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Review

Understanding metabolism with flux analysis: From theory to application

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ABSTRACT

Quantitative and qualitative knowledge of metabolic rates (i.e. fluxes) over a metabolic network and in specific cellular compartments gives insights into the regulation of metabolism and helps to understand the contribution of metabolic alterations to pathology. In this review we introduce methodology to resolve metabolic fluxes from stable isotope labeling and relevant techniques in model development, model simplification, flux uncertainty analysis and experimental design that together is termed metabolic flux analysis. Finally we discuss applications using metabolic flux analysis to elucidate mechanisms pertinent to tumor cell metabolism. We hope that this review gives the readers a brief introduction of how flux analysis is conducted, how technical issues related to it are addressed, and how its application has contributed to our knowledge of tumor cell metabolism.

1. Introduction

Metabolism is essential to cell physiology by providing energy, building blocks, signaling molecules and redox reagents that together are indispensable for cell survival and growth. Metabolic networks for multiple organisms, including human tissues, have been annotated (Thiele et al., 2013; Thiele and Palsson, 2010) and other 'omics' datasets, including genomics, transcriptomics, proteomics and metabolomics, have expanded rapidly in the last decade (Yizhak et al., 2015). However, the most biologically and physically relevant aspects of metabolism, metabolic fluxes, cannot be completely determined directly from other omics data. In analogy to the traffic condition in a city (Hiller and Metallo, 2013; Sauer and Zamboni, 2008), the city is in a traffic jam if its streets are filled with slowly moving cars. Knowing how many cars are on the road (concentration of metabolites), how many people are driving on each road (abundance of transcripts or proteins), the reasons for why people would be driving at a certain time (genetic events and environmental factors) are insufficient to know exactly whether the cars are stalled or are able to move freely (flux configuration in the metabolic network).

Due to the inability of molecular measurements to completely determine metabolic fluxes, technical approaches towards resolving metabolic fluxes are indispensable. Here we refer to metabolic flux analysis as the collective set of techniques and information related to rates of metabolic reactions taking place inside a cell and exchange of metabolites between the cell and the extracellular environment or between intercellular compartments. For exchange fluxes through which metabolites are absorbed or released by the cells, the fluxes can be determined by time-dependent profiles of metabolites in the media since the exchange flux is the only factor contributing to the dynamics of metabolites in media (Jain et al., 2012). However, in the case of intracellular fluxes, metabolite kinetics are determined by multiple chemical reactions producing or consuming the metabolite or by its exchange into and out of numerous cellular compartments, which hinders direct evaluation of fluxes from isolated metabolite dynamics alone. On the other hand, most metabolic network models contain more fluxes than metabolites, resulting in an underdetermined system if only information on the level of metabolites is considered.

Stable isotope labeling experiments, or often referred to as tracing experiments, in which incorporation of labeled substrates into intermediates of the metabolic network is utilized to resolve the fluxes, afford a solution. Most frequently used tracers in tracing experiments are ^{13}C-labeled substrates, but other tracers including ^{2}H, ^{15}N and ^{18}O are also used (Klein and Heinzle, 2012). Besides its application in quantitatively determining the flux configuration in a metabolic network, tracing experiments are also applicable in qualitative evaluation of metabolic fluxes such as the dependence of metabolic pathways on certain carbon sources and the partitioning of fluxes into diverging pathways at a branch point (Buescher et al., 2015). At the most descriptive level, a pathway's activity that originates from a substrate is implied if intermediates in this pathway become significantly enriched with when that labeled substrate is supplemented. This strategy is widely applied in identifying nutrient dependencies of metabolic pathways in cancer, which will be discussed further in Section 4. Partitioning of metabolic flux into multiple pathways at a branch point could also be qualitatively evaluated if the products of the alternative

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pathways have different labeling patterns. Examples include the glycolysis versus oxidative branch of pentose phosphate pathway traced by [1-^{13}C]-glucose (Zhao et al., 2016), oxidative versus reductive metabolism of glutamine through the TCA cycle traced by [U-^{13}C]-glutamine (Mullen et al., 2012), and pyruvate entering TCA cycle through pyruvate dehydrogenase (PDH) versus through pyruvate carboxylase (PC) traced by [U-^{13}C]-glucose (Sellers et al., 2015) or [1-^{13}C]-pyruvate (Cheng et al., 2011). In order to achieve a quantitative evaluation of fluxes, labeling patterns obtained from tracing experiments must be combined with detailed information of the metabolic network including both stoichiometry and atom-transition matrices. There are several techniques that vary in model, algorithm and experimental data used, which have been introduced by previous reviews and protocols (Buescher et al., 2015; Niedenführ et al., 2015; Shestov et al., 2013; Yuan et al., 2008; Zamboni, 2011; Zamboni et al., 2009) and will be discussed in detail in the next section.

