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^{13}C metabolic flux analysis of recombinant expression hosts

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Identifying host cell metabolic phenotypes that promote high recombinant protein titer is a major goal of the biotech industry. ^{13}C metabolic flux analysis (MFA) provides a rigorous approach to quantify these metabolic phenotypes by applying isotope tracers to map the flow of carbon through intracellular metabolic pathways. Recent advances in tracer theory and measurements are enabling more information to be extracted from ^{13}C labeling experiments. Sustained development of publicly available software tools and standardization of experimental workflows is simultaneously encouraging increased adoption of ^{13}C MFA within the biotech research community. A number of recent ^{13}C MFA studies have identified increased citric acid cycle and pentose phosphate pathway fluxes as consistent markers of high recombinant protein expression, both in mammalian and microbial hosts. Further work is needed to determine whether redirecting flux into these pathways can effectively enhance protein titers while maintaining acceptable glycan profiles.

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Introduction

Industrial bioprocesses place high demands on the intermediary metabolism of host cells to meet the biosynthetic requirements for maximal growth and protein expression. ^{13}C metabolic flux analysis (MFA) has become the premier approach to quantitatively assess the metabolic phenotypes of cultured cells and is now playing a growing role in host cell engineering and bioprocess optimization. Although transcriptomics [1] and proteomics [2] can be used to infer metabolic pathway alterations *indirectly* from changes in enzyme expression, this can be misleading

since metabolic enzymes are largely regulated by allosteric feedback and substrate availability, and mRNA or protein abundances often do not correlate strongly with pathway fluxes [3,4]. On the other hand, ^{13}C MFA relies on least-squares regression of *direct* metabolite measurements (isotope labeling patterns and extracellular exchange rates) to determine the adjustable flux parameters in a mathematical model of cellular metabolism (Figure 1). The resulting output is a comprehensive flux map that depicts the flow of carbon throughout intracellular metabolism under the experimental conditions of interest. Recent theoretical and experimental advances are pushing the boundaries of ^{13}C MFA to new heights of precision and flexibility, while increased availability of public software tools is streamlining MFA workflows and providing improved access to these technologies within the biotech research community. In this article, we provide an overview of these advances and highlight some important findings that have been obtained through the application of ^{13}C MFA to industrial expression hosts. We also point to emerging areas where ^{13}C MFA is likely to provide new insights for improving the quantity and quality of recombinant proteins produced by these hosts.

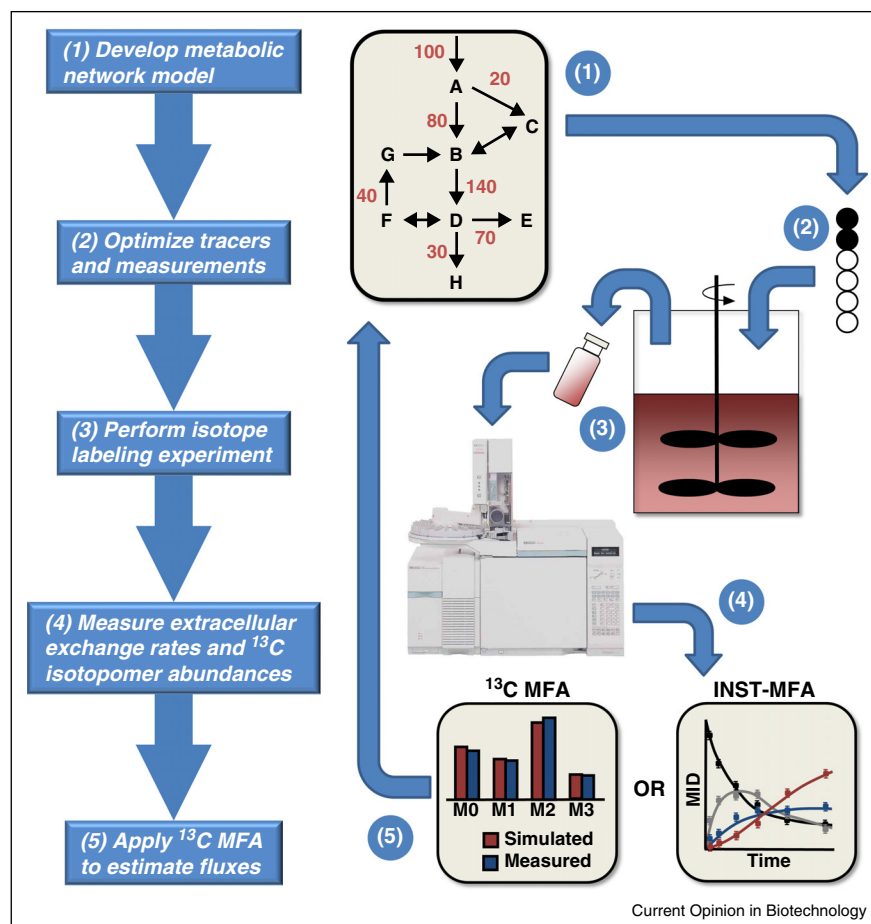
Recent advances in ^{13}C MFA tools and methodologies

