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Current Opinion in Biotechnology

# Recent advances in high-throughput $^{13}$C-fluxomics

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The rise of high throughput (HT) strain engineering tools accompanying the area of synthetic biology is supporting the generation of a large number of microbial cell factories. A current bottleneck in process development is our limited capacity to rapidly analyze the metabolic state of the engineered strains, and in particular their intracellular fluxes. HT $^{13}$C-fluxomics workflows have not yet become commonplace, despite the existence of several HT tools at each of the required stages. This includes cultivation and sampling systems, analytics for isotopic analysis, and software for data processing and flux calculation. Here, we review recent advances in the field and highlight bottlenecks that must be overcome to allow the emergence of true HT $^{13}$C-fluxomics workflows.

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**Current Opinion in Biotechnology** 2017, **43**:104–109

This review comes from a themed issue on **Analytical biotechnology**

Edited by **Jurre J Kamphorst** and **Ian A Lewis**

For a complete overview see the Issue and the Editorial

Available online 10th November 2016

http://dx.doi.org/10.1016/j.copbio.2016.10.010



## Introduction

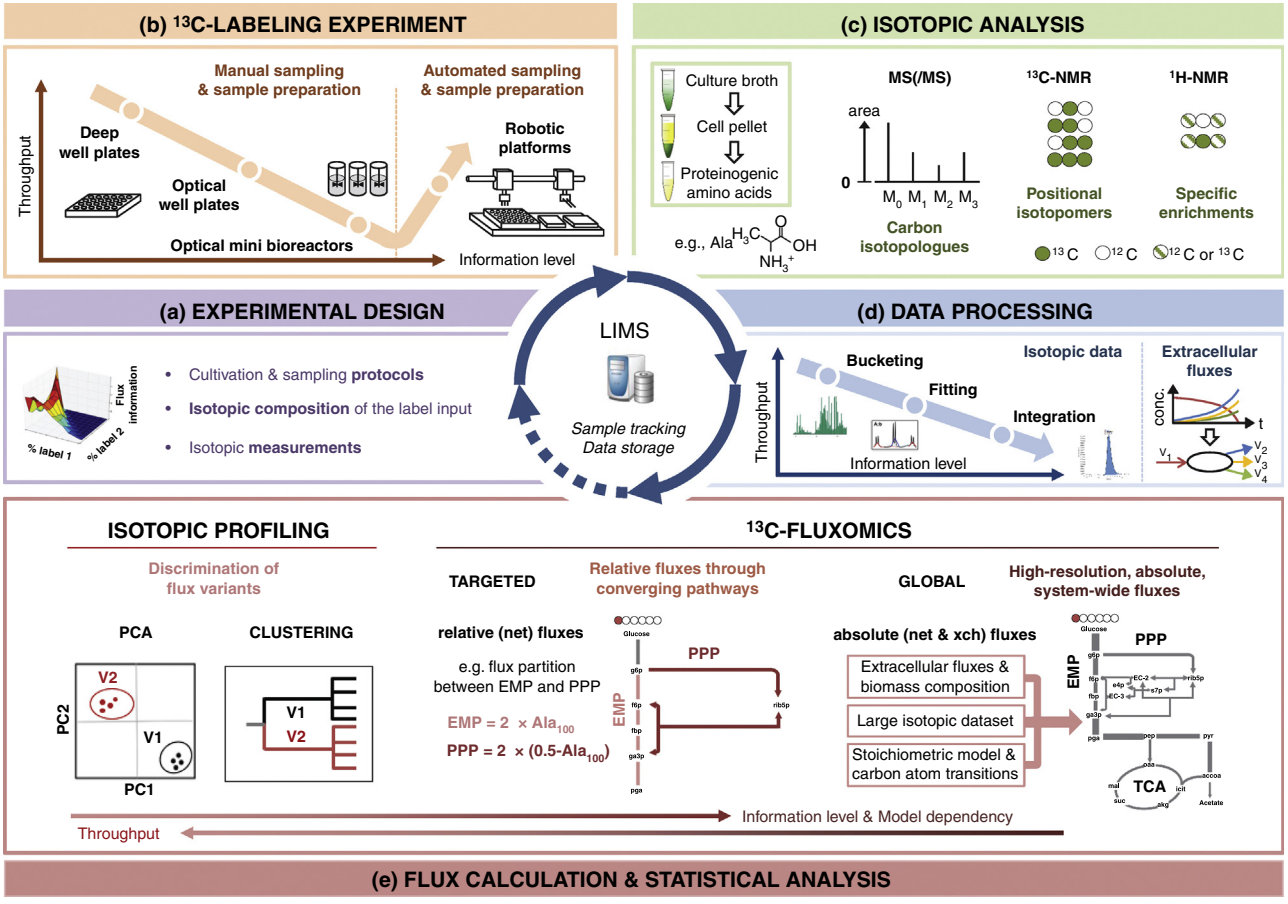
Fluxomics is the determination of the actual reaction rates within metabolic networks. It is increasingly exploited to probe metabolic activity in biological, biotechnological and medical sciences [1–4]. It is recognized in biotechnology as a valuable tool to identify metabolic pathways [5], to assist the discovery of novel regulatory interactions [6], to screen metabolic variants [7,8], to quantify flux responses to environmental and genetic perturbations [9••,10], to support the construction of genome-scale metabolic models [11], or to identify metabolic bottlenecks to increase productivity [12]. The rise of this area is accompanied by advances in molecular biology enabling the creation of a large number of 'cell factories' [1]. Hence, there is increasing need for innovative, automated high throughput (HT) solutions to profile cellular phenotypes and support biotechnological processes.

