^{13}C-Isotope-Assisted Assessment of Metabolic Quenching During Sample Collection from Suspension Cell Cultures

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ABSTRACT

Metabolomics and fluxomics are core approaches to directly profile and interrogate cellular metabolism in response to various genetic or environmental perturbations. In order to accurately measure the abundance and isotope enrichment of intracellular metabolites, cell culture samples must be rapidly harvested and cold-quenched to preserve the *in vivo* metabolic state of the cells at the time of sample collection. When dealing with suspension cultures, this process is complicated by the need to separate the liquid culture media from cellular biomass prior to metabolite extraction. Here, we examine the efficacy of several commonly used metabolic quenching methods, using the model cyanobacterium *Synechocystis* sp. PCC 6803 as an example. Multiple ^{13}C-labeled compounds, including ^{13}C-bicarbonate, ^{13}C-glucose and ^{13}C-glutamine, were used as tracers during the sample collection and cold-quenching process to assess the extent of metabolic turnover after cells were harvested from culture flasks. We show that the combination of rapid filtration followed by 100% cold (−80°C) methanol quenching exhibits the highest quenching efficiency, while mixing cell samples with a partially frozen 30% methanol slurry (−24°C) followed by centrifugation is slightly less effective at quenching metabolism but enables less laborious sample processing. By contrast, rapidly mixing the cells with a saline ice slurry (~0°C) is less effective as indicated by high isotope-labeling rates after sample harvest, while mixing the cells with 60% cold methanol (−65°C) prior to centrifugation causes significant metabolite loss. This study demonstrates a rigorous, quantitative, and broadly applicable method for assessing the metabolic quenching efficacy of protocols used for sample collection in metabolomics and fluxomics studies.

INTRODUCTION

Metabolomics and fluxomics are powerful techniques for directly profiling metabolic activities under defined conditions, and have been applied to interrogate regulatory mechanisms, discover disease biomarkers, and identify metabolic bottlenecks[1, 2]. Specifically, metabolomics focuses on quantifying metabolite concentrations, while fluxomics relies on measurements of isotope enrichment and extracellular exchange rates of metabolites to calculate intracellular metabolic fluxes. Tremendous advances in sophisticated measurement techniques, such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), have enabled precise measurement and subsequent annotation of increasing numbers of metabolites. However, the success of any metabolomics or fluxomics workflow starts with careful sample preparation, which typically necessitates rapid collection of cell culture samples followed by immediate quenching of enzymatic activities while minimizing metabolite leakage so that metabolites can be accurately measured under the controlled temporal and physiological conditions of the experiment. Since metabolism remains active, at least partially, until cellular enzymes are removed or denatured, some turnover of metabolites during sample collection is almost inevitable, which contributes to systematic measurement errors of metabolite abundance and/or isotope enrichment. However, quantifying the extent of these measurement errors and how they compare across different sampling and quenching procedures has been a persistent challenge. This is largely due to a lack of generalizable methods for rigorous assessment of metabolic quenching protocols.

Commonly used metabolic quenching methods include directly mixing cell culture samples with a quenching solution selected from a variety of possible options, such as cold methanol solutions[3, 4], cold media[5], or cold saline ice slurries[6-8]. Once quenched, the cell biomass can be physically separated from the spent medium and quenching solution prior to freezing or immediate metabolite extraction. Alternatively, the spent medium can be initially removed by centrifugation or rapid filtration prior to freezing or quenching in cold methanol, hot methanol or ethanol, or perchloric acid[6, 9, 10]. Prior studies have compared an array of quenching methods for metabolomics analysis of bacteria[8, 11, 12], yeast[12, 13], algae[14] and mammalian cells[6], but the optimal method was often found to be metabolite- and organism-specific. Therefore, refining and expanding upon current methods to assess metabolic quenching efficiency is expected to facilitate the application of metabolomics to new organisms and experimental systems.

To date, assessment of metabolic quenching methods has centered around evaluating several criteria such as (i) leakage of metabolites due to loss of cell integrity during quenching[6, 12], (ii) recovery of certain rapidly turned over intracellular metabolites[4, 6, 12, 15, 16], (iii) breadth of detectable metabolites[13, 14] and, (iv) abundance of metabolites recovered in cell extracts[6, 8, 13, 17]. However, these criteria do not directly reflect the extent of metabolite turnover that occurs during sample removal and quenching, which may mislead researchers to adopt protocols that cause dramatic shifts in metabolite concentrations or isotopic labeling prior to measurement and thereby lead to invalid conclusions. In a recent study aimed at comparing metabolic quenching efficiency of different sampling protocols, researchers injected ^{13}C-bicarbonate into cultures of the cyanobacterium *Synechococcus* sp. PCC 7002 followed by rapid sampling, quenching, extraction and ^{13}C-enrichment analysis. The ^{13}C-enrichment trajectories of targeted metabolites did not show differences between four different quenching protocols, suggesting similar quenching efficiencies of the examined methods. However, because ^{13}C-bicarbonate was provided to cells prior to sample collection, it was not possible to rigorously assess the extent of postharvest metabolite turnover. In the same study, significant metabolite leakage was observed when cold methanol was used as the quenching solution[8].

