Next Generation Macromolecular Crystallography Experiments

ANSTO Australian Synchrotron
Future Light Sources Virtual Symposium

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Structural Molecular Biology
Stanford Synchrotron Radiation Lightsource
SLAC National Accelerator Laboratory

Next Generation Crystallography Experiments

1) New structures of increasingly larger proteins and multi-protein complexes

Challenges:

- Difficult to obtain protein sample and crystals, small crystals with high-solvent content, radiation sensitive, delicate, difficult to cryo-preserve, mosaic, weakly diffracting
- Membrane proteins – LCP media requires high-speed X-ray grid searches to locate
- Large unit cells – small beams with low divergence to separate diffraction spots

Connection to CryoEM facilities

2) Studies of protein dynamics to understand the atomic positions and motions involved in biological function

- Understand the relationships involved in molecular recognition, transition state stabilization, allostery and other aspects of biological processes
- Study key details of the active site of metalloenzyme intermediate states for the development of efficient biomimetic catalysts or to understand and optimize engineered enzymes/organisms

3) Rapid screening and structure solution therapeutics development

- Target proteins with drug-like compounds and fragments for structure-based drug design
- Understanding the mechanisms of action of new drugs entering clinical trials
- Visualization of pathogen protein-antibody interactions for vaccine and therapeutics development
- Synchrotron is a resource rapid pandemic response

The Synchrotron as a Tool for Pandemic Response

The Covid-19 Pandemic has highlighted the role of Light Sources as an important tool for biomedical research and development:

- Fragment screening for structure-based drug design
- Viewing protein-antibody interactions for vaccine development
- Deciphering the action of new drugs
- Studying the pathology of disease progression

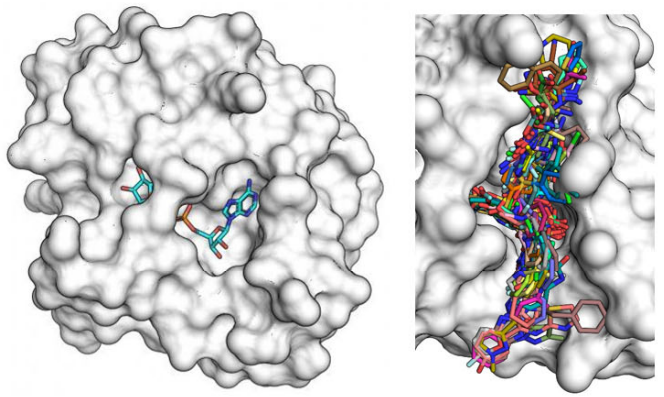
Structure-guided SARS-CoV-2 Macrodomain Inhibition

SLAC

Fragment screening at SSRL (SLAC) & ALS (LBL) & NSLS-II (BNL) & Diamond Light Source (UK) characterizes the binding site of the SARS-CoV-2 macrodomain for targeted inhibition

- The SARS-CoV-2 macrodomain-1 is a promising antiviral target because catalytic mutations of the macrodomain have rendered other viruses non-pathogenic
- Massive fragment screening efforts were undertaken by researchers at UCSF, University of Oxford, SSRL, ALS, BNL and the Diamond Light Source towards the development of a strong inhibitor for the treatment of Covid-19 infection
- Computational docking was used to screen more than 2,500 compounds yielding over 200 X-ray crystallographic structures of fragments bound in the active site of the macrodomain
- Structures revealed a large degree of macrodomain flexibility in the active site
- X-ray data collection at SSRL to ultra-high resolution and at physiological temperature revealed a significant increase in the width of the active site cleft which could provide additional conformations for fragment lead discovery

It should now be possible to use pairs of fragments to make joined complexes with combined affinities as strong lead candidates for Covid-19 therapeutics



***Left**: Surface representation of Nsp3 macrodomain with its natural substrate (colored cyan and orange) bound in the active site cleft.*
***Right**: Close up view of ~200 fragments (colored) that bind at the macrodomain active site indicating a large degree of flexibility associated with molecular recognition.*

