excitation processes was shown in this paper.) The attraction of using femtosecond pulses is that it lets you separate different possible pathways in the time domain, which becomes important if there are intermediate states and the time ordering of the relaxation pathways is key to understanding the molecular photophysics or photochemistry.

1 M. Shapiro and P. Brumer, *Rep. Prog. Phys.*, 2003, **66**(6), 859–942.
2 S. Mukamel and R. F. Loring, *JOSA B*, 1986, **3**(4), 595–606.

Mike Ashfold asked: Can someone briefly summarise the recent evidence for a long lived component to the excited state decay of DNA multimers? If correct, this finding surely necessitates some reappraisal of the photostability of the DNA bases – traditionally attributed to the efficiency of the radiationless processes by which their excited state population transfers back to the ground state.

Artur Nenov responded: From the Kerr-gated time-resolved pump–probe transient absorption and fluorescence experiments in (dA)20, a single-stranded adenine polynucleotide, Phillips and co-workers obtained three different time-constants, 0.39, 4.3 and 182 ps, the latter being the one responsible for the long-lived spectroscopic signal, which in this case was measured with time-resolved fluorescence detecting at ~390 nm.[1] Kohler and co-workers performed pump–probe transient absorption measurements (pump at ~266 nm/probe at ~252 nm) in adenine-based polynucleotides, obtaining different results depending on the length of the strand: 105 ps for ApA[2] and 207 ps for d(A)4.[3] Newer works from Kohler and co-workers[4,5] dwell on the relationship between the stacking motifs and the long-lived signal by constraining the adenosine chains with large groups to create steric hindrance and enhance the stacking, thereby relying on circular dichroism experiments to relate ground state conformations to the long-lived excited state. Markovitsi and co-workers have also studied adenine based polynucleotides by means of time-resolved fluorescence upconversion, obtaining similar results as those found by Kohler for natural DNA samples.[6,7]

Regarding double-stranded chains, the effect of the Watson–Crick base pairs (*e.g.* poly(dA-dT) *vs.* poly(dA))[8,9] quickly accelerates the decay lifetimes through proton–hydrogen transfer mechanisms that are ultrafast in nature.[10] Specific sequences, such as those alternating G/A in a double-stranded chain[11] have been reported to accelerate the decay times, especially those linked with G–C Watson–Crick base pairs.[10] Some recent works by Zinth and co-workers do point towards the quenching effect of the double-strand on the long-lived lifetime.[12]

1 W.-M. Kwok, C. Ma and D. L. Phillips, *J. Am. Chem. Soc.*, 2006, **128**, 11984.
2 T. Takaya, C. Su, K. de La Harpe, C. E. Crespo-Hernández and B. Kohler, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 10285.
3 C. Su, C. T. Middleton and B. Kohler, *J. Phys. Chem. B*, 2012, **116**, 10266.
4 J. Chen, A. K. Thazhathveetil, F. D. Lewis and B. Kohler, *J. Am. Chem. Soc.*, 2013, **135**, 10290.
5 J. Chen and B. Kohler, *J. Am. Chem. Soc.*, 2014, **136**, 6362.
6 D. Markovitsi, T. Gustavsson and I. Vayá, *J. Phys. Chem. Lett.* , 2010, **1**, 3271.
7 I. Vayá, T. Gustavsson, T. Douki, Y. Berlin and D. Markovitsi, *J. Am. Chem. Soc.* , 2012, **134**, 11366.
8 C. Greve, N. K. Preketes, R. Costard, B. Koeppe, H. Fidder, E. T. J. Nibbering, F. Temps, S. Mukamel and T. Elsaesser, *J. Phys. Chem. A*, 2012, **116**, 7636.
9 C. Greve, N. K. Preketes, H. Fidder, R. Costard, B. Koeppe, I. A. Heisler, S. Mukamel, F. Temps, E. T. J. Nibbering and T. Elsaesser, *J. Phys. Chem. A*, 2013, **117**, 594.
10 N. K. Schwalb and F. Temps, *J. Am. Chem. Soc.*, 2007, **129**, 9272.
11 N. K. Schwalb and F. Temps, *Science*, 2008, **322**, 243.
12 D. B. Bucher, A. Schlueter, T. Carell and W. Zinth, *Angew. Chem. Int. Ed.*, 2014, **53**, 11366.

