Chapter 11

Protein Turnover Methods in Single-Celled Organisms: Dynamic SILAC

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Abstract

Early achievements in proteomics were qualitative, typified by the identification of very small quantities of proteins. However, as the subject has developed, there has been a pressure to develop approaches to define the amounts of each protein – whether in a relative or an absolute sense. A further dimension to quantitative proteomics embeds the behavior of each protein in terms of its turnover. Virtually every protein in the cell is in a dynamic state, subject to continuous synthesis and degradation, the relative rates of which control the expansion or the contraction of the protein pool, and the absolute values of which dictate the temporal responsiveness of the protein pool. Strategies must therefore be developed to assess the turnover of individual proteins in the proteome. Because a protein can be turning over rapidly even when the protein pool is in steady state, the only acceptable approach to measure turnover is to use metabolic labels that are incorporated or lost from the protein pool as it is replaced. Using metabolic labeling on a proteome-wide scale in turn requires metabolic labels that contain stable isotopes, the incorporation or loss of which can be assessed by mass spectrometry. A typical turnover experiment is complex. The choice of metabolic label is dictated by several factors, including abundance in the proteome, metabolic redistribution of the label in the precursor pool, and the downstream mass spectrometric analytical protocols. Key issues include the need to control and understand the relative isotope abundance of the precursor, the optimization of label flux into and out of the protein pool, and a sampling strategy that ensures the coverage of the greatest range of turnover rates. Finally, the informatics approaches to data analysis will not be as straightforward as in other areas of proteomics. In this chapter, we will discuss the principles and practice of workflow development for turnover analysis, exemplified by the development of methodologies for turnover analysis in the model eukaryote *Saccharomyces cerevisiae*.

Key words: Metabolic labeling, amino acids, mass spectrometry, stable isotope, cell culture, yeast metabolism, protein synthesis, protein degradation, turnover rate.

1. Introduction

Metabolic incorporation of stable isotope labels is a fundamental methodological approach in comparative proteomics, particularly

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applied to isolated cells grown in culture and most commonly known as SILAC (stable isotope labeling with amino acids in cell culture) experiments (1). In SILAC, one cell culture is labeled with, for example, an amino acid in which every carbon atom is carbon-13 (^{13}C, "heavy", abbreviated H), and a second culture is labeled with the same amino acid in which every carbon atom is carbon-12 (^{12}C, "light", abbreviated L). Often the labeled cells are grown under normal conditions, and the corresponding unlabeled cells are exposed to a differing physiological, or pathological, environment. This has the advantage of a single control sample for multiple perturbed samples, without the need for additional expensive labeled amino acids. Conversely, the perturbed samples could be labeled and compared to a single unlabeled control. Moreover, different isotopes, such as [$^{13}C_6$]arginine and [$^{13}C_6$][$^{15}N_4$]arginine, allow for a degree of multiplexing (the first is 6 Da heavier than the unlabeled counterpart and the second is 10 Da heavier). The two (or more) cell cultures are combined, post-labeling, and the mixture subjected to downstream processing for proteomics. Subsequently, every peptide that contains at least one instance of that amino acid will appear as a H–L doublet in mass spectrometric analysis. The relative intensities of the H and L ions will indicate the relative expression of the protein when the cell cultures are compared. A fundamental goal that is implicit in the SILAC approach is that the labeling is complete – each label can only report on the entire population of proteins. In practice, this is not formally necessary, but with single cells grown in culture, this is attainable and makes downstream analysis much simpler.

Thus, an assumption with SILAC experiments is the completeness of labeling of the control culture. If any unlabeled proteins remain, their constituent peptides will add to the peak intensity of the peptides representing protein from the unlabeled culture, and therefore peak ratios will not indicate true biological protein abundance ratios without more detailed analysis. Incomplete labeling, whether through the use of partially labeled precursors or as a consequence of sampling cells before they are fully labeled, can compromise a SILAC experiment if not spotted or complicate the data analysis if known. In this regard, a rapid method of checking incorporation is valuable (2).

