Quantitative Proteomics by Metabolic Labeling of Model Organisms*

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In the biological sciences, model organisms have been used for many decades and have enabled the gathering of a large proportion of our present day knowledge of basic biological processes and their derailments in disease. Although in many of these studies using model organisms, the focus has primarily been on genetics and genomics approaches, it is important that methods become available to extend this to the relevant protein level. Mass spectrometry-based proteomics is increasingly becoming the standard to comprehensively analyze proteomes. An important transition has been made recently by moving from charting static proteomes to monitoring their dynamics by simultaneously quantifying multiple proteins obtained from differently treated samples. Especially the labeling with stable isotopes has proved an effective means to accurately determine differential expression levels of proteins. Among these, metabolic incorporation of stable isotopes *in vivo* in whole organisms is one of the favored strategies. In this perspective, we will focus on methodologies to stable isotope label a variety of model organisms *in vivo*, ranging from relatively simple organisms such as bacteria and yeast to *Caenorhabditis elegans*, *Drosophila*, and *Arabidopsis* up to mammals such as rats and mice. We also summarize how this has opened up ways to investigate biological processes at the protein level in health and disease, revealing conservation and variation across the evolutionary tree of life.   *Molecular & Cellular Proteomics 9:11–24, 2010.*

Well before the genomics era, the foundation for our current understanding of genetics was largely established by biological research performed using model organisms. Early genetics discoveries such as the chromosome theory of heredity and bacterial conjugation were first described in the fruit fly *Drosophila melanogaster* (1) and the bacterium *Escherichia coli* (2), respectively. Apart from these organisms, most of the current knowledge of development, evolution, and genetics originates from other classical model organisms including the bakers' yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the mouse *Mus musculus*. Nowa-

days, they hold a primary position in the analysis of biological, disease, and pharmaceutical processes in modern biology and probably claim an even more promising position in future biological research. With increasing numbers of completed genome annotations, however, the focus is also shifting somewhat from these classical model organisms toward organisms that have unique genetic properties, are economically interesting, or are more directly related to human disease such as puffer fish, rice, and *Plasmodium*, respectively. Consequently, the definition of a model organism has broadened over the past decade, and today model organisms are found in nearly all branches of the "tree of life," providing extensive means to further investigate conservation or diversification of biological principles through evolution (3). This has gained momentum tremendously by the completion of genome sequencing efforts in hundreds of organisms. In relatively simple organisms (bacteria and yeast), this has allowed the systematic investigation of multiple basic biological processes conserved through evolution (*e.g.* apoptosis (4) and vacuolar transport (5)). Higher organisms are highly useful for the study of complex traits, which is facilitated by large collections of mutant strains (6, 7). This is of particular relevance where model systems of human physiology, either in a healthy or diseased state, are studied. Fruit flies and *C. elegans*, the "classical" model organisms, have been used as models for a variety of diseases (8) but also for natural processes like aging (9, 10), sleep (11, 12), and olfaction (13). Mouse and rat models have been a longstanding model for human biology (14), especially in cancer (15). Particularly the availability of strains engineered to represent human diseases has increased our understanding of pathological processes tremendously (16).

So far, the focus has primarily been on genetic and genomic aspects of these processes and disorders, but with the maturation of proteomics techniques, ways to study these at the protein level in a meaningful way are coming within reach. Over the last decade, proteomics research has experienced significant advances and has evolved into an indispensable technology to investigate the proteomic composition of biological samples. Proteomics has shifted from the analysis of small sets of proteins toward the comprehensive investigation of a much larger number of proteins expressed in a cell, tissue, or organism (17). Nowadays, a typical proteomics experiment is peptide-centric and starts with the enzymatic digestion of a protein mixture followed by fractionation using one or more chromatographic steps to reduce sample complexity (18, 19) as illustrated in Fig. 1. Peptides are fragmented
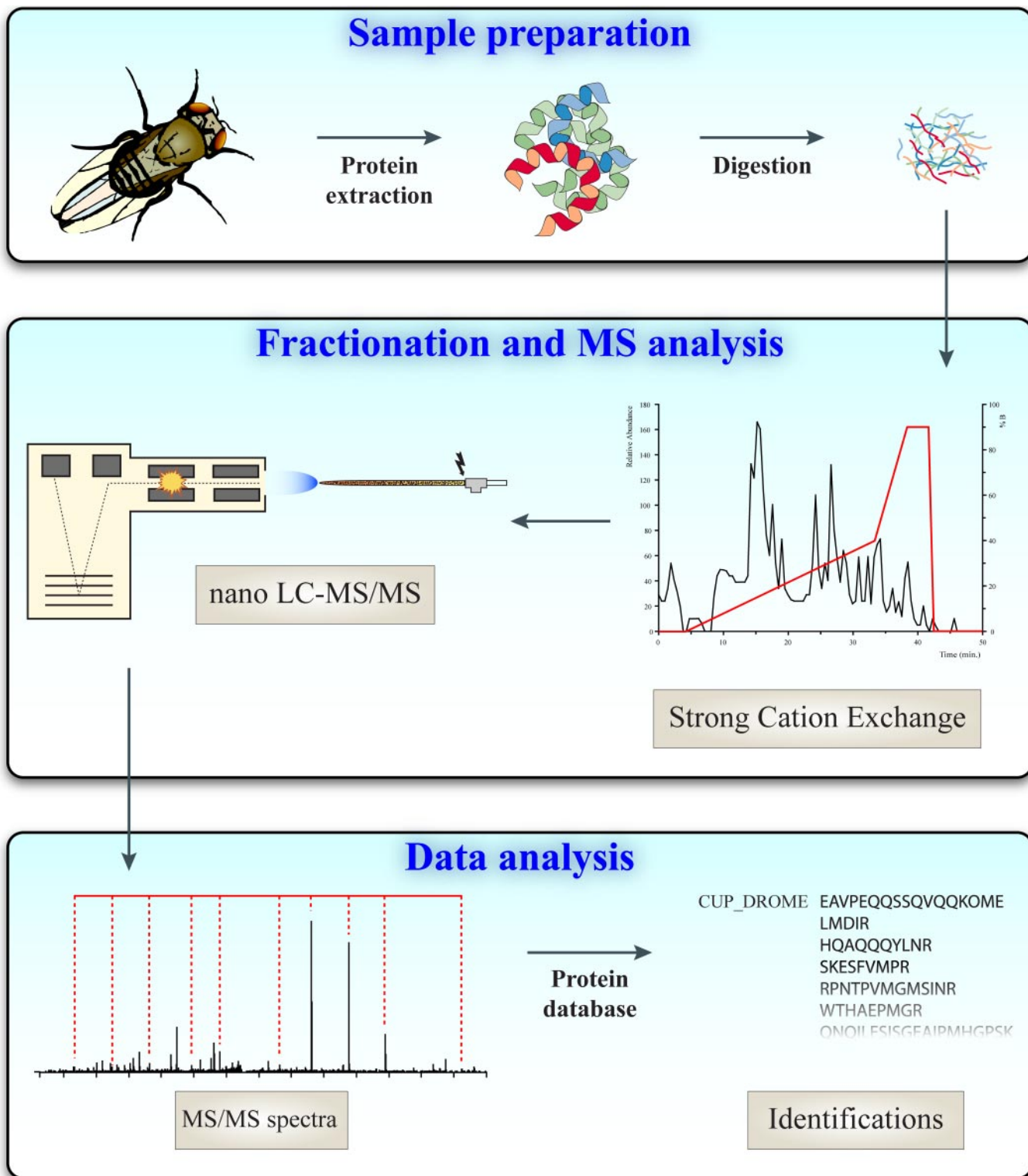
Fɪɢ. 1. **Qualitative proteomics work flow.** Proteins are extracted, digested, and separated by strong cation exchange. Each strong cation exchange fraction is then analyzed by nano-LC-MS/MS. Peptide fragment spectra are used in a database search to identify the peptide sequence and the corresponding protein.

