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Optimal tracers for parallel labeling experiments and ^{13}C metabolic flux analysis: A new precision and synergy scoring system

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Abstract

^{13}C -Metabolic flux analysis (^{13}C -MFA) is a widely used approach in metabolic engineering for quantifying intracellular metabolic fluxes. The precision of fluxes determined by ^{13}C -MFA depends largely on the choice of isotopic tracers and the specific set of labeling measurements. A recent advance in the field is the use of parallel labeling experiments for improved flux precision and accuracy. However, as of today, no systemic methods exist for identifying optimal tracers for parallel labeling experiments. In this contribution, we have addressed this problem by introducing a new scoring system and evaluating thousands of different isotopic tracer schemes. Based on this extensive analysis we have identified optimal tracers for ^{13}C -MFA. The best single tracers were doubly ^{13}C -labeled glucose tracers, including [1,6- ^{13}C]glucose, [5,6- ^{13}C]glucose and [1,2- ^{13}C]glucose, which consistently produced the highest flux precision independent of the metabolic flux map (here, 100 random flux maps were evaluated). Moreover, we demonstrate that pure glucose tracers perform better overall than mixtures of glucose tracers. For parallel labeling experiments the optimal isotopic tracers were [1,6- ^{13}C]glucose and [1,2- ^{13}C]glucose. Combined analysis of [1,6- ^{13}C]glucose and [1,2- ^{13}C]glucose labeling data improved the flux precision score by nearly 20-fold compared to widely use tracer mixture 80% [1- ^{13}C]glucose + 20% [U- ^{13}C]glucose.

Keywords

Optimal tracer experiment design; isotopic labeling; *E. coli*; integrated flux analysis; GC-MS

1. INTRODUCTION

The significance of a judicious selection of isotopic tracers for ^{13}C metabolic flux analysis (^{13}C -MFA) has been known since the early years of ^{13}C -MFA (Follstad and Stephanopoulos, 1998; Mollney et al., 1999; Wittmann and Heinzle, 2001). In recent years, in the quest for ever increasing accuracy and precision in ^{13}C -MFA, parallel labeling experiments have emerged as the new state-of-the-art technique (Antoniewicz, 2015a;

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Antonieiwicz, 2015b; Crown and Antonieiwicz, 2013a; Crown and Antonieiwicz, 2013b). This powerful flux analysis approach presents new opportunities for metabolic engineering studies; however, it also brings with it new challenges in the identification of optimal tracers. Significantly, parallel labeling experiments require careful selection of complementary tracers that take full advantage of the additional experimental effort that is required (Antonieiwicz, 2013a). A bottleneck in the selection of optimal tracers is the lack of a systematic approach to evaluate the results of *in silico* simulations and *in vivo* labeling experiments to identify complementary tracers. For example, while it is relatively straightforward to determine the optimal tracer for a single flux of interest in a model, the question is more challenging when multiple fluxes must be estimated with high precision (Crown and Antonieiwicz, 2012). Often, selecting a particular tracer that improves the precision of one flux in the model results in decreased precision of another flux (Crown et al., 2012). Parallel labeling experiments offer a potential solution to this problem by tailoring specific isotopic tracers for different parts of metabolism. However, it is still not clear how optimal tracers should be selected for parallel labeling experiments.

One of the predominant methods for tracer selection and experiment design is based on a grid-search strategy combined with the use of linearized statistics. The process involves calculating the parameter covariance matrix for various isotopic tracers of interest. For this analysis the following information is needed: an assumed network model, a set of fluxes (measured or assumed), and an assumed measurement set. To compare between different tracers and determine which tracer is optimal for a given system, the D-optimality criterion is commonly applied (Mollney et al., 1999). The D-optimality criterion is related to the covariance matrix of the free fluxes and provides a measure of single parameter confidence intervals and correlations between estimated parameters. A relative information score for each tracer is then determined from the D-optimality criterion, given an assumed reference tracer experiment. The tracer scheme that produces the highest information score is then selected as the optimal tracer (Arauzo-Bravo and Shimizu, 2003; Noh and Wiechert, 2006; Yang et al., 2006). A drawback of this approach is that it inherently relies on the assumption that the underlying non-linear ^{13}C -isotopomer balances can be approximated by linearization near the optimal solution, which is not always valid (Antonieiwicz et al., 2006).

