

DISCUSSIONS

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Nanoscale and bio imaging: general discussion

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Martin McCoustra opened the discussion of the paper by **Chris Jacobsen** by asking: We have heard many comments on the idea of molecular movies in this Discussion. What likelihood do we have of what might be referred to as biomolecular movies showing the temporal evolution of biomolecules in a living organism?

Chris Jacobsen communicated in reply: The challenge here involves radiation damage. For repeatable phenomena in measurements that involve statistical measures of systems, one avoids radiation damage by measuring at a variety of time points after a stimulus (repeatability) with the irradiation hitting a number of biomolecules so that the dose to each individual biomolecule is reduced (statistical measures). This might apply for example to small angle scattering. When it comes to imaging larger and thus non-identical objects such as organelles and whole cells where there is considerable overall structural variation (no two cells look alike), direct real-space statistical averaging seems difficult to apply. Also, when one is doing even 20 nm resolution imaging, the radiation dose is in the 10^8 Gray range which is sufficient to break about one bond per molecule so the movie is far more likely to show the biomolecule being damaged rather than undergoing a “normal” temporal evolution. Of course, pump-probe crystallography can be used for stroboscopic (repeatable sample) and ensemble-averaged (crystal with many identical unit cells) measurements of the time evolution of a photostimulable biomolecule—which in the case of proteins represents a very small though important subset of all known proteins.

Henry Chapman asked: Would it be possible to use some sort of dynamic response in a pixel of an STXM scan as a contrast mechanism, for example to map the distribution of chemical activity in a sample?

Chris Jacobsen responded: Interesting thought. Of course X-ray photon correlation spectroscopy (XPCS) involves using the rearrangement of the coherently scattered intensity distribution to tell one about the timescale of rearrangements of optical phases of the material within the coherently illuminated beam spot. The challenge of course is one of radiation damage, in that when one goes from a large-beam-spot XPCS experiment to a nano focus scanning transmission X-ray microscope (STXM) beam spot, the number of photons per area goes up to very high levels and one is as likely to see a dynamic response caused by beam damage as a dynamic response caused by a more "natural" process in a pixel. The per pixel dose in 20–50 nm resolution STXMs is in the 10^6 – 10^8 Gray range, and at around 10^8 Gray one has broken about one bond per molecule. At the same time, there are some indications that chemical damage takes on the order of a millisecond or so to be made manifest in diffraction patterns, so maybe there is some hope? Interesting question.

Grigory Smolentsev remarked: You use the regularization method to include *a priori* knowledge into your model. Are there any other methods that allows you to include *a priori* knowledge into the model, for example, those based on the linear combination of components obtained using principal component analysis? Could you compare your method with the alternative algorithms?

Chris Jacobsen replied: In fact the spectra arising from cluster analysis (which we include as the "cluster similarity" regularizer in eqn. 10–13 in our Faraday Discussion paper) are exactly what you suggest: a linear combination of components obtained from principal component analysis (PCA). As described in our earlier paper (Lerotic *et al.*, *Ultramicroscopy*, **100**, 35 (2004)), we use PCA to obtain a basis set with desirable properties like orthogonality and noise-suppression. We then find weightings among these components by seeking clusters of pixels (independent spectral measurements) in this component space, yielding spectra that are linear combinations of PCA spectra. However, as explained in Section 2 of this Faraday Discussion paper, the resulting set of cluster spectra can be incomplete as a basis set and can lead to errors in analysis. What NNMA gives us is a way to use the cluster spectra as one part of the cost function (one regularizer). However, it is used in combination with the data-matching cost of eqn. 3, and the sparseness regularizer of eqn. 7–9, to yield an improved solution as shown by the lack of unphysical "negative absorption" values in the NNMA solutions shown in Fig. 6 of our paper.

Jonathan Underwood communicated: The regularization method presented in your paper bares some similarity to other widely used strategies such as Tikhonov regularization and particularly Projected Landweber regularization in terms of the non-negativity constraint. The convergence properties of these strategies are reasonably well studied and understood (*e.g.* *Num. Funct. Anal. Opt.*, **13**, 413 (1992)). Is anything known about the convergence properties of your regularization strategy?