Kenneth Ghiggino said: I would like to raise an issue about nomenclature. In your paper you have referred to CT excimer states. How do these states differ from the usual definition for an excimer or a CT state? Is it necessary to introduce the term CT excimer state?

Artur Nenov answered: Thanks to Prof. Ghiggino's comment we realized that the "CT excimer state" notation was inadequate. In fact, we refer to the existence of a low lying CT state formed through an electron hopping from an adenine to an adjacent one. As a consequence an excimer is formed, characterized by the shortening of the inter-base distance by approx. 0.5 Angstrom,[1] which is accompanied by geometrical deformations in both bases aiming at stabilizing the opposite charges. Thanks to the comment raised by Prof. Ghiggino we corrected the nomenclature in our paper.

1 I. Conti, A. Nenov, S. Hoefinger, S. F. Altavilla, I. Rivalta, E. Dumont, G. Orlandi and M. Garavelli, *Phys. Chem. Chem. Phys.*, 2015, **17**, 7291–7302.

Helena J. Shepherd opened the general discussion of the paper by **Marylise Buron-Le Cointe**: (1) You state that "...in the intermediate phase, a spin state concentration wave (SSCW) appears resulting from a symmetry breaking (cell doubling) associated with a long-range order of alternating high spin and low spin molecular states". Do you have any comments regarding the relationship (causal/non-causal, *etc.*) between the different symmetry breaking processes and the appearance/disappearance of the SSCW? This is a potentially interesting discussion in light of the fact that when the SSCW disappears (in the LS state), a further symmetry breaking transition occurs to a phase that is different from either the high spin or intermediate phases (*i.e.* the phase transition is not re-entrant). (2) You carried out time-resolved studies at temperatures corresponding to the plateau associated with the intermediate phase. Is it possible to perform similar experiments at *e.g.* ~90 K (in the LS state but above T(LIESST)), and what do you/would you expect to observe in this case (*e.g.* fraction of molecules excited, symmetry breaking, SSCW, relaxation times, *etc.*)?

Marylise Buron-Le Cointe replied: (1) There are different degrees of freedom in crystals which can break the symmetry. The SSCW is associated with a long-range order of molecules in the HS and LS states, which are symmetry-equivalent in the high temperature HS phase. Indeed, there are some SCO materials in the literature for which the HS and LS phases have the same symmetry, *i.e.* a re-entrant phase transition occurs (see ref. 26 and 27 of our paper for example). In the present case, the symmetry breaking in the LS phase results from a molecular tilting. This is a purely structural ordering as all the molecules have the same LS electronic state. This is also true for the photoinduced state where an ordering of tilted molecules appears: the photoinduced HS state is of lower symmetry than the HS state stable at high temperatures (see our ref. 4). Therefore the SSCW is a symmetry breaking process but in these materials other types of more conventional structural symmetry breaking can also appear, even though the SCO is usually an isostructural process.[1] (2) In the investigated material it is difficult to perform such a study (optical or X-ray diffraction) from the LS phase because the first-order phase transition from the SSCW plateau to the LS phase decreases the crystalline quality. In addition, the recovery time to the LS state increases at low temperatures and may be too long at 90 K for performing pump–probe studies with a 40 Hz repetition rate. This may not be a problem for other stepped SCO compounds for which the SSCW forms at higher temperatures and it might be interesting to investigate this aspect. We expect then to observe a similar multi-step out-of-equilibrium dynamics. From the LS phase, a fs light excitation should promote locally some molecules to the HS state within 160 fs and the elastic and thermal steps will promote more molecules to the HS state. Then, it is possible that the SSCW forms on the few ms time-scale, when a transient temperature is reached and it should disappear in 10s ms when the sample relaxes to the initial temperature.

