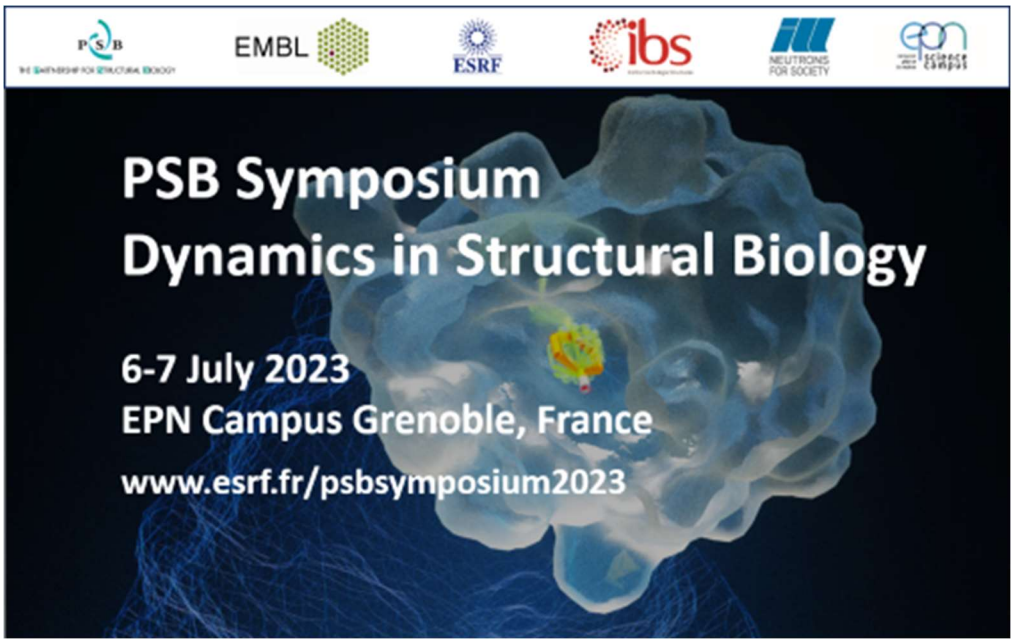

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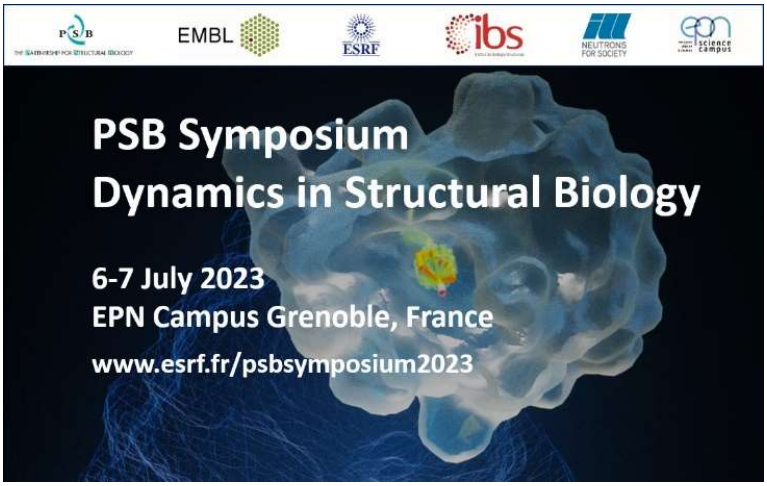


DAY 1 - Thursday 6th July 2023

09.15-13.45	Registration	
09:40-12:15	Tours of PSB facilities - (only if you are already registered)	
11.30-13:30	Lunch in the canteen	
13:45	**Kristina Djinovic Carugo** EMBL Grenoble, FR	*Conference Welcome*
14:00-18:25	**Session 1: Dynamics across membranes** Chairs: Matthew Bowler (EMBL) & Malene Jensen (IBS)	
14:00	**Jonas Ries** EMBL Heidelberg, DE	*Super-resolution microscopy for dynamic structural cell biology*
14:35	**Cécile Breyton** Institut de Biologie Structurale, FR	*Structural basis of bacteriophage T5 host recognition, infection trigger and E. coli cell wall perforation*
14:50	**Luca Costa** Centre de Biologie Structurale, FR	*Correlative AFM and Fluorescence Microscopy/Spectroscopy for Structural Biology*
15:25	**Andrea Florres Ibarra** Jagiellonian University, PL	*Fixed-target time-resolved serial femtosecond crystallography on a Light-Oxygen-Voltage 1 (LOV1) domain*
15:40	**Alexey Amunts** Stockholm University, SE *(EMBO Young Investigator Lecture)*	*How genes become machines in mitochondria*
16:15	coffee break	
16:45	**Enrica Bordignon** University of Geneva, CH	*Effects of pH and point mutations on the kinetics of the prepore-to-pore transition of a Tc toxin*
17:20	**Guillaume Tresset** Université Paris Sud, FR	*Energetics and kinetic assembly pathways of Hepatitis B virus in the presence of antivirals*
17:35	**Sophie Korn** Goethe University, DE	*The SARS-CoV-2 Nucleocapsid NTD prefers transient RNA stemloops*
17:50	**Gabriel Waksman** Birkbeck - University College London, UK	*Structural and Molecular Biology of Conjugative Transfer Systems in Gram-negative bacteria*
18:25	*end of the session*	
18:30 - 21:30	Cocktail reception Buffet dinner + poster session	

DAY 2 - Friday 7th July 2023

09:00-12:30	**Session 2: Observing molecular dynamics in biological systems** Chairs: Daniele de Sanctis (ESRF) & Malene Jensen (IBS)	
09:00	**Toshio Ando** Kanazawa University, JP	*Directly watching protein molecules in dynamic action by high-speed AFM*
09:35	**Jörg Standfuss** Paul Scherrer Institute, CH	*Watching the release of a photopharmacological drug from tubulin using time-resolved serial crystallography*
09:50	**Karin Kornmueller** Medical University of Graz, AT	*The quest for order in a dynamic system: Low density lipoprotein structure and dynamics in the light of different scattering techniques*
10:25	Coffee break + group picture	
11:05	**Marius Schmidt** University of Wisconsin-Milwaukee, US	*Time-resolved serial crystallography at XFELs*
11:40	**Stéphanie Hutin** CEA Grenoble, FR	*Order in the disordered - Molecular determinants of phase separation and its physiological role in plant temperature sensing*
11:55	**Hashim Al-Hashimi** Columbia University, US	*RNA conformational propensities determine cellular activity*
12:30-13:30	Buffet lunch – EMBL/ILL Hallway	

13:30-18:45	**Session 3: *Dynamics of biological processes*** Chairs: Judith Peters (UGA/ILL) & Matthew Bowler (EMBL)	
13:30	**Irina Gutsche** Institut de Biologie Structurale, FR	*Tricks, turns and secrets of the Respiratory Syncytial Virus nucleocapsid from a cryo-EM standpoint*
14:05	**Jaime Martin-Benito Romero** Centro Nacional Biotecnologia, ES	High-Speed Atomic Force Microscopy Reveals Real-Time Dynamics of Influenza A Virus Transcription
14:20	**Fabio Sterpone** Institut de Biologie Physico-Chimique, FR	*Multiscale modelling of biological systems: crowding and aggregation processes*
14:55	**Nikolai Klena** Human Technopole, IT	Correlating protein localization in cryo-electron tomograms using ultrastructural expansion microscopy
15:10	**Héloisa Bordallo** University of Copenhagen, DK	*The same, but different: Structural and dynamical interplay in bio-interfaces tracked using scattering*
15:45	Coffee break	
16:15	**Hugo Van Ingen** Utrecht University, NL	*NMR-driven integrative studies of nucleosome assembly & remodelling*
16:50	**Amit Meir** MRC-University of Glasgow, UK	*Cryo-EM studies of the SARS-CoV-2 nucleocapsid-gRNA interaction reveal its dynamic assembly mechanism*
17:05	**Antonino Caliò** European Synchrotron Radiation Facility, FR	*Unravelling the mechanisms of adaptation to high pressure in proteins*

17:20	**Ilme Schlichting** Max Planck Institute for Medical Research, DE	*Insight into the workings of fatty acid photodecarboxylase*
17:55	**Stephen Cusack** EMBL Grenoble, FR	*Closing lecture - Conformational dynamics of the influenza transcription/replication machine*
18:45	*Closing remarks*	
	Transfer from EPN to Conference Dinner venue by tram and cable car	
20:00	Cocktail and Conference Dinner at Le Restaurant O^2 Téléphérique	

PSB Symposium

Dynamics in Structural Biology

Invited speakers

Hashim Al-Hashimi
Columbia University, US
website

Karin Kornmueller
Medical University of Graz, AT
website

Alexey Amunts
Stockholm University, SE
website

Jonas Ries
European Molecular Biology Laboratory, DE
website

Toshio Ando
Kanazawa University, JP
website

Ilme Schlichting
Max Planck Institute for Medical Research, DE
website

Héloisa Bordallo
University of Copenhagen, DK
website

Marius Schmidt
University of Wisconsin-Milwaukee, US
website

Enrica Bordignon
University of Geneva, CH
website

Fabio Sterpone
Institut de Biologie Physico-Chimique, FR
website

Luca Costa
Centre de Biologie Structurale, FR
website

Hugo Van Ingen
Utrecht University, NL
website

Stephen Cusack
European Molecular Biology Laboratory, FR
website

Gabriel Waksman
Birkbeck - University College London, UK
website

Irina Gutsche
Institut de Biologie Structurale, FR
website

Abstracts of the selected short talks

(by order of presentation)

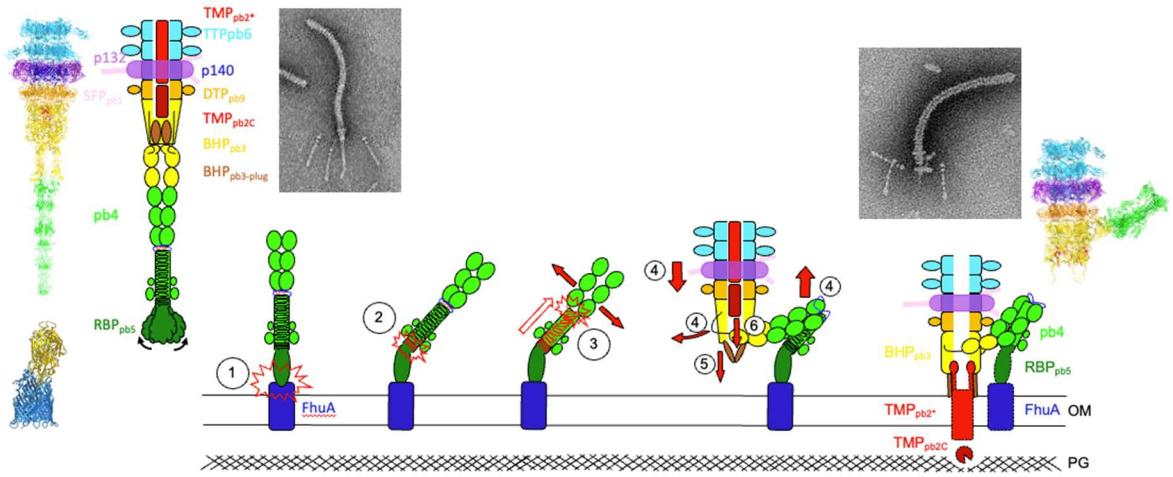
Structural basis of bacteriophage T5 host recognition, infection trigger and *E. coli* cell wall perforation

R. Linares, S. Degroux, G. Effantin, C.A. Arnaud, E. Boeri-Erba, G. Schoehn and C. Breyton

Institut de Biologie Structurale, Grenoble, France. **Cecile.Breyton@ibs.fr**

The vast majority of bacteriophages (phages) - bacterial viruses - present a tail that allows host recognition, cell wall perforation and safe channelling of the viral DNA from the capsid to the cytoplasm of the infected bacterium. The majority of tailed phages bears a long flexible tail (*Siphoviridae*) at the distal end of which a tip complex, often called baseplate, harbours one or more Receptor Binding Protein·s (RBPs). Interaction between the RBPs and the host surface triggers cell wall perforation and DNA ejection, but little is known on these mechanisms for *Siphoviridae* [1]. We determined the structure of siphophage T5 tip at high resolution, by electron cryo-microscopy, before and after interaction with its *E. coli* receptor FhuA reconstituted into nanodisc, allowing to trace most of its constituting proteins [2]. It brings out the dramatic conformational changes underwent by T5 tip upon infection, *i.e.* bending of the central fibre on the side, opening of the tail tube and its anchoring to the membrane, and formation of a transmembrane channel. We also determined the structure of T5 RBP in complex with FhuA [3]. Altogether, these new structures allow us to propose a mechanism of host recognition and activation of viral entry for *Siphoviridae*, including the cascade of events that commits T5 to cell wall perforation and DNA ejection (Figure 1).

1.



2. Figure 1: Proposed mechanism for phage T5 trigger for infection, from host recognition to membrane anchoring and outermembrane channel formation (from [1 and 2]).

References

1. Linares R, Arnaud CA, Degroux S, Schoehn G, Breyton C. (2020) Structure, function and assembly of the long, flexible tail of siphophages. *Curr Opin Virol*. 45:34-42. doi: 10.1016/j.coviro.2020.06.010.
2. Linares R, Arnaud CA, Effantin G, Darnault C, Epalle NH, Boeri Erba E, Schoehn G, Breyton C Structural basis of bacteriophage T5 infection trigger and E. coli cell wall perforation. (2023) *Sci Adv.* Mar 24;9(12):eade9674. doi: 10.1126/sciadv.ade9674.
3. Degroux S, Effantin G, Linares R, Schoehn G, Breyton C. (2023) Deciphering Bacteriophage T5 Host Recognition Mechanism and Infection Trigger. *J Virol*. Feb 13:e0158422. doi: 10.1128/jvi.01584-22.

Fixed-target time-resolved serial femtosecond crystallography on a Light-Oxygen-Voltage 1 (LOV1) domain

G. Gotthard[1]*, A. Flores-Ibarra[2]*, M. Carrillo[1], B. Olasz[2], M. Pachota[2], M. Kepa[1], J.H. Beale[1], P. Nogly[2]

[1]Division of Biology and Chemistry, Laboratory for Biomolecular Research, Paul Scherrer Institute, Villigen
[2]Dioscuri Centre for Structural Dynamics of Receptors, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow
*Authors contributed equally. Presenter's email: **andrea.ibarra@uj.edu.pl**

X-ray free electron lasers have allowed for the rapid growth of time-resolved structural experiments. We set to perform experiments that made use of a Light-Oxygen-Voltage 1 (LOV1) domain, for pump-probing and X-ray data collection at the Cristallina endstation of the SwissFEL using their novel MISP-chips produced from opaque materials.

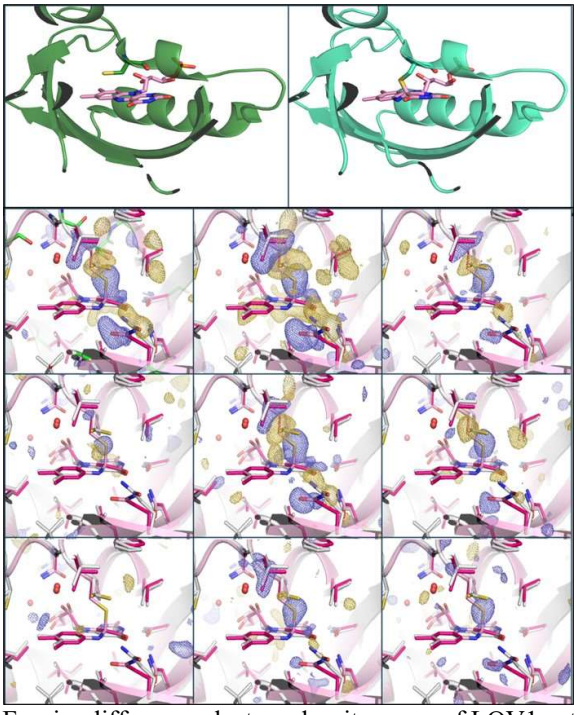


Figure. Fourier difference electron density maps of LOV1 activation.

When the LOV domain is activated, it forms a photoadduct between the FMN cofactor and a neighboring Cys. By pump-probing the protein, we expected to find activation (light state) from the wells of the chips that were laser-probed and a lack of activation (dark state) from the wells that were not. Our results show that this experiment is already a proof of principle, albeit the need for further fine-tuning, for pump-probed measurements using fixed-target delivery while preserving the difference between the light and dark states when using the opaque MISP-chips.

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Energetics and kinetic assembly pathways of Hepatitis B virus in the presence of antivirals

K. Kra,[1,2] S. Li,[3] L. Gargowitsch,[1] J. Degrouard,[1] J. Pérez,[4] R. Zandi,[3] S. Bressanelli,[2] , G. Tresset[1]

[1]Université Paris-Saclay, CNRS, Laboratoire de Physique des Solides, 91405 Orsay (France), [2]Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198 Gif-sur-Yvette (France), [3]Department of Physics and Astronomy, University of California, Riverside (United States), [4]SOLEIL Synchrotron, 91192 Gif-sur-Yvette (France)

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Capsid assembly modulators (CAMs) are antiviral molecules that disturb the formation of icosahedral viral capsids, in particular, those of the Hepatitis B virus (HBV) [1]. We report an integrated, physics-driven study elucidating quantitatively the effects of two classes of CAMs on HBV capsid assembly [2]. Time-resolved small-angle X-ray scattering measurements [3] revealed accelerated self-assembly processes that implied the increase of subunit binding energy from 9- up to 18-fold the thermal energy due to CAMs. Cryotransmission electron microscopy images showed that both classes induce various changes in capsid morphology: from a slight elongation, unrecognized in previous work, to a strong deformation with a capsid size more than twice as large. The observed capsid morphologies were closely reproduced in coarse-grained simulations by varying the Föppl-von-Kármán number, thus pointing out the role of CAMs in altering the capsid elastic energy. Our results illuminate the mechanisms of action of CAMs on HBV capsid assembly at high spatiotemporal resolution, and may open up perspectives on virus-derived nanocapsules with tunable morphologies.

