Parallel labeling experiments for pathway elucidation and ^{13}C metabolic flux analysis

Maciek R. Antoniewicz

Department of Chemical and Biomolecular Engineering, Metabolic Engineering and Systems
Biology Laboratory, University of Delaware, Newark DE 19716, USA

*Corresponding author:

Maciek R. Antoniewicz

Department of Chemical and Biomolecular Engineering

University of Delaware

150 Academy St

Newark, DE 19716

Tel.: 302-831-8960

Fax.: 302-831-1048

Email: mranton@udel.edu

Abstract

Metabolic pathway models provide the foundation for quantitative studies of cellular physiology through the measurement of intracellular metabolic fluxes. For model organisms metabolic models are well established, with many manually curated genome-scale model reconstructions, gene knockout studies and stable-isotope tracing studies. However, for non-model organisms a similar level of knowledge is often lacking. Compartmentation of cellular metabolism in eukaryotic systems also presents significant challenges for quantitative ^{13}C -metabolic flux analysis (^{13}C -MFA). Recently, innovative ^{13}C -MFA approaches have been developed based on parallel labeling experiments, the use of multiple isotopic tracers and integrated data analysis that allow more rigorous validation of pathway models and improved quantification of metabolic fluxes. Applications of these approaches open new research directions in metabolic engineering, biotechnology and medicine.

Introduction

Quantitative studies of cellular metabolism are of central importance in modern biological sciences, including in metabolic engineering, medicine, systems and synthetic biology [1-4]. ^{13}C -metabolic flux analysis (^{13}C -MFA) is the most rigorous and widely used method for quantifying *in vivo* metabolic fluxes [5]. Initially developed in mid-1990s [6], ^{13}C -MFA has been continually refined and improved over the past two decades [7]. In ^{13}C -MFA, a tracer experiment is performed where a ^{13}C -labeled substrate, e.g. [1,2- ^{13}C]glucose, is added to the culture medium and the cells are allowed to metabolize it. This results in the incorporation of ^{13}C atoms into intracellular metabolites, biomass macromolecules, e.g. proteins, lipids, RNA and DNA; and

secreted metabolic products, e.g. acetate, lactate. The incorporation of ^{13}C is then measured by mass spectrometry (MS), tandem mass spectrometry (MS/MS), or nuclear magnetic resonance (NMR) [8-11]. After correction for natural isotope abundances, the labeling data are fit to a metabolic model using one of several available software tools for ^{13}C -MFA that return the estimated metabolic fluxes and several statistical metrics [5].

Initially, ^{13}C -MFA was applied to a handful of model organisms that were of immediate importance to the biotechnology industry [7]. In most cases, greatly simplified models were used for flux calculations due to computational and modeling limitations. These limitations were later eliminated with the development of the EMU framework [12], which allowed more realistic pathway models to be considered for ^{13}C -MFA [13]. In recent years, ^{13}C -MFA has been applied increasingly to study a wide range of non-model organisms for which pathway models are not nearly as well understood [14]. ^{13}C -MFA is also increasingly applied to study mammalian systems [15], plant systems [16,17], and other eukaryotic organisms [18], where complications related to compartmentation of metabolism, e.g. duplication of metabolic pathways in multiple compartments, and slow labeling dynamics present significant challenges for ^{13}C -MFA [19]. In this paper, we review recent advances in methods for pathway elucidation and ^{13}C -MFA based on parallel labeling experiments, where multiple isotopic tracers are performed in parallel and the data are analyzed concurrently. We highlight important experimental and computational approaches and discuss how these can be applied to extend the capabilities of ^{13}C -MFA for pathway analysis.

