Faraday Discussions
Cite this: *Faraday Discuss.*, 2015, **177**, 517

Future challenges: general discussion

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DOI: 10.1039/C5FD90019K

Martin Zanni opened a general discussion of the paper by **Shaul Mukamel:** Shaul – the experiments that you are proposing are fascinating. Your idea of doing a "Raman" style experiment with X-ray pulses strikes me as a terrific idea because it will be much simpler to implement experimentally yet yield very informative data. I had a few questions. A Raman pulse will presumably be on-resonance to improve signal strengths. Are there differences in the electronic states of the atoms that would make one excited state preferable over another?

Shaul Mukamel answered: In addition to being stronger, Resonance stimulated X-ray Raman signals (SXRS), have another notable advantage: spatial selectivity. A core resonant Raman process creates a wavepacket of valence excitations localized in the vicinity of the selected atom. Other Raman pulses, including the detection pulse, can similarly watch valence excitations around other selected atoms. Thus the signals are very sensitive to the selected resonances and offer an unusual spatial resolution. Stated differently: in an analogous manner to the way resonant optical/UV Raman selects vibrations belonging to a specific chromophore, Resonant X-ray Raman selects valence excitations localized near the atom where the core hole resides.[1]

1 J. Biggs, D. Healion, Y. Zhang, and S. Mukamel, Multidimensional Attosecond Resonant X-ray Spectroscopy of Molecules; Lessons from the Optical Regime, *Ann. Rev. Phys. Chem.*, 2013, **64**, 101–127.

Elangannan Arunan followed up by asking: Could you comment on the difference between X-ray Raman scattering and Compton scattering? Raman scattering was considered the 'visible analog' of Compton scattering observed in X-ray.

Shaul Mukamel replied: Fundamentally there are two types of scattering of radiation by matter: elastic (Rayleigh) and inelastic (Raman). The former is

coherent and scales as N^2 with the number of scatterers, and the latter is incoherent and N scaling. Historically the elastic (inelastic) scattering of electromagnetic radiation by a free charged particle has been named Thompson (Compton). Compton scattering was an important demonstration that X-ray photons can be treated as particles. Taking a broader viewpoint, it is an example of Raman scattering. Similarly, Brillouin scattering is simply a Raman process involving acoustic phonons .

Kenneth Ghiggino remarked: You mentioned briefly in your presentation about results for a Zn and Ni porphyrin dimer. Can you expand briefly on the information that was obtained for this system using the approach outlined?

Shaul Mukamel responded: Using the REW-TDDFT computational protocol,[1] simulated SXRS signals of various Zn–Ni porphyrin heterodimers with different linkers and linking conformations were obtained.[2,3] The time-domain signals can be directly connected to the motion of the excited state wavepacket created by the pump pulse. Both the one-color (pump and probe at the same energy edge) and two-color (pump and probe at different edges) SXRS signals show a real time image of a back-and-forth excitation energy transfer (EET) in the system. This is not available from time-resolved fluorescence anisotropy decay measurements. This study demonstrates that SXRS could be a powerful tool for revealing EET mechanisms in molecular aggregates and could help the design of highly efficient solar energy devices.

1 Y. Zhang, J. D. Biggs, D. Healion, N. Govind and S. Mukamel, Core and Valence Excitations in Resonant X-ray Spectroscopy using Restricted Excitation Window Time-dependent Density Functional Theory, *J. Chem. Phys.*, 2012, **137**, 194306.
2 J. D. Biggs, Y. Zhang, D. Healion and S. Mukamel, Watching Energy Transfer in Metalloporphyrin Heterodimers with Stimulated X-ray Raman Spectroscopy in Real Time, *Proc. Natl. Acad. Sci.*, 2013, **110**, 15597.
3 Y. Zhang, J. D. Biggs and S. Mukamel, Understanding Excitation Energy Transfer in Metalloporphyrin Heterodimers with Different Linkers, Bonding Structures and Geometries through Stimulated X-Ray Raman Spectroscopy, *J. Mod. Opt.*, 2014, **61**, 558.

Wolfgang Junge asked: Shaul, how does your approach compare in signal-to-noise with the UV-vis experiments on a similar topic by Harry Gray[1–3]?

1 J. N. Onuchic, D. B. Beratan, J. R. Winkler and H. B. Gray, *Annu. Rev. Biophys. Biomol. Struct.*, 1992, **21**, 349–377.
2 H. B. Gray and J. R. Winkler, *Annu. Rev. Biochem.*, 1996, **65**, 537–561.
3 J. J. Regan, B. E. Ramirez, J. R. Winkler, H. B. Gray and B. G. Malmstrom, *J. Bioenerg. Biomembr.*, 1998, **30**, 35–39.

Shaul Mukamel answered: The X-ray measurements are more difficult. The signal-to-noise depends on the pulse characteristics and detection modes, which are being improved continuously, so it is hard to give a definite answer. The important point is that they provide qualitatively different information on many electronic states which are not available from narrower band UV/VIS experiments.

Debabrata Goswami questioned: When you have an extreme ultrafast pulse interaction that has a huge bandwidth, it may no longer allow us to use the Born–Oppenheimer approximation, nor the Frank–Condon principle. How do you get the separation of variables? With these kinds of transitions how do you reconcile them with your theory? When you are no longer in that regime, how do you separate them?

Shaul Mukamel replied: I would state the problem differently. A broadband pulse creates a superposition of many electronic states. The standard expansion in adiabatic states given by products of electronic and nuclear states does not necessarily fail but becomes tedious and impractical. In our applications we looked at very short times where nuclear motions may be neglected and only considered the purely electronic response. Treating nuclear dynamics classically pauses no major difficulty. However, developing efficient simulation protocols that treat both electrons and nuclei quantum mechanically is an open challenge. Cederbaum has addressed this issue in recent work.

Himangshu Prabal Goswami commented: Can we not capture the Auger transitions by starting from the original TRPES Hamiltonian in your eqn 1 and going to a higher order in perturbation in the $H_{\mathbf{p}}$, $H_{\mathrm{v}}(t)$, $H_{\mathrm{x}}(t)$ or a combination of the interacting Hamiltonians, instead of introducing the operators $H_{\mathbf{k}}$ and A, as introduced in eqn 44 of your paper, separately in the Hamiltonian.

Shaul Mukamel responded: The interactions represented by H_{x} and H_{v} are field-matter interactions and while a suitable combination could accomplish the same orbital re-arrangement as the Auger process, it would alter the field (the transition would be radiative). The Auger process, in contrast, is a non-radiative transition mediated by Coulomb matrix elements (as opposed to transition dipoles or combinations thereof).

Artur Nenov asked: How is the dephasing during t_1 and t_3 described for the TRPES signal (eqn 30 and 37 of your paper)? If a constant dephasing was used, what values were used for generating the TRPES spectrum?

Shaul Mukamel answered: The spectrum in our Fig. 3 is created by using eqn 37 in our paper, which averages over a set of semi-classical trajectories on a signal level. The nuclear degrees of freedom are treated classically, implying that the dephasing due to the vibrational motion is included in the model. By applying the surface hopping method and ionizing from a specific state the dephasing is implicitly included. Coherence between electronic states is encoded in the fluctuating gap coordinate. In Fig. 3, both initial pumping and photoionization were taken to be impulsive and thus the dephasing was taken into account only for time period (t_0). Equation 30 is more general and uses the fluctuating gap coordinates to recast the coherences into populations during the time periods.

Artur Nenov remarked: The expression for the TRPES signal (your eqn 30) is obtained under the assumption of a coherent superposition during the coherence time t_1 since one follows the fluctuation of the energy gap along each trajectory. Does this assumption hold for ultrafast dynamics where the conical intersection is reached from the excited state within few tens of fs, while it is inaccessible from the ground state? Does one need to correct for the decoherence of the wave-packets propagating on the ground and excited states?

Shaul Mukamel replied: If the conical intersection (CI) can be reached during the pumping pulse, then it is possible for the ket (or bra) to reach the CI before the bra (or ket) can interact with the field to generate the presumed population at time t_2. Relaxing the assumption of a population-Green's function for the time period t_2 would include the processes indicated by the question. This could be done by introducing a fluctuating gap coordinate as was done for t_1 and t_3 .

E. D. Jemmis opened the discussion of the paper by **John R. Helliwell**: Your results clearly indicate that the Pt–Br bond-breaking process in the protein–$PtBr_6$ complex is faster than the Pt–I bond-breaking process. Is this in tune with the bond strengths involved? Are there any computational or experimental data available on bond strengths? Are the observed differences a result of an unusual coincidence of the cavity size available to the protein fitting well for the PtI_6 complex and rather loosely for the $PtBr_6$ complex?

John R. Helliwell responded: As you indicate the expectation would be that the Pt–I bond breaking would be quicker than the Pt–Br as the former bond, I expect, is weaker than the latter, *i.e.* the Pt to bromide and Pt to iodide bond distances are respectively 2.4 *versus* 2.7 Angstrom (see our PDB codes 4owh and 4owc described in ref. 1). Secondly, I like your observation that the observed differences in X-ray radiation sensitivity might be a result of an unusual coincidence of the protein surface cavity size available to the PtI_6 complex matching it better than for the $PtBr_6$ complex.

1 S. W. M. Tanley, L.-V. Starkey, L. Lamplough, S. Kaenket and John R. Helliwell, *Acta Cryst.*, 2014, **F70**, 1132–1134.