Metallo et al. (Metallo et al., 2009) introduced another approach for tracer selection using a precision scoring system that captures the nonlinear behavior of ^{13}C -MFA. The suggested precision scoring method relies on calculating accurate nonlinear confidence intervals for free fluxes (Antonieiwicz et al., 2006). The method computes a score for each flux, based on the upper and lower bounds for the confidence intervals and using a flux weighting parameter. If a flux has a score of zero, the flux is unidentifiable; if the score is one, the flux is optimally identifiable. The precision score is then calculated as the sum of the scores for each flux. Similar to the grid-search approach, precision scores are compared for various tracers of interest and the tracer that has the highest score is selected as optimal. More recently, Walther et al. (Walther et al., 2012) proposed a genetic algorithm for tracer selection. Despite addressing the nonlinearities of flux confidence intervals, the proposed approach potentially introduces biases due to normalization of flux confidence intervals with respect to flux values (i.e. pathways with small fluxes values such as ED pathway and glyoxylate shunt were weighted more heavily than pathways with large fluxes such as

glycolysis, PP pathway and TCA cycle), as well as the method's reliance on empirically derived parameters to determine scores.

In this work, we propose a new precision scoring metric that captures the nonlinear behavior of flux confidence intervals, i.e. similar to the methods by Metallo et al. and Walther et al., but does not rely on empirically derived parameters and avoids potential biases due to flux normalization. We also propose a new synergy scoring metric that allows, for the first time, optimal tracers to be selected for parallel labeling experiments. Through the use of these two new scoring metrics we have identified new optimal tracers for high-resolution ^{13}C -MFA studies and have validated the performance of these tracers experimentally.

2. THEORY

2.1. The precision score

Here, we propose the following precision scoring metric to evaluate the precision of estimated fluxes:

$$P = \frac{1}{n} \sum_{i=1}^n p_i$$

with

$$p_i = \left(\frac{(UB_{95,i} - LB_{95,i})_{ref}}{(UB_{95,i} - LB_{95,i})_{exp}} \right)^2$$

The precision score (P) for a given tracer experiment is calculated as the average of individual flux precision scores (p_i) for n number of fluxes of interest. An individual flux precision score is calculated as the squared ratio of the 95% flux confidence interval obtained for a reference tracer experiment ("ref") relative to the tracer experiment that is being evaluated ("exp"). An individual flux precision score is thus roughly equivalent to the fold-improvement in flux variance relative to the reference tracer experiment, i.e. in linear statistics: variance = (standard deviation)². An individual flux precision score of 1.0 indicates that the flux precision for a particular tracer experiment is the same as for the reference tracer experiment. A precision score greater than one is desirable, as this means that the tracer experiment results in a narrower confidence interval compared to the reference experiment. Since individual flux precision scores can vary from flux to flux, i.e. a tracer experiment may perform better than the reference for some fluxes and worse for other fluxes, the ultimate gauge of tracer performance is the total precision score for the fluxes of interest. A precision score greater than one indicates that the tracer experiment on a whole outperforms the reference tracer experiment. The larger the precision score, the more substantial the increase in flux precision is compared to the reference tracer experiment. To make sure that a handful precision scores don't dominate the total precision score, we set a maximum value of 9 for any individual precision score when evaluating single tracer experiments (and 30 for parallel labeling experiments).

If necessary, the precision score can be tailored further by applying different weighting factors, w_i , for different fluxes of interest:

$$P = \left(\sum_{i=1}^n w_i * p_i \right) / \left(\sum_{i=1}^n w_i \right)$$

2.2. The synergy score

In addition to the new precision scoring metric described above, we also propose a new synergy scoring metric that quantifies the increase in flux information obtained as a result of conducting multiple parallel labeling experiments and simultaneously fitting the data for ^{13}C -MFA:

$$S = \frac{1}{n} \sum_{i=1}^n s_i$$

with

$$s_i = \frac{p_{i,1+2}}{p_{i,1} + p_{i,2}}$$

The synergy score (S) is calculated as the average of individual flux synergy scores (s_i). An individual flux synergy score is calculated by dividing the precision score for the parallel labeling experiment, denoted by $p_{i,1+2}$, by the sum of the precision scores for the respective individual experiments, $p_{i,1}$ and $p_{i,2}$. Note that this definition of the synergy score can be expanded for more than two parallel labeling experiments by adding additional terms in the denominator.

An individual flux synergy score of 1.0 indicates that no additional information is gained by fitting multiple parallel labeling experiments simultaneously. Intuitively, by performing N number of parallel labeling experiments the precision score is expected to increase by about N -fold. For non-linear problems such as ^{13}C -MFA, the synergy score can be smaller than one, or greater than one. A synergy score greater than 1.0 indicates a greater than expected gain in flux information, while a synergy score of 1.0 or less indicates a smaller than expected improvement in flux precision. As with the precision scores, synergy scores can vary from flux to flux. For parallel labeling experiments it is desirable to have a total synergy greater than one, as this indicates that the global flux resolution is improved synergistically through the use of complementary tracers.