Pathway elucidation in non-model organisms

The costs of genome sequencing have dropped dramatically in the past decade, resulting in an explosion in the number of available genomes and genome-scale models (GSMs) [20]. The majority of current GSMs have been automatically generated and have not undergone rigorous experimental validation [21,22]. This raises justifiable concerns about the accuracy of these models [23,24]. In a number of cases where GSMs were subjected to experimental validation, major mistakes and omissions were identified. As an example, in 2008 two manually-curated GSMs were published for the industrially important bacterium *Clostridium acetobutylicum* [25,26]. In subsequent years, several validation studies were conducted [27-29], where parallel labeling experiments were applied to establish the direction of carbon flow in the metabolic pathways. It was identified that both GSMs incorrectly predicted the structure of the TCA cycle and missed the presence of *Re*-citrate synthase as the first enzyme of the TCA cycle in *C. acetobutylicum*. This example illustrates the importance of experimental validation of GSM and the practical utility of applying stable-isotope tracing approaches for this purpose. Commonly used tracers for pathway analysis include: positionally-labeled ^{13}C -glucose, ^{13}C -acetate, ^{13}C -pyruvate, ^{13}C -aspartate, and ^{13}C -glutamate tracers, as well as ^{13}C -formate and $^{13}\text{CO}_2$. Additionally, ^{13}C -carbohydrate tracers can be applied to determine the relative activity of glycolysis pathway, pentose phosphate pathway and Entner–Doudoroff pathway [30].

Pathway validation studies were recently completed for a wide range of non-model organisms, including *Dehalococcoides* sp. [31,32], *Thermus thermophilus* [33], *Geobacillus* sp. [34,35], *Thermoanaerobacter* sp. [36], *Synechocystis* sp. [37], *Sulfolobus* sp. [38], *Heliobacterium modesticaldum* [39], and *Chlorobaculum tepidum* [40]. Important new insights from these studies include the identification of *Re*-citrate synthase, as opposed to the better known *Si*-citrate

synthase, as the first enzyme of the TCA cycle in many organisms, and the identification of citramalate pathway as an important pathway for isoleucine biosynthesis in a much larger set of organisms than previously assumed. One-carbon metabolism was also extensively studied in non-model organisms through parallel labeling experiments. Insights from these studies provide a solid foundation for future research on these organisms, as well as serving as a guide to improve future genome annotations and construction of GSMs [20].

Compartmentation of metabolism in eukaryotes

In eukaryotic cells, compartmentation of metabolites and metabolic reactions in different organelles (e.g. cytosol, mitochondrion, peroxisome) presents a significant challenge for ^{13}C -MFA [41]. Currently, there is no general approach that allows compartment-specific fluxes to be determined with high precision. While it has been recognized that metabolism of certain ^{13}C -tracers can in theory produce different labeling patterns in different compartments from which then compartment-specific fluxes could be determined [42], application of this approach has been hampered by difficulties in obtaining compartment-specific labeling measurements. With current analytical techniques, only average labeling patterns and metabolite levels from all compartments within a cell can be measured.

As an alternative approach, compartment-specific labeling patterns can be inferred from labeling of so-called proxy metabolites that are produced only in one compartment. For example, it has been suggested that under certain experimental conditions lactate labeling is a good proxy for the labeling of cytosolic pyruvate pool, whereas alanine reflects largely the labeling of mitochondrial pyruvate pool [42]. With this assumption, it is possible to determine certain compartment-

specific fluxes in eukaryotic cells. It would be valuable to identify additional proxy metabolites, and thus expand the amount of compartment-specific labeling information. This would undoubtedly improve our ability to measure compartment-specific metabolic fluxes in eukaryotic systems.

A second alternative solution may be found in a new framework that was recently developed for ^{13}C -MFA of co-cultures. Gebreselassie et al. demonstrated that precise metabolic fluxes can be estimated in mixed microbial cultures without physically separating the cells [43]. In this approach, species-specific fluxes are determined from total biomass labeling obtained using conventional mass spectrometry. To quantify species-specific fluxes the species are treated as separate compartments in the model, each with its own unique metabolism and metabolite labeling. To describe the labeling of total biomass, a mixing parameter is introduced that captures the fractional contribution of each species' specific labeling to total biomass labeling. The mixing parameter(s) are estimated concurrently with the fluxes for each separate species in the co-culture. In theory, a similar approach could be applied to determine compartment-specific fluxes in eukaryotic cells. This would eliminate the need to physically separate molecules from different compartments, or rely on indirect isotopic labeling information through proxy metabolites. If successful, this would be the first general approach for determining compartment-specific metabolic fluxes in eukaryotic cells. Initial studies applying this strategy have been encouraging [44,45].

