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Table of contents

Prokaryotic Synthetic Biology

A synthetic communication system uncovers self-jamming of M13 transmission

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Intercellular communication enables coordinated action by cells of microbial communities and multicellular organisms, often mediated by molecular exchange of information. Inspired by their success, synthetic biologists have recently started implementing population-level controls in engineered organisms with the aim of expanding circuit size and complexity. Yet, realising the true potential of multicellular synthetic biology requires an expanded communication alphabet as well as quantitative models to predict complex behaviour. Towards that aim, here we repurpose the M13 bacteriophage machinery for cell-to-cell communication between Escherichia coli cells and characterise the signalling dynamics. The fitted quantitative model includes the growth burden of the communication machinery, the relationship between cellular growth phase and the secretion-infection kinetics, and concurrent antibiotic selection. Limitations of deterministic models are demonstrated, with stochastic effects playing a key role in reproducing the observed infection kinetics. Surprisingly, we discover that the M13 minor coat protein pIII is released into the medium to confer extracellular immunity to uninfected cells. In a simulated gut environment, this mechanism enables the phage to farm uninfected bacterial cells for the future, increasing the overall success of both M13 and E. coli. In addition to establishing a tool for intercellular communication, our work uncovers the mutualistic nature of a phage-bacterial relationship that has evolved over long-term coexistence.

Keywords: Phage secretion, M13, Simulations, coexistence

*Speaker

A synthetic trans-envelope signaling system for multiplexed protein detection in E. coli

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Circulating Tumour Cells (CTCs) split away from solid primary tumours and end up in the blood until eventually forming metastases. CTCs are an interesting target for diagnostics because they share phenotypical traits of solid tumours and have the advantage to be harvested non-invasively, by liquid biopsy. However, their low abundance in blood makes robust and accurate phenotypic characterisation challenging. Here, we propose to engineer *E. coli* as a biosensing platform for the detection of CTC markers at single-cell level. Single CTCs will be captured and isolated using a proprietary microchip, in which single cells will be exposed to our biosensing *E. coli* strain. Depending on the markers present at the surface of CTCs, our sensor will produce different small molecules distinguishable by Surface-Enhanced Raman Spectroscopy (SERS). The resulting SERS spectra will be analysed using a machine-learning algorithm to determine the phenotype of single CTCs.

Keywords: Biosensors, E. coli, CTCs

*Speaker

Acoustic bacteria as live reporter for therapeutic bacteria in tumors

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Engineered therapeutic bacteria offer great hope in the treatment of several pathologies, in particular cancer. In that context, therapeutic bacteria localization, growth and activation need to be tightly monitored from the outside to ensure that therapeutic action has maximal efficacy and safety. Current imaging strategies include luminescence which lacks resolution, PET-scan which require heavy-equipement, and fluorescence imaging which is fundamentally limited by light absorption through biological tissues. Recent developments have demonstrated how to use acoustic waves to image engineered bacteria that produce gas vesicles repurposed from cyanobacteria and, thus, act as acoustic reporters. Our project is to assess whether gas vesicles in therapeutic bacteria can be conditionally produced directly in tumors as a response to its microenvironment at sufficient level to be detected with acoustic imaging. For that, we will evaluate the impact of gas vesicles production on bacteria physiology in microfluidic devices such as the Mother Machine. In various cancer cell spheroids, we will evaluate the bacteria tumor colonization and the activation of gas vesicle producing synthetic circuits. Finally, will measure the acoustic signal generated using ultrafast ultrasound imaging for an optimal spatial resolution.
This project is conducted in collaboration with Jérôme Bonnet's team from CBS Montpellier who will design and optimize synthetic gene circuits, and Mickael Tanter's team from ESPCI who will provide support for acoustic imaging.

Keywords: Therpautic bacteria, ultrasound imaging, cancer

*Speaker

Addressable Phase-separated RNAs in E. coli

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Biochemical processes often require spatial regulation and specific microenvironments. The general lack of organelles in bacteria limits the potential of bioengineering complex intracellular reactions. Here we demonstrate synthetic membraneless organelles in Escherichia coli termed Transcriptionally Engineered Addressable RNA Solvent droplets (TEARS). TEARS are assembled from RNA-binding protein recruiting domains fused to poly-CAG repeats that spontaneously drive liquid-liquid phase separation from the bulk cytoplasm. Targeting TEARS with fluorescent proteins revealed multilayered structures with composition and reaction robustness governed by non-equilibrium dynamics. We show that TEARS provide organelle-like bioprocess isolation for sequestering biochemical pathways, controlling metabolic branch points, buffering mRNA translation rates and scaffolding protein-protein interactions. We anticipate TEARS to be a simple and versatile tool for spatially controlling E. coli biochemistry. Particularly, the modular design of TEARS enables applications without expression fine-tuning, simplifying the design-build-test cycle of bioengineering.

Keywords: liquid, liquid phase separation, synthetic organelles, membraneless organelles, metabolic engineering, RNA condensates

*Speaker

Combination of molecular approaches to access homogeneous glycoconjugates for vaccine purposes.

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Glycoconjugate vaccines are effective, safe and well tolerated, especially in young children. They consist in the conjugation of purified or synthesized bacterial surface polysaccharides (PS) to a protein, called carrier protein. The latter allows the induction of antibodies directed against the PS through the recruitment of CD4+ T lymphocytes. PS length, PS/carrier protein ratio and bioconjugation chemistry are key parameters that have a direct impact on the immune response. Previous work in the laboratory initially aimed at random conjugation of a bacterial PS to a carrier protein indicated that the optimal number of conjugations to induce an anti-PS response in our model was 4. As this random glycoconjugate induces a strong recognition of PS epitopes but a weak recognition of protein epitopes, the team investigated site-selective conjugation. Conversely, the targeted glycoconjugate showed a strong anti-protein response but a weak anti-PS response. These previous results gave new perspectives on the development of these glycoconjugates by highlighting the need to improve the ratio of anti-PS to anti-protein response. Given the importance of the coupling site, site-directed mutagenesis and non-canonical amino acid incorporation (ncAA) appear to be an attractive strategy to access homogeneous conjugates and study the relationship between immunogenicity and structure. We aim to test this strategy using pneumococcal surface adhesin A (PsaA) as carrier protein and a synthetic dodecasaccharide mimicking the capsular PS of Pn serotype 14 as a model. The PsaA is a highly conserved immunogenic protein expressed by Streptococcus pneumoniae (Pn) and responsible for colonization of the upper host tract. To date, ncAA with biorthogonal function (azide) has been incorporated into the PsaA allowing a coupling by strain-promoted azide-alkyne cycloaddition, a copper free process ensuring the selectivity of the click reaction. Taking advantage of the absence of cysteine, four lysines were mutated to cysteines for subsequent bioconjugation to PS (thiol-maleimide reaction). These mutations were made within the T epitopes to induce T helper cell proliferation. The final carrier protein, i.e. the quadruple cysteine mutant with ncAA is in production. The later will then be conjugated to PS by click chemistry and its efficiency will be evaluated via biological tests. The response will be compared to that observed for Prevnar13® (commercial vaccine) used as positive control.

Keywords: glycoconjugate, site, directed mutations, non, canonical amino acid incorporation, vaccine, recombinant protein, bioconjugation

*Speaker

Controlling probiotic lactobacillus behavior by confinement in mechanically tunable hydrogels

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Lactobacilli form one of the largest family of probiotics and are found as commensals at many sites in human body such as in the oral cavity, gastrointestinal tract, upper respiratory tract, skin and reproductive organs. At these sites they have also been found to provide health benefits such as anti-inflammatory and anti-pathogenic effects. There is a growing interest to engineer these lactic acid bacteria as live biotherapeutics that produce and deliver drugs at these sites in the body. To improve their biosafety and survival within the body, they are being encapsulated within porous materials like hydrogels. This gives rise to Engineered Living Materials (ELMs) in the form of bacterial hydrogels capable of drug delivery in the body. Recent studies with E. coli have highlighted that spatial confinement and mechanical properties of the external matrix can directly impact the metabolism, growth, and inducible gene expression rates of the encapsulated bacteria. However, such effects have not been studied with lactobacilli. In this work, we encapsulate L. plantarum in mechanically tunable hydrogels and study their growth, metabolism and inducible gene expression. The hydrogel environment is conducive for the survival, growth and functionality of these bacteria. It is possible to chemically induce the protein production and the secreted proteins can diffuse through the matrix to be released into the surrounding medium. Notably, we show that the viscoelastic properties of the gels have a significant effect on the growth and metabolism of lactobacilli and can be used to tune their functionality. These results and the fundamental insights derived from them will greatly help to understand the effect of mechanical forces on the behavior of lactobacilli, apart from providing a guideline to improving their performance when developed as ELMs.

Keywords: Probiotic lactobacilli, Engineered living materials, Drug delivery

*Speaker

Designing synthetic receptors for multi-input detection and orthogonal signalling

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Whole cell biosensors are synthetic biological devices that take advantage of the natural capacity of bacteria to self replicate, process multiple signals and respond to environmental cues. In E. coli, artificial receptors with programmable specificity are being developed to detect multiple natural and synthetic molecules with internal orthogonal signalling responses. These engineered bacterial strains will serve in cost effective and rapid diagnosis, ligand-receptor screening and ultimately therapeutic applications.
The synthetic receptor design is based on receptor activation via Ligand-induced Dimerization (Chang et al, *ACS Synth. Biol.* 2018) using E.coli's CadC DNA-Binding domain (DBDs), domain connectors (cytosolic and transmembrane forms), and modular dimerizing ligand binding domains (LBDs).

Synthetic receptors are cloned in E. coli, activated with ligand and the system response is characterised by flow cytometry measuring fluorescence, or chemical outputs. Such approach was used to construct Bile salts biosensors that were shown able to detect endogenous levels of these components within clinical samples. New DBD and LBD candidates are now being investigated by screening size variations and connector sequences libraries using CadC for DBD and a caffeine responding nanobody (VHH) for LBD as scaffolds for prototyping. Preliminary

*Speaker

results reveal an important improvement potential. Finally, a DBD-VHH candidate showed promising dose-dependent responses in the cytosol while the transmembrane form is still under investigation.
Expanding the available modules for transmembrane dimerizing synthetic receptors will lead to quick and straightforward method for receptor development to screen ligand-receptor pairs and develop novel biosensors.