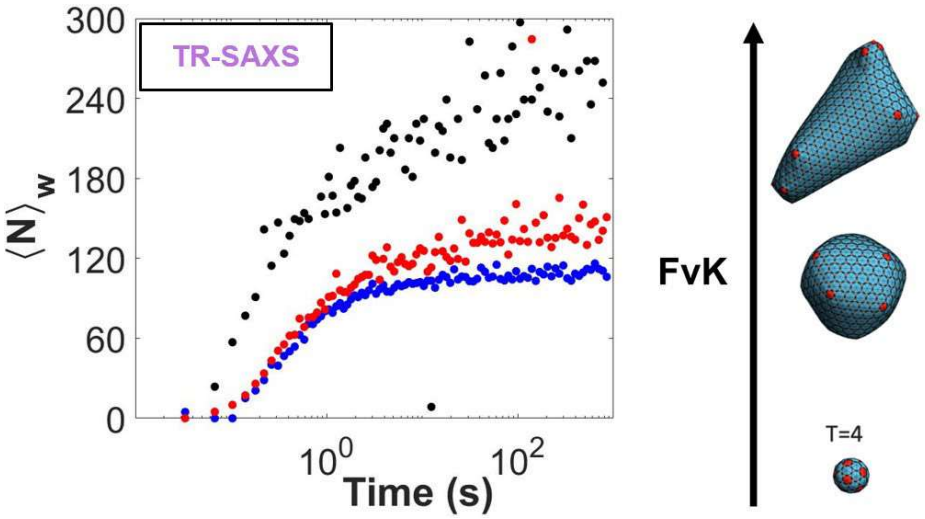

Figure 1: (Left) Mean aggregation number of self-assembling capsids versus time in the presence of CAMs inferred from TR-SAXS. (Right) Simulated coarse-grained capsids with increasing Föppl-von-Kármán (FvK) number.

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The SARS-CoV-2 Nucleocapsid NTD prefers transient RNA stemloops

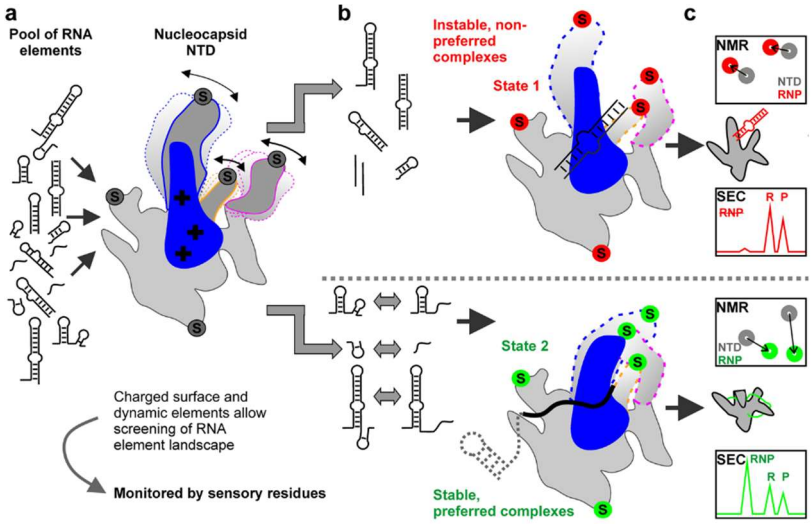
S.M. Korn[1], K. Dhamotharan[1], C.M. Jeffries[2], A. Schlundt[1]

[1]Institute for Molecular Biosciences, Goethe University, 60438 Frankfurt and [2]European Molecular Biology Laboratory Hamburg Site, c/o DESY, 22607 Hamburg, Germany, **korn@bio.uni-frankfurt.de**

The Nucleocapsid (N) of SARS-CoV-2 is the most abundant coronaviral protein and is centrally involved in viral RNA-transcription and packaging of the large genome into virus particles. N manages the enigmatic balance of bulk RNA-coating versus precise binding to designated RNA cis-regulatory elements, but how N organizes the inevitable recognition of specific motifs remains unanswered. To this end, we systematically analysed the interactions of N's N-terminal RNA-binding domain (NTD) with individual SARS-CoV-2 cis RNA elements using solution-based structural methods to allow integrating the dynamic aspects of specific RNA-protein complex formation [1].

We here for the first time unravel the NTD RNA-binding preference pattern in the natural SARS-CoV-2 genome context. NMR spectroscopy revealed categorizable chemical shift perturbation patterns, related to a combination of structured RNA elements, in which a transient stem-loop represents the preferred target site for the NTD. Together with comprehensive NMR relaxation data, structural information obtained from small-angle X-ray scattering and a broad set of biophysical data on RNA-protein complex formation, we show that the NTD's flexible loops read the intrinsic signature of preferred RNA elements for selective and stable complex formation within the large pool of available motifs.

We suggest the NTD is responsible for specific RNA recognition by SARS-CoV-2 N and targets a combination of RNA sequence and transient structure, which hints at a functional role of these RNA elements adopted to the temperature range of virus-infected tissues.



3.

4. Figure 1: a) The N NTD shows promiscuous binding to a large variety of candidate RNA elements, reasoned by its large positively charged surface as the palm of a hand. B) Dynamic fingers sense ('S') preferred RNA elements (green) and disfavor irrelevant RNAs (red). c) The fingers' sensory residues report about specific interactions, e.g. via characteristic NMR CSP signatures. Preferred complexes (RNP) reveal physical stability, as e.g. seen in co-elution during aSEC runs, as compared to non-preferably bound RNAs. Taken from [1]

References

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Watching the release of a photopharmacological drug from tubulin using time-resolved serial crystallography

M. Wranik[1], T. Weinert[1], C. Slavov[2], T. Masini[3], A. Furrer[1], N. Gaillard[1], D. Gioia[3], M. Ferrarotti[3], D. James[1], H. Glover[1], M. Carrillo[1], D. Kekilli[1], R. Stipp[1], P. Skopintsev[1], S. Brünle[1], T. Mühlethaler[1], J. Beale[1], D. Gashi[4], K. Nass[4], D. Ozerov[5], P. J.M. Johnson[4], C. Cirelli[4], C. Bacellar[4], M. Braun[2], M. Wang[4], F. Dworkowski[4], C. Milne[4], A. Cavalli[3,6], J. Wachtveitl[2], M. O. Steinmetz[1,7], J. Standfuss[1]

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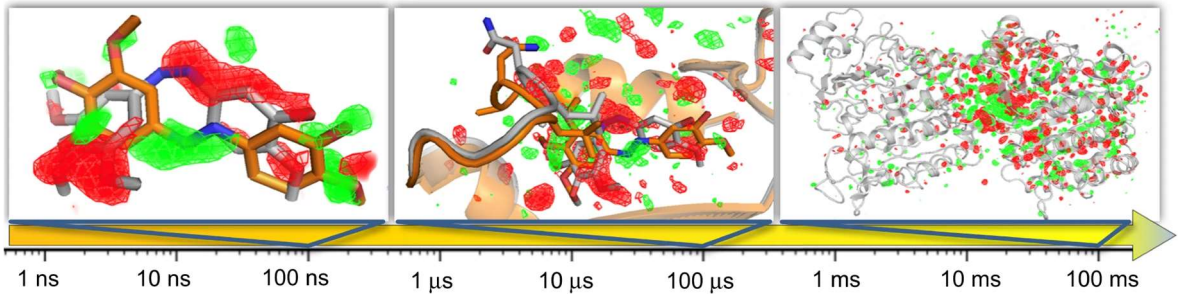
The binding and release of ligands from their protein targets is central to fundamental biological processes as well as to drug discovery. Photopharmacology introduces chemical triggers that allow the changing of ligand affinities and thus biological activity by light. Insight into the molecular mechanisms of photopharmacology is largely missing because the relevant transitions during the light-triggered reaction cannot be resolved by conventional structural biology. Using time-resolved serial crystallography at the Swiss Light Source and the Swiss X-ray Free Electron Laser, we captured the release of the anti-cancer compound azo-combretastatin A4 and the resulting conformational changes in tubulin. Nine structural snapshots logarithmically spaced in time from 1 ns to 100 ms complemented by molecular dynamics simulations and time-resolved spectroscopy show how *cis*-to-*trans* isomerization of the azobenzene bond leads to a switch in ligand affinity, opening of an exit channel, and collapse of the binding pocket upon ligand release. The resulting global backbone rearrangements are related to the action mechanism of microtubule-destabilizing drugs.



5. Figure 1: Time-resolved snapshots of azo-CA4 release from tubulin. The panels from left to right show isomorphous difference maps (negative (red) and positive (green) densities at 3 σ) obtained at 100 ns with changes centered on the ligand, 100 µs with changes centered on the binding pocket, and 100 ms with conformational changes propagating throughout the tubulin alpha subunit. The structure in the given time range (colored in orange) is compared to that of the previous time range (colored in gray).

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Order in the disordered - Molecular determinants of phase separation and its physiological role in plant temperature sensing

S. Hutin[1], A. Miranova[1], A. Popov[2], JR. Kumita[3], P.Wigge[4], Y. Stahl[5], L. Costa[6], M. Tully[2], C. Zubieta[1]

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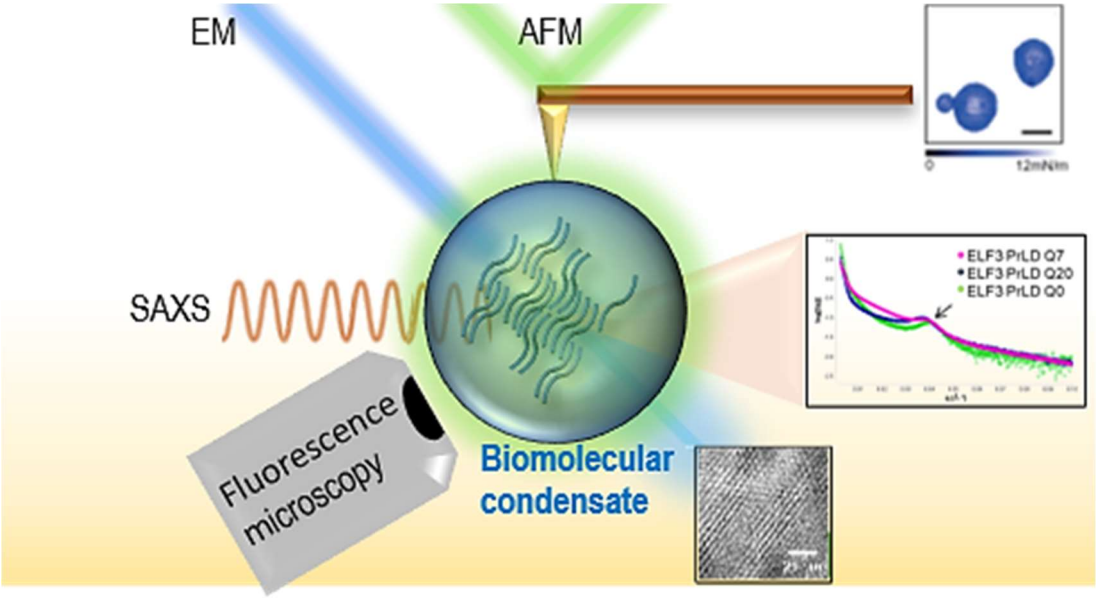
[2] European Synchrotron Radiation Facility, Structural Biology Group, 71 Avenue des Martyrs, 38000 Grenoble, France.
[3]Dept. of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, UK, CB2 1PD.
[4]Leibniz-Institut für Gemüse- und Zierpflanzenbau, Großbeeren, Germany and Institute of Biochemistry and Biology, University of Potsdam, Potsdam, Germany.
[5]Institute for Developmental Genetics and Cluster of Excellence on Plant Sciences, Heinrich-Heine University, Universitätsstraße 1, D-40225 Düsseldorf, Germany.
[6] Centre de Biologie Structurale (CBS), Univ Montpellier, CNRS, INSERM, 29 Rue des Navacelles, 34090 Montpellier, France

Liquid-liquid phase separation (LLPS) is an important mechanism enabling the dynamic compartmentalization of biological macromolecules, such as proteins and nucleic acids, as a function of the cellular environment. *In vitro*, phase separation is sensitive to pH, ionic strength and, perhaps most notably, temperature [1]–[3]. This suggests that, *in vivo*, LLPS may act as a wide-ranging sensing mechanism, allowing a fine-tuned response to the changing physicochemical environment. Recent studies, by myself and others, established that protein-mediated LLPS serves as an environmental sensing mechanism in response to external stimuli including temperature, water and nutrient availability, pathogen challenges and stress conditions, demonstrating that it is likely an important mechanism for directly sensing biotic and abiotic variables [4]–[11]. However, the underlying molecular mechanisms, the different physicochemical variables that trigger LLPS *in vitro* and *in vivo*, and its physiological role are the subject of ongoing debate due to the challenges in studying this complex biophysical phenomenon. We have identified two environmental sensing proteins involved in temperature sensing and response via LLPS; EARLY FLOWERING 3 (ELF3) and PLETHORA 3 (PLT3) [4], [12]. Both proteins contain largely unstructured prion-like domains (PrLD) that act as drivers of LLPS *in vivo* and *in vitro*. The PrLDs contain poly-glutamine (polyQ) tracts, whose length, in case of ELF3, varies across natural Arabidopsis accessions. In roots and *in vitro*, ELF3 and PLT3 interact directly and co-localize to condensates.
We use a combination of biochemical, biophysical and structural techniques to investigate the dilute and condensed phases of the ELF3 PrLD with varying polyQ lengths and its interactions with the PLT3 PrLD. We demonstrate that the dilute phase of the ELF3 PrLD forms a monodisperse higher order oligomer that does not depend on the presence of the polyQ sequence. This species undergoes LLPS in a pH and temperature-sensitive manner and the polyQ region of the protein tunes the initial stages of phase separation [13]. We show that ELF3 PrLD can recruit PLT3 to the condensates. Interestingly, while the PLT3 PrLD is highly dynamic in the condensates ELF3 molecules show a less dynamic behaviour. Surprisingly, the presence of PLT3 does not influence the overall biophysical properties of the condensates, which are dominated by the ELF3 PrLD. Additionally, the condensed phase rapidly undergoes aging and forms a hydrogel as shown by fluorescence and atomic force microscopies. Furthermore, we demonstrate that the ELF3 PrLD hydrogel assumes a semi-ordered structure using small angle X-ray scattering, electron microscopy and X-ray diffraction [13] (Figure 1).

These experiments demonstrate a rich structural landscape for a PrLD protein and provide a framework to analyse the structural and biophysical properties of biomolecular condensates.



6. Figure 1: Using AFM, EM, SAXS, and X-ray diffraction, we provide a new experimental pipeline to study biomolecular condensates,

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High-Speed Atomic Force Microscopy Reveals Real-Time Dynamics of Influenza A Virus Transcription

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The Influenza A virus genome is composed of eight different single-stranded viral RNA (vRNA) segments assembled into viral ribonucleoprotein complexes (vRNP) in which the RNApol binds to the 5' and 3' ends of the vRNA, while NPs cover the remaining vRNA [1]. The vRNP are the essential units for transcription and replication of the viral genome and represents the minimal transcriptional machinery of influenza viruses [2]. Recent studies support the Processive Helical Track model which suggests that the RNApol pulls the vRNA template from the NPs into its active site while it's still bound to the vRNA ends and without disassembly of the vRNP structure [3]. This mechanism involves significant restructuring of the vRNP, but the highly dynamic interactions between its components allow it to regain its initial shape after each transcription cycle, enabling it to perform several consecutive rounds of transcription. Monitoring these putative conformational changes is crucial for a better understanding of the mechanistic processes that take place during vRNA synthesis. Here, we aimed to address this challenge by following with High-Speed Atomic Force Microscopy (HS-AFM) the conformational dynamics of individual recombinant vRNP (rRNP, Figure 1A and 1B) during transcription. Our results support the Processive Helical Track model, showing reversible conformational changes in the rRNP (Figure 1C) and events of multiple consecutive rounds of such changes. In addition, we provide the first estimations of the average transcription rates of the RNApol within the rRNP complex and its modulation by NTP analogs that affect the structure of the nascent RNA.

