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Stable isotope labeling tandem mass spectrometry (SILT): Integration with peptide identification and extension to data- dependent scans

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Abstract

Quantitation of relative or absolute amounts of proteins by mass spectrometry can be prone to large errors. The use of MS/MS ion intensities and stable isotope labeling, which we term stable isotope labeling tandem mass spectrometry (SILT), decreases the effects of contamination from unrelated compounds. We present a software package (SILTmass) that automates protein identification and quantification by the SILT method. SILTmass has the ability to analyze the kinetics of protein turnover, in addition to relative and absolute protein quantitation. Instead of extracting chromatograms to find elution peaks, SILTmass uses only scans in which a peptide is identified and that meet an ion intensity threshold. Using only scans with identified peptides, the accuracy and precision of SILT is shown to be superior to precursor ion intensities, particularly at high or low dilutions of the isotope labeled compounds or with low amounts of protein. Using example scans, we demonstrate likely reasons for the improvements in quantitation by SILT. The appropriate use of variable modifications in peptide identification is described for measurement of protein turnover kinetics. The combination of identification with SILT facilitates quantitation without peak detection and helps to ensure the appropriate use of variable modifications for kinetics experiments.

Keywords

Quantitative mass spectrometry; algorithm; software; kinetics; error analysis

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Note to editors and reviewers: A review-only version of SILTmass is included as a supplementary material in a zipped archive. Instructions for running the program are included in the archive. The program will stop functioning after August 17, 2008.

Financial Disclosure and Conflict of Interest

RJB is a co-founder of C2N Diagnostics and is a consultant for Bristol-Myers Squibb and Pfizer. Other authors do not have financial interests or conflicts of interest to disclose.

Supporting information online

A description of the peptide identification method implemented in SILTmass is included in supplementary methods, along with a detailed description of the analysis of fixed and variable modifications and an accompanying figure. A screenshot of the SILTmass GUI showing settings specific to SILT is included as a supplementary figure. Three figures showing example MS/MS scans referenced in the manuscript are included as supplementary materials, along with a table showing the variance found when comparing individual b and y ions.

Introduction

Tandem mass spectrometry has proved to be a powerful technique for the identification of proteins in complex biological samples. However, robust quantitative mass spectrometric techniques have been more elusive.¹ The challenges faced in developing quantitative methods are numerous, including ion suppression,² coelution of unrelated molecules and variability in both low-flow rate chromatography and electrospray. A variety of methods have been developed for quantification, controlling for inherent variability via the number of replicates, the programming of the mass spectrometer and often, but not always, with the use of stable isotopic labels.

Quantification without isotopic labels can be quite robust. Relative protein amounts can be quantified by comparing precursor ion currents for particular peptides in multiple chromatographic runs.^{1,3} With spectral counting, the relative abundance of a particular protein in a data-dependent scan is derived from the number of MS/MS scans matched to a particular protein.^{4,5} Label-free methods perform well in head-to-head comparison with other methods.^{1,6}

Nonetheless, the use of stable isotope labels in mass spectrometry should minimize the effects of run-to-run variability by comparing signals in adjacent MS/MS scans within a single chromatographic run. Stable isotopes can be incorporated into proteins through chemical or metabolic labeling. With chemical labeling, cysteine groups (e.g. ICAT) or lysine groups (e.g. ICPL and iTRAQ) are commonly targeted.^{7–11} Metabolic labeling of proteins can be accomplished by incubating cultured cells with isotope-labeled amino acids (e.g. SILAC).¹² If metabolic labeling of protein is not feasible, peptides may be chemically synthesized with isotope-labeled amino acids (AQUA).¹³ Typically, the elution peak for the peptide of interest is detected and the ion currents of the precursor (parent) ions are summed (integrated) over the elution period.³ The integrated precursor ion currents are then compared for the labeled and unlabeled peptides.