Note that the synergy score can also be expressed as:

$$S = \frac{1}{n} \sum_{i=1}^n \frac{(UB_{95,i} - LB_{95,i})_1^{-2}}{(UB_{95,i} - LB_{95,i})_1^{-2} + (UB_{95,i} - LB_{95,i})_2^{-2}}$$

From the above equation it is clear that the synergy score is independent of the reference tracer experiment. Similar to the precision score, the synergy score can be tailored further by applying different weighting factors, w_i , for different fluxes of interest:

$$S = \left(\sum_{i=1}^n w_i * s_i \right) / \left(\sum_{i=1}^n w_i \right)$$

3. MATERIALS AND METHODS

3.1. Materials

Media and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Tracers were purchased from Cambridge Isotope Laboratories: [1-¹³C]glucose (99.6 atom% ¹³C), [1,2-¹³C]glucose (99.8%), [1,6-¹³C]glucose (99.2%), and [U-¹³C]glucose (99.3%). M9 minimal medium was used for all tracer experiments. All solutions were sterilized by filtration.

3.2. Strain and growth conditions

E. coli BW25113 (GE Healthcare Dharmacon OEC5042) was used in this study. Four parallel labeling experiments were performed with 2 g/L of [1,2-¹³C]glucose; [1,6-¹³C]glucose; 51.5% [1,2-¹³C]glucose + 48.5% [1,6-¹³C]glucose; and 81% [1-¹³C]glucose + 19% [U-¹³C]glucose, as described previously (Crown et al., 2015a). The isotopic purity of glucose tracers and the composition of glucose tracer mixtures was validated by GC-MS. Cells were grown in aerated mini-bioreactors with a working volume of 10 mL at 37°C (Leighty and Antoniewicz, 2013). The cultures were inoculated from the same pre-culture that was grown overnight in a shaker flask at 37°C.

3.3. Analytical methods

Samples were collected during the exponential growth phase to monitor cell growth and glucose consumption. Cell growth was monitored by measuring the optical density at 600nm (OD₆₀₀) using a spectrophotometer (Eppendorf BioPhotometer). The OD₆₀₀ values were converted to cell dry weight concentrations using a pre-determined OD₆₀₀-dry cell weight relationship for *E. coli* (1.0 OD₆₀₀ = 0.32 g_{DW}/L) (Long et al., 2016b). After centrifugation, the supernatant was separated from the biomass pellet and glucose concentration was measured with a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH). Acetate was measured by HPLC (Au et al., 2014).

3.4. Gas chromatography-mass spectrometry

GC-MS analysis was performed on an Agilent 7890B GC system equipped with a DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 µm-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977A Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230°C, the MS quad temperature at 150°C, the interface temperature at 280°C, and the inlet temperature at 250°C. GC-MS analysis of *tert*-butyldimethylsilyl (TBDMS) derivatized proteinogenic amino acids was performed as described in (Long and

Antoniewicz, 2014b). Labeling of glucose was determined using the aldonitrile propionate derivatization method described in (Antoniewicz et al., 2011).

3.5. Metabolic network model and ^{13}C -metabolic flux analysis

The metabolic network model used for ^{13}C -MFA was described previously (Crown et al., 2015a), and is given in Supplemental Materials. The model includes all major metabolic pathways of central carbon metabolism, lumped amino acid biosynthesis reactions, and a lumped biomass formation reaction. The model also accounts for dilution of intracellular labeling from incorporation of unlabeled CO_2 (Leighty and Antoniewicz, 2012). All simulations and ^{13}C -MFA calculations were performed using the Metran software (Yoo et al., 2008) which is based on the elementary metabolite units (EMU) framework (Antoniewicz et al., 2007a). Fluxes were estimated by minimizing the variance-weighted sum of squared residuals (SSR) between the measured and model predicted mass isotopomer distributions using non-linear least-squares regression (Antoniewicz et al., 2006). For integrated analysis of parallel labeling experiments, the data sets were fitted simultaneously to a single flux model as described in (Leighty and Antoniewicz, 2013). Flux estimation was repeated 10 times starting with random initial values for all fluxes to find a global solution.