Keywords: receptors, biosensor, bacteria, engineering, diagnosis

Exotic and xenobiotic deviations from the set of canonical nucleotides

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The main objective of our team is to design, construct and evolve genetically modified microorganisms that pose no threat to health and the environment ("safe GMOs"). These organisms will use an artificial genetic system based on xenobiotic nucleic acids (XNA) to transmit genetic information. We are also studying the diversification of nucleic acids in the natural living world, focusing on the genomes of bacteriophages which have a wide chemical diversity of non-canonical nucleic bases. Some viruses infecting hosts such as proteobacteria, cyanobacteria and actinobacteria have a DNA genome in which adenine is completely replaced by aminoadenine (Z). The elucidation of the biosynthetic pathway of this non-canonical nucleotide and the discovery of bacteriophage polymerases dedicated to the incorporation of this nucleotide into DNA pave the way for the propagation of chemically modified genomes in bacteria (Pezo et al, Science 2021).

Keywords: xenobiology bacteriophages, aminoadenine

*Speaker

Genomics driven discovery of anti-phage systems.

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All cells face the threat of viral infections and develop anti-viral systems to fight them off. Due to the negative selective pressure exerted on bacteria, anti-phage defense evolved, followed by phages strategies to counteract such systems. This so-called "arms race" is considered as old as bacteria themselves and its study led to fundamental discoveries in biology as well as the development of impactful biotechnologies such as Restriction-modification (R-M). The discovery of CRISPR-Cas in the 2000s marked a drastic shift and prompted a renewed interest in finding novel anti-phage systems. How many more remain unknown? In this seminar, I will discuss how methods inspired by synthetic and systems biology led to the discovery of more than a 100 novel anti-phage mechanisms and how these are transforming our understanding of , phage-bacteria interactions and immunity across domains of life.

Keywords: Phages, anti, phage systems, heterologous expression, comparative genomics

*Speaker

Implementation of a synthetic formate assimilation pathway in Escherichia coli by adaptive evolution

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We report the successful implementation of formatotrophy in *Escherichia coli* by means of a stepwise adaptive evolution strategy through the reductive glycine pathway, described as the most energetically favorable synthetic route of aerobic formate assimilation. Medium swap and turbidostat regimes of continuous culture were applied to force the channeling of carbon flux through the synthetic pathway to pyruvate establishing growth on formate and CO2 as sole carbon sources. Genetic analysis of intermediate isolates revealed a mutational path followed throughout the adaptation process. Mutations were detected affecting the copy number (gene *ftfL*) or the coding sequence (genes *folD* and *lpd*) of genes, which specify enzymes implicated in the three steps forming glycine from formate and CO2, the central metabolite of the synthetic pathway. The mutation R191S present in methylene-tetrahydrofolate dehydrogenase/cyclohydrolase (FolD) abolishes the inhibition of cyclohydrolase activity by the substrate formyl-tetrahydrofolate. The mutation R273H in lipoamide dehydrogenase (Lpd) alters substrate affinities as well as kinetics at physiological substrate concentrations likely favoring a reactional shift towards lipoamide reduction. In addition, genetic reconstructions proved the necessity of all three mutations for formate assimilation by the adapted cells. The largely unpredictable nature of these changes demonstrates the usefulness of the evolutionary approach enabling the selection of adaptive mutations crucial for pathway engineering of biotechnological model organisms.

Keywords: one carbon metabolism, evolution, continuous culture, mutation analysis, Escherichia coli

*Speaker

Optimization of lignocellulosic fermentation by Clostridium phytofermentans for the production of new fermentation products

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Lignocellulose, an abundant feedstock for the renewable production of value-added biochemicals, is composed of a matrix of polysaccharides and aromatic polymers. *Clostridium phytofermentans* is a gram-positive, anaerobic, mesophilic bacterium that efficiently ferments the diverse polysaccharides that compose lignocellulose. Our laboratory has developed a suite of genome engineering methods for *C. phytofermentans* and explored its molecular biology by high-throughput profiling of its transcriptome and proteome.
Our aim is to do metabolic engineering of *C.phytofermentans* to produce novel fermentation products, including n-butanol. As butanol is toxic, we have evolved strains of *C. phytofermentans* with enhanced butanol tolerance by long-term continuous culture. In a second step, we are developing a MoClo-based combinatorial hierarchical assembly of the butanol pathway to obtain the best possible butanol yield. Finally, a CRISPR-dCpf1 (dCas12a) system has been implemented to repress fermentation genes and thus redirect the flow towards the production of n-butanol or any molecule of interest. In the future, all three approaches should be combined to create a butanol-resistant strain that optimally produces butanol.

Keywords: Clostridium, Metabolic engineering, CRISPR interference, Cas12a, Butanol, Directed evolution

*Speaker

Programming lactic acid bacteria for cancer therapy : a part toolbox

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In the recent years, bacteria have been genetically engineered to detect and treat several pathologies in vivo, including infections, metabolic disorders and inflammatory bowel diseases. Recently, numerous studies have been conducted to modify bacteria to treat cancer. The strategy of anti-cancer bacteria consists in genetically modifying bacteria in order to make them recognize, colonize, and proliferate in the tumor microenvironment and finally produce in situ therapeutic molecules in a controlled manner. A potential advantage of using bacteria as cargo is to counter the side effects of chemotherapy and immunotherapy treatments, which are still generally delivered systemically.

Our project ONCOLAC is to engineer *Lactobacillus gasseri ATCC 33323* as a new chassis for therapeutic delivery in cold tumors. As precision engineering of LAB (lactic acid bacteria) is currently limited by the lack of tools enabling reliable control of gene expression, a part of our project aims at building a collection of well-characterized genetic parts to control transcription, translation and secretion levels. To do so we developed a promoter and RBS library as well as a MoClo kit for vector construction for lactic acid bacteria. In parallel, we are implementing new reporter genes for in vivo tracking of LAB and we are optimizing the production of cytotoxic and immunomodulatory proteins in *Lactobacillus gasseri*. Ultimately, bacterial therapeutic activity will be controlled by sensors responding to signals from the tumor microenvironment. In order to test, improve and validate our recombinant strains, we are combining in vitro spheroid-based screening with animal models for colonization assays.

*Speaker

Keywords: Lactic acid bacteria, cancer, tumor, reporter gene, plasmids

Shape control of minimal cells by reconstitution of Spiroplasma citri cytoskeleton

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Bottom-up synthetic biology aims at building a basic living unit made exclusively of non-living components. In this context, our goal is to build an artificial cell capable of self-movements. Spiroplasmas are helical and motile Mollicutes, bacteria which stand for a near-minimal genome. Their envelop is made of a single membrane with no peptidoglycan cell-wall, a feature shared with lipidic vesicles. We therefore chose to reproduce the *Spiroplasma* motility in liposomes. *Spiroplasma citri* has no external appendages such as pili or flagella, structural elements responsible for motility in other bacteria. Its helicity and motility solely relies on the presence of an internal cytoskeleton that extends from one pole to the other following the shortest path of the helical cell body. Our main approach is based on the heterologous production of *S. citri* cytoskeleton proteins in *E.coli*, their purification followed by their encapsulation/assembly in liposomes produced by microfluidics. To get information on the set of cytoskeleton proteins required, a second and complementary approach lies in the heterologous expression of these proteins in *Mycoplasma capricolum* subsp. *capricolum*, a pleiomorphic, non-motile mollicute lacking the *S. citri* specific cytoskeleton proteins. The overall objectives are to identify the minimal set of proteins allowing morphology and motility in artificial biomimetic systems and minimal cells, and to acquire structural and functional information on the mechanisms underlying bacterial helical morphology and motility. In addition, obtaining complex shapes from spherical synthetic compartments is expected to be a first step towards the design of artificial cells capable of morphological reconfiguration and motility.

Keywords: minimal and synthetic cells / cytoskeleton / microfluidics / synthetic biology / cell shape

*Speaker

SynBio at a global scale: from the Petri dish to planet Earth

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Synthetic Biology has evolved in the last few years in the context of a major climate crisis characterized by unacceptably high atmospheric levels of green house gases, the worrying pollution of the oceans with very recalcitrant plastics and microplastics and the noxious effects of micropollutants on many ecosystems. Global problems ask for global solutions and the environmental microbiome-because of its dimension and its amazing activities-may end up being out best instrument to both counter the impact of industrial development and enable a new, sustainable interplay with the natural world. While the whole planet is afflicted at a global scale by chemical pollution and anthropogenic emissions, the ongoing development of systems and synthetic biology, modern chemistry and some key concepts from ecological theory allow us to tackle this phenomenal challenge and entertain large-scale interventions aimed at reversing and even improving this state of affairs. This involves (i) identification of key reactions or processes that need to be re-established (or altogether created) for ecosystem reinstallation, (ii) implementation of such reactions in natural or designer hosts able to self-replicate and deliver the corresponding activities when/where needed in a fashion guided by sound ecological modelling, (iii) dispersal of niche-creating agents at a global scale-what has been called *Environmental Galenics*-and (iv) containment, monitoring and risk assessment of the whole process. The pillar of this new scenario includes a deep engineering of microorganisms as live chassis for delivering beneficial activities and multi-scale environmental interventions for pollution prevention/remediation (including climatic change). Current advances in the use of environmental bacteria (eg *Pseudomonas putida*) as SynBio chassis of choice for meeting some of these objectives will be addressed.

Keywords: Climate crisis, chassis, greenhouse gases, containment, barcoding, Pseudomonas putida, scaling up

*Speaker

Synthetic toxin-intein combinations as genetic weapons for specific killing of pathogenic bacteria in complex populations.

Didier Mazel * [1]

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Antibiotic resistance is becoming a major concern worldwide. The development of new antimicrobials is urgently needed. We constructed a synthetic system, which can perform killing of specific pathogenic bacteria within mixed populations.
Our system is based on the coupling of artificially split toxins (non-functional) with split intein technology. Inteins are protein sequences embedded inside a *host protein* (called extein) from where they can be self excised in a process called protein splicing. Naturally, inteins exist also as split modules. Each half of toxin-intein fusion was cloned in two different plasmids that, separately, do not kill the cell. Together, intein halves recognize each other in the cytoplasm and, after protein splicing, the toxin is reconstituted, killing the bacterium.