In marked contrast, proteome-level turnover measurements differ from standard comparative proteomics experiments in one fundamental way – the proteins *cannot* be fully labeled, as it is the time dependence of the process of labeling of each protein that defines the turnover rate. We have referred to such studies as "dynamic SILAC" experiments (3) (for a summary of the few studies of this type, *see* **Table 11.1** and references therein). Although single time point experiments are possible, the preferred experiments are those in which the proteome is sampled

Table 11.1
Summary of current literature detailing turnover experiments using stable isotope labels with cells in culture

Organism	Label	Labeling protocol	Analytical protocol	First author (ref.)	Date
Saccharomyces cerevisiae (BY4743)	DL-[$^{2}H_{10}$]Leucine	Glucose-limited chemostat culture. Once cells were fully labeled with leucine, the growth medium was changed to contain unlabeled leucine. Samples taken regularly during the light chase.	2D SDS-PAGE gel followed by in-gel tryptic digestion and MALDI-ToF MS analysis	Pratt (4)	2002
Escherichia coli (K-12)	[$^{13}C_6$]Glucose	Cells were grown in unlabeled growth medium and harvested 30 min after addition of labeled glucose.	1D SDS-PAGE gel followed by in-gel tryptic digestion. Analysis using MALDI-ToF/ToF and LCQ ion-trap MS	Cargile (5)	2004
HeLa cells	^{15}N-enriched medium	Cells were cultured in natural isotope containing medium for 2 days, then transferred to ^{15}N-enriched growth medium. Samples collected at intervals after transfer. Experiments included two heat-shock exposures of 30 min.	2D SDS-PAGE gel followed by in-gel tryptic digestion and MALDI-ToF/ToF analysis	Gustavsson (6)	2005

(continued)

Table 11.1 (continued)

TAP-tagged yeast	TAP tag	Cells grown to exponential (log) phase. Samples collected following cycloheximide treatment after 0, 15, and 45 min.	SDS-PAGE and Western blotting followed by chemiluminescence at three exposure times using a CCD camera	Belle (7)	2006
Mycobacterium smegmatis (mc2 155)	[^{15}N]Ammonium sulfate	*For acid shock*: cultured at mid-log phase doped with [^{15}N]ammonium sulfate and grown for one doubling time before harvesting. *For iron starvation*: cells grown in heavy medium and harvested at mid-log phase. Cell pellets then resuspended in medium containing ^{14}N and harvested after one doubling time.	Tris–HCl SDS-PAGE gel followed by in-gel digestion and nanoLC/LTQ-FT	Rao (8)	2008
Human adenocarcinoma A549 cells	L-[$^{13}C_6$]Arginine	Cells grown in labeled medium for 13 days, then transferred to unlabeled medium. Samples taken regularly.	GeLC-MS using 1D SDS-PAGE and analysis using LTQ ion-trap	Doherty (3)	2009

along the labeling trajectory, to allow the rate of change in labeling profile to be assessed (3–8).

1.1. Fundamental Structure of a Turnover Experiment

Because turnover can operate even in the absence of any change in the protein pool size, it follows that tracer labels must be used to measure flux through the protein pool. Methods that measure regression of a protein pool after poisoning of protein synthesis with cycloheximide, for example, are of limited value; the toxic effects limit the time frame over which loss of protein in the absence of protein synthesis is physiologically relevant. As such, tracer methods to follow turnover in the steady state are best used with living cells. Turnover experiments invoke an added degree of complexity such that comparative turnover analyzes have yet to become routine, and in the first instance, preliminary studies have concentrated upon building a profile of turnover rates for individual proteins. In all cases, the experimental outline is essentially the same.

Protein turnover can be monitored in two different ways using the same general approach: labeling to completeness followed by a "light" chase using non-labeled amino acid, or alternatively labeling over time from the initial unlabeled culture. In the latter, unlabeled (L) cells are exposed to the heavy labeled (H) precursor, and the incorporation of the heavy isotope reflects the rate of synthesis. Alternatively, the cells can be pre-labeled with the H precursor, and at the start of the turnover experiment, the precursor is swapped to the L counterpart. Although the two experiments are formally equivalent, there are some advantages of taking the latter approach, related to the need for control of the relative isotope abundance (RIA) of the precursor (9, 10). In a turnover study, the transition of the precursor pool from labeled to unlabeled, or vice versa, is an obligatory component. Ideally, this transition will be from an RIA of 1 to an RIA of 0 (a "light chase"), or from an RIA of 0 to an RIA of 1 (a "heavy chase"). Whilst not essential, management of the transition such that it is between 0 and 1, whether in a light or a heavy chase experiment, simplifies the analysis and maximizes the range of labeling. One incidental benefit of a light chase experiment is cost; an excess of amino acid is required to ensure that any labeled amino acid returned to the precursor pool from protein degradation and then reincorporated is negligible, thus rapidly fixing the RIA at 0. If this excess is prepared from unlabeled precursor, the costs will be dramatically lower.