Three methods were used to calculate 95% confidence intervals of fluxes. The first method, described in (Antoniewicz et al., 2006), calculates accurate nonlinear 95% confidence intervals by evaluating the sensitivity of the minimized SSR to flux variations. The second method is based on Monte Carlo simulations, where random errors from a normal distribution (here we assumed 0.4 mol% measurement errors for all GC-MS measurements) are introduced and flux estimation is repeated with the corrupted data sets. In this work, we have performed 1,000 Monte Carlo simulations to determine 95% confidence intervals of fluxes. The third method is based on linearized statistics, where 95% confidence intervals of fluxes are obtained from the parameter covariance matrix (Mollney et al., 1999).

To model fractional labeling of biomass amino acids G-value parameters were also included in ^{13}C -MFA. As described previously (Antoniewicz et al., 2007b), the G-value represents the fraction of a metabolite pool that is produced during the labeling experiment, while 1-G represents the fraction that is naturally labeled (e.g. from inoculum). By default, one G-value parameter was included for each measured amino acid in each data set. Reversible reactions were modeled as separate forward and backward fluxes. Net and exchange fluxes were determined as follows: $v_{\text{net}} = v_f - v_b$; $v_{\text{exch}} = \min(v_f, v_b)$. To determine the goodness-of-fit, ^{13}C -MFA fitting results were subjected to a χ^2 -statistical test. In short, assuming that the model is correct and data are without gross measurement errors, the minimized SSR is a stochastic variable with a χ^2 -distribution (Antoniewicz et al., 2006). The number of degrees of freedom is equal to the number of fitted measurements n minus the number of estimated independent parameters p . The acceptable range of SSR values is between $\chi^2_{\alpha/2}(n-p)$ and $\chi^2_{1-\alpha/2}(n-p)$, where α is a certain chosen threshold value, e.g. 0.05 for 95% confidence interval.

3.6. Assumption of no kinetic isotope effect

A common assumption in ^{13}C -MFA is that there is no ^{13}C kinetic isotope effect; essentially, that transporters and enzymes don't discriminate between ^{12}C and ^{13}C (Feng and Tang, 2011). The assumption of no kinetic isotope effect was also applied in this study for all tracers. Support for this assumption comes from several studies. Sandberg et al. (Sandberg et al., 2016), for example, demonstrated that there was no measurable difference in the uptake of ^{12}C glucose and ^{13}C glucose by wild-type *E. coli* and several evolved *E. coli* strains. Additionally, massively parallel labeling experiments have been used to test this assumption. In a recent study by Crown et al. (Crown et al., 2015a), 14 parallel labeling experiments were successfully combined into one global flux solution. This would not be possible if the different tracers had caused a significant change in metabolism. Thus, based on best available methods, the assumption of no kinetic isotope effect appears to be valid for ^{13}C -MFA.

4. RESULTS AND DISCUSSION

4.1. Evaluation of single glucose tracers

First, we performed *in silico* simulations to evaluate the performance of 19 commercially available glucose tracers (Table 1). We also evaluated the performance of two commonly used glucose tracer mixtures: 80% [$1\text{-}^{13}\text{C}$]glucose + 20% [$\text{U-}^{13}\text{C}$]glucose (which was selected as the reference tracer experiment in this study), and 20% [$\text{U-}^{13}\text{C}$]glucose + 80% natural glucose. These two tracer schemes are widely used because of the relatively low cost of the tracers involved, i.e. [$1\text{-}^{13}\text{C}$]glucose (~\$100/g) and [$\text{U-}^{13}\text{C}$]glucose (~\$200/g), compared to the cost of other glucose tracers (Table 1).

Flux precision for each glucose tracer was determined as follows: 1) for each tracer, GC-MS measurements of proteinogenic amino acids were simulated (see Supplemental Materials for the list of simulated fragments) using a previously determined flux map for wild-type *E. coli* (Crown et al., 2015a); 2) ^{13}C -MFA was performed on the simulated data. Glucose influx was fixed at 100 and acetate yield was 70 ± 5 mol/mol (Long et al., 2016b). No other external constraints were imposed. A constant measurement error of 0.4 mol% was assumed for all GC-MS measurements; 3) 95% confidence intervals of fluxes were determined using three different methods: i) using the method described in (Antoniewicz et al., 2006), which produces accurate nonlinear confidence intervals by evaluating the sensitivity of SSR to flux variations; ii) using 1000 Monte Carlo simulations; and iii) using linearized statistics that approximate 95% confidence intervals at the optimal solution; 4) Precision scores were calculated as the average of individual precision scores for the following eight key fluxes in central carbon metabolism: upper glycolysis (v_2 , $\text{G6P} \rightarrow \text{F6P}$), oxidative pentose phosphate pathway (v_{10} , $6\text{PG} \rightarrow \text{Ru5P} + \text{CO}_2$), non-oxidative pentose phosphate pathway (v_{14} , $\text{F6P} \rightarrow \text{E4P} + \text{E-C2}$), Entner–Doudoroff pathway (v_{18} , $6\text{PG} \rightarrow \text{KDPG}$), TCA cycle (v_{21} , $\text{AcCoA} + \text{OAC} \rightarrow \text{Cit}$), glyoxylate shunt (v_{29} , $\text{ICit} \rightarrow \text{Glyox} + \text{Suc}$), cataplerosis (v_{31} , $\text{Mal} \rightarrow \text{Pyr} + \text{CO}_2$), and gluconeogenesis (v_{34} , $\text{OAC} \rightarrow \text{PEP} + \text{CO}_2$).