We cloned our toxin-intein combinations under promoter sequences controlled by specific transcriptional regulators from the genome of *Vibrio cholerae* and *Shigella flexneri* and involved in their pathogenicity. We then engineered a plasmid -the genetic weapon- to spread through conjugation into mixed population of bacteria, identifying and killing specifically either *V. cholerae or S. flexneri.* We tested the *in vivo* potential of this approach in zebrafish larvae infected by *V. cholerae* and showed that conjugation of our genetic system and the specific killing of the targeted bacteria take place in this context (Nat Biotechnol. 2019 Jul;37(7):755-760).

Our system precisely recognizes and kills bacteria in a natural complex community that could help in the resolution of main problems of classical antibiotic treatments: indiscriminate killing of beneficial bacteria and the emergence of resistance.

Keywords: toxin, antibiotic resistance, intein

*Speaker

Eukaryotic Synthetic Biology

Anti-CRISPR protein technologies for precision genome editing

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Anti-CRISPR (Acr) proteins, natural inhibitors employed by phages to suppress the CRISPR immunity in their microbial hosts, have gained increasing attention over the past years. On the one hand, this is due to the key role of Acrs in the evolutionary battle between phages and microbes. On the other hand, Acrs can also be harnessed to control and fine-tune the activity of CRISPR-Cas effectors in different cell types and even animals, and hence facilitate various genome editing applications. In this talk, I will first summarize several Acr-based technologies pioneered by our group, including (i) opto- and chemogenetic Acr variants enabling spatially- and temporally-confined CRISPR genome perturbations and (ii) microRNA-dependent Acr transgenes for cell-type and tissue restricted genome editing. Last, I will highlight our recent work on dissecting the mutational fitness landscape of Acrs via deep mutational scanning, which provides insights into Acr evolution and will aid future Acr optimization.

Keywords: protein engineering, optogenetics, genome editing, CRISPR, Cas, viral vectors

*Speaker

Improving CAR-T cell safety and efficacy through computational protein design

Leo Scheller * [1], Greta Giordano-Attianese [2], Sailan Shui [1], Rocio Castellanos [3], Raphaël Di Roberto [3], Anthony Marchand [1], Sai Reddy [3], Melita Irving [2], George Coukos [2], Bruno Correia [1]

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Chimeric antigen receptors (CARs) enable immune cells to recognize and kill cancer cells. However, CAR-T cell therapy is high risk and, to date, has been effective against few types of cancer only. I will describe our current progress in using computational protein design to generate CARs that can be turned 'on' or 'off' in response to approved small-molecule drugs. In this way, we aim to widen the therapeutic window of CAR-T cell therapy, as the drugs allow precise dosing of CAR-T cell activity. This could reduce side effects as well as bring currently too risky therapies closer to clinical use.

We developed several strategies to generate on- or off-switch CARs responding to the small molecules Venetoclax, A1155463, and caffeine. We tested and optimized these systems in dimerization dependent receptors in HEK-293T cells and in a CAR display platform in a murine T-cell line, allowing for higher throughput. By mutating interface residues, we made variants of the switches that respond to a wide range of drug concentrations.
Structure-based design was crucial for optimizing protein performance in these systems, highlighting it's potential for improving diverse applications in synthetic biology. I will present the current state of our results and discuss how these and additional design considerations of the CAR architecture can improve the efficacy of switch domains in CAR-T cells.

Keywords: Mammalian cells, CAR, T cells, Computational protein design, Receptor engineering

*Speaker

Optogenetic control of beta-carotene bioproduction in yeast: strain development across lab-scales.

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Optogenetics arises as a valuable tool to precisely control genetic circuits in microbial cell factories. Light control holds the promise of optimizing bioproduction methods and maximize yields, but its implementation at different steps of the strain development process and at different culture scales remains challenging. In this study, we aim to control beta-carotene bioproduction using the EL222 optogenetic system in the yeast Saccharomyces cerevisiae and investigate how its performance translates across culture scales. For this, we adapted and built four lab-scale illuminating devices each handling different culture volumes, and each having specific illumination characteristics and cultivating conditions. We first evaluated how optogenetic activation and beta-carotene production behave across devices and optimized them both independently. Then, optogenetics and beta-carotene production were combined to make a light-inducible beta-carotene producer strain. This was achieved by placing the transcription of the bifunctional lycopene cyclase / phytoene synthase CrtYB under the control of the pC120 optogenetic promoter regulated by the EL222-VP16 light-activated transcription factor, while other carotenogenic enzymes (CrtI, CrtE, tHMG) were expressed constitutively. We show that illumination, culture volume and shaking impact differently optogenetic activation (mostly due to illumination efficiency) and beta-carotene production (via changes in metabolism and beta-carotene photosensitivity) individually and across devices. Nevertheless, those identified constraints were found to converge in both genetic systems. This enabled us to determine the best culture conditions to maximize light-induced beta-carotene production in each device, reaching a content of up to 880 μg/gCDW. Overall, we used beta-carotene production controlled by the EL222 optogenetic system as a case study to exemplify the stakes of scaling up optogenetic in devices of different lab scales and identify the interplays and potential conflicts between optogenetic control and metabolic pathway efficiency. Proceeding by first optimizing both components of the system independently is necessary to avoid extensive troubleshooting and will ultimately result in timesaving. Our results can then help understand and predict future larger scale issues in an effort to bring optogenetics to the industrial scale.

Keywords: Optogenetics, bioproduction, Saccharomyces cerevisiae, beta, carotene, synthetic biol-

*Speaker

ogy, metabolic engineering

Photocaged Transcriptional Activators for Optogenetic Gene Expression Control

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Tight control of gene expression is critical to proper cellular function and the capacity of a cell to respond to environmental cues. In addition, misregulation and disruptions of gene expression are implicated in a number of disease states. The ability to manipulate gene expression with fine spatiotemporal control is therefore integral to understanding these dynamic processes on the timescales by which they occur.
Light activated, genetically encoded switches are well suited to address these issues given the high degree of speed, precision, and tunability that they provide to regulate effector proteins upon light stimulation. Common strategies to enable the light inducible transcriptional activation of targeted genes rely on protein n-hybrid approaches in which light induces a conformational change in a photoswitching protein to enable the inhibition or recruitment of an effector molecule such as a transcriptional activation domain or DNA binding protein to a gene of interest. While the dynamic range of such systems to induce gene expression is undeniable, trade-offs such as off-target effects, background transcriptional activation, and stoichiometric differences between protein components can compromise the extensive use of current systems.
To address these issues, we developed a highly compact, single component transcriptional activation system which utilizes a truncated variant of the blue light sensing LOV2 domain from phototropin-1 of Avena sativa (AsLOV2) to photocage the transcriptional activation domain VP16 from the Herpes Simplex Virus. Biochemical and structural similarity with the J-alpha helix region of the AsLOV2 domain allowed for its partial replacement with the transcriptional activation peptide from VP16. Experimentation with Photocaged VP16 (PcVP16) in HEK293T cells demonstrates successful upregulation of a luciferase reporter gene in the absence of 470nm wavelength blue light, an effect nearly completely abolished when illuminated with blue light. In ongoing work, we are validating our system for highly controllable and rapid induction of targeted genes when fused to DNA binding domain proteins.

Keywords: Optogenetics, Transcription, Light Activated, Photocaged, AsLOV2, VP16, GAL4, CRISPR

*Speaker

Quantitative Characterisation and Modelling of Genetic AND-Gates: From Robust Molecular Models to Predictive Design of Synthetic Gene Circuits

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In the past few decades, synthetic gene circuits have grown from simple transcriptional cascades systems to very sophisticated architectures involving complex gene networks, often built using multiple boolean logic gates. This development has opened the way to new potential therapeutic strategies, with notable examples in gene therapy and cancer treatment.
Synthetic gene circuits are commonly engineered using well-described building blocks, promising a highly tunable and reliable behaviour for high-specificity cell targeting. However, implementing circuits in mammalian cells remains challenging and precise response functions are still hard to predict. An important effort is now given to the rationalisation of network design, coupling model-based prediction and systematic measurement of circuits responses, in order to optimise existing circuits or create *de novo* functional devices.

To this aim, we are developing an experimental platform combining multi-colour fluorescence imaging, pixel-correlation analysis and thermodynamic modelling, in order to build a predictive model of AND-gate like circuits based on the dimerisation of two independent proteins into a synthetic transcription factor (sTF). First results, extracted from a simplified gene cascade, give interaction energies between active/inactive sTF and different output genes. The variation of single molecular parameters allows us to gain insight on how our system works, thus increasing the robustness of our theoretical model.
Using the combinatorial assembly of dimer mutants, we are currently characterising variants of AND-gate circuits. This ongoing work will allow the construction of a comprehensive model able to predict the design of circuits with advanced functionalities such as complex cell-state classifiers, multi-output switches or oscillators.

Keywords: Synthetic gene circuits, mammalian synthetic biology, modelling

*Speaker

Synthetic Mycology: Investigations in bioelectricity and genetic transformation in Ganoderma lucidum

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While historically understudied compared to its animal, plant, and bacterial counterparts, the fungal kingdom has demonstrated interesting characteristics in the fields of both basic and applied research in recent years. Filamentous fungi are capable of forming large underground mycelial networks that sequester carbon and transport nutrients between myriad plant species. As such, they require intraorganism communication across vast distances. Recent work demonstrating electrical activity in fungi may provide a key to the mechanism of this communication. Additionally, entrepreneurial interests have begun to take advantage of many unique properties of filamentous fungi and are working to replace products such as polystyrene packaging and animal leather with more sustainable alteratives. To this end, this work aims to investigate two potential avenues of research: to uncover the biological mechanisms behind filamentous fungi action potential formation and to develop a genetic manipulation toolkit to enhance their potential as future biomaterials.