While the integrated ion current of a precursor ion should be highly correlated to the relative protein or peptide amounts, these peaks may also contain signals from unrelated compounds. Precursor ion intensities may also depend on the quality/reproducibility of chromatography and electrospray ionization, potentially affecting comparisons between chromatographic runs. The intensities of the b and y fragment ions in the MS/MS spectra of isotope-labeled peptides can yield precise and accurate results across a wide range of isotope labeling ratios.^{6,14–18} We call the analysis method Stable Isotope Labeling Tandem mass spectrometry (SILT), to avoid confusion with other MS/MS quantitation techniques such as iTRAQ.

Application of the SILT method has perhaps been limited by the extensive processing required to implement the method, although an automated analysis program, Census, has recently been released that extracts chromatograms from fragment ions.⁶ Our prior use of the SILT method was limited to ion-specific experiments, with a single peptide analyzed per chromatographic run. In a typical ion-specific experiment, all of the MS/MS scans in a chromatographic run alternated between the unlabeled peptide and singly-labeled peptide. Using the SILT method, the peptide sequence, known *a priori*, was used to predict the m/z of b and y ions for the labeled and unlabeled peptides. The elution peak for the peptide of interest was manually identified in the chromatogram and the intensities at the m/z of the predicted b and y-ions in the selected MS/MS spectra were analyzed in Microsoft Excel. The total intensities of b and y ions for the unlabeled and isotope-labeled peptide were used to determine the ratio of unlabeled to labeled peptide with high accuracy.¹⁸

We describe an extension of the SILT method to data-dependent scans, using a custom-written program (SILTmass). SILTmass performs both peptide identification (by ion

matching^{19–25}) and SILT analysis automatically on tandem mass spectrometric data input in the mzXML format²⁶. Using data-dependent scans, the labeling of multiple proteins can be quantified in a single chromatographic run, allowing the SILT method to be applied to more traditional proteomics experiments, including SILAC-style experiments. One major focus of the software development effort, however, is the analysis of protein synthesis and degradation kinetics in animals, including humans,¹⁷ which requires specific mass spectrometric protocols and the judicious use of variable modifications in peptide identification. Overall, SILTmass provides a generalized method for the absolute and relative quantitation of protein labeling with a high degree of accuracy even for complex protein mixtures.

Methods

Production of ¹³C-ApoE standards

Immortalized murine astrocytes expressing human ApoE3 were used to prepare ¹³C-leucine-labeled ApoE standards. Cells were grown to near confluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and geneticin (G418, Invitrogen) as previously described.²⁷ The medium was changed to serum-free DMEM/Ham's F-12 lacking leucine, supplemented with 105 mg/L leucine (0–20% ¹³C₆-leucine, 98% ¹³C₆, Cambridge Isotopes) and 1% N-2 supplement (Invitrogen).²⁷ Medium was collected every 24 h and replaced with fresh serum-free medium, for 2 days.

Isolation of ApoE and digestion

WUE4, an anti-human ApoE monoclonal antibody was coupled to CNBr-activated Sepharose beads per the manufacturer's instructions (GE Healthcare). ApoE was isolated from 1 mL of cell culture media with WUE4-beads (100 µL, 1:1 beads: phosphate-buffered saline) in the presence of protease inhibitors leupeptin and aprotinin (Calbiochem). Samples were rotated overnight at 4 °C. The beads were then centrifuged at 16,000 RCF and the supernatant was removed. The beads were washed with 1 mL phosphate-buffered saline three times, and then washed three times with 1 mL 25 mM ammonium bicarbonate (pH 7.8). After the final wash, the beads were resuspended in 9:1 ammonium bicarbonate: acetonitrile solution. Sequencing grade-trypsin (Promega) in 25 mM ammonium bicarbonate was added (1:50 trypsin to protein by weight) and incubated overnight at 37 °C.

Expression of HepG2 proteins

HepG2 cells were cultured in lysine-free medium (Washington University School of Medicine Tissue Culture Support Center) containing either 105 µg/mL of L-lysine or ¹³C₆, ¹⁵N₂- L-lysine hydrochloride. Cells were initially grown in medium supplemented with 10% dialyzed fetal calf serum and 1 µM all-trans retinoic acid until reaching 50–60% confluency. The culture medium was then switched to serum free medium containing primary hepatocyte growth supplements (Lonza), 1 µM all-trans retinoic acid, and 1 µg/mL aprotinin. Medium was collected every 2 days, supplemented with protease inhibitor cocktail (Roche) and filtered through a 0.22 µm filter (Millipore). The filtered medium was concentrated to about 1 mL using 10K MWCO centrifuge filters and stored at –80°C.