Figure 1 shows the calculated standard deviations of key fluxes in central carbon metabolism for the different glucose tracers, and Figure 2 shows the calculated precision scores, sorted

from the best to the worst performing glucose tracer. Tracers with a precision score greater than 1.0 performed better than the reference tracer experiment, 80% [1-¹³C]glucose + 20% [U-¹³C]glucose; tracers with a precision score smaller than 1.0 performed worse than the reference tracer experiment. Figures 2A, 2B and 2C compare the precision scores determined using the three different methods for determining 95% confidence intervals of fluxes. Overall, there was excellent agreement between the accurate nonlinear confidence intervals method (Figure 2A) and the Monte Carlo simulations method (Figure 2B). In contrast, linearized statistics produced significantly different precision scores (Figure 2C). For example, the first two methods determined that the reference tracer experiment was one of the worst performing tracers. Both methods determined that the mixture 80% [1-¹³C]glucose + 20% [U-¹³C]glucose performed worse than 100% [1-¹³C]glucose, which was recently validated experimentally (Crown et al., 2015a). In contrast, the linearized statistics method predicted the opposite result. Overall, this method overestimated the performance of tracer mixtures. It is important to note that nearly all studies thus far have relied on linearized statistics to identify optimal tracers. Consistent with our simulation results, in many cases mixtures of tracers have been predicted to perform better than pure glucose tracers (Arauzo-Bravo and Shimizu, 2003; Mollney et al., 1999). Here, we demonstrate that using linearized statistics may not be appropriate for tracer selection. In the remainder of this study, we used the accurate nonlinear confidence intervals method by Antoniewicz et al. (2006) to determine 95% confidence intervals of fluxes, since this method produced the same results as the Monte Carlo simulation method, but was computationally much more efficient.

Of the 19 commercially available glucose tracers, 14 performed better than the reference tracer experiment (Figure 2A). The highest precision score of 3.7 was obtained for [1,6-¹³C]glucose, which was particularly good at determining fluxes of Entner–Doudoroff pathway and glyoxylate shunt (Figure 1). Interestingly, the top six best performing tracers were all doubly labeled glucose tracers: [1,6-¹³C]glucose (precision score = 3.7), [1,2-¹³C]glucose (precision score = 2.8), [5,6-¹³C]glucose (precision score = 2.8), [2,3-¹³C]glucose (precision score = 2.3), [4,5-¹³C]glucose (precision score = 1.9), and [3,4-¹³C]glucose (precision score = 1.8). Doubly labeled tracers have not been widely used for ¹³C-MFA, with the exception of [1,2-¹³C]glucose (Ahn and Antoniewicz, 2013; Crown and Antoniewicz, 2013b; Murphy et al., 2013; Walther et al., 2012). The tracers that performed the worst were 20% [U-¹³C]glucose (precision score = 0.4) and [4-¹³C]glucose (precision score = 0.1).

4.2. Optimal tracers are not sensitive to flux values

In theory, different tracers could be optimal for different flux maps. To evaluate the sensitivity of optimal tracer selection with respect to flux values, we generated 100 random flux maps and repeated the analysis described above (the 100 random flux maps are provided in Supplemental Materials). The results are summarized in Figure 2D. The 100 random flux maps captured a wide range of possible flux scenarios, with the glycolysis flux ranging from 7 to 98 (normalized to glucose uptake of 100), the oxidative pentose phosphate flux ranging from 0 to 92, the Entner–Doudoroff pathway flux ranging from 0 to 38, the TCA cycle flux ranging from 0 to 64, the glyoxylate shunt flux ranging from 0 to 43, and the

acetate secretion flux ranging from 0 to 99. We found that the precision scores calculated for wild-type *E. coli* flux map (Figure 2A) and 100 random flux maps (Figure 2D) were very similar, thus suggesting that optimal tracer selection doesn't depend strongly on the actual flux values used for tracer selection. For example, in both cases: 1) [1,6-¹³C]glucose was determined to be the best tracer; 2) doubly labeled glucose tracers produced the highest precision scores; 3) the reference tracer experiment with 80% [1-¹³C]glucose + 20% [U-¹³C]glucose performed very poorly overall; and 4) 20% [U-¹³C]glucose and [4-¹³C]glucose were the two worst performing tracers.

