The next phase in the development of $^{13}\text{C}$ isotopically nonstationary metabolic flux analysis

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Highlight

This viewpoint devises recommendations for future studies utilising $^{13}\text{C}$ isotopically nonstationary metabolic flux analysis to characterise plant metabolism. Most importantly, it highlights the necessity for model validation.
\(^{13}\)C isotopically nonstationary metabolic flux analysis (INST-MFA) is an emerging technique for estimations of metabolic fluxes and pool sizes. Within the plant sciences, two studies utilising this technique to characterise carbon metabolism have been published so far. Here, I examine these studies carefully. Readers unfamiliar with \(^{13}\)C-INST-MFA will obtain a critical understanding of the method and its findings. Readers working with \(^{13}\)C-INST-MFA are recommended to enter a phase of model validation to devise clear-cut protocols enabling robust estimations of specific fluxes.

\(^{13}\)C isotopically nonstationary metabolic flux analysis (INST-MFA) is an isotope tracer method for \textit{in vivo} characterisation of metabolism following a two-step protocol (Ma \textit{et al.}, 2017; Cheah and Young, 2018). First, progressive \(^{13}\)C labelling of metabolite pools in plants fed \(^{13}\)CO\(_2\) is measured over time (pre-steady-state kinetics). Second, measured labelling patterns are modelled as function of carbon flux rates and pool sizes by an iterative optimisation algorithm (starting from random or pre-defined flux and pool size values and finding the best fit by iteratively modifying these values). The software program INCA provides a powerful and user-friendly modelling environment (Young, 2014). Best-fit solutions between model and measured data yield flux and pool size estimates. Thus, a fundamental question is whether the statistical solution is physiologically reasonable/correct. To date, two INCA-based \(^{13}\)C-INST-MFA studies were published. Ma \textit{et al.} (2014; Study 1) estimated the response of Rubisco oxygenation-to-carboxylation (\(v_O/v_C\)) in \textit{Arabidopsis thaliana} rosettes to changes in light intensity. Xu \textit{et al.} (2021; Study 2) estimated day respiration (\(R_D\)) including flux through the oxidative pentose phosphate pathway (OPPP, \(v_{\text{OPPP}}\)) in \textit{Camelina sativa} leaf chloroplasts. Here, I examine these reports and derive recommendations for future studies.

\textbf{Realistic reaction networks}

In \(^{13}\)C-INST-MFA, a list of coded reactions specifies by which routes carbon can move from labelled or unlabelled sources through metabolic networks into sinks (Fig. 1). The reaction network of both studies allows direct export of 3-phosphoglycerate (3PGA) from chloroplasts to the cytosol. In the light however, 3PGA export is believed to be restricted due to the chloroplast-to-cytosol pH gradient (Flügge \textit{et al.}, 1983, and references therein). Additionally, cytosolic reactions catalysed by glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase are missing (conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate to 3-phosphoglycerate; TP to 3PGA). Thus, \(^{13}\)C flux into glycolysis, and the tricarboxylic acid cycle may follow unrealistic routes and has no cytosolic connection with sucrose biosynthesis. Furthermore, fractional refixation of respired CO\(_2\) is not considered (Loreto \textit{et al.}, 1999), and numerous reversible reactions were programmed as irreversible or \textit{vice versa}. This includes reactions of the Calvin-Benson cycle catalysed by phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (conversion of 3PGA to 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate; 3PGA to TP), fructose bisphosphatase (conversion of fructose 1,6-bisphosphate to fructose 6-phosphate; FBP to F6P), and fructose-
bisphosphate aldolase (conversion of dihydroxyacetone phosphate and erythrose 4-phosphate to sedoheptulose 1,7-bisphosphate; TP and E4P to SBP). Lastly, mesophyll chloroplasts reportedly lack enolase (van der Straeten et al., 1991; Prabhakar et al., 2009; Fukayama et al., 2015). Thus, stromal conversion of 3PGA to phosphoenolpyruvate (PEP) is likely infeasible, and fatty acid biosynthesis likely relies on PEP import from the cytosol. Future studies are encouraged to implement more realistic reaction networks representing carbon metabolism with all its intrinsic restrictions and freedom. Incorporation of cytosolic glyceraldehyde-3-phosphate dehydrogenases and phosphoglycerate kinase may enhance the utility of the model since these reactions proposedly constitute a central hub in leaf energy metabolism (Wieloch, 2021).

