

## DISCUSSIONS

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## Instrumentation and methods: general discussion

Jochen Küpper, Gwyn Williams, Henry Chapman, Katharine Reid, Jeppe Christensen, Hans Jakob Wörner, Elaine Seddon, Gopal Dixit, Eleanor Campbell, Jonathan Underwood, Michael Woerner, John Spence, Jasper van Thor, Martin Wolf, Oriol Vendrell, De Ninno Giovanni, Christian Bressler and Nora Berrah

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**Jochen Küpper** opened the discussion of the paper by John Spence by commenting: Dear Speakers, following the discussions of relevant time-scales in your papers and especially based on the suggestion of John Spence, that biology is made up of "slow processes", I would like to hear your opinion on what are the relevant timescales to understand nature. What range of time-scales are relevant for the understanding of complex chemical and biological systems? What is the most important timescale (maybe in terms of SI prefix) to understand? And which is the most important timescale to investigate over the next years?

**John Spence** communicated in reply: For biological systems we can take the problem of protein folding as an example. Experiments must be undertaken on hydrated samples to be meaningful. Measurements of folding time have been simulated using atomic potentials and molecular dynamics (for shorter times and small proteins only, due to computer limitations). A large protein contains tens of thousands of atoms, almost entirely C, O, N, H, in the form of a series of any of the 20 small amino acid molecules (residues), each of known structure, in some sequence defined by DNA. Proteins at RT in solution differ from small molecules in that entropy is a large term in their free energy, and hydrogen bonding and van der Waals forces play important roles in folding, as does hydrophobic interactions (residues that hate water hide in the middle). A recent study found the folding time to be approximately  $N/100$  microseconds for  $N$  amino acids. These times are long because of the time needed for the structure to explore stochastically the huge configuration space (Levinthal's paradox) in order to find a local total energy minimum. The study of shorter times (sub picosecond) would certainly be important for studies of the chemistry of individual amino acids, where entropy is not the dominant term in the free energy.



**Oriol Vendrell** responded: In chemical dynamics, the time-scale in which measurable changes can be detected by some probe scheme after a reactive event is triggered lies in the order of tens to hundreds of femtoseconds. This is the natural time-scale for bond vibrations and nuclear rearrangements, which is the consequence of the usual energy differences between vibrational levels in molecules. This comes as a natural thing after the great developments in the field of femtochemistry over more than two decades.<sup>1</sup> Most femtochemistry studies though are related to photochemical reactions, which can be triggered by ultra-short laser pulses and probed at well defined time-delays. However, the dynamics of ground-state chemical processes, meaning the time it takes for a individual reactive events to connect reactants to products *via* a transition state, is also of the order of tens to hundreds of femtoseconds.<sup>2</sup> Such thermally activated events, even if individually fast, are often very rare depending on the energy barrier height between reactants and products and cannot be triggered easily. This makes femtochemistry investigations of thermally activated reactions, the vast majority of chemistry, scarce with only a few exceptions.<sup>3</sup> A great challenge and opportunity for the next years is in my opinion the extension of usual femtosecond spectroscopy studies, which constitute an indirect probe of structural and electronic rearrangements, towards femtosecond structural determination in complex environments and for complex structures, where accurate theory predictions are often beyond reach. In this respect, we have been investigating opportunities for transferring large amounts of energy to liquid phases with short and intense THz pulses as a possible way to trigger thermal chemical reactions of dissolved molecular species.<sup>4</sup>

1. A. H. Zewail, *Science*, 1988, **242**, 1645.
2. B. J. Gertner, R. M. Whitnell, K. R. Wilson and J. T. Hynes, *JACS*, 1991, **113**, 74–87.
3. D.M. Newmark, *Acc. Chem. Res.*, 1993, **26**, 33–40.
4. P. K. Mishra, O. Vendrell and R. Santra, *Angew. Chem. Int. Ed.*, 2013, **52**, 13685.

**Jasper van Thor** communicated in reply: The femtosecond time domain is key to studying activation processes in biological materials. The fundamental chemistry of bond rearrangement and dynamics occurs on ultrafast time scales, which subsequently trigger slower processes. From an experimental point of view both the ultrafast as well as the slow processes are of biological interest, and resolving the full cascading interconversion processes structurally is one important goal for XFEL science. It is of interest to note that XFELs have already shown particular utility also for resolving slow processes, beyond the nanosecond regime, taking advantage of the ability to conduct radiation damage free experiments as well as, in principle, detect single turnover mechanisms. For femtosecond time resolved studies, discussed in our paper, the key issue is the detection sensitivity in light of fundamental limits to femtosecond population transfer and intrinsic noise characteristics of the XFEL source. In order to develop the necessary signal-to-noise to detect the small structure factor amplitude differences technical details for a three-pulse probe–pump–probe scheme are considered and previously presented.<sup>1</sup> The paper by John Spence also references parts of this previous discussion.<sup>1</sup> In this scheme I proposed an internally referenced measurement of photoinduced femtosecond dynamics, which



requires several geometrical and timing characteristics to be implemented. A pulse replica is generated from a monochromatic source with a small angle of incidence, in addition to focusing both beams to integrate over the rocking curve and the mosaic block which we have shown may lead to additional structure factor amplitude noise if the source otherwise has a small convergence (as discussed in our paper). By introducing also a time delay in between the pulse replicas, adding an optical pump will record both an un-pumped and a pumped diffraction pattern on the area detector in a single frame. A ratiometric measurement will thus provide the photoinduced differences in principle within the detector dynamic range. A Serial Femtosecond Crystallography application would need substantial attenuation to achieve non-destructive sampling of the first interaction, while attenuated defocused measurements of large crystals are shown to be non-destructive. Critical parameters of a split and delay unit include the stability of the intensity ratio and energies of the pulse replicas, also with pre-monochromation of either a self-seeded source or in SASE mode. A geometrical splitter may be insufficiently stable while also optical splitters based on thin crystals give rise to noise. The performance of the split and delay instrument will therefore likely dominate the sensitivity with which the photo induced structure factor amplitude differences are determined in a ratiometric manner with a probe–pump–probe scheme.

1. J.J. van Thor, (21 Feb 2014) 1st Ringberg Workshop on Structural Biology with FELs, “Considerations for ultrafast pump–probe X-ray crystallography: Non-linear cross sections dominate femtosecond time resolution and the rotation method for large crystals”, Ringberg Castle, Germany.