Preparation of ¹³C₆, ¹⁵N₂-HepG2 protein standards

Concentrated isotope-labeled and unlabeled media were mixed into 10 µL volumes at various ratios. Samples were mixed with 10 µL of 2X SDS-PAGE loading buffer, boiled for 5 min at 100°C and separated by SDS-PAGE. Individual bands were excised, reduced with 50 mM TCEP, alkylated with 100 mM iodoacetamide, and in-gel digested with 250 ng of sequence-grade trypsin (Princeton Separations) for 16 h at 37°C. The trypsin reaction was

stopped by the addition of 0.1% TFA in DI water and the peptide solutions were evaporated to dryness in an Eppendorf Vacuufuge. Dried samples were stored at -20°C until analysis by mass spectrometry.

Preparation of H4 APP695 Δ NL cell lysates

H4 APP695 Δ NL neuroglioma cells expressing human A β were used to prepare ^{13}C -leucine-labeled A β standards *in vitro*. Cells were grown until near confluency in DMEM supplemented with 10% dialyzed fetal bovine serum. The cells were then labeled with ^{13}C -leucine as described for ApoE above (0–100% $^{13}\text{C}_6$ -leucine, 98%). Medium containing secreted proteins was collected after 2 and 3 days, replacing with fresh medium. Labeled medium was filtered, pooled, aliquoted and stored at -80°C until use. A β protein was purified from the cell culture medium using a mid-domain anti-A β antibody (HJ5.1) covalently bound to Sepharose beads.

After three days of labeling, about 250 μg of H4 APP695 Δ NL cells were lysed with a sonicator in a 1 mL solution containing 200 mM ammonium bicarbonate, complete protease inhibitor cocktail, 0.8% sodium azide, 4.8 mg of RNase and 2000 U of DNase. The proteins were dried after a BCA quantitation assay and resuspended in 20 μL 8 M urea and 100 mM Tris, pH 8.5, reduced with 4.5 mM TCEP and alkylated with 9 mM iodoacetamide. Excess TCEP was quenched with 4.7 mM DTT and the sample was desalted overnight by dialysis. Lysyl-endoproteinase C was added to the samples at an enzyme: substrate ratio of 1:100 and incubated for 12 h at 37°C , with shaking, followed by sequence-grade trypsin to a final concentration of 1:25 for another 3 h. The fraction of newly synthesized protein was estimated from linear regression of labeling percentages reported by SILTmass, obtained from a series of samples labeled with 0–100% ^{13}C -leucine. Standard errors in the slopes were determined with GraphPad. Significance was determined from confidence intervals ($\alpha = 0.05$), with α corrected for multiple comparisons (Bonferroni correction).

Nano-LC tandem MS (MS/MS2)

Experiments were performed on a Thermo Finnigan LTQ equipped with a New Objective nanoflow ESI source. Peptides were separated by RP-HPLC using an Eksigent 2D-LC nanoflow pump operating in 1D mode at a flow of 200 nL/min. The autosampler temperature was 4°C . Samples (5 μL) were injected onto a New Objective Picofrit column (75- μm diameter) packed to 10 mm with 5 μm XBridge C8 resin (Waters). Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The gradient was 5% B to 50% B in 30 min followed by 50% B to 95% B in 10 min, then, 95% B for 5 min and re-equilibration for 5 min. The LTQ was operated in positive ion mode using a spray voltage of 1.8 kV, and a capillary temperature of 250°C . Data were acquired in top-5, data-dependent acquisition mode with mass tags enabled (either ± 3 or ± 4 m/z) using a collision voltage of 35 V. The collision gas was helium. The LTQ scans were converted to mzXML format using ReAdW: (http://sashimi.sourceforge.net/software_glossolalia.html), 28 which was modified to increase the tolerance for the precursor ion search within the survey scan from 0.05 amu to 0.2 amu.