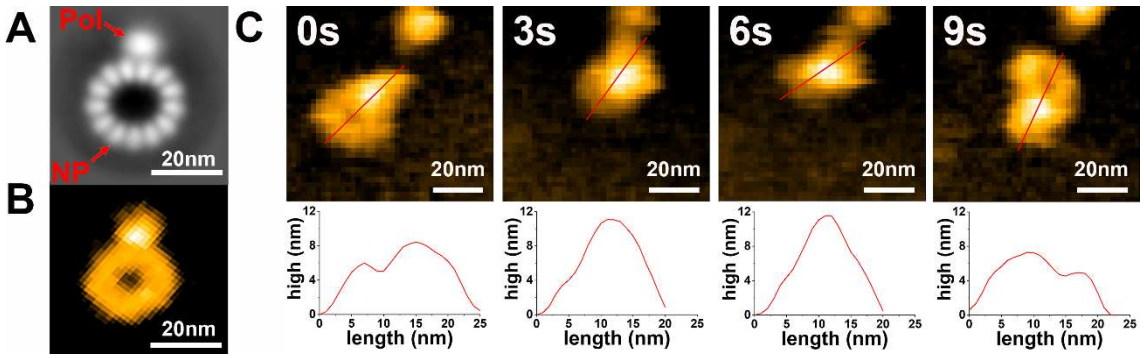

Figure 1: Conformational changes of Influenza rRNP during transcription. (A) CryoEM average image of the rRNP. (B) AFM image of the rRNP. (C) HS-AFM real time image sequence (top) and measured high profiles (bottom) of the rRNP during transcription.

Correlating protein localization in cryo-electron tomograms using ultrastructural expansion microscopy

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Cryo-electron tomography (cryo-ET) allows for the native visualization of macromolecular complexes. Although advances in sample acquisition and subtomogram averaging have pushed resolution limitations to new heights, determining the protein identity and composition of moderate-resolution cryo-ET structures remains a challenge. Correlated light and electron microscopy (CLEM) allows for the contextualization of protein fluorescence coupled with structural information; however, performing super-resolution imaging by light microscopy remains highly specialized in cryo-conditions. It is thus difficult to obtain the nanoscale-level fluorescence resolution sought after for cryoCLEM applications.

We developed a *quasi-correlative* approach to assess protein localization in densities within cryo-electron tomograms through ultrastructural expansion microscopy (U-ExM), a fluorescence microscopy technique which physically expands a biological sample embedded in a hydrogel approximately 4X, allowing for super-resolution acquisition.

Using cells distinct from cryo-ET acquisition, we performed U-ExM to correlate protein identity to structure along the *Chlamydomonas reinhardtii* basal body-flagellar apparatus, an evolutionarily conserved, microtubule-based structure with well characterized dimensions by electron microscopy, using tubulin labelling to standardize positional coordinates between tomograms and U-ExM micrographs. The correlation between U-ExM and cryo-ET allowed us to determine the localization of post-translational modifications of tubulin along the microtubule triplets, doublets, and singlets of the basal body-flagellar apparatus, as well as identifying the front-loaded position of the anterograde intraflagellar transport (IFT) train motor, kinesin-2, on these assembling polymers. Lastly, we could determine that the microtubule sleeve, a novel flagellar structural element determined by subtomogram averaging, is directly involved in flagellar abscission through the U-ExM of deflagellated *C. reinhardtii*.

We have further developed the coupling of cryo-ET and U-ExM to perform *direct* correlation between fluorescence staining and cryo-ET density by performing U-ExM on electron microscopy grids retrieved immediately following cryo-ET. To this end, we could directly image the *same* flagellar complexes, containing IFT trains, motility components, and microtubules by cryo-ET and U-ExM. The combinatorial and direct visualization of the same cellular compartment is among the first super-resolution cryoCLEM applications and offers a promising and accessible avenue for visualizing protein identity and organization in cryo-ET data.

Cryo-EM studies of the SARS-CoV-2 nucleocapsid-gRNA interaction reveal its dynamic assembly mechanism

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The coronavirus SARS-CoV-2 and its respiratory disease, Covid-19, have impacted billions of people worldwide. Understanding the factors mediating SARS-CoV-2 infection are of high priority; One of those factors is the viral ribonucleocapsid, a protein (Nucleocapsid)-RNA (SARS-CoV-2 genomic RNA, gRNA) complex. The Nucleocapsid-gRNA interaction is essential for proper packaging of the viral RNA in the virion, and without it, the gRNA molecule cannot be contained inside the virus.

So far, high-resolution structure of the full-length molecule is unavailable, due to the nucleocapsid's dynamic nature, a result of flexible regions between the N-terminus RNA binding domain and the C-terminus oligomerization domain. Here, using single-particle Cryo-EM, we revealed the ribonucleocapsid structure and assembly mechanism at low resolution, by investigating the Nucleocapsid-gRNA interaction during several steps of formation. Our observations show the ribonucleocapsid forms large rings, ~150Å diameter, that assemble into higher-order structures (HOS) of multiple rings in a capsid-like shape. These HOS form only in the presence of the gRNA; in its absence, the nucleocapsid alone does not form them. Furthermore, incubating the ribonucleocapsid complex with ribonucleases resulted with the disassembly of the HOS into individual rings and further into the nucleocapsid's primary tetramers. The vast Cryo-EM dataset allowed us to reconstitute multiple 3D classes of HOS, suggesting regions along the gRNA dictate variations in the HOS shape and assembly. We further identified potential packaging signals; the gRNA regions protected by the nucleocapsid from nuclease degradation and show their effect on the ribonucleocapsid oligomeric state. Overall, we were able to construct an assembly sequence of the ribonucleocapsid formation upon viral RNA binding.

Understanding the structure of the ribonucleoprotein complex and its assembly mechanism will lead toward the development of novel antiviral drugs, with high specificity towards SARS-CoV-2.

Unravelling the mechanisms of adaptation to high pressure in proteins

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The adaptation of proteins to high pressure is still an open debate, but understanding it could shed light on the origins of life [1], lead to a better understanding of protein dynamics, and deliver new tools to engineer pressure-resistant enzymes for biotechnological purposes. While the thermodynamic and dynamical properties of model proteins under pressure have been extensively studied [2], the evolutionary aspects of their adaptation are still unclear.
Disentangling the contributions of pressure adaptation from those of another adaptation, such as high or low temperature, is a difficult task. In fact, genomic studies could not determine a clear pattern among the order of Thermococcales.
Recent experiments by our group focused on whole cells of two closely related species (*Thermococcus barophilus,* Tba, and *Thermococcus kodakarensis,* Tko) that grow at the same optimal temperature (85°C) but differ only for the optimum pressure (400 bar for Tba, 1 bar for Tko), and they highlighted the differences in the dynamics of the two organisms' proteomes [3]. To take this investigation to the molecular level, we studied the *Phosphomannose Isomerase* and the *Ribosomal protein S24e* from the two organisms with Elastic and Quasi-elastic Incoherent Neutron Scattering, 2-D NMR Spectroscopy, X-ray crystallography and Molecular Dynamics Simulations. Our results evidence that the substitutions of amino acids enhancing pressure stability are those in the hydrophobic core, which eliminate cavities, and those on the surface, which modulate the interaction of the proteins with the surrounding water layer and give them the right flexibility to perform their function under high pressure (fig. 1). Therefore, the study of the dynamics of these proteins enabled us to gain detailed structural information, to describe their behaviour under extreme conditions and characterize their adaptation.

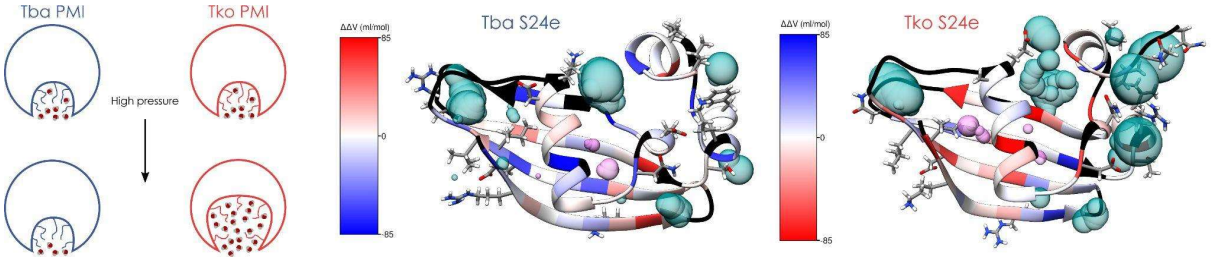

Figure 1: Left, a summary of the EINS and QENS results about the effects of high pressure on Tba PMI and Tko PMI. Center and right, residue-specific difference of denaturation volume (ΔΔV) between Tba S24e and Tko S24e as measured by 2-D NMR, represented as the colour of the ribbon. Internal cavities are represented in magenta, while solvent-accessible cavities are in cyan.

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PSB Symposium - list of posters

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P2. **Borislav Angelov -** *Time resolved X-ray diffraction study of ice formation in lipid membranes under cryogenic cooling*

P3. **Rocio Arranz -** *A Novel Monoclonal Antibody Targeting a Large Surface of the Receptor Binding Motif Shows Pan-neutralizing SARS-CoV-2 Activity Including BQ.1.1 Variant*

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P19. **Jessica Harich -** *Residue Size Dependency of the In-cage Geminate Recombination Dynamics of the Biologically Relevant Disulfide Moiety after UV-cleavage investigated by time resolved X-ray Spectroscopy*

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P53. **Jip Wulffele -** *Single Particle Tracking PALM Provides Insight into Nucleoid Remodeling in Deinococcus radiodurans*

Thriving in the heat – Lysine acetylation stabilizes the quaternary structure of a Mega-Dalton hyperthermoactive PEP-synthase

P. Albanese[1,2,$], W.Song[3], S.van Keulen[4], J. Koendjbiharie[5], F. Koh[6], B. Steigenberger[1,2], T. Vincent[7], A. Konijnenberg[6], A. J.R. Heck[1,2], S.W.M. Kengen[5], A.M.J.J. Bonvin[4], F. Forster[3], R. A. Scheltema[1,2]

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Over the evolutionary time, structural adaptations enabled proteins and enzymes to have sufficient stability and flexibility to perform the basic functions of Life under various environmental conditions. The catalytic cores of key metabolic enzymes of hyperthermophilic Archaea work at extreme temperatures, even above 100°C, similar to the conditions where the earliest life forms may have thrived.

Here, we characterize the structural dynamics of a key enzyme of the central carbon metabolism in *Pyrococcus furious* through an integrative approach. We combined structural mass spectrometry, cryo-electron microscopy, mass photometry and molecular modelling with molecular dynamics simulations to unveil the flexible organization and catalysis of the conserved phosphoenolpyruvate synthase (PPSA) in hyperthermophilic conditions. Its 24-meric assembly - weighing over 2 MDa - harbours flexible distal domains, whose proper functioning and coordination depends on widespread chemical acetylation of lysine residues. Intriguingly, this post-translational modification, along with other types of spontaneous lysine acylation, is also widespread in most of the major protein complexes of *P. furiosus*. We further speculate that these modifications may therefore have originated in the chemically favourable primordial conditions, where the metabolic reactions were not compartmentalized into organelles, and gradually became highly specialized and enzyme-driven in more distantly related mesophilic Eukaryotes.

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Time resolved X-ray diffraction study of ice formation in lipid membranes under cryogenic cooling

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Time-resolved structural studies of water crystallization in lipid membranes at cryogenic temperatures are important for understanding ice nanocrystal nucleation and can lead to a better understanding of how to postpone ice formation. We investigated 40,000-frame time-resolved synchrotron microfocus X-ray diffraction (TR-XRD) kinetics of water-to-ice crystallization in a lipid/protein/salt mesophase exposed to cryostream cooling at 100 K. The choice of monoolein/hemoglobin/salt/water membrane assemblies represented model compositions of protein-loaded lipid cubic phases (LCP), which are widely utilized for protein crystallization. The results revealed that the in situ recorded Braggs peaks, characterizing the kinetics of ice crystallization, arise from different ice crystal polymorphs, which accompany the crystallization of the lipid/protein mesophase. Under restrictions in nanoscale geometry, metastable short-living cubic ice (I_c) formed far before hexagonal ice (I_h) [1].

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A Novel Monoclonal Antibody Targeting a Large Surface of the Receptor Binding Motif Shows Pan-neutralizing SARS-CoV-2 Activity Including BQ.1.1 Variant

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During previous COVID-19 waves, therapeutic administration of monoclonal antibodies (mAbs) has been highly effective in preventing COVID-19-related hospitalization and death. However, there are currently no approved therapeutic mAbs capable of neutralizing the new variant BQ.1.1. In this multidisciplinary work, we characterize, from a functional and structural point of view, 17T2, a new mAb with high and exceptionally broad neutralization activity against pre-Omicron and Omicron SARS-CoV-2 variants, including the highly immune evasive variants BA.5 and BQ.1.1.

Cryo-electron microscopy (cryo-EM) analysis showed that 17T2 binds the Omicron BA.1 spike protein with the three RBD domains in "up" position and recognizes a large epitope overlapping with the receptor binding motif. According to its type of interaction, 17T2 is considered a class 1 antibody, as it binds to the left shoulder-neck region of the RBD. A comparison with a structurally similar neutralizing mAb (S2E12) was carried out to understand the differences in the neutralizing activity against newest Omicron sublineages which led to the detection of point mutations in key residues that may lead to a higher interaction with the RBD, emphasizing a salt bridge between the CDR 3 of 17T2 heavy chain and the RBD that stabilizes the complex.

Altogether, the results highlight the impact of small and unique structural antibody changes, in both the heavy and the light chains, on neutralizing performance. As a result, 17T2 mAb could be considered a potential candidate for future therapeutic and prophylactic interventions.

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Developing advanced models of biological membranes with hydrogenous and deuterated natural glycerophospholipids

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Cellular membranes are complex systems including hundreds of different lipid species. Their investigation often relies on simpler model systems including fewer synthetic lipid species. Glycerophospholipids (GPLs) extracted from cells are a valuable resource to produce more advanced models of biological membranes. Here we present the optimization of a method previously reported by our team for the extraction and purification of GPLs from P. pastoris. The implementation of an additional purification step by High Performance Liquid Chromatography-Evaporative Light Scattering Detector enabled a better separation of the GPLs from neutral lipids such as sterols, and also allowed the GPLs to be purified according to their different polar head classes. For this study we produced phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylglycerol (PG) mixtures. These exhibit a single composition of the polar head, i.e. PC, PS or PG, but contain acyl chains of varying lengths and unsaturation's, which was determined by Gas Chromatography- Flame Ionization Detection. The lipid mixtures were produced both in their hydrogenous and deuterated versions and used to form lipid bilayers both on solid substrates and as vesicles in solution. The supported lipid bilayers were characterized by quartz crystal microbalance with dissipation monitoring and neutron reflectometry, whereas the vesicles by small angle X-ray and neutron scattering. Our results show that despite a small difference in the acyl chain composition, the hydrogenous and deuterated extracts produced bilayers with very comparable structure, which makes them valuable to design experiments involving selective deuteration with techniques such as NMR, neutron scattering or infrared spectroscopy.

The onset of molecule-spanning dynamics in heat shock protein Hsp90

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Protein dynamics have been investigated on a wide range of time scales. Nano- and picosecond dynamics have been assigned to local fluctuations, while slower dynamics have been attributed to larger conformational changes. By investigating the heat shock protein 90 (Hsp90), we can assign molecule-spanning dynamics on the 100 ns to 200 ns time scale to dynamic modes on this time scale. To obtain a comprehensive picture of these global real-space movements (Figure 1), we apply a combination of nanosecond fluorescence correlation spectroscopy (ns-FCS), time-resolved anisotropy (TRA), neutron spin echo spectroscopy (NSE), neutron backscattering spectroscopy (NBS), small angle neutron scattering (SANS) and molecular dynamic (MD) simulations [1].

The dynamic modes of Hsp90, affected to various degrees by a co-chaperone Sba1, and its time scales depend on the conformational state of the Hsp90 dimer. Altogether, our data is best described by fast molecule-spanning dynamics, which precede larger conformational changes in Hsp90 and might be the molecular basis for allostery. Our integrative approach provides comprehensive insights into molecule-spanning dynamics on the nanosecond time scale for a multi-domain protein.

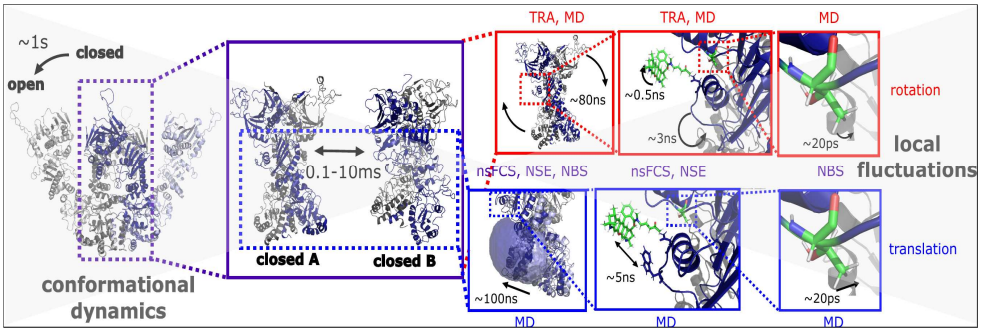

Figure 1: Representation of the molecule spanning dynamics. The different time-scales as well as the corresponding techniques used for their investigation are displayed in the different boxes [1].

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Mesh-and-collect approach for the time-resolved studies of rhodopsins

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Rhodopsins is a family of light-sensitive membrane proteins that consist of 7 transmembrane helices and contains retinal as a cofactor. Rhodopsins perform many vital functions, including light-sensing and transport of ions and hydrogens. Recently, they have received a lot of attention due to their application in optogenetics - field which uses light-sensitive proteins to restore neuron signaling with light and can potentially cure blindness and other diseases. Rhodopsins are activated by a visual light (typically at 500-560 nm), after which undergo structural changes that result in the either pumping, channeling, or sensoring activities. The knowledge of structure at each particular time point, molecular movie, is a goal of this research. MAR is a light-driven proton pump that can give good quality crystals is an ideal target for developing the time-resolved (TR) technique at the ESRF. The TR structure of this protein at this resolution can potentially have a huge impact on optogenetics.

