Thursday, September 22, Session II

L7

TOWARDS UNDERSTANDING AND INHIBITING THE *HOOK-INTO-GROOVE* INTERACTION OF THE HERPESVIRAL NUCLEAR EGRESS COMPLEX

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Herpesviruses are ubiquitous human pathogens. After genome replication and capsid assembly, transport of the capsids through the nuclear membrane and into the cytoplasm is mediated by the conserved *nuclear egress complex* (NEC) with its signature *hook-into-groove* interaction (Fig. 1).

We have recently solved the crystal structure of the *varicella-zoster virus* (VZV) NEC composed of the proteins Orf24 and Orf27 (PDB: 7PAB, Fig. 1), which closely resembles the NECs of *herpes simplex virus* (HSV-1), *pseudorabies virus* (PRV), *human cytomegalovirus* (HCMV) and *Epstein-Barr virus* (EBV) [1]. Computational and biophysical characterisation of the interaction revealed distinct differences between - (VZV), - (HCMV) and -herpesviruses (EBV).

The *hook-into-groove* interaction presents itself as a promising target to combat herpesvirus infections. To investigate this, we have adopted a fluorescence-based screen utilising *Split-superpositive GFP* (Sp-spGFP) to identify short peptides from large libraries that could constitute potential inhibitors of the *hook-into-groove* interaction [2]. To that end, the respective halves of Sp-spGFP were fused to 15- to 22-residue libraries with six randomised positions as well as pUL50 and co-expressed in *E. coli* (Fig. 2). Cells with the highest fluorescence were sorted and enriched multiple times. Binder sequences were obtained using next generation sequencing and are currently being analysed *in vitro*.

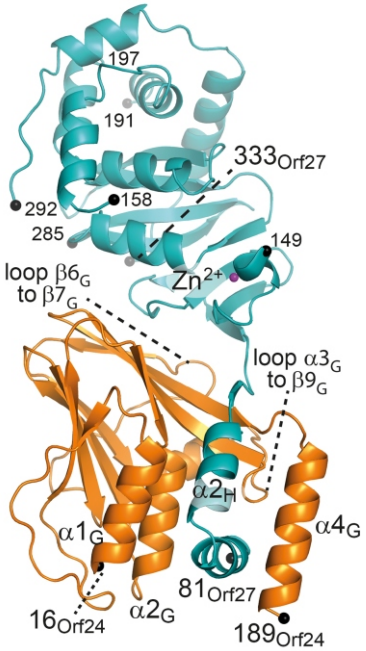

Figure 1. VZV NEC composed of Orf24 and Orf27 with *hook-into-groove* interaction.

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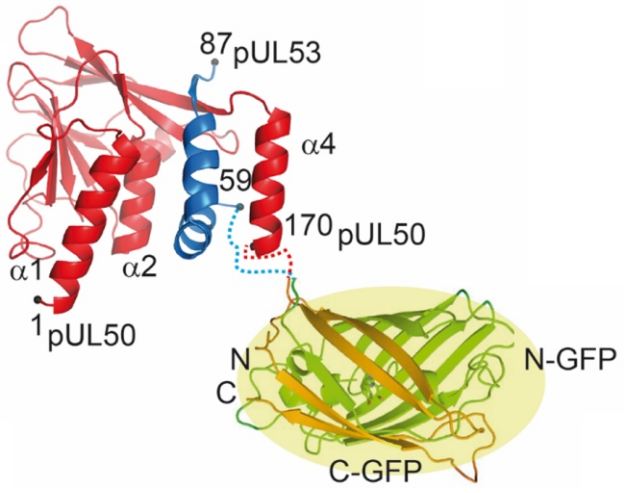

Figure 2. Schematic representation of pUL50 and the pUL53 hook (PDB: 6T3X) fused to GFP (PDB: 1EMA).

L8

CRYSTAL STRUCTURE OF SMOLSTATIN – PROTEASE INHIBITOR FROM THE MYXOZOAN PARASITE *SPHAEROSPORA MOLNARI*

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The cystatin superfamily is a large group of cysteine protease inhibitors (including type 1 cystatins – stefins, type 2 cystatins – true cystatins and type 3 cystatins – kininogens), present in various organisms. Parasite cystatins are involved in the active parasitism in the host by supressing host immune responses. Thus, cystatins are critical for the interactions between host and parasite during the infection [1, 2].