Chris Jacobsen responded: To our understanding, the cited paper deals with the problem of determining A in $Af=g$ when f and g are known, subject to constraints; it shows good convergence properties. In our case we know neither A (the set of spectra) or f (the thickness maps) in advance; we only know the measured data g . We are not aware of analyses on convergence rates for global solutions, though the steepest-descent type approach we have used (based on derivatives of cost functions) at least aides in finding local solutions. We have carried out some work on the optimization of the regularizing parameter λ ; we will report on that in a separate paper.

Henry Chapman addressed Chris Jacobsen and Jasper van Thor: I noticed that the update algorithm you present in your paper (eqn. 3–5) is essentially the algebraic reconstruction technique (ART) used in tomography (also called the Kaczmarz algorithm, used in Caterina Vozzi's tomographic reconstructions paper). Presumably regularisation has been applied in tomography too, or perhaps your developments could be of interest for that application. Your method is also immediately applicable to solving structural kinetics problems, and would be a big improvement upon the SVD method developed by Marius Schmidt and others for time-resolved WAXS and time-resolved crystallography,¹ just replacing energy (in your case) for time (in theirs). I have not seen a discussion in those applications how they impose the constraint that populations are positive. It seems to me that your regularisation technique would be a big improvement, especially sparsity which will enforce populations to occur more in a sequence than a jumble, and allowing one to avoid model bias.

1 M. Schmidt, S. Rajagopal, Z. Ren, and K. Moffat, Application of singular value decomposition to the analysis of time-resolved macromolecular X-ray data, *Biophys. J.*, 2003, **84**(3), 2112–2129.

Jasper van Thor communicated in reply: Dear Henry, in Singular Value Decomposition or other methods of matrix factorisation, a separation is made purely on the basis of orthogonality. The fundamental assumption is one of linearity. Thus, any individual measurement, or single column, of the input matrix must then be some linear combination of the left singular vectors. The basis spectra, or left singular vectors in themselves, have no physical meaning: these do not represent spectra for pure species. Therefore, it is always required to apply a model or connectivity scheme that relates to the populations found in the right singular vectors (see for example reference 1). By requiring amplitudes to be non-negative, as Chris has implemented, such a model is effectively being used.

1. L. J. G. W. van Wilderen, C. N. Lincoln, J. J. van Thor, *PLOS One*, **6**, 2011, e17373.

Chris Jacobsen answered: Good suggestions. I hasten to add that by no means did we invent regularization methods! We simply applied them to the case of spectromicroscopy analysis, where to our knowledge these methods had not been

applied before (except for the PhD work of Holger Fleckenstein, who is a coauthor on the paper).

Jasper van Thor asked: Dear Chris, in the matrix decomposition did you perform a matrix inversion which presents the basis spectra μ_{SxN} which you show to have all the significant amplitude? Or did you perform a Singular Value Decomposition $D = \mu S t^T$, where the singular values represent the amplitudes and also provide the statistical significance of the contributions of the basis spectra μ . The thickness maps as well as the basis spectra would need to be weighed by the respective singular value S . Does that correspond to the amplitudes which you have shown?

Chris Jacobsen replied: In the cluster analysis approach described in the first part of the paper, our procedure is as follows: (1) perform an eigenvector analysis from the covariance of the data matrix D , and then limit the vectors to those with statistical significance; and (2) carry out cluster analysis to find pixels with similar eigenspectra weightings. The spectra at those pixels are then averaged together to yield a matrix μ_{SxN} which is then inverted using singular value decomposition (SVD) as you describe. This is described in the 2004 Ultramicroscopy paper by Lerotic *et al.* cited in the manuscript. These spectral solutions are then used as just one of two regularizations in the non-negative matrix analysis (NNMA) approach described, where the main cost function is the data matching error (optical density matrix minus the product of the present guess of the set of spectra times the present guess of the weightings or thicknesses; that is, minimizing the square of $D - \mu t$). I hope this answers the question.

Michael Woerner communicated: I see some similarities of your method with the so called maximum entropy method (MEM). Is it possible to combine your method with the MEM?