In this study, we compared the efficacy of four most commonly used metabolic quenching methods by using isotopic tracers to assess the extent of metabolic turnover after sample collection. We supplied ^{13}C-glucose, ^{13}C-bicarbonate and ^{13}C-glutamine to quantify the residual metabolic activity of cell samples after removal from suspension cultures of the cyanobacterium *Synechocystis* sp. PCC 6803, used here as an example organism. We demonstrate that these isotopic tracers could be readily incorporated into the cellular metabolome of unquenched samples via multiple entry points distributed across the metabolic network. A tracer cocktail including all three isotopes was then used to compare the ability of the selected quenching methods to effectively inhibit enzyme activities based on the extent of ^{13}C-labeling that occurred after sample removal. For example, effective sample quenching should result in minimal ^{13}C enrichment, while ineffective quenching should lead to increased labeling of the intracellular metabolome. We concluded that a pure saline ice slurry (0°C) was not as effective as a 30% methanol slurry (about −24°C) or rapid filtration (followed by quenching in −80°C methanol) in terms of preventing metabolite turnover after sample collection, while the rapid filtration method also exhibited the highest capability to preserve metabolite abundances during processing of cyanobacterial samples. The strategy demonstrated in this study could be further applied to test and optimize metabolic quenching procedures for a variety of other organisms or cell culture systems.

EXPERIMENTAL SECTIONS

Bacterial strains and growth conditions. The glucose-tolerant cyanobacterium *Synechocystis* sp. PCC 6803 was grown in liquid BG11 medium supplemented with 10 mM HEPES-NaOH (pH 8.2) and 10 mM $NaHCO_3$. Specifically, one liter of medium contains the following components in deionized water: 1.5 g $NaNO_3$, 75 mg $MgSO_4 \cdot 7H_2O$, 36 mg $CaCl_2 \cdot 2H_2O$, 6.6 mg citric acid$\cdot H_2O$, 1 mg NaEDTA, 20 mg Na_2CO_3, 30.5 mg K_2HPO_4, 6 mg ferric ammonium citrate, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2 \cdot 4H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.39 mg $Na_2MoO_4 \cdot 2H_2O$, 0.08 mg $CuSO_4 \cdot 5H_2O$, and 0.049 mg $Co(NO_3)_2 \cdot 6H_2O$, 10 mM HEPES-NaOH (pH 8.2) and 10 mM $NaHCO_3$. The medium was sterilized by passing through sterile 0.22 µm filters before use. Cyanobacterial liquid cultures were aerated with 1% CO_2 and grown under constant light of about 120 µE m^{-2} s^{-1} on a rotary water-bath shaker at 130 rpm and 30°C. When cyanobacteria were grown on solid medium, 15 g L^{-1} agar was supplemented to the BG11 medium, and sterilized by autoclaving at 121°C for 30 min before 3 g L^{-1} thiosulfate was added.

Preparation of quenching solutions. (1) For preparing the saline ice slurry, 30 mL of 40 mM sodium chloride (NaCl) in water was transferred into a 50-mL centrifuge tube. The solution was then kept at −20 °C until it became partially frozen (i.e., over 50% ice). The tube was vigorously shaken by hand to form an ice slurry before being placed in an ice bath at ~0°C. The NaCl ice slurry was then used within half an hour. (2) For preparing the 30% methanol ice slurry, 9 mL methanol was mixed with 21 mL of a 40 mM NaCl solution in a 50-mL centrifuge tube and subsequently kept at −80°C until the solution started to solidify (at a freezing temperature of approximately −20°C). The tube was then vigorously shaken by hand to form an ice slurry and stored at −20°C. The 30% methanol slurry was then used within 1-2 hours. (3) For preparing the 60% methanol slurry, 18 mL methanol was mixed with 12 mL of a 40 mM NaCl solution in a 50-mL centrifuge tube and subsequently kept at −80°C for at least 2 hours until the solution started to gel (at a freezing temperature of approximately −40°C). The 60% methanol slurry was then used immediately. (4) For quenching samples after membrane filtration, 2 mL of pure methanol was pipetted into a 47-mm petri dish, covered with a lid, and kept at −80°C for at least 1 hour before use.

Sample collection for comparison of metabolite recovery. *Synechocystis* sp. PCC 6803 was inoculated from late exponential growth phase culture (OD_{730} at about 1.0) to an initial OD_{730} of 0.1 in 250 mL of fresh medium in a 1-L flask. The culture was grown for approximately one day, reaching an OD_{730} of about 0.6. Then, 20 mL of culture broth was drawn from the culture flask using a syringe and injected into a 50-mL centrifuge tube containing 30 mL of either NaCl ice slurry at ~0°C, 30% methanol ice slurry at −24°C, or 60% methanol ice slurry at −65°C (Table S1). The centrifuge tube was immediately inverted twice and placed on ice. The samples were subsequently centrifuged at 4600 g for 5 min at 1°C. Ten mL of supernatant was passed through a filter with 0.22 µm pores and stored at −80°C, and the rest was vacuum aspirated; the cell pellet was flash frozen in liquid nitrogen and stored at −80°C for subsequent metabolite extraction. A fourth method involved subjecting 20 mL of culture broth to filtration through a 47-millimiter-diameter membrane with 0.8 µm pores, and then immediately transferred into 2 mL of pure methanol in a 47-millimiter-diameter petri dish sitting on dry ice. The cells were scraped off the membrane and then transferred into a 15-mL centrifuge tube and kept at −80°C for subsequent metabolite extraction and analysis. Ten mL of filtrate was stored at −80°C for later use. It typically took about 10–15 seconds to withdraw 20 mL of sample from the culture flask and mix it with the cold quenching solutions by inverting twice, and it took approximately 20 seconds to withdraw 20 mL of sample from the culture flask and filter the broth through the 0.8-µm membrane and then transfer the membrane into cold methanol.

Sample collection for assessment of postharvest isotope enrichment without quenching. A 20-mL syringe was pre-loaded with either (i) 0.5 mL of 1 M ^{13}C sodium bicarbonate, (ii) 0.16 mL of 1 M [U-$^{13}C_6$]glucose, (iii) 0.2 mL of 0.1 M [U-$^{13}C_5$]glutamine, or (iv) a tracer cocktail containing all three isotopes. The syringe was used to draw a 20-mL sample from the culture flask, and the syringe contents were immediately injected into a 50-mL tube containing 30 mL of 40 mM NaCl solution kept at room temperature. Then, the tube was capped, inverted twice, and kept on ice at ambient light for approximately 10 min. Subsequently, the samples were centrifuged at 4600 g for 5 min at 1°C. The supernatants were vacuum aspirated, and the cell pellets were flash frozen in liquid nitrogen and stored at −80°C until subsequent analysis.

