**Program**

**TSRC Workshop on Macromolecular Crowding June 5-9, 2023**

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| Organizers:  | Kristin Slade and Andrea Soranno |
| TSRC Hosts:  | Cindy Fusting (970) 708-5069; Mark Kozak, (970) 708-4426  |
| Talk:  | ~30 min talk + 10 min questions/discussions; questions are encouraged during talks  |

**Sunday June 4, 2023**

5:00-6:30 pm Meet & Greet and Check in

Location: The Liberty (**121 South Fir St**, next to Baked in Telluride)

A Telluride staff member will be on hand to welcome you and distribute badges.

**Monday Morning, June 5th**

7:30 - 8:30 AM Breakfast at the school every morning (Monday- Friday)

**Session #1:**

8:30-8:45 AM Welcome and Opening Remarks (TSRC staff)

8:45-9:25 AM: Debabrata Dey (Weizmann Institute of Science, Israel) – Virtual Talk

**Small molecules are not so small inside the cell: charge plays a critical role in dictating the diffusion behavior of molecules**

9:25-9:55 AM:Andrea Soranno (Washington University in St. Louis)

**Crowding effects on disordered proteins**

10 AM-10:15 AM Coffee Break

10:15-10:55 AM Gary J. Pielak (University of North Carolina at Chapel Hill)

**Protein stability in living cells & under crowded conditions *in vitro***

10:55-11:35 AMDaniel Harris (The Hebrew University, Israel)

**Decoding the enthalpic and entropic contributions to macromolecular crowding**

**Session #2: Joint Session with the Biological Physics of Chromosomes**

1:00-1:45 pmArnold Boersma (Utrecht University)

**Sensing self-assembly in crowded solutions.**

1:45-2:30 pm Olga Dudko (University of California San Diego)

**What phase(s) of matter is the chromosome — and why should the cell care?**

**15 min break**

2:45-3:30 pm Arohan R. Subramanya (University of Pittsburgh)

**Cell volume rescue via crowding-induced phase separation**

3:30-4:15 pm Alexandra Zidovska (New York University)

 TBA

**Tuesday Morning, June 6th**

7:30 - 8:30 AM Breakfast at the school every morning (Monday- Friday)

**Session #3:**

8:30-9:10 AM: Kristin Slade (Hobart and William Smith Colleges)

**Influence of crowded conditions on glutamate dehydrogenase kinetics**

9:10-9:50 AM: Eva Pluhařová (J. Heyrovsky Institute of Physical Chemistry, Czech Academy of Science)

**Molecular simulations of the influence of crowders on enzymes: from domain motion**

**to the active site**

10 AM-10:15 AM Coffee Break

10:15-10:55 AM Fabio Sterpone (French National Center for Scientific Research, Paris)

**Simulation of proteins and fluid at mesoscale: crowding, aggregation and other**

**amenities**

10:55-11:35 AMStepan Timr (J. Heyrovsky Institute of Physical Chemistry, Czech Academy of Science)

**Proteins in a heated crowd: linking the dynamics and structure of the cellular interior**

**to cell metabolism and thermal death**

11:35-11:25 AM Peter Chung (University of Southern California)

 **Reconciling the existence of tau-mediated microtubule bundles with polymer physics**

**Wednesday Morning, June 7th**

7:30 - 8:30 AM Breakfast at the school every morning (Monday- Friday)

**Session #4:**

8:30-9:10 AM: Ben Schuler (university of Zurich)

 **Crowding in a biomolecular condensate**

9:10-9:50 AM: Shahar Sukenik (UC Merced)

 **Extreme crowding: what happens to proteins when water leaves the cell?**

10:00 AM-10:15 AM Coffee Break

10:15-10:55 AM: Huan-Xiang Zhou (University of Illinois Chicago)

 **Atomistic modeling of liquid-liquid phase equilibrium explains dependence of critical**

**temperature on γ-crystallin sequence**

10:55-11:35 AM: Stephen Fried (Johns Hopkins University)

**Photo-crosslinking mass spectrometry captures protein complexes at residue-level**

**resolution in their native environment**

**Wednesday 5:30pm - 7:30pm: All Telluride Science Picnic**

Free BBQ, Beer, Wine and Non-Alcoholic Beverages. Friends & Family are invited free of charge.