Optimal experiment design (OED)

The earliest ^{13}C MFA experiments involved feeding either $[1-^{13}\text{C}]$ or $[\text{U}-^{13}\text{C}_6]\text{glucose}$ as a single tracer. Nowadays, the increased precision obtainable from combining multiple tracers, whether fed simultaneously or in parallel experiments, is widely understood and accepted. However, it is not always appreciated that the optimal tracer combination can depend strongly on the network topology and the available measurements, which vary from system to system. This implies that there is a need to tailor the labeling strategy to the system of interest rather than naïvely following prior conventions. Furthermore, the use of ^2H or ^{15}N tracers to probe redox or nitrogen metabolism, respectively, has proven to be a powerful complement to ^{13}C MFA. For example, the Metallo and Rabinowitz labs have recently applied an arsenal of different ^2H and ^{13}C tracers to assess the contributions from multiple pathways to compartment-specific NADPH production in the cytosol and mitochondria of mammalian cells [5^{••},6^{••}].

The first systematic treatment of optimal design of isotope labeling experiments was introduced by Möllney

Figure 1



Typical workflow of ^{13}C MFA studies. **(Step 1)** Development of a mathematical model comprising a system of mass balances and isotopomer balances on all metabolites in the biochemical reaction network is the initial step of MFA. **(Step 2)** This model can be applied to perform simulation studies and optimal experiment design (OED) computations to select tracer/measurement combinations that maximize flux resolution. **(Step 3)** A series of isotope labeling experiments is then performed by introducing the selected tracer(s) into culture and collecting cell and media samples at various time points during the growth phase of interest. **(Step 4)** MS and/or NMR is used to analyze the isotope labeling of target metabolites in these samples, while a parallel set of biochemical analyses is applied to profile the exchange rates of extracellular metabolites, as well as cell growth and product formation rates. **(Step 5)** These data sets are regressed using either stationary ^{13}C MFA or INST-MFA, depending on whether the labeling reaches equilibrium, which enables the adjustable flux parameters in the model to be uniquely determined. A series of statistical tests can be performed to assess the goodness-of-fit of the model and to calculate uncertainties for each of the estimated parameters. Recent theoretical advances and improvements in the availability of MFA software tools have enhanced the efficiency of this workflow, especially steps 1, 2, and 5. At the same time, better analytical methods for obtaining isotopomer measurements have provided increased flexibility and precision in steps 3 and 4.

et al. [7]. Their approach was based on the classical OED formulation of minimizing a scalar objective function computed from the flux covariance matrix of a reference flux map. Recent studies have applied more sophisticated OED algorithms to tailor ^{13}C MFA experiments to mammalian cell cultures, which pose a greater challenge due to their use of complex media containing multiple carbon sources. For example, Metallo *et al.* [8] examined flux identifiability of a carcinoma cell line using a variety of single tracers. Rather than using the parameter covariance matrix as a local estimate of flux uncertainty, they applied the parameter continuation method of Antoniewicz *et al.*

[9] to compute accurate nonlinear confidence intervals on all fluxes. They identified $[1,2-^{13}\text{C}_2]\text{glucose}$ and $[\text{U}-^{13}\text{C}_5]\text{glutamine}$ as the most useful single tracers for flux determination in glycolysis and citric acid cycle (CAC) pathways, respectively. Walther *et al.* [10] later extended this approach to examine mixtures of tracers identified using a genetic algorithm.

