

Building a Bridge between Cell-Free Experimentation and Cellular Metabolic Engineering

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Project Goals: We will address the challenge of designing, building, and optimizing biosynthetic pathways in cells in a new interdisciplinary venture that establishes the clostridia Foundry for Biosystems Design (cBioFAB). Working both *in vitro* and *in vivo*, the goal is to interweave and advance state-of-the-art computational modeling, genome editing, omics measurements, systems-biology analyses, and cell-free technologies to expand the set of platform organisms that meet DOE bioenergy goals. cBioFAB will (i) reconceive how we engineer complex biological systems by linking pathway design, prospecting, validation, and production in an integrated framework, (ii) enable systems-level analysis of the David T. Jones collection, one of the largest collections of clostridia strains in the world, to uncover novel metabolic pathways, regulatory networks, and genome editing machinery, and (iii) open new paths for synthesis of next-generation biofuels and bioproducts from lignocellulosic biomass.

Speeding up design-build-test (DBT) cycles is a fundamental challenge facing metabolic engineering. We plan to address this challenge by establishing the clostridia Foundry for Biosystems Design (cBioFAB) (Figure 1). To this end, we report a new *in vitro* prototyping and rapid optimization of biosynthetic enzymes approach (termed iPROBE) to inform cellular metabolic engineering. In our approach, cell-free cocktails for synthesizing target small molecules are assembled in a mix-and-match fashion from crude cell lysates selectively enriched with pathway enzymes. This approach reconstructs pathways in two steps where the first step is enzyme synthesis via cell-free protein synthesis and the second step is enzyme utilization via substrate and cofactor addition to activate small molecule synthesis. We demonstrate that iPROBE can quickly study pathway enzyme ratios, tune individual enzymes in the context of a multi-step pathway, screen enzyme variants for high-performance enzymes, and discover enzyme functionalities. The rapid ability to build pathways *in vitro* using iPROBE allows us to generate large amounts of data describing pathway operation under several operating conditions. However, to date no easy method of analysis provides informative bridging of cell-free data to cellular metabolic engineering. In this work, we address this limitation by developing a

quantitative metric that combines titer at reaction completion, rate during the most productive phase of pathway operation, and enzyme expression as measured by protein solubility (TREE score). By reducing the complexity of available cell-free data to one value we can now quickly screen and rank pathways in the cell-free environment and provide useful information for cellular metabolic engineering. We demonstrate iPROBE and the use of the TREE score for the production of 3-hydroxybutyrate and *n*-butanol in *Clostridium*, an industrially relevant non-model organism. This work shows that iPROBE can be used for multiple enzymatic pathways and for non-model organisms. We anticipate that iPROBE will facilitate efforts to define, manipulate, and understand metabolic pathways for accelerated DBT cycles in the cell-free environment before engineering organisms.

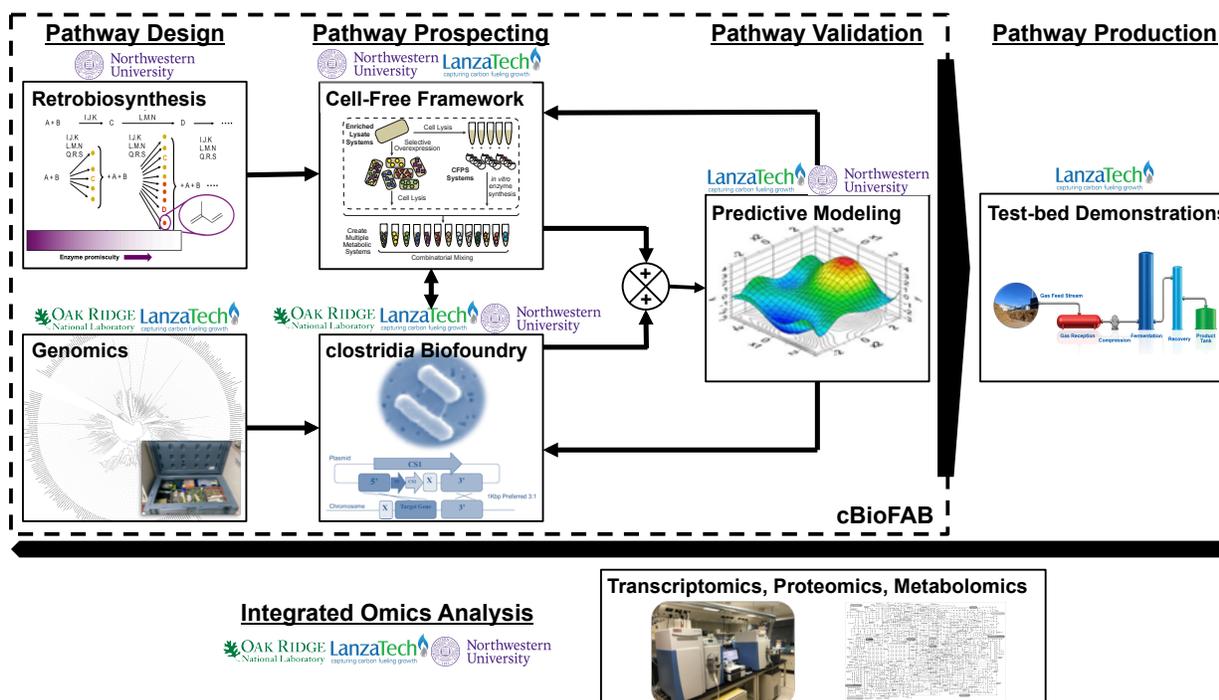


Figure 1. Proposed workflow for this project and participating units.

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