

¹³C-FLUXOMICS: A TOOL FOR MEASURING METABOLIC PHENOTYPES

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Introduction

Systems biology combines datasets for gene expression, such as transcriptomics and proteomics, with metabolite profiles (metabolomics) and flux distributions (fluxomics) in order to achieve a holistic view of the system under study (1). This combination of techniques potentially represents the most powerful approach for understanding and manipulating cellular metabolism (2). Where transcriptomics and proteomics provide a measure of metabolic network capacities (i.e., enzyme activities) and metabolomics a measure of thermodynamic driving forces, fluxomics provides a direct measure of the metabolic phenotype, namely the *in vivo* enzyme activity measured as the molar flux through each reaction in the network.

Apart from the most peripheral fluxes, such as uptake and secretion rates, it is not possible to directly measure fluxes in a metabolic network, although they can be inferred from stoichiometric models (3). Over the last two decades, three major approaches for quantitating metabolic flux analysis have been developed: dynamic flux modelling (4), flux balance analysis (FBA) (3,5), and ¹³C metabolic flux analysis (¹³C-MFA)(6). Dynamic flux modelling relies on knowledge of kinetic parameters of all enzymes under study. These parameters are usually very hard or even impossible to determine *in vivo* and therefore the application of dynamic flux modelling will probably remain limited to small local networks.

FBA, also commonly called metabolic flux analysis (MFA), allows the calculation of net fluxes in the cell using mass balancing, which means that, overall, the sum of all molar fluxes entering and leaving a metabolite pool must be zero. It can be applied to underdetermined networks with the use of constraints (reversibility of reactions, upper and lower boundaries of rates, etc), the inclusion of co-factor balances and/or the application of an optimisation function (e.g., to maximise growth). It fails to resolve parallel pathways (7), as well as reversibility of enzymes (8), but is applicable to very large networks (>500 reactions) such as genome scale models. Most importantly, it can be used in a predictive way to study 'what if' scenarios prior to any lab work, for instance, gene essentiality by *in silico* gene knockout.

¹³C-MFA is an extension of FBA. In this approach, precursors (i.e., glucose, amino acids) enriched in the stable carbon isotope ¹³C are introduced into the metabolic system under study. ¹³C label is subsequently incorporated into all metabolites that are directly connected to the original labelled precursor. The level of ¹³C enrichment in metabolites is measured via mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. These enrichments

are used together with stoichiometric constraints of the network to quantify intracellular metabolic fluxes. This enables resolution of parallel pathways and reversibility of enzymes. In contrast to traditional FBA, co-factor balances are not used as constraints for flux calculations, but can be inferred from flux results. Typically, ¹³C-MFA focuses on central carbon metabolism, delivering a flux estimate that describes the metabolic state of a cell at a given time. Several detailed reviews cover ¹³C-MFA (9-11). ¹³C-MFA was first developed for bacteria (12) and yeasts (13), and has predominantly been used for the study of microbial growth in defined medium on a single carbon source, which greatly simplifies determination of uptake and secretion rates, closure of mass balances and labelling analysis of target metabolites. In recent years, ¹³C-MFA has been combined with other '-omics' techniques for in-depth characterisation of microbial systems (14,15). This combination of techniques provides a deeper understanding of the underlying regulation and has been used variously for metabolic engineering, drug design or basic biological research. With more sophisticated quantitative metabolomics arising and faster modelling algorithms available, ¹³C-MFA will be more readily applicable to more complex systems.