Molecular Insights into the Dynamic Allosteric Regulation of Mycobacterial Inosine-5′-Monophosphate Dehydrogenase

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Inosine-5′-monophosphate dehydrogenase (IMPDH) is a conserved purine metabolism enzyme that is considered a promising drug target against mycobacterial infections. However, most previous biochemical and structural studies were performed with IMPDH without its regulatory CBS domain. The aim of this project is to describe the allosteric regulation of full-length IMPDH and its underlying molecular mechanism. First, we isolated full-length and ΔCBS variants of IMPDH from *Mycobacterium smegmatis* and performed a detailed *in vitro* biochemical characterisation. The impact of selected purine nucleotide compounds on IMPDH activity was tested, showing dramatic inhibitory effect of their combination at biologically relevant concentrations. Next, we utilised single particle cryo-EM analysis and successfully obtained a series of datasets of IMPDH in complex with combinations of substrates and its allosteric regulators bound in the CBS domain. The cryo-EM data, supported by HDX-MS experiments, demonstrated extensive dynamic rearrangements of the IMPDH octameric assemblies necessary for the full activity. Structural changes in the active/inhibited forms of IMPDH enabled us to propose interdomain crosstalk that leads to changes in the mobility of the catalytic core of the enzyme, and thus locking it in a rigid-inactive conformation. The described mechanism represents an example of how IMPDH, an enzyme at a crossroad of two branches of purine metabolism, can integrate several allosteric signals to fine-tune its activity. This mechanism could potentially be exploited in the design of more selective antimycobacterial IMPDH-targeting drugs.

Workflows for complex biological experiments at eBIC

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Electron Bio-imaging centre (eBIC) at Diamond Light Source (DLS) is offering ever expanding service to the user community. In addition to the standard data collections for single particle cryo-EM, cryo- electron tomography (cryo-ET) and micro- 3D crystal electron diffraction (3DED), eBIC now offers user access to an automated Focused Ion Beam (FIB) sample milling and correlative fluorescent cryo-microscopy. Access to the-state-of-the-art blot-free, rapid sample freezing Chameleon device [1] is also available, in collaboration with SPT Labtech and the Rosalind Franklin Institute. Significant evolution in the user access mode is an emerging ability to group experiments requiring various instruments into workflows. Examples include optimisation of rapid sample freezing conditions for Chameleon using sample screening on a 200 keV transmission electron microscope (TEM), followed by data collection from the best samples on a 300 keV TEM; FIB milling combined with cryo-ET data collections [2] and on-the-fly tomogram reconstruction; correlative fluorescent and electron microscopy, which can also include FIB milling with in-chamber fluorescence; semi-automated 3DED data collection and analysis with optional FIB milling of larger crystals [3] or correlative fluorescent microscopy for crystal finding. Experiments at eBIC can be complemented by the use of other DLS facilities like B24 beamline for structured illumination microscopy/soft X-ray microscopy [4], VMXm micro/nanofocus X-ray crystallography beamline, or services offered at the Membrane Protein Lab (MPL) [5], and Research Complex at Harwell (RCaH). Continuous instrument upgrades at eBIC have seen up to 20-fold increase in the speed of data collections over the last 5 years. Increased throughput allows data collections on samples prepared at different conditions within a single session [6]. Automated data analysis pipelines offer real time feedback to users about the quality of their samples and the data being acquired [7]. Almost every type of experiment can be conducted either with on-site visit or fully remote [8]. Online systems are available for sample shipping and automated tracking, remote instrument access and data analysis (ISPyB/SynchWeb). Scientists at eBIC continuously work to further expand the variety and quality of the experiments and workflows offered on the user programme. Significant effort is invested in communication with the community and user training to enable exploitation of the eBIC beamlines to their full potential.

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End-to-end accurate and high-throughput modeling of antibody-antigen complexes

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Antibodies are produced by the immune system in response to infection or vaccination. While sequencing of the individual antibody repertoire is becoming routine, identifying the antigens they recognize requires costly low-throughput experiments. Even when the antigen is known, epitope mapping is still challenging: experimental approaches are low-throughput and computational ones are not sufficiently accurate [1].

Recently, AlphaFold2 [2] has revolutionized structural biology by predicting highly accurate protein structures and complexes. However, it relies on evolutionary information that is not available for antibody-antigen interactions. Traditional computational epitope mapping is based on structure modeling (folding) of the antibodies [3], followed by docking the predicted structure to the corresponding antigen [4]. The problem with this sequential approach is that the folding step does not consider the structural changes of the antibody upon antigen binding and the docking step is inaccurate because the antibody is considered rigid.

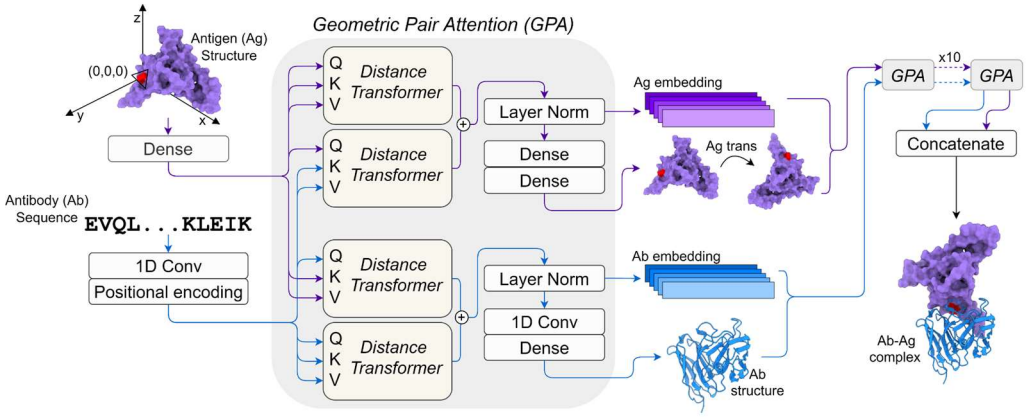

Figure 1: The Neural Network architecture.

Here, we develop a deep learning end-to-end model, that given an antibody sequence and its corresponding antigen structure can simultaneously perform folding and docking tasks. The model produces the 3D coordinates of the entire antibody-antigen (Ab-Ag) or nanobody-antigen (Nb-Ag) complex, including the side chains. An accurate model is detected among the Top-5 and Top-100 predictions for 28% and 70% of the test set, respectively. In addition to mining antibody repertoires, such a method can have the potential to be used in antibody-based drug design, as well as in the vaccine design.

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Structural study of M. kandleri malate deshydrogenase: emergence of allosteric capacity using evolution-guided punctual mutations

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Allostery is a mode of regulation of the activity of a protein by which the binding of an effector molecule at one site modifies the conditions of binding of another molecule, at another distant site of the same protein. Many extensive studies have shown that allosteric regulation relies on protein dynamics. In this model, the allosteric capacity of an enzyme depends on the reorganization of the conformational landscape of the protein, induced by various events such as interactions with ligands and physicochemical variations of the environment.

Protein superfamilies encompassing both allosteric and non-allosteric enzymes offer great opportunities to reveal the molecular mechanisms of allosteric regulation. This is particularly the case for the malate (MalDH) and lactate dehydrogenase (LDH) superfamilies, the study of which has allowed to identify the different evolutionary steps that led to the emergence of allosteric regulation in this superfamily. Our study focuses mainly on the M Kandleri malate dehydrogenase and the introduction of essential mutations allowing the enrichment of conformational substates and the emergence of allosteric properties.

Xtrapol8: automatic elucidation of low-occupancy intermediate states in crystallographic studies

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Keywords: Time-resolved crystallography; Fourier difference density maps; Extrapolated structure factors

The ultimate goal of structural biology is to link structure to function but this connection remains difficult to make when only static structural models are available. Accordingly, the structural characterization of reaction intermediates is of high interest and pursued by an increasing group of structural biologists. A big leap forward was taken with the advent of timeresolved serial crystallography at XFELs and synchrotrons, which enables researchers to follow the structural evolution of the crystalline structure after a specific trigger. However, a major limitation remains that the occupancy of the intermediate state has to be large enough to become visible in the electron density map. This is generally not the case, with triggered crystals existing as mixtures of initial, intermediate and final state(s). Fortunately, data processing strategies exist that can extract the intermediate state signal. Indeed, differences between the triggered and untriggered data can be visualized in Fourier difference ($F_{obs,triggered}$-$F_{obs,untriggered}$) electron density maps. Furthermore, extrapolated structure factor amplitudes (ESFAs)[1] can be calculated that solely describe the intermediate state and can be used in crystallographic structure refinement.

Here we will present Xtrapol8, a program that makes these approaches accessible to a wide audience of structural biologists, from well-experienced crystallographers to newcomers in the field. Briefly, Xtrapol8 calculates Bayesian-weighted[2-3] Fourier difference maps, estimates the occupancy of the intermediate state(s) in the crystals, and generates extrapolated structure factor amplitudes (Fig. 1). With the possibility to launch Xtrapol8 via the command line or graphical user interface, and to control various parameters of which defaults are carefully chosen, the program is highly adapted to the user's expertise. We anticipate that it will ease and accelerate the handling of time-resolved structural data, and thereby the understanding of molecular processes underlying function in a variety of proteins.

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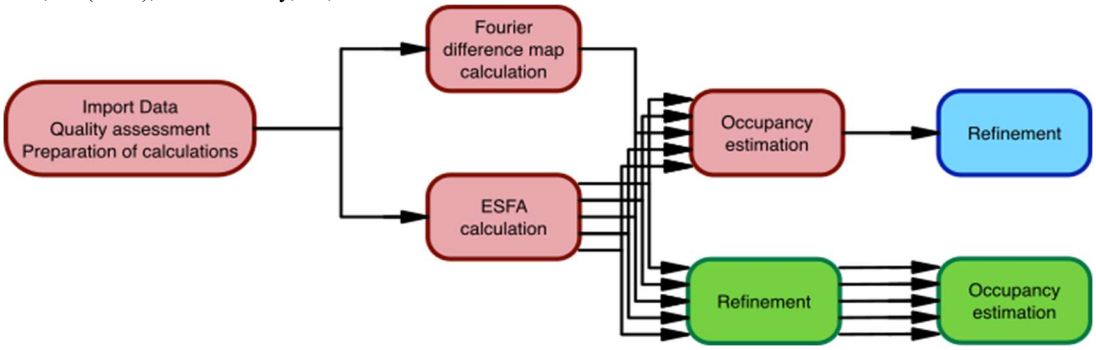

Fig. 1. The main steps followed by Xtrapol8, colored according to usage (red: main steps, blue: fast-and-furious mode, green: calmand-curious mode).

Deciphering the role of Dual Leucine Zipper Kinase in the c-Jun N-terminal kinase cell signalling pathway

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Activation of the pro-apoptotic c-Jun N-terminal kinase (JNK) cell signalling pathway requires the signal to be transmitted from initiators, which bind to the scaffold protein POSH (Plenty Of SH3s), to several downstream kinases [1]. The Dual Leucine Zipper Kinase (DLK) has been identified as a critical player in this process, being able to bind both POSH and the downstream scaffold protein JIP1 [1,2]. DLK contains a 380-amino acid intrinsically disordered regulatory tail believed to harbour interaction sites for several of the SH3 domains of POSH. Here, we obtain a full structural characterization of the regulatory tail of DLK, and we study the interaction with the SH3 domains of POSH by NMR spectroscopy. Using titration experiments and nuclear relaxation rates to delineate the binding site, we demonstrate that the interaction does not involve the classical proline-rich motifs, but that the second and third SH3 domain of POSH recognize a pre-formed helical molecular recognition element in DLK [3]. In our efforts to reveal the structural basis for this interaction, we have obtained diffracting crystals of the second SH3 domain of POSH, which likely displays a non-canonical SH3 fold with an additional C-terminal helix crucial for maintaining the protein fold. We compare our experimental data with AlphaFold2 predictions of the DLK-POSH interaction illustrating the challenges associated with predicting structures of complexes involving intrinsically disordered proteins and underlining the necessity of acquiring experimental data in order to understand the regulatory mechanisms associated with this class of proteins.

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Light atoms identification and location by anomalous scattering

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More than a third of all known proteins bind metal ions [1]. Metal ions play key roles in a broad range of cellular processes, they are involved in protein structure stability and catalysis; with traditional examples of zinc fingers in transcription factors and iron in haemoglobin. Therefore, identifying metal ion-binding sites is important for understanding the biological functions of proteins and further helps in designing potent therapeutics.
Experimental and computational methods have been developed to identify or predict metal ion ligand-binding residues. However, experimentally identifying and locating metal ions, such as calcium and potassium in protein structures can be challenging. The unique wavelength range of the macromolecular crystallography beamline I23 at Diamond Light Source [2] allows identification and location of metal ions and lighter atoms of biological relevance (Ca, K, S, P and Cl) using X-ray anomalous scattering in crystal structure analysis.
In a typical experiment, anomalous datasets are collected at two wavelengths, above and below the ion or element absorption edge, and then processed to calculate phased anomalous Fourier difference maps. The difference in anomalous peak heights between these two datasets allows the direct identification and visualisation of the ion in the protein structure. We successfully used this method in different projects to experimentally map ions in crystal structures and some examples will be discussed.

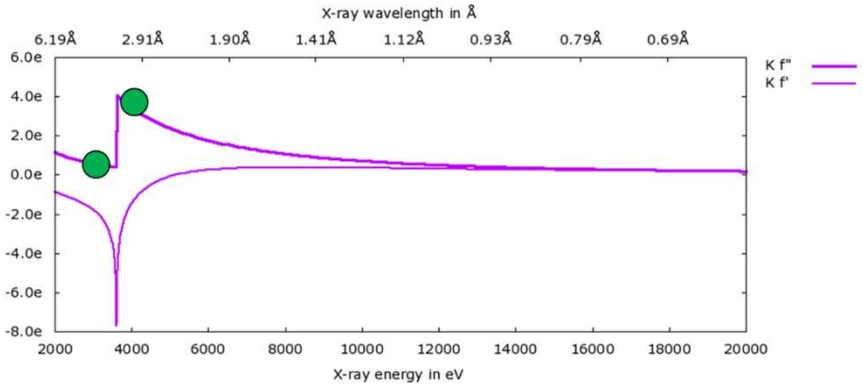

Figure 1: Variation of coefficients f' and f'' with X-ray wavelength (or energy) for potassium.

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Slow protein dynamics probed by time-resolved oscillation crystallography at room temperature

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The development of serial crystallography over the last decade at XFELs and synchrotrons has produced a renaissance in room-temperature macromolecular crystallography (RT-MX), and fostered many technical and methodological breakthroughs designed to study phenomena occurring in proteins on the picosecond-to-second timescale. However, there are components of protein dynamics that occur in much slower regimes, of which the study could readily benefit from state-of-the-art RT-MX. Here, the room-temperature structural study of the relaxation of a reaction intermediate at a synchrotron, exploiting a handful of single crystals, is described. The intermediate in question is formed in microseconds during the photoreaction of the LOV2 domain of phototropin 2 from Arabidopsis thaliana, which then decays in minutes. This work monitored its relaxation in the dark using a fast-readout EIGER X 4M detector to record several complete oscillation X-ray diffraction datasets, each of 1.2 s total exposure time, at different time points in the relaxation process. Coupled with *in crystallo* UV-Vis absorption spectroscopy, this RT-MX approach allowed the authors to follow the relaxation of the photoadduct, a thio-ether covalent bond between the chromophore and a cysteine residue. Unexpectedly, the return of the chromophore to its spectroscopic ground state is followed by medium-scale protein rearrangements that trigger a crystal phase transition and hinder the full recovery of the structural ground state of the protein. In addition to suggesting a hitherto unexpected role of a conserved tryptophan residue in the regulation of the photocycle of LOV2, this work [1] provides a basis for performing routine time-resolved protein crystallography experiments at synchrotrons for phenomena occurring on the second-to-hour timescale.

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Hunting crystals like an eagle: *In-situ* data collection offers a wide field of view before focusing on the promising target.

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Eagles have an outstanding field of view, spanning 270°, and their visual acuity is 2.5 times greater than that of humans. They can rapidly scan large landscapes and focus on even the smallest, fast-moving objects. These skills can also be beneficial in the field of macromolecular crystallography. Efficient exploration of crystallisation diversity is crucial in finding the best conditions for diffraction experiments and for dynamic structural studies it is therefore optimal to screen around experimental conditions close to the final ones (*i.e.*: room temperature).

In-situ data collection plays a crucial role in this process, acting as the "eagle eye" that helps discover important details. The *in-situ* approach can efficiently characterise samples at room temperature. It allows each step, from initial screening to structure determination, to be performed directly on the crystallisation plate.

In-situ experiments enable the easy and rapid determination of key parameters such as diffraction quality, maximum resolution, cell parameters, and space group. These parameters are essential for optimising subsequent steps before conducting time-resolved experiments.

Furthermore, with the development of serial approaches (such as SSX), structure determination can be a by-product of crystal screening. *In-situ* experiments can also allow for the exploration of photoactivity efficiency in time-resolved approaches that rely on laser illumination.

To offer the best of *in-situ* capabilities, we have designed a comprehensive integrated workflow on the ESRF beamline MASSIF-1. As pioneers in single crystal automation, we provide a wide range of experiments, starting from *in-situ* crystal screening to structure determination. Our approach combines robotics and algorithms for automated decision making, offering diverse experiments beyond crystal characterization. The workflow integrates *in-situ* data collection, automated harvesting, and both room-temperature and cryo-temperature data collection. It is flexible and suitable for all projects, particularly the most challenging ones.