Mike Ashfold asked: I was very struck by the sequence of images showing the progressive X-ray induced loss of Br atoms from the room temperature hen egg white lysozyme bound $PtBr_6$ species in your Fig. 4. Given that these are held in a non-spherical pocket, is it fanciful to imagine that one might be able to recognise site specific Br atom loss – now or in the future?

John R. Helliwell answered: The bromines obviously go somewhere as they depart the platinum hexabromide. Since this crystal form of hen egg white lysozyme is grown from NaCl as precipitant, the bromines would compete with the

chlorides already bound to the protein, so most likely the bromines will go into the crystal solvent channels and thereby will not be visible to X-ray crystal structure analysis. The other aspect is that we expect the bromines to be steadily replaced with waters as per our Fig. 5. These, at fractional occupancy, are not obviously visible in the electron density of our Fig. 4.

Neil Hunt commented: The abstract of your article refers to lattice-free single molecule diffraction. What are the challenges facing the practical achievement of this?

John R. Helliwell replied: The macromolecular structure determination studies at the Stanford LINAC Coherent Light Source, as I explained in my Science Perspective article (reference 7 of our article), are currently working with a microcrystal size range of samples. The next step would be nanocrystals, then nanoclusters and then finally single molecules (or complexes such as my own research sample alpha crustacyanin with a 320 kDa molecular weight). To reach these successive stages of sample volume capability is the challenge, one that our article discusses in detail. I would add though that it was openly discussed at The Royal Society Discussion Meeting held in October 2013 (ref. 5 of our paper is a collection of most of those articles; from these see my book review at *J. Synchrotron Rad.*, 2015, **22**, 191–192) that a higher peak X-ray pulse flux would help the objective of measuring X-ray diffraction data from smaller samples. However a counter argument was made by Dr Abbas Ourmazd that ultra weak X-ray diffraction patterns should also lead to interpretable electron density maps from samples smaller than microcrystals (see especially his abstract at https://royalsociety.org/events/2013/xray-lasers-satellite/).

Elangannan Arunan addressed **John R. Helliwell**: In his talk Prof. Helliwell mentioned finding 'electron density within a molecule' by using X-ray methods. Two slides on the recent Atomic Force Microscope images of chemical bonds were shown at the end of this session. One of them had a beautiful image of a 'hydrogen bond'.[1] This image has been questioned recently in another study by Swart and coworkers[2] who have titled their paper 'Intermolecular contact in AFM images without intermolecular bonds'. I welcome any comments about whether this question can be unambiguously answered by any of the techniques discussed during this meeting. X-ray, attosecond, ...?

1 J. Zhang *et al.*, *Science*, 2013, **342**(6158), 611–614.
2 S. K. Hämäläinen, N. van der Heijden, J. van der Lit, S. den Hartog, P. Liljeroth and I. Swart, *Phys. Rev. Lett.*, 2014, **113**, 186102.

John R. Helliwell answered: Thank you for drawing my attention to these two articles on AFM results. In the short time available to me set by the Faraday Discussion deadline I have looked to understand how AFM might allow quantitative structural chemistry studies (bond distances and angles having standard

deviations that allow bond order discrimination for example). The two articles you quote seem not to provide such details. Nevertheless, from the reference list of the *Phys. Rev. Lett.* article, I found the associated article: *Science*, 2012, **337**(6100), 1326–1329 entitled "Bond-Order Discrimination by Atomic Force Microscopy" by L. Gross *et al.*. Their abstract states "The greater electron density in bonds of higher bond order led to a stronger Pauli repulsion, which enhanced the brightness of these bonds in high-resolution AFM images. The apparent bond length in the AFM images decreased with increasing bond order because of tilting of the CO molecule at the tip apex." These are certainly impressive studies by AFM. The AFM approach seems to allow a semi-quantitative scrutiny of the structural chemistry unlike crystal structure analysis by X-rays (or neutrons) where detailed molecular crystal structure refinement is undertaken and standard deviations on bond distances and angles in a molecule can be derived. [*N.B.* on a point of nomenclature standard deviations are now referred to as standard uncertainties.]

Neil Hunt continued the general discussion of the papers by **John R. Helliwell**, **Shaul Mukamel** and **Martin Meedom Nielsen**: In light of the session being one that addresses future directions, can you comment on how you envision your respective research areas evolving over the next decade?

John R. Helliwell commented: I have no budget for the methods development and structural biology (on the 320 kDa alpha crustacyanin multi-macromolecular complex) research that I have outlined in my article, however the fact that the UK is now a formal participant in the Euro XFEL encourages me to think that it should be possible to undertake the work, *i.e.* it might be funded.

Shaul Mukamel responded: Single molecule diffraction is an exciting future development made possible by recently developed ultrafast and intense free electron laser pulses. The connection of diffraction to spectroscopy should be clarified. Classically, diffraction patterns are thought of as the interference of elastically scattered X-ray wave-fronts emanating from different particles.[1] A fundamental difficulty in extending diffraction to single molecules is that X-ray scattering (as any light scattering) from single molecules may not be simply described as "diffraction". There are two basic microscopic mechanisms for light scattering from an assembly of N particles in the ground state. The first is incoherent, where the entire process occurs with a single particle. The second is coherent, and involves a pair of particles each generating a scattering amplitude.[2] These two components of the X-ray scattering signal come with different form factors, unlike in the classical theory.[3] Additionally, they scale as N and N^2, respectively, with the number of particles. Coherent scattering from the ground state is elastic, whereas incoherent scattering contains both elastic and inelastic components. This classification is based on the fundamental way in which the field and matter interact (how the detected field mode is populated from the vacuum) and holds regardless of the initial state of the system (be it stationary, pure or mixed). In optical spectroscopy, elastic and inelastic scattering is known

as Rayleigh and Raman, respectively, whereas elastic and inelastic scattering of X-rays from a free charged particle are historically known as Thompson and Compton, respectively. In the spectroscopic language, X-ray diffraction is thus an example of Rayleigh scattering. For a single molecule , $N = 1$, elastic and inelastic signals may be comparable and the scattering process is better labeled as Rayleigh and Raman rather than diffraction.

1 J. Als-Nielsen and D. McMorrow, Elements of Modern X-Ray Physics, Wiley, Hoboken, 2011.
2 K. E. Dorfman, K. Bennett, Y. Zhang and S. Mukamel, Nonlinear Light Scattering in Molecules Triggered by an Impulsive X-Ray Raman Process, *Phys. Rev. A*, 2013, **87**, 0853826.
3 K. Bennett, J. D. Biggs, Y. Zhang, K. E. Dorfman and S. Mukamel, Time-, Frequency-, and Wavevector-Resolved X-Ray Diffraction from Single-Molecules, *J. Chem. Phys.*, 2014, **140**, 204311.

Martin Meedom Nielsen answered: For an ultrafast time-resolved molecular structure, I see a very interesting development towards using combinations of different spectroscopic and scattering techniques, simultaneously in the same experiment. This has the potential of providing not only complementary information about spin and structural dynamics of the system, but interestingly, as the measurements are truly simultaneous, it will enable to cross correlate findings between the methods and enhance the ability to extract quantitative results. For the sample systems, I see a move towards ever more complex systems. Also systems that are close to 'real' applications in chemistry, materials science, and electronics. This is a very important development for the field, which has for a long time been limited to a set of model systems.

Judith Howard asked: Where do you think the subject will be in another 10–20 years with respect to membrane proteins?

John R. Helliwell replied: As an objective measure the website http://blanco.biomol.uci.edu/MP_Structure_Progress.html monitors and analyses the PDB deposition statistics for membrane protein structures in detail and the growth rate of this category of depositions in the PDB suggests that "we can expect the number of new membrane protein structures by 2025 to be over 2000". In January 2015 the number of membrane protein structures in the PDB, mainly due to X-ray crystallography, logged by the same website were: "unique proteins in database = 522; coordinate files in database = 1592; published reports of membrane protein structures in database = 931 (These counts do not include pre-publication structures)." Clearly the progress in macromolecular crystallisation techniques for this previously challenging category of protein structures for crystal structure analysis has therefore been substantial. It would also be likely that the new X-ray lasers and ultra-bright upgraded synchrotron radiation X-ray sources, such as the world leading ESRF and initiatives like the MASSIF (massively automated sample selection integrated facility) for the automatic screening of huge numbers of samples to assess their single crystal diffraction characteristics, are likely to accelerate the rate of membrane protein crystal structure determinations further. Also most major research infrastructure synchrotron sites in Europe have, or are developing,

multidisciplinary centres, like the Partnership for Structural Biology (Grenoble), the Centre for Structural Systems Biology (Hamburg), the Membrane Protein Laboratory and the Oxford Protein Production Facility (RAL). In the USA, the APS is building its Advanced Protein Crystallization Facility, which will allow the production, characterization and crystallization of proteins. All these, often multi-facility/multi-laboratory partnerships, stretch facility impact beyond the supply of X-rays and/or neutrons to helping users prepare proteins, perform quality control and crystallize them, and refine conditions.[1]

1 J. R. Helliwell and E. Mitchell, Synchrotron radiation macromolecular crystallography: science and spin-offs, *IUCrJ*, 2015, **2**, 283–291.