1.2. Overall Experimental Strategy

As metabolic labeling involves the incorporation of a stable isotope label into newly synthesized proteins in vivo, it also enables the rate of protein synthesis, not just change in abundance, to be monitored by assessing the peak intensity ratio between corresponding labeled and unlabeled peptides over time. Any peptide

that could contain the label can be used as a surrogate to measure turnover for the entire protein, as turnover kinetics are the same for all peptides from the same protein (3).

1.3. Choice of Label

As stable isotopes are chemically identical to their non-labeled counterparts, they respond identically in a mass spectrometer so that if peptides containing either the labeled or non-labeled forms are present in the same quantity in a sample, they will have the same intensity in a mass spectrum. The most common stable isotope labels used are ^{13}C, ^{15}N, and ^{2}H, which are often integrated into amino acids or other compounds such as glucose (11). The benefit of using a stable isotope-labeled amino acid, such as [$^{13}C_6$]arginine or [$^{2}H_{10}$]leucine, instead of glucose, is that the mass offset created by the label is constant and therefore H–L pairs are easy to identify. When [^{15}N]H_4Cl is used as the sole source of nitrogen, all amino acids incorporate the label at the α-nitrogen position, and those with nitrogen-containing side chains (Asn, Gln, His, Trp, Lys, and Arg) will also incorporate different numbers of heavy nitrogen atoms into the side chain. The outcome is that peptides of differing chain length and amino acid composition have different mass offsets from the unlabeled counterpart. Compare this complexity of labeling to the use of [$^{13}C_6$]arginine, where a peptide containing a single arginine residue will always show a 6 Da separation between peaks representing the labeled and unlabeled forms. A di-arginine peptide will show a 12 Da separation and so on.

Similar is true of deuterated amino acids, but transamination of the α-carbon deuteron can lead to a mass shift less than that expected. For example, [$^{2}H_{10}$]leucine can be transaminated and lose the label at the α-carbon atom, generating a 9 Da difference between labeled and unlabeled peptides. Additionally, hydrogen is more hydrophilic than deuterium, which can lead to elution of peptides labeled with [^{2}H]amino acids from reversed-phase chromatography columns before their unlabeled counterpart. This could result in inaccurate intensity ratios between H–L pairs, although the deuterium interaction with the column is dependent on the properties of the other amino acids in the sequence of the peptide (12).

Stable isotope-labeled precursors are not isotopically pure, and isotope purities of 98.5–99%, often referred to as 98.5 or 99APE (atom percent excess), are typical. For the incorporation of a single labeled amino acid, such as [$^{13}C_6$]arginine, every peptide containing a single arginine residue will, even if "fully" labeled with the heavy isotope, demonstrate about 1% ion intensity from the unlabeled contaminant. In most circumstances this is not a major issue, although it could introduce quantitative errors when only a few percent of unlabeled peptide is present in the analyte. By contrast, nitrogen labeling is more complex

and requires analysis and correction. To illustrate, the peptide LVFHSASTEDNNQLIMEGR has the elemental composition $C_{91}H_{145}N_{27}O_{32}S_1$ and thus at an isotopic purity of 99% ^{15}N (therefore 1% ^{14}N), there are 27 different ways to construct a peptide with a single "light" nitrogen atom, all of which have a mass 1 Da less than the monoisotopic true heavy labeled peptide. This peak is therefore about 27% of the height of the fully labeled peptide, representing a significant portion of the heavy synthesized material, and has to be factored into calculations.

