Goal | Breakthough Capability | Milestone

## Cell-free systems capable of natural and/or non-natural reactions.

### Ability to build reproducible and comparable cell-free systems for practical applications in bioengineering and biomanufacturing from multiple organisms, including non-model hosts.

- Complete characterization of the general effects of cell-growth harvest conditions and extract preparation parameters on bacterial cell-free extract behavior.
- Complete standardization of common-use bacterial cell-free system.
- Complete library of user-defined reaction components that could be used in a customizable cell-free system.
- Consistent ability to generate cell-free systems from any organism or a subset of organisms that make all types of desired products.

### Ability to build a cell, including the molecular subsystems that enable the processes of DNA replication, transcription, translation, energy regeneration, and membrane construction.

- Demonstrated ability to synthesize all components encoded by a (minimal/synthetic) cell using cell-free systems.
- Demonstrate and design a minimal genome that could support the construction of a cell, including regulation.
- Ability to build metabolic modules capable of supporting long-lasting energy regeneration.
- Ability to have ribosomes make ribosomes in a cell-free system.
- Expand the chemistry of living systems to make chemical reactions not possible with biological chemistry alone.
- Engineer compartmentalization and communication strategies for the design of (synthetic) cells.
- Replace test tubes with chemically-defined, standardized micro-vesicles to compartmentalize processes.

### Long-lasting, robust, and low-cost cell-free system for protein synthesis and biomanufacturing.

- Identify reagent instabilities in cell-free systems across multiple organisms and all biological kingdoms.
- Alleviate reagent instabilities and prolong the half-life of cell-free reagents from a few hours to several days using inexpensive substrates.
- Avoid inhibition (poisoning) of cell-free reactions by byproducts or the desired products.
- Stabilize catalysts to facilitate cell-free reactions on the order of weeks.
- Robust and scalable production of cell-free systems that last for weeks.

### Ability to use cell-free systems to inform cellular design of genetic parts and circuits.

- Ability to use next-generation sequencing read-outs to quantitatively map performance of genetic designs in cell-free systems.
- Ability to identify new genetic parts in cell-free systems for any bacterial host to facilitate forward engineering in cells.
- Ability to identify new genetic circuits in cell-free systems for any bacterial host to facilitate forward engineering in cells.
- Ability to identify new genetic circuits in cell-free systems for any eukaryotic host to facilitate forward engineering in cells.
- Accelerate the development of any non-model host into useful chassis organisms for engineering biology with cell-free systems.

### Decentralized, portable, on-demand sensing and manufacturing using cell-free systems.

- Ability to use safe lysates low in endotoxin for sensing and manufacturing objectives.
- Demonstrate portability (two-year storage duration of freeze-dried reactions without loss of functionality) of cell-free systems.
- Increase productivity and rate of cell-free reactions.
- Point-of-care cell-free protein production system ready for validation by the Food and Drug Administration.
- Point-of-care cell-free protein therapeutic and vaccine production system ready for validation by the Food and Drug Administration.

### Ability to manufacture any targeted glycosylated protein or metabolite using cell-free biosynthesis.

- Ability to build modular, versatile cell-free platforms for glycosylation pathway assembly.
- Expanded set of glycosylation enzyme-variants that efficiently install eukaryotic glycans.
- Production of bacterial glycoconjugate vaccines in cell-free systems.
- Expanded set of enzymes capable of glycosylating metabolites *in vitro*.
- Cell-free pipelines to produce and assess the functionality of diverse, human glycosylated protein therapeutics
- Ability to produce any glycosylated protein therapeutics and vaccines at the point-of-care in less than one week.

## On-demand production of single-cell hosts capable of natural and non-natural biochemistry.

### Ability to grow any host, anytime, in a controlled and regulated setting.

- Establish protocols for the development of media that support cellular viability for non-model organisms.
- Develop robust, high-throughput screens for rapidly assaying useful properties in libraries of organisms.
- Robust screening of useful chassis beyond model organisms.
- Use output of high-throughput screens/sensors and computer control to amplify a signal or expand a cell line that produces a product of interest.

### Routine domestication of non-model organisms through DNA delivery and genetic modification.

- Catalog and assay current methodologies and tools for carrying out DNA delivery in microbial/mammalian systems and plant systems.
- Development of well-characterized and robust insertion sites in plant genomes.
- Develop high-throughput, targeted editing and rapid-genome-evolution tools that couple genetic changes to phenotypic changes.
- Develop high-throughput methods that can be done in parallel for DNA delivery (using standard methods) into non-model hosts.
- Develop high-throughput, genome-wide editing tools for non-model organisms.
- Routine genetic manipulation of any non-model host in less than one week from first isolation.
- Establish a suite of gene-editing tools for the rapid insertion and/or deletion of genetic elements in diverse primary mammalian cells.
- Establish robust temporal and/or spatial control of gene expression in mammalian cells.
- Develop universal approaches to transforming any plant.
- Characterize basic DNA parts for expression strength in non-model organisms.
- Develop broad-host-range vectors for a variety of model and non-model organisms.

