Isotopically nonstationary metabolic flux analysis (INST-MFA): Putting theory into practice

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Abstract

Typically, ^{13}C flux analysis 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 metabolic flux analysis (INST-MFA) can be used to estimate fluxes. This requires solution of differential equations that describe the time-dependent labeling of network metabolites, while iteratively adjusting the flux and pool size parameters to match the transient labeling measurements. INST-MFA holds a number of unique advantages over approaches that rely solely upon steady-state isotope enrichments. First, INST-MFA can be applied to estimate fluxes in autotrophic systems, which consume only single-carbon substrates. Second, INST-MFA is ideally suited to systems that label slowly due to the presence of large intermediate pools or pathway bottlenecks. Finally, INST-MFA provides increased measurement sensitivity to estimate reversible exchange fluxes and metabolite pool sizes, which represents a potential framework for integrating metabolomic analysis with ^{13}C flux analysis. This review highlights the unique capabilities of INST-MFA, describes newly available software tools that automate INST-MFA calculations, presents several practical examples of recent INST-MFA applications, and discusses the technical challenges that lie ahead.

Introduction

Isotope tracer methods for assessing metabolic fluxes have far-reaching applications in both biotechnology and medicine. Fluxes represent the ultimate outcome of metabolic control across multiple mechanistic levels and, therefore, provide a quantitative representation of the cellular metabolic state [1]. As a result, knowledge of metabolic fluxes is useful for improving biocatalysts through metabolic engineering or developing diagnostics and therapies for metabolic diseases. ^{13}C metabolic flux analysis (MFA) has been used to study metabolic phenotypes of numerous microbial, plant, and animal systems under well-controlled experimental conditions [2,3]. However, the underlying assumptions of metabolic and isotopic steady state required for rigorous ^{13}C MFA studies are continually being challenged by applications involving more complex systems and shorter experimental time scales. Isotopically nonstationary MFA (INST-MFA) has thus emerged in response to the need to obviate the assumption of isotopic steady state so that fluxes can be estimated from isotopically transient labeling data.

Though INST-MFA offers several advantages over steady-state ^{13}C MFA [4,5], its increased computational and experimental demands have previously hindered its widespread adoption [6]. Fortunately, much progress has been made to streamline INST-MFA workflows over the past three years. The purpose of this review is to highlight the unique capabilities of INST-MFA and present recent examples of how advances in theory and methodology have been put into practice. In particular, we describe newly available software tools that automate the computational modeling steps required for INST-MFA, which have provided a major step forward in the field. Finally, we conclude with a discussion of impending challenges that must be overcome to spur INST-MFA ahead into new application areas. We refer readers to other well-written reviews on

the fundamentals of ^{13}C MFA [7,8] or detailed descriptions of the experimental workflow of INST-MFA (Figure 1A) [6,9].

Essential concepts of INST-MFA

Metabolic pathways rearrange substrate atoms in unique and predictable ways. Therefore, administration of a stable isotope tracer, such as ^{13}C-labeled glucose, to a biological system enables the relative contributions from different pathways (and ultimately their fluxes) to be determined by monitoring the patterns of isotope incorporation that emerge in downstream metabolites over time. This process is analogous to adding a colored dye into a network of stirred tanks such that the rate of labeling in each tank is determined by its residence time constant. In a similar manner, the isotope labeling dynamics (Figure 1B, circles) of each metabolite pool will depend on its turnover time constant. Eventually, the system will reach an isotopic steady state wherein the labeling of each metabolite is fully equilibrated and will remain constant as long as the metabolic state is unperturbed.

Because many metabolic pathways often act in concert to rearrange substrate atoms in complex ways, it is typically necessary to use a mathematical model to infer information about fluxes from isotope labeling experiments (ILEs). These metabolic models are specific for each system under study. Every reaction in the model should be associated with an annotated enzyme or transport process, and all atom rearrangements should be confirmed from the biochemical literature. This information is used to enumerate mass balances and isotopomer balances that describe the conservation of atoms within the network. Once constructed, the model is used to simulate isotope enrichments by iteratively guessing the values of metabolic fluxes. The

simulated enrichments are then compared to those measured experimentally. In the early iterations, differences between simulated and measured metabolite enrichments are usually rather large (Figure 1B, orange line). This difference is gradually minimized after subsequent iterations (Figure 1B, green line) until the model converges to a best-fit solution that is confined by the measured isotope enrichments, as well as any directly measureable fluxes and pool sizes (Figure 1B, blue line). This unique flux solution provides a snapshot of the system's metabolism during the ILE.

Mathematical models used for both MFA and INST-MFA assume that the system is at metabolic steady state. In other words, the reaction fluxes and metabolite pool sizes remain constant throughout the duration of the ILE. The critical difference between the two approaches is that INST-MFA applies ordinary differential equations (ODEs) to simulate how isotope labeling measurements change over time. Therefore, transient labeling measurements collected across multiple time points can serve as inputs for INST-MFA, while measurements used for MFA must be collected after isotope labeling has fully equilibrated. Even though the metabolic networks may be similar in size, the simulation times for INST-MFA are usually significantly longer due to 1) the need to repeatedly solve ODEs rather than algebraic balance equations and 2) the increased number of measurement time points that need to be simulated. For example, a single steady-state flux estimation for a large *E. coli* network with measurements at a single time point (taken from [4]) took less than 10 seconds, while a single INST-MFA flux estimation with measurements at nine time points took approximately 10 minutes.