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Dual sensor diguanylate cyclases

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The ability to adapt to environmental changes is an integral property of organisms across all kingdoms of life. To this end, receptors capable of perceiving different signals and featuring the ability to forward these to various effectors are employed by nature.
The receptor proteins studied in this work feature two input domains, that respond to different types of signals. An N-terminal receiver domain responds to phosphorylation and is connected via a long linker to a LOV domain, which reacts to blue light via adduct formation with its FMN cofactor. Finally, a C-terminal GGDEF domain acts as the effector domain since it provides diguanylate cyclase activity (DGC) required for the formation of the bacterial second messenger c-di-GMP. The level of this second messenger typically influences lifestyle decisions in bacteria and regulates the transition between motile and sessile forms. We are investigating the *in vitro* properties of these enzymes and want to unravel the process of signal integration and transduction on a molecular level. An integrative structural biology approach is being followed, combining functional assays with X-ray crystallography and Hydrogen-Deuterium exchange coupled to mass spectrometry (HDX-MS).
Investigating several naturally occurring homologs with a Rec-LOV-GGDEF architecture [Figure 1], we have shown that the activation of the LOV domain leads to upregulation of DGC activity. The fold-change in activity varies between homologs and is typically in the range of 2- to 8-fold. Pseudophosphorylation of the receiver domain using BeF_3^- has not shown a significant increase or even subtle inhibition of enzymatic activity. Therefore, the effect of small molecule phosphodonors is currently being tested. Efforts toward solving the structure of one homolog are currently in progress but are complicated by the inherent dynamics of the multi-domain protein.

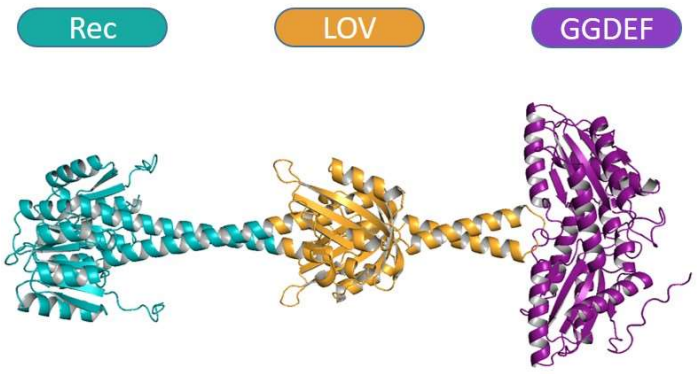

Figure 1: AlphaFold2 model of a dual sensor diguanylate cyclase.

submitted by Maximilian Fuchs

Nanobodies allosterically modulate the GTPase activity of the Parkinson's disease related LRRK2 homolog and aid its cryo-EM characterisation

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Mutations in the gene coding for the LRRK2 protein lead to Parkinson's disease (PD). LRRK2 is a so-called Roco protein, composed of multiple domains including a RocCOR supradomain bearing GTPase activity and a kinase domain. Common PD mutations decrease the GTPase activity and increase the kinase activity. To study certain detailed mechanistic aspects of LRRK2 we also use a homologous Roco protein from the bacterium *C. tepidum* Roco (CtRoco), which displays a simpler architecture lacking the kinase domain. Using biophysical and biochemical approaches we discovered that the RocCOR domain regulates transitions between monomer-dimer states through its GTPase cycle [1]. We found that one PD-related mutation changes this equilibrium towards a dimer, thus blocking the protein in an inactive state. Conformation-specific nanobodies (Nbs) against CtRoco were generated, and we identified one Nb (Nb_{Roco1}) that modulates the cycle of the protein. This Nb reverts the effect on the mutation and restores the normal monomer-dimer equilibrium and GTPase activity in an allosteric way (Figure 1) [2]. Structural studies to obtain detailed insights in the exact mechanism by which these Nbs induce the shift in dimer-monomer equilibrium are currently ongoing. Nbs were used to stabilize the active conformations for structural studies and a cryo-EM structure of the active conformation bound to two Nbs is presented. The structural analysis reveals the allosteric action of these two Nbs. Together these studies provide new mechanistic understanding of Roco proteins and how they are affected by PD mutations, and potential routes for therapeutic intervention are put forward.

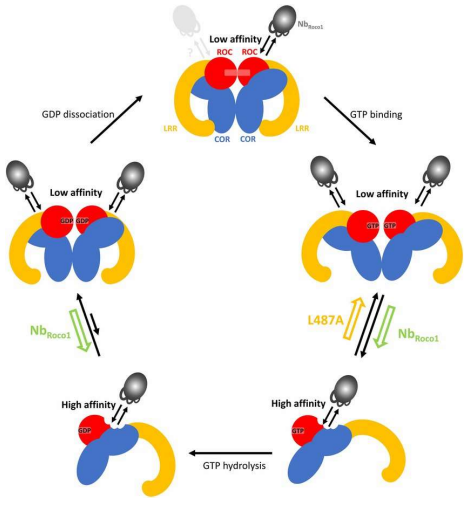

Figure 1: Proposed mechanism of action of Nb_{Roco} on CtRoco mutant.

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MAGEA4 alters the auto-ubiquitination activity of Rad18 to enhance trans-lesion synthesis

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MAGE-A4 is a cancer-testis antigen (CTA) that primarily expresses in testis in healthy adults but aberrantly gets overexpressed in many human cancers. MAGEA4 belongs to the Type-I melanoma antigens (MAGE) family which have been shown to act as oncogenic and tumor markers for various cancers [1]. In its cancer-specific role, MAGE-A4 interacts with, and stabilises, the RING E3 ligase Rad18 [2]. RAD18, along with the E2 RAD6, plays a key role in the DNA damage response through the mono-ubiquitination of the DNA sliding clamp PCNA upon the encountering of DNA damage during DNA replication, promoting a polymerase switching event and allowing replication to continue in an error-prone manner, without the risk of detrimental double stranded DNA breaks, in a process known as trans-lesion synthesis (TLS) [3]. MAGEA4 mediated RAD18 stabilisation could result in TLS and error-prone DNA synthesis in the absence of DNA damage, potentially favouring tumor evolution. However, the precise mode of the interaction between Rad18 and MAGE-A4 and mechanism of aberrant TLS activation in cancer cells remained unknown. Here, we employed Alphafold and NMR to find that the Rad6-binding domain (RBD) of Rad18 interacts with a groove in the c-terminal winged-helix domain of MAGE-A4. Mutations disrupting the interaction between MAGE-A4 and Rad18 compromise MAGE-A4-induced Rad18 stability. Additionally, we show that MAGEA4 binds to the RBD of RAD18 with a 50-fold greater affinity than RAD6. Finally, we found that MAGE-A4 stabilizes Rad18 by displacing Rad6 from near the ubiquitination sites of Rad18 thereby blocking the degradative autoubiquitination of Rad18. Our results shed light on the mechanism of activation of Rad18 by MAGE-A4 and pave the way for potential therapeutic targeting of this cancer-specific interaction.

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Residue Size Dependency of the In-cage Geminate Recombination Dynamics of the Biologically Relevant Disulfide Moiety after UVcleavage investigated by time resolved X-ray Spectroscopy

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The tertiary structure of proteins is stabilized by disulfide bonds formed from two spatially adjacent L-cysteinyl residues. But these disulfide bridges are prone to UV radiation damage with potentially adverse effects to the stability of a protein's tertiary structure. We demonstrated the use of time resolved X-ray spectroscopy (TRXAS) - uniquely useful for the identification of photoproducts with high chemical specificity due to probing the sulfur core-level transitions - at the sulfur K edge to observe the UV photochemistry of small organosulfur compounds in nonpolar solvent environments [2-3]. From these models we moved to the natural amino acid dimer L-Cystine [1] and the tripeptide Glutathione (GSSG) in aqueous solution to understand the photochemistry under physiological conditions with increasing chain length. In the latest step of this bottom-up approach, we have first exciting insights into the UV-photochemistry of the disulfide bridges within the protein hen egg white Lysozyme (Fig.1).

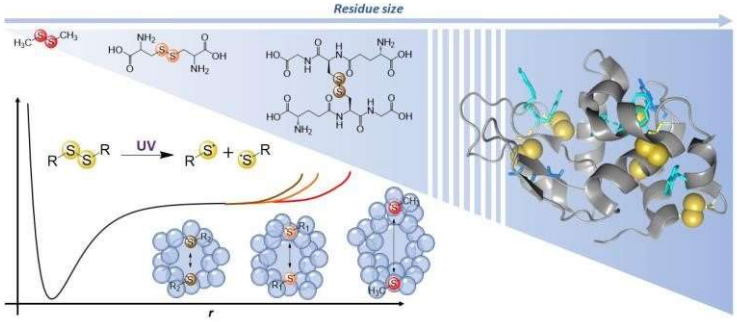

Figure 1: Top: Aliphatic disulfides with increasing residue size: dimethyl disulfide (DMDS, red S atoms), Lcystine (orange S atoms), glutathione disulfide (brown S atoms) and the protein Lysozyme, which has four disulfide bonds. Bottom: Breaking of the disulfide bond using UV light. Schematic on the influence of residue size on photoproduct expulsion and recombination dynamics within the solvent cage.

We find that upon UV irradiation, apliphatic disulfides immediately undergo S-S bond cleavage, leading to the formation of two identical thiyl radicals, followed by fast geminate recombination indicating a very effective recombination process for thiyl radicals to the ground state. This process is only possible in condensed phases and its speed increases with chain length. Our results show that L-Cystine already captures the essence of the ultrafast photochemistry of the disulfide bridge, but that the size of the residue adjacent to the disulfide bonds has a strong influence on the immediate recombination dynamics of the photoproducts.

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Human HSP90 a contortionist drug target: Modulation of its energy landscape upon ligand binding

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HSP90 is a major chaperone required for folding of various client proteins that has been reported as a therapeutic target for cancer [1]. This chaperone is a highly flexible protein with structural variability, particularly in its ATP-lid domain that covers the nucleotide/drug binding site, as shown by over 300 crystallographic structures of the HSP90 N-terminal domain. We used NMR to characterize the ATP-binding domain of the human protein HSP90α in solution [2]. We demonstrated that the ATP-binding domain of HSP90 samples different conformations on the millisecond time scale, and we were able to elucidate the structures of the major state and a minor state. While the major state is found with the ATP-lid in an open conformation as predicted by previously determined X-ray structures of N-HSP90, we established that the ATP-lid also samples a closed conformation up to 30 Å away from the major state [3]. Using NMR CPMG relaxation dispersion experiments, we kinetically and thermodynamically characterized the exchange between the different conformations sampled by the N-terminal domain of HSP90 and investigated how resorcinol derivatives modulate the energy landscape of HSP90. We demonstrated that most resorcinol ligands bind to pre-existing conformations. Interestingly, a few resorcinol derivatives, differing only by one substituent, are able to induce the formation of a new helix in the ATP-lid major state conformation and have a residence time on target increased by two orders of magnitude [4]. Although these ligands stabilize a new conformation in the ground state, we have also shown that they slow down the conformational exchange.

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Structure modeling and specificity of peptide-MHC class I interactions using geometric deep learning

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Major Histocompatibility Complex (MHC) plays a major role in the adaptive immune response by recognizing foreign proteins through binding to their peptides. In humans alone there are several hundred different MHC alleles, where each allele binds a specific subset of peptides. The peptide-MHC complex on a cell surface is identified by a T-cell receptor (TCR) and this binding invokes an immune response. Therefore, predicting the binding specificity of peptide-MHC pairs is necessary for understanding the immune recognition mechanism. Here, we develop an end-to-end novel deep learning model, MHCfold, that consists of structure and specificity prediction modules for simultaneous modeling of peptide-MHC class I (pMHCI) complexes and prediction of their specificity based on their modeled structure. MHCfold produces highly accurate structures of pMHCI complexes with mean Cα RMSD of 0.98Å and 1.50Å for the MHC α chain and the peptide, respectively. The binding specificity is also predicted with high accuracy (mean AUC of 0.94). Furthermore, the structure modeling component is orders of magnitudes faster than state-of-the-art methods (modeling of 100,000 pMHCI pairs in four hours on a standard computer), enabling high-throughput applications for large immunopeptidomics datasets. While peptide-MHC specificity can be accurately predicted from the sequence alone, TCR specificity prediction likely requires modeling of the 3D structures. We anticipate our model can be further used in structure-based prediction of TCR specificity.
MHCfold is available @https://github.com/dina-lab3D/MHCfold

Assembly mechanism of a multidomain ribozyme revealed by an integrated structural biology approach

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Self-splicing group II intron ribozymes (GIIi) are present in bacteria and in organelles of yeast and plants. GIIi sequentially assemble their structural domains into a tertiary conformation that brings distal motifs in proximity to form the conserved active site for self-splicing. Previous studies captured X-ray crystal structures of two assembly intermediates of the GIIi from *O. iheyensis*. The first structure described the architecture of the 5'-terminal domain 1 (D1) and the second structure described the fully assembled and biochemically active domains 1 to 5 (D1-5). Interestingly, when isolated (D1 structure), D1 displays a closed (inactive) conformation that would not be able to harbor the active site motifs in its core, as is instead required for catalysis (D1-5 structure). Therefore, after folding autonomously in the closed state, D1 then needs to open its core. This conformational toggling between the D1 closed and open states is likely to be triggered by the interaction between D1 and D2, D3, D4, and/or D5. However, how this assembly happens mechanistically still remains unknown.

To investigate the GIIi assembly mechanism, we employed an integrated structural biology approach encompassing cryo-EM, SAXS and MD simulations. Our data showed that GIIi assembly is not sequential, with each domain docking on the previously assembled ones in a 5'- to 3'-direction, as it was proposed earlier, according to a "first come, first fold" mechanism. Instead, GIIi follow a more intricate assembly pathway. Initially, D1 is stable in the closed state captured crystallographically, as confirmed by our new MD data. D2 then assembles onto D1 *via* a tetraloop-tetraloop receptor tertiary interaction, as shown by our new cryo-EM structures of D12. At this point, the D1 "closed" state is destabilized, enabling D1 to explore previously-uncharacterized discrete "very-open" conformations, in which the D1 core is much wider than in the catalytically-folded state. These novel conformations are maintained even after addition of further domains, as shows by our new cryo-EM structures of D1-3 and D1-4. Curiously, D3 does not assemble onto D1 until D4 is present, though D4 is unstructured. This late assembly of D3 may be a strategy necessary for the intron to exclude D4 from its core, because D4 harbors a very large and flexible open reading frame which would otherwise risk the disruption of the pre-folded D1 and impair active site formation. Only upon synthesis of D5, which contributes the active site motifs, does the ribozyme get locked into its fully assembled enzymatically active conformation.

Based on our 4 new cryo-EM structures and multi-microsecond MD simulations, we thus propose a new "open-door" mechanism for GIIi multidomain assembly, whereby D1 transits from a closed conformation unable to harbor the active site (resembling a closed door) to a dynamic wide-open conformation forming a cavity significantly larger than the active site (resembling a wide-open door), and only locks into its final state when the active site motifs from D5 are in place. For these transitions, the contribution of D2, D3, and D4, which we visualize at high-resolution, in solution and *in silico* is crucial and precisely regulated by specific and evolutionary-conserved long-range tertiary interactions. By determining this novel RNA assembly mechanism, we present the first near-atomic resolution movie of a large multidomain RNA in the process of acquiring its functionally active conformation.

Roodmus: A framework for benchmarking heterogeneous reconstruction methods in cryo-EM single particle analysis

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Single particle cryo-EM data sets are assumed to be representative of the different molecular conformations accessible under the experimental conditions prior to vitrification. If true, they carry information about conformational flexibility. Traditionally, most of this conformational heterogeneity is filtered out through 2D and 3D classification such that a reconstruction of a single state can be produced at high resolution. Recently, there has been increasing interest to exploit the rich source of information on conformational heterogeneity and this has led to a surge of new methods developed to retrieve a continuum of structural states from the observed particle images. Methods such as cryoDRGN [1] or cryoSPARC's flexible refinement [2] rely on encoding particles into a continuous latent space that provides an abstract representation of the heterogeneity contained in the data.

It can be unclear what type of heterogeneity is extracted from the data by the model and more importantly, whether the latent space embedding is representative of the true structural dynamics present in the sample.

To aid in the development and analysis of heterogeneous reconstruction methods, we have created a benchmarking tool called Roodmus. Our package takes as input a molecular dynamics simulation from which a representative ensemble of structural states is sampled. Cryo-EM micrographs containing the sampled particle conformations are then simulated using Parakeet [3], a forward model based on the multislice algorithm. Knowledge of the ground truth allows evaluation of heterogenous reconstruction algorithms as to their ability to sort conformations based on physically plausible trajectories.

