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Isotopically non-stationary metabolic flux analysis: complex yet highly informative

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Metabolic flux analysis (MFA) using isotopic tracers aims at the experimental determination of *in vivo* reaction rates (fluxes). In recent years, the well-established ^{13}C-MFA method based on metabolic and isotopic steady state was extended to INST-MFA (isotopically non-stationary MFA), which is performed in a transient labeling state. INST-MFA offers short-time experiments with a maximal information gain, and can moreover be applied to a wider range of growth conditions or organisms. Some of these conditions are not accessible by conventional methods. This comes at the price of significant methodological complexity involving high-frequency sampling and quenching, precise analysis of many samples and an extraordinary computational effort. This review gives a brief overview of basic principles, experimental workflows, and recent progress in this field. Special emphasis is laid on the trade-off between total effort and information gain, particularly on the suitability of INST-MFA for certain types of biological questions. In order to integrate INST-MFA as a viable method into the toolbox of MFA, some major challenges must be addressed in the coming years. These are discussed in the outlook.

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Introduction

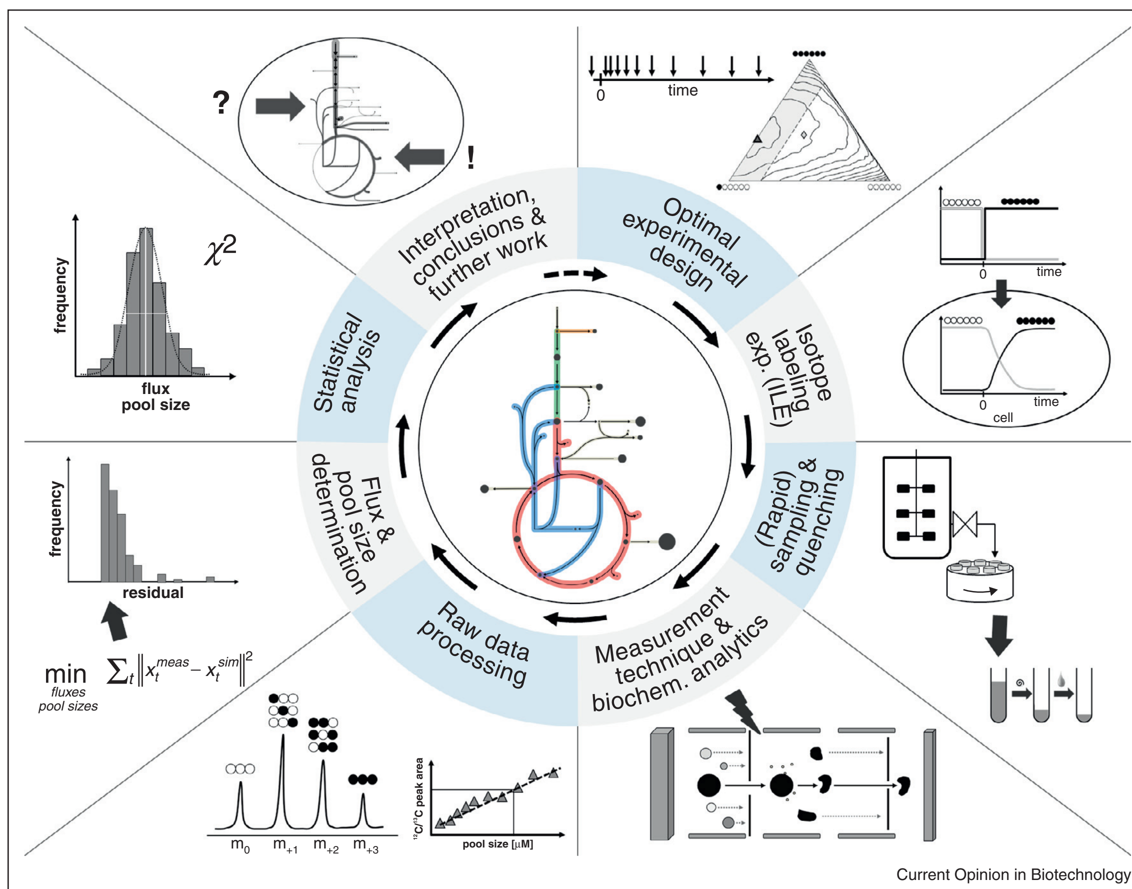
The physiological phenotype of a cell is constituted by its metabolic fluxes [1]. The *in vivo* measurement of as many intracellular fluxes as possible provides invaluable information for understanding cellular regulation in response to genetic interventions or changed environmental conditions. The result of a metabolic flux analysis (MFA) — seen as an experimental protocol [2] — is a flux map which gives a quantitative picture of metabolism in action (Figure 1, center).