SILTmass – Data processing

Settings and input files are specified using a graphical user interface (GUI) and console application developed in Microsoft Visual C++ 2008 Express. The SILTmass GUI records the user-specified settings in the Windows registry and launches SILTmass, the console application. SILTmass reads the settings for the analysis from the Windows registry. Data are read from the specified mzXML files using RAMP (http://sashimi.sourceforge.net/software_glossolalia.html), 28 modified to read precursor ion intensities from the mzXML file. The MS/MS data are sorted by the precursor ion m/z in ascending order to expedite

database searching. The charge of a precursor ion for SILT analysis is currently assumed to be +2. For the current analyses, only the human, mouse and bovine sequences in the NCBI non-redundant database were searched. After peptides are identified by ion matching, a probabilistic score is calculated as described previously.^{29, 30} Additional details on the search engine, including a detailed description of the processing of variable modifications, are included as online supplementary methods.

SILTmass – quantitation

SILTmass quantifies the amount of labeled peptides using two methods, depending on the experiment performed (see Figure 1). The first type of experiment applies to a mixture of unlabeled proteins and very highly labeled internal standards, as would be the case in SILAC or AQUA experiments. This analysis is performed if the ‘SILAC/AQUA’ checkbox is selected in the SILTmass GUI. The second type of experiment applies to measurement of the kinetics of protein turnover. In studying kinetics, degradation of older, unlabeled protein may be incomplete and the fraction of labeled amino acid within a cell might be much less than one, particularly *in vivo*. Thus, a statistical distribution of labeled and unlabeled residues will be present for any newly synthesized protein, which will be mixed with older, unlabeled protein. SILTmass is designed to account for the unique aspects of kinetics experiments,^{31–33} which may enhance the measurement of protein synthesis and degradation rates in cell culture, animal models, and human studies.

To calculate the fractional incorporation of isotope-labeled amino acids into a protein, a series of MS/MS scans are recorded for the top five most intense peaks in the survey scan with mass tags enabled. Typically, in the first MS/MS scan ions are fragmented at the precursor ion m/z (i.e. +0 amu relative to the precursor m/z). In the second MS/MS scan, ions are present at the precursor ion m/z plus $1/2 \times \text{SIMD}$, where ‘SIMD’ is the mass difference between the isotope-labeled amino acid used in the experiment and the unlabeled amino acid. However, some complexity exists using mass tags on a Thermo Finnigan instrument running Xcalibur. After a precursor ion is selected, the most intense peak that is *plus or minus* the specified mass tag is selected. The scans are then ordered such that the lower molecular weight precursor ion is always in the first scan, thus the selected precursor ion may be fragmented in the second scan. If the ‘SILT amino acid’ variable modification is used and an intense ion is identified in the second scan, a percent labeling is calculated for this peptide even if no identification is made in the first scan. To avoid confusion, +0 will refer to the peptide in the first scan (the lower m/z scan), regardless of which scan contains the identified peptide.

When the trap is gated at the precursor m/z plus $1/2 \times \text{SIMD}$, doubly-charged peptides containing exactly one isotope-labeled residue will be analyzed. As an example, we will discuss the case in which the isotope-labeled amino acid is $^{13}\text{C}_6$ -leucine, although any isotope-labeled natural amino acid can be analyzed by SILTmass. For peptides with a single leucine, the fractional labeling of protein, p , is calculated as:

$$p = \frac{I_{b,y}^{+3}}{I_{b,y}^{+3} + I_{b,y}^{+0}} \quad [1]$$

where $I_{b,y}^{+0}$ and $I_{b,y}^{+3}$ are the total intensities of the singly and doubly charged b and y ions in the unlabeled peptide and the singly-labeled peptide, respectively. Note that equation 1 actually gives the fraction of labeled protein/peptide in a SILAC/AQUA experiment, while it yields the fraction of tRNA loaded with labeled amino acid in kinetics experiments when only newly synthesized proteins are present. Additionally, p calculated by equation 1, after

division by the fraction of labeled amino acid loaded on tRNA, yields the fraction of newly synthesized protein in a kinetics experiment where protein turnover is not complete.