Sample collection for assessment of metabolic quenching efficacy. Twenty milliliters of culture broth was drawn from the flask using a syringe containing the aforementioned ^{13}C-tracer cocktail comprised of 0.5 mL 1 M ^{13}C sodium bicarbonate, 0.16 mL 1 M [U-$^{13}C_6$]glucose and 0.2 mL [U-$^{13}C_5$]glutamine, and then was immediately processed following each of the four quenching procedures described in the section **Sample collection for comparison of metabolite recovery**. Then, the cells were stored at −80°C until subsequent analysis.

Extraction and derivatization of metabolites for GC-MS analysis. Cell pellets were each resuspended with 2 mL −20 °C methanol and transferred into a 15-mL tube, after which 4 mL of cold chloroform and 6 µL of 10 mM L-norvaline (internal standard) was added. The tubes were capped and vortexed at 1300 rpm for 30 min at 2°C. Then, 1.5 mL of ice-cold deionized water was added to each sample followed by vortexing at 2°C for another 10 min. Then, samples were centrifuged at 4600 g for 20 min at 1°C, and 1.4 mL of supernatant from each sample was pipetted into a clean Eppendorf tube and dried overnight on a drying block at room temperature. The dried cell extract samples were either derivatized immediately or kept at −80°C for later derivatization. Specifically, 50 µL of 2% methoxyamine hydrogen chloride (MOX) in pyridine (ThermoFisher Scientific) was added into each sample and sonicated for 30 min followed by incubation at 40°C for 90 min. After a brief centrifugation, 70 µL of MTBSTFA + 1% TBDMCS (Regis Technologies, Inc., IL, USA) was added to each sample and incubated at 70°C for 30 min to convert metabolites to their MOX-TBDMS derivatives. In parallel, a series of standards spanning a range of metabolite concentrations was dried and derivatized to establish a calibration curve for quantifying the metabolite concentrations in

samples. The derivatized samples were centrifuged at 17000 x g for 5 min at room temperature, and then 70 µL of clear supernatant from each sample was transferred into a GC vial with a polypropylene insert.

GC-MS analysis of metabolites. Derivatized samples were analyzed using an Agilent 7890 gas chromatograph (GC) connected to an Agilent 5977A mass spectrometer (MS) following a previously published procedure[18].

Analysis of isotope enrichment of metabolites. The following fragment ions containing the entire carbon skeletons of the targeted metabolites were monitored: *m/z* 585 for 3-phosphoglycerate (3PGA), *m/z* 453 for phosphoenolpyruvate (PEP), *m/z* 174 for pyruvate (PYR), *m/z* 246 for alanine (ALA), *m/z* 441 for 2-phosphoglycolate (2PGC), *m/z* 246 for glycine (GLY), *m/z* 390 for serine (SER), *m/z* 459 for citrate (CIT), *m/z* 346 for α-ketoglutarate (AKG), *m/z* 432 for glutamate (GLU), *m/z* 289 for succinate (SUC), *m/z* 418 for aspartate (ASP), *m/z* 419 for malate (MAL), *m/z* 287 for fumarate (FUM). The compositions of these fragment ions are described in Table S2. Mass isotopomer distributions (MIDs)[19] were calculated for the targeted fragment ions. Average percent enrichment (APE) of each fragment ion was calculated by first correcting the MID for natural abundance of stable isotopes[20] and then applying the formula $APE = \sum_{i=0}^{N} \frac{M_i \times i}{N} \times 100\%$, where N is the number of metabolite carbon atoms in the fragment ion and M_i is the fractional abundance of the *i*th mass isotopomer[21]. The theoretical APE for fragment ions with natural background abundance of stable isotopes is zero, while the theoretical APE for fragment ions fully labeled with ^{13}C is 100%.

RESULTS AND DISCUSSION

Cyanobacterial metabolites exhibit extensive turnover in the absence of metabolic quenching as determined by their postharvest ^{13}C labeling. Three metabolic tracers, e.g., ^{13}C-glucose, ^{13}C-bicarbonate and ^{13}C-glutamine, were administered immediately after withdrawal of broth samples from cultures of the cyanobacterium *Synechocystis* sp. PCC 6803 to examine the turnover of metabolites in samples collected without any method of metabolic quenching. These three tracer substrates are able to label central metabolic pathways through distinct entry points that span the metabolic network of *Synechocystis* (Figure 1). Therefore, we hypothesized that the tracers could be used to assess the extent of residual enzymatic activity within different sections of the metabolic network, i.e., glycolysis, Calvin-Benson-Bassham cycle, photorespiration, and the tricarboxylic acid (TCA) cycle. It was found that ^{13}C-glucose was able to effectively label most of the targeted metabolites, reaching up to a maximum enrichment of 59.3% for phosphoenolpyruvate (PEP); however, relatively low enrichments were observed in the TCA cycle metabolites citrate, α-ketoglutarate (AKG), glutamate, and succinate. By contrast, ^{13}C-glutamine was able to confer ~10% enrichment to AKG, glutamate and succinate, but did not confer any significant isotopic enrichment to fumarate, malate, or aspartate, indicating that the succinate dehydrogenase activity was extremely low in *Synechocystis* 6803 under the postharvest conditions examined. ^{13}C-bicarbonate was also able to label all targeted metabolites but resulted in lower APEs than ^{13}C-glucose, probably due to the requirement of energy (light) input for bicarbonate assimilation and CO_2 fixation via the CBB cycle. Note that harvested samples were exposed to ambient light (<10 µE m^{-2} s^{-1}) that was much weaker than the illumination (120 µE m^{-2} s^{-1}) during cultivation. Administering a combination of all three isotope tracer compounds, i.e., ^{13}C-cocktail, had a somewhat additive effect, which resulted in the highest APEs for all the targeted metabolites in the absence of quenching (Figure 1). Note that the ^{13}C-enrichments provided by the ^{13}C-cocktail were typically less than the sum of the ^{13}C-enrichments provided by the three individual ^{13}C-tracers (Figure 1), possibly due to competition between tracers entering the same metabolic pathways. Although ^{13}C-glucose, ^{13}C-bicarbonate and ^{13}C-glutamine were