Finally, it is worth noting two other recent studies where a combination of ^2H and ^{13}C -tracers were applied to determine compartment-specific NADPH metabolism in mammalian cells

[46,47]. These studies highlight the potential of using multiple different isotopes (e.g. ^{13}C , ^2H , ^{15}N , ^{18}O) for enhanced pathway elucidation that should be explored more in the future.

Parallel labeling experiments for ^{13}C -flux analysis

^{13}C -MFA is traditionally conducted using a single tracer experiment. An alternative approach for ^{13}C -MFA is to use multiple parallel labeling experiments, which can offer a number of advantages [48]. For a single tracer experiment, the choice of isotopic tracer is known to impact significantly which fluxes in a pathway model can be observed, as well as imposing a theoretical limit on the flux precision. Thus, optimal tracer selection is now well-recognized as an important step in ^{13}C -MFA [49]. In recent years, improved approaches were developed for selection of optimal isotopic tracers [50-52]. Based on these studies, however, it has also become increasingly clear that for realistically sized metabolic models there is no single best tracer that allows all fluxes in the model to be determined with high precision [53]. Parallel labeling experiments provide a practical solution to this problem with only a modest increase in experimental, analytical and computational effort.

In the parallel labeling approach, two or more tracer experiments are conducted in parallel, where in each experiment a different isotopic tracer is used. The cultures are generally started from the same inoculum to minimize biological variability. The data from the different experiments are then integrated for ^{13}C -MFA by concurrently fitting all measurements to a single flux model [48,54] (Figure 1). This approach allows individual tracers to be tailored for specific parts of the network model so that all fluxes of interest can be estimated with high precision [50].

In microbial systems, where minimal media containing a single carbon source are often used, different isotopic forms of the same substrate are chosen (Figure 2). For example, parallel labeling experiments with [1,2- ^{13}C]glucose and [1,6- ^{13}C]glucose tracers are particularly useful for precise flux analysis in microbial cells [55]. In mammalian systems, where complex media with many carbon sources are used, different substrates are typically chosen as ^{13}C -tracers. For example, parallel labeling experiments with [1,2- ^{13}C]glucose and [U- ^{13}C]glutamine have been shown to be especially valuable for high flux resolution [52,56]. An additional advantage of the parallel labeling experiments approach is that the labeling time can be reduced significantly by introducing multiple entry-points of isotopes into metabolism. With carefully selected ^{13}C -tracers isotopic steady-state can be achieved within a reasonable time frame; for example, ^{13}C -glucose tracers are known to label metabolites in the upper part of central metabolism typically within 1 hr, while ^{13}C -glutamine tracers allow fast labeling of TCA cycle metabolites, typically within 3 hrs [56]. Compared to a single ^{13}C -glucose tracer experiment that may require 24 hrs or longer to reach isotopic steady state [57], parallel labeling experiments can reduce the length of a tracer study by several fold [56]. The improved temporal resolution provides new opportunities to explore dynamics of flux changes in mammalian cell cultures [19], for example, changes in fluxes occurring during the transition from growth phase to stationary phase, or during differentiation of e.g. stem cells.

The parallel labeling approach was recently termed COMPLETE-MFA, short for complementary parallel labeling experiments technique for metabolic flux analysis [53]. The key strength of the COMPLETE-MFA approach is the potential to generate complementary labeling information that significantly improves precision of fluxes in complex systems. The number of parallel

labeling experiments to be performed is an important consideration, which depends on: the desired flux precision, the number and type of available labeling measurements, the complexity of the metabolic network model, the availability and cost of isotopic tracers, and the effort required to perform multiple labeling experiments [48]. In the ideal case, two to three parallel labeling experiments with complementary tracers would be sufficient. To date, the most extensive COMPLETE-MFA study performed considered integrated analysis of 14 parallel labeling experiments in *E. coli* [58]. Other comprehensive COMPLETE-MFA studies are highlighted in Table 1. An important insight from these studies is that performing more than two to three parallel labeling experiments does not significantly improve flux precision [58]. Thus, a point of diminishing returns is reached beyond which it may no longer be justifiable to perform additional experiments, i.e. assuming optimal ^{13}C -tracers were identified and applied in the first place.

