EBRC Engineering Biology Research Consortium

2025 Annual Meeting Program

May 1-2, 2025

University of Washington

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EBRC Engineering Biology Research Consortium

ANNUAL MEETING AGENDA

University of Washington

Alder Hall
1315 NE Campus Pkwy
Seattle, WA 98105

Wednesday, April 30, 2025

4:00-5:30 PM	**SPA Site Visit** *Email helix@ebrc.org for more information*
7:30-8:30 PM	**Industry Member Mixer** *Email helix@ebrc.org for more information*
7:30-9:30 PM	**SPA Social** *Email helix@ebrc.org for more information*

Thursday, May 1, 2025

Alder Hall: Alder Auditorium	
8:00 AM	**Arrival & Registration** *Coffee and tea available* *Poster set up in Alder Commons*
8:30 AM	**WELCOME TO THE EBRC ANNUAL MEETING** *India Hook-Barnard (EBRC)*

8:45 AM	**SESSION 1: RESEARCH TALKS - "Social and Educational Dimensions of Engineering Biology Research"** *Moderator: Cameron Kim (Duke University)* • Eunseo Lee (Washington University in Saint Louis): Evaluating large language models' impact on graduate-level bioengineering education • Callie Chappell (Stanford University): LABraries and LABrarians support biotechnology for everyone • Nasa Sinnott-Armstrong (Fred Hutchinson Cancer Center): IndiGROW: Art as a platform for synthetic biology education • Cameron Kim (Duke University): LABEEs -Laboratory Automation in Bio(medical) Engineering Education
9:45 AM	**National Security Commission on Emerging Biotechnology (NSCEB)** *Highlights from the Commission's Report, and call to action*
10:45 AM	**BREAK** *Light refreshments are located in Alder 105.*
11:15 AM	**Policy Focus Area Update** *Garrett Dunlap (EBRC)*
11:30 AM	**SESSION 2: RESEARCH TALKS - "Toward Waste Valorization for Future Bioeconomy"** *Moderator: Tae Seok Moon (J. Craig Venter Institute)* • Ariane Mora (California Institute of Technology): Enzyme-tk: A computational and experimental pipeline for the identification of engineerable enzymatic starting points for wastewater bioremediation • Ross Klauer (University of Delaware): Discovery and engineering of DyP peroxidases for LDPE oxidation to initiate deconstruction • Hansen Tjo (Princeton University): Engineering sugar uptake in the lignocellulolytic, extreme thermophile *Anaerocellum bescii* for producing renewable fuels and chemicals • Kathryn Hoyt (Northeastern University): 13C-metabolic flux analysis in the methanol-consuming acetogen *Eubacterium limosum*
12:30 AM	**LUNCH** *Boxed lunch provided in Alder 105*

1:30 PM	***JSPG* Showcase Talks** *Moderator: Julietta Sheng (EBRC)* ● Casey Isabelle (Arizona State University): Governance challenges for direct to consumer genetically engineered organisms ● Avery Brewer (Arizona State University): Genetically engineered microbes for bioremediation: opportunities and limitations in the emerging bioeconomy
1:50 PM	**Student and Postdoc Flash Talks** *Moderator: TBD*
2:30 PM	**BREAK** *Light refreshments are located in Alder 105.*
3:00 PM	**Enacting EBRC Roadmaps** *Moderator: Emily Aurand (EBRC)* *Panel discussion with guest experts on the landscape and challenges for translating and implementing foundational engineering biology research and tools across applications.* ● Larisa Rudenko (BioPolicy Solutions) ● Rob Carlson (Planetary Technologies) ● Arden Yang (Allen Institute)
3:50 PM	**Roadmapping Focus Area Update** *Emily Aurand (EBRC)*
4:00 PM	**Keynote Address** *Dr. Kate Rubins (NASA)* Sequencing, Sensing, and Synthetic Biology in Space
5:15 PM	**Poster Session** *Posters in Alder Commons, Food in Alder 105* ● *Odd*-numbered Poster Presentation Time: 5:15 - 6:15 ● *Even*-numbered Poster Presentation Time: 6:15 - 7:15
7:30 PM	**ADJOURN**

Friday, May 2, 2025

Alder Hall: Alder Auditorium	
8:00 AM	**Day 2 Arrival** *Coffee and tea available*
8:30 AM	**WELCOME TO DAY 2** *India Hook-Barnard (EBRC)*
8:45 AM	**Security and Responsibility in Engineering Biology** *Becky Mackelprang (EBRC)*
8:45 AM	**Opening up "Containment"** Introduction ● Emma Frow, Dalton George (*Arizona State University*) Panel ● Richard Murray (*Caltech*) ● James Chappell (*Rice University*) ● Becky Mackelprang (*EBRC*)
9:15 AM	**Security at the intersection of AI and engineering biology** ● Sebastian Rivera (*EBRC*)
9:30 AM	**Security Focus Area**
9:45 AM	**BREAK** *Light refreshments are located in Alder 105.*

10:10 AM	**SESSION 3: RESEARCH TALKS - "Regulation of Cells and Materials"** *Moderator: Theresa Loveless (Rice University)* ● Shiyi Li (University of California, Los Angeles): Programmable artificial RNA condensates in mammalian cells ● Gun Woo Byeon (University of Washington): Design of overlapping genes using deep generative models of protein sequences ● Elise Zimmerman (Rice University): Pathogen-selective antibiotics created using a trans-splicing ribozyme triggered by native mRNA ● Xiangting Lei (Georgia Institute of Technology): Light-induced reversible assembly and actuation in ultrafast Calcium-driven chemomechanical protein networks
11:00 PM	**TBA**
11:45 AM	**LUNCH** *Boxed lunch provided in Alder 105*
12:45 PM	**Education Focus Area Update** *Emily Aurand (EBRC)*
12:50 PM	**Student & Postdoc Association (SPA) Update** *Ava Karanjia, SPA President (University of Washington)*
1:00 PM	**Community BioFutures** ● Johnathan O'Neil, Julietta Sheng (EBRC) ● Janet Standeven (Georgia Tech): Creating local synthetic biology pipelines based on our experiences at Georgia Tech and regional K-12 schools

1:30 PM	**SESSION 4: RESEARCH TALKS - "Advances in High-Throughput Synthetic Biology"** *Moderator: Ilenne Del Valle (Oak Ridge National Laboratory)* ● David Ross (National Institute of Standards and Technology): A strategy for data collection to solve the protein function problem ● Harry Adamson (Pennsylvania State University): A scalable, low-cost, high-throughput design-build-test pipeline for complex genetic systems ● Jacob Brandner (University of Washington): Pooled microbial CRISPR screens using single-cell RNA sequencing ● Jiwoo Kim (Rice University): Using synthetic and systems biology to compare the effects of osmotic and matric potential on a soil bacterium ● Corey Husdon (The Align Foundation): Open data and public benchmarking to accelerate the field of protein engineering
2:50 PM	**CLOSING REMARKS**
3:00 PM	**ADJOURNMENT**

NATIONAL SECURITY COMMISSION ON EMERGING BIOTECHNOLOGY

Moderator: Dr. India Hook-Barnard (EBRC)
Thursday, May 1

Alexander Titus (NSCEB)

Alexander Titus is a technologist working at the intersection of data science and biotechnology. His career has woven between the private sector, public sector, and academia, and he is currently the VP of Artificial Intelligence & Machine Learning at Avidity Biosciences and Research Faculty at the University of Southern California.

Sam Weiss Evans (NSCEB)

Sam's work focuses on the governance of security concerns and other broader social aspects of emerging research and innovation, especially biology. He is currently a Senior Policy Advisor for the US National Security Commission on Emerging Biotechnology, where he leads the Commission's work on safety, security, and responsible innovation. Previously, Sam was at Harvard University, in a joint appointment with the School of Government and the School of Engineering and Applied Sciences.

JSPG SHOWCASE TALKS

Moderator: Dr. Julietta Sheng (EBRC)
Thursday, May 1

"Governance challenges for direct to consumer genetically engineered organisms"

Casey Isabelle (Arizona State University)

In this briefing, we propose that direct-to-consumer (DTC), live genetically engineered (GE) organisms represent a novel and non-obvious category of biotechnology. The context of use for these products raises new governance and regulatory questions. Unlike most GE organisms to date, which have been marketed to large agro-industrial players, direct-to-consumer organisms are marketed directly to individuals. The four case examples of GE DTC organisms explored in this briefing - which include the Glofish, Firefly Petunia, Norfolk Purple Tomato, and Zbiotics probiotic - showcase niche products directed towards at-home lifestyle, ornamental, or wellness applications. Challenges for governing these products in society include 1) a lack of information available to consumers about broader risks, impacts, and responsible use, 2) deployment and stewardship is under the purview of the individual consumer in society and without explicit containment mechanisms, and 3) accountability or responsibility for addressing any unintended consequences is unclear. We suggest that these challenges could be addressed by considering the context of use as a key part of federal agency product reviews, supporting more comprehensive post-market surveillance of these products, and creating greater transparency mechanisms via a public registry for DTC products.

Co-authors: Casey Isabelle, Dalton R. George

"Genetically engineered microbes for bioremediation: opportunities and limitations in the emerging bioeconomy"

Avery Brewer (Arizona State University)

With the global bioremediation market anticipated to grow by $8.29 billion dollars between 2023 and 2028, this method of reducing pollutants represents a rapidly expanding sector of the bioeconomy (Johnson 2024). Millions of tons of pollutants now contaminate soil and groundwater, posing severe threats to human and environmental health (Münzel et al. 2022). Bioremediation is an effective way to immobilize or transform many pollutants. However, as emerging contaminants such as microplastics, xenobiotics, and per- and polyfluorinated alkyl substances (PFAS) prove increasingly resistant to treatment with naturally occurring organisms, it may be necessary to expand bioremediation's toolkit. There has been long-standing interest in developing genetically engineered microbes for bioremediation (GEMBs) to enable faster remediation times and address a wider range of contaminants. Despite decades of investigation and development of GEMBs, none have been commercialized. Historically, the perceived need for GEMBs has not been sufficient to justify the investment and risk in the context of an uncertain regulatory environment and a paucity of fundamental knowledge of GEMBs. However, as industries, environments, and human health experience disruptions from increasingly recalcitrant, widespread, and hazardous pollutants, the value proposition of GEMBs is more compelling than ever before. The contemporary challenges with managing environmental pollutants coupled with advances in genetic engineering methods and renewed interest from researchers, developers, and policy makers signal an opportunity to realize the potential for GEMBs. To support safe and efficient development, characterization, and commercialization of GEMBs as a means of addressing the urgent crisis of pollution, we propose (1) clarifying and restructuring the regulatory process for GEMBs, (2) developing research and testing infrastructure for GEMBs, and (3) establishing an interagency coordination office.

Co-authors: Avery M. Brewer, Dalton R. George

ENACTING EBRC ROADMAPS

Moderator: Dr. Emily Aurand (EBRC)
Thursday, May 1

Rob Carlson (Planetary Technologies)

Dr. Carlson is a Managing Director at Planetary Technologies and is well-known in our community as the creator of the Bioeconomy Dashboard, among many other bioeconomy resources. Rob has written numerous books-and-blogs on biotechnology solutions, scaling technology for sustainability, and strategies for investment and making the most out of biology.

Larisa Rudenko (BioPolicy Solutions)

Dr. Rudenko is a co-founder of BioPolicy Solutions and is known as one of the foremost experts in considering the opportunities and risks of emerging biotechnologies. Larisa has worked in the public and private sector, including as a senior advisor for biotechnology at the FDA and consulting on biotech regulations globally.

Arden Yang (Allen Institute)

Dr. Yang is the Vice President for Innovation at the Allen Institute, where he leads their collaborations and strategic alliances towards maximizing the impact of their research and discoveries. Arden has led biotechnology strategy and innovation programs at AstraZeneca, Genentech, and Adaptive Biotechnologies.

KEYNOTE ADDRESS

Dr. Kate Rubins
Thursday, May 1

Photo Credit: NASA

Kate Rubins was selected by NASA in 2009. Rubins completed her first spaceflight on Expedition 48/49, where she became the first person to sequence DNA in space. She holds a Bachelor of Science in Molecular Biology from the University of California, San Diego and a Ph.D. in Cancer Biology from Stanford University Medical School Biochemistry Department and Microbiology and Immunology Department. Dr. Rubins conducted her undergraduate research on HIV-1 integration in the Infectious Diseases Laboratory at the Salk Institute for Biological Studies. She worked as a Fellow/Principal Investigator at the Whitehead Institute for Biomedical Research and headed 14 researchers studying viral diseases that primarily affect Central and West Africa. Rubins served aboard the International Space Station as flight engineer on Expedition 63/64. Across her two long-duration spaceflights, she has four spacewalks and a total of 300 days in space.

RESEARCH TALK ABSTRACTS

Thursday, May 1

SESSION 1: RESEACH TALKS - "Social and Educational Dimensions of Engineering Biology Research"
Moderator: Cameron Kim (Duke University)

"Evaluating Large Language Models' Impact on Graduate-Level Bioengineering Education"
Eunseo Lee (Washington University in Saint Louis)

Artificial Intelligence (AI) and large language model (LLM) use is becoming prevalent in college education and workforce development in the bioengineering field today. While LLMs can answer a specific query, their capability to answer quantitative problems or bioprocess engineering reasoning problems is in question. Despite the wide usage of LLM among students, there is little data on the LLM's impact on higher education (e.g., whether it may discourage independent thinking and learning). We conducted a quantitative comparison study in a graduate-level bioprocess course by focusing on three popular large language models: US OpenAI and Chinese Qwen and DeepSeek.

We studied if the use of AI can improve student performance on an exam with conceptual and calculation problems related to bioprocess. The students took 2 exams of the same content, first without using LLM and second using LLMs provided by the university. In the exam where LLM use was allowed, the average score was 36% higher compared to a textbook-and-notes-only exam. Our findings suggest that AI helps everyone equally - students with disability and students with and without an undergraduate background in biology. Even high school students using LLMs scored average of 72, outperforming the average graduate students with average score of 66.3. We also compared different LLMs' performance. Under best practices, OpenAI o1 achieved a score of 89 out of 100, and DeepSeek R1 achieved a score of 84.5, both surpassing 96% of human test-takers, while cheaper LLM models DeepSeek V3, OpenAI GPT-4o and Qwen 2.5 14B scored lower than 73. This disparity among LLMs on test performance can lead to social inequality if students cannot have access to paid LLM resources.

These findings suggest that as the AI can provide instant answers, traditional course format focusing on conceptual knowledge is becoming outdated. To make higher education relevant, we found strategies to design problems that are difficult for LLMs to answer, by adding extra details in the question statement and incorporating the latest research. This approach encourages students to engage deeply with the literature and course material rather than relying heavily on LLM-generated content. We also developed an AI-literacy course module that introduces AI/LLM hands-on activities for students to learn LLM applications in biology. This activity equips students with essential skills for the future job market in the biomanufacturing field.

Co-authors: Zhengyang Xiao, Eunseo Lee* (presenter), Roland Ding, Sophia Yuan, Yinjie J. Tang

"LABraries and LABrarians support biotechnology for everyone"
Callie Chappell (Stanford University)

To ensure that everyone can learn, explore, and innovate responsibly with biology, we must have publicly accessible labs, LABraries and community-based science leaders, LABrarians. In this talk, we will lay out a vision for a LABrary system and a new profession, LABrarians.

Although initially for biology, LABraries will eventually serve as centers of knowledge and learning for all natural sciences. Just as the public library system transformed the 20th century, a 21st century LABrary system will empower millions starting with bioliteracy.

Spaces such as community biology labs, nature centers, and museums already excite the public to explore biology in their everyday lives. Yet, they are not accessible to all. A LABrary in every city and town will equip everyone to support their families and communities by providing good jobs, new innovations, and education for a better tomorrow. The public can learn new skills, conduct research, and develop new inventions at their local LABrary. These public spaces for science will equip everyone to be leaders and participants in the domestic and global bioeconomy, not just passive consumers and observers.

Co-authors: Callie R. Chappell, Ana Quiroz, Kevin Cordero, Angel Millard-Bruzos, Alex Agris, Esteban Cervantes, David Kong, Drew Endy

"IndiGROW: Art as a platform for synthetic biology education"
Nasa Sinnott-Armstrong (Fred Hutchinson Cancer Center)

Art is a crucial medium for promoting conversations about engineering biology in society. Here, we present a recent art project, IndiGROW, that explores the relationship between bioindustrial manufacturing and the history of indigo dye. For the piece, we grew bioengineered yeast that produces an indigo-like substance, indigoidine. It features a number of artworks based around microbial production of indigoidine dye and examines our understanding of biotechnology as a force of global change. This project was exhibited in Los Angeles as part of Emergence, a group show by Fathomers in the broader series of Pacific Standard Time (PST) ART: Science and Art Collide galleries.

In this talk, I will discuss the process of developing and presenting this artwork, the legal and logistical challenges of doing so as an assistant professor, and the impact of science-art collaborations like these beyond academic and industry spaces. In addition to the art exhibition, this project represents new, technical improvements in bioengineered dye production and is integrated into a series of community workshops for the public. By merging science and art, this project increases awareness of and access to engineering biology.

Co-authors: Nasa Sinnott-Armstrong, Callie Chappell, Dezmond Goff, Ashlyn Kamin

"LABEEs -Laboratory Automation in Bio(medical) Engineering Education"

Cameron Kim (Duke University)

The next revolution in biotechnology is being led by the advent of low-cost, high throughput lab automation systems. Scientists and engineers of tomorrow will need to master these concepts, yet there is a lack of good training material for students currently studying at the university level. These skills will require an understanding of the hardware and software necessary to implement new protocols, troubleshoot designs, and innovate new automation technologies. We begin to address this need via a multifaceted approach. Using the OpenTrons OT2 liquid handling platform and the Open-Source Python software PyLabRobot, we designed curriculum materials in the form of guided Jupyter notebooks for students at both the undergraduate and graduate levels. Our notebook suite exposes students to concepts in lab automation, such as serial dilution and our Food Coloring Academy, guides them through protocol design using PyLabRobot, and incorporates a browser-based visualizer for students to simulate their designed protocols on a wide array of robots used in both industry and research labs. As students complete training notebooks, we pose open-ended technical and reflective questions to be answered in a two-semester senior capstone class. Specifically, we demonstrated that the ELISA assay can be performed on the OT2 robot, and collected preliminary data on how it can be optimized to reduce both reagent and plastic waste, advancing an integration of sustainability in biotechnology research. This project will be continued as a capstone by undergraduate students in the F24-S25 school year. Our vision is integrated within a larger education ecosystem of first-year engineering design and communication that introduces custom automation components, a biotechnology makerspace and authentic undergraduate research experiences, and ethical awareness in limitations and biases of laboratory automation. As a student-led initiative, we hope to report on the increased self-efficacy of biomedical engineering students in comfort not only in lab robotics and automation, but also design thinking, algorithmic thinking, and sustainable practices. We invite an open dialogue at our poster to get input from industry leaders, lab managers, students, and faculty as to how our curriculum should be shaped for future iterations of the course materials, appropriate scaffoldings, and garnering industry support for education advances in laboratory automation that prepares the incoming workforce with the relevant knowledge, skills, and abilities (KSAs) in this field. Our poster will be interactive to better understand the educational state-of-art and ultimately, develop open-source educational resources that are hardware-agnostic and appropriately scaffolded for any level of university education.

Co-authors: Joseph Laforet Jr, Margaret Gatongi, Stefan Golas, Cameron M. Kim, PhD, Emma J. Chory, PhD.

SESSION 2: RESEARCH TALKS - "Toward Waste Valorization for Future Bioeconomy"
Moderator: Tae Seok Moon (J. Craig Venter Institute)

"Enzyme-tk: A computational and experimental pipeline for the identification of engineerable enzymatic starting points for wastewater bioremediation"
Ariane Mora (California Institute of Technology)

Anthropogenic chemicals are ubiquitous: they are used in agriculture (e.g. pesticides), present in everyday materials (e.g. plastics), and personal care products (e.g. siloxanes). Pollutants impact human health, adversely affect crop yield, and contaminate water supplies. Enzymes, nature's catalysts, present a benign and efficient approach to break down toxic chemicals and thus enable novel bioremediation approaches. However, finding an enzyme capable of catalyzing a specific, desired reaction remains a challenge. To accelerate bioremediation efforts, we developed a computational and experimental toolkit, Enzyme-tk, to identify engineerable enzymes to degrade a specific pollutant of interest. First, machine learning and bioinformatic approaches are used to identify bacterial and archaeal enzymes for a desired reaction. These in silico predictions are then validated by an experimental pipeline, and sequence-function data are collected for model finetuning and directed evolution campaigns. Using our toolkit, we discovered an enzyme from a thermophilic bacterium that degrades bis(2-ethylhexyl) phthalate, an endocrine-disrupting pollutant found at high concentrations in biosolids. Overall, this work builds the foundations of an experimental and computational toolkit that accelerates enzyme discovery for bioremediation.

Co-authors: Ariane Mora*, Julia C. Reisenbauer*, Helen Schmid, Ikumi Miyazaki, William Rieger, Yueming Long, Ryen O'Meara, Sabine Brinkmann-Chen, Frances H. Arnold (* joint first authors)

"Discovery and engineering of DyP peroxidases for LDPE oxidation to initiate deconstruction"
Ross Klauer (University of Delaware)

Polyethylene (PE) is the most widely produced plastic globally due to its favorable high ductility, mechanical strength, and bond homogeneity. However, these properties make PE highly resistant to deconstruction. This high recalcitrance means that there is not a robust recycling infrastructure for PE, as it is less expensive to produce PE from virgin petroleum feedstocks. PE upcycling approaches can offer a more economically viable alternative, but chemical deconstruction and upcycling approaches have high energy requirements and limited product specificity that limit their economic feasibility. Biological PE deconstruction and upcycling can improve PE waste management by allowing high-yield conversion of PE waste to valorized products with low energy requirements resulting from operation near ambient conditions. However, biological PE deconstruction is not yet possible, as polyethylene active enzymes are yet to be discovered. In this work, we mine the gut of LDPE fed yellow mealworms and discovered Dye Decolorizing Peroxidases (DyPs) that oxidize LDPE, initiating deconstruction. We identify a hydrophobic loop region that is required for DyP LDPE activity and demonstrate its importance by tuning DyP activity by modulating loop length and hydrophobicity. Moreover, we show that a non-canonical active site comprised of solvent exposed aromatic amino acid side chains is responsible for activity on LDPE chains that are too large to fit into the canonical enzyme active site. Engineering efforts for improved DyP stability, flexibility, and catalytic activity have led to improvements in enzymatic activity towards traditional DyP dye substrates and towards LDPE, moving the enzyme class closer to industrially relevant activity levels. This work provides a starting point for a completely biological PE deconstruction pathway by demonstrating changes in oxidation state on PE chains without chemical pre-treatment. This critical first step towards biological PE upcycling provides an additional carbon source for (bio)manufacturing to reduce reliance on petroleum based feedstocks for chemical production.

Co-authors: Ross R. Klauer, D. Alex Hansen, Zoé O.G. Schyns, Lummy Oliveira Monteiro, Jenna A. Moore-Ott, Mekhi Williams, Megan Tarr, Jyoti Singh, Ashwin Mhadeshwar, LaShanda T.J. Korley, Kevin V. Solomon, Mark A. Blenner

"Engineering uptake of lignocellulose-derived sugars in the extremely thermophilic, lignocellulolytic bacterium Anaerocellum bescii for producing renewable fuels and chemicals"

Hansen Tjo (Princeton University)

Lignocellulosic biofuels and biochemicals are the building block to sustainable, bio-based supply chains. However, plant biomass is physically and chemically recalcitrant to degradation and conversion into value-added products. Anaerocellum bescii, a non-model extremely thermophilic bacterium (T_opt ~ 75 ℃), is a promising metabolic engineering chassis organism due to its highly effective carbohydrate-active enzymes (CAZymes) and rich inventory of ATP-Binding Cassette (ABC) sugar transporters enabling simultaneous fermentation of a broad range of oligosaccharides. Nevertheless, the potential of A. bescii as a model microbial platform for biofuels production is constrained by a limited understanding of how diverse sugars from CAZyme-mediated lignocellulose deconstruction are transported into the cell.

Here, we utilize in vitro experiments and in vivo genetics to investigate the hitherto unknown substrate preferences for lignocellulose-important ABC transporters in A. bescii. We have identified the sole ABC transporter for cellulose utilization in A. bescii, genetically deleted it, and shown that the absence of this transporter disrupts growth on cello-oligosaccharides and cellulose. Then, using well-established protein expression and biophysical screening workflows i.e. differential scanning calorimetry and isothermal titration calorimetry, we have determined the substrate specificity of the two cello-oligosaccharide - binding proteins associated with this ABC transporter. We complement our findings on cellulose transport with structural and biophysical insights into the uptake of xylan and xyloglucan substrates from hemicellulose. Notably, we have solved the crystal structure of the xyloglucan-binding protein in A. bescii, which possesses affinity for xyloglucan oligosaccharides as well as cello-oligosaccharides. We also discuss ongoing efforts to knockout sugar transporters specific to lignocellulosic substrates for metabolic control of CAZyme and chemical production. By shedding light on, and perturbing the entry points for lignocellulosic sugars into the cell, our results will accelerate the development of A. bescii as an industrially-relevant workhorse for converting plant biomass into renewable fuels and chemicals.