**Jeppe Christensen** asked: In your paper you say that the time resolution of FELs are far better than needed for biological studies where processes happen on a micro- to millisecond time scale. At the same time you commented on the problems with shot-to-shot stability of the FELs. My question is, why go through the hassle of performing an FEL experiment, when synchrotrons work at the desired time scale and are much more stable. One could work at room temperature and just do one shot per crystal to avoid radiation damage.

**John Spence** replied: We use the XFEL in biology to outrun radiation damage, not to obtain high time resolution. Previously, samples were frozen to minimize damage at synchrotrons, which prevents us from studying dynamics. Radiation damage has always limited the quality of diffraction data from biomolecules, and in particular the resolution. Thus the XFEL opens the way to the study of dynamics at room temperature in a native environment, at atomic resolution, without damage.

**Michael Woerner** communicated: If you are only interested in the time-dependent relative positions of the nuclei I think the concept of “diffract before destroy” might work. However, X-ray diffraction gives information about the electron density map. Thus, the strong X-ray pulse might modify the electron density during diffraction. Do you consider such phenomena in your analysis ?



**John Spence** answered: Radiation damage is due to impact ionization by photoelectrons, which takes time to develop. It is found that with pulses shorter than about 70 fs, the atomic structures determined using an XFEL are the same as those obtained on a synchrotron, so the "diffract before destroy" method does indeed work, as shown in many papers. Elastic scattering commences instantaneously, and for short pulses, some of the atom images will be ionized, however this has little effect on a density map at a 3 Å resolution, especially if the phases are obtained by molecular replacement from models in the protein data base. By avoiding the need to freeze samples on a synchrotron (to avoid damage), it therefore opens the way to the analysis of dynamics at room temperature. The comparison of XFEL and synchrotron structure determination for the same sample is given in *Science*, 2012, 337, 362, the evolution of the damage in time can be understood by studying the intensity of Bragg beams as a function of pulse duration (see *Nature Photonics*, 2011, 6, 35) and a new protein structure is determined by XFEL in Weierstall, *Nature Comms.*<sup>1</sup> It is important to understand that the effects of radiation damage depend on resolution – fine detail is destroyed first, and high order Bragg beams fade first with increasing dose.

1. Uwe Weierstall, Daniel James, Dingjie Wang, Wei Liu, John C.H. Spence, R. Bruce Doak, Garrett Nelson, Petra Fromme, Raimund Fromme, Ingo Grotjohann, Christopher Kupitz, Nadia A. Zatsepin, Shibom Basu, Daniel Wacker, Chong Wang, Sébastien Boutet, Marc Messerschmidt, Garth J. Williams, Jason E. Koglin, M. Marvin Seibert, Cornelius Gati, Robert L. Shoeman, Anton Barty, Henry N. Chapman, Richard A. Kirian, Kenneth R. Beyerlein, Raymond C. Stevens, Dianfan Li, Syed T.A. Shah, Nicole Howe, Martin Caffrey, Vadim Cherezov, Lipidic cubic phase injector facilitates membrane protein serial femtosecond crystallography, *Nature Commun.*, 2014, 5, 3309.

**Jonathan Underwood** addressed John Spence and Jasper van Thor: There has been much discussion during this conference on the opportunities presented by X-ray FEL technology for structural (diffractive) imaging of static and dynamic molecular structures, and the results presented in this session show that this technique holds great promise. A complementary and proven technique for measuring structural dynamics is offered by electron diffraction. In comparison with X-ray diffraction, electron diffraction has several appealing features: (1) scattering cross sections for electrons are typically 4–6 orders of magnitude larger due to the Coulombic interaction with both the electrons and nuclei in the target; (2) the inelastic/elastic scattering cross section ratio for electrons is lower for electrons than for X-rays; and (3) the energy deposited into the target per inelastic collision is lower for electrons than X-ray photons. The net result of these factors is that 3 orders of magnitude less energy is deposited per useful scattering event for electrons than for X-rays, significantly reducing the problem of sample damage. Historically, when employing electrons in the 30–200 keV range, the temporal resolution in such experiments has typically been limited to *ca.* 0.5 ps by several factors: (1) the velocity mismatch between the laser and electron pulses as they traverse the sample; (2) the space-charge repulsion which acts to temporally broaden the electron bunch (and also may stochastically blur the observed image leading to reduced spatial resolution); and (3) the spread of initial electron velocities (corresponding to the energy spread of the electrons produced from the photocathode) which leads to broadening of the electron bunch as it travels to the sample. In addition, the space-charge repulsion also places an upper limit on the



electron bunch charge requiring many electron bunches to be scattered in order to build up a diffraction pattern.

More recently, Hastings and co-workers (*Appl. Phys. Lett.*, 2006, **89**, 184109) demonstrated that electron diffraction is possible with relativistic electrons in the few MeV energy range. At this energy, the limitations described above are removed, and so this brings the possibility of electron diffraction with sub-100 fs time resolutions with high bunch charge, potentially giving single shot images. Do you think this approach holds promise for the sorts of problems currently being targeted by X-ray diffraction at FELs? Where do you see the role, if any, of ultrafast relativistic electron diffraction in the study of structural dynamics?

**John Spence** responded: Many points need to be made within the context of Henderson's *Quart Rev Biophysics*, 1995, **28**, 171, comparison of X-rays, neutrons and electrons as probes for structural biology, the considerable volume of literature on the use of MeV TEM in materials science and biology in the 1970's, and work published by the few groups now operating either fast electron diffraction cameras or fast electron microscopes (which solve the phase problem by direct imaging). In addition, it is important to specify if one is imaging single particles in ice, gas diffraction from small molecules (not viruses or large proteins), 2D crystals in ice, or solution scattering. Further important distinctions must be made between single-shot and stroboscopic methods, and between 2D projections and 3D images, which required data to be merged, perhaps from shots from identical objects in different orientations. Crystalline redundancy reduces sensitivity to damage by periodic averaging, so that if large enough crystals can be made, it is very difficult to compete with X-ray crystallography. Note that for biological significance, samples must be wet, frozen or otherwise hydrated.

