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Extending the Range of SLIM-Labeling Applications: From Human Cell Lines in Culture to *Caenorhabditis elegans* Whole-Organism Labeling

Laurent Lignieres,[¶] Nicolas Sénécaut,[¶] Tien Dang, Laura Bellutti, Marion Hamon, Samuel Terrier, Véronique Legros, Guillaume Chevreux, Gaëlle Lelandais, René-Marc Mège, Julien Dumont, and Jean-Michel Camadro*



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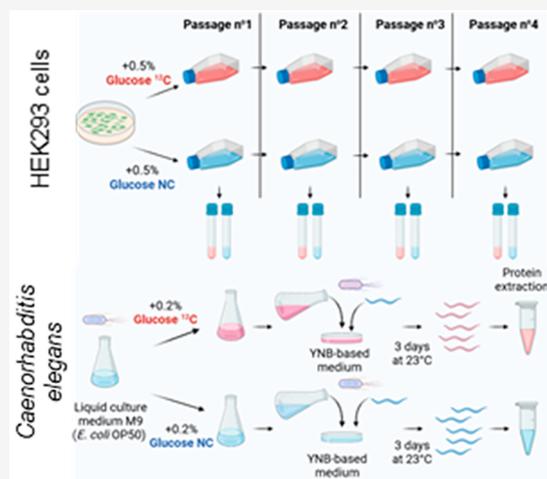
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ABSTRACT: The simple light isotope metabolic-labeling technique relies on the *in vivo* biosynthesis of amino acids from U-¹²C-labeled molecules provided as the sole carbon source. The incorporation of the resulting U-¹²C-amino acids into proteins presents several key advantages for mass-spectrometry-based proteomics analysis, as it results in more intense monoisotopic ions, with a better signal-to-noise ratio in bottom-up analysis. In our initial studies, we developed the simple light isotope metabolic (SLIM)-labeling strategy using prototrophic eukaryotic microorganisms, the yeasts *Candida albicans* and *Saccharomyces cerevisiae*, as well as strains with genetic markers that lead to amino-acid auxotrophy. To extend the range of SLIM-labeling applications, we evaluated (i) the incorporation of U-¹²C]-glucose into proteins of human cells grown in a complex RPMI-based medium containing the labeled molecule, considering that human cell lines require a large number of essential amino-acids to support their growth, and (ii) an indirect labeling strategy in which the nematode *Caenorhabditis elegans* grown on plates was fed U-¹²C]-labeled bacteria (*Escherichia coli*) and the worm proteome analyzed for ¹²C incorporation into proteins. In both cases, we were able to demonstrate efficient incorporation of ¹²C into the newly synthesized proteins, opening the way for original approaches in quantitative proteomics.

KEYWORDS: simple light isotope metabolic labeling, mass spectrometry, bottom-up proteomics, human cell line, *Caenorhabditis elegans*, essential amino acids, auxin



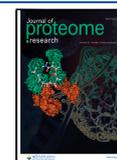
INTRODUCTION

The simple light isotope metabolic (SLIM)-labeling strategy¹ is an original method to address the dynamics of proteome variations *in vivo* using mass spectrometry-based technologies. The method relies on the ability of cells to synthesize amino acids through the catabolism of carbon sources, such as glucose, the almost universal fuel in cellular bioenergetics. Our approach has been, therefore, to provide U-¹²C]-glucose to prototrophic cells, for which we demonstrated that all amino acids produced *in vivo* show homogeneous U-¹²C] isotopic composition of the carbon atoms. This has important consequences for mass spectrometry (MS)-based proteomics, as the resulting isotope cluster of the labeled peptides exhibits a strong increase in monoisotopic ion intensity, with better signal-to-noise ratio, making it easier to identify the corresponding peptide. We further applied this labeling method to quantify differences in peptide abundance in experiments in which we mixed equal amounts of total

peptides from a control condition (cells grown on glucose of normal carbon composition, NC condition) and from a ¹²C condition (cells grown in the presence of U-¹²C]-glucose as the sole carbon source). Precise analysis of the intensities in the isotope cluster showed a strict correlation with the amount of ¹²C incorporated into the peptides from the ¹²C condition. This allows determination of the abundance relative to the NC condition. The SLIM-L method was developed using the prototrophic yeast *Candida albicans*,¹ where it allowed to determine the half-life of the proteins at the whole-proteome scale, in a control condition and in the presence of MG132, a