2. Overview of methods

2.1. Isotopic steady-state methods

Among the myriad of techniques for flux analysis, isotopic stationary metabolic flux analysis, or ^{13}C metabolic flux analysis (^{13}C MFA), is the most commonly used method (Buescher et al., 2015; Wiechert et al., 1999, 1997; Wiechert and de Graaf, 1997; Zamboni et al., 2009). By feeding cells with ^{13}C labeled substrates, different distributions of carbon fluxes into branched and convergent metabolic pathways result in different steady state distribution of isotopomers for intermediate metabolites, which can be determined by techniques like mass spectrometry (MS), or nuclear magnetic resonance (NMR) spectroscopy and utilized in inferring the flux ratio at branching points or absolute fluxes, with the supplementary knowledge of absolute exchange fluxes with the extracellular environment. Here we refer to isotopomers as metabolites that only differ in isotope distribution. For a molecule with N carbon atoms, there are 2^N isotopomers in total. Two points regarding the metabolic network are essential considerations: the stoichiometry and atom mapping information for each reaction. With this knowledge and under the assumption of metabolic steady state (metabolite concentrations do not change with time) and isotopic steady state (isotopomer distributions do not change with time), algebraic equations connecting metabolic fluxes and steady state isotopomer distributions can be derived by balancing the rate of production and consumption of each isotopomer:

$$\frac{dM_{ij}}{dt} = \sum_{k\in In(i)} v_k \sum_{\Theta\in Gen(k,i,j)} \prod_{(l,m)\in\Theta} r_{lm} - \left(\sum_{k\epsilon Out(i)} v_k\right) r_{ij} = 0. \quad (1)$$

In this equation, M_{ij} is the abundance of the jth isotopomer of metabolite M_i, $In(i)$ and $Out(i)$ are the sets containing all indexes of fluxes producing and consuming M_{ij}, respectively. $Gen(k, i, j)$ is the set containing all substrate isotopomer combinations that produce M_{ij} via the flux v_k, r_{ij} is the fraction of isotopomer M_{ij}, $\boldsymbol{v}$ is the flux vector which is also constrained by the metabolic steady state assumption and nonnegative constraints:

$$\boldsymbol{S}*\boldsymbol{v} = 0, \boldsymbol{v} \geq 0. \quad (2)$$

Here, S is the stoichiometric matrix describing a metabolic network.

By solving the algebraic equations, the flux vector $\boldsymbol{v}$ can be calculated from the isotopomer distribution $\boldsymbol{r}=\{r_{ij}\}$. With efficient algorithms for simulating profiles of steady state isotopomer distributions available, the unknown flux variables can be evaluated by solving a large-scale constrained non-linear least squares problem, in which the difference between isotopomer distribution profiles simulated from assumed flux configurations and measured by experiments is minimized (Fig. 1):

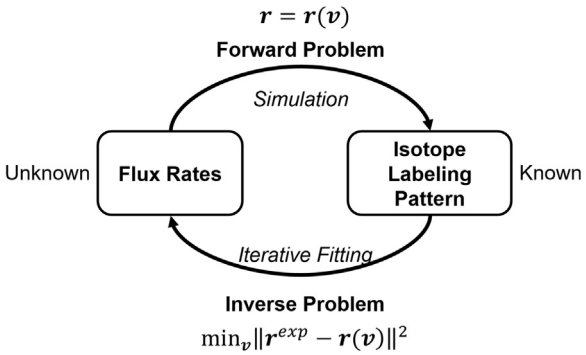

Fig. 1. Metabolic flux analysis as an inverse problem. Inferring metabolic fluxes from isotope labeling profiles is typically done by minimizing the difference between isotope labeling patterns simulated from fluxes and measured by experiments.

$$\min_{\boldsymbol{v}} \|\boldsymbol{r}^{exp} - \boldsymbol{r}(\boldsymbol{v})\|^2 . \quad (3)$$

The non-linear least squares problem could be solved using optimization algorithms such as sequential quadratic programming, the Levenberg-Marquardt algorithm and so on (Boggs and Tolle, 1995; Marquardt, 1963; Wiechert et al., 1997). Note that since the algebraic Eq. (1) and the metabolic steady state constraints (2) are both linear to the flux variables, solutions of the Eqs. (1) and (2) form a convex cone, that is, all linear combinations of some feasible flux configurations with nonnegative coefficients will still be feasible solutions. Thus, absolute flux rates cannot be determined solely by solving (1) and (2). In order to infer absolute fluxes, tracing experiments must be combined with metabolite exchange rates measured by other techniques or assumptions on metabolic objective functions, which are commonly used in flux balance analysis.

A simpler alternative of ^{13}C-MFA, flux ratio (FR) analysis, has some practical advantages over ^{13}C-MFA when the goal is to determine relative forward fluxes of converging pathways. Mass isotopomer balance equations that have very simple forms are easily written at branch points of the metabolic network, which enables direct calculation of the relative fluxes from mass isotopomer distribution vectors (MDVs) of the product and the alternative substrates (Fischer and Sauer, 2003; Zamboni et al., 2009). Besides its simplicity in computation, FR doesn't require the complete topology of metabolic network to be constructed, thus facilitating application where there is incomplete knowledge of the metabolic reaction network.

2.2. Isotopic non-steady state methods

A limitation of ^{13}C-MFA is that it is based on the assumption of isotopic steady state, which is hard to determine and can take several hours upon addition of the labeled substrate in a mammalian system. This analysis is also difficult to interpret when cells are undergoing a response to a perturbation such as a drug treatment or growth factor withdrawal. By relaxing the assumption of isotopic steady state while keeping the assumption of metabolic steady state, we have isotopically non-stationary metabolic flux analysis (INST-MFA), which essentially treats the metabolic network as a dynamical system. Instead of isotopomer balance equations at the steady state which are solved in ^{13}C-MFA, ordinary differential equations (ODEs) describing how the isotopomer distributions change with time are solved to simulate the dynamical isotopic profile, which is fit to corresponding experimental data (Wiechert and Nöh, 2013). In INST-MFA, since isotopomer distribution is measured on multiple time points and isotopomer dynamics is simulated by solving ODEs instead of making measurements on only one time point and solving linear equations, it is apparently more demanding in computational time than ^{13}C-MFA.

A special case of INST-MFA, termed kinetic flux profiling (KFP), is also applied in resolving metabolic fluxes due to its simplicity in data