in the mass spectrometer as they elute, and subsequent matching of fragmentation profiles against a protein database leads to peptide and protein identification. When performed at a large scale, this can be used for the identification of thousands of proteins in cells or subcellular structures (20–23). Although such qualitative approaches are fruitful in providing

information on proteins present in cells or tissues, they largely ignore the dynamics of protein expression when different conditions are to be investigated. This is highly relevant because in general only the proteins that differ between biological states (*e.g.* healthy/diseased) are likely to be of primary interest. Because mass spectrometry is not inherently quantitative, it is beneficial to add an internal standard as a reference for the peptide of interest. For large scale experiments, often all proteins or peptides in one sample are modified with a stable isotope-coded mass label. After mixing the labeled sample with an unmodified sample, the intensity ratio between the modified peptide and the unlabeled peptide accurately reflects the change in expression level.

Various approaches have been developed for the incorporation of stable isotopes into proteins that can be divided into *in vivo* and *in vitro* methods. In the former, isotope-enriched compounds (salts or amino acids) are added to the growth media that can be metabolized by the cell and incorporated into proteins. *In vitro* labeling can be established using chemical derivatization of proteins or peptides after protein extraction. The choice for either approach depends on the biological system under investigation, but there are a few considerations that should be taken into account because of their impact on the experimental work flow. In Table I, some of the strengths and weaknesses of both metabolic and chemical labeling methods are summarized.

One major consideration for labeling *in vivo* (metabolic) or *in vitro* (chemical) critically depends on whether the biological sample in question can metabolically incorporate the isotope label. Metabolic labeling requires the addition of an isotopically enriched element (*e.g.* ^{13}C, ^{15}N, or ^{18}O in salts or amino acids) to the growth media in a form that makes it available for incorporation into the entire organism, tissue, or cell. In contrast, chemical labeling occurs after protein extraction and therefore is completely independent of the source and preparation of the sample. This has the advantage that virtually any type of biological sample can be labeled, including human tissue or body fluids. Additionally, the time needed for this type of labeling is in general much shorter than when a label is incorporated metabolically where it may take weeks to *in vivo* label organisms or cells depending on the growth rate. This can even increase to a few months if a secondary labeling step is required such as is the case in the ^{15}N labeling procedure of fruit flies and worms by feeding them on labeled yeast and *E. coli*, respectively.

The great advantage of metabolic labeling becomes clear when the proteomics work flow is considered. Fig. 2 gives an overview of the different positions in the experimental work flow where the internal standard can be introduced. Clearly, the best place to introduce an internal standard is by metabolically incorporating the stable isotope into living organisms or cells, thereby producing the lowest variation before any sample processing occurs (Fig. 2, *left*). When the internal standard is introduced further downstream in the work flow,

higher levels of variation can be expected due to parallel sample processing as is the case with chemical derivatization of intact proteins (*e.g.* with ICAT and isotope-coded protein labeling (ICPL)[1] (24, 25)) (Fig. 2, *middle*) or with chemical labeling of peptides such as isobaric tag for relative and absolute quantitation (iTRAQ) and stable isotope dimethyl labeling procedures (26–28) or proteolytic digestion in ^{18}O-labeled water (29, 30) (Fig. 2, *right*).

CHEMICAL AND LABEL-FREE APPROACHES FOR QUANTITATIVE PROTEOMICS

The advantage of using iTRAQ labeling over other (chemical) labeling strategies is the possibility to simultaneously analyze up to eight biological samples in one experiment by labeling peptides (primary amino groups) with isobaric tags that differ in reporter and balancer groups (26, 31). Quantitative information is obtained by comparing the unique reporter groups in the fragmentation spectrum, and therefore this labeling strategy is fully MS/MS-dependent, and quantitative information is only obtained from peptides that were subjected to fragmentation. The only other quantitative method that exclusively relies on fragmentation data is the label-free method of spectral counting. The abundance of a protein can be estimated by counting the number of sampling events of a peptide from this protein. It has been observed that the number of assigned MS/MS spectra directly correlates with protein abundance. In this approach, samples are analyzed and processed separately, and protein lists are compared in terms of sampling events per protein. Although spectral counting has proven to be very reliable at measuring large changes between proteins, reliability drops dramatically when smaller changes are estimated (32). In contrast, the other (chemical) labeling strategies are based on chromatographic intensities of intact peptide signals and do not require fragmentation for quantitation. However, not necessarily all peptides can be used for quantitation depending on the labeling strategy. For instance, in ICAT labeling only cysteine-containing peptides can be used for quantitation, and only lysine-containing peptides and protein N termini can be used in ICPL. Additionally, when trypsin is used as the proteolytic enzyme in ICPL, possibly longer peptides may be expected due to the modification of lysine residues. Trypsin will only cut after arginine residues, and the resulting longer peptides may be more difficult to identify. This is not the case for labeling strategies based on peptide labeling or label-free quantitation based on the ion intensity (MS mode) of peptides. Similarly to spectral counting, samples are analyzed and processed separately, but this strategy relies on extracting mass spectrometric peak areas for all peptides that are subsequently integrated for their