Here, we structurally characterized stefin Smolstatin from *Sphaerospora molnari*, which is a myxozoan parasite of common carp *Cyprinus carpio*. From evolutionary point of view, myxozoans are parasites that stand at the base of the metazoan evolution, therefore structural information can bring insights into the evolution of the cystatin superfamily as well as it can contribute to the aquaculture field for studying host- parasite interactions [3]. Smolstatin is a 13.5 kDa large single domain protein, which consists of typical cystatin-like domain, but unlikely for stefins, it also carries a signal peptide.

Smolstatin was recombinantly produced, purified and crystallized using sitting drop vapour diffusion technique. Diffraction data were collected on BL14.1 at the BESSY II electron storage ring operated by the Helmholtz-Zentrum Berlin [4]. Smolstatin crystallized as a domain swapped dimer. The crystal structure was determined by molecular replacement, refined and deposited to the PDB database under the accession code 8and.

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This research was supported by GAČR 21-16565S, GAJU 106/2021/P and ERDF No. CZ.02.1.01/0.0/0.0/15_003/000041.

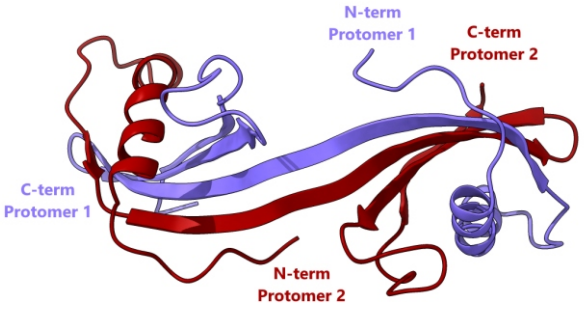

Figure 1: Crystal structure of Smolstatin.

L9

STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF THE HISTONE DEACETYLASE-LIKE AMIDOHYDROLASE FROM *KLEBSIELLA PNEUMONIAE*

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N -Lysine acetylation participating in the regulation of protein function was first discovered by histones and is now detected on various proteins in all three domains of life. The acetyl group of the acetylated substrates can be erased by two types of deacetylase, Zn^{2+} dependent classic deacetylase and NAD^{+}-dependent sirtuin. Besides proteins, small molecules like sugars, polyamines, or antibiotics can also be acetylated and deacetylated. *Klebsiella pneumonia* is an opportunistic pathogen frequently causing acute nosocomial infection. It is genetically closely related to *Escherichia coli*. However, only one sirtuin deacetylase was confirmed in the *E. coli* K12 strain, while *K. pneumonia* has, in addition, a class II classic deacetylase homolog, which is annotated as histone deacetylase-like amidohydrolase (HdaH). We confirmed its deacetylase activity with synthetic substrates but so far did not find any endogenous bacterial protein substrate. Further enzymatic and structural investigations imply it might be a native polyamine deacetylase. The aim of our research is a deeper understanding of the bacterial acetylation system, and providing knowledge for preventing and treating *K. pneumonia* infection.

L10

ARCHITECTURE AND FUNCTIONAL DYNAMICS OF A LARGE, VIRULENCE-ASSOCIATED DNA HELICASE FROM ENTEROHAEMORRHAGIC *ESCHERICHIA COLI*