R. J. Dwayne Miller continued the discussion of the paper by **Martin Meedom Nielsen**: I am sure you did not go into these experiments wanting to get into the details of the detector response. Characterizing the detector was really heroic work. Your method of correcting for the nonlinear response of the detector was key to pulling out the dynamics of interest. However, the detector is still intrinsically nonlinear. The noise characteristics are not the same as a linear detector under which the detector can operate within the shot noise limit. For most experiments, the biggest source of noise is the laser excitation and probe contributions to the overall signal detection, as well as sample issues. At XFELs, one is using a SASE source in which the shot to shot variations are nearly chaotic with variances over 100%. In a time-resolved measure, one is doing a differential measurement for time-resolved diffraction or diffuse scattering experiments and must detect signal changes on the level of 1% or less. Even if the normalization for shot to shot variations, timing jitter, spectrum fluctuations are handled perfectly, there are noise contributions in the detection used for normalization, in addition to the nonlinear detector response and associated increased sensitivity to noise in the low intensity detection range. The very significant difference in noise amplitude in the low intensity relative to the nonlinear higher intensity regimes of the detector response may lead to problems in trying to detect very small changes in signal. For just simple femtosecond pump–probe spectroscopy, one needs around the order of 0.5% rms noise, from all sources (laser, detectors) within the detection bandwidth. In this case, the laser excitation/probe are the largest contributor to the noise and laser operation around 0.5% rms is needed to pull out changes in signal of the order of 0.1%. Can you comment on the effect of noise on the measurement, notably the contributions from the X-ray probe noise (after all the normalizations) and the nonlinear noise contributions to the detector? Given the noise limits, can you give an approximate estimate of the concentration and fraction excited that is needed to clearly resolve structure changes above background scatter from the solvent? This estimate will of course depend on the Z contrast of the photoactive system under study. If you could give a range for different Z values it would be appreciated to help guide future experiments.

Tim Brandt van Driel replied: The challenges at XFELs, as opposed to synchrotron sources, have been the systematic errors and nonlinear effects, as well as artifact-like fluctuations, that could not be corrected for or averaged out.

This is an area of constant development both with regards to detector development and data processing. After the presented corrections have been applied, the CSPAD detector still exhibits common mode fluctuations that do not average out with the number of images usually measured for X-ray diffuse scattering. After corrections, the remaining fluctuations are common mode and Poisson distributed counting noise similar to CCD cameras.

The scattering power of the sample depends on Z, but the difference scattering varies in the measured Q-range and depends on the Fourier transform of the changes in the electron density distribution. The background noise depends on the Fourier transform of the full electron density. Therefore the resulting difference signal is given by the absolute number of excited molecules and the noise is given by the total number of molecules in the beam path. From recent experiments a difference signal of at least 0.1% is needed for the easy identification of a difference scattering signal. For solute molecules containing heavy metal centers (Ir or Pt), or even polypyridyl metal-centered complexes (Fe, Co and Ru), these signal levels are currently achievable with 10 mM concentrations and excitation fractions around 10%, but it depends on the experiment.

Martin Meedom Nielsen added: Regarding the nonlinearity of the detector, referring to Fig. 8b and 8c in our paper, the detector is linear to better than 1% over a quite wide range of beam intensities. Even so, some of our analysis has been enabled by grouping measurements according to relatively narrow intensity ranges allowing a less than 10% variation in measured intensity in the groups. It is true that the shot-to-shot variations of the SASE source may lead to problems in detecting small changes in the signal. These problems can be checked for by comparing the difference signals obtained over a range of variations in incoming intensity. The difference signal is observed to converge as this range is systematically reduced. In this way, we further reduce the effect of non-linearities and can significantly enhance the ability to observe small changes in the signal. Referring to Fig. 14 in our paper, we can reliably detect changes in signal intensity well below 0.1% after applying the corrections described in this paper.

Comparing our Fig. 14 with Fig. 4 in reference 1, a sensitivity of order 0.1% is more than adequate for distinguishing between solvent and solute contributions to the data. We have not performed quantitative simulations of this, but based on recent experience with 100 μm thick liquid jets we find that around 10 mM concentration is fully adequate for Fe and Co-based compounds such as $Fe(bpy)_3$ and $Co(terpy)_2$. Typical excitation fractions under beamline experimental conditions have been in the range of 20–40% for these compounds.

1 K. Haldrup, G. Vankó, W. Gawelda, A. Galler, G. Doumy, A. M. March, E. Kanter, A. Bordage, A. Dohn, T. van Driel, K. Kjær, H. Lemke, S. Canton, J. Uhlig, V. Sundstrom, L. Young, S. Southworth, M. Meedom Nielsen, C. Bressler, Guest-Host Interactions Investigated by Time-Resolved X-ray Spectroscopies and Scattering at MHz Rates: Solvation Dynamics and Photoinduced Spin Transition in Aqueous $Fe(bipy)_3^{2+}$, *J. Phys. Chem. A*, 2012, **116**, 9878–9887.

John R. Helliwell said: On page 20 of your article you make the point that the SVD type of correction method you have harnessed can be used for any

fluctuations relating to (variations in the) detector electronics. Once you move away from the detector prototype that you used thus far, *i.e.* to more mature devices, such detector instabilities are unlikely. The effects on the X-ray diffraction intensities of changes in air scattering will be small in any case. Can you please comment on both these two points?

Martin Meedom Nielsen replied: Ideally, one should never be forced to use correction schemes, such as the ones we have developed. However, the area detectors used since the start of operation of the XFEL facilities have all been suffering from electronic fluctuations and non-linearities, making the interpretation of difference scattering signals very challenging. It is certainly a hope, and often also true, that fluctuations in detector electronics become less and less important as the technology matures. In the latest generation of the CSPAD detector, we can directly observe that the relative magnitude of the correction terms become smaller compared to the older versions. Regarding changes in air scattering, it is a concern when measuring the difference in scattering signals. Often the sample is held in a He environment, but some amount of atmospheric air is always present, and if this amount changes, *e.g.* due to leakage, it changes the difference scattering signal. Also without He enclosures, differences due to air scattering are readily observed if the air surrounding the sample is moving, for example if air is blown over the sample from an air conditioning unit. Using difference scattering, we are extremely sensitive to changes in the scattering signals. This is a great benefit for analysing changes in molecular systems, but also a challenge when phenomena not related to the molecular structure are fluctuating and causing changes in the scattering signal. Hence we need tools to identify different sources of such changes, and here we have had good experience using the tools described in the paper.

Neil Hunt queried: How do you see access to large facilities, such as XFELs evolving as technology advances?

Martin Meedom Nielsen responded: Access to making experiments at XFELs will presumably always be more limited than access to making experiments at synchrotron sources, as long as there are fewer XFEL facilities and as long as only a limited number of their experimental stations are capable of operating simultaneously. The latter will improve. LCLS has developed and is already operating a 'beam sharing' mode, and the European XFEL is from the design already envisaged to be able to operate more than one experiment at the same time. Additionally, I see a big potential in development of software for accelerating the output of results from XFELs and making the experiments more efficient. Virtual experiments will help users design and optimize their experiments, and standardised software solutions for data reduction and analysis will help the users convert their data into results much more rapidly and reliably. This will undoubtedly also be an important parameter in opening XFEL science to a wider community.

Wolfgang Junge opened a general discussion of the paper by **R. J. Dwayne Miller** by communicating: Dwayne, how do you overcome a possible limitation of time-resolution by Coulomb spread of the electron pulse?

R. J. Dwayne Miller communicated in reply: Thanks for this question as it allows me to expand on this key enabling development. This question is related to the one on linear chirp on the electron pulse. We discovered that it is possible to minimize Coulombic repulsion effects on the spatial-temporal resolution by properly managing the longitudinal Coulombic or space charge effects on pulse broadening, without affecting the transverse beam divergence significantly. The longitudinal broadening (defined by the pulse propagation direction) leads to loss in time-resolution. The transverse broadening to this direction leads to increased beam divergence and loss of spatial resolution. The epiphany moment in how to optimize electron pulses for femtosecond structural studies came through an effectively exact solution to the coupled equations of motion for up to 10 000 electrons.[1] It should be noted that with the very high scattering cross section of electrons, this number of electrons is sufficient for single shot atomic structure determination of many solid state systems. For a 100 micron beam parameters needed to match typical laser excitation conditions, the space-charge broadening was confined primarily to longitudinal pulse broadening. This detail needs to be fully appreciated. Moreover, there were two solutions that jumped out of these calculations that were completely missed in previous work using solely analytical methods with overly simplified approximations. The first most obvious solution was that if the electron pulse broadens with propagation, then simply don't let it propagate very far. In this work, we could determine exactly the electron gun dimensions needed to achieve 100 femtosecond electron pulses without sacrificing the spatial resolution. This point was obviously understood qualitatively but we could not determine precisely the spatial scale for electron acceleration. Now we can. This new insight led to the development of the "compact electron gun", which is capable of over 10^5 electrons with sub-100 fs pulse durations, and over 5 nm transverse coherence, in our present designs. These systems are by far the simplest and most robust sources for structural dynamics. With new developments in photocathodes giving lower emittance, or higher transverse spatial coherence, these sources will soon be capable of studying systems as large as proteins. The second solution is more involved but it was also immediately apparent from these calculations. We discovered that the longitudinal space charge effects still conserve the space-time correlation. The electrons at the front of the electron pulse experience the electron charge distribution at the back of the pulse and are accelerated; whereas the electrons at the back of the pulse are decelerated. The electrons at the front of the pulse stay at the front and the electrons at the back stay at the back. For nonrelativistic electrons, the higher energy electrons from this process move forward in time relative to the retarded electrons. The big surprise was that this "chirp" or variation in space-time is effectively perfectly linear. This effect was completely unexpected and certainly missed by analytical methods prior to this work. When one sees such a linear chirp, it is clear that simple dispersive elements can be used to temporally refocus the electron pulse down to near its initial conditions at birth and place a sample at the re-focusing point. Basically any dispersive element that makes the

higher energy electrons travel a longer path than the lower energy electrons will work, with the condition that the spatial overlap should be reconstituted at the sample position. These elements are well known in the accelerator community. The best dispersive element for this application has been developed by the Luiten group using a so-called pill lens for rf pulse compression. This device creates a close to linear chirp of opposite sign, without affecting the emittance or spatial properties of the pulse, to recompress the pulse at the sample position. Pulses with up to 10^6 electrons and pulse durations as short as 30 fs can be generated with this approach. There are technical challenges associated with timing jitter between the laser excitation and the rf that limit the time-resolution to approximately 200–300 fs, the exact same problem as incurred with XFELs for the same reason. Time stamping methods have been developed that enable correction of this jitter to 30 fs.[2]