Co-authors: Hansen Tjo, Anherutowa Calvo, Kelly Blundin, Jonathan M. Conway

"13C-Metabolic Flux Analysis in the Methanol-Consuming Acetogen Eubacterium limosum"

Kathryn Hoyt (Northeastern University)

Acetogens are particularly promising microbial biocatalysts, as they are anaerobic bacteria that grow on a range of C1 compounds (CO2, CO, methanol, formate) at high carbon yield and energetic efficiency. Eubacterium limosum is a particularly flexible acetogen, as it can grow on all of these C1 compounds, although growth is slow which limits productivity. Cofeeding glucose has been reported to increase the growth rate of E. limosum on methanol, specifically reaching a 7.5-fold increase in specific growth rate, and a temporary 33% increase in specific methanol uptake rate during co-feeding, compared to growth on methanol alone [1]. The reason behind the cofeeding benefit has not been previously understood. We interrogated the underlying mechanism by conducting the first 13C metabolic flux analysis (MFA) for a methylotrophic acetogen, and the first for E. limosum. To conduct the growth experiments in parallel, we adapted the eVOLVER mini-reactor platform [2] into a benchtop anaerobic mini-chemostat platform (AneVO). Over the course of these experiments, we expanded knowledge of central carbon metabolism in E. limosum, identifying an unannotated enzyme connecting glycolysis with the bifurcated TCA cycle, and revealing the unexpected impact of cysteine uptake on the direction of carbon flux through pyruvate ferredoxin oxidoreductase (PFOR). We also calculated in vivo Gibbs free energies for the reaction steps most important to mixotrophic cofeeding, which showed high reversibility, and operation close to equilibrium for all three substrate conditions. These pathway steps with low driving forces require more enzyme to be generated to maintain flux and are potential locations for engineering. These insights are indispensable for ongoing engineering of E. limosum into a microbial biocatalyst for C1 conversion into product compounds.

References:

1. Loubiere et al. J. Gen. Microbiol. 138, 979–985 (1992).
2. Wong et al. Nat. Biotechnol. 36, 614–623 (2018).

Co-authors: Kathryn Hoyt, Liam Jenkins Sánchez, William Gasparrini, Maxwell Nyadeva, Dr. Benjamin Woolston

RESEARCH TALK ABSTRACTS

Friday, May 2

SESSION 3: RESEARCH TALKS - "Regulation of Cells and Materials"
Moderator: Theresa Loveless (Rice University)

"Programmable artificial RNA condensates in mammalian cells"
Shiyi Li (University of California, Los Angeles)

Artificial biomolecular condensates have emerged as powerful tools to modulate cellular behavior. In this work, we describe a strategy for constructing artificial condensates in living mammalian cells using single-stranded, short RNA motifs. These condensates are RNA-rich compartments that form spontaneously and remain distinct from their surroundings. The designed RNA sequences contain stem-loop structures that fold during transcription and phase separate through loop-loop interactions in the nucleus and cytoplasm. By tuning these sequences, we can generate distinct condensate populations that can be directed to specific subcellular locations. Furthermore, these RNA motifs can be engineered to recruit small molecules and proteins into the RNA-rich phase. Introducing linker RNAs enables the formation of multiple subcompartments, where organization can be precisely controlled by adjusting the relative abundance of different RNA species. This approach offers a versatile platform for investigating and manipulating molecular processes within cells.

Co-authors: Shiyi Li, Eric John Payson, Yuna Kim, Anli A. Tang, Dino Osmanovic, Kevin Wang, Alexandra Bermudez, Wen Xiao, Neil Lin, Kathrin Plath, Douglas L. Black, Elisa Franco

"Design of overlapping genes using deep generative models of protein sequences"
Gun Woo Byeon (University of Washington)

Overlapping genes (OLGs) that encode separate proteins in two different reading frames of the same nucleotide sequence are surprisingly common in nature and found in a majority of viruses. But since the discovery of OLGs by Sanger and co-workers in 1977, their very existence has posed a long standing biophysical and evolutionary puzzle given that the sharing of codon nucleotides severely restricts their sequence space. Are naturally observed OLGs merely individual evolutionary idiosyncrasies, or on the contrary, is overlap-encoding readily feasible? We investigate this question by engineering synthetic OLG sequences using state-of-the-art generative models of protein sequences. To evaluate the approach, we designed overlapped sequences that fall into two different protein families. We also encoded distinct highly ordered de novo protein structures and observed surprisingly high in silico and experimental success rates. This demonstrates that the overlap constraints under the structure of the standard genetic code do not significantly restrict simultaneous accommodation of well defined 3D folds in alternative reading frames. Our work suggests that OLG sequences may be frequently accessible in nature and could be readily exploited through synthetic biology approaches to provide new strategies for engineering genetic stability, bio-containment and compression of genetic information.

Co-authors: Gun Woo Byeon, Marc Expòsit Goy, David Baker, Georg Seelig

"Pathogen-selective antibiotics created using a trans-splicing ribozyme triggered by native mRNA"

Elise Zimmerman (Rice University)

The rise of antimicrobial-resistant pathogens due to the overuse and non-specific nature of chemical antibiotics is predicted to result in >10 million deaths annually by 2050. Additionally, current pipelines to discover antibiotics are depleted. Thus, there is an urgent need for novel antibiotics that are easy to produce, cell-selective, and simple to diversify as drug-resistant microbes evolve. To address this, we have developed a new class of RNA therapeutics. Our platform, called RNA-Activated Protein by Trans-splicing Ribozyme (RAPTR), is a modular ribozyme-based system that encodes a death-inducing protein output that is translated only when spliced into a pathogen-specific target mRNA, enabling the use of the host translational machinery to express the protein output. RAPTRs are concise RNA sequences encoded in a single RNA strand that can be easily incorporated into delivery vectors. We developed a selection platform to perform high-throughput library screenings coupled with next-generation sequencing to identify potent RAPTR designs for any given mRNA target. RAPTR can be engineered to produce toxic outputs fit for each pathogenic strain thus reducing escape rates. Additionally, we successfully engineered P1 bacteriophage to deliver RAPTR to E. coli while maintaining RAPTR's mRNA-specific activity. Finally, we demonstrated the potential applications of RAPTR by targeting a clinically-derived pathogenic E. coli strain and achieved 99.99% killing efficiency with no observable effect on a non-pathogenic E. coli strain. We further tested the pathogen-specific RAPTR in a synthetic consortium, containing both wild-type and pathogenic E. coli, and observed selective removal of the pathogen. This work creates a novel RNA-based antimicrobial system capable of killing bacteria with high efficiency, low escape rate, and high selectivity that can be used to combat antimicrobial-resistant bacteria and selectively control fitness in microbial consortia.

Co-authors: Maria Claudia Villegas Kcam, Kim Wai Mo, Dorsa Sattari Khavas, Li Chieh Lu, Jonathan Silberg, James Chappell

“Light-induced reversible assembly and actuation in ultrafast Calcium-driven chemomechanical protein networks”

Xiangting Lei (Georgia Institute of Technology)

Programming ultrafast, reversible motions in soft materials has remained a challenge in active matter and biomimetic design. Here, we present a light-controlled chemomechanical network based on \textit{Tetrahymena thermophila} calcium-binding protein 2 (Tcb2), a Ca^{2+}-sensitive contractile protein. These networks, driven by Ca^{2+}-triggered structural rearrangements, exhibit dynamic self-assembly, spatiotemporal growth, and contraction rates up to tenfold faster than ATP-driven actomyosin systems with non-muscle myosin II motors. By coupling light-sensitive chelators for optically triggered Ca^{2+} release, we achieve precise, reversible growth and contractility of Tcb2 networks, revealing emergent phenomena such as boundary-localized active regions and density gradient-driven reversals in motion. A coupled reaction-diffusion and viscoelastic model explains these dynamics, highlighting the interplay between chemical network assembly and mechanical response. We further demonstrate active transport of particles via network-mediated forces \textit{in vitro} and implement reinforcement learning to program sub-second, spatiotemporal actuation \textit{in silico}. These results establish a platform for designing responsive active materials with ultrafast chemomechanical dynamics and tunable optical control, with applications in synthetic cells, sub-cellular force generation, and programmable biomaterials.

Co-authors: Carlos Floyd, Jerry Honts, Saad Bhamla

SESSION 4: RESEARCH TALKS - "Advances in High-Throughput Synthetic Biology"
Moderator: Ilenne Del Valle (Oak Ridge National Laboratory)

"A Strategy for Data Collection to Solve the Protein Function Problem"
David Ross (National Institute of Standards and Technology)

The success of AlphaFold2 and similar AI models in predicting protein structure from sequence has demonstrated the potential revolutionary impact of AI applied to biotechnology. A key ingredient in that success was the protein structure data in the PDB, which took over four decades and $10B to collect. To enable future Bio-AI models with similar impact (but in less than 40 years), we need strategies to collect the appropriate high-quality data. NIST and The Align Foundation have been collaborating with several other labs to develop a strategy and measurement platform to collect large datasets to enable a solution to the Protein Function Problem: the quantitative prediction of protein function from sequence. The resulting growth-based quantitative sequencing (GROQ-seq) platform can be adapted to a variety of different protein function types and can produce quantitative functional characterization data for hundreds of thousands of proteins per experiment at a cost of approximately $0.05 per sequence. In this presentation, we will discuss the key features of the GROQ-Seq platform that enable quantitatively reproducible measurements with distributed data collection at multiple biofoundries, including: 1) the use of shared laboratory automation protocols, 2) "protein function ladder" calibration standards, and 3) quantitative assessment and validation of data quality. We will also give an overview of the progress in the ongoing development of GROQ-Seq assays for different types of proteins including allosteric transcription factors, proteases, tRNA synthetases, RNA polymerases, histidine kinases, single-chain antibody fragments, and metabolic enzymes. Finally, we will highlight some examples of the quantitative accuracy of GROQ-Seq and use of the resulting data to engineer difficult-to-achieve and multi-objective functional changes, including changes in ligand specificity, functional tuning, and inversion of function.

Co-authors: Aviv Spinner, Simon d'Oelsnitz, Svetlana Ikonomova, Olga Vasilyeva, Nina Alperovich, Eugenia Romantseva, Dana Cortade, Erika DeBenedictis, Peter Kelly, Paul Campitelli, Liskin Swint-Kruse, Banu Ozkan, Ross Thyer, Zachary Jansen

"A Scalable, Low-Cost, High-Throughput Design-Build-Test Pipeline for Complex Genetic Systems"

Harry Adamson (The Pennsylvania State University)

High-throughput genetic engineering remains constrained by high synthesis costs, assembly inefficiencies, and challenges in expressing structurally complex proteins. To address these limitations, we developed a massively parallel, cost-effective pipeline for the design, assembly, and functional screening of complex genetic systems, enabling the rapid construction of hundreds to thousands of protein expression plasmids (2 days per work unit, ~$18 per plasmid). Our pipeline integrates multi-objective computational design, utilizing Promoter Calculator and RBS Calculator to optimize promoter strength, ribosome binding sites, codon usage, and genetic stability. A Genetic Systems Builder (GSB) algorithm automates sequence fragmentation, overhang optimization, and primer design for high-fidelity oligo pool-based assembly. By combining ssDNA and a Golden Gate assembly workflow, this approach enables low-cost construction of highly repetitive and GC-rich sequences that are difficult to synthesize using traditional polymerase cycling assembly methods. Context Aware AutoAlign, a novel bioinformatics algorithm, performs automated quality control and gene expression error identification using long-read nanopore sequencing. Functional characterization is performed with a translationally coupled in vivo biosensor, providing real-time, label-free quantification of protein expression without cell lysis or protein modifications.

As proof of concept, we applied this pipeline to assemble and characterize highly repetitive and GC-rich structural proteins, achieving a 78% build efficiency and screening distinct subpopulation phenotypes across constructs. We subsequently adapted this workflow for high-throughput application, constructing a diverse genetic library of 209 sequences (0.7kb – 5.6kb) in parallel. We show this DBT framework overcomes key bottlenecks in high-throughput synthetic biology workflows while reducing costs by an order of magnitude compared to traditional cloning methods.

Co-authors: James R. McLellan, Khushank Singhal, Melik C. Demirel, Howard M. Salis

"Pooled microbial CRISPR screens using single-cell RNA sequencing"
Jacob Brandner (University of Washington)

High-content pooled CRISPR screens using single-cell RNA sequencing allow massive-scale interrogation of transcriptional networks. Despite their utility, pooled CRISPR screens in bacteria have been limited to measuring simple phenotypes such as cell growth. Here, we present CRISPR-microSPLiT, a pooled microbial single-cell CRISPR activation and interference (CRISPRa/i) screening platform capable of deciphering hundreds of gene regulatory networks simultaneously by targeting endogenous transcription factors. Our platform uses microbial split-pool ligation transcriptomics (microSPLiT), a high-throughput and low-cost single-cell RNA sequencing technology, to measure the transcriptome-wide impact of each perturbation within a pool. Each CRISPR perturbation is encoded by a specific guide barcode construct (GBC) transcript which links the identity of the guide to the transcriptional response. To benchmark our platform, we performed CRISPRa/i on transcriptional activators and repressors in E. coli and measured expression of downstream genes in the regulatory network. Many promoters are controlled by multiple transcription factors and therefore multiple guides are necessary to dissect these complex regulatory interactions. In a screen of 18 multi-guides either targeting two or three genes simultaneously, we found the dominant transcription factor for promoters controlled by multiple regulators. Lastly, in a pooled library of over 100 guides targeting 52 genes, we uncovered direct regulatory network connections for 14 known and 3 putative transcription factors across 28 CRISPRa/i guides. For regulators with no prior characterization (lgoR, ytfA, phnF), we identified novel transcriptional responses. Our results demonstrate that CRISPR-microSPLiT is a scalable and versatile platform for uncovering gene regulatory networks. We expect that CRISPR-microSPLiT will be portable to industrially-relevant microbes for applications in metabolic engineering and biotherapeutics.

Co-authors: Jacob R. Brandner, Quoc Tran, Dmitry Sutormin, Karl D. Gaisser, Jesse G. Zalatan, Georg Seelig, James M. Carothers, Anna Kuchina

"Using synthetic and systems biology to compare the effects of osmotic and matric potential on a soil bacterium"

Jiwoo Kim (Rice University)

Soil water availability affects microbial community structure, nutrient availability, and respiration. Soil water potential describes the force at which the soil is holding onto the water, which controls the direction of the water flow. Currently, it is not known if the soil osmotic potential (Ψo) and matric potential (Ψm) lead to similar gene expression responses in microbes. To study this question, we studied the activity of a putative osmotic stress response promoter in Variovorax beijingensis, a microbe isolated as part of a model soil consortium that grows on chitin. By examining the activity of this promoter using an indicator gas reporter, we show an increase in indicator gas production as water potential increases in both liquid medium and soil matrix. Analysis of Variovorax growth across a range of osmotic and matric potentials revealed that this microbe can respire and grow in liquid and soil conditions that span -240 to -2630 kPa, which exceeds plant wilting point (-1500 kPa). Ongoing work is investigating how the transcriptome of Variovorax responds to osmotic and matric pressure stresses of -240 and -1400 kPa. We will present the results of this transcriptome analysis, which is expected to reveal how V. beijingensis adapts differently to high Ψo and Ψm at the transcription level. It will also reveal transcription regulators that may be targeted for engineering soil microbial communities that remediate fertile land affected by droughts and floods.

Co-authors: Caroline A. Masiello, Jonathan J. Silberg

"Open data and public benchmarking to accelerate the field of protein engineering"
Corey Hudson (Align Bio)

The Align Foundation is a nonprofit organization that works to propel biology into a new era of reproducible, scalable, and shareable research. Our vision is a world where tedious experimentation can be supplanted with powerful predictive models that accelerate progress in the field. To get there, we host a suite of programs and tournaments designed to bring people together and enable the execution of large, collaborative, data-intensive projects supported by automated methods. By doing so, we hasten the 'industrial revolution' of life science research, helping the field mature into a robust, agile, well-understood discipline.

Our work is accomplished largely by two complementary research thrusts: the creation of novel, public datasets and creating metascientific challenges for the synthetic biology community. Our public datasets are collaboratively generated through coordinated methods development and data acquisition powered by partnerships with automated research facilities, including cloud labs, contract research organizations and biofabs. We then monitor the impact of our data by hosting community benchmarking competitions and machine learning challenges.

To assess the state of engineering methods, we have implemented a protein function engineering tournament for creating clear problem definitions and benchmarks for success. Inspired by The Critical Assessment of Protein Structure Prediction (CASP), a competition that led to the development of Alphafold2, we created a Protein Engineering Tournament for benchmarking computational protein design techniques. Our initial iteration of this Tournament concluded with teams competing from around the world in both in silico and wet lab validation rounds on the topic of enzyme design. The next instance of this tournament will begin later in 2025, with expanded scope and additional impactful challenge questions. We hope the Protein Engineering Tournament will provide a transparent platform with which to evaluate progress in this field and mobilize the scientific community. Data from the initial tournament has been open sourced and provided for community use. The next instance of this tournament will make data sharing and model evaluation centerpiece outcomes, in order to further the goal of furthering more powerful protein function prediction.

Co-authors: Corey M. Hudson, Peter Kelly, and Erika DeBenedictis, The Align Foundation

POSTER SESSION LIST

Session 1 (5:15 – 6:15 pm)

1. **Nathan Miller, University of Delaware**
   "Investigating the role of the gut microbiome from yellow mealworm larvae in LDPE deconstruction"

3. **Aditya Sarnaik, Arizona State University**
   "Bioprocess engineering for manufacturing sunscreen components from non-GMO cyanobacteria"

5. **Lukas Oesinghaus, University of Washington**
   "Quantitative design of cell type-specific mRNA stability from microRNA expression data"

7. **Stephanie Heard, University of Illinois Chicago**
   "Engineering of Burkholderiales bacteria for the discovery of new polyketide-nonribosomal peptide natural products"

9. **Megan McSweeney, Stanford University**
   "A modular cell-free protein biosensor platform using split T7 RNA polymerase"

11. ***Poster Withdrawn***

13. **Nicholas Kaplan, University of Washington**
    "Quantifying replication fidelity of unnatural base pairs with nanopore sequencing and deep learning"

15. **Leah Anderson, University of Washington**
    "Enhancing freeze-thaw tolerance in heat-resilient brewing yeast through experimental evolution"

17. **Quoc Tran, University of Washington**
    "CRISPRi-based synthetic circuits for neural network-like computing in mammalian cells"

19. **Yan Zhang, California Institute of Technology**
    "Designing the Cell-Free Gene Expression Environment with the One-Pot PURE System: Insights into Reaction Composition and Efficiency"

21. **Cholpisit Kiattisewee, Massachusetts Institute of Technology**
    "How Many Plasmids Can Bacteria Carry? A Synthetic Biology Perspective"

23. **Cholpisit Kiattisewee, SynBio4ALL Africa**

"SynBio4ALL Africa: Accessible Synthetic Biology Education Across the Continent"

25. James McLellan, The Pennsylvania State University
"Predicting & controlling T7 RNAP transcription rates to increase IVT yields & eliminate undesired transcription products"

27. Ian Faulkner, University of Washington
"DBTL cycles for engineering aromatic bioproduction in Pseudomonas putida with multi-node CRISPRa/i gene regulatory programs"

29. P. C. Dave Dingal, The University of Texas at Dallas
"Identification and engineering of highly active proteases using co-evolutionary models"

31. Rory Majule, University of Washington
"eChromatin: Developing a cell-free gene expression platform using targeted induced steric hinderance within DNA arrays"

33. Zhengyang Xiao, Washington University in St. Louis
"Glucose-acetate co-utilization in two nonmodel oleaginous yeasts for efficient bioproduction"

35. Sadikshya Rijal, Arizona State University
"Mitigating Winner-Takes-All Resource Competition with CRISPR-Driven Multi-Module Negative Feedback"

37. Efraín Rodriguez Ocasio, University of Wisconsin-Madison
"Computer aided identification and production of replacement bioproducts for the decarbonization of the chemical industry"

39. Zachary Martinez, California Institute of Technology
"Declarative Bioengineering Platform for the Simplification of Composing Physics and Deep Learning-Based Workflows"

41. Rohita Roy, Stanford University
"Engineering Pulsatile Population Dynamics: A Paradoxical Feedback Circuit for Synthetic Biology and Therapeutics"

43. Hinako Kawabe, University of Washington
"Harnessing Non-standard Nucleic Acids for Highly Sensitive Icosaplex (20-Plex) Detection of Microbial Threats for Environmental Surveillance"

45. Ben Alexander, University of Delaware
"Modulating CAZyme Activity Through Histone Modification in Anaerobic Gut Fungi"

47. Katie Denecke, University of Washington

"A RNA scaffold-based CRISPR system for orthogonal gene activation and repression in immune cells"

49. Amanda Ro, Lauren Ackermann, University of Washington
"Using Experimental Evolution to Select Against Mother-Daughter Cell Separation Defects in Brewing Yeast"

51. Tracy Mallette, University of Washington
"Overcoming Slow Reaction Kinetics in Hybrid DNA:RNA Strand Displacement Reactions"

53. Jayson Sumabat, University of Washington
"High accuracy nanopore sequencing of xenonucleic acids using deep learning"

55. Andrea Garza Elizondo, Oak Ridge National Lab
"Physical automation of the domestication of non-model bacteria"

57. Luis Montalvo González, Purdue University
"Modular construction of biocontainment circuits for non-traditional rhizobia chassis"

59. Siyuan Feng, Northwestern University
"Enhancing transcription-based sensing with complex coacervates"

61. Amanda Robert, University of Washington
"Developing toolkits for genome engineering and transcriptional programming in purple nonsulfur bacteria"

63. Arren Liu, Johns Hopkins University
"Developing Genetic Manipulation Tools for The Gut Microbiota Member Turicibacter sanguinis"

65. Kira Olander, University of Washington
"Programmable Molecular Recording Using CRISPR-Based Circuits for Enhanced Bioproduction in Microbes"

67. Karly Liebendorfer, Georgia Institute of Technology
"Optimizing the Limit of Detection for the TLISA Point-of-Care Biosensor Platform to Achieve Clinical Relevancy"

69. Gage Owens, University of Washington
"Appropriating the biosynthesis of nucleoside natural products for biocatalysis"

71. Randon Serikawa, University of Washington
"LTTR Late Than Never: High Throughput Characterization of LysR-Type Transcriptional Regulators"

73. Randi Avery, University of Washington
"yEvo: Leveraging a high school teaching lab to study antifungal resistance mutations"

75. Margaret Cook, University of Washington
"Developing kinetic models of cell-free lysate metabolism for carbon-conserving pathway engineering"

77. Angie Aguirre-Tobar, Fred Hutchinson Cancer Center
"Lil Labs: Bridging Biotechnology to Community for Local Action and Innovation"

79. Emily Heckard, Georgia Institute of Technology
"Portable Glucose Monitor-Based Field Deployable Sensing"

81. Haoxian Xu, University of Washington
"Family-wide functional annotation of transcriptional regulators"

83. Jackson Comes, University of Washington
"Harnessing the Metabolic Flexibility of Purple Non-Sulfur Bacteria for Bioproduction Applications"

85. Jiho Seok, Georgia Institute of Technology
"Reducing False Positives with an AND-Gate Rolling Circle Amplification for Pathogen Detection"

87. Jason Cain, University of Washington
"An in silico multi-scale model of combinatorial regonition circuit design in CAR T cell-based therapies"

89. Jonathan Klonowski, EBRC
"Accelerating Engineering Biology Innovation through Advanced Automation"

91. Michael Guzman, University of Washington
"Conjugation-Compatible Serine-integrase Assisted Genome Engineering (cSAGE) in Non-Model Bacteria"

93. Barbara Dunn, University of Washington
"The Rise and Fall of Brewing Yeasts: Harnessing the Awesome Power of Experimental Evolution to Select for Increased Flocculation in Ale and Lager Yeasts"

POSTER SESSION LIST

Session 2 (6:15 – 7:15 pm)

2. **Meagan Olsen, Northwestern University**
   "Towards economical on-demand biomanufacturing of biologics with minimal cell-free systems"

4. **Adjo Elodie Kadjo, University of Illinois Chicago**
   "The Role of Epigenetics in Bacterial Secondary Metabolite Production"

6. **Anne Villacastin, Lawrence Berkeley National Laboratory**
   "Enhancing Sorghum Transformation for Bioenergy Crop Improvement Using Morphogenic Chimera GRF-GIF"

8. **Vitor Lourenzon, University of Illinois at Chicago**
   "Engineering marine sponge-associated bacteria for enhanced production of antibiotic antidotes."

10. **August Staubus, Rice University**
    "High-throughput domain insertion libraries produce ligand-activated splicing ribozymes for broad-host range inducible control, genetic recordings, and biosensing"

12. **Rachel L. Powell, University of Washington**
    "One CEN to Rule Them All: Characterization of S. cerevisiae and S. uvarum centromeric sequences to engineer the Saccharomyces panCEN"

14. **Talia Jacobson, University of Florida**
    "Plant and yeast bioengineering unveils glycosyl hydrolases for soluble fiber production"

16. **Sulogna Chatterjee, University of Florida**
    "Bioengineering Cupriavidus necator to produce advanced polyhydroxyalkanoate from levulinic acid"

18. **Sebastian Castillo Hair, University of Washington**
    "Generalizable design of human cell type-specific expression via deep learning models of genomic accessibility"

20. **Asher Vokoun, The Pennsylvania State University**
    "High-throughput assembly and evaluation of nitroreductase enzymes for 2,4,6-trinitrotoluene degradation"

22. **Cholpisit Kiattisewee, Massachusetts Institute of Technology**

"Political and Bureaucratic Instability - the Elephant in the Room Hindering Thailand's Bioeconomy"

24. Logan Spicer, University of Florida
"Marchantia polymorpha as a Synthetic Biology Chassis for Plant Glycobiology"

26. Morgan Bean, University of Washington
"A compact CRISPR recorder for high-resolution mapping of clonal immune cell lineage decisions"

28. Ania-Ariadna Baetica, Drexel University
"Efficient Exploration of Synthetic Gene Circuit Design Spaces: A Multi-Objective Simulated Annealing Approach"

30. Cameron Roots, University of Texas at Austin
"EFM Calculator 2.0: Characterizing and predicting the presence of short repeated sequences to improve genetic stability"

32. Matt Demelo, Rice University
"Profiling Nrf2 Activity across Cell Lineages with Analog DNA Memory Recorders for Prediction of Cisplatin Resistance Emergence in Head and Neck Squamous Cell Carcinomas"

34. Natanya Villegas, University of Oregon
"Optimizing in vitro Transcribed CRISPR-Cas9 Single-Guide RNA Libraries for Improved Uniformity and Affordability"

36. Johnathan O'Neil, Engineering Biology Research Consortium
"Bioinspiration: An Argument for Investing in Basic Biological Research in the U.S."