M. Schuller, *et al.*, "Fragment Binding to the Nsp3 Macrodomain of SARS-CoV-2 Identified Through 2 Crystallographic Screening and Computational Docking", *bioRxiv* doi: 10.1101/2020.11.24.393405 (2021)

U.S. DEPARTMENT OF ENERGY | Office of Science | SLAC | SSRL | BERKELEY LAB | ALS | BROOKHAVEN NATIONAL LABORATORY | NSLS-II | UCSF | UNIVERSITY OF OXFORD | diamond | XCHEM Diamond Fragment Screening

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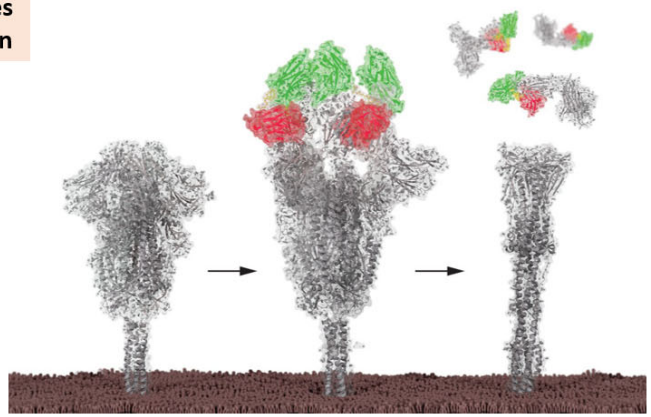
Structure-based Development of Multivalent Nanobodies

SLAC

Experiments conducted at SSRL (SLAC) & APS (Argonne) examines the effectiveness of nanobodies against SARS-CoV-2 spike protein

- Monoclonal antibodies that bind to the SAR-CoV-2 spike protein and block cell entry are difficult to produce in large quantities at low cost
- Single-domain antibodies – nanobodies – are easier to produce, can be stored at RT and have the potential to be administered by inhalation
- Complexes of the spike protein with a bound nanobody have been structurally characterized using X-ray crystallography (SSRL and APS)
- Base on the structural results, improved nanobodies that bind to two distinct epitopes on the spike protein were engineered
- The resulting bivalent nanobody neutralizes SARS-CoV-2 more potently than a single nanobody; the dual binding mode stabilizes the spike in an active conformation triggering a premature post-fusion conformation, which irreversibly inactivates the spike protein

Multivalent nanobodies promise to have a significant impact on vaccine development, deployment and ultimately vaccine access



***Mechanism of inhibition**: Inactive SARS-CoV-2 spike protein on cell surface (left). Bivalent nanobody binding stabilizes the spike in an active conformation (middle). This triggers a premature induction of the post-fusion conformation, irreversibly inactivating the spike protein (right).*

P.-A. Koenig, *et al.*, "Structure-guided Multivalent Nanobodies Block SARS-CoV-2 Infection and Suppress Mutational Escape", *Science*, 10.1126/science.abe6230 (2021)

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Feline Antiviral Drug Inhibits SARS-CoV-2

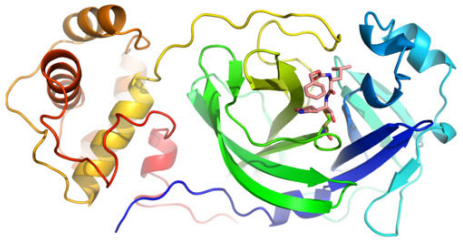
SLAC

Experiments conducted at SSRL BL12-2 (SLAC) examine a feline antiviral drug that inhibits SARS-CoV-2 replication

- Once inside host cells, the SARS-CoV-2 virus uses cell machinery to produce viral polyproteins, which are then cleaved by proteases to generate further proteins that are essential for viral replication
- Small dipeptide viral protease inhibitors developed to treat coronavirus in cats are strong target candidates for the SARS-CoV-2 main protease (SARS-CoV-2-Mpro) in humans
- X-ray crystallography data on SARS-CoV-2-Mpro in complex with a strong inhibitor (gc376) were collected on SSRL BL 12-2
- The resulting structure revealed a strong covalent bond is formed between gc376 and the protease complex, thereby making it a strong inhibitor.