Methodology for ¹³C Metabolic Flux Analysis

¹³C-MFA relies on an assumption that the system is at pseudo-steady state, i.e., the molar flux through a metabolite pool is orders of magnitude larger than changes in concentration of this metabolite over time. This assumption is generally valid, except for extreme transient states or accumulation of compatible solutes in the system. In addition, it is assumed that there is no isotope effect, meaning no significant preference of enzymes for labelled or non-labelled substrates and that the system is well mixed. Finally, simulation becomes easier if the system under study is also at isotopic steady state, which means that the labelling distributions of the metabolites are stable. Consequently, the pool size of metabolites and the fluxes through the pool determine how long it takes to reach isotopic steady state. For free intracellular amino acids, this can be reached within minutes to hours (14), while proteinogenic amino acids derived from protein hydrolysates will take several cell doublings before isotopic steady state is reached. For a more dynamic analysis, measurement of isotope incorporation into free intracellular pools of low abundant metabolites are ideal (e.g., sugar phosphates in glycolysis), because isotopic steady state is achieved quickly and the measurement of enrichment kinetics is not necessary.

Performing a ^{13}C Metabolic Flux Analysis

^{13}C -MFA can be separated in four different phases (Fig. 1):

i. Experimental design: Design is important because very costly tracer substrates may be ineffective when used in a suboptimal design. For design, the stoichiometry and the atom transitions need to be known. By performing labelling experiments *in silico* for the expected range of fluxes, it is possible to determine the most suitable labelled substrate(s) to use and the most suitable labelled metabolites to analyse by MS or NMR, i.e., which metabolites have labelling patterns that are most responsive to changes in fluxes. These metabolites can be derived from macromolecules, such as proteinogenic amino acids or free intracellular metabolites. The choice depends on available sample size and equipment sensitivities.

ii. Performing the experiment: Pseudo-steady-state conditions require balanced growth throughout the tracer experiment. It is essential that no nutrient limitations, e.g., oxygen in aerobic experiments, occur during the experiment, biomass composition should be fairly constant and mass balances of the system for at least one macro-nutrient, e.g., carbon or nitrogen, should be closed. Bioreactors guarantee such a controlled environment with balanced growth, but it has been shown that shake flasks and even microplate systems can deliver these conditions within certain limits (16).

iii. Quantitative metabolomics: All major substrates and products need to be accurately quantified. For MFA, the biomass itself is an important product; its major composition (DNA/RNA, protein, carbohydrate, lipid contents) determines a whole range of anabolic fluxes. Finally, MS (17) or NMR (12) equipment is required to quantify the labelling enrichment in the target metabolites (e.g., gas chromatography, mass spectrometry analysis of proteinogenic amino acids). Accurate isotope ratios, but not absolute quantification, of those metabolites is essential.

iv. Flux estimation and sensitivity analysis: Flux estimation is an iterative process, where the stoichiometric and atom mapping networks are used to calculate labelling outputs while achieving the experimentally observed uptake, secretion and growth rates. The calculated labelling outputs are compared to the experimentally determined ones. The fluxes are modified until the differences between calculated and measured labellings are minimised. After this fitting process, the sensitivity of fluxes is further evaluated using statistical procedures. As a result, each flux is obtained with a certain confidence interval.

While phases (ii) and (iii) demand sophisticated experimental and analytical procedures, phases (i) and (iv) require sophisticated modelling software. We recently implemented one of the fastest algorithms for ^{13}C -MFA, the EMU approach (18), in an easy to use, open source software package, OpenFLUX (19) (<https://sourceforge.net/projects/openflux>). This software can be used for experimental design, as well as for flux estimation and sensitivity analysis. It currently supports the most common platform for labelling analysis, mass spectrometry, but since it is supplied open source, users can include their own code in order to use NMR fine spectral information. It also supplies the atom transitions for common reactions of central carbon metabolism, but can be custom designed for any network.