Location: Tent behind the Intermediate School (*which is the location for all workshops:*

 *725 W Colorado Ave*)

**Thursday Morning, June 8th**

7:30 - 8:30 AM Breakfast at the school every morning (Monday- Friday)

**Session #5: Crowding, phase separation and assemblies**

8:30- 9:10 AM: Klaus Huber (University of Paderborn)

**Mimicking nature – self-assembly of a synthetic dyestuff under crowding conditions**

9:10-9:50 AM:Simon Ebbinghaus (TU Braunschweig) – Virtual Talk

**Folding and self-assembly in cells and condensates**

10 AM-10:15 AM Coffee Break

10:15-10:55 AM Caitlin Davis (Yale University)

**Stability vs. environmental sensitivity: The trade-off faced by proteins *in vivo***

10:55-11:35 AM Stéphane Longeville (CEA Saclay)

 **Polymerization and diffusion of hemoglobin in the sickle cell disease (SCD)**

**Thursday 5:30pm - 7:30pm: Pizza Party $45/person**

**Available Abstracts**

**Monday June 5th**

**Small molecules are not so small inside the cell: Charge plays a critical role in dictating the diffusion behaviour of molecules**

Debabrata Dey, and Gideon Schreiber\*

Department of Biomolecular Sciences, Weizmann Institute of Science, Israel

Living cells are densely packed conglomerate of macromolecules, where diffusion is essential for their function. The crowded conditions may affect diffusion both through hard (occluded space) and soft (weak, non-specific) interactions. Diffusion serves as a good proxy to measure the binding or aggregated state of the molecule of interest (proteins or small molecules) in the crowded environment. We have previously developed Line-FRAP [1], a method based on measuring recovery of photobleaching under a confocal microscope that allows diffusion coefficient measurements in a variety of environments, from *in vitro* to *in vivo*. The use of Line mode greatly improves the time resolution in FRAP data acquisition, from 20-50 Hz in the classical mode to 800 Hz in the line mode. We evaluated the method using 20 different bacterial proteins chemically labelled or by fusion to YFP. The calculated diffusion rates were comparable to literature data as measured *in vitro*, in HeLa cells and in *E.coli*. Diffusion coefficients in HeLa was ~2.5-fold slower and in *E.coli* 15-fold slower than measured in buffer and were comparable to previously published data. However, the diffusion behaviour of small molecule drugs is very different under different crowding conditions, including living cells [2-3]. Changing the protein crowder concentration has a marked influence on some of the drug’s behaviour depending on its nature to aggregated state or surface adsorption properties [2]. However, inside the cell, we found a strong correlation between the drug p*K*a and intracellular diffusion and distribution [3]. Weakly basic, small-molecule drugs displayed slower fraction recovery after photobleaching and 10 to 20-fold lower diffusion rates in cells than in aqueous solutions, suggesting sequestration. But while our imaging studies showed that acidic organelles, particularly the lysosome, captured these molecules, blocking lysosomal import did not improve diffusion. Instead, blocking protonation enhanced their diffusion, as shown by their N-acetylated analogues [3]. Moreover, co-administration with basic quinacrine, which is accumulated in lysosomes, prevented the sequestration of the anti-cancer drug mitoxantrone, thereby improving its biodistribution. These results suggest combining N-acetylation with co-administration may improve the intracellular diffusion and distribution of weakly basic, small-molecule drugs [3]. Diffusion behaviour of small organic molecules inside the complex environment of a living cell can also be very useful, considering drug efficacy depends not only on delivery but also on intracellular diffusion, distribution, and availability.

**References:**

1. Line-FRAP, a versatile method to measure diffusion rates in vitro and in vivo by D. Dey, S. Marciano, A. Nunes-Alves, V. Kiss, R.C. Wade, G. Schreiber, *Journal of Molecular Biology*, *433*, 9, article number 166898 (**2021**).
2. The effects of protein crowders on small molecule drug diffusion by D. Dey, A. Nunes-Alves, R. C. Wade, G. Schreiber, *iScience*, *25*, 10, article number 105088 (**2022**).
3. Reversing protonation of weakly basic drugs greatly enhances intracellular diffusion and decreases lysosomal sequestration by D. Dey, S. Marciano, A. Poryvai, O. Groborz, L. Wohlrábová, T. Slanina, G. Schreiber, *bioRxiv*, (**2023)** <https://doi.org/10.1101/2023.04.19.537456>