Applications of INST-MFA

For systems where steady-state isotope labeling is informative and can be obtained within the timeframe of a typical ILE, stationary ^{13}C MFA is usually the preferred approach because of its relative simplicity. However, there are important situations where fluxes cannot be determined at isotopic steady state, and hence INST-MFA is required.

Autotrophic systems

First, INST-MFA can be applied to estimate fluxes in autotrophic systems, which consume only single-carbon substrates [9,10]. 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 become uniformly labeled at isotopic steady state (Figure 2A, final time point) [10-12]. Hence, steady-state labeling measurements are independent of fluxes and cannot be used to estimate their values. However, since unique labeling patterns exist during the transient period that precedes isotopic steady state (Figure 2A, intermediate time points), these measurements can be used to calculate a unique flux solution using INST-MFA.

Since the first autotrophic network was mapped using INST-MFA in the model cyanobacterium *Synechocystis sp.* PCC6803 [10], there has been a surge in applications of INST-MFA to define the diverse and intricate capabilities of photoautotrophic metabolism. For instance, INST-MFA has been recently used to expose enhanced carbon assimilation capabilities in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 [13] and a surprisingly flexible metabolism of a recombinant ethylene-producing strain of *Synechocystis sp.* PCC6803 [14]. Besides prokaryotes, researchers have also applied INST-MFA to characterize the

photoautotrophic metabolism in compartmentalized eukaryotes such as plant leaves [15], diatoms [16], and algae [17]. In another study, the first use of INST-MFA to examine the effects of nitrate deprivation on photoautotrophic metabolism was also successfully conducted [18].

INST-MFA has also immensely benefited the biotechnology of algae and cyanobacteria (recently reviewed in [19]) as it ties genome engineering efforts to changes in photoautotrophic metabolic fluxes, and vice versa. For instance, recent work by Jazmin et al. [20] demonstrates the ability to identify metabolic bottlenecks in a recombinant aldehyde-producing cyanobacterial strain by using INST-MFA to guide rational metabolic engineering. A recent INST-MFA study in the diatom *Phaeodactylum tricornutum* elucidated the change in photoautotrophic metabolism in response to increased lipid production caused by overexpression of diacylglycerol acyltransferase [16]. In another example, Hendry et al. [21] shed light upon the metabolic effects of disrupting a major carbon storage pathway by performing INST-MFA on a cyanobacterium with inactivated glycogen synthesis genes. Their study was particularly interesting as it showed that mutants were capable of redistributing carbon towards alternative sinks, which helps explain why inactivating glycogen synthesis has had mixed outcomes when applied to different production platforms [22-25].

Systems that are slow to label

Second, INST-MFA is ideally suited to systems that label slowly due to the presence of large intermediate pools or pathway bottlenecks. Slow labeling poses a challenge for the design of ILEs because isotopic steady state may not be achieved within the period that the system can be maintained at metabolic steady state (Figure 2B). This scenario is most pertinent in mammalian

systems, which often experience a switch from lactate production during exponential growth phase to lactate consumption during stationary growth phase [26]. Several earlier studies in mammalian cell cultures have applied INST-MFA to determine fluxes from transient labeling patterns measured during the short-lived periods of metabolic steady state [27-30]. Similar approaches have been recently applied to murine embryonic stem cell lines [31] and immortalized human amniocytes [32].

The use of INST-MFA in cell culture systems has the added benefit of avoiding the additional time and cost of feeding isotope tracers over extended periods [33,34]. As a result, INST-MFA is expected to become an indispensable tool for extending ^{13}C MFA approaches to studies of industrial bioprocesses. Besides INST-MFA, slow labeling dynamics can also be circumvented through parallel labeling experiments with multiple tracers that probe different parts of the metabolic network [35]. For instance, parallel ILEs with labeled glucose and glutamine were applied to overcome the slow labeling of TCA cycle metabolites in CHO cells such that stationary MFA could be applied [36]. A recent study has further combined parallel labeling strategies with INST-MFA [32] to maximize the information content of each ILE.

Systems that require increased precision

Finally, INST-MFA provides increased sensitivity to estimate reversible exchange fluxes, futile cycles, and metabolite pool sizes [37-39]. It is important to note that the latter capability provides a potential framework for integrating and cross-validating metabolomic analysis with flux analysis [40]. Many net fluxes are estimated with greater precision by INST-MFA due to the shear increase in number of measurement inputs that are regressed [39]. Reversible exchange

fluxes [41,42], on the other hand, are often poorly estimated by steady-state MFA [38] and can introduce instability to the simulations if they are too large [43]. INST-MFA has been used to precisely determine some exchange fluxes that were otherwise unobservable, even in systems that achieve rapid isotopic steady states (Figure 2C). In a few instances, unique reaction mechanisms such as metabolite pool buffering [44,45] and futile ATP cycling [46] were discovered based on exchange fluxes determined by INST-MFA.