A Simple Example

The basic principles underlying ^{13}C -MFA can be illustrated with a small stoichiometric model that feeds on glucose and only produces serine and CO_2 (Fig. 2). The network contains upper glycolysis and the pentose-phosphate pathway (PPP) without any reversible reactions for simplification. After sketching out the network, mass balances can be drawn around the internal metabolites. The mass balances reveal that the fluxes v_6 , v_7 , and v_8 must be identical, as must v_4 and v_5 . It is obvious that unless CO_2 is quantified, it is not possible to resolve the fluxes through glycolysis or PPP by pure mass balancing. However, atom mapping reveals that in the first step of PPP, C_1 of glucose-6-phosphate (G6P) is lost to CO_2 and this information can be used for a tracer experimental design. Using glucose labelled at C_1 position, the label is retained through glycolysis, yielding one mole labelled glyceraldehyde-3-phosphate (G3P) per mole G6P entering glycolysis. On the other hand, no label is retained if G6P is catabolised via PPP. So by measuring the ^{13}C enrichment in G3P or its derivative serine (SER), the split-ratio between glycolysis and PPP can be calculated (Fig. 2). Since C_1 of G6P becomes C_3 of one of the G3Ps, labelling analysis would in fact only need to quantify the enrichment in this single atom. In order to fully resolve all the fluxes, the only additional measure needed is either the glucose uptake rate or the serine production rate. In addition to the optimal flux value, the sensitivity analysis will then provide a certain confidence interval for each flux parameter based on the measurement errors involved in the analytics.

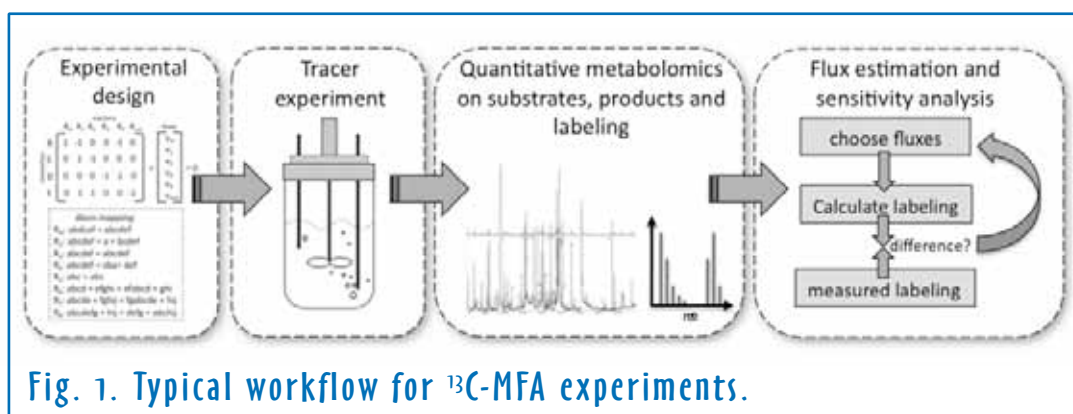


Fig. 1. Typical workflow for ^{13}C -MFA experiments.