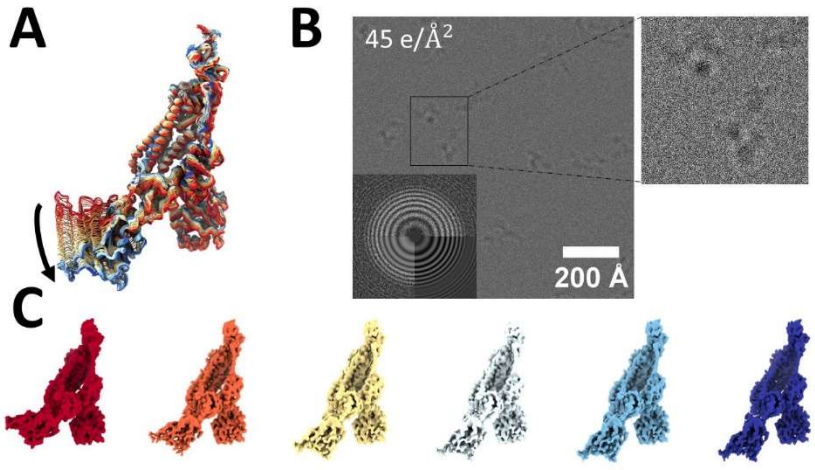

Figure 1: **A** an MD trajectory visualized as an ensemble. **B** Individual states are simulated as molecules in a micrograph. **C** Heterogeneous reconstruction recovers states from the ensemble

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The structure of the MKK6/p38α interaction reveals the details of MAPK specificity and activation

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The MAP kinases (MAPKs) are central components of cellular signalling but the molecular details of the activation of a MAPK by its upstream MAP2K remain unknown.

Through a multidisciplinary approach we generated the first model of the interaction between the MAPK p38α and its activating MAP2K MKK6, two important members of the family involved in the inflammatory response. Combining a 4 Å cryo-EM structure with HDX-MS, MD simulations and *in cellulo* experiments, we shed light onto the dynamic multi-step phosphorylation mechanism leading to the MAPK activation, revealing new catalytically relevant interactions, and showing that MAP2K disordered N-termini determine pathway specificity.

We also discovered a novel phosphorylation mechanism involving ADP: the dual specificity MAP2Ks are able to phosphorylate and activate their substrate MAPK using the β-phosphate of ADP. This alternative route of phosphorylation could confer an adaptive advantage to stress in case of ATP deprivation to maintain the signalling pathway.

Our work captures, for the first time, a fundamental step of cell signalling: a kinase phosphorylating its downstream target kinase; and exposes a non-canonical phosphorylation mechanism.

Towards automation of serial crystallography using a multi-reservoir high viscosity extruder

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Instrumentation development is crucial in overcoming technical challenges when solving fundamental scientific questions. This cannot be overstated, in the advent of the 4th generation synchrotrons as well as X-ray free electron lasers (XFELs), where new sample manipulation and delivery techniques including liquid jets, high-viscosity extrusion (HVE) injectors, fixed target supports and acoustic 'drop-on-demand' systems [1-3] are necessary to utilise the full power of these X-ray sources in the field of structural biology.

As one of our latest developments, I will describe the multi-injector device [4], shown below, which constitutes an important step towards automation and efficient sample delivery system for XFEL beamlines and potentially for the 4th generation synchrotrons. Compared to a standard single extruder, sample exchange time was halved and the workload of users was greatly reduced. In-built temperature control of samples facilitated optimal extrusion and offered a path forward for the observation of temperature-dependent structural changes. After commissioning the device with lysozyme crystals, we collected time-resolved data using crystals of a membrane-bound, light-driven sodium pump. Static data were also collected from the soluble protein tubulin that was soaked with a series of small molecule drugs. Using these data, we identified low occupancy (as little as 30%) ligands using a minimal amount of data from a serial crystallography experiment, a result that could be exploited for structure-based drug design.

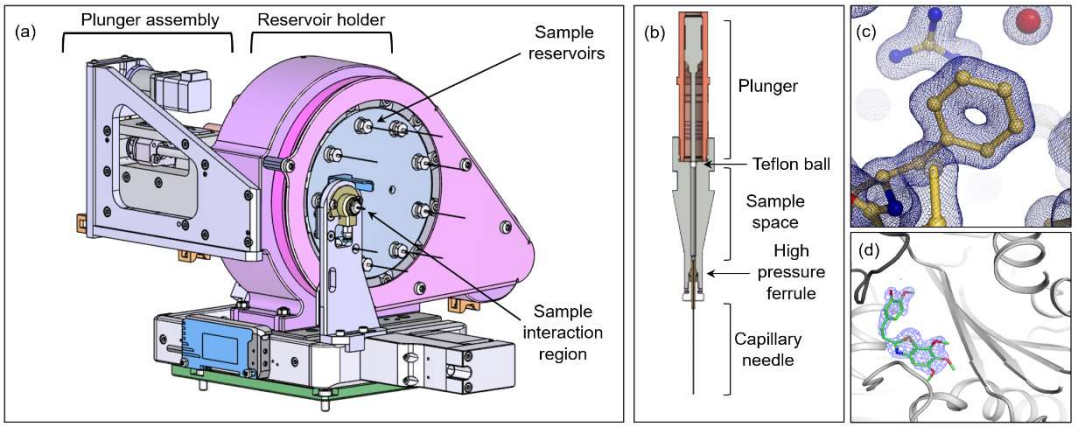

Figure 1: (a) Multi-reservoir high viscosity extruder. The temperature-controlled rotating drum contains slots for nine individual sample reservoirs. (b) Single 130 µL sample reservoir with supporting components. (c) High resolution map (1.43 Å, $2F_o$-F_c at 1.5σ) of lysozyme focused on PHE34 in lipidic cubic phase (LCP). (d) High resolution map (1.65Å, $2F_o$-F_c at 1.5σ) of α-β tubulin bound SBTubA4 for *in vivo* photo control of microtubule dynamic.

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Visualizing the multi-step folding trajectory of an intrinsically disordered scaffold protein upon binding to the small GTPase Rac1

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Activation of the pro-apoptotic c-Jun N-terminal kinase (JNK) cell signalling pathway is initiated by binding of the small GTPase Rac1 to the intrinsically disordered scaffold protein POSH (Plenty Of SH3s).[1-3] Here we identify a novel recognition mode for Rac1 binding to a non-canonical CRIB motif in the intrinsically disordered region of POSH. Using NMR nuclear relaxation rates to delineate the precise binding site, we demonstrate that the interaction involves two molecular recognition elements (MRE1 and MRE2) covering an impressive 55 amino acids of POSH. Using high-throughput crystallization screening, CrystalDirect harvesting and automated crystal diffraction at the MASSIF-1 beamline at the ESRF, Grenoble, we obtained the crystal structure of the POSH-Rac1 complex at 1.2 Å resolution showing complete folding of both MREs of POSH upon binding to Rac1. Using an extensive set of chemical exchange saturation transfer (CEST) NMR experiments, we map the kinetic details of the folding trajectory of POSH upon binding to Rac1. We show that the interaction initially proceeds through binding and instantaneous folding of MRE1 followed by a reversible folding event of MRE2 on the seconds timescale on the surface of Rac1. Extending our approach to the oncogenic splice variant Rac1b, which displays 100-fold lower affinity for POSH, we were able to attribute this affinity difference to a reduction in the association rate between the GTPase and POSH supporting the hypothesis that Rac1b is unable to stabilise its binding-competent closed-switch conformation.[4] Taken together, our work identifies a novel recognition mode by Rac1 and has implications for understanding how Rac1b becomes defective in downstream signalling pathways and contributes to tumorigenesis in multiple human cancers.

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Changes in GlpG dynamics upon inhibitor binding go far beyond the active site

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GlpG is a transmembrane serine protease from *E. coli* catalysing the cleavage of intramembrane protein substrates. While there are inhibitors and substrates available binding to it, the native substrate, the mechanism of recognition, and the full cleavage process are not yet fully understood. Among others, TM5 and loop 5 have been suggested to orchestrate substrate binding [1,2]. After optimizing several expression and purification conditions, we use selected methyl groups of GlpG in different positions around TM5 to study chemical shift perturbations and fast ms motion comparing GlpG in apo- and inhibitor-bound state via solution NMR spectroscopy. Our data indicate that upon inhibitor binding, changes in the conformational ensemble are observed up to around 18 Å away from the active site, suggesting long-range modulation of the conformations around the putative substrate entrance by the occupied active site.

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Single-molecule FRET study of conformational dynamics of CRABP1 under denaturing conditions in crowded environments

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The cellular retinoic acid binding protein 1 (CRABP1) is a transport protein composed primarily of a β-barrel scaffold into which retinoic acid as a hydrophobic ligand enters for binding.
It plays a crucial role in the regulation of retinoic acid-dependent gene transcription through the cellular transport of retinoic acid to the nucleus [1]. Furthermore, CRABP1 can prolong the cell cycle as it activates extracellular signal-regulated kinases 1 and 2, which are important components of the cell cycle process [2].

In this work, the conformational dynamics of CRABP1 under denaturing conditions in the absence and presence of crowding agents are studied by single-molecule FRET using a diffusion-based confocal microscope. In order to adequately probe the conformational dynamics, a FRET pair (AL488/AL647) is chosen that offers a high FRET efficiency contrast in denaturing environment. Urea is used to ensure chemical denaturation of CRABP1. To mimic the dense cellular environment around CRABP1, PEG2000 and PEG4000 are used as crowding agents.

CRABP1 is first studied under native conditions in the absence of crowding agents to investigate the folded state, which is characterized by a high fret efficiency with no visible conformational dynamics in the corresponding FRET histogram.
Upon stepwise increase in urea concentration, conformational dynamics between the folded and the denatured states are observed, indicated by a shift to lower FRET efficiencies. The urea titration series also allows the determination of the transition mid-concentration (C_m) at which the folded and denatured states are equally occupied.
At the urea concentration C_m, the influence of crowding agents on the conformational exchange is studied, which is expressed in a reduced FRET contrast and a population shift towards the folded state, indicating a restricted movement of CRABP1 in the denatured state on the one hand and stabilization of the folded state on the other.
The kinetics of the underlying dynamics at C_m in presence and absence of crowding agents are revealed by photon distribution analysis, suggesting a two-state dynamic exchange on the time scale of hundreds of microseconds.

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Towards molecular understanding of the UbiJ-UbiK$_2$ protein complex by multiscale molecular modelling

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Ubiquinone (UQ) is a redox-active prenol localized in the membranes [1] and conserved eukaryotes and many proteobacteria. UQ is composed of two main part, a redox active aromatic group forming a polar head and a hydrophobic polyisoprenoid tail. In *Escherichia coli*, the UQ biosynthesis is performed via a cytosolic complex, called the Ubi metabolon [2]. This latter is composed of 5 enzymes (methyltransferases UbiG and E, hydroxylases UbiI, H and F) and 2 structural proteins (UbiJ and K). UbiJ is assumed to bind the hydrophobic tail of UQ via a domain called SCP2 located at the N-term and known to interact with the lipid bilayer, notably to enable lipid transport [3]. Moreover, experimental evidences have revealed an interaction between UbiJ and an UbiK dimer, but also between UbiK and the cellular membrane in *E. coli* [2]. Considering the biological context and the importance of UbiJ and UbiK to have a functional assembly, we investigated the molecular complex UbiJ-UbiK$_2$ using multiscale molecular modelling approaches such as Alphafold2 modelling [4], Coarse-Grained (Martini3 FF) and all-atom (CHARMM36 FF) MD simulations, and Free Energy Calculations using Umbrella Sampling method.

So, our computational study led to: (i) the identification of the key amino acid residues involved in the interaction between UbiJ-UbiK$_2$ complex and the membrane, (ii) validation of UQ binding mode into the SCP2 domain of UbiJ through free energy calculations along its release towards the membrane.

Finally, this work provides new insight of the functional role of UbiJ and UbiK for UQ biosynthesis, more specially for UQ release into the membrane. [5]

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Influence of the membrane-mimicking environment on structure and dynamics of bacterial outer membrane proteins

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Structural investigations of membrane proteins require isolation of the target molecule from its cellular environment, which may affect the protein's structure and dynamics. Outer membrane proteins (OMP) of Gram-negative bacteria may be particularly affected by the membrane mimetics as they have a peculiar native environment, notably composed by a charged and densely packed layer of lipopolysaccharides (LPS) [1].

Among the different biophysical techniques used to study membrane proteins, Magic-Angle Spinning (MAS) NMR is particularly suited to determine structure, dynamics and interactions in a large variety of environments [2]. In particular, continuous advances in MAS speeds availability and in methodological developments pushes forward the number of systems available to this technique [3].

We present here investigations of two different OMPs, the alkane transporter of *P. putida* AlkL [4] and *K. pneumoniae* OmpA [5], which illustrate the influence of different membrane mimicking strategies (detergent micelles, liposomes, outer membrane vesicles) on their molecular organization. The surrounding effects depend on the different chemical properties of the two proteins. Our data however indicate on both proteins crucial changes on the organization and dynamics of the outer loops by increasing the hydrophobic thickness, and the crowding in the extracellular region.

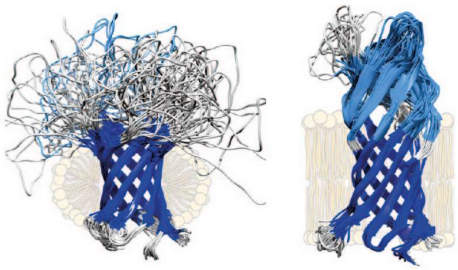

Figure 1: Structure of *P. putida* AlkL in detergent micelles (left) and in liposomes (right).

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Conformational heterogeneity in the chromophore pocket of mEos4b impacts the observed photoswitching and photoconversion properties

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Photoconvertible fluorescent proteins (PCFPs) are an essential tool for PALM-type super-resolution microscopy. Upon application of UV light, the initially green fluorescence of PCFPs irreversibly turns red. This is the result of a light-induced extension of the chromophore conjugated electron system. One of the main factors limiting the maximal achievable resolution in PALM is the limited photoconversion efficiency (PCE) of the PCFPs, ie only a fraction of the molecules typically reach the red state. In order to improve PCE, detailed mechanistic knowledge of the photoconversion process is crucial, which remains actively debated till date. Using NMR spectroscopy coupled with in-situ light-illumination and fluorescence microscopy we studied the Green-to-Red photoconversion mechanism of mEos4b, a popular PCFP. As PCFPs also show pronounced reversible photoswitch between their green state and a metastable dark state, we also studied this photoswitching mechanism. We discovered that mEos4b (as well as other PCFPs) exhibits a well-defined two-state heterogeneous population in its green form. We found marked differences in these two states in terms of their structure and local chemistry around the chromophore pocket. We studied the conformational exchange between the two states, notably under UV light. I will describe how this hitherto undetected conformational partitioning impacts photoconversion and reversible photoswitching of mEos4b.

Site-Directed Spin Labelling and Electron Paramagnetic Resonance for the study of the human flavoprotein Cytochrome P450 reductase

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Human cytochrome P450 reductase is a membrane protein located in the cytoplasmic side of the endoplasmic reticulum. It contains two binding domains for FAD and FMN, connected by a flexible region, enabling electron transfer (ET) from NADPH to cytochrome P450.[1] Two crystallographic structures were obtained for *Rattus norvegicus* showing "closed" and "open" conformations, showing different distances between the flavin cofactors.[2] Indeed, for inter-flavin ET, FAD and FMN domains must be nearby ("closed" conformation), but inter-protein ET (FMN to CYPs) requires a more "open" conformation. The understanding and characterization of domain movement associated with ET is essential and will be studied by measuring distances between two paramagnetic centres via two approaches: 1) using endogenous probes by isolating the flavin radical, and 2) by Site-Directed Spin Labelling and EPR. Classically, cysteines are labelled with specific nitroxide probes.[3] However, C566 is involved in the enzymatic activity and cannot be modified. The incorporation of non-natural amino acids and their labelling with specific nitroxides is a powerful alternative.[4] First results on human soluble CPR concerning the labelling of non-natural amino acids, as well as the trapping of flavin centres, will be presented.

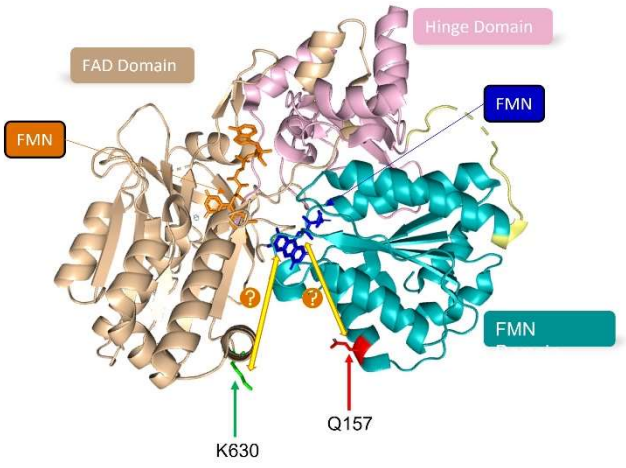

Figure 1: Structure of the soluble part of *R. norgevicus* CPR with highlights on the labelling positions.

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Disassembling a complex I assembly factor: investigating the root causes of neurodegeneration

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The Mitochondrial Complex I Assembly (MCIA) complex is essential for the biogenesis of respiratory Complex I (CI), the first enzyme in the respiratory chain, which has been linked to Alzheimer's disease (AD) pathogenesis. However, how MCIA facilitates CI assembly, and how it is linked with AD pathogenesis, is poorly understood. The MCIA complex is composed of three core proteins: ACAD9, ECSIT and NDUFAF1. Previous research found that upon ECSIT binding, ACAD9, which also plays a role in fatty acid β-oxidation (FAO), loses the FAD cofactor essential for FAO activity1. We have determined that ECSIT binding induces a major conformational change in the FAD-binding loop of ACAD9, releasing the FAD cofactor and converting ACAD9 from an FAO enzyme to a CI assembly factor2. We also provide evidence that ECSIT phosphorylation downregulates its association with ACAD9 and is reduced in neuronal cells upon exposure to amyloid-β (Aβ) oligomers. These findings advance our understanding of the MCIA complex assembly and suggest a possible role for ECSIT in the reprogramming of bioenergetic pathways linked to Aβ toxicity, a hallmark of AD.

