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Title: Repurposing the *Saccharomyces Cerevisiae* Peroxisome for Compartmentalizing Multi-enzyme Pathways
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Progress/Accomplishments:
Our project goal was to repurpose the *Saccharomyces cerevisiae* peroxisome to be a synthetic organelle as a generalizable strategy for compartmentalizing metabolic pathways. We chose the peroxisome because it is not required for yeast viability; thus, much of the endogenous luminal proteins could be replaced by engineered machinery. Towards this goal, we have improved heterologous protein import with an efficient and modular targeting sequence, characterized peroxisome membrane permeability to small molecules, and developed a synthetic protein trafficking strategy. This enabled the compartmentalization of a small metabolic pathway; however, it also illustrated a natural size-dependent permeability to small molecules. We have determined this permeability to be molecule-dependent, but in the range of 400 to 700 Daltons. Thus, the peroxisome can be used for some applications, but a reduced permeability limit will be required for more long-term application goals of altering the chemical environment to enable chemistry to be performed that would not be feasible in the cytoplasm.

An important early achievement was the development of metabolic enzyme sequestration providing a high-throughput quantitative assay for heterologous protein import. When the metabolic enzyme is sequestered, production of a green pigment is prevented. We used this assay to screen a linker library immediately upstream of the conventional peroxisome targeting sequence (PTS1). A sequence (ePTS1) enabling dramatically improved protein import was isolated. Critically, ePTS1 appears to be modular – broadening the range of protein cargoes than the conventional PTS1. The ePTS1 was integrated as a C-terminal tag on four proteins necessary for progression through the cell cycle and observed that induction of peroxisome import prevents growth. Additionally, we determined that the rate of ePTS1-mediated import is extremely high such that very little enzymatic activity is observed in the cytoplasm prior to import.

A long-standing debate in the peroxisome field has been the extent of permeability of the peroxisome to small molecules. Previous work has investigated this question using

purified peroxisomes where permeability up to approximately 700 Daltons was observed. However, an alternative hypothesis is that this observed permeability was an artifact of the purification process. The ability to efficiently target heterologous protein cargo with the ePTS1 tag combined with the metabolic enzyme sequestration assay provided a means to test this question *in vivo* with minimal perturbation. To keep the chemical properties of a small molecule substrate as consistent as possible while increasing the size of the molecule, we used a betaglucosidase sequestration assay with X (5-bromo-4-chloro-indoxyl)-glucoside substrates where the number of glucose molecules is varied to the molecular weight. Upon betaglucosidase-mediated hydrolysis, the X-glucoside increases fluorescence. By comparing fluorescence in a strain where betaglucosidase is compartmentalized in the cytoplasm to compartmentalization in the peroxisome, we were able to determine that there is, indeed, a size dependency to peroxisome permeability. The 571 Dalton X-cellobioside (i.e., containing two glucose molecules) can gain access to the peroxisome compartmentalized betaglucosidase, whereas the 733 Dalton X-cellotrioside (i.e., containing three glucose molecules) does not gain access to the peroxisome lumen.

We demonstrated that the two enzymes of this pigment-producing pathway can be compartmentalized in the peroxisome using the engineered ePTS1 tag. Thus, demonstrating enzymatic activity can be achieved in the organelle's lumen. Further, we showed that under certain conditions increases in production can be achieved with the increased local concentration in the peroxisome.

Most of these results are described in (Deloache, Russ, & Dueber, 2016). Additionally, an enzyme-coupled biosensor developed from this project was critical for the additional paper (Deloache et al., 2015).

Publications:

Deloache, W. C., Russ, Z. N., & Dueber, J. (2016). Towards repurposing the yeast peroxisome for compartmentalizing heterologous metabolic pathways. *Nature Communications*, *7*, 11152. http://doi.org/10.1038/ncomms11152

Deloache, W. C., Russ, Z. N., Narcross, L., Gonzales, A. M., Martin, V. J. J., & Dueber, J. (2015). An enzyme-coupled biosensor enables (S)-reticuline production in yeast from glucose. *Nature Chemical Biology*, *11*(7), 465–471. http://doi.org/10.1038/nchembio.1816