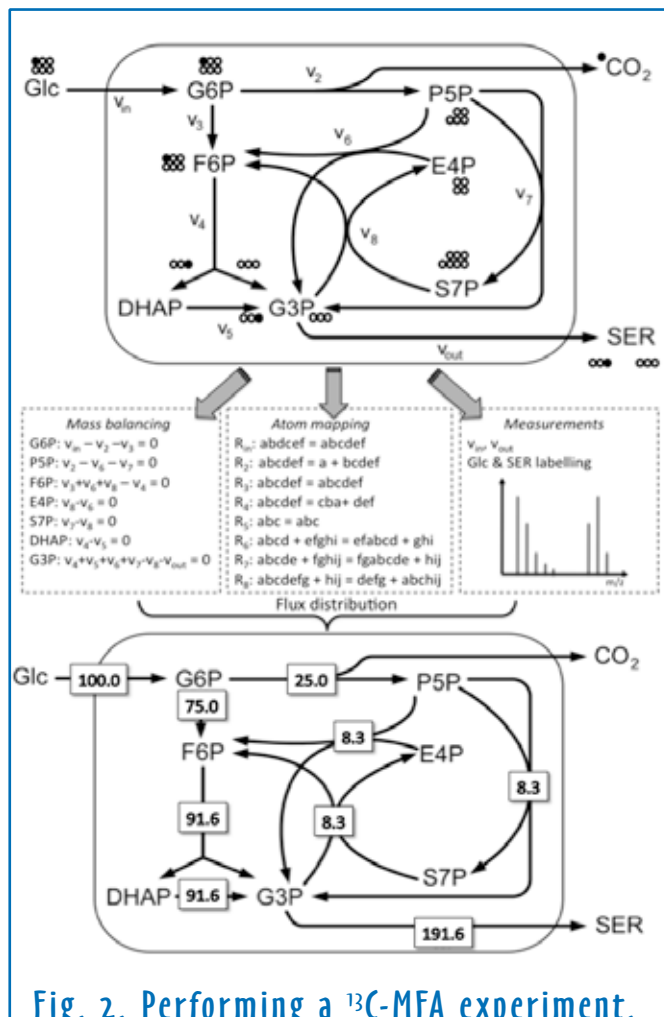


Fig. 2. Performing a ¹³C-MFA experiment.

Designing the network under study, infer mass balances and atom mapping from the network, perform a tracer experiment on labelled substrate (e.g., ¹⁻¹³C glucose), and quantify measurable rates and labellings. Combining all the information in flux analysis software allows the estimation of the *in vivo* flux distribution. Glc = glucose, G6P = glucose-6-phosphate, F6P = fructose-6-phosphate, DHAP = dihydroxy-acetonephosphate, G3P = glyceraldehyde-3-phosphate, SER = serine, S7P = sedoheptulose-7-phosphate, E4P = erythrose-4-phosphate, P5P = pentose-5-phosphate (lumped pool of xylose-5-phosphate, ribose-5-phosphate and xylulose-5-phosphate).

A Complex Example: Flux Analysis in *Saccharomyces cerevisiae* on Two Carbon Sources

Although ¹³C-MFA has been broadly applied to microbial systems such as bacteria or baker's yeast on a single carbon source, performing such an analysis poses additional challenges when looking at compartmentalised eukaryotic systems and/or multiple carbon substrate scenarios. Metabolites present in two compartments will display two distinct labelling patterns based on the reactions in these compartments and cell extraction leads to a mixing of these patterns with unknown ratios, since the concentrations of metabolites in individual compartments cannot be accurately determined. Alternative carbon substrates, on

the other hand, will dilute the label applied and can reduce the sensitivity of labelling towards a change in fluxes.

As an example of a more complex situation, we have studied the addition of glutamate as a nitrogen and alternative carbon source to a standard glucose-based minimal medium for the growth of baker's yeast (Fig. 3).

The network was modelled in OpenFlux with 403 reactions. Of these, 386 were used for mass balancing and 120 for isotopomer balancing. Prior analysis revealed that the network could be resolved using [¹⁻¹³C] glucose (i.e., glucose labelled at C₁), together with unlabelled glutamate, when measuring the uptake and secretion rates of glucose, glutamate, 2-oxoglutarate, ethanol, pyruvate, succinate and fumarate, along with the ¹³C enrichments in thirteen proteinogenic amino acids (alanine, glycine, valine, leucine, isoleucine, methionine, serine, threonine, phenylalanine, aspartate, glutamate, histidine and tyrosine). Sensitivity analysis showed that the fluxes around glycolysis and the pentose-phosphate pathway were more accurately determined than the fluxes around the TCA cycle. Greater accuracy of TCA cycle fluxes would be achieved using additional tracers and adding labelling analysis of selected free intracellular metabolites that more accurately reflect the labelling state of metabolites in cytosol and mitochondria.

Outlook

Since the early 1990s, ¹³C-MFA has been used routinely for the analysis of microbial metabolism on minimal media. Combined with other '-omics' datasets, fluxomics provides a deep understanding of the regulation involved in microbial metabolic networks. The speed of the new algorithms for flux modelling alongside the rapid development of new instrumentation for quantitative metabolomics enables a second generation of ¹³C-MFA experiments applicable to a wider range of increasingly complex systems, such as mammalian cell culture systems. The use of new metabolomics technologies for the labelling analysis of fast turnover intracellular metabolites will allow a more dynamic ¹³C-MFA. We foresee that ¹³C-MFA will evolve into a routine technology within future systems biology frameworks.