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potent inhibitor of the ubiquitin-proteasome protein degradation machinery,² or in the presence of phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine-proteases. In these experiments, all the amino acids required for protein synthesis can be produced by the catabolism of U-¹²C]-glucose. It was further extended to *Saccharomyces cerevisiae* cells with a limited number of mutations used as genetic markers, making the cells auxotrophic for certain amino acids,³ therefore introducing the notion of essential amino acids. These amino acids must be supplied in the growth medium of the cells in a natural, nonlabeled form. We developed open-source data processing workflows that efficiently take into account such auxotrophy to make the method broadly available to the scientific community.⁴ It was, however, necessary to be able to extend the range of applications of SLIM-L. We, thus, evaluated the method on a human cell line in culture (HEK293) and through an indirect labeling strategy on the multicellular nematode *Caenorhabditis elegans*.

MATERIALS AND METHODS

Growth of HEK293 Cells and Labeling Strategy

HEK293 cells were grown to confluence (7×10^4 cells/cm²) in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco ref 31966-021), 10% fetal calf serum (FCS, VWR ref S1810-500), and 100 U/mL penicillin/100 µg/mL streptomycin (100× stock solution Gibco ref 15140122). The cells were washed with phosphate-buffered saline (PBS), trypsinized using 0.05% trypsin-ethylenediaminetetraacetic acid 1× solution (Gibco ref 25300-054), and 1×10^4 cells/cm² were used to inoculate fresh T-75 flasks containing 14 mL of the labeling medium. The labeling medium consisted of modified RPMI 1640 medium without L-glutamine, amino acids, or glucose (MyBioSource, ref MBS652918), 5% FCS, L-glutamine (Gibco ref 25030149, 200 mM diluted 1/100), essential amino-acid cocktail (MEM Amino Acids Solution, ref 11130, ThermoFisher Scientific, 50×, diluted 1/30), 100 U/mL penicillin/100 µg/mL streptomycin, and either 0.5% (w/v) regular glucose (Sigma-Aldrich ref G7021-100G) (NC-medium; NC-condition) or U-¹²C]-glucose (Cambridge Isotope Laboratories ref CLM-4819-1) (12C-medium; 12C-condition). Cells were incubated in a humidified atmosphere at 37 °C with 5% CO₂ in air.

Cells were first grown in the NC-condition up to 90% confluence (3.5×10^4 cells/cm²). Cells were washed with the same medium, scraped, collected by centrifugation at 1000 r.c.f, and washed again with sterile PBS. They were separated into two batches, one used to inoculate a flask with NC-medium and the other a flask with 12C-medium at a cell density of 1×10^4 cells/cm². The passages were repeated 4 to 6 times. The main portion of the cells collected after every passage (2.5×10^6 cells) was used for proteomics studies. After two washes with cold PBS, cells were centrifuged at 1000 r.c.f. Pellets were resuspended in RIPA buffer (ref 89900, ThermoFisher scientific, Waltham, MA, USA) containing a protease inhibitor cocktail (cOmplete Protease Inhibitor Cocktail, Roche, Basel, Switzerland) following the manufacturer's protocol.

Growth of *Caenorhabditis elegans* and Labeling Strategy

The *C. elegans* N2 Bristol strain (wild-type background) is classically grown on Petri dishes prepared with nematode growth medium (NGM) agar overlaid with a fresh culture of *Escherichia coli* cells, as described in (http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html).

To develop our labeling strategy, the *E. coli* uracil auxotroph strain OP-50⁵ was grown in liquid culture on a synthetic minimal M9 medium⁶ containing either 0.2% regular glucose or 0.2% U-¹²C]-glucose as the sole carbon source. After overnight growth, the bacteria were plated on NGM Petri dishes, which were allowed to dry before being stored at 4 °C.