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[1] The abbreviations used are: ICPL, isotope-coded protein labeling; iTRAQ, isobaric tag for relative and absolute quantitation; SILAC, stable isotope labeling with amino acids in cell culture; SMIRP, subtle modification of isotope ratio proteomics.

Comparison of strengths and weaknesses of various metabolic (in vivo) and chemical (in vitro) labeling techniques
ETD, electron transfer dissociation; ECD, electron capture dissociation; +, beneficial; −, hampering.

Labeling method	Cost	Strengths	Weaknesses
Metabolic labeling (*in vivo*)			
SILAC	+	Incorporation at the organism level (lowest variation). Available (free) quantitation software.	Not applicable to human samples. Arginine-to-proline conversion. Expensive and slow. Enzymes other than trypsin and/or Lys-N may produce non-quantifiable peptides. Auxotroph for the labeled amino acid(s).
^{15}N labeling	+	Incorporation at the organism level (lowest variation). All peptides can be used for quantitation regardless of the enzyme	Not applicable to human samples. Expensive and slow. Available quantitation software. Unknown mass difference prior to identification.
^{13}C labeling	+	Incorporation at the organism level (lowest variation). All peptides can be used for quantitation regardless of the enzyme.	Not applicable to human samples. Expensive and slow. Available quantitation software. Unknown mass difference prior to identification. Isotope distribution might hamper identification.
SMIRP	+/−	Incorporation at the organism level (lowest variation). All peptides can be used for quantitation regardless of the enzyme.	Not applicable to human samples. Slow. Available quantitation software.
Isotope-depleted labeling	+	Incorporation at the organism level (lowest variation). All peptides can be used for quantitation regardless of the enzyme.	Not applicable to human samples. Expensive and slow. Available quantitation software. Identification requires ECD or ETD. Quantitation at the protein level.
Chemical labeling (*in vitro*)			
ICAT	+/−	Applicable to any sample. Fast.	Incorporation at the protein level (moderate variation). Only Cys-containing peptides can be used for quantitation.
ICPL	+/−	Applicable to any sample. Fast.	Incorporation at the protein level (moderate variation). Only Lys-containing peptides and the protein N terminus can be used for quantitation. Trypsin cleaves C-terminal to arginine residues only.
iTRAQ	+/−	Applicable to any sample. Fast. Simultaneous analysis of 8 labeled samples. No increase in complexity at the MS level.	Incorporation at the peptide level (high variation). Quantitation is based on 1 or a few tandem mass spectra. Requires mass spectrometers that can analyze the low *m/z* region.
^{18}O labeling	−	Applicable to any sample. Cheap and fast.	Incorporation at the peptide level (high variation). Difficult to reach complete labeling. Available quantitation software.
Dimethyl labeling	−	Applicable to any sample. Cheap and fast. Automation is possible.	Incorporation at the peptide level (high variation). Identification issues due to the number of variable modifications.

respective retention times (33). A more detailed description of chemical and label-free approaches in quantitative proteomics and a comparison between them are not the focus of this perspective, especially as a number of excellent reviews have been published in the last few years covering these subjects (17, 34–37). Instead, we will focus specifically on strategies using metabolic incorporation of mass labels *in vivo* in whole organisms.

METABOLIC LABELING FOR QUANTITATIVE PROTEOMICS

Recently, the development of strategies to label cells in culture with stable isotopes (stable isotope labeling with amino acids in cell culture (SILAC)) has found broad application and continues to expand to a wide range of cell lines. A number of first rate studies have been published (38, 39) and reviewed elsewhere (35, 40) that show the power of SILAC. Nevertheless, some limitations of this labeling procedure should be considered. For instance, the cells or organism should be auxotroph for the labeled amino acid(s), either by mutations or by using essential amino acid(s), to ensure that the labeled amino acid is the only source for protein synthesis. The widespread metabolic conversion of arginine to proline is an undesired side effect when arginine is used in SILAC