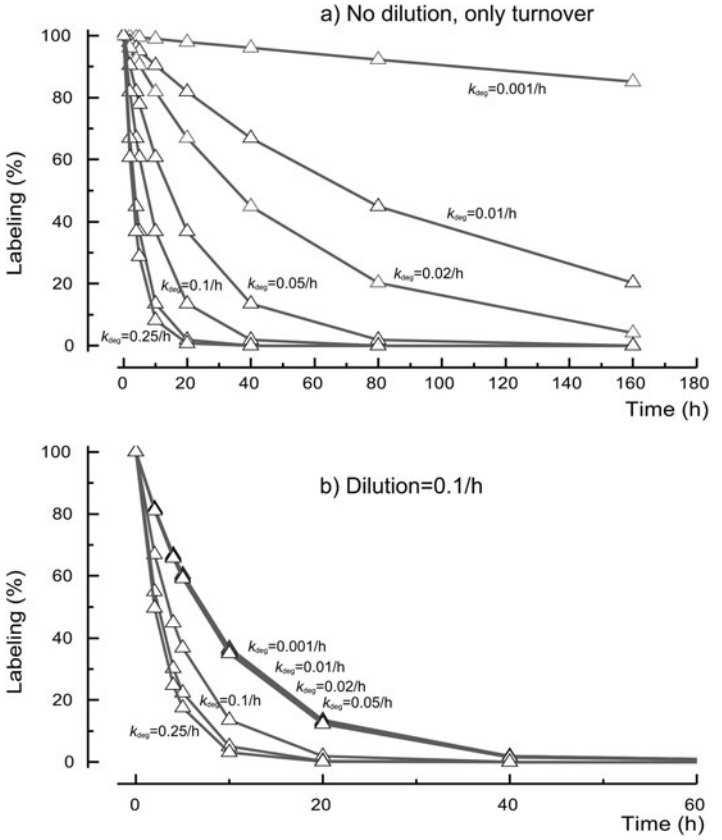


Fig. 11.1. Accurate determination of degradation rate requires multiple points to define the rate of loss of material, and the range of degradation rates that are anticipated defines the sampling strategy (**Panel a –** degradation in the absence of any pool expansion). However, if the system is growing rapidly (e.g., cells in exponential growth or a chemostat operating at a dilution rate of 0.1/h), then the change of labeling within any protein is a combination of degradation and dilution (**Panel b**). The outcome of this complication is that lower degradation rates are not readily discerned, yielding similar rates of loss of labeling irrespective of degradation rate, and therefore placing extra demands on the quality of isotopic data. In this example, it is likely that only those proteins with degradation rates more than two times the dilution rate would yield satisfactory degradation rates.

1.4. Limitations of Sampling Frequency

To access quantitative data on proteins that are turned over within minutes, samples must be taken rapidly and at very early time points in the chase. How rapidly this can occur is determined mostly by the practicalities of sampling and of arresting the cells metabolically. Moreover, sampling usually disrupts the mixing and aeration in batch culture, and thus the cells are changed. A continuous chemostat culture has the advantage that the eluted cells can be collected, but large samples will be collected over a significant time window. To illustrate, a 1 L chemostat culture operating at a dilution rate (flow/volume ratio; $D=F/V$) of 0.1/h will produce 100 mL of cell suspension/h, or 1.6 mL/min. If biomass equivalent to 20 mL of cells were required, cells would have to be collected for 15 min, during which time some high turnover proteins could be fully replaced. Sampling frequencies should be geometrically distributed in order to cover the broadest range of degradation rates. For example, a sampling regimen of 0, 2, 5, 10, 20, 40, 80, 160 min will yield at least five data points containing between 100 and 5% undegraded material for all rates of degradation from 0/min (no degradation) to 0.25/min: a half life of 2.7 min. A second complication derives from growth. If cells are actively growing (such as in exponential phase), then proteins are lost from cells in part by degradation and in part by dilution into daughter cells. This sets a limit on the range of degradation rates that can be reliably assessed (**Fig. 11.1**). Low turnover proteins will transition from labeled to unlabeled (or vice versa) mostly by dilution, and the closer the degradation rate is to zero, the more difficult it will to be to resolve losses due to degradation from those due to dilution.

2. Materials

2.1. Labeling of Cells in Culture

1. This chapter aims to give details on the analysis of proteomic data to determine protein turnover rates in yeast; for a more comprehensive guide to culture methodology, please refer to other chemostat culture methods, also (3, 8, 13).
2. Downstream analysis will be simplified if a yeast strain auxotrophic for the amino acid to be labeled is used, e.g., BY4743, a leucine auxotroph. This is not critical however, especially if an essential amino acid is chosen, as the organisms may be able to use the amino acid provided in the growth medium (in labeled form) (8) and not dilute the pool with biosynthesis de novo.
3. Additional materials required include the stable isotope-labeled amino acids, for example, [$^{2}H_{10}$]leucine or

[$^{13}C_6$]lysine and [$^{13}C_6$]arginine (Cambridge Isotope Laboratories) and the corresponding unlabeled amino acid (Sigma-Aldrich) (*see* **Notes 1** and **2**):

i. For a "light" chase experiment, 100 mg stable isotope-labeled amino acid is added to the culture for the initial labeling phase.

ii. The unlabeled amino acid is then added (1 g/50 mL medium) to the culture and the inflowing medium changed to also contain unlabeled amino acid (50 mg/L).