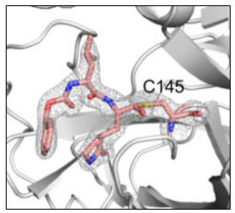
This work laid the framework for clinical drug trials with Anivive Lifesciences Inc.

https://www.prnewswire.com/news-releases/anivive-initiates-two-pre-clinical-studies-of-investigational-antiviral-gc376-for-the-treatment-of-covid-19-301087309.html



X-ray crystallographic structure *of the SARS-CoV-2 main protease with the gc376 inhibitor (red sticks) bound in the active site.*

Close up view *of the active site; gc376 (red sticks) interacts covalently with the active site cysteine (C145) of the SARS-CoV-2 main protease complex making it a strong inhibitor.*



W. Vuong, *et al.*, "Feline Coronavirus Drug Inhibits the Main Protease of SARS-CoV-2 and Blocks Virus Replication", *Nat. Commun.* **11**, 4282 (2020)

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SLAC SSRL UNIVERSITY OF ALBERTA

The Synchrotron as a Tool for Pandemic Response

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Covid-19 Related Results:

- Over 1000 SARS-CoV-2 related structures deposited into the Protein Data Bank (PDB)
- Over 3/4 of these were determined using crystallography at a synchrotron
  (In comparison, 22% used cryoEM and 3% a home X-ray source)
- Over 40% of these were done using a synchrotron within the USA

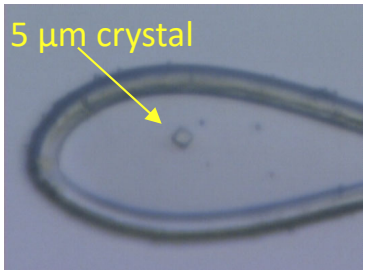
US DOE Light Sources rapidly developed protocols and training to reopen for Covid-19 research with minimal staff onsite following CDC safety guidelines

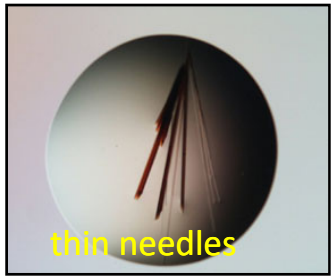
- Remote-accessible macromolecular crystallography beamlines enabled numerous SARS-CoV-2 related structural studies - without outside researchers traveling to the synchrotron
- Investment in onsite epidemiological laboratory facilities at the synchrotron could avoid delays in sample transport and production – as individual off-site labs develop protocols to restart work

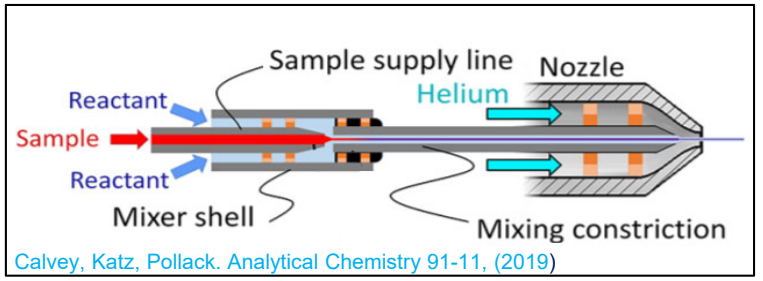
Smaller Brighter X-ray Beams - Smaller Crystals

1) New structures of increasingly larger proteins and multi-protein complexes

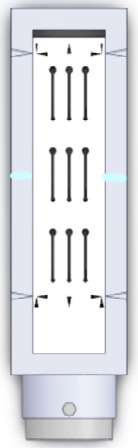
- Difficult and expensive to produce large amounts of sample
- Often only very small crystals are obtained
- Micro-beam grid-scans to locate the best diffracting areas of larger crystals