Extracellular fluxes between cells and their environment can be easily derived from time-dependent changes of extracellular metabolite concentration. By contrast, intracellular fluxes are not directly measurable but can be inferred from $^{13}$C-isotope tracer experiments [13••]. When cells are grown on a $^{13}$C-enriched substrate, $^{13}$C atoms propagate through the metabolic network according to the metabolic pathways and their activity. The $^{13}$C-labeling patterns of metabolic intermediates and cellular components thus depend on the intracellular fluxes. The labeling information can be measured by mass spectrometry (MS) or nuclear magnetic resonance (NMR). The isotopic data can be exploited in three different ways (Figure 1), including the discrimination of metabolic variants (isotopic profiling), the measurement of specific fluxes (targeted flux analysis) or the system-wide investigation of the fluxome (global fluxomics). The quantification of metabolic activity — i.e. flux measurement — is inferred from the labeling data with the help of appropriate mathematical approaches and models that describe isotope propagation through the metabolic network investigated. Finally, $^{13}$C-fluxomics was initially developed to investigate metabolic and isotopic steady-states, but is currently expanding to analyze isotopic and metabolic dynamic states [14,15]. The development of HT fluxomics has focused so far on stationary $^{13}$C-fluxomics, which is well established, robust, and amenable to automation.

## Requirements for an ideal HT $^{13}$C-fluxomics workflow

A typical $^{13}$C-fluxomics workflow (Figure 1) includes the combination of several complex experimental and computational steps. Developing HT methods in this context will inevitably lead to looking for a compromise between the throughput (i.e. number of experiments within a given time period) and information level (number of fluxes and precision of flux values). Hence, the development of an efficient HT $^{13}$C-fluxomics workflow is hindered by several technical challenges. As discussed elsewhere [9••], this includes (1) the careful design of the $^{13}$C-labeling experiments (choice of cultivation medium, label input, isotopic measurements, among others) to maximize the flux information with respect to the biological question to be addressed by the study (Figure 1a); (2) the parallelization of $^{13}$C-labeling experiments to increase the number of conditions that can be investigated (Figure 1b); (3) the miniaturization of the cultivation volume to reduce the costs of the experiments ($^{13}$C-substrates can be very expensive); (4) the strict control of the cultivation conditions to establish and