utilized as isotope tracers in the present study, the choice of tracers is not necessarily limited to these three and should be re-evaluated for other species. A minimum requirement for an appropriate tracer compound is that it should be efficiently imported and metabolized by the target cells of interest.

Postharvest ^{13}C labeling reveals variations in efficacy of different metabolic quenching procedures. The quenching efficacy of four representative quenching methods were investigated using the aforementioned ^{13}C-cocktail administered to cyanobacterial culture samples immediately upon withdrawal from culture flasks. These quenching methods were: (1) 20 mL culture mixed with 30 mL NaCl ice slurry at 0°C, (2) 20 mL culture mixed with 30 mL 30% methanol at −24°C, (3) 20 mL culture mixed with 30 mL 60% methanol at −65°C, and (4) 20 mL culture vacuum-filtered and immersed in 2 mL 100% methanol at −80°C. We found that the NaCl ice slurry (method 1) exhibited the lowest quenching efficacy as it led to the highest isotopic labeling of a majority of examined metabolites, with 3-phosphoglycerate (3PGA) reaching the highest APE of 18.0% (Figure 2). The other three quenching methods showed improved quenching effectiveness compared to that of the NaCl ice slurry in the CBB and glycolytic pathway, resulting in lower APEs for most of the targeted metabolites. It is possible that both smaller ice crystals and lower initial temperatures of the methanol solutions could have led to faster quenching relative to that of NaCl slurry. Surprisingly, 60% methanol quenching led to the highest APEs for GLY, AKG, GLU and SUC among the four compared quenching protocols, indicating its relatively low efficiency in quenching the activities of associated enzymes. Our results suggest that these quenching procedures vary in quenching efficacy across different metabolites or pathways within the metabolic network, although the 30% methanol protocol and filtration protocol generally exhibited comparable metabolic quenching efficiency.

While substantially lower than the enrichments obtained without any quenching procedure ("Unquenched"), these quenching methods still indicate postharvest turnover as high as ~15% for certain metabolites such as 3PGA and ASP, which may seem unacceptable for some metabolomics and fluxomics studies. However, these turnover estimates may be exaggerated by the introduction of exogenous glucose and glutamine substrates that were not present in the culture broth, as well as the increased osmotic pressure exerted by the ^{13}C-cocktail (leading to final concentrations of 8 mM ^{13}C-glucose, 25 mM ^{13}C-bicarbonate, and 1 mM ^{13}C-glutamine within the syringe used for sample withdrawal; Figure 2), which would not normally be present post-sampling. These factors may also serve to amplify the transport of ^{13}C tracers into cells and thus stimulate metabolism due to a sudden influx of available substrates.

To reduce the osmotic pressure and possible metabolic perturbations caused by the ^{13}C-cocktail, we re-examined the four quenching methods using only ^{13}C-bicarbonate as a metabolic tracer. Because sodium bicarbonate was already present in the culture broth, this tracer was expected to provide a more realistic estimate of metabolite turnover during sample collection. However, ^{13}C-bicarbonate provides less dynamic range for comparison of the different quenching methods since it does not enrich metabolism as extensively as the ^{13}C-cocktail does. These studies confirmed that method 1 (NaCl ice slurry) still resulted in the highest enrichments for all the targeted metabolites, with 3PGA, PEP, pyruvate and alanine reaching >5% APEs (Figure 3). By contrast, methods 2-4 resulted in much lower APEs for the measured metabolites when compared to that of method 1. For these methods, APEs were generally <5% for CBB cycle and glycolytic intermediates, <2% for photorespiration pathway and TCA cycle intermediates. Note that exposing the solution to ambient CO_2 has a negligible effect to dilute the isotope tracer (Figure S2), so the low isotope enrichment of metabolites following the filtration protocol was not due to exchange with ambient CO_2.

Nevertheless, enrichment in downstream metabolites would not be observed if there was not some residual enzymatic activity remaining during or after sample collection, which serves as a basis for comparing the different quenching methods. Note that although we performed the sample withdrawal and quenching as rapidly as possible, it still typically took about 10–15 seconds to withdraw 20 mL of

sample from the culture flask and mix it with the cold quenching solutions, and it took approximately 20 seconds to withdraw 20 mL of sample from the culture flask and filter the broth through the 0.8-µm membrane and then transfer the membrane into cold methanol. The 10–20 second time window for sample handling prior to cold quenching is expected to contribute to the isotopic labeling of targeted metabolites as shown in Figures 3 and 4. To avoid metabolic turnover during sample withdrawal and filtration, automated fast sample collection techniques could be implemented in the future[22]. Even if such techniques were used to minimize sample collection times, the approach described in this study could still be applied to rigorously compare the efficiency of different sampling and metabolic quenching methods.