Unfortunately, no direct measurement principle exists for observing the *in vivo* flow of metabolites through a biochemical network [3,4]. For this reason fluxes must be calculated indirectly from other, measurable information. Here, simple stoichiometric methods solely based on measurements of the extracellular fluxes between cell and environment (growth/uptake/production rates) are strongly limited because they crucially rely on critical biological assumptions [5]. Complementing extracellular data by intracellular isotopic labeling information requires a higher effort but is found to be more reliable theoretically and practically [1,6,7]. After almost two decades of development, the ^{13}C method is now established as a quasi-standard for MFA and is still experiencing new applications and extensions [8–14,15••,16,17•,18].

Generally, the isotope labeling experiment (ILE) underlying a MFA is initiated by a feed change from an isotopically unlabeled to labeled substrate followed by the observation of dynamic labeling profiles within the intracellular metabolite pools (see Figure 2) [2]. Here, it makes a big difference whether steady-state or dynamic information is required. So far, both ^{13}C-MFA and isotopically non-stationary MFA (INST-MFA) assume a metabolic steady state (i.e. constant fluxes). In the case of the much faster INST-MFA this also allows for the investigation of quasi-stationary processes with constant fluxes over short time intervals [19••]. Moreover, some initial concepts for both metabolically and/or isotopically dynamic methods have been published [20–24] which will not be discussed in more detail.

While the evaluation of a classical ILE is based on proteinogenic labeling information, it is now possible to measure labeling directly in metabolic intermediates [19••,25–27]. Unfortunately, the protein pool is coupled to metabolism in both directions (synthesis/degradation), which means that even in intracellular metabolite pools it can take a rather long time to reach an isotopic steady state [28,29]. Thus, the cell doubling time is still a lower bound for the duration of an ILE [30]. This situation changes significantly with the INST-MFA method. This novel approach relies on the time-resolved measurement of labeling enrichment in intracellular metabolite pools (cf. Figures 1 and 2). On the one hand — as it is unnecessary to wait for an isotopically steady state — this, under optimal conditions, enables the metabolic fluxes to be determined within some few minutes [19••,31•]. On the other hand, INST-MFA is clearly much more demanding than conventional ^{13}C-MFA with respect to experimental, analytical and computational effort.

Figure 1



General workflow of INST-MFA involving well-planned experiments, experimentation under well-controlled and monitored conditions, advanced bioanalytics, accurate sampling devices, efficient sample handling, extensive raw data processing, and advanced model-based data evaluation, usually in an iterative modeling experimental procedure (see main text for details).

Currently, only a few fully quantitatively evaluated INST-MFA experiments have been reported. After establishing the dynamic theory [32], the experimental concept of INST-ILE experiments was proposed in [30,33], and the first experiments were published in [19••,34•] almost simultaneously. Since then, there have been a few further applications to diverse biological systems [7,11,35,36•,37–41]. Other recently reported INST experiments have not yet been quantitatively evaluated on the basis of a full-scale network model [15••,17•,42,43,44•]. These rather specific or explorative approaches are not discussed here in detail. It should also be noted that the terminology is not yet used consistently [30,35,43,44•].

For the historical development and broader field of ^{13}C-MFA, the reader is referred to general reviews [5,8,45,46•]. The principles, methodology, and analytics of INST-MFA were recently reviewed [31•]. As a complement to this review, after briefly outlining the general procedure of INST-MFA, the present contribution discusses in detail in which situations the new method is superior to conventional methods or there is even no alternative at all. After that we focus on the novel methods' challenges, open questions, and the trade-off between effort and information gain.

Work flow of INST-MFA

Because of the limited number of full-fledged INST-MFA examples and their heterogeneity with respect to biological system, underlying time scale, measurement equipment and computational framework, there is currently no consensus or even standard. However, the following general work flow (see Figure 1) is — more or less — common to all examples.

Optimal experimental design (*OED*): The expected information gain of an INST-ILE can be strongly influenced by several freely configurable parameters (see Figure 2a–d). These are labeled substrate composition, sampling times, measured intracellular metabolites and measurement configuration. Informative experimental designs

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