Keywords: Filamentous Fungi, Bioelectricity, Eukaryotic Transformation, Biomaterials

*Speaker

Natural Product discovery and Metabolic engineering

Engineered E. coli to produce Raman Reporters (RaRs) that can be detected by Surface-enhaced Raman Scattering (SERS)

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A critical milestone in BIOCELLPHE isthe selection of SERS-active molecules acting as reporters (i.e. Raman reporters, RaRs). Three criteria were applied in order to select appropriate RaRs: (i) High SERS activity, (ii) unambiguous identification in mixtures, and (ii) their biosynthetic pathways must be expressed and optimized in *E. coli*.
To this aim, a shortlisting of RaRs was obtained out by running machine learning programs trained on literature data, and by the implementation of retrosynthesis software to assess the engineering and optimization of their biosynthesis in *E. coli*. The SERS activity of potential candidates identified was evaluated. Additionally, in order to realize unambiguous identification of the selected metabolites, a multivariate statistical analysis of the spectral data generated for each particular candidate was implemented.
We identified four SERS-active bacterial metabolites, namely Prodeoxyviolacein, deoxyviolacein, Violacein, and pyocyanin that can potentially be used as RaRs as they meet all the above requirements. We are currently evaluating whether a fifth one (proviolacein) can also be used as a RaR.

Keywords: SERS, Metabolic engineering, RaR, CTCs

*Speaker

Galaxy-SynBioCAD: tools and automated pipelines for Synthetic Biology Design and Metabolic Engineering

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There's a substantial number of tools released around Synthetic Biology and Metabolic Engineering- related questions and needs. This population of tools is difficult to comprehend and use together, main reasons being complexity and interoperability issues. Indeed, a high level of expertise could be required for installing codes, and execution for real life use cases could be computationally resource demanding. Plus some tools, although complementary, use different inputs and outputs which prevent easy chaining.
The Galaxy-SynBioCAD portal (1) is a growing toolshed for synthetic biology, metabolic engineering, and industrial biotechnology. The tools and workflows currently shared on the portal enable one to build libraries of strains producing desired chemical targets covering an end-to-end metabolic pathway design and engineering process: from the selection of strains and targets, the design of DNA parts to be assembled, to the generation of scripts driving liquid handlers for plasmid assembly and strain transformations.

Tools are made available on GitHub, anaconda.org and the Galaxy Tool Shed, opening to the greatest number access and utilization throughout the SynBio community, and significant ef-

*Speaker

fort has been granted for adopting FAIR principles. As a community effort helped by funded projects, the scope covered by tools is expected to expand over time.

The poster will give an overview of the Galaxy-SynBioCAD portal in the context of prediction and construction of E. coli lycopene-producing pathways. The poster will open the discussion around good practices guiding releases of tools through continuous integration.

A – lightweight – testing instance of Galaxy-SynBioCAD is available at https://galaxy-synbiocad.org.

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Keywords: Design Automation, Biosynthetic Pathway Engineering, Galaxy workflows, Standards, Web Application

Scalable microbial metabolite discovery through synthetic biology

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Secondary metabolites produced by microorganisms have been a critical source of bioactive molecules with applications in diverse therapeutic areas, from infectious diseases to cancer. Despite an enormous unexplored diversity, discovering new bioactive metabolites has become increasingly difficult with standard approaches. Genomic technologies can now be leveraged to discover secondary metabolites by focusing on the genes responsible for their synthesis, which typically form biosynthetic gene clusters (BGCs) within microbial genomes. Sequencing and bioinformatics allow prioritization of BGCs predicted to encode unexplored natural products, and cloning and heterologous expression of such BGCs can lead to discovering novel therapeutically relevant molecules. Here, a method to massively parallelize these processes will be presented. The streamlined interrogation of a large number of BGCs contained in a strain collection will be showcased, leading to the discovery of several previously uncharacterized natural products, including a novel antibiotic. The importance of leveraging economies of scale with such strategies will be discussed.

Keywords: Multiplexing, Cloning, Biosynthetic pathways, Secondary metabolites, Antibiotics

*Speaker

The Automated Galaxy-SynBioCAD Pipeline for Synthetic Biology Design and Engineering

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We introduce the Galaxy-SynBioCAD portal, a toolshed for synthetic biology, metabolic engineering, and industrial biotechnology. The tools and workflows currently shared on the portal enables one to build libraries of strains producing desired chemical targets covering an end-to-end metabolic pathway design and engineering process from the selection of strains and targets, the design of DNA parts to be assembled, to the generation of scripts driving liquid handlers for plasmid assembly and strain transformations. Standard formats like SBML and SBOL are used throughout to enforce the compatibility of the tools. In a study carried out at four different sites, we illustrate the link between pathway design and engineering with the building of a library of *E. coli* lycopene-producing strains. We also benchmark our workflows on literature and expert validated pathways. Overall, we find an 83% success rate in retrieving the validated pathways among the top 10 pathways generated by the workflows.

Keywords: Synthetic Biology, Metabolic Engineering, Strain Engineering Automation, Galaxy Workflows

*Speaker

Molecular programming and Systems Biology

A deep unsupervised language model for protein design

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Protein design has the potential to tackle many environmental and biomedical problems. Recent progress in the field of natural language processing (NLP) has enabled the implementation of ever- growing language models capable of understanding and generating text with human-like capabilities. Given the many similarities between human languages and protein sequences, the use of NLP models offers itself for predictive tasks in protein research. Motivated by the evident success of generative Transformer-based language models such as the GPT-x series, we trained ProtGPT2, a language model trained on protein space that generates *de novo* protein sequences that follow the principles of natural ones. In particular, the generated proteins present amino acid propensities which resemble natural proteins, whereas disorder and secondary structure prediction show that 88% of ProtGPT2- generated proteins are globular, in line with natural sequences. Sensitive sequence searches in protein databases demonstrated that ProtGPT2 sequences are distantly related to natural ones, and similarity networks further evidenced that ProtGPT2 is sampling unexplored regions of the protein space. AlphaFold prediction of sequences revealed well-folded structures with high pLDDT scores. Therefore, ProtGPT2 has the potential to generate *de novo* proteins in a high-throughput fashion in a matter of seconds. The model is easy-to-use and available to the community.

Keywords: machine learning, natural language processing, language model, protein design, protein sequence space

*Speaker

Artificial Metabolic Networks: enabling neural computations with metabolic networks

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Metabolic networks have largely been exploited as mechanistic tools to predict the behavior of a strain in different environments. However, the performance of this constraint-based modeling approach relies on labor-intensive experiments to determine media intake fluxes. In this paper, we show how neural methods can surrogate constraint-based modeling, make a metabolic network suitable for backpropagation, and consequently be used as an architecture for machine learning. We showcase the performance of our hybrid - mechanistic and neural - model, fitted with an experimental dataset of Escherichia coli growth rates in different media compositions, reaching a regression coefficient of 0.76 on cross-validation aggregated test sets. We expect Artificial Metabolic Networks to provide easier discovery of metabolic insights and prompt new biotechnological applications.

Keywords: Artificial Neural Network, Metabolic Network, Mechanistic Modeling, Metabolic Flux Analysis, Scientific Machine Learning, Hybrid Modeling, Artificial Metabolic Network

*Speaker

Design and implementation of de novo biosynthetic cascades for organic synthesis

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The combination of sequential biocatalytic reactions in non-natural synthetic cascades is a rapidly developing field and leads to the generation of complex valuable chemicals from simple precursors. (for a recent review see *Nat Rev Methods Primers* 2021, 1, 46). As the toolbox of available biocatalysts continues to expand, so do the options for biocatalytic retrosynthesis of a target molecule, leading to new routes employing enzymatic transformations. To facilitate the design stage, we have established RetroBioCat, a computer-aided synthesis planning tool for biocatalytic reactions and cascades (*Nat Catal* 2021, 4, 98). The implementation of such cascade reactions requires careful consideration, particularly with respect to whether the pathway is constructed *in vitro* or *in vivo*, in batch format or in flow *(Angew Chem Int Ed* 2021, 60(34), 18660). This lecture will showcase several successful *de novo cascades* and discuss the relative merits of *in vitro*, *in vivo* or hybrid approaches to building biocatalytic cascades. A particularly important reaction class for enzyme cascades is C-H activation, which allows for the stereoselective functionalisation of simple organic substrates and biological feedstocks, such as fatty acids.

Keywords: Biocatalysis, enzyme cascades

*Speaker

Latent Dirichlet Allocation for Double Clustering (LDA-DC): Discovering patients phenotypes and cell populations within a single Bayesian framework

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Introduction

Human disorders have a highly multifactorial nature and depend on genetic, behavioral, socioeconomic, and environmental factors. The number of metabolic diseases, cancer, and autoimmune pathologies has increased significantly in recent years, making research in this field a public health priority. In parallel, bioclinical routine datasets have expanded in conjunction with all kind of "omics" data, from both the host and microbiota, as well as metabolomic, proteomic, and cytometry data (1). All these types of data have some underlying structure on their own, taking values on different scales, with different variability, and are differently distributed. In addition, human patients are an equally important source of variability even among carefully selected cohorts: phenotypic variability (age, gender, previous conditions), dietary habits, bad vs good responders to the treatment, etc. In particular, new types of data have emerged which yield description at the cell level ie cytometry of scRNA seq. These data add a new layer of structuration that needs to be taken into account.

Motivations and Results

From the analytical viewpoint, the single cell data are huge-dimensional matrices produced for each subject. The data dimension, i.e., the number of cells, vary from one individual to another, and note that cell types, as well as the correspondence between the cell populations of the subjects, have to be identified before applying any statistical machine learning method. We refer to the challenge we introduce and consider here as to a double clustering problem, where the aim is to simultaneously,
purely from observations without any prior knowledge determine cell types, as well as stratify patients in order to study mechanisms of pathologies explained by particular cell subpopulations. We propose a novel approach to stratify cell-based observations within a single probabilistic framework, i.e., to extract meaningful phenotype from both patients and cells simultaneously. Our method is a practical extension of the Latent Dirichlet Allocation and is used to solve the Double Clustering task. The first step of our framework is the identification of the cell types. Once the cell types are fixed, we can efficiently estimate both probability of a phenotype given

*Speaker

a patient and the probability of a cell type given a phenotype. We tested our method on different datasets ranging from simulated patients to whom with AML (acute myeloid leukemia) or Crohn's disease, and were able to identify simultaneously clusters of patients and clusters of cells related to patients' conditions. Furthermore, using a network approach, we were able to stratify patients and identify groups of patients with specific phenotypes.

