

Dynamics of positional enrichment: Theoretical development and application to carbon labeling in *Zymomonas mobilis*

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Abstract

Positional enrichment analysis has become an important technique for assessing detailed flux distributions and the fates of specific atoms in metabolic pathway systems. The typical approach to positional enrichment analysis is performed by supplying specifically labeled substrate to a cell system, letting the system reach steady state, and measuring where label had arrived and accumulated. The data are then evaluated mathematically with the help of a linear stoichiometric flux distribution model. While this procedure has proven to yield new and valuable insights, it does not address the transient dynamics between providing label and its ultimate steady-state distribution, which is often of great interest to the experimentalist (pulse labeling experiments). We show here that an extension of a recent mathematical method for dynamic labeling analysis is able to shed light on these transitions, thereby revealing insights not obtained with traditional positional enrichment analyses. The method traces the dynamics of one or more carbons through fully regulated metabolic pathways, which, in principle, may be arbitrarily complex. After a brief review of the earlier method and description of the theoretical extension, we illustrate the method with an analysis of the pentose phosphate pathway in *Zymomonas mobilis*, which has been used for traditional positional enrichment analyses in the past. We show how different labeling schemes result in distinctly different transients, which nevertheless eventually lead to a steady-state labeling profile that coincides exactly with the corresponding profile from traditional analysis. Thus, over the domain of commonality, the proposed method leads to results equivalent to those from state-of-the-art existing methods. However, these steady-state results constitute only a small portion of the insights obtainable with the proposed method. Our method can also be used as an “inverse” technique for elucidating the topology and regulation of pathway systems, if appropriate time series data are available. While such dynamic data are still rather rare, they are now being generated with increasing frequency and we believe it is desirable, and indeed necessary, to accompany this trend with an adequate, rigorous method of analysis.

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1. Introduction

Isotopomers are molecules consisting of the same atomic structures but differing in the positions of isotopic substitutions. Radioactive labeling in specific positions and novel sophisticated tracking techniques have made it possible to customize isotopomers and use them to determine the fates of individual atoms during metabolic processes [1–3]. For instance, using glu-

cose input that is labeled in one specific position, it has become possible to determine exactly how the hexose is split into trioses during glycolysis and how specific carbon atoms move through the citric acid cycle. For a molecule of n isotopic positions, the complete isotopomer set contains 2^n possible labeling states if two isotopes such as ^{12}C and ^{13}C are considered. If complex pathways are considered, the number of “labeling states” grows quickly and becomes an impediment to experimental and theoretical studies alike, rendering specific tracking tasks *in vivo* complicated. Nonetheless, great strides have been made, at least for analyses of metabolic systems that are allowed to reach steady state. On the experimental side, nuclear magnetic resonance [4] and mass spectrometry [5] have proven to be very effective.

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Dictated primarily by the complexity of mathematically representing general dynamic labeling processes, theoretical analyses have mostly focused on the distribution of fluxes in stoichiometric network models at steady state [1,3,6–8]. Nonetheless, the potential benefit of dynamic analyses has been acknowledged and addressed in rudimentary form [9], for instance, for kinetic investigations of individual enzymes [10,11] and in the analysis of secondary metabolism [12]. In fact, early studies that described the rate of change in metabolites with differential equations and multiplied the fluxes with appropriate tracer-to-trace ratios ([9,13]; see also [14–18]) may be seen as the foundation of a general formulation of the mathematical tracking problem, as it is proposed here.