labeling (41, 42). This effect is illustrated in Fig. 3A where a mass spectrum shows the desired unlabeled and double labeled peptide (+16 Da, heavy lysine and arginine) but also additional unwanted peaks at positions that correspond to incorporation of single (+6 Da) and double (+12 Da) labeled proline residues in the peptide. A significant amount of signal can reside in these peaks and can negatively affect quantitation. This phenomenon is not apparent in the case of metabolic ^{15}N labeling using salts and can be visualized by comparing the mass spectrum in Fig. 3A with that in Fig. 3B where the same peptide was ^{15}N-labeled (11 nitrogen atoms, +11 Da). Although the arginine-to-proline conversion is most likely still taking place, the effect is eliminated because in this type of labeling the mass of the proline-converted peptide is similar to the mass of the precursor peptide. However, the mass difference between an unlabeled and ^{15}N-labeled peptide is *a priori* unknown during mass spectrometric analysis and becomes apparent only after identification. This might hamper sophisticated acquisition software that only selects regulated peptides for fragmentation and/or automated quantification prior to identification. When using ^{15}N, suboptimal labeling (exemplified by the *red* colored isotope at *m/z* 410.73 in Fig. 3B) is a recurrent issue that should be accounted for to obtain correct abundance ratios (43). Nevertheless, the procedure of ^{13}C or ^{15}N substitution in living organisms is very attractive because little to no side effects (cytologically or morphologically) have been reported (44, 45). Moreover, it is not essential to achieve complete labeling. The incorporation of for in-
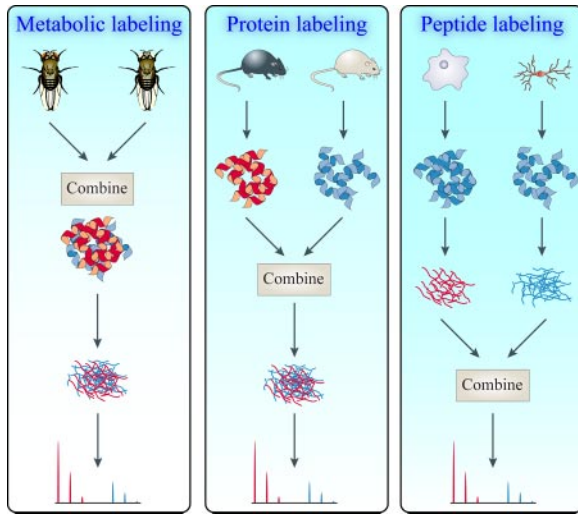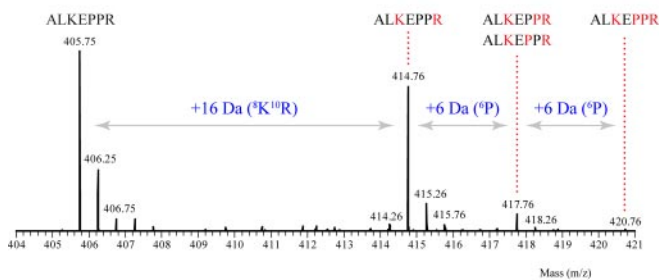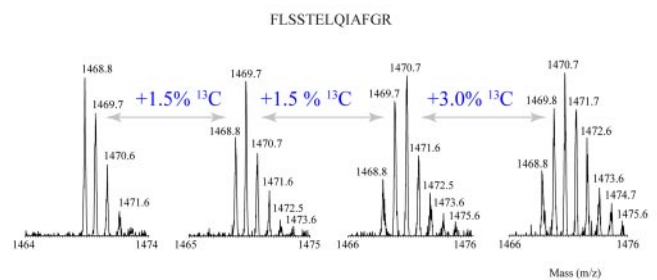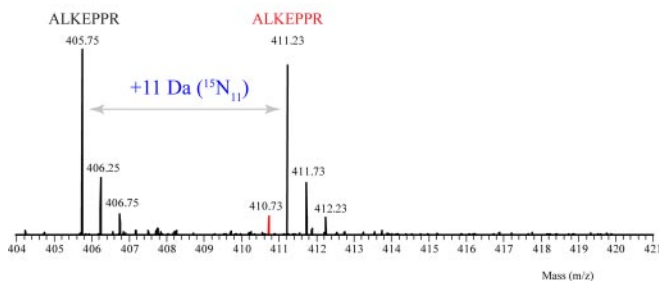

FIG. 2. **Strategies for quantitative proteomics.** Stable isotopes can be incorporated at different stages of the quantitative work flow and are indicated in *black*. The methods are metabolic labeling (*left*), protein labeling (*middle*), and peptide labeling (*right*). Relative expression levels are obtained by mass spectrometry where the signal of the unlabeled peptide is compared with that of the labeled peptide.

FIG. 3. **Overview of typical mass spectra that are obtained from different metabolic labeling strategies.** In SILAC (*A*), labeled arginine can be converted to proline, indicated in *red* in the peptide sequence, resulting in peaks that are 6 Da higher than the labeled peptide. If such peaks are formed in heavy nitrogen labeling (*B*), they appear at the same position as the ^{15}N-labeled peptide because they are isobaric. The *red* colored isotope at *m/z* 410.73 is a product of suboptimal ^{15}N labeling. Minor enrichment of ^{13}C in SMIRP leads to quantifiable peptide signals (*C*), whereas higher incorporation is used to determine protein synthesis and degradation (*D*).

stance 1.5% ^{13}C, a strategy termed subtle modification of isotope ratio proteomics (SMIRP) (Fig. 3*C*), is already enough to obtain good quantitative data, but automated quantitation is more difficult because of the lack of available software. Another application of incomplete labeling is to determine protein synthesis and degradation by monitoring the incorporation of labeled ^{13}C in a time-dependent manner (Fig. 3*D*). Also, in these cases, the lack of suitable software hampers data processing so far.

The availability of software solutions for the analysis of quantitative proteomics data generated by LC-MS is extensive but still limited. This is caused by the fact that only a few solutions might be available for a specific type of labeling analyzed on a particular mass spectrometer. Software tools to analyze quantitative LC-MS data are usually developed for specific types of mass spectrometers, thereby restricting these tools to analyze data from only those instruments. Some labeling strategies such as SMIRP and isotope-depleted labeling even lack software solutions to automatically perform quantitation, whereas others (^{15}N and ^{13}C labeling) seem restricted to a few available software tools. An overview of software solutions has been published (46).