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Keywords: Double Clustering, Bayesian topic modelling, Latent Dirichlet Allocation, Precision medicine, Unsupervised learning

Paclitaxel Causes Cardiac Failure by Inhibiting Voltage-Gated Potassium Current: Insight from an In Silico Study

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Introduction:
Paclitaxel, an alkaloid, is used for chemotherapy of various cancers including ovarian, gastric, and lung cancers. Accumulated clinical and experimental evidence support that Paclitaxel causes peripheral neuropathy, which influences the quality of life. Cardiotoxicity due to the use of Paclitaxel is still under clinical investigation. The purpose of this study is to clarify the propensity of Paclitaxel concentration in modulating cardiac electrophysiological properties.

Material and Methods:

This in-silico model for the sinoatrial node (SAN) comprises the inward rectifier ion channels, voltage-gated sodium channel, voltage-gated potassium channel (Kv2.1), L-type calcium channel, calcium-dependent potassium channel, funny current channel, and calcium diffusion mechanisms. Concentration-dependent Paclitaxel (10μM to100μM) profile for 200 ms is interpreted as the altered conductance of Kv2.1 channel and then incorporated into the SA node electrophysiology. Both current-clamp and voltage-clamp protocols are applied to record electrophysiological activities.

Results:

After injecting a current stimulus (Istim) of varying magnitude (0.1-0.10 nA) and duration (10-50 ms), action potentials (AP) are reproduced by the SA node. The modulating effects of Paclitaxel concentration on the SA node's electrophysiological properties are investigated in two folds. First, we reproduced the current-voltage (I-V) curve profile of the Kv2.1 ion channel with respect to multiple doses of Paclitaxel under the voltage clamp protocol. It showed the continuous decrease of outward current because of multiple doses of Paclitaxel. At the highest concentrations (100μM), the peak of the outward current reduced to 26% of its' control value. The I-V curve is shifted to a 20% more positive side and the half-activation potential is increased by 28%. Then, the altered outward current is incorporated into the whole-cell model to investigate the AP firing patterns. For 100μM of Paclitaxel, the repolarization phase of AP was prolonged and the frequency of the firing pattern was reduced.

Conclusions:

*Speaker

Our in-silico study suggests that Paclitaxel at a higher concentration reduces the frequency rate of the spontaneous AP firing by suppressing the Kv2.1 current. Therefore, the dosage of Paclitaxel should be controlled to avoid cardiac toxicity. Further clinical trials are essential to analyze its' subcellular mechanisms.

Keywords: Ion channels, Cancer drugs, Cardiotoxicity, In Silico Study

Protein Design with Automated Reasoning and Deep Learning

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Protein Design with Automated Reasoning and Deep Learning

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In this talk, we introduce a method for Computational Protein Design (CPD) based on two different Artificial Intelligence technologies: automated reasoning, augmented with Deep Learning. The goal of CPD is to design proteins with enhanced or new properties or functions. We formulate CPD as an optimization problem: given an input backbone (crafted to carry out the desired function/property), we want to find the most suitable sequence, i.e., the sequence minimizing the energy of the backbone. Exploring the huge sequence space to find the optimal sequence requires an efficient optimization algorithm. We will present our exact algorithm that optimizes a decomposable pairwise energy of the backbone (1).

This pipeline offers options useful for real-case designs, and therefore it has been successful in several protein engineering problems. Indeed, it makes it possible to consider additional design requirements (for instance in terms of protein composition), to redesign only a part of the protein or to simultaneously consider several conformational/molecular states. For example, this AI-based CPD approach has been successfully applied to design self-assembling symmetrical proteins and enzymes with optimized and new properties/activity of interest for bio(nano)technologies as well as novel nanobody scaffolds for diagnosis. Specifically, we will present an application case on the engineering of a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase to use the NAD+ cofactor instead of NADP+ for cell-free biocatalysis.

Despite its successes, our pipeline is limited by the energy function optimized. Existing decomposable energy functions are based on simplified physic force-fields or on statistics. Here, we tried to directly learn a pairwise decomposable fitness function on known protein structures using Deep Learning techniques. We in silico tested the quality of the learned energy function, and found it to be competitive with state-of-the art hybrid and statistical functions such as

*Speaker

those available in Rosetta (2) or KORP (3).

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Keywords: Computational Protein Design, Deep Learning, Enzyme Engineering

Spatio-temporal optogenetic control of yeast sucrose catabolism

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Microbial communities are the siege of complex, spatio temporal metabolic interactions in which cooperation and competition take place. Building on these properties , there is a growing interest in engineering microbial consortia for bioproduction, fabrication of living materials or medical applications based on live therapeutics. In addition, optogenetics has been proposed as an unprecedented opportunity to shape metabolic interactions within microbial communities in space and time. Here we demonstrate how to determine microbial interaction length scale within a cooperator/cheater model system using optogenetics to control cooperation in space. We designed and optimised a *Saccharomyces cerevisiae* yeast strain for optogenetic control of the SUC2 invertase production with a high induction range, allowing yeast to locally grow on sucrose with blue light. Thanks to a custom-built device, we then applied light patterns of increasing spatial wavelength to investigate the spatial filtering properties of a cooperator/cheater consortium. We found that such a consortium can be understood as a spatial bandpass filter with a low cut-off wavelength due to cheater-cooperator competition for hexoses and a high cut-off wavelength evidencing intra-cooperator competition for sucrose. Such interactions were found to be conditions dependent, acting at up to the centimetre scale. We anticipate that our optogenetic setup and strains can be used to quantitatively shape and study metabolic interactions within complex microbial consortia. In particular, it should be of interest for the design of light responsive Engineered and Hybrid Living Materials.

Keywords: Optogenetic, Yeast, Cooperation, Consortium, Microfluidic, Spatial Patterning

*Speaker

Towards engineering a native bacterial microcompartment system in Escherichia coli.

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Bacterial microcompartments (BMCs) represent prokaryotic counterparts to the eukaryotic organelles. BMCs act as intracellular microbioreactors that spatially insulate targeted pathways and enhance their kinetics through metabolic channelling effects. Some strains of *E. coli* natively bear an *ethanolamine utilization* (*eut*) operon with all the genetic elements required to produce Eut BMCs. The *E. coli* Eut BMCs could represent powerful tools for synthetic biology applications, such as encapsulating heterologous pathways *in vivo* to improve their yields. However, they remain poorly characterized. We have thus implemented a systems biology approach to evaluate whether *E. coli* can produce well-assembled and functional Eut BMCs, focusing on the laboratory strain *E. coli* K12 W3110. Using 13C-tracer fluxomics data and modelling based approaches, we first characterised the ethanolamine metabolism. We then observed the Eut BMCs by transmission electron microscopy as well as cryotomography. Moreover, we generated novel insights into the Eut BMCs composition through proteomics analysis. Altogether, these results allowed us to understand how the *E. coli* Eut BMC operates and how to hack its function while maintaining its integrity.

Keywords: Prokaryote, Microcompartment, Systems Biology

*Speaker

Towards the computational design of genetically encodable nanomachines

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The realization that biological systems generate nanoscale mechanical forces by leveraging biochemical energy, has brought many to contemplate the design of synthetic biomolecular machines. Yet, decades after Feynman's insights, the capability to perform useful work with artificial machines remains mostly limited to the macroscale. While theoretically simple, the physical principles that govern their operation have remained inaccessible to synthetic approaches to biology: designing dynamic nanoscale protein architectures requires a subtle control over the structural energetics and self-assembly kinetics of symmetric multicomponent systems. We propose to leverage computational protein design to fabricate genetically encodable machinery de novo. We develop novel computational tools based on Deep Learning and use them to design de novo protein mechanical systems. Starting from in silico blueprints we generate a library of protein components and design their interfaces to direct self-assembly into mechanically constrained symmetric and quasi-symmetric heteromultimeric complexes. We then investigate the design of a Brownian motor mechanism capable of generating torque, by computationally installing catalysis and tailoring the energy landscape in order to convert biochemical energy stored in a small molecule fuel into work. Using cryo-electron microscopy we demonstrate that protein rotary machines can be designed accurately to fold and self-assemble according to specifications, and single molecule microscopy shows that their rotational properties depend on the energetic landscape, which is tuned computationally. Our results indicate that computational design can be used to fabricate protein based mechanical systems operating at the nanoscale, opening avenues towards the systematic design of genetically encodable nanomachines.

Keywords: Computational protein design, de novo protein, nanomachines, motors, bottom, up synthetic biology, deep learning, computational biochemistry, structural biology

*Speaker

Cell-free systems and Build-a-cell

A New Spin on Efficient Reconstitution of Biological Systems in GUVs

Kristina Ganzinger * [1]

[1] AMOLF, Amsterdam – Netherlands

Reconstitution of biological processes from well-defined, individual components has enabled researchers to study increasing complex biological systems at the molecular and mechanistic level. To mimic cell or organelle membranes in these minimal systems, giant unilamellar vesicles (GUVs) are often used as simple models for membrane-bound compartments. As researchers aim to build a minimal synthetic cell from the bottom up, reconstitution of cellular processes in GUVs will be particularly valuable to include transport processes across membranes and a mechanically responsive compartment. However, reliable GUV fabrication remains a major experimental bottle-neck, in particular if they are to encapsulate functional biomolecules. Here, we will present our recent work on optimising a double-emulsion method for producing functionalised GUVs called continuous droplet interface crossing encapsulation (cDICE). By optimising the protocol and tightly controlling experimental conditions, we improved the efficiency, ease, and, most importantly, reproducibility of cDICE-based GUV formation. We show cDICE can produce GUVs containing a multitude of minimal systems, including a minimal actin cytoskeleton and bacterial chromosomes. By allowing the formation of bespoke liposomes with good yields and reproducibility, optimised cDICE promises to become a standard method for making functionalised vesicles for the biophysics and synthetic biology communities.