Another exciting development was the introduction of the EMU basis vector (EMU-BV) approach, which can be used to express the labeling of any metabolite in the network as a linear combination of labeled substrate

atoms [11^{*}]. This allows the influence of fluxes on the isotopomer measurements to be decoupled from substrate labeling, and thereby enables a fully *a priori* approach to tracer selection that does not depend on the choice of a reference flux map. Crown *et al.* [12] subsequently applied the EMU-BV method to identify two novel tracers for quantifying oxidative pentose phosphate pathway (OPPP) flux and anaplerotic pyruvate carboxylase flux using a metabolic model of HEK-293 cell metabolism. The EMU-BV approach also suggests that maximal flux information can be obtained by integrating data sets from multiple parallel tracer experiments, each of which is designed to elucidate specific reactions in the network [13]. Several recent studies have applied parallel labeling strategies to reduce the time of tracer experiments in mammalian cell cultures [14^{**}], to validate assumptions of biochemical network models [15], and to enhance precision of ^{13}C flux estimates [16]. These and other advances in OED of isotope tracer studies have been recently reviewed [17].

Isotopically nonstationary (INST) and dynamic MFA

Typically, ^{13}C MFA relies on assumptions of both metabolic and isotopic steady state. If metabolism is steady but isotope labeling is not allowed to fully equilibrate, isotopically nonstationary MFA (INST-MFA) can be used to estimate fluxes [18,19]. This requires solution of ordinary differential equations (ODEs) that describe the time-dependent labeling of network metabolites, while iteratively adjusting the flux and pool size parameters to match the transient labeling measurements. If measurements are obtained under both isotopically *and* metabolically nonstationary conditions, a fully dynamic modeling approach is required to estimate fluxes. This has been referred to as dynamic MFA (DMFA), but the majority of prior DMFA studies have not attempted to utilize ^{13}C labeling information [20]. Aside from a few proof-of-concept studies [21,22] or isolated examples that assume quasi-stationary labeling [23–25], there are currently no established methods for performing fully dynamic ^{13}C MFA.

^{13}C INST-MFA holds a number of unique advantages over approaches that rely solely upon steady-state isotopomer measurements. First, ^{13}C INST-MFA can be applied to estimate fluxes in autotrophic systems, which consume only single-carbon substrates [26,27]. This task is impossible with stationary ^{13}C MFA due to the fact that all carbon atoms in the system are derived from the same source and therefore will become uniformly labeled at steady state. Second, INST-MFA is ideally suited to systems that label slowly due to the presence of large intermediate pools or pathway bottlenecks. This approach not only avoids the additional time and cost of feeding isotope tracers over extended periods [28,29], but is critically necessary in cases where metabolic steady state is short-lived [30,31]. As a result, INST-MFA is

expected to become an indispensable tool for extending ^{13}C MFA approaches to studies of mammalian systems and industrial bioprocesses. Finally, INST-MFA provides increased measurement sensitivity to estimate reversible exchange fluxes and metabolite pool sizes [32,33], which represents a potential framework for integrating metabolomic analysis with ^{13}C MFA.

New measurement strategies

In addition to theoretical advances, the field of ^{13}C MFA is also rapidly expanding due to new isotopomer measurement capabilities. Traditionally, these measurements have been obtained from mass spectrometry (MS) [34] or nuclear magnetic resonance (NMR) [35] analysis of amino acid, fatty acid, or sugar constituents of cellular biomass. More recently, attention has shifted to the analysis of soluble metabolites that quickly approach isotopic steady state and enable dynamic changes in metabolism to be readily tracked. Furthermore, application of tandem MS/MS instruments is enabling enhanced accuracy [36] and increased position-specific labeling information [37–39] to be obtained from mass isotopomer analysis, which can significantly improve the precision of flux estimates obtained from ^{13}C MFA [40]. Others have shown how careful selection of target analytes that are biosynthetically derived from different organelles [41] or from different cell/tissue types [42,43] can be used to resolve compartment-specific fluxes that reflect cellular or subcellular metabolic heterogeneity. MFA approaches that leverage hyperpolarized ^{13}C substrates for real-time NMR imaging of labeling dynamics [44] or ultra-high resolution MS instrumentation [45,46] have also been recently developed. These and other advances in isotopomer measurement capabilities are paving the way for more sophisticated ^{13}C MFA approaches that provide increased precision, robustness, and flexibility.