2.2. Harvesting Soluble Proteins

1. To suspend protein synthesis at the instant of collection, cycloheximide (Sigma-Aldrich, freshly prepared in water or buffer at neutral pH) is added to each sample taken at a final concentration of 100 μg/mL.
2. Before lysis, 20 mM HEPES (pH 7.5) (Sigma-Aldrich) containing 1 protease inhibitor cocktail tablet (EDTA-free) (Roche Diagnostics) per 10 mL is required.
3. Glass beads (Sigma-Aldrich) are used to lyse the cells.
4. Make solutions of DNase and RNase (Sigma-Aldrich) at 1 mg/mL.

2.3. Sample Preparation

1. Ammonium bicarbonate (50 mM; Analar grade) is required as the buffer for tryptic proteolysis. For best results, all solutions used here should be made fresh.
2. For in-solution proteolysis, a detergent is added to the sample to denature the proteins present. RapiGest^{TM} surfactant (Waters) is made up to 1% (w/v) using 50 mM ammonium bicarbonate.
3. Reduction of disulfide bonds between cysteine residues during in-gel and in-solution proteolysis is achieved using a final concentration of 10 and 3 mM dithiothreitol (DTT) (Sigma-Aldrich), respectively, in 50 mM ammonium bicarbonate. For the in-solution protocol outlined below, this is achieved with a 9.2 mg/mL DTT starting solution.
4. Alkylation with final concentration of 60 and 9 mM iodoacetamide (IAA) (Sigma-Aldrich) in 50 mM ammonium bicarbonate is also required. The 9 mM IAA for in-solution proteolysis requires a 33 mg/mL starting solution.
5. For in-gel proteolysis, 25 mg lyophilized trypsin (Roche Diagnostics) is resuspended in 250 μL of 50 mM acetic acid. This is then diluted 1:9 with 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.01 μg/μL.
6. For the in-solution digestion protocol, 125 mL of 10 mM acetic acid is used to resuspend the trypsin, which is then

added to the sample for proteolysis at 0.2 μg/μL concentration at a 1:50 trypsin:protein ratio.

7. All other solutions (acetonitrile and trifluoroacetic acid) are obtained from VWR.

2.4. Mass Spectrometry (MS)

1. For matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) MS, the matrix α-cyano-4-hydroxycinnamic acid should be made up to 8 mg/mL using 60% acetonitrile and 1% trifluoroacetic acid. Best results are obtained when 1 μL sample is dried onto the MALDI target and then 1 μL matrix is added and allowed to crystallize.

The buffers used for reversed-phase liquid chromatography (RP-LC) are made from HPLC-grade acetonitrile and HPLC-grade water (VWR) plus 0.1% formic acid. A linear gradient is most commonly used, for example, 0–50% buffer B over 30 min, when using a 75 μm capillary reversed-phase (C18) column.

3. Methods

3.1. Labeling of Cells in Culture

The main details covered in this section will not be the growing of the yeast but the subsequent mass spectrometric analysis of labeled peptides following protein isolation from the yeast cells. However, the basic method for a protein turnover experiment, using deuterium-labeled leucine as the stable isotope-labeled amino acid, is detailed below (3, 13) (*see* **Note 3**):

1. Grow cells in a glucose-limited chemostat culture using complete synthetic medium, supplemented with 100 mg DL-[$^{2}H_{10}$]leucine (98.5APE) at a dilution rate of 0.1/h (*see* **Note 4**):
   i. To ensure cells are fully labeled, maintain cells in this growth medium for at least seven doubling times (*see* **Note 5**).
   ii. Following this, add the 50 mL solution of unlabeled L-leucine to the culture and change the inflowing medium to also contain unlabeled L-leucine (50 mg/L).
   iii. Begin sampling of the yeast cells at appropriate time points, e.g., 0, 10, 40 min, and then continue with fairly regular sampling (e.g., seven samples every 1–2 h for the first 12 h) reducing frequency until the two final samples at approximately 24 and 51 h.
   iv. If the chemostat is sampled directly, and large volumes are removed, the apparent dilution rate will be modified (*see* **Note 6**).