In a kinetics experiment, if a peptide from a newly synthesized protein contains two leucines, the probability that both leucines are labeled is p^2 , that neither is labeled is $(1-p)^2$ and that one of the two leucines is labeled is $2p(1-p)$. For comparing unlabeled peptide (0 out of 2 leucines labeled) to a singly-labeled peptide (1 out of 2 leucines labeled), the ratio, r , of the total intensities of singly and doubly charged b and y ions in the second scan and the first scan should equal:

$$r = \frac{I_{b,y}^{+3}}{I_{b,y}^{+0}} = \frac{2p(1-p)}{(1-p)^2} = \frac{2p}{(1-p)} \quad [2]$$

Using this formula, p can be calculated from the measured ratio r :

$$p = \frac{r}{(r+2)} \quad [3]$$

With SILAC/AQUA experiments, equation 3 will yield a value close to zero, because very few singly-labeled peptides will be present if the internal standard is highly labeled. Thus, this comparison will not be made if the 'SILAC/AQUA' checkbox is selected in the SILTmass GUI. In a kinetics experiment, equation 3 is useful for determining p if it can be assumed that all of the protein is newly synthesized (i.e. has reached steady state). If the sample contains a mixture of new and old proteins, equation 3 must be corrected using the percent labeling of newly synthesized protein (i.e. fraction of tRNA with labeled amino acid), which can be obtained by examining the ratio of $I_{b,y}^{+6}$ and $I_{b,y}^{+3}$ for peptides with two leucines, as described below.

The ratio of doubly-labeled ($I_{b,y}^{+6}$; 2 out of 2 leucines labeled) to singly-labeled peptides ($I_{b,y}^{+3}$; 1 out of 2 leucines labeled) can be used to calculate the fraction of labeled leucine loaded on tRNA. These doubly- and singly-labeled peptides are by necessity newly synthesized and their ratio is not affected by the presence of older, unlabeled protein. This analysis is implemented by selecting 'SILT amino acid' as a variable modification and the 'SILAC/AQUA' box unchecked. The ratio of the total intensities of b and y ions is calculated when the singly-labeled peptide is present in the first MS/MS scan and the doubly-labeled peptide is present in the second MS/MS scan. This ratio is calculated as:

$$r_{new} = \frac{I_{b,y}^{+6}}{I_{b,y}^{+3}} = \frac{p_{new}^2}{2p_{new}(1-p_{new})} = \frac{p_{new}}{2(1-p_{new})} \quad [4]$$

Using this formula, the fraction of labeled amino acid in the newly synthesized protein, p_{new} , can be calculated from the measured ratio r_{new} :

$$p_{new} = \frac{r_{new}}{r_{new} + 1/2} \quad [5]$$

A discussion of the combinatorics involved can be found in the description of MIDA (mass isotopomer data analysis) from Hellerstein and colleagues.^{31,34,35}

Peptides with two leucines are quite common and use of these peptides in determining labeling percentages is desirable. However, with highly-labeled samples, the +0, +1/2 × SIMD series and equation 3 are prone to large errors due to the paucity of peptides with 1 out of 2 leucines labeled. An MS/MS series consisting of +0, +1 × SIMD is more useful but requires programming the mass spectrometer with a +1 × SIMD mass tag instead of a +1/2 × SIMD mass tag. For this mass tag setting, doubly-labeled peptides are compared to unlabeled peptides using the following formulas:

$$r = \frac{I_{b,y}^{+6}}{I_{b,y}^{+0}} = \frac{p^2}{(1-p)^2} \quad [6]$$

$$p = \frac{\sqrt{r}}{(1 + \sqrt{r})} \quad [7]$$

Equation 7 assumes that all of the protein is newly synthesized. If this is not the case, the fraction of proteins that are newly synthesized protein is:

$$\frac{[\text{new protein}]}{[\text{new protein}] + [\text{old protein}]} = \frac{1}{\left(\frac{1}{p^2} - \frac{2}{p}\right)(p_{\text{new}})^2 + 2p_{\text{new}}} \quad [8]$$

where p is the value calculated from equation 7 and p_{new} is the percent labeling of newly synthesized protein, which may be obtained by knowledge of the fraction of labeled amino acid to which the cell is exposed or from an experiment that can be analyzed using equation 5. A similar correction exists for equation 3:

$$\frac{[\text{new protein}]}{[\text{new protein}] + [\text{old protein}]} = \frac{p}{p_{\text{new}}(1 + p - p_{\text{new}})} \quad [9]$$

The applicability of the various equations to different experiments is summarized in Table 1.

To estimate the percent labeling of a particular protein, a weighted average is calculated for all the peptides identified within a protein that meet certain minimum criteria (see Figure 2). The weighting factor is simply the total intensity of b and y ions in the first MS/MS scan. For peptides with two leucines, the weighting factor is normalized to account for the distribution of peptides among unlabeled, singly- and doubly-labeled peptides, to allow direct comparison with peptides containing a single leucine. SILTmass also estimates labeling percentages using the intensities of precursor ions in MS scans as recorded by ReAdW. Similar to the SILT analysis, only data from scans containing identified peptides that meet the criteria in Figure 2 are used to calculate the labeling percentage from the precursor ion intensities (i.e. the elution peaks are not reconstructed). Additionally, labeling percentages for a protein are only used if 5 or more scans from the protein meet the criteria in Figure 2.

Software distribution

Please visit <http://labs.seas.wustl.edu/bme/elbert/SILTmass.htm> for updates on distribution of SILTmass.

Results and Discussion

Characteristics of SILTmass

Analysis options are set in a Windows-based GUI, which allows specification of the number of missed cleavages, fixed and variable modifications, tolerances for matches to MS and MS/MS scans, minimum probability-based score for matches, etc. Parameters specific to SILT that can be entered using the GUI are shown in Supplementary Figure 1. In particular, the identity of the isotope-labeled amino acid and the SIMD of the heavy amino acid can be specified. In applying the SILT method, different mass spectrometric experiments are possible, each with a unique series of MS/MS scans for each precursor ion (some examples are listed in Table 2). Series of MS/MS scans that examine the $+1 \times \text{SIMD}$ precursor ion may be useful for comparing unlabeled peptides with doubly-labeled peptides (Experiment B), while series of increasing molecular weights may be used to study the kinetics of isotope labeling by examining the distribution of isotopomers.^{34,35} SILTmass currently assumes that the precursor ions are doubly charged. In Experiment A, precursor ions that are singly or triply charged will not be found in the $+1/2 \times \text{SIMD}$ scan. However, Experiment B may benefit from analysis of singly-charged precursor ions that contain one labeled amino acid and this will be implemented in future versions of SILTmass.

In practice, mass tags are implemented on Thermo Finnigan instruments as follows. After a high-intensity ion is selected in the survey scan, the most intense ion that is *plus or minus* the mass tag is selected for fragmentation. However, the lower molecular weight ion is always fragmented in the first scan. One consequence is that for highly labeled samples, the high intensity ion selected in the survey scan might actually be found in the second scan. Thus, if SILTmass identifies an isotope-labeled peptide in the second scan in an MS/MS series, the series will still be analyzed, since the first scan in the series will contain a peptide that is $-1/2 \times \text{SIMD}$ relative to the identified peptide. However, the isotope-labeled peptide can only be identified if 'SILT amino acid' is selected as a fixed or variable modification. Thus analysis of variable modifications is an important part of SILTmass, allowing data analysis over the full range of labeling percentages. In practice, SILTmass accounts for the number of unlabeled and isotope-labeled amino acids in each identified peptide to ensure that only plausible comparisons are made. Although the SILT method in Excel or Census focus on extraction of b and y ion intensities during the elution peak, SILTmass simply analyzes scans for which a peptide identification was obtained. The correlation between the two methods is quite high ($r = 0.99992$), despite substantial difference in the methodology (Figure 3A). This is probably due to the use of several SILT analysis criteria to eliminate scans that are likely to yield poor quantitation.