Publicly available software for INST-MFA

There are presently two publicly available Matlab software packages designed to perform INST-MFA: INCA [47] and OpenMebius [48]. The most useful feature of these tools is that they greatly simplify the network specification and model building steps of INST-MFA by automatically generating metabolite balances and isotopomer balances from user-defined reactions and atom transitions. Moreover, mass isotopomer distribution data, gathered from either GC-MS or LC-MS/MS, can be conveniently imported into these programs from excel worksheets. Once the experimental inputs and metabolic network are specified, users are guided through the analysis workflow leading to the calculation of flux results (Figure 1A).

With either software, the flux estimations occur through iterative simulations performed within the Matlab computing environment. In each iteration, reaction fluxes are guessed to solve the metabolite and isotopomer balance equations in order to generate simulated isotope enrichment data. The sum of squared residuals (SSR), which is a measure of discrepancy between the simulated and experimentally measured enrichment data, is assessed after each run and minimized in subsequent runs. The iterations are terminated once no further improvements in

SSR can be achieved. In INCA, a goodness-of-fit assessment is available to help users identify major sources of discrepancies between the model simulations and experimental measurements. The fit of the experimental data to the model is considered “acceptable” when the SSR falls within the expected chi-square distribution range [49].

To determine the precision of an acceptable solution, statistical metrics are applied to assess the 95% confidence intervals of each calculated flux and pool size. In doing so, INCA uses either parameter continuation [49] or Monte Carlo analysis [50] while OpenMebius utilizes a grid search method. Once any of these statistical procedures is completed, the lower and upper bounds for each flux and pool size estimate are computed. In addition, a confidence interval plot is generated for each reaction that describes the sensitivity of the SSR to the estimated flux value. A flux can be estimated precisely when the SSR is sensitive to perturbations in its estimated value.

Finally, it is also worth noting that the simulation run times for many INST-MFA models can be rather long, and hence parallelization capabilities are essential to ensure that total analysis times are not prohibitive [47]. Using a cyanobacterial INST-MFA model consisting of 55 reactions and 34 metabolites [20], running 50 flux estimations from random initial guesses required ~20 minutes using INCA on a parallel cluster of 5 quad-core Linux computers (Intel Core i7 2600 processor with 4 GB of memory). Calculation of all 95% confidence intervals using parameter continuation required ~6 hours of run time. These same calculations would have required approximately 20 times longer if run in serial mode. For basic program setup and

troubleshooting in INCA, users are encouraged to make use of its comprehensive user manual [51].

Conclusions and challenges ahead

In recent years, INST-MFA has advanced from a theoretical curiosity to a practical method of flux assessment. It has proven useful for quantifying metabolism of systems that cannot achieve isotopic steady state or do not produce informative steady-state labeling patterns. The release of generalized software tools [47,48,52] implementing advanced computational algorithms [4,5] has now made INST-MFA more widely available to the scientific community.

Despite its advantages, the increased complexity of INST-MFA introduces additional difficulties at both the computational and experimental levels. First, introducing isotopically nonstationary measurements adds further complexity to experimental design. In addition to the design parameters that must be considered in the steady-state case, INST-MFA requires careful selection of sampling time points and possibly metabolite pool size measurements [53]. These new dimensions make the search for an optimal experimental design even more difficult and time-consuming. Several computational approaches have been developed to efficiently traverse this design space, including parameterized sampling and *a posteriori* ranking of measurement time points [54,55]. Adebiyi et al. [12] recently applied these principles to examine the optimal design of ILEs for determination of fluxes in cyanobacteria. They found that a minimum of three sample time points were needed for precise flux estimation. Because some fluxes were more sensitive to early time points and others were more sensitive to later time points, it was concluded that sampling should span the entire ILE time course from the early transient period

until the labeling approaches steady state. This was best accomplished using an exponential sampling strategy, where the time intervals between successive samples were gradually increased through repeated scaling by a constant factor.

Adebiyi et al. [12] also examined the importance of pool size measurements on the precision of flux estimates. Unlike related approaches such as kinetic flux profiling [53,56], direct measurements of intracellular metabolite pool sizes are not critical for flux estimation by INST-MFA. The pool sizes are treated as adjustable model parameters, which are optimized to match the experimental labeling dynamics during the data regression. This is a significant advantage of INST-MFA over other modeling approaches that depend explicitly on pool size measurements or kinetic parameters that are not reliably obtained *in vivo*. For instance, it is often difficult to achieve absolute quantification of intracellular pool sizes due to losses during metabolite extraction or unknown subcellular compartmentation of metabolites. Therefore, most prior INST-MFA studies have not supplied pool size measurements to the data regression [10,13,18,20,21,31,32]. In fact, studies using both simulated data sets [12,15] and actual experimental data sets [13] have concluded that the addition of pool size measurements made negligible contributions toward flux determination by INST-MFA.