Keywords: minimal cells, reconstitution, bottom, up synthetic biology

*Speaker

Building a synthetic cell via evolution

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Our group is engaged in the long-term effort to build an entire living cell using a bottom-up synthetic biology approach. We envision the chassis of a synthetic cell as a reconstituted gene expression system encapsulated inside a lipid vesicle compartment. Our latest results on DNA replication, phospholipid biosynthesis, and liposome constriction will be presented. Moreover, we will see how directed evolution will accelerate the optimization and functional integration of these biological modules. The challenges to create an autonomously replicating synthetic cell and the great opportunities that may arise by joining forces with other (French) groups will finally be discussed.

Keywords: Minimal cell, cell, free protein synthesis, synthetic genomes, directed evolution

*Speaker

Cell-free biosynthesis combined with deep learning accelerates de novo development of antimicrobial peptides

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The rise of antibiotic resistance requires immediate antimicrobial strategies against multi-drug resistant pathogens. Antimicrobial peptides (AMPs) provide alternatives to traditional antibiotics with advantages e.g., lower resistance evolution. Here, we used cell-free transcription-translation systems for the cost-, time-, and labor-effective ribosomal production of de novo-designed AMPs via deep learning. Overall, we produced 500 AMP sequences by encoding the peptides directly from DNA in vitro and tested them on gram-negative and gram-positive bacteria. We found 30 new-to-nature (BLAST) AMPs, mostly alpha-helical (Alphafold) rich in cationic and hydrophobic amino acids that are properties similar to natural AMPs. Atomistic simulation of the interaction between these AMPs with both bacterial and human cell membranes showed that i) these peptides are most likely to act through membrane and ii) they have a closer interaction with bacterial than human cell membrane. We then characterized these 30 AMPs for minimum inhibitory concentrations on a variety of bacteria, hemolysis, and cytotoxicity on human cells, leading us to promising AMPs with high activity and low toxicity which do not evolve resistance in E. coli.

Keywords: Cell free systems, Antimicrobial peptides, Deep learning

*Speaker

Dynamic behavior of light-responsive coacervate droplets in microfluidics

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Liquid-liquid phase separation is ubiquitous in biology since it is the underlying mechanism to form membraneless organelles. Coacervate droplets produced in vitro between oppositely charged polyelectrolytes are promising models of membraneless organelles. These droplets are highly-crowded and completely-open compartments, thus easily respond to environmental stimuli and recapitulate essential processes occurring inside living cells. To mimic the dynamic behavior of membraneless organelles, we designed light-responsive coacervate droplets capable of dissolution and reformation under irradiating with light of different wavelengths. Droplet-based microfluidics is used to provide a stable and clear platform for our observation, which allows us to extract the kinetics of light-induced droplet formation and dissolution. Based on our results, we are able to rationalize the behavior of these coacervate droplets when they are pushed away from thermodynamic equilibrium. These light-responsive systems could open perspectives for the reversible control of biomolecular sequestration and biocatalytic reactions, and serve as dynamic artificial membraneless organelles in synthetic cells.

Keywords: Dynamic coacervates, photo, responsive systems, membraneless organelles, microfluidics

*Speaker

Enzyme reactions in coacervates droplets as artificial membraneless organelles

Nicolas Martin * [1]

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Living cells are complex compartmentalized chemical systems. Membrane-less organelles have recently emerged as ubiquitous compartments used by cells to orchestrate biological processes in space and time. Coacervate microdroplets produced *in vitro* by liquid-liquid phase separation between oppositely charged polyelectrolytes are now gaining increasing interest as artificial surrogates of membraneless organelles. We will here quantitatively show that coacervate droplets provide a favourable environment for enzyme reactions, and that enzyme reactions can be used to drive coacervate formation and dissolution. Our results emphasize the key role of the coacervate matrix on the catalytic activity of model enzymes, and highlight the possibility to control phase separation using biocatalytic reactions. Overall, these studies open perspectives to develop dynamic and catalytically active artificial membraneless organelles as controllable modules for synthetic cell research.

Keywords: coacervates, enzyme reactions, liquid, liquid phase separation, membraneless organelles

*Speaker

Optimized cell-free protein synthesis using unprotected linear dna from exonuclease-deficient cellular extracts

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Cell-free protein synthesis (CFPS) has recently become very popular in the field of synthetic biology due to its numerous advantages. The use of linear DNA templates in cell-free systems promises to accelerate the prototyping and engineering of synthetic gene circuits. However, linear DNA templates are rapidly degraded by exonucleases that are naturally present in the cell extracts. Current approaches tackle the problem by adding exonuclease inhibitors and DNA-binding proteins to protect the linear DNA, requiring additional time- and resource-intensive steps. Here, we delete the recBCD exonuclease gene cluster from the Escherichia coli BL21 genome. By using cell extracts from exonuclease-deficient knockout cells, linear DNA templates remain intact without requiring any end-modifications. When using linear or plasmid DNA templates at the buffer calibration step, the optimal potassium glutamate concentrations obtained when using linear DNA were consistently lower than those obtained when using plasmid DNA for the same extract. We demonstrate the robustness of the exonuclease deficient extracts across seven different batches and a wide range of experimental conditions across two different laboratories. Finally, we illustrate the use of the ΔrecBCD extracts for two applications: toehold switch characterization and enzyme screening.

Keywords: CFPS, Linear DNA, Buffer optimization, Strain modifications, DNA prototyping, Biosensing engineering

*Speaker

PeroxiHUB: A Modular Cell-Free Biosensing Platform Using H2O2 as Signal Integrator

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Cell-free systems have great potential for delivering robust, inexpensive, and field-deployable biosensors. Many cell-free biosensors rely on transcription factors responding to small molecules, but their discovery and implementation still remain challenging. Here we report the engineering of PeroxiHUB, an optimized H2O2-centered sensing platform supporting cell-free detection of different metabolites. H2O2 is a central metabolite and a byproduct of numerous enzymatic reactions. PeroxiHUB uses enzymatic transducers to convert metabolites of interest into H2O2, enabling rapid reprogramming of sensor specificity using alternative transducers. We first screen several transcription factors and optimize OxyR for the transcriptional response to H2O2 in a cell-free system, highlighting the need for preincubation steps to obtain suitable signal-to-noise ratios. We then demonstrate modular detection of metabolites of clinical interestlactate, sarcosine, and cholineusing different transducers mined via a custom retrosynthesis workflow publicly available on the SynBioCAD Galaxy portal. We find that expressing the transducer during the preincubation step is crucial for optimal sensor operation. We then show that different reporters can be connected to PeroxiHUB, providing high adaptability for various applications. Finally, we demonstrate that a peroxiHUB lactate biosensor can detect endogenous levels of this metabolite in clinical samples. Given the wide range of enzymatic reactions producing H2O2, the PeroxiHUB platform will support cell-free detection of a large number of metabolites in a modular and scalable fashion.

Keywords: synthetic biology, cell, free systems, biosensor, hydrogen peroxide, H2O2, enzymatic transducer, computer aided design

*Speaker

Philosophy

Function: the concept that (almost) everyone uses but no one really knows - and why it matters

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Biologists attribute functions to the traits of living beings. Engineers assign functions to the objects they design and to their parts. Chemists assign functionalities to certain groups of atoms in organic molecules. Physicists, by contrast, have absolutely no use for functions. Synthetic biology is at the crossroads of biology, chemistry, physics and engineering, and its core business is the manipulation, redesign and creation of functions. But what is a function? When we look at how this notion is defined in each of these disciplines respectively, it seems that they use totally different concepts that bear the same name. This poses a problem for understanding what synthetic biology is really about. It may also have profound practical and ethical implications.

Keywords: Philosophy, function

*Speaker

Workshop - iGEM presentations

A synthetic biology toolkit to interface genetic circuits with electronics

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[1] Centre de Recherches Interdisciplinaires [Paris] – iGEM Paris-Bettencourt – France

Language originating from the field of electronics is often employed in synthetic biology to describe genetic logic circuits, meanwhile, the real intersection of electronics with genetics remains largely unexplored. Our iGEM team is developing a toolkit of genetic parts with the concomitant open-source hardware to enable synthetic biologists to interface bacterial gene expression with electronics. Our toolkit incorporates a multitude of genetic parts from previous research in electro-microbiology that have been standardised to allow for a wide variety of applications. We are conversely researching new means of bioelectronic control using hyperpolarization of bacterial membranes for gene induction. Our toolkit will open doors for foundational research in gene expression and control and accelerate new ideas in biomanufacturing, biomonitoring, and biorobotics.

Keywords: Genetic Engineering, Electromicrobiology, Synthetic Biology Toolkit, Electrogenetics, Electronics

*Speaker

Electricia coli

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According to the World Health Organization, around 10 million people died of cancer globally in 2021. Cancer remains the most life-threatening disease all over the world. Therefore, early-stage cancer detection is critical for timely intervention needed to save the patient's life. Here we propose a system for more accurate and less expensive detection of cancer by linking together techniques of biomarker sensing and the external electron transfer through a microbial fuel cell. Consequently, we would detect an electrical signal in the presence of a cancer biomarker.

Our project relies on the use of a toehold switch sensor to detect the cancer biomarker PANTR1 long non coding RNA (lncRNA), and subsequently express a downstream gene that leads to an electron flow outside the cell resulting in an electric signal.

To make sure that we get a significant difference in current generation, we have built our own microbial fuel cell device, where electrons released in the media will react with an electrode (anode) in a first chamber, get conducted through an external circuit, and finally get released into a second chamber electrode (cathode).

The external circuit is linked to an Arduino chip that allows real-time electron transfer measurements with a higher accuracy than fluorescent molecules or chromophores commonly used in such devices.

Our setup is highly sustainable as the device can be reused for a number of years. It can be developed into a biosensor kit for analytical laboratories and a smartphone application to closely monitor real-time measurements.

Keywords: biosensor, toehold, MFC, CymA, Mtr CAB, Arduino, chip, electron transfer, PANTR1, lnc RNA

*Speaker

Lighting up the future of agriculture: Fiat Lux, a biosynthetic tool to track pathogenic bacteria in plants

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[1] Student – Institut National des Sciences Appliquées (INSA) - Lyon – France

Bacterial bioluminescence is a useful tool for studies. It is commonly used as a biosensor for detecting toxic chemicals. Our project, Fiat Lux, based on the work of Carola Gregor and her team in 2017, aims at extending and simplifying the use of luminescence as a reporter gene, notably for in vivo infection studies. Our team has focused the proof of concept on the soft rot disease in potatoes, caused by the Dickeya species. Our tool, Fiat Lux, is a promising way of finding a solution to combat this disease, and would contribute towards building more sustainable and responsible agricultural practices. This proof of concept will explain to all how to use Fiat Lux as a response to new pathogenic threats. This presentation will provide an overview of our team's ambition and first results.