38. Joseph Vath, Georgia Institute of Technology
"Modular Communication in Synthetic Protocells"

40. Samiha Zaman, University of Delaware
"Engineering romaine lettuce leaf surface yeast for human pathogen detection"

42. Yunqing Wang, California Institute of Technology
"Targeted DNA Insertion in Plants by CRISPR-associated Transposases"

44. Raine Hagerty, University of Delaware
"Development of tools for stable genetic engineering of the lignocellulolytic anaerobic gut fungus Neocallimastix frontalis"

46. Madelyn Shelby, University of Washington
"Deciphering the Human Splicing Code with Massively Parallel Reporter Assays"

48. Beau Lonnquist, University of Washington - Seattle
"Bending the Rules: de novo Transcription Factor Design for Targeted Gene Regulation"

50. Arjun Aditham, Fred Hutchinson Cancer Center
"Deep mutational scanning defines mutational constraint and antibody-escape mutations of rabies glycoprotein"

52. Ziqi Zheng, Georgia Institute of Technology
"Modeling and Optimization of Linear Expression Template Stability in E. coli-Based Cell-Free Systems"

54. Nidhi Mehta, University of Washington
"Redesigned single chain IL-12 enhances its secretion in E. coli for cancer immunotherapy"

56. Abdelrahman Youssef, Arizona State University
"Bistability Driven by Resource Competition in Cascading Gene Circuits"

58. Shifeng Xu, Georgia Institute of Technology
"Low-Cost Point-of-Care Diagnostic for Early Preeclampsia Detection via TLISA-Based Protein Sensing"

60. Theresa Loveless, Rice University
"Toward long-term recording of cell history"

62. Hannah Roberts, University of Florida
"Biomanufacturing performance and transcriptomic response of engineered Escherichia coli in both simulated and spaceflight variable gravity"

64. Sarah Grube, University of Washington
"Transcriptome-Guided Landing Pad Selection for Stable Bioproduction in Rhodobacter sphaeroides 2.4.1"

66. Widianti Sugianto, University of Washington
"Harnessing immobilized cell-free multi-enzyme systems as reusable biocatalysts"

68. Alexandra Patterson, Georgia Institute of Technology
"MEDIFLOW (Multiplexed and Engineerable Diagnostics using Integrated Flow)"

70. Zekun Li, Worcester Polytechnic Institute
"Oleochemical cell factories enabled by synthetic biology and metabolic engineering in the extremophile yeast Debaryomyces hansenii CBS 767"

72. Brian Darst, University of Washington

"MOD-GAP: Modular genetic design for one-pot Golden Gate Assembly of multi-guide CRISPR programs in bacteria"

74. Ross Jones, University of British Columbia
"Understanding upstream dominance in synthetic gene circuits in human cells"

76. Bria Metzger, University of Washington
"Lil Lab Network: Local to Global Co-Design For Biotechnology Futures"

78. Andrew Holston, University of Oregon Knight Campus
"Engineering fusion variants to optimize chimeric histidine kinase functionality"

80. Yujia Huang, University of Washington
"Profiling transcriptional regulatory networks at high resolution and scale in non-model bacteria using a pooled CRISPR-microSPLiT screening platform"

82. Dezmond Goff, SoundBio Lab
"Science in Action: How a Local Community Lab Bridges Research and the Common Good"

84. Stephen Fedak, University of Washington
"Mapping bacterial sRNA regulatory networks with single-cell RNA sequencing and CRISPR interference"

86. Tommy Primo, University of Washington
"Tunable Synthetic Promoters for CRISPRa-Mediated Multi-Gene Expression in Pseudomonas putida for Optimizing Chemical Bioproduction"

88. Caleb Hoffman, Southern Utah University
"Characterizing Clostridium novyi-NT Spore Surface Composition: A Detection Toolkit"

90. Felicia Oentoro, Georgia Institute of Technology
"Protein degradation as a tool in cell-free systems"

92. Jessica Caruso, University of Washington
"Directed Evolution of a Non-Heme Iron Enzyme Enabled by Computational Stabilization for Amine Hydroxylation"

94. Vinoo Selvarajah, iGEM

POSTER ABSTRACTS

1. ***"Investigating the role of the gut microbiome from yellow mealworm larvae in LDPE deconstruction"***

Nathan Miller (University of Delaware)

More than 20 million tons of plastic waste annually accumulates in our environment leading to environmental and human health issues. Polyethylene (PE) is the most abundant polymer in waste streams (37%) and is considered highly resistant to degradation. Industrial efforts, including mechanical and chemical processes, are energetically intensive and generate reduced-value products. Biological systems are a promising alternative to PE recycling as they operate at physiological conditions and may be engineered to generate value-added products. However, no complete pathway for PE degradation has been elucidated. To address this, we study the yellow mealworm (larvae of Tenebrio molitor), which possesses the ability to consume and deconstruct PE over rapid timescales (<30 days). We hypothesize that the mealworm's gut microbiome is inhabited by a diverse group of bacterial and fungal taxa that contain an array of enzymes that participate in PE deconstruction. While it is known that the composition of the gut microbiome shifts significantly with a PE diet, the functional significance of these changes has not been evaluated. We utilize high-temperature gel permeation chromatography (HT-GPC) to directly track how the microbiome changes the size of ingested PE polymer chains. However, low molecular weight hydrocarbon (~C20-C120) products from the mealworm itself are convoluted with PE degradation products creating the potential for false deconstruction claims. We report several strategies to remove this contamination prior to and/or following HT-GPC analysis. Equipped with a robust method of analysis, we plan to investigate if the mealworm microbiome is either (i) necessary and/or (ii) sufficient for PE deconstruction and determine the functional contribution of this diverse microbial community.

Co-authors: Nathan Miller, Ross Klauer, Alex Hansen, Dr. Zoe Odile Schyns, Dr. Jenna Ott Moore, Dr. Mark Blenner, Dr. Kevin Solomon

2. *"Towards economical on-demand biomanufacturing of biologics with minimal cell-free systems"*

Meagan Olsen (Northwestern University)

There is a significant global need for rapid, on-demand production of medical therapeutics to address emergent biological threats. Current manufacturing processes for protein-based biologics are often time-consuming, expensive, and inaccessible to rural and developing regions. In contrast to established in vivo production platforms, cell-free protein synthesis systems take advantage of the cellular machinery in crude cell lysates to express proteins without the need for living cells and can be freeze-dried to readily store, distribute, and activate production by simply adding water. However, high reagent costs and comparatively low protein yields (~$3,500 for common reagents per gram protein) limit the widespread use of cell-free systems. Here, we seek to optimize cell-free protein synthesis systems to achieve low-cost, high-yielding, and scalable production of medically relevant proteins. We first analyze cell-free reaction metabolomics to identify problematic reagent degradation and accumulation associated with the use of low-cost, minimal reagent formulations. We then apply Design of Experiments-based optimization strategies to develop a novel reagent mixture that uses non-phosphorylated energy substrates and is capable of producing >2 g/L of protein product at <5% the cost of traditional cell-free systems. This minimal cell-free system is demonstrated across a panel of medically relevant protein products, including conjugate vaccines, full-length antibodies, and therapeutic enzymes. We also explore alternative approaches to improving cell-free protein synthesis yields, including introduction or removal of effector proteins and activation of key metabolic and energy regeneration pathways. This work furthers the development of cost-effective cell-free biomanufacturing for rapid protein therapeutic production.

Co-authors: Caroline E. Copeland, Chad A. Sundberg, Geuncheol Gil, Ryan N. Rezvani, Manesh Chand, Weston K. Kightlinger, Antje Krüger, Govind Rao, James R. Swartz, Ashty S. Karim, & Michael C. Jewett

3. *"Bioprocess engineering for manufacturing sunscreen components from non-GMO cyanobacteria"*

Aditya Sarnaik (Arizona State University)

Moving from just sun protection to safe for the skin cosmetics, sunscreen UV-filters, regulated as over-the-counter drugs, are increasingly scrutinized for human health impacts. Furthermore, their ecological impacts are also being questioned, creating significant new regulatory barriers and motivation to find safer and more eco-friendly alternatives. Cyanobacteria can naturally produce UV-absorbing mycosporine-like amino acids (MAAs) offering a biologically- and ecologically-safer solution over chemically synthesized UV-filters. This work highlights a pilot scale framework for cultivation of a cyanobacterial consortium for biomanufacturing of MAAs, concurrent with harvesting of cyanobacterial exopolysaccharides (EPS).

A stable bioproduction consortium has been developed, under this work, with a N-fixing Cyanothece sp. HCC 1134, Picosynechococcus sp. PCC 7002 and Synechococcus bacillaris CCMP 1333. The consortium was maintained in F/2 medium and scaled up to 15 L, indoors under diurnal light conditions. It was further scaled up in duplicate, to 110 L in outdoor green-house flat panels, with and without NaNO3. Results show that the cultures produce intra- and extra-cellular MAAs, absorbing in both the UVA and UVB ranges. UVB absorbing MAAs are more abundant than UVA absorbing MAAs extracellularly, while both types of MAAs are in relatively equivalent concentration intracellularly. In the absence of UV light, MAA production was influenced by the C:N ratio, where N-supplemented cultures produce double the MAAs compared to the cultures using only atmospherically fixed nitrogen. Similarly, EPS production was predominantly influenced by the C:N ratio, as well as the metabolic state of the culture. EPS production was equivalent under both the tested conditions. The consortium was further scaled up to 750 L in a tubular photobioreactor (LGEM), under natural sunlight, with intermittent NaNO3 spikes for sustaining growth and bioproduction. MAA and EPS productions were higher in the LGEM than the flat panels, in part due to higher light intensities.

The results indicate that 1) the consortium naturally produces MAAs and EPS, 2) MAA yields can be improved through UV exposure, and 3) EPS yields can be improved by modulating the environmental conditions of light, temperature and nutrients. Overall, this process for economical production of MAAs and EPS highlights the potential for a new era of safe and effective, eco-friendly sunscreens.

Co-authors: Aditya P. Sarnaik, Rocco Mancinelli, John McGowen, David Smernoff, Taylor L. Weiss

4. *"The Role of Epigenetics in Bacterial Secondary Metabolite Production"*

Adjo Elodie Kadjo (University of Illinois Chicago)

Microorganisms compete for survival in microbial communities by producing natural products (NPs). This ability of microorganisms to make NPs can be harnessed for pharmaceutical and agricultural applications. However, the cues present in microbial communities to produce NPs may no longer be present under laboratory conditions. Despite methods that aim to replicate environmental conditions, such as adding elicitors to the culture media and co-cultivation, most predicted NPs remain inaccessible. We are particularly interested in the potential role of epigenetics in bacterial NP activation.

Epigenetics are chemical modifications on the DNA that can change gene function without altering the DNA sequence. Epigenetics has been explored to improve NP production in fungi where histone deacetylase and DNA methyltransferase inhibitors have allowed upregulation of NP biosynthesis. In bacteria, DNA methylation is the principal epigenetic modification. Methylation of two of the canonical base pairs, adenine and cytosine, to yield N6-methyladenine (m6A), N4-methylcytosine (m4C), and C5-methylcytosine (m5C) are known. DNA methylation has been shown to affect gene transcription, replication repair, and natural product production.

Preliminary data from two deletion mutants of Burkholderia sp. FERM BP-3421 showed global metabolomic changes in the mutants compared to the wild type. For example, new HPLC peaks and new mass features are detected in the mutant that were not observed in the wild type, whereas other peaks and features disappeared. After performing RNA sequencing, we also observed changes in the transcription of several biosynthetic gene clusters encoding NPs. We intend to understand the mechanisms leading to transcriptomic and metabolomic changes and to access the potential of the generated mutants for the discovery of new NPs. Our latest results will be presented.

Co-authors: Adjo Elodie Kadjo and Alessandra Eustaquio

5. *"Quantitative design of cell type-specific mRNA stability from microRNA expression data"*

Lukas Oesinghaus (University of Washington)

Limiting expression to target cell types is a longstanding goal in gene therapy, which could be met by sensing endogenous microRNA. However, an unclear association between microRNA expression and activity currently hampers such an approach. Here, we probe this relationship by measuring the stability of synthetic microRNA-responsive 3'UTRs across 10 cell lines in a library format. By systematically addressing biases in microRNA expression data and confounding factors such as microRNA crosstalk, we demonstrate that a straightforward model can quantitatively predict reporter stability purely from expression data. We use this model to design constructs with previously unattainable response patterns across our cell lines. The rules we derive for microRNA expression data selection and processing should apply to microRNA-responsive devices for any environment with available expression data.

Co-authors: Lukas Oesinghaus, Sebastian Castillo-Hair, Nicole Ludwig, Andreas Keller, Georg Seelig

6. *"Enhancing Sorghum Transformation for Bioenergy Crop Improvement Using Morphogenic Chimera GRF-GIF"*

Anne Villacastin (Lawrence Berkeley National Laboratory)

Sorghum's drought tolerance, ease of cultivation, high biomass content, and adaptability to marginal lands make it an attractive candidate for sustainable energy sources. However, its limited potential for genetic transformation remains a huge barrier to its use in biotechnological applications, hindering the development of improved sorghum varieties. Our study addressed this challenge by focusing on enhancing regeneration - a pivotal step in the transformation process. We introduced developmental regulators from wheat GROWTH REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) into sorghum using Agrobacterium-mediated transformation. GRF-GIFs in tandem have been shown to enhance regeneration efficiencies of monocot wheat, rice, triticale, citrus, and grape. Our findings demonstrate that the stable expression of wheat GRF4 and GIF1 tandem genes positively influenced sorghum transformation with up to a 48% increase in regeneration capacity. Future work includes exploring sorghum-specific GRF-GIF tandem genes and using diverse sorghum varieties, particularly biomass cultivars. We hypothesize that the same positive effect could be observed in other sorghum cultivars due to the highly conserved nature of the morphogenes across the plant kingdom. Optimizing this method using other sorghum varieties, particularly biomass cultivars, could streamline the process of utilizing sorghum as a bioenergy crop.

Co-authors: Anne J. VIllacastin, Henrik V. Scheller, Jenny C. Mortimer

7. *"Engineering of Burkholderiales bacteria for the discovery of new polyketide-nonribosomal peptide natural products"*

Stephanie Heard (University of Illinois Chicago)

Natural products are specialized small molecules produced in nature that have had an undeniable impact on society, and rising drug resistance has led to a need for new drug scaffolds with unique mechanisms of action. We focus on the underexplored Burkholderiales order of bacteria, which is the third most biosynthetically diverse, and we maintain a library of 115 sequenced strains containing undiscovered chemistry. Using a combination of genomics, metabolomics and synthetic biology approaches, this work aims to enable the identification, isolation and characterization of new natural products. The polyketide-nonribosomal peptide class of natural products is of particular interest to us, as all three Burkholderiales natural products that have advanced in clinical trials belong to this class. We have identified two gene clusters of interest, one from an industrial Burkholderia sp. host and the other found in 23 strains in our Burkholderiales collection. In-frame gene deletions are underway in the native producer strains to correlate compounds to each cluster. In some cases, endogenous restriction-modification systems were exploited to improve the transfer of foreign DNA. Once putative masses are identified, titers will be optimized by engineering the pathways to facilitate isolation and structure elucidation. Finally, both clusters of interest contain putative resistance genes that may hint at the molecular targets of the encoded metabolites, making biological activity assays more targeted. The synergy of genomics, metabolomics and synthetic biology can have a profound impact on pharmaceutical research by accelerating progress and evading common pitfalls.

Co-authors: Thomas Pavey, Roger Linington, Alessandra Eustaquio

8. *"Engineering marine sponge-associated bacteria for enhanced production of antibiotic antidotes."*

Vitor Lourenzon (University of Illinois at Chicago)

The top prescribed antibiotics that are used today are broad spectrum. Broad-spectrum antibiotics target not only the intended bacterial pathogen but also disrupt our healthy microbiota. One potential approach to circumvent this collateral damage of broad-spectrum antibiotics is to combine them with selective antibiotic antidotes that would allow the antibiotic to target the pathogen while preserving the commensal microbiota (Maier et al., 2021). Pseudovibriamides are hybrid nonribosomal peptide, polyketide natural products isolated from the marine sponge-associated bacterium Pseudovibrio brasiliensis Ab134. These compounds share structure similarity with previously described antibiotic antidotes such as detoxin D1 (Yonehara et al., 1968). We have shown that pseudovibriamides present antibiotic antidote activity, being able to protect Bacillus cereus against the broad spectrum antibiotic blasticidin S whereas a marine sponge pathogen is not protected (unpublished).

We hypothesize that the antibiotic antidote activity of pseudovibriamides has a protective role for the marine sponge microbiota against broad spectrum antibiotics produced in the environment and that this ecological role may be translated to human application. However, pseudovibriamides were isolated in low yields, limiting their exploration.

To better understand the biosynthesis of pseudovibriamides, we generated a library of knock-out mutants, deleting 12 genes annotated as part of the biosynthetic gene cluster (Dai & Lourenzon et al., 2024). Guided by the biosynthesis knowledge obtained, we were able to engineer the wild-type strain, enhancing the production by >4 fold. In addition, we optimized culture conditions, extraction, and purification methods, significantly improving compound yields, which allowed us to acquire material to explore the antibiotic antidote activity. The biosynthesis of pseudovibriamides, the strain optimization and the biological activities will be presented.

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Co-authors: Vitor Lourenzon, Yitao Dai, and Alessandra S. Eustaquio.

9. *"A modular cell-free protein biosensor platform using split T7 RNA polymerase"*

Megan McSweeney (Stanford University)

Conventional laboratory protein detection techniques are not suitable for point-of-care (POC) use because they require expensive equipment and laborious protocols, and existing POC assays suffer from long development timescales. Here, we describe a modular cell-free biosensing platform for generalizable protein detection that we call TLISA (T7 RNA polymerase–linked immunosensing assay), designed for extreme flexibility and equipment-free use. TLISA uses a split T7 RNA polymerase fused to affinity domains against a protein. The target antigen drives polymerase reassembly, inducing reporter expression. We characterize the platform and then demonstrate its modularity by using 16 affinity domains against four different antigens with minimal protocol optimization. We show that TLISA is suitable for POC use by sensing human biomarkers in serum and saliva with a colorimetric readout within 1 hour and by demonstrating functionality after lyophilization. Altogether, this technology has the potential to enable truly rapid, reconfigurable, modular, and equipment-free detection of diverse classes of proteins.

Co-authors: Megan A. McSweeney, Alexandra T. Patterson, Kathryn Loeffler, Regina Cuellar Lelo de Larrea, Monica P. McNerney, Ravi S. Kane, and Mark P. Styczynski

10. *"High-throughput domain insertion libraries produce ligand-activated splicing ribozymes for broad-host range inducible control, genetic recordings, and biosensing"*

August Staubus (Rice University)

RNA has been found to be a highly tractable platform for developing biotechnologies. RNA devices have found great utility in this area because they have both information storage and catalytic activity, work effectively across diverse hosts, and impose low cellular burden. One of the most complex naturally occurring RNA devices is the group I intron, a splicing ribozyme that catalyzes its own removal from an RNA strand, joining the flanking sequences into one contiguous strand. Here, I describe my work using high-throughput domain insertion engineering to develop ligand-activated splicing ribozymes (LASRs) that respond to a variety of inputs ranging from nucleotides to metal cations. These LASRs can regulate the translation of any protein coding sequence and function in diverse bacterial hosts. Moreover, by coupling LASRs with a genetic recorder, I demonstrate we can report on the intracellular concentration of a target chemical within a microbial consortium that can be read out by sequencing. Thus, LASRs deliver a versatile biosensing platform for the creation of new diagnostic tools, high-throughput screens and selections, and inducible genetic-control elements for biotechnology.

Co-authors: August Staubus, Ella Ramamurthy, Anika Gupta, James Chappell

11. *Poster Withdrawn*

12. *"One CEN to Rule Them All: Characterization of S. cerevisiae and S. uvarum centromeric sequences to engineer the Saccharomyces panCEN"*

Rachel L. Powell (University of Washington)

Saccharomyces cerevisiae is one of the most important experimental systems for modern genomics research and biological engineering. Many applications of *S. cerevisiae* rely on the use of plasmids or yeast artificial chromosomes (YACs), which both require a centromere in order to promote segregation. Budding yeasts utilize "point centromeres" composed of three highly conserved centromere DNA elements in a relatively small chromosomal region. This feature makes them easy to clone onto plasmids and YACs; the most commonly used centromeric sequence for yeast expression vectors is a 125 bp fragment *of S. cerevisiae CEN6*, denoted *ScCEN6*. However, it is uncertain if *ScCEN6* is the optimal sequence for high fidelity plasmid segregation, particularly when used across diverse yeast strains and species. For example, previous work demonstrates that *ScCEN6* cannot effectively function in *S. uvarum*; *ScCEN6* has a ~20% loss per generation when measured through a classic plasmid loss rate assay. Interestingly, a larger *CEN* fragment improves this rate to ~5% loss per generation, indicating that *S. uvarum CENs* may require additional flanking sequences for full functionality. To achieve the most optimized *CEN* across all *Saccharomyces* strains and species, a *panCEN* sequence will be engineered based on data from **two major aims: 1.** Determine optimal *CEN* sequences for *S. cerevisiae* and *S. uvarum*; and **2.** Characterize chromatin accessibility for *CEN* sequences across different *Saccharomyces* to understand how *CEN* sequences behave *in situ*.

Co-authors: Rachel L. Powell, Monica R. Sanchez, Anja R. Ollodart, Ivan Liachko, Maitreya J. Dunham

13. *"Quantifying replication fidelity of unnatural base pairs with nanopore sequencing and deep learning"*

Nicholas Kaplan (University of Washington)

Innovations in sequencing, synthesis, and manipulation of nucleic acids–built around the 4-letter DNA alphabet (A, T, G, C)–have driven major advances in medicine, genomics, and synthetic biology. Yet, research over the last three decades has shown that expanding the genetic alphabet to six or more letters is possible, offering new opportunities for biotechnology. Unnatural base pairing xeno nucleic acids (ubp XNAs) are synthetic nucleotides that maintain base pairing complementarity while remaining orthogonal to the natural bases. These ubp XNAs exhibit diverse structures, ranging from isomers of standard bases (isoG:isoC) to entirely novel hydrophobic pairs. However, the absence of next-generation sequencing tools for ubp XNAs has prevented high-throughput omics studies, limiting research progress. Further, replication errors often revert ubp XNAs to natural bases during PCR, posing a significant challenge for routine molecular biology workflows.

Here, we show how next-generation sequencing and deep learning can solve both sequencing and amplification problems. Using nanopore sequencing, we train recurrent neural network models to sequence hydrogen bonding and non-hydrogen bonding ubp XNAs with high accuracy (90-99%). We then apply these models to study ubp XNA loss during PCR amplification by tailoring to detect known replication error-modes. Using high-throughput, multiplexed condition screening (polymerase, pH, nucleotide concentration, etc.) we find conditions that lead to enhanced replication fidelity of the highly error-prone isoG:me-isoC pair in a 6-letter PCR reaction (>96% fidelity). This work closes a critical technological gap of XNA-compatible sequencing techniques, paving the way for fundamental discoveries in xenonucleic acid research with broader implications for synthetic biology, medicine, and molecular evolution.

Co-authors: Nicholas A. Kaplan, Jayson R. Sumabat, Jorge A. Marchand

14. *"Plant and yeast bioengineering unveils glycosyl hydrolases for soluble fiber production"*

Talia Jacobson (University of Florida)

The β-1,4-linked mannans are the most ancient and abundant hemicellulosic components found in plant cell walls. These versatile hydrocolloids serve as gelling agents in food, feed, biomedical, and bioenergy sectors. Despite their widespread applications, how mannans are assembled and modified remains poorly understood, hindering our ability to improve their structure in crops and microbial cell factories. Indeed, increasing mannan content to enhance the industrial utility of plant fibers has been a long-standing goal; however, prior efforts have remained unsuccessful and cause significant perturbations to plant development. Endo-β-1,4-mannanases (MANs) are crucial in breaking β-1,4-glycosidic bonds, thereby mobilizing mannans and enabling a diverse array of biological processes. Contradictory to the current hypothesis on MANs role in degrading the cell wall, we report that Arabidopsis man2 man5 double mutant plants produce less mannan in the seed coat epidermis. Overexpression of glucomannan synthase CELLULOSE SYNTHASE-LIKE A (CSLA) in the man double mutant partially restored mannan content but at the detriment of other seed mucilage polysaccharides. Utilizing a synthetic biology approach, we assessed the functional link between CSLAs and MANs in a yeast system engineered to produce plant mannans. By non-invasively monitoring mannans with a newly developed MannanTrack probe, the solubility and secretion of the polymers increased when CSLAs were co-expressed with MAN2/5. This effect could be abolished by a single amino acid change in the catalytic core of MAN2. In both plant and yeast systems, MAN2 localizes to intracellular punctate structures. We propose that MAN2/5 maintain hemicellulose production in the Golgi apparatus by cleaving insoluble mannan polymers into hydrophilic molecules that are more easily exported to the apoplastic space. Collectively, we present a novel mechanism of hydrolytic enzymes to sustain hemicellulose production that could be engineered to make enhanced plant biomaterials.