Second, the labeling of some intracellular metabolites can exhibit very short isotopic transients, on the order of minutes to even seconds. Rapid sampling and quenching must be applied in these situations to obtain meaningful data. The field of metabolomics has witnessed considerable progress in this area, and some of these measurement techniques have already been successfully adapted for INST-MFA studies in *E. coli* [37,38]. If downstream sample processing and data

analysis can be streamlined and automated, INST-MFA could soon become the basis for high-throughput MFA studies [37,57]. Continued progress to overcome these technical hurdles will undoubtedly open the door to further innovations that extend the scope and reach of INST-MFA into new application areas.

Acknowledgments

This work was supported by Department of Energy award DE-SC0008118.

Disclosure

Dr. Young is a co-founder of Metalytics, LLC.

Special interest

[18]* Nitrogen starvation induces significant carbon flux redistribution in cyanobacteria. Since this response was too rapid for practical application of INST-MFA, the authors examined the effects of a partially recapitulated nitrogen starvation phenotype in a mutant strain deficient for nitrate import. Using a 1-liter photobioreactor, the mutant was successfully maintained in exponential growth to enable INST-MFA studies. The results suggest that cyanobacterial responses to nitrate starvation involve increasing flux through anaplerotic reactions and elevating ATP consumption.

[20]* By comparing the fluxes in an isobutyraldehyde producer to a non-producing strain under photoautotrophic conditions, differences in metabolic fluxes through precursor pathways were identified and subsequently targeted for overexpression, leading to improved strain performance.

[21]* Redirecting flux of fixed carbon from major carbon sinks toward synthetic production pathways is a long-standing goal of metabolic engineering in photoautotrophs. Glycogen storage consumes significant amounts of fixed carbon that could be otherwise directed to product synthesizing pathways. This study revealed that cyanobacterial mutants that are incapable of accumulating glycogen redistribute carbon toward other storage molecules, such as sucrose.
[32]* This study combined parallel labeling with [1,2-$^{13}C_2$]glucose and [U-$^{13}C_5$]glutamine with INST-MFA to characterize metabolism of adenovirus infection in human amniocyte-derived cells.

Outstanding interest

[14]** By comparing the kinetic ^{13}C-labelling patterns in wild-type and ethylene-producing *Synechocystis* strains, the Yu lab found that metabolic flux flowing into the TCA cycle was dramatically enhanced in the ethylene-producing strain. The results showed that about 13% of total fixed carbon flowed into the TCA cycle in the wild-type strain, whereas flux entering the TCA cycle reached 37% of total fixed carbon in a strain with optimized ethylene-forming enzyme (EFE) expression. Concomitantly, the predominantly bifurcated TCA cycle flux changed to a cyclic pattern in the EFE-expressing strain.
[15]** This study describes the application of INST-MFA to a terrestrial plant system for the first time. Through comparison of plants that had been acclimated to “normal” and “high” light conditions, metabolic flux maps were used to identify changes in photorespiratory and biosynthetic fluxes that occurred in plants that were adapted to high-light conditions.
[17]** This study performed comprehensive characterization of a new strain of eukaryotic algae. Photoautotrophic and photoheterotrophic metabolism were studied with INST-MFA and

compared side-by-side. Biomass components and macromolecular compositions were measured. This is also the first study for which INST-MFA was used to characterize photoautotrophic metabolism across four compartments; cytosol, chloroplast, mitochondria and peroxisome.

References

1. Nielsen J: **It is all about metabolic fluxes**. *J Bacteriol* 2003, **185**:7031-7035.
2. Cheah YE, Hasenour CM, Young JD: **13C Flux Analysis in Biotechnology and Medicine**. In *Systems Biology*. Wiley-VCH Verlag GmbH & Co. KGaA; 2017:25-70.
3. Dai ZW, Locasale JW: **Understanding metabolism with flux analysis: From theory to application**. *Metabolic Engineering* 2017, **43**:94-102.
4. Young JD, Walther JL, Antoniewicz MR, Yon H, Stephanopoulos G: **An Elementary Metabolite Unit (EMU) based method of isotopically nonstationary flux analysis**. *Biotechnology and Bioengineering* 2008, **99**:686-699.
5. Jazmin LJ, Young JD: **Isotopically Nonstationary 13C Metabolic Flux Analysis**. In *Systems Metabolic Engineering: Methods and Protocols*. Edited by Alper HS. Humana Press; 2013:367-390.
6. Wiechert W, Noh K: **Isotopically non-stationary metabolic flux analysis: complex yet highly informative**. *Current Opinion in Biotechnology* 2013, **24**:979-986.
7. Wiechert W: **C-13 metabolic flux analysis**. *Metabolic Engineering* 2001, **3**:195-206.
8. Yang TH: **13C-Based Metabolic Flux Analysis: Fundamentals and Practice**. In *Systems Metabolic Engineering: Methods and Protocols*. Edited by Alper HS. Humana Press; 2013:297-334.