Metabolite recovery varies inversely with the methanol content of solutions used for cold-quenching. Pre-cooling quenching solutions to temperatures below 0°C requires the addition of an organic solvent, such as methanol, which typically causes some leakage of metabolites from cells into the quenching solution[6, 8, 14]. When centrifugation is used to remove the spent media prior to extracting the cell biomass, this leakage can result in sampling losses that reduce measurement sensitivity and hinder accurate quantification of metabolite abundances. Because fast filtration removes the media from suspension cultures prior to quenching, the organic solvent does not need to be separated from the cell biomass prior to extraction and thus there is less opportunity for losses to occur. As minimizing metabolite leakage and thereby preserving metabolite abundance is another important goal of the sample collection method, in addition to rapid metabolic quenching, we compared the amounts of target metabolites in cell extracts obtained using each of the four quenching procedures, as well as a control that involved simply centrifuging unquenched samples ("Centrifuge"). We found that method 4, i.e., fast filtration followed by quenching in −80°C methanol, provided the overall highest metabolite abundances, presumably because the media was removed prior to quenching and subsequent extraction. Methods 1-3, which involve cold-quenching followed by media removal by centrifugation, provided significantly lower recovery of some metabolites, especially 3PGA, PEP, pyruvate, 2PGC, fumarate and aspartate (Figure 4). For these methods, metabolite abundance was inversely related to the methanol content of the quenching solution. In particular, method 3 led to consistently low metabolite signals compared to the other three quenching methods. This result could indicate increased cell damage during sample processing due to the high methanol content of the quenching solution. After the cell culture sample was mixed with the 60% methanol solution, the final methanol concentration of the mixture reached 36%, which could be sufficient to permeate the cell membrane and cause leakage of intracellular metabolites into the quenching solution. The low temperature of the 60% methanol solution (i.e., −65°C) could also induce additional metabolite leakage due to an enhanced cold shock response of the cells[23]. Indeed, we detected lower concentrations of metabolites extracted from cell pellets and significantly higher concentrations of metabolites in the supernatants of the quenched samples when using method 3 relative to the other three methods (Figure S1; Table S3—S4). We noticed that the total pool sizes of some metabolites, such as PEP, from the methanol quenching protocols were substantially lower than from the filtration method (Table S4). Similar results of metabolite loss following methanol quenching were reported previously[6, 24], but the underlying mechanism is still unclear and needs to be explored in the future. Interestingly, collecting cells via centrifugation without quenching (i.e., "Centrifuge" method in Figure 4) led to higher levels of recovered metabolites, such as glycine, serine and aspartate, and lower levels of other metabolites, such as 3PGA, PEP, pyruvate and fumarate, from cell extracts when compared to the filtration method followed by cold quenching (Table S3). We suspect this was due to the postharvest metabolic turnover as revealed in Figures 1—3. Overall, these data suggest that simply focusing on metabolite recovery could be misleading when comparing different quenching methods.

CONCLUSIONS

Metabolomics and fluxomics necessitate rapid and effective quenching of intracellular enzyme activities so that the measured metabolite profiles reflect the actual *in vivo* metabolic phenotype under defined temporal and physiological conditions. Nevertheless, rigorous methods for quantitatively assessing the efficacy of various sample collection and metabolic quenching procedures are lacking. Here, we present a practical method that leverages stable isotopes to evaluate four different quenching procedures for processing cell culture samples from suspension cultures of a model cyanobacterium. We found that mixing cell cultures with a ^{13}C tracer cocktail consisting of ^{13}C-glucose, ^{13}C-bicarbonate and ^{13}C-glutamine immediately after sample withdrawal was an effective strategy to distinguish the efficiency of the examined quenching methods. It was concluded that (i) fast filtration coupled with pure methanol quenching at −80°C or (ii) 30% methanol quenching at −24°C coupled with centrifugation are superior in both halting enzyme activities and preserving intracellular metabolites during sample collection from photoautotrophic *Synechocystis* 6803 cultures. By contrast, the use of a cold NaCl saline ice slurry (0°C) is inefficient at quenching enzymatic activities, and 60% methanol quenching at −65°C coupled with centrifugation resulted in substantial metabolite leakage. While fast filtration provided slightly better performance in preserving metabolite abundances, filtering and scraping cells off individual membranes makes this method more laborious relative to the other methods involving cold-quenching followed by centrifugation. The merits and demerits of the quenching methods examined in this study are summarized in Table S5. For certain applications, such as ^{13}C flux analysis of photosynthetic cyanobacteria, a number of samples need to be withdrawn and quenched within a short period of time (i.e., a few minutes). Furthermore, accurate measurement of metabolite ^{13}C labeling profiles is more important than quantification of absolute metabolite abundance for many ^{13}C flux studies[25]. In such cases, 30% methanol quenching at −24°C coupled with centrifugation could be a more practical and less laborious approach, albeit at the expense of some metabolite losses (Figure 4). The method demonstrated in this study requires the skillset of stable isotope labeling and mass spectrometry-based metabolite analysis, which is a well-established technique and is accessible to most analytical chemists. With minimal modification, the strategy could be readily applied to compare and optimize metabolic quenching protocols for other types of suspension cultures, thus providing a versatile approach to improve sample collection for metabolomics and fluxomics investigations.

Supporting Information

Additional experimental details, materials, and methods. Presence of metabolites in the cell-free supernatants following various quenching procedures (Figure S1). Exposing the solution to ambient CO_2 has little isotope tracer dilution effect (Figure S2). Temperatures of quenching solutions before and after mixing with the culture medium (Table S1). Fragment ions of targeted metabolites in the central metabolic network (Table S2). Intracellular metabolite recovery rates relative to that of "Filtration" method (Table S3). Total detected metabolite pool sizes relative to that of "Filtration" method (Table S4). Merits and demerits of the quenching methods examined in this study (Table S5).