Co-authors: Talia Jacobson, Mair Edwards, Moni Qiande, Madalen Robert, Julia Moncrieff, Catalin Voiniciuc

15. ***"Enhancing freeze-thaw tolerance in heat-resilient brewing yeast through experimental evolution"***

Leah Anderson (University of Washington)

Microbreweries typically store beer yeast by freezing, but many brewing strains are freeze-sensitive and significantly lose viability when repeatedly frozen and thawed. Furthermore, heat-induced die-off events, which are increasing with climate change, may inadvertently select for yeast that are even more vulnerable to freeze stress. This highlights the need for brewing yeast that can withstand both high and low temperature extremes. One promising approach to achieving this is experimental evolution, which has been shown to enhance freeze-thaw tolerance in Saccharomyces cerevisiae laboratory strains through genetic adaptation. We are now applying this approach to a unique group of Norwegian farmhouse brewing strains, known as Kveik yeast, which can ferment at high temperatures without producing off-flavors in the beer. Using a freeze-thaw-growth experimental evolution protocol, we subjected Kveik yeasts to cycles of freezing, thawing, and high-temperature growth at 37°C. After 30 cycles, the Kveik strains demonstrated enhanced freeze-thaw survival; stationary phase cultures were able to survive up to 7 freeze-thaw events at -80°C without the use of glycerol. We are now conducting whole genome sequencing to pinpoint genetic mutations that contribute to the increased freeze-thaw tolerance. Our findings will open new avenues for strain engineering, enabling the targeted introduction of these genetic changes to create beer yeasts that are both heat and freeze tolerant. By combining the natural heat resilience of Kveik strains with engineered freeze-thaw tolerance, we can develop more robust and adaptable brewing yeasts for industrial applications.

Co-authors: Leah Anderson, Rachel Powell, Elinor Washington, Barbara Dunn, Maitreya Dunham

16. *"Bioengineering Cupriavidus necator to produce advanced polyhydroxyalkanoate from levulinic acid"*

Sulogna Chatterjee (University of Florida)

Bio-based plastics offer a sustainable alternative to conventional plastics by replacing fossil fuel-based materials with renewable resources. Their use can lower carbon footprints, while biodegradable variants could enhance recycling efficiency and reduce plastic waste's environmental impact. Specifically, polyhydroxyalkanoates (PHA), a class of biological thermoplastic polyester that can be produced from various renewable feedstocks, combine these features. Poly(3-hydroxybutyrate) [P(3HB)], a short-chain-length PHA (scl-PHA) that consists of 3-hydroxybutylate (3HB) is the most common bio-polyester, however it has limited commercial application due to poor material properties, such as brittleness and high melting temperature. This can, however, be overcome by incorporation of other monomers into the polymeric chain. For instance, co-polymerization of hydroxybutyrate with hydroxyvalerate, reduces the polyester's crystallinity, lowers the melting temperature and increases the flexibility as.

While the co-polymerization of 3-hydroxybutyrate (3HB) with 3-hydroxyvalerate (3HV) has been extensively studied, the incorporation of 4-hydroxyvalerate (4HV) has seldomly been studied in detail. This is most likely because few metabolic routes to 4HV formation exist. We discovered that Cupriavidus necator, a well-established PHA producer, carries a pathway that forms both 3HV- and 4HV-CoA from levulinic acid (LevA) as its sole carbon-substrate. LevA is a C5 hydroxy acid that can be readily obtained from waste biomass such as cellulose-rich plant residues. If no viable source of LevA exists, the compound could potentially also be biosynthesized de novo from protocatechuate via the β-ketoadipate pathway, a metabolic route present in certain microorganisms. We found that C. necator wild-type can naturally co-polymerize 3-hydroxyvalerate but incorporates 4-hydroxybutyrate only at minor fractions into PHAs. The major limitation appears to be the native PHA synthase (phaC), which is specific to only a few scl-PHAs. To overcome this bottleneck and enable the production of PHAs with diverse monomer compositions and enhanced physical properties, we introduced heterologous phaC with broader substrate specificity. Unexpectedly, a highly promiscuous Class-II phaC from Pseudomonas sp. MBEL 6-19 did not lead to significantly higher 4HV incorporation, while a Class-I PHA synthase from Aeromonas caviae formed a PHA with approx. equimolar fractions of 3HB, 3HV, and 4HV. By tuning the expression-level of the PHA synthase, we were further able to modulate the composition, as well as tune molecular weight of the formed polyesters from 300 to 1000 kDa. The formed polyesters appeared transparent and tough, suggesting potential for applications that reach far beyond those of common PHB. In-depth characterization of the material properties is underway.

17. *"CRISPRi-based synthetic circuits for neural network-like computing in mammalian cells"*

Quoc Tran (University of Washington)

Cells are decision-making machines, integrating environmental and endogenous inputs to make complex decisions. The engineering of synthetic gene circuits attempts to replicate and understand this phenomenon, drawing inspiration from electrical circuits to build synthetic information processing networks inside of living organisms. One type of information processing network, the artificial neural network, has broadly proven to be powerful tools for predictive modeling and classification tasks across many fields. Here, we construct an artificial neural network (ANN) as a synthetic gene circuit in HEK-293T cells. We model and demonstrate a general ANN architecture composed of CRISPRi-regulated transcriptional artificial neurons with sgRNA connections. Each CRISPRi-based artificial neuron can sum the contributions of up to twelve individually-weighted input signals and be cascaded up to three layers to form a cellular artificial neural network with input, hidden, and output layers. The weights of the sgRNA connections to the neurons can be independently tuned across three orders of magnitude. Taking advantage of existing machine learning software packages, we can model our cellular artificial neural networks with biologically-constrained parameters and train them in silico to implement arbitrary digital logic functions and classify between two cell states given a synthetic dataset of cellular features. We are currently working to test these networks in cells. The CRISPRi-based artificial neural network leverages the capabilities and tools of computational neural networks, and, in the future, could be used for tasks such as classifying cell states in a diagnostic device.

Co-authors: Quoc Tran, Georg Seelig

18. *"Generalizable design of human cell type-specific expression via deep learning models of genomic accessibility"*

Sebastian Castillo Hair (University of Washington)

Enhancer sequences regulate eukaryotic gene expression with spatio-temporal and cell type specificity. Engineering enhancers that restrict expression to specific cell types and tissues could improve our ability to design more specific gene therapies with reduced side effects. However, enhancer identification and design remain limited by our understanding of their regulatory grammar. Here, we develop and experimentally validate deep learning-designed cell type-specific enhancers. We trained deep learning models on genomic accessibility data, based on the rationale that active enhancers reside in genomic regions with an open, accessible chromatin state. These data are available for hundreds of human cell types and tissues—including single-cell resolution datasets— and thus offers a vast resource for enhancer design. Using data of >3 million DNase-hypersensitive sites across 733 cell types and tissues from the ENCODE and Roadmap Epigenetics projects, we trained deep learning models to predict cell type-specific accessibility from sequence. We then applied gradient descent-based sequence optimization and generative neural networks to design de novo sequences predicted to maximize cell type-specific accessibility. Notably, synthetic sequences generated via deep learning exhibited a more complex regulatory grammar with a higher density of putative regulatory elements than genomic sequences. To validate our designs, we tested a library of 9,000 synthetic enhancers in a panel of 10 human cell lines—including HepG2 (liver), K562 (lymphoid), SJCRH30 (muscle), WERI-Rb1 (retina), and MCF7 (breast)—as well as in vivo in mouse retinas. In most cases, synthetic sequences displayed significantly higher enhancer activity and specificity in their target cells compared to putative genomic enhancers. Our results demonstrate that functional enhancers can be designed directly from models of genomic accessibility, highlighting the potential of deep learning-driven sequence design for synthetic biology.

Co-authors: Sebastian M. Castillo-Hair, Christopher H. Yin, Leah VandenBosch, Timothy Cherry, Wouter Meuleman, Georg Seelig

19. *"Designing the Cell-Free Gene Expression Environment with the One-Pot PURE System: Insights into Reaction Composition and Efficiency"*

Yan Zhang (California Institute of Technology)

Cell-free expression systems, which enable gene transcription and translation to occur outside living cells, have become foundational tools in synthetic biology. Using a reconstituted protein expression system, such as the One-Pot PURE system, over lysate-based systems further advances our ability to precisely control the gene expression environment. We can dictate the gene expression machinery and the energy regeneration mechanism to execute gene expression programs in a defined manner that might not be possible with a lysate-based system.

However, since the introduction of the One-Pot PURE approach in 2019, achieving consistent productivity with this system has remained a challenge. In this work, we systematically identified and addressed key sources of variability affecting One-Pot PURE system performance. During the recombinant expression of PURE proteins for the One-Pot PURE reaction, we found that genetic instability led to spontaneous protein dropouts, which we mitigated by optimizing growth media with catabolite repression. Additionally, we identified a critical set of proteins whose expression was significantly impaired when expressed in E. coli M15, the host strain recommended in the original approach. Replacing this host with a more suitable host increased the PURE protein abundance in the final mixture and improved system productivity. Beyond optimizing protein composition, we discovered that variations in energy formulations, likely influenced by the tRNA pool, could compensate for deficiencies in the PURE system. These findings suggest that designing the tRNA pool could further enhance protein synthesis efficiency.

This work highlights the complex biochemical interplay influencing protein expression capacity in the design of a minimal, reconstituted protein expression system. By addressing key bottlenecks to improve system productivity, our work advances the engineering of robust cell-free gene expression systems for synthetic biology applications.

Co-authors: Yan Zhang, Matas Deveikis, Yanping Qiu, Lovisa Björn, Zachary A. Martinez, Tsui-Fen Chou, Paul S. Freemont, Richard M. Murray

20. *"High-throughput assembly and evaluation of nitroreductase enzymes for 2,4,6-trinitrotoluene degradation"*

Asher Vokoun (The Pennsylvania State University)

2,4,6-trinitrotoluene (TNT) is a major soil pollutant at sites where it has been manufactured, tested, and stored. Bioremediation is one possible approach to large-scale removal. Here, we present a method for the high-throughput assembly and screening of nitroreductase enzymes (NTRs) for TNT degradation. From literature, we identified four NTRs known to effectively degrade TNT. Using AFDB Clusters, we generated a list of over 1000 enzymes that were structurally similar to these literature-knowns. From this list of enzymes, we randomly selected 300 for assembly and evaluation. We used the Genetic Systems Builder (GSB), a new pipeline for oligopool design and streamlined low-cost, high-throughput cloning, to design an oligopool containing the genetic information for all 300 candidate enzymes. The GSB's high-throughput Golden Gate assembly workflow was then used to clone the enzymes individually into plasmids containing a T7 promoter. We expressed these enzymes in E. Coli and cultured in media containing TNT, generating growth curves. From these growth curves, the specific growth rate was computed as a proxy metric for TNT degradation efficiency. We analyzed the degradant profiles for several top-performing candidate enzymes using LC-MS^3, and discovered a range of products. These profiles can be further affected by growing cell cultures containing combinations of enzymes.

Co-authors: Howard Salis

21. *"How Many Plasmids Can Bacteria Carry? A Synthetic Biology Perspective"*

Cholpisit Kiattisewee (Massachusetts Institute of Technology)

Plasmid is a pinnacle tool in synthetic biology and other biotechnological applications. It serves as a simplest approach to introduce recombinant DNA which then transcribed into RNA that functions as is or gets translated into a protein of interest. Despite its widespread utility, the question "How many plasmids can be used in this bacteria?" remains underexplored in existing literature. In this article, I discuss multiple plasmids maintenance in bacteria through a microbial synthetic biology perspective. I delve into the theoretical aspect and existing evidence of multi-plasmid systems, aiming to pinpoint the possible maximum number of unique plasmids a single microbe can carry. Furthermore, I highlight how the existing applications of multi-plasmid systems drive novel discovery and development in metabolic engineering and synthetic biology. Lastly, a new DNA data storage strategy is proposed by combining multi-plasmid DNA carriage in a single organism with the whole-plasmid sequencing platform.

Co-authors: Cholpisit Kiattisewee

22. *"Political and Bureaucratic Instability - the Elephant in the Room Hindering Thailand's Bioeconomy"*

Cholpisit Kiattisewee (Massachusetts Institute of Technology)

Political instabilities and subsequent bureaucratic challenges can significantly impact the bioeconomy. In this policy position paper, I discussed these challenges in the context of Thailand's bioeconomy development. Thailand has a unique history of political instability, including frequent military coups, which has deeply influenced its science policy landscape. Major establishments and reforms of the national science governance body are often correlated with political transitions. Frequent shifts in policy focus led to bureaucratic instability, hindering the long-term development of bioeconomy. Using the Bio-Circular-Green (BCG) economic model as a reference, key challenges were presented together with policy recommendations. Finally, the long-term Thailand 4.0 Strategy was revisited for its potential to foster national science policy frameworks within the global south ecosystem.

Co-authors: Cholpisit Kiattisewee

23. *"SynBio4ALL Africa: Accessible Synthetic Biology Education Across the Continent"*

Cholpisit Kiattisewee (SynBio4ALL Africa)

SynBio4ALL Africa is a non-profit organization dedicated to advancing education and research in synthetic biology and emerging areas, such as gene-editing for crop resilience and biodiversity conservation, across Africa. A key step to achieving this goal is offering accessible, resource-aware training to the African community. To do this, our team of volunteers, largely comprised of EBRC SPA members, has offered online lectures in real-time over Zoom and available asynchronously on our website. Students earn certificates by completing a minimum number of homeworks that review concepts covered in lectures and receive meticulous feedback on their work from graduate-level practitioners. Our six-week beginner's course reached over 200 interested individuals in which more than 50 participants from at least five different countries completed the course requirement, and our 2024 intermediate course grew to ten weeks with over 100 weekly participants, culminating in original group presentations on synthetic biology solutions to independently chosen research problems. In 2025, supported by a microgrant from the Experiment Foundation, we will offer a course in plant synthetic biology to support student interest in sustainable agriculture, biodiversity conservation, and hands-on experiences. In addition to the usual lectures, students will write proposals for a native plant to sequence addressing both biological and technical considerations, whose genome will be sequenced and deposited following the course. By collaborating with global partners, especially with the Engineering Biology Research Consortium (EBRC) and local African institutions, SynBio4ALL Africa aims to promote knowledge exchange, mentorship, and research capacity building. We want to extend our invitation to the EBRC community to join the mission of cultivating a synthetic biology workforce in Africa as a growing player in the global bioeconomy.

Co-authors: Emmanuel Sebunya Kato, Heidi Klumpe

24. *"Marchantia polymorpha as a Synthetic Biology Chassis for Plant Glycobiology"*

Logan Spicer (University of Florida)

Plant cell walls are a complex network of polysaccharides that affect metabolic function, structurally support, and protect the cell. Inherent recalcitrance, complexity of cell walls, and genetic redundancy is a challenging limitation to engineering their structures in most land plants. The common liverwort, Marchantia polymorpha, is a non-vascular land plant and a promising model system for synthetic biology. Marchantia has a haploid dominant life cycle, low genetic redundancy, and rapid growth. We have implemented protocols to stably transform Marchantia thallus cuttings or spores using Agrobacterium tumefaciens and have demonstrated transgene insertions as well as CRISPR/Cas9 genome editing. For example, the Arabidopsis thaliana genome contains nine Cellulose Synthase-Like A (CSLA) genes encoding putative (gluco)mannan synthases, but Marchantia only encodes one CSLA homolog, greatly simplifying its functional characterization. Despite the abundance of mannose-rich polymers in bryophytes (over 10% of Marchantia wall carbohydrates), we regenerated the csla mutant plants that are essentially devoid of mannan. Surprisingly, the three independent Marchantia csla alleles, including two independent frameshift mutations, grew similar to wild-type during vegetative, haploid development and bypasses the embryo lethality previously reported for an Arabidopsis csla7 mutant. Therefore, Marchantia offers efficient transformation and rapid regeneration that will facilitate multiplexed genome editing strategies to decipher cell wall biosynthesis by altering glycan structure and function.

Co-authors: Logan Spicer, Catalin Voiniciuc

25. *"Predicting & controlling T7 RNAP transcription rates to increase IVT yields & eliminate undesired transcription products"*

James McLellan (The Pennsylvania State University)

T7 transcription systems are a versatile tool for high-yield mRNA production for both research and at-scale industrial applications. Such systems may be employed in mass production of mRNA-based therapeutics, such as during the COVID-19 pandemic, in which rapid development and distribution of SARS-CoV-2 mRNA vaccines is estimated to have saved 19.8 million lives during the first year of public availability. However, DNA template sequences used for mRNA production may contain unintended partial or non-canonical cryptic T7 promoter sequences, resulting in off-target transcription start sites (TSS). Such cryptic TSSs create in truncated mRNA transcripts, T7 RNAP interference, and RNA-duplexes that reduce yields and purity.

We developed a machine learning model to predict the T7 promoter transcription rate of a given DNA sequence. We designed, cloned, and carried out in vitro transcription on a library of 11,588 single and tandem T7 promoter variants and used barcoded reads to calculate transcription and total Gibbs Binding Free Energy (ΔGtotal) of each variant. Our model performs well on an unseen test dataset (R2 = 0.77) and accurately identifies nucleotides with known significant contributions to transcriptional activity (-11G, -9C, -8T, -7C) as highly important features in the model. We use our model to identify cryptic TSSs in the Pfizer COVID-19 vaccine sequence (BNT162b2), the human epidermal growth factor receptor gene, and the coxsackievirus and adenovirus receptor gene, and use synonymous codon substitution to propose new sequences with reduced off-target transcriptional activity.

Co-authors: Howard Salis

26. *"A compact CRISPR recorder for high-resolution mapping of clonal immune cell lineage decisions"*

Morgan Bean (University of Washington)

T cells circulate throughout the body surveying environments, countering threats, and storing information about past pathogenic experience. During immune challenges, naive T cells expand and differentiate into distinct functional states, including effector cells, which drive pathogen clearance, and memory cells, which persist to enable rapid anti-pathogen responses upon reexposure. To discover how T cells differentiate, and to design immunotherapies that bolster strong immune responses, it is critical to study the mechanisms and trajectories of T cell differentiation. Currently, cell differentiation states are readily measured by single cell RNA sequencing (scRNA-seq). However, single-cell snapshots do not reveal how individual cells transition between states, which is critical in understanding the mechanisms driving cell fate decisions. To overcome this challenge, we built immGESTALT, a CRISPR recorder for high-resolution, non-invasive tracing of primary immune cell differentiation and lineages in vivo. immGESTALT is a compact, dual retroviral system optimized for delivery to Cas9-expressing primary mouse T cells. immGESTALT maps clonal and subclonal lineages with (1) a static barcode to label clonal T cell populations and (2) CRISPR/Cas9 editing to generate unique scratchpads across T cell expansion and differentiation. The barcode and scratchpad are positioned on an mRNA transcript, enabling T cell clonal and subclonal lineage as well as differentiation state readouts via scRNA-seq. immGESTALT enables mapping of greater than 10^6 T cell lineages with sufficient editing efficiencies, scratchpad allele diversity, and gRNA independence for high-resolution tracing of lineage decisions over extended experimental timelines. With immGESTALT, we can link T cell differentiation and ancestry information to non-invasively map T cell state trajectories in mouse models.

Co-authors: Morgan Bean, Yufei Gao, Jesse Zalatan, Hao Yuan Kueh

27. *"DBTL cycles for engineering aromatic bioproduction in Pseudomonas putida with multi-node CRISPRa/i gene regulatory programs"*

Ian Faulkner (University of Washington)

Design of microbial strains engineered for bioproduction can be accelerated by combining tools, like CRISPR gene regulation and multi-omics data analysis, with strategies like iterative incorporation of machine learning (ML). CRISPR systems can be crucial tools for controlling multi-gene expression programs, but unpredictability of guide RNA (gRNA) folding can disrupt expression control. We recently described a correlation (rS = 0.8) between a computational parameter describing folding rate into the active structure and gRNA efficacy for CRISPR activation (CRISPRa), and now make use of that correlation to build large arrays of gRNAs pre-screened for efficacy. This screening criterion also enables forward design of gRNAs and multiple synthetic CRISPRa promoters matched to those guides. In E. coli, we have recently used this system to systematically profile production throughout a three-dimensional design space of enzyme stoichiometry (Nat Comm 15:6341, 2024). In Pseudomonas putida, we expand the gRNA array to include a number of perturbations of endogenous genes in central metabolism and amino acid anabolism. These perturbations are intended to boost metabolic flux toward 4-aminocinnamic acid, a precursor for advanced polymers produced from chorismate using a three-operon heterologous pathway. It is difficult to rationally predict the most effective combinations of perturbation targets, so we incorporate modeling approaches into target selection and combination. In particular, we are developing an iterative design-build-test-learn framework using multi-omics data analysis and ML-directed combination recommendations to increase 4-ACA production through incremental addition of new perturbations. By engineering multi-guide CRISPRa/i programs simultaneously controlling these genomic targets and the pathway genes, we are developing new data-driven, model-guided approaches to strain optimization with accelerated DBTL cycles, observing some improvement even in early cycles.

Co-authors: Jason Fontana, David Sparkman-Yager, Ryan Cardiff, Cholpisit Kiattisewee, Aria Walls, Tommy G. Primo, Patrick C. Kinnunen, Allan Scott, Tijana Radivojevic, Alex Beliaev, Hector Garcia Martin, Jesse G. Zalatan, and James M. Carothers

28. *"Efficient Exploration of Synthetic Gene Circuit Design Spaces: A Multi-Objective Simulated Annealing Approach"*

Ania-Ariadna Baetica (Drexel University)

Synthetic biological circuits are subject to constraints on their performance and resource usage. Computationally exploring the design space of synthetic circuits can be helpful in prototyping their experimental implementations. In this work, we computationally prototype designs of synthetic circuits with constraints on their sensitivity to variation in parameters, resource usage, and incorporation of feedback regulation. These three different types of constraints require an algorithm that can find the best solutions in parameter space.

In previous work, we have considered the problem of computationally prototyping circuits with constraints on their sensitivity to variations in parameters and we have discovered regions of parameter space using a grid search method — by sampling the parameter space at discrete grid points with sufficiently high resolution. While it is effective for simple circuits, this brute-force approach becomes computationally expensive and time-consuming as circuit complexity increases, making it challenging to explore high-dimensional design spaces efficiently. To address this limitation, we propose a novel method to explore the synthetic biological circuit design space. Our approach leverages multi-objective simulated annealing (MOSA). By integrating the MOSA algorithm, we identify promising parameter regions without exhaustively sampling the entire space. This not only reduces computational costs but also preserves solution quality by focusing on optimal trade-offs between our sometimes-competing design objectives. In this work, we present a step-by-step workflow for applying MOSA to synthetic gene circuit design and systematically compare its performance against the conventional grid search method. The results demonstrate that MOSA achieves superior computational efficiency, significantly lowering the number of required simulations, while maintaining or even improving optimization accuracy. Our findings highlight the potential of MOSA as a powerful tool for exploring complex, high-dimensional parameter spaces, offering a scalable alternative to traditional brute-force strategies.

Co-authors: Nguyen Hoai Nam Tran, Quang Luan Dang Tran, and Ania-Ariadna Baetica

29. *"Identification and engineering of highly active proteases using co-evolutionary models"*

P. C. Dave Dingal (The University of Texas at Dallas)

Proteases from the family Potyviridae are widely popular choices for cleaving peptide epitopes off recombinant proteins and for engineering synthetic biological circuits. More than 3,800 Potyviral proteases are predicted to cleave peptides in a sequence-specific manner, making characterization of the specificity and efficiency of each protease experimentally challenging. To accelerate functional determination of substrate specificity, we built statistical models via direct coupling analysis (DCA) to predict and developed cell-based assays to validate protease performance. DCA modeling revealed several features of the Potyviral protease family: First, we identified several proteases that performed better in cell-based assays than the commercially available tobacco etch virus protease. Second, DCA predictions correlated well with experimental data on crosstalk interactions between multiple proteases. Third, the predictive power of DCA is sufficient to resolve the effect of single amino-acid changes on protease specificity and efficiency. We leveraged this substrate-specificity resolution to demonstrate the ability of model-specified proteases in detecting mutations that may arise in the cellular proteome. Current efforts are focused on directed evolution of de novo protease-substrate pairs and targeting substrates in vivo that are of biomedical relevance.

Co-authors: Medel B. Lim Suan Jr., Cheyenne Ziegler, Zain Syed, Ajay Tunikipati, Rodrigo Raposo, Jaimahesh Nagineni, Jaideep Kaur, Faruck Morcos, and P. C. Dave P. Dingal

30. *"EFM Calculator 2.0: Characterizing and predicting the presence of short repeated sequences to improve genetic stability"*

Cameron Roots (University of Texas at Austin)

Evolution often presents a challenge for metabolic engineering. Under many circumstances, engineered devices impose a burden to the cell by changing expression profiles and diverting cell resources, lowering their chassis's growth rate and enabling mutants with broken genetic devices to overtake the population. One approach to increase the time to failure is to reduce the evolvability of breaking mutations. A source of these mutations is when DNA polymerase slips on nearby repeated sequences, producing a deletion or duplication. While the effects of large sequence repeats are well understood, to our knowledge no comprehensive studies have quantified the mutation rates of small, noncontiguous repeats caused by polymerase slippage. To address this gap, we performed fluctuation tests to empirically evaluate the deletion rate for short repeated sequences of 6 to 11 bases in length up to 500 bases apart in Escherichia coli. We then built and evaluated a generative additive model to predict the mutation rate from repeat sequence length and intervening sequence length. This model was incorporated into the Evolutionary Failure Mode Calculator 2.0, expanding the coverage of mutational hot spots from the original EFM Calculator. Further, EFM Calculator 2.0 improves upon the prior version by implementing multithreading, allowing bulk input, providing a more comprehensive user interface, and being permissively licensed. We used EFM Calculator 2.0 to evaluate the prevalence of short repeated sequences in the iGEM Registry and predicted a large number of biobricks with modestly elevated instability from short repeated sequences. This work provides a new computational method for identifying mutational hotspots, improving the reliability of engineered genetic constructs.