9. Jazmin LJ, O'Grady JP, Ma F, Allen DK, Morgan JA, Young JD: **Isotopically nonstationary MFA (INST-MFA) of autotrophic metabolism**. *Methods Mol Biol* 2014, **1090**:181-210.

10. Young JD, Shastri AA, Stephanopoulos G, Morgan JA: **Mapping photoautotrophic metabolism with isotopically nonstationary ^{13}C flux analysis**. *Metab Eng* 2011, **13**:656-665.

11. Shastri AA, Morgan JA: **A transient isotopic labeling methodology for C-13 metabolic flux analysis of photo auto trophic microorganisms**. *Phytochemistry* 2007, **68**:2302-2312.

12. Adebiyi AO, Jazmin LJ, Young JD: **C-13 flux analysis of cyanobacterial metabolism**. *Photosynthesis Research* 2015, **126**:19-32.

13. Abernathy MH, et al.: **Deciphering cyanobacterial phenotypes for fast photoautotrophic growth via isotopically nonstationary metabolic flux analysis**. *Biotechnology for Biofuels* 2017, **10**:273.

14. Xiong W, Morgan JA, Ungerer J, Wang B, Maness PC, Yu JP: **The plasticity of cyanobacterial metabolism supports direct CO2 conversion to ethylene**. *Nature Plants* 2015, **1**:6.

15. Ma FF, Jazmin LJ, Young JD, Allen DK: **Isotopically nonstationary C-13 flux analysis of changes in Arabidopsis thaliana leaf metabolism due to high light acclimation**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:16967-16972.

16. Dinamarca J, Levitan O, Kumaraswamy GK, Lun DS, Falkowski PG: **Overexpression of a diacylglycerol acyltransferase gene in Phaeodactylum tricornutum directs carbon towards lipid biosynthesis**. *Journal of Phycology* 2017, **53**:405-414.

17. Wu C, Xiong W, Dai JB, Wu QY: **Genome-Based Metabolic Mapping and C-13 Flux Analysis Reveal Systematic Properties of an Oleaginous Microalga Chlorella protothecoides**. *Plant Physiology* 2015, **167**:586-599.

18. Nakajima T, Yoshikawa K, Toya Y, Matsuda F, Shimizu H: **Metabolic Flux Analysis of the Synechocystis sp. PCC 6803 ΔnrtABCD Mutant Reveals a Mechanism for Metabolic Adaptation to Nitrogen-Limited Conditions**. *Plant and Cell Physiology* 2017, **58**:537-545.

19. Knoot CJ, Ungerer JL, Wangikar PP, Pakrasi HB: **Cyanobacteria: promising biocatalysts for sustainable chemical production**. *Journal of Biological Chemistry* 2017.

20. Jazmin LJ, Xu Y, Cheah YE, Adebiyi AO, Johnson CH, Young JD: **Isotopically nonstationary 13C flux analysis of cyanobacterial isobutyraldehyde production**. *Metabolic Engineering* 2017, **42**:9-18.

21. Hendry JI, Prasannan C, Ma F, Möllers KB, Jaiswal D, Digmurti M, Allen DK, Frigaard N-U, Dasgupta S, Wangikar PP: **Rerouting of carbon flux in a glycogen mutant of cyanobacteria assessed via isotopically non-stationary 13C metabolic flux analysis**. *Biotechnology and Bioengineering* 2017, **114**:2298-2308.

22. Jacobsen JH, Frigaard NU: **Engineering of photosynthetic mannitol biosynthesis from CO2 in a cyanobacterium**. *Metabolic Engineering* 2014, **21**:60-70.

23. van der Woude AD, Angermayr SA, Puthan Veetil V, Osnato A, Hellingwerf KJ: **Carbon sink removal: Increased photosynthetic production of lactic acid by Synechocystis sp. PCC6803 in a glycogen storage mutant**. *Journal of Biotechnology* 2014, **184**:100-102.

24. Work VH, Melnicki MR, Hill EA, Davies FK, Kucek LA, Beliaev AS, Posewitz MC: **Lauric Acid Production in a Glycogen-Less Strain of Synechococcus sp. PCC 7002**. *Frontiers in Bioengineering and Biotechnology* 2015, **3**:48.

25. Davies FK, Work VH, Beliaev AS, Posewitz MC: **Engineering Limonene and Bisabolene Production in Wild Type and a Glycogen-Deficient Mutant of Synechococcus sp. PCC 7002**. *Frontiers in Bioengineering and Biotechnology* 2014, **2**:21.

26. Young JD: **Metabolic flux rewiring in mammalian cell cultures**. *Current Opinion in Biotechnology* 2013, **24**:1108-1115.

27. Ahn WS, Antoniewicz MR: **Metabolic flux analysis of CHO cells at growth and non-growth phases using isotopic tracers and mass spectrometry**. *Metabolic Engineering* 2011, **13**:598-609.

28. Nicolae A, Wahrheit J, Bahnemann J, Zeng A-P, Heinzle E: **Non-stationary 13C metabolic flux analysis of Chinese hamster ovary cells in batch culture using extracellular labeling highlights metabolic reversibility and compartmentation**. *BMC Systems Biology* 2014, **8**:50.