Chris Jacobsen responded: Maximum entropy involves minimizing some norm that measures contrast or fluctuations in the solution. These can include norms such as L_1 or L_2 . In our case, we used an L_1 norm on the thickness solution t , so in some sense we have included a regularization that is representative of what is used in some Maximum Entropy methods.

Majed Chergui opened the discussion of the paper by **Michael Woerner** by asking: Your set-up for fs powder diffraction is quite unique. How do you compare it with ultrafast electron diffraction where the electron beam can be focussed down to a single grain size, or also to the developments for nanodiffraction at synchrotrons, which may lead to ultrafast studies too?

Michael Woerner communicated in reply: First, I would like to mention, that table top femtosecond X-ray diffraction can be also performed using the rotation method as we have recently demonstrated (B. Freyer *et al.*, *Optics Express*, **19**, 15506 (2011)). However, the diffraction efficiency for X-rays is much smaller than that for electrons. Thus, femtosecond nanodiffraction of X-rays will be the playground of the big accelerator machines (at least in the near future). Recently, we started a collaboration with Andrius Baltuska of the TU Vienna to develop schemes for a table top femtosecond X-ray source using a long wavelength driver (*i.e.* an OPCPA system working at $\lambda = 4 \mu\text{m}$). Such experiments turned out to be very promising in the sense that we can expect for future table top systems a hundred times higher X-ray flux.

Jasper van Thor asked: Dear Michael, from your presentation it is clear that you apply a maximum entropy method to find the charge density distribution from your experimental data. Firstly, could you please reiterate the phasing method that was used, and the accuracy of the structure factors in terms of crystallographic quality indicators and *R* factors. Secondly, can you show that the pumped data are truly isomorphous with the unpumped data? From the strength of the charge density which you derive and have shown is surprising that no bond length modification is apparently observed, which would likely dominate the transient structure factor amplitude differences. Further, transient difference density is present only within the duration of the pump and is not seen after. For the field driven signal, would THz pumping then retrieve the time-domain response to the field?

Michael Woerner communicated in reply: First, I would like to mention that we use the maximum entropy method (MEM) (instead of using a direct Fourier transform in the truncated *q*-space) just as a tool to derive the electron density map. Thus, the MEM has nothing to do with phase problem. How we deal with the phasing problem is detailed at the beginning of section 3 of our article. In summary, our method of analysis determines the average unit cell of the entire powder sample which has per definition the symmetry properties of the initial structure. An analysis using the Patterson method (Stingl *et al.*, *Phys. Rev. Lett.*, **109**, 147402 (2012)) shows that the latter is the dominant contribution to the change of the electron density map. In general, an excitation using a polarized electric field might also change the intensity along a particular diffraction ring in powder diffraction. Our diffraction efficiency is too low to address such questions in more detail. Within an optical cycle of the 800 nm driving pulse the change of bond lengths are negligible. Impulsive Raman excitation, however, leads to changes of the bond lengths in LiBH_4 at later times as evident from Fig. 3(c–f) in our article. Similar to impulsive Raman excitation THz excitation might also lead to bond length changes on the longer times scales of vibrations.

Martin McCoustra commented: One wonders what might happen to the charge exchange as LiH is compressed? Would measurements in a diamond anvil cell be possible to experimentally address this question?

Michael Woerner responded: In the literature one finds stationary X-ray diffraction experiments on compressed LiH up to 250 GPa (Lazicki *et al.*, *Phys. Rev. B*, **85**, 054103 (2012)) using a diamond anvil cell. Thus, in principle femto-second X-ray diffraction under pressure should be possible. In practice, however, one expects additional problems in time-resolved experiments due to both X-ray absorption and dispersion of pump light in the anvil cell itself. So far, I did not find an analysis of stationary diffraction experiments concerning a pressure dependent ionicity of LiH. Depending on the ionicity of the pressure dependent ground state of LiH I expect a field induced change of the ionicity as discussed in our article.

Christian Bressler communicated: You have succeeded in extracting results *via* fs-powder diffraction in a laser-driven X-ray plasma source. Do you see the need to apply for beam-time at an XFEL source? What benefit from this source would you expect against your ample in-house beam-time? Or why did you not apply for XFEL beam-time anywhere so far?