Conclusions

Metabolic network models play an important role in modern biological sciences, serving as the foundation for quantitative studies of cellular metabolism. With the ever-accelerating pace of genome sequencing and annotation, generation of metabolic pathway models has outpaced the capacity to experimentally validate the models. In the future, standardization of procedures for model validation through stable-isotope tracing techniques and implementation of best-practices for ^{13}C -MFA will facilitate the construction a high-quality pathway models [7]. Parallel labeling experiments and other approaches for measuring compartment-specific fluxes will also open new opportunities to investigate the complexities of eukaryotic metabolism. This may be employed, for example, to identify new drug target interventions in medicine and investigate the

metabolism of complex multi-species cultures in biotechnology. Finally, as the scope of genome-scale models increases beyond carbon metabolism, new opportunities are realized for more rigorous integration of omics-data for applications in metabolic engineering and beyond [59-61].

Acknowledgements

This work was supported by NSF CAREER award (CBET-1054120).

Tables

Table 1. Examples of large-scale COMPLETE-MFA studies

Number of parallel labeling experiments	Isotopic tracers used*	Organism studied	Reference
14	[1,2]gluc, [2,3]gluc, [4,5,6]gluc, [2,3,4,5,6]gluc, [1]gluc+[4,5,6]gluc, [1]gluc+[U]gluc (1:1), [1]gluc+[U]gluc (4:1), 20% [U]gluc; [1]gluc; [2]gluc; [3]gluc; [4]gluc; [5]gluc; [6]gluc	<i>E. coli</i>	[58]
6	[1]gluc; [2]gluc; [3]gluc; [4]gluc; [5]gluc; [6]gluc	<i>E. coli</i>	[53]
5	[1]xyl; [2]xyl; [3]xyl; [4]xyl; [5]xyl	<i>Geobacillus</i> LC300	[35]
5	20%, 40%, 60%, 80%, 100% [U]gluc	<i>E. coli</i>	[54]
5	[1]gluc; [2]gluc; [6]gluc; [U]gluc; [U]gln	HEK-293	[62]

* Abbreviations: gluc = glucose; xyl = xylose; gln = glutamine; [1,2]gluc = [1,2-¹³C]glucose; [U]gluc = [U-¹³C]glucose.

Figure captions

Figure 1. Parallel labeling experiments approach for ^{13}C -metabolic flux analysis (^{13}C -MFA).

Two or more tracer experiments are conducted in parallel. In each experiment a different isotopic tracer is applied. The isotopic labeling data from all experiments are then integrated for ^{13}C -MFA by concurrently fitting all measurements to a single flux model.

Figure 2. Parallel labeling experiments in microbial cells and mammalian cells for ^{13}C -MFA. In microbial systems, where minimal media with a single carbon source are often used, different isotopic forms of the same substrate are chosen. In mammalian systems, where complex media with multiple carbon sources are used, different substrates are chosen as ^{13}C -tracers.

References

Papers of special interest (*)

Ref. 7. Crown and Antoniewicz.

This paper describes current best-practices for performing stable-isotope tracing studies and ^{13}C -MFA. The guidelines outlined in this paper facilitate rigorous data analysis, data sharing and validation of metabolic pathway models.

Ref. 14. Zhang et al.

This paper describes the Central Carbon Metabolic Flux Database (CeCaFDB), a continually updated, manually curated, literature-derived fluxomics database. In addition to serving as a central repository of fluxomics data, CeCaFDB supports interactive visualization of flux distributions for improved data interpretation.

Ref. 20. Saha et al.

This paper provides an overview of the available biological databases and automated/semi-automated tools for reconstruction of comprehensive metabolic pathway models, as well as methodologies for integration of omics data in metabolic models.