Co-authors: Cameron Roots, Sreya Das, Kevin Yang, Vishy Rao, Avyay Katre, Microbe Hackers Evolutionary Stability Students, Dennis Mishler, Jeffrey Barrick

31. *"eChromatin: Developing a cell-free gene expression platform using targeted induced steric hinderance within DNA arrays"*

Rory Majule (University of Washington)

Cell-free gene expression systems can be used in the development of hybrid functional materials, to design synthetic biological and bio-computational systems, and to reduce biological complexity when investigating genetic pathways found in nature. Such systems are not without their limitations, however, such as resource depletion, limited mechanisms for real-time control of reaction timing and dynamics, and the inability to integrate spatial reaction dynamics. To address these limitations, we are building a small, automated and modifiable platform for coordinated cell-free gene expression in a biologically minimal, electronically-actuated context. We envision a design of discrete self-assembled monolayers (SAMs) of DNA on an chip array, where cell-free expression rates of surface-bound gene fragments are controlled by steric hinderance of RNA polymerase action within a desired section or sections of the array. We seek to achieve this local steric hinderance by way of electrophoretic forces, but are also exploring other means such as the predictable and reversible induction of secondary structures and the integration of reversible, light-inducible cross-linkers. Thus far, we have designed a physical system for parallel DNA monolayer generation and testing. We have also characterized the densities and compositions of these monolayers and show that we can achieve cell-free transcription-translation (TXTL) activity using specifically bound DNA monolayers. Our current efforts are focused on the reduction of noise in our physical system and on achieving consistently distinguishable TXTL rates through electronically or light-induced changes in steric hinderance.

Co-authors: Kyvalya Reddy, Oren Fox, Chris Takahashi, Jeff Nivala

32. *"Profiling Nrf2 Activity across Cell Lineages with Analog DNA Memory Recorders for Prediction of Cisplatin Resistance Emergence in Head and Neck Squamous Cell Carcinomas"*

Matt Demelo (Rice University)

Cisplatin resistant cancer is a pressing concern in the clinic, as cisplatin-centric chemotherapy is the primary treatment strategy for numerous cancers, such as Head and Neck Squamous Cell Carcinomas (HNSCCs). The master regulator of the antioxidant response, Nrf2, has been identified as a key driver of resistance acquisition, but its specific role is convoluted by the complexity of natural Nrf2 flux and the heterogeneity of Nrf2 activity between cells within tumor populations. Chronic, highly dynamic patterns of dysregulated Nrf2 activity are established drivers of resistance acquisition, but there is a lack of feasible strategies to profile these patterns for effective prediction of resistance in tumors. Here, we develop a genetic circuit for recording Nrf2 activations across cellular lineages via a prime editing-mediated analog DNA memory recorder, peCHYRON. To facilitate recording of Nrf2 "OFF" and "ON" cell states, we have also developed a redox-sensitive repressor, "neh2-TetR", allowing differentiation of distinct Nrf2 activation events over time in the DNA record. In our prototype circuit, we achieve three-fold higher recording of Nrf2 activity in HEK293T cells supplemented with a constitutive Nrf2 expression vector, demonstrating the feasibility of this recording strategy. Additionally, we achieve modest recording of Nrf2 activity in un-supplemented cells, indicating our recording circuit is sufficiently sensitive to detect natural fluctuations in Nrf2 activity. Low overall recording efficiency remains a significant bottleneck, resulting in inefficent continuous recording activity, necessitating further optimization of the recording circuit and prime editor protein. Further optimization of this circuit will facilitate profiling of Nrf2 activity patterns across cellular lineages during cisplatin resistance acquisition in cells, allowing for prediction of the emergence of drug resistance in tumors "primed" for resistance acquisition by Nrf2.

Co-authors: Matt Demelo, Mitchell J. Frederick, Vlad Sandulache, Theresa Loveless

33. *"Glucose-acetate co-utilization in two nonmodel oleaginous yeasts for efficient bioproduction"*

Zhengyang Xiao (Washington University in St. Louis)

Acetate metabolism in microbial systems is inefficient, with substantial carbon loss (>50% as CO2) and heat dissipation due to high enzyme usage and alternative oxidase (AOX). This study characterizes acetate utilization in eukaryotic yeasts Yarrowia lipolytica and Lipomyces sp. In Yarrowia, acetate catabolism showed low ATP/ADP ratios and high enzyme usage burdens, leading to suboptimal poly-3-hydroxybutyrate (PHB) synthesis. Despite acetyl-CoA abundance from acetate, PHB synthesis was constrained by insufficient reducing power. However, co-utilizing glucose with acetate resolved these challenges by providing reducing power and energy (41% higher ATP/ADP ratios), reducing flux rigidity, and segregating glucose (glycolysis and pentose phosphate pathway) and acetate (TCA cycle) metabolism without catabolite repression. Glucose-acetate ratio was tunable, and nitrogen limitation increased acetate uptake by 53%. Overexpressing acetyl-CoA synthetase enhanced PHB synthesis by 77%. On the other hand, Lipomyces tetrasporous showed good acetate/formate tolerance and highly efficient acetate co-utilization with glucose and xylose. For the first time, 13C metabolic flux analysis was done in Lipomyces' glucose, xylose, and acetate metabolism, identifying potential strain engineering targets for biosynthesis (such as lycopene). These findings underscore the advantage of eukaryotic yeasts in VFA utilization. To make them promising platforms for volatile fatty acid (VFA) valorization, we proposed strategies to optimize acetate utilization. (1) Take advantage of segregation of sugar phosphate pathways and TCA cycle to balance energy and cofactors. (2) Use nitrogen limitation to regulate acetate uptake kinetics. (3) Target bottleneck enzymes (e.g., ACS, AOX) to enhance carbon efficiency. This work advances the rational design of low-carbon biomanufacturing using renewable VFAs.

Co-authors: Zhengyang Xiao, Xiaochao Xiong, Yufei Sun, Masoud Tourang, Shulin Chen, Yinjie J. Tang

34. *"Optimizing in vitro Transcribed CRISPR-Cas9 Single-Guide RNA Libraries for Improved Uniformity and Affordability"*

Natanya Villegas (University of Oregon)

We describe a scalable and cost-effective sgRNA synthesis workflow that reduces costs by over 70% through the use of large pools of microarray-derived oligos encoding unique sgRNA spacers. These sub-pool oligos are assembled into full-length dsDNA templates via Golden Gate Assembly before in vitro transcription with T7 RNA polymerase. RNA-seq analysis reveals severe biases in spacer representation, with some spacers being highly overrepresented while others are completely absent. Consistent with previous studies, we identify guanine-rich sequences within the first four nucleotides of the spacer, immediately downstream of the T7 promoter, as the primary driver of this bias. To address this issue, we introduced a guanine tetramer upstream of all spacers, which reduced bias by an average of 19% in sgRNA libraries containing 389 spacers. However, this modification also increased the presence of high-molecular-weight RNA species after transcription. We also tested two alternative bias-reduction strategies: compartmentalizing spacers within emulsions and optimizing DNA input and reaction volumes. Both methods independently reduced bias in 2,626-plex sgRNA libraries, though to a lesser extent than the guanine tetramer approach. These advancements enhance both the affordability and uniformity of sgRNA libraries, with broad implications for improving CRISPR-Cas9 screens and optimizing guide RNA design for other CRISPR and nuclease systems.

Co-authors: Yukiko Gaudreault, Abigail Keller, Phillip Kearns, James Stapleton, Calin Plesa

35. *"Mitigating Winner-Takes-All Resource Competition with CRISPR-Driven Multi-Module Negative Feedback"*

Sadikshya Rijal (Arizona State University)

Cellular resource limitations create unintended interactions among synthetic gene circuit modules, compromising circuit modularity. This challenge is particularly pronounced in circuits with positive feedback, where uneven resource allocation can lead to Winner-Takes-All (WTA) behavior, favoring one module at the expense of others. In this study, we experimentally implemented our previously proposed Negatively Competitive Regulatory (NCR) controller using CRISPR interference (CRISPRi) and evaluated its effectiveness in mitigating WTA behavior in Escherichia coli. We integrated a tunable dCas9 gene into the E. coli genome and designed guide RNAs (gRNAs) for each module, with each module producing its own gRNA to repress its expression. The genome-integrated dCas9 controller was induced minimally, such that the sgRNAs compete for the limited availability of dCas9. This mechanism not only introduces strong negative feedback to the high-activity module but also redistributes resources to the low-activity module, ensuring more balanced resource allocation. We observed that the NCR controller enhanced the coactivation of the modules, while WTA behavior was evident in the control group where dCas9 repression was absent. Our quantitative experiments demonstrate that the NCR controller significantly attenuates WTA behavior in both dual self-activation and cascading bistable switches circuits, offering an effective strategy to regulate resource competition and enhance circuit modularity.

Co-authors: Sadikshya Rijal, Kylie Standage-Beier, Austin Stone, Abdelrahman Youssef, Rong Zhang, Xiao Wang, Xiao-Jun Tian

36. *"Bioinspiration: An Argument for Investing in Basic Biological Research in the U.S."*

Johnathan O'Neil (Engineering Biology Research Consortium)

The U.S. must sustain strong funding for basic biological research to maintain global innovation leadership, promote sustainability, and enhance public appreciation of science. Bioinspiration, where scientists are inspired by nature to develop innovation, demonstrates how biological research has translated to societal benefit within and far outside the biological sector. For example, researchers within the U.S. studied how honeybees collect pollen as a swarm and applied that knowledge to make internet browsing more robust and efficient. Japanese Shinkansen trains were inspired by how a kingfisher's beak allows it to dive into the water with minimal splash to make the trains faster but quieter. These innovations and discoveries exist because of continued financial support for basic biological research and the bioinspiration that came from it. Continued investment in biological research within academia and small businesses will lead to new bioinspired innovations that can promote public health, job growth, sustainable energy sources, and other societal benefits. Additionally, continued investment in bioinspiration education and outreach will increase public awareness and appreciation of how biological research drives innovation, sustainability, and economic growth, ultimately stimulating greater future engagement in biology.

37. *"Computer aided identification and production of replacement bioproducts for the decarbonization of the chemical industry"*

Efraín Rodriguez Ocasio (University of Wisconsin-Madison)

A significant challenge for conversion researchers is the identification of economically viable target chemicals for bioproduction. Much of the previous and current work has focused on the bioproduction of existing commodity chemicals to replace petroleum-based production methods for these chemicals. In many cases, the cost comparison of producing these chemicals through a biological process is unfavorable compared to the petroleum-based process. This economic challenge suggests that an alternative avenue of investigation would be the identification of promising replacement chemicals and the biological means to produce these replacement chemicals. This project integrates genome scale metabolic models and process models to assess promising replacement chemicals and guide the selection of new target molecules for bioproduction. This project looks beyond the traditional platform chemicals to identify molecules that can be produced from lignocellulosic biomass with an economically competitive process and a lower carbon footprint than petroleum-based chemicals.

38. *"Modular Communication in Synthetic Protocells"*

Joseph Vath (Georgia Institute of Technology)

Many in the engineering biology community are working towards the development of techniques for studying biological processes and utilizing them for the development of novel, bio-inspired technologies. Here we describe the collaborative effort of our group to develop a platform for modeling bacterial communication and signaling mechanisms for eventual use as a component of a point-of-care (POC) multiplex diagnostic tool. Using an aqueous two-phase separation (ATPS) system for creating communities of simplified "protocells", each lacking a lipid membrane, we model bacterial signaling systems. Our protocells communicate by intracellular signaling molecules including cyclic AMP (cAMP), bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), and homo-serine lactone (HSL)-based quorum sensing systems, and implement more diverse, structurally dissimilar communication molecules such as pyrones. Each is linked to a reporter protocell capable of producing a visible indicator of successful communication between protocells. By including multiple protocells with different communication systems, we are able to observe potential crosstalk between signaling molecules and receptors distinct from their cognate receptor molecules, allowing us to better design protocell communities with orthogonal communication mechanisms. To replicate the more complex stimuli response mechanisms seen in live organisms and simplify any future POC tools, we plan to combine these orthogonal communication systems using self-assembling fragmented RNA polymerase for multi-input, single output signal integration. The development of this platform for modeling social bacterial communities presents opportunities for studying a range of communication systems within a modular, controllable environment and for implementing communication-based biological circuits in novel applications such as diagnostic tools and biological computation.

Co-authors: Joseph Vath, Felicia Oentoro, Mark Styczynski, Brian Hammer

39. *"Declarative Bioengineering Platform for the Simplification of Composing Physics and Deep Learning-Based Workflows"*

Zachary Martinez (California Institute of Technology)

Deep-learning models have transformed many scientific disciplines, powered by the explosion of available data. In biology, petabases of sequencing data have enabled protein language models (pLMs) to learn the "language of life." Yet despite their potential, these models often remain inaccessible to researchers lacking machine learning expertise. TRILL (TRaining and Inference using the Language of Life) is a modular, open-source platform designed to bridge this gap. It allows researchers to build scalable protein analysis and engineering workflows using cutting-edge models like ESM2, LigandMPNN, and Boltz-1, without requiring coding or ML experience. Whether on a laptop via Google Colab or on a multi-GPU supercomputer, TRILL uses optimized strategies like ZeRO-Offload and distributed data parallelism to efficiently run models with millions to billions of parameters. TRILL includes modules for embedding protein sequences, training classifiers, predicting 3D structures, molecular docking, and more. Its interactive "exploration engine" supports dimensionality reduction and visualization, producing shareable HTML documents for data exploration. Users can fine-tune general pLMs on specific proteins to generate variants or use inverse folding and diffusion models to design proteins from structural inputs. By chaining together various models and methods, TRILL enables the creation of bespoke, declarative workflows tailored to specific goals. As a proof of concept, TRILL enabled the creation of a neurotoxic protein classifier, discovery of novel neurotoxins by mining millions of proteins, and the in-silico design of therapeutic anti-toxin binders. Community-driven extensions like "foldtuning" further showcase TRILL's flexibility in generating functional protein analogues. By combining machine learning and physics-based tools, TRILL lowers barriers and provides a unified environment for modern protein design and discovery.

Co-authors: Richard Murray, Matt Thomson

40. *"Engineering romaine lettuce leaf surface yeast for human pathogen detection"*

Samiha Zaman (University of Delaware)

Foodborne illnesses in the United States, particularly those associated with leafy greens like romaine lettuce, remain a significant public health concern with traditional detection methods. Current gold-standard techniques, such as selective culturing and plating coupled with downstream polymerase chain reaction (PCR) steps, offer great sensitivity and specificity. However, these methods suffer from long read out times of several days that result in detection long after the produce is sold and can only be performed via subsampling that misses contaminated produce in large lots. Existing portable assays and biosensors improve speed but frequently fall short in sensitivity, cost-effectiveness or adaptability to real food matrices. To address this, we propose the development of a whole-cell biosensor using Sporobolomyces lactuca, a yeast species naturally residing on the surface of romaine lettuce leaves. By engineering S. lactuca to produce easily detectable pigment indigoidine in response to pathogen-specific signals, this system can enable in situ and continuous monitoring of contamination. As a novel species, S. lactuca lacks established genetic tools, necessitating the development of a genetic toolkit to enable its use as a functional biosensor platform. Flow cytometry analysis confirms that S. lactuca is haploid and amenable to standard genome editing approaches. To facilitate SpCas9 ribonucleoprotein (RNP) complex delivery for targeted genetic engineering, I established a protoplast generation method for S. lactuca. In parallel, I also evaluated the sensitivity of heterologous indigoidine expression from pathway genes to establish a target for sensor control. Indigoidine expression is most limited by BpsA, indicating that pathogen-sensing promoters should regulate this single gene for effective signal output. Future work will focus on differential transcriptome analysis to identify pathogen-sensing promoters which will function as the sensing module for pathogenic E. coli and on enabling CRISPR-based genome modifications of S. lactuca. This approach offers a Generally Recognized as Safe (GRAS) platform for in-situ biosensing, leveraging the natural presence of S. lactuca on romaine lettuce to provide continuous, real-time monitoring for food safety.

Co-authors: Kevin Solomon

41. *"Engineering Pulsatile Population Dynamics: A Paradoxical Feedback Circuit for Synthetic Biology and Therapeutics"*

Rohita Roy (Stanford University)

Synthetic biology enables the precise control of bacterial population dynamics through engineered genetic circuits, paving the way for innovative therapeutic and biosensing applications. Here, we present a paradoxical feedback circuit that employs AHL (acyl-homoserine lactone) quorum sensing coupled with the ΦX174 lysis protein to generate a pulsating population profile. This state-of-the-art circuit design integrates positive feedback activation of AHL synthesis with a delayed negative feedback loop, where AHL accumulation eventually triggers bacterial lysis through the controlled expression of ΦX174 lysis protein. The pulsatile nature of this system allows for dynamic population control, preventing premature clearance while maintaining a functional bacterial density, making it an adaptable framework for diverse synthetic biology applications. This tunable oscillatory behavior offers significant advantages in therapeutic interventions, particularly in self-regulating drug delivery systems, inflammation-responsive probiotic therapies, and targeted bacterial therapy for infections and tumors. Furthermore, the modular nature of this genetic circuit allows for seamless integration into various host species and therapeutic contexts, offering fine-tuned population dynamics that enhance bacterial viability and efficacy. Our work highlights the potential of paradoxical feedback-driven pulsation as a robust strategy for next-generation therapeutic bacterial consortia and dynamic biosensors, with broad implications for microbiome engineering, infectious disease treatment, and synthetic microbial ecosystems.

42. *"Targeted DNA Insertion in Plants by CRISPR-associated Transposases"*

Yunqing Wang (California Institute of Technology)

The genetic improvement of crop plants is crucial for enhancing the sustainability of global agricultural systems. Plant synthetic biology has developed powerful methods for precise and spatiotemporal gene expression and editing, minimizing fitness costs in engineered organisms. However, the inability to control the genomic insertion site in plants given tool limitations have significantly hampered plant bioengineering efforts. Researchers have used CRISPR-Cas9 homology-directed repair (HDR) for targeted gene insertion in plants to express exogenous DNA1. Yet, this still has a single-digit efficiency hindering progress.

Here, we developed a high efficiency targeted gene insertion toolkit in plants to better understand, control, and improve plant bioengineering. We establish the CRISPR-associated transposase (CAST) system for programmable and high efficiency DNA integration, merging CRISPR RNA-guided targeting with high insertion efficiency of transposases. CAST was discovered in bacteria and has recently been adapted to human cells2,3, while its feasibility and efficiency in plants is unknown.

We reconstituted the Type I-F CASTs from Vibrio cholerae and Pseudoalteromonas in model plants Arabidopsis thaliana and Nicotiana benthamiana. We achieved episomal and chromosomal integration of a ~1 kb fluorescent reporter DNA in single plant cells in solution and in intact plant leaf cells, respectively, with bi-directional integration confirmed by nested-PCR and NGS. Ongoing efforts focus on increasing the cargo size, insertion efficiency, performing heritable transformations using CASTs, and translating to crops.

An efficient and targeted gene insertion tool facilitates fundamental biological understanding of plant genomes and identifies genomic safe harbor sites for inserting genes of interests with optimal outputs in plant biomanufacturing and trait improvement.

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Co-authors: Kimberley Muchenje

43. *"Harnessing Non-standard Nucleic Acids for Highly Sensitive Icosaplex (20-Plex) Detection of Microbial Threats for Environmental Surveillance"*

Hinako Kawabe (University of Washington)

The emergence and evolution of pathogens continuously threaten global public health. To understand and track disease burden through environmental surveillance, there is an urgent need for a molecular assay that is sensitive, specific, and highly multiplexable. Many existing assays rely on the polymerase chain reaction (PCR), including quantitative and digital PCR (q/dPCR). However, these assays are constrained in multiplexability due to the limited number of available fluorophores, along with reduced sensitivity and specificity from off-target effects such as primer dimerization and non-specific amplification. Here, we present an icosaplex (20-plex) PCR assay capable of simultaneously detecting 18 enteropathogen and two antimicrobial resistance genes. The primers in this assay incorporate two sets of non-standard nucleotides: the self-avoiding molecular recognition systems (SAMRS) reduce primer dimerization, and the artificially expanded genetic information systems (AEGIS) reduce non-specific amplification. PCR amplification was subsequently followed by next-generation sequencing using both nanopore and Illumina platforms. The SAMRS-AEGIS 20-plex assay was evaluated on 10 wastewater, 10 soil, and 10 fecal samples. Compared to a standard DNA 20-plex, the SAMRS-AEGIS 20-plex had between 2-8 times more target alignment and 2-4 times fewer primer alignment. We also compared this assay to the TaqMan array card (TAC), a qPCR-based assay, and found 90% agreement on positive calls and 89% agreement on negative calls. Of the discrepancies, 47 out of 62 were targets the SAMRS-AEGIS 20-plex detected, but the TAC did not. Moreover, the sequencing data enabled subspecies and allelic variant differentiation, highlighting the advantages of sequencing over a fluorophore-based readout. This work demonstrates the benefits of integrating non-standard bases in a highly multiplexable assay, offering a versatile tool to support environmental monitoring and diagnostics.

Co-authors: Hinako Kawabe, Luran Manfio, Sebastian Magana Pena, Nicolette A. Zhou, Steven A. Benner, Karen Levy, Zunyi Yang, Jorge A. Marchand, Erica R. Fuhrmeister

44. *"Development of tools for stable genetic engineering of the lignocellulolytic anaerobic gut fungus Neocallimastix frontalis"*

Raine Hagerty (University of Delaware)

Anaerobic fungi (AF) native to the digestive tracts of large herbivores efficiently process diverse plant biomass, potentially increasing access to renewable carbon for the bioeconomy. While AF have been demonstrated to be genetically tractable, current approaches only result in transient transformation of AF. Developing a toolkit for stable, permanent transformation would enable new opportunities to engineer AF for sustainable biomanufacturing. To address this gap, I propose using CRISPR-Cas ribonucleoprotein (RNP) complexes to facilitate stable genomic integration in AF. As a first steps towards a proof of principle, we have demonstrated electroporation-mediated uptake of an exogenous fluorescent protein (mCherry) into the model AF Neocallimastix frontalis, validated through flow cytometry and confocal microscopy. The ability to localize these internalized exogenous proteins to the nucleus of AF via fusions with previously identified nuclear localization sequences (NLS) is currently being evaluated. This work establishes a protein delivery system in Neocallimastix frontalis that may enable the internalization and nuclear localization of a CRISPR-associated gene-editing complex. This could facilitate genomic integration, enhancing access to and utilization of this highly efficient biomass-degrading platform.

Co-authors: Kevin Solomon

45. *"Modulating CAZyme Activity Through Histone Modification in Anaerobic Gut Fungi"*

Ben Alexander (University of Delaware)

Lignin, a complex polymer found in plants, represents a large waste stream of carbon that cannot be utilized effectively due to its recalcitrance. Anaerobic gut fungi (AGF) play a crucial role in breaking down these complex plant substrates in herbivorous animals, yet the mechanisms regulating their metabolic activity remain poorly understood. Understanding how these modifications influence AGF metabolism could provide new strategies for enhancing lignocellulose degradation and improving bioprocessing efficiency. In many eukaryotic microbes, histone modifications are key regulators of gene expression, but their specific roles in AGF have not been characterized. I used ELISA assays to quantify the baseline abundances of key histone modifications in untreated AGF cultures. Cultures were then treated with histone-modifying enzyme (HME) inhibitors—suberoylanilide hydroxamic acid (SAHA), sodium valproate, and nicotinamide—and shifts in histone modification levels were measured. The functional consequences of these changes were assessed using a dinitrosalicyclic acid (DNS) assay to evaluate xylan and cellulose degradation. Treatment with HME inhibitors resulted in distinct shifts in histone modification levels, which are suspected to influence the expression of carbohydrate-active enzymes (CAZymes). Consistent with this hypothesis, DNS assays showed that treated cultures exhibited increased xylan degradation, with the most substantial effect observed under SAHA treatment, a potent histone deacetylase inhibitor. These results suggest that altering histone modification patterns can modulate CAZyme activity and, by extension, AGF lignocellulose-degrading enzymes. These findings suggest that histone modifications regulate AGF carbohydrate metabolism, potentially influencing the expression of CAZymes. Identifying which histone modifications are linked to CAZyme activity could enable the development of targeted epigenetic tools to enhance lignocellulose degradation.

46. *"Deciphering the Human Splicing Code with Massively Parallel Reporter Assays"*

Madelyn Shelby (University of Washington)

Alternative splicing (AS), a key contributor to proteome diversity, is regulated by a cis-regulatory code that is interpreted by trans-acting RNA Binding Proteins (RBPs) with tissue and cell type-specific expression. Splice-affecting genetic variants contribute to many genetic diseases including various neurological diseases and cancers. A deeper understanding of the role of RBPs in alternative and cell-type specific splicing will enable improved variant effect prediction and engineering of therapeutics to correct aberrant splicing. Here we use a Massively Parallel Reporter Assay (MPRA) to screen potential splice-altering variants linked to diseases using reporter gene constructs. Our MPRA focuses on exon skipping, the most prevalent form of alternative splicing, and spans 2,107 exons from 1,741 genes. Near-site saturation mutagenesis variants were included to provide insights beyond the scope of naturally occurring mutations. We have characterized this MPRA library in five human cell lines: HEK293, K562, HeLa, HMC3 and MCF7, measuring variant impacts on exon skipping as well as differential exon skipping between cell types. Using this MPRA, we were able to evaluate the effects of modifying RBP binding motifs, allowing us to identify relevant RBPs and further understand the trans-regulatory code. For example, in the BIN1 gene, which is associated with late-onset Alzheimer's disease, disruption of the EWSR1, PUM1, and FUS binding motifs creates a significant increase in exon inclusion. We have also used these datasets coupled with RBP expression data from the Protein Atlas to investigate the relationships between RBP expression and cell-type specific splicing behavior. We evaluated individual cell-type specific sequences and created motif-knockouts to further probe the relationship between RBPs and cell-type specific splicing. A exon from the COLQ gene has differential exon skipping between HEK293 and HELA cells and contains binding motifs for SRSF3, SRSF9 and HNRNPH2. By creating sequences that knock-out these binding motifs, we can identify the role each RBP plays in determining cell-type specific splicing. We plan to use these findings to generate a second-generation library as well as create a single-cell assay to further probe RBP behavior in alternative splicing. We anticipate that this work can be used to design programable cell-type specific splicing for treatment of human disease.