1. We use the XFEL to outrun radiation damage, not to obtain high time resolution. Consider a pulse which is a delta-function in time. The elastic diffraction pattern would be recorded before the onset of the damaging photoelectron cascade, so that damage-free diffraction would result. Historically, radiation damage has always limited the resolution and data quality in most biological imaging methods at high resolution.
2. An important difference between X-rays and electron beams is that, after losing energy in the sample, an electron continues to the detector to create inelastic background (unless an Omega filter is used for diffraction; see Spence and Zuo, *Electron Microdiffraction*, Plenum Press, New York, 1992). No MeV Omega filter has been built, and these do not exclude phonon-scattering losses. X-rays are annihilated (in the creation of photoelectrons) during the most probable inelastic interactions, so that inelastic background is then not created.
3. While Henderson shows that the ratio of image-forming elastic scattering to damaging inelastic scattering (and the amount of energy dumped in the sample) are favorable to electron beams over X-rays, the XFEL is capable of outrunning damage altogether (see Barty *et al.*, *Nature Photonics*, 2012, **6**, 35), so these considerations do not apply. Using this "diffract-and-destroy" capability it becomes possible to study dynamics at room temperature and high resolution, without the need for freezing, as in cryo-EM, which prevents the study of dynamics (unless "quenching" methods are used). Although the elastic cross section for electrons is relatively much greater than that of X-rays, a 0.1 micron diameter XFEL hard X-ray beam contains about  $10^{12}$  photons in 50 fs, whereas an electron field emission gun produces about 20 electrons per picosecond. Larger photocathodes for electron beams degrade spatial coherence, and the chromatic stability of the multi-MeV electron beams used in accelerators is far worse (as seen in Hasting's paper) than those used in *e.g.* a 1MeV electron microscope, where an energy spread of 1 part in  $10^6$  or better is obtained using high stability voltage doublers.
4. Can femtosecond electron beams outrun radiation damage? Under the spatial and chromatic coherence conditions needed for single-particle imaging one has much less



than one beam electron in each 50 fs pulse from a field-emission electron source. Using the periodic averaging available in a 2D organic crystal in ice it may be possible, but would not have obvious advantages over existing 2D crystal cryo-EM methods, for which frozen samples normally do not allow study of dynamics. Zewail's group have shown how stroboscopic methods can be used to build up an electron diffraction pattern or image from the repeated excitation of a reversible process in a sample for which a sharp optical trigger exists. Space charge effects in the beam can in principle be eliminated by working with one electron per pulse and MHz repetition rates.

5. The effects of coherent multiple elastic electron scattering in thin samples have been extensively studied (see Spence, *High Resolution Electron Microscopy*, OUP, 4th edn, 2014). For protein nanocrystals we find (Subramanian and Spence, *Ultramic.*, 2014, submitted) a maximum tolerable thickness of about 70 nm thickness at 1 MeV. Since most of the information in a density map comes from phases, this limit may be increased by modeling from the PDB to get phases (molecular replacement method). At high energies, where ionization damage decreases, damage due to ballistic "knock-on" processes increases. (This factor, plus cost, lead to the demise of HVEM microscopy in materials science in the 1980's.)
6. Cryo-electron microscope imaging of two-dimensional protein crystals in a thin (*e.g.* 50 nm thick) film of ice, combining Bragg diffraction (to measure structure factors) and TEM imaging (to solve the phase problem) has been highly successful, and offers the highest resolution of any cryo-EM method. These crystals are typically less than 10 nm thick, while 0.5 MeV electron beams are commonly used. These conditions avoid multiple scattering and allow the study of dynamics by quenching the crystal in different intermediate states. The samples are hydrated, as required. I believe it would be very difficult to compete with this approach using the beam from an accelerator, which does not allow imaging for phasing, and causes knock-on damage.

**Henry Chapman** asked: Regarding the issue of reducing crystal size to the point that electron penetration is not an issue, it is quite easy to make a 20 nm crystal (of one unit cell), it is called a single molecule. How does electron diffraction from single molecules in the gas phase compare with X-ray FEL single-molecule diffraction?

**John Spence** communicated in reply: Gas-phase electron diffraction has a long history, largely restricted to the small molecules which can easily be vaporized, unlike proteins. Using electrospray or similar methods, it is now possible to create a vapor of large hydrated molecules such as proteins in the vacuum conditions needed for electron diffraction. The possibility of undertaking serial electron diffraction from a stream of molecules was discussed in Spence and Doak, *Phys Rev Lett.*, 2004, **92**, 198102. Any water jacket will add significantly to the thickness of the molecule, which needs to be less than about 20 nm to avoid multiple scattering perturbations to the data. My comments elsewhere on background due to inelastic scattering also apply (electrons which loose energy in the sample continue on to the detector). Fast electron diffraction from gas-phase small organic molecules has been developed extensively in Zewail's laboratory at Caltec. A field emission electron source produces about 40 electrons per picosecond, but may be readily focussed down to nanometer dimensions. Since protein unfolding times are long, if a method could be found for launching proteins from liquids into vacuum without a thick water jacket, and provided an Omega type parallel-detection energy filtering device were used to reduce inelastic background, then electron diffraction at perhaps 400 keV of gas-phase proteins would be worthwhile.



**Jonathan Underwood** raised the question: Are the data you presented the results of calculations or experiments? How do you expect the results to scale to say 6–7 MeV electron energies?

**John Spence** answered: Our paper (Subramanian and Spence, *Ultramic*, submitted, 2014) gives multiple scattering electron diffraction simulations for protein nano crystals up to 1 MeV beam energy. Beyond that the strength of the interaction does not change significantly (see Spence, *High Resolution Electron Microscopy*, OUP, 4th edn, 2014, Figure 6.8 gives the dependence of phase-contrast images of an atom with beam energy, not diffracted Bragg beam intensities). The appropriate theoretical form for scattering at several MeV is the Moliere High Energy Approximation (see T.-Y. Wu and T. Ohmura, *Quantum theory of scattering*, Prentice Hall, 1st edn, 1962, p. 50). The design of electron microscopes for energies up to 3MeV has been described in detail in the literature, and several 1 MeV machines are currently operating. The difficulties in designing a diffraction camera to operate above 1 MeV are likely to be: 1. The design of the required area detector. Those currently used in 1MeV TEMs should be carefully studied, based on 45 degree mirrors. 2. The very small Bragg angles, and correspondingly powerful very high current lenses needed to magnify these patterns up to the pixel size of the detectors. (Quadrupoles might be used instead, to reduce current.) 3. The stability of the accelerating potential, which causes chromatic aberration. This effect must be less than the Bragg angle. 4. The effects of knock-on ballistic damage. 5. The construction of transfer stages for hydrated or cryo-cooled samples in a suitable goniometer, if crystals must be used to obtain a sufficient intensity of high angle scattering. 6. The effects of inelastic scattering, causing background.