Of the two technical achievements in optimizing electron pulses for structural dynamics, the compact electron gun is the simplest to use and by far the most robust. It reduces the real time observation of atomic motions to experiments no more difficult than conventional pump–probe experiments that are routinely done in the femtosecond laser community. I would only recommend using rf pulse compression or other pulse compression methods if the higher time-resolution (<100 fs) is warranted to solve the problem of interest.

1 B. J. Siwick, J. R. Dwyer, R. E. Jordan and R. J. D. Miller, *J. Appl. Phys.*, 2002, **92**, 1643–1648.
2 M. Gao, Y. Jiang, G. H. Kassier *et al.*, *Appl. Phys. Lett.*, 2013, **103**(3), 033503.

John R. Helliwell commented: The 10^6 gain of electrons as a probe over X-rays in their scattering efficiency by matter is indeed very attractive, but traditionally this strong interaction has made interpretation difficult (due to dynamical scattering effects) and in particular standard deviations on atomic positions are either not quoted or are quite a lot larger than those derived from X-rays or neutrons as probes of the structure of matter. Can you please comment on this as it will underpin how well electron diffraction can be harnessed in quantitative structural chemistry or structural biology studies?

R. J. Dwayne Miller answered: The high scattering cross section and the very short de Broglie wavelengths are major distinguishing features of electron probes in relation to X-ray probes of structure. The much shorter wavelength actually transforms to higher spatial resolution (in the limit the samples are of sufficient quality and the diffraction occurs in the kinematic scattering regime). This much higher spatial resolution and scattering cross section is explicitly exploited in gas phase diffraction from which most of the bond length information is determined. I make this distinction to point out that intrinsically electrons have a much higher information content per scattering event and a much higher spatial resolution. The real problem as you point out is the effect of multiple electron scattering for samples that are too thick relative to the electron elastic mean free path and/or too thick relative to the inelastic electron mean free path that limits electron transmission and creates background scatter. In the past, it has been very difficult to attain samples sufficiently thin to avoid this problem. There have been only a

few classic examples of solving protein structures using electrons for systems such as bacteriorhodopsin that naturally form 2D crystals. However, with the latest developments in nanofabrication methods, this problem is no longer restricting the use of electron probes. Moreover, it is becoming apparent that some of the most important protein targets, such as G-protein coupled receptors, intrinsically do not grow large crystals of sufficient quality for structure determination, but it is possible to attain submicron or nanocrystals of these and related systems. The ability to do protein nano-crystallography has been one of the main scientific motivations for XFELS. If protein nano-crystallography is indeed as important a new direction for structural biology as currently thought, electrons have a decided advantage. This length scale is perfect for electrons to avoid multiple electron scattering. The real challenge for both X-rays and electron approaches is sample delivery.

To specifically address your point, we deliberately did a detailed study of the effect of multiple electron scattering on the problem. In our Fig. 1, we did a first principles calculation of the electron scattering cross section using Al as a well-defined test case. In Fig. 6 and 7, we show the effect of different sample thicknesses on the observed diffraction pattern and compare to the ideal single scatter limit. It is clear that we can still resolve diffraction intensities well correlated to structures up to 3–4 times the electron mean free path. For relativistic electron regimes, this means samples close to micron thickness can be studied. This finding may seem surprising. However, issues raised over multiple electron scatterings do not consider the diffuse scatter of the crystal itself, even within the single scatter limit. It is only for perfect crystals that one could state that multiple electron scattering is limiting the resolution. In fact, it is usually crystal quality and not multiple scattering that will limit the ultimate spatial resolution for a given sample. This statement will be especially true for nanoprotein crystals where it is unclear if such systems will be inherently poorly diffracting systems due to the surface strain and disorder effects. The spatial resolution one can attain for protein nanocrystals remains an open question. There is no fundamental limitation in the resolving power of electrons in this case. High resolution structure determination will be possible. There is also the prospect that the new high coherence electron sources could even provide atomic resolution for poorly diffracting nanocrystals as there is additional information in the scattering process that has yet to be exploited.

I should also respond to your question with respect to extracting transient structures for photoinduced structural transitions and photochemical processes, which is the main emphasis of this work. We are not trying to resolve unknown static structures. We know the starting structure and in most cases the final fully relaxed structure. We can use *ab initio* phasing methods such as charge flipping methods, but given the enormous number of diffraction orders and constraints on the structural changes, it is simplest to model the structural changes and do a correlation analysis.[1] We have found a robust convergence in tracking the structural changes. The variances to the fits are obtained from the coefficients in the Pearson correlation analysis. For cases where there is a singularly important parameter such as the bond elongation in undergoing a spin transition or metal–ligand charge transfer band, your point is well taken. We have not reported on such systems as yet. It would be helpful to explicitly state the error bars in regard to specific displacements, especially for comparison to theoretical predictions.

1 M. Gao, C. Lu, H. Jean-Ruel, L. C. Liu, A. Marx, K. Onda, S.-ya Koshihara, Y. Nakano, X. Shao, T. Hiramatsu, G. Saito, H. Yamochi, R. R. Cooney, G. Moriena, G. Sciaini and R. J. Dwayne Miller, *Nature*, 2013, **496**, 343–346.

Siva Umapathy asked: When you observe the motion of particles, if there is a solvent effect (such as inertial solvation) on the dynamics, which would be at the same time scale as pulse evolution, can you get information of the coordinates?

R. J. Dwayne Miller replied: We have developed nanofluidic cells to enable the study of solution phase chemistry. We have demonstrated path-length control to achieve a stable 100 nm flow, which is sufficient for even non-relativistic electrons to be used to probe solution phase processes and even real space imaging within conventional TEMs. As discussed the development of REGAE was in fact motivated to take advantage of the much higher penetration depth of relativistic electrons to enable direct observation of atomic motions in liquid environments. This feature is important as most chemistry occurs in the solution phase. However, to date we have not studied solution phase chemical processes as we are further refining the nanocell concept. I can however comment that the time-resolution is sufficient to follow even the fastest solvation dynamics as exemplified in water. The solvation dynamics have an inertial contribution as you point out. For water the fastest motions involve hindered librational motions with an approximate density of states weighted relaxation time of 70 fs. This time scale can be resolved. The specific motions may be very difficult as this is a collective response involving small net displacements of some 80 water molecules within a typical solvation volume. The effect is larger in terms of the relaxation energetics but the driving force is spread out over a large number of molecules. There are similar relaxation processes within the solid state in which adjacent moieties or unit cells undergo a concerted relaxation around photoproduct states and here one can observe the motions. The motions of the PF_6-counterion in EDO–TTF is a case in point in which we were able to well resolve its motions.

Martin Meedom Nielsen commented: You describe multiple scatterings of electrons as a problem for understanding the scattering signal. However for Low Energy Electron Diffraction (LEED), multiple scattering has been used to enhance the sensitivity of the method, by measuring the scattered intensity as a function of the acceleration voltage of the electron beam (the so called Intensity–Voltage (IV) curves), see *e.g.* the books by J. B. Pendry, *Low Energy Electron Diffraction*, 1974, and M. A. van Hove and S. Y. Tong, *Surface Crystallography by LEED*, 1979. Would it be possible to change the energy of the electrons in order to measure IV curves and retain the time-resolution in your ultrashort electron pulses? Would it limit the time-resolution when using electrons at low energies?

R. J. Dwayne Miller answered: To be clear, my main point is that multiple electron scattering can be well handled by proper care in preparing the samples. For time-resolved studies, one knows the starting, and in most cases, the end

point structures, so that less information is needed for determining the changes in structures relative to the static structure determination. This feature is due to the differential detection with and without laser excitation that removes much of the inelastic scattering contributions that are more or less constant for chemical reactions, especially on picosecond to subpicosecond time scales for which there is negligible non-radiative relaxation into lattice heating. From our studies of the thickness dependence on the diffraction intensities for different diffraction orders, samples with thicknesses at least 2–3 times the inelastic mean free path will be sufficient. We generally try to keep the sample closer to the single scatter limit as we did for the organic systems we have studied. I quite like your comment about scanning the electron energy to exploit multiple scattering to enhance sensitivity and by that structural resolution. This approach can easily be done as previously with LEED. The time-resolution is challenging as it is difficult to keep the electron propagation path short enough for low energy electrons to in turn keep the pulses subpicosecond with sufficient electrons. I expect it should be possible to attain picosecond time-resolution with much averaging of the low electron bunch charge to keep the pulses as short as possible. If a reversible surface process can be found then 100 femtosecond time-resolutions will be achievable. I am most interested in surface chemistry, which is generally non-reversible. Studies of surface chemistry will require low excitation and sampling rates with sufficient times for the regeneration of the surface between excitation events – all under UHV conditions. It is challenging but possible.