Keywords: bioluminescence, bacteria, iLux, pathogenesis

*Speaker

The CO2CURE project: Development of autotrophic Streptomyces capable of producing molecules of interest such as antibiotics by carbon fixation

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[1], Alicia Lemaire [1], Baptiste Ortheau [1], Alexandre Pradignac [1], Antoine Regardin [1], Yasmine Socratoff [1], Tiroumagale Tillay [1], Raphaël Vatin *

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Synthetic biology for bioproduction is a rapidly booming field and a promising option for growing industries. However, the majority of strains used in this field are not autotrophic, which means that they cannot fix carbon dioxide (CO2) from the atmosphere. As it turns out, CO2 is the most common greenhouse gas that overheats our planet. Here we present the proof of concept for making such strains autotrophic by implementing the Calvin cycle. We choose strains of *Streptomyces*, a genus of bacteria as an example which produce antibiotics along with other highly valuable molecules and specialized metabolites used in laboratories.
Here, in the CO2CURE project, we propose to produce antibiotics from atmospheric CO2, leading to the recycling of CO2 to produce antibiotics more ecologically and cost-effectively. In this project, we introduce new chassis to the iGEM competition that belong to the genus *Streptomyces* which are multicellular bacteria. They have linear genomes that are very GC-rich (72%) and are compartmentalized, with a central compartment that is highly expressed and silent terminal ones. To turn any *Streptomyces* into autotrophs, we began by transforming the genes encoding RubisCO (Ribulose-1,5-bisphosphate Carboxylase Oxygenase) and PRK (Phos-

*Speaker

phoribulokinase), as these genes represent a huge part of the Calvin cycle. We have developed a genetic tool for the insertion of *rubisco* and *prk* genes into the *Streptomyces* genome.

Furthermore, to obtain antibiotic production in the minimal medium, we had to repress the expression of the *lsr2a* gene which is an exogenous silencer. This silencer represses the gene clusters such as *SMBGC*s (specialized metabolite biosynthetic gene clusters) and stops biosynthesis of different metabolites in *Streptomyces*. We have engineered genetic tools via the Crispr-dCas9 system to switch off *lsr2a* and force antibiotic production in a low carbon environment. We took advantage of the same methodology (Crispr-dCas9) to switch off the *glycerophosphate mutase* genes to force the appearance of the Calvin cycle in their primary metabolism. Finally, we compared numerous *Streptomyces* strains, their phenotypic characteristics, and genomes to determine the best strains for this project and selected GC-rich reference chassis.
The CO2CURE project will enable faster, more affordable, and sustainable bioproduction of antibiotics and many other useful derivatives, as we build a universal vector to transfer the Calvin cycle into various *Streptomyces* species. Our project also introduces fundamental progress in the field of synthetic biology. We hope that the CO2CURE project will encourage the synthetic biology community to introduce more cycle-based processes into the industry, as they are greatly environmentally sustainable.

Keywords: Streptomyces, bioproduction, carbon fixation, antibiotics, Calvin cycle

The NAWI Project

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[1], Lara Pérez Gorgol [1], Léa Maati [1], Luca Donati *

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The world is facing major problems, namely the increase of the population and a current unsustainable agriculture promoting global warming and soil degradation. Thus feeding future populations is a challenge. The idea behind our iGEM project called NAWI for New Algae for World Improvements is therefore to produce an innovative food source for the future.
To achieve this goal, our strategy is to produce a green alga with added value, by modifying *Chlamydomonas reinhardtii* and making it an iron-rich organism which could help fight iron deficiency.

Our strategy is to transfect the algae with a plasmid which increases the production of the truncated hemoglobin THB1, a protein that releases iron upon digestion. In order to have control over THB1 expression, firstly we will use an inducible promoter. Moreover, we will rely on insulator sequences, which are enhancer-blocking and also heterochromatin spreading blocking sequence, which is useful during stable genomic transformation. Insulators from a variety of species have been tested and shown to be functional in plants. We have decided to focus on the gypsy-like sequence found in the *Arabidopsis thaliana* genome.
Finally, to complete our project, we will give it an interesting taste for its consumption. For that, we will express a taste protein (monellin, neoculin, miraculin...). But also, we would like to achieve a sustainable production. In order to achieve this, the idea would be to use bioreactors, avoiding the use and the depletion of soils; and also to use agro-food waste as source of carbon for their growth.

Keywords: Algae, innovative food source, iron rich organism, iron deficiency, hemoglobin THB1, insulator sequences, taste protein, bioreactors, agro food waste.

*Speaker

iGEM IONIS Paris - StarchLight

Solène Galpy * [1], Chloé Bonef *

[2], Alexandre Trubert *

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[4], Martin Pezous *

[5]

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[3] Trubert – iGEM IONIS Paris – France
[4] Dieumegard – iGEM IONIS Paris – France
[5] Pezous – iGEM IONIS Paris – France

Starchlight aims at designing and making a microbial fuel cell (MFC) device able to extract energy from starch-based brewery wastes, store it, and release it in the form of an electric current. For that purpose, StarchLight team plans to genetically engineer a strain of Escherichia coli and use Schewanella oneidensis bacteria as a final electricity producer.

Keywords: Upgrading of a waste, environment, synthetic biology

*Speaker

Workshop - Synthetic Biology of lactic acid bacteria (Applications of recombinant LAB)

Engineered Lactococcus lactis with simultaneous tumor antigen targeting and proinflammatory cytokine binding ability

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Lactococcus lactis is regularly used as a starter in food fermentation and is the most prevalent lactic acid bacterium in the gut microbiota. Due to its safety, it has been engineered as a vector for delivery of therapeutic proteins in inflammatory bowel disease, infections, allergies, diabetes and cancer, with some examples already in clinical trials. We have co-displayed cytokine-binding proteins and ligands for tumor antigens to facilitate the specific interactions of the bacteria with the cancer cells and targeted delivery of cytokine-binding proteins.
We prepared multifunctional *L. lactis* by using dual-expression plasmid pNZDual and by introducing BglBrick cloning for straightforward assembly of up to three expression cassettes. IL-8-binding evasin and IL-6-binding affibody were displayed on *L. lactis* for cytokine removal, while EpCAM-binding affitin and HER2-binding affibody were displayed for targeting tumor antigens. Infrared fluorescent protein was concomitantly expressed to enable bacterial detection and imaging. Engineered *L. lactis* was able to bind IL-8 and IL-6 from human colon adenocarcinoma cells Caco-2 and HT-29 and from monocyte-like cells THP-1 and U-937. The engineered *L. lactis* removed $>$ 65% of IL-8 from the supernatant of Caco-2 and HT-29 cells, while the removal of IL-6 was even more effective, with specific removal of 99% of recombinant IL-6 and up to 94% of IL-6 from the supernatant of THP-1 and U-937 cells. Specific adhesion of the engineered bacteria was observed in HEK293 cells transfected to overexpress EpCAM or HER2 receptors, as well as in Caco-2 and HT-29 cells. Fluorescence microscopy revealed app. 40 *L. lactis* cells bound per single EpCAM- and app. 10 *L. lactis* cells bound per single HER-2-expressing HEK293 cell. Apart from static conditions, targeting ability of engineered *L. lactis* was also demonstrated under constant flow in microfluidic system to better simulate the conditions in the gastrointestinal tract.
In summary, multifunctional *L. lactis* was engineered by simultaneously expressing proteins with different functionalities, including tumor antigen binders, cytokine binders, and infrared fluorescent protein. Activity of the recombinant bacteria was confirmed in several cell models, and the microbe-based approach resulted in novel therapeutic strategy against colorectal cancer.

Keywords: Lactococcus lactis, cytokines, IL, 6, IL, 8, tumor antigens, binders, surface display

*Speaker

Potential and opportunities for use of recombinant lactic acid bacteria in the gut

Catherine Daniel * [1]

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The safety status of lactic acid bacteria (LAB) and their capacity to survive the passage through the gastrointestinal tract have rendered them excellent candidates for the production of therapeutic proteins and their delivery *in situ* to the gut. For the last two decades, major health beneficial effects of recombinant LAB have been successfully demonstrated after mucosal administration, predominantly using animal models. Indeed, we have shown that modulation of the immune response by antigen/allergen production and the delivery of therapeutic molecules to treat intestinal diseases are successful applications of recombinant LAB. The field has recently moved into the era of human clinical trials with the potential of recombinant LAB as therapeutic tools for their safe and efficient use in human health. Moreover, as the gastrointestinal tract is the main ecological niche in which LAB provide health benefits, there is currently a need to characterize host-microbe interactions in space and time by tracking these bacteria *in vivo*. Therefore, we have set up new tools allowing us to directly trace microorganisms in the gut of mice after oral administration. We developed different luciferase-expressing LAB strains to follow them in the digestive tract of living mice by non-invasive bioluminescence *in vivo* imaging technologies. Finally, we have constructed a fluorescent *Lactobacillus* strain and combined non-invasive whole-body imaging with *ex vivo* fluorescence confocal microscopy imaging to monitor the impact of intestinal inflammation on the persistence of orally administered *Lactobacillus* in healthy and inflamed mouse colons. We showed that an anti-inflammatory *Lactobacillus*, orally administered, persists for longer and at higher counts in the inflamed colon than in the healthy colon. We confirmed these results by *ex vivo* confocal imaging of colons from mice with experimental colitis. Moreover, extended orthogonal view projections enabled us to localize individual *Lactobacillus* in sites that differed for healthy *versus* inflamed guts. In healthy colons, orally administered bacteria were localized in the lumen in close contact with commensal bacteria and sometimes in the crypts but very rarely in contact with intestinal cells. In contrast, *Lactobacillus* bacteria in the inflamed colon were found in direct contact with damaged epithelial cells. Taken as a whole, recombinant LAB will definitely contribute to disease prevention and individualized therapies in future clinical practice, and to inspire novel microbial strategies utilizing both probiotics and their products in the fields of biology and medicine.