3.2. Harvesting Soluble Proteins

1. When cells (40 mL) reach an A_{600} of about 1.6, collect into ice-cold Falcon tubes containing cycloheximide (100 μg/mL final concentration).
2. Centrifuge at 4°C for 5 min at 7,000 × *g* and discard the resulting supernatant.
3. Resuspend the pellet in 1 mL ice-cold double distilled water and transfer to 1.5 mL microcentrifuge tube.
4. Centrifuge again at 16,000 × *g* and discard the supernatant:
   i. Pellets can be stored at –80°C at this point.
5. Resuspend the pelleted cells in 300 μL of 20 mM HEPES, pH 7.5, plus protease inhibitor solution.
6. Vortex with glass beads to lyse cells in six bursts of 45 s (allowing 45 s cooling).
7. Add 6 and 2 μL of the DNase and RNase solutions, respectively.
8. Incubate at 4°C for 1 h.
9. Centrifuge the cell lysate at 4°C for 10 min at 2,500 × *g* and collect the supernatant.

3.3. Sample Preparation

For ease of analysis, and to increase information gained, the sample must be separated before analysis using mass spectrometry. Gel-based methods can be used, with separation of the proteins by 1D or 2D SDS-PAGE before in-gel proteolysis; if a gel-free approach is taken, an in-solution proteolysis step usually precedes separation.

3.3.1. In-Gel Proteolysis Following Gel-Based Separation

1. Excise spots corresponding to the same protein from each gel and perform standard in-gel digestion technique:
   i. De-stain gel plug of protein spot with 25 μL of 1:1 acetonitrile:50 mM ammonium bicarbonate ammonium bicarbonate for 15 min or until all color removed.
   ii. Reduce disulfide bonds between cysteine residues using 25 μL of 10 mM DTT. Incubate at 60°C for 60 min, then discard the liquid.
   iii. Alkylate with 25 μL of 60 mM IAA. Incubate in the dark at room temperature for 45 min, then discard the liquid.
   iv. Dehydrate the gel plug by incubating at 37°C in 10 μL acetonitrile, leaving the microcentrifuge tube lid open. After 15 min, if the gel plug is white, remove the remaining liquid and leave for a further 10 min.
   v. Rehydrate the gel plug in 10 μL of 0.01 μg/μL trypsin solution and incubate overnight at 37°C.

3.3.2. In-Solution Proteolysis for Gel-Free Separation

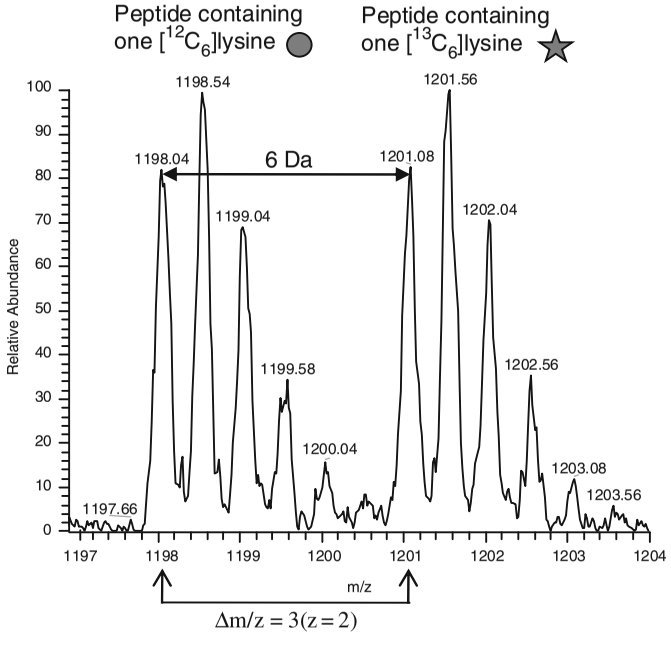
1. To 100 μg protein in 160 μL (final volume) of 50 mM ammonium bicarbonate, add 10 μL of 1% (w/v) RapiGest^{TM} surfactant. Incubate at 80°C for 10 min.
2. Add 10 μL of 3 mM (final concentration) DTT and incubate at 60°C for 15 min.
3. Alkylate with 10 μL of 9 mM (final concentration) IAA for 30 min in the dark.
4. To this add 10 μL of 0.2 μg/μL trypsin and incubate overnight at 37°C.
5. Inactivate the detergent by the addition of 1 μL trifluoroacetic acid (0.5%, v/v, final concentration) and incubate for 45 min at 37°C.
6. Before analysis, remove all precipitation formed at this stage by centrifugation.