New software tools

Unlike other ‘omics’ approaches, ^{13}C MFA requires least-squares regression of raw measurements to an appropriate metabolic network model. This imposes a significant computational burden that has previously restricted MFA studies to a small number of specialist labs. However, many of these groups have now made their in-house software tools publicly available, beginning with 13CFLUX in 2001 [47]. This was followed by several other software packages that were similarly developed to facilitate steady-state ^{13}C MFA calculations (Table 1). Only recently, however, have public software tools become available to automate ^{13}C INST-MFA. The INCA [48^{**}] and OpenMebius [49] packages now provide the flexibility to perform either steady-state MFA or INST-MFA using a single modeling platform. Furthermore, INCA provides capabilities to perform tracer simulations, OED calculations, and constraint-based pathway analyses. In addition, a variety of software packages are

Table 1**List of publicly available software tools for ¹³C MFA**

Name	Capabilities	License	Accessibility	Ref.
¹³C MFA				
13CFLUX2	Steady-state MFA	Free non-commercial (NC)	UNIX/Linux	[75]
FIA	Steady-state MFA	Open source	Cross-platform	[76]
FiatFlux	Metabolic flux ratio analysis	Free academic, Requires Matlab	Cross-platform	[77]
INCA	Steady-state and INST-MFA	Free NC, Requires Matlab	Cross-platform	[48**]
influx_s	Steady-state MFA	Open source	Cross-platform	[78]
Metran	Steady-state MFA	Free NC, Requires Matlab	Cross-platform	[79]
OpenFLUX	Steady-state MFA	Open source, Requires Matlab	Cross-platform	[80]
OpenMebius	Steady-state and INST-MFA	Open source, Requires Matlab	Cross-platform	[49]
Data processing				
ETA	Regression of extracellular exchange rates	Free, Requires Matlab	Cross-platform	[81]
FFC	Identify chemical composition of MS fragment ions	Open source	Linux or Windows	[82]
iMS2Flux	Processing and correction of MS data	Open source	Cross-platform	[83]
IsoCor	Correction of MS data	Open source	Cross-platform	[84]
IsoDesign	Optimize tracer combinations	Open source	Cross-platform	[51]
MetaboliteDetector	GC-MS peak detection, identification, alignment, and integration	Open source	Linux or Windows	[85]
NTFD	Non-targeted detection of isotopically enriched metabolites	Open source	Linux or Windows	[50*]
Data visualization				
Cytoscape	Network visualization	Open source	Cross-platform	[86]
Omix	Network visualization	Free 'Light Edition', Fee to upgrade	Cross-platform	[87]

now available to automate the ancillary data processing and visualization steps associated with MFA (Table 1). Other tools have been developed that use non-targeted profiling to discover downstream metabolites that are enriched by a given tracer [50*] or to optimize tracer selection [51] as a prelude to further ¹³C MFA studies. Although an experienced pathway modeler is still required to design and interpret MFA experiments, the availability of public software packages that streamline the data analysis workflow and reduce the burden of developing custom software code is making these techniques increasingly available to the broader scientific community. This is also leading to increased standardization of MFA practices, as the tools and methodologies become more widely established [52,53]. However, the capabilities of public software packages still lag somewhat behind state-of-the-art ¹³C MFA approaches. For example, tools for handling tandem MS/MS data [54] or ultra-high resolution MS data [55] have only recently begun to appear in the MFA literature.