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Deal with the crowd: protein structural dynamics monitored by nitroxide-based SDSL-EPR spectroscopy directly inside cells.

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Understanding how the intracellular medium modulates protein structural dynamics and protein-protein interactions is an intriguing but required topic scientists search to address by studying biomolecules in their native environment. As the cellular environment cannot be reproduced in vitro, investigation of biomolecules directly inside cells has attracted a growing interest in the past decade. Indeed, efforts in magnetic resonance spectroscopies have enabled important improvements in the study of structural dynamics directly in the cellular context.

Among magnetic resonances approaches, site-directed spin labeling coupled to electron paramagnetic resonance spectroscopy (SDSL-EPR) has demonstrated to be one of the powerful approaches to study structural properties of biomolecules [1]. In particular, nitroxide-based SDSL-EPR couples the benefits of high sensitivity and the lack of size constraints for the biomolecule of interest with the ability to study protein structural transitions and interactions at physiological temperature.

In this talk, we will discuss the results achieved in the investigation of the structural dynamics features of a cytosolic protein directly inside cells under conditions ensuring the preservation of cell integrity, by combining the use of nitroxide labels and EPR (cw-EPR and pulsed dipolar experiments) spectroscopy. In particular, we will focus on NarJ protein [2], for which we investigated its structural features directly in *E. coli* cells, in cell lysates and in the presence of different synthetic crowding agents. Furthermore, we were able to follow NarJ activity directly inside cells, by evaluating its ability in restoring the activity of its biological partner, the nitrate reductase. [3]

These results represent a step forward in the development of EPR-based cellular structural biology that goes beyond the feasibility and opens new perspectives to address biological questions directly inside the cell.

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Linking Sequence, Structure, Dynamics, and Function of a Pheromone Binding Protein

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The ability to respond to chemical stimuli is a fundamental behavior of all organisms. Lepidoptera males have an exquisitely sensitive olfactory system that is capable of perceiving airborne pheromone molecules released by females and responding to them over great distances. Pheromone binding proteins (PBPs) located in the antennae of the males play an important role in olfaction. They are carrier proteins that pick up volatile hydrophobic pheromone (odor) molecules at high pH and transport them across the aqueous sensillar lymph releasing at low pH near the membrane-bound olfactory neuron. Unraveling the mysteries of pheromone binding and release controlled by changes in pH is critical not only to our understanding of animal olfaction but also for any future investment on control of the olfactory behavior of deleterious insects that are voracious agricultural pests of many important crops through pheromone based integrated pest management.

Ostrinia furnacalis aka Asian Corn Borer is an invasive agricultural pest that damages not only crops of economic value but 300 other types of garden vegetables and fruits. Pheromone binding protein2 of *Ostrinia furnacalis* (OfurPBP2) plays a key role in mating and reproduction. NMR structure, thermal unfolding, and functional studies reveals that OfurPBP2 releases its ligand in a novel mechanism via partial unfolding through a molten globule state at acidic pH near the dendritic neuron. Furthermore, the apoprotein is more dynamic than the holoprotein under identical conditions. Along with structure, function, change in protein dynamics upon ligand binding (or release) will be discussed.

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Dissecting *Plasmodium falciparum* myosin A function and regulation: paving the way to develop novel antimalarial strategies

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Malaria is the most important human infectious disease. The last WHO report estimates 241 million Malaria cases and 627000 deaths worldwide in 2020. This mosquito-borne disease results from infection caused by Apicomplexan parasites of the *Plasmodium* genus. Combining X-ray crystallography, transient kinetics, molecular dynamics and parasitology, we characterized PfMyoA, an atypical class XIV myosin from *Plasmodium falciparum*. We demonstrated that the unique N-terminal extension of PfMyoA tunes the motor properties by allowing it to move at high speed (during the motile stage) or produce high force at the expense of the speed (during the stages of invasion) depending on its phosphorylation state. Our results include the atomic structure of full-length PfMyoA revealing that the two light chains PfELC and MTIP stabilize a more primed pre-power stroke state through a novel motor domain/lever arm interface. Finally, we demonstrated that both the heavy chain and the PfELC are essential for the infectivity of the parasite. These results shed light on PfMyoA as a first order target to design a new generation of selective antimalarial compounds that would target specifically PfMyoA motor activity or block the docking of the light chains to the lever arm.

Understanding the photoactive properties of the cyanobacterial Orange Carotenoid Protein and its role in photoprotection

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Cyanobacteria are considered the most abundant photosynthetic organisms on the planet and are known to thrive across a diverse range of aquatic habitats. Much of this success is attributed to their photosynthetic capabilities, and how tight regulation of these processes can be environmentally tuned across the phylum, such that different species can accommodate for varying light intensities. While photosynthetic input is to be maximised in order to increase the metabolic yield, high-light intensities can over-excite the protein-pigment light harvesting complexes which will produce harmful singlet oxygen species, greatly damaging the cell. Many groups of cyanobacteria have sought to address this balance through Non-Photochemical Quenching (NPQ) and the evolution of the Orange Carotenoid Protein (OCP).

OCP is a 35kDa reversibly photoactive protein, which utilises a keto-carotenoid as it's chromophore, which sits tethered between its two main domains [1]. In functionalising a single carotenoid, OCP can scavenge singlet oxygen produced by over-excitation, converting it back into harmless molecular oxygen. Additionally, the presence of a blue-green photon can instigate structural changes in the carotenoid, inspiring further large-scale conformational changes in the surrounding protein scaffold. This newly activated OCP can then bind to the light-harvesting protein-pigment complexes, and through NPQ, the carotenoid is able to quench excess-excitation energy into the environment as heat. Upon absorption of a blue-green photon, OCP is transformed from the conformationally closed, spectroscopically orange OCP (OCP^O), into the open, spectroscopically red, light-adapted state (OCP^R). The protein marks itself as a great candidate for use in optogenetics and in the optimisation of photosynthetic pathways, due to domain-dissociation and its role in photoprotection, respectively. However, the active yield sits at only 0.15% following photon absorption [2], rendering structural studies of the photoactivation mechanism incredibly difficult, and making future application almost infeasible.

We are employing the use of several crystallographic techniques to provide a structural basis towards the study of this unique photoactivation mechanism, and to probe the photocycle which so far is based solely on spectroscopic findings. Both static and time-resolved crystallographic findings, in combination with other biophysical techniques, foster an understanding of the protein, allowing us to make mutants with altered photophysical properties, providing key insight into the critical roles of certain residues which informs the overall sequence of events. Through comparing our structural findings relating the wild-type protein to selected mutants, we are provided with a working understanding detailing the structural dynamics seldom seen between proteins and carotenoids.

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Structural Basis of Human Melanogenic Enzymes

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The production of the pigment melanin in human requires the activity of at least three melanogenic enzymes, tyrosinase (TYR) and tyrosinase-related proteins 1 (TYRP1) and 2 (TYRP2), which regulate the type and amount of melanin produced. Despite their essential role, the catalytic mechanism and specificity are still under strong debate. The lack of structural data hampers the understanding of their molecular bases and the design of specific inhibitors to treat pigmentation disorders or melanoma[1]. Recently, the solved crystal structure of human TYRP1 challenged previous assumptions claiming it is a redox enzyme[2,3]. In view of the existence of pathological mutations for all three melanogenic enzymes, it is thus very likely that each enzyme plays a unique function in human melanogenesis[4]. Therefore, it is crucial to obtain 3D structures of all three melanogenic enzymes at atomic level as well as to identify their respective metabolites in order to improve our current knowledge on the human melanin biosynthesis pathway. With all these information, compounds of great efficiency and specificity can then be designed to treat melanogenesis disorders.

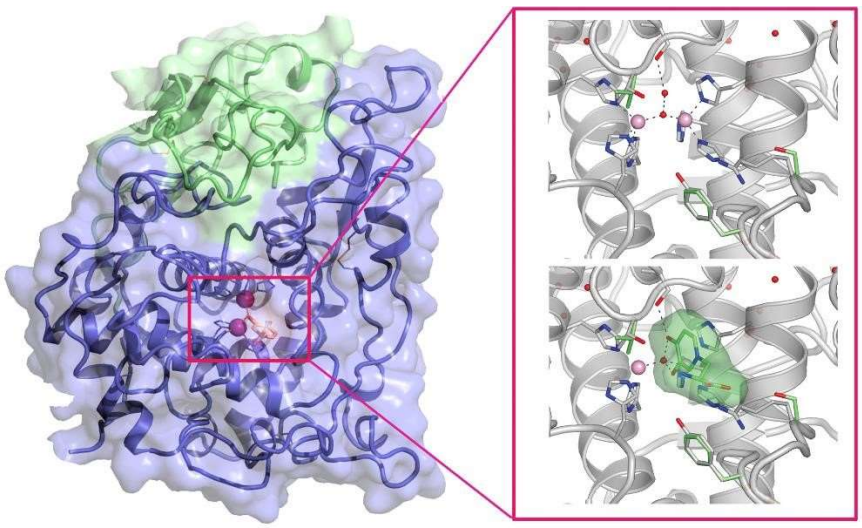

Figure. Cartoon representation of TYRP1 protein with close-up view of the active pocket. *On left, the tyrosinase-like domain and cysteine-rich domain of TYRP1 protein are coloured in blue and green respectively. The two metal ions at the active pocket are represented as magenta spheres. Close-up of the active site structure, as displayed in the inset, reveals metal-coordinating histidine residues (shown as stick models), metal bridging water molecules (red spheres) and substrate mimosine (stick model with green surface). Hydrogen-bonding interactions are also shown as black dotted lines.*

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Teichoic Acids and Peptidoglycan Synthesis at the Nanoscale

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Gram-positive bacteria such as *Streptococcus pneumoniae* possess a thick layer of peptidoglycan (PG), a 3D network of glycan chains cross-linked by short peptide chains, which maintains cell morphology and structural strength to resist osmotic pressure [1]. Anchored to this layer are teichoic acids (TA), complex anionic polymers that play important roles in morphogenesis and virulence processes [2]. While both components are essential for a healthy cell wall, TA are still poorly understood compared to PG, and little is known about their assembly during cell growth. Past studies have showed that in *S. pneumoniae*, PG and TA are seemingly synthesized concomitantly in a mid-cell annular region of only 100 nm width-wise; however, since the resolution of conventional fluorescence microscopy is limited to 250 nm due to the diffraction of light, it remains a great challenge to investigate the area of active cell wall synthesis in details [3] [4]. To elucidate the relative dynamics of TA insertion into the PG layer, we employ a labelling method using chemically-modified metabolites probes, and observe labelled cells with the single-molecule localization microscopy technique called dSTORM (direct STochastic Optical Reconstruction Microscopy), which permits a resolution of about 30 nm. Labelled regions in wild-type and mutant cells are then analysed and compared at the nanoscale, demonstrating interesting variations as the cell cycle progresses.

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Impact of self and distinct pair correlations on the pico-second dynamics probed by neutron scattering in protein powders

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Neutron scattering is a key technique to investigate the average motions of hydrogen nuclei in a protein solution or in a hydrated powder, relying on the huge incoherent scattering cross-section of hydrogen compared to all other nuclei. However, the advent of polarisation analysis to investigate protein dynamics challenges the assumption that hydrogens' self-dynamics overtake all other contributions, namely self and collective dynamics of heavy atoms and deuterated water. We investigate to what extent the neutron probe is sensitive to the hydration layer of a protein hydrated in D_2O, even at the low hydration level of $h = 0.4$. We also question the use of per-deuterated proteins as a tool to study proteins' internal collective motions [1].

Our study compares both a protonated and a per-deuterated Green Fluorescent Protein powder hydrated in D_2O, combining polarised neutron diffraction studies and polarised and non-polarised Quasi Elastic Neutron Scattering (QENS) experiments at the pico-second scale, investigating momentum transfers from $Q = 0.5\text{Å}^{-1}$ to $Q = 2A^{-1}$.

Our calculations of the diffraction pattern of the GFP protein, compared to static structure factors obtained with polarisation analysis, unveil the very fast exchange of hydration water as well as the effective coherent contribution to scattering at this Q scale [2]. Moreover, for the first time, coherent dynamics corresponding to information on self and pair correlations are investigated in proteins. Their influence on fitted dynamical parameters, compared toconventional QENS studies, is quantified. We especially point out the impact of the solvent's self-dynamics, contaminating the signal through a non-negligible coherent contribution.

This study also highlights the sensitivity of a minimal model based on Fractional BrownianDynamics [3] to understand such a complex system, besides its appropriate physical meaning [4]. Three parameters are enough to catch scale dependent time and heterogeneity information, and convey valuable meaning for both incoherent and coherent scattering functions without requiring the assumption of a model. This is of particular relevance to investigate coherent dynamics since theoretical models are still poorly available.

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Targeting the splicing site with small molecule modulators

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Splicing is a key step in RNA maturation and its defects cause cancer and genetic diseases [1,2]. Indeed, abnormal spliced mRNA isoforms have been found in different types of cancer and have been proven to play a crucial role in tumour progression and invasion, as well as in cell proliferation, apoptosis, angiogenesis and metabolism. Thus, splicing modulation is emerging as a viable path to treat genetic diseases and cancers. But so far, splicing modulation has mostly targeted spliceosomal proteins through gene-unspecific, often toxic approaches [3,4]. Thus, we still need better targets and drugs to achieve personalized, lower-toxicity treatments. In our work, we are using a bacterial ancestor of the human spliceosome to develop and characterize small molecules that target the splicing active site. Our model system is a self-splicing ribozyme, called the group II intron, whose active site structure and reaction chemistry are highly similar to those of the catalytically active states of the spliceosome, i.e. the B_{act} and C_i states. Based on this evolutionary, structural, and functional conservation, we expect that designing modulators of the group II intron will inform the design of novel, more specific spliceosomal modulators, for the treatment of severe human diseases. More broadly, our project will help expand the chemical space of RNA-directed small molecules, with broader biotechnological and pharmacological implications.

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Capturing enzyme catalysis at the new ID29 from the ESRF Extremely Brilliant Source

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Fourth generation synchrotron sources create new opportunities to expand research in structural biology and in protein crystallography in particular. The ESRF Extremely Brilliant Source upgrade programme was completed with the construction of the new ID29, the first beamline in the world completely dedicated to room temperature experiments and time-resolved macromolecular serial crystallography. The combination of two choppers will generate a pulsed beam allowing data collection from the microsecond to higher time delay with a photon flux around 10^{15} ph/sec; the double multilayer mirror monochromator (DMM) allows to tune ID29 beam on a wide energy range (from 10 to 35 keV) with a larger bandwidth; a Jungfrau 4M detector has been integrated in the ESRF data acquisition pipeline and can be operated up to 1 khz data acquisition rate. A new diffractometer, the MD3upSSX, has been developed to match the specificity of the beamline. It presents a flexible sample environment, that accommodates fixed target [1-3], viscous injectors [4-6], microfluidics [7] or tape drive [8-11]. This high versatility will make possible to perform mixing experiments to study enzyme catalysis. Pump-probe experiments can also be performed with the support of a high repetition rate laser, which runs synchronously with the pulsed beam. In addition to the beamline, users have access to a laboratory in order to prepare their samples and characterise them offline before their beamtime.

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Cellular dynamics of the human NTH1 DNA glycosylase and its partner YB1 by confocal and super-resolution microscopy

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Most cytotoxic chemotherapies aim to eliminate cancer cells, notably by, inducing severe damage to the DNA. Depending on the level of DNA damage, certain DNA damage response (DDR) systems are activated by these therapies. However, some cancer cells become resistant to these treatments due to the stimulation of DNA repair pathways. One of the DNA repair pathways known as the Base Excision Repair (BER) pathway plays a central role in the stability of the genome and efficient repair of oxidised DNA base lesions and DNA damage induced by cancer treatment. They are therefore, considered as the targets of choice for the development of new generation anti-cancer drugs. The BER pathway is initiated by DNA glycosylases such as the endonuclease III enzyme, NTH1. This enzyme recognises and eliminates oxidative, hydrated or reduced pyrimidine base damages and apyrimidinic sites. In humans, the repair activity of NTH1 is stimulated by the multifunctional oncoprotein, YB1. Thus, the hNTH1/YB1 interface has emerged as a promising target in chemoresistance. In order to study the cellular dynamics of these two factors at the highest possible spatial resolution, preliminary work involving optimization of antibody labelling for NTH1, YB1, APE1 and histones were performed by western blot and immunofluorescence via confocal microscopy. Photo-transformable fluorescent protein (PTFP) fusion constructs of NTH1 and YB1 were cloned, sequenced and transfected into the HeLa cell line demonstrating expression levels of our exogenous proteins of interest. Preliminary super-resolution microscopy data on exogenously expressed NTH1 fused to mEos4B acquired by PALM (PhotoActivated Localization Microscopy) and on endogenous NTH1 obtained by immunostaining and STORM (Stochastic Optical Reconstruction Microscopy) begins to reveal the nanoscale localizations of this repair enzyme within the nucleus of fixed HeLa cells. This data will contribute to a better understanding of the cellular and molecular mechanisms underlying the functions of NTH1 and YB1 at the nanoscale level. Further studies of its localization in response to DNA damage agents could potentially allow us to map the sites of oxidative DNA damage in chromatin.

