2023 BioXFEL 10th International Conference San Juan, Puerto Rico Welcome Packet

Conference Chairs

Juan Lopez Garriga, Ph.D.
Alexandra Ros, Ph.D.
Edward Snell, Ph.D.

Program Committee

Petra Fromme, Ph.D.
Mark Hunter, Ph.D.
Henrike Müller-Werkmeister, Ph.D.
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10TH ANNUAL BIOXFEL INTERNATIONAL CONFERENCE

Thank you for attending the BioXFEL 10th International Conference in San Juan, Puerto Rico. The following booklet contains useful information regarding the 2023 International conference. We have an array of speakers representing countries around the world. It is our hope that you find the conference informative and enjoyable.

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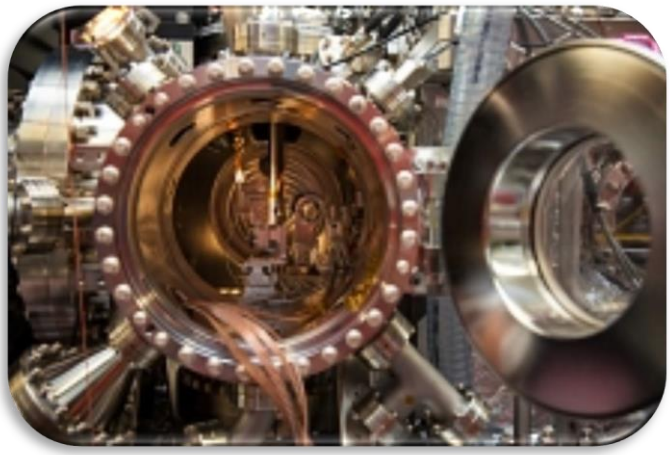
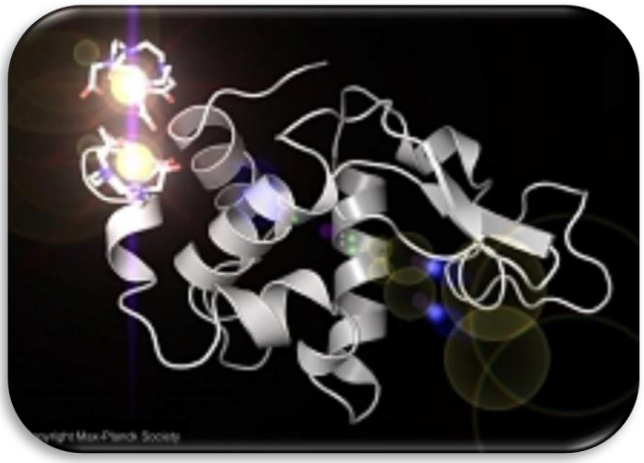
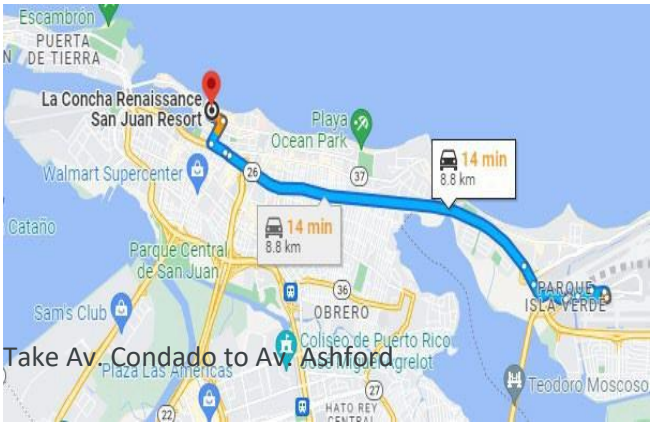




Transportation & Hotel



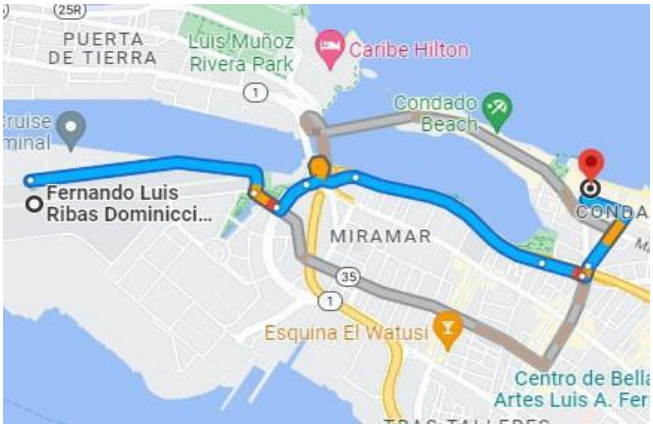
Take Av. Condado to Av. Ashford

Luis Munoz Marin International

Distance from hotel: 5.4 miles

Drive time: 14 minutes

Directions: Get on Expreso Román Baldorioty de Castro/PR-26 from Av. Aeropuerto and Marginal Aeropuerto
4 min (2.5 km) Follow Expreso Román Baldorioty de Castro/PR-26 to Marginal Rd in Santurce, San Juan. Take the exit toward Ave.R.H. Todd/Centro/Pda. 18/Santurce/Condado from Expreso Román Baldorioty de Castro/PR-26 4 min (5.4 km)



Fernando Luis Ribas Dominicci Regional Airport

Distance from hotel: 7.45 miles

Drive time: 10 minutes

Directions: Follow C, Muelle to CII Guamani/PR-16
Take Expreso Roman Baldorioty de Castro/PR-26 to Av. Ashford in Condado.

There are ubers, taxis and buses available at the airport to take you to the hotel.

Hotel

We will be staying at the **La Concha Renaissance San Juan Resort** located on the eastern coast of the island. The resort is located just 5 minutes from Condado Beach along the water. Amenities include:

- State-of-the-art fitness center, Spinning, Zumba, Yoga & Meditation classes
- Casino featuring 360 slots, sportsbook and tables open 24 hours
- 3 swimming pools and whirlpool
- 9 restaurants and bars to accommodating all palettes
- The Spa at Condado Vanderbilt
- Golf at Bahia Beach Resort (30 minutes away)
- Pet-Friendly

Address and Phone Numbers:

La Concha Tel: 1-787-721-7500 1077 Ashford

Avenue Fax: 1-787-977-4053 San Juan,

Puerto Rico 00907

Sponsorships

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Introduction

Welcome the 2023 BioXFEL conference in the beautiful city of San Juan in Puerto Rico. We are very happy that after 2 online conferences in 2021 and 2022 this conference is back "in person" and we welcome scientists from all over the world to this exciting conference.

This BioXFEL conference series has now a decade long history going back to October 2013, when Prof. John Spence and Henry Chapman chaired the first meeting at the Royal Society in London on X-ray lasers In Biology. At the same time, the NSF supported Science and Technology Center, BioXFEL started to advance the application of emerging X-ray Free Electron lasers to biological problems. That first BioXFEL meeting has been followed by a meeting each year. The conference has taken place, in Puerto Rico a number of times, then Las Vegas, New Orleans, and San Diego. In 2020 we were back in Puerto Rico at what turned out to be the very start of the COVID-19 pandemic and we are very happy to be back in person, in San Juan, Puerto Rico, with strong student and faculty participation from Puerto Rico.

The field has grown tremendously over the last decade. We have seen the new XFELs opening for users in Japan, S Korea, Germany and Switzerland, and we see the next generation of XFEL on the rise in the USA with LCLS II. The last decade has pioneered Biology with XFEL with many new and unexpected developments and breathtaking new discoveries, with the newest ones being presented at this year's conference.

BioXFEL as an organization has contributed to almost 900 papers in the field and deposited almost 1200 structural models as part of its efforts and those of collaborators and is well connected to the scientific community with many new collaborations established. The science is important but so is the community. BioXFEL has a strong educational component and we have witnessed the development of many new rising stars in the field with prospering academic careers. Importantly, The BioXFEL community has impacted the career of many scientists from Puerto Rico, and we are proud of our contribution to their career. Unfortunately, we have also lost some members of the community, especially John Spence who passed away in 2021 is greatly missed. To a large extent, BioXFEL is built as a community open to all and trained many who are now involved with XFEL-based research all over the world. We hope this community will grow and prosper.

We welcome all of you to this 10th BioXFEL conference. We hope you will enjoy the science presented, the opportunity to meet many in the field, and the opportunity to develop new ideas and collaborations that make the next decade of XFEL research just as exciting and sometimes as surprising as the previous decade.

Welcome to the BioXFEL annual conference.

Sincerely,

Petra Fromme and Edward Snell

Statement of Appropriate Conduct

The BioXFEL holds professional conferences and meetings to enable its members to receive continuing education, build professional networks, and discover new products and services for professional use. To provide all participants – members and other attendees, speakers, exhibitors, staff and volunteers – the opportunity to benefit from the event, the BIOXFEL is committed to providing a harassment-free environment for everyone, regardless of gender, sexual orientation, gender identity, gender expression, disability, physical appearance, ethnicity, religion or other group identity.

NSF seeks to provide a conference environment in which diverse participants may learn, network and enjoy the company of colleagues in an environment of mutual human respect. *We recognize a shared responsibility to create and hold that environment for the benefit of all.* **Some behaviors are, therefore, specifically prohibited:**

- Harassment or intimidation based on race, religion, language, gender, sexual orientation, gender identity, gender expression, disability, appearance, or other group status.
- Sexual harassment or intimidation, including unwelcomed sexual attention, stalking (physical or virtual), or unsolicited physical contact.
- Yelling at or threatening speakers (verbally or physically).

Speakers are asked to frame discussions as openly and inclusively as possible and to be aware of how language or images may be perceived by others. Participants may – and do – exercise the "law of two feet." Exhibitors must follow all BioXFEL Exhibits rules and regulations and BioXFEL policies.

All participants are expected to observe these rules and behaviors in all conference venues, including online venues, and conference social events. Participants asked to stop a hostile or harassing behavior are expected to comply immediately. Conference participants seek to learn, network and have fun. Please do so responsibly and with respect for the right of others to do likewise.

Please contact Conference staff if you believe you have been harassed or that a harassment problem exists. All such reports will be directed immediately to the BioXFEL Managing Director, Michael Logar, who will determine and carry out the appropriate course of action, and who may consult with and engage other conference staff, leaders and legal counsel as appropriate. Event security and/or local law enforcement may be involved, as appropriate based on the specific circumstances. A follow-up report will be made to individuals who report being harassed.

BioXFEL

2023 Annual Conference General Agenda

Tuesday, May 16th
Location - Salon Indigo

ITEM	TIME	PRESENTER(S)	TOPIC
Breakfast & Registration	8:00 - 8:55	Conference Registration and Breakfast in Ocean Lounge	
Opening Remarks	9:00 - 9:10	Lopez-Garriga, Ros, Snell, Fromme	Conference Welcome
Session I: Exciting Biology **Session Chair Henrike Müller-Werkmeister** **Session Co-Chair Tek Narsingh Malla**	9:10 - 9:30	Juan Lopez-Garriga	Sulfhemeproteins Scenarios and Mysteries
	9:30 - 10:00	Emina Stojkovic	High-Resolution Crystal Structures of Transient Intermediates in the Phytochrome Photocycle
	10:00 - 10:30	Thomas Barends	Ultrafast Structural Changes in Myoglobin: Influence of Pump Laser Fluence
	Break 10:30 - 10:45		
	10:45 - 11:15	Rebecca Jernigan	Hiding COVID: How Understanding an Accessory Protein Can Advance Biology and Medicine
	11:15 - 11:45	Lainey Williamson	Using Serial Femtosecond Crystallography to Solve the Structures of Pesticidal Proteins from Natural Crystals
	11:45 - 12:05	Matthew Rodrigues	Ultrafast Structural Changes Direct the First Molecular Events of Vision
Lunch	12:05 - 1:30	Served at Ocean Lounge BioXFEL Scholars ONLY - Interview with Sarah Woodruff in the Salon Del Mar	
Keynote Lecture	1:30 - 2:30	Tabbetha Dobbins	Learning the Language of Light: Key Points from My Journey from Physics Undergrad to Materials Science Grad to Physics Professor and University Leadership
Session II: Dynamical Studies **Session Chair Marius Schmidt**	2:30 - 3:00	Lois Pollack	Time-Resolved Solution Scattering Monitors Chemically Triggered Biomolecular Reactions at LCLS
	3:00 - 3:30	Michael Thompson	Studying Dynamic Enzymes with Temperature-Jump Crystallography
	3:30 - 4:00	Jan Kerns	Combined X-ray Studies on Metalloenzymes at Room Temperature - Insights into Photosystem II and Other Systems
	Break 4:00 - 4:15		
	4:15 - 4:45	Michel Steinmetz	Development and Use of Anti-tubulin Compounds for Time-Resolved Serial Crystallography at Synchrotrons and XFELs
	4:45 - 5:05	Kara Zelinski	A New Approach to Mix-and-Inject Serial Synchrotron Crystallography Resolves the Function of DJ-1
Poster Blitz	5:05 - 5:20	Conference Attendees	TBD
Poster Session	5:30 - 7:00	Conference Attendees	In Salon Atlantiko with appetizers provided

2023 Annual Conference General Agenda

Wednesday, May 17th
Location - Salon Indigo

ITEM	TIME	PRESENTER(S)	TOPIC
Breakfast	8:00 - 8:55	Served at Ocean Lounge	
Session III: Sample Delivery **Session Chair Richard Kirian**	9:00 - 9:30	Alexandra Ros	Overcoming Sample Amount Limitations in Static and Dynamic Serial Crystallography with XFELs
	9:30 - 10:00	Hao Hu	Serial Femtosecond/Millisecond Crystallography (SFX/SMX) Sample Delivery with LCP Injectors
	10:00 - 10:30	Meghan Shelby	Fixed Target Tools to Address Sample Environment and Delivery Hurdles in Room Temperature Serial Crystallography
	Break 10:30 - 10:50		
	10:50 - 11:20	Mark Hunter	Advancing Sample Delivery for LCLS Experiments
	11:20 - 11:40	Roberto Alvarez	Nano-Drop Fluctuation X-ray Scattering
	11:40 - 12:00	Christina Schmidt	Sample Characterization and Laboratory Support for Time-Resolved Studies of Biomolecules at the European XFEL
Lunch	12:00 - 1:30	Served at Ocean Lounge Recruitment Luncheon Event from 12:30 - 1:30 in Salon Del Mar	
Session IV: Facility and Instrumentation **Session Chair Petra Fromme**	1:30 - 2:00	Matthias Kling	Bioscience Opportunities with LCLS-II and LCLS-II-HE
	2:00 - 2:30	Shibom Basu	ID29: A Time-Resolved Serial Crystallography Beamline Offers New Opportunities at EMBL-ESRF
	2:30 - 3:00	Robert Kaindl	CXFEL Labs – New Horizons for Ultrafast X-ray Science
	Break 3:00 - 3:20		
	3:20 - 3:50	Darya Marchany-Rivera	Anaerobic Crystallography, In situ UV-Vis Spectroscopy and Serial Crystallography of Proteins
	3:50 - 4:20	Raphael de Wijn	Capabilities and Developments at the SPB/SFX Scientific Instrument at the European XFEL
	4:20 - 4:50	Florian Dworkowski	Closing the Gap - Integrated Time-Resolved Crystallography at the SwissFEL and Swiss Light Source
BioXFEL Dinner	6:00 pm	Conference Attendees	Annual Conference Dinner at Marena Pool Area

BioXFEL 2023 Annual Conference General Agenda

Thursday, May 18th
Location – Salon Indigo

ITEM	TIME	PRESENTER(S)	TOPIC
Breakfast	8:00 - 8:55	Served at Ocean Lounge	
Session V: Complementary, Computational, and Enabling Approaches	9:00-9:30	Roberto Alonso Mori	X-ray Spectroscopy at LCLS: Elucidating the Electronic and Structural Dynamics of Metalloproteins
	9:30-10:00	Tek Narsingh Malla	Heterogeneity in the Mycobacterium Tuberculosis β-Lactamase Inhibition by Sulbactam
	10:00-10:30	Chenghua Shao	Supporting Deposition of XFEL/SX Structures at the Protein Data Bank
	Break 10:30 - 10:50		
Session Chair Edward Snell	10:50 - 11:10	Henrike Müller- Werkmeister	Multiscale Femtosecond Spectroscopy as Complementary Tool for Time-Resolved Serial Crystallography
	11:10 - 11:30	Mitchell Miller	Testing Deep Learning-Based Predictions of Electron Density Maps from Input Patterson Maps
Closing Remarks	11:30 - 11:40	Lopez-Garriga, Ros, Fromme, and Snell	Closing Session
Lunch	11:40 - 1:00	Served at Ocean Lounge	

Speaker Abstracts

10TH ANNUAL BIOXFEL INTERNATIONAL CONFERENCE

DAY ONE CONFERENCE SPEAKER ABSTRACTS

Sulfhemeproteins Scenarios and Mysteries

Lopez-Garriga, J., [1] Marchany-Rivera, D., [1] Ríos-González, B.,[1] Rodriguez-Mackenzie, A.,[1] Santos-Velazquez[1], L., Arbelo-Lopez, H.,[1], Wymore, T.[2]

[1]University of Puerto Rico, Mayagüez Campus, Mayagüez P.R. 00680 [2] Laufer Center Stony Brook University, **Stony Brook, N.Y.** 11794-5252

The discovery that hydrogen sulfide (H_2S) is produced endogenously by four enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase, 3-mercapto pyruvate sulfurtransferase, and D-amino acid oxidase have raised questions about H_2S biological function, transport, and chemical reactions. Variations in its concentrations from 50 µM to 150µM have been implied in cancer, diabetes, chronic kidney problems, cardiovascular, and other diseases. Also, it plays a role in neuromodulation, neuroprotection, vasodilation, inflammation, and cytoprotection. Moreover, H_2S interacts with myoglobin (Mb), hemoglobin (Hb), lactoperoxidase (LPO), myeloperoxidase, and other heme proteins. Experimentally, the reaction between Oxy-Mb and H_2S at pH 5.00 and 6.60 showed that the heme active site doubly protonated His64$_{\varepsilon\delta}$ promotes a faster sulfMb formation than the mono-protonated tautomer, His64$_{\varepsilon}$. Thus, as pH increases, there is a preference for transforming from the triplet state to the singlet state, explaining the intensity decrease of the 622 nm transition. Hybrid quantum/molecular mechanical (QM/MM) reaction methods between OxyMb and H_2S reveal favorable energy for sulfheme formation. The synchronized transformation accompanies the homolytic cleavage of H_2S followed by the favorable (-11.2 kcal/mol) transient intermediates Cpd-0, and the thiyl radical. The overall process leads to the stable ΔE of -69.1 kcal/mol, five-member ring met-aquo-sulfMb structure. The results also show that Mb in the presence of H_2S and H_2O_2 is energetically more favorable than its native analog by -130 Kcal/mol. The mechanism suggests that H_2S inhibits the formation of stable radical intermediate species, Cpd-III and Cpd-I, and no ROS species are present as reaction products. Native LPO in aerobic conditions (O_2 or H_2O_2) in the presence of H_2S leads to SulfLPO with electronic transitions at 638 nm and 727 nm. Interestingly, throughout the reactions, a turnover of ferrous and ferric sulfLPO followed by a recovery LPO was observed. Product analysis suggests that the turnover process generates oxidized sulfur species, most likely sulfate (SO_4^{-2}). Despite these results, no structural experiment data show the presence of these hemeprotein intermediates and sulfur products. A three-minute aerobic reaction between a Met-AquoMb single crystal and H_2S shows electronic transitions at 406 nm, 428 nm, 542 nm, 578 nm, 623 nm, and 644 nm, respectively. Even though there is a mixture of H_2O-MbFe(III) and other heme derivatives, they were cryo-trapped, and X-ray information collection was possible. Data processing showed partially undefined density in the iron vicinity with the possible presence of multiple heme species. Water or H_2S used as the six heme ligands did not help define the unaccounted electron density and its planarity distortion. Thus, X-ray and Bio-XFEL techniques applied to explore the reactions between Mb, Hb, LPO, and HRP and H_2O_2 or O_2 in the presence of H_2S offer a series of advantages to unravel the Sulfheme scenario. Initial results show

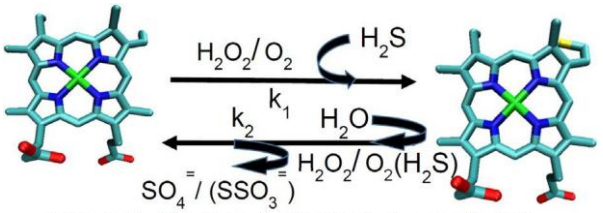

Figure 1. Heme to Sulfheme turnover (k_1/k_2)

that doping of Met-Mb (FeIII) crystals with H_2O_2 and H_2S generates green crystals associated with the Sulfheme derivatives, suggesting that it is possible to follow such reaction by traditional X-ray, XFEL, and XANES Sulfur and Iron techniques.