1 N. Bréfuel, E. Collet, H. Watanabe, M. Kojima, N. Matsumoto, L. Toupet, K. Tanaka and J. P. Tuchagues, Nanoscale self-hosting of molecular spin states in the intermediate phase of spin-crossover materials, *Chem. Eur. J.*, 2010, **16**, 14060–14068.

R. J. Dwayne Miller asked: The time scale you observe and assign to the relaxation within the spin manifold to reach the fully relaxed spin state (recovery to the low spin state) is comparable to the thermal diffusion. How do you separate the lattice relaxation from the thermal diffusion effects? One would think that an extremely fast forward transition, fast low spin to high spin transition, would have similarly fast high spin to low spin transitions upon relaxing back to the ground electronic state and relaxation of the Fe–N bond (the key coordinate) back to its original position, based on microscopic reversibility.

Marylise Buron-Le Cointe answered: Indeed, the recovery to the LS state is driven by the time it takes to reach thermal equilibrium with the sample environment and this is governed by the heat exchange with the cryostat and is therefore sample shape and size dependent. The recovery 10s ms time scale observed here for this stochastic dynamics is much longer than that of more elementary physical processes (molecular switching, unit-cell deformations, ...).

These are indeed hidden in a statistical average. We can only reveal the intrinsic time-scale of these elementary processes during the transformation, as their dynamics is clocked by the ultrashort laser pulse. The recent study of reverse LIESST has also shown that the photoinduced LS state is reached within 40 ps from the HS state. This photoinduced LS state recovers the HS state within less than 1 ms (see our ref. 16).

Debabrata Goswami queried: Since the concerned experiments involve Spin based processes – you would start seeing differences of linear *versus* circular polarization that could help with what you are looking at. The other approach is magnetic, can you isolate the temperature aspect? I feel the use of different polarizations would help.

R. J. Dwayne Miller replied: We use polarized excitation in some cases to maximally excite the sample. Since the sample orientations with respect to the transition dipole moments are not often known, at least not without detailed parallel studies, we also used circularly or depolarized excitations to provide constant excitation conditions independent of crystal orientation. The spin to which you refer is related to the magnetic spin transitions, say in the intersystem crossing or spin cross over phase transitions, which is not covered in this work. It is certainly of interest. In this case, we would have to use spin polarized electron sources to provide a contrast sufficient to definitively determine the spin coupled lattice dynamics. It is possible to generate spin polarized electron sources using negative affinity GaAs photocathodes. We would love to do such studies and I hope we can pursue such studies in the near future. There are important outstanding issues as discussed in the paper presented by M. Buron-Le Cointe *et al.* on spin crossover systems in these proceedings.

Marylise Buron-Le Cointe commented: The LIESST effect is driven by light absorption of the diamagnetic LS species. The use of polarization in such low symmetry crystals is therefore of interest only for optimizing the penetration depth of light (this was used in our ref. 14 and 15 for example). In the present sample, the light penetration depth dependence with polarization is weak and we observed similar responses for random orientations of the crystals with regard to light polarization. In addition, the HS state is paramagnetic (there is no magnetic order, at least in the investigated temperature range). There are other systems, such as the [Fe(1-propyltetrazole)6](BF4)2 SCO compound, where a single-molecule magnet behavior is observed in the photoexcited HS state.[1] However, this process only occurs at very low temperatures (around 20 K), where the lifetime of the photoinduced HS state is too long to perform kHz stroboscopic pump–probe studies.