4.3. Evaluation of mixtures of glucose tracers

Next, we evaluated if mixtures of glucose tracers would perform better than pure glucose tracers. Note that the reference tracer experiment is an example of a mixture of glucose tracers: 80% [1-¹³C]glucose + 20% [U-¹³C]glucose. In a previous study, we experimentally evaluated four different mixtures of glucose tracers: [1-¹³C]glucose and [U-¹³C]glucose (1:1 mixture), [1-¹³C]glucose and [U-¹³C]glucose (4:1 mixture), [1-¹³C]glucose and [4,5,6-¹³C]glucose (1:1 mixture), and [U-¹³C]glucose and unlabeled glucose (1:4 mixture), in addition to a large number of pure glucose tracers (Crown et al., 2015a). Experimentally, we found that pure glucose tracers performed better than mixtures of glucose tracers. Here, we wanted to determine if this observation could be generalized. Thus, we evaluated all possible dual mixtures of glucose tracers, as well as mixtures of all individual glucose tracers and unlabeled glucose. For all mixtures, nine different ratios were evaluated: 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, 80/20, and 90/10.

The results of this extensive analysis are summarized in Figure 3. Overall, we found that in the vast majority of cases pure tracers performed better than mixtures of tracers. In fact, mixing different tracers often resulted in a significantly lower precision score. For example, a 50/50 mixture of [1,2-¹³C]glucose and [1,6-¹³C]glucose produced a precision score that was less than half of the precision scores of the respective pure tracers. There were only 6 cases where a mixture of tracers performed slightly better than the respective pure tracers (highlighted in red in Figure 3); however, in none of these cases did the precision score approach that of the best pure glucose tracers identified in the previous section. Thus, based on this exhaustive analysis we can conclude that pure glucose tracers in general perform better than mixtures of tracers for ¹³C-MFA in *E. coli*.

4.4. Identifying optimal tracers for parallel labeling experiments

Next, we set out to identify optimal tracers for parallel labeling experiments. We examined all possible combinations of two parallel labeling experiments with the 19 commercially available glucose tracers. Mixtures of tracers were not included in this analysis based on the results in the previous section. For consistency, the tracer experiment with 80% [1-¹³C]glucose + 20% [U-¹³C]glucose was used as the reference experiment. The calculated precision scores and synergy scores are shown in Figure 4. The highest precision score was obtained for parallel labeling experiments with [1,6-¹³C]glucose and [1,2-¹³C]glucose, which had a precision score of 7.8. This precision score was higher than the sum of the precision scores for the respective single pure tracers, i.e. 3.7 for [1,6-¹³C]glucose and 2.8 for [1,2-¹³C]glucose. Thus, there was significant synergy in the use of these two tracers. In

this case, the synergy score was 1.2 ($=7.8/(3.7+2.8)$). There were several other combinations of parallel labeling experiments that produced a high precision score and synergy score. Interestingly, all of the best performing parallel labeling experiments (with precision scores above 7.0) included at least one doubly labeled glucose tracer.

The precision and synergy scores on the diagonal in Figure 4 correspond to performing the same tracer experiment twice. We found that synergy scores on the diagonal were all less than 1, thus indicating that no synergistic flux information is gained by performing the same tracer experiment twice, as might be expected. Of course, there could be other good reasons for performing the same tracer experiment multiple times, e.g. to evaluate biological variability (Au et al., 2014); however, from the perspective of improving flux precision, it is always better to use two different tracers in parallel. Several tracers had particularly high synergy scores, e.g. [2,5- ^{13}C]glucose, suggesting that this tracer brings significant complementarity in parallel labeling experiments. Overall, the synergy scores varied from values less than 1 (no synergy) to values much greater than 1 (high synergy). This result indicates that judicious selection of tracers for parallel labeling experiments is important, since some (but not all) parallel labeling experiments produce synergistic improvements in flux precision. To our knowledge, this is the first time that synergies resulting from the use of parallel labeling experiments have been rigorously quantified.

