**02/09/2020**

“A molecular rheostat maintains ATP levels to drive a synthetic biochemistry system.”

By Paul Opgenorth…James Bowie

* Rheostat that regulates ATP levels by controlling flow down either an ATP generating or non ATP generating pathway according to the free phosphate concentration
* Implemented for production of isobutanol from glucose
* Maintains adequate ATP concentration even in the presence of ATPase contamination
* Used in design of continuously operating, self-sustaining synthetic biochemistry systems
* Need high yield, high productive for economic competitiveness of low-value chemicals such as biofuels
* Constraints imposed by having to maintain cell viability
* We want to build cell free pathways that can economically sustain high flux for long periods of time without the metabolic regulatory systems that exist in cells – requires new design principles
* Must consider generation, regulation, and recycling of high-energy cofactors such as ATP, NADH, and NADPH.
* High energy cofactors generated in catabolic or breakdown phase (glycolysis), used and regenerated in anabolic or biosynthetic phase
* Demand perfect stoichiometry – if 2 atp generated in breakdown, 2 atp used in biosynthetic
* What is a rheostat?
  + A contrivance for adjusting or regulating the strength of electrical currents, operating usually by the intercalation of resistance which can be varied at will

**A stoichiometric isobutanol pathway**

* The 2-ketoacid isobutanol biosynthetic phase employs two equivalents of pyruvate and two equivalents of NADPH, but no ATP
  + Canonical pathway produces 2 equivalents of NADPH and ATP
* They designed a 14 enzyme pathway to make the cofactor use stoichiometric
* They use COPASI to model and identify likely key steps in the pathway
  + An open source software app for creating and solving mathematical models of biological processes such as metabolic network, cell-signaling pathways, regulatory networks, infectious diseases, etc
* Found that Hex and Pfk were critical for achieving flux through the system
* Can focus on detecting only phosphate
  + Can we assume that phosphate concentration is indicative of ATP concentration? Yes if that’s the only thing being phosphorylated in the artificial cell
* Based on high or low P concentrations, certain pathways are preferred

**Design of an ATP rheostat**

* Results showed that ATP depletion is major problem for the long-term sustainability of the stoichiometric reaction system
* Design something that restores and regulates ATP levels as an intrinsic feature of the system
* Molecular rheostat has two comp. pathways that transform G3P into 3PG
* One generates atp, other doesn’t
* mGapDH-PGK branch produces an additional equivalent of ATP compared to the GapN-only branch
* rheostat responds to the depletion of ATP and acts to restore ATP by using the phosphorylating mGapDH branch
* The molecular rheostat reaches a steady state at any time the flux through mGapDH and PGK is greater than the ATPase activity being introduced
  + Steady state can be achieved with wide range of ATPase activities

**Engineering mGapDH**

* They introduced basic residues to help create (30 fold difference) affinity for Nadp+ over NAD+

**Isobutanol production with the molecular rheostat**

* Rheostat reaction held and ATP steady state concentration at round 600 µM for 48 h

**Enzyme stability limits production**

* The reaction completely stopped after 72 hours
* A precipitate began to form after 24 hrs
  + Conincided w decrease in isobutanol production
  + Enzymes were denaturing over time, bc of increasing isobutanol conc
* Complete inactivation of GapN and KivD to isobutanol
* Identified a handful of enzymes that are high priority targets for improving their stability to increase the overall sustainability of the system

**Discussion**

* We can quickly design, implement, and further optimize systems in cell-free synthetic biochemistry environments
* Can quickly identify and isolate
* Synthetic biochemistry systems should run longer, be more flexible
* Rheostat is like metabolite proofreading
* Molecular purge valve – regulates production and consumption of nadp+/nadph
* Major practical limitation for commercial feasibility remains the procurement of enzymes that are sufficiently stable to warrant the added initial investment required to produce them

“Light-driven ATP production promotes mRNA biosynthesis inside hybrid multi-compartment artificial protocells.”

