Kinetic flux profiling for quantitation of cellular metabolic fluxes

Jie Yuan, Bryson D Bennett & Joshua D Rabinowitz

241 Carl Icahn Laboratory, Department of Chemistry and Lewis–Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey 08544, USA. Correspondence should be addressed to J.D.R. (joshr@princeton.edu).

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This protocol enables quantitation of metabolic fluxes in cultured cells. Measurements are based on the kinetics of cellular incorporation of stable isotope from nutrient into downstream metabolites. At multiple time points, after cells are rapidly switched from unlabeled to isotope-labeled nutrient, metabolism is quenched, metabolites are extracted and the extract is analyzed by chromatography–mass spectrometry. Resulting plots of unlabeled compound versus time follow variants of exponential decay, with the flux equal to the decay rate multiplied by the intracellular metabolite concentration. Because labeling is typically fast ($t_{1/2} \leq 5$ min for central metabolites in *Escherichia coli*), variations on this approach can effectively probe dynamically changing metabolic fluxes. This protocol is exemplified using *E. coli* and nitrogen labeling, for which quantitative flux data for ~15 metabolites can be obtained over 3 d of work. Applications to adherent mammalian cells are also discussed.

INTRODUCTION

Kinetic flux profiling (KFP) aims to provide a practical experimental approach for measuring metabolic fluxes in live cells. The central idea of KFP is that larger metabolic fluxes are associated with faster transmission of isotopic label from added nutrient to downstream metabolites. The half-time of labeling of a metabolite will depend directly on the speed of transmission of label into the metabolite (i.e., the flux) and inversely on the size of the metabolite pool (i.e., the intracellular metabolite concentration). The KFP approach has been used to investigate nitrogen assimilation fluxes in exponentially growing *E. coli*[1], metabolic flux changes accompanying onset of carbon starvation in *E. coli*[2], carbon fluxes in *E. coli* fed with glucose versus acetate (Daniel Amador-Noguez and J.D.R., unpublished data), aromatic amino-acid pathway flux in nitrogen-starved *E. coli* and yeast[3] and carbon flux in growing, quiescent and virally infected human fibroblasts (Hilary Coller, Johanna Scarino, Joshua Munger, Thomas Shenk, B.D.B., J.D.R., unpublished data).

This concept is illustrated in **Figure 1**. During exponential growth, the rate of production of each metabolite (influx) should match its rate of consumption (efflux) so that the intracellular concentration remains constant. Under this pseudosteady state, if an external nutrient is instantaneously switched from natural to isotopically labeled, for a metabolite X directly downstream of nutrient assimilation, unlabeled X (X^U) will be replaced over time by its labeled counterpart (X^*) and the fraction of unlabeled X (X^U/X^T) will decay exponentially (**Fig. 1**). The rate constant of this decay (k_X) is determined by the ratio of the flux through X (f_X) to the total pool size of X (X^T) as shown in **Figure 1**. Therefore, one can calculate the flux through X (f_X) from k_X and X^T. k_X can be obtained experimentally by the protocol described here and X^T by a diversity of literature approaches[4–6], including the protocol of Bennett *et al.*[7]. For an example of quantitative analysis of data from more complex cases (which realistically involve metabolites not immediately downstream of labeled nutrient and being formed by multiple reactions), see the ANTICIPATED RESULTS section.

Experimental design

Accurately measuring k_X is most easily achieved by rapid and complete switch of the nutrient of interest from unlabeled to isotope-labeled form, followed by fast sampling of cells[1]. For reliable flux measurements, both steps must be accomplished without perturbing cellular metabolism. Otherwise, the artifacts induced by the handling steps will mask the true cellular metabolic state[8–12]. To meet this need for nonadherent cells (e.g., *E. coli*[1–3,13], *Saccharomyces cerevisiae*[3]), we developed a filter culture technique in which cells are grown on a membrane filter sitting on top of agarose plates loaded with media. The cells are fed by nutrient diffusion from the underlying medium up through the filter. This technique enables isotope switching by transferring the filter between agarose plates of different composition. It also allows fast metabolic quenching by transferring the filter into cold organic solvent, which stops metabolism (initially due to the temperature drop and subsequently by denaturing enzymes) and simultaneously initiates the extraction process by disrupting the cell membrane. Movements of the filter can be done in ~1 s. Minor deviations in the transfer time tend to have minimal impact, as the cells continue to receive nutrients from the filter during the transfer.

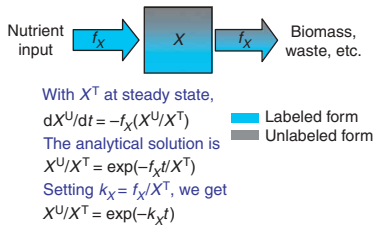

Figure 1 | Illustration of the basic concept of KFP. Metabolite X is generated directly from nutrient and is consumed during biosynthesis (eventually leading to biomass production). At metabolic steady state, the influx and efflux of X pool are both f_X. The differential equation describes the rate of disappearance of the unlabeled form of X when unlabeled nutrient is substituted with isotope-labeled nutrient. X^U denotes unlabeled X, X^T the total pool size of X and f_X the flux through metabolite X.

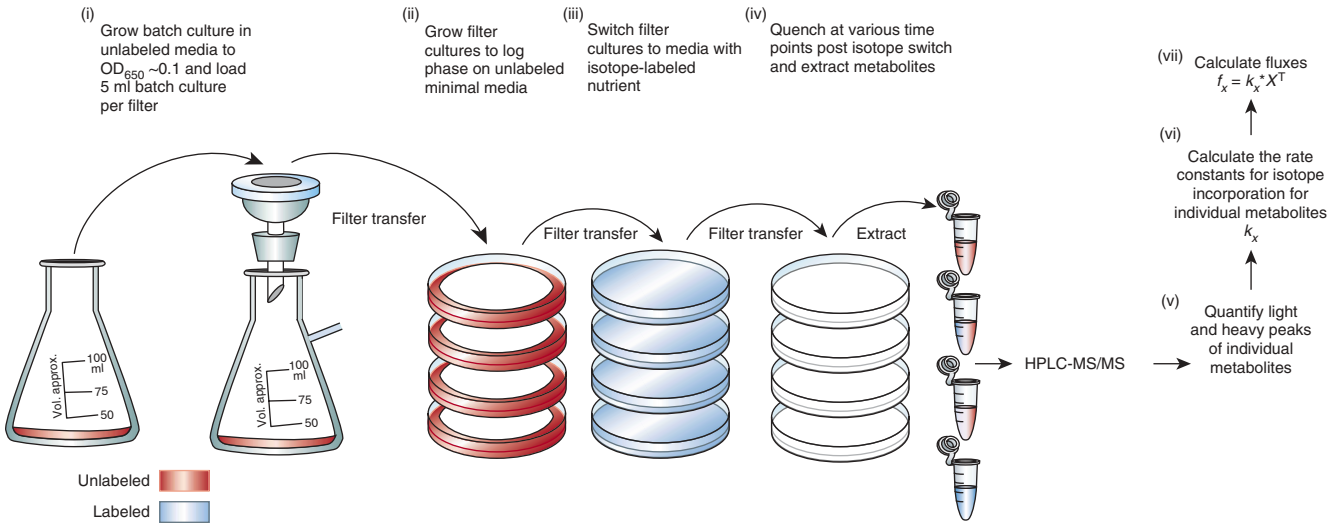

Figure 2 | Overview of experimental procedure for KFP (Steps 1–8).

Although the overall strategy is the same, the filter culture approach is not necessary for adherent cell types like human fibroblasts. Instead, medium removal via quick aspiration is followed by addition of isotope-labeled medium for isotope switch or cold organic solvent for metabolic quenching and extraction. Specific cell-handling steps are provided in the protocol.

Overview of the workflow and experimental setups for KFP are shown in **Figure 2**. The step of quantifying heavy and light (i.e., labeled and unlabeled) isotopic forms can be achieved by any appropriate form of mass spectrometry (MS). Typically, we use liquid chromatography (LC)–electrospray ionization (ESI)–triple-quadrupole MS operating in multiple reaction monitoring (MRM) mode. MRM is a targeted form of tandem mass spectrometry (MS/MS) with the advantage of excellent sensitivity and linear dynamic range. LC separation before the MS/MS analysis is valuable for separating isobaric compounds. Other forms of chromatography-MS should also be applicable. These include gas chromatography–MS and LC–time-of-flight–MS. For further information on analytical options, see the protocol of Bennett *et al.*[7]. The rate constants obtained from KFP can then be combined with intracellular concentrations of metabolites determined separately to calculate fluxes.