3.4. Mass Spectrometry (MS)

If a gel-based strategy is used, analysis of individual protein spots by MALDI-ToF MS is sufficient to acquire data for individual proteins, which also avoids any possible complications introduced by the differential elution of deuterium-labeled peptides. An in-solution tryptic digest of the whole-cell lysate is however too complex to analyze by MALDI-ToF MS alone. Online liquid chromatography (LC) separation into an electrospray (ESI) mass spectrometer, such as a Q-ToF or linear ion-trap instrument, is preferred. Alternatively, offline LC followed by MALDI-ToF MS can be used, taking care to combine all spectra for labeled and unlabeled peptides:

1. Acquire protein identification data:
   i. For MALDI-ToF MS analysis, peptide mass fingerprinting (PMF) will identify the protein present in the spot.
   ii. Analysis using ESI-MS can take advantage of MS/MS capabilities and sequence information can also be obtained for database searching (*see* **Note 7** and **Fig. 11.2**).
2. The protein identifications can then be used to validate H–L peptide pairs, separated by a mass difference corresponding to the number of labeled amino acids present (*see* **Note 8**). The number of labeled amino acids present can also be used to confirm and support the protein identification (14).
3. Record isotopomer peak intensities for MS peaks representing L and H peptides or the corresponding peak areas from extracted ion chromatograms (*see* **Notes 9** and **10**).
4. The RIA of the label in each specific protein at each time (RIA_t) is calculated as follows:

$$RIA_t = \frac{\text{Labeled isotopomer}}{\text{Labeled isotopomer} + \text{Unlabeled isotopomer}} = \frac{H}{H + L}$$



1. P32497|EIF3C_YEAST **Mass:** 93375 **Score:** 365 **Queries matched:** 5 **emPAI:** 0.12
Eukaryotic translation initiation factor 3 subunit C OS=Saccharomyces cerevisiae GN=NIP1 PE=1 SV=2

☐ Check to include this hit in error tolerant search or archive report

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	228	1198.04	2394.07	2394.18	-0.11	0	(110)	5.4e-010	1	K.DQLDSADYVDNLIDGLSTILSK.Q ●
☑	233	1201.53	2401.05	2400.20	0.85	0	118	7.4e-011	1	K.DQLDSADYVDNLIDGLSTILSK.Q + (PFG)_6HeavyLys (K) ★

Fig. 11.2. An example of protein identification from MS/MS data searched using the MASCOT search engine with [$^{13}C_6$]lysine set as a variable modification in the search parameters. The data available from the search enables an extracted ion chromatogram to be obtained to assess peak area or a zoom scan (shown) to obtain peak intensity values from the isotopomer envelope.

5. Plot these values over time for each protein.
6. Using non-linear curve fitting, the rate of loss of the label (k_{loss}) can be calculated from the gradient of the line when the RIA at the beginning (RIA_0) and the end (RIA_∞) of the experiment are taken into consideration:
   i. RIA_0 can be determined using a random selection of proteins taken before the start of the light chase. Although this RIA should be 1, it is possible that the value may be slightly less due to the purity of the label used and degradation of unlabeled proteins releasing amino acids into the precursor pool.
   ii. The RIA of the culture at the end of the labeling period can be taken as 0 as long as there has been more than seven doubling times (but this should be directly observable).
   iii. When $RIA_\infty = 0$, $RIA_t = RIA_0 \times \exp^{-kt}$, where $k = k_{loss}$.
7. From this it is possible to determine the rate of protein degradation (k_{deg}) by factoring in the effect of the rate of dilution (k_{dil}) into the value of k_{loss}, i.e., $k_{deg} = k_{loss} - k_{dil}$:
   i. As k_{dil} is constant, it does not affect the error in the parameter estimates.
8. It is also possible to measure the rate of loss, and thence, turnover from a single time point t using the equation $k_{loss} = (-\log_e(RIA_t/RIA_0))/t$.

   If single time point experiments are to be used, an increased number of peptides per protein would be recommended to decrease the error. However, a true time course is valuable as it defines the trajectory of the loss of label from the proteins and, for example, allows it to be assessed as formally first order.