Certainly before embarking on a large construction project for a multi-MeV diffraction camera for biology, the samples of interest should be studied in an existing 1 MeV machine, fitted with cryo-EM sample handling facilities. This exists in Japan. In the USA, a time-resolved high energy machine is being considered for materials science. My answer to the question on whether electron beams can outrun radiation damage is also relevant.

**Jochen Küpper** asked: Dear John, thank you very much for the detailed explanation of the issues with high-energy-electron diffraction. Now, with your calculations, what are the prospects of the investigation of ultrafast chemical dynamics in relatively small gas-phase molecules, let us say isolated molecules with a size up to 10 nm, using coherent electron diffractive imaging with few-femtosecond few-MeV electron beams from accelerator-based electron sources? Will it be possible to obtain images of intact molecules, maybe in a diffract-only-from-intact-molecules approach as described for small-molecule X-ray diffraction (Küpper *et al.*, *Phys. Rev. Lett.*, 2014, **112**, 083002)?

**John Spence** replied: Much of my answer to Henry Chapman's question applies, but the need for hydration is removed for small molecules. The Zewail group has pioneered this type of fast gas-phase electron diffraction at lower beam





energies. In principle, the use of MeV beams eliminates the "space-charge" problem, since for relativistic reasons the electric and magnetic components of the Lorentz force between charges in the beam cancel at infinite energy. In practice, one has the challenges of high voltage engineering to obtain a small energy spread (sufficient chromatic coherence), and the problems of building an efficient area detector which is not damaged by the beam (one design uses a phosphor on a pellicle screen, viewed by a CCD at 90 degrees through a 45 degree mirror with a hole in it for the central beam. The screen is replaced as it damages, and one must consider the X-ray background from the beam striking anything, getting into the detector). In addition the de Broglie wavelength of the electron is so small that scattering angles become extremely small, requiring strong magnetic lenses to magnify the diffraction pattern, and very high collimation (much less than a Bragg angle for any crystalline sample), which reduces fluence. Finally, one has the problem of radiation damage to the sample, in the form of knock-on ballistic damage, which probably cannot be out-run by an electron beam. If the plan is for the beam to span many randomly oriented molecules per pulse, then data analysis becomes a headache, although in principle the method of angular correlations due to Z. Kam can disentangle the orientational disorder (see Kirian, *J. Phys. B: At. Mol. Opt. Phys.*, **45**, 223001 (2012)). This requires a coherence width shorter than the distance between molecules. If the plan is for a coherent beam whose size is about equal to that of one molecule ("isolated molecules"?), using a pulsed photofield source and lenses to demagnify the beam to nanometer dimensions, then there will not be much signal, or even one scattered electron per picosecond, despite the high elastic cross section (see question 609).

**Michael Woerner** commented: Electron diffraction scatters elastically off the Coulomb potentials of the nuclei whereas in X-ray diffraction photons scatter elastically off the electron density. A combination of both experiments might give insight into non-Born Oppenheimer effects. How far are we still away from studying such phenomena in combined time-resolved experiments?

**John Spence** answered: Time-resolved Bragg diffraction from protein nano crystals involved in photosynthesis using the pump-probe method is described in Aquila *et al.*, *Optics Express*, **20**, 2706 (2012) and Kuptiz *et al.*, *Nature*, 2014, doi: 10.1038/nature13453, just out. These papers use the diffract-and-destroy method, and look for changes in X-ray structure factors due to illumination by visible light to measure atomic motion on the microsecond time scale. Motion is slow in biological systems because the dominant contribution to free energy is configurational entropy, not electron transfer. The differences between electron scattering (from the coulomb potential) and X-ray scattering (from the electron density) can be used to provide very sensitive images of chemical bonding between atoms in crystals by electron diffraction, not possible using X-ray diffraction, as shown in Zuo *et al.*, *Nature*, 1999, **401**, 49, for the ground state of copper oxide. Failures of the B-O approximation would require very high time resolution, which is not possible using electron diffraction due to source





brightness and emittance limitations (the degeneracy of field emission sources is about  $1\text{E-6}$ ).

**Gopal Dixit** asked: Dear John, I am concerned about your idea to use attosecond X-ray pulses for time-resolved X-ray scattering (TRXS). The Fourier limited attosecond X-ray pulse has unavoidable finite energy bandwidth due to energy-time uncertainty relation. As you make your pulse shorter and shorter, the bandwidths will become larger and larger. Now, if you consider TRXS from a single molecule (not crystal), your time-resolved scattering signal will contain a significant amount of incoherent X-ray scattering and the energy resolution could not be better than the finite bandwidth of the pulse. In other words, it is impossible to disentangle the coherent and incoherent X-ray scattering contributions to the total signal within the finite bandwidth of the attosecond X-ray pulse. This scenario makes the analysis of the signal more complicated in the vicinity of avoided crossing and conical intersection for probing an ultrafast chemical reaction.<sup>1–3</sup>

1 G. Dixit, O. vendrell and R. Santra, *PNAS*, 2012, **109**, 11636–11640.

2 G. Dixit and R. Santra, *J. Chem. Phys.*, 2013, **138**, 134311.

3 G. Dixit, J. M. Slowik and R. Santra, *Phys. Rev. A*, 2014, **89**, 043409.

**John Spence** responded: I agree with your analysis for “single particles”, as the cryo-EM community calls your gas-phase molecules, so it would not work (in biology, these would have to be hydrated, which introduces considerable experimental complications). My analysis was for a crystalline sample, where the sample then acts as its own monochromator, picking out only those wavelengths which satisfy Bragg’s condition for diffraction into the same direction. Then the coherent amplification of intensity due to Bragg scattering gives enough scattering to obtain an atomic resolution image (unlike that from a single particle), while the interference between these two wavelengths (frequencies) provides information to solve the phase problem. If the bandwidth is sufficient to span two different such Bragg conditions, then the pulse duration must be shorter than the period of beating between them.

**Jochen Küpper** addressed Jasper van Thor and John Spence: Dear Jasper, John Spence told us again that biological processes are slow. But what is your opinion on the need to look at ultrafast (femtosecond) timescales even for large biological molecules? Don’t we need to understand the short-time dynamics to understand the (slow) function?