Martin Meedom Nielsen added: Is it possible to tune the energy of the electron bunches, and if yes, could one use this to make spectroscopic studies?

R. J. Dwayne Miller responded: It is rather simple to tune the voltage in a programmable way. The only issue is the field gradient. We have developed a tunable gap to keep the gradient constant as one scans the electron energies. This question really represents an excellent suggestion. The answer is yes.

Debabrata Goswami asked: You have used linearly compressed pulses, do you know what happens if you do a systematic study as a function of chirp on the results? Are the results only dependent on the pulse width?

R. J. Dwayne Miller communicated in reply: There are both the pulse widths, or time-resolution, and the spatial resolving power that require attention when using compressed electron pulses. The chirp that naturally develops for non-relativistic electrons is exceptionally linear, amazingly so. For relativistic electrons, it is very close to linear within the field gradient used. Small nonlinear terms can be corrected in the rebunching cavity. The only effect is that one can broaden, linearly, the pulse to stretch it and enable more electrons per pulse without incurring a space charge time broadening of the pulse. In principle, there is no limit to the pulse compression in the linear chirp regime. The only problem is that the path-length at the sample position over which the pulse is compressed.

If you inspect the inset to our Fig. 5 (panel d), you will see that the pulse is compressed at the sample position and then increases in pulse duration after this point. For REGAE parameters, the length over which the pulse is optimally compressed is in the order of a few centimeters so it is relatively easy to temporally focus at the sample position. However, if we increase the electron bunch charge more, the compression results in space charge effects that lead to a loss in transverse spatial coherence and the optimally compressed region becomes smaller than practical. We see this effect as a loss of spatial resolution or diffraction quality. The limit to electron bunch density is very easy to determine. The numbers we have presented are for optimal conditions with the maximum electrons per pulse without sacrificing spatial resolving power for following molecular processes for systems as large as proteins.

Tim Brandt van Driel communicated: When using electron diffraction to record 10 fs resolution movies in a single shot, is this only doable for large structural changes such as sample melting? Are the 10 fs the full instrument response function and what are the main contributions such as laser and electron pulse duration? Additionally, do you have similar problems as X-rays with sample damage, sample charging or Coulomb explosion?

R. J. Dwayne Miller communicated in reply: The concept of using a streak camera was not discussed in our paper but is mentioned here to give some perspective on future directions. This idea of obtaining full movies in a single shot has been discussed previously. This prospect reduces sample requirements by nearly two orders of magnitude in terms of having a sufficient surface area to collect the atomically resolved dynamics relative to stroboscopic, single time point sampling. In this regard, I would like to particularly point out the work of Heinrich Schwoerer's group where they have shown significantly improved signal-to-noise using a streaked time base to collect all the dynamics at once.[1] They show full movies captured for the photoinduced modulation of the structure order parameter for the charge density waves in TaS2. This is a rather complex diffraction pattern and the motions involved are very small (0.1 Å or less depending on the excitation level). The time-resolution they achieved was approximately 200 fs, limited by the field gradient they could apply. Similar demonstrations of the concept have been given by Musumeci's group where they gave a proof of principle study of melting using relativistic electrons in which a 400 fs time-resolution was demonstrated.[2] The latter study did not take into account the nonlinear features of the rf field used for streaking. We have modelled the parameters for REGAE and have developed a more linear sweep field. We expect to achieve a 10 fs time-resolution based on the streak velocity and pixel resolution of our detector. We have also developed analytical methods that take advantage of the known initial structure to extract the dynamics from the 2D streaked diffraction image. It is clear we can use this approach for molecular systems as large as EDO-TTF with unit cells up to several nm.[3] The question is what is the minimum amount of information one needs to construct an image? It is not inconceivable that this approach could scale to systems as large as proteins given the known starting structures and fully relaxed structure or end points to

constrain the fits. The streak camera approach provides sufficient time-resolutions to capture the fastest possible nuclear motions while enabling the study of precious samples, *i.e.* irreversible systems that cannot be obtained in sufficiently high quality crystals for multiple sampling methods. It effectively increases the source brightness by another factor of 100 or more depending on the dynamic range sampled by the streak field. One can keep the electron bunch charge per unit time constant and simply stretch the pulse by a factor of 100 or more and increase the total number of electrons accordingly to sample the dynamic range of interest in a single shot. This is truly a unique feature of electron sources that should be fully exploited.

As per the electron induced damage of the samples, we have never observed this effect. In a conventional TEM, the areal beam current is many orders of magnitude higher than the average currents used for femtosecond electron diffraction. We have observed sample damage in seconds within a TEM yet we have collected data for months on the same sample without any noticeable changes using our femtosecond electron sources (EDO-TTF as a case in point). We often inspect samples for diffraction quality first in a TEM before committing beam time for femtosecond diffraction studies. We can attest that electron induced damage is not an issue as it is for X-rays or TEM focusing conditions. Basically, the peak current for the femtosecond electron pulses is high but the average current is many orders of magnitude less than conventional TEMs. There is essentially no change in the lattice temperature from the inelastic scattering of the electrons at the average currents used, which completely eliminates thermally induced lattice damage. In terms of electron induced ionization effects, we are many orders of magnitude below the 1–10 e^-/atom flux where this effect dominates. The sole source of sample damage is due to the optical excitation, which has been the driving force for developing the brightest electron sources possible to collect atomically resolved structures for even complex systems in a single shot – and single shot atomically resolved full movies are now possible.

1 M. Eichberger, N. Erasmus, K. Haupt, G. Kassier, A. von Flotow, J. Demsar and H. Schwoerer, *Appl. Phys. Lett.*, 2013, **102**(12), 121106.
2 P. Musumeci, J. T. Moody, C. M. Scoby, M. S. Gutierrez, M. Westfall and R. K. Li, *J. Appl. Phys.*, 2010, **108**(11), 114513.
3 M. Gao, C. Lu, H. Jean-Ruel, L. C. Liu, A. Marx, K. Onda, S.-ya Koshihara, Y. Nakano, X. Shao, T. Hiramatsu, G. Saito, H. Yamochi, R. R. Cooney, G. Moriena, G. Sciaini and R. J. Dwayne Miller, *Nature*, 2013, **496**, 343–346.

Priyadarshi Roy Chowdhury asked: What are the basic factors that should be taken into consideration during the mapping motions with ultra bright electrons in the cases of different types of molecules in solid, semi-solid or volatile samples?

R. J. Dwayne Miller responded: Let me first address solid state samples for femtosecond electron diffraction studies where one has the highest space-time-resolution. The machine physics for attaining a sufficiently bright electron source has been solved in that it is no longer limiting problem selection. The real challenge now is the samples. Generally speaking, one would like to probe different aspects of

fundamental issues related to structural dynamics and material properties. I am personally mostly interested in chemical reaction dynamics as this methodology allows the observation of the relative atomic motions that propagate the system from one distinct molecular structure to another, *i.e.* a direct observation of the defining moments that lead to chemistry. It also allows us to observe how a complex many body system reduces to a few key motions at the far from equilibrium points involved in barrier crossing. In this respect, the relative figures of merit for samples to probe these issues are critical considerations. You need to find a system that probes the fundamental issue in question and this process must be capable of being photoinitiated with femtosecond laser pulses, otherwise it is not possible to address the relevant timescales for the atomic motions. Basically, it must be possible to prepare a system optically on an excited state potential energy surface that is effectively barrierless to the process of interest and the quantum yield must be close to unity. As a rule of thumb, it is necessary to have approximately 10% of the lattice undergoing the structural change of interest to get above the background diffraction and diffuse scatter. In the femtosecond time domain, there are peak power limitations that prevent exciting 100% of the sample as this necessarily leads to multiphoton ionization artifacts. It is for this reason that the system should exhibit a high quantum yield for the photoinduced structural process of interest, and certainly should be above 10% as a threshold for consideration. In addition, the sample must form high quality crystals that can be prepared as thin as 20–200 nm thick, depending on Z of the dominant lattice host atom and associated electron scattering cross section. We either grow crystals directly to meet this condition or microtome them to the desired thickness. The biggest problem is with water soluble crystals where microtoming is not possible as the high surface tension of water is used to pick up a sample after microtoming thin sections. The real frontier of this field now is in designing crystals to probe different aspects of chemistry (and biology) with the above constraints.