Co-authors: Samantha Koplik, Angela Yu, Charles Roco, Yue Zhang, Nicholas Bogard, Alex Sabo, Johannes Linder, Georg Seelig

47. *"A RNA scaffold-based CRISPR system for orthogonal gene activation and repression in immune cells"*

Katie Denecke (University of Washington)

To generate the diverse cell types that comprise the immune system, progenitor cells must activate and maintain expression of key lineage specifying genes. Gene activation requires the action of transcription factors that act coordinately on cis-regulatory elements at target gene loci. CRISPR activation or interference systems (CRISPRa/CRISPRi), which facilitate recruitment of transcriptional effectors to specific genomic loci can be used to perturb both transcription factors and cis-regulatory elements; thus, yielding unique biological insights into their interactions within gene regulatory networks. However, these systems remain challenging to implement in immune cells. Further, concurrently implementing activation and interference in the same cell, is particularly challenging as it requires orthogonal recruitment of distinct effectors to independent loci. To fill this gap, we are developing a CRISPRai system that utilizes scaffold gRNAs (scRNA) with additional RNA hairpins to recruit distinct RNA binding protein-effector fusions to target gene loci, enabling activation and repression at distinct loci in the same cell. This system utilizes truncated gRNAs that do not induce editing upon binding, making it compatible for use in primary murine immune cells from Cas9-expressing mouse strains.

In preliminary studies, we have tested and validated key components of this CRISPRai system in a murine pro-T cell line. Specifically, we have employed the CRISPRai system in a mouse T cell progenitor line (SCID.adh2C2) with both CRISPRa and CRISPRi independently. Using this scaffold gRNA system we have achieved robust up-regulation of multiple genes using a CRISPRa system with truncated gRNAs. Furthermore, we have employed an orthogonal KRAB-based CRISPRi system in this cell line with a knock-in YFP reporter for Bcl11b. Using this CRISPRi system, we have achieved repression of Bcl11b expression through scRNA-mediated recruitment of KRAB to the promoter region. Moving forward, we will elucidate transcription factors and cis-regulatory elements underlying the activation of Bcl11b, a key developmental gene essential for progenitor cell commitment to the T cell lineage. While individually, these CRISPRa and CRISPRi systems will provide novel insights, they can be employed at the same time in the same cell to illuminate new biological insights into complex gene regulatory networks.

Co-authors: Kathryn Denecke*, Morgan Bean*, Paul Leanza, Ethan Cheney, Linda Guo, Jesse Zalatan, Hao Yuan Kueh

48. *"Bending the Rules: de novo Transcription Factor Design for Targeted Gene Regulation"*

Beau Lonnquist (University of Washington - Seattle)

Transcription factors (TFs) capable of binding specific DNA sequences are integral to targeted genetic regulation in both natural and synthetic contexts. However, the design of de novo TFs has proven challenging despite major advancements in computational tools for protein design. Preliminary efforts to design de novo TFs yielded a small library of dimers composed of two protein subunits in complex with one another, mimicking a common structural conformation of native TFs. Although these de novo TFs induced genetic repression, the magnitude of repression was relatively modest compared to natural repressors. Furthermore, the design characteristics indicative of the highest performing de novo TFs were unclear, suggesting that TF-induced repression was more complex than just allosteric inhibition of the RNA polymerase. To create TFs capable of higher levels of repression, machine learning tools including RFdiffusion, ProteinMPNN, AlphaFold3, and RoseTTAFoldNA were used to design de novo homodimeric TFs able to bend the DNA upon binding. In doing so, designed TFs could further inhibit the function of the cellular machinery involved in transcription by altering the structure of the DNA promoter region. The top designs were selected, synthesized, and tested for efficacy as genetic inhibitors in Escherichia coli, with preliminary results suggesting that these DNA-bending TFs successfully magnified repression of the target gene. These TFs represent a major advancement in engineering protein-DNA interactions and could have a variety of applications across synthetic biology and genetic engineering. In particular, successful designs could have applications in synthetic gene circuits, as biosensors for various cellular processes, and even therapeutics for a wide range of genetic diseases.

Co-authors: Beau Lonnquist, Cameron Glasscock, David Baker

49. *"Using Experimental Evolution to Select Against Mother-Daughter Cell Separation Defects in Brewing Yeast"*

Amanda Ro, Lauren Ackermann (University of Washington)

While flocculation is a desirable trait for brewing yeast because it eases the removal of cells from beer after fermentation, other modes of cell-to-cell adhesion can be detrimental to the brewing process. Mother-daughter separation defects cause cells to form large aggregated clusters which use more oxygen, produce a lower fermentative yield, and require more head space during fermentation. These defects can be caused by mutations to a number of genes, which makes a targeted genetic approach challenging. In this work, we used experimental evolution to eliminate mother-daughter separation defects present in a widely used brewing strain. Cells with this defect are less buoyant and settle faster than non-adhering cells. We used this property to select against cells with this defect by letting the cultures settle and propagating only cells present in the top layer of the media. We propagated top-layer cells for approximately 300 generations (about two months), collected daily optical density measurements, and conducted settling assays. Over time, we found that large, branched cell clusters decreased in frequency in our top-layer samples while the amount of single cells increased, which we confirmed through microscopy and optical density measurements. We characterized the mutations that drive this strain's separation defect using whole genome sequencing of the evolved and ancestral populations. This project demonstrates how experimental evolution can be used to select against less desirable traits in commercially important yeast strains. Future research could implement similar or reciprocal methods to evolve for decreased or increased flocculation respectively.

Co-authors: Amanda Ro, Lauren Ackermann

50. *"Deep mutational scanning defines mutational constraint and antibody-escape mutations of rabies glycoprotein"*

Arjun Aditham (Fred Hutchinson Cancer Center)

Rabies lyssavirus (RABV) infects nearly all terrestrial mammals and causes nearly 60,000 human deaths annually. The glycoprotein of RABV, which mediates cell entry, is the target of existing vaccines and antibody-based post-exposure prophylaxis. However, the RABV glycoprotein exhibits substantial natural sequence variation, limiting the potency of therapeutics and prophylactic measures. Measuring the functional effects of mutations in the glycoprotein and their response to antibodies is crucial both for triaging prospective antibody therapeutics and vaccine design.

Here, we leverage a high-throughput, pseudovirus platform to perform a deep mutational scan (DMS) of the rabies glycoprotein to measure how all single amino-acid mutations affect cellular entry and antibody escape. Our DMS reveals areas of mutational constraint in the rabies glycoprotein, likely due to protein folding and conformational change requirements for function. We also experimentally identify escape mutations for 8 antibodies, including for those in clinical use or development. Escape mutations for most antibodies are already present in some circulating rabies strains. We also use our DMS to hypothesize mechanisms of action for antibodies lacking structural characterization. In summary, we expect this information will guide development of antibody therapeutics resilient to genetic variation and inform pathways for engineering stabilized vaccine antigens.

Co-authors: Arjun K Aditham, Caelan E Radford, Caleb R Carr, Naveen Jasti, Neil P King, Jesse D Bloom

51. *"Overcoming Slow Reaction Kinetics in Hybrid DNA:RNA Strand Displacement Reactions"*

Tracy Mallette (University of Washington)

DNA based molecular computing has widespread potential as a research tool and for biomedical applications like disease detection or treatment. Many applications in synthetic biology, molecular diagnostics, and oligonucleotide therapeutics require DNA computing circuits to interface with RNA necessitating DNA:RNA hybrid complexes. Recent studies have shown that hybrid reaction kinetics vary significantly relative to the purine content of the invading strand. In the case of a high purine (A/G) invader, RNA replacing DNA (RNA>DNA) is shown to be much faster than DNA>DNA. Conversely, a high purine DNA invader displacing an RNA incumbent (DNA>RNA) has relatively slow kinetics. In the cases where the signal to be detected is a natural RNA or the output is an RNA meant to bind a natural target, the sequence is defined by nature and cannot be altered to improve the kinetic situation, thus different methods are needed to facilitate these reactions.

This work explores different strategies to overcome slow reaction kinetics in hybrid systems that are created due to high or low purine sequence composition. First, we utilize RNA wobble base pairs to design a system where the RNA version has secondary structure, but the DNA strand does not. We show that adding this structure can help to overcome kinetic barriers to the hybrid reaction. Another strategy demonstrated is to add kinetic and thermodynamic driving force via strategic placement of mismatched base-pairs in the pre-formed duplex. These mismatches can then be corrected by the invading strand forming a more favorable complex. Finally, many relevant RNA output strands, such as an antisense oligonucleotide, have chemical modifications to make them more nuclease resistant. These modifications also change the kinetic and thermodynamic landscape for strand displacement reactions. We demonstrate that the mismatch approach can also be used in these cases.

Co-authors: Tracy Mallette, Tiernan Kennedy, Chris Thachuk

52. *"Modeling and Optimization of Linear Expression Template Stability in E. coli-Based Cell-Free Systems"*

Ziqi Zheng (Georgia Institute of Technology)

Cell-free systems, composed of cell lysate, energy substrate buffer, and template DNA, provide a versatile platform for gene expression by bypassing cellular membrane constraints, enabling efficient energy allocation and simplifying reaction dynamics. These characteristics establish cell-free systems as robust prototyping tools for applications in various fields including synthetic biology and biomanufacturing. Another unique advantage is their compatibility with both plasmid and linear expression templates (LETs), which drastically reduces template preparation time compared to conventional plasmid-based approaches. However, it was found that endogenous exonucleases in cell lysates rapidly degrade LETs, leading to low template stability and reduced expression yields, limiting their practical utility.

This research focuses on developing a computational model of transcription and translation pathways in an Escherichia coli lysate-based cell-free system optimized for LET-based gene expression. By integrating kinetic data and mechanistic insights, the model aims to investigate the DNA degradation pathways that govern LET instability. Specifically, we investigate the roles of exonucleases in template degradation, providing a framework to predict expression dynamics. Building on this foundation, we can explore protection strategies for LETs, including synthetic DNA analogs, sequence template design and exonuclease inhibitors, to enhance template stability and boost expression efficiency. Future work may also involve applying machine learning to optimize LET sequences and protection strategies, accelerating the development of robust cell-free platforms. These advances will not only deepen our understanding of cell-free system dynamics but also unlock the potential of LET-based systems for rapid prototyping and scalable biotechnological applications.

53. *"High accuracy nanopore sequencing of xenonucleic acids using deep learning"*

Jayson Sumabat (University of Washington)

Unnatural base-pairing xenonucleic acids (ubp XNA, or XNA) are synthetic nucleotides that form orthogonal base pairs to the standard bases. By expanding chemical and structural diversity, XNAs can improve a wide range of biotechnologies. As notable examples, in nucleic acid therapeutics, aptamer libraries evolved with XNAs have been shown to have higher binding affinities than their natural counterparts; in diagnostics, XNAs enable design of highly-specific, high-multiplexable assays; and in synthetic biology, XNAs can be used to expand the genetic code by increasing the number of orthogonal codons. However, XNA sequencing technology currently lags contemporary technology used for standard DNA sequencing and are often low throughput, costly, or not generalizable to any sequence context.

One third generation sequencing platform, protein-based nanopore sequencing, has shown promise as a general solution for sequencing XNAs. With nanopore sequencing, XNAs can be directly sequenced at the single-molecule level without amplification or need for specialized reagents. This advancement is enabled by specialized basecalling models that handle the conversion of an observed ionic current to nucleic acid sequence.

In this work, we present deep learning models trained to basecall hydrogen-bonding and non-hydrogen bonding XNAs from raw data collected using commercial nanopore devices. Two sets of basecalling models were trained – (1) task-specific, single-context models that can be used for quantifying XNA fraction in XNA-DNA sequence mixtures; (2) generalized, all-context models that can perform de novo discovery of XNAs within a DNA sequence. To demonstrate use cases, we applied these models to measure polymerase error rates when replicating XNAs (single-context) and de novo discovery of XNAs in aptamer libraries (all-context).

This work establishes a robust framework for XNA sequencing that decreases technological barriers to developing XNA-based biotechnology.

Co-authors: Nicholas Kaplan, Jorge A. Marchand

54. *"Redesigned single chain IL-12 enhances its secretion in E. coli for cancer immunotherapy"*

Nidhi Mehta (University of Washington)

Bacteria offer a promising, cost-effective alternative to human live-cell cancer immunotherapies due to their ability to colonize tumors and thrive in the tumor microenvironment (TME). Nonpathogenic E. coli can be engineered to secrete immunomodulatory proteins to their extracellular medium. However, large and multimeric immunomodulatory proteins remain a challenge to express and secrete in E. coli. Here, we rationally redesign the pro-inflammatory, heterodimeric cytokine Interleukin-12 (IL-12) to improve its expression and secretion while maintaining therapeutic function. We used the deep learning-based tool ProteinMPNN and Alphafold3 structural models to redesign IL-12 as a stabilized, single-chain protein. With this approach, we obtained redesigned single chain IL-12 (scIL-12) variants with improved bacterial expression and secretion compared to the wild-type scIL-12. In functional assays, bacterially-secreted scIL-12 redesigns were able to polarize primary human T cells with activity comparable to recombinant native IL-12. The efficacy of redesigned bacterial IL-12 will be further evaluated in mouse tumor models. This strategy could be used to redesign other therapeutically potent proteins with similar structural challenges to IL-12, widening the scope of therapeutic secretion in E. coli.

Co-authors: Nidhi Mehta, Valentina Chiavarini, Roger Geiger, Jesse Zalatan

55. *"Physical automation of the domestication of non-model bacteria"*

Andrea Garza Elizondo (Oak Ridge National Lab)

In the field of synthetic biology, "domestication" can be defined as the process of taking a wild-type organism and developing methods to genetically engineer it for a desired purpose. To achieve domestication, three main sequential barriers must be overcome – the introduction, maintenance, and functioning of genetic material. However, optimizing methods for the introduction of genetic material is often time-consuming and laborious. To accelerate this process, we have developed protocols that physically automate steps of DNA transformation methods. Specifically, we show the optimization of electroporation pulse conditions, using a robotic liquid handler to electroporate, dilute, and plate cells. We then outline our future plants to automate further steps in the transformation process, including the preparation of electrocompetent bacterial cells.

Co-authors: William Alexander, Adam Guss, Carrie Eckert

56. *"Bistability Driven by Resource Competition in Cascading Gene Circuits"*

Abdelrahman Youssef (Arizona State University)

In synthetic biology, one of the primary objectives is to design robust and stable genetic circuits that exhibit accurate, predictable behavior. Modularity is a central principle in circuit design, enabling the construction of complex systems from smaller, well-characterized components. However, a major challenge to achieving modularity is resource competition, which can significantly disrupt circuit performance by altering gene expression dynamics and modules behavior. Here, we investigate resource competition within a direct inhibitory circuit, where the inhibited module unexpectedly competes with its upstream regulatory module for shared cellular resources. When cells were initialized in an OFF state, we observed a decrease in the expression of the regulatory module as the inhibited module's expression increased indicating a nontrivial feedback loop driven by resource limitation. We further explored the impact of promoter strength on circuit performance by varying the promoter driving the regulatory module. The system displayed high sensitivity to promoter strength, highlighting its critical role in modulating resource allocation. Additionally, we evaluated the influence of growth dilution by testing the circuit in various media compositions. Resource competition persisted across different conditions, though the extent varied with the growth environment. Interestingly, distinct expression patterns and dynamics emerged when cells were initialized in an ON state, suggesting that the system's history and initial conditions play a key role in shaping its behavior.

Co-authors: Xiaojun Tian, Rong Zhang

57. *"Modular construction of biocontainment circuits for non-traditional rhizobia chassis"*

Luis Montalvo González (Purdue University)

Biocontainment strategies for environmentally released genetically engineered microorganisms (GEMs) are gaining importance due to their potential value in enhancing agricultural productivity and sustainability. Rhizobia, a group of nitrogen-fixing, root-nodulating bacteria, represents an ideal chassis for engineering the rhizosphere and the nodule microenvironment in a variety of legume crops. However, two major challenges remain when engineering rhizobia for agricultural applications: (1) their status as non-traditional chassis organisms complicate the construction and testing of genetic circuits, and (2) there is insufficient documentation and study of the escape ratios and environmental persistence of engineered rhizobia. This study aims to address these gaps by testing the openCIDAR 3G method to construct evolutionarily stable biocontainment circuits in selected rhizobia strains. Additionally, we are evaluating several transformation methods to identify the most efficient and reliable means for introducing genetic circuits into rhizobia. Finally, we will assess the stability of these circuits at different genomic levels, including plasmid, mega-plasmid, and chromosomal. Our work seeks to advance the engineering of rhizobia while mitigating the potential environmental risks associated with the unintended release of GEMs.

Co-authors: Luis G. Montalvo González, Grace Cook, Leopold Green

58. *"Low-Cost Point-of-Care Diagnostic for Early Preeclampsia Detection via TLISA-Based Protein Sensing"*

Shifeng Xu (Georgia Institute of Technology)

Timely and accurate diagnosis of preeclampsia is essential to reduce maternal mortality, especially in low- and middle-income countries where clinical infrastructure is limited. Current diagnostic methods are often inaccurate and require expensive equipment to quantify readouts, limiting their availability at the point of care.

To address this, we use TLISA (T7 polymerase-Linked ImmunoSensing Assay), a modular, cell-free platform that enables low-cost, semi-quantitative detection of proteins without instrumentation. In TLISA, fragments of an engineered RNA polymerase are fused to affinity domains specific to a target biomarker. When both affinity domains bind the same target protein, the fragments colocalize, reactivating polymerase activity and triggering a visible colorimetric response.

We aim to develop TLISA-based sensors for PlGF1 and sFLT1, two angiogenic protein biomarkers clinically validated for early preeclampsia risk assessment. For PlGF1, we screen published nanobody and peptide affinity domains using a cell-free ELISA and evaluate all polymerase fragment combinations in TLISA. For sFLT1, we repurpose VEGFR-1-binding peptides, validating their binding and TLISA performance. We then optimize sensor designs to enhance signal strength and specificity.

This work supports the development of an affordable (<$1), rapid (<45 min), and user-friendly diagnostic for early preeclampsia detection in low-resource settings. By targeting clinically relevant thresholds of PlGF1 and sFLT1 in fingerstick blood volumes, TLISA may facilitate earlier intervention and improve pregnancy outcomes.

Co-authors: Felicia Oentoro, Mark Styczynski

59. *"Enhancing transcription-based sensing with complex coacervates"*

Siyuan Feng (Northwestern University)

Cell-free biosensors are powerful platforms that leverage ligand-responsive transcription factors to detect chemicals of interest. While inexpensive, modular, and distributable, these biosensing platforms are typically constrained by slow readouts and variable limits of detection. In this work, we address these limitations by interfacing transcriptional biosensors with complex coacervates, membrane-less compartments shown to concentrate solutes, enhance catalysis, and buffer unfavorable environments. First, we demonstrate that complex coacervates can accelerate in vitro transcription by T7 RNA polymerase. Next, we show that these systems can support sensing mechanisms that regulate transcription, and accelerate sensing kinetics by 2x at room temperature. By integrating complex coacervates with a range of transcriptional sensors, we discover that phase separation can enhance the sensitivity of ligand sensing, with improvements related to ligand polarity. Lastly, we demonstrate that coacervate-mediated enhancements may be preserved after single pot lyophilization and rehydration. To our knowledge, this is the first study that demonstrates enhanced enzymatic transcription using complex coacervates, as well as the first to show transcription-based biosensing in any phase separated system. We envision that this work contributes to a growing understanding of phase separation in biology and advances the use of membrane-less compartmentalization to enhance cell-free systems.

Co-authors: Siyuan Feng, Antonio Garcia IV, Samanvaya Srivastava, Julius B. Lucks

60. *"Toward long-term recording of cell history"*

Theresa Loveless (Rice University)

DNA recorders are synthetic biology tools that enable new kinds of developmental biology experiments. Cells of interest are engineered to express genome editors that make mutations at one or more recording loci in each cell's genome. If random mutations are made constitutively, recording loci can be sequenced to reveal cell lineage. If mutations are made inducibly, recording loci can be sequenced to reveal cells' gene expression history. A successful DNA recording architecture has combined constitutively-expressed prime editor with inducible pegRNAs (1,2). Each pegRNA can edit the same locus, in temporal order, but each adds a slightly different sequence. After multiple rounds of recording, then, each recording locus reflects which pegRNAs edited it in which order, allowing inferences about the cells' exposure to those pegRNAs' inducers. We have previously described one DNA recording architecture of this type, peCHYRON2, which adds, in each round of recording, the target site for the next round. Therefore, peCHYRON may be especially appropriate for very long-term recording applications, or applications for which it is critical to record at a specific endogenous genomic locus. Here, we will present three projects aimed at improving the recording efficiency and general applicability of this tool. First, we have assayed the effects on cells of long-term recording. We saw little change in cell viability or growth rate, but significant silencing of prime editor. Second, we have assessed how improved prime editors affect the recording efficiency of peCHYRON. Finally, we have used MinsePIE3, a machine learning model that predicts pegRNA insertion efficiency, to optimize the pegRNA components of peCHYRON, and will present initial results.

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Co-authors: Matt C. Demelo, Catalina A. Dentzel Helmy, Erik Rodriguez Teran, Sara K. Ross, Kyle P. Murrray

61. *"Developing toolkits for genome engineering and transcriptional programming in purple nonsulfur bacteria"*

Amanda Robert (University of Washington)

Purple nonsulfur bacteria (PNSB) are promising chassis for sustainable biomanufacturing due to their metabolic versatility and their ability to utilize diverse feedstocks. However, engineering PNSB is challenging due to their complex metabolism and the lack of efficient genetic tools. To address this, we have developed a genome engineering toolkit that enables both efficient genome engineering and precise transcriptional control. For genomic integration, we have developed a conjugation-compatible variant of SAGE (cSAGE), which leverages conjugation for DNA delivery and enables the efficient integration of large payloads (>10 kb) and up to 10 distinct payloads within a single genomic landing pad 1. Using cSAGE, we characterized a set of 12 distinct promoters spanning a 90-fold expression range, providing a tunable system for heterologous gene expression. To complement stable integration with dynamic gene regulation, we established CRISPR-based transcriptional control in PNSB by implementing CRISPR interference (CRISPRi). We successfully ported a CRISPRi system into two PNSB strains and demonstrated targeted knockdown of a heterologous gene, achieving up to 92-fold repression. By integrating cSAGE for stable genome engineering with CRISPRa/i for multi-gene regulation, we have developed a comprehensive toolkit for precise metabolic control in PNSB. This system enables systematic perturbation and modification of PNSB metabolism, providing a route to unlock their potential as microbial platforms for industrial bioproduction.

Co-authors: Amanda M. Robert , Michael S. Guzman , Cholpisit Kiattisewee, Jackson Comes, Allan Scott, Joshua Elmore, Brian H. Darst, Ryan A.L. Cardiff, Diego Alba Burbano , Stella Anastasakis , Sarah Grube , Jesse G. Zalatan , and James M. Carothers

62. *"Biomanufacturing performance and transcriptomic response of engineered Escherichia coli in both simulated and spaceflight variable gravity"*

Hannah Roberts (University of Florida)

Engineered microbes offer a powerful approach to biological in situ resource utilization (ISRU) for in-space manufacturing. However, culture challenges imposed by the space environment such as variable gravity and radiation exposure impact space biomanufacturing by altering microbial physiology and genetic regulation. Because space biomanufacturing can facilitate independence from difficult supply lines for missions to the Moon and Mars, there is a need to characterize in-space biomanufacturing performance at various gravity levels. Unfortunately, responses in widely characterized Escherichia coli vary across published modeled and spaceflight microgravity experiments under different hardware and environmental conditions, even before any biomanufacturing occurs. Thus, we sought to establish the quantitative and transcriptomic effects of different gravity levels on biomanufacturing yield and titer across analog devices and repeated spaceflights. We engineered E. coli to produce colorimetric □-carotene, a vitamin with space-relevant properties including vision health promotion and radioprotection. After characterizing the effects of simulated lunar, Martian, and microgravity on biomanufacturing performance (yield and titer) using two analog devices (High Aspect Rotating Vessels and a Random Positioning Machine), we translated these results to the spaceflight environment on two SpaceX missions, CRS-27 and Crew-7. For the same engineered E. coli strains, we determined biomanufacturing performance and gene expression in microgravity aboard the International Space Station (ISS), and in low gravity in the Rhodium Variable Gravity Simulator, a calibrated low speed centrifuge on the ISS that simulates lunar and Martian gravity. Additionally, we characterized the effects of cell motility, selection pressure, and repeated spaceflight on biomanufacturing performance and differential gene expression. Our results firmly baseline future E. coli space biomanufacturing performance.