### Ability to build and control small molecule biosynthesis inside cells by design or through evolution.

- Identify model organisms for performing specific types of chemistries or organisms that have native precursor biosynthesis pathways for specific classes of molecules.
- Construct a limited number of model host organisms for synthesizing all-natural products.
- Construction of single-cell organisms for production of unnatural derivatives of natural products.
- Software and hardware for optimizing titer, rate, and yield of any product produced by any host.
- On-demand construction of single cell organisms for production of nearly any molecule of interest, including organic chemicals and polymers.
- Precise temporal control of gene expression for well-studied systems.
- Temporal control over multiplexed regulation of many genes in parallel.

### Spatial control over, or organization of, metabolic pathways in cells and construction of unnatural organelles.

- Tools to target heterologous proteins to various subcellular compartments.
- Inducible synthesis of organelles.
- Methods and tools to reprogram transport of metabolites and compartmentalization of biochemical reactions.
- Alter chemical conditions within the organelle/microcompartment.
- Gain-control for selective permeability in and out of the organelle.
- Multiple orthogonal organelles/microcompartments in the same cell for compartmentalizing different parts of a pathway.

### Production and secretion of any protein with the desired glycosylation or other post-translational modifications.

- One or more microbial hosts capable of producing laboratory-scale quantities of a single glycoform of a desired protein.
- A few microbial hosts capable of secreting functional versions of proteins with post-translational modifications.
- Ubiquitous control of post-translational modification in a diverse array of hosts.

## On-demand fabrication and modification of multicellular organisms.

### Ability to control differentiation and de-differentiation of cells within a population.

- On-demand, reproducible functionalization of simple micro-tissues or micro-consortia made up of two or more engineered cell types.
- Programmable and regulatable pathways that can be induced to differentiate or de-differentiate somatic cells.

### Ability to characterize and control the three-dimensional architecture of multicellular systems.

- Characterize existing tissue components and standardize measurements to evaluate function.
- Identification of novel 3D scaffold designs that can lead to desirable cellular properties.
- Create modular, synthetic communication circuits that can be implemented in tissues to allow for control of new or existing cellular communication systems.
- Bottom-up design and construction of whole organs at the centimeter-length scale.

### Ability to achieve stable non-heritable changes in somatic cells.

- Routine delivery of biomolecule "effectors" (i.e., DNA, RNA, proteins) into slowly-dividing or non-dividing cells.
- Generation of effective artificial epigenetic chromosomal states and maturation of the emerging field of chromatin engineering.
- Ability to generate cell states that are stable and effective after the inducer/effector is removed in certain model tissues.
- Nimble adaptation of somatic cell engineering technologies to any natural tissue at any developmental stage.

### Ability to make predictable and precise, targeted, heritable changes through germline editing.

- Complete sequence of select host genomes to allow design of targets for gene editing.
- Efficient germline transformation systems developed in targeted hosts.
- Ability to deliver transgene constructs to most (>90%) somatic cells in a higher organism to rapidly prototype transgenic phenotypes.
- Ability to coordinate engineered multicellular functions in intact organisms via orthogonal communication systems.
- Routine, on-demand, efficient germline editing for any targeted host of interest at high-throughput scale.
- Define and validate tissue-specific DNA parts in plants.
- Temporally controlled transgene expression that works on the scale of generations.
- Efficient gene editing in differentiated cells.
- Ability to domesticate engineered biological parts to confer immune tolerance in immunocompetent organisms.
- On-demand gene editing of organisms with desired traits.

## Generation of biomes and consortia with desired functions and ecologies.

### Ability to control cell-to-cell communication between different species.

- Tightly-controlled promoter-response regulator systems that enable intra- and inter-species cellular communication.
- Synthetic cell-to-cell communication elements and networks that function in a broad range of host organisms.
- Signal-response pathways that function in synthetic communities of 5-10 organisms, employing a variety of pathway types and host species.
- Ability to produce engineered microbes that can reliably invade and coexist within a complex community and manipulate the consortium/biome function and behavior.

### Ability to characterize, manipulate, and program three-deminsional architecture of the biome.

- Use of existing technologies to better understand the species composition and collective components of microbial communities and consortia.
- Non-destructive, 3D visualization of microbial communities from a broad range of environments.
- Ability to manipulate the 3D architecture of natural or engineered communities using external inputs.
- Programmed communities that self-assemble into a desired 3D architecture.

### Ability to control and/or define the function of an engineered microbial community/biome.

- Ability to combine species with specialized functions to enable the production of desired products.
- Assembly of consortia to produce desired molecules/products, considering community-level metabolic flux.
- Plug-and-play assembly of consortia to produce desired molecules/products from specific starting materials, considering community level metabolic flux and organism-to-organism communication.
- On demand assembly of consortia that are programmed to respond dynamically.

### Targeted modification of an existing microbiome to enable new functions or address dysbiosis through the addition, removal, or reorganization of the community members.

- Use of existing technologies to characterize functions of microbial communities from a broad range of environments.
- Characterize how select microbiomes respond to changes in the environment.
- Predictive models of microbiome function and response to a broad range of environmental and ecological changes.
- Ability to modify an existing biome or consortia as desired.

2 Years | 5 Years | 10 Years | 20 Years