Ref. 53. Leighty and Antoniewicz.

This paper describes the COMPLETE-MFA approach for comprehensive analysis of parallel labeling experiments. In this study, six parallel labeling experiments were successfully integrated for high-resolution flux analysis in *E. coli*.

Papers of outstanding interest (**)

Ref. 42. Buescher et al.

This paper provides general guidelines for interpreting ^{13}C metabolite labeling patterns from steady-state and dynamic stable-isotopic tracer experiments. Important pitfalls and challenges in the analysis of isotopic labeling data are discussed.

Ref. 48. Crown and Antoniewicz.

This paper provides a thorough review of past and present methodologies for stable-isotope tracing, pathway analysis and ^{13}C -metabolic flux analysis, with a special focus on parallel labeling experiments.

Ref. 58. Crown et al.

This paper describes the most extensive parallel labeling experiments study performed so far. Here, 14 parallel labeling experiments were successfully integrated for high-resolution ^{13}C -MFA

in *E. coli*. Optimal ^{13}C -tracers were identified for measuring fluxes in different parts of metabolism.

Ref. 59. King et al.

This paper describes the evolution of genome-scale models beyond metabolism for applications in metabolic engineering. A new generation of genome-scale models and methods are being developed that enable improved omics-data integration and new types of modeling predictions.

1. Antoniewicz MR, Stephanopoulos G, Kelleher JK: **Evaluation of regression models in metabolic physiology: predicting fluxes from isotopic data without knowledge of the pathway.** *Metabolomics* 2006, **2**:41-52.
2. Metallo CM, Vander Heiden MG: **Understanding metabolic regulation and its influence on cell physiology.** *Mol Cell* 2013, **49**:388-398.
3. Hiller K, Metallo CM: **Profiling metabolic networks to study cancer metabolism.** *Curr Opin Biotechnol* 2013.
4. Toya Y, Shimizu H: **Flux analysis and metabolomics for systematic metabolic engineering of microorganisms.** *Biotechnol Adv* 2013, **31**:818-826.
5. Antoniewicz MR: **Methods and advances in metabolic flux analysis: a mini-review.** *J Ind Microbiol Biotechnol* 2015, **42**:317-325.
6. Zupke C, Stephanopoulos G: **Intracellular flux analysis in hybridomas using mass balances and in vitro (^{13}C) nmr.** *Biotechnol Bioeng* 1995, **45**:292-303.

- * 7. Crown SB, Antoniewicz MR: **Publishing ^{13}C metabolic flux analysis studies: a review and future perspectives.** *Metab Eng* 2013, **20**:42-48.
8. Antoniewicz MR, Kelleher JK, Stephanopoulos G: **Accurate assessment of amino acid mass isotopomer distributions for metabolic flux analysis.** *Anal Chem* 2007, **79**:7554-7559.
9. Choi J, Grossbach MT, Antoniewicz MR: **Measuring complete isotopomer distribution of aspartate using gas chromatography/tandem mass spectrometry.** *Anal Chem* 2012, **84**:4628-4632.
10. Szyperski T: **Biosynthetically directed fractional ^{13}C -labeling of proteinogenic amino acids. An efficient analytical tool to investigate intermediary metabolism.** *Eur J Biochem* 1995, **232**:433-448.
11. Antoniewicz MR: **Tandem mass spectrometry for measuring stable-isotope labeling.** *Curr Opin Biotechnol* 2013, **24**:48-53.
12. Antoniewicz MR, Kelleher JK, Stephanopoulos G: **Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions.** *Metab Eng* 2007, **9**:68-86.
13. Ravikirithi P, Suthers PF, Maranas CD: **Construction of an E. Coli genome-scale atom mapping model for MFA calculations.** *Biotechnol Bioeng* 2011, **108**:1372-1382.
- * 14. Zhang Z, Shen T, Rui B, Zhou W, Zhou X, Shang C, Xin C, Liu X, Li G, Jiang J, et al.: **CeCaFDB: a curated database for the documentation, visualization and comparative analysis of central carbon metabolic flux distributions explored by ^{13}C -fluxomics.** *Nucleic Acids Res* 2014.
15. Young JD: **Metabolic flux rewiring in mammalian cell cultures.** *Curr Opin Biotechnol* 2013, **24**:1108-1115.