High-resolution Crystal Structures of Transient Intermediates in the Phytochrome Photocycle

Stojković, E. A.[1], Malla, T.N.[2], Aldama, L.[1], Pandey, S.[2], Noda, M.[1], Carrillo, M.[1], Feliz, D.[1], Westenhoff, S.[3] and Schmidt, M.[2]

1. Department of Biology, Northeastern Illinois University, Chicago, IL 60625, USA; 2. Department of Physics, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, USA; 3. Department of Chemistry, University of Gothenburg, Göteborg 41390, Sweden.

Phytochromes are red/far-red light photoreceptor enzymes in bacteria to plants, which elicit a variety of important physiological responses. They display a reversible photocycle between the resting (dark) Pr state and the light-activated Pfr state. The light signals are transduced as structural change through the entire protein to modulate the enzymatic activity. It is unknown how the Pr-to-Pfr interconversion occurs as the structure of intermediates remain elusive. Here, we present crystal structures of the bacteriophytochrome from myxobacterium *Stigmatella aurantiaca* captured by two different X-ray Free Electron Lasers, the Spring-8 Angstrom Compact free electron LAser (SACLA) and the Linac Coherent Light Source (LCLS). The structures were determined at early time points after light illumination of the Pr state. We observe large structural displacements of the covalently bound bilin chromophore, which trigger a bifurcated signaling pathway. The snapshots show with atomic precision how the signal progresses from the chromophore towards the output domains, explaining how plants, bacteria and fungi sense red light.

The research was funded by NSF-STC 'Biology with XFELs (BioXFEL)', award number 1231306. E.A.S. is supported by NSF-MCB-EAGER 1839513, NIH T34GM 105549 and an NSF STC BioXFEL center sub-award 6227.

Ultrafast structural changes in myoglobin : influence of pump laser fluence

Thomas R.M. Barends[1], Swarnendu Bhattacharyya[2], Alexander Gorel[1], Giorgio Schiro[3], Camila Bacellar[4], Claudio Cirelli[4], Jacques-Philippe Colletier[3], Lutz Foucar[1], Marie Luise Grünbein[1], Elisabeth Hartmann[1], Mario Hilpert[1], Philip J.M. Johnson[4], Marco Kloos[5], Gregor Knopp[4], Bogdan Marekha[6], Karol Nass[4], Gabriela Nass Kovacs[1], Dmitry Ozerov[4], Miriam Stricker[7], Martin Weik[3], R. Bruce Doak[1], Robert L. Shoeman[1], Christopher J. Milne[4], Miquel Huix-Rotllant[2], Marco Cammarata[8], Ilme Schlichting[1]

[1] Max Planck Institute for Medical Research, Heidelberg, Germany ; [2] Institut de Chimie Radicalaire, CNRS, Aix Marseille Univ, Marseille, France ; [3] Institut de Biologie Structurale, Grenoble, France ; [4] Paul Scherrer Institute, Villigen, Switzerland ;[5] European XFEL GmbH, Schenefeld, Germany; [6] Laboratoire de Chimie, ENS de Lyon, Lyon, France; [7] Department of Statistics, University of Oxford, Oxford, UK ; [8] ESRF, Grenoble, France

The high-intensity femtosecond pulses generated by X-ray free-electron lasers enable pump-probe studies of electronic- and nuclear changes during light-induced reactions. On time scales from femtoseconds to milliseconds and for a variety of biological systems, time-resolved serial femtosecond crystallography (TR-SFX) has provided detailed structural data on processes such as light-induced isomerization, breakage or formation of chemical bonds and electron transfer. However, to date, most if not all ultra-fast TR-SFX studies have employed such high pump laser energies that nominally, several photons were absorbed for each chromophore. As such multiphoton absorption processes may force the protein response

into nonphysiological pathways, this is of considerable concern as it poses the question whether this experimental approach allows valid inferences to be drawn about biological processes, which are likely single-photon.

Here we describe an ultrafast pump-probe SFX study of the photodissociation of carboxymyoglobin, which shows that different pump laser fluences result in strikingly different dynamics. In particular, these concern the mechanistically important coherent oscillations of the Fe-CO bond distance (predicted by recent quantum wavepacket dynamics) which are seen to depend strongly on pump laser energy. While our results confirm both the feasibility of performing TR-SFX pump probe experiments in the linear photoexcitation regime, they also show the necessity of doing so. We propose this to be a starting point for the reassessment of the design and interpretation of ultrafast TR-SFX pump probe experiments, to ensure any emergent insights are biologically relevant.

Hiding COVID: How Understanding an Accessory Protein Can Advance Biology and Medicine

Rebecca Jernigan[1,7], Dhenugen Logeswaran[1], Sabine Botha[1], Nirupa Nagaratnam[1], Diandra Doplar[1], Mokul Sonker[1], Manashi Sonowal[1], Michelle Sheikh[1], Gihan Ketawala[1], Megan Shelby[2], Mimi Cho Young[2], Matthew Coleman[2], Julius Chen[1], Matthew Coleman[2], Emily Kaschner[1], Sebastien Boutet[3], Mark Hunter[3], Meng Liang[3], Ray Sierra[3], Jay-How Yang[1], Raimund Fromme[1], Michelle Zacks[1], David Larwood[6], Lisa Shubitz[7], Matthias Frank[2], Jose Martin Garcia[1,4], Marius Schmidt[5], Alexandra Ros[1], Debra Hansen[1], Julius Chen[1], Petra Fromme[1]

1. School of Molecular Science and Biodesign Center for Applied Structural Discovery, Arizona State University, Tempe, Az, 85287-1604 2. Lawrence Livermore National Laboratory, 7000 East Avenue, Livermore, CA 94550, USA 3. LCLS SLAC National Accelerator Laboratory 4. Institute Physical-Chemistry Rocasolano, Spanish National Research Council, Serrano street 109, 28006, Madrid, Spain 5. Physics Department, University of Wisconsin-Milwaukee, 3135 N. Maryland Ave, Milwaukee, Wisconsin 53211, USA, 6. Valley Fever Solutions, Tucson, Az 7. University of Arizona, College of Veterinary Medicine, 1580 E Hanley Blvd, Oro Valley, AZ 85737

The uridine-specific endoribonuclease, NendoU, from SARS-CoV-2 enables the evasion of the innate immune system by cleaving the polyuridine leader sequence of antisense viral RNA. Using serial femtosecond crystallography (SFX) at X-ray Free Electron Lasers (XFELs), the objectives are to better understand the catalytic mechanism of NendoU, its function related to viral pathogenesis, and application to therapeutic drug design. The first room-temperature structure of NendoU by SFX to 2.6 Å reveals insights into the flexibility, dynamics, and other intrinsic properties of NendoU, with indications that the enzyme functions as an allosteric switch. While NendoU is highly conserved among coronaviruses, crystallographic and functional studies of NendoU mutants from SARS-CoV-2 variants were conducted to elucidate the impact of how structural changes may relate to the disease process. To fully encompass the impact of structural biology in medicine, inhibitors of NendoU were explored as potentials for prophylactic treatment. One potential therapeutic is the uridine analog, NikkomycinZ, a well-tolerated drug targeted towards the fungal disease endemic to the southwest United States, coccidioidomycosis. While still being explored, the connection between the uridine-specific endoribonuclease of a virus and the uridine analog targeting fungal disease highlights the benefits of interdisciplinary work. NendoU is protein of exciting biological relevance for its mechanism in RNA processing, application to the study of infectious disease, and the methodologies used to accomplish these objectives.

Using serial femtosecond crystallography to solve the structures of pesticidal proteins from natural crystals

Lainey J. Williamson [1], Marina Galchenkova [2], Hannah L. Best [1], Richard J. Bean [3], Anna Munke [2], Salah Awel [2], Gisel Pena [2], Juraj Knoska [2], Robin Schubert [3], Katerina Doerner [3], Hyun-Woo Park [4], Dennis K. Bideshi [4], Alessandra Henkel [2], Viviane Kremling [2], Bjarne Klopprogge [2], Emyr Lloyd-Evans [1], Mark Young [1], Joana Valerio [3], Marco Kloos [3], Marcin Sikorski [3], Grant Mills [3], Johan Bielecki [3], Henry Kirkwood [3], Chan Kim [3], Raphael de Wijn [3], Kristina Lorenzen [3], P. Lourdu Xavier [2,5], Aida Rahmani [2], Luca Gelisio [2], Oleksandr Yefanov [2], Adrian P. Mancuso [3,6], Brian Federici [7], Henry N. Chapman [2,8,9], Neil

Crickmore [10], Pierre J. Rizkallah [11], Colin Berry [1] and Dominik Oberthür [2]

[1] School of Biosciences, Cardiff University, UK; [2] Center for Free Electron Laser Science CFEL, Deutsches Elektronen-Synchrotron DESY, Notkestr. 85, 22607 Hamburg, Germany; [3] European XFEL GmbH, Schenefeld, Germany; [4] Department of Biological Sciences, California Baptist University, USA ; [5] Max-Planck Institute for the Structure and Dynamics of Matter, 22761 Hamburg, Germany; [6] Department of Chemistry and Physics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria 3086, Australia; [7] Department of Entomology and Institute for Integrative Genome Biology, University of California, USA; [8] Centre for Ultrafast Imaging, Universität Hamburg, Hamburg, Germany; [9] Department of Physics, Universität Hamburg, Hamburg, Germany; [10] School of Life Sciences, University of Sussex, Falmer, UK; [11] School of Medicine, Cardiff University, UK

Bacillus thuringiensis (Bt) and *Lysinibacillus sphaericus* (Ls) are gram-positive, spore-forming bacteria that produce several classes of structurally diverse pesticidal proteins, many of which are produced naturally as crystalline inclusions. These proteins constitute the major factors in bioinsecticides and transgenic crops. The general mode of action begins with dissolution of the pesticidal crystal proteins in the acidic or alkaline environment of the target insect larvae gut, followed by proteolytic processing to the active form. The activated protein oligomerizes and binds specifically to receptors present on the midgut epithelial cells, leading to pore-formation, cell lysis, and ultimately, insect death. While some protein structures are available, many are uncharacterised, particularly in the protoxin forms. In addition, there is currently a limited understanding surrounding the mechanism of natural crystallisation and the processes contributing to subsequent dissolution. Here, we utilized serial femtosecond crystallography (SFX) at the European X-ray Free Electron Laser facility to determine the structures of pesticidal proteins from natural crystals isolated from spores.

SFX was employed to solve structures including Tpp49Aa1 and two forms of Cry8Ba2 produced naturally in the same preparation. The Cry8Ba2 structure represents the first structure of a non-mutated Cry toxin in the long protoxin form and sheds light on the arrangement of Cry toxins into natural crystals. In addition, mixing experiments conducted at varied pH enabled investigations of the early events leading up to the dissolution of natural Tpp49Aa1 crystals. This work will pave the way for further investigations on the structure and dynamics of bacterial insecticides, with the goal of developing better and greener insecticides for agricultural pest-control.

Ultrafast structural changes direct the first molecular events of vision.

Rodrigues, M.J.[1], Gruhl, T.[1], Weinert, T.[1], Schertler Group[1,2], Nango group[3], Neutze group[4], Nogly group[2], SwissFEL group[5], SACLA group[6], Standfuss Group[1], Panneels, V.[1], Schertler, G.F.X.[1,2]

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Mammalian rhodopsin is our receptor for vision. It belongs to the highly druggable G protein-coupled receptor family. Upon light illumination, the photoreceptor binds and activates the intracellular G protein transducin, triggering a cascade of signalling events to the brain via the optic nerve within milliseconds. However, the intramolecular initial events transforming the rhodopsin resting state [1-2] (dark state) into the transducin-binding activated state [3-5] (Meta II state) are not completely understood.

Recently, we captured snapshots of the native bovine rhodopsin at room temperature using time-resolved ultrafast serial femtosecond crystallography, already successfully used for the proton pump bacteriorhodopsin[6-7], at the SACLA and SwissFEL X-ray free electron lasers (XFELs). Thousands of rhodopsin microcrystals grown in the dark were successively injected in to the light of a pump laser and probed after various time-delays from femtoseconds to milliseconds using an XFEL. After correction of our diffraction data for a lattice-translocation defect, we were able to resolve the structures of dark and light-activated states[8].

Upon photon absorption, the 11-cis retinal chromophore of rhodopsin undergoes one of the fastest events in biology: its isomerisation into the all-trans conformation. After 1 picosecond, we observe a highly distorted all-trans retinal that has induced a few changes in its binding pocket while the excess photon energy dissipates anisotropically inside rhodopsin as a protein breathing motion towards the extracellular domain. Interestingly, some amino acids known to be key elements in the transduction of the signal are involved in the protein breathing motion[9].

The same type of experiment was applied at later time-delays from 100 ps to early microseconds showing a relaxation of the whole structure followed by the first major retinal conformational changes modifying its binding pocket.

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Learning the Language of Light: Key Points from My Journey from Physics Undergrad to Materials Science Grad to Physics Professor and University Leadership.

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This talk describes my professional journey as an example which can inspire students who are passionate about Science, Technology, Engineering and Mathematics (STEM). It uses the synchrotron studies performed on hydrogen storage materials to derive key points of how the scientific career can be pursued with passion. Any student coming from a 1st generation college student background can take home points that they can use to persist in their scientific endeavors.

Time-resolved solution scattering monitors chemically triggered biomolecular reactions at LCLS

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Recently, we extended time-resolved solution scattering studies at XFELS from light activated reactions to include chemically triggered reactions. Using LCLS beams in conjunction with rapid fluid mixers initially developed for mix-and-inject serial crystallography (2), we have monitored the folding of a small RNA from a dynamically varying, completely unstructured ensemble to a fully structured state, following the addition of Mg^{2+} ions by rapid diffusion. Reaction time scales from single milliseconds to seconds are accessible to this technology. Distinctive features displayed in the scattering profiles are easily connected with real space RNA structural features, allowing us to watch transient structures form and change, until molecular interactions secure a final, stable fold (1). Given the exploding interest in RNA, and the relative difficulty of measuring its dynamic structures, this new information can help determine both static and dynamically varying RNA structures. In addition, this new capability, uniquely facilitated by the XFEL, opens an entirely new area of research monitoring biomolecular folding and interactions.

Investigator funding for this work (beyond facilities and equipment) was provided by the National Science Foundation through the BioXFEL STC award number 1231306 and through grant DBI-1930046 to L.P.. Funding was also provided through the National Institutes of General Medical Sciences, National Institutes of Health (NIH) award R35-GM122514 to L.P. and R01-GM133998 to T.D.G..

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Studying Dynamic Enzymes with Temperature-Jump Crystallography

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The catalytic cycles of many enzymes rely on conformational dynamics to facilitate substrate binding, catalysis, and product release. Time-resolved crystallography is ideally suited to study the functional motions of enzymes, and we are developing infrared laser-induced temperature-jump (T-jump) as a rapid perturbation for these experiments. I will present initial work, in which we used T-jump crystallography to map structural motions in lysozyme, a dynamic enzyme, demonstrating the feasibility of the technique. Next, I will describe ongoing efforts to apply T-jump crystallography beyond model systems such as lysozyme. Specifically, I will show how we are using T-jump to understand the complex catalytic mechanism of soybean lipoxygenase, and share how we are developing instrumentation for T-jump experiments across both synchrotron and XFEL beamlines.

Combined X-ray studies on metalloenzymes at room temperature - insights into Photosystem II and other systems

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Obtaining information about changes in the electronic and geometric structure of the active site of a metalloenzyme during catalytic turnover is essential to obtain a detailed understanding of its reaction mechanism. Utilizing femtosecond X-ray pulses from XFELs it is possible to record "undamaged" snapshots of metalloenzymes at room temperature. Given adequate reaction triggering options these can be collated to a "movie" that shows the sequence of events at the catalytic site necessary for the reaction to take place. We developed a drop-on-demand sample delivery system that can be combined with various triggering options and allows for time resolved recording of X-ray diffraction and X-ray spectroscopic data at XFEL sources [1]. Here we will describe our recent results using this system on a number of metalloenzymes including photosystem II [2,3], ribonucleotide reductases, hydrogenases, methyl coenzyme M reductase [4], and isopenicilin N synthase [5] and drop-on-drop mixing results [6]. We will also highlight the advantages of collecting X-ray emission data concomitantly with the diffraction data, allowing to follow changes of the oxidation state of the metal site over the reaction cycle and correlate these with the structural snap shots obtained from the diffraction measurements. Results for membrane bound O_2 tolerant [Ni/Fe] hydrogenase (MBH) from *R. eutrophia* for example show clear changes in both the Ni and Fe oxidation state at different points in the O_2 induced inactivation as well as the H_2 induced reactivation reaction of the enzyme correlated with different structural changes in the Ni-Fe active site as well as in the proximal FeS cluster. We will also cover our latest results on the last reaction step in the water oxidation reaction in PS II, the S_3-S_0 transition, where molecular oxygen is formed and released.

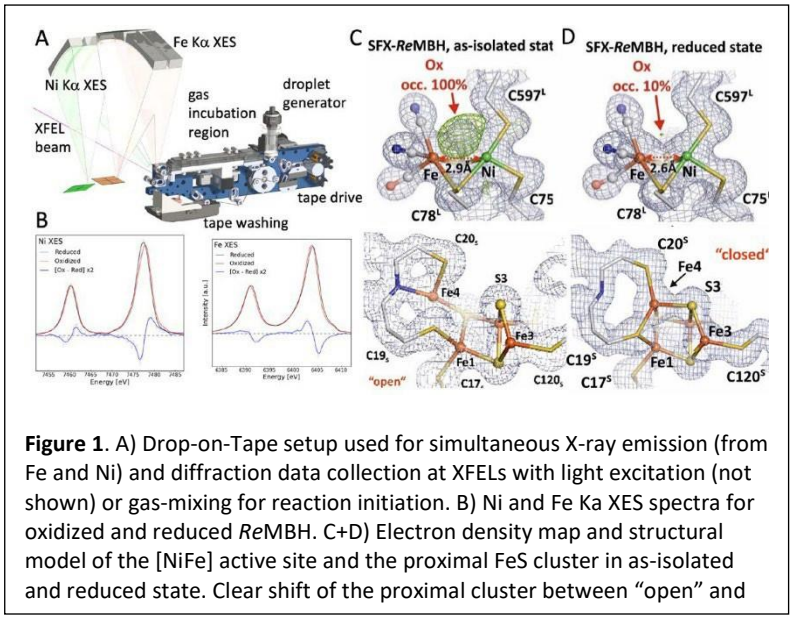

Figure 1. A) Drop-on-Tape setup used for simultaneous X-ray emission (from Fe and Ni) and diffraction data collection at XFELs with light excitation (not shown) or gas-mixing for reaction initiation. B) Ni and Fe Ka XES spectra for oxidized and reduced *Re*MBH. C+D) Electron density map and structural model of the [NiFe] active site and the proximal FeS cluster in as-isolated and reduced state. Clear shift of the proximal cluster between "open" and

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Development and use of anti-tubulin compounds for time-resolved serial crystallography at synchrotrons and XFELs

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Tubulin is a highly dynamic protein and a validated anticancer drug target. Using a combined computational and crystallographic fragment screening approach, we recently identified several novel binding sites in tubulin, some of which are connected allosterically through a dynamic network of amino acids and secondary structure elements. Based on our results and using straightforward chemistry, we for the first time fully rationally designed several small molecule tubulin inhibitors that display unique molecular mechanisms of action. We further exploited the light-induced conformational switch properties of a designed tubulin inhibitor to assess its unbinding using time-resolved serial crystallography at the Swiss Light Source and SwissFEL. Our results provide detailed information on the dynamic nature of tubulin, which is fundamental to its functional role in several biological processes including cell division, cell polarity, cell migration, signaling, and intracellular transport.