Typically, a single replicate of KFP is informative (as even a single replicate contains multiple time points). For quantitative work where small differences between conditions are involved or precise flux estimates are desired, 3–4 replicates are preferred. As KFP experiments are internally controlled (by the multiple isotopic forms), external controls are not required. It is often useful, however, to conduct switches from unlabeled to unlabeled media to ensure against unanticipated metabolic shifts due to media change rather than isotope switch.

Application and limitations

Kinetic flux profiling provides an approach for experimentally quantifying metabolic fluxes in live cells. The principal starting information required is the structure (connectivity) of the metabolic network being investigated (see ANTICIPATED RESULTS for an example). An advantage of the technique is that the experimental data provide a check on the pathway architecture, based on the requirement for precursor metabolites to become labeled before their downstream products. Quantitative analysis of fluxes by KFP is facilitated when the fluxes are at steady state; however, differential KFP (described below) can provide quantitative data even outside of the steady-state condition. Further quantitative assumptions are not required, as fluxes are calculated directly from the experimental results obtained as described here (k_X) and in the companion protocol of Bennett *et al.*[7] (X^T).

One can choose to use basic nutrient(s) (e.g., glucose, ammonia, etc.) to introduce isotopic labels or to use special tracers for a specific pathway. We have successfully applied KFP to measure fluxes involved in nitrogen metabolism in *E. coli*[1,2], and for this reason, this protocol uses the example of $^{15}NH_4Cl$ as the labeled nutrient in this model organism. The KFP approach is, however, also widely applicable to other labeled nutrients and cell types, as demonstrated by published work from our laboratory using ^{13}C-glucose as the tracer in both *E. coli* and *S. cerevisiae*[3] and unpublished data from our laboratory with cultured human cells.

It is important to note that KFP measures gross fluxes: the labeling kinetics is related to the sum of all fluxes feeding into (or, equivalently at steady state, flowing out of) a metabolite. In the case of reversible reactions, this means that the flux measured by KFP may differ significantly from the net pathway flux.

A limitation to the KFP approach is that quantitative flux information is reliably obtained only for metabolites that turn over slowly relative to their upstream precursor. Consider a case in which the isotope label is relayed from a precursor metabolite *X* to a downstream metabolite *Y*. If *X* turns over faster than *Y*, then the labeling kinetics of *Y* will reliably reflect the flux through *Y*, based on the magnitude of k_Y. In contrast, if *X* turns over at a much slower rate than *Y*, then the labeling kinetics of *Y* will mainly depend upon the labeling of *X*, rendering measurement of k_Y, and accordingly flux through *Y*, imprecise (a more quantitative treatment of this important case is provided in the ANTICIPATED RESULTS). For a similar reason, the uptake of the nutrient or the tracer needs to be

efficient, and the nutrient must not accumulate significantly internally. Otherwise, the rate of labeling of metabolites will be largely determined by the rate of turnover of the internal pool of the nutrient/tracer, rendering it impossible to accurately quantitate the downstream fluxes.

Differential KFP

Differential KFP provides an example of a KFP variant suitable for quantitative analysis of dynamically changing fluxes. It was developed to evaluate changes in biosynthesis and macromolecule degradation when cells are exposed to an environmental perturbation. It has been used to demonstrate that carbon starvation in *E. coli* results in rapid turning off of *de novo* biosynthetic fluxes, with protein degradation becoming the major source of intracellular amino acids[2]. As shown in **Figure 3**, differential KFP consists of two (or more) sets of the KFP experiment, with the isotope switch initiated at different times with respect to the perturbation (e.g., preceding or following the perturbation). The kinetic patterns of isotope incorporation into metabolites obtained from these experiments, in addition to the knowledge of metabolite concentration changes triggered by the perturbation, can often be used to determine the effect of the perturbation on metabolic fluxes. Carbon source withdrawal in *E. coli* is used as an example of a perturbation in this protocol.

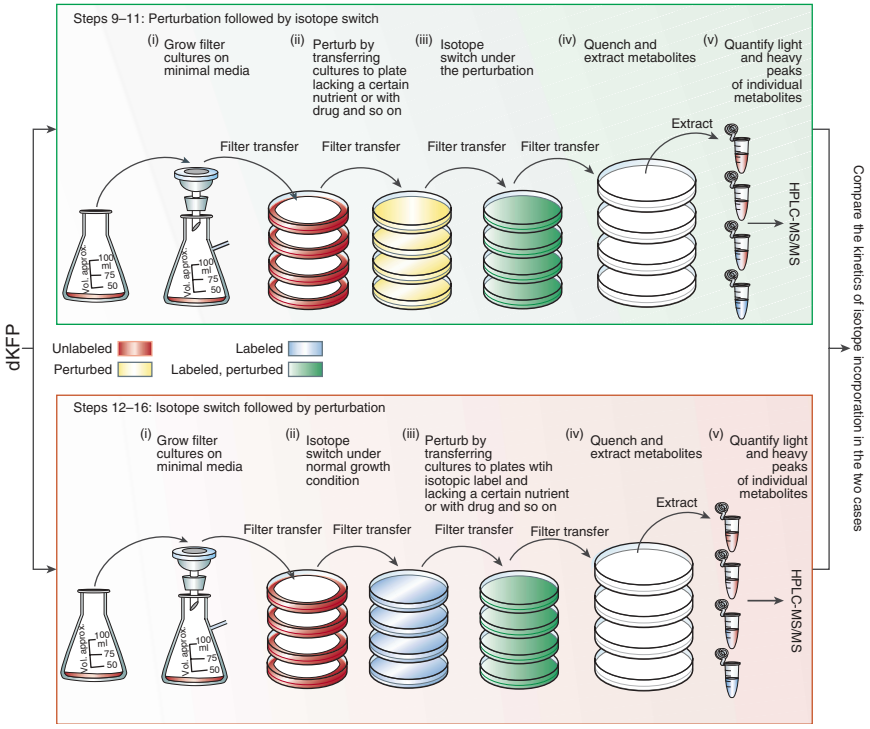

Figure 3 | Overview of experimental procedure for differential KFP (Steps 9–16, optional).

Alternative approaches

Efforts at measuring cellular metabolic fluxes have been ongoing for decades and a diversity of valuable tools have been developed[14–20]. Several of these contain elements similar to the current KFP approach. Instead of detailing these related approaches, here we focus on two conceptually distinct alternatives: flux balance analysis (FBA)[21] and metabolic flux analysis (MFA)[22].

Flux balance analysis is a constraints-based computational approach that requires little experimental data and offers an estimation of the range of feasible flux distributions in steadily growing cells[21,23]. Although it has proven powerful, especially for *E. coli*[24], the precise determination of fluxes by FBA relies on an objective function and related assumptions (e.g., that *E. coli* maximizes biomass yield per molecule of carbon source consumed[23–25]). For most organisms, a validated objective function is not available, limiting the ability of FBA to make quantitative flux predictions in the absence of experimental data[26–28].

Metabolic flux analysis is an experimental approach that typically involves feeding cells with a mixture of different ^{13}C-labeled glucose species (e.g., uniformly labeled and only one carbon labeled) for a prolonged period under metabolic steady state[29], until the isotopic labeling pattern of the compounds to be measured (most typically proteic amino acids) reaches a steady state (>1 h). From the labeling pattern of proteic amino acids[30] or primary free metabolites[31], metabolic fluxes (mostly of central carbon metabolism) are then deconvolved with the aid of computer modeling[32,33].