Applications to recombinant protein manufacturing

¹³C MFA of industrial expression hosts

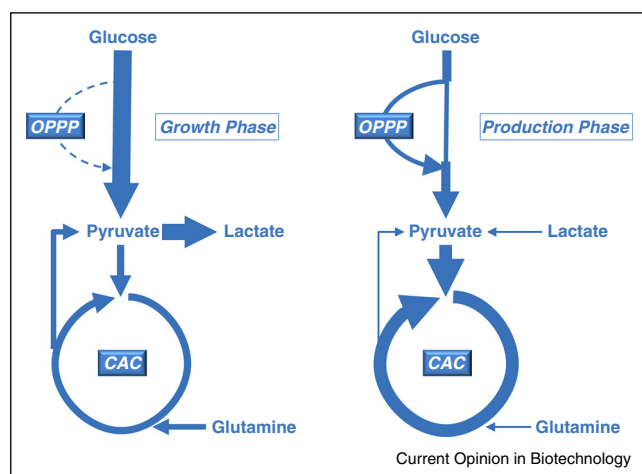
¹³C MFA is an important tool for guiding host cell engineering efforts, as well as media and bioprocess optimization [56]. Although some of the earliest ¹³C MFA studies were performed on antibody-producing hybridoma cultures nearly two decades ago [57,58], there has been a recent resurgence of efforts aimed at applying modern ¹³C MFA approaches to these and other

industrial expression hosts. In particular, several recent studies have examined how metabolic fluxes of Chinese hamster ovary (CHO) cells respond to different stages of fed-batch culture [14**,59**,60], different nutrient feeds [61], inducible expression of a recombinant protein [62], or expression of anti-apoptotic factors [63*] (Table 2). These studies have consistently observed that CHO cells rely more heavily on CAC flux and oxidative phosphorylation to supply energy when the cell-specific production rate (CSPR) of recombinant protein is highest (Figure 2) [59**,60–62]. This often involves a shift from lactate production to lactate consumption during the later stages of fed-batch growth when CSPR typically peaks [14**,59**,63*]. Furthermore, several studies have reported that enhanced OPPP flux coincides with the transition from growth phase to production (or stationary) phase [14**,59**,60,63*]. Similar phenotypes have been observed in microbial hosts such as *Pichia pastoris* [64,65**], *Bacillus subtilis* [66], and *Aspergillus niger* [67*], which typically upregulate CAC and/or OPPP flux in response to increased recombinant protein expression. It is still unclear, however, whether these metabolic adaptations are actually driving increased CSPR or whether they are simply side effects of the decrease in growth rate that typically coincides with an increase in recombinant protein expression. Also, cellular mechanisms that regulate the 'lactate shift' in mammalian cells are still being elucidated [68]. Few studies have attempted to directly manipulate flux into these pathways based on the findings of ¹³C MFA. As a result, further work is needed to determine whether ¹³C MFA can

Table 2**List of recent ^{13}C MFA studies using industrial expression hosts**

Year	Host	Product	Culture	Significant findings	Ref.
<i>Mammalian hosts</i>					
2014	CHO	None	Batch	Expression of Bcl-2 Δ anti-apoptotic factor enhanced pyruvate entry to CAC and increased lactate consumption during stationary phase.	[63*]
2014	CHO	mAb	Fed-batch	High-glutamine feed resulted in improved VCD and protein titer. Low-glutamine feed enhanced CAC flux and CSPR.	[61]
2013	CHO	mAb	Fed-batch	Induction of protein expression was associated with increased relative partitioning of pyruvate into the CAC.	[62]
2013	CHO	mAb	Fed-batch	Glycolytic flux to lactate dominated during the initial growth phase. Culture shifted to lactate uptake and enhanced CAC and OPPP during production phase.	[59**]
2013	CHO	None	Fed-batch	Relative partitioning of flux to OPPP and CAC was increased during stationary phase, although total carbon uptake was greatly reduced relative to growth phase.	[14**]
2011	CHO	mAb	Fed-batch	Nearly all glucose carbon was directed to OPPP and CAC during late non-growth phase.	[60]
2010	CHO	mAb	Perfusion	Approximately equal partitioning of carbon between glycolysis/OPPP and lactate/CAC.	[88]
<i>Microbial hosts</i>					
2014	<i>P. pastoris</i>	Enzyme	Batch	CAC flux was elevated in production strain relative to parental strain.	[65**]
2014	<i>B. subtilis</i>	Enzyme	Batch	OPPP and CAC fluxes were elevated under conditions where CSPR was highest.	[66]
2014	<i>S. pombe</i>	Enzyme	Chemostat	Substituting glycerol for glucose and supplementing acetate to culture medium enhanced CSPR while increasing CAC flux and NADPH production.	[89]
2012	<i>A. niger</i>	Enzyme	Batch	NADPH supplied by OPPP and malic enzyme was elevated in production strain but CAC flux was decreased relative to parental strain.	[67*]
2010	<i>P. pastoris</i>	Enzyme	Fed-batch	CAC flux was elevated in production strain relative to parental strain.	[64]