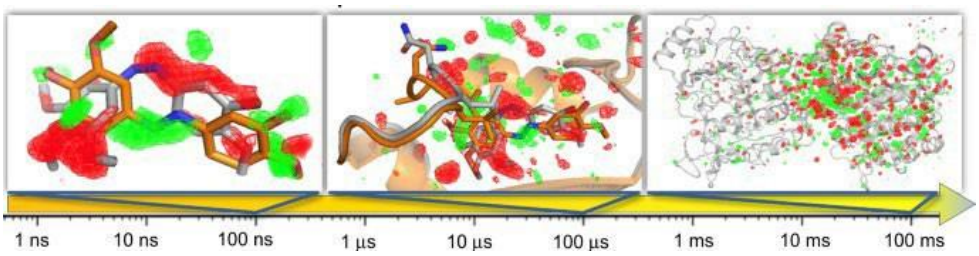

Figure. Time-resolved structural snapshots of azo-combretastatin A4 release from tubulin. The time arrow depicts the investigated time regime. The panels from left to right show the isomorphous difference maps obtained at 100 ns with changes centered on the ligand, 100 µs with changes centered on the binding pocket, and 100 ms with conformational changes propagating throughout the protein. All panels show isomorphous difference maps in red (negative) and green (positive) at 3 σ. 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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A New Approach to Mix-and-Inject Serial Synchrotron Crystallography Resolves the Function of DJ-1

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Serial crystallography, enabled by the high brilliance available at X-ray Free Electron Lasers (XFELs) and Third Generation Synchrotrons, allows for the room temperature measurement of protein structures. This has fostered the development of numerous time-resolved techniques to capture snapshots of biological reactions while they are in progress. Of particular interest, the mix-and-inject serial crystallography (MISC) technique utilizes microfluidic mixers to rapidly combine protein microcrystals with small molecule ligands just prior to data collection. At XFELs, MISC is typically combined with Gas Dynamic Virtual Nozzles (GDVNs) to deliver freshly mixed crystals via liquid jet to the X-ray interaction region to be compatible with the 10s of femtosecond exposure time. Past XFEL work captured reaction timepoints from the single millisecond to the second range. There is considerable interest in adapting the technique to synchrotrons, but changes in sample delivery are needed to accommodate the increased X-ray exposure time required, usually on the scale of milliseconds at monochromatic beamlines. The polychromatic BioCARS beamline at the APS, however, offers a high incident flux in sub-ms exposures. Additionally, Laue Crystallography has the advantage of acquiring more information per frame, allowing for relatively rapid data collection. Here, we demonstrate a new MISC co-flow sample delivery scheme in which flow-focusing diffusive microfluidic mixers are constructed with concentric capillaries. Freshly mixed crystals remain confined by tubing for data collection. By varying the flowrates or changing the position of the X-ray beam relative to the tip of observation region, many timepoints can be reached in a single sample cell. We used this new technology to study DJ-1, an important protein in oxidative stress response. Interestingly, DJ-1's function has been heavily disputed. This demonstration of synchrotron MISC allowed for direct observation of DJ-1 acting on its substrate, methylglyoxal, confirming its role as a glyoxalase rather than a deglycase.

DAY TWO CONFERENCE SPEAKER ABSTRACTS

Overcoming Sample Amount Limitations in Static and Dynamic Serial Crystallography with XFELs

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The advances of X-ray free electron lasers (XFEL) have enabled serial femtosecond crystallography (SFX) and structure determination for complex proteins such as membrane proteins at high resolution and in time-resolved (TR) mode. The pulsed nature of all current XFELs stipulate on demand sample delivery matching the respective pulse frequency to increase sample efficiency. Liquid jets – early developed for the delivery of crystal slurries into the path or an XFEL beam – still serve as a reliable injection method for SFX. However, injection through a liquid jet wastes >90% of sample, limiting macromolecular structure determination in SFX to proteins which can be isolated at high yields (hundreds of mg). In addition, TR experiments require sample amounts proportional to the time points probed.

To address this bottleneck, the Ros lab and collaborators have developed droplet injectors that are compatible with traditional jet injection through a gas dynamic virtual nozzle (GDVN). Our approach is based on microfluidic droplet generators producing a segmented flow, where crystal laden droplets of mother liquor are partitioned by an immiscible oil. The oil phase serves as a sacrificial liquid and maintains the jet injection in a continuous manner. Generating the droplets at a frequency matching a particular XFEL beam repetition rate and synchronizing their arrival in time with the XFEL pulses, is the principle behind this novel strategy to save precious protein crystal sample. We have demonstrated this droplet injection approach both at the Standford LCLS and the European XFEL (EuXFEL) for four different proteins including the first room temperature structure of the enzyme NQO1 (NAP(P)H:quinone oxidoreductase 1), an important anti-cancer drug target. The droplet generators can be integrated in He atmosphere and in vacuum chambers. So far a 4- fold reduction in sample consumption could be demonstrated with diffraction resolution matching the continuous GDVN injection for all protein crystals tested. Most importantly, the droplet injectors can be coupled to mix-and-inject TR-SFX through the employed 3D-printing fabrication strategies and the capabilities of microfluidic mixing. Implementation of this approach in a TR-SFX experiment at the EuXFEL has provided the first NADH bound NQO1 structure obtained at an XFEL. The segmented droplet approach thus has a high potential to serve a critical role in static and dynamic SFX experiments overcoming the severe limitations in sample amounts currently required for complete data sets.

Serial Femtosecond/Millisecond Crystallography (SFX/SMX) Sample Delivery with LCP Injectors

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The lipidic cubic phase (LCP) injector (or high viscosity injector) developed at ASU has been successfully applied to many structural studies of proteins at XFEL and Synchrotron sources across the world. This lead to important contributions to the structure determination of pharmacologically relevant G-protein-coupled receptors (GPCR), which can be crystallized

in LCP. The injector system has been improved towards the goal of easier operation for users and expanded suitability for different proteins.

One of the main benefits of the LCP injector is its low sample consumption. It can produce a slow moving micron sized extrusion stream of highly viscous liquid containing micron sized protein crystals with a typical sample flow rate between 0.05 and 1 µL/min. However, applying it to non-LCP-grown crystals, such as those obtained in solution, is one of the challenges. Many crystals cannot survive embedding in LCP with two coupled syringes due to the high shear forces applied during the process. In addition, LCP produces relatively high background scattering. Therefore, we have explored several other viscous media to better fit a variety of sample needs. Recently, we have focused on a hydrophilic medium, Polyethylene oxide (PEO); it is compatible with many solution-grown crystals, including the Photosystem II protein complex, one of the most delicate protein crystal samples. Moreover, the PEO jet diameter can be reduced by coflowing sheath gas, which results in a faster jet speed, while maintaining low sample flow rate. This can open up possibilities for time-resolved (TR-SFX) studies with many more protein crystals, especially those with low yield. Relative to standard SFX, TR-SFX studies require at least 5 to 10 times faster jet speed. For most media, increasing the sample flow rate is the only option, which greatly increases sample consumption; however, the PEO jet's tunable diameter achieves fast jet speed without increasing sample flow rate. Additionally, since the jet diameter is not tied to the capillary inner diameter (ID) anymore, large ID capillaries can be chosen for bigger crystals without increasing sample consumption, which can reduce issues with clogging.

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Fixed target tools to address sample environment and delivery hurdles in room temperature serial crystallography

Shelby, M.L.,[1] Liu, Z.,[2] Gu, K.K.,[2] Narayanasamy, S.R.,[1] Jernigan, R.,[3] Kashipathy, M.,[3] Lyubimov, A.,[4] Botha, S.,[3] Bowman, S.,[5] Russi, S.,[4] Cohen, A.E.,[4] Poitevin, F.P.,[6] Hunter, M.S.,[6] Batyuk, A.,[6] Fromme, P.,[3] Coleman, M.A.,[1,2] Kuhl, T.L.,[2] and Frank, M.[1,2]

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Sample delivery remains a massive, frequently limiting challenge for XFEL and synchrotron-based room temperature serial crystallography, especially where applied to samples that are difficult to purify and crystallize in large quantities or prove unstable under conventional jetting conditions. This is largely due to several conflicting requirements: sample environment maintenance for optimal sample stability, minimal contribution to background, and the need to constantly refresh the microcrystalline sample into the X-ray beam path. A fixed target sample delivery approach provides key solutions by lowering sample consumption and maintaining a hydrated environment while allowing greater flexibility in sample distribution and handling. In recent years we have demonstrated highly adaptable all-polymer microfluidic chips for on-chip sample crystallization, delivery, encapsulation, and support in both synchrotron and XFEL serial diffraction experiments. Importantly, this approach removes common obstacles to SFX data collection by significantly reducing the sample volume required and the need for in-jet stability, providing a robust, flexible, low-background approach to protein SFX.

Advancing sample delivery for LCLS experiments

Mark Hunter

The high spatiotemporal resolution afforded by LCLS has enabled many new and exciting studies in the biological sciences. With the upcoming upgrades to LCLS-II and LCLS-II-HE, the facility continues to push the boundaries for our x-ray delivery, increasing average brightness and enabling experiments utilizing tender x-rays as two examples. For many of our science programs, however, careful attention must also be given to the state of the sample for the experiment and how we introduce it to the x-ray beam. As a result, the R&D program at LCLS is working on the design, testing, and deployment of novel and advanced sample characterization and delivery methods and hardware to support our Science program. Sample recirculation systems with integrated diagnostics, liquid sheet jets, as well as automated droplet systems are at different stages of development and show promise for different experiments at LCLS. These efforts will be introduced with a discussion of next steps and plans with a particular focus on opportunities for the community to engage and collaborate in our efforts.

Nano-drop Fluctuation X-ray Scattering

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Nearly fifty years ago Zvi Kam proposed that short x-ray exposures – shorter than a particle's rotational diffusion time – would contain fluctuations with sufficient structural information to enable direct 3D computational imaging [1]. Kam's proposal avoids a priori modeling and cryogenic cooling or crystallizing samples, but the ultra-bright ultra-short pulses required have only recently been achieved through the advent of the X-ray Free Electron Laser. While the technique has been demonstrated on large samples (e.g., icosahedral viruses in a liquid jet [4]), there has not yet been a convincing demonstration of Fluctuation X-Ray Scattering (FXS) signals from proteins that do not form large complexes. We propose nano-drops to overcome the limiting factors [3] faced by previous efforts. Here we report on the signal-to-noise ratio improvements provided by nano-drops, reduced sample consumption, and our progress towards reproducible generation of nano-drops.

Acknowledgement

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Sample Characterization and Laboratory Support for time-resolved studies of biomolecules at the European XFEL

Schmidt, C.[1] and the Sample Environment & Characterization group (SEC) at the European XFEL[1]

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The study of ultrafast time-resolved structural changes of biomolecules at free electron lasers entails special sample production and delivery requirements that remain a bottleneck for successful experiments for new and experienced crystallographers.

To enable successful user experiments, the Sample Environment & Characterization group (SEC) at the European XFEL offers a well-equipped bio-/physics- and chemistry lab infrastructure. We provide extensive user support for sample preparation, characterization, quality-control and delivery. We offer specialized equipment for sample characterization, such as DLS, SONICC, and TEM. Moreover, a sample delivery test jetting station helps to save valuable beamtime.

As there is an ongoing need for improving sample delivery methods as well as data analysis of diffraction images for serial crystallography and time-resolved experiments, our group is running several development projects that aim to provide better methods and instrumentation for sample preparation, characterization, quality control and delivery at the instruments. Different examples are presented on this poster, as well as an overview about the sample preparation and characterization strategies.

Bioscience opportunities with LCLS-II and LCLS-II-HE

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X-ray free electron lasers such as the LCLS have been revolutionary in structural biology, allowing unique experiments on diverse biological targets. LCLS is currently undergoing major upgrades (LCLS-II and LCLS-II-HE) that will dramatically increase the flux of ultrashort soft- and hard-x-ray pulses by extending the repetition rates from currently 120 Hz to up to 1 MHz. Along with upgrades of the accelerator and the development of advanced beam modes, a new suite of instruments will enable studies of matter on atomic time and length scales in unprecedented detail. I will report on several of these upgrades that will impact our ability to conduct bioscience at LCLS. The applications range from crystallography to imaging with powerful complimentary components in spectroscopy that will be discussed in the broader context of facility progression.

ID29: a time-resolved serial crystallography beamline offers new opportunities at the EMBL–ESRF

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The European Synchrotron facility – ESRF has recently been upgraded to Extremely Brilliant Source (EBS) in 2020 [1]. EBS-ESRF storage ring produces an extremely low horizontal emittance and 10^3 times more brilliant than 3rd generation sources, paving the scope and nature of Macromolecular X-ray crystallography (MX). After EBS upgrade, the highly focused and intense X-ray beam bridges the gap between any 3rd generation synchrotron and X-ray free electron lasers (XFELs), enabling data collection from micron-sized biomolecular crystals. Following the advances in serial crystallography at the XFELs [2], ESRF identified 8 scientific themes to address after EBS-upgrade. This includes development of a dedicated time-resolved serial crystallography (TR-SSX) beamline. Thus, old ID29, a formerly experimental phasing microfocus MX beamline [3], was entirely re-built to World's first of its own kind of beamline towards µs-time-resolved serial crystallography. The beamline produces 10-µs pulsed X-ray beam with a continuous flux of ~10^{15} photons/sec and equipped with multi-layer monochromator resulting in 1% bandwidth. ID29 – TR-SSX beamline enables to explore the uncharted territory between 3rd generation source and XFEL. Two chopper systems produce the pulsed X-ray beam synchronized with the reference

frequency of the storage ring. ID29 is equipped with KB-mirrors to produce 1 µm X-ray beam, state-of-the-art MD3up-SSX diffractometer, a nanosecond tunable high-repetition laser, and Jungfrau 4M detector, enabling data acquisition rate of 925 Hz.

In the talk, new ID29 beamline together with developments in sample-delivery, data acquisition system, time-resolved setup will be introduced and opportunities of various SSX data collection at the ID29 will be highlighted.

Reference:

[1] Raimondi, ***J. Synch. Rad.*** 2016
[2] Chapman *et al.,* ***Nature***, 2011
[3] de Sanctis D., *et al.,* ***J. Synch. Rad.***, 2012

CXFEL Labs – New Horizons for Ultrafast X-ray Science

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The advent of X-ray free electron lasers (XFELs) has spawned powerful new means to probe the structure and dynamics of complex matter on intrinsic scales, enabling new discoveries across molecular biology, atomic and molecular physics, quantum materials, and other scientific fields. While current XFELs entail large kilometer-scale facilities, ASU has embarked on a path to develop new *compact* accelerator-based sources of ultrashort X-rays, whose much smaller footprint and cost make them accessible to a university campus as well as to medical, defense, or industrial laboratory settings. In this talk, I will overview the development of these compact sources, which harness interactions between electrons and intense infrared light pulses for X-ray generation. Here, the Compact X-ray Light Source (CXLS) employs inverse Compton scattering to provide femtosecond incoherent hard X-ray pulses in the 2–20 keV range. Having produced first X-rays in early 2023, CXLS is undergoing commissioning and will provide capabilities for macromolecular crystallography, phase-contrast imaging, and ultrafast materials studies. In turn, the next-generation Compact X-ray Free Electron Laser (CXFEL) has started construction and will build on the latest advances in laser technology and electron beam physics to produce a room-sized X-ray laser with full coherence in time and space. CXFEL employs electron-beam patterning for control of X-ray time structure and bandwidth, enabling durations of few femtoseconds down into the attosecond regime, multi-pulse sequences, and high pulse-to-pulse stability. The capabilities of this fully-coherent soft-X- ray laser source will enable completely new pathways to directly observe, manipulate, and control quantum dynamics and fundamental processes in complex matter. Once operational, CXFEL will serve and expand a large user community in advancing the frontiers of biology, AMO, and quantum materials along with other fundamental and appliedsciences.

Acknowledgment: This work is carried out together with W. S. Graves, P. Fromme, M. Holl, S. Teitelbaum, A. Sandhu, and numerous collaborators at ASU and partnering institutions. Construction of CXFEL is funded by the National Science Foundation (NSF) via the Mid-scale RI-2 program under Cooperative Agreement No. 2153503. CXFEL design was supported by NSF Grant No. 1935994.

Anaerobic crystallography, *in situ* UV-vis spectroscopy and Serial crystallography of proteins

Marchany-Rivera, Darya[1] representing the entire SSRL-SMB team.

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UV-Visible Absorption microspectrophotometry (UV-Vis AS) can characterize color changes within crystals to examine ligand binding, electronic states and reaction rates. A vast number of biomolecules contain metal centers that are key for their biological function. Accurate determination and assignment of metal oxidation states both before and during x-ray diffraction data collection (especially for radiation sensitive metals) is important to derive correct conclusions about structure-function relationships. To minimize radiation-induced structural artifacts, UV-Vis AS is useful in and in developing data collection strategies. In addition to x-ray exposure, in some cases the oxidation state is affected by environmental conditions such as oxygen sensitivity, for which anaerobic environments can be crucial for appropriate structure interpretation. By observing reactions within single crystals, UV-Vis AS can help optimize experimental design for time-resolved crystallography, including use of serial crystallography methods at XFELs and serial synchrotron beamlines. We offer the capability to measure UV-Vis spectra from single crystals *in situ* at SSRL beamline BL9-2 and in the laboratory, as well as offering anaerobic setups for crystal growth, characterization and mounting. Practical applications of the technologies to study specific protein systems will be described, including methyl coenzyme-M reductase (MCR), myoglobin (Mb) and isocyanide hydratase (ICH).

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Capabilities and developments at the SPB/SFX Scientific Instrument at the European XFEL

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The unique capabilities of X-ray Free Electron Lasers (XFELs), and especially high repetition-rate XFELs, offer an unprecedented ability to generate large quantities of serial crystallography data. At the European XFEL (EuXFEL), the megahertz repetition rate and the very short and ultra-bright X-ray pulses provide a fertile ground for the development and usage of innovative experiment designs and methods. The Single Particles, Clusters, and Biomolecules & Serial

Femtosecond Crystallography (SPB/SFX) scientific instrument is dedicated to conducting SFX and single particle time resolved experiments, by providing optimised configurations exploiting the unique EuXFEL capabilities. At the same time, the instrument provides the flexibility and setups for new developments. This presentation will give an overview of the capabilities of the SPB/SFX scientific instrument and associated user facilities at EuXFEL. In addition, some of the science performed to date will be presented, focusing on SFX and including megahertz repetition rate serial crystallography, time-resolved studies, as well as new methods. An outlook to future capabilities and upgrades will also be shown.

Closing the Gap - Integrated Time-Resolved Crystallography at the SwissFEL and Swiss Light Source

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The SwissFEL currently provides the hard X-ray endstations Alvra, Bernina, and Cristallina. Especially Alvra is a forerunner in the crystallographic community, having successfully performed many serial femtosecond crystallography experiments, using both, high-viscosity extruders (HVE) and GDVN jets as the sample delivery method [1-3]. With the recently commissioned SwissMX experiment based at Cristallina, there is now also a dedicated setup for fixed-target experiment, both in high-throughput and pump-probe mode, with a mixing setup on the horizon. However, available beamtime at FELs is sparse and entry barrier for new teams is high.

To alleviate these issues and close the probe-time-gap in the millisecond-to-second regime, we build the VESPA endstation at the Swiss Light Source (SLS), dedicated to multi-time-resolved serial millisecond crystallography [4], acoustic levitation goniometry [5], and kilohertz data acquisition serial crystallography [6]. The latter allowed us to push the achievable time resolution at a synchrotron source to microseconds, without the need for choppers. In combination with different pump methods, including dedicated *cw* and nanosecond Lasers, as well as temperature control, and ligand mixing, this will enable our research community to investigate an even larger array of protein samples.

We will introduce the recently formed PSI focus team for time resolved crystallography, which is dedicated to facilitating easy access to facilities and instruments, as well as providing training and support for research teams interested to get into the field. We will also present an overview of available techniques and expertise, including results from our experimental portfolio, at both SwissFEL and the SLS, and will present an outlook on novel techniques and instruments, especially in light of the upcoming SLS 2.0 upgrade.

DAY THREE CONFERENCE SPEAKER ABSTRACTS

Heterogeneity in the *Mycobacterium tuberculosis* β-lactamase inhibition by Sulbactam

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For decades, researchers have been determined to elucidate essential enzymatic functions on the atomic lengths scale by tracing atomic positions in real time. Here, we report in atomic detail and real time the mechanism of enzyme - ligand binding for *Mycobacterium tuberculosis* β-lactamase (BlaC) inhibition by sulbactam. Our work builds on new possibilities unleashed by mix-and-inject serial crystallography (MISC) at X-ray free electron laser (XFEL) facilities. In this approach,

enzymatic reactions are triggered by mixing substrate solution with enzyme crystals. The reaction of the BlaC with the cephalosporin antibiotic substrate Ceftriaxone was previously investigated with 5 ms time resolution. Here, we investigate the BlaC reaction with the irreversible inhibitor sulbactam (SUB) with a similar time resolution using mix-and-inject serial crystallography. A total of six different timepoints spanning 3 ms to 700 ms were collected. Our results reveal changes along the reaction pathway, detailing how SUB approaches the catalytic cleft, binds to the enzyme non-covalently before forming a trans-enamine.