Structural insights into the modular architecture of the Integrator complex

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Integrator is a metazoan-specific, multi-component co-factor of the RNA Polymerase II (RNAPII), that was initially discovered as the snRNA 3'-end processing endonuclease. Recent reports revealed that it has a broader role in the transcription of both non-coding and coding RNAs, where it regulates promoter-proximal disassembly of paused RNAPII. This is achieved via two independent enzymatic activities within the endonuclease and phosphatase modules. While the structures of the core Integrator bound to the paused RNAPII have been determined, many of its accessory subunits are not resolved in those reconstructions and consequently, their function in the context of the entire Integrator remains elusive.

Recently, we and others have shown that INTS15 (formerly known as C7ORF26) is a novel component of the Integrator complex that may play a role in maintaining its structural integrity. We employed AlphaFold2 deep-learning structure prediction algorithm to screen all possible binary interactions between Integrator subunits to uncover that INTS15 is predicted to interact directly with INTS10 and INTS5.

Here we report two cryo-EM reconstructions of INTS15-containing Integrator sub-complexes: INTS10/13/14/15 and INTS5/8/10/15. Our structures reveal that INTS15 plays a pivotal role in bridging the peripheral Arm module to the core of the Integrator, via its interactions with INTS5. Superposition of the newly described sub-complexes onto the structure of the Integrator bound to the RNAPII paused elongating complex (PEC) places INTS13/14 in a position that would in principle allow it to bind directly or indirectly the DNA upstream from the transcription pause site.

By combining AlphaFold2 modelling with the new and previously available cryo-EM reconstructions, we propose an integrative model of the fully assembled Integrator complex and discuss its possible implications for transcription attenuation.

Improving serial crystallographic data using a Genetic Algorithm

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In Structure-based Drug Design, determining the structure of ligands in complex with target proteins is critical. X-ray Crystallography is a tool in structural biology, used to investigate these molecular structures at high resolution. The aim of this project is to improve the electron density maps around these ligands, thereby improving the atomic models. Diffraction data from these complexes are collected using serial X-ray crystallography from many micro crystals. We are developing algorithms such as Genetic algorithms and Hierarchical cluster analysis that can identify isomorphic groups from these large pools of data to improve ligand electron density.

The *ic*OS Lab: providing complementary (time-resolved) spectroscopic data to decipher protein dynamics

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Photoactive protein dynamics is being studied in the crystalline state at synchrotron sources for timescales ranging from microseconds to minutes. *In crystallo* optical spectroscopy (*ic*OS) can be used to complement time-resolved (TR) X-ray crystallography experiments: to estimate the optimal number of photons needed to trigger a given photoreaction, to detect potential artefacts resulting from the crystalline state and to identify the most relevant time points. At the ESRF, a dedicated laboratory, named the *ic*OS Lab [1], has been developed over the years to record optical spectroscopy data from protein crystals. The experiments can be performed in a static or 'slow' time-resolved manner (~40 ms time resolution) using the versatile *ic*OS setup (Figure 1). We have recently developed the dedicated TR-*ic*OS setup for time-resolved spectroscopic experiments using a pump-probe scheme with a time resolution of a few microseconds. The latter has been designed to assist diffraction measurements on the newly-built serial crystallography beamline ID29 in its TR operation mode. To illustrate the capabilities of our various instruments, a number of applications on biological systems will be presented, including the photocycle of the archaeal proton pump bacteriorhodopsin and the photoactive state of a plant photoreceptor domain, of which we have monitored the build-up and relaxation on various time scales [2,3].

Figure 1: Sample environment of the *ic*OS setup (left) and the TR-*ic*OS setup (right).

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NMR Investigations of the Let-7 pre-miRNA Conformational Plasticity

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Non-coding RNAs are essential elements for the cellular life. However, despite their critical biological role, the way they perform their function remains elusive. Micro-RNAs (miRNAs) are key regulators of gene expression by silencing messenger RNAs. miRNA precursors (pre-miRNAs) are typically stem loop structures of ~70 nucleotides and are essential intermediates in the biogenesis and regulation of miRNA.

Here, we aim at extending current methodologies to describe RNA conformational dynamics towards a complex, biologically relevant RNA, the let-7 pre-miRNA. Due to its large size and the presence of a long fairly unstructured loop, this system remains a challenging target. By describing the conformational plasticity of the large let-7 pre-miRNA, we aim at better understanding its physico-chemical behaviour and connect it to its biological function.

By using a combination of high field and advanced labeling schemes, we could measure on this system a significant number of experimental data, including phage-induced and field-induced Residual Dipolar Couplings (RDCs) and chemical shifts. Those experimental data were combined with advanced molecular dynamic simulations, using a Sample and Select strategy.

With our approach, we could propose a conformational ensemble that depict the dynamics occurring with the let-7 pre-miRNA at timescales up to the millisecond. Our strategy allowed us to visualize large conformational changes and identify key structural features of this system.

With our approach, we were able to demonstrate the possibility to probe large RNA dynamics at timescales up to the millisecond and to reveal the potential importance of this conformational plasticity for its capability to interact with its biological partners.

Dynamic hubs of cis-regulatory elements in mRNA untranslated regions

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mRNA fate is substantially regulated via their 3'-untranslated regions (3'-UTRs). Cis-regulatory elements represent binding sites for trans-acting RNA-binding proteins (RBPs), which account for mRNA transport, translation, and degradation. They can be short sequences or folded RNA motifs and occur in variable numbers, sequential or functional redundancy. Thus, they pose particular challenges to specific recognition by RBPs.
RNA structure in 3'-UTRs provides an additional layer of mRNA regulation, translating the 1D-arrangement of individual cis-trans pairs into 3D-space. 3'-UTRs cluster cis-elements in regulatory hubs that integrate binding by numerous RBPs, which themselves, in addition, use multiple domains for multivalent RNA-binding. However, the extent of cis-trans interactions and their spatial arrangement within an mRNA is incompletely understood.
A particular challenge is the structural depiction of entire regulatory hubs, if not complete mRNA 3'-UTRs in their native forms. This appears particularly critical when considering the inherent dynamics of RNA and their potential influence, e.g. on the availability of folded cis-elements, as exemplified by us here [1] (Figure 1A). Further, we here provide selected insights into combinatory structural biology approaches centred on solution NMR spectroscopy to analyse cis-element structures, dynamics and binding by regulatory RBPs. In doing so, the integration of high-resolution information from single cis-trans pairs into full 3'-UTR secondary and tertiary structures is accomplished. In particular detail, a recent example is provided for the recognition of multiple 3'-UTR cis-elements by the immunoregulatory multi-domain RBP Roquin [2] (Figure 1B).

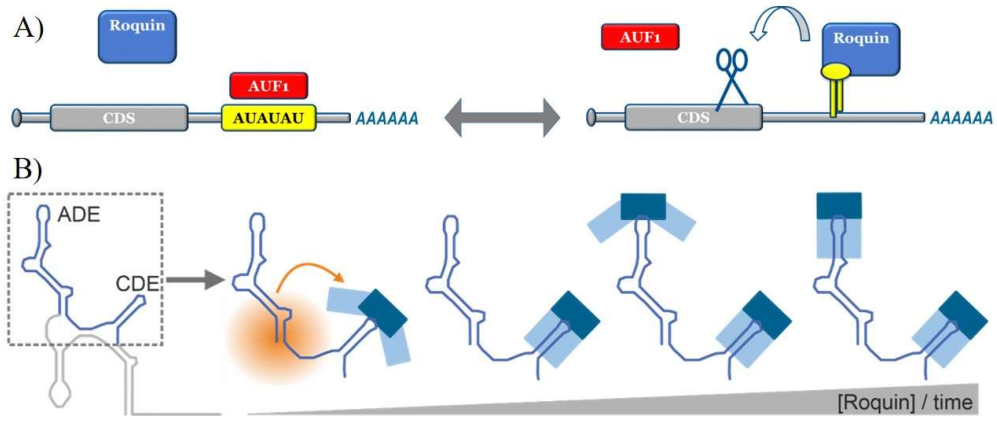

Figure 1: A) Scheme of an AU-rich mRNA 3'-UTR cis-element in dynamic exchange between single-stranded and stem-looped conformers, each bound by the highly specific RBPs AUF1 or Roquin [1]. B) Secondary structure scheme of the full-length *Ox40* 3'-UTR, which harbors different types of mRNA-decaying folded cis-elements (ADE, CDE) sequentially bound by Roquin and involving various RNA-binding domains [2].

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Dynamics of Intrinsically Disordered Proteins in Complex Environments by NMR spin relaxation and MD simulation

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Dynamics of Intrinsically Disordered Proteins are key to their numerous functions in biology. Although progress has been made to characterize their dynamics in solution [1], we still need a clear picture of their functional motions and mechanisms, such as in cells or in membraneless organelles delimited by liquid-liquid phase separation. Here, using the C-terminal domain of the N protein of Measles Virus as model system, we investigate the dynamics of this prototypical IDP in biomolecular condensates. Extensive NMR spin relaxation measurements combined with Molecular Dynamics simulations allow us to characterize the dynamics of this IDP in dilute, crowded as well as condensed environments [2]. An accurate analysis of protein dynamics in these environments will allow us to better understand the functional role of liquid-liquid phase separation as a widespread feature of many biological systems.

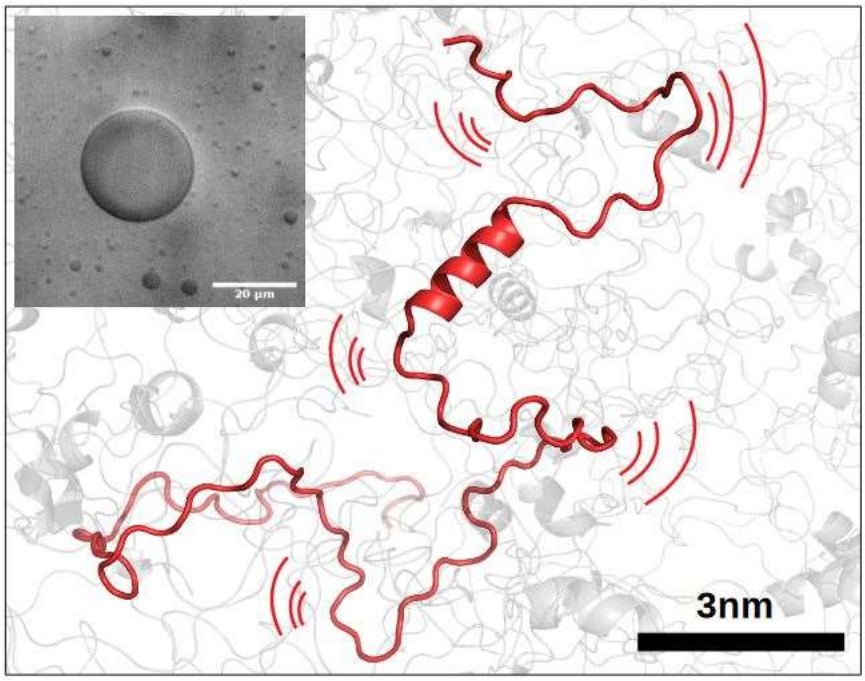

Figure 1: Illustration of an intrinsically disordered protein in a condensed phase. Top left: DIC image of a droplet of MeV Ntail condensed phase.

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Title: Predicting structures of large protein assemblies using combinatorial assembly algorithm and AlphaFold2

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Deep learning models, such as AlphaFold2 and RosettaFold, enable high-accuracy protein structure prediction. However, large protein complexes are still challenging to predict due to their size and the complexity of interactions between multiple subunits. Here we present CombFold, a combinatorial and hierarchical assembly algorithm for predicting structures of large protein complexes utilizing pairwise interactions between subunits predicted by AlphaFold2. CombFold accurately predicted (TM-score > 0.7) 72% of the complexes among the Top-10 predictions in two datasets of 60 large, asymmetric assemblies. Moreover, the structural coverage of predicted complexes was 20% higher compared to corresponding PDB entries. We applied the method on complexes from Complex Portal with known stoichiometry but without known structure and obtained high-confidence predictions. CombFold supports the integration of distance restraints based on crosslinking mass spectrometry and fast enumeration of possible complex stoichiometries. CombFold's high accuracy makes it a promising tool for expanding structural coverage beyond monomeric proteins.

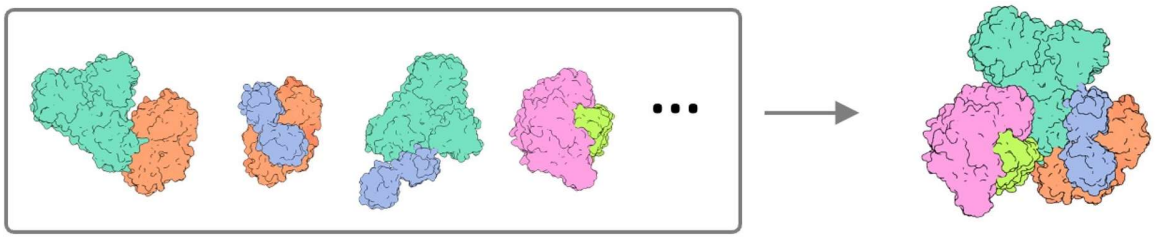

Figure 1: CombFold assembly based on pairwise AlphaFold2 predictions

Legionella pneumophila effector SidH exerts host toxicity through interaction with tRNA

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Legionella pneumophila (LP) is the most pathogenic strain of the genus *Legionella* and amounts to 90% of the diagnosed Legionnaires' disease which is a severe form of pneumonia. *Legionella* infects human lung macrophages, resides inside Legionella-containing vacuole where it secretes more than 300 effector proteins into the host cytosol using type IV Secretion system. These effectors interfere with diverse host cell pathways and help in maturation of LCV and *Legionella* replication. Many of these effectors are cytotoxic when ectopically expressed in yeast and human cells. SidH is a toxic effector of LP that is 253 kDa in size with no sequence similarity to the proteins of known function. It is included in SdhA family of effectors along with SdhA and SdhB[1]. SidH is regulated by the *Legionella* metaeffector LubX which targets SidH for degradation in a temporal manner during *Legionella* infection[2]. Mechanism behind the toxicity of SidH and its role in LP infection are unknown. We anticipate that structural studies on this family of effectors may likely reveal a novel structure-function relationship in addition to providing unique insights into human bactericidal pathways. We have determined the cryo-EM structure of SidH at 2.7Å resolution, revealing a unique alpha helical arrangement with no overall similarity to known protein structures. Surprisingly, purified SidH came bound to *E. coli* EF-Tu/t-RNA/GTP ternary complex which could be modeled into the cryo-EM density. Mutation of residues disrupting SidH-tRNA/EF-Tu interface abolishes the toxicity of overexpressed SidH in human cells, a phenotype confirmed in infection of *Acanthamoeba castellani*. We also present the cryo-EM structure of SidH in complex with a U-box domain containing ubiquitin ligase LubX providing insights into the regulation of SidH. The importance of LubX-mediated degradation was further shown in infection assays, as *LP* SidH mutants resistant to LubX-mediated degradation are more toxic to host cells. Our data provide basis for the toxicity of SidH and its regulation by the metaeffector LubX.

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Exploring the reaction coordinate of the AAA+ protein NSF with cryo-EM

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Members of the conserved AAA+ protein superfamily often couple the energy released upon hydrolysis of ATP to large-scale conformational change in order to carry out mechanical remodelling within the cell. One such essential protein is NSF, which, together with SNAP adaptor proteins, plays a key role in disassembling various SNARE complexes, including the neuronal SNAREs following vesicle fusion and neurotransmitter release. While previous EM structures revealed the overall configuration of NSF and aSNAP together with one particular SNARE complex, the general principles of NSF action—from substrate recognition to disassembly to release—remain unclear.

Here, we present the first results from the analysis of a large, multidimensional single particle cryo-EM dataset containing a mixture of both SNARE protein substrates and catalytic states of NSF at atomic resolution. We explore the roles of various conformational states in both recognizing substrate and connecting nucleotide hydrolysis to substrate disassembly using multiple machine learning based approaches.

I also will briefly describe a new series of experiments designed to explore protein dynamics on the picosecond timescale with a combination of ultrafast spectroscopy and time-resolved diffraction experiments at the LCLS.

Single Particle Tracking PALM Provides Insight into Nucleoid Remodeling in *Deinococcus radiodurans*

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Single molecule fluorescence imaging has become an indispensable tool for biological research, enabling investigation of molecular structures and dynamics at the nanoscale. Single molecule imaging adds a particular advantage to the study of bacteria, which due to their small size present a challenge for conventional fluorescence microscopy methods. Here, we employed photoactivated localization microscopy (PALM) and single particle tracking PALM (sptPALM) to visualize stress-induced nucleoid remodeling in *Deinococcus radiodurans.* Nucleoid remodeling has emerged as a generic stress response in bacteria and is thought to facilitate DNA repair and protect the genomic DNA against further damage. The molecular mechanisms driving this remodeling, however, are not well understood. We addressed this question by studying the stress induced changes in nucleoid morphology and in diffusion dynamics of histone-like protein HU, the most conserved and one of the most abundant nucleoid associated proteins in bacteria. PALM imaging revealed that the nucleoid of *D. radiodurans* adopts a rounded and compacted morphology during stationary phase and during recovery from UVC induced DNA damage as compared to during exponential phase. However, despite the morphological similarities, sptPALM showed that the diffusion dynamics of HU are drastically different in stationary phase cells and cells recovering from DNA damage: while HU diffusion is decreased during stationary phase, it is increased after exposure to UVC irradiation. We discuss how to interpret these changes in HU diffusion with respect to the altered nucleoid morphology.

PSB Symposium

Dynamics in Structural Biology

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