In recent years, increasing numbers of model organisms are being used for comparative proteomics, and various methodologies have been developed to metabolically label them. Shown in Fig. 4 is a compendium of the current "tree of metabolically labeled life," and it can be seen that species in almost any branch can be and have been labeled. Table II gives a more detailed overview of these species together with the type of metabolic labeling applied. Quantitative proteomics in (model) organisms, either by metabolic or chemical labeling, can be extremely powerful to elucidate biological processes. Most of these studies focus on the effect of an isolated treatment (*e.g.* stress condition or growth factor) of cells grown in a Petri dish, which should be regarded as a somewhat artificial system because cells have often been maintained for thousands of cell doublings over many years. Moreover, any cell system neglects the differences in responses that would normally occur in an organism (because of the nature of the cells or organs) or communication between them. If the aim is to understand cells in their natural environment, one should aim to perform studies in the intact organism. There is no doubt that quantitative approaches will be essential in uncovering relevant players, and consequently it is important to develop quantitative proteomics methods in model organisms. Therefore, here we present an overview of intact model organisms that have been metabolically labeled for the purpose of quantitative proteomics. Per species we provide the current status of the methodology as well as examples of their application to biological problems.
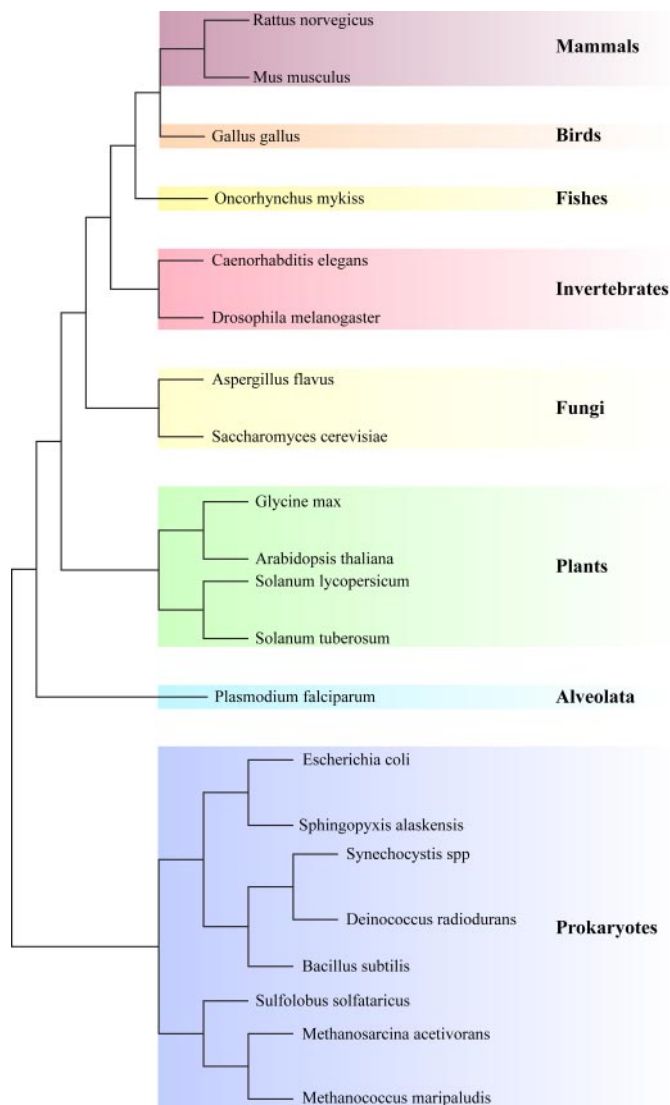

FIG. 4. **Tree of metabolically labeled life.** *Branch lengths* are not proportional to evolutionary distance.

OVERVIEW OF METABOLICALLY LABELED SPECIES

Prokaryotes

The prokaryotes *E. coli*, *Deinococcus radiodurans*, and *Synechocystis* spp. were initially used to establish different ^{15}N metabolic labeling methodologies. In one of the first studies reported to accomplish this, unstressed and Cd^{2+}-stressed *E. coli* were grown on normal and heavy isotope (^{13}C, ^{15}N, and ^{2}H)-depleted media, respectively (47). After mixing aliquots from both cultures, relative expression levels of intact proteins were determined by fitting the experimental results with the predicted shapes of the calculated isotopic envelopes. Although this procedure readily lends itself for the determination of relative expression levels, identifying these proteins is somewhat more complicated and requires more sophisticated fragmentation methods such as electron capture dissociation and electron transfer dissociation to obtain

Class and species	SILAC	^{15}N	^{13}C	Refs.
Prokaryotes				
E. coli	√	√[a]	√[a]	47, 56, 60–64
D. radiodurans		√		49
Synechocystis spp.		√	√[b]	51
S. solfataricus		√	√	52–54
M. maripaludis		√		55
M. acetivorans		√		58
B. subtilis	√	√		59
S. alaskensis		√		57
Alveolata				
Plasmodium falciparum	√			146
Plants				
G. max		√		101
S. tuberosum		√		102
A. thaliana	√	√		105–113
S. lycopersicum		√		114
Fungi				
S. cerevisiae	√	√		23, 71–83, 85, 87–90
A. flavus	√			95, 97
Drosophila* and *C. elegans				
D. melanogaster		√		43, 126, 128, 129
C. elegans		√		43, 126, 127
Fishes				
Oncorhynchus mykiss		√		144
Birds and mammals				
G. gallus		√		130
Rattus norvegicus			√	131–133
M. musculus		√		134

[a] These labeling strategies are based on heavy isotope depletion (47).

[b] This strategy is based on subtle isotope enrichment ($\sim$1–2%) (51).

sequence information for identification purposes (48). To overcome this identification issue, proteins can be digested into peptides for both quantitative and qualitative information. This procedure was developed by growing *D. radiodurans* on fully ^{15}N-enriched (>98%) media after which proteins were digested into peptides to facilitate protein identification and quantitation (49, 50). A third method to determine protein expression levels uses the labeling of proteins with a subtle enrichment ($\sim$1–2%) of a stable isotope instead of using fully enriched (>8%) labeled media. This approach, referred to as SMIRP, has been used to label *Synechocystis* spp. with various ^{13}C/^{12}C isotope ratios to determine the feasibility of this approach. Optimal results were obtained with enrichments as low as $\sim$1–2% ^{13}C that have no effect on either data-dependent acquisition or database searching algorithms but yield a measurable effect on peptide isotopic distribution of which an isotope ratio can be inferred (51). A method similar to the labeling procedure of *D. radiodurans* (*i.e.* using fully enriched heavy nitrogen) included the incorporation of stable isotope-labeled ^{13}C atoms in the hyperthermophilic crenarchaeon *Sulfolobus solfataricus*. In this approach, three different versions of the same protein are produced (unlabeled, ^{15}N-, and ^{13}C-labeled), allowing for the analysis of three different samples in a single experiment (52). The same group also investigated the applications and limitations of ^{15}N labeling and showed that stable isotope labeling with heavy nitrogen in combination with mass spectrometric detection provides an excellent tool for studying protein dynamics (53, 54). This was further corroborated in another study where the archaeon *Methanococcus maripaludis* was ^{15}N-labeled, and protein expression levels were validated using real time PCR (55). An additional benefit of ^{15}N labeling is its use to increase the number and confidence of protein identifications. In ^{15}N labeling, the number of nitrogen atoms in a peptide can easily be determined from the mass difference between labeled and unlabeled peptide pairs, which can be used as an additional criterion to accept or refute peptide identification (56). Recently, *Sphingopyxis alaskensis* has been ^{15}N-labeled to investigate normalization and statistical analysis in quantitative proteomics-generated data (57). Two of the first applications utilizing metabolic labeling of a prokaryote included ^{15}N labeling and SILAC or a combination thereof. In one study, *Methanosarcina acetivorans* was ^{15}N-labeled to investigate acetate *versus* methanol growth conditions (58). In the other study, changes in the membrane proteome during stationary phase adaptation of *Bacillus subtilis* were monitored, and both techniques showed similar valuable data for quantification of bacterial membrane proteins (59). The SILAC procedure has also extensively been investigated and applied to *E. coli* cells. Several different labeled amino acids were used to metabolically label *E. coli*, including lysine (60, 61), leucine (61–63), glycine (61), and methionine (61, 64).