To assist in the choice of the appropriate experimental approach, MFA and KFP are compared here. MFA is well suited to measuring the ratio of fluxes at branch points when the alternative branches yield different labeling patterns of a downstream metabolite[30,31]. It is also suitable for large-scale studies with respect to the number of species/strains[29], as the fluxes (relative to glucose uptake) of multiple pathways can be obtained from a single sample. No time courses or pool size data are needed, which reduces the experimental demand compared with KFP. However, MFA (at least in its most commonly practiced form) is largely limited to carbon metabolism, as labeling by other elements rarely produces the rich spectrum of labeling patterns of metabolites required for flux deconvolution (e.g., there are 32 theoretical C-labeling states but only 2 possible N-labeling states of glutamate—labeled or unlabeled). In contrast, KFP is more versatile in terms of what pathways can be monitored. In addition, KFP also has the following strengths compared with MFA: easy data deconvolution (in many cases, the differential equations of KFP have analytical solutions in the form of exponential functions with few free parameters, enabling direct parameter determination); short labeling time (no requirement for incorporation of isotope labels to reach steady state, which allows effective probing of dynamically changing fluxes using variants like differential KFP); and KFP can provide absolute fluxes instead of just split ratios. Each individual approach has limitations, and combining multiple approaches will generally yield the most complete understanding.

MATERIALS

REAGENTS

- HPLC-grade water
- HPLC-grade acetonitrile ! CAUTION Wear gloves and safety glasses when handling acetonitrile.
- HPLC-grade methanol ! CAUTION Wear gloves and safety glasses when handling methanol.
- Ammonium hydroxide (for buffering of HPLC solvents) ! CAUTION Wear gloves and safety glasses when handling ammonium hydroxide.
- Ammonium acetate (for buffering of HPLC solvents) ! CAUTION Wear gloves and safety glasses when handling ammonium acetate.
- Formic acid (optional, depending upon the extraction solution) ! CAUTION Wear gloves and safety glasses when handling formic acid.
- Ammonium bicarbonate (optional, depending upon the extraction solution; use to neutralize extract if initially extracting in the presence of formic acid)
- Minimal medium components (for microbial culture)
- $^{15}NH_4Cl$ (and/or other isotope-labeled nutrients)
- Ultrapure agarose (Invitrogen) (for microbial filter culture)
- DMEM medium without the nutrient you wish to use to introduce isotope label (for mammalian cell culture)
- HEPES buffer (for mammalian cell culture)
- 100 mm × 15 mm sterile Petri dishes (Fisher Scientific Ltd., cat. no. 08-757-12) (for making agarose plates for filter cultures; for culturing of adherent mammalian cells, select instead the Petri dish surface chemistry most appropriate to growth of your specific cell line)
- 15-ml sterile polypropylene conical tubes for growing 5-ml overnight cultures (for microbes)
- Sterile flask (for growing liquid cultures of the microbes, for preparation of subsequent filter culture)
- 82-mm nylon membrane filters for microbial filter cultures (pore size 0.45 μM, Millipore or GE Water & Process Technologies); note that nitrocellulose filters are adequate if using methanol to extract, but will dissolve in acetonitrile-containing solution
- 1.7-ml microcentrifuge tubes (e.g., Bioexpress, cat. no. C-3269-1)
- Dry ice if using methanol extraction
- Cell scraper (for adherent cells)

EQUIPMENT

- Shaker table, for growing liquid microbial cultures (typically placed in a 37 °C warm room or incubator for *E. coli*)
- Spectrophotometer (e.g., Thermo Electron Corp., Genesys 10 uv) used at 650 nm for measuring OD with plastic cuvettes (e.g., Fisher Scientific Ltd., cat. no. 14-955-127)
- Pipette filler/dispenser (a motorized one is recommended) and 5-ml pipettes
- Tweezers for manipulating the membrane filters
- Microcentrifuge, for pelleting cell debris after extraction (if not available, a normal centrifuge can also be used)
- Vortex mixer
- HPLC–ESI–MS/MS and matching HPLC vials (see EQUIPMENT SETUP)
- −20 °C freezer (only when using 40:40:20 acetonitrile:methanol:water for extraction)
- Metal pan and icepacks to fill, precooled to −20 °C (when using 40:40:20 acetonitrile:methanol:water extraction mixture)
- N-evap evaporation system (Organomation Associates Inc.) (optional, use only if sample concentration is required to get adequate signal; this is not generally recommended)
- Glass vacuum filtration apparatus, components listed below:
  - Microanalysis vacuum filter holders, stopper, perforated, no. 8 (Fisher Scientific Ltd., cat. no. 09-753-32A)
  - Microanalysis vacuum filter holders, sintered support base, diameter: 90 mm (Fisher Scientific Ltd., cat. no. 09-753-27B)
  - Vacuum filtering flask, 1 liter

REAGENT SETUP

Cells Before initiation of an experiment, cells should be handled as per typical laboratory protocols tailored to the cell type of interest. To initiate this protocol, a starter culture is required for microbes and 10^6–10^7 cells in culture for mammalian cells.

Washed agarose Wash the Ultrapure agarose three times with HPLC-grade water to remove trace impurities. For 30 g of agarose, use 1 liter of water for each wash. For each wash, stir the agarose–water mixture for 10 min and leave aside to settle for ~1 h. Aspirate the water with care to avoid loss of agarose. The resulting washed agarose can be used to make minimal media plates with 1.5% (wt/vol) agarose. *Note*: this is not necessary for adherent cell types.

Minimal liquid media Combine sterile salts, glucose (or other carbon source) and water according to the media recipe. (The exact composition of the complete minimal media we use is as follows: KH_2PO_4 4.7 g liter^{-1}, K_2HPO_4 13.5 g liter^{-1}, K_2SO_4 1 g liter^{-1}, $MgSO_4 \cdot 7H_2O$ 0.1 g liter^{-1}, NH_4Cl 10 mM, glucose 0.4%. Use isotope labeled nutrient when appropriate.)

Minimal media plates Combine minimal liquid medium with 1.5% (wt/vol) washed agarose. Autoclave and pour into the sterile Petri dishes to make media plates. Use 20 ml of agarose–medium mixture per 10-cm plate. Four types of plates will be used in the protocol: two types for KFP (Steps 1–8) and additional two types for differential KFP (Steps 9–22). Compositions specific to $^{15}NH_4Cl$-labeling are as listed below:

(A) Regular (or unlabeled) plates: salts + glucose + water + agarose
(B) ^{15}N-labeled plates: salts + glucose + water + agarose, substitute NH_4Cl with $^{15}NH_4Cl$
(C) No-glucose plates: salts + water + agarose (optional, Steps 9–22 only)
(D) ^{15}N-labeled no-glucose plates: salts + water + agarose, substitute NH_4Cl with $^{15}NH_4Cl$ (optional, Steps 9–22 only)

Note that, as carbon starvation is used as the example of perturbation in this protocol, glucose is removed from the plate content for this purpose in Type C and D plates. Exact content of the Type C and D plates will depend on your experiment and perturbation of interest. Agarose plates are not necessary for adherent cell types.

Extraction solutions Different groups of metabolites are extracted with different efficiency depending upon the extraction solution mixture[34]. Choice of extraction solution should be made according to which metabolites are of the greatest interest. Among the seven solution systems we have tested for extracting filter cultures, 40:40:20 acetonitrile:methanol:water solvent system works the best for extracting filter cultures in general; addition of formic acid to a final concentration of 0.1 M provides additional protection of nucleotide triphosphates against degradation[13]. NH_4HCO_3 solution is used to neutralize the formic acid immediately after extraction. Methanol (100% methanol at −75 °C for the first round, 80:20 methanol:water at 4 °C for the two subsequent rounds) extracts amino acids effectively while extracting fewer other components than acetonitrile:methanol:water; it is accordingly preferred for studies focused solely on amino acids and was used for our KFP study of nitrogen metabolism[1]. For extracting human fibroblasts, we have obtained adequate results with 80:20 methanol:water for all the three extractions. Pending more definitive studies, we recommend this solvent mixture for them.

HPLC mobile phase Solvent A: 20 mM ammonium acetate + 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45; Solvent B: acetonitrile. Note that this is our mobile phase of choice when working with aminopropyl column in hydrophilic interaction chromatography mode; there are many other chromatography choices available[31,35–37]. Many of these have important advantages relative to the aminopropyl approach for certain classes of compounds. For more information, see Lu *et al.*[38].

EQUIPMENT SETUP

HPLC system Hydrophilic interaction chromatography is performed on a 2-mm inner diameter column packed with 5-μm aminopropyl resin to 250 mm in length, using an LC-10A HPLC system (Shimadzu). The column is maintained at 15 °C with a solvent flow rate of 0.15 ml min^{-1}, and the gradients are as follows: $t = 0$, 85% B; $t = 15$ min, 0% B; $t = 28$ min, 0% B; $t = 30$ min, 85% B; $t = 40$ min, 85% B. Other chromatography approaches and/or HPLC systems can be used depending on their availability and the metabolites of interest.