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Production of flagella in enterohaemorrhagic Escherichia coli (EHEC) supports EHEC virulence and requires the EHEC-specific nucleic acid-dependent NTPase, Z5898[1]. Z5898 has been suggested to represent a separate clade of DExH-box RNA helicases in EHEC and related bacteria [2]. Here, we find that Z5898 actually exhibits efficient 3'-to-5' directional DNA helicase activity, but lacks RNA helicase activity. Cryogenic electron microscopy-based structural analyses of Z5898 in complex with DNA and an ATP analog revealed core helicase domains as also found in the antibiotics resistance DNA helicase, MrfA [3]. Although MfrA-like domains in Z5898 are interspersed with and expanded by a dimerization domain, a duplex binding domain, three zinc-binding domains and two phospholipase D-like domains, they assemble a MrfA-like core structure in 3D (Fig. 1). The dimerization motif can flexibly connect two Z5898 molecules, and the oligomeric state depends on the chemical environment and the DNA-binding mode. Comparison of Z5898 in various structural states revealed a dynamic interplay between several domains, structural motifs and bound DNA. Structure-guided mutagenesis in conjunction with functional assays pinpointed a DNA-binding rope, an ATPase activation clamp and a DNA gate as key molecular tools of the helicase. Our results reveal that Z5898-like nucleic acid-dependent NTPases have to be re-classified as DNA helicases and hint at possible strategies to interfere with the virulence-related activities of these enzymes.

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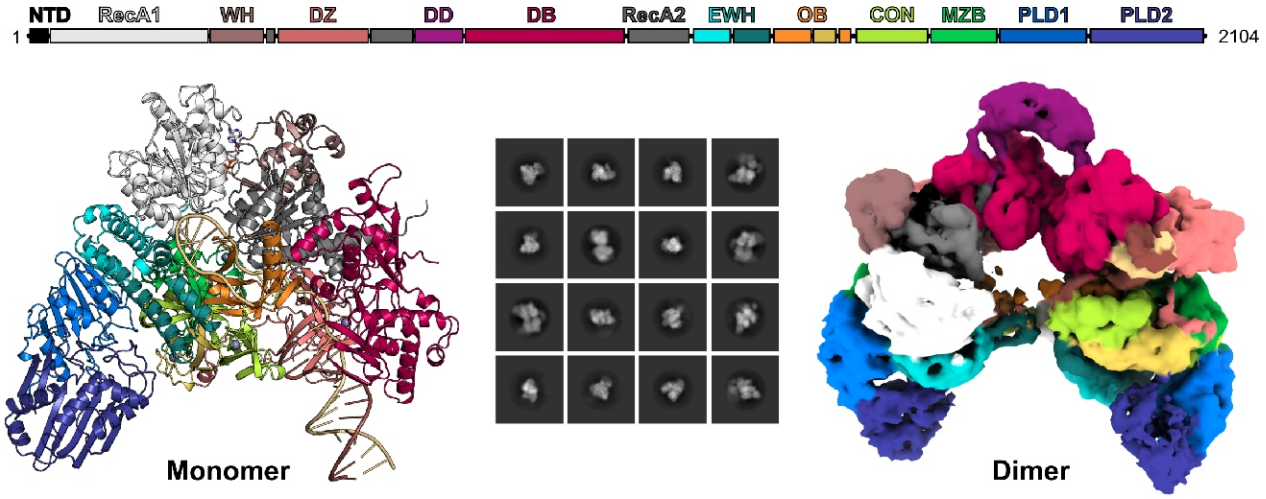

Figure 1: Top, domain composition of the *EHEC* DEAH/RHA-type helicase Z5898. Left, cartoon representation of monomeric Z5898 in complex with $ATP_{\gamma}S$ and DNA. Middle, 2D class averages of picked particle images during cryoEM analysis. Right, cryoEM density map of dimeric Z5898 in complex with $ATP_{\gamma}S$ and DNA.

L11

MECHANISM OF PROTEIN-PRIMED TEMPLATE-INDEPENDENT DNA SYNTHESIS BY ABI POLYMERASES

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Bacterial reverse transcriptases (RTs) can be classified into over 20 lineages, out of which only retrons, type II introns and diversity generating retroelements have been extensively characterized so far [1]. Among RTs which are not yet well-understood, there is a distinct group of Abi polymerases. They are involved in abortive infection — antiviral defense strategy leading to suicide of the infected cell [2]. Abi polymerases can be divided into three separate clads – AbiA, Abi-P2 and AbiK. They are unique in their ability to synthesize DNA products without either a template or a primer, utilizing hydroxyl groups of their own amino acids for priming instead [3].