**Protein stability in living cells & under crowded conditions *in vitro***

Gary J. Pielak

Department of Chemistry

University of North Carolina at Chapel Hill

The crowded and complex environment in cells is predicted to affect protein behavior compared to dilute buffer. My laboratory and that of our collaborator, Daniel Harries, are examining the stability of proteins and their complexes in cells and *in vitro* under crowded conditions. A challenge in these endeavors is detecting the test protein in a sea of crowders. 19F-NMR is ideally suited to overcome this challenge and provide high quality data on folded- and unfolded- proteins as well as free- and bound- forms of complexes. I will focus on experiment-based equilibrium-thermodynamic protein-stability data acquired in living *Escherichia coli*cells, *Xenopus laevis*- and *Danio rerio*- oocytes and under crowded conditions *in vitro*. The latter efforts focus on concentrated polyethylene glycol (PEG) solutions using data acquired as a function of temperature, PEG molecular weight and PEG concentration. The results show that crowding affects protein stability in ways not always correctly predicted by simple models. Daniel Harries will then describe a new model to explain the effects of crowding on protein stability.

**Cell volume rescue via crowding-induced phase separation**

Arohan R. Subramanya, MD

Departments of Medicine and Cell Biology

University of Pittsburgh School of Medicine

During hypertonic stress, acute cell volume contraction threatens cellular homeostasis. To counter this problem, cells coordinate a rapid volume recovery response termed regulatory volume increase (RVI). For decades, physiologists proposed that cells trigger RVI by detecting the molecular crowding that accompanies acute shrinkage, though the underlying mechanisms involved were obscure. In this presentation, I will describe how this crowding sensing function is mediated by With-No-Lysine (WNK) kinases, a family of serine-threonine kinases that coordinate electroneutral cation chloride flux via phosphorylation. During volume contraction, a massive intrinsically disordered region (IDR) encoded by the WNK1 C-terminal domain (CTD) drives the phase separation (PS) of WNK1 into biomolecular condensates, which activate signaling and net ion influx. I will discuss conserved features within WNK IDRs that facilitate their crowding-induced phase behavior and will describe how the WNK CTD triggers condensate-dependent volume recovery within minutes in response to small changes in tonicity. The discovery that WNK kinases function as physiologic phase separating crowding sensors provides a clear example of functional condensed phase signaling in biology, with implications for the design of crowding induced biomolecular condensates that work within the natural range of stress.

**Tuesday Morning, June 6th**

**Influence of Crowded Conditions on Glutamate Dehydrogenase kinetics**

Kristin Slade

Department of Chemistry,

Hobart and William Smith Colleges

To study the consequences on macromolecular crowding on enzyme kinetics, the Michaelis-Menten parameters of glutamate dehydrogenase (GDH) were monitored in the presence of high concentrations of polyethylene glycol (PEG), dextran, or bovine serum albumin (BSA). Given this enzyme’s central role at interface of nitrogen and carbohydrate metabolism, GDH is highly regulated. Our results suggest that crowding may influence this regulation. For example, crowding enhances substrate inhibition of GDH in a pH-dependent manner. The effects of crowding on the enthalpy ΔH҂ and entropy ΔS҂ of activations determined from Eyring plots were also pH-dependent. Overall, these results highlight the importance of studying enzymes especially heavily regulated ones like GDH under crowded conditions that more realistically mimic the crowded cellular environment.

**Molecular Simulations of the Influence of Crowders on Enzymes: From Domain Motion to the Active Site**

Eva Pluhařová

J. Heyrovsky Institute of Physical Chemistry, Czech Academy of Science, Czech Republic

The cellular environment is crowded, because macromolecules occupy up to 40 % of the volume of the cells. Proteins are frequently studied in aqueous solutions without the presence of other macromolecules both in vitro and in silico. However, conclusions drawn from these studies may not be fully applicable, because crowding influences the overall stability of proteins, conformational equilibria, binding, enzyme kinetics, etc.