The advantages of extending analyses beyond the steady state are clear. First, the steady-state distribution of fluxes does not provide any information about the speed with which material distributes throughout the system. In fact, given only one measurement of the flux distribution, it is not clear whether the system even has reached steady state so that present methods are valid. From a biochemical point of view, steady-state analysis is useful for evaluating levels of metabolites as well as degradation/clearance rates. On the other hand, pulse labeling studies provide unique information on rates of synthesis and incorporation. For these and other reasons, many biochemists prefer pulse labeling and combination studies over exclusive steady-state analyses. Second, a dynamic analysis allows a much refined exploration of control and regulation in the system. This exploration may be achieved through simulation studies or, at least in principle, through nonlinear regression in cases where experimental time series measurements are available. In particular, if former experimentation had concluded that a pathway could be organized in one of two ways, a targeted experiment, evaluated with the method proposed here, could permit the final determination. Third, the proposed method allows a true evaluation and interpretation of positional labeling experiments, if they are executed as time series, which is not possible with traditional steady-state analysis. Fourth, in some situations, only the labeling dynamics yields true insight, as is the case for CO₂ assimilation during autotrophic photosynthesis [19]. In other cases, such as batch cultures, where continuous labeling may be intractable or impractical, the system may not even reach a steady state. Fifth, further complications with traditional methods arise if the system contains flux cycles, if ubiquitous metabolites like ATP or NADH are involved in the labeling process, or if the pathway simply becomes too complex [19]. Recently, Wiechert and collaborators proposed an approach called *instationary metabolic flux analysis* [20–22], which allows to some degree the tracking of isotopomer dynamics of metabolites during transitions. This approach is not generally applicable, though, because it requires that the fractions of labeled metabolites are in a (pseudo)-steady state and that the input label pulse does not modify the internal metabolite levels. Sixth, given good data, the method can be used to explore the connectivity and regulation of pathway systems, by virtue of inverse engineering and the matching of simulated labeling results computed for hypothesized model structures against observation data. In this mode, the method not only elu-

cidates flux rates and flux split ratios, but has the potential of discovering the full dynamics of the system. In fact, since our method involves several sets of equations, but no more parameters than a simple pathway model, this inverse task is not any more complicated than other reverse engineering tasks that have been discussed widely in the recent literature [23–27].

Pursuing a different and more general strategy, we proposed a mathematical method for following a radioactive label throughout a fully dynamic metabolic pathway system [28]. The method combined probabilistic arguments with kinetic modeling based on generalized mass action (GMA) equations and predicted the likely distributions of labeled and unlabeled molecules within each metabolite pool throughout the experimental period of time. The method was shown not to require assumptions on metabolic effects of the input pulse, the size of the pulse, or the structure or complexity of the system. We tested the method first with artificial data and subsequently through simulations of yeast sphingolipid metabolism, where we predicted the dynamics and fate of a labeled palmitate substrate. Validation experiments showed a high degree of semi-quantitative concordance with the model predictions [29].

Our earlier approach to assessing the dynamics of label was designed to characterize how much of a given metabolite pool is labeled at a given point in time. However, this method does not allow a distinction between different degrees of labeling within a given molecule or between labels at different atomic positions. This latter distinction is at the core of *positional enrichment* (also referred to as *atom positional labeling* or *fractional enrichment*) analysis and the topic of the model extension presented here. As in the original paper, the method is independent of a particular kinetic modeling framework, and we will illustrate it with “canonical” GMA models within biochemical systems theory (BST; [30–33]). In these models, each flux is represented as a product of power-law functions that contains all variables contributing to the dynamics of this flux, along with a rate constant. The variables are raised to powers, called kinetic orders, whose signs indicate whether their effect is augmenting (positive sign) or diminishing (negative sign). The repertoire of dynamic responses that can be captured by GMA models is vast [34], and GMA models have been used successfully in many contexts, within and outside metabolic pathway analysis. The wide applicability and flexibility of these models is important, because, as Ratcliffe and Shachar-Hill [9] remarked in the recent review: “Dynamic labeling has not seen such rapid progress as steady-state labeling [22], and it still suffers from inherent limitations in complex networks [19].” In the following, we first describe the extension of the previous method to the dynamics of positional enrichment and subsequently illustrate the method with the pentose phosphate pathway of *Zymomonas mobilis*, as presented in Wiechert and de Graaf [35]. This system has particular appeal because the authors characterized it well and analyzed its steady state after positional enrichment, thereby affording us with direct data for comparison at least for some aspects of our method. Secondly, the pathway contains several splitting reactions that are the drivers of distributed atom positions. Finally, kinetic information characterizing this system was recently made available [36].

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