16. Junker BH: **Flux analysis in plant metabolic networks: increasing throughput and coverage.** *Curr Opin Biotechnol* 2014, **26**:183-188.
17. Kruger NJ, Ratcliffe RG: **Fluxes through plant metabolic networks: measurements, predictions, insights and challenges.** *Biochem J* 2015, **465**:27-38.
18. Niklas J, Schneider K, Heinzle E: **Metabolic flux analysis in eukaryotes.** *Curr Opin Biotechnol* 2010, **21**:63-69.
19. Ahn WS, Antoniewicz MR: **Towards dynamic metabolic flux analysis in CHO cell cultures.** *Biotechnol J* 2012, **7**:61-74.
- * 20. Saha R, Chowdhury A, Maranas CD: **Recent advances in the reconstruction of metabolic models and integration of omics data.** *Curr Opin Biotechnol* 2014, **29**:39-45.
21. Kim J, Reed JL: **Refining metabolic models and accounting for regulatory effects.** *Curr Opin Biotechnol* 2014, **29**:34-38.
22. Heavner BD, Price ND: **Transparency in metabolic network reconstruction enables scalable biological discovery.** *Curr Opin Biotechnol* 2015, **34C**:105-109.
23. Tang JK, You L, Blankenship RE, Tang YJ: **Recent advances in mapping environmental microbial metabolisms through ¹³C isotopic fingerprints.** *J R Soc Interface* 2012, **9**:2767-2780.
24. Feng X, Zhuang WQ, Colletti P, Tang YJ: **Metabolic pathway determination and flux analysis in nonmodel microorganisms through ¹³C-isotope labeling.** *Methods Mol Biol* 2012, **881**:309-330.
25. Senger RS, Papoutsakis ET: **Genome-scale model for *Clostridium acetobutylicum*: Part I. Metabolic network resolution and analysis.** *Biotechnol Bioeng* 2008, **101**:1036-1052.

26. Lee J, Yun H, Feist AM, Palsson BO, Lee SY: **Genome-scale reconstruction and in silico analysis of the *Clostridium acetobutylicum* ATCC 824 metabolic network.** *Appl Microbiol Biotechnol* 2008, **80**:849-862.
27. Au J, Choi J, Jones SW, Venkataramanan KP, Antoniewicz MR: **Parallel labeling experiments validate *Clostridium acetobutylicum* metabolic network model for C metabolic flux analysis.** *Metab Eng* 2014, **26**:23-33.
28. Amador-Noguez D, Feng XJ, Fan J, Roquet N, Rabitz H, Rabinowitz JD: **Systems-level metabolic flux profiling elucidates a complete, bifurcated tricarboxylic acid cycle in *Clostridium acetobutylicum*.** *J Bacteriol* 2010, **192**:4452-4461.
29. Crown SB, Indurthi DC, Ahn WS, Choi J, Papoutsakis ET, Antoniewicz MR: **Resolving the TCA cycle and pentose-phosphate pathway of *Clostridium acetobutylicum* ATCC 824: Isotopomer analysis, in vitro activities and expression analysis.** *Biotechnol J* 2011, **6**:300-305.
30. Klingner A, Bartsch A, Dogs M, Wagner-Dobler I, Jahn D, Simon M, Brinkhoff T, Becker J, Wittmann C: **Large-Scale ¹³C flux profiling reveals conservation of the Entner-Doudoroff pathway as a glycolytic strategy among marine bacteria that use glucose.** *Appl Environ Microbiol* 2015, **81**:2408-2422.
31. Tang YJ, Yi S, Zhuang WQ, Zinder SH, Keasling JD, Alvarez-Cohen L: **Investigation of carbon metabolism in "*Dehalococcoides ethenogenes*" strain 195 by use of isotopomer and transcriptomic analyses.** *J Bacteriol* 2009, **191**:5224-5231.
32. Zhuang WQ, Yi S, Bill M, Brisson VL, Feng X, Men Y, Conrad ME, Tang YJ, Alvarez-Cohen L: **Incomplete Wood-Ljungdahl pathway facilitates one-carbon metabolism**