 We focus on two enzymes: glutamate dehydrogenase and citrate synthase whose activities are influenced by crowding. By means of classical molecular simulations, we study these enzymes in aqueous solutions of glucose and dextran. Our simulations show that crowding slightly alters protein structure and fluctuations in comparison with aqueous solution. Principal component analysis identified dominant domain motion around the active site. We observed that the crowder molecules preferentially interact with the polar amino acid residues at the protein surface as well as with the substrate molecules present in the active site. The dynamics of the protein-crowder interaction quantified as a residence time is pronouncedly heterogeneous. Finally, substrate molecules in the active site are flexible. The results shall provide deeper understating of enzymes' functioning in crowded cells.

**Proteins in a heated crowd: linking the dynamics and structure of the cellular interior to cell metabolism and thermal death**

Stepan Timr

Department of Computational Chemistry
J. Heyrovsky Institute of Physical Chemistry, Czech Republic

The importance of accounting for the crowded conditions in the cell interior is now well recognized. However, we are only beginning to understand how changes in the local structure and dynamics of the cytoplasm allow cells to control metabolic processes and respond to changing external conditions. In this presentation, I am going to describe multiscale simulations establishing a link between the temperature-dependent dynamics of the E. coli cytoplasm and the folding state of the E. coli proteome. By combining the computational results with those of neutron scattering measurements, we clearly show that only a minor fraction of the proteome unfolds at the cell death. We find that these unfolded proteins form an entangling interprotein network—dominated by hydrophobic interactions—across the cytoplasm. Finally, the combined insights from experiments and simulations prove that the dynamical state of the E. coli proteome is an excellent proxy for the temperature dependent bacterial metabolism and death.

**Simulation of proteins and fluid at mesoscale: crowding, aggregation and other amenities**

Fabio Sterpone

French National Center for Scientific Research, Paris
In this talk we discuss the potentiality of multi scale simulations of crowded systems via an unified framework combining particle and fluid dynamics. We show how the transport properties in crowded protein solutions result from a delicate balance among inter-protein interactions. We explore the combination of coarse-grained and atomistic simulations to quantify the thermal stability of proteins under different crowding states.  We explore how the explicit inclusion of hydrodynamics affects protein dynamics at different scales and opens the route for in-silico rheology of cytoplasm-like systems. Finally, we move outside the cell and discuss how extra-cellular crowding and fluid motion participate in pathological processes as amyloid aggregation.

**Reconciling the existence of tau-mediated microtubule bundles with polymer physics**

Peter Chung

Department of Physics and Astronomy

 Tau, an intrinsically disordered protein unequivocally linked to Alzheimer's disease, is known to bind to and bundle microtubules into higher-order lattices. These lattices maintain their wide spacings (~25-30 nm) under macromolecular crowding conditions in both *in vitro* and cell free experiments, a surprising result that is inconsistent with the intrinsically disordered nature of Tau. Here, I will propose different models to reconcile these inconsistencies and incorporate recently published findings on the ability of Tau to form condensates on microtubules surfaces. Proposed experiments may provide a novel, molecular function of Tau that potentially allows molecular trafficking to occur under crowded cell conditions.

**Wednesday Morning, June 7th**

# Atomistic Modeling of Liquid-Liquid Phase Equilibrium Explains Dependence of Critical Temperature on γ-Crystallin Sequence

# Huan-Xiang Zhou

Department of Chemistry and Department of Physics

# University of Illinois Chicago

Liquid-liquid phase separation of protein solutions has regained heightened attention for its biological importance and pathogenic relevance. Coarse-grained models are limited when explaining residue-level effects on phase equilibrium. Here we report phase diagrams for γ-crystallins using atomistic modeling. The calculations were made possible by combining our FMAP method [1] for computing chemical potentials and Brownian dynamics simulations for configurational sampling of dense protein solutions, yielding the binodal and critic temperature (*T*c). We obtain a higher *T*c for a known high-*T*c γ-crystallin, γF, than for a low-*T*c paralog, γB. The difference in *T*c is corroborated by a gap in second virial coefficient [2]. Decomposition of inter-protein interactions reveals one amino-acid substitution between γB and γF, from Ser to Trp at position 130, as the major contributor to the difference in *T*c. This type of analysis enables us to link phase equilibrium to amino-acid sequence and to design mutations for altering phase equilibrium.