Mass spectrometer A Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corporation) is run in MRM mode and coupled to the HPLC via electrospray ionization. Electrospray ionization spray voltage is 3,200 V in positive mode. Nitrogen is used as sheath gas at 30 psi and as the auxiliary gas at 10 psi, and argon as the collision gas at 1.5 mTorr, with a capillary temperature of 325 °C. MRM scan time is 0.1 s and scan width is 1 *m/z*. Other forms of MS (e.g., single quadruple, time of flight, ion trap) generally provide slightly less optimal quantitation (e.g., worse signal-to-noise and reproducibility) but can nevertheless be used.

MRM scans Reactions should be optimized for metabolites of interest using standards before the quantification experiment. A list of reactions used in our

experiments has previously been published[1]. Optimization of the product ion and collision energy for a given unlabeled metabolite is achieved by infusing purified compound standard into a triple quadrupole mass spectrometer. Collision energy should be identical for labeled and unlabeled forms. For ^{15}N labeling, the parent ion mass should be increased by the number of nitrogen atoms in the metabolite, and the product ion mass should be increased by the number of nitrogen atoms in the product ion (product ion structures can be obtained from the literature for common metabolites, or otherwise estimated based on common routes of fragmentation and confirmed experimentally by MS/MS of the labeled forms). For partially labeled forms, more than one product ion mass may be possible for each parent ion mass. The different product ion masses arise from labeling at different positions within the parent. As an example, consider the possibilities for a compound with two nitrogen atoms that gives a product ion with 1 nitrogen (e.g., glutamine): with $0 \times {}^{15}N$ in the parent, there cannot be ^{15}N in the product ion; with $1 \times {}^{15}N$ in the parent, there can be 0 or $1 \times {}^{15}N$ in the product ion; with $2 \times {}^{15}N$ in the parent, there must be $1 \times {}^{15}N$ in the product ion.

PROCEDURE

▲ CRITICAL Steps 1–8 enable measurement of steady-state metabolic fluxes. Steps 9–22 (Differential KFP, optional) are used for probing flux changes in response to an environmental perturbation. Steps 9–11, 12–16 and 17–22 are logistically independent of each other and can be carried out in any order.

1| Grow cells, carry out isotope switch and prepare cell extracts. Option A is designed for nonadherent microbes and has been tested for *E. coli* and *S. cerevisiae*. Option B is designed for adherent mammalian cells and has been tested for primary human fibroblasts.

(A) For nonadherent microbial cells (grow cells and prepare for extraction (i–xi); carry out isotope switch and generate metabolite extracts (xii–xxiii))

(i) Prepare agarose plates. One Type A plate and one Type B plate for each time point will be needed, except that the zero time point needs only a Type A plate. Prepare two or three additional Type A plates to determine OD_{650} (the OD measurement will be used to determine when cells have reached an appropriate density for conducting the isotope switch).

(ii) Inoculate your *E. coli* strain (or other nonadherent cell types) into 5 ml of regular minimal media in a 15-ml conical centrifuge tube (i.e., no isotopic agent added) and grow overnight at 37 °C.

(iii) Before use, warm all the plates to the temperature at which the cells are grown (i.e., 37 °C for *E. coli*), by placing them in a warm room or incubator. Dilute the overnight culture into fresh minimal media to $OD_{650} \sim 0.03$ (roughly 1:50 dilution) and allow to grow in batch to early exponential phase (OD_{650} of ~ 0.1).

(iv) Determine the total volume of batch culture according to the number of Type A plates needed; 5 ml of the batch culture will be needed for each plate. Allow some extra culture for OD measurements that preceed creating the filter cultures. OD can be measured by pipetting 1 ml of the liquid culture into spectrophotometer cuvette and measuring absorbance at 650 nm, using fresh media as blank.

(v) Assemble the vacuum filter support, stopper and vacuum filter flask. Connect the side arm of the filter flask to a vacuum valve or pump (get the assembly ready before the batch culture reaches $OD_{650} = 0.1$). If possible, place the whole assembly in a warm room to avoid temperature perturbation while preparing your filter cultures.

(vi) Open the vacuum to a moderate level. Lay a piece of membrane filter on the filter support with a pair of tweezers. Pass 5 ml of the liquid culture ($OD_{650} \sim 0.1$) through the membrane filter carefully using a pipette and dispenser. Slowly drip your culture in drops as evenly as possible onto the filter. For photographs of this step, see Bennett *et al.*[7].

(vii) Remove the membrane filter from the filter support once the 5 ml of culture is loaded. Place it with the cell side face up onto a Type A agarose plate, being careful to ensure that the filter makes good contact with the agarose over the entire cell-containing area. This step usually takes 30–60 s per filter.

(viii) Repeat until loading all Type A plates with the filter cultures is completed.

▲ CRITICAL STEP Be consistent when loading cells onto the filter to minimize variations between your filter cultures. Pay attention to the vacuum level, the speed at which you drip the liquid onto the filter and so on.

(ix) Allow the filter cultures to grow to mid-log phase. Determine OD_{650} by thoroughly washing a filter with 5 ml of water and measure the OD of the wash using the spectrophotometer. (To wash the cells off the filter completely, one effective approach is to tilt the filter slightly and pipette the water onto the filter with mild pressure strong enough to produce a steady stream but not to cause a splash.) *E. coli* typically take 2–2.5 h to grow to an OD_{650} of 0.4 after completion of Step 1A(vii).

(x) For each time point, prepare ~ 4 ml of extraction solution (3.5 ml for the first extraction and 0.1 ml for each of the two subsequent extractions), one clean empty Petri dish, two microcentrifuge tubes and one 15-ml conical tube. Label the dishes and tubes to avoid confusion. If different extraction solution is needed for the second and third round of extraction, prepare accordingly.

(xi) Approximately 15 min before isotope switch, prepare for extraction by transferring 2.5 ml of the extraction solution into every Petri dish. Bring extraction solution and Petri dishes to the desired temperature by, for the example of methanol extraction, placing them on dry ice.

(xii) Set timers for desired lengths of time.

(xiii) Using a pair of tweezers, peel the filter culture off the Type A plate and immediately place it with the cells face-up on a fresh Type B plate and start the timer.

▲ CRITICAL STEP Try to control the length of time that the filter culture sits on the labeled media as accurately as possible. For multiple points in a small window, deal with only one or two filters at a time and keep the extraction solution nearby for prompt quenching. Otherwise, transfer the filter cultures onto Type B plates one by one in a warm room until completed.

(xiv) After the filter cultures have been grown on labeled media (Type B plates) for the targeted length of time, peel the filter off with tweezers and immerse it in the extraction solution in the Petri dish, with cells face-down. Tilt the dish to ensure that the filter is completely covered by the extraction solution. Allow it to remain for 15 min at the extraction temperature (e.g., on dry ice for methanol extraction).

▲ CRITICAL STEP Move the cells from the agarose plate to the cold organic solvent quickly to avoid metabolic perturbations.

(xv) Rinse the filter with the extraction solution in the dish and transfer the resulting solution, together with cell remnants suspended in it, into two microcentrifuge tubes. Add another 1 ml of extraction solution to the dish and use it to re-rinse the filter. Combine with the original 2.5-ml cell extract in the microcentrifuge tubes.

(xvi) Centrifuge in a microcentrifuge at full speed (16,000*g*) and 4 °C for 5 min to pellet the insoluble materials. (Microcentrifuge and matching tubes are used here for easier pelleting of the cellular debris; they can be substituted with a regular tabletop centrifuge and matching tubes if desired.)

(xvii) Transfer the supernatant from the two microfuge tubes into one clean conical tube and set aside (e.g., on dry ice for methanol extraction).

(xviii) Resuspend the pellets in 100 µl (50 µl per pellet) of extraction solution (80:20 methanol:water for methanol extraction). Let sit for 15 min at an appropriate temperature (e.g., 4 °C for methanol extraction). If using 40:40:20 methanol: acetonitrile:water as extraction solution, little pellet will form here. You may choose to skip one or more of the extraction steps (Steps 1A(xviii–xxi)) depending on the size of the pellet.

(xix) Pellet in a microcentrifuge at full speed (16,000*g*) and 4 °C for 5 min.

(xx) Combine the supernatant with the original one on dry ice.