- in organohalide-respiring *Dehalococcoides mccartyi*. *Proc Natl Acad Sci U S A* 2014, 111:6419-6424.**
33. Swarup A, Lu J, DeWoody KC, Antoniewicz MR: **Metabolic network reconstruction, growth characterization and ¹³C-metabolic flux analysis of the extremophile *Thermus thermophilus* HB8. *Metab Eng* 2014, 24:173-180.**
  34. Tang YJ, Sapra R, Joyner D, Hazen TC, Myers S, Reichmuth D, Blanch H, Keasling JD: **Analysis of metabolic pathways and fluxes in a newly discovered thermophilic and ethanol-tolerant *Geobacillus* strain. *Biotechnol Bioeng* 2009, 102:1377-1386.**
  35. Cordova LT, Antoniewicz MR: **¹³C Metabolic flux analysis of the extremely thermophilic, fast growing, xylose-utilizing *Geobacillus* strain LC300. *Metab Eng* 2015:DOI: 10.1016/j.ymben.2015.1006.1004.**
  36. Feng X, Mouttaki H, Lin L, Huang R, Wu B, Hemme CL, He Z, Zhang B, Hicks LM, Xu J, et al.: **Characterization of the central metabolic pathways in *Thermoanaerobacter* sp. strain X514 via isotopomer-assisted metabolite analysis. *Appl Environ Microbiol* 2009, 75:5001-5008.**
  37. You L, Berla B, He L, Pakrasi HB, Tang YJ: **¹³C-MFA delineates the photomixotrophic metabolism of *Synechocystis* sp. PCC 6803 under light- and carbon-sufficient conditions. *Biotechnol J* 2014, 9:684-692.**
  38. Nunn CE, Johnsen U, Schonheit P, Fuhrer T, Sauer U, Hough DW, Danson MJ: **Metabolism of pentose sugars in the hyperthermophilic archaea *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*. *J Biol Chem* 2010, 285:33701-33709.**

39. Tang KH, Feng X, Zhuang WQ, Alvarez-Cohen L, Blankenship RE, Tang YJ: **Carbon flow of heliobacteria is related more to clostridia than to the green sulfur bacteria.** *J Biol Chem* 2010, **285**:35104-35112.
40. Feng X, Tang KH, Blankenship RE, Tang YJ: **Metabolic flux analysis of the mixotrophic metabolisms in the green sulfur bacterium *Chlorobaculum tepidum*.** *J Biol Chem* 2010, **285**:39544-39550.
41. Wahrheit J, Nicolae A, Heinzle E: **Eukaryotic metabolism: measuring compartment fluxes.** *Biotechnol J* 2011, **6**:1071-1085.
- ** 42. Buescher JM, Antoniewicz MR, Boros LG, Burgess SC, Brunengraber H, Clish CB, DeBerardinis RJ, Feron O, Frezza C, Ghesquiere B, et al.: **A roadmap for interpreting C metabolite labeling patterns from cells.** *Curr Opin Biotechnol* 2015, **34**:189-201.
43. Gebreselassie NA, Antoniewicz MR: **¹³C-Metabolic flux analysis of co-cultures: A novel approach.** *Metab Eng* 2015, doi: 10.1016/j.ymben.2015.07.005.
44. Wasylenko TM, Ahn WS, Stephanopoulos G: **The oxidative pentose phosphate pathway is the primary source of NADPH for lipid overproduction from glucose in *Yarrowia lipolytica*.** *Metab Eng* 2015, **30**:27-39.
45. Vacanti NM, Divakaruni AS, Green CR, Parker SJ, Henry RR, Ciaraldi TP, Murphy AN, Metallo CM: **Regulation of substrate utilization by the mitochondrial pyruvate carrier.** *Mol Cell* 2014, **56**:425-435.
46. Lewis CA, Parker SJ, Fiske BP, McCloskey D, Gui DY, Green CR, Vokes NI, Feist AM, Vander Heiden MG, Metallo CM: **Tracing compartmentalized NADPH metabolism in the cytosol and mitochondria of mammalian cells.** *Mol Cell* 2014, **55**:253-263.