Co-authors: Hannah I. Roberts, Anya Volter, Jithran Ekanayake, Meghan L. Jones, Heath J. Mills, Olivia Gámez Holzhaus, Amor A. Menezes

63. *"Developing Genetic Manipulation Tools for The Gut Microbiota Member Turicibacter sanguinis"*

Arren Liu (Johns Hopkins University)

The mammalian microbiome is home to trillions of microbes that impact aspects of host physiology. One microbiota genus of interest are Turicibacter, which interact with neurotransmitters, SSRIs, lipids, and cholesterols found in their hosts. Although Turicibacter have been implicated in several aspects of host physiology, compatible genetic manipulation tools have yet to be reported, making mechanistic studies difficult to conduct. The work presented here aims to develop genetic tools capable of manipulating gene expression in Turicibacter sanguinis. To start, we assessed the minimal inhibitory concentration of T. sanguinis MOL361 to antibiotics that will be used selection markers for transformants. T. sanguinis MOL361 growth was inhibited at 2 µg/mL chloramphenicol, 50 µg/mL kanamycin, and 450 µg/mL spectinomycin. Next, we developed an electroporation method to deliver plasmid DNA and assessed how various conditions impacted transformation efficiency. The best condition tested using unmethylated DNA, resulted in a transformation efficiency of 2.7 x 10 cfu/µg DNA (about 20-30 colonies per reaction). In accordance with our goal to manipulate gene expression in T. sanguinis, we identified multiple compatible origins of replication (pBP1, pCD6, and pIP404) and are developing a library of plasmids containing promoters with different activities. To assess the compatibility of the tools developed from this study, they will be assessed in other Turicibacter strains found to also influence host-physiology. All in all, this work is the first to report a method capable of transforming DNA into the Turicibacter genus. Future work will look to expand the toolkit to include gene editing methods (i.e. CRISPR-technologies) and improve DNA electroporation efficiency. This work will enable future studies to specifically investigate mechanisms employed by T. sanguinis and opens the potential to engineer them as biotherapeutics for host health.

Co-authors: Arren Liu, Yutong Zhu, Jonathan Lynch

64. *"Transcriptome-Guided Landing Pad Selection for Stable Bioproduction in Rhodobacter sphaeroides 2.4.1"*

Sarah Grube (University of Washington)

Purple non-sulfur bacteria (PNSB) are promising hosts for sustainable bioproduction due to their versatile metabolisms. In order to engineer these non-model microbes, high-efficiency tools such as SAGE (Serine recombinase-Assisted Genome Engineering) have been developed. SAGE relies on integrations into a stable genomic location, so-called "landing pads", which contain an array of 10 distinct integrase recognition sequences. These landing pads are located at neutral genomic sites that enable stable expression of heterologous genes without disrupting host physiology. Since neutral sites are poorly characterized in PNSB, we employed a genome-wide transcriptomics-guided approach to identify ten candidate SAGE landing pad locations spanning both chromosomes and plasmids in Rhodobacter sphaeroides 2.4.1. Candidates were selected using RNA-seq and a custom Python script, which searched for regions longer than 500 bp with fewer than 25 expressed transcripts under aerobic or anaerobic conditions. Two-step allelic exchange was used to integrate the landing pads, and integration was verified with PCR and whole-genome sequencing. The resulting strains are being characterized under both aerobic and anaerobic conditions, assessing fitness and expression of SAGE-integrated fluorescent reporters. Upon validation of the neutral landing pads, we will employ SAGE to introduce heterologous biosynthetic pathways targeting the production of industrially relevant metabolites. These include the mevalonate and 4VP pathways, which require stable multi-gene expression for effective production. Overall, this work establishes a foundation for predictable, high-efficiency metabolic engineering in PNSB and supports their broader usage as chassis for C1-based biomanufacturing.

Co-authors: Sarah Grube, Michael Guzman, Stella Anastasakis, Amanda Robert, Cholpisit Kiattisewee, Jackson Comes, Ryan Cardiff, Diego Alba Burbano, Allan Scott, Joshua Elmore, James Carothers

65. *"Programmable Molecular Recording Using CRISPR-Based Circuits for Enhanced Bioproduction in Microbes"*

Kira Olander (University of Washington)

Transforming molecular recording technologies that store information about cellular events into transcriptional responses can significantly enhance our ability to engineer microbes for next-generation bioproduction applications. Circuitry that is responsive to the timing, order, and intensity of molecular events can give engineers control over how a bacterial cell processes and transforms information into downstream responses. In this work, we show that we can incorporate base editors as molecular recorders into existing CRISPR circuitry in bacteria. We demonstrate the recruitment of both activators and base editors to a single deactivated Cas9 (dCas9) in a guide-dependent manner. Utilizing RNA hairpin and RNA-binding protein pairs, we create two orthogonal CRISPR complexes: one that recruits a base editor and another that recruits an activator. We tested this system using "broken" CRISPRa reporters, where the CRISPRa spacer target has a non-functional PAM. The base editor introduces A-to-G edits in the non-functional PAM, enabling the CRISPRa complex to bind and activate gene expression. This reporter can sense the presence of a given input, resulting in a permanent response that can control any desired transcriptional output. Additionally, we show that split guide RNAs can be used for base editing with this reporter system. This approach requires each piece of the guide to be present simultaneously for functional base editing, allowing for the temporal association of two different inputs. Furthermore, we construct circuits where transient mRNA expression triggers a base edit to activate a permanent transcriptional response. Our findings demonstrate that CRISPR base editing and activation can be paired to create programmable and permanent transcriptional responses, offering a powerful tool for synthetic biology applications.

Co-authors: Ava Karanjia, Mia Giallorenzi

66. *"Harnessing immobilized cell-free multi-enzyme systems as reusable biocatalysts"*

Widianti Sugianto (University of Washington)

Bacterial lysate-based cell-free systems enable the expression of biosynthetic pathways from DNA templates, offering great potential for modular and deployable bioproduction in low-resource or remote settings. Yet, their broader use is limited by challenges in sustaining enzyme activity, maintaining reusability, and separating enzymes from the bulk reaction mixtures. Here, we present a method to co-immobilize multiple cell-free expressed enzymes in poly(ethylene glycol) diacrylate (PEGDA) hydrogels to create reusable biocatalysts. Proteins can be chemically ligated within these hydrogels, and small-angle X-ray scattering (SAXS) reveals that the hydrogel mesh size is suitable for protein entrapment, effectively retaining cell-free proteins for at least a week. Using three heterologous enzymes expressed in cell-free systems, the immobilized multi-enzyme systems catalyzed the conversion of pyruvic acid into malic acid, a versatile chemical precursor. By decoupling cell-free pathway expression from the immobilization step, we preserved the activity of both heterologously-expressed and endogenous lysate enzymes within the hydrogels. These enzyme-laden hydrogels can be transferred to new vessels and reused to catalyze multiple bioproduction cycles. In some cases, the immobilized enzymes also outperformed their free, unbound counterparts in both activity and longevity. Overall, this work provides a straightforward and scalable approach to immobilize cell-free multi-enzyme systems for sustainable chemical bioproduction using widely-available biomaterials, without compromising cell-free expression.

Co-authors: Widianti Sugianto, Ryan A. L. Cardiff, Claire Benstead, Gokce Altin-Yavuzarslan, Lilo Pozzo, Alshakim Nelson, James M. Carothers

67. *"Optimizing the Limit of Detection for the TLISA Point-of-Care Biosensor Platform to Achieve Clinical Relevancy"*

Karly Liebendorfer (Georgia Institute of Technology)

Timely diagnostic tools are essential for the rapid and efficient detection of a wide range of diseases and conditions. One key challenge in developing these tools is establishing a limit of detection (LOD) that can detect clinically relevant levels of a target substance. Our lab has recently developed biosensor platforms for protein detection utilizing cell-free systems (CFS). CFS are processed cell lysates that enable in vitro transcription and translation (TXTL) machinery to detect various molecules. The TLISA (T7 polymerase-Linked ImmunoSensing Assay) serves as a central tool in this work, which employs an engineered RNA polymerase split into two fragments, each fused to small antibody-like domains targeting the same protein. Only in the presence of the target protein do the fragments colocalize, triggering reassembly and subsequent TXTL that generates a colorimetric reporter. The intensity of the color produced reflects the amount of the protein biomarker present in a given sample, facilitating its application in diagnostics. Currently, the TLISA platform has a LOD between 50-500 nM, while clinically relevant concentrations of specific proteins of interest range between 0.05-0.5 nM. Aiming to reach these desired concentrations, this project targets to significantly lower the LOD for improved, clinically relevant point-of-care (POC) diagnostics. To accomplish this, mutations shown in literature to enhance T7 RNAP activity will be introduced into the polymerase fragments to improve their performance as it is hypothesized that polymerase inefficiencies after reassembly are leading to higher LODs. Additionally, modifications will be made to the reporter gene to enhance polymerase binding, boosting TXTL mechanisms. Together, these modifications should improve the function of the TLISA system, achieve lower LOD levels, and lead to a sensor platform suitable for POC diagnostic applications.

Co-authors: Karly Liebendorfer, Alexandra Patterson, Mark Styczynski

68. *"MEDIFLOW (Multiplexed and Engineerable Diagnostics using Integrated Flow)"*

Alexandra Patterson (Georgia Institute of Technology)

Although the United Nations has defined universal access to medical care as a primary goal, the inaccessibility of a critical healthcare tool—diagnostics—has significantly impeded global medical access. While conventional point-of-care (POC) diagnostic platforms are effective for detecting simple biomarkers, such as those used in diagnosing pregnancy or COVID-19, they often fall short in complex diagnoses due to sensitivity, specificity, and multiplexing requirements. Promisingly, synthetic biology-inspired diagnostics are a prime alternative to conventional systems, due to their laboratory-grade capabilities and multiplexed detection. However, full translation of these approaches to the POC has been impeded by a lack of low-resource, multiplexed reporters (i.e. a color change) to indicate disease presence. Here, we address this critical need via MEDIFLOW (Multiplexed and Engineerable Diagnostics using Integrated FLOW), an 8-plexed POC diagnostic platform that can be seamlessly plugged into established synthetic biology sensors. Briefly, MEFILOW uses expanded lateral flow and cell-free systems to achieve 8 localized color-change reactions, enabling the sensitive and rapid indication of biomarker presence. Through optimization, we achieved a greater than 20-fold reduction in sensitivity compared to traditional multiplexed reporters and demonstrate modularity through simple integration with established synthetic biology sensors. In total, these efforts have the potential to enable rapid development of globally impactful next-generation diagnostics by uniquely enabling multiplexing at the POC.

Co-authors: Alexandra Patterson, Jiho Seok, Andrew Wheeler, Mark Styczynski

69. *"Appropriating the biosynthesis of nucleoside natural products for biocatalysis"*

Gage Owens (University of Washington)

Nucleoside analogs serve an important role as essential therapeutics use for the treatment of various cancers, viral infections, and fungal infections. Many synthetic nucleoside analog drugs derive their bioactivity from functional groups that are either inspired by, or convergently similar to, those found in nucleoside natural products (NNPs). The biosynthetic pathways that produce NNPs offer step-efficient, potentially greener routes to synthesize both known and novel therapeutics. For various classes of NNPs, the enzymes responsible for their biosynthesis remain either uncharacterized or undiscovered. This work explores the biosynthesis of Ascamycin, an NNP produced by Streptomyces sp. JCM 9888, that contains an unusual 2-chloroadenine moiety. 2-chloroadenine is a key pharmacophore used in the FDA-approved anti-cancer drugs cladribine and clofarabine. We aim to elucidate the enzymatic steps within the ascamycin biosynthetic gene cluster (BGC) responsible for the formation of the 2-chloroadenine moiety and to explore their utility in the biosynthesis of chlorinated nucleoside drugs. Initial fermentation experiments have confirmed the production of chlorinated and brominated nucleosides and nucleobases, including previously unknown substrates. Ongoing work includes the generation of gene knockout strains and heterologous expression of candidate genes in Streptomyces spp. to probe enzyme function and substrate flexibility.

Co-authors: Sarah Eykel, Jorge A. Marchand

70. *"Oleochemical cell factories enabled by synthetic biology and metabolic engineering in the extremophile yeast Debaryomyces hansenii CBS 767"*

Zekun Li (Worcester Polytechnic Institute)

High production of sustainable oleochemicals requires the development of optimal strains as cell factories. The CTG clade yeast D. hansenii CBS 767 has attractive catabolic, anabolic, and tolerance phenotypes. It naturally grows on the major monosaccharides of lignocellulosic biomass, specifically glucose, xylose, and arabinose. It can also overproduce free fatty acids, key precursors oleochemicals. However, the genetic basis for these advantageous phenotypes remains poorly understood. Therefore, we conducted a cross-species transcriptomic experiment comparing D. hansenii stress response to the oleaginous model yeast Yarrowia lipolytica. A metabolic network analysis reaffirmed that Yarrowia upregulates lipid biosynthesis pathways under nitrogen-limiting conditions and revealed that Debaryomyces yeasts produce the greatest amount of lipids in unstressed conditions. We then used the genomic data to derive functional D. hansenii promoters and terminators, and formatted them in a modular cloning system. Using this system, we integrated several pathways for fatty acid-derived chemicals including fatty alcohols and alkanes. Through screening of specific pathway enzymes, endogenous acetylating acetaldehyde dehydrogenase (ACDH) and fatty acyl-CoA reductases (FAR), we constructed efficient pathways for producing hexadecanol (224.6 mg l−1). We also constructed a three-gene alkane biosynthesis pathway, with 18 possible variants resulting from a fully factorial combination of two thioesterase (TES) variants, three carboxylic acid reductase (CAR) variants, and three aldehyde deformylating oxygenase (ADO) variants. The best pathway achieved 38.3 mg L−1 heptadecane. We then engineered the elongation pathway to produce medium-chain alkanes by heterologous expression of acyl-transferase (phaG) and increased growth on xylose by overexpression of xylose reductase (XR). Therefore, we were able to achieve medium-chain alkane production in D. hansenii on all three biomass – glucose, xylose, and arabinose. This demonstrates D. hansenii is an attractive host for producing oleochemicals from lignocellulose. In summary, this work highlights the potential of D. hansenii CBS 767 as a saltwater cell factory to produce fatty acids derived products.

Co-authors: Zekun Li, Nilesh Kumar Sharma, Sarah Weintraub, Nicole Petersen, Eric Young

71. *"LTTR Late Than Never: High Throughput Characterization of LysR-Type Transcriptional Regulators"*

Randon Serikawa (University of Washington)

Lys-R type transcriptional regulators (LTTRs) are one of the largest families of bacterial transcriptional regulator proteins with over 850,000 known members. Many of these LTTRs are enriched in our gut microbiota, whose metabolic processes affect human health outcomes. LTTRs regulate gene expression through the binding of specific ligands to their ligand binding domain. Currently, less than 500 of them have been studied which represents a severe knowledge gap that conventional methods of characterization are unable to keep up with. We aim to create a high throughput methodology to characterize LTTRs by their corresponding ligands that regulate gene expression. We are currently developing an assay to use chimeric LTTRs, or engineered LTTRs that share the same DNA binding domain yet a variable ligand binding domain. The use of chimeric LTTRs, which will all bind to the same DNA promoter, will potentially allow dozens of LTTRs to be tested in one assay. Our work thus far has demonstrated that chimeric LTTRs can be expressed in E.coli cells and purified using affinity chromatography and magnetic bead purification. We have also demonstrated that their ligand binding domains are functional and specific via differential scanning fluorimetry, and that their DNA binding domains are functional using an electromobility shift assay using SYBR green and SYPRO ruby dyes. Future work will explore their ability to regulate gene expression when their proper ligands are introduced with a substrate-induced gene expression reporter assay. Then uncharacterized LTTR candidates to be made into chimeras will be selected via a bioinformatic sequence similarity network analysis for assay piloting. If successful, this assay has potential to elucidate new metabolic pathways of our gut microbiota allowing for better understanding of their complex relationship with the human body.

Co-authors: Hoaxian Xu, Lauren Rajakovich

72. *"MOD-GAP: Modular genetic design for one-pot Golden Gate Assembly of multi-guide CRISPR programs in bacteria"*

Brian Darst (University of Washington)

Multi-target CRISPR-Cas activation (CRISPRa) and inhibition (CRISPRi) programs can help overcome complex challenges in bacterial metabolic engineering. However, previous methods of cloning multi-target guide RNA (gRNA) constructs have proved tedious and time-consuming, motivating us to develop a strategy for rapid, scalable construction of CRISPRa/i programs in bacteria. Here we present MOD-GAP, a modular genetic design approach that enables the one-pot assembly of multi-gene CRISPR-Cas9 gene regulation programs functional in bacteria. We first developed a modular framework for designing and generating genetic parts to express multiple guide RNAs from the same plasmid. We then show that this approach allows the assembly of plasmids expressing at least 9 guide RNAs from individual transcriptional units in E. coli and at least 8 guide RNAs in Pseudomonas putida, a useful bioproduction chassis. To demonstrate the utility of this method in constructing functional genetic circuits, we assembled a 7-layer CRISPR activation cascade in a single step via MOD-GAP and observed propagation of an inducible input signal through the entire cascade. By combining easy-to-use Python code with a toolkit of genetic parts, MOD-GAP now provides a route for rapidly constructing multi-guide CRISPRa/i expression programs for a wide range of applications in basic science, bioproduction and biotherapeutics.

Co-authors: Cholpisit Kiattesewee, Diego Alba Burbano, Semira Beraki, Ian D. Faulker, Jesse G. Zalatan, James M. Carothers

73. *"yEvo: Leveraging a high school teaching lab to study antifungal resistance mutations"*

Randi Avery (University of Washington)

Evolution is a topic that is particularly difficult for high school students to understand, with many common misconceptions continuing into the general population. With today's political climate, it is increasingly important to properly teach high school students evolution in an accessible manner, as programs serving underrepresented students are likely to decrease. Toward this goal, the Dunham lab has developed a curriculum implementing a hands-on approach where students can see evolution in real-time using a yeast experimental evolution and genomics lab named "yEvo." Briefly, students passage eight ancestral strains of Saccharomyces cerevisiae, which have been engineered to express various color phenotypes, in increasing concentrations of an antifungal drug. Since its inception, yEvo has been performed in dozens of classrooms and hundreds of students have studied both the azole and echinocandin classes of antifungal drugs. Evolved strains that show an antifungal-resistant phenotype compared to their ancestors are whole-genome sequenced by the Dunham lab. We then return data on the de novo mutations in the students' evolved strains to the students via an original Mutation Browser website (https://yevo.org/mutation-browser/). The students can view the types of mutations found, which genes developed mutations, and the functions of those genes. We in the Dunham lab then leverage the data on recurring mutations to draw conclusions about antifungal resistance, as those mutations are robust to batch effects due to the various approaches the different high school classes take. Not only is yEvo beneficial for educating the next generation of adults, it also provides us with a high-throughput approach for elucidating antifungal resistance mutations.

Co-authors: Leah Anderson, Renee Geck, Joe Armstrong, Randi Avery, Jasmine Schoch, Virginia Wang, Zilong Zeng, Sayeh Gorjifard, UW Genome Sciences Hackathon Team, Maitreya Dunham

74. *"Understanding upstream dominance in synthetic gene circuits in human cells"*

Ross Jones (University of British Columbia)

Context-dependence in gene expression muddies our ability to predictably design robust synthetic genetic circuits in cells. A significant and poorly-understood context-dependency is how the expression of one gene affects that of neighboring genes in the genome, especially in mammalian cells. In collaboration with the Galloway Lab at MIT, we are studying this problem through the lens of genomic "syntax", the relative position and orientation of synthetic genes, and its effects on gene expression. For example, we have found that when integrating two genes into human cells in a tandem orientation: {Promoter1:Protein1_PolyA1 // Promoter2:Protein2_PolyA2}, expression of a given gene will be much stronger when in the upstream position than the downstream position. This pattern of "upstream dominance" holds for different integration modalities (PiggyBac transposon, lentivirus, and CRISPR knockin), different promoters (both constitutive and induced), and different human cell types (e.g. HEK-293 and pluripotent stem cells [PSCs]). To further understand the mechanistic basis for upstream dominance, we engineered human PSCs with an array of two-gene constructs where we tested how combinations of different promoters, poly-A signals, gene orientations, and chromatin insulators affect gene expression. Our results suggest that several factors including DNA supercoiling, DNA looping, and silencing by the human silencing hub (HUSH) complex likely contribute to upstream dominance. Further improvements in our understanding of upstream dominance in human cells and the development of methods to predict and counteract its effects will enable much more precise control of complex gene circuits integrated into human cells. Our focus on engineering PSCs, which have vast potential to differentiate into diverse cells and tissues, will have significant implications for advancing regenerative medicine.

Co-authors: Ross D. Jones, Jiyoung Yun, Ali Murtaza, Matthew Chan, Christopher P. Johnstone, Kasey S. Love, Kate E. Galloway, Peter W. Zandstra

75. *"Developing kinetic models of cell-free lysate metabolism for carbon-conserving pathway engineering"*

Margaret Cook (University of Washington)

Model-driven engineering promises to accelerate the use of cell-free lysate-based systems for pathway prototyping and next-generation bioproduction applications by allowing us to predict how engineered pathways will interact with lysate biochemistry and impact the synthesis of target molecules. We developed a semi-automated pipeline that integrates proteomics and metabolomics data to build, parameterize, and fit a kinetic model of lysate metabolism. The pipeline uses KEGG to identify reactions catalyzed by detected enzymes, BRENDA or machine learning models to estimate kinetic parameters, and Equilibrator to calculate thermodynamics. Preliminary models are refined by fitting to time-course metabolomics data using particle swarm optimization. Using this approach, we were able to construct a kinetic model of endogenous metabolism in E. coli-based 'TXTL' lysate. The final model includes 33 enzymes, 53 reactions, 63 metabolites, and 438 parameters, and was trained on 338 time-course metabolite measurements and tested on 91. The model accurately captured dynamic shifts in concentrations of key metabolites, demonstrating the pipeline's ability to fit large-scale models to rich experimental data. We identified dominant metabolic modules, cofactor usage, and metabolite sinks. Simulations of interventions such as dilution, inhibitor addition, and heterologous gene expression revealed how perturbations reshape network behavior and how endogenous flux interacts with engineered pathways. This work provides a foundation for the rapid, automated construction of kinetic models for cell-free systems, enabling model-guided pathway design and optimization for crude lysates from various source microbes, and supporting the engineering of carbon-conserving bioproduction pathways.

Co-authors: Diego Alba Burbano, Ryan Cardiff, Adel Heydarabadipour, Herb Sauro, James Carothers

76. *"Lil Lab Network: Local to Global Co-Design For Biotechnology Futures"*

Bria Metzger (University of Washington)

Biotechnology conceives of itself as a branch off of academic research. In reality, this is only a fraction of the biotechnology practiced worldwide. What becomes possible when we open our definition of biotechnology? What is achieved we not only include, but prioritize scientific practices that are community-driven and culturally relevant? We launched the Lil Lab Network to explore these questions. With support from Experiment.com and local funding, we launched a microgrant application to build Lil Labs worldwide. We intentionally reached out to community scientists, educators, advocates, and creatives with broad expertise beyond academia. Each microgrant recipient ("Steward") received $500 to build and a Lil Lab: a neighborhood-scale hub of scientific practice. Each Lil Lab shares resources, holds workshops, and sparks collaborations unique to the needs of the local community. We organized a series of workshops to support Stewards in establishing and evaluating their Lil Labs. In turn, Stewards provided feedback about their needs, challenges, and successes. The organizational structure of the Lil Lab Network itself emerged out of this extended process of co-design. Together, we defined six core values: every Lil Lab is community-driven, respectful, accessible, independent, safe, and fun. In addition, we we have created a community driven biosafety protocol that ensures we meet the needs of both the Lil Labs community as well as local, national, and international laws. Finally, to respond most effectively to each Steward's needs, we established the following working groups: administration, public relations, biosafety, and resource repository. Currently, the Lil Lab Network includes 16 Lil Labs across 5 countries. The process of establishing the Lil Lab Network emphasizes the power of community-led co-design for inclusive and innovative biotechnology futures.

Co-authors: Bria Metzger, Prasanna Padmanabham, Rangarajan Bharadwaj, Angie Aguirre-Tobar, Ashley Herrera, Callie R. Chappell, Nasa Sinnott-Armstrong

77. *"Lil Labs: Bridging Biotechnology to Community for Local Action and Innovation"*

Angie Aguirre-Tobar (Fred Hutchinson Cancer Center)

Envisioning an expansion on biotechnology takes collective imagination and a model that integrates interdisciplinary expertise. Lil Labs is a model that fuses academic, industrial and community experience with biological systems into a creative and neighborly exchange. Led by organizers passionate about science, these hubs support community science with publicly available scientific tools. So we ask, how can individual Lil Labs interact, spark the exchange of resources, and ultimately expand resilience and capacity at the regional scale? To explore these relationships locally, we worked with community leaders and education programs to establish 12 Lil Labs across Seattle and connected sites. Lil Labs affiliated with University of Washington center bio-art and hands-on workshops where people exchange protocols for home-made kombucha, dye clothing with yeast-produced indigo and hold spaces for creative projects in academic settings. In the greater Seattle area, Lil Labs have become a nexus of climate resilience and environmental health advocacy. From distributing water filters, masks, and at-home tests for mold, to workshops contextualizing environmental risks of disease development for disproportionately affected populations, these Lil Labs foster community advocacy and integration of biotechnology into real-time changes for neighbors. In rural places facing ecological changes, Lil Labs can support action centered in cultural fluency of the land. In the San Juan Islands, community members have expressed concern of the increasing tick population. Following the model of co-creation, academics from the University of Washington and scientists from the Lopez Island-based community biolab Kwiáht started a workshop series specific to island tick ecology. More than just public, physical boxes, Lil Labs are nodes where the lived experiences of community members and biotechnology innovation meet to build a dynamic understanding of our changing neighborhoods.