Rapid mixing micro-crystal injector



Sui, S., ...Perry, S.. J. Appl. Cryst. 54 (2021)

Multi-crystal microfluidic holder

2) Studies of protein dynamics

- Small crystals enable rapid diffusion of reactants for time-resolved mixing studies
- Smaller crystals minimize optical absorbance for homogenous triggering of light activated processes *in crystallo*. Photo-release of bound caged reactants
- Brighter X-rays for shorter exposure times required to study short-lived intermediate structures

3) Rapid screening and structure solution therapeutics development

- Easier to soak/diffuse drug-fragments and small molecules into small crystals without cracking
- Use of small crystals saves the time required to optimize crystallization conditions
- Small crystals can be used with multi-crystal holders to further save time.

Room Temperature Crystallography

Includes controlled elevated temperatures and controlled humidity crystallography

1) Experimental Considerations / Advantages

- Direct measurement of the innate diffraction quality of a crystal, free from potential damage or crystal degradation introduced during cryo-protection.
- Some crystals such as large complexes are difficult to cryo-preserve and cryo-preservation can be a time-consuming process – eliminates this process
- Sample dehydration experiments to improve the diffraction resolution of ill-behaved macromolecular crystals
- Increased protein mobility within crystals - enables triggered reactions and time-resolved studies

2) Expands Structural Knowledge

- Structure determination at near physiological temperatures can provide a structure closest to that for the protein *in vivo*.
- Enable visualization of functionally relevant water networks and alternate conformations disrupted by cryopreservation
- The conformational flexibility retained provides greater access to ligand binding pockets, facilitating binding studies and fragment-based drug discovery
- Produce molecular movies – to visualize biological function
- Intermediate state structures from time-resolved crystallography can provide unique electronic and spatial features to aid in the modeling of new drug-like compounds

Experimental Opportunities at Physiological Temperatures

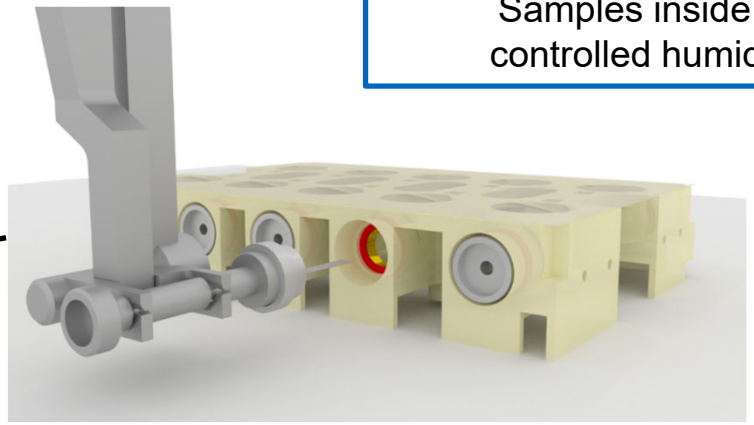
Making powerful room temperature and time-resolved methods accessible to the wider user community requires a combination of brighter X-ray sources and new automation to efficiently delivery multiple crystals and meet stringent timing requirements

New Automation to Support Remote Experiments at Elevated Temperature and Controlled Humidity

Robotic Sample Mounting of Samples at Controlled Humidity

- Plate is useful for in-situ crystallization and diffraction data collection
- Tools developed for the safe shipping of samples to the synchrotron
- Can be used with capillaries and micro-fluidics