1 X. Feng *et al.*, *J. Am. Chem. Soc.*, 2013, **135**, 15880.

John R. Helliwell asked: On page 8 of your article and in your talk you emphasized that (only) "0.2% of the molecules in your sample switch from the LS to HS state at the ps timescale". You could of course use a smaller sample, to

try and enhance this, and in any case preserve the X-ray diffraction intensities by using Laue diffraction rather than monochromatic diffraction.[1] However that is not the focus of your study, rather, it is seeing the doubled cell nicely evident in Figure 7 of your article and highlighted in your abstract. Am I correct?

1 Z. Ren, D. Bourgeois, J. R. Helliwell, K. Moffat, V. Srajer and B. L. Stoddard, Laue crystallography: coming of age, *J. Synchrotron Rad.*, 1999, **6**, 891–917.

Marylise Buron-Le Cointe responded: Yes it is correct. When one is interested in the structure of the photo-excited state, a matching of the sample size and of the laser penetration depth is necessary for photoswitching a large fraction of molecules. When this penetration depth is small, one has to use thin crystals and it is then necessary to increase the X-ray flux by using a broad band or "pink" X-ray spectrum. This is the Laue diffraction technique and for example P. Coppens *et al.* applied this technique to study photo-switching in different molecular crystals by using the ratio method.[1] This ratio method is well-adapted for small changes on Bragg peak intensities without considering changes of lattice parameters. In other words, it is not well adapted for observing the second elastic step and the third macroscopic heating step (see our Fig. 6b). In the out-of equilibrium dynamics of SCO systems investigated here, several steps are involved. During the ps non-thermal photo-switching step, we photo-excite typically 0.2% of the molecules, because in the μs delays the crystals warms up and the HS state is then thermally populated. On other SCO compounds, we could increase the fraction of photo-excited molecules up to a fraction closer to 1% and more (when the deposited laser energy increases). The crystal warming can then reach 20–30 K or more.[2,3] In the present study, we wanted to limit such heating effects to investigate the symmetry breaking aspects of the process, not the thermally-induced one. The process investigated here is not limited by the penetration of light since the symmetry changes globally in the crystal, as indicated by the almost complete disappearance of the peaks characteristic of the symmetry breaking. In addition, the use of Laue is not appropriate in the present study as we focus on the cell doubling aspects. We need therefore to separate the diffracted intensity by the Bragg peaks related to the symmetry breaking indexed (h k 2p + 1) from the diffracted intensity by the other Bragg peaks. With Laue techniques, some peaks indexed (h k 2p + 1) and (h′ k′ 2p′) can superpose on the same spot of the detector (if the X-ray spectrum is broad enough). It is therefore more difficult to reconstruct the diffracted intensity in the reciprocal space (see our Fig. 7) and especially for the (h k 2p + 1), directly related to the amplitude of the symmetry breaking order parameter.

1 P. Coppens, M. Pitak, M. Gembicky, M. Messerschmidt, S. Scheins, J. Benedict, S. Adachi, T. Sato, S. Nozawa, K. Ichiyanagi, M. Chollet and S. Koshihara, The RATIO method for time-resolved Laue crystallography, *J Synchrotron Rad.*, 2009, **16**, 226–230.
2 M. Lorenc, C. Baldé, W. Kaszub, A. Tissot, N. Moisan, M. Servol, M. Buron-Le Cointe, H. Cailleau, P. Chasle, P. Czarnecki, M. L. Boillot and E. Collet, *Phys. Rev. B*, 2012, **85**, 054302.
3 W. Kaszub, M. Buron-Le Cointe, M. Lorenc, M.-L. Boillot, M. Servol, A. Tissot, L. Guérin, H. Cailleau. and E. Collet, *Eur. J. Inorg. Chem.*, 2013, **5–6**, 992–1000.