**Constrained fluxes**

INCA allows users to constrain fluxes and pool sizes, e.g., based on independent physiological measurements or theoretical considerations. Users may specify constants or intervals or choose to not impose any constraints. In all models (both studies), net CO₂ assimilation was fixed at predetermined values scaling fluxes up to reasonable values (supporting interpretation of results) while maintaining flux ratios. Additionally, study 1 fixed the absolute flux into starch (Fig. 1, \( \nu_{\text{Starch}} \)) and flux ratios between sucrose and amino acid biosynthesis according to physiological measurements. Similarly, study 2 fixed absolute fluxes into starch, sucrose (\( \nu_{\text{Sucrose}} \)), and amino acid biosynthesis, and the ratio of Rubisco oxygenation-to-carboxylation. This practice is potentially problematic since it may affect modelled flux ratios. Additionally, the necessity for constraints poses an important question. If large fluxes need to be fixed (\( \nu_{\text{Starch}} \), \( \nu_{\text{Sucrose}} \), \( \nu_{O/C} \)), can one rely on \(^{13}\)C-INST-MFA to return credible results for unconstrained fluxes including smaller fluxes of interest (\( \nu_{\text{OPPP}} \))? Thus, future studies are encouraged to present models without constraints alongside constrained models to show that data-driven flux estimation is feasible. Ideally, \(^{13}\)C data should drive the estimations with a minimum of imposed constraints.

**Effects of constrained fluxes on fluxes of interest**

In principle, constraining fluxes or pool sizes can affect estimates of fluxes or pool sizes of interest due to interconnectivities within the reaction network (Fig. 1). In study 2, \( \nu_{O/C} \) was constrained to be in between 0.2 and 0.25. Modelling returned a \( \nu_{O/C} \) ratio of 0.2, and \( R_L \) and \( \nu_{\text{OPPP}} \) of 5.2 and 4.6 μmol CO₂ g⁻¹ FW hr⁻¹, respectively. When left unconstrained, modelling returned a physiologically unrealistic \( \nu_{O/C} \) ratio of 0.09, and \( R_L \) and \( \nu_{\text{OPPP}} \) of 12.1 and 10.5 μmol CO₂ g⁻¹ FW hr⁻¹, respectively. This indicates negative correlations between \( \nu_{O/C} \) and \( R_L \) and \( \nu_{O/C} \) and \( \nu_{ \text{OPPP} } \) (the lower photorespiration, the higher day respiration). Hence, fixing \( \nu_{O/C} \) at values >0.2 may cause \( R_L \rightarrow 0 \) and \( \nu_{\text{OPPP}} \rightarrow 0 \). Note that under normal growth conditions, \( \nu_{O/C} \) ratios of 0.34 are common (Sharkey, 1988; Cegelski and Schaefer, 2006; Pärnik et al., 2007). Thus, future studies are encouraged to include sensitivity analyses investigating dependences between constrained fluxes and fluxes of interest.
Validation of results by independent methods

INCA-based $^{13}$C-INST-MFA returns a comprehensive dataset containing estimates of (i) forward and reverse fluxes of all reactions, and (ii) pool sizes of all metabolites specified in the reaction network (Fig. 1). Some of these items are accessible to other analytical techniques which, in principle, enables independent validation of $^{13}$C-INST-MFA results. Study 1 made no attempt to confirm modelled $v_O/v_C$ estimates by independent methods. However, estimated ratios were within the physiologically reasonable range. By contrast, study 2 tested the model estimate for $R_L$ (5.2 μmol CO$_2$ g$^{-1}$ FW hr$^{-1}$) by the Laisk method which returned an $R_L$ estimate of 9.3 μmol CO$_2$ g$^{-1}$ FW hr$^{-1}$ (Brooks and Farquhar, 1985). However, corresponding 95% confidence intervals showed no overlap (3.5-8.05 versus 8.1-10.7 μmol CO$_2$ g$^{-1}$ FW hr$^{-1}$). Thus, these estimates are statistically different at the 0.05 significance level. Additionally, the model estimate for $v_{OPP}$ in chloroplasts was compared with an estimate of flux through the cytosolic OPPP (Sharkey et al., 2020). However, there is no reason to believe that these pathways carry the same flux. Thus, validation of estimates from $^{13}$C-INST-MFA by independent methods has not yet been achieved. However, independently determined fluxes currently used as constraints ($v_{Starch}$, $v_{Sucrose}$) can be used to test the method by leaving them unconstrained and comparing modelled and measured values. Additionally, $v_O/v_C$ ratios may help to test the method since several alternative methods can provide independent estimates (Busch, 2013).

Metabolically inactive pools or injection of carbon from unlabelled sources

In $^{13}$C-INST-MFA, $^{12}$C is progressively flushed out of the metabolic network and replaced by $^{13}$C from the labelling compound, e.g., $^{13}$CO$_2$ (Fig. 1). Both studies reported fast initial labelling of metabolite pools. After several minutes however, labelling slowed and, even after one hour, a significant fraction of the pools remained unlabelled. This was attributed to metabolically inactive pools (i.e., metabolite pools disconnected from the flux of incoming $^{13}$C) and modelled accordingly by including a dilution term for each metabolite (accounting for apparently constant offsets between measured and modelled $^{13}$C enrichments). Alternatively, labelling lags may be explained combinedly by breakdown of weakly-labelled cytosolic sucrose into glucose and fructose, phosphorylation by hexokinase and fructokinase, and reinjection of glucose-6-phosphate-derived carbon into chloroplasts via a cytosolic OPPP not shown in figure 1 (Sharkey et al., 2020). Figure 2 shows reported $^{13}$C enrichments of metabolites of the Calvin-Benson cycle, and starch and sucrose biosynthesis one hour into $^{13}$CO$_2$ labelling of Arabidopsis thaliana rosettes (Szecowka et al., 2013). Additionally, these authors reported subcellular distributions of metabolites given on the x-axis from fully plastidial (x=0) to fully cytosolic (x=1). Interestingly, plastidial metabolites are more strongly $^{13}$C labelled than cytosolic metabolites. Metabolite distribution explains 55% of the labelling variability (p<0.01, n=11). Since it is not apparent why sizes of metabolically inactive pools would correlate with plastid-cytosol metabolite distribution, this corroborates the idea of injection of weakly labelled carbon into...
cytosolic metabolism. Future $^{13}$C-INST-MFA studies are encouraged to further explore this by expanding their reaction networks by sucrose breakdown pathways and a cytosolic OPPP. Additionally, sucrose, glucose, and fructose are large carbon pools with significant vacuolar contributions (Szecowka et al., 2013). Thus, cytosol-vacuole transmembrane transport of these metabolites may need to be considered.