In our study, we present structures of two Abi polymerases, belonging respectively to AbiK and Abi-P2 families. They form C-shaped, bilobal structures with RT-like domain on one side and a unique helical domain on the other. AbiK adopts a hexameric form, while Abi-P2 adopts a trimeric one, which is a feature unprecedented for RTs. These oligomeric states have been confirmed both by cryo-electron microscopy and MALS studies. Activity assays were performed to confirm priming role of an AbiK tyrosine covalently attached to DNA and to identify priming residue of Abi-P2. In summary, our data reveal a structural basis for a unique template independent, protein-primed polymerization mechanism in Abi RTs.

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FT2

IDENTIFICATION OF SELECTIVE INHIBITORS OF *S*-ADENOSYL-L-HOMOCYSTEINE HYDROLASE FROM *PSEUDOMONAS AERUGINOSA* VIA HIGH-THROUGHPUT SCREENING AND CRYSTALLOGRAPHIC APPROACHES

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S-adenosyl-L-methionine (SAM)-dependent methylation reactions are crucial for numerous vital processes in prokaryotic and eukaryotic organisms. In human cells, as well as its opportunistic pathogen, *Pseudomonas aeruginosa*, these reactions are controlled by only one enzyme, namely *S*-adenosyl-L-homocysteine hydrolase (SAHase). Therefore, SAHase is an essential element of cell metabolism. Thus, selective inhibition of the bacterial enzyme is an excellent possibility for drug intervention at the molecular level of cell metabolism. On the one hand, the goal is challenging because SAHases are conserved proteins, especially at the active site. However, our recent study indicates that the activity of SAHases is influenced by affecting the enzyme dynamics [1]. Therefore, targeting SAHase dynamics is an alternative strategy for developing new selective inhibitors of SAHase from *Pseudomonas aeruginosa* (PaSAHase) that do not bind in the highly-conserved active site but on the protein surface regions.

We started our research with a high-throughput inhibition study of PaSAHase, where we tested around 5000 compounds from the Pilot European Chemical Biology Library (EU-OPENSCREEN). This step identified 34 compounds that inhibited PaSAHase activity (hit rate 0.68%). However, after their validation, we limited the number of confirmed inhibitors to 22. To evaluate if these compounds exhibit any selectivity against the bacterial enzyme, we determined their IC_{50} values, testing their influence on both P. aeruginosa and human SAHases. Parallelly, we determined crystal structures of PaSAHase-ligand complexes to characterize the binding modes of the analyzed inhibitors. It allowed us to distinguish between compounds that affect the enzyme activity through the binding in the active site or on the protein surface region involved in the enzyme dynamics. A combination of biochemical and structural methods allowed us to select several lead compounds that inhibit PaSAHase in a highly selective manner. Based on our structural studies, we characterized determinants that ensure the selectivity of these compounds against PaSAHase. These results will constitute the basis for further optimization of lead compounds.

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This work has been funded by National Science Centre, Poland, grant number SONATA BIS 2018/30/E/NZ1/00729.

FT3

NEW CHEMICAL ENTITIES FOR MODULATING SARS-COV-2 ACTIVITY

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Even after over two and a half years the SARS-CoV-2 pandemic is still a global threat and while there are effective approved vaccines against the coronavirus, emerging new variants as the Omicron variant are more resistant towards the vaccine1. Moreover, there is a general decline in vaccine protection over time which together results in the urgent need to develop drugs against SARS-CoV-2. With enormous effort made, two therapeutics, Molnupiravir and Paxlovid, could recently be approved. However, it is still of great interest to find potent drugs against the virus, foremost to increase efficiency of treatment, which is far from ideal yet, and also to prepare for upcoming variants that could evade established treatments.

The NECESSITY (New chemical entities for modulating SARS-CoV-2 activity) project aims to find inhibitors against the main protease of SARS-CoV-2 (M^{PRO}). M^{PRO} is vital for the viral life circle, has a relatively low mutation rate and was proven to be druggable¸. The NECESSITY project is a trilateral project between the Innsbruck Medical University, the Palacký University Olomouc and the Helmholtz-Zentrum Berlin. For the project, a library of over 8000 small-molecule compounds and peptide analogous, most of them synthesized as kinase and protease inhibitors will be employed. Focused selections will be screened using high-throughput X-ray crystallography. Initial hits will further be developed into strong binders and analysed with biophysical methods like MST, ITC, NMR, and TSA and virological assays.