By Emiliano Altamura…Fabio Mavelli

* Hybrid multi compartment approach to build Artificial Simplified-Autotroph Protocells (ASAPs)
* Chromatophores from *Rhodobacter sphaeroides* performs photophosphorylation of ADP to ATP functioning as nanosized photosynthetic organellae when encapsulated inside artificial giant phospholipid vesicles
* Under continuous illumination chromatophores produce ATP that in turn sustains the transcription of a DNA gene by t7 RNA polymerase inside ASAPs
* CryoEM and spectroscopy for characterizing chromtaphore morphology and orientation of photphosphorylation proteins
  + Allow high ATp production rates
* mRNA biosynthesis inside individual vesicles has been determined by confocal microscopy
* hybrid multi compartment approach – time convenient , effective, promising
* We want autonomous generation of energy!
* ATP is generated in vivo by photosynthetic phosphorylation, oxidative phosphorylation, and substrate-level phosphorylation
* Photosynthetic and oxidative phosphorylations share a common design that requires the establishment of a proton gradient and a difference of chemical potential across the lipid membranes of cells - triggers ATP synthase
* Photosynthesis – gradient generated by absorption of light
* Single-compartment approach – vesicles functionalized with >90% oriented protein complexes able to transduce light energy in chemical energy in form of a proton gradient
* Hybrid Multi-Compartment Approach (HyMCA)
  + Entrapment of nanosized biological vesicles (< 100 nm) inside a giant artificial vesicle (> 1 µm)
* R sphae. Chromatophores used as light driven ATP synthesizing organellae trapped inside phospholipid giant unilamellar vesicles (GUVs)

**Obtaining functional chromatophores from R sphae**

* CryoEm and cryo electron tomography confirm present of closed spheroidal chromatophores
* Cryo em reveals presence of F0F1 ATP synthase complex in the functional orientation on the membrane of closed chromatophores

**Assaying ATP production by chromatophores in bulk**

* Externally added ADP and Pi
* Continuous illumination
* Amount of synthesized ATP quantified by luciferin-luciferase bioluminescence assay

**Assembling ATP-producing multi-compartment protocells**

* Use GUVs prepared by droplet transfer method using POPC as phospholipid
* Water-in-oil droplets transformed into GUVs

**Photosustaining mRNA biosynthesis in ASAPs**

**Conclusions**

* We have presented a prototype of a photoactive Artificial Simplified-Autotroph Protocell (ASAP), made of GUVs that encapsulate *R. sphaeroides* chromatophores able to transduce light energy in chemical energy. Chromatophores act as natural photosynthetic organellae enclosed in an artificial protocell converting ADP and Pi into ATP at the sole expense of light energy.

**2/12/2020**

**Ppt sent by Murray**

* Attempt at doing Tx Tl life extension
* Goal: increase longevity of Tx Tl rxn
  + Also assess output (total protein produced)
* Replicate what is reported in literature and improve on it
* Construct used – JoVe paper (*Sun et al* 2013)
  + Sigma 70 promoter with lambda phage operators
* Was not able to recreate Vincent paper results
* Consider the role of oxygen on Tx Tl, required for oxidative phosphorylation, to make ATP
* The basic buffer requirements are considered and explained
  + Tested different buffer environments and their fraction of total output over time
* Seem to be some base conditions that cause synergistic effects
* Adding reagents does not greatly enhance the overall longevity of TX TL, a much more consistent expression level is maintained
* Longevity of the reaction does not change with or without holes (oxygen access)
  + But affects are seen with the total output of the system
  + Higher reporter concentrations
* Kinda confusing why oxygen changes rate not longevity
* What happens if you increase DNA concentration?
* Effects of air are not as obvious
* Longevity of tx tl can be extended using bis-tris
  + Less linear expression
  + But also less absolute protein amount
* Attempting to increase tx/tl by manipulating reaction conditions most likely not the way
* Better to manipulate biochemical/metabolic pathways active during tx/tl
* Something about pH acidification/stress
* Or incorporate synthetic biochemistry modules from Bowie lab
  + Add NoxE to recycle NADH
  + Add GapM6 for continuous reduction of Nadp+ to Nadph
  + Add PHB pathway enzymes for carbon sink since PHB is a polymer that falls out of solution (pulling force)

**Protocols for Implementing an *Escherichia coli* Based TX-TL Cell-Free Expression System for Synthetic Biology**

<https://www.jove.com/video/50762/protocols-for-implementing-an-escherichia-coli-based-tx-tl-cell-free>

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