In SILTmass, the analysis criteria that must be met before an identified peptide is analyzed using the SILT method are shown in Figure 2. One criterion concerns the magnitude of the total ion current in the MS/MS scans. We have observed that low intensity MS/MS scans have higher absolute errors (Figure 3B). This is most noticeable in ion-specific runs (i.e. a single precursor m/z analyzed repeatedly for the entire run). In ion specific runs, peptides identified in scans outside of the elution peak are generally associated with low intensities and large absolute errors. For data-dependent runs, low intensity MS/MS scans are rarely observed because only high intensity MS peaks are selected for MS/MS fragmentation. In fact, we have yet to observe this criterion used in a data-dependent scan. However, this criterion allows SILTmass to be used equally well with both data-dependent and ion-specific runs. Another criterion is related to very low total ion counts that are occasionally observed in second MS/MS scans of small peptides (molecular weights less than about 1000 Da; Figure 3C). The cause of this is not fully understood, but may relate to the chromatography of small peptides.

Analysis of isotope-labeled human ApoE

Using SILTmass, we quantified incorporation of isotope-labeled leucine into Apolipoprotein E (ApoE) that was produced by transfection of human ApoE into immortalized murine astrocytic cells.²⁷ The mouse cells were cultured in medium containing 0–20% $^{13}\text{C}_6$ -leucine for 24 h. The secreted proteins were collected and human ApoE was purified by immunoprecipitation. The samples were analyzed by LC-MS/MS2 on a Thermo Finnigan LTQ linear ion trap mass spectrometer in triplicate (three injections of the same sample) in data-dependent mode. The mass spectrometric data were analyzed using SILTmass and an average percent incorporation of labeled leucine was calculated for all identified peptides and proteins. As ApoE is a secreted protein, the vast majority of the protein that was collected was newly synthesized.

SILTmass accurately and precisely measured the extent of protein labeling. The absolute error in the labeling percentage was less than 2% (Figure 4A). However, the corresponding relative percent errors increased as the labeling percentage decreased (Figure 4B). We found relative errors for the samples with 10% or 20% labeling of less than 10%. For the 1.25% sample, the relative percent error was about 50%. Use of the precursor ion intensity from the same scans for quantitation was much less accurate or precise, particularly for <10% labeling.

Simultaneous measurement of labeling percentages for other proteins in the ApoE sample

Use of data-dependent scans allowed the analysis of multiple proteins per sample. Although secreted human ApoE was purified from the cell culture medium by immunoprecipitation, other proteins produced by the mouse cells were usually identified. In particular, mouse fibronectin (gi|46849812) was consistently observed along with IgG from the immunoprecipitation. Using SILTmass, mouse fibronectin was found to be labeled to a similar extent as the transfected human ApoE, while IgG was found to be essentially unlabeled, as expected (Figure 5A). The accuracy of the measurements was much worse when the same scans were analyzed using precursor ion intensities (Figure 5B). For both methods, the percent labeling for a protein was used only if at least five scans from the protein met all the criteria in Figure 2. Other proteins were observed in the immunoprecipitated samples, but yielded fewer useable scans. Compared with the ApoE measurements in the same sample, larger variances were found for fibronectin using precursor ion intensity. This may reflect the lower signal found for fibronectin compared to ApoE (probably due to a lower protein concentration) and noise and non-specific signals may have been larger compared to the signal from fibronectin peptides. SILTmass, on the other hand, had similar variances for ApoE and fibronectin.

SILAC-style experiment with C3 complement protein and alpha-2 macroglobulin

In the ApoE experiment, the percent labeling was controlled by the ratio of labeled to unlabeled amino acid in the cell culture medium. This is useful for following the kinetics of protein synthesis and turnover, but many experiments have the goal of identifying the relative amount of a protein versus a highly-labeled internal standard. In a SILAC-style experiment, one cell sample is labeled to nearly 100% and another is left unlabeled.¹² A