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Authors' Contributions

B.W. designed and performed the experiments and drafted the manuscript. J.D.Y. supervised the research and revised the manuscript.

Competing interests

J.D.Y is a co-founder and shareholder of Metalytics, Inc

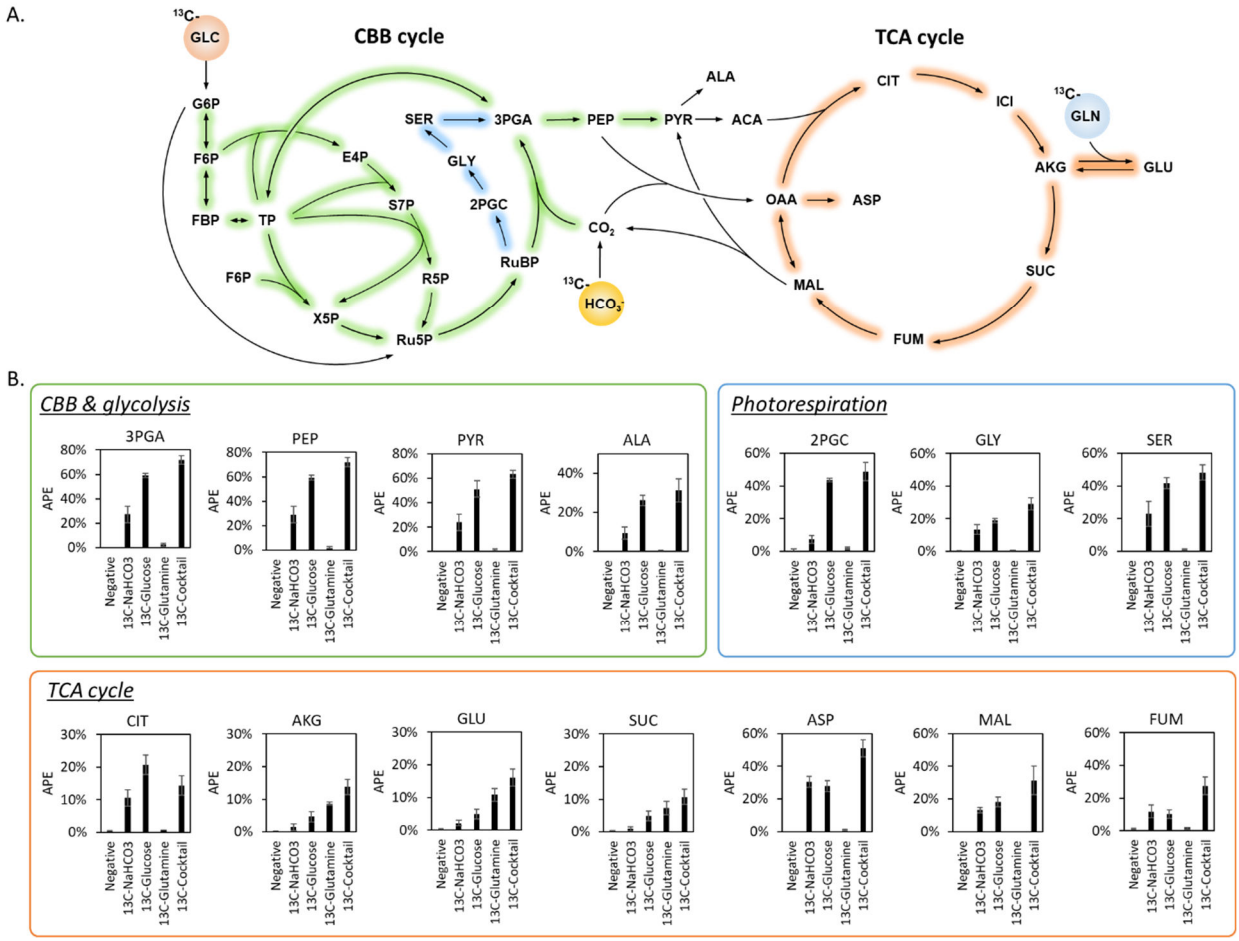

Figure 1. Assessment of postharvest isotopic labeling of metabolites using ^{13}C-glucose (GLC), ^{13}C-bicarbonate and ^{13}C-glutamine (GLN) in samples collected without metabolic quenching. (A) A schematic diagram showing the entry sites of the ^{13}C tracers into the central metabolic network of cyanobacterium *Synechocystis* sp. PCC 6803. Green arrows indicate reactions involved in the Calvin-Benson-Bassham (CBB) cycle and glycolysis. Blue arrows indicate reactions involved in photorespiration. Orange arrows indicate reactions involved in tricarboxylic acid (TCA) cycle. (B) Average percent enrichment (APE) of representative metabolites after cyanobacterial samples were mixed with ^{13}C tracers and injected into room-temperature NaCl solutions. Neg, negative control without using ^{13}C tracers. G6P, glucose-6-phosphaste. F6P, fructose 6-phosphate. FBP, fructose 1,6-bisphosphate. TP, triose phosphate (glyceraldehyde 3-phosphate or dihydroxyacetone phosphate). E4P, erythrose 4-phosphate. X5P, xylulose 5-phosphate. S7P, sedoheptulose 7-phosphate. R5P, ribose 5-phosphate. Ru5P, ribulose 5-phosphate. RuBP, ribulose 1,5-bisphosphate. 3PGA, 3-phosphoglycerate. 2PGC, 2-phosphoglycolate. GLY, glycine. SER, serine. PEP, phosphoenolpyruvate. PYR, pyruvate. ALA, alanine. CIT, citrate. ICI, isocitrate. AKG, α-ketoglutarate. GLU, glutamate. SUC, succinate. OAA, oxaloacetate. ASP, aspartate. MAL, malate. FUM, fumarate. Data represent means and standard deviations of three biological replicates.