Drug-design in academia is usually bound to individual groups and methodologies initiating the process and acquire collaboration partners stepwise once suitable first starting points are identified. This can slow down to the academic drug discovery process considerably. Therefore, the NECESSITY project is designed from the start as a closely interconnected consortium with a shared vision of finding lead structures for new antivirals. The project could be a blueprint for increasing collaboration in academic compound development approaches, and thereby the speed of how fast global health threats can be combated, not only during the current SARS-CoV-2 pandemic but as well in the future for other viral diseases.

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FT4

STRUCTURAL BASIS OF CO-TRANSCRIPTIONAL rRNA FOLDING AND PROCESSING

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Biosynthesis of ribosomal (r) RNA is a prime example for co-transcriptional RNA folding and processing and it is coordinated with co-transcriptional RNP assembly. During the transcription of primary transcripts containing 16S, 23S, 5S rRNA and intervening tRNAs, RNA polymerase (RNAP) is modified by transcription factors NusA, NusG, NusB, NusE and SuhB to form a transcription anti-termination complex (*rrn*TAC) and a multi-factor RNA chaperone at the RNA exit channel of RNAP that supports the folding of the nascent RNA [1, 2]. Co-transcriptional rRNA folding is a prerequisite for subsequent processing of the primary transcript by several nucleases to yield mature 16S, 23S, 5S rRNA and tRNA [3]. RNase III is a double strand-specific endoribonuclease and the nuclease that acts first during rRNA maturation [4, 5]. Following completion of 16S rRNA transcription, a long, double-stranded RNA stem is formed (16S stem) that harbors the recognition motif for RNase III cleavage to generate pre-16S rRNA. While the role of RNase III during ribosome biogenesis has been well characterized [3-5], the molecular details of RNA recognition and processing during active transcrip-