Michael Woerner answered: I will apply for beam-time at an XFEL source once those devices provide >10 keV X-ray pulses at a repetition rate of 1 kHz or higher with a timing jitter between laser pump and X-ray probe pulses of less than 50 fs. So far the research we would like to do can be easily performed with our table top fs X-ray source (despite having much less X-ray photons per shot). The main reason I did not apply for XFEL beam-time is simply the lack of free personnel and working hours from our side. The experiments we are doing in our lab are very time consuming and personnel intensive. If this situation changes in the future (*i.e.* more personnel) we are happy to apply for beam-time at an XFEL.

Jonathan Underwood opened the discussion of the paper by **Jochen Küpper** by commenting: Prof Marangos asked what would be needed to extend the technique presented by Küpper and colleagues for diffractive imaging of ground state (static) molecular structures to the measurement of excited state structural dynamics. If one considers an experiment where a resonant pump pulse electronically excites molecules, and then the resulting structural dynamics are probed *via* diffraction with a time delayed X-ray pulse, then the optimal situation would be that the molecular alignment is maximal at the instance of probing. So, one can imagine an approach in which a strong non-resonant laser field is applied prior to the pump pulse to create a rotational wave packet in the ground state of the molecule. By careful design of the alignment laser field parameters (pulse shape, time-dependent polarization) and time-delay between the alignment and pump laser fields it may then be possible to transfer the rotational coherence to the excited electronic state where the subsequent wave packet evolution will produce the desired alignment at the instance of probing. However, this is clearly a very challenging proposition, as one needs to understand how the rotational wave packet will evolve in the excited state which may have very different (and time-dependent) rotational constants to the ground electronic state. Also, the alignment field parameters would need to be varied each time the pump and

probe time delay was changed. There is very clearly a need to consider this problem in further detail as current understanding of what is and is not possible is currently poor.

Jochen Küpper communicated in reply: This experimental protocol suggested by Dr. Underwood is interesting and should, in principle, work. There are a few comments I wish to make below.

At the same time, I want to point out that we should really make big efforts to look at ground state chemistry. While there is very important electronically-excited state (photo)chemistry going on in chemistry and biology, most chemical dynamics does not happen in excited electronic states, but in the ground state. This involves almost all "classical", thermally activated chemistry and biology and comprises by far the largest part of all chemical and molecular dynamics. Therefore, we need to develop protocols to trigger and time chemical reactions on the ground state potential energy surface, and methods to investigate, image them with spatial and temporal atomic resolution. It might be possible to define the starting point of such ground-state reactions with (resonant or non-resonant) THz or IR photons. An alternative approach would be to use highly sensitive detection methods that are capable of simply observing the few, rare, reaction events in a thermally activated "soup" of reactants. While the latter is a dream, maybe solely a thought, the former approach of photo-triggering ground state reactions seems in reach. First experiments are already emerging^{1,2} and need to be transferred to the ultrafast-time and structure resolved domain of imaging experiments.

Regarding the suggested transfer of coherence from the electronic ground state to the electronically excited state, a few points discussed at the meeting were related to the change of the inertial tensor upon excitation, *e.g.* the changes of rotational constants and the reorientation of the principal axes of inertia. These are generally important considerations, but we might be lucky in many cases: axis tilting effects are often very small ($<1^\circ$) for valence excitations, rotational constants typically change by one to a few percent, and sometimes even the electronic properties, such as dipole moment and polarizability, change only weakly. Moreover, all these details can be derived from eigenstate resolved spectroscopies –for the prototypical molecule indole this has been shown in detail, including the details of strong vibronic cooling between different electronically excited states.^{3–5} Regarding the alignment process, which is in the short pulse "impulsive" regime, I wish to point out that for the alignment to be visible in the coherent diffraction pattern, one has to obtain very strong degrees of alignment, *i.e.*, $\langle \cos^2\theta \rangle \gg 0.8$.⁶ This will be very hard to achieve in an impulsive alignment approach, although multi-pulse alignment might be able to achieve it (Cryan *et al.*, *Phys. Rev. A*, 2009, **80**, 063412). Overall, it might be easier to implement a quasi-adiabatic alignment protocol, in which a moderately-long-pulse ac field angularly "permanently" confines the molecule—with significantly stronger degrees of alignment and independent of the molecule's electronic state. However, the presence of the field clearly has to be considered regarding the stability of the molecule in various states as well with respect to its influence onto the investigated dynamics (see also the next question for further details). Then, the truncated pulse method mentioned by Jonathan Underwood elsewhere might