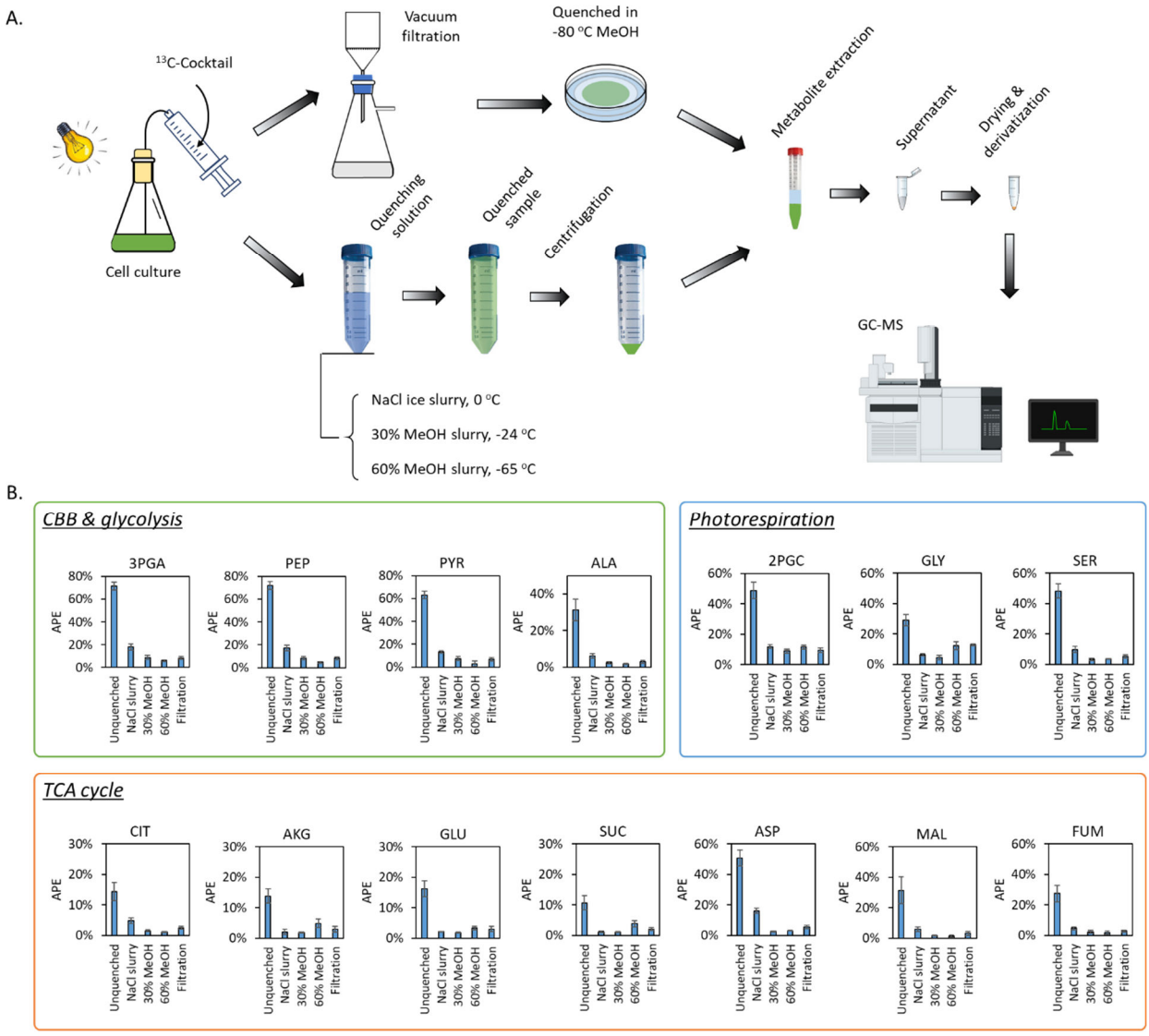

Figure 2. Comparison of metabolite turnover after sample harvest for various procedures using a ^{13}C-tracer cocktail including ^{13}C-glucose, ^{13}C-bicarbonate and ^{13}C-glutamine. (A) A schematic diagram illustrating the process for assessing quenching efficacy of various quenching methods. The tracer cocktail was added to the syringe prior to sample collection and labels any metabolites that are newly synthesized during sample processing. 20 mL of culture broth was drawn from the culture flask and either *i*) filtered through a 0.8-µm membrane and immediately quenched in 2 mL −80°C methanol, or *ii*) directly plunged into 30 mL quenching solution, followed by centrifugation and metabolite extraction and analysis. Unquenched control samples were collected as described in Fig. 1. (B) Average percent enrichment (APE) of targeted metabolites. Data represent means and standard deviations of three biological replicates.