29. Maier K, Hofmann U, Bauer A, Niebel A, Vacun G, Reuss M, Mauch K: **Quantification of statin effects on hepatic cholesterol synthesis by transient 13C-flux analysis**. *Metabolic Engineering* 2009, **11**:292-309.

30. Murphy TA, Dang CV, Young JD: **Isotopically nonstationary C-13 flux analysis of Myc-induced metabolic reprogramming in B-cells**. *Metabolic Engineering* 2013, **15**:206-217.

31. Sa JV, Kleiderman S, Brito C, Sonnewald U, Leist M, Teixeira AP, Alves PM: **Quantification of Metabolic Rearrangements During Neural Stem Cells**

Differentiation into Astrocytes by Metabolic Flux Analysis. *Neurochemical Research* 2017, **42**:244-253.

32. Carinhas N, Koshkin A, Pais DAM, Alves PM, Teixeira AP: **13C-metabolic flux analysis of human adenovirus infection: Implications for viral vector production**. *Biotechnology and Bioengineering* 2017, **114**:195-207.

33. Zhao Z, Kuijvenhoven K, Ras C, van Gulik WM, Heijnen JJ, Verheijen PJ, van Winden WA: **Isotopic non-stationary 13C gluconate tracer method for accurate determination of the pentose phosphate pathway split-ratio in Penicillium chrysogenum**. *Metab Eng* 2008, **10**:178-186.

34. Horl M, Schnidder J, Sauer U, Zamboni N: **Non-stationary (13)C-metabolic flux ratio analysis**. *Biotechnol Bioeng* 2013, **110**:3164-3176.

35. Antoniewicz MR: **Parallel labeling experiments for pathway elucidation and (13)C metabolic flux analysis**. *Curr Opin Biotechnol* 2015, **36**:91-97.

36. Ahn WS, Antoniewicz MR: **Parallel labeling experiments with 1,2-C-13 glucose and U-C-13 glutamine provide new insights into CHO cell metabolism**. *Metabolic Engineering* 2013, **15**:34-47.

37. Schaub J, Mauch K, Reuss M: **Metabolic flux analysis in *Escherichia coli* by integrating isotopic dynamic and isotopic stationary ^{13}C labeling data**. *Biotechnol Bioeng* 2008, **99**:1170-1185.

38. Noh K, Gronke K, Luo B, Takors R, Oldiges M, Wiechert W: **Metabolic flux analysis at ultra short time scale: isotopically non-stationary ^{13}C labeling experiments**. *J Biotechnol* 2007, **129**:249-267.

39. Noack S, Nöh K, Moch M, Oldiges M, Wiechert W: **Stationary versus non-stationary 13C-MFA: A comparison using a consistent dataset**. *Journal of Biotechnology* 2011, **154**:179-190.

40. McAtee AG, Jazmin LJ, Young JD: **Application of isotope labeling experiments and C flux analysis to enable rational pathway engineering**. *Curr Opin Biotechnol* 2015, **36**:50-56.

41. Wiechert W: **The Thermodynamic Meaning of Metabolic Exchange Fluxes**. *Biophysical Journal* 2007, **93**:2255-2264.

42. Wiechert W, de Graaf AA: **Bidirectional reaction steps in metabolic networks: I. Modeling and simulation of carbon isotope labeling experiments**. *Biotechnology and Bioengineering* 1997, **55**:101-117.

43. Wiechert W, Möllney M, Isermann N, Wurzel M, de Graaf AA: **Bidirectional reaction steps in metabolic networks: III. Explicit solution and analysis of isotopomer labeling systems**. *Biotechnology and Bioengineering* 1999, **66**:69-85.

44. Jordà J, Cueto Rojas H, Carnicer M, Wahl A, Ferrer P, Albiol J: **Quantitative Metabolomics and Instationary (13)C-Metabolic Flux Analysis Reveals Impact of Recombinant Protein Production on Trehalose and Energy Metabolism in Pichia pastoris**. *Metabolites* 2014, **4**:281-299.

45. Jordà J, Suarez C, Carnicer M, ten Pierick A, Heijnen JJ, van Gulik W, Ferrer P, Albiol J, Wahl A: **Glucose-methanol co-utilization in Pichia pastoris studied by metabolomics and instationary (13)C flux analysis**. *BMC Systems Biology* 2013, **7**:17-17.

46. Zhao Z, ten Pierick A, de Jonge L, Heijnen JJ, Wahl SA: **Substrate cycles in Penicillium chrysogenum quantified by isotopic non-stationary flux analysis**. *Microbial Cell Factories* 2012, **11**:140.

47. Young JD: **INCA: a computational platform for isotopically non-stationary metabolic flux analysis**. *Bioinformatics* 2014, **30**:1333-1335.

48. Kajihata S, Furusawa C, Matsuda F, Shimizu H: **OpenMebius: An Open Source Software for Isotopically Nonstationary C-13-Based Metabolic Flux Analysis**. *Biomed Research International* 2014:10.