**Jasper van Thor** communicated in reply: The quantum yield of biological reactions is determined in the femtosecond time domain. Therefore, ultrafast coherent processes may determine the outcome of much slower processes that occur thermally. The example of excited state dynamics in the Photoactive Yellow Protein includes a photoisomerisation that has a time constant of about 400



femtoseconds. This is within or comparable with the vibrational dephasing time in biomolecules. We consider the possibility of direct detection of a coherent wave packet motion by high resolution X-ray crystallography. With the approximately 200 femtosecond pump–probe jitter we would expect to observe such motion only very imprecisely, however with few-femtosecond time stamping techniques and enough data redundancy there are no physical limitations to recording such a coherent wave packet motion. This also presents compelling motivation for theory development, particularly for the application of biomolecular processes.

**John Spence** responded: Please see my answer to Jochen Küpper's question about time-scales in biology.

**Jochen Küpper** asked: Dear John, you mention in your paper that you would like to see 10 as pulses, but as far as I understand it, you only want the corresponding bandwidth. The latter might be easier, at least conceptually it is much easier to get the bandwidth than to also temporally compress the pulse. However, now thinking about the parameters: 1  $\mu\text{J}$  in 10 as focused to 100 nm creates a field of about  $10^{20} \text{ W cm}^{-2}$ . This looks like a pretty strong field which will instantaneously destroy the molecule (Lorenz *et al.*, *Phys. Rev. E*, 2012, **86**, 051911, and Chapman *et al.*, *New Journal of Physics*, 2012, **14**(11), 115015). Will strong ultra-short pulses like these really be useful for diffractive imaging of chemical and biological systems and processes?

**John Spence** answered: I agree we only want the bandwidth, and this can be an incoherent superposition of energies for Laue diffraction, it does not need to be coherent (coherence makes possible the phasing method I also suggest). The existing theory and experiment for our "diffract-then-destroy" experiments suggest that, for the purposes of finding atomic positions, the instantaneous elastic scattering will terminate even for attosecond pulses before the damaging photoelectron cascade gets going. Note that it is not necessary to destroy the sample to avoid damage. If the attosecond pulses are weak, one can still out-run radiation damage.

**Michael Woerner** opened the discussion of the paper by Jasper van Thor by commenting: Typically, chemical reactions are performed in the liquid phase. We investigated an intra-crystalline acid–base reaction in ammonium sulphate: *The Journal of Chemical Physics*, 2010, **133**, 064509.

**John Spence** responded: Using snap-shot X-ray scattering from molecules in our micron-sized liquid jet, running across the pulsed XFEL beam in the diffract-then-destroy mode, it is possible to track chemical reactions by fast solution scattering (FSS) or fast WAXS. These reactions can be triggered using a mixing jet, or optical pumping. See Arnlund *et al.* (*Nature Comms*, doi: 10.1038/NMeth.3067) and Wang *et al.*, *J. Synchrotron Radiat.*, in press.



**Jasper van Thor** answered: Protein function requires the presence of water. The typical water content of protein crystals is about 40–60%, and biological function is very often conserved in crystalline form. Crystals of the Photoactive Yellow Protein undergo photoinduced reactions which strongly resemble those in liquid phase.<sup>1–3</sup>

1. C. N. Lincoln, A. E. Fitzpatrick and J. J. van Thor, *Phys Chem Chem Phys*, 2012, **14**, 15752–15764.
2. P. Ramachandran, J. Lovett, P. Carl, M. Cammarata, J. H. Lee, Y. O. Jung, H. Ihee, C. Timmel and J. J. van Thor, *J Am Chem Soc*, 2011, **133**(24), 9395–9404.
3. K. Ng, E. D. Getzoff and K. Moffat, *Biochemistry*, 1995, **34**(3), 879–90.

**Jochen Küpper** commented: As I understand your experiment, you are really performing a strong-field coherent control experiment to start the dynamics. Is that correct? If so, are you not really trying to find out how you get most of the population into the excited state, the starting point of the dynamics, and then follow the field induced dynamics, instead of the observation of weak-field single-photon-induced dynamics that occur in nature. Now, after all, I am still wondering how much can you learn about the actual biological process from the strong-field-initiated process despite these conceptional problems? More generally, I dare to ask the question of how we can follow photochemical dynamics after the absorption of a single photon from a weak source? Can we, and do we have to, look at a sample, in a ultrafast stroboscopic approach, of many molecules with individual ones reacting stochastically and be able to see the randomly-timed change of a single molecule?

**Jasper van Thor** replied: Dear Jochen, indeed, we are applying strong field coherent control. Femtosecond time resolved pump–probe protein X-ray crystallography requires both a very sensitive detection of the photoinduced structure factor differences as well as an optimal control of photochemical dynamics in crystals. In the case of femtosecond photoexcitation of crystals of the Photoactive Yellow Protein, I have shown a multilevel electronic scheme that illustrates the need for strong field coherent control. We are dealing with a heterogeneous singlet excited state with interconversions and relaxations on the femtosecond timescale, all having different branching ratios to generate the primary photoproduct which has a photoisomerised biological chromophore in 400 femtoseconds. Two photon processes with strong cross sections in the blue edge of the absorption spectrum result in photoionisation processes which are a loss channel, whereas pumping in the red edge readily results in pump-dump scenarios. We have previously shown<sup>1</sup> that modification of the pulse duration, peak power, center wavelength and importantly addition and control of second order dispersion is required to optimize the femtosecond population transfer to the photoproduct state. From systematic studies with power titrations for shaped pulses we were able to extract all the non-linear cross sections for several regimes.<sup>1</sup> An important aspect is the presence of a second order dispersion that chirps against the dynamic Stokes shift, which has a ~200 fs time constant, to minimise the stimulated emission. The resulting optimised optical conditions are those with suppressed non-linear cross sections. These high field conditions



are therefore the best representative for one-photon processes of single molecules under weak illumination: under such conditions internal conversions appear in approximately ~70% of excitations whereas ~30% undergoes photoisomerisation. The strong-field coherent control conditions are designed to approach this as closely as possible, while achieving maximised and detectable levels of photo-intermediate to above the detection limit of the time resolved pump-probe X-ray crystallographic measurements. Pulse shaping with high peak power, manipulating the quantum interferences between multiple pathways such as considered in the 'Brumer-Shapiro' scheme has been well understood.<sup>2</sup> In addition, P6<sub>3</sub> crystals of PYP are monoaxial and we must also consider birefringent optical propagation in the medium, as well as the photolysed depth.

With regard to your final question, I believe that just as in isotropic solutions as seen by ultrafast spectroscopy, coherence could be observable also in the crystalline state, within the experimental bandwidth, resolution and dephasing time.