4.5. Experimental validation of optimal tracers

To validate the predictions described in the previous sections, we performed four parallel labeling experiments with wild-type *E. coli*. Specifically, labeling experiments were performed with the following tracers: 100% [1,2- ^{13}C]glucose; 100% [1,6- ^{13}C]glucose; 51.5% [1,2- ^{13}C]glucose + 48.5% [1,6- ^{13}C]glucose; and 81% [1- ^{13}C]glucose + 19% [U- ^{13}C]glucose. The first two tracers were used to validate the prediction that [1,6- ^{13}C]glucose and [1,2- ^{13}C]glucose are optimal tracers for ^{13}C -MFA when single tracer experiments are used (see section 4.1), and to test the synergy of these two tracers in parallel labeling experiments (see section 4.4). The third tracer was chosen to validate the prediction that mixing tracers will result in a dramatically poorer performance compared to using the respective pure tracers (see section 4.3). The fourth tracer experiment was used as the reference tracer experiment.

E. coli was grown aerobically in parallel batch cultures and mass isotopomer distributions of proteinogenic amino acids were measured by GC-MS (the data is given in Supplemental Materials). Data from the four tracer experiments were then first analyzed separately by ^{13}C -MFA. In all cases a statistically acceptable fit was obtained. The estimated fluxes agreed well with those previously reported for the closely related *E. coli* K-12 MG1655 strain (Crown et al., 2015a; Leighty and Antoniewicz, 2013). Next, we fitted the experiments with [1,2- ^{13}C]glucose and [1,6- ^{13}C]glucose in parallel, which also resulted in a statistically acceptable fit. Figure 5 shows the calculated precision scores. As predicted, the tracer experiments with [1,2- ^{13}C]glucose and [1,6- ^{13}C]glucose performed significantly better (precision scores of 6.1 and 5.4, respectively) than the reference tracer experiment. Moreover, as predicted, when the two tracers were mixed (51.5% [1,2- ^{13}C]glucose + 48.5% [1,6- ^{13}C]glucose), a significantly lower precision score of 2.1 was obtained. Finally, parallel

fitting of [1,2-¹³C]glucose and [1,6-¹³C]glucose tracer experiments resulted in the highest precision score (18.3) with a synergy score of 1.6 ($=18.3/(6.1+5.4)$). The higher than expected precision score obtained here (i.e. compared to simulation results) was mainly due to a poorer than expected performance of the reference tracer experiment. Taken together, the experimental results described in this section confirmed all important predictions of our *in silico* simulations.

5. CONCLUSIONS

In this contribution, we have introduced a new scoring system for identifying optimal tracers for ¹³C-MFA. Unlike previous efforts (Arauzo-Bravo and Shimizu, 2003; Metallo et al., 2009; Walther et al., 2012), the proposed precision scoring metric accounts for nonlinear flux intervals and is not biased due to normalization by flux values. The new synergy score introduced here provides information whether it is beneficial to conduct tracer experiments in parallel. Through extensive *in silico* simulations, 19 commercially available glucose tracers were evaluated for ¹³C-based flux analysis. We demonstrated that the current standard tracer, 80% [1-¹³C]glucose + 20% [U-¹³C]glucose, performs poorly in general. A large number of pure glucose tracers, especially doubly ¹³C-labeled tracers such as [1,6-¹³C]glucose and [1,2-¹³C]glucose, performed significantly better. Flux precision was dramatically improved through the use of parallel labeling experiments. For example, we demonstrated an 18-fold improvement in the precision score (compared to the reference tracer experiment) by using parallel experiments with [1,6-¹³C]glucose and [1,2-¹³C]glucose.

In this work, we have focused on identifying optimal tracers for ¹³C-flux analysis in *E. coli* because of the significant importance of this organism in both academia and industry (Long and Antoniewicz, 2014a). The optimal tracers identified here may not be optimal for other organisms, especially if the structure of the metabolic pathways are dramatically different from *E. coli*. However, if the metabolic pathways are similar then likely the same tracers will be optimal, given the fact that we have evaluated a very wide range of possible flux scenarios and consistently found the same tracers to perform optimally.