**Figure 1**




Overview of a high-throughput $^{13}$C-fluxomics workflow. (a) **Experimental design** allows maximizing the flux information depending on the biological question addressed by the study. Besides defining appropriate cultivation conditions (medium composition, temperature, pH, among others), the label input must be optimized according to the isotopic data that can be measured, the metabolic systems investigated, and the experimental costs. (b) Robotic and manual systems are available to perform **$^{13}$C-labelling experiments** and sample preparation. A trade-off between throughput and fine control of growth is determined by the cell cultivation system used and by the degree of workflow automation. (c) NMR-based and/or MS-based **isotopic analyses** are then carried out to measure the $^{13}$C-incorporation into metabolites. (d) **Data processing** is necessary to extract meaningful isotopic information from the raw data. In contrast to isotopic profiling or targeted $^{13}$C-fluxomics, global $^{13}$C-fluxomics also requires measuring extracellular (production and consumption) fluxes from the time-course variations of extracellular concentrations. (e) Finally, different computational approaches for **flux calculation and statistical analysis** are applied according to the purpose of the investigations (i.e. isotopic profiling, targeted fluxomics or global fluxomics) and the level of biological knowledge required to extract the flux information.
MS: Mass spectrometry, MS/MS: Tandem mass spectrometry, NMR: Nuclear magnetic resonance, conc.: Concentration, xch: Exchange, LIMS: Laboratory information management system, EMP: Embden-Meyerhof-Parnas pathway, PPP: Pentose phosphate pathway, TCA: Tricarboxylic acid cycle, Ala: Alanine, PCA: Principal component analysis.

maintain metabolic and isotopic steady-states; (5) the permanent monitoring of growth to collect labeled biomass once appropriate steady-state conditions have been reached; (6) the rapid and automated sampling of labeled material; (7) an automated sample preparation process to cope with the large number of samples; (8) a fast and sensitive analytical technique for isotopic measurements to cope with the low amount of biological material (Figure 1c); (9) an automated isotopic data extraction tool ensuring reproducibility and reliability to alleviate tedious manual processing (Figure 1d); (10) tools for data interpretation (i.e. enabling variant discrimination, flux calculations) with reduced user supervision (Figure 1e); and (11), the integration of all the individual tools into a unified workflow. Below, we discuss the recent advances in HT $^{13}$C-fluxomics and highlight bottlenecks that must be overcome to allow the emergence of more efficient HT workflows.

## Cultivation and sampling

The ability to perform $^{13}$C-labeling experiments with cells grown in controlled and monitored environments is crucial for flux analysis (Figure 1b). Indeed, the tight control of cultivation parameters (i.e. pH, temperature, dissolved oxygen) is necessary to maintain stable physiological conditions, and monitoring the growth parameters

allows to ensure the labeled material is collected under appropriate conditions (metabolic and isotopic steady states). A large number of simultaneous flux experiments can be conducted using multiwell plates [10,16,17•,18]. By performing the cultivation in parallel and miniaturizing the working volumes, these devices significantly reduce the experimental costs. By contrast, they do not allow a tight control of cultivation conditions. HT cell cultivation systems have been developed to overcome this limitation [19–23]. These systems rely on the use of fluorescent probes sensitive to oxygen or pH to monitor cultivation parameters. However manual interventions for monitoring growth and for sampling labeled material are required, which strongly hamper their utilization — as such — for HT flux analysis. Robotic cultivation platforms, which integrate cultivation devices (i.e. microtiter plates or micro-scale bioreactors) into workstations, allow fully automated monitoring of growth parameters while ensuring tight control of cultivation conditions [20,24,25]. These systems were not designed to perform $^{13}$C-labeling experiments or to sample metabolites, which is a crucial task in fluxomics. The full automation of all cultivation and sampling steps has been recently achieved by integrating a block of 48 microscale bioreactors into an automated workstation designed specifically to fulfill flux analysis requirements [9••]. The machine can operate 24 hours a day; and up to 96 samples can be collected and processed in parallel, continuously and with little human supervision [9••]. To cope with the large amount of samples generated, automation of sample preparation is the next logical step. Robotic liquid handlers able to wash, centrifuge, filter and distribute samples in a fully automated manner already exist, and are used for preparing a variety of biological samples [26]. Such devices can be adapted to prepare labeled samples and distribute them in containers (multiwell plates, vial trays, NMR tubes, among others) adapted for MS or NMR analysis.