be a very viable compromise. Overall, I conclude by fully supporting the statements of Jonathan Underwood that “there is very clearly a need to consider this problem in further detail as current understanding of what is and is not possible is currently poor”. Even the *sole* development of an understanding of the right approach would yield tremendous insight into the intricate workings of molecules.

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5. J. Küpper, D. W. Pratt, W. L. Meerts, C. Brand, J. Tatchen and M. Schmitt, *Phys. Chem. Chem. Phys.*, 2010, **12**(19), 4980–4988.
6. F. Filsinger, H. N. Chapman, G. Meijer, H. Stapelfeldt and J. Küpper, *Phys. Chem. Chem. Phys.*, 2011, **13**(6), 2076.

Jonathan Underwood commented: Jochen Küpper makes very good points about the need to find ways to measure structural dynamics during ground state (chemically relevant) processes in molecules. My main concern about using the approach presented by Küpper and colleagues for structural imaging of ground state molecules undergoing chemical change is that the presence of the adiabatically applied alignment laser field could well be expected to change the observed chemical processes (beyond that expected from steric/geometric considerations). The laser field intensities used for producing molecular axis alignment typically lie in the region of 10^{12} W cm $^{-2}$ corresponding to an electric field of about 10^9 V m $^{-1}$. Such high fields strengths are expected to significantly distort the electronic structure of even ground state molecules – indeed the quantum mechanical expression for the molecular polarizability expresses the field-induced mixing of electronic states. As such, I am concerned that the alignment laser field would directly influence the distribution of charge in the molecules under study, affecting their reactivity.

Jochen Küpper responded: Again, I also consider this a very insightful and important comment by Jonathan Underwood. On the quantitative detail I wish to point out that the quantum-state-selection exploited in our work^{1,2} not only allows for the selection of distinct molecular species,³ but it allows the achievement of very strong alignment (and orientation) at moderated field strengths of only 10^{10} W cm $^{-2}$.⁴ Nevertheless, while this strongly mitigates the problem, it does not solve it and one has to consider the (ac) Stark effect onto the chemical dynamics. Typical Stark energy shifts are of the order of 1 meV under the specified conditions. These shifts, clearly, could change the dynamics in a state-to-state resolved experiment where degeneracies might be lifted or induced, but it is likely not relevant in terms of the chemical energies in typical reactions, which could be of the order of 1 eV. Therefore, our current investigations of statistically averaged (*versus* quantum states) dynamics should not be much influenced.

At the same time, one should also relate these fields ($\sim 1 \times 10^8$ V m $^{-1}$) to the “internal fields” from the reactions partners, which are of the order of the atomic

unit of the electric field ($\sim 5 \times 10^{11} \text{ V m}^{-1}$). Again, this is a difference of more than three orders of magnitude and it will take some effort and progress to be able to see these effects. In how far the more subtle effects hinted at, such as charge redistributions, *e.g.*, through the Stark-coupling of orbitals or states, are a different issue, is related to the discussion of the validity of the Born–Oppenheimer approximation, elsewhere in this Discussion.

Overall, the presence of an alignment laser will surely have an influence on the molecular system under investigation. Personally, I would be very happy to see this effect as it would show that we had finally arrived at the observation of intricate details of the dynamics of molecular systems. Under these circumstances we would first learn what this influence is and, subsequently, would surely be able to mitigate or avoid it altogether. The presented X-ray diffraction probe^{1,2,5} should, at least, be a largely unaffected probe to observe these effects –once it is a routine tool with sufficient temporal and spatial resolution.

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