We also observed substrate tunnelling-and-gating like mechanism which has not yet been discovered in any published structures of BlaC. This results in different reaction rate coefficients for different monomers in the asymmetric unit although the monomers are identical. Within the four subunits (A-D), the access to active site of subunits B and D is wide open while that of subunits A and C is occluded. Two residues (which we call the guardian residues) parametrize the opening and closing of the access to the active sites in A/C. This lowers the velocity of β-lactamase reaction with sulbactam. At 30ms after reaction initiation, the sulbactam in subunit B/D already reacts with Ser70 to form a covalently bound trans-enamine, while the intact sulbactam persists in subunits A/C. The results give insight how the shape of the active site determines rate coefficients and reaction mechanisms of a biomedically relevant reaction.

By using a newly developed singular value decomposition (SVD) program that remains functional even if unit cell parameters change during the reaction, the kinetics of the inhibitor binding was extracted from the XFEL data. From the MISC data, diffusion times and rate coefficients can be estimated by applying reasonable, chemically meaningful constraints and tying the analysis to observed occupancy variations of the enzyme-inhibitor complexes in the different subunits. In addition, we have characterized the time dependent concentration of reacting species inside enzyme crystals along the reaction coordinate by factoring the results of SVD analysis, occupancy values of ligands and the gating mechanism.

This work is led by Prof. Marius Schmidt (m-schmidt@uwm.edu) and his lab at University of Wisconsin-Milwaukee in collaboration with Cornell University, Northeastern Illinois University, Rice University, Arizona State University, and Linac Coherent Light Source.

Supporting Deposition of XFEL/SX Structures at the Protein Data Bank

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The Protein Data Bank (PDB) was established in 1971 as the first open-access digital data resource in biology with just seven X-ray crystallographic structures of proteins. Today, the single global PDB archive houses more than 200,000 experimentally-determined three-dimensional (3D) structures of biological macromolecules that are made freely available to millions of users worldwide with no limitations on usage. This information facilitates basic and applied research and education across the sciences, impacting fundamental biology, biomedicine, biotechnology, bioengineering, and energy sciences. The Worldwide Protein Data Bank (wwPDB, wwpdb.org) consists of organizations (RCSB PDB, PDBe, PDBj, BMRB, and EMDB) that jointly manages the PDB, EMDB, and BMRB Core Archives and is committed to making data Findable, Accessible, Interoperable, and Reusable (FAIR).

Consistent year-on-year growth in the number of structures coming into the PDB from X-ray Free-Electron Laser (XFEL) facilities has been observed. Current PDB holdings include 700+ XFEL structures. The XFEL development has significantly boosted Serial X-Ray Crystallography (SX) study on dynamic protein structure changes. Although most of SX structures in the PDB were resolved with XFEL data, there are also ~300 SX structures resolved from diffraction data collected on conventional synchrotron light sources. To help archiving the XFEL and SX structures and their metadata, wwPDB has extended data model in the PDBx/mmCIF Dictionary with new data categories/items that support metadata collection for XFEL structures deposited via wwPDB OneDep structure deposition, validation, and biocuration system. We are also working on extending data model of the PDBx/mmCIF Dictionary for the inclusion of unmerged XFEL/SX diffraction data and the parameters describing reflection data collection and process.

Here we will present an overview of statistics, features, and classification within the PDB archive and deposition, validation and biocuration of XFEL/SX structures with wwPDB OneDep system (https://deposit.wwpdb.org/). We will also present metadata collection specific to XFEL/SX experiments such as light source, sample delivery, and reflection data processing. wwPDB is committed to working closely with federal funders and XFEL/SX users to ensure faithful preservation and representation of their structures, experimental data, and metadata in the PDB archive. We are actively seeking collaboration with XFEL/SX software development groups to enable log data parsing in order to facilitate PDB deposition.

RCSB PDB is funded by the National Science Foundation (DBI-1832184), the US Department of Energy (DE-SC0019749), and the National Cancer Institute, National Institute of Allergy and Infectious Diseases, and National Institute of General Medical Sciences of the National Institutes of Health under grant R01GM133198.

Multiscale femtosecond spectroscopy as complementary tool for time-resolved serial crystallography

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Time-resolved serial crystallography at synchrotrons and XFELs has paved the way for detailed studies of structural changes during biochemical reactions over many orders of magnitude in time and enabled real multiscale experiments in structural biology.

However, many time-resolved SFX experiments rely on light as trigger for the reaction, either because a light-sensitive system, like fluorescent or retinal proteins, a light-induced reaction or ligand dynamics, like in Heme proteins or a photocaged substrate are used.

For all those cases, a detailed spectroscopic investigation of the dynamics is necessary prior and in parallel to XFEL experiments. Multiscale optical spectroscopies can not only provide guidance for crucial timepoints of the reaction, but also are useful to study laser-induced damage processes.

We are working on the characterisation of photocaged compounds and expand to use similar sample delivery for ultrafast transient UV/VIS and IR than in SFX/SSX experiments (so far fixed targets, jets planned).

Testing Deep Learning-based Predictions of Electron Density Maps from Input Patterson Maps.

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We have been testing the ability of deep learning to predict electron density maps given the Patterson map. Hurwitz (DOI:10.48550/arXiv.2003.13767) reported success in determining the positions of 10 randomly positioned, identical atoms from their Patterson maps via a convolutional neural network. We have conducted trials using atom arrangements extracted from short peptides. After training U-Net and 3D vision transformer-based machine learning models implemented in the Torch Python framework, we found that we can predict a low-resolution electron density estimate from the Patterson maps. The histogram of the predicted phase error is more accurate than the random distribution. From a recent trial, we estimate that roughly 85% of the output maps were accurate enough for building a startling model to start refinement of the peptide. We are looking at how far various training approaches can take us. Towards that end, we are exploring ways to expand the to account for symmetry and are continuing to make our training and test model data more like actual crystal data.

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*Presenting author

X-ray spectroscopy at LCLS: elucidating the electronic and structural dynamics of metalloproteins

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The study of metalloproteins has greatly benefited from the advent of X-ray free electron lasers (XFELs) in the last decade. Metalloproteins, containing metal centers that efficiently catalyze chemical processes under ambient conditions, are found widely in nature. Synchrotron radiation (SR) sources worldwide rely on X-ray diffraction (XRD) as a standard technique for determining the geometric structure of crystallized proteins, while X-ray spectroscopic techniques provide information on the electronic structure of the active site. However, despite the great progress and maturity of the field, experiments using SR-based measurements face challenges, mainly due to radiation-induced damage caused by X-rays, which can make it difficult to collect a signal from intact samples, especially those that are sensitive to radiation.

In contrast, at XFELs, the fs X-ray pulses are short enough to probe the sample and generate a detectable signal that outpaces radiation damage at the ps scale typical of synchrotrons. Consequently, cryogenic conditions are no longer needed and sensitive systems can be studied under ambient functional conditions, making it possible to observe catalytic

reactions and associated electronic and geometric changes in real-time with fs time resolution. In this presentation I will describe the methods developed at the Linac Coherent Light Source (LCLS) in recent years for studying metalloproteins with a focus on the advances in hard X-ray spectroscopy techniques and instrumentations. X-ray Absorption Spectroscopy can be used to study the ultrafast photoinduced electronic and geometric changes occurring in the metal center in metalloproteins. Additionally, X-ray emission spectroscopy can serve as an in-situ method for monitoring the integrity of metal catalytic centers and to follow their electronic structure dynamics while collecting XRD data simultaneously. Finally, I'll also introduce future spectroscopic developments projected for the near and mid future of LCLS including new instrumentation and opportunities in the soft and tender X-ray range.

POSTER ABSTRACTS

Cardiolipin- Based Nanocarrier for effective delivery of Cytochrome c in Cancer Cells

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It is imperative to develop effective strategies to address current cancer treatment challenges. The current classes of anticancer drugs available on the market feature rich structural diversity but exhibit a narrow spectrum of effects and limited timespan of application due to toxicity, side effects, and acquired resistance. The lack of specificity and flexibility to withstand modifications without loss of activity of small molecule drugs has led to increased interest in developing cytotoxic protein drugs because they could be readily modified given their large size and multiple potential attachment points. Cytochrome c (Cyt c) is one such protein that has attracted the attention of research groups due to its potential to be developed into a potent and selective anticancer drug. Cyt c, a multifunctional protein, is crucial to regulate the life and death decisions of the cell. Apoptosis inhibition cancer cell hallmarks have been described in many cancer cells: proteins that regulate the outer mitochondrial membrane permeability for Cyt c release are rendered dysfunctional by mutations, which decreased levels of Cyt c in the cytoplasm. Therefore, this project aims to introducing Cyt c from an external source into the cytoplasm of cancer cells to overcome the upstream blockage caused by mutations in the

apoptotic pathways. However, like most other proteins, Cyt c cannot enter the cell making its delivery to the cytoplasm quite challenging. Lipid nanoparticles confer stability and protection to therapeutic proteins and nucleic acids, with applications ranging from cancer treatment to vaccine delivery. While it is hypothesized that cardiolipin (CL), a mitochondrial lipid that facilitates the activation of Cyt c's apoptotic activity by Cyt c detachment from the inner mitochondrial membrane and its release from the mitochondria to the cytoplasm, we aim to leverage the native interaction between CL and Cyt c by producing CL-based nanoparticles carrying Cyt c, a delivery system that preserves the proapoptotic activity and structure of Cyt c. To test, we prepared CL-based nanoparticles carrying Cyt c, characterized the Cyt c-CL formulation and evaluated the cytotoxic properties of the nanoparticle. Using UV-Vis, ^{1}HNMR, DLS and SEM we demonstrate the existence of the Cyt c-CL formulation of interest, in comparison to data from the free Cyt c and CL. Furthermore, the Cyt c-CL demonstrate high potency and broad spectrum antiproliferative behavior against the A549 cancer cell line. Additionally, fluorescent microscopy measurements have been preferment to understand Cyt c-CL antiproliferative behavior against MDA-MB-231 cancer cells. This project's long-term goal is to create an innovative broad-spectrum drug strategy for hard-to-treat cancers.

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Mitigating Oxidative Stress via p47 Interaction with Plasma Membrane

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The overall focus of this project is the structure of the PX domain of p47 and its binding to potential inhibitors. p47 is one protein within the NADPH oxidase 2 (NOX2) enzyme complex. This peripheral membrane protein produces reactive oxygen species, compounds that can trigger apoptosis. Oxidative stress induced diseases include cancer and cardiovascular disease. Determining molecules that block p47 activation would be the first auspicious step in the development of an anti-cancer drug.

Structural studies of the PX domain of p47 were conducted using both X-ray crystallography and NMR spectroscopy techniques. Using this data, fragment screenings for ligand binding were performed. The principles of fragment-based drug design were implemented in this study.

Several fragments show strong binding to p47 (K_d < 1 mM). These ligands will be studies and developed further to yield more energetically favorable and efficient binding. Once their interactions with p47 are fully characterized, novel ligands will be synthesized and tested for inhibition ability.

Survey of Potential Sample Delivery Problems with CXFELs

Emmanuel Aneke

Compact X-ray Free-Electron Lasers (CXFELs) are a promising technology for generating intense and bright X-ray laser beams in a smaller and more affordable package than traditional XFELs. One application of XFELs is Serial Femtosecond Crystallography (SFX), a technique for studying the atomic structure of proteins and biomolecules. In SFX, sample delivery refers to the process of delivering a stream of small crystals of the protein or biomolecule of interest into the path of the X-ray laser beam. CXFELs may face some challenges in sample delivery for SFX-style experiments. These challenges include low repetition rates, less stable X-ray beams, radiation damage, cooling requirements, and compatibility issues with existing delivery systems. I will cover current state of the art sample delivery at XFELs and other light sources that might be useful next steps for sample introduction to CXFELS.

Improved Aerosol Delivery with Electrospray with the use of an optimized convergent nozzle

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Single-Particle Imaging (SPI) and Fluctuation X-ray scattering (FXS) may become powerful techniques of the future for studying the structural dynamics of biological macromolecules without crystallization or cryogenic freezing. These techniques require ultra-bright X-Ray free electron lasers (XFELs) and consequently efficient sample delivery methods to position either isolated molecules (SPI) or liquid nanodroplets (FXS) within nano-focused x-ray beams.

Conventional approaches for aerosol injection at XFEL sources use a differential Aerodynamic lens stack with a preceding skimmer stage to reduce the background gas scattering noise. In literature, the use of a 100-micron convergent nozzle allows for aerosol injection without the need for a differential skimmer. The convergent nozzle produces a tight beam of nanodroplets with low divergence and high density, suitable for FXS or SPI experiments. However, nozzle clogging is one of the challenges that limits the reliability of this aerosol beam. Also, the particle ejection speed limits the hit rate.

We present simulations that agree with the experimental beam profile and suggest ways to optimize the nozzle design and operation. We also demonstrate the feasibility of using electrospray instead of gas dynamic virtual nozzle (GDVN) with a nebulizer as the aerosol source to reduce the nozzle clogging problem and allow for a more reliable sample delivery source. The transmission efficiency of aerosol delivery is reduced due to charge repulsion, image charge effects and evaporation effects. To overcome this limitation, we are developing a novel approach that combines electrospray with a convergent nozzle near the source to enhance the aerosol density and hit rate for FXS and SPI experiments. Since the aerosols are charged, they can be slowed down using electrostatic fields to increase particle density.

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NMR-Based Structural Biology Laboratory at the University of Puerto Rico and the Molecular Sciences Research Center

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This presentation highlights the structural biology research projects supported by the nuclear magnetic resonance (NMR) laboratory of the Molecular Sciences Research Center (MSRC) and the University of Puerto Rico, Rio Piedras Campus. NMR spectroscopy is a powerful technique for the analysis of macromolecular structure, dynamics, and interactions. The MSRC NMR Laboratory houses a 700MHz spectrometer with both solution and solid-state capabilities and a 500MHz spectrometer equipped with a cryoprobe for high-throughput solution NMR analysis. In collaboration with local and non-local researchers, we apply solution and solid-state NMR to a range of systems where insight on structure and dynamics is critical. Examples include HIV protein assemblies, nanocrystalline proteins, protein-inhibitor interactions for anti-cancer therapeutic development, nanoparticle drug-delivery formulations, protein-loaded liposomes, RNA aptamers, and biomineralization assemblies. NMR analysis is combined with complementary techniques also available at the MSRC, including scanning electron microscopy, X-ray diffraction, optical microscopy, and protein synthesis and purification. We showcase examples of these applications and present opportunities for further collaborations with the scientific community in Puerto Rico.

Elucidation of a new pharmacological strategy against cancer through the inhibition of ribonucleotide reductase by titanium (IV).

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Current anticancer drug development efforts focus on targeting specific molecular pathways that enable cancer cells to survive. Given the role of iron in cancer growth and metastasis, reducing intracellular iron bioavailability may lead to the development of a broad spectrum of these anticariogenic agents against iron dependent enzymes. This investigation is intended to accomplish this by inhibiting the activity of the R_2 subunit of the ribonucleotide reductase (RNR), the enzyme that produces the building blocks of DNA replication. E. coli was used as the basis for designing an efficient protocol for histag-RNRR2 expression and Nickel-NTA column purification. Using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI TOF techniques, it has been determined that R_2 has a molecular weight of 47 kDa, which matches the weight of the protein plus the His tag. Minimum concentration of 25 µM of the apo protein is required to assemble of the tyrosyl radical-dinuclear iron cluster cofactor of RNRR2.The addition of excess Fe(ll) (at least five equivalents) and must activate the reaction with an oxygenated buffer that is already saturated with oxygen to ensure that all the iron is bound completely. This work explores the idea that metal ions of Ti(IV)-cTfm compounds contribute to RNR inhibition by directly coordinating with diiron cofactor sites. This Ti(IV) coordination is expected to have sufficient affinity to effectively block Fe binding, because it is a redox functionally inactive metal, it will not be able to activate RNR by adding a second dimension of inhibition. To demonstrate the affinity and inhibition of Fe by Ti(IV), transmetallation studies were carried out using UV-Vis spectroscopy adding different proportions. To determine the concentration of the metals bound to R_2, an analysis by inductively coupled plasma mass spectrometry (ICP-MS) has been carried out.

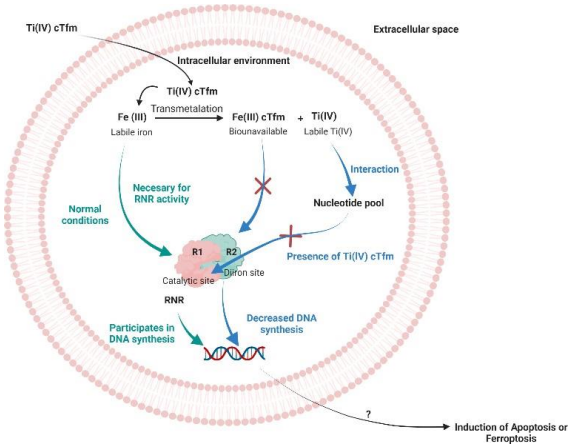

Figure 1. cTfm strategy, where the cTfm ligands release Ti(IV) and then capture Fe(III) via a transmetalation process in cancer cells. The cytotoxic effect combines the decrease in bioavailable Fe with Ti(IV) binding to intracellular targets.

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Synthesis and Characterization of Chemical Probes to Elucidate the Mechanism of HIV-1 Maturation Inhibitors

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The principal focus of our research is the inhibition of Gag maturation, a main protein of the HIV-1 virus. The Gag domains MA-CA-SP1-NC-SP2-P6 form assemblies with various roles in its retroviral replication. The protein first self-assembles into an immature virus particle. For the maturation of the virus, proteolytic cleavage of Gag is followed by a major structural rearrangement that prepares the virus particles for infection. Some antiretroviral drugs work against virus binding, fusion, reverse transcription, integration, budding, and proteolytic cleavage, but none yet against maturation. Each of them focuses on a step, process, or enzyme that is part of a cycle. On the other hand, the small molecule antiretroviral drug Bevirimat has been shown to bind between the CA and SP1 domains during the viral cycle, blocking the access of the enzyme interacting with Gag. Bevirimat (BVM) is the first-in-class maturation inhibitor. Nevertheless, BVM clinical trials were halted due to reduced effectiveness in almost half of the patients. Ongoing research efforts are based on modifications to the molecule due to its antiretroviral promise. Studies have shown that substitutions at carbon 28 increase the antiretroviral activity and drug-target interactions of the synthesized derivatives. Here, we outline our efforts in making such derivatives.