(xxi) Repeat Steps 1A(xviii–xx) to generate complete cell extract of one filter culture (i.e., one time point).

(xxii) Neutralize the extract in each tube with 300 µl of 15% NH_4HCO_3 if using acidic acetonitrile:methanol:water extraction. Centrifuge one more time after neutralization to remove precipitates.

(xxiii) Repeat Steps 1A(xiii–xxii) for all time points.

(B) For adherent mammalian cells (grow cells and prepare for extraction (i–vi); carry out isotope switch and generate metabolite extracts (vii–xi))

(i) Prepare 24 ml of media without isotope label and 6 ml of media with isotope-labeled nutrient for each sample to be generated. The medium should be buffered with HEPES to a final concentration of 10 mM to keep the pH relatively constant before and after isotope switch.

(ii) Culture cells in a 10-cm Petri dish with 8 ml of unlabeled media for each sample. Allow to grow until 24 h before the experiment, typically 2–7 d depending upon cell culture growth rate.

(iii) Twenty-four hours before the isotope switch, aspirate the media in the Petri dishes and replace with 8 ml of fresh unlabeled media each. The fresh media used in this step should be equilibrated in the incubator overnight before the media change.

(iv) Equilibrate the rest of the media prepared in Step 1B(i) in the incubator overnight before the isotope switch experiment.

(v) Prepare 5 ml of extraction solution for each sample (4 ml for the first extraction and 0.5 ml for each of the two subsequent extractions) and bring to appropriate temperature. Prepare clean cell scrapers.

(vi) Repeat Step 1B(iii) 1 h before the isotope switch. The two media changes carried out (24 h and 1 h) before the experiment are to avoid artifacts caused by changing media chemical composition (e.g., by removing extensive amounts of built-up cellular waste products) during the isotope switch.

(vii) Remove the unlabeled media by tilting the dish slightly and allowing the media run toward the side as much as possible. Aspirate thoroughly and quickly (<10 s), replace with labeled media and start the timer.

▲ CRITICAL STEP Try to control the length of time the cells are exposed to labeled media as accurately as possible. For multiple points in a small window, deal only with one or two dishes at a time and keep the extraction solution nearby for prompt quenching. Including the time to get the dish out of the incubator, it usually takes $\sim$1 min to completely change the media for one dish.

(viii) After the cultures have been grown in labeled media for the targeted amount of time, aspirate the media completely and add 4 ml of the extraction solution. Let sit for 15 min at the extraction temperature (e.g., on dry ice for methanol extraction). Scrape the cells off the dish with a cell scraper. Transfer the cell suspension into a 15-ml conical tube. Centrifuge for 5 min at 2,000*g* and 4 °C to pellet the cells. Transfer the supernatant into a new 15-ml tube and set aside.

(ix) Resuspend the pellet in 500 µl of extraction solution by vortexing, and let sit at 4 °C for 15 min. Centrifuge for 5 min at 2,000*g* and 4 °C, combine the supernatant with the supernatant obtained from Step 1B(viii).

(x) Repeat Step 1B(ix) for a third round of extraction.

(xi) Repeat Steps 1B(vii–x) for all time-point samples.

2| (Optional) For each sample, evaporate the cell extract under N_2 gas flow (using an N-Evap system) until dry. Resuspend in 200 µl of 1:1 methanol:water solution. Be aware that, while this step will increase the metabolite concentration in the sample, it could also potentially cause increased ion suppression and loss of less stable metabolites. This step is generally unnecessary for microbial cell cultures, but it may have value for some mammalian ones.

3| Transfer the cell extract into HPLC vials and load the vials into the LC-MS autosampler.

Analyze by HPLC-MS/MS

4| Analyze the samples by LC-MS, using 10-µl injection volume (more if necessary; be aware that this may result in increased ion suppression), preprogrammed LC gradient and (for triple quadrupole MS) preprogrammed MRM scan events.

■ **PAUSE POINT** LC-MS runs can be done overnight.

5| For individual metabolites, quantify peaks of unlabeled and isotopically labeled forms using predefined MRM channels and appropriate program (or alternative MS-based approach). Quantitation based on peak height or peak area generally gives similar results.

Data analysis

6| Determine the fraction of unlabeled form for each metabolites in each sample by the formula below:

$$\text{Fraction unlabeled} = \frac{\text{Peak height}_{\text{unlabeled form}}}{\text{Sum of peak height}_{\text{every isotopic form}}}$$

This simplest 'fraction unlabeled' formula neglects the effect both of natural isotopes (e.g., naturally occurring ^{13}C) and of unlabeled nutrient that may contaminate the labeled nutrient stock. Although naturally occurring isotopes will lead to chronic underestimation of fraction unlabeled (i.e., fraction unlabeled will not start at 100%), for N labeling, the effect is consistent throughout a labeling time course and thus does not affect interpretation of kinetics. Impurities in the labeled nutrient can lead to underestimation of the total extent of labeling, but do not affect the labeling rate. For experiments involving carbon labeling and more sophisticated data interpretation, correction for naturally occurring ^{13}C can be valuable (see TROUBLESHOOTING section for formulas).

7| Derive equations according to the relevant pathways (see Quantitative treatment of KFP data in ANTICIPATED RESULTS) to calculate apparent first-order rate constant k_X for metabolite X.

8| Using the apparent first-order rate constants obtained above and the intracellular metabolite concentrations (determined separately), calculate fluxes: flux through metabolite X equals the product of k_X and the intracellular concentration of X, i.e., $f_X = k_X \times X^T$. This concludes the steps of standard kinetic flux profiling. Steps 9–22 encompass differential KFP and are optional.

Perturbation followed by isotope switch (optional)

9| Follow Steps 1A(i–xi). Prepare three types of plates beforehand. (See REAGENT SETUP for details. Note that the composition of the Type C and D plates will need to be modified according to your planned perturbation.)

- Type A, for growing filter cultures
- Type C, for introducing the perturbation
- Type D, for introducing isotope label after the perturbation has been initiated

10| Transfer filter cultures from the Type A plates onto the Type C plates one by one and let grow for 10 min (or desired length of time).

11| Follow Steps 1A(xii) to 6, moving from a Type C plate to a Type D plate in Step 1A(xiii).

Isotope switch followed by perturbation (optional)

12| Follow Steps 1A(i–xi). Prepare three types of plates beforehand (see REAGENT SETUP for details). Again, note that the composition of the Type B and D plates will need to be modified according to your planned perturbation: Type A, for growing filter cultures; Type B, for introducing the isotope label; and Type D, for triggering perturbation after the isotope switch.

13| Transfer filter cultures from Type A plates onto Type B plates.

14| (Optional) Take time points and quench filter cultures as in Step 1A(xiii–xiv). (This step is not essential but offers a check of isotope incorporation before initiating the perturbation and is recommended.)

15| Five minutes (or desired length of time) after the isotope switch, transfer the rest of the filter cultures from Type B plates to Type D plates. Note that at this step it is desirable to achieve labeling of the majority of free metabolites but none or little of the macromolecules before introducing the perturbation. The time provided here is for N-labeling in *E. coli*. You will need to determine appropriate timing for your organism and condition.

16| Quench, extract and analyze, following the instructions for Steps 1A(xiv) to 6.

Effect of perturbation on metabolite concentrations (optional, provides concentration not flux information)

17| Follow Steps 1A(i–xi). Prepare Type A and Type C plate beforehand (see REAGENT SETUP for details). Note that neither of these plates use isotopic label.

18| Transfer filter cultures from the Type A plates onto Type C plates one by one.

19| Quench and extract at various time points after Step 18 as per Step 1A(xiv–xxii). Make sure to cover at least the same length of period as in Steps 9–11 and 12–16.

20| Follow instructions for Steps 2 to 4 to analyze cell extracts.

21| For individual metabolites, quantify peaks using predefined MRM channels and appropriate program (or alternative MS-based approach).

22| Combine the results from Steps 9–11, 12–16 and 17–22 to evaluate flux changes caused by the environmental perturbation. One effective approach for evaluation is via computational modeling and data simulation.

? TROUBLESHOOTING

● **TIMING**

Many of these steps have time periods dependent upon the growth rate of the cell culture. As such, these time periods may vary substantially with the cultures used.