**Future focus**

To date, evidence that $^{13}$C-INST-MFA returns reliable flux and pool size estimates is not available. Therefore, the field is recommended to enter a phase of validation of the complex models used in $^{13}$C-INST-MFA to devise clear-cut protocols enabling robust estimations of specific fluxes.

**Keywords:** $^{13}$C labelling; $^{13}$CO$_2$; $^{13}$C tracer experiments; carbon flux estimation; complex models; labelling lag; metabolic flux analysis; model validation; photosynthesis; carbon metabolism
Abbreviations: 3PGA, 3-phosphoglycerate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; INCA, isotopomer network compartmental analysis; INST-MFA, isotopically nonstationary metabolic flux analysis; OPPP, oxidative pentose phosphate pathway; PEP, phosphoenolpyruvate; $R_L$, day respiration; SBP, sedoheptulose 1,7-bisphosphate; TP, triose phosphate; $\nu_{O/N}$, Rubisco oxygenation-to-carboxylation ratio; $\nu_{OPPP}$, OPPP flux; $\nu_{Sucrose}$, flux into sucrose; $\nu_{Starch}$, flux into starch

Data Availability: The data supporting the findings of this study have been published previously by Ma et al. (2014), Szecowka et al. (2013), and Xu et al. (2021).
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Figure legends

Figure 1 Reaction networks of published $^{13}$C-INST-MFA studies. Black: Network as programmed by Ma et al. (2014; Study 1) including reactions of the Calvin-Benson cycle, photorespiration, starch and sucrose biosynthesis, glycolysis, the tricarboxylic acid cycle, and amino acid biosynthesis. Blue: Add-ons to the network of study 1 by Xu et al. (2021; Study 2) including reactions of the oxidative pentose phosphate pathway, fatty acid biosynthesis, and the tricarboxylic acid cycle. Orange: $^{13}$C enriched compound (label) entering the reaction network. Reactions inside the green box were programmed as chloroplast-localised, while reactions outside the box were programmed as either cytosolic or without compartment identifier. Abbreviations: 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; 6PG, 6-phosphogluconate; ACA, acetyl coenzyme A; ADPG, ADP-glucose; AKG, α-ketoglutarate; ALA, alanine; ASN, asparagine; ASP, aspartate; CIT, citrate; E4P, erythrose 4-phosphate; EC2, enzyme-bound two-carbon fragment; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; FUM, fumarate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GA, glycerate; GLN, glutamine; GLU, glutamate; GLY, glycine; ICI, isocitrate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PRO, proline; PYR, pyruvate; R5P, ribose 5-phosphate; RUP5, ribulose 5-phosphate; RUBP, ribulose 1,5-bisphosphate; S6P, sucrose 6-phosphate; S7P, sedoheptulose 7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; SER, serine; SFrc, fructosyl moiety of S6P; SGlc, glucosyl moiety of S6P; SUC, succinate; THR, threonine; TP; triose phosphate; UDPG, UDP-glucose; X5P, xylulose 5-phosphate; $v_C$, Rubisco carboxylation flux; $v_O$, Rubisco oxygenation flux; $v_{OPPP}$, OPPP flux; $v_{Sucrose}$, flux into sucrose; $v_{Starch}$, flux into starch.

Figure 2 $^{13}$C enrichment of metabolite pools in Arabidopsis thaliana rosettes one hour into $^{13}$CO$_2$ labelling as function of the intracellular metabolite distribution from fully plastidial (x=0) to fully cytosolic (x=1). Abbreviations: 3PGA, 3-phosphoglycerate; ADPG, ADP-glucose; DHAP, dihydroxyacetone phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; RuBP, ribulose 1,5-bisphosphate; S6P, sucrose 6-phosphate; S7P, sedoheptulose 7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; UDPG, UDP-glucose. Reanalysed data from Szecowka et al. (2013). Sucrose and glucose 1-phosphate were excluded from the analysis since the former has a large vacuolar fraction and the latter reportedly exhibits an anomalous labelling behaviour (Szecowka et al., 2013; Xu et al., 2021).
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