## Isotopic analyses

Isotopic data for HT flux analysis are typically collected from proteinogenic amino acids (obtained by hydrolyzing proteins) by MS or NMR (Figure 1c). Analyzing proteins, which are the most abundant molecules in microbial biomass, offers an opportunity for reducing sample size. They are chemically stable, which avoids degradation issues during the sampling process. The amino acids released by acidic hydrolysis of biomass proteins contain rich isotopic information, which reflects the labeling patterns of key intermediates of central metabolic pathways. Hence, the labeling patterns of proteinogenic amino-acids provide valuable information to infer metabolic fluxes within central carbon metabolism [27]. Besides proteins, nucleic acids, which are also abundant and stable in microbial cells, could also be valuable material for the purpose of HT fluxomics although they carry limited isotopic information [28,29]. Isotopic analyses of proteinogenic amino-acids are performed with well-established and robust GC–MS and 2D NMR methods which have many attractive, albeit specific, features for $^{13}$C labeling analysis. GC–MS is a sensitive technique requiring only few mg of cells, allowing an excellent separation of the analytes within only 20–30 min, and providing precise measurements of isotopologue distributions [19,30••]. Albeit not yet applied to HT fluxomics, direct infusion methods might be highly amenable to strongly alleviate sampling and sample preparation in HT protocols [31,32]. By contrast to MS, NMR provides quantitative information on the incorporation of the $^{13}$C label in specific positions of molecules, and can be applied to complex mixtures of metabolites without the need to separate them [33••]. Several NMR experiments have been developed to quantify specific (sub)sets of isotopomer by exploiting $^{1}$H–$^{13}$C and $^{13}$C–$^{13}$C couplings [33••]. The acquisition time varies from few minutes to hours depending on the NMR experiment.

Continuous efforts have enhanced the sensitivity, speed, and robustness of both NMR-based and MS-based isotopic analyses [34–39]. To help alleviate the increasing number of samples and thus the amount of data generated by NMR and MS, automated processing tools have been developed for isotopic data extraction [9••,40] and for post-processing steps, such as correction of MS(/MS) data for naturally occurring isotopes [41] (Figure 1d). These methods are suitable for fast, robust, and reproducible isotopic analysis, and they are now sufficiently mature to be integrated into HT platforms [8,9••,42–44].

## Model-dependent and model-independent data interpretation

The interpretation of $^{13}$C-labeling data requires mathematical and computational tools [13••] (Figure 1e). Different computational approaches have been developed. The most effective tool depends primarily on the biological system and objective of the study, but also on other important factors, such as accessible isotopic data, cultivation conditions, and number of experiments. Current approaches allow (*i*) to discriminate strains or conditions (isotopic profiling), (*ii*) to estimate local ratio of fluxes in converging pathways (targeted $^{13}$C-fluxomics), and (*iii*) to finely quantify the absolute system-wide fluxes (global $^{13}$C-fluxomics).

Isotopic profiling exploits unsupervised, multivariate statistical classification (such as clustering or principal component analyses) to discriminate strains or conditions based on $^{13}$C-labeling data, without a priori knowledge of the metabolic system [7,9••,45,46]. Isotopic profiling only provides information about differential metabolic activity — that is, similar fluxomes are grouped together, thus highlighting flux variants. Biological interpretations are supported by the isotopic variables contributing to group discrimination. As a major advantage, isotopic profiling does not rely on mathematical models of the metabolic

system investigated, and can be applied to any biological system and condition. This approach is particularly relevant for comparing a very large number of strains and conditions in functional screening studies [7,8,9••].

Beyond isotopic profiling, targeted $^{13}$C-fluxomics provides quantitative information on the relative contribution of converging pathways to the formation of a particular metabolite, based on straightforward analytical interpretations of $^{13}$C-labeling patterns [13••,47]. The estimated flux ratios can then be used as additional constraints in (undetermined) stoichiometric models to estimate (net) fluxes at the network level.