Step 1A for *E. coli*:

Step (i): 15 min + 2 h to cool
Step (ii): overnight, 12–16 h
Steps (iii–iv): ~2 h, depending on the culture growth rate
Step (v): 5 min
Steps (vi–viii): 1 min per filter culture plate
Step (ix): 2–3 h
Step (x): 10–20 min
Step (xi): 10 min
Steps (xii–xiv): 15–75 min, depending on time points chosen
Steps (xv–xxiii): ~1–2 h

Step 1B for human fibroblasts:

Step (i): 15 min
Step (ii): 2–7 d
Step (iii): 10 min
Step (iv): overnight
Step (v): 10 min
Step (vi): 10 min + 60 min incubation
Steps (vii–xi): 15–135 min, depending on the time points chosen

Steps 2–8 (analysis):

Step 2 (optional): 3 h
Steps 3 and 4: 15 min + LC-MS running time
Steps 5 and 6: 10 min per metabolite, desk work (no laboratory component); can be broken into pieces as convenient
Steps 7 and 8: 1–3 d desk work (no laboratory component); can be broken into pieces as convenient

Differential KFP (optional steps):

Step 9: see Steps 1A(i–xi)
Step 10: 2–30 min, depending on the length of perturbation
Step 11: see Steps 1A(xii) to 6
Step 12: see Steps 1A(i–xi)
Steps 13 and 14: 5–30 min, depending on the length of preperturbation isotope switch
Step 15: 2–30 min, depending on the length of perturbation
Step 16: see Steps 1A(xiv) to 6
Step 17: see Steps 1A(i–xi)

Step 18: 2–30 min, depending on the length of perturbation
Step 19: 5–75 min, depending on the time points chosen
Step 20: see Steps 1A(xiv) to 4
Step 21: 10 min per metabolite, desk work (no laboratory component); can be broken into pieces as convenient
Step 22: 1–3+ d desk work (no laboratory component); can be broken into pieces as convenient

? TROUBLESHOOTING

Signal for labeled compounds is smaller than anticipated. Check that MRM scan events are appropriate for labeled forms of interest. A good check is to grow cells for an extended period in the labeled nutrient and make sure that the signal for the labeled forms (in the labeled cells) is similar to the signal for the unlabeled forms (in the unlabeled cells). This also provides a useful check as to whether isotopic purity of the nutrient is acceptable (such purity can also be directly measured by MS). Note that high purity is required to obtain complete labeling of compounds that assimilate the label into many positions (e.g., 98% ^{15}N-ammonia will lead up to 98% full labeling of glutamate, 96% full labeling of glutamine (with most of the residual in the single-labeled state) and less than 13% full labeling of a 100 amino-acid protein).

Isotope-labeled forms of different metabolites share the same nominal mass. Rely on chromoatographic separation. Alternatively, separate based on fragmentation pattern (product ion mass in MRM analysis) or based on exact mass (if using a mass spectrometer with very high resolving power, such as a Fourier transform instrument).

Isotope switch results in marked deviations from metabolic steady state. This is evident as large changes in the sum of all isotopic forms upon isotope switch. The cell handling described herein is specifically designed (and, in our hands, validated) to avoid this problem. Closely adhere to the instructions herein to avoid it. Also, avoid holding the cells in the same unlabeled media for long time periods before the switch (this can lead to accumulation of waste products in the media or depletion of nutrients) and be careful to match media pH, temperature, CO_2 content and so on. Check the adequacy of cell handling via switches from unlabeled to identical unlabeled media.

All compounds in a pathway seem to label at the same rate. This occurs when a step upstream of the pathway is slower than any of the pathway steps (i.e., the turnover of some pool between the added nutrient and the pathway is slower than turnover of any of the pathway pools). This precludes quantitative flux analysis of the pathway. There are three options: (i) accept the lower bound on the pathway flux provided by the observed data, (ii) find a way to circumvent the slow upstream step (in particular, find a way to expedite the isotope switch if it is rate limiting); (iii) apply an alternative flux measurement approach.

Data do not follow an exponential pattern. Make sure that the cells are at metabolic steady state at the time of the isotope switch. If the cells are not at steady state, use differential KFP to understand the ongoing flux changes. Quantitative analysis of differential KFP data does not follow a standard protocol, but an example can be found in Yuan *et al.*[2].

Correction for natural abundance of carbon 13 is required. When drawing biological conclusions based on partial labeling that is far from complete, correction for natural abundance of carbon 13 is required. Such corrections are generally unimportant with N-labeling of *E. coli*. However, we have found that they can be important in interpreting TCA cycle ^{13}C-labeling patterns in mammalian cells. Appropriate corrections (assuming ^{13}C labeling) are as follows:

$$M^{0}_{\text{real}} = \frac{M^{0}_{\text{measured}}}{0.989^{N}}$$

$$M^{+1}_{\text{real}} = \frac{M^{+1}_{\text{measured}}}{0.989^{N-1}} - 0.011N \times M^{0}_{\text{real}}$$

$$M^{+2}_{\text{real}} = \frac{M^{+2}_{\text{measured}}}{0.989^{N-2}} - 0.011^{2} \times N/2(N-1) \times M^{0}_{\text{real}} - 0.011 \times (N-1) \times M^{+1}_{\text{real}}$$

where M^0 is the amount of the monoisotopic compound, M^{+1} is the amount of the compound with one ^{13}C atom, M^{+2} is the amount of the compound with 2 $\times$ ^{13}C atom and so on; N is the number of carbon atoms in the molecule; and 'real' refers to values corrected for coincidental labeling and 'measured' refers to raw values determined by LC-MS/MS. The subtraction operations serve to remove the naturally occurring isotopic envelop of the less-labeled species from the 'real' values of the more extensively labeled species. The division operations serve to add back the isotopic envelope of the indicated species that is otherwise lost as more extensively labeled species due to the natural abundance of ^{13}C.

ANTICIPATED RESULTS

KFP

Superimposed chromatograms of unlabeled and ^{15}N-labeled glutamate from one KFP experiment, demonstrating the replacement of the unlabeled form of glutamate by its labeled counterpart when *E. coli* are switched from unlabeled to isotopically labeled ammonia, are shown in **Figure 4a**. Each chromatogram corresponds to one time point post the isotope switch. Note that glutamate has only one nitrogen atom and therefore only one labeled form. When multiple labeled forms exist for a metabolite

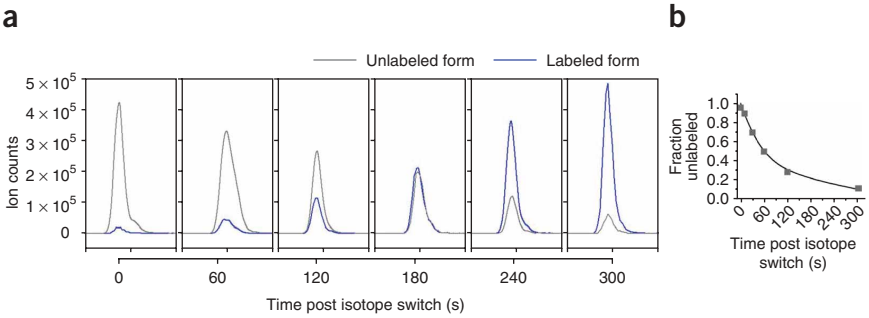

Figure 4 | Sample chromatograms and results for KFP. (**a**) Overlaid chromatograms of unlabeled (gray) and labeled (blue) glutamate after the nitrogen source was switched from $^{14}NH_4Cl$ to $^{15}NH_4Cl$. (**b**) Kinetics of disappearance of unlabeled glutamate after the nitrogen isotope switch. Each data point corresponds to the 'fraction unlabeled' calculated from the chromatograms in panel **a**.

(e.g., metabolites containing multiple nitrogen atoms for nitrogen labeling), all possible isotopically labeled forms should be taken into consideration. From each chromatogram, 'fraction unlabeled' is calculated as described in PROCEDURE and plotted against time in **Figure 4b**. This data can then be used to calculate the apparent first-order rate constant k.