Time-Resolved Enzymatic Structure Elucidation Enabled by 3D-Printed Droplet Microfluidics for Reduced Sample Consumption During Serial Femtosecond Crystallography

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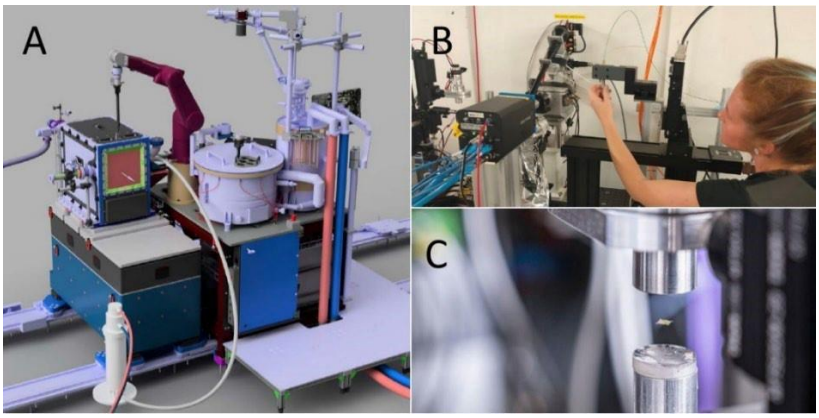
Resolving the catalytic mechanism of enzymes at atomic resolution has been the principal goal of structural biologists and recent developments of time-resolved serial femtosecond crystallography (TR-SFX) have provided insights for resolving these mechanisms. One such enzyme is NQO1, a flavoenzyme overly expressed in tumors found in the thyroid, breast, and lung, making this mechanism interesting for quinone-based chemotherapeutics. However, NQO1, like many proteins of interest, are difficult to purify and crystalize in large quantities. As a result, delivering crystals of these precious proteins to an XFEL using traditional continuous injection techniques, like gas dynamic virtual nozzles (GDVNs), can vastly limit the application of these instruments for structure determination due to the large amount of protein required. To solve this major challenge of sample consumption, we've designed droplet injector devices (DID), capable of generating and stimulating protein crystal laden droplets segmented by an immiscible oil before injecting the sample into the XFEL via a 3D-printed GDVN. Integrated with a unique electrical triggering feedback mechanism, these devices enabled the successful tuning of the droplet generation frequency and delivery of protein crystals to the Linear Coherent Light Source (LCLS) XFEL, solving the first room temperature structure of NQO1 to 2.7 Å with 75% less sample waste than classic injection. [1] Furthering the applicability of these novel DIDs, we improved the droplet generator by increasing the channel dimenions from 100 to 150 µm to produce droplets at 10 Hz compatable with the European XFEL (EuXFEL) pulse structure. Two designs were made DID-A and DID-B, where the dispersed aqueous channel in DID-B was kept at 100 µm leading to droplets nearly half the size of DID-A, further conserving sample at 10Hz. Additional improvement of the flow rate stability at rates <5 µL/min using a syringe pump resulted in 10 Hz droplet generation for periods of several hours in the vacuum

droplets nearly half the size of DID-A, further conserving sample at 10Hz. Additional improvement of the flow rate stability at rates <5 µL/min using a syringe pump resulted in 10 Hz droplet generation for periods of several hours in the vacuum chamber of the SPB/SFX instrument. The increased flow stability also eliminated the need for a complex feedback mechanism and instead only a continuous electrical trigger was needed for the alignment of droplets in time with the XFEL. When DID-B was combined with an integrated mixer (M-DID-B), we achieved 10 Hz droplets while conducting time-resolved mixing experiments with NQO1 crystals and the substrate NADH. DID-A and M-DID-B were employed at EuXFEL in 2022 (P3083) and during three sixteen-hour shifts, we consumed less than half a mililiter of NQO1. Furthermore, we collected 37,000 diffraction patterns from the apo NQO1 structure and over 40,000 indexable patterns from a 264ms time point upon mixing NQO1 with NADH. Based on this data set, we have determined the structure of NQO1 in complex with its substrate NADH at 2.6 Å, which exhibits the first structural evidence of the reduction of the NQO1 enzyme byNADH.

Figure 1: (A) The SwissMX fixed target module for the Cristallina endstation. (B) HVE mode at VESPA at SLS. (C) Acoustic levitation goniometry using spinning discs.



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An easy-to-use polymeric fixed target chip for serial crystallography at XFELs

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Efficient sample delivery is the keystone to successful macromolecular experiments at both X-ray free electron laser (XFEL) and synchrotron sources, with ideal delivery systems allowing for fast data collection, low sample consumption, and low

background contributions. Fixed target methods have been shown to check these boxes, while minimizing sample handling and providing the option for in-situ crystallization. However, current fixed-target systems are difficult to fabricate or modify, with specific emphasis on silicon or polymer-based systems that require difficult etching and other clean room technology.

Here, we present a novel design for fixed target sample delivery using a fully polymer based microfluidic chip that is highly modifiable for a variety of experiment types, protein types, or beamline requirements. The chip demonstrates an easy-to-use, easy-to-fabricate, inexpensive, and stable system with low background scatter suitable for both SFX and SSX. The sample imaging window is composed of 2-5µm thick cyclic olefin copolymer (COC) with a maximum continuous imaging area of 3mm x 30mm. The design is highly modifiable, with alterations to chip and window dimensions, flow layer thickness, and window material type possible to best match the sample or beamline requirements. Crystals of two model proteins, lysozyme, and thaumatin were used to validate the design's effectiveness at a synchrotron and XFEL (XFEL, lysozyme only), yielding complete datasets with resolutions of 1.42, 1.48, and 1.70 Å (XFEL), respectively. A full lysozyme dataset was obtained at XFEL using one chip and ~25 minutes of data collection.

A Mathematical Algorithm for Estimating the Two-Dimensional Rotational Diffusion Coefficient from X-ray Photon Correlation Data

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X-ray Photon Correlation Spectroscopy (XPCS) is a promising experimental technique for investigating disordered systems (see Figure 1). This approach involves illuminating the sample with coherent X-ray beams, and then recording the scattered images. The heterogeneity within the sample causes spatial variation of the image brightness, known as speckle. Fluctuations within the sample result in temporal variations of the speckle patterns, which can be analyzed to extract valuable information about the sample dynamics. The typical analysis of XPCS images involves calculating the temporal autocorrelation between the collected images.

Studying diffusion is fundamental in scientific domains like molecular biology and materials science. Diffusion can be broken down into two main components: translational and rotational. The translational diffusion coefficient and rotational diffusion coefficient characterize the magnitude of each component respectively. While the translation diffusion coefficient can be predicted from the temporal autocorrelation, there was previously no algorithm using XPCS data to estimate the rotational diffusion coefficient, which is essential for understanding protein dynamics, material synthesis, and more.

To address this gap, we have developed a numerical methodology for predicting the rotational diffusion coefficients in two-dimensional cases based on XPCS data (Hu, 2021). Our approach involves using the angular-temporal cross-

correlation of the images to generate a 4-way data tensor. To estimate the rotational diffusion coefficient from this tensor, we developed a numerical algorithm called "Multi-tiered Estimation for Correlation Spectroscopy (MTECS)." This algorithm breaks down the complex optimization problem into several sub-parts that can be efficiently inverted or approximated iteratively. We have tested this approach on simulated data. MTECS was able to estimate a range of rotational diffusion coefficiesavents with only a few percent relative errors. It can also filter the input cross-correlation (see Figure 2), as it incorporates additional filtering methods.

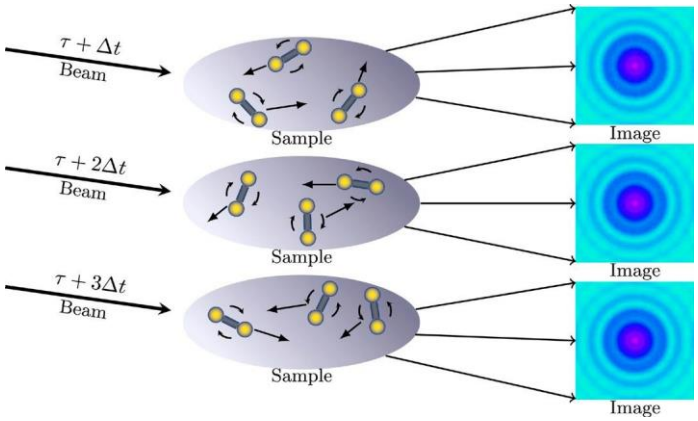

Figure 1: Schematic illustration of XPCS experiments

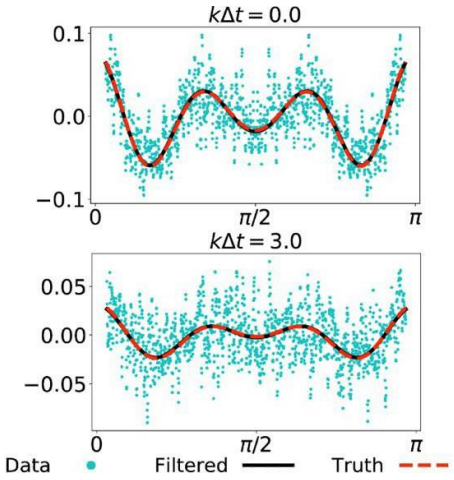

Figure 2: Examples of filtered data

"A novel GUI for serial data classification using Machine Learning approaches"

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The structure solution of biological structure is being revolutionized by serial X-ray diffraction employing X-ray free-electron laser (XFEL) sources, particularly the advent of serial femtosecond crystallography (SFX). The measurements of micrometer-sized crystals at room temperature allow time-resolved studies to trace the path of biochemical reactions at unprecedented temporal resolution, made possible by X-ray pulses that outrun the effects of radiation damage. These pulses can deliver X-ray doses that are more than a thousand times higher than those that are possible with conventional X-ray sources.[1] The large amounts of data produced by these studies (up to multiple TBs for a single experiment) must be quickly processed and analysed. While software for online data monitoring and data reduction have been designed over the past decade (e.g., OM[2], Cheetah[3], CrystFEL[4]), these are targeted towards finding crystal hits and not classifying data by spurious, often unquantifiable data artifacts. The state-of-the-art X-ray detectors are undergoing continuous development, and experimental parameters can push them beyond their reliable operating regime for individual frames

within a single run of data collection. Including intensities from these frames into the merged structure factors can lead to inaccuracies in the final reported intensities, particularly detrimental for anomalous phasing or time-resolved difference density calculations where highly accurate recordings are required.

Here, we report a new data sorting tool that offers a variety of Machine Learning algorithms to sort data trained on either manual sorting by the user, or by profile fitting the expected intensity distribution on the detector based on the experiment. This is integrated into an easy-to-use graphical user interface (*GUI*), specifically designed to support the detectors, file formats, and software available at the Linac Coherent Light Source (US) and the European XFEL (Germany).

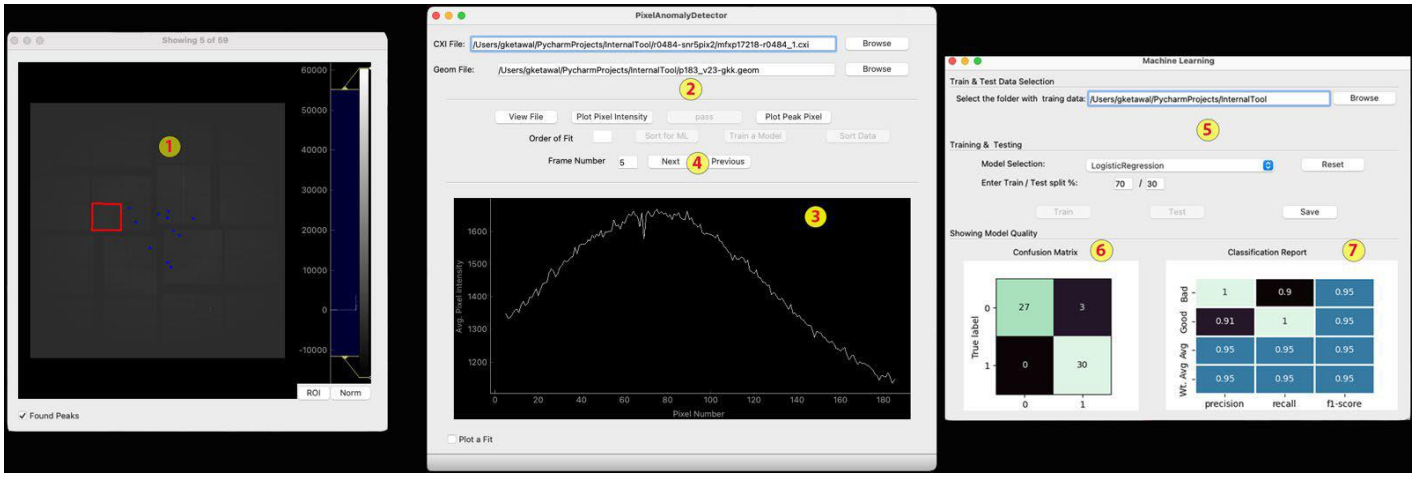

Figure 3. The user interface of the PixelAnomalyDetector. *The* loaded *image file* is displayed in the image viewer (1)*,* and a vertically averaged pixel intensity plot (3) is readily available in the *main window* (2). Seamlessly navigate through HDF5 files with the next and previous buttons (4). The *tool* offers a wide range of options to train your model, including model selection and customizable train-test splits (5)*, w*ith the ability to display model performance via a confusion matrix (6) and classification report (7).

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A Comprehensive and Automated Python-Based Pipeline for Microjet Characterization: Pushing the Boundaries of GDVN Performance

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Gas Dynamic Virtual Nozzles (GDVNs) play a crucial role in XFEL crystallography and solution scattering experiments, yet their comprehensive characterization has been limited due to the tedium and complexity of both data collection and analysis procedures. To address this issue, we have developed a versatile pipeline, written in Python, that streamlines the characterization of GDVN micro- and nanojets. The pipeline offers multiple usage examples, including image cleaning with

standard Python image processing libraries, jet speed measurement, jet stability assessments, and the capability to determine jet diameters with precision that is better than the microscope resolution.

In addition to image analysis code, the pipeline includes a simple high-level Python interface to popular hardware such as Photron high-speed cameras, Sensirion liquid flowmeters, Bronkhorst gas flowmeters, Shimadzu HPLC pumps, and other devices. This allows a user to easily script automated scans of liquid and gas flow rates with the full flexibility of the Python language. A PyQt5 graphical interface is also provided for convenience. Such automation of both data collection and image processing allows for the rapid production of diagrams that map gas/liquid flow conditions to jet properties (speed, diameter, angular variance, jetting/dripping status, etc.) and dimensionless numbers such as Weber, Reynolds, and Capillary numbers.

This comprehensive toolbox (measurement, control, analysis) equips GDVN users with the necessary tools to robustly characterize the "jetability" of their samples before conducting experiments. One can also conduct robust systematic characterizations of 3D-printed GDVN designs according to geometric design parameters along with liquid properties such as density, viscosity, surface tension, and non-Newtonian perturbations such as protein crystals. We are now in the process of applying our pipeline to various nozzles and liquids with the aim to fully optimize GDVN design. We also present our efforts to identify the smallest measurable jet, pushing the boundaries for nanojet production. We expect that users can eventually consult the relevant phase diagrams to identify the most suitable GDVN design for their specific sample characteristics, and thereby mitigate costly GDVN failures during XFEL beamtimes.

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Microfluidic System for Fixed-target Serial Crystallography and Space Travel

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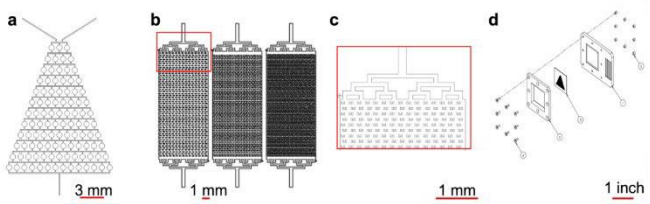
X-ray crystallography is one of the major tools for discovering new insights into protein structure and function with a huge impact on progressing the field of medicine and targeted therapeutics. Research efforts in the field of protein crystallography include optimizing sample delivery methods enabling collection of high-quality diffraction data as well as conserving precious protein crystal samples. One of the major sample delivery methods implements a fixed-target where the crystals are embedded on a solid support and mounted perpendicular to the X-ray beam path. Here, we are reporting a fixed-target microfluidic device for sample delivery to the newly developed first-of-its-kind compact X-ray light source (CXLS) at Arizona State University.[1] In addition, robust, thick versions of the fixed-target devices were used in preliminary experiments for crystallization in outer space.

A previously designed microfluidic device[2] was used for developing a fixed-target sample delivery device using a thermoplastic material, cyclic olefin copolymer (COC). Recently, COC has gained immense interest as a material for fabricating fixed-target devices due to its high X-ray transparency and low gas permeability. The initial device design (figure 1a) contained 102 circular nanowells with a diameter of 750 µm each. These two-layer devices were fabricated with one layer of imprinted COC and another featureless COC layer, both having a thickness of 1 mm. These devices were filled with

lysozyme crystals and mounted inside a cube satellite which has been launched into space with SpaceX (FalconX transporter 6) on 2nd January 2023.

Furthermore, an alternative design (figure 1b) of a fixed-target device was developed for capturing crystal in up to 1400 traps in a single chip andholding the crystals in predetermined positions. This design will enable ease of automation for the fixed-target



stage scanning the crystals in the path of the Figure 1: a. Initial fixed-target design with nanowells of a diameter X-ray source. The current requirement of of 750 µm, b. Current fixed-target design with 3 symmetric units placing large crystals (~50 µm in all arranged within an area of 1 inch red highlighted area with the inlets and traps to hold the crystals, ´ 1 inch, c. Enlarged area of the dimensions) in the CXLS lead to the d. Disassembled cassette design with cassette base each having a improvement of the device fabrication 1 inch ´ 1 inch window (1), cassette cover (2), fixed-target device process which can yield a channel depth of (3), 2-56 ´ 0.25 FHS screw 316 (4),2-56 brass insert small (5) ~50 µm. This goal was achieved by switching to a new set of Si wafers which facilitated longer wafer etch time, a crucial step for augmenting the channel depth in the final devices. A Fixed-target device with the alternative design was successfully fabricated having an overall thickness of ~400 µm. In addition, preliminary experiments of capturing crystals in the device traps were also demonstrated successfully with small (~30 µm) NQO1 crystals. Finally, a fixed-target holder (figure 1d) cassette was designed, and 3D printed for holding the fixed-target devices on the stage inside the CXLS experimental chamber. This two-layer holder cassette has a base layer with an indent for mounting the device and a cassette cover which is tightened on the former layer with screws. With the generation of first X-rays from the CXLS and further improvement of fixed-target devices, this system offers high potential for the first fixed-target serial crystallography experiments planned at ASU.

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Intracellular detection of carbon and noble metal-based nanoparticle drug delivery platforms.

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Numerous multifunctional nanomaterials have been designed and tested as anti-cancer agents, bringing to focus a set of challenges such as system stability, localization, biodistribution, biocompatibility and efficiency. As a solution to these challenges, we propose nano-systems composed of Graphene Quantum Dots (GQDs) and GQDs covering a Gold-Silver (AuAg) alloy core to aid in anti-cancer therapy and imaging. GQDs belong to carbon-based nanomaterials, known to be efficient drug delivery platforms with high biocompatibility. Applications for alloy nano-systems include being used for surface-enhanced Raman spectroscopy (SERS) and colorimetric detection, both serving for early cancer detection as well as drug delivery tracking. Because of these properties and applications, this project explores GQD-covered AuAg nanoparticles and lone GQD systems as a promising alternative to tackle present cancer treatment, drug delivery and diagnosis challenges. In this study, nanomaterial cell internalization was detected via confocal microscopy and a SERS substrate, using prostate cancer cells (PC-3) as an in vitro model. The Raman signal obtained when using a SERS substrate

was strong enough to identify the nanomaterial it was incubated with and confirm the time it entered the cell, complimenting the results of nanomaterial aggregation locations found with confocal microscopy tests.

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Sample Optimization for the Analysis of Conformation and Nucleotide Exchange in Rac1 by Solution NMR Spectroscopy.

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One of the most promising approaches in treating cancer is the targeting of the Rho family GTPases Rac due to its key role in the proliferation of cancer cells leading up to its metastasis. The continuous activation of Rac by guanine nucleotide exchange factors (GEFs) is what gives the protein its migrating and invasive properties and due to this, the targeting and inhibiting of GEFs receptors has become a subject of study in developing novel cancer therapy. An understanding of the interaction between the protein and the inhibitor is still unknown and thus, analyzing its structure before and after the addition of it is of great interest. NMR methods have been adapted to study these conformation changes resulting from the GTPases-GTP and GTPases-GDP interactions giving further understanding for potential anti-cancer treatment. Therefore, to be able to conduct the analysis, the purification and stability retention of Rac must be successful. The main goal is to provide and environment that allows the protein to survive in its most stable conformation in a way that the results are as accurate as possible, and the protein yield is sufficient to conduct the studies. Variables including the usage of a detergent, the amount of proteases and concentration of buffers among others are key factors in a purification of a complex tagged protein like Rac-1 and its Glutathione S-Transferase (GST) tag. In this presentation, we will focus on the purification of the isotopically labeled Rac-1 and how altering the conditions in which the purification is normally performed could increase the yield of the protein allowing for further studies including liquid state NMR while providing evidence of the behavior of GDP bound Rac in comparison with GTP bound Rac.

Whipping Jet, a novel aerosol generator makes droplets March to beat: a new XFEL sample environment technology.

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Well-defined aerosols pave the way for versatile basic and applied research using X-ray Free Electron Lasers (XFEL). We have demonstrated a unique whipping instability that is generated from a high-aspect-ratio microfluidic device resulting in a unique steady-state gas-focused whipping jet (WJ) without any need for electrification. This WJ device emanates a

multi-monodisperse whipping spray jet with a two-dimensional (2D) profile. We demonstrate this phenomenon based

on various fluidic parameters theoretically and experimentally. The 2D WJ's unique behavior is derived using analytical fluid dynamics to explain jet diameter, whipping regime, and spreading angle. The phenomenon is further characterized experimentally by measuring the angle with respect to the flow rate, the distances between droplets, the droplet shapes, and the reproducibility of these parameters. We also explained the precise fabrication of such inexpensive devices. Lastly, we highlight these devices' potential use as sample environments in versatile applications ranging from cryo-electron microscopy over mass spectrometry to drug formulation and structural studies at XFELs. In droplet-droplet interaction/colliding based mixing time-resolved structural biology experiments at XFELs, the buffer injector can be replaced by WJ device to probe various mixing time-steps. Secondly, WJ device can be utilized for transmission-type scattering/spectroscopy experiments at XFELs.