In the first series of experiments, 10 adult worms were placed on NC or 12C agar plates seeded with either control or ¹²C-labeled *E. coli* as the nutrient, respectively. Following the production of fertilized eggs, the adult worms were removed and the embryos grown for 3 days at 23 °C until they became adults. In total, 100 worms per dish were collected for proteomics experiments.

In the second series of experiments, a similar protocol was used but auxin (4 or 10 mM, final concentration) was included in the NGM to inhibit residual bacterial growth; worms at the L4 larval stage were transferred to fresh plates containing 4 mM auxin at a higher density (200–400 worms per plate).

Finally, we developed a new synthetic solid medium, to avoid any metabolic interference of the medium with either *E. coli* or worm development, consisting of (quantities for 1 L of medium): 3 g of NaCl, 1 g of yeast nitrogen base (Difco, with ammonium sulfate, without amino acids), and 17 g of agar. After autoclaving, we added the following amounts of sterile solutions to the medium: 1 mL of cholesterol (5 mg/mL in EtOH); 1 mL of CaCl₂ (1M); 1 mL of MgSO₄ (1M), 25 mL of KH₂PO₄ pH 6, and 2 g of glucose (0.2 g/100 mL). The worms were grown as described in the second set of experiments.

For all experiments, cell-free extracts were prepared in lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.4, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM MgCl₂, 100 mM KCl, 10% glycerol, 0.05% NP40) using either a FastPrep bead-beating system (MP Biochemicals) or manual Potter homogenization or by extraction in Laemmli buffer. For each sample, 100 adult worms were washed in M9 buffer (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, and 1 mM MgSO₄·7H₂O) supplemented with 0.1% Triton X-100. Samples were then resuspended in 15 µL of Laemmli buffer (1× final), heated at 98 °C for 15 min, vortexed at 4 °C for 15 min, and heated again at 98 °C for 15 min.

Growth of *Escherichia coli* and Labeling Strategy

E. coli wild-type strain K12 substr. MG1655⁷ was grown in liquid culture on synthetic M9 minimal medium⁶ containing either 0.5% regular glucose or 0.5% U-¹²C]-glucose as the sole carbon source. After overnight growth at 37 °C on a rotating shaker (300 rpm), cells were washed two times with cold PBS. After centrifugation at 1000 r.c.f, cells were resuspended in a 25 mM NH₄HCO₃ buffer containing a protease inhibitor cocktail (cOmplete Protease Inhibitor Cocktail, Roche, Basel, Switzerland), and lysed using a French press (One Shot instrument, CellD Sarl, Nîmes, France).

Sample Preparation Prior to LC-MS/MS Analysis

C. elegans cell lysates were loaded onto precast NuPage Bis-Tris (4–12% poly acrylamide, ThermoFisher Scientific) sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, migrated no more than 5 mm, and stained with InstantBlue staining solution (Sigma-Aldrich ref ISBL1). Gel plugs were destained using a solution of 50 mM ACN/NH₄HCO₃ (50/50) for 15 min with agitation. Plugs were

reduced with a solution of 10 mM DL-dithiothreitol (DTT, Sigma-Aldrich, Saint Louis, MO, USA) for 30 min at 56 °C and then alkylated using a 20 mM solution of iodoacetamide (IAA, Sigma-Aldrich, Saint Louis, MO, USA) for 30 min at ambient temperature in the dark. After a washing step, the plugs were dehydrated for further trypsin treatment.

Proteins (10 µg protein extract) from HEK293 cell lysates were precipitated with six volumes of cold acetone (−20 °C). Samples were maintained overnight at −20 °C and then centrifuged for 10 min at 13,000 r.c.f., 4 °C. The protein pellets were dissolved in 8 M urea prepared in 25 mM NH₄HCO₃ buffer and reduced with a solution of 10 mM DTT for 30 min at 37 °C. The reduced protein samples were then alkylated using a 20 mM IAA solution for 30 min at ambient temperature in the dark. Then, the samples were diluted in 25 mM NH₄HCO₃ buffer to obtain a final urea concentration below 1M.