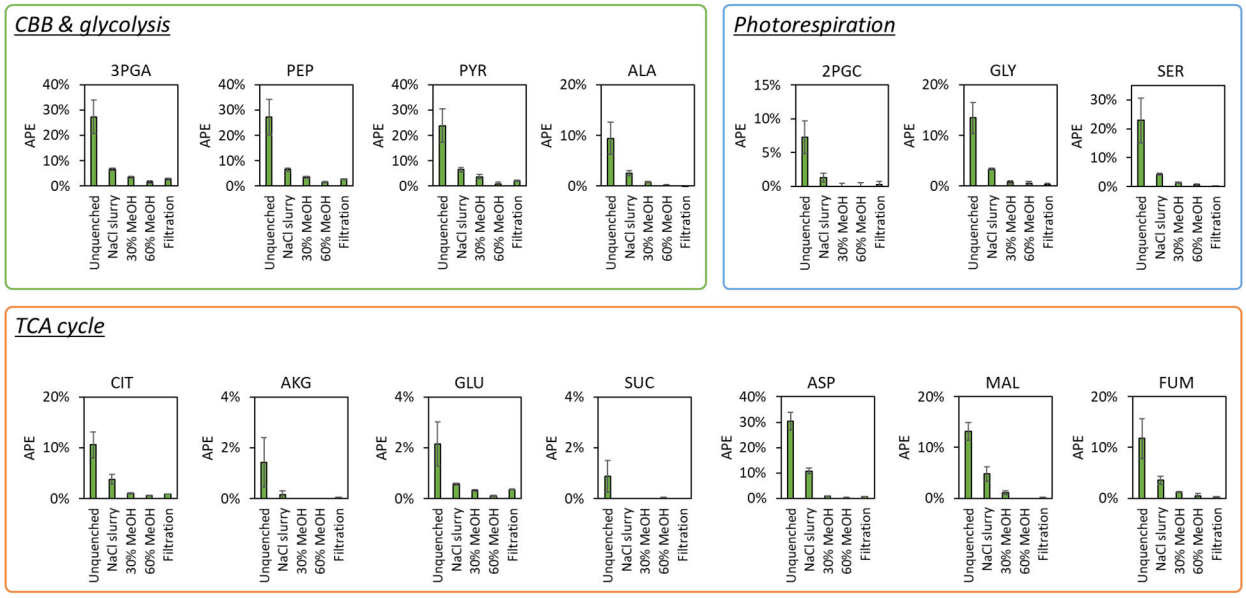

Figure 3. Comparison of ^{13}C enrichment after sample harvest for various procedures using ^{13}C-bicarbonate as metabolic tracer. The ^{13}C-bicarbonate tracer was added to the syringe prior to sample collection. Since bicarbonate was already present in the culture broth, mixing culture broth with ^{13}C-bicarbonate tracer was expected to exert minimal osmotic effects and metabolic perturbations to the cyanobacterial cells. APE=average percent enrichment. Data represent means and standard deviations of at least three biological replicates.

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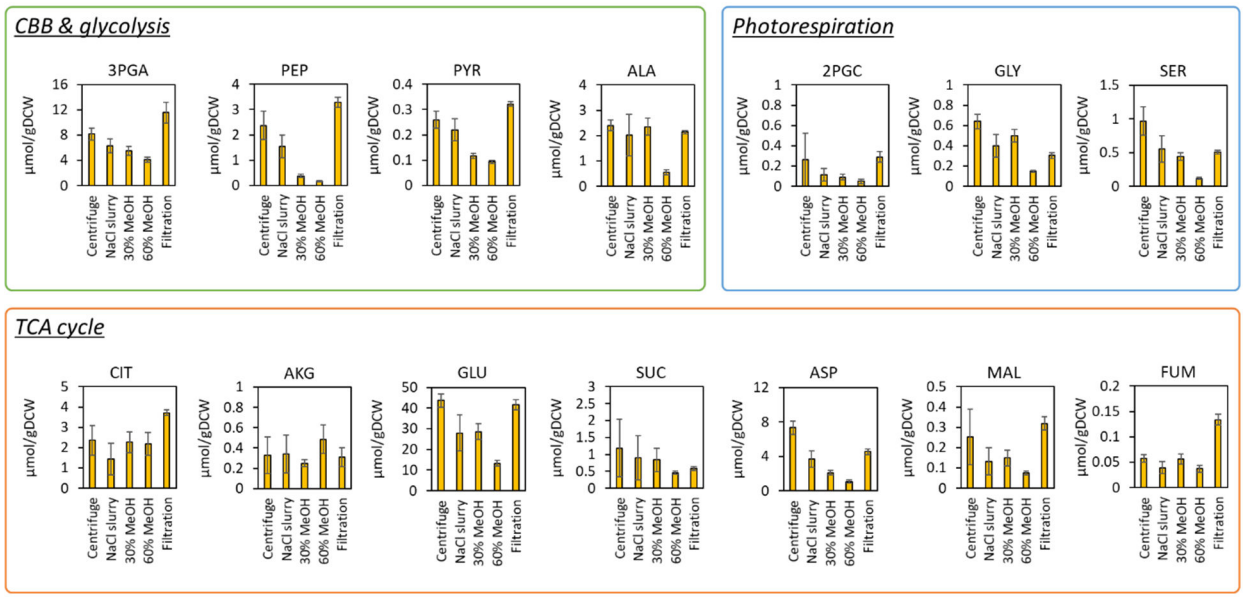

Figure 4. Recovery of metabolites from cell extracts obtained from various sample collection procedures. 20 mL of culture broth was drawn from the culture flask using a syringe without ^{13}C tracer and immediately subjected to membrane filtration and quenching in −80°C methanol or directly injected into a 30 mL quenching solution followed by centrifugation. Control samples were collected by simply centrifuging unquenched culture to separate cells from media (labeled "Centrifuge"). The extracted metabolites were derivatized and quantified using GC-MS, and normalized to gram dry cell weight (gDCW). Data represent means and standard deviations of four biological replicates.

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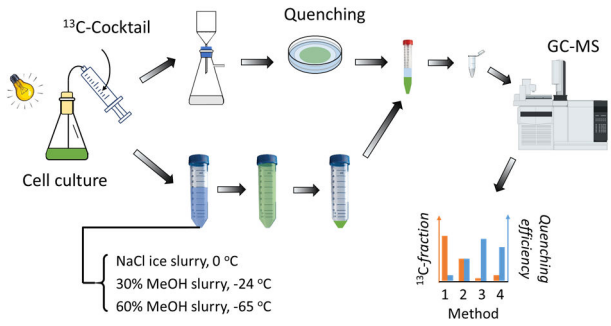

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