Samples inside at controlled humidity

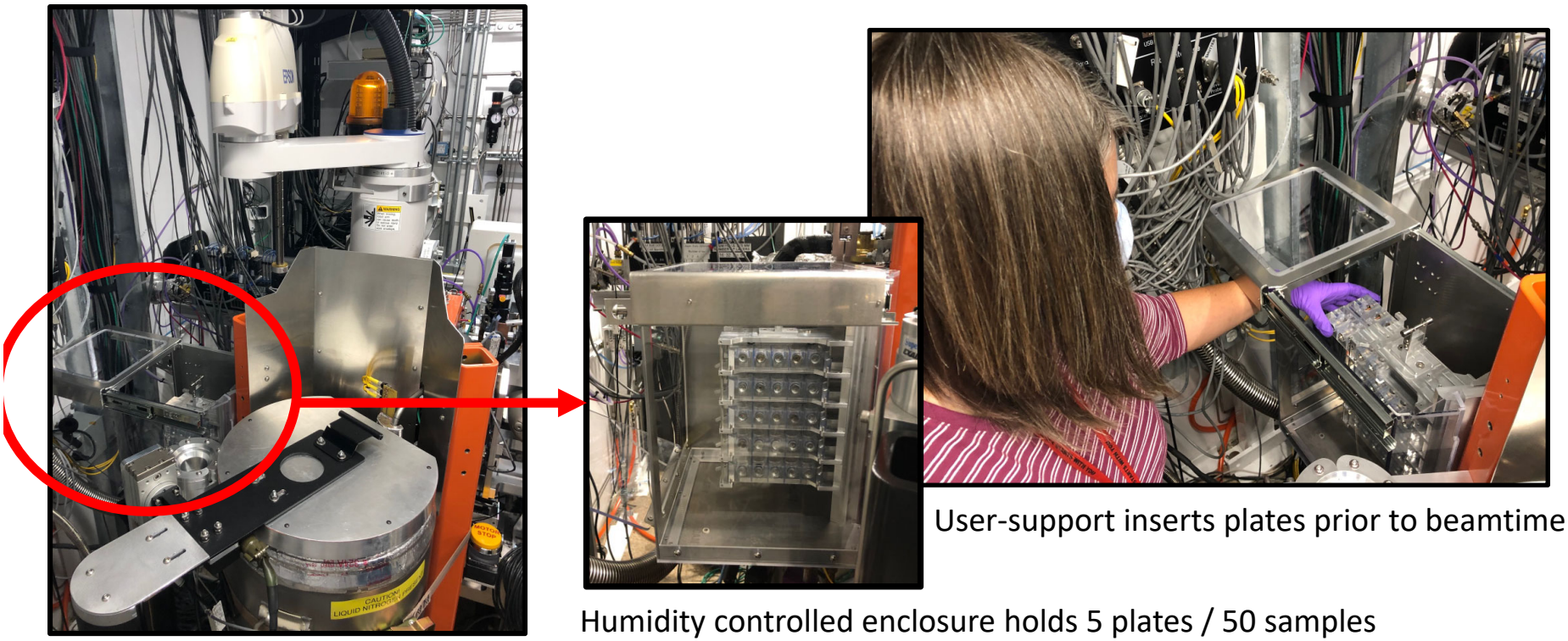


https://www-ssrl.slac.stanford.edu/smb-mc/content/users/manuals/remote-access-at-elevated-temperatures-and-controlled-humidity

New Automation to Support Remote Experiments at Elevated Temperature and Controlled Humidity

Robotic Sample Mounting of Samples at Controlled Humidity

- First used at LCLS-MFX
- Upgrade installed at 12-1 over the short August SSRL shutdown
- Enclosure holds 5 plates, each with 10 sample bases at controlled humidity
- Plate kits are being supplied to commissioning users – 10 groups so far.



User-support inserts plates prior to beamtime

Humidity controlled enclosure holds 5 plates / 50 samples

https://www-ssrl.slac.stanford.edu/smb-mc/content/users/manuals/remote-access-at-elevated-temperatures-and-controlled-humidity