In both cases, the samples were digested overnight at 37 °C with sequencing grade trypsin (Promega, Madison, WI, USA; ratio Enzyme:Sample 1:20). The digested peptides were loaded and desalted either on Evotips, provided by Evosep (Odense, Denmark), or using ZipTip µ-C18 pipette tips from Pierce Biotechnology (Rockford, IL, USA), according to the manufacturers' procedures.

Proteomics Analysis: Liquid Chromatography Coupled to Mass Spectrometry Data Acquisition

Samples were analyzed using an Orbitrap Fusion Tribrid mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) coupled either to an Evosep One system (Evosep, Odense, Denmark) or a Proxeon 1200 system from ThermoFisher Scientific (Waltham, MA, USA). The Evosep One system was operated using the 30SPD method developed by the manufacturer. Briefly, the method is based on a 44 min gradient and a total cycle time of 48 min with a C18 analytical column (0.15 × 150 mm, 1.9 µm beads, ref EV-1106) equilibrated at room temperature and operated at a flow rate of 500 nL/min. H₂O/0.1% FA was used as solvent A and ACN/0.1% formic acid (FA) as solvent B. On the Proxeon 1200, peptides were separated by chromatography using the following settings: Acclaim PepMap100 C18 precolumn (0.075 × 20 mm, 3 µm, 100 Å), Pepmap-RSLC Proxeon C18 column (0.075 × 750 mm, 2 µm, 100 Å), 300 nL/min flow rate, a 120 min gradient from 95% solvent A (H₂O/0.1% FA) to 40% solvent B (100% ACN/0.1% FA), followed by column regeneration, giving a total acquisition time of 140 min. The mass spectrometer was operated in the data-dependent MS/MS mode. Peptide masses were analyzed in the Orbitrap cell in full ion scan mode with a resolution of 120,000, a mass range of *m/z* 350–1550, and an AGC target of 4 × 10⁵. MS/MS analyses were performed in the top speed 3s mode. Peptides were selected for fragmentation by higher-energy C-trap dissociation (HCD) with a normalized collisional energy of 27% and a dynamic exclusion of 60 s. Fragment masses were measured in an ion trap in the rapid mode, with an AGC target of 1 × 10⁴. Monocharged peptides and those with unassigned charge states were excluded from the MS/MS acquisition. The maximum ion accumulation times were set to 100 ms for MS and 35 ms for MS/MS acquisitions.

Certain samples were also analyzed using a Q-Exactive Plus Orbitrap mass spectrometer coupled to a Nano-LC Proxeon 1000, both from ThermoFisher Scientific (Waltham, MA,

USA). Peptides were separated by chromatography as described above when using the Proxeon 1200 settings. Peptides were analyzed in the Orbitrap cell in positive mode at a resolution of 70,000, with a mass range of *m/z* 200–2000 and an AGC target of 3 × 10⁶. MS/MS data were acquired in the Orbitrap cell in the Top20 mode. Peptides were selected for fragmentation by HCD with a normalized collisional energy of 27%, dynamic exclusion of 60 s, a quadrupole isolation window of 1.4 Da, and an AGC target of 2 × 10⁵. Peptides with unassigned charge states or those that were monocharged were excluded from the MS/MS acquisition. The maximum ion accumulation times were set to 50 ms for MS acquisition and 45 ms for MS/MS acquisition.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository⁸ with the data set identifier PXD28880.

Data Analysis

All MS/MS data were processed using the SequestHT (v1.1.0.189) node of Proteome Discoverer 2.4. MS/MS spectra were searched against the UniprotKB protein database of *C. elegans* (UniprotKB, release 2020_02, 26956 entries), *Homo sapiens* (UP000005640, release 2021_02, 20380 entries), or *E. coli* (UniprotKB, Strain K12 release 2019_01, 4391 entries). A maximum of two missed cleavages were authorized and precursor and fragment mass tolerances were either set to 15 ppm and 0.5 Da for the Orbitrap Fusion or 10 ppm and 0.02 Da for the Orbitrap Q-exactive Plus. The following post-translational modifications were included: carbamidomethyl (C) as fixed and oxidation (M) and deamidation (NQ) as variable. Peptide spectrum matches (PSMs) were filtered at a 1% false discovery rate. Contaminants brought by the growth medium in the HEK123 cell cultures were searched using a dedicated protein sequence database.⁹