Acknowledgement:
This work has been supported by the Cluster of Excellence "The Hamburg Center for Ultrafast Imaging-Structure, Dynamics and Control of Matter at the Atomic Scale" of the Deutsche Forschungsgemeinschaft (CUI, DFG-EXC1074, project ID 194651731). This work was conducted through the Berkeley Synchrotron Infrared Structural Biology (BSISB) Imaging program, supported by the US Department of Energy, Office of Biological and Environmental Research, under contract no. DE-AC02-05CH11231. This work was performed under the auspices of the US Department of Energy by Lawrence Livermore National Laboratory under contract DE-AC52-07NA27344. The authors would like to acknowledge the cleanroom facility provided by the Center for Free-Electron Laser Science (CFEL) at Deutsches Elektronen-Synchrotron (DESY).

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Time Resolved Structural Elucidation of Photosystem I at EuXFEL

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Photosynthesis is the process by which oxygen is produced in our atmosphere and it essentially fuels all life on earth. This process is driven by multiple large membrane protein-pigment complexes including photosystem I (PSI). It mediates the light-driven electron transfer from plastocyanin at the lumenal side of the thylakoid membrane to ferredoxin at the stromal side. PSI exists as a trimer in cyanobacteria while it is a monomer with four light harvesting complex (LHC-I) antenna proteins attached in higher plants. Though PSI being a key component in photosynthesis and bioenergetics in general, the

mechanism of light capture and electron transfer in PSI is not fully understood yet. Structures of PSI have been published using both X-ray crystallography and cryo electron microscopy (cryo-EM) but there has been no time resolved study published on its mechanism till date. In this investigation, the dynamics of electron transfer reactions in PSI is studied by time resolved serial femtosecond crystallography (TR-SFX) at EuXFEL. The electron transfer from quinones to the first 4FeS4 cluster, Fx, is of special interest as it represents the rate limiting step in the electron transfer chain. PSI was isolated and purified from *Thermosynechococcus elongatus* and crystals of PSI were grown onsite at EuXFEL. TR-SFX data was collected with 400 nm nanofocus beam. Overall, the dynamic changes of the quinones, the water clusters, the protein and cofactor environment during electron transfer can be revealed from this study.

Assessing the Stabilization Effect of Maturation Inhibitors in HIV-1 CA-SP1 Protein Structure

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Gag polyprotein is the main structural constituent of the human immunodeficiency virus type 1 (HIV-1) and it is responsible for virus particle assembly and maturation. Viral maturation is a restructuring process that converts immature, non-infectious virions newly released from the cell into mature virions capable of entering and infecting a new host cell. HIV-1 maturation is mediated by Gag processing by the viral protease. Specifically, the region of capsid (CA) and spacer peptide 1 (SP1) of Gag is vital for the regulation of the assembly and maturation. Moreover, it is the target of a new class of antiretroviral drugs called maturation inhibitors (MIs) that halt the maturation process.

Current therapies to prevent HIV-1 replication target viral enzymes, however, these novel MIs target Gag directly. Bevirimat (BVM) was the first HIV-1 maturation inhibitor characterized at the clinical and molecular levels. Bevirimat's hypothesized mode of action is the hyper-stabilization of Gag, however, the physical determinants of this protein-drug interaction and its effect on the protein structure and function remain to be elucidated. The triterpene skeleton of BVM has significant anti-HIV properties and properly modifying the structure at the C-28 carboxylic acid position has resulted in more potent MIs. These improved compounds have not been rationalized at a molecular level. We hypothesize that BVM derivatives will have a stabilizing effect like Bevirimat and can be physically measured by Circular Dichroism (CD) and with state-of-the-art nuclear magnetic resonance (NMR) spectroscopy.

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Guanosine-based hydrogel as a new injection matrix for serial femtosecond crystallography

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Serial femtosecond crystallography (SFX) at X-ray free electron lasers (XFEL) prevents chemical and structural X-ray radiation-damage to crystalline proteins and allows *in crystallo* studies of protein dynamics on ultra-short timescales[1,2]. In SFX, the consumption of protein microcrystals can be minimized by the use of viscous matrices in high-viscosity extrusion injection (HVE)[3]. Here, we report the characterization of a guanosine hydrogel as a new crystal-carrier matrix for SFX experiments. Guanosine and its derivative guanosine-5'-monophosphate can self-assemble to form square planar structures known as G-quartets *via* Hoogsteen hydrogen bonds.[4] At the right conditions, these two-dimensional structures associate through π–π stacking interactions forming large three-dimensional tetra-helicoidal strands called G-quadruplexes (G4). G4 supramolecular networks spontaneously form stable and viscous hydrogels that can contain up to 99% of water.[4] We explored the ability to generate micro-streams at various viscosities of the hydrogel and determined the stream velocity and the stability of the hydrogel by mixing it with protein precipitant compounds. Once embedded in the G4 matrix, microcrystals remain stable and light-sensitive systems can be photoactivated. Finally, we performed an SFX experiment at Japan's Spring-8 Angstrom Compact Free-electron Laser (SACLA) embedding lysozyme microcrystals in the G4 hydrogel. Our results demonstrate the usability of G4-based hydrogels for SFX studies, including time-resolved experiments.

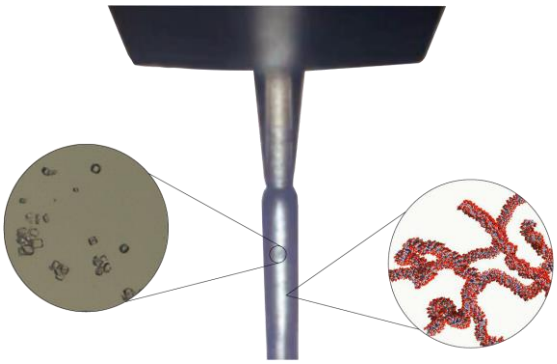

Figure 1. High-viscosity extrusion of lysozyme microcrystals embedded in a G4 hydrogel.

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Validation of Multi-State Enzyme Design by Multi-Temperature X-ray Crystallography

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Computational protein design enables the modification of natural proteins to perform new or altered functions. In this study, a novel multi-state design method was used to create variants of the enzyme aspartate aminotransferase (AAT) with tailored substrate specificity. By tuning AAT's conformational ensemble to favor either a "closed" or an "open" conformation, its activity can be shifted to prefer either aspartate (closed) or phenylalanine (open) as a substrate. Thus, the ability to engineer control over the enzyme conformational ensemble allows for rational tuning of function. To validate the design outcomes, we used multi-temperature x-ray crystallography (5-30 °C) to determine the structures of the closed and open AAT variants. The structures confirmed that the intended conformational preferences were achieved. In addition to validating the computational protein design strategy, our structures further demonstrate the power of non-cryogenic x-ray crystallography for exploring protein conformational ensembles in atomic detail.

Structural Characterization and Structure-Guided Engineering of MfnG SAM-dependent MTase

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Methylation has drawn interest for its role in various bioprocesses, its effect on the chemical properties of compounds, and its use in the development of new pharmaceuticals. S-Adenosyl-L-Methionine (SAM)-dependent methyltransferases (MTases) catalyze the transfer of methyl groups. MfnG is a SAM-dependent MTase that methylates oxygen in tyrosine's side chain. Many MTases have accepted SAM analogues and synthesized new products, but MfnG has been shown unable to do this. Characterization of MfnG with bound tyrosine and SAH, the degraded product of SAM, has been done; however, the crystal structure of MfnG with SAM in the catalytic site has not been determined. In order to characterize MfnG's methylation mechanism, time-resolved crystallography experiments will be done to generate a motion picture for the methylation of MfnG's tyrosine. We also performed structural analysis to make MfnG more promiscuous by expanding the SAM binding pocket through the mutation of key residues. The development of these mutants proves our ability to perform structure-guided protein engineering on other MTases that could create new natural product-based compounds that may have therapeutic relevance

Uncovering the determinants of chromatin remodeler-nucleosome interactions: High-resolution cryo-EM structure of the human BAF-nucleosome complex

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The regulation of human gene expression is a complex process that involves a range of molecular players, transcription factors, chromatin modifying and repair proteins. The BAF (BRG1/BRM associated factor) complex is a critical component of this process, playing a key role in regulating gene expression through its ability to remodel chromatin structure. Given its importance in gene regulation in growth and embryonic development, the BAF complex has emerged as an attractive

target for understanding its role in cell proliferation and cancer. However, to fully understand the mechanisms underlying the complex's function and to design more effective drugs, a detailed structural understanding of the complex's interactions with genomic nucleosomes and the transcription factors important for their genomic targeting is essential. In this study, we present the high-resolution cryo-EM structure of the human BAF complex bound to a nucleosome found in a genomic region important for human embryonic development. This nucleosome is specifically targeted in the process of reprogramming differentiated cells to the pluripotent state. Our results provide new insights into the molecular mechanisms underlying BAF complex engagement with nucleosome. We further performed in vitro experiments that explored the contributions of histone tail modifications and transcription factor binding in BAF-nucleosome interaction. Our findings reveal the importance of these factors in facilitating BAF complex-nucleosome interactions. It is worth noting that while several studies have investigated the interaction of the BAF complex with nucleosomes, all currently known BAF nucleosome structures involve artificial nucleosome sequences like 601. Therefore, our study presents the first high-resolution structure of the human BAF complex bound to a biologically relevant nucleosome sequence, providing a more physiologically relevant insight into the BAF-nucleosome interaction.

Retrieving Biological Function from Time-Resolved Single-Particle cryoEM.

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Recent progress in data analysis of single-particle cryogenic Electron Microscopy (cryoEM); such as Geometric Machine Learning algorithms, allows one to retrieve the conformational spectrum of heterogeneous molecular ensembles. Until recently, most efforts have been attempted under equilibrium conditions [1,2], where the conformational spectrum is time-independent, and thus directly yields the free-energy landscape. However, most biological systems operate far from equilibrium to sustain the processes that constitute life.

Under non-equilibrium conditions, the functional pathways are time-dependent, and the evolution of the conformational spectrum can be described by a Fokker-Planck equation, however with an unknown operator. State-of-the-art developments in Machine Learning, the so-called Physics-Informed Neural Networks (PINN) [3], allows one to retrieve the underlying Fokker-Planck operator from sparse observations alone [4], providing a complete physics-based description of the non-equilibrium process. Moreover, time-resolved cryoEM experiments have recently become practical [5]. Together, this enables us to combine the advantages of time-resolved serial crystallography ('non-equilibrium processes') with the advantages of single-particle methods ('avoid averaging over unlike particles').

Based on these opportunities, we establish a conceptional and algorithmic framework to extract functional pathways from non-equilibrium from a collection of time-resolved single-particle images. This constitutes an unexplored route in studying biological function and structural dynamics under non-equilibrium conditions.

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Modular Droplet Injectors for Sample Conservation Providing New Structural Insight for the Conformational Heterogeneity in the NQO1 Enzyme at LCLS

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Droplet-based injection strategies are a promising tool to reduce the large amount of sample consumed in serial femtosecond crystallography (SFX) measurements at X-ray free electron lasers (XFELs) with continuous injection approaches. Here, we demonstrate a new microfluidic modular droplet injector (MDI) design (shown in Figure 1) that was successfully applied to deliver microcrystals of the human NAD(P)H: Quinone oxidoreductase 1 (NQO1) and phycocyanin. We investigated droplet generation conditions through electrical stimulation for both protein samples and implemented hardware and software components for optimized crystal injection at the Macromolecular Femtosecond Crystallography (MFX) instrument at the Stanford Linac Coherent Light Source (LCLS). Various electrical stimulation conditions were tested and an increase of up to 4-fold in obtained indexed patterns per µL of the sample compared to continuous injection was observed in some cases. Under such optimized droplet injection conditions, we demonstrate that up to 4-fold sample consumption savings can be achieved with the droplet injector when compared to continuous injections.

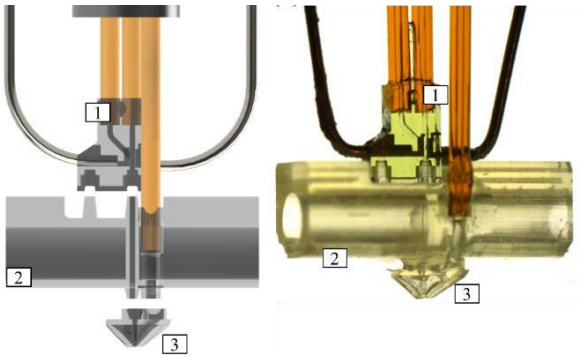

Figure 4: Graphic (left) and microscopic image (right) of the three MDI components: 1) droplet generator, 2) droplet detector, 3) 3D-printed GDVN connected via silica capillaries (orange) to deliver sample, oil, and He gas,

In addition, we collected a full data set with droplet injection for NQO1 protein crystals with a resolution up to 2.5 Å, leading to the first room-temperature structure determination of NQO1 at an XFEL. NQO1 is a flavoenzyme associated with cancer, Alzheimer´s, and Parkinson´s disease and our results reveal for the first time that residues Tyr128 and Phe232, which play key roles in the function of the protein, show an unexpected conformational heterogeneity at room temperature within the crystals. These results suggest that different substrates exist in the conformational ensemble of NQO1 with functional and mechanistic implications for the enzyme's negative cooperativity through a conformational selection mechanism. Our study thus demonstrates that microfluidic droplet injection constitutes a robust sample-conserving injection method for SFX studies on protein crystals that are difficult to obtain in amounts necessary for continuous injection and time-resolved mix-and-inject studies at XFELs.

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Structural Studies of Non-structural protein 15 (Nsp15) Endoribonuclease from original SARS-CoV-2 and its Variants for Therapeutic Intervention

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SARS-CoV-2 is responsible for the ongoing global pandemic of COVID-19. Since first identified in Wuhan, China in December 2019, SARS-CoV-2 has caused 6.6 million deaths worldwide. The ongoing COVID-19 pandemic has shown the urgency to investigate the structural and functional aspects of important viral proteins for the development of more effective vaccines and therapeutic drugs. Nsp15, an endoribonuclease from SARS-CoV-2 plays active roles in immune evasion[1] and hence emerged as a drug target for COVID-19. Here we report the identification and characterization of a high frequency mutation in Nsp15 from the SARS-CoV-2 variant, Epsilon. First detected in California, USA in July 2020, Epsilon exhibited increased transmissibility compared to other SARS-CoV-2 variants circulating at the time.[2] We performed multiple sequence alignment of 126 genomes of epsilon and identified four non-synonymous amino acid changes in Nsp15 (V66L, A81V, D183N and E266Q). We created these four single mutants to study their impact on Nsp15 catalysis. We also created a catalytically inactive mutant (H234A) for comparative analysis. Initial protein expressions revealed, E266Q which had a mutation rate of 4.76% exhibited very low expression levels compared to wild type (WT) whereas the inactive H234A exhibited very higher expression levels. This observation was reiterated by large scale protein purification as well. We hypothesize that being an endoribonuclease, Nsp15 might be cleaving its own mRNA during recombinant expression, thus the activity levels of E266Q, WT and H234A directly correlate to their corresponding expression levels. Comparative tests with other mutants like T112I from the omicron variant was also done. We performed a preliminary activity assay which demonstrated catalytic efficiency of E266Q is equal to or even slightly higher than WT. Enzyme kinetics experiments are underway to confirm this phenomenon. Currently we are pursuing comparative structural studies on E266Q, T112I, WT and H234A using both cryo-electron microscopy (cryo-EM) and X-ray crystallography techniques to understand how mutation in Nsp15 influences increased transmissibility of SARS-CoV-2. This will lead to mitigation strategies and therapeutic interventions not only against Epsilon but also other emerging variants.

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Exploring the Conformational Landscape of Cyclin Dependent Kinase 2 using Multi-Temperature Crystallography

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Cyclin Dependent Kinase 2 (CDK2) is a key regulator of the cell cycle, and mutations that hyperactivate Cdk2 can lead to overactive cell division, a defining feature of cancerous cells. Kinases are dynamic proteins that can exchange between an ensemble of active and inactive conformational substates. Nonetheless, prior CDK2 crystal structures were solved at cryogenic temperatures, potentially limiting insights into the ensemble of active and inactive conformations. My goal is to use structural perturbations, in the form of temperature and ligands, to explore the conformational landscape of CDK2. I have successfully crystallized CDK2 and performed multi-temperature X-ray diffraction experiments in the presence and absence of an ATP analog. Initial analysis using ensemble refinement has revealed diverse conformations for functional motifs in the kinase N-lobe, including the DFG motif, which is important for defining the active and inactive states of kinases. The conformation of the DFG loop is variably modulated by different CDK2 inhibitors, and therefore our results may provide unique insight into the design of specific and potent CDK2 inhibitors.

3D Printed Microfluidics for Improved Sample Delivery at the European XFEL

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Advancements in X-ray free electron lasers (XFELs) offer a rapidly emerging technology that can be used to determine the structures of proteins and protein complexes using serial femtosecond crystallography (SFX). Transforming the ability to image proteins provides possibilities beyond traditional single-crystal goniometer crystallography by enabling measurements on micro- and nano-sized crystals as well as time-resolved experiments that measure dynamics at unprecedented temporal resolution at room temperatures. Because these labor-intensive crystals are destroyed after interacting with a single XFEL pulse, they need to be serially replenished into the path of the X-ray beam. This permits the collection of full data sets to reconstruct protein electron density maps using the obtained diffraction patterns. To resolve this sample consumption challenge in preparation for the November 2022 experiments at the European XFEL (EuXFEL) in Germany, we characterized a revolutionary method that conserves sample[2] during delivery to the pulsed X-ray beam. Moreover, by fine-tuning the electric stimulation and phase shift with an improved geometry, we aim to adjust the droplet generation frequency to match that of the EuXFEL at 10Hz and study the influence of viscous properties of aqueous sample mediums.

Previously, using a 2-photon polymerization 3D-printing technique, we designed 3D-printed droplet-based injection devices coupled with a gas dynamic virtual nozzles (GDVNs)[1], to produce aqueous crystal-laden droplets contained in an immiscible oil phase. Here, we modified the diameter of the microfluidic channels of the devices to generate droplets at a lower frequency, to match the 10Hz pulse train repetition rate[2] at the EuXFEL. Because the flow rate ratio of the aqueous and oil mixtures can be controlled to produce specific droplet shape, size, and generation frequency, we investigated optimal oil (F_o) to aqueous (F_a) flow rate ratios that would produce droplets at the desired frequency and droplet volume. We further tested different viscosities of aqueous sample at 10%, 18%, and 20% concentrations of polyethylene glycol 3350 to assess the influence of medium viscosity on droplet generation. Finally, we utilized quinone oxidoreductase 1

(NQO1) crystals to understand the droplet generation behavior with the presence of protein crystals in the aqueous droplets.

Our study demonstrates that the optimal flow rate ratio to generate droplets at a frequency of 10 Hz was 19:1 (F_o/F_a). This was possible due to the change in the width of the channel, as predicted by theory. Moreover, more viscous aqueous samples produced larger average frequencies of droplet generation, and higher total flow rate ($F_o + F_a$). In conclusion, a medium viscosity of 18% PEG and a total flow rate of 20 µL/min with 19:1 (F_o/F_a) generated droplets at 10 Hz most consistently. Using these parameters, the feedback mechanism was extended to aqueous samples containing NQ01 crystals, which when electrically stimulated, successfully recorded synchronized droplets at 10Hz. Most importantly, the droplet injection reduced the aqueous flow rates that contained protein crystals down to 1.5 µL/min, meaning that our droplet generator devices now save 92.5% of the sample compared to continuous injection methods at the same total flow rates. This work was essential to the preparation and conduction of the November 2022 experiments at the EuXFEL, and this study will allow tuning to other XFEL pulse repetition rates in thefuture.

Acknowledgments: This work was funded by the National Science Foundation STC award number 1231306 during a summer BioXFEL internship.

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Proteolytic cleavage of the HIV-1 maturation switch domain by generic proteases

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The Gag polyprotein is critical for the HIV-1 virus infection cycle. Capsid is the Gag domain responsible for most self-assembly interactions. It is subdivided in CTD and NTD. Following the CTD there is a peptide of approximately 14 amino acids, SP1. HIV protease cleaves the Gag protein in the maturation process. The last cleavage site is between CTD and SP1.