Fungi

S. cerevisiae—The yeast *S. cerevisiae* fulfills an important dual role, being an industrially applied organism in itself while serving as one of the prime model organisms for higher eukaryotes. First, this microorganism is extensively used in industry as it has the ability to produce for instance alcohol and carbon dioxide. In addition, it can be used as a host for the production of proteins and small molecules exploited by pharmaceutical companies for the production of insulin or penicillin (65). Second, yeast has been used for a long time as a model organism in the field of molecular biology. Properties such as the rapid adaptation and easy growth under numerous conditions, its lack of pathogenicity, and the availability of powerful genetic manipulation techniques make this organism perfectly suited for laboratory experiments. In addition, it was the first eukaryote to have its genome sequenced in 1996, resulting in the prediction of $\sim$6000 protein-coding genes (66). Since then, large amounts of data based on genome, transcriptome, proteome, and metabolome studies have been generated. A great deal of this information is curated, stored, and managed in the on-line database *Saccharomyces* Ge-

nome Database (67). In the postgenomics era, information from studies at different genomic levels is combined in a systems biology approach, resulting in a comprehensive understanding of eukaryotic cell biology. In this context, it is expected that yeast will continue to provide new knowledge and insights (68–70).

Metabolic labeling for quantitative proteomics is extensively applied in yeast and can be accomplished by two different approaches. Stable isotope-labeled atoms such as ^{15}N can be incorporated by growing yeast in media containing labeled ammonium sulfate as the sole nitrogen source (71). The other method involves the incorporation of one or more stable isotope-labeled amino acids into the proteome of yeast. The former methodology was used in 1999 by Oda *et al.* (72) to uniformly ^{15}N label yeast, which they used to investigate protein expression as well as site-specific changes in phosphorylation levels. They reported that relatively small changes in phosphorylation ($>20\%$) can be reliably detected from small amounts of gel-separated proteins (72). Yates and co-workers (73–78) extensively used ^{15}N-labeled yeast to establish methodologies for the analysis of quantitative proteomic samples as well as to address biological questions. For instance, by combining metabolic labeling with multidimensional protein identification technology, they described a system useful for detailed quantitative proteomics analysis (73) that they used to investigate the correlation between mRNA and protein expression levels (74). This approach was complemented with a correlation algorithm called RelEx for the automated analysis of quantitative data (75) and an algorithm called The Atomizer to determine isotope enrichment levels (76). Both approaches were validated using complex mixtures with known enrichment levels of ^{15}N-labeled yeast, and moreover, the RelEx algorithm was applied to the study of NaCl osmotic stress on the protein level in yeast. An alternative data acquisition method that was developed using ^{15}N-labeled yeast by Yates and co-workers (77) relies on data-independent analysis where quantitative results are determined directly from tandem mass spectra (MS^2) using a modified version of RelEx. In addition, this group also evaluated the use of an LTQ-Orbitrap hybrid mass spectrometer for quantitative analysis using labeled yeast (78). Other groups that focused on more fundamental research using ^{15}N-labeled yeast investigated top-down quantitative proteomics approaches (79) and the correlation between spectral counting and metabolic labeling (80, 81). Besides this important basic research, biological effects like response to nutrient limitations (82), adaptation to anaerobiosis (83, 84), or phosphorylation level changes related to glucose activation (85) were studied using ^{15}N-labeled yeast.

The other metabolic labeling strategy established in yeast involves the incorporation of one or more labeled amino acids. The first studies describing such an approach used deuterium-labeled amino acids such as triple deuterated methionine (Met-d_3) and serine (Ser-d_3) and double deuterated tyrosine

(Tyr-d_2) (86). Deuterated leucine was also used, but it differed in the number of deuterium atoms in the amino acid. In one case, triple deuterated leucine (Leu-d_3) was used (87), whereas in the other case, decadeuterated leucine (Leu-d_{10}) was used (88). In both cases, a yeast strain was used that is auxotrophic for leucine, ensuring that all leucine was replaced by labeled leucine. Once established, the Leu-d_{10} metabolic labeling approach was used to investigate the response of the yeast proteome to H_2O_2 (89). Besides labeled leucine, also stable isotope-labeled arginine and lysine were used to label yeast. A double auxotroph strain was used to uniformly label yeast to study the changes in pheromone-induced phosphorylation (90). However, a disadvantage of using labeled arginine in eukaryotes is that the accuracy of peptide ratio calculation is compromised by the metabolic conversion of arginine to proline, which seems to be quite efficient in yeast (41, 42). Several solutions have been published (91, 92) to correct for this effect to ensure accurate quantitation. A study that used labeled lysine only focused on determining factors that prevent complete proteome analysis (23). The sequencing speed of the mass spectrometer was found as one of these factors. The sequencing speed was determined by comparing the number of identified "light" and "heavy" peptides. If the sequencing speed is sufficiently high, both forms of a SILAC pair should be sequenced and identified, but this was shown to be true only for the more abundant peptides. The other factor that limited complete proteome coverage was the restricted dynamic range of the mass spectrometer compared with the dynamic range of the yeast proteome (*i.e.* 100 *versus* 10,000) (93). The dynamic range of the mass spectrometer was determined by comparing the intensity of the most abundant SILAC pair with the least abundant pair in the same mass spectrum (23). Recently, the same group investigated and quantified the proteomes of haploid and diploid yeast. By using the same labeling strategy in combination with several different fractionation techniques, they increased the number of identified proteins to a level of 4,399 proteins. In addition, they showed that mass spectrometry is as sensitive as other protein detection techniques such as green fluorescent protein and tandem affinity purification tagging (94).