1. C. N. Lincoln, A. E. Fitzpatrick and J. J. van Thor, *Physical Chemistry Chemical Physics*, 2012, **14**, 15752–15764.
2. W. Wohlleben, T. Buckup, J. L. Herek and M. Motzkus, *Chemphyschem*, 2005, **6**, 850–7.

**Michael Woerner** communicated: By using strong THz sources for triggering electric field induced events in matter one can also explore chemical reaction in the electronic ground state. Is your future planning of experiments also along those lines?

**Jasper van Thor** answered: THz excitation of macromolecules would similarly allow access to ground state dynamics. A particular challenge may be the existence of a congested Density of States in the low frequency region, such that selective pumping, or explicit mode assignment, is not straightforward. Experimentally, considering the possibility to extend studies as you have demonstrated for small molecules to macromolecules is appealing and I hope that in the future instrumentation will be available to allow for the exploration of this regime.

**Jochen Küpper** asked: On page 2 of your manuscript, you mention various structures (I0, I1, I2', I2, *etc.*). Can you explain to us what these structures look like, what shape or structure they exhibit, according to current belief?

**Jasper van Thor** replied: Dear Jochen, the species which is called 'I0' is the primary photoproduct which is the target state for femtosecond time resolved crystallography. It is an electronic ground state species in which the p-coumaric acid chromophore of the protein has undergone photoisomerisation. The time constant of this photoisomerisation process is ~400 fs. The further intermediates that are thermally populated in picosecond to millisecond time scales in the 'photocycle' of the Photoactive Yellow Protein are characterised by reorganizations of the protein and solvent parts of the macromolecule. Eventually, the



system recovers the dark ground state, making stroboscopic measurements possible. Resolving the structural features that belong to the pure species that interconvert requires methods of Singular Value Decomposition and Global Analysis from extensive series of time resolved measurements. For the purpose of our work we are primarily focused on the excited state dynamics which form the primary photoproduct '10'.

**Jochen Küpper** commented: Do I understand it correctly that the actual structure, that is, the three-dimensional arrangement of atoms and the surrounding electron densities, are not known for these species?

**Jasper van Thor** answered: Currently, the earliest structural information obtained using pump-probe Laue X-ray crystallography using synchrotron radiation is 100 ps (Jung *et al.*, *Nat. Chem.*, 2013, 5(3), 212–220, Jung *et al.*, *Nat. Chem.*, 2014, 6(4), 259–260, Schotte *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, 109(47), 109–147, Kaila *et al.*, *Nat. Chem.*, 2014, 6(4), 258–259). Whereas techniques are being developed that use synchrotron pulses to achieve increased time resolution, and accessing femtosecond and few-picosecond delays will be possible using XFEL sources, as we have discussed in our contribution (van Thor *et al.*, *Faraday Discuss.*, 2014, DOI: 10.1039/C4FD00011K).

**Hans Jakob Wörner** opened the discussion of the paper by Oriol Vendrell by asking: I have two questions related to the feasibility of the experiment that you propose. First, how does the electronic structure of neutral water clusters  $(\text{H}_2\text{O})_n$  differ from that of protonated water clusters  $\text{H}^+(\text{H}_2\text{O})_n$  that you calculate? Second, what optical densities would be required in this experiment and how do they compare to what can be achieved?

**Oriol Vendrell** responded: Related to the first question, ionized neutral water clusters have been studied at the ADC(3) level of theory up to  $n=4$  water molecules.<sup>1</sup> For  $n=4$ , the outer-valence ionization potential spans from about 12.1 to 19.5 eV whereas the inner valence starts at about 31 eV and the double ionization threshold starts at about 28.2 eV. In the large protonated cluster considered by us,  $n = 21$ , the outer valence ionization potential spans the range 12 to 25 eV, 12 eV corresponding to ionization from far away from the extra proton and 25 eV to ionization from its vicinity. Therefore, we think that in the bulk or in the clusters one should be able to ionize from the vicinity of the extra proton and observe the correlated proton-hole dynamics discussed in our contribution without reaching the double ionization threshold at about 28 eV that would result in autoionization dynamics.

Related to the second question, an experiment based on absorbance measurements would be very hard or even impossible because of the original positive charge of the clusters and the corresponding very low densities. Photo-ionization experiments conducted at FLASH on  $\text{H}^+(\text{H}_2\text{O})_2$  used detection of charged fragments at a mass spectrometer, which is then extremely sensitive to



individual events.<sup>2</sup> In a similar way, an experiment related to our calculations may be realizable if, after the X-ray probe step, secondary processes such as total Auger yield or total fluorescence from the core-hole relaxation are measured. In this mode of operation, the bandwidth of the X-ray probe should remain at most in the 1 to 2 eV range, since the hole relaxation dynamics occur in the 10 eV energy scale. For Fourier limited pulses this corresponds to pulse lengths beyond 1 fs and the relaxation time in the large cluster is of the order 100 fs. Therefore, an *e.g.* 10 fs (limited) X-ray probe would be sufficient to observe the hole relaxation.

1. I. B. Müller and L. S. Cederbaum, *JCP*, 2006, **125**, 204305.

2. L. Lammisch, *et al.*, *Phys. Rev. Lett.*, 2010, **105**, 253003.

**Katharine Reid** asked: From comments I have heard at this meeting it seems that in an ideal world we would like to have a light source with a tunable pulse duration, tunable bandwidth, tunable intensity and tunable repetition rate. As it seems unlikely that such a source can ever be realized in practice, would it make sense for the community to develop an international strategy for light source development whereby sources with complementary specifications are available in different locations?

**Martin Wolf** responded: This would be a wonderful source. For many problems one would like to operate with pulses with a optimum time-bandwidth product preserving energy resolution and having appropriate time resolution at the same time. Having ultimate time resolution is not always beneficial as, for example, the bandwidth of attosecond pulse in the range of several eV to even 10–12 eV often hinders certain spectroscopic applications.

**Christian Bressler** communicated in reply: European XFEL seeks to deliver just that! (1) Variable pulse filling patterns could allow us to perform experiments from 10 Hz, over 10 kHz (10 pulses only) to 4.5 MHz (up to 2700 pulses, all at a 10 Hz burst repetition rate), all according to user demand. (2) Variable pulse duration: LCLS, but also European XFEL (and SACLA as well) can tailor the X-ray pulse width from 100 fs (or more) down to few fs (and possibly below). (3) Variable pulse intensity: every SASE FEL can attenuate the beam at will from the full single pulse intensity (which is largest at LCLS and European XFEL, somewhat lower at SACLA and soon at both SwissFEL and Pohang FEL). (4) Focussability of the beam on the sample at XFEL sources is also important.