The purpose of this study was to develop and evaluate an in vitro system that captures the structure and enzymatic behavior of the HIV maturation complex. We found conditions in which a non-viral protease demonstrates preference for the CTD-SP1 cleavage site and does not hydrolyze amino acids from the globular domain.

The assay we have developed facilitates the examination of maturation inhibitors currently under active investigation as potential HIV therapies.

Structural photoactivation of phytochromes and photolyases studied by femtosecond time-resolved crystallography.

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Photoactive proteins act in many organisms, i.e. in photosynthesis, light sensing, and in DNA repair. Upon absorption of light, the chromophore and surrounding protein undergo a series of structural changes on multiple time and length scales, so that the protein becomes activated. We use femtosecond time-resolved crystallography to characterize these structural changes. Here, I will discuss two recent examples of this work.

The first example are phytochromes, which sense ambient red-light conditions in plants, fungi and bacteria. The structures of the resting and light-activated states of bacteriophytochromes are known, but the structural mechanism for interconversion remains elusive. I will present crystallographic snapshots of two bacterial phytochromes, time-resolved from femto- to milliseconds at room-temperature by serial X-ray crystallography at the Japanese X-ray free electron laser (SACLA).[1,2] From our recent (unpublished) snapshots on femtosecond time scales, a revised photochemical mechanism emerges, with implications for the primary photoresponse of phytochrome proteins.[3]

The second example will be on the photoactivation of a DNA photolyase. In these enzymes a FAD co-factor is photoreduced by electron transfer along a conserved chain of tryptophans. Our time-resolved structures on picosecond time scales follow thís electron transfer reaction in real time. They reveal how protein motions guide the charge transfer actively through small, but notable changes. We newly imply a conserved methionine residue in the charge transfer reaction.[4] The results provide a basis for understanding of how protein dynamics control electron transfer, which is relevant for all charge transfer reactions in proteins, such as in photosynthesis and cellular respiration.

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2) Carillo et al., Structure 2021
3) unpublished.
4) unpublished.

SLO Data Processing – When Indexing Ambiguity, Sample Heterogeneity and a Perturbation Collide.

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Soybean Lipoxygenase (SLO) serves as a prime example of the coupling between solvent thermal motions and enzyme catalysis[1]. Nonetheless, the precise mechanism of this energy transfer is an open question and well-suited to investigation using time-resolved temperature-jump crystallography[2]. We set out to answer this question, collecting temperature-jumped serial crystallography datasets, with time delays in the nanosecond regime. Before investigating time-resolved differences we processed laser-off data, and this is where the trouble began. Due to similarities in the length of the a and b axes of the crystallographic unit cell, we encountered a fourfold indexing ambiguity. While working to resolve this indexing ambiguity we discovered unit cell heterogeneity within samples, similar to challenges seen in Photosystem-II[3].

Compounding these problems with the fact that temperature is known to induce unit-cell expansion[4], we realized that real-time feedback on dataset completion and merging datasets for structural analyses would prove challenging. We're still resolving these puzzles, but current efforts have disentangled a fair portion of the heterogeneity and we anticipate shining a light on the inner workings of one of nature's most fascinating enzymes soon.

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Grant Acknowledgement:
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cctbx.xfel: a Data Processing Pipeline for XFEL Crystallography Experiments.

Young, I.D.[1]; Paley, D.W.[1]; Oklejas, V.[1]; Schriber, E.A.[1]; Mendez, D.[1,2]; Mittan-Moreau, D.W.[1]; Ganapati, V.[1]; Tchon, D.[1]; Blaschke, J.[1]; Wittwer, F.[1]; Brewster, A.S.[1]; Sauter, N.K.[1]

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As the field of biological crystallography at XFELs has matured, data processing for these experiments has advanced from jury-rigged rotation crystallography software to dedicated serial crystallography software capable of natively handling such concepts as still shots, reflection partiality, shot-to-shot differences in incident spectra, shot-to-shot differences in crystal parameters, and so forth. A number of options are now available to users, with *cctbx.xfel*[1], *CrystFEL*[2], and *nXDS*[3] representing the options most thoroughly fine-tuned to XFEL experiments and the most often used at beamlines. Our software, *cctbx.xfel*, is part of the Computational Crystallography Toolbox (cctbx)[4] ecosystem and is built on the *DIALS*[5] software, a collaboration between Lawrence Berkeley National Laboratory, Diamond Light Source, and CCP4, implementing object-oriented, modular crystallography data processing tools. Although there is no such thing as a "routine" XFEL experiment, the challenges arising during data processing are now relatively well-understood, and data reduction is becoming more accessible to users beyond software developers. Here we seek to provide an overview of the *cctbx.xfel* data processing framework for new users, including pointers to additional resources.

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A New Approach to Mix-and-Inject Serial Synchrotron Crystallography Resolves the Function of DJ-1

Zielinski, K.A.[1], Dolamore, C.[2], Dalton, K.M.[3], Hekstra, D.R.[3,4], Henning, R.[5], Srajer, V.[5], Wilson, M.A.[2], and Pollack, L.[1]

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2. Department of Biochemistry and the Redox Biology Center, University of Nebraska, Lincoln, NE 68588, USA
3. Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA
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5. BioCARS, Center for Advanced Radiation Sources, The University of Chicago, Lemont, IL 60439, USA

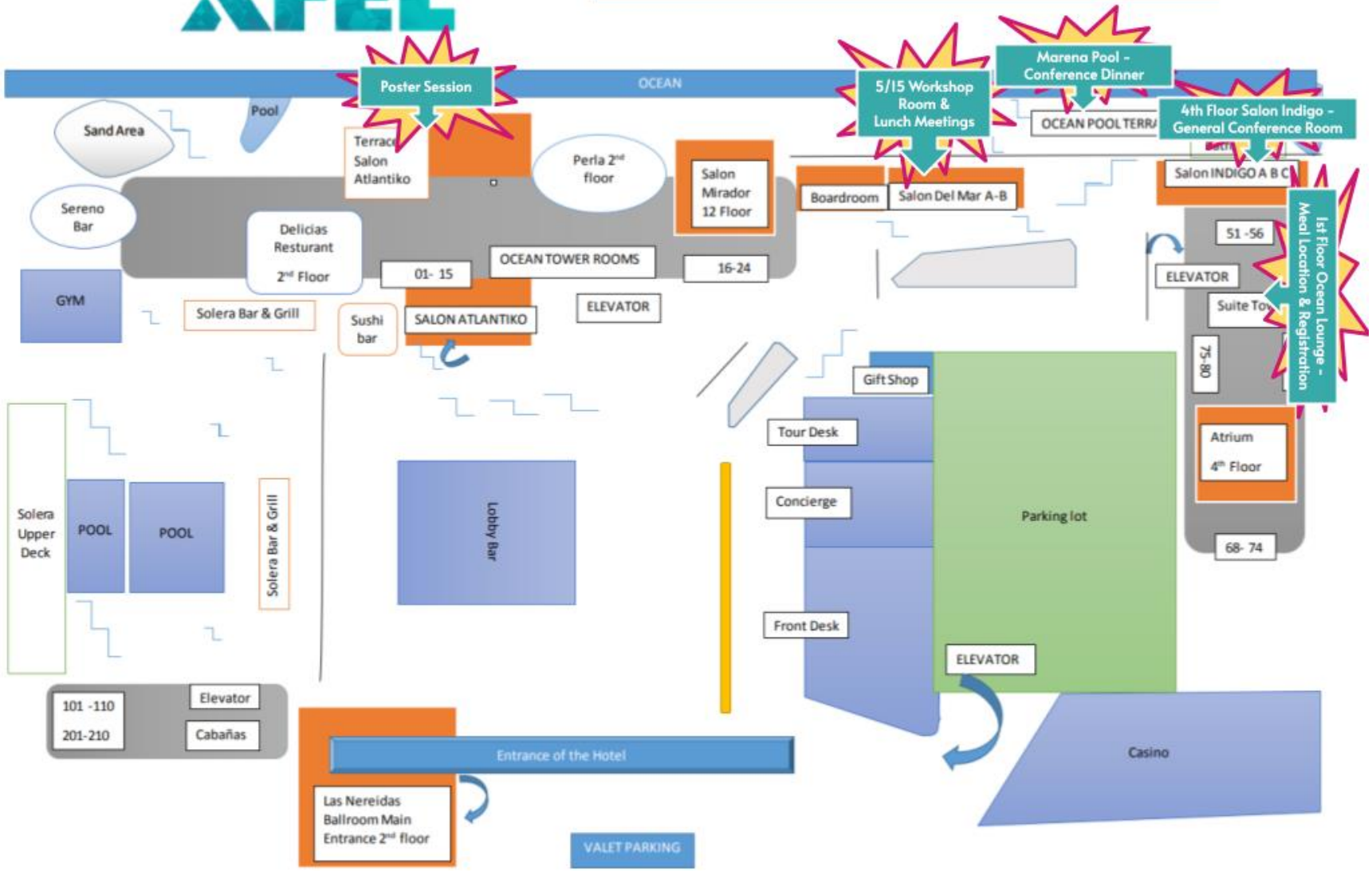
Serial crystallography, enabled by the high brilliance available at X-ray Free Electron Lasers (XFELs) and Third Generation Synchrotrons, allows for the room temperature measurement of protein structures. This has fostered the development of numerous time-resolved techniques to capture snapshots of biological reactions while they are in progress. Of particular interest, the mix-and-inject serial crystallography (MISC) technique utilizes microfluidic mixers to rapidly combine protein microcrystals with small molecule ligands just prior to data collection. At XFELs, MISC is typically combined with Gas Dynamic Virtual Nozzles (GDVNs) to deliver freshly mixed crystals via liquid jet to the X-ray interaction region to be compatible with the 10s of femtosecond exposure time. Past XFEL work captured reaction timepoints from the single millisecond to the second range. There is considerable interest in adapting the technique to synchrotrons, but changes in sample delivery are needed to accommodate the increased X-ray exposure time required, usually on the scale of milliseconds at monochromatic beamlines. The polychromatic BioCARS beamline at the APS, however, offers a high incident flux in sub-ms exposures. Additionally, Laue Crystallography has the advantage of acquiring more information per frame, allowing for relatively rapid data collection. Here, we demonstrate a new MISC co-flow sample delivery scheme in which flow-focusing diffusive microfluidic mixers are constructed with concentric capillaries. Freshly mixed crystals remain confined by tubing for data collection. By varying the flowrates or changing the position of the X-ray beam relative to the tip of observation region, many timepoints can be reached in a single sample cell. We used this new technology to study DJ-1, an important protein in oxidative stress response. Interestingly, DJ-1's function has been heavily disputed. This demonstration of synchrotron MISC allowed for direct observation of DJ-1 acting on its substrate, methylglyoxal, confirming its role as a glyoxalase rather than a deglycase.

Attendee List

Last Name:	First Name:	Organization:
Acosta-Mercado	Jemily	University of Puerto Rico, Río Piedras Campus
Ali	Nadia	Georgetown University
Alonso-Mori	Roberto	SLAC National Accelerator Laboratory
Alvarez	Roberto	Arizona State University
Aneke	Emmanuel	Northwestern University
Ansari	Adil	Arizona State University
Barends	Thomas	Max Planck Institute for Medical Research
Basu	Shibom	European Molecular Biology Laboratory (EMBL)
Bayro	Marvin	University of Puerto Rico at Rio Piedras
Botha	Sabine	Arizona State University
Bussiere	Dirksen	Eli Lilly and Company
Caffrey	Martin	Trinity College Dublin
Claudio Ares	Oscar	University of Puerto Rico at Rio Piedras
Cohen	Aina	SLAC National Accelerator Laboratory
de Wijn	Raphael	European XFEL
Diaz Corretjer	Carla	University of Puerto Rico at Rio Piedras
Doppler	Diandra	Arizona State University
Doyle	Ross	Rayonix, L.L.C.
Dworkowski	Florian	Paul Scherrer Institut
Etemadpour	Roshanak	University of Wisconsin-Milwaukee
Frank	Matthias	Lawrence Livermore National Laboratory and UC Davis
Fromme	Raimund	Arizona State University
Gellerson	Madison	Hauptman-Woodward Medical Research Institute
Gorel	Alexander	Max Planck Institute for Medical Research
Gu	Kevin	University of California, Davis
Hochberg	Tanner	Arizona State University
Hu	Hao	Arizona State University
Hu	Zixi	Lawrence Berkeley National Laboratory
Hudson	Creed	Arizona State University
Hunter	Mark	SLAC National Accelerator Laboratory
Jernigan	Rebecca	Arizona State University
Kaindl	Robert	Arizona State University
Keable	Stephen	Lawrence Berkeley National Laboratory
Kern	Jan	Lawrence Berkeley National Laboratory
Ketawala	Gihan	Arizona State University
Kling	Matthias	SLAC National Accelerator Laboratory
Logar	Michael	Hauptman-Woodward Medical Research Institute
Lopez Garriga	Juan	University of Puerto Rico at Mayagüez
Makita	Hiroki	Lawrence Berkeley National Laboratory
Manatou	Dimitra	Arizona State University
Manna	Abhik	Arizona State University
Marchany-Rivera	Darya	SLAC National Accelerator Laboratory - SSRL-SMB
Marchesini	Stefano	SLAC National Accelerator Laboratory

Medina	Nataniel	University of Puerto Rico at Rio Piedras
Menendez	David	Northeastern Illinois University
Miller	Mitchell	Rice University
Monteiro	Diana	Hauptman-Woodward Medical Research Institute
Morales Perez	Myrna Luz	University of Puerto Rico at Rio Piedras
Muchmore	Christine	Rayonix, L.L.C.
Muller-Werkmeister	Henrike	University of Potsdam
MUNIYAPPAN	SRINIVASAN	University of Wisconsin-Milwaukee
Narayanasamy	Sankar Raju	Lawrence Livermore National Laboratory & Berkeley Lab
Narsingh Malla	Tek	University of Wisconsin-Milwaukee
Nijhum	Najia Qayyum	Arizona State University
Nockideneh	Hannah	Arizona State University
Pollack	Lois	Cornell University
Pulavarti	Surya	University of Grenoble
Reyes-Colon	Kiara	University of Puerto Rico
Rios-Santacruz	Ronald	Institut de Biologie Structurale (IBS)
Rodrigues	Matthew	Paul Scherrer Institute
Rodriguez	Joshua	University of California Merced
Ros	Alexandra	Arizona State University
Sánchez Ortiz	Keishla	Scripps Research Institute
Santiago-Russe	Ana Sofía	University of Puerto Rico at Mayagüez
Saxena	Manoj	BBK ISMB
Schmidt	Christina	European XFEL GmbH
Schmidt	Marius	University of Wisconsin-Milwaukee
Schwander	Peter	University of Wisconsin-Milwaukee
Shao	Chenghua	Rutgers University
Sierra	Ray	SLAC National Accelerator Laboratory
Shelby	Megan	Lawrence Livermore National Laboratory
Sonker	Mukul	Arizona State University
Sonowal	Manashi	Arizona State University
Souder	Andree	University of California, Merced
Sripati	Manasa	Arizona State University
Steinmetz	Michel	Paul Scherrer Institute
Stojkovic	Emina	Northeastern Illinois University
Tamayo	John	Arizona State University
Terranova	Nicole	Hauptman-Woodward Medical Research Institute
Thompson	Michael	UC Merced
Velez	Yesenia	University of Puerto Rico at Rio Piedras
Velez Crespo	Grace	Universidad Central del Caribe
Wakatsuki	Soichi	SLAC National Accelerator Laboratory
Westenhoff	Sebastian	University of Gothenburg
Williamson	Lainey	Cardiff University
Wolff	Alexander	UC Merced
Woodruff	Sarah	Miami University
Young	Iris	Lawrence Berkeley National Laboratory
Zielinski	Kara	Cornell University

La Concha Map for BioXFEL Annual Conference



Local Activities

Guided LED Night Kayak Excursion in Condado Lagoon (2 minutes)

- Located in the Virgin Islands of Puerto Rico, our award-winning Night Kayak tour is sure to be the highlight of your entire vacation! Enjoy are clear-bottom kayaks or SUPs that have been outfitted with powerful LED lights. These lights illuminate the water all around you, creating an ideal "window into the sea". Don't miss out. Paddle out and experience the GLOW today!
- **Admission: From $59 per adult**.
- **Duration: 75 mins**
- **Phone: 787-248-4569**
- **Website: https://www.nightkayak.com/night-tours/puerto-rico/**

El Yunque Natural Waterslide & Rainforest Hiking with Transportation (39 minutes)

- Experience the stunning El Yunque Rainforest, just outside of San Juan, during this exciting small-group excursion. Spend the day exploring the rain forest, stopping at watering holes and natural rock slides to swim, free dive, and cliff jump. At the end of the tour, tuck into a delicious lunch of Puerto Rican food at a local restaurant.
- **Admission: From $73 per adult**.
- **Duration: 420 mins**
- **Phone: N/A**
- **Website: https://www.tripadvisor.com/AttractionProductReview-g147319-d15593291-El_Yunque_Natural_Waterslide_Rainforest_Hiking_with_Transportation-Puerto_Rico.html**

Old San Juan City WALKING TOUR (18 minutes)

- Explore the historic area of Old San Juan on a small-group walking tour. Step back in time, over 500 years, and learn about the island of Puerto Rico from your guide. As you walk, pass by important landmarks while your guide regales you with history and tidbits about the local culture.
- **Admission: From $40 per adult**.
- **Duration: 120 mins**
- **Phone: N/A**
- **Website: https://www.viator.com/tours/San-Juan/Old-San-Juan-Walking-Tour/d903-16292P1**

San Juan Guided Snorkel Tour at Escambron Beach (7 minutes)

- Take advantage of the opportunity to explore Puerto Rico's underwater landscapes on this private snorkeling excursion off the coast of San Juan. No previous snorkeling experience is necessary, as your guide offers a pre-departure lesson and provides all the equipment you need. Enjoy personal attention from your private guide and take home complimentary a GoPro video to commemorate the outing.
- **Admission: From $59 per adult**.
- **Duration: 90 mins**
- **Phone: 787-305-1020**
- **Website: https://tryscubadiving.com/san-juan/**

Jet Snorkel-Snorkeling with Turtles Escambron Beach (7 minutes)

- Take advantage of the opportunity to explore Puerto Rico's underwater landscapes on this private snorkeling excursion off the coast of San Juan. No previous snorkeling experience is necessary, as your guide offers a pre-departure lesson and provides all the equipment you need. Enjoy personal attention from your private guide and take home complimentary a GoPro video to commemorate the outing.
- **Admission: From $100 per adult**.
- **Duration: 90 mins**
- **Phone: N/A**
- **Website: https://www.viator.com/tours/Puerto-Rico/Jet-Snorkel-Snorkeling-with-a-boost/d36-174461P1**

***2* Hours Private UTV Can-am Adventure Tour (11 minutes)**

- Come with your group and enjoy a 2 hour "Road and Beach Areas" experience in an ATV. You will discover the beauties of our Island and you will be able to appreciate our flora and fauna. Live a unique experience, full of adrenaline and cultural diversity on the routes that we have already established for the enjoyment and safety of you and your family. You will explore the gastronomic route, Beach, Coasts and the tourist area of San Juan. We offer our trips between good weather and bad weather We are a company committed to customer satisfaction and we provide highly trained staff so that you and your group have a pleasant experience. Your Caribbean Experience is about to begin!
- **Admission: From $259 per group (3)**.
- **Duration: 120 mins**
- **Phone: N/A**
- **Website: https://www.tripadvisor.com/AttractionProductReview-g147319-d25183602-2_Hours_Private_UTV_Can_am_Adventure_Tour_in_San_Juan_Puerto_Rico-Puerto_Rico.html**

Sunset Sail by San Juan Historical Bay (6 minutes)

- Add an extra layer of relaxation to your vacation on a sunset sailing cruise from San Juan. It's the perfect opportunity to take a break from the traffic and city crowds. With space for maximum six passengers, your evening experience is guaranteed to be intimate. The sun, sea, and shore are the perfect backgrounds for your vacation portraits. Unlimited beer, wine, and soft drinks are served on board the boat.