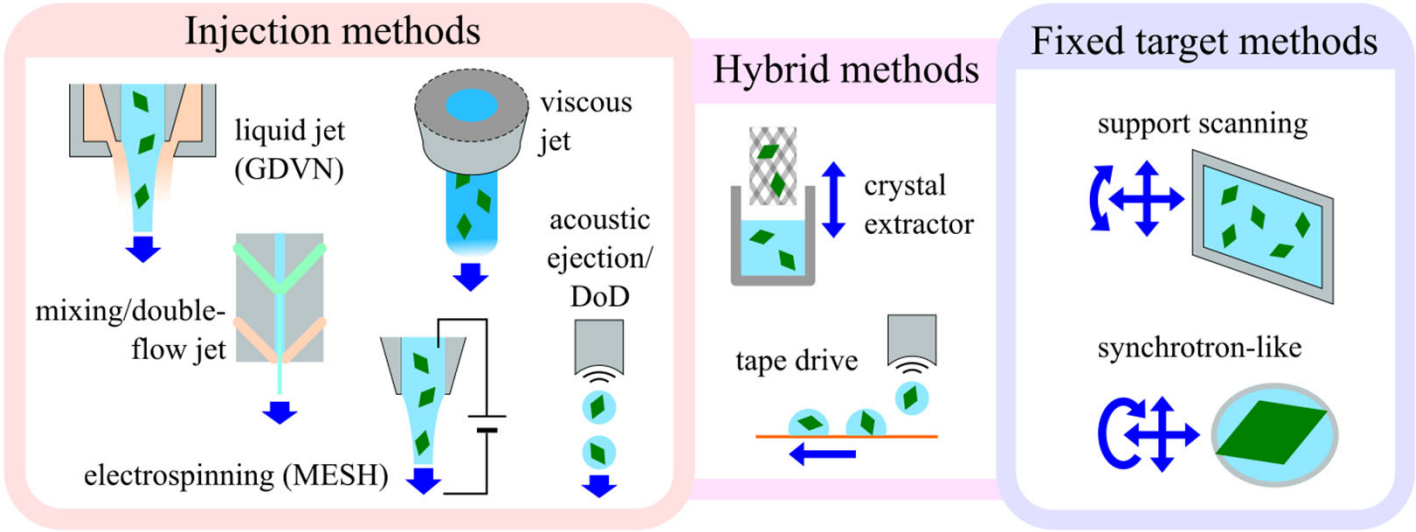
Overcoming Radiation Damage at Elevated Temperatures

1) Multi-crystal to serial crystallography at the synchrotron

- Spreads the X-ray dose required for a dataset to many crystals to reduce radiation induced artifacts in structural results.

Serial Methods originally developed at the XFEL can be applied at the synchrotron

- Many micro-crystals (or areas of larger crystals) are rapidly exposed
- While the XFEL is a pulsed source, at the synchrotron all crystals translated into the beam path are exposed for more efficient sample use
- Beam time at the synchrotron more accessible and specialized beamlines can provide fully automated complex setups to enable advanced techniques and combined methods.



Martiel, Muller-Werkmeister & Cohen (2019) Acta Cryst. D75, 160-177.

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2) Other Techniques

The combination of small X-ray beams with small crystals (<1 µm) – enables the escape of a significant amount of damaging high-energy photoelectrons before damaging the crystal

Nave & Hill, J. Synchrotron Rad. (2005). 12, 299-303

>3X increase in diffraction intensity / unit dose by use of High Energy X-rays (12.4 - 25 keV)

- First use of a Cadmium Telluride Eiger2 detector
- Higher energy data had an increase in high-resolution limit of up to 0.3 Å
- New beamlines optimized for Higher Energy Measurements

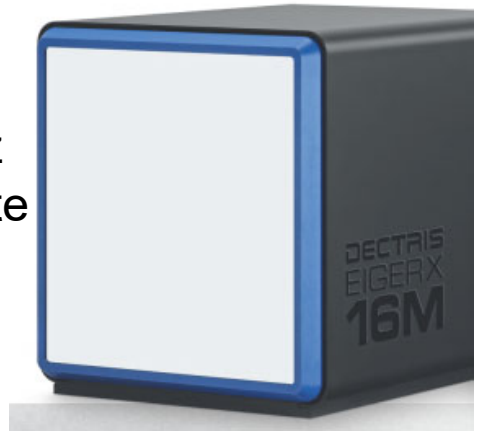
Storm, Axford & Owen (2021) https://www.biorxiv.org/content/10.1101/2021.01.21.427633v1