Quantitative treatment of KFP data

The general concepts for treatment and interpretation of KFP data are discussed in the Introduction. Here, we present a more quantitative and complex example of data analysis. Imagine a pathway as shown in **Figure 5**: metabolite X is produced both from nutrient (by *de novo* synthesis) and from degradation of some macromolecules (e.g., protein, nucleic acids, glycogen and so on), and the two influxes are f_1 and f_2, respectively. Similarly, Y is down stream of X in the pathway and can also be produced directly from macromolecules; the fluxes are f_4 and f_5, respectively. Additionally, the fluxes directing X and Y out of the pathway (for biomass production and so on) are f_3 and f_6, respectively, as shown. Under steady state, the following conditions exist (f_X and f_Y denote total fluxes through the pools of X and Y, respectively, superscript U stands for 'unlabeled', * for 'labeled' and T for 'total'):

$$f_1 + f_2 = f_3 + f_4 = f_X,\ X^U + X^* = X^T;$$
$$f_4 + f_5 = f_6 = f_Y,\ Y^U + Y^* = Y^T$$

Decreasing unlabeled X and Y after switching the nutrient from natural to isotopically labeled can be described by the following differential equations (which assume that the nutrient switch is so fast as to be effectively instantaneous relative to the labeling times of X and Y):

$$dX^U/dt = f_2 - f_X(X^U/X^T) \quad (1)$$

$$dY^U/dt = f_4(X^U/X^T) + f_5 - f_Y(Y^U/Y^T) \quad (2)$$

The apparent first-order rate constant (k) for X and Y are defined as

$$k_X = f_X/X^T, \text{ and } k_Y = f_Y/Y^T$$

and for simplified results, set

$$f_2/f_X = \alpha, \text{ and } f_5/f_Y = \beta$$

The analytical solution to equations (1) and (2) is

$$X^U/X^T = (1-\alpha)\exp(-k_X t) + \alpha \quad (3)$$

$$Y^U/Y^T = [(1-\alpha)(1-\beta)/(k_X - k_Y)][k_X \exp(-k_Y t) - k_Y \exp(-k_X t)] + [1-(1-\alpha)(1-\beta)] \quad (4)$$

Therefore, by measuring fraction unlabeled (X^U/X^T and Y^U/Y^T) versus time, the apparent first-order rate constants (k_X and k_Y) can be calculated. Combined with the concentrations (X^T and Y^T), the fluxes f_X and f_Y through X and Y can be obtained. For cases in which $X^T \gg Y^T$ and $f_X \approx f_Y$, this implies $k_X \ll k_Y$, and therefore equation (4) reduces to equation (3) and k_Y cannot be determined. In this situation, one can only conclude that $f_Y \gg Y^T \times k_X$.

The biexponential nature of equation (4) arises from passage of label into Y being delayed by labeling of X. For metabolites further downstream from the added nutrient, full mathematical treatment is yet more complex and often best handled by computer simulation. Alternatively, one can apply a variant of equation (4) that is more broadly applicable.

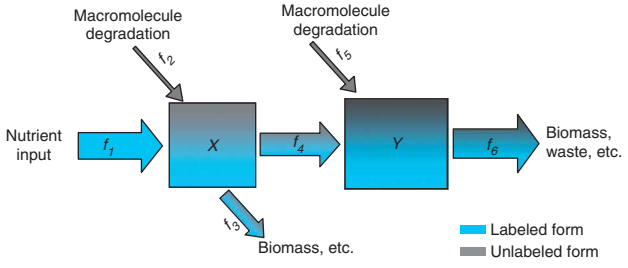

Figure 5 | Example pathway for derivation of equations and quantitative treatment of KFP data. A metabolic pathway consists of metabolites X and Y. X is made directly from nutrient (f_1) and from the degradation of macromolecules (proteins, nucleic acids and so on) (f_2). In addition to producing Y (f_4), X is also used for biomass production (f_3). Y can also be generated from macromolecules (f_5). As only metabolites X and Y are considered in this case, all effluxes from Y are combined regardless of their specific destinations for simplicity (f_6).

To understand this variant, consider metabolite *W*, downstream of metabolite *V* (where *V* is not directly downstream of the labeled nutrient). Assume that, consistent with most data that we observe in the lab, the unlabeled form of *V* shows approximately single exponential decay. Passage of label into *W* will be delayed by *V*, just as passage of label into *Y* is delayed by *X*. However, equation (3) (which assumes a direct connection to labeled nutrient) will not apply to *V*. Fit the data for *V* instead to a variant of equation (3), except replacing k_X with k'_V (equation (5)). k'_V is not designed to give the flux through *V*, but to lay the groundwork for measuring flux through *W*. Knowledge of k'_V allows one to fit the data for *W* to equation (6). This equation is equivalent to equation (4), with k_X replaced by k'_V and k_Y by k_W. The parameter k_W gives flux through *W* from $f_W = k_W \times W^T$. This approach can be applied repeatedly. It is important, however, to always solve for k' for the precursor based on a single exponential approximation (equation (5)) and k based on equation (6) (i.e., to determine flux through *Z*, a product of *W*, one needs to calculate k'_W and k_Z, use of k_W is not correct).

$$V^U/V^T = (1-\alpha)\exp(-k'_V t) + \alpha \quad (5)$$

$$W^U/W^T = [(1-\alpha)(1-\beta)/(k'_V - k_W)]\,[k'_V \exp(-k_W t) - k_W \exp(-k'_V t)] + [1-(1-\alpha)(1-\beta)] \quad (6)$$

Example of nitrogen assimilation pathway in *E. coli*

To demonstrate the quantitative application of KFP to a real set of metabolic pathways, we use here the nitrogen assimilation system of *E. coli*. Before embarking on KFP analysis *per se*, we provide a small amount of background that is essential to understanding the network: The central pathways of nitrogen assimilation in *E. coli*, as well as selected effluxes to amino acid and nucleotide biosynthesis, are shown in **Figure 6**. Ammonia can be directly assimilated into glutamate via the enzyme glutamate dehydrogenase (GDH). Alternatively, ammonia can condense with glutamate to form glutamine, catalyzed by the ATP-consuming enzyme glutamine synthetase (GS). Glutamine then yields two molecules of glutamate via glutamate synthase (commonly referred to as GOGAT, which stands for glutamine amide 2-oxoglutarate aminotransferase)[8,40]. The GS-GOGAT cycle has the same net effect as GDH, but differs in (i) burning ATP and (ii) having a lower K_m (i.e., higher affinity) for ammonia[8]. Glutamate is the major nitrogen distributor in the cell, feeding most of the amino-acid biosynthesis via transamination[41]. Glutamine feeds selected other pathways[41]. **Figure 6** shows one example of a typical transamination reaction (glutamate + α-keto-isovalerate → α-ketoglutarate + valine), and one example of a typical glutamine-consuming pathway (pyrimidine biosynthesis; glutamine enters at the first step, formation of carbamoyl phosphate).

Switching of exponentially growing *E. coli* into ^{15}N-ammonia followed by kinetic sampling and LC-MS/MS analysis (as per Steps 1 option A to 8) yields data adequate to deduce most of the fluxes in **Figure 6**, despite the presence of cycles, branch points and compounds that cannot be directly measured by LC-MS/MS due to low abundance.

Before analyzing the KFP data itself, it is necessary to determine the turnover time of ammonia following the isotope switch. This was approximated by directly measuring the time for ammonia to diffuse onto an empty filter (**Fig. 7a**) and fitting the data to a single exponential saturation-binding equation, that is, $A(t) = A_{max}(1 - \exp(-k't))$ (the fit gives $k' = 7.5\ min^{-1}$).

The observed value of k' for ammonia was used in equation (6) to calculate flux through glutamine based on the data in **Figure 7a**. This reflected a conscious decision to treat single-labeled glutamine (the first form that appeared after the isotope switch) as the product of unlabeled glutamate and labeled ammonia. This was based on the empirical observation that glutamine labeled much faster than glutamate ($t_{1/2}$ approximately 10 and 60 s, respectively) and the MS/MS-based determination that the single-labeled form of glutamine contained ^{15}N predominantly in the amide position. The resulting k for glutamine was 14.3 min^{-1} and multiplication by the pool size of 3.9 µmol g_{CDW}^{-1} gave a flux of 3.4 mmol $(g_{CDW}\ h)^{-1}$.