- **Admission: From $93 per adult**.
- **Duration: 120 mins**
- **Phone: N/A**
- **Website: https://www.viator.com/tours/San-Juan/Sunset-Sailing-Trips/d903-92366P2**

Arenales Caves, River and Waterfalls Plus Hidden Springs Full Day Adventure (47 minutes)

- Spend an adventurous day exploring rivers and caves with this small-group tour of the Arenales Caves. You'll hike through rivers, visit an underground cave, and get the chance to do some swimming. You'll finish off your day of adventure with a hike to a small hidden spring before returning to yourhotel.
- **Admission: From $110 per adult**.
- **Duration: 300 mins**
- **Phone: N/A**
- **Website: https://www.tripadvisor.com/AttractionProductReview-g147319-d23795052-Arenales_Caves_Charco_Azul_River_Caves_and_Waterfall_Exploration_Adventure-Puerto_.html**

Rum Tasting Tour – Casa BACARDI (20 minutes)

- For spirits enthusiasts, nothing beats sampling a producer's creations, with a master blender, at the actual distillery where the spirits are crafted. Sample some of the best, premier Bacardí rum with this connoisseur-quality experience at Casa Bacardí in San Juan. Join your host to sample several styles and vintages of Bacardí products. Highlights include tastings of the Gran Reserva Maestro de Ron, Reserva Limitada, and the coveted Casa Bacardí Special Reserve—only available at the distillery.
- **Admission: From $75 per adult**.
- **Duration: 75 mins**
- **Phone: N/A**
- **Website: https://www.bacardi.com/casa-bacardi/tickets/**

EcoAdventure Ziplining (27 minutes)

- Try this ziplining adventure at Campo Rico, just a few minutes from San Juan. Choose a morning or afternoon slot to get into your harness and zip through the sky over the canopy, with gorgeous ocean views all around you. There will be a series of hanging bridges and ziplines on this adventure, and the final zipline drops you off at a mojito bar where you can treat yourself to a refreshing beverage.
- **Admission: From $129 per adult**.
- **Duration: 120 mins**
- **Phone: 1-888-651-9785**
- **Website: https://www.viator.com/tours/San-Juan/Campo-Rico-Ziplining-Adventure/d903-42873P1**

Luquillo Beach Horse Ride from Carabali Rainforest Adventure Park (40 minutes)

- Travel through the Puerto Rican countryside on this guided horseback riding tour. Along the way, you'll cross the Mameyes River, trot through the tropical forest, and ride along the shores of Luquillo Beach. Plus enjoy free time on the beach to relax and grab a cocktail (at your own expense).
- **Admission: From $54 per adult**.
- **Duration: 120 mins**
- **Phone: 1-787-889-4954**
- **Website: https://carabalirainforestpark.com/adventures/horseback-yunque/**

Beginner Surf Lessons (12 minutes)

- Learn to surf in the warm waters of San Juan with a beginner surf lesson. Meet your instructor and small group and learn the basics of surfing on the beach. Then, head into waist-deep water and try to catch some waves. Your guide is always there to correct your form and make sure you have a great experience out on the water.
- **Admission: $65 per adult**.
- **Duration: 90 mins classes: 12pm, 1pm, 2pm, 3pm**
- **Phone: 1-787-791-0404**
- **Website: https://www.marriott.com/en-us/hotels/sjuiv-courtyard-isla-verde-beach-resort/experiences/**

San Juan Ghost Walking Tour (12 minutes)

- Uncover the sordid history of San Juan as you embark on a walking tour through the streets of the old town. Listen to stories from your guide about hauntings, the Bermuda Triangle, pirates, the Fountain of Youth, and local folklore; you may even spot some paranormal activity as you walk around.
- **Admission: $45 per adult**.
- **Duration: 150 minutes**
- **Phone: 1-833-446-7813**
- **Website: https://www.americanghostwalks.com/tour/san-juan-nighttime-ghost-walk**

Museo De Arte De Ponce

- Featuring the finest collection of European and Latin American art in the Caribbean, this museum was donated to the people of Puerto Rico by Luis Ferre, a former governor. The collection represents the principal schools of American and European art of the past 5 centuries. Among the nearly 400 works on display are exceptional pre-Raphaelite and Italian Baroque paintings. Visitors will also see artworks by other European masters, Puerto Rican and Latin American paintings, graphics, and sculptures. On display are some of the best works of the two "old masters" of Puerto Rico: Francisco Oller and Jose Campeche. The museum also contains a representative collection of the works of the old masters of Europe, including Gainsborough, Velazquez, Rubens, and Van Dyck. The museum is best known for its pre-Raphaelite and Baroque paintings and sculpture — not only from Spain, but from Italy and France as well. Both the Whitney Museum in New York and the Louvre in Paris have borrowed from its collection. Temporary exhibitions are often featured.

- Admission:
  - $6
  - Audio guide $2
- Hours:
  - Wednesday – Monday 10AM – 5PM
  - Sundays 12PM – 5PM
  - closed Tuesday
- Phone: 787-840-1510 / 787-848-0505
- Website: www.museoarteponce.org

Museo de la Musica Puertorriqueña – Museum of Puerto Rican Music (9 minutes)

- Located just east of the Plaza de las Delicias lies the Museo de la Musica Puertorriqueña displaying musical instruments of Puerto Rico with Spanish and African influence. Discover the rich history of Puerto Rican musicians, see a performance by local artists in the courtyard, or take a music lesson for an afternoon.
- Admission: Free
- Hours: Wednesday – Sunday 8:30AM – 4:00PM
- Phone: 787-844-7016

Parque de Bombas – Old Firehouse Museum (9 minutes)

- The first thing you may notice is this museum's unique black and red appearance. It was constructed in 1882 as the centerpiece of a 12-day agricultural fair intended to promote the civic charms of Ponce. Within one year, El Parque de Bombas was designated as the island's first permanent headquarters for a volunteer firefighting brigade.
- Admission: Free
- Hours: 9AM – 5PM **tours given in English daily**
- Phone: 787-284-3338

Museo de la Historia de Ponce – Ponce Museum of History - Casa Salazar (9 minutes)

- Here you can discover the history of the city of Ponce dating back from the Taino tribes to the present day. The museum features interactive displays, a conservation library, souvenir and gift shop, cafeteria, and conference facilities.
- Admission: Free
- Hours: Tuesday – Sunday 8:30AM – 4:30PM **tours given in English daily**
- Phone: 787-844-7071 / 787-844-7042

Tibes Indian Ceremonial Center (10 minutes)

- Known as the most important archaeological deposit in the Caribbean, Tibes represents the room of continuous indigenous life by more than thousand years before the arrival of Columbus to the Island. Bordered by the Portugues River and excavated in 1975, this is the oldest pre-Hispanic cemetery in the Antilles. It contains some 186 skeletons, dating from A.D. 300, as well as pre-Taino plazas from A.D. 700. The site also includes a museum, an exhibition hall, and a re-created Taino village.
- Admission: $5

- Hours: Tuesday – Sunday 8AM – 4PM
- Approximate time required at the site: 1 ½ hours
- Phone: 787-840-5685 / 787-840-2255

Teatro La Perla (10 minutes)

- Built in the neoclassical style of 1864; this theater is the largest and most historic of the Spanish-speaking Caribbean. Its renowned acoustics are so clear that microphones are not necessary. The theater was nearly completely destroyed in the 1918 earthquake and fires. It reopened in 1941 with most of its original features. Come learn the entire history of this ancient theater and enjoy a journey to a time gone by.
- Admission: Free
- Hours: Monday – Friday 8:00AM – 4:30PM **hours subject to change due to performances**
- Phone: 787-843-4322

Our Lady of Guadalupe Cathedral (10 minutes)

- Located in the Plaza de las Delicias, and adjacent to the Parque de Bombas, this cathedral is often referred to as Ponce Cathedral. The original structure was built in the 1660's and identically situated. It has a history of destruction over the centuries having been greatly affected by earthquakes and fires. The current structure was built in 1931 but has remaining elements dating back to the 1800's. It has two steeples and three domes which create a striking façade that complements the beauty of the surrounding plaza.
- Admission: Free
- Hours:
    - Monday – Friday 6:00AM – 1PM
    - Saturday – Sunday 6:00AM – 12Noon & 3:00PM – 8:00PM
- Phone: 787-842-0134

El Museo Castillo Serralles – Serralles Castle Museum (15 minutes)

- Take a tour of the largest and most imposing building in the city of Ponce – the Serralles Castle is known as one of Puerto Rico's most treasured architectural masterpieces. Guides will lead you through this Spanish Revival House where you'll see panoramic courtyards, a baronial dining room, and much more. Built on El Vigia Hill during the 1930's by local rum distiller owners, the Serralles family, the castle is the best evidence of the wealth produced by the turn-of-the-century sugar boom. Along with the Castle Museum, there is a Japanese Garden, Butterfly Nursery, and Vigia Cross.
- Admission:
    - Japanese Garden and Vigia Cross: $5.50
    - Castle Gardens and Butterfly Nursery: $7.00
    - Castle Museum, Castle Gardens, and Butterfly Nursery: $8.50
    - Combo Package including all areas: $12.80
- Hours:
    - Thursday – Sunday 9:30AM – 5:30PM
    - Last tour 5:15PM

- Phone: 787-259-1774
- Website: www.castilloserralles.org Spanish only

Places Nearby 30-40 minutes by Taxi

Hacienda Buena Vista – Coffee Plantation (27 minutes)

- Once one of the most successful plantations in Puerto Rico, this hacienda showcases the era of 19th century farm production. It was a working coffee plantation until the 1950's, and 86 of the original 500 acres are still part of the estate. Tour the beautiful grounds and see authentic artifacts from the 1850's.
- Admission: $10
- Hours: English tours are given ONLY at 1:30PM Wednesday – Sunday **call or go online to make a reservation**
- Phone: 787-722-5882
- Website: http://www.paralanaturaleza.org/home-eng.html

Guanica Dry Forest (36 minutes)

- A United Nations designated International Biosphere Reserve, this forest showcases a historic lighthouse, a lookout tower, 700 types of plants and trees, the richest population of birds on the Island (136 different species), a lignum vitae tree that is over 1,000 years old, 14 hiking trails, several beaches, and offshore cays where you can kayak and snorkel. The Guanica Forest Reserve is a coastal mangrove forest and has been a UNESCO World Biosphere Reserve because of several endangered species found only in Puerto Rico. Gilligan's Island and Whale Island can be easily reached by ferry (departing every hour) for snorkeling, lying on the beach, or having a picnic.
- Admission: Free
- Hours:
  - Forest: Tuesday – Sunday 9:00AM – 5:00PM | Islands: Tuesday – Sunday 10:00AM – 5:00PM
- Phone: 787-821-5706

Local Restaurants

Serafina San Juan Restaurant (8 minutes)

- Serafina Restaurant is located within La Concha Resort & Hotel in the Condado area of San Juan. Just steps from the beach, this area offers some of the best museums, nightclubs and natural attractions on the island. Take in the enchanting views, breathe the fresh salt air and experience something truly delicious with us.
- Cuisine: Modern Italian
- Price range: $15 - $30
- Address: 1077 Ashford Avenue
- Phone: 787-722-5050
- Website: https://www.serafinasj.com

Pico Rico BBQ (9 minutes)

- Pico Rico BBQ serves a delicious meal. It is in a high traffic area in the heart of the tourist area of San Juan. It was interesting to watch the tourists walk right past while the locals walked right in for their lunches and dinners.
- Cuisine: barbeque
- Price range: $25 - $40
- Address: 1407 Ashford Avenue
- Phone: 939-313-0450
- Website: https://www.picoricobbq.com

Tacos & Tequila (4 minutes)

- Tacos and Tequila is a tantalizing culinary tribute to the most beloved dishes of Mexico. With a menu authored by Executive Sous Chef José Alvarez, this laid-back yet vibrant venue includes favorites like tacos al pastor, tinga chicken tacos and – for vegetarian foodies – tacos starring Mushroom & Sweet Potato. Quench your tropical thirst with a refreshing Cerveza, a cool margarita (there's a wide array to choose from!), or one of our specialty cocktails, garnished with fresh local herbs, fruits, and spices.
- Cuisine: Mexican
- Price range: $15 - $30
- Address: 1055 Avenue Doctor Ashford Avenue
- Phone: 787-985-7799
- Website: https://www.condadovanderbilt.com

Ola Ocean Front Bistro (4 minutes)

- Ola" – the Spanish word for "wave" – welcomes visitors with a relaxed, family-friendly vibe and authentic fare brimming with the traditional flavors of Puerto Rico. With the sun-splashed Atlantic as a backdrop, Ola serves made-to-order dishes for breakfast, lunch and dinner, made with only the freshest of locally-sourced ingredients. Care for an exotic, island libation or hand-crafted cocktail? Pull up a seaside seat at the Mojito Bar for a soothing refresher.
- Cuisine: Puerto Rican
- Price range: $25 - $50
- Address: Pool Level, 1055 Ashford Avenue
- Phone: 787-985-7845
- Website: https://www.condadovanderbilt.com/dine/ola-oceanfront

XO Asian Cuisine (5 minutes)

- Ola" – the Spanish word for "wave" – welcomes visitors with a relaxed, family-friendly vibe and authentic fare brimming with the traditional flavors of Puerto Rico. With the sun-splashed Atlantic as a backdrop, Ola serves made-to-order dishes for breakfast, lunch and dinner, made with only the freshest of locally-sourced ingredients. Care for an exotic, island libation or hand-crafted cocktail? Pull up a seaside seat at the Mojito Bar for a soothing refresher.
- Cuisine: Asian
- Price range: $5 - $25
- Address: 1300 Ashford Avenue
- Phone: 939-633-3233
- Website: https://www.foodbooking.com/ordering/restaurant/menu?company_uid=d002974b-bb67-40b3-b78e-3393b2ed1cba&restaurant_uid=0a00c067-0ffd-4562-bb97-a3b3660233b9&facebook=true

Cayo Blanco Seafood Restaurante (4 minutes)

- A creative Puerto Rican seafood and Caribbean restaurant located in Condado area in San Juan, Puerto. Delicious to the last bite. Starting with the empanadas we got the assorted that included crab, lobster and steak. All three had their own powerful flavors that makes you think about ordering another round. We followed it up with swordfish and lobster. Both had flavors and textures that combined to make a lasting impression. Both temperatures and wellness were to perfection.
- Cuisine: Seafood & Caribbean
- Price range: $25 - $50
- Address: 1050 Ashford Avenue
- Phone: 939-337-0397
- Website: https://www.cayoblancopr.com

The Oyster Shack (2 minutes)

- We serve freshly opened oysters, clams, crudo, and refreshing ceviche. The Oyster Shack is for the dedicated seafood lover and those with a curious palate that like trying new, interesting dishes. Come visit us with your family and have the experience of dining at our fine establishment for yourself.
- Cuisine: Seafood
- Price range: $20 - $35
- Address: 1108 Magdalena
- Phone: 939-227-8674
- Website: https://www.theoystershackpr.com/menu

POKEBAR Hawaiian Kitchen (6 minutes)

- Our philosophy is from "Sea to Bowl". That means that in sourcing our fish we aim to use the highest-quality and most sustainable raw fish we can find while still maintaining a reasonable price point. This philosophy extends into all areas of our offerings - from our veggies to our sauces.
- Cuisine: Hawaiian
- Price range: $12 - $25
- Address: 1302, #3 Ashford Ave
- Phone: 939-337-7653
- Website: https://www.pokebarpr.com/menu

Sage Steak Loft (5 minutes)

- Nestled within the quaint and charming Olive Boutique Hotel is Italian steakhouse that packs a big punch when it comes to freshly sourced ingredients expertly prepared by a team handpicked by renowned chef Mario Pagan. Chef Mario Pagán brings to Sage his creative flair and energy by presenting a classic steakhouse fare and mixing it with playful Italian dishes. Afterward, make sure to go to the roof and enjoy some after-dinner cocktails at the wonderful al fresco bar overlooking the Condado Lagoon.
- Cuisine: Steak & Pasta
- Price range: $18- $62
- Address: 55 CII Aguadilla
- Phone: 787-728-3535
- Website: https://oliveboutiquehotel.com/food/

STK Steakhouse Loft(5 minutes)

The Lodge by STK Rooftop is a spectacular winter oasis, tucked away in the center of the Meatpacking District. The Aspen-like vibes and enclosed rooftop experience makes you feel as if you are wining and dining in a snow globe. The customized seasonal food and cocktail menus fits the lodge theme perfectly.

- Cuisine: Steak & Pasta
- Price range: $18- $62
- Address: 1055 Ashford Ave.
- Phone: 787-722-2828
- Website: https://stksteakhouse.com/venues/san-juan/

Lola (9 minutes)

- Lola is located in the inner courtyard of the Hotel Ramada Ponce. Lola's menu consists of about 15 entrees including stuffed sausage, banana stuffed beef tenderloin, risotto croquettes, or lobster eggrolls, wrapped in bacon, lemon, and cilantro mahi-mahi with coconut sauce appetizer, and their native trio (mini fritters, mini bacalaitos, and fried cheese).
- Cuisines: International
- Price range: $4 - $32
- Address: Plaza de Ponce, Calle Unión, Esq. Reina y Mendez Vigo, Ponce, PR 00731
- Phone: 787-813-5033
- Website: http://www.lolacuisine.com/index.php

El Negocio de Panchi (14 minutes)

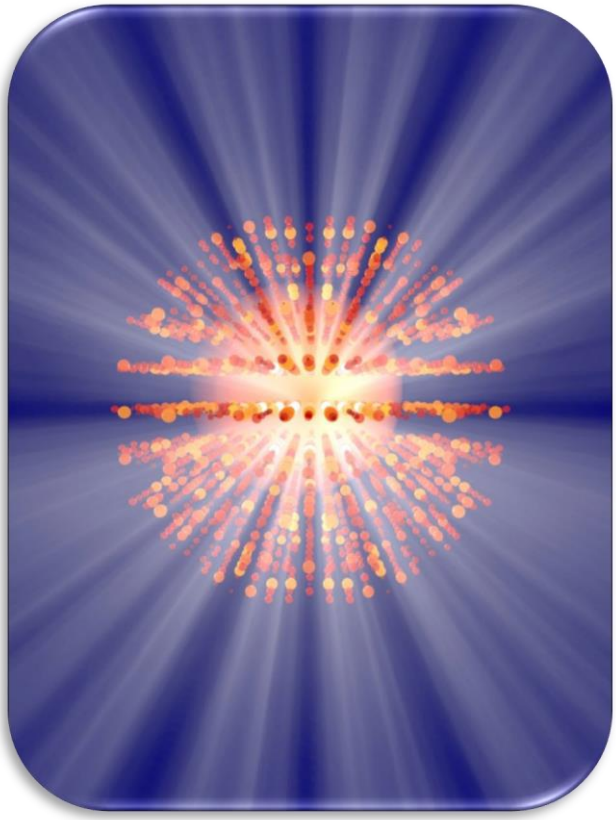
- El Negocio de Panchi offers exquisite international proposals with a creole twist. They are constantly changing the menu to offer customers innovative dishes. Various cuts of fresh fish and beef, veal osso bucco, three-cheese risotto, and crusted halibut with sausage are among a few of the options on the menu. Do not leave without trying one of their homemade desserts.
- Cuisines: Mediterranean, International
- Price range: $10 - $50
- Address: Coto Laurel, 00780, Puerto Rico
- Phone: 787-848-4788

Pito's Seafood Café and Restaurant (15-20 minutes)

- Here you'll indulge in the freshest seafood sitting at the water's edge on the southern coast of Puerto Rico. With three levels of dining, open-air views of the sea for casual dining, an authentic wine cellar for very intimate parties and two air conditioned rooms for elegant dining, you'll experience the best of Caribbean dishes. You can also enjoy their cigar room which offers a menu of fine local and international cigars.
- Cuisine: Caribbean
- Price range: market price
- Address: Rd. No. 2 "Las Cucharas" Ponce, P.R. 00731
- Phone: 787-841-4977
- Website: http://www.pitosseafoodpr.com/index.html

Chez Mademoiselle Coco (18 minutes)

- Beautiful French restaurant along Riego Lake with indoor and outdoor seating. Authentic French cuisine in a tropical setting with cool and relaxing ambiance.
- Cuisine: French
- Price range: $6.50 - $39
- Address: Barrio Singapur Calle 6, Juana Diaz, Ponce 00795
- Phone: 787-604-9791
- Website: http://www.chezmademoisellecoco.com/default.html



Other Information

Puerto Rico has the third largest population in the Greater Antilles at approximately 3.6 million. It is an unincorporated territory of the United States and uses the U.S. dollar as its form of currency. The official languages are Spanish and English. Puerto Rico has a tropical climate. The average high in January is 83°F with an average low of 72°F. The average humidity at that time of year is about 78%.

Tipping is customary. A good tip is considered to be 15%-20% for servers, hotel staff, and cab drivers. Be sure to check your bill at hotels and restaurants as such places often include gratuity especially for larger groups.

Linac Coherent Light Source at SLAC National Accelerator Laboratory