47. Fan J, Ye J, Kamphorst JJ, Shlomi T, Thompson CB, Rabinowitz JD: **Quantitative flux analysis reveals folate-dependent NADPH production.** *Nature* 2014, **510**:298-302.
- ** 48. Crown SB, Antoniewicz MR: **Parallel labeling experiments and metabolic flux analysis: Past, present and future methodologies.** *Metab Eng* 2013, **16**:21-32.
49. Antoniewicz MR: **¹³C metabolic flux analysis: optimal design of isotopic labeling experiments.** *Curr Opin Biotechnol* 2013, **24**:1116-1121.
50. Crown SB, Ahn WS, Antoniewicz MR: **Rational design of (1)(3)C-labeling experiments for metabolic flux analysis in mammalian cells.** *BMC Syst Biol* 2012, **6**:43.
51. Crown SB, Antoniewicz MR: **Selection of tracers for ¹³C-metabolic flux analysis using elementary metabolite units (EMU) basis vector methodology.** *Metab Eng* 2012, **14**:150-161.
52. Walther JL, Metallo CM, Zhang J, Stephanopoulos G: **Optimization of ¹³C isotopic tracers for metabolic flux analysis in mammalian cells.** *Metab Eng* 2012, **14**:162-171.
- * 53. Leighty RW, Antoniewicz MR: **COMPLETE-MFA: complementary parallel labeling experiments technique for metabolic flux analysis.** *Metab Eng* 2013, **20**:49-55.
54. Leighty RW, Antoniewicz MR: **Parallel labeling experiments with [U-¹³C]glucose validate E. coli metabolic network model for ¹³C metabolic flux analysis.** *Metab Eng* 2012, **14**:533-541.
55. Sandberg TE, Long CP, Gonzalez JE, Feist AM, Antoniewicz MR, Palsson BO: **Evolution of E. coli on [U-¹³C]glucose reveals a negligible isotopic influence on metabolism and physiology.** *Metab Eng* (submitted).

56. Ahn WS, Antoniewicz MR: **Parallel labeling experiments with [1,2-(13)C]glucose and [U-(13)C]glutamine provide new insights into CHO cell metabolism.** *Metab Eng* 2013, **15**:34-47.
57. Ahn WS, Antoniewicz MR: **Metabolic flux analysis of CHO cells at growth and non-growth phases using isotopic tracers and mass spectrometry.** *Metab Eng* 2011, **13**:598-609.
- ** 58. Crown SB, Long CP, Antoniewicz MR: **Integrated 13C-metabolic flux analysis of 14 parallel labeling experiments in Escherichia coli.** *Metab Eng* 2015, **28**:151-158.
- ** 59. King ZA, Lloyd CJ, Feist AM, Palsson BO: **Next-generation genome-scale models for metabolic engineering.** *Curr Opin Biotechnol* 2015, **35C**:23-29.
60. O'Brien EJ, Palsson BO: **Computing the functional proteome: recent progress and future prospects for genome-scale models.** *Curr Opin Biotechnol* 2015, **34C**:125-134.
61. Long CP, Antoniewicz MR: **Metabolic flux analysis of Escherichia coli knockouts: lessons from the Keio collection and future outlook.** *Curr Opin Biotechnol* 2014, **28**:127-133.
62. Henry O, Jolicoeur M, Kamen A: **Unraveling the metabolism of HEK-293 cells using lactate isotopomer analysis.** *Bioprocess Biosyst Eng* 2011, **34**:263-273.

Figure 1

Parallel labeling experiments for ^{13}C -MFA

Figure 2

Graphical Abstract

Parallel labeling experiments for ^{13}C -MFA