We measured ¹²C incorporation into the identified peptides using our recently described workflow,³ in which the intensity of every isotopologue in the isotopic clusters is extracted from the .pdresults SQL database containing the data from the Minora node of Proteome Discoverer 2.4 software.⁴ For each peptide identified, the sequence was decomposed into the essential amino acids (not labeled) and the nonessential amino acids (labeled), and we extracted their elemental composition. The number of carbon atoms was split into those originating from essential amino acids, with a probability of presence of ¹²C set to 0.9893, and those originating from the nonessential amino acids with a probability of presence of ¹²C set to 1. Taking into account the experimental intensity of the monoisotopic ion (M₀) and the +1 isotopologue (M₁), the composition of each peptide and the associated probabilities of occurrence their stable isotopes, it is possible to compute the corresponding ¹²C enrichment,³ expressed as the probability of presence of the ¹²C element. The workflows implemented in the KNIME environment¹⁰ are available on the Zenodo web server (<https://zenodo.org>) under the DOI: 105281/zenodo.4467828.

The boxplots to visualize the level of ¹²C incorporation for each experiment were generated using R (version 4.1.2, 2021-11-01) with the following packages: ggplot2 (version 3.3.6), dplyr (version 1.0.9), and tidyverse (version 1.3.2).

RESULTS AND DISCUSSION

We designed a growth medium of controlled composition to evaluate the efficiency of the SLIM-labeling strategy in the HEK293 human cell line in culture. The medium contains a lower than usual concentration of FCS (5% instead of 10%) in RPMI and was manually supplemented with the essential amino acids (E-AAs), glutamine, and glucose to force the cells to synthesize the nonessential amino acids from this carbon source.

Human cells require several E-AAs for growth. The definition of essential and dispensable amino acids is an ongoing subject of debate¹¹ and Reeds¹² stated “From a strictly metabolic perspective, there are only three indispensable amino acids (lysine, threonine, and tryptophan) and two dispensable amino acids (glutamate and serine)”. However, there is a generally accepted broader view of the essential nature of amino acids, in particular when in the context of cell lines in culture. It is often stated that these amino acids are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, whereas arginine, glycine, and proline are considered to be precursors of conditionally essential amino acids and cysteine and tyrosine are metabolized from essential amino acids.¹² Cells are able to synthesize the nonessential amino acids (NE-AAs) alanine, aspartic acid, asparagine, glutamic acid, glycine, proline, and serine. Glutamine, often considered as a NE-AA, is, however, usually added to the growth media as a source of nitrogen. We used a commercially available essential amino acid solution that contained all amino acids except glycine, alanine, asparagine, serine, proline, glutamine, aspartic acid, and glutamic acid, but glutamine was also added to the medium as a source of nitrogen.

Our modified medium did not alter the basic properties of the HEK293 cells in terms of cell size, morphology, growth rate, or cellular yield (Supplementary Figure S1 and data not shown). The total protein content was similar whether the cells were grown in normal glucose or U-¹²C]-glucose (Supplementary Figure S2).

The analysis of ¹²C incorporation into the proteins showed that the labeling could be measured from the first passage and that it remained at a similar level throughout successive passages (Figure 1).

This result indicates that the neo-synthesis of amino acids from exogenous glucose is efficient and reaches its maximum level from the first passage, i.e., from the first 3 days of growth. Furthermore, the labeling reached during the first passage remained relatively constant over successive passages at the individual peptide level (data not shown).

Our experimental results show large variations in the amount of incorporated ¹²C throughout the entire proteome. This can be explained by the fact that the amino-acid composition of the tryptic peptides within every protein sequence varies widely and, therefore, the ratio of NE-AAs (neosynthesized from glucose) to E-AAs also varies accordingly. We evaluated the observed variability by computing the amino-acid composition of each protein from its sequence using an automated procedure, taking into account the composition of the growth medium we used, with the E-AAs arginine, cysteine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, and asparagine, whereas the NE-AAs were glycine, alanine, serine, proline, glutamine, lysine, aspartic acid, and glutamic acid. Based on the numbering of the

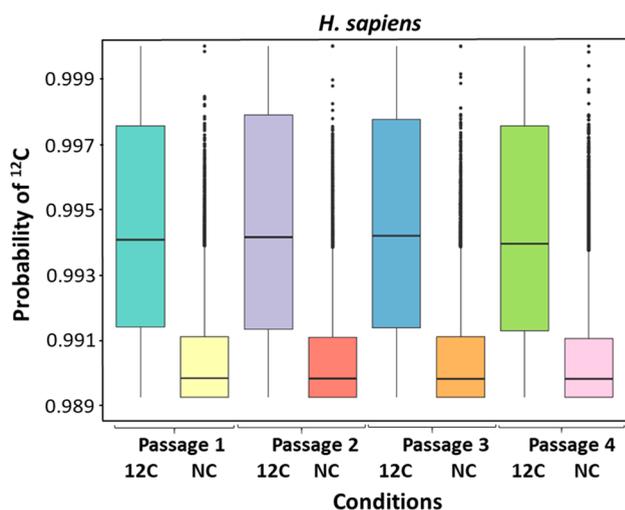


Figure 1. Measurement of the efficiency of ¹²C incorporation into tryptic peptides from HEK293 cells over four passages on the modified RPMI medium with normal glucose (NC, P(¹²C) = 0.9893) or U-¹²C]-glucose (12C, P(¹²C) = 1).

amino acids from the sequences of the human reference proteome, there was almost the same number of E-AAs (13,816,788) and NE-AAs (11,280,854) (Supplementary Figure S3). The ratio of E-AAs to NE-AAs was 1.225. However, there was a bias in the amount of carbon in the E-AAs relative to NE-AAs after taking into account the elemental composition of the amino acids (Supplementary Figure S4), with a mean ratio of 1.90, instead, and a strong bias toward E-AA composition for certain proteins (Supplementary Figure S5). This indicates that the number of labeled carbon atoms must be lower than the number of nonlabeled carbon atoms. We wondered whether it may be necessary to further weight these values by estimating the actual amino-acid requirement to sustain the theoretical total synthesis of the proteome. Using data retrieved from the PAXdb protein abundance database^{13,14} (<https://pax-db.org/>), we computed the number of carbons for the 19,339 proteins representing the 90% most abundant proteins, of which the copy number per cell (expressed as ppm) varied from 37,104 ppm for P62328 (thymosin beta-4) to 0.003 ppm for Q2M2E5 (C5orf64). The ratio of E-AAs to NE-AAs remained close to two (1.96), indicating that the parameter of protein abundance did not affect our measurement of ¹²C incorporation into the human proteome.

The nematode *C. elegans* has been reported to have similar amino-acid requirements as human cell lines,¹⁵ except for arginine, which is nonconditional and therefore essential in *C. elegans*, and cysteine, which is nonessential in *C. elegans*. Feeding the worms with bacteria fulfills all of their nutritional requirements without adding individual constituents to the growth medium. This prompted us to evaluate an indirect SLIM-labeling strategy in which the worms were fed ¹²C labeled *E. coli* and ¹²C incorporation into the worm proteins, resulting from digestion of the bacterial proteins, measured.

The first experiments on *C. elegans* were disappointing, as we observed only weak incorporation of ¹²C in the 12C condition (Figure 2A). Control experiments in which *E. coli* was grown in a U-¹²C]-glucose-containing synthetic medium showed the bacterial proteins to be fully labeled, with complete incorporation of ¹²C (Figure 2B).

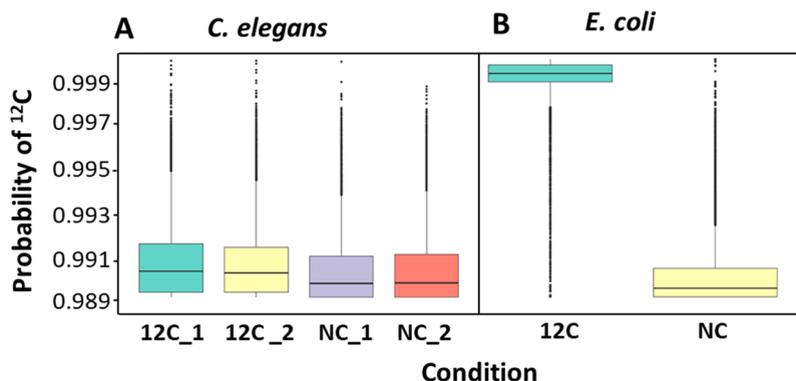


Figure 2. (A) Incorporation of ^{12}C into *C. elegans* grown on the standard NGM medium and fed ^{12}C *E. coli*. (B) Incorporation of ^{12}C into *E. coli* grown on a synthetic medium containing glucose (NC or 12C conditions) as the sole carbon source.

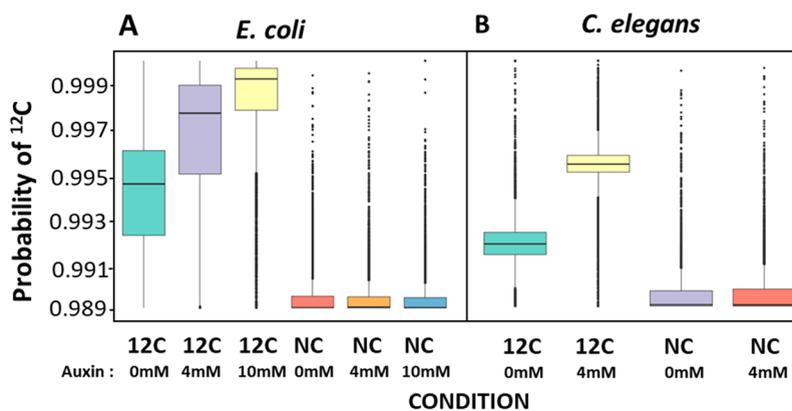


Figure 3. (A) Incorporation of ^{12}C into *E. coli* cells grown on NGM medium with or without auxin and (B) in *C. elegans* grown on NGM medium with or without auxin fed ^{12}C labeled *E. coli*.

These results indicate that isotopic dilution occurred in the bacterial cells during some step of the process in the first experiment. As the NGM medium contains a small amount of peptone, the most obvious explanation is that this medium may have been sufficient to support the growth of the bacteria, with a population of newly grown bacteria greatly exceeding the initial population of labeled bacteria. The resulting population of bacteria, therefore, contained nonlabeled carbon that were further incorporated into the *C. elegans* proteins through digestion and growth.

We redesigned the experimental protocol, taking into account that bacterial RNA and protein synthesis can be restricted by auxin,¹⁶ a plant hormone now routinely used in *C. elegans* biology associated with the targeted degon strategy (auxin-induced degradation).^{17,18} We therefore evaluated isotope dilution in ^{12}C -labeled *E. coli* cells after 3 days of culture on NGM plates containing various concentrations of auxin. The yield of remaining ^{12}C in *E. coli* cells was increased by 40% in the presence of 4 mM auxin and by 65% in the presence of 10 mM auxin relative to controls without auxin (Figure 3A). In parallel, the amount of ^{12}C incorporated into the worms fed ^{12}C -grown *E. coli* plated on NGM with 4 mM auxin increased by approximately 40% (Figure 3B). Although these results contribute to the demonstration that worm proteins can be labeled by amino acids derived from the bacterial proteins, the experimental conditions were not fully satisfactory because of the toxicity of high doses of auxin and the cost of the experiments.

It has been reported that *C. elegans* can be grown under axenic conditions on defined media.^{15,19} Typically, these media contain salts, cholesterol, heme, vitamins, oligo elements, and the necessary amino acids to sustain the growth of the worms. *C. elegans* has been reported to require almost the same essential amino acids as human cell lines. We wished to avoid the addition of any amino acids in our experiments. Therefore, we combined the preparation of a defined synthetic medium based on yeast nitrogen base, of which the composition is very close to that of the previously mentioned chemically defined media in terms of salts, vitamins, and oligo elements (https://legacy.bd.com/europe/regulatory/Assets/IFU/Difco_BBL/233510.pdf), but supplemented with cholesterol and, as described for the previous experiments, plated with *E. coli* cultures prepared under either the NC-condition or 12C-condition. Under these conditions, the worm proteome was completely labeled (Figure 4), showing that the efficiency of the degradation of the bacterial proteins and reincorporation of the resulting amino acids into *C. elegans* proteins was nearly 100%.

CONCLUSION

Here, we show that it is possible to efficiently incorporate ^{12}C -amino acids into complex biological systems, such as human cell lines in culture or the multicellular nematode *C. elegans*. For human cell lines, the ^{12}C -amino acids are provided by *in situ* synthesis from U- ^{12}C]-glucose, whereas for *C. elegans*, the ^{12}C -amino acids are directly provided from the digestion of the

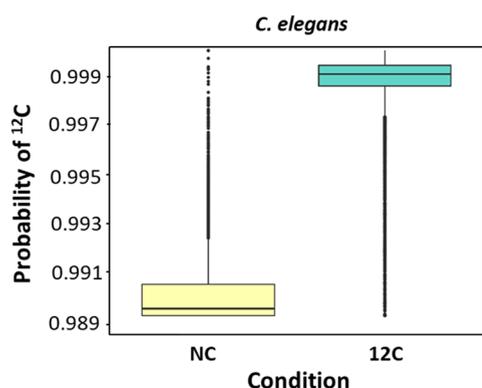


Figure 4. Incorporation of ¹²C into *C. elegans* peptides from worms grown on YNB medium with *E. coli* with normal glucose (NC) or U-¹²C]-glucose (12C).

proteins by the worms of the U-¹²C]-bacteria used to feed them and sustain their growth.

Our results show that the HEK293 cells in culture retain their metabolic ability to synthesize amino acids from glucose. The yield of incorporated ¹²C was consistent with the theoretical expected yield of incorporation, suggesting that the other components of the growth medium, especially the fetal calf serum, did not significantly contribute to the production of nonessential amino acids. The high variability of the yield of incorporated ¹²C into the HEK293 cell proteins is fully explained by the large heterogeneity of the amino-acid composition of the human proteome and the resulting tryptic peptides. The growth medium used contained 13 of the 20 amino acids, representing approximately 70% of the total carbon incorporated into the proteins. This did not prevent accurate measurement of the incorporation of ¹²C into the proteome. However, the SLIM-L approach would obviously benefit considerably from the availability of individual U-¹²C]-amino acids. Although providers such as Cambridge Isotope Laboratories, BocSciences Isotopes, or Sigma-Aldrich provide ¹³C-labeled amino acids, mostly for metabolomic studies or nuclear magnetic resonance analysis of proteins, none of them are currently providing ¹²C-labeled amino acids. It would be a positive result of this study if it helped to promote the production of such compounds.

Our experiments on the *C. elegans* proteome provided a number of interesting results on the interaction of the worms with the bacteria used to feed them and sustain their growth. The first observation is that, despite the care taken to control bacterial growth (type of strain, temperature, composition of the agar plates), there was significant isotope dilution in the bacterial population, resulting in the initial U-¹²C]-bacteria being outnumbered by bacteria able to grow on the nonlabeled medium in the Petri dishes. Adding auxin to the medium only partially limited the observed isotope dilution, restricting the bacterial growth and consequently significantly increasing the ¹²C labeling of the proteins in *C. elegans*. This prompted us to design a synthetic medium on which to plate the bacteria and the worms using the well characterized YNB medium supplemented with cholesterol, with the U-¹²C]-bacteria remaining as the only source of amino acids through the digestion of the bacterial proteins and the utilization of the resulting amino acids. This medium, which is easy to prepare and use, may be very useful for addressing a number of important questions in *C. elegans* biology.

■ ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository⁸ with the data set identifier PXD28880.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.2c00699>.

Figure S1: optical micrographs of HEK263 cells grown in the different media; Figure S2: SDS-PAGE analysis of total protein extracts from HEK293; Figure S3: Distribution of the number of essential and nonessential amino acids in the human proteome; Figure S4: Distribution of the number of carbon atoms in essential and nonessential amino acids in the human proteome; Figure S5: Distribution of the ratio of the number of carbons in essential over nonessential amino acids in the human proteome (PDF)

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Notes

The authors declare no competing financial interest.

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