49. Antoniewicz MR, Kelleher JK, Stephanopoulos G: **Determination of confidence intervals of metabolic fluxes estimated from stable isotope measurements**. *Metab Eng* 2006, **8**:324-337.

50. Schmidt K, Nørregaard LC, Pedersen B, Meissner A, Duus JØ, Nielsen JØ, Villadsen J: **Quantification of Intracellular Metabolic Fluxes from Fractional Enrichment and ^{13}C-^{13}C Coupling Constraints on the Isotopomer Distribution in Labeled Biomass Components**. *Metab Eng* 1999, **1**:166-179.

51. Jazmin LJ, Beckers V, Young JD: **User Manual for INCA**. 2014.

52. Murphy TA, Young JD: **ETA: Robust software for determination of cell specific rates from extracellular time courses**. *Biotechnol Bioeng* 2013, **110**:1748-1758.

53. Heise R, Fernie AR, Stitt M, Nikoloski Z: **Pool size measurements facilitate the determination of fluxes at branching points in non-stationary metabolic flux analysis: the case of Arabidopsis thaliana**. *Front Plant Sci* 2015, **6**:386.

54. Nöh K, Wiechert W: **Experimental design principles for isotopically instationary 13C labeling experiments**. *Biotechnology and Bioengineering* 2006, **94**:234-251.

55. Noh K, Wahl A, Wiechert W: **Computational tools for isotopically instationary ^{13}C labeling experiments under metabolic steady state conditions**. *Metabolic Engineering* 2006, **8**:554-577.

56. Yuan J, Bennett BD, Rabinowitz JD: **Kinetic flux profiling for quantitation of cellular metabolic fluxes**. *Nat Protoc* 2008, **3**:1328-1340.

57. Sauer U: **High-throughput phenomics: experimental methods for mapping fluxomes**. *Curr. Opin. Biotechnol.* 2004.

Figure captions

Figure 1. Summary of the flux estimation procedure using INST-MFA.

(A) Simplified workflow of INST-MFA studies. Currently available software tools, namely INCA and OpenMebius, are used for model specification, flux estimation, and statistical analysis. Initial guesses of fluxes and pool sizes are needed for the first iteration (iter) only. The flux map generated is a snapshot of metabolism during the course of the ILE.

(B) Circles indicate a typical enrichment profile of a metabolite following introduction of an isotope tracer. Lines represent model-simulated measurements. After numerous iterations (orange and green lines), the model converges on a solution that minimizes the SSR between the experimentally determined and model-simulated measurements (blue line).

Figure 2. Practical applications of INST-MFA (cases A, B, and C) versus MFA (case C only).

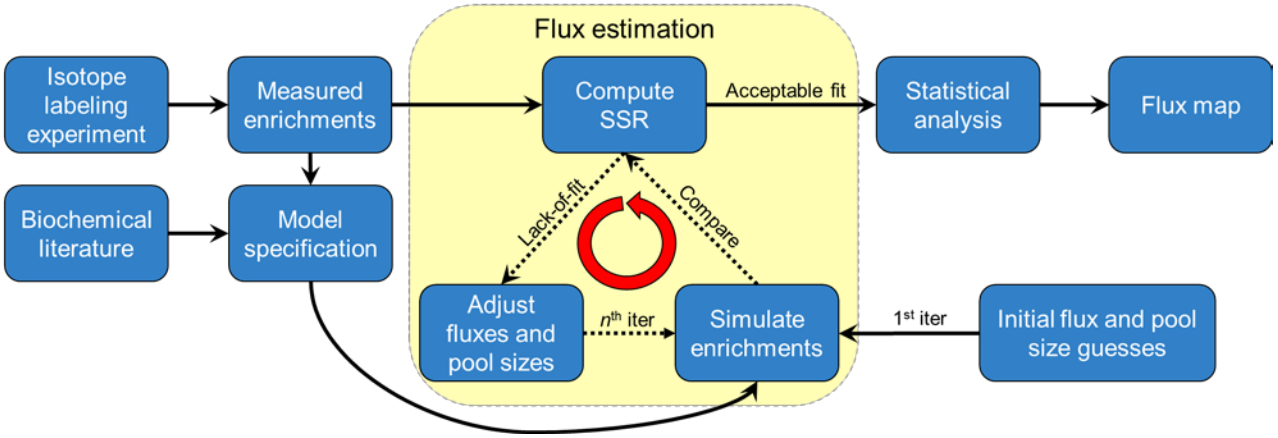
(A) Autotrophic systems. Due to the use of a single-carbon tracer, no unique flux solution can be calculated at isotopic steady state because all metabolites become uniformly labeled.

(B) Slow labeling dynamics. The labeling of some metabolites may be too slow to achieve isotopic steady state within the timeframe that metabolic steady state can be maintained.