**Jochen Küpper** addressed Oriol Vendrell and John Spence: Following earlier comments by Oriol Vendrell and others, I would like to comment on the Born–Oppenheimer approximation and its relevance for the topic of this session and the conference:

In simple words, the Born–Oppenheimer (BO) approximation assumes that the kinetic energy of the nuclei is negligible (Demtröder, *Experimentalphysik 3*, Springer, Berlin Heidelberg New York, 4 ed., section 9.1, vol. 3, pp. 1–668 (2010)).



Within that approximation, we can then separate the molecules' Hamiltonian(s) into the electronic and the nuclear (potential energy) part. Now, this approximation is a good one for large parts of a molecule's phase space, but it does break down, by definition, when we look at fast nuclear dynamics. Processes where nuclear dynamics are slow might be best investigated with high-resolution eigenstate spectroscopy (Herzberg, *Molecular Spectra and Molecular Structure: Spectra of Diatomic Molecules*, Krieger, 1989, vol. 1–3, Küpper *et al.*, *Physical Chemistry Chemical Physics*, 2010, **12**(19), 4968–4979, and Küpper *et al.*, *Physical Chemistry Chemical Physics*, 2010, **12**(19), 4980–4988).

What we are after here are dynamical processes, where the nuclei move fast, and where obviously the BO approximation does break down. Now, what we really want to understand are dynamical processes, such as the isomerization or folding of molecules, the breaking and forming of bonds, *etc.* The BO approximation might not be the right picture to look at these processes.

**John Spence** communicated in reply: See my answer to question 601. In structural biology, as for a rubber band or a polymer, it is the entropy which matters.

**Hans Jakob Wörner** answered: The key assumption of the Born–Oppenheimer approximation (BOA) is not that the kinetic energy of the nuclei is negligible, but rather that derivatives of the electronic wave function with respect to nuclear coordinates are negligible.<sup>1</sup> This is in general fulfilled when the electronic energy-level intervals are much larger than the vibrational ones. Highly excited vibrational levels of an isolated electronic state are well described within the BOA, whereas the vibrational ground state of a molecule is not when it lies energetically close to a conical intersection, as is frequent in Jahn–Teller-active systems, see *e.g.* **ref. 2**.

Switching to the time domain, a wave packet can always be expanded in eigenstates of the molecular Hamiltonian. Therefore, the velocity of nuclei in a wave packet, defined by the energy intervals, has no impact on the validity of the BOA. For example, ultrafast isomerization on an energetically isolated electronic ground-state surface is well described by the BOA, whereas arbitrarily slow wave packet dynamics across a conical intersection is not. Hence, I do not expect a significant difference in the applicability of the BOA between time- and frequency-domain spectroscopies.

1. M. Born and R. Oppenheimer, *Annalen der Physik*, 1927, **389**, 457–484.

2. H. J. Wörner and F. Merkt, *Angew. Chem. int. ed.*, 2009, **48**, 6404–6424, and references therein.

**Oriol Vendrell** commented: I would like to add a few comments on the topic of the Born–Oppenheimer (BO) approximation. Hans Jakob Wörner correctly points out that the BO or adiabatic approximation consists in neglecting the coupling terms between different electronic states. These are small when the potential energy gap is large but must be explicitly considered when electronic states are close in energy. It is nowadays very well established that, as soon as molecular





systems leave the ground electronic state, conical intersections and avoided crossings between potential energy surfaces are ubiquitous and fully determine the dynamics of the system.<sup>1</sup>

In a previous comment, which triggered the remark of Jochen Küpper, I stated that the BO approximation is a very good one, which is true for molecules in their ground state but not so true anymore for electronically excited molecules. I should have been more precise, for what I meant was the group-BO approximation.<sup>2</sup> The group-BO approximation implies that for the group of states of interest all couplings are considered but no couplings are taken into account to states outside this group. This is the theoretical setup in which virtually all molecular dynamics and spectroscopy is performed. In it, the exact expansion of the molecular wavefunction in terms of an infinite number of electronic states is truncated to a matrix Schrödinger equation of the size of the number of electronic states of interest.

The main point that I wanted to make though, is that the key idea of nuclei evolving in potential energy surfaces as the key concept to understand chemical dynamics does not need to be abandoned even if one is dealing with large numbers of coupled electronic states and strong non-adiabatic effects. This is the case *e.g.* in our contributed paper and it is also true for large bandwidth attosecond pulses applied to molecular systems to trigger joint electronic and nuclear dynamics, as in attosecond charge migration studies.

1. D. G. Truhlar and C. A. Mead, *Phys. Rev. A*, 2003, **68**, 032501.

2. G. A. Worth and L. S. Cederbaum, *Annu. Rev. Phys. Chem.*, 2004, **55**, 127–58.

**Jochen Küpper** addressed all the attendees: Following up on the comment of Michael Wörner, I want to point out that THz radiation cannot only be used in a strong field regime to trigger reactions, but also as a resonant weak-field THz trigger. Following our original demonstration of conformer separation (Filsinger *et al.*, *Phys. Rev. Lett.*, 2008, **100**(13), 133003) there have been initial calculations by Ingo Barth and Jörn Manz (FU Berlin, private communication of unpublished results) on the conformer interconversion of 3-aminophenol. These calculations hinted at the possibility to resonantly climb up the ladder of internal-rotation states of the OH torsion, overcome the barrier, and possibly even resonantly climb it down on the other side.

This should be generalized to an approach where we resonantly excite molecules into a reactive vibrational state in the electronic ground state – and then follow the subsequent chemical dynamics by the wonderful “imaging” experiments discussed at this meeting. In a statistical limit, repeating the experiments for many different excited vibrational states would allow us to determine the chemical reactivities for large parts of the molecule phase space. Overall, such a weak-field approach seems to be a challenging approach, but the hope is that it provides direct “molecular movies” of actual chemical processes, including the initial fast nuclear and possibly even the corresponding electronic dynamics of chemical reactions in the electronic ground state – corresponding to “plain chemistry”.

**Henry Chapman** opened the discussion of the paper by Nora Berrah by commenting: Regarding the difference between the X-ray FEL pulse duration and the



electron bunch duration (which is what is reported on the LCLS status screen) we also saw in Bragg termination measurements that the X-ray pulses were considerably shorter than the electron bunch, as described by Barty *et al.*, *Nature Photon*, 2012, 6, 35. We should point out that LCLS now has a tool that measures the energy loss vs. time of the electron bunch which tells you which part of the bunch produced X-rays.