suggest valid metabolic engineering targets for enhancing recombinant protein production or reducing byproduct accumulation in industrial bioprocesses.

Figure 2

Metabolic phenotypes during growth and production phases of fed-batch cultures. Exponentially growing cultures exhibit high glycolytic flux and rely on elevated glutamine consumption to fuel mitochondrial metabolism. This results in lactate and ammonium accumulation in the culture medium. High-titer cultures shift to oxidizing glucose primarily in the OPPP and CAC pathways during production phase, which provides increased NADPH and ATP yields and reduced accumulation of inhibitory byproducts. In many cases, these cultures will consume lactate that was previously produced during the growth phase. Low-titer cultures typically fail to achieve this metabolic shift and continue to exhibit a glycolytic phenotype even after growth has slowed and recombinant protein production has peaked. (Arrow size is scaled in proportion to carbon flux. Dotted arrow indicates negligible flux.)

From quantity to quality improvements

There is now a proliferation of success stories in the biotechnology industry where recombinant protein titers have been improved beyond the 5 g/L level. While further improvements in product titers are still achievable and would provide additional manufacturing capacity and cost savings, a broader view of cell culture engineering has now firmly taken root that encompasses improved product quality and consistency, in addition to yield improvements. There has been much effort to understand and engineer protein glycosylation pathways [69], but few studies have directly examined how rewiring of central carbon metabolism impacts the supply of nucleotide-sugars and other precursors needed for synthesis of complex glycans [70]. It is likely that future ^{13}C MFA experiments and metabolic engineering studies will place equal emphasis on understanding the role of central metabolism in controlling both the quality as well as the sheer amount of product that can be manufactured by a recombinant host. Indeed, Burleigh *et al.* [71] recently applied stoichiometric MFA to investigate how changes in CAC and glycolytic fluxes correlate with changes in product glycosylation by CHO cells. Other studies have applied ^{13}C isotopomer analysis to determine the relative contributions of different biosynthetic pathways to the production of N-acetyl-glucosamine [46] or to assess the turnover of nucleotide-sugars required for protein glycosylation in cancer cells [72]. These studies

are leading the way toward a more systematic treatment of glycosylation pathways that relies on ¹³C tracing and MFA to examine how host cell metabolism impacts recombinant protein quality.

Conclusions

The capabilities of ¹³C MFA are rapidly expanding due to improved theoretical approaches for designing and modeling isotope labeling experiments. In particular, INST-MFA experiments are already conducted in a fraction of the time required for steady-state MFA and could soon become the basis for high-throughput MFA approaches [32,73]. These applications will gradually push the boundaries of INST-MFA toward fully dynamic ¹³C MFA [74]. At the same time, new MS and NMR approaches for obtaining isotopomer measurements are allowing more information to be extracted from ¹³C labeling experiments. This progress is enabling researchers to adapt flux analysis methodologies to a wider range of experimental systems or to tailor their studies to selectively monitor specific pathways of interest. Furthermore, the increased availability of public software tools for processing, analyzing, and visualizing MFA data sets is reducing the computational barriers that have limited access to these approaches in the past. As a result, there has been a surge of new ¹³C MFA studies examining industrial expression hosts in the last several years. These studies point to enhanced OPPP and CAC fluxes as consistent markers of high protein productivity in both mammalian and microbial hosts. However, much uncertainty remains as to whether and how these pathways can be engineered to drive improved protein expression, and whether these manipulations will have important side effects on protein quality. These questions remain at the forefront of cell culture research, and their answers will depend heavily on continued development and application of ¹³C MFA methodologies as an important component of the cyclic ‘model-build-test-learn’ metabolic engineering paradigm.

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