Keywords: digestive tract, mucosal administration, animal models, in vivo imaging technologies, delivery of therapeutic molecules

*Speaker

Potential use of recombinant biocontained lactococci to deliver the antiprotease elafin in IBD patients

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[2] Interactions des Bactéries Commensales et Probiotiques avec lHôte – MICrobiologie de lALImentation au Service de la Santé – France

Elafin, a natural protease inhibitor expressed in healthy intestinal mucosa, has pleiotropic anti-inflammatory properties *in vitro* and in animal models. We found that mucosal expression of Elafin is diminished in patients with inflammatory bowel disease (IBD). This defect is associated with increased elastolytic activity (elastase-like proteolysis) in colon tissue. We engineered two food-grade strains of lactic acid bacteria (LAB) to express and deliver Elafin to the site of inflammation in the colon to assess the potential therapeutic benefits of the Elafin-expressing LAB (Motta *et al*, 2012). In mouse models of acute and chronic colitis, oral administration of Elafin-expressing LAB decreased elastolytic activity and inflammation and restored intestinal homeostasis. These results suggest that oral delivery of LAB secreting Elafin may be useful for treating IBD in humans. We recently constructed a biocontained elafin-secreting *Lactococcus lactis* strain which could be potentially administered to IBD patients.

Keywords: Lactic acid bacteria, IBD

*Speaker

Use of recombinant LAB as cDNA delivery vehicle and its applications

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The advantage of DNA vaccines relies in their ability to induce both cellular and humoral Th1 immune responses. In contrast to bacteria-mediated delivery of protein antigens, bacteria-mediated delivery of DNA vaccines leads to the expression of post-translationally modified antigens by host cells resulting in presentation of conformationally restricted epitopes to the immune system. As for protein delivery, the use of food-grade lactococci and lactobacilli as cDNA delivery vehicles has been demonstrated *in vitro* and *in vivo* to be a good alternative to attenuated pathogens. Plasmid transfer occurs as well in intestine than colon and mainly in epithelial cells and can be increased by the use of invasive lactic acid bacteria. The cDNA delivery strategy allow us to modify locally and transitory the phenotype of the host. Since ten years now we have delivered different proteins as antigens, cytokines or anti-microbial peptides to decipher their role in allergy, Inflammatory Bowel Disease, Irritable Bowel Syndrome or Type 2 Diabetes.

Keywords: cDNA delivery, plasmid transfer

*Speaker

Workshop - Synthetic Biology of lactic acid bacteria (Tools for LAB engineering)

Genetic toolbox for lactic acid bacteria

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Broadly speaking, *Lactobacilli* holds great promise for various biotechnological applications (1). Flagship examples to date include the development of engineered probiotic treatments (2) and in situ production of mucosal vaccines (3). Although providing an effective way to widen industrial applicability, genetic engineering of these highly phylogenetically diverse *Lactobacilli* species remains vastly limited by the lack of **cognate genetic elements** to support and precisely **control gene expression** such as **plasmids, promoters, RBS, or reporter genes.** In order to generalize the use of *lactobacilli* as an engineering platform, it is necessary to develop genetic tools allowing the reliable control of gene expression but also transposable between different species.

For this purpose we have developed different tools using **synthetic biology approaches** to obtain standard collections of regulatory elements for *lactobacillus*. Here we present i. A library of over **3000 constitutive and synthetic characterized promoters and RBS** functioning in various lactobacillus species; ii. A small collection of **pH tolerant reporter genes**; iii. A **modular cloning kit (MoClo) of shuttler-vectors** including replication origin (gram positive and gram negative), antibiotic resistances and transcription units (including promoters). This **LactoClo kit** allows the rapid construction of expression vectors for a wide range of gram positive bacteria.

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Keywords: Lactobacillus, cancer, promoter, plasmid, moclo

Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic Lactobacillus

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The *Lactobacillus* genus comprises many species that are commonly used in the food industry mainly due to their preservative action. Notwithstanding, several *Lactobacillus* species have been regarded as probiotics because of the beneficial effects they trigger in the human body. Therefore, there exists a growing interest in engineering these probiotic bacteria for healthcare applications in animals and humans.
Nevertheless, there are several drawbacks when it comes to genetically engineering these probiotic bacteria and using them in the human body: i) high amounts of recombinant plasmids are generally required to transform these bacteria, which means that an intermediate host such as *E. coli* is needed to obtain enough plasmid amount, ii) the genetic toolboxes available are typically very poor, and iii) alternatives to antibiotic-based plasmid retention strategy have not been explored yet.

In this work, we first describe a novel Gibson-based Assembly direct cloning method in *Lactiplantibacillus plantarum* WCFS1 that does not involve other bacteria whatsoever. Secondly, we also describe the results concerning a promoter from a very phylogenetically distant bacteria, *Salmonella typhimurium*, which can drive the expression of a reporter gene 5-fold higher than previously reported promoters. Thirdly, an easy-to-implement plasmid retention strategy based on multiple toxin-antitoxins has been explored for the first time in *Lactobacillus* and showed promising results.
Taken altogether, we believe that all these novel insights might positively contribute to enhancing the genetic programmability of *Lactobacillus* for healthcare applications.

Keywords: Diret cloning, toxin, antitoxin, super strong promoter

*Speaker

The pSIP-system as a tool for genetic engineering of Lactobacillales

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Many species of the *Lactobacillales* order are natural inhabitants of the human gastrointestinal tract and have the Generally Recognized As Safe (GRAS) status. Therefore, these bacteria are being extensively studied as potential vectors for *in situ* delivery of heterologous proteins. Additional advantages of lactobacilli include general robustness, beneficial modulatory effects on the immune system and the ability to survive the passage through the gastrointestinal tract. The quorum sensing-based inducible pSIP-system is a versatile tool that enables inducible high-level production of heterologous proteins in lactobacilli. The multiple cloning-site delimited modules of a SIP-plasmid permits easy and fast exchange of the promoter, replicon, antibiotic resistance gene or target gene during vector construction. Today, the system is widely used for secretion and precision surface anchoring of heterologous proteins in lactobacilli. Target proteins can be anchored to the surface both covalently or non-covalently, to either the cell membrane or the peptidoglycan layer in the cell wall. The secretion and anchoring systems have been optimized by characterization and functional testing of several SecA signal peptides, LysM anchoring domains, lipoprotein anchors, N-terminal transmembrane anchors and LPxTG cell wall anchoring domains.
The pSIP-system has successfully been used for secretion and surface display of enzymes, cancer antigens and a wide variety of antigens from pathogenic bacteria and viruses in *L. plantarum*. Recently, we showed that an engineered *Lactobacillus* strain carrying a surface-located *Mycobacterium tuberculosis* antigen induced antigen-specific immune responses in the lungs and splenocytes of mice. This study clearly indicated that optimized surface display of the antigen outperformed intracellular localization of the antigen.

Keywords: surface display, pSIP system, expression

*Speaker

The phage mv4 recombination system: towards a reprogrammable tool for bacterial genome engineering?

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One of the challenges of synthetic biology is to produce new microorganisms able to degrade or synthetize complex compounds, requiring the transfer of entire or synthetic metabolic pathways into bacterial chassis made up of model bacterial species known for their robustness in technological processes. Through their ability to integrate large DNA fragments into the genome of their host bacterium, recombination systems of temperate bacteriophages, consisting of an integrase (Int) that catalyses the recombination between a phage DNA site (*attP*) and a bacterial DNA site (*attB*), could constitute relevant tools for the incorporation of these metabolic pathways into bacterial frameworks. Their use is however limited by the impossibility of choosing the integration site in the host genome because each integrase depends strictly on its *attP* and *attB* sites sequences. The mv4 phage integration module, which targets one of the *Lactobacillus bulgaricus* serine tRNA, has been described as atypical (1, 2) because it did not require any host factor and the *attP* and *attB* sites showed atypical organization.
Through the use of random DNA libraries, *in vitro* recombination, and NGS sequencing of the reaction products, we demonstrated that the *attB* site and the core region of the *attP* site are longer (21-bp) than previously described, and exhibit an organization similar to tyrosine recombinases recombination systems, with two 7-bp inverted repeat regions surrounding the 7-bp central region (the region where the strand exchange occurs). We also demonstrated that mv4 integrase have some flexibility in the nucleotide sequence of the recombination sites, allowing us to reprogram the recombination specificity *in vitro* toward different natural or synthetic target sites by simple nucleotide modification of the 21-bp *attP* core-site (3). The recombination specificity was also redirected *in vivo* to target the serine-tRNA of *E. coli* and *L. lactis*, two model bacteria of biotechnological interest (3). These results pave the way for the development of a transgenesis tool combining the advantages of integrases and the ease of targeting CRISPR-Cas type systems.

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Keywords: genome engineering, site, specific recombination, tyrosine recombinase, bacteriophage

Use of recombinant Lactic Acid Bacteria to treat infectious diseases: a focus on human papillomavirus (HPV)-induced cancer

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Food-grade lactic acid bacteria (LAB) are good candidates for the development of mucosal vectors, and are attractive alternatives to attenuated pathogens. Indeed, for decades, these non-pathogenic bacteria have been used for fermentation, food production and preservation; as such, they are considered generally recognized as safe (GRAS) microorganisms. In addition, there is now strong evidence to support interest in the use of these microorganisms for the development of novel live vectors for human and animal health purposes. Most of this work has been based on the LAB model, *Lactococcus lactis*, which is suitable for heterologous expression of therapeutic proteins and some probiotic strains of *Lactobacillus* spp. Thus, recombinant strains of lactococci and lactobacilli expressing bacterial and viral antigens, anti- or pro-inflammatory cytokines, antiproteases and antioxidant enzymes have been successfully tested for their beneficial effects in different murine models. In this presentation, I will summarize some of our most outstanding results on the use of LAB to deliver medical proteins to mucosal surfaces, in particular HPV-16 antigens to treat cervical uterine cancer.

Keywords: Lactic acid bacteria, cancer, HPV, cytokines

*Speaker