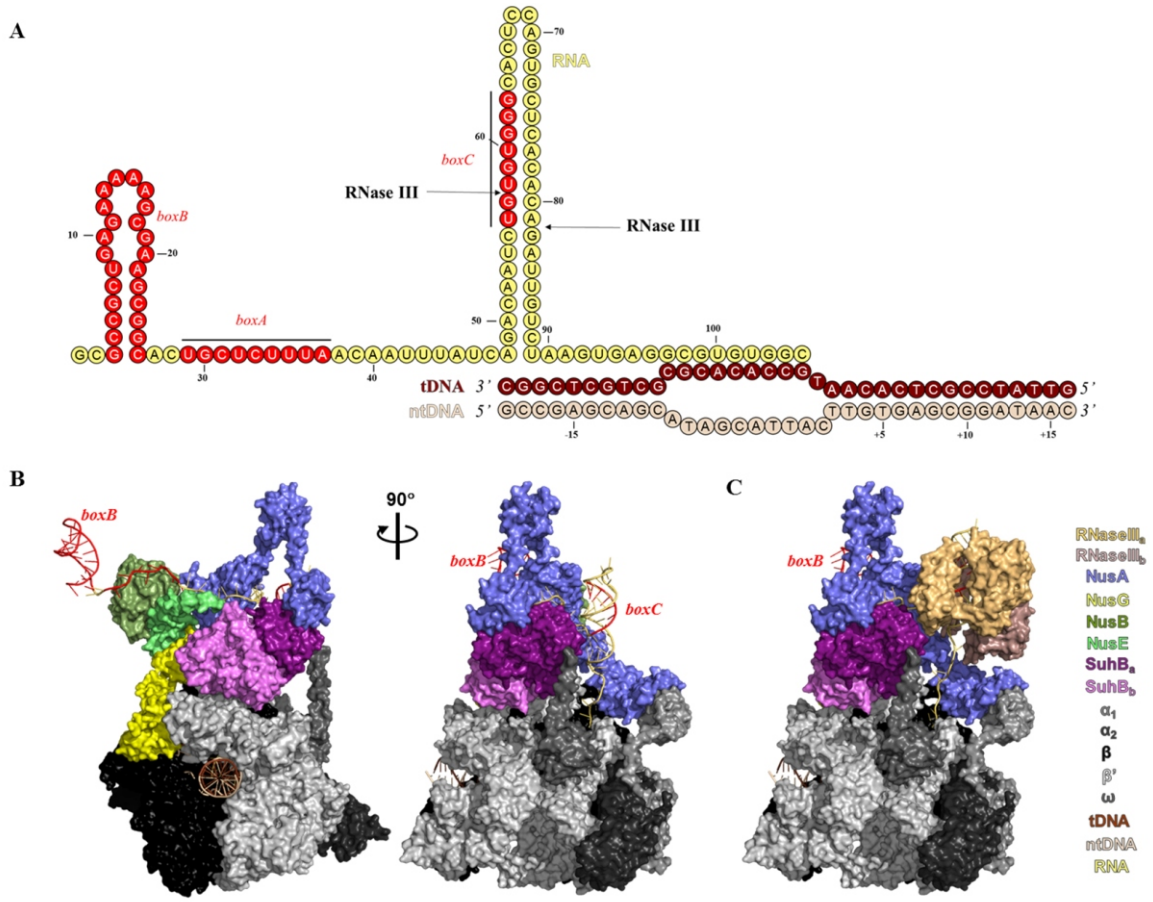

Figure **1**. Cryo-EM structure of *rrn*TAC in the absence and presence of RNase III inactivated variant.

tion and co-transcriptional RNA folding still remain elusive.

By using single-particle cryogenic electron microscopy (cryo-EM), we have determined several structures of *rrn*TACs that contain variants of the 16S stem, harboring the RNase III cleavage motif and resembling the pre-processed transcript of pre-16S RNA (Fig. 1A, B). The structures reveal further insights into the chaperoning activity of the modifying RNP. By fixing the 5' end of the 16S stem close to the RNA exit channel, NusA and the SuhB dimer provide a platform for the formation of dsRNA in a locally defined region. We also elucidated the structure of an *rrn*TAC associated with an inactive variant of RNase III bound to the 16S stem (Fig. 1C). The dimeric RNase III engages the 16S stem on the opposite side of the RNAP-associated RNA chaperone and forms direct contacts not only to the dsRNA, but also to NusA and to one of the SuhB subunits. Structure-informed functional analyses revealed that SuhB residues forming direct contacts to nascent RNA are important for efficient rRNA folding and/or processing as mutating these residues reduced RNase III cleavage efficiency.

Our results underline the importance of co-trans-Hcriptional RNA folding to obtain native and functional RNAs or targets for further maturation. We uncovered the structural basis of co-transcriptional, long-range RNA secondary structure formation by keeping the 5' part of the nascent RNA fixed to a modifying RNP and close to the emerging 3' part to support the formation of dsRNA.

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CL1

GETTING IT RIGHT FROM THE START – COLLECTING BETTER DATA WITH THE D8 VENTURE

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The performance of integrated home-lab X-ray systems for protein crystallography has increased substantially in the last decade. Improvements in source technology like the IμS-DIAMOND or the ultra-bright METALJET have made data collections faster. Large area photon-counting detectors such as the PHOTON III [1] are now affordable options for the home-lab and allow accurate data to be collected from very small, poorly diffracting crystals. Together with the possibility to operate the systems remotely and fully autonomously with the SCOUT sample changer, the user experience closely resembles the experiences at a modern protein beamline, but with less time pressure plus the great benefit of the vicinity to the laboratory to facilitate experiment optimization.

While Default settings in our PROTEUM3 software enable also the non-expert user to generate excellent data in most cases, an optimized data collection on challenging systems is required to get the best data quality which can be crucial to solve the structure.

Here we will show how the properties of the X-ray beam delivered by focussing optics compares to those from synchrotron beamlines and how the experimental parameters must be considered differently to get the good data from poorly diffracting crystals with long unit cells.

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Figure 1. The D8 VENTURE system for macromolecular crystallography.