With respect to gas phase systems, the major limitation is the number density of gas molecules one can deliver, with the above caveats for quantum yield for the process of interest fully respected. For any given system there will be a limit to the number density and this further stipulates a sample path-length to achieve sufficient electron diffraction. This aspect to the problem is well known in the gas electron diffraction community where the emphasis is on high resolution structures. However for time-resolved studies, the temporal resolution is limited by the velocity mismatch between the speed of the laser excitation pulse to induce the structure changes and the speed of the electron pulse probing the structural changes. For typical molecular beam dimensions and electron energies of less than 100 keV, as typically used for gas phase studies, the time-resolution is limited to a few picoseconds, with 10 picoseconds being more typical when electron broadening effects in propagation to the molecular beam are taken into account. Basically the time-resolution is limited by the difference in propagation time for the electrons to traverse the excited volume relative to the laser excitation. It is possible to use tilted phase fronts for the laser excitation to minimize the velocity mismatch but this is rather difficult to implement and has not been done as yet for gas phase samples. It is precisely in this domain where relativistic electron sources will open up a 10 femtosecond time-resolution to chemical reaction dynamics. There is effectively no velocity mismatch, *i.e.* the electrons travel very near the speed of light to the point that path length limitations are no

longer a concern. REGAE was designed to meet this goal for the gas phase to take advantage of the higher velocity and to also open up solution phase studies for atomically resolving reaction dynamics to take advantage of the longer electron mean free paths in the relativistic regime. We are now in the process of introducing different gas and liquid cell concepts based on our work using nanofluidics[1] to tune the desired sample path-length for a given problem from 100 nm (liquid) to 100 microns (gas). It would be a dream come true if we could compare the reaction dynamics for the same system under isolated collision free conditions of the gas phase to that in the solution phase where most chemistry occurs to truly separate the role of the solvent in directing chemical processes.

1 C. Mueller, M. Harb, J. R. Dwyer and R. J. Dwayne Miller, *J. Phys. Chem. Lett.*, 2013, **4**(14), 2339–2347.

Priyadarshi Roy Chowdhury queried: How does the bucket size increase during the snapshots of your molecules?

R. J. Dwayne Miller answered: Depending on the dynamics of interest, we control the bunch charge density, or bucket as you term it, to give us the most electrons possible without losing the required time-resolution. Control over the "bucket size" is readily achieved by controlling the intensity of the laser excitation used for electron photoinjection. For 100 femtosecond dynamics, we typically use between 10^5 to 10^6 electrons per pulse in an approximately 100 micron spot. For the sample thicknesses used, this source intensity is comparable to 10^{11} to 10^{12} X-ray photons from an XFEL in terms of detected signal counts. For this comparison as a calibration, these are very bright sources. The main limitation is in the transverse coherence where we are currently limited to transverse coherence lengths of a few nanometers, which limits the size of the unit cell one can study with atomic resolution to the ensuing dynamics. To date, we have achieved very high space-time-resolutions for unit cells of approximately 4–5 nm, which is approaching protein crystal unit cell dimensions. REGAE in this respect holds promise to go to unit cells larger than 10 nm.

Again, the bunch charge density must be adjusted to attain the required space-time-resolution. Higher time-resolution generally means lower electron numbers per pulse that are compensated for by averaging more laser-electron pump–probe shots to achieve the desired signal-to-noise in the diffraction pattern. Since the laser excitation generally damages the crystal (not the electron pulse as in the X-ray case), this requirement puts great demands on the samples. In the case of our studies on the ring closing reaction for diarylethene we used literally hundreds of crystals to acquire full atomic movies of the key features in the reaction dynamics.[1] Each shot may give a sufficient diffraction intensity to correlate to the structure, however, to distinguish different types of relatively small motions with respect to the interatomic separation requires acquiring a higher signal-to-noise ratio to better constrain the fits.

1 H. Jean-Ruel, M. Gao, M. A. Kochman, C. Lu, L. C. Liu, R. R. Cooney, C. A. Morrison and R. J. Dwayne Miller, *J. Phys. Chem. B.*, 2013, **117**(49), 15894–15902.

Priyadarshi Roy Chowdhury communicated: What are the precautions that must be taken during mapping motions with ultra bright electrons? What is the maximum resolution that has been achieved to date in case of mapping motions with ultra bright electrons?

R. J. Dwayne Miller replied: With regard to the first question, I am going to assume that one is using optimized electron pulses for the dynamics of interest and the sample issues discussed are under control. In terms of execution of the experiment, the main precaution is that the laser excitation should be below 100 GW cm^{-2} to avoid excessively high peak powers that lead to multiphoton ionization artifacts. There is always a tendency to go to higher excitations to get further above the background to observe a structural change. Ionization will ensure you observe structural changes but they will not be related to electronic surfaces of interest that can be connected to the chemistry of interest, as opposed to Coulombic repulsion or trivial electrostatic charging effects on the lattice. There is an equally important issue that photoionization leads to photoemission with electrons moving away from the surface region creating large surface fields that also modify the diffraction pattern and can be confused with diffraction from lattice motion.[1] There are both lattice charging effects and surface field artifacts at the high power that must be avoided. This peak power issue is a major concern in which control experiments as a function of peak power are needed to be sure this effect does not contribute to the observed dynamics. Such controls are often not conducted due to limited signal-to-noise in the use of low brightness or unstable sources for the structural probe. It is in this respect that the use of ultrabright electron sources has significant advantages. The higher brightness and robust, stable nature of these sources enables the attainment of a higher signal-to-noise ratio for any given excitation level relative to other sources at the present time. These features enable studies at sufficiently low peak powers/intensities to avoid this problem.

With respect to your second question regarding maximum resolution, there are both spatial and temporal aspects to consider. The maximum time-resolution achieved to date to a structural transition is in the order of 100 fs. For examples important to condensed matter physics, see the works of Sciaini *et al.*[2] on the study of the electronically induced melting of Bi, and Morrison *et al.*[3] on the photoinduced phase transition of VO2 as representative examples. For molecular processes, see Gao *et al.*[4] on a formally photoinduced charge transfer process in a charge ordered organic system and Jean-Ruel *et al.* for an example of a ring closing reaction.[5] There are faster nuclear motions possible but it must be born in mind that structural transitions are dominated by the lowest frequency, mostly anharmonic, motions coupled to the reaction or structural transition, that undergo the largest net displacement along the reaction coordinate. The relaxation or dissipation processes to propagate the system to the new structural minimum dominate the dynamics and these modes tend to have half periods in the order of 100 fs. To date, the spatial-temporal resolution has achieved the necessary limits to follow the primary dynamics involved in the structural transitions studied. There are structural transitions such as the primary processes in vision that will necessitate a 10–20 fs time-resolution with better than 0.1 Å spatial resolution, but here there are sample issues. In principle, the current generation of electron sources are up to this task. It is really

the sample that is most limiting in terms of what dynamics can be probed. In this respect, it is interesting to also note that the spatial resolution of the structural changes is in the order of 0.01 Å or less. This estimate can be gleaned from gas phase electron diffraction studies and from signal-to-noise analyses of solid state diffraction patterns. We typically see diffraction out to better than 0.1 Å (for *e.g.* charged ordered molecular crystals such as EDO-TTF)[4] with good signal-to-noise, relative to 1–2 Å for X-ray diffraction. (We generally cut the camera off at 0.2 Å but will extend the reciprocal space for higher resolution in the future.) This difference is a reflection of the much shorter wavelength of electrons relative to X-rays typically used in diffraction studies. Again, the sample quality must be good enough to support this higher intrinsic spatial resolution. It is interesting to note that the spatial resolution is in principle high enough to literally observe nuclear vibrations associated with say IR transitions. The root mean square (rms) motion in going from $v = 0$ to $v = 1$ is in this order . The problem is that for a harmonic oscillator there is no net motion; the mean remains the same. The anharmonicity is the key factor and far from equilibrium motions must explore higher degrees of anharmonicity in the interatomic potential to observe these net displacements along the potential. It will be interesting to use high power IR excitation for strong field control of atomic motions to probe this region and directly follow the light matter interactions with femtosecond electron diffraction probes. We are just in the process of such studies and I expect we will be able to significantly push the spatial resolution limits to the fundamental space-time limits of the source technology in this class of experiments.

1 H. Park and J.-M. Zou, *Phys. Rev. Lett.*, 2010, **105**, 059603.
2 G. Sciaini, M. Harb, S. G. Kruglik, T. Payer, C. T. Hebeisen, F.-J. Meyer zu Heringdorf, M. Yamaguchi, M. Horn-von Hoegen, R. Ernstorfer and R. J. Dwayne Miller, *Nature*, 2009, **458**, 56–59.
3 V. R. Morrison, R. P. Chatelain, K. L. Tiwari, A. Hendaoui, A. Bruhács, M. Chaker, B. J. Siwick, *Science*, 2014, **346**(6208), 445–448.
4 M. Gao, C. Lu, H. Jean-Ruel, L. C. Liu, A. Marx, K. Onda, S.-ya Koshihara, Y. Nakano, X. Shao, T. Hiramatsu, G. Saito, H. Yamochi, R. R. Cooney, G. Moriena, G. Sciaini and R. J. Dwayne Miller, *Nature*, 2013, **496**, 343–346.
5 H. Jean-Ruel, M. Gao, M. A. Kochman, C. Lu, L. C. Liu, R. R. Cooney, C. A. Morrison and R. J. Dwayne Miller, *J. Phys. Chem. B.*, 2013, **117**(49), 15894–15902.

Elangannan Arunan opened the discussion of **Martin Zanni**'s paper: The discussion on your Fig. 4, in particular the assignments of the A, B and C peaks, is confusing to me. On page 9, line 8, it is mentioned that peak C is caused by coupling between esters. On the same page, in line 40, it is mentioned that peak C is caused by coupling between bright and dark eigen states (in general 'dark and bright states' are used to refer to the states in one isolated molecule while discussing IVR). Are these two statements contradicting?