It is also important to note that flux results not only depend on the selected tracers, but also on the labeling measurements used for ¹³C-MFA. In this work, we have assumed that proteogenic amino acids (measured by GC-MS) were used for ¹³C-MFA. GC-MS is a wide used technology in the ¹³C-MFA field (Antoniewicz, 2015a). However, several alternative measurement techniques are available, and in some cases, may be preferred: tandem mass spectrometry (Antoniewicz, 2013b; Choi and Antoniewicz, 2011; Choi et al., 2012), LC-MS/MS (McCloskey et al., 2016), and NMR (Masakapalli et al., 2014; Szyperski, 1995; Tang et al., 2007). Moreover, in addition to measuring labeling of protein-bound amino acids, it may be advantageous to measure ¹³C-labeling of carbohydrates (McConnell and Antoniewicz, 2016), fatty acids (Crown et al., 2015b), nucleosides (Miranda-Santos et al., 2015), glycogen and RNA (Guzman et al., 2014; Long et al., 2016a), and intracellular metabolites (Ahn and Antoniewicz, 2013; Millard et al., 2014). Finally, a practical consideration is the cost associated with performing ¹³C-MFA studies: the cost of tracers (Table 1), analytical instruments, sample preparation, analysis time, and the cost of

performing parallel labeling experiments vs. single tracer experiments (Hollinshead et al., 2016). All of these factors should be considered collectively to determine the best or most convenient strategy for completing a ^{13}C -MFA study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Precision of estimated fluxes in central carbon metabolism obtained with ¹³C-MFA for 19 commercially available glucose tracers and two common glucose tracer mixtures, 20% [U-¹³C]glucose; and 80% [1-¹³C]glucose + 20% [U-¹³C]glucose. ¹³C-MFA was performed using simulated GC-MS data.

Figure 3.

Precision scores for mixtures of glucose tracers. For each combination of two glucose tracers, nine mixing ratios were evaluated, ranging from 10%/90% to 90%/10%. Blue squares correspond to cases where the precision score monotonically increased (or decreased) with respect to the mixing ratio. Yellow squares correspond to cases where mixing pure tracers resulted in a significantly reduced precision score. Red squares correspond to cases where mixing of tracers resulted in an improved precision score compared to pure tracers. ^{13}C -MFA was performed using simulated GC-MS data and assuming wild-type *E. coli* fluxes.

Figure 4. Precision and synergy scores for parallel labeling experiments with pure glucose tracers. Synergy scores above 1 (positive synergy) are highlighted in green, and synergy scores below 1 (no synergy) are highlighted in red. ¹³C-MFA was performed using simulated GC-MS data and assuming wild-type *E. coli* fluxes.

Figure 5.

Experimentally determined precision scores for four different tracers, and for the parallel fit of tracer experiments with [1,2-¹³C]glucose and [1,6-¹³C]glucose. The precision score for the reference tracer experiment, 80% [1-¹³C]glucose + 20% [U-¹³C]glucose, is by definition 1 (highlighted in yellow).

Table 1

19 commercially available ^{13}C -glucose tracers.

Glucose tracers	Abbreviation	List Price (\$/g)*
[1- ^{13}C]Glucose	[1]Gluc	\$87 (Isotec)
[2- ^{13}C]Glucose	[2]Gluc	\$200 (Omicron)
[3- ^{13}C]Glucose	[3]Gluc	\$1200 (Omicron)
[4- ^{13}C]Glucose	[4]Gluc	\$1600 (Omicron)
[5- ^{13}C]Glucose	[5]Gluc	\$1700 (Omicron)
[6- ^{13}C]Glucose	[6]Gluc	\$700 (Omicron)
[1,2- ^{13}C]Glucose	[12]Gluc	\$650 (Omicron)
[1,3- ^{13}C]Glucose	[13]Gluc	\$1700 (Omicron)
[1,6- ^{13}C]Glucose	[16]Gluc	\$1500 (Omicron)
[2,3- ^{13}C]Glucose	[23]Gluc	\$1800 (Omicron)
[2,5- ^{13}C]Glucose	[25]Gluc	\$2600 (Omicron)
[3,4- ^{13}C]Glucose	[34]Gluc	\$3200 (Omicron)
[4,5- ^{13}C]Glucose	[45]Gluc	\$2550 (Omicron)
[4,6- ^{13}C]Glucose	[46]Gluc	\$9760 (Omicron)
[5,6- ^{13}C]Glucose	[56]Gluc	\$2600 (Omicron)
[1,2,3- ^{13}C]Glucose	[123]Gluc	\$1700 (Omicron)
[4,5,6- ^{13}C]Glucose	[456]Gluc	\$3200 (Omicron)
[2,3,4,5,6- ^{13}C]Glucose	[23456]Gluc	\$9400 (Omicron)
[U- ^{13}C]Glucose	[U]Gluc	\$195 (Isotec)

* Shown is the lowest listed price per gram of tracer on 3/27/2016.

* Omicron, <http://www.omicronbio.com/index.html>

* Isotec, <http://www.sigmaaldrich.com/chemistry/stable-isotopes-isotec.html>

* Cambridge Isotope Laboratories, <http://www.isotope.com>