4. Notes

1. Stable isotopically labeled compounds are often named informally, according to the nomenclature such as [$^{13}C_6$]lysine. In this instance, there are only six carbon atoms and therefore, all six atoms must be replaced – the compound is referred to as (U-$^{13}C_6$ lysine) where "U" refers to "uniformly labeled". However, it is also possible to purchase labeled lysine variants such as "2-^{13}C, 99%; ε-^{15}N, 99%" that is only labeled at the α-carbon atom and the side chain nitrogen atom. Care must be taken in defining exactly the precursor form being used.

2. Trypsin is the endopeptidase most commonly used to generate peptides for mass spectrometric analysis and has a clearly defined specificity (cleavage at Arg-X and Lys-X, Arg-Pro and Lys-Pro excepted). Labeling with [$^{13}C_6$]arginine and [$^{13}C_6$]lysine has the advantage that almost all tryptic peptides are identically labeled with the +6 Da variant. The exceptions are peptides that contain internal arginine or lysine residues, either because of missed cleavages or because the peptide contains the uncleavable Arg-Pro or Lys-Pro bonds.
3. Experiments, especially in chemostat culture, that use stable isotope-labeled amino acids can be costly. There is merit in performing an entire experiment as a "dry run" using unlabeled amino acids throughout. In a perfect experiment, the switch from heavy to light amino acid (or vice versa) will be near instantaneous, complete and have no effect on the growth rate or gene expression profile of the organism. In particular, a dry run would identify the bottlenecks in the period of rapid sampling and allow appropriate measures to be taken to avoid these bottlenecks.
4. L-amino acids are the forms that are metabolically active and the precursors for protein synthesis. To reduce costs further, it is possible to use the DL racemic mixture of a labeled amino acid, and to assume that label from the D-amino acid does not make a significant contribution to the labeling pattern.
5. It is possible that during exponential growth, some proteins have zero degradation, and thus, label can be incorporated into the protein only during pool expansion, i.e., growth. After each doubling time, half of that protein in each cell would have been replaced by newly synthesized proteins containing the stable isotope-labeled amino acid from the growth medium. Therefore, after one generation, at least 50% of the proteins will be labeled, after two generations 75% etc., until after seven generations over 99% of proteins with the slowest rate of turnover will contain a heavy amino acid.
6. The changes in dilution rate can be caused by the removal of cells from a chemostat culture during sampling. When a set volume is removed from the culture, the rate of cell loss from the outflow is reduced, while the volume is replenished by new medium. The remaining cells therefore remain in the chemostat system for an extended period of time. This can be avoided by sampling directly from the culture outflow, as long as the flow rate is fast enough

to deliver sufficient cells for analysis, or by using a batch culture.

7. Proteomic search engines (MASCOT, SEQUEST, etc.) allow the use of variable modifications when analyzing mass spectrometric data. If the chosen labeled amino acid is set as a variable modification when identifying proteins using these search tools, a comprehensive list indicating labeled and unlabeled peptides will be created which helps to reduce analysis time (**Fig. 11.2**). In addition, some software, such as ProteinLynx Global Server (Waters), will also give the intensity values of the identified peaks.
8. Fully deuterated amino acids are relatively inexpensive but will be labeled at the α-carbon atom. This atom is metabolically labile, and the deuteron would be lost by the reversible process of transamination. In our experience, the α-deuteron of [$^{10}H_2$]leucine was completely lost in a yeast labeling experiment, and turnover was assessed by a 9 Da separation between unlabeled and single labeled peptides.
9. A critical step in any turnover study is the need for an instantaneous transition as the experiment is switched between the two labeled variants. Two things can go wrong here; the changeover might be slow or the changeover might be incomplete. If the changeover is incomplete, the precursor pool will possess an RIA that is neither zero nor unity. In this instance, some of the precursor pool will be labeled and some unlabeled. This is detectable by the inability of the labeling curve to move between 0 and 1, or vice versa, and by the appearance of intermediate labeled forms of peptides where the peptide contains two instances of the amino acid. It is advisable to deliberately look at such peptides to ensure that there is complete replacement of the precursor pool.
10. There are a number of bioinformatic tools that have been created to automatically acquire information regarding peak intensities and relative abundances in stable isotope-labeled samples (not discussed in this review), each with their advantages and disadvantages (15).

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