Martin Zanni responded: We believe that the C peak is caused by coupling between the bright and dark eigenstates of the esters, in analogy to the a^+ and a^- peaks of a beta-sheet. The "dark" eigenstate may still carry a little oscillator strength, but not very much since it is not prominent along the diagonal.

John R. Helliwell asked: In Fig. 5 of your article you give calculated spectra for different monolayer structures. Have you considered using these to interpret the experimental spectrum of Fig. 4, which could perhaps be explained by the fractional occupancies of Fig. 5A, 5B and 5C, *i.e.* Fig. 4 could be due to a mixture of those monolayer structural states?

Martin Zanni answered: The simulations I presented in my paper are very rudimentary. Not only did we not consider fractional occupancies, which is a good suggestion, but we have also only simulated a small range of possible structures and used quite course models. So, the comparison to the experiments can definitely be improved and the structures refined.

Abhishek Shahi communicated: Your article is written very clearly. I have four questions:

(i) Can you help us in understanding the a^- and a^+ modes, are they normal modes of vibration?

(ii) If we can see the cross peak G in the high intensity, why can we not see both the parent peaks (a^- and a^+) from where the peak G originates?

(iii) Why is there inconsistency in the intensity of predicted and observed spectra (particularly for peak G)? Is there another factor which determines the intensity in experiments? I guess in the model, only the change in dipole moment is accounted for.

(iv) How is the FGAIL oriented in the absence of MMB ? Is it clearly random or can the a^-/a^+ ratio give some information about the orientation?

Martin Zanni replied:

(i) Yes, the a^+ and a^- are normal modes. See my book "Concepts and methods of 2D infrared spectroscopy".[1]

(ii) The cross peaks can sometimes be much stronger than one of the two diagonal peaks. The intensity of a cross peak is given by the mu1*mu1*mu2*alpha2, where mu is the infrared transition dipole and alpha is the polarizability tensor for a given mode 1 or 2, respectively. The diagonal peaks are given by mu*mu*mu*alpha. One way that the diagonal peak can be very weak but the cross peak strong, is if alpha1 is large but alpha2 is small.

(iii) Please keep in mind that our work is preliminary. Our simulations are quite rudimentary and we have not tested a wide range of possible monolayer or peptide structures. Thus, our conclusions may change. If you are thinking of modeling these spectra, please feel free to come to your own conclusions about our assignments!

(iv) In the absence of the membrane we did not measure the SFG signal for FGAIL. The peptides definitely deposited, but there was no signal, meaning either that they did not form fibers or that the fibers were isotropically distributed. In principle the a^+ and a^- features can give information about the orientation, but their relative intensities are also influenced by the size of the sheet and the structural disorder, which are two factors that are difficult to characterize accurately.

1 P. Hamm and M. Zanni, *Concepts and Methods of 2D Infrared Spectroscopy*, Cambridge University Press, Cambridge, UK, 2011.

Debabrata Goswami followed the discussion by asking: Could you please comment on how your Femtosecond Pulse Shaped 2D-IR distinctively stands out compared to the more conventional interferometer based time delayed 2D-IR spectrometers?

Martin Zanni answered: 2D-IR *via* pulse shaping is much more versatile and has faster data collection than conventional interferometers. A conventional interferometer moves a translation stage to increment the time delay between two laser pulses. Physically moving the stage is slow and most stages are not precise enough to make accurate time delays. The simplest use of a pulse shaper is to replace the delay stage, because the shaper can increment the delays instantaneously up to laser repetition rates of 100 kHz. Updating the delay shot-to-shot is advantageous not only because data collection is faster, but also because the signal-to-noise is improved by shifting the signal frequency further from the noise spectrum. That is why commercial FTIR spectrometers dither their interferometers as fast as possible, but no mechanical device can compare to the speed of an acousto-optic modulator based pulse shaper. There are additional benefits with a pulse shaper as well. The phase of the pulses can also be modified in order to phase cycle. That enables data collection to be collected in the rotating frame, as Goswami and Warren showed many years ago, as well as removing background scatter without using chopping. In my own research laboratory, we now have 5 pulse shaping 2D-IR spectrometers in operation and not a single interferometric system.

Sankarampadi Aravamudhan commented: In your paper you state that "Thus, rather than use a single picoseconds mid-IR pulse, we use two femtosecond laser pulses, as shown in Fig. 1A. The time-delay, t_1, between the pulses is scanned and the data Fourier transformed to give the second frequency axis but with no loss in time-resolution and improved line shapes." Are the pulses specified in the excerpt mid IR and which are femtosecond visible region pulses? Clearly specify the above with respect to the t_1 parameter. You also mention "Second, the signal must be heterodyne detected, otherwise phase distortions are present that make it difficult to interpret the spectra or to compare to either FTIR or 2D IR spectra which are always heterodyne detected. For the samples studied here, heterodyne detection is automatically accomplished with the non-resonant signal from the gold interface." With the specification in the previous extract, a more vivid explanation/description of the content of lines is highly desirable if the real advantage is to become well appreciated by one and all among the interested readers.

Martin Zanni communicated in reply: Generally speaking, the local oscillator should be the same frequency as the signal that is emitted from the sample. For

2D-IR spectroscopy, that means that the local oscillator is identical to any of the three pulses preceding it and so can be easily generated with a beam splitter. For SFG, it is a little more difficult, because the emitted signal is at a shorter wavelength than any of the excitation pulses. Thus, the local oscillator needs to be generated by summing the visible and infrared excitation pulses, similar to the SFG process of the sample itself, but using some other material with a non-resonant response so that the local oscillator does not contain molecular signals but is just a short and smoothly varying envelope. One can use a non-linear crystal or even a piece of quartz. In the experiment that I presented in this Faraday Discussion, we used the gold substrate under the sample to generate the local oscillator. Not only does it provide a nice pulse at the correct frequency, but the local oscillator is also aligned with the signal.

Volker Deckert asked: What are the limitations of your depth resolution, with respect to the surface sensitivity?

Martin Zanni communicated in reply: The "depth resolution" will be set by the system itself. If the interface is a single monolayer, then it will be detected, even if it is buried between two large masses. For example, the interface between two liquids or the interface between a liquid and solid. That is because the SFG signal only arises from non-centro symmetric places. As a result, the signal will arise for whatever portion of the sample is non-centro symmetric. So, if the interface is many layers thick, they will all give a signal just so long as they are all oriented the same. The extreme case would be a non-linear crystal like BBO that is designed to sum frequencies together very efficiently. Thus, the "depth resolution" for SFG cannot be answered generally or compared to microscopies without more specifics.

Keisuke Tominaga queried: Is it possible to study the liquid gas interface? It is far away from the metal surface, and the IR pulse is absorbed by the liquid.

Martin Zanni answered: Yes, you can study liquid gas interfaces. As you point out, the IR pulse is absorbed by the liquid, and so the IR beam should impinge on the interface from the gas side. 2D-SFG experiments on gas–liquid interfaces have been performed by Tahei Tahara and Mischa Bonn.

Keisuke Tominaga followed up by asking: Could you explain the selection rules for your 2D-SFG measurements?

Martin Zanni communicated in reply: The selection rules for 2D-SFG are quite interesting. In 1D-SFG, the modes have to be both IR and Raman active, which is also true for the diagonal peaks in 2D-SFG, but not so for the cross peaks. This fact is briefly alluded to in our Faraday Discussion paper. It was first

presented in ref. 1. In other words, a cross peak in a 2D-SFG spectrum can appear even if one of the two diagonal peaks that it connects is completely SFG inactive. The reason is the following. The two photon IR pump pulse will excite a mode even if it is SFG inactive, because they are absorbed due to the infrared transition dipole, even if it does not emit. If that SFG inactive mode is then coupled to a SFG active mode, then a cross peak will appear because the active mode will emit. Thus, the cross peaks will map out modes that are invisible in standard SFG. There are other unintuitive situations that arise in 2D SFG, which are explained in ref. 2.

1 J. E. Laaser, D. R. Skoff, J.-J. Ho, Y. Joo, A. L. Serrano, J. D. Steinkruger, P. Gopalan, S. H. Gellman and M. T. Zanni, *J. Am. Chem. Soc.*, 2013, **136**(3), 956–962.
2 J. E. Laaser and M. T. Zanni, *J. Phys. Chem. A*, 2013, **117**, 5875–5890.

Siva Umapathy communicated: Could you please comment on a comparative aspect of vibrational SFG *vs.* 2D-IR-SFG. In the experiment 2D coupling that you monitor, would you observe similar coupling in vibrational 2D-SFG?

Martin Zanni responded: Our paper "Extracting Structural Information from the Polarization Dependence of One- and Two-Dimensional Sum Frequency Generation Spectra" provides a good comparison of SFG, 2D-SFG and 2D-IR spectroscopy.[1] 2D-SFG is to SFG like 2D-IR is to FTIR spectroscopy. The 2D versions visualize the couplings through the cross peaks. In addition, 2D-IR and 2D-SFG will both measure the same couplings for a given sample. The spectra might look a little different because the 2D-SFG spectra are weighted by a Raman tensor, but if both the 2D-SFG and 2D-IR spectra are collected with heterodyne detection, like was first done by my group in 2011,[2] then the comparison of the 2D-IR and 2D-SFG spectra is very informative. A 2D-SFG spectrum provides information that cannot be obtained by standard vibrational SFG. It can resolve relative molecular orientations and SFG forbidden peaks, for example. Please see our paper above for more details.