**Nora Berrah** responded: This is a good addition to the LCLS beam diagnostics.

**Eleanor Campbell** commented: Care should be taken when interpreting mass spectra in terms of dynamics that occur on the fs timescale. The mass spectra probe the ion distributions that are present on a timescale of microseconds – *i.e.* much longer than the initial excitation pulse. The model used to interpret the C<sub>60</sub> experiments considers only direct ionisation and secondary electron collision processes in one fullerene molecule. Under the conditions of the experiment, many energetic electrons are produced and escape from the ‘nanoplasma’. The absolute cross-sections for electron impact ionisation/fragmentation were studied in detail during the 90s (*e.g.*, Foltin *et al.*, *Chem. Phys. Lett.*, 1998, 289, 181, Hathiramani *et al.*, *Phys. Rev. Lett.*, 2000, 85, 3604) and are large with a plateau for electron energies of a few hundred eV. It is quite possible that secondary ionisation/fragmentation of other fullerene molecules in the target is making a significant contribution to the observed mass spectra.

**Nora Berrah** answered: This is indeed a good point.

**Jochen Küpper** communicated: Nora, I have a naive question regarding the very good match of your experiment and the quite classical modeling. Obviously, the electrons in molecules are strongly correlated, or entangled, but you and your collaboration can nicely model this using a very classical description. This is indeed an interesting finding and also a helpful one. However, I wonder where/when the correlation/entanglement is lost – at least it seems to be lost. Considering the good match of the classical modeling, this collapse of entanglement must happen very early, *e.g.*, does it do so with the first photon absorbed, or similar?

**Nora Berrah** replied: The good match of experimental data with classical modeling is valid only in the case of ultra-strong fluence and also at an ultra-short timescale (4 fs in our case). In these cases, electron correlations do not seem to be important as demonstrated by the excellent agreement between experimental data and classical modeling. This is not the case at intermediate fluence where molecular effects are more important as revealed by the lack of good agreement between experimental data and classical modeling. I also assume that the collapse of entanglement may occur very early, with the first photon absorbed since modeling shows that the dynamics occurs at the first few fs. Note that the



classical modeling treats the particles (electron and ions) classically but the cross sections and rate equations are generated using quantum mechanics.

**Michael Woerner** addressed Nora Berrah and Jochen Küpper: During the discussion of Nora Berrah's paper the question was raised from when the classical picture can be applied. The quantum to classical transition has something to do with the decoherence and the measurement process. We published a paper on interband tunnelling of electrons in GaAs: *Phys. Rev. B*, 2010, **82** 75204. The **refs. 37–41** therein give valuable insight into the decoherence and the rate of the latter process on the relative distance between particles.

**Nora Berrah** answered: Thank you for the information.

**Katharine Reid** addressed Nora Berrah and Giovanni De Ninno: You both referred to the possibility of performing time-resolved pump–probe experiments, but very few experiments presented at this conference have used such a scheme (though many have aspired to). Would you be able to comment on the kinds of time-resolved pump–probe experiments (wavelengths, time resolution) that are possible at your respective light sources (FERMI and LCLS) and on the prospects for pump–probe experiments at such sources in the future?

**Giovanni De Ninno** replied: At FERMI, we can carry out pump–probe experiments using different (complementary) setups. In the ‘standard’ configuration, the pump pulse is generated by taking a fraction of seed laser (wavelength: 800 nm, pulse duration: adjustable in the range 400–100 fs FWHM), or of one of its low-order harmonics (*e.g.* the third one), while the probe is provided by the FEL itself (fully tunable in range between 80–4 nm, with pulse duration adjustable in the range 50–200 fs FWHM). If required by users, the sample can be pumped by the FEL and probed by the laser. The typical jitter between the pump and the probe is quite small (*i.e.* about 5 fs). The seeded nature of our FEL permits the implementation of two additional ‘exotic’ configurations, both allowing FEL–pump–FEL–probe experiments in the XUV range, with a temporal resolution of several tens of fs. The first one, described in **reference 44** of our manuscript, relies on seeding the electron beam with a strongly frequency-chirped laser pulse; this naturally leads to the generation of two FEL sub-pulses (*i.e.* the pump and the probe), characterized by the controlled temporal and frequency separations. The second exotic configuration is the one exploited in the experiment reported in our paper. In this case, the electron beam is seeded with two separated laser pulses, characterized by a predetermined temporal and frequency separation. All the above configurations are routinely used at the FERMI beamlines. For prospects about future pump–probe experiments at FERMI, see <http://www.elettra.trieste.it/lightsources/fermi.html>.

**Nora Berrah** commented: There have been time resolved experiments at FLASH, FERMI and at LCLS using optical laser pump X-ray probe and using X-ray



pump-X-ray probe. Data from these experiments are being analyzed but also some have been published. For example the following LCLS papers are the result of optical laser pump X-ray probe but there are more papers in the literature from other FELS, so this is only representative:

1. B. F. McFarland *et al.*, *Nature Communications*, 2014, **5**, 4235.
2. V. S. Petrovic *et al.*, *Phys. Rev. Lett.*, 2012, **108**, 253006.

**Gwyn Williams** opened the discussion of the paper by Giovanni De Ninno by asking: Do the two colour photon pulses come from the same electron bunch, and if so, what is the length of this electron bunch?

**Giovanni De Ninno** answered: Here you have our answer to the comment “Do the two colour photon pulses come from the same electron bunch, and if so, what is the length of this electron bunch?”:

Yes, the two-colour FEL pulses are generated by the same electron bunch, seeded by two seed pulses. For the presented experiment, the length of the ‘smooth’ part (*i.e.* flat, both in energy and current) of the electron bunch was about 500 fs.

**Elaine Seddon** communicated: Your paper records that the intensity jitter in the seeded FEL pulses is around 15%. I would like to know if this is expected to be a problem for some users and if so is there currently a drive to improve the jitter?

**Giovanni De Ninno** communicated in reply: In general, an intensity fluctuation around 15% is not an issue for the large majority of our users, who have the possibility to keep track of the FEL intensity behaviour on a shot-to-shot basis. This allows them to normalize the obtained results. In order to get a better stability, we are currently following two directions, *i.e.* a further reduction of the electron-beam *vs.* laser jitter, and the improvement of the electron-beam longitudinal flatness.