Co-authors: Ashley Herrera-Augustiano, Bria Metzger, Nasa Sinnott-Armstrong, Callie H Chappell

78. *"Engineering fusion variants to optimize chimeric histidine kinase functionality"*

Andrew Holston (University of Oregon Knight Campus)

Bacteria deploy millions of sensor histidine kinases (HKs) to detect nutrients, antibiotics, pH, and diverse ions, yet <0.01% have been functionally mapped, and the rules governing transmembrane signaling remain unclear. We present a multiplexed gene-library and screening platform that accelerates HK deorphanization while laying the groundwork for plug-and-play biosensors. Variable sensor, transmembrane, and HAMP domains from 3,061 different natural HK sensors were grafted onto the conserved kinase domain of E. coli EnvZ, and for each chimera we synthesized up to eight phase variants that add or delete residues at the fusion junction to sample phase space. The resulting designs (21,726 genes) were expressed in an E. coli ΔenvZ ΔompR strain using FACS and NGS to separate and identify chimeric HK variants in different basal activation states, recovering functional data representing 2,456 of the 3,061 sensor domains. Analysis of the genotype–phenotype links reveals sequence and register motifs that lock receptors in high-activity states, enabling predictive models that enrich for functional designs. Comparative statistics across sensor domain classes highlight systematic differences in signaling behavior. By rationally tuning phase and domain composition, we aim to create a toolbox of chimeric HKs that convert diverse chemical cues into a unified multiplexable gene expression readout, providing a versatile scaffold for whole-cell biosensors in environmental monitoring, diagnostics, and metabolic control. Our approach offers a path towards comprehensive functional annotation of the millions of HKs in metagenomic space and the rapid assembly of bespoke sensing circuits for synthetic biology.

Co-authors: Andrew Holston, Philip Jimenez, Samuel Hinton, Luca Lippert, Calin Plesa

79. *"Portable Glucose Monitor-Based Field Deployable Sensing"*

Emily Heckard (Georgia Institute of Technology)

Point-of-care (POC) diagnostics offer the potential to transform healthcare by providing fast, affordable, and accessible testing outside traditional lab settings. Blood glucose monitors (BGMs) exemplify this technology, widely used for measuring glucose levels in individuals with diabetes. These devices are highly accurate and can provide real-time results. However, their use has the potential to extend beyond glucose detection to diagnose various health conditions.

To enable this, we have developed a novel method combining BGMs with cell-free expression (CFE) systems. CFE systems are biological platforms that can detect specific substances or produce proteins without living cells. By integrating CFE with BGMs, we can measure a wide range of biomarkers, turning a simple device into a powerful diagnostic tool. While this method is affordable and sensitive, its complexity limits its practical use in resource-limited settings.

My objective is to simplify this system, making it user-friendly and deployable in global health applications. I plan to automate the necessary steps using a microfluidic device to control reagent delivery and modify the process to work with whole blood, eliminating the need for serum separation. These advancements will allow for easy, cost-effective diagnostic testing at the point of care, expanding access to vital health testing, especially in underserved regions.

Co-authors: Mark Styczynski, Yan Zhang, Tabitha Rosenbalm, Mike Farrell, Mohamed Badawy

80. *"Profiling transcriptional regulatory networks at high resolution and scale in non-model bacteria using a pooled CRISPR-microSPLiT screening platform"*

Yujia Huang (University of Washington)

Understanding transcriptional regulatory networks (TRNs) is fundamental for engineering non-model bacteria to improve their performance in biotechnological applications. However, these networks remain poorly characterized due to limited genetic tools and high-throughput approaches. Here, we extend the CRISPR-microSPLiT platform—originally developed in E. coli—to two non-model bacteria widely used in industrial and environmental biotechnology: Pseudomonas putida and Bacillus subtilis. CRISPR-microSPLiT combines CRISPR activation/interference (CRISPRa/i) with microbial split-pool ligation transcriptomics (microSPLiT) single-cell RNA sequencing to profile transcriptome-wide responses to gene perturbations at high resolution and scale. In this platform, a unique guide barcode construct (GBC) associates each CRISPR perturbation with its corresponding transcriptional response. We established effective 1-plasmid systems for CRISPRa/i in P. putida and CRISPRi in B. subtilis, each included guide expression programs for CRISPRa/i machinery with compatible GBCs. To benchmark our ability to uncover single-cell regulatory network responses in P. putida, we targeted one heterologous reporter gene (sfGFP) using CRISPRa and three known transcription factors (cra, pdhR, and gclR) associated with carbon source metabolism using CRISPRi. From 13,906 recovered cells with a median of 34 transcripts per cell, over 50% of cells were successfully assigned to specific perturbations. This allowed us to discover direct regulatory connections for all four perturbations, including sfGFP upregulation from CRISPRa, activation of the fruBKA operon upon cra repression, lldP upregulation with pdhR repression, and notably, upregulation of 13 out of 19 genes in the gclR regulon after gclR knockdown. These results demonstrate the scalability and versatility of CRISPR-microSPLiT in non-model systems, establishing a powerful framework for genome-wide interrogation of bacterial gene regulation.

Co-authors: Yujia Huang, Jacob Brandner, Stephen Fedak, Anna Kuchina, James Carothers

81. *"Family-wide functional annotation of transcriptional regulators"*

Haoxian Xu (University of Washington)

The rapid advancement of sequencing technologies has resulted in over 250 million unreviewed protein sequences, far outpacing our ability to functionally characterize genes—reflected by only ~572,000 experimentally validated entries in the SwissProt database. This gap represents a major bottleneck in microbial discovery. LysR-type transcriptional regulators (LTTRs), one of the largest and most widespread families of bacterial transcription factors, are a prime example. Although LTTRs play central roles in regulating diverse cellular processes, most of their sequences remain functionally uncharacterized.

To address this, we aim to uncover how LTTRs regulate gene expression in response to cellular signals. We begin by leveraging the concept of gene clusters, which enables more efficient functional prediction by analyzing groups of genes with shared regulatory contexts rather than investigating individual genes in isolation. LTTRs often regulate genes located adjacent to their own coding sequence, frequently forming divergent operons with their target genes. By identifying the ligand that binds to a specific LTTR, we can often infer the substrate or signal associated with the entire gene cluster, providing critical insight into its functional role within the cell.

We have developed a scalable fluorescence-based platform to detect ligand binding to LTTR proteins. Our workflow incorporates auto-induction media for protein expression and magnetic bead purification, enabling high-throughput and parallel processing of LTTR candidates. We use Differential Scanning Fluorimetry (DSF) to measure changes in protein thermal stability upon ligand binding, where ligand interaction typically stabilizes the protein structure and increases its melting temperature. We have validated this approach by demonstrating elevated melting temperatures for well-characterized LTTRs in the presence of their native ligands, confirming both specificity and measurable affinity in these interactions. These results establish DSF as a reliable and sensitive readout for LTTR-ligand binding.

This platform will enable large-scale functional screening of uncharacterized LTTRs against diverse ligand libraries, offering valuable insights into bacterial physiology and paving the way for novel applications in biotechnology and antimicrobial development.

Co-authors: Randon Serikawa

82. *"Science in Action: How a Local Community Lab Bridges Research and the Common Good"*

Dezmond Goff (SoundBio Lab)

Public-access science spaces are an emerging topic of interest for researchers and community stakeholders. Here we present SoundBio Lab, a non-profit community biology lab in Seattle, as an example and model for open science spaces that increase scientific literacy, boost workforce development, further research innovation, and leverage scientific resources to meet community needs.

Founded in 2016, SoundBio Lab maintains a Biosafety Level 1 (BSL-1) wet lab space for learners, hobbyists, and early-career researchers. We offer hands-on biology instruction and skills training, including new courses funded by Experiment.com. Our community-led projects have ranged from explorations of kombucha microbiology to local salmon DNA barcoding, and now include ongoing initiatives like yeast evolution experiments (with support from the yEvo project) and a DIY HPLC development effort. We support increasing youth exposure and access to STEM education, including partnerships with local schools and mentored research experiences such as our Life Science Explorers program or National Youth Laboratory internship. Participants from our programs routinely cite our programs as their first opportunity to engage in authentic research or lab work.

Looking ahead, SoundBio Lab is expanding programming through our upcoming biomanufacturing course collaboration with Shoreline Community College and launching open-science initiatives for biomaterials (IndiGROW) and biosensor development. By lowering barriers to entry and making science visible and accessible, SoundBio Lab demonstrates how grassroots spaces are instrumental in supporting a healthy, diverse, and accessible bioeconomy and biology research community.

83. *"Harnessing the Metabolic Flexibility of Purple Non-Sulfur Bacteria for Bioproduction Applications"*

Jackson Comes (University of Washington)

Purple non-sulfur bacteria (PNSB), such as Rhodopseudomonas palustris, are emerging as promising chassis for sustainable bioproduction due to their metabolic versatility, including photosynthetic carbon fixation and aromatic catabolism. However, this metabolic versatility is underpinned by highly branched and conditionally regulated networks, posing challenges for predictive metabolic engineering. To rationally rewire these networks, we require both improved genome-scale models to understand flux distribution and regulatory dynamics, and precise genetic tools to manipulate and interrogate these systems.

We are constructing a genome-scale metabolic model (GEM) of R. palustris to predict flux distributions and identify pathway bottlenecks under diverse growth modes. This model will be refined using multi-omic data and used to guide metabolic engineering strategies. Additionally, CRISPR interference and activation (CRISPRi/a) systems are being explored to enable tunable control of native gene expression without permanent genomic edits. For stable integration of heterologous pathways, we employ conjugation-compatible SAGE and CRAGE systems.

As a proof of concept, we are engineering R. palustris to produce 4-vinylphenol (4VP) and isoprenoid precursors via the para-coumaric acid decarboxylation and mevalonate pathways. To enhance carbon retention and flux through the glyoxylate cycle, we are introducing the malyl-CoA-glycerate (MCG) pathway. Together, these efforts aim to establish PNSB as genetically tractable, metabolically flexible platforms for light-powered, carbon-negative bioproduction.

Co-authors: Jackson Comes, Michael Guzman, Amanda Robert, Diego Alba, Ryan Cardiff, Brian Darst, Stella Anastasakis, Sarah Grube, James Carothers

84. *"Mapping bacterial sRNA regulatory networks with single-cell RNA sequencing and CRISPR interference"*

Stephen Fedak (University of Washington)

Bacteria respond to environmental cues through transcriptional regulation and noncoding small RNAs (sRNAs), which modulate translation of specific mRNAs through basepairing. Although hundreds of sRNAs have been identified or predicted across bacterial genomes, the binding partners and regulatory functions of most remain unknown. Elucidating these sRNA regulons can identify targets to control bacterial metabolism for therapeutic or bioproduction purposes.

High-throughput approaches like single-cell transcriptomics are powerful tools to study sRNA because they capture the heterogeneity in transcriptional states associated with multiple targets of a given sRNA. We used the bacterial single-cell RNA sequencing platform microSPLiT to explore sRNA expression patterns in two systems undergoing cell state transitions: biofilm formation in Pseudomonas aeruginosa and CRISPR interference of three transcription factors in Pseudomonas putida.

In P. aeruginosa, we observed at least one sRNA in 3,622 of 18,807 cells, covering 22 of 44 annotated sRNA. Genes coexpressed with biofilm regulatory sRNAs crcZ and phrS included known binding targets, showing microSPLiT's capability as an sRNA detection platform. We also identify potential new targets for the recently discovered sRNA sicX, further defining its role in adaptation to hypoxia. In P. putida, 7,900 of 13,906 cells expressed sRNA, with 54 of 64 known species present. Expression of many sRNA was context-specific. Upon CRISPRi of gclR, a negative regulator of alternative carbon source usage, we observed upregulation of the catabolite-repression-alleviating crcY/Z pair. Perturbing pdhR, the repressor of pyruvate dehydrogenase, led to expression of uncharacterized PP_mr02, suggesting it may be involved in osmotic stress response. These results highlight the utility of single-cell RNA sequencing to expand knowledge of sRNA regulation, and lay the foundation for future work defining non-coding regulons via dCas13 perturbation of sRNA.

Co-authors: Stephen Fedak, Yujia Huang, Jacob Brandner, Karl Gaisser, James Carothers, Anna Kuchina

85. *"Reducing False Positives with an AND-Gate Rolling Circle Amplification for Pathogen Detection"*

Jiho Seok (Georgia Institute of Technology)

Reducing false positives is crucial for biosensor reliability, especially in point-of-care detection of pathogens from complex samples. Isothermal amplification is widely adopted in point-of-care diagnostics because it operates without the need for thermal cycling or specialized equipment. However, the high frequency of false positives caused by non-specific amplification remains a critical challenge in isothermal nucleic acid diagnostics. To address this challenge, we developed a novel Multi-key Rolling Circle Amplification (RCA) technique, which detects multiple genes from a single pathogen in parallel. This cross-verification approach reduces false positives by ensuring that amplification only occurs when all target genes are present. We present a user-friendly diagnostic platform by integrating this technique with a Lateral Flow Assay (LFA). We showed that this approach is compatible with a wide range of nucleic acid targets, including RNA and DNA-RNA hybrids. Additionally, the system supports simultaneous detection of three or more gene targets, further improving specificity through multi-gene cross-verification. Furthermore, we demonstrated the feasibility of field application by showcasing a master mix that integrates each reaction step of the multi-key RCA and by demonstrating its long-term storage capability through lyophilization. This novel technique has the potential to enable development of POC biosensors with minimized false positives.

Co-authors: Jiho Seok, Mark Styczynski

86. *"Tunable Synthetic Promoters for CRISPRa-Mediated Multi-Gene Expression in Pseudomonas putida for Optimizing Chemical Bioproduction"*

Tommy Primo (University of Washington)

CRISPR-mediated gene activation (CRISPRa) tools can be combined with data-driven modeling into a platform for rapidly engineering multi-gene expression programs. When applied to bioproduction, these approaches optimize metabolic flux, bringing titer, rate and yield (TRY) closer to industrial levels. Additionally, they can overcome metabolic engineering challenges like accumulation of toxic intermediates and undesired side products. Pseudomonas putida (P. putida) is a gram-negative soil bacterium with a versatile metabolism capable of tolerating harsh bioprocesses, making it a suitable chassis for bioproduction of toxic aromatic compounds. Recent work has found CRISPRa to work in P. putida. To increase the sizes and complexities of multi-gene transcriptional programs implemented in P. putida, we engineered 4 tunable synthetic promoters. Starting with well-characterized E. coli promoters, using sequence-design and in-vivo screening, we adapted their architecture to have similar functions but different sequences for optimal gene expression. We found that the GC content upstream of the minimal promoter and promoter identity were important for obtaining low basal levels and high activated levels. Screening effective guide-RNA target sites, we adapted the predefined promoter architecture into additional, sequence-orthogonal and tunable tools that are functional in P. putida. We now have a set of 5 orthogonal promoters. They generate up to 31-fold differences in combinatorial genetic output and when combined, provide a toolbox for simultaneously varying the expression levels of single-gene or multi-gene transcriptional units. These tools provide a modular and robust control mechanism for precise tuning of multi-gene expression programs. New workflows combining multi-gene CRISPRa programs with machine learning models will allow us to profile biosynthetic pathways and improve production of novel compounds of industrial and medicinal relevance in a semi-automated fashion.

Co-authors: Brian H. Darst, Ian D. Faulkner, James M. Carothers

87. *"An in silico multi-scale model of combinatorial regonition circuit design in CAR T cell-based therapies"*

Jason Cain (University of Washington)

Designing cellular therapies to combat cancer is an inherently complex systems engineering problem. On-target off-tumor (OTOT) toxicity is a key obstacle in applying chimeric antigen receptor (CAR) T cell-based therapies to solid tumors. Combinatorial recognition circuits applied to CAR T cell-based cancer therapies could extend the current state-of-the-art treatments from hematologic and bone marrow contexts to the heterogeneous and dynamic solid tumor microenvironment. However, these recognition synthetic networks introduce both an increasing parameter space as well as additional temporal and spatial dynamics that must be considered for successful prediction, mitigation, and control of OTOT toxicity. To inform design targets, we are actively building and interrogating a multi-scale spatio-temporal computational model of the tumor microenvironment built using an agent-based framework (ARCADE). We have extended this framework to include logical signaling cascades in CAR T cells for two proposed logical operands: (1) inducible AND gates (e.g. synNotch-CAR therapies) and (2) inhibitory AND NOT gates (e.g. Inhibitory CAR therapies, iCAR). We show that our models are consistent with literature observations and extrapolate our findings to generate hypothetical parameter criteria (e.g. antigen heterogeneity thresholds, signaling kinetics, inflammation control) towards identifying therapeutic windows that balance toxicity with recognition, control, and proliferation resistance. We believe that the successful application of multi-scale models capable of linking subcellular processes to tissue behavior are the next generation of synthetic design tools that will accelerate the design of the next generation of synthetic cellular therapies.

Co-authors: Jason Y. Cain, Allison W. Li, Neda Bagheri

88. *"Characterizing Clostridium novyi-NT Spore Surface Composition: A Detection Toolkit"*

Caleb Hoffman (Southern Utah University)

Pancreatic cancer remains one of the deadliest cancers, with a five-year survival rate of only 13% in the United States. One of the main challenges in treating pancreatic tumors is their avascular nature, which prevents effective delivery of traditional therapies through passive diffusion, especially for tumors encased in a fibrous desmoplastic layer. Additionally, the harsh biological microenvironment (i.e. hypoxia, acidity) of these tumors diminishes therapeutic efficacy.

Clostridium novyi-NonToxic (NT), an attenuated oncolytic bacterium that does not cause sepsis or bacteremia, offers a promising approach for treating pancreatic tumors. These bacteria selectively target and destroy cancer cells in a hypoxic solid-state tumor while stimulating an immune response. Genetic modifications to C. novyi-NT spores can improve tumor targeting and penetration. However, advancing pre-clinical development requires the establishment of standardized methods to track biodistribution and tumor localization.

The chemical composition of the C. novyi-NT surface remains poorly understood, and traditional staining techniques may not be effective for evaluating biodistribution. This study aims to assess common fluorescent dyes for their ability to interact with the C. novyi-NT surface.

We have developed a method for incubating C. novyi-NT spores with fluorescent dyes such as Nile Red and carboxyfluorescein succinimidyl ester (CFSE) to examine surface interactions in a time course study. Preliminary results suggest esterase activity and the presence of lipid functional groups on the C. novyi-NT spore surface. Further experiments on will refine these findings, offering insights into the spore's biochemical properties and enhancing in vivo biodistribution analysis.

Co-authors: Caleb Hoffman, Jackson Sandberg, Jack Stevenson, Caleb J. Bussard, Jessica E. Pullan, Kaitlin M. Dailey

89. *"Accelerating Engineering Biology Innovation through Advanced Automation"*

Jonathan Klonowski (EBRC)

The bioindustry sector is experiencing unprecedented growth and innovation, driven by advances in genomics, engineering biology, and bioprocessing. However, traditional manual processes often limit scalability, reproducibility, and throughput. Advanced automation technologies, including robotics, microfluidics, and machine learning, offer transformative solutions to overcome these bottlenecks. Utilizing interviews with stakeholders at the CTO and director levels, we explore the deployment of these technologies, highlighting their potential to advance biotechnologies through the research, development, and manufacturing phases and into commercial products. Robotics enhances reproducibility, reduces human error, and enables high-throughput experimentation, while data analytics and machine learning algorithms optimizes data use, standardizes processes, and advises next steps. The combination of these systems allow them to feed off one another, increasing the rate of advancement through the design, build, test, learn (DBTL) cycle. This offers ventures advantages when refining biological systems and increases their bandwidth to explore new avenues of inquiries or market segments. We also present challenges associated with automation adoption, such as initial investment costs, integration complexities, and the need for coordinating skilled teams spanning disciplines. Finally, we identify factors that are needed to improve the ecosystem, accelerate adoption, and unleash the potential for biotechnology to address global sustainability challenges in healthcare and industry.

90. *"Protein degradation as a tool in cell-free systems"*

Felicia Oentoro (Georgia Institute of Technology)

In vivo protein degradation via proteases has been studied extensively and used in multiple applications, from controlling protein expression levels to implementing biosensors. An increasingly popular alternative chassis for applications like these are cell-free systems (CFS), powerful in vitro tools made from cell lysates that can execute transcription and translation. However, the characterization of proteases in cell-free systems is more limited, likely because cell-free applications often aim to maximize protein expression levels rather than reduce them. Nevertheless, there are many potential beneficial applications of proteases in CFS. As uses of cell-free systems become more complex, proteases could provide a powerful tool to control gene expression. In this work, we explore the impacts of supplementing endogenous and heterologous proteases into a protease-deficient E. coli cell-free extract. The use of orthogonal proteases is explored to limit impacts on the cell-free proteome and to enable complete control over protease-induced degradation. We aim to express and confirm functionality of proteases native to E. coli as well as proteases from other organisms. Using GFP as our reporter, we have successfully degraded protein with endogenous ClpXP protease and E. coli lon protease. The extract for our cell-free systems is deficient in lon protease, so it is externally added via enriched extract. We are currently working on demonstrating functionality of lon proteases from other organisms, such as M. florum, and are also working on demonstrating the versatility of this system by implementing them in a variety of application contexts.

Co-authors: Mark P. Styczynski

91. *"Conjugation-Compatible Serine-integrase Assisted Genome Engineering (cSAGE) in Non-Model Bacteria"*

Michael Guzman (University of Washington)

Efficient genomic integration is essential for engineering microbial systems, particularly in non-model bacteria and environmental isolates, where low transformation efficiencies often pose significant barriers. While tools like Serine Recombinase-Assisted Genome Engineering (SAGE) enable site-specific integration, they rely on electroporation—restricting their use in strains that are not easily transformable. To overcome this, we developed Conjugation-compatible SAGE (cSAGE), a highly portable and modular platform for stable, multi-payload genomic integration. By leveraging bacterial conjugation—a broadly conserved DNA transfer mechanism—cSAGE expands site-specific genome engineering to a wider range of bacterial hosts, including those recalcitrant to electroporation. We standardized cSAGE within the SEVA (Standard European Vector Architecture) framework and transitioned all components to low-copy, narrow-host-range vectors for modularity and reproducibility. To evaluate and optimize cSAGE across diverse hosts, we constructed a library of ~50 plasmids and systematically tested multiple integrases, antibiotic resistance markers, and reporter genes across five genera of purple nonsulfur bacteria (PNSB). Our screening across five PNSB species confirmed robust, site-specific integration with high efficiencies (up to 10^7 CFU). We further demonstrated stable multi-cargo integration using two serine recombinases in two species and are expanding to three recombinases per strain to increase integration capacity. Markerless genome engineering was achieved in several species using sucrose counterselection, with 100% curing efficiency. As a proof of concept, we integrated biosynthetic pathways for heterologous production of isoprenoids and phenylpropanoids in Rhodopseudomonas palustris CGA009 and Rhodobacter sphaeroides 2.4.1. These results highlight the potential of cSAGE to accelerate metabolic engineering and strain development in previously intractable bacterial systems.

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92. *"Directed Evolution of a Non-Heme Iron Enzyme Enabled by Computational Stabilization for Amine Hydroxylation"*

Jessica Caruso (University of Washington)

Non-heme iron-α-ketoglutarate dependent (Fe(II)/αKG) oxygenases are a powerful class of enzymes for use in industrial biocatalysis. Fe(II)/αKGs catalyze a regioselective C(sp3)–H hydroxylation at unactivated positions which is synthetically challenging. Generating industrially relevant products requires engineering enzymes for increased activity with non-native substrates. However, mutagenesis towards higher activity can have a negative tradeoff with stability, which can limit the utility of evolved enzymes. Our lab has previously shown we can overcome this stability tradeoff by using the deep learning-based tool ProteinMPNN to generate a stable starting variant for directed evolution. Starting with a ProteinMPNN-stabilized variant allowed for efficient evolution of a non-native Fe(II)/αKG reaction. A critical step in this process was identifying the criteria necessary to retain catalytic activity in stabilized redesigns. We have now applied this method to generate a stabilized variant of a new Fe(II)/αKG, PolL, while maintaining C-H hydroxylation activity for non-native free amine substrates. These reactions generate small molecule building blocks that can be used in the synthesis of more complex pharmaceuticals and other products. Initial attempts to perform directed evolution with wild-type PolL generated enzymes that were too unstable to characterize. We expect that the computationally-stabilized PolL variant will enable efficient directed evolution for non-native free amine hydroxylation reactions. This general approach may be useful for a wide variety of biosynthetically-useful enzymatic reactions.

Co-authors: Brianne King, Daniel Ong, Jolene Nguyen, Jesse Zalatan

93. *"The Rise and Fall of Brewing Yeasts: Harnessing the Awesome Power of Experimental Evolution to Select for Increased Flocculation in Ale and Lager Yeasts"*

Barbara Dunn (University of Washington)

Flocculation is a process whereby microbial cells strongly clump together after having initially grown as single cells; the process is facilitated by changes to the cell walls in response to nutritional changes in the medium during growth. Flocculation is a major aid in the brewing industry because it allows the finished beer to easily clear, with less filtering or longer settling times needed; ideally it should happen only near the end of fermentation, so that dispersed single cells can efficiently carry out the majority of the fermentation. Many brewing yeast strains have low levels of flocculation, so we have begun a series of experimental evolution studies to select for increased flocculation without losing the sensory and fermentation behavior characteristics of the original strains. For our initial experiments we used a West-coast style ale strain and a German-style lager strain. We performed long-term evolution experiments in triplicate for each strain, using small scale (3-5 ml) cultures with sterile 10% malt extract as the growth medium. After either 1 (ale) or 2 (lager) days of growth, the cultures were centrifuged at 100 x g very briefly, and a small volume from the bottom of the tube was transferred to fresh medium; this was repeated until we observed significant increases in clumping behavior, occurring after 50+ transfers. We obtained novel clones, from both the ale and lager strains, that demonstrate strongly increased flocculation. We sequenced their genomes, as well as those of the ancestor strains, to detect nucleotide and copy number changes. Because very flocculent brewing strains have been shown to cause increased levels of diacetyl, an undesired flavor molecule, we have also been exploring ways to minimally genetically modify these and other strains to reduce diacetyl levels, using CRISPR. We hope to use such combinations of experimental evolution and genetic modification to develop novel brewing strains with a variety of desired properties.

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