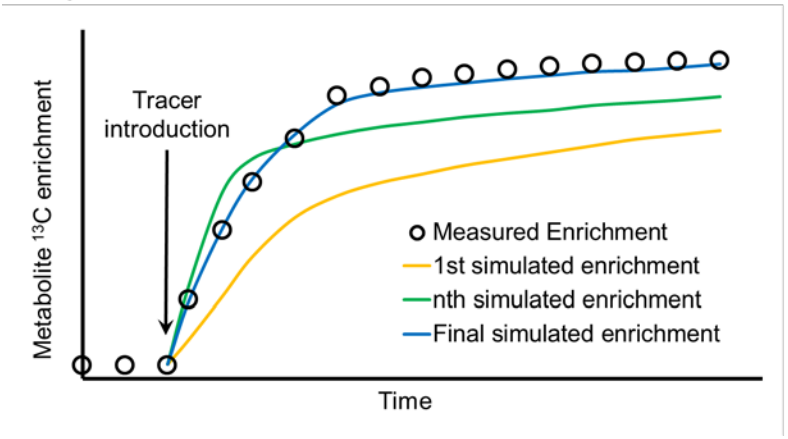
(C) Rapid isotopic steady state. Although stationary MFA can be used to determine fluxes, INST-MFA can be used in some situations to improve estimates of exchange fluxes and pool sizes if rapid sampling is available.

Arrows represent fluxes and tanks represent pool sizes at each time point.

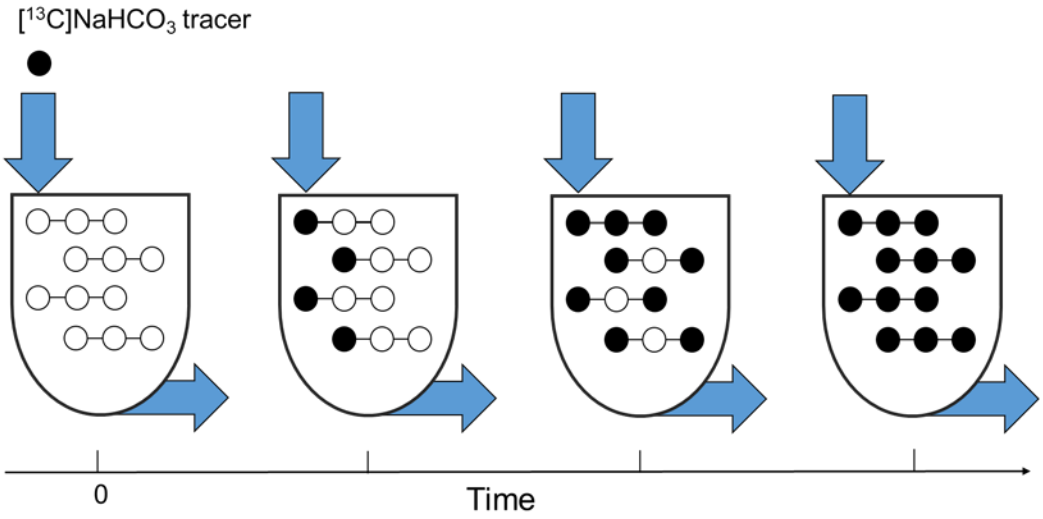
A) Simplified INST-MFA workflow



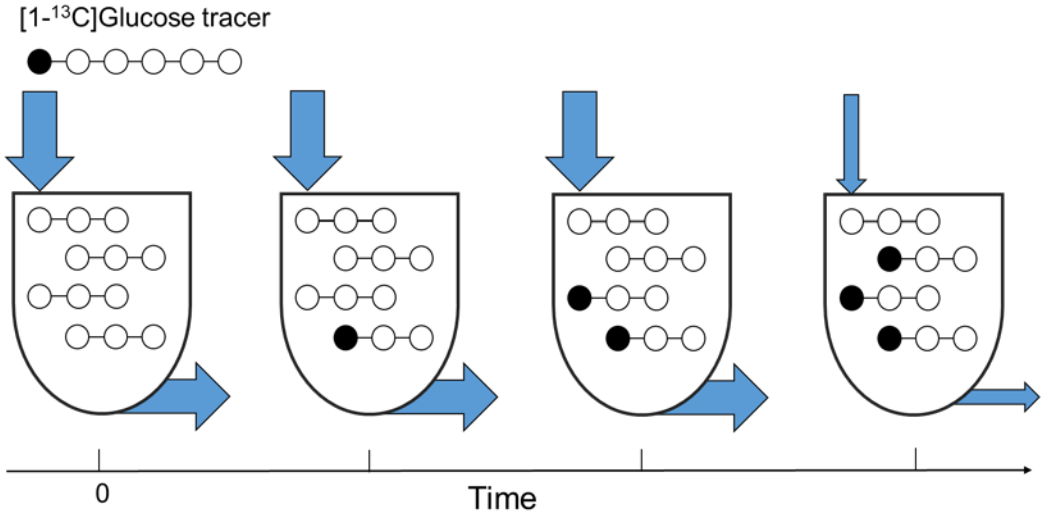
B) Transient isotope enrichment



A) Autotrophic system



B) Slow labeling dynamics



C) Rapid isotopic steady state