Flux through glutamate was calculated using equation (6), with k' based on either ammonia or glutamine amide labeling (k' =7.5 min^{-1} and 3.6 min^{-1}, respectively). Both approaches yield similar $k_{glutamate}$ (1 min^{-1} and 1.2 min^{-1}, respectively) because $k' \gg k$ in either case. As mentioned in the previous section, when $k' \gg k$, equation (6) reduces to equation (3),

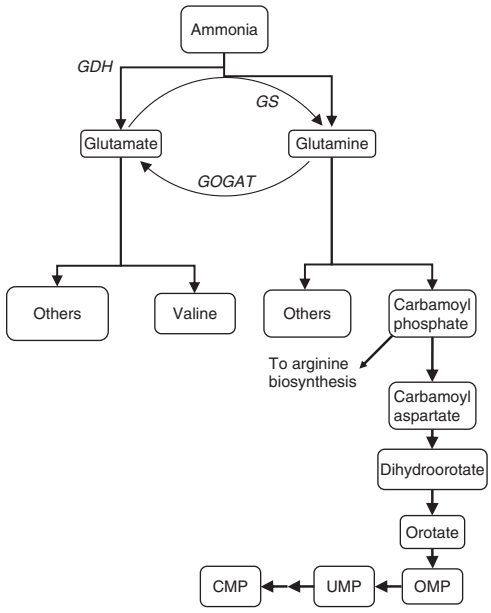

Figure 6 | Central nitrogen-assimilation pathways and selected nitrogen-consuming pathways in *E. coli*. Italics show the abbreviated names of the central nitrogen-assimilation enzymes glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT). Only transfer of nitrogen is shown; carbon skeletons and energy molecules are omitted. Note that GOGAT generates two molecules of glutamate from one molecule of glutamine.

and fitting glutamate data to equation (3) gives $k_{glutamate}$=0.8 min^{-1}, which is close to the k calculated from equation (6) given fitting error. With a pool size of 101 µmol g_{CDW}^{-1}, the fluxes for glutamate calculated from the three $k_{glutamate}$ values are 6, 7.2 and 4.8 mmol (g_{CDW} h)$^{-1}$, respectively.

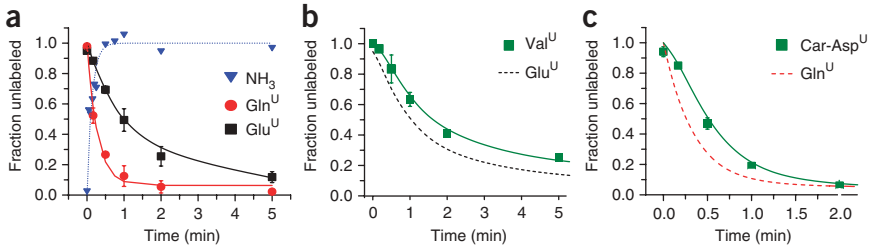

Figure 7 | Selected nitrogen KFP results for *E. coli*. (**a**) Ammonia diffusion and decay of the unlabeled signal for glutamine and glutamate following isotope switch into $^{15}NH_3$. (**b**) Decay of the unlabeled valine signal. (**c**) Decay of the unlabeled carbamoyl aspartate signal. All reprinted with permission from Yuan *et al.*[1].

At steady state, the total consumption and production fluxes of each metabolite must be equal. The total glutamate flux, measured to be 6 mmol (g_{CDW} h)$^{-1}$, therefore equals the sum of its two consumption fluxes: glutamine synthetase flux, measured to be 3.4 mmol (g_{CDW} h)$^{-1}$, and the flux of glutamate to biomass, which by subtraction must be 2.6 mmol (g_{CDW} h)$^{-1}$. The flux of glutamine to biomass is known (based on overall metabolic stoichiometry and the composition of *E. coli*) to be ~15% of the glutamate flux to biomass. Thus, it must be ~ 0.4 mmol (g_{CDW} h)$^{-1}$. The total glutamine influx of 3.4 mmol (g_{CDW} h)$^{-1}$ equals the sum of its efflux to biomass of 0.4 mmol (g_{CDW} h)$^{-1}$ and to glutamate via GOGAT, which by subtraction must be 3 mmol (g_{CDW} h)$^{-1}$. GOGAT flux from glutamine of 3 mmol (g_{CDW} h)$^{-1}$ yields 6 mmol (g_{CDW} h)$^{-1}$ of glutamate, which is the measured glutamate influx. Thus, KFP was adequate to determine that GOGAT is the major source of glutamate, despite the complexities introduced by metabolic cycling.

Flux through valine can be calculated by straightforward application of equation (5) and equation (6) to the data in **Figure 7a** and **b** (glutamate is the sole nitrogen parent of valine, and k' is accordingly based on a fit of equation (5) to observed results for glutamate; $k' = 0.8$ min^{-1}, $k_{valine} = 3.9$ min^{-1}, pool size = 2.4 µmol g_{CDW}^{-1}, flux =0.6 mmol (g_{CDW} h)$^{-1}$). Note that transamination reactions are rapidly reversible and the valine flux determined by KFP is the gross, rather than net, flux.

Calculation of pyrimidine pathway flux is complicated by the many steps involved and the inability to measure some components. It is facilitated, however, by flux into carbamoyl aspartate being essentially irreversible and by the requirement for flux at steady state to be equal for all steps of the linear portion of the pathway linking carbamoyl aspartate and UMP. Carbamoyl aspartate labels quickly after ammonia isotope switching, with the label first appearing in the carbamoyl nitrogen. Carbamoyl aspartate flux would typically be calculated using k' based on carbamoyl phosphate. The level of carbamoyl phosphate is, however, generally too low for us to detect it in *E. coli*. As we have validated the method for carbamoyl phosphate, with a detection limit of 100 ng ml^{-1}, this implies that the cellular carbamoyl phosphate concentration is very low. Therefore, the small cellular carbamoyl phosphate pool can only slightly delay the passage of nitrogen from glutamine to carbamoyl aspartate. Accordingly, pathway flux (from carbamoyl aspartate to UMP) was calculated by equation (6), with k' based on fit of equation (5) to experimental results for glutamine and fitting to the carbamoyl aspartate data in **Figure 7c** ($k' = 3.6$ min^{-1}, $k_{carbamoyl\ aspartate} = 4.1$ min^{-1}, pool size = 0.8 µmol (g_{CDW})$^{-1}$, flux =0.2 mmol (g_{CDW} h)$^{-1}$). This yields net pathway flux, as the step being monitored is essentially irreversible. Extension of quantitative analysis to CMP was not feasible, however, as data on the parent of CMP (with RNA likely one major source) was not available.

Differential KFP

Figure 8 shows a 'fraction unlabeled' graph generated in the same fashion as **Figure 4b**, from chromatograms of proline in a differential KFP experiment. Data corresponding to Steps 9–11 are shown in red and those corresponding to Steps 12–16 are shown in blue. The two data sets are aligned according to the time of isotope switch. The black arrows indicate the time of the perturbation event for each set. Note that both curves had positive slope for $t > 10$ min, indicating that the unlabeled fraction is increasing even though only labeled nutrient is being provided. As proline (and most other metabolites) can be produced via both *de novo* synthesis (which will generate labeled proline) and protein degradation (which will produce unlabeled proline), this behavior suggests that the fraction of

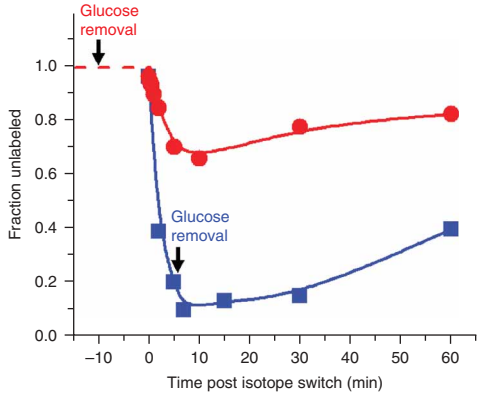

Figure 8 | Sample result for differential KFP. The red trace represents the kinetics of disappearance of unlabeled proline when carbon source was removed 10 min before the nitrogen isotope switch. The blue trace represents the kinetics of disappearance of unlabeled proline when carbon source was removed 5 min after the nitrogen isotope switch. The two traces were aligned by the time of isotope switch, with the time corresponding to glucose removal indicated by black arrows.

proline production from protein degradation is increasing during carbon starvation. The fact that the two curves did not converge even at about 1 h postperturbation and isotope switch implies that the flux through proline pool is small. More quantitative information and rough estimation of flux changes can be obtained when combining these pieces of kinetic data with the concentration changes obtained via Steps 17–22. For an example of detailed data treatment and analysis in this fashion, see Yuan *et al.*[2].

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