1 J. E. Laaser and M. T. Zanni, *J. Phys. Chem. A*, 2013, **117**, 5875–5890.
2 W. Xiong, J. E. Laaser, R. D. Mehlenbacher and M. T. Zanni, *PNAS*, 2011, **108**(52), 20902–20907.

Kenneth Ghiggino opened a general discussion of the paper by **Robert Pal**: You have reported results using a 355 nm excitation light source. Can you expand on the reasons for using this excitation wavelength in your work considering potential issues with optics transmission and increased light scattering? Can multiphoton excitation of the sample be used with this technique and would it offer any advantages?

Robert Pal answered: Initially PhMoNa and its associated methodology was established using functionalised lanthanide(III) complexes. The reason behind

this is that these Ln(III) complexes upon excitation at 355 nm possess long lived sensitized emissive lifetimes (orders of ms). In this case the initial electro-optical modulation of the excitation beam and galvanometric sample raster scan speeds could be synchronized in time scales as slow as 1Hz. After successful hardware configuration it was facile to perform enhanced resolution scans using commercial cellular stains (*e.g.* MitoTracker Green) at 400Hz using its corresponding (488 nm for MTG) excitation laser. It is well known that commercial optics could display lower optical performance below 400 nm, however, in our case LambaBlue (Leica) objectives with excellent >350nm optical performance were initially used. Light scattering and UV induced sample bio-auto-fluorescence (BAF) was eliminated by carefully adjusting the scanning parameters *via* unstained control experiments and time-gated dye detections in case applied Ln(III) complexes could be facilitated too (ns BAF *vs.* ms Ln(III) lifetimes). Sample photobleaching was not observed as we maintained live cell sustainable laser parameters throughout (Ln(III) dye photobleaching has been extensively studied in ref. 1). This technique could be transferred to a multiphoton excitation setup, by simply employing a tunable laser and suitable 2PE dye combination, however, it could not overcome the inherent resolution limitations of multiphoton excitation using low NA objectives to achieve deeper sample penetrations.

1 S. J. Butler *et al.*, *Chem. Sci.*, 2014, 5, 1750.

Christoph Schnedermann asked: Optical microscopy is often employed to track nanoscopic objects in time. What are the limitations of your technique in this regard and how does it compare to more routinely used techniques?

Robert Pal replied: The limitation of tracking nanoscopic objects is limited in this set up by the mechanical vibration of the applied electro-optical modulator, generating standing waves. This has a maximum limiting value of 400 Hz per line (minimum 1024 pixel per line). Combine this with the required automatic reconstruction of a 4 line averaging scan, due to the raster scanning of the *in situ* formed improved PSF excitation cluster, this line scan speed is technically 100 Hz per line, which could give the best scenario of 9.76 ns/pixel excitation and readout/cycle. Further proposed improvements in and the development of a novel non-mechanical electro-optical modulator could foresee a 10 fold improvement in the above limiting scan speed.

Sankarampadi Aravamudhan commented: In Fig. 1c of your paper (see Fig. 1 below), slice-level 1 (the lower trace) has to a greater extent the possibility for the circles to overlap than what appears to be possible at slice-level 2 (the upper trace). Hence the resolution must be defined with an appropriate convention, so that it becomes applicable without ambiguity in all contexts encountered. In the above two levels one must specify the spectrometer settings unambiguously for a comparison of resolution.

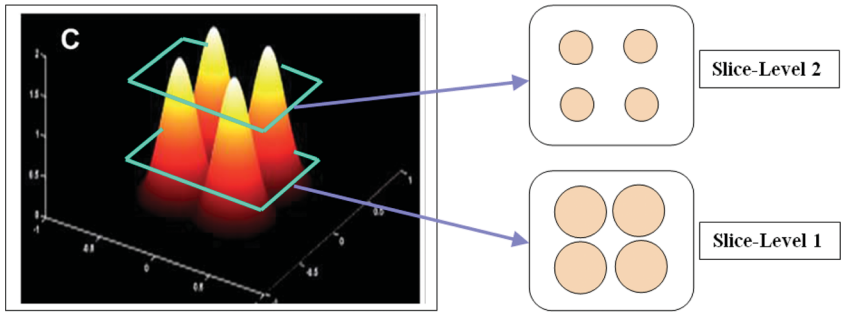

Fig. 1 Qualitative Resolution depending on the image slicing-level.

Robert Pal communicated in reply: In this form of structurally modulated enhanced confocal microscopy the achieved 2 fold improvement in the *x*,*y* (lateral) resolution allows the user to harness the advantages presented by the variable confocal pinhole of the system and set it to the corresponding airy disk size which will result in the 2 fold improvement in optical sectioning and subsequent *z* (axial) resolution. Any observed *z* saturation could also be eliminated if needed by a set 10% overlap in *z* sections applying a cross section eliminating algorithm, although due to the default line averaging nature of the set up this has not been needed. The answer to this question in my response herein is referring to axial resolution, for the lateral resolution element of this question my explanation is detailed in my response to the question asked by Siva Umapathy below.

Siva Umapathy asked: What is your prediction of the best resolution you could get spatially?

Robert Pal responded: Currently this version of PhMoNa provides half of the diffraction limited resolution (at a given excitation wavelength and objective NA, ~60nm lateral at 355 nm and 1.40 NA; axial resolution is governed by the applied pinhole size which is a function of lateral resolution), providing an eight times reduced detectable voxel size. This is a result of the 2 by 2 cluster generated in the excitation beam profile. Further splitting can be achieved, however, this will not improve the resolution at this point any further as the generated peaks will overlap at their origin more than 20%, which will produce destructive wave-patterns prohibiting good signal-to-noise and Nyquist sampled emission detections.

Debabrata Goswami queried: Is it true that as you make the confocal point bigger, you have less *z* resolution? However, can you still do slicing along the *z*-axis to get the 3D image?

Robert Pal communicated in reply: It is in design principle true that in confocal microscopy with increased detection pinhole size the studied optical

thickness is increased, but the set-up (NA of objective, applied excitation wavelength, *etc.*) determined axial resolution will determine the *xyz* 'clarity' of the image. 3D slicing is possible even without a pinhole (*e.g.* wide-field fluorescence microscopy), however, maximum image clarity and *z* profile accuracy can only be achieved if this axial resolution limit is used to determine the applied *z* step size (and keeping the Rayleigh criterion, Sparrow limit in mind for efficient true contrast). If the optical sections have been set to be 'thinner' than the achievable axial resolution would permit, over-sampling (bigger axial PSF component than *z* parameter of the confocal voxel) will occur. This will actually reduce the resolution in the reconstructed 3D image due to neighboring sections 'bleeding' into each other saturating the voxels as the image is being formed along the *z* axis. This, for the same principle, could subsequently also reduce the observed lateral resolution.

Volker Deckert queried: Is there a difference in lateral resolution if you compare your combination of SIM and confocal with bright field SIM?

Robert Pal answered: The achievable lateral resolution is at the same levels as with wide-field SIM, however in this case it is done *via* a variable raster cluster scanning pattern harnessing the advantageous superior optical sectioning (axial resolution) of a confocal setup. Theoretically the lateral resolution could be further improved (by at least a further factor of 2) and surpass the performance of a WF-SIM setup, but due to prolonged scanning times, complications in optical components compatibility and reduced signal-to-noise ratio this has not been pursued further.

Debabrata Goswami commented: In context to the work presented in the paper by Dr Pal, the use of an idea like the PALM super-resolution technique introduced by Dr Eric Betzig (based on the switchable fluorophores of Dr W. E. Moerner)[1,2] would be more appropriate compared to the STED microscopy technique of Dr Stefen Hell.[3] All these have been recognized with the 2014 Chemistry Nobel prize. In the particular case of Dr Pal, the multiple image idea of PALM would work very well.

1 E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, Imaging Intracellular Fluorescent Proteins at Nanometer Resolution, *Science*, 2006, **313**, 1642–1645.
2 R. M. Dickson, A. B. Cubitt, R. Y. Tsien, W. E. Moerner, On/off blinking and switching behavior of single molecules of green fluorescent protein, *Nature*, 1997, **388**, 355–358.
3 S. W. Hell and J. Wichman, Breaking the diffraction resolution limit by stimulated emission depletion microscopy, *Opt. Lett.*, 1994, **19**, 780–782.

Robert Pal responded: Thank you for the valuable comment, yes indeed the 2014 Nobel prize has been given for the 'physicochemically approached' (fluorophore modulation and localisation) resolution enhancement to achieve sub-diffraction limit (predominantly 2D) images. 3D reconstruction in all these pointilistic techniques is already achieved. In the case of PALM/STORM this has

been done *via* the methodology of interferometric determination of the tracked fluorophores' axial position. These methods could be detailed further, however the vital point of PhMoNa is not to compete with these superior techniques, but to combine the advantageous properties on LSCM and SIM. Therefore by doing so this auxiliary technique can literally half the current diffraction limited resolution of a given confocal microscope without the need of applying a range of novel compatible fluorophores, expensive new equipment and often disruptive experimental parameters (such as high laser powers). The achieved aim was to provide the broad imaging community with a 'way' of performing live cell experiments with higher resolutions by keeping both existing LSCM instrumentation and applied experimental techniques in place.

Priyadarshi Roy Chowdhury asked: What precautions should be followed during slicing?

Robert Pal communicated in reply: There are no precautions to address during optical slicing since it is experimentally identical to a standard confocal microscope.