John R. Helliwell commented in reply: I fully accept your clarification of the aim of your experiment, thank you. Regarding deconvoluting the overlapping of Laue diffraction spots, either exactly (namely 'multiplets') or spatial overlaps due to the finite diffraction spot size, the accurate estimation of the individual spot intensities was indeed a challenge but circumvented by several techniques, and indeed the measured accuracy of the deconvoluted multiples or the spatial overlaps is now as good as 'singlet' Laue spots,[1] which of course predominate.[2]

1 Y. P. Nieh, J. Raftery, S. Weisgerber, J. Habash, F. Schotte, T. Ursby, M. Wulff, A. Haedener, J. W. Campbell, Q. Hao and J. R. Helliwell, Accurate and highly complete synchrotron protein crystal Laue diffraction data using the ESRF CCD and the Daresbury Laue software, *J. Synchrotron Radiat.*, 1999, **6**, 995–1006.
2 D. W. J. Cruickshank, J. R. Helliwell and K. Moffat, Multiplicity Distribution of Reflections in Laue Diffraction, *Acta Cryst. A*, 1987, **43**, 656–674.

R. J. Dwayne Miller said: This question is aimed at trying to gain some physical insight into the mechanism for this extremely fast spin transition, giving rise to the formation of a spin concentration wave. For spin cross over materials, the transition from low to high spin occurs within a few 10s of a femtosecond. It seems clear that the spin transition is related to the bond displacement along the Fe–N coordinate as you discuss in your paper. What I find unusual is that the mechanism must involve a break down in the Born–Openheimer approximation in treating this electronic transition. The physics for the coupling of the bond displacement to the spin is not evident. For classic treatments of intersystem crossing of photoexcited molecular systems, the chemistry community has rationalize the spin transition in terms of <L.S><FC>, *i.e.* the coupling between the orbital angular momentum (L) and spin (S) weighted by the Franck–Condon (FC) overlap between the low spin and high spin transition electronic manifolds. This relationship gives the perturbative term to the Hamiltonian in which the force acting on the spin system is derived from the fluctuating magnetic moments associated with the photoexcited changes in electron distribution and the associated orbital angular momentum. The transition probability associated with this mechanism is generally many orders of magnitude smaller than what is observed for the photoexcited spin cross over materials. Can you give us some insight into the physical mechanism coupling the spin to the bond displacement coordinate? There needs to be some induced change in the local magnetic moments to couple to the spin, *i.e.* there needs to be a force to flip the spin.

Marylise Buron-Le Cointe replied: It is true that the mechanism behind the extremely fast LIESST, between states with different spin and structure, defies conventional descriptions. All the recent studies of the ultrafast LIESST effect indicate a breakdown of the Born–Oppenheimer approximation (see our ref. 16 and 20). The electronic and nuclear wave functions are strongly coupled during the process which occurs on a time-scale corresponding to elementary molecular deformations or vibrational periods (160 fs). LIESST in SCO systems is thus mediated by the spin–orbit coupling. There is also a vibronic coupling between the excited states involved during LIESST. The LS to HS LIESST can be induced from the relaxation of the initially excited $^1T_1$ ligand field state to the HS state or

from the relaxation of ^{1}MLCT states, as is the case here. van Veenendaal discussed theoretically the role of the significant changes of the metal–ligand (Fe–N) distance between LS and HS states: the coupling between the electronic state and the structure (the HS state is less bonding and the Fe-N bond expands) is similar to a constant force that displaces the equilibrium position of the ligand.[1] The ultrafast inter-system crossing was proposed to result from the dephasing of the photoexcited electronic state into the HS phonon states. This mechanism was recently evidenced by femtosecond pump–probe techniques, which revealed coherent breathing vibrations accompanying the LIESST and the change of molecular structure occurring within 160 fs (see our ref. 20). We should underline that the reverse LIESST process is different as it occurs on the 40 ps time-scale (see our ref. 16) and in this case the Born–Oppenheimer approximation seems valid.

1 M. van Veenendaal, J. Chang and A.J. Fedro, *Phys. Rev. Lett*, 2010, **104**, 067401.