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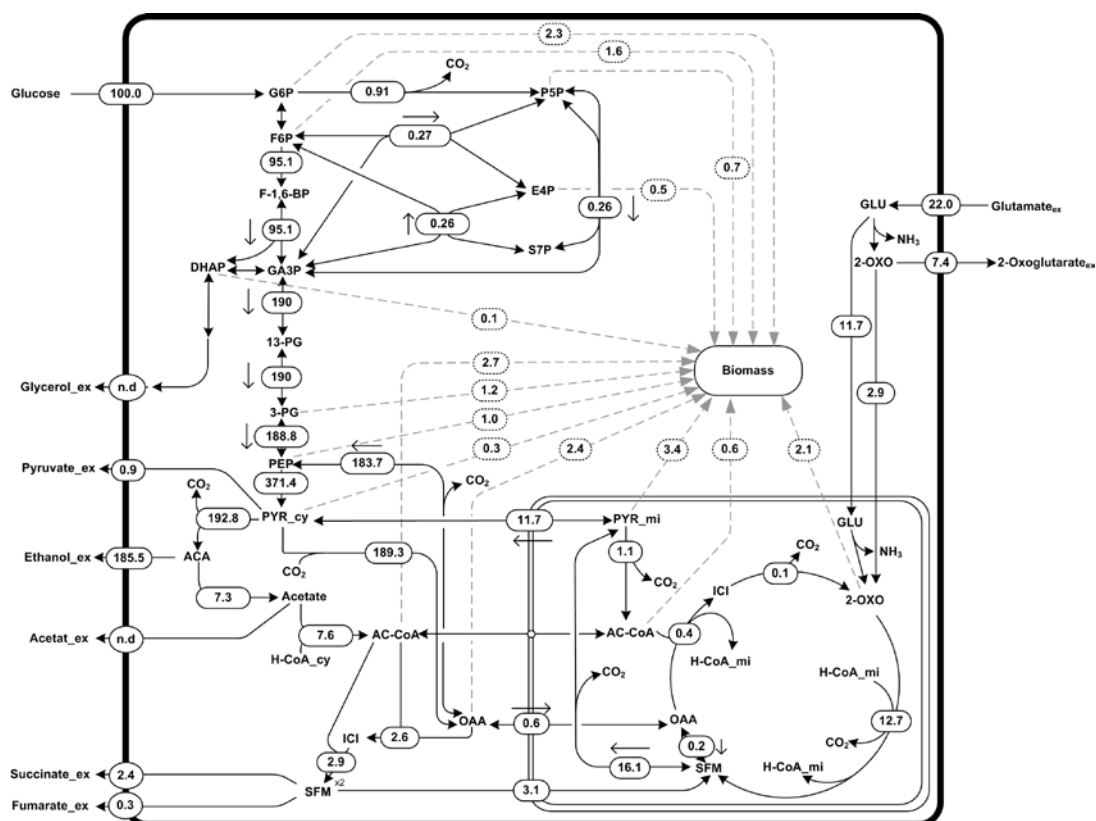


Fig. 3. Simplified flux map describing the flux distributions in *S. cerevisiae* grown on [1-¹³C] glucose and glutamate as the carbon and nitrogen source

The experiment was performed during exponential growth in small scale (10 ml shake flask cultures, 150 rpm, pH=5.8, 30°C, chemically defined medium). The original model contained 403 metabolic reactions. SFM is the lumped pool of succinate, fumarate and malate. The fluxes are shown as net-fluxes in % of the glucose uptake rate [$\text{mmol g}_{\text{CDW}}^{-1} \text{h}^{-1}$]. Direction of reversible reactions is indicated by an arrow pointing towards the overall flux. For simplification of the figure, all anabolic demands are summarised in the respective total drain from central carbon metabolism precursors.

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