The combination of single crystal spectroscopic techniques and diffraction techniques

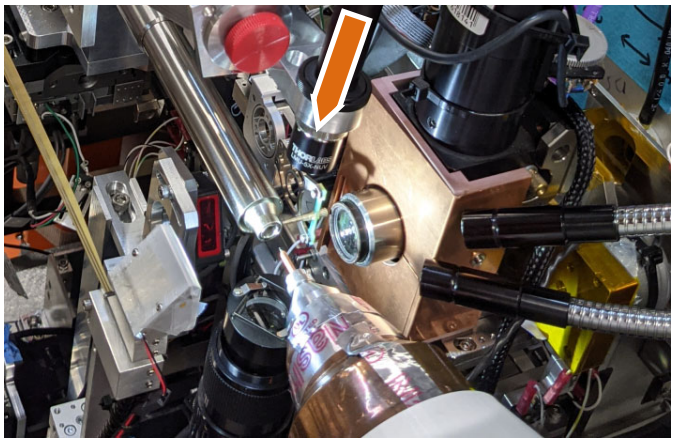
Time-Resolved Studies at the Synchrotron

- Experiments can provide **µs** temporal resolution using monochromatic X-rays with fast-frame rate detectors
- Pink beam methods with choppers or single-bunch ring operation can provide **sub-ns** temporal resolution
  - New sources could incorporate pseudo-single-bunch orbits for routine measurements in this regime Sun, Robin, Steier, & Portmann, G. doi:10.1103/PhysRevSTAB.18.120702
- Rapid time-resolved crystal **rotation** methods – provide an opportunity not possible at the XFEL.
  - Reactions can be triggered in crystals and full rotation datasets can be collected in seconds (90 degrees/second)
  - Rotation data can be divided into individual time points and data from multiple crystals combined to produce molecular movies

~500 Hz frame rate



EIGER 16M 2XE PAD



Option for UV-light (or other light sources) for time-resolved measurements

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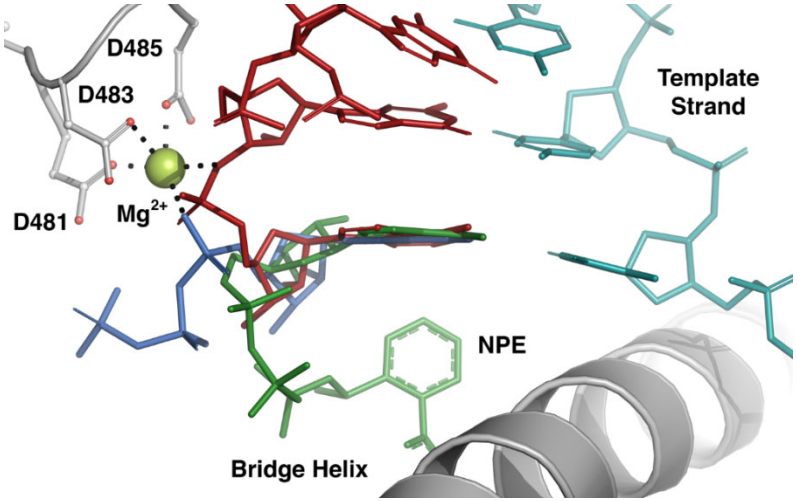

Time-resolved studies of transcription

The Calero Lab (University of Pittsburgh) has demonstrated that photoactive caged ATP (green) can bind to RNA-Pol II and that illumination with a UV source can break the nitrobenzyl group (NPE) *in crystallo* allowing ATP release, metal coordination (blue) and phosphodiester bond formation (red).

These experiments were performed remotely using SSRL beam line 12-1 where crystals were exposed to UV light (to break the cage), followed by a temperature increase from 100K to 170K (above the glass transition temperature of water), followed by rapid helical-rotation data collection (2 seconds per data set).

Thank you for your attention