Aspergillus flavus—Recently, two studies were published that described the SILAC labeling of the fungus *A. flavus* by growing this multicellular prototroph on media containing heavy arginine. This amino acid was chosen over lysine because it occurs at a frequency of about 6% in the proteome (95). By determining the incorporation efficiency of 50 proteins, they found an average enrichment level of 78%, which is similar to the enrichment level of 81% reported by the SILAC labeling of *Arabidopsis* suspension cells (96) (see above). This suboptimal enrichment level was sufficient to quantify protein changes in response to environmental stimuli regulating biosynthesis of the carcinogen aflatoxin (95). Another study by the same group, however, showed that this suboptimal enrichment hampered the quantification of intact

proteins that have a large number of arginine residues. Here, they established a quantitative top-down proteomics approach using this labeled fungus and showed that proteins with few arginines can readily be identified and quantified (97). However, they suggest to reduce endogenous amino acid incorporation by using an arginine auxotroph strain, thereby facilitating straightforward quantification of proteins that contain larger number of arginines (97).

Plants

Metabolic labeling of plants dates back to the mid-sixties of the last century where labeled nitrogen (in the form of ammonium sulfate) was added in tracer amounts to a fertilizer and used to evaluate the efficiency of fertilizer applications in rice (*Oryza sativa*) (98). Metabolic incorporation of labeled nitrogen was accomplished by growing rice hydroponically (*i.e.* using mineral nutrient solutions instead of soil) and used to monitor how the heavy nitrogen accumulated into specific parts of the plant. Although only ~30% enriched labeled nitrogen was used (($^{15}NH_4)_2SO_4$ and $K^{15}NO_3$) to investigate the uptake and distribution of nitrogen, it was sufficient to spectroscopically determine the amount of heavy nitrogen in different organs of the rice plant (99). The same method was used to label the potato plant (*Solanum tuberosum*) (100). Likely the first report of a highly enriched, uniformly labeled plant was published in 1994 where soybean plants (*Glycine max*) were labeled with ~98% ^{15}N to investigate nutrient absorption and metabolism in human or animal studies (101). Next, potato plants were uniformly ^{15}N-labeled (>98%) for the purpose of structural proteomics (102). Intact potato plants were obtained by growing aseptic seed potato tubers using a hydroponics setup for a period of 93 days in a climate-controlled greenhouse on a nutrient solution containing 99% potassium nitrate ($K^{15}NO_3$) as the sole nitrogen source. Although *S. tuberosum* was the first plant to be fully ^{15}N-labeled, *Arabidopsis thaliana* is considered the prime model species in plant biology, and the genome of this plant was the first plant genome to be sequenced (103). Complemented with methods to incorporate stable isotopes *in vivo*, this model has facilitated mass spectrometry-based quantitative proteomics applied to plant biology.

The genome of the flowering plant *Arabidopsis* is one of the smallest among plants and was sequenced in 2000. It was concluded that this plant has 11,000–15,000 protein families, a number that is similar to other multicellular eukaryotes like *C. elegans* and *Drosophila* (103). The current estimate is that the *Arabidopsis* genome contains ~27,000 genes encoding 35,000 proteins, but these numbers still change continuously due to combined efforts by The *Arabidopsis* Information Resource that updates the *Arabidopsis* genome annotation annually (104). *Arabidopsis* is an autotrophic species synthesizing all amino acids from inorganic nitrogen. This makes it particularly difficult to metabolically incorporate labeled amino

acids into the proteome of the plant at high efficiency. However, labeling enrichments of 70–80% have been reported when suspension cells were grown in the presence of exogenously supplied heavy arginine for 7 days (96). In this experiment, SILAC was used to identify regulated glutathione *S*-transferases and 14-3-3 proteins in response to treatment with salicylic acid. Disadvantages of this approach include incomplete labeling; the use of only one labeled amino acid, reducing the number of quantifiable peptides; and expensive labeled amino acids that are needed to reach efficient enrichments. This method is therefore limited to plant cell culture. Alternatively, these drawbacks can be circumvented by growing cell cultures on media containing highly enriched ^{15}N as the sole nitrogen source (105–108). Typically, cells were grown for around 21 days in modified liquid media containing >98% ^{15}N, and complete uniform incorporation was achieved. No detrimental isotopic effects were observed because the morphology and growth rate of the ^{15}N-labeled cells were indistinguishable from their ^{14}N counterparts (105). In some cases inverse labeling was performed to confirm results (107, 108). Besides proteomics, also metabolomics can be studied using this type of labeling, but this application is evidently limited to metabolites that contain nitrogen (105, 106). Labeling with heavy carbon (^{13}C) is therefore considered to be more efficient for this purpose.