[1] Qin & Zhou, J Phys Chem B 120, 8164 (2016)

[2] Qin & Zhou, J Phys Chem B 123, 8203 (2019)

[3] A preprint is available in bioRxiv: [https://doi.org/10.1101/2023.04.25.538329](https://es.sonicurlprotection-mia.com/click?PV=2&MSGID=202305032035000188164&URLID=5&ESV=10.0.21.7607&IV=7AE38E7ABA3D895069984D2D03563063&TT=1683146106501&ESN=DS93AQ2KrfMEzug%2Bo2mknyywU3NXb%2FshcDShVdIEUJc%3D&KV=1536961729280&B64_ENCODED_URL=aHR0cHM6Ly9kb2kub3JnLzEwLjExMDEvMjAyMy4wNC4yNS41MzgzMjk&HK=22D93359836BB951EA16DD694E699503023615A5D365169CEE7E95D2C2784BBB)

**Photo-crosslinking Mass Spectrometry Captures Protein Complexes at Residue-level Resolution in their Native Environment**

Anneliese M. Faustino,1 Stephen D. Fried1,2

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Structural methods such as x-ray crystallography and cryo-EM have played prominent roles in our understanding of protein complexes. However, many protein complexes function in intricate, multifaceted, and sometimes surprising ways in the cellular context, and high-resolution structural information of protein complexes *in situ* is challenging to obtain. Presently, cryo-electron tomography (cryo-ET) has emerged as a leading technique to address these questions. Though powerful, this method is most effective for large protein complexes with distinctive shapes (such as the ribosome). Complementary to cryo-ET are crosslinking mass spectrometry (XL-MS) approaches which excel at resolving molecular identities, and with recent advances are now amenable to analyzing complex samples (i.e., whole proteomes). Photo-crosslinkers have the theoretical advantage of possessing greater structural and temporal resolution than chemical crosslinkers, but due to technical challenges, they have yet to be applied to complex samples. We have recently introduced a new type of photo-crosslinker and workflow which together enable reliable proteome-wide identification of photo-crosslinked peptides. In these experiments, cells are treated with a photo-activatable crosslinker, rapidly crosslinked *in situ*, and the resulting crosslinked peptides sequenced, revealing protein-protein interaction networks captured in their native cellular context at residue-level resolution. Piloting this method on *E. coli*, we have discovered some interesting interactions, some novel (such as evidence for coupling between RNA polymerase to bacterial histone-like proteins) and some providing new structural insight into known protein complexes (such as the degradosome and the mannose phosphotransferase system importer). Scaling up these methods, we anticipate that photo-crosslinking will be able to resolve quinary interaction networks at high resolution in a variety of systems, and in combination with cryo-ET, help us to decode the “molecular sociology” of the cell.

**Thursday Morning, June 8th**

**Stability vs. environmental sensitivity: The trade-off faced by proteins *in vivo***

Caitlin M. Davis

Department of Chemistry

Yale University

Although biomolecules evolved to function in the cell, most biochemical and biophysical studies have been carried out in dilute aqueous solution *in vitro.* Such studies neglect differences in the local environments of cells that can impact protein stability and function. Here we have experimentally tested the role of the local environment on the folding of FRET-labeled phosphoglycerate kinase (PGK) *in vitro, E. coli,* U-2 OS cancer cells, and in differentiated tissues of zebrafish. To investigate cellular pressures on protein sequence evolution, we compared a consensus PGK with four extant sequences. Temperature-dependent fluorescence microscopy was used to monitor thermodynamics and kinetics via the single-cell FRET signal. Strikingly, we found the folding of PGK-FRET to be dependent on tissue and cell type. To interpret *in vivo* and in-cell results, an *in vitro* systematic series of solvation environments distinguished contributions from non-steric chemical interactions and steric crowding interactions to stability, compactness, and kinetics. Analysis of extant homologs of PGK revealed that sequence differences are predominantly located at surface residues. We find that PGK stability is strongly correlated with more negative net surface charge that leads to repulsive interactions, whereas environmental sensitivity is correlated with high positive surface charge that promotes sticking in the cytosol. We conclude that residues at the surface positions are not weakly conserved because they are less important for stability in the cell than the highly conserved interior residues, but rather that their lack of conservation results from unique selective pressures imposed by the intracellular environment. Taken together, our results demonstrate that different cellular environments of different tissues contribute to different protein sequences, stabilities, and kinetic phenotypes.