Global $^{13}$C-fluxomics has been developed to increase both the number of fluxes that can be calculated and the precision on the estimated fluxes. This approach requires the integration of large isotopic data sets and additional measurements (extracellular uptake and production rates and chemical composition of biomass) using a stoichiometric model of the network that contains carbon atom transitions for each reaction [13••,48]. System-wide isotopic mass balances are constructed to simulate the propagation of $^{13}$C-label through the network, and absolute fluxes are estimated through an iterative procedure by minimizing the difference between experimental and simulated data. Appropriate statistical methods are eventually applied to estimate the flux uncertainty with respect to the measurements and their precision [48,49].

Several methods and software are now available for isotopic profiling [7,9••] and for flux calculation in targeted [47,50,51] or global [52,53•,54–56] approaches. Global $^{13}$C-fluxomics is more computationally demanding than targeted $^{13}$C-fluxomics and was thus initially limited to the analysis of a few conditions. More efficient mathematical frameworks have been developed to speed up simulations according to the type of isotopic data that are measured (MS, MS/MS, $^{1}$H NMR, $^{13}$C NMR) [41,57–61], novel optimization algorithms have improved both calculation times and accuracy of the convergence process [53•,55,62], new modeling frameworks have drastically increased the size of networks that can be investigated [63•], and the computational burden associated to HT studies has been reduced by exploiting multicore CPUs and grid computing [53•,54,55]. These computational and mathematical advances have made global $^{13}$C-fluxomics now applicable on a large scale [8,10,46,64,65].

## Towards an integrated $^{13}$C-fluxomics workflow

To achieve the full potential of HT fluxomics, efficient workflows that integrate the different steps of the analytical process are needed not only to accelerate the application of fluxomics but also to make it more robust and more reliable. A range of software is available that perform the main computational aspects of a fluxomics experiment, including experimental design, (isotopic) data analysis, flux calculations, and data (flux) visualization [66,67]. A crucial step towards integrated fluxomics workflows is the standardization of data and processes in fluxomics, which has received attention recently [13••,68••]. Standardization of models and model formats, as well as integration of isotopic modeling in the more global context of metabolic network modeling (e.g. constraint-based modeling, kinetic modeling, among others), needs to receive specific attention in the near future. Finally, the most crucial step is the integration of all these software in comprehensive pipelines available to the non-specialized scientists through dedicated interfaces, similar to what is currently observed in the field of metabolomics. This can be achieved by integrating current software into flexible and interactive tools that facilitate their automated application [69]. The most advanced work in the field of integrated fluxomics workflow was recently published by Dalman *et al.* [70••]. This work is establishing the foundations of HT fluxomics workflows by providing not only access to a complete library of tools for flux simulation and calculation, but also to tools for the storage and management of data, metadata, models, versioning, protocols, to advanced visualization tools, and to a complete service-oriented interface. This work represents a milestone in the development of integrated and flexible fluxomics workflows that can allow either highly detailed investigations of a few fluxomes or automated, large-scale investigations by HT fluxomics.

## Concluding remarks

Significant progress in the development of HT fluxomics made in the last few years has contributed to deepening our understanding of cellular metabolism. As illustrative examples, HT fluxomics has allowed to identify transcription factors that control metabolic fluxes in yeast under a given condition [17•], to reveal the conservation of the Entner–Doudoroff pathway as a common glycolytic strategy among glucose-using marine bacteria [8], or to elucidate the control exerted by key enzymes on metabolic fluxes [9••].

Being a very complex process requiring many heterogeneous tasks and sub-tasks to be performed and iterated, improvements still remain to be made to automatize the complete workflow, to increase the number of investigations, and to improve the quality of flux measurements. Further developments should be made by keeping flexibility in mind, since each fluxomics investigation needs to be carefully adapted to the considered organism, biological question and physiological state. All together, this will undoubtedly contribute to the expansion of HT $^{13}$C fluxomics applications.

## Acknowledgements

We acknowledge support from the INRA (Institut National de la Recherche Agronomique) and MetaToul (Metabolomics & Fluxomics facilities, Toulouse, France, http://www.metatoul.fr), which is part of the national

infrastructure MetaboHUB-ANR-11-INBS-0010 (The French National Infrastructure for Metabolomics and Fluxomics, www.metabohub.fr). We thank Ed Kent for careful reading of the manuscript.

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