As for the labeling of cell cultures, heavy nitrogen has been used as the prime method to metabolically label intact plants *in vivo* with high levels of enrichment. The first publication of labeling of intact *Arabidopsis* for proteomics experiments was in 2007 (109). Since then, several different ^{15}N labeling techniques have been investigated that include the comparison of partial *versus* full labeling (110), the automated analysis of uniformly labeled proteins using Mascot peptide identification in conjunction with the trans-proteomic pipeline (111), and a procedure referred to as hydroponic isotope labeling of entire plants (112). A notable aspect in the method to metabolically incorporate heavy nitrogen in intact plants is that almost all proteomics studies on *Arabidopsis* grow plants in liquid media. It can be argued that biologically meaningful results can only be obtained when plants are grown on solid medium, thereby simulating growth conditions as natural as possible. Leaf senescence was investigated in *Arabidopsis* by growing the plants on solid labeled and unlabeled media (113). Recently, the approach known as stable isotope labeling *in planta* was published where the tomato plant *Solanum lycopersicum* was ^{15}N-labeled by growing it for 2 months on solid media (114). Besides metabolic labeling, most other quantitative proteomics methods such as iTRAQ, ICAT, and ^{18}O labeling have been established in plants as well. For an overview of these techniques and their application in plants see Thelen and Peck (115).

D. melanogaster and C. elegans

Since the introduction of the nematode *C. elegans* and the fruit fly *D. melanogaster* as model organisms in biological research (1, 116, 117), many fundamental biological principles have been disclosed using these species. They were the first multicellular organisms to have their genomes sequenced in 1998 and 1999, respectively (118, 119), and nowadays, truly outstanding resources are available for both species (120, 121) such as the on-line databases Wormbase (122) and Flybase (123). The cellular complexity and the conservation of biological pathways between these invertebrates and higher organisms, including humans, opened the door for using them as tools to study comparative human genetics (7, 8, 124, 125). Indeed, many biomedical discoveries were fueled by *C. elegans* and *D. melanogaster* research. Of note, the processes underlying diabetes and Alzheimer disease were first discovered in *C. elegans* (for a review, see Ref. 8).

Although quantitative genetics and functional genomics have been established in these model organisms for some time now, quantitative proteomics based on metabolic labeling is only beginning to be introduced. Flies and worms can be metabolically ^{15}N-labeled by feeding them on uniformly ^{15}N-labeled *S. cerevisiae* and *E. coli*, respectively (126). Whereas flies are completely labeled after one generation, worms need to be grown on labeled media for at least one more generation after which second generation worms are completely labeled. However, due to different protein turnover rates in different tissues, it is necessary to analyze the enrichment of different proteins to ensure complete labeling. Labeled worms have been used to identify targets of insulin signaling (127), and labeled flies have been used to identify novel seminal fluid proteins (128) and proteins involved in the maternal-to-zygotic transition (129). In addition, both species have been used to optimize the identification and quantitation of ^{15}N-labeled proteins in comparative proteomics (43). The method for metabolic labeling of both *Drosophila* and *C. elegans* should be easily adoptable by many fly and worm laboratories because it requires only minor adaptations compared with routine protocols for growing flies and worms. Therefore, we expect this approach to find broad application in fly and worm developmental biology and beyond.

Birds and Mammals

One of the first reports of stable isotope labeling in birds involved the partial SILAC labeling of the chicken *Gallus gallus*. A diet that consisted of 50% labeled valine was fed to 6-day-old chickens to monitor the incorporation of the heavy amino acid and thereby facilitate the determination of protein turnover rates. Although these chickens are not fully labeled, such an approach allows for the accurate detection of protein turnover rates *in vivo* (130). Wu *et al.* (131) even extended the metabolic ^{15}N labeling approach to mammals by labeling rats using a diet source that was supplemented with ^{15}N-enriched (>99%) algal cells. After feeding the rats for 44 days, enrichment levels ranged from 74 to 92% depending on the type of tissue (131). Using an improved labeling strategy, they increased the enrichment to 94% throughout all tissues of the rat, and these tissues can serve as internal standards to facilitate the quantitative proteomics analyses of complex mammalian tissue samples (132). For instance, labeled rat brain was used to investigate the synaptosomal proteome of the rat cerebellum during postnatal development (133). In addition to labeled rats, other mammals such as mice can be metabolically labeled by using a SILAC diet where second generation mice showing complete labeling without obvious effects on growth, behavior, or fertility were observed (134). In these experiments, ^{13}C-labeled lysine was used; it is an essential amino acid, thus preventing synthesis from other (unlabeled) sources of amino acids. As a result, it is not straightforward to use trypsin as the preferred protease because this enzyme produces both C-terminal arginine and lysine peptides of which only the lysine-containing peptides can be used for quantitation. The endoprotease Lys-C, in contrast, produces only peptides that have a C-terminal lysine, making Lys-C the preferred choice as the proteolytic enzyme. For such studies, the recently introduced protease Lys-N may provide a very elegant alternative in the future (135, 136).

A limitation to the use of labeled rats or mice could be the investment that is required to produce isotope-enriched offspring. However, the amount of protein that can be collected from basically every tissue, even from a single animal, is sufficient for hundreds of proteomics experiments. Furthermore, if this material is produced from a wild type, commonly used strain it could be distributed and used as an internal standard even in facilities without resources to label these animals themselves.

OUTLOOK

Although stable isotope labeling has been achieved across many species in almost all branches of life (Fig. 4), there are still some model organisms where the introduction of a labeling strategy would be very useful. For example, *Danio rerio* (zebrafish) has been established as an important model organism to study vertebrate biology (137, 138), especially in immunology (139), cancer (140), neurological disorders (141), and toxicology (142). This research is facilitated by the development of excellent genetics techniques in zebrafish such as the targeted knockdown of genes by morpholino antisense technology (143). There are several reasons accounting for the fact that this organism has not been metabolically labeled yet. Notably, the extensive developmental period and the absence of a labeled food source limit straightforward metabolic labeling. However, metabolic labeling of fish is not unprecedented with the incorporation of ^{15}N into rainbow trout to study protein turnover (144).

The last but certainly not least important species that has not been metabolically labeled so far is humans. Up to now,

SILAC approaches to metabolically label human cell lines have been firmly established (40), but the next logical step would be to extend this to human tissue. Tissue or organs grown in the laboratory from stem cells may become a relevant source for such experiments (145). However, the holy grail of quantitative proteomics is to metabolically incorporate stable isotopes into intact humans. Heavy nitrogen would be an excellent candidate because there are delightful products for everybody including vegetarians. The menu consists of plants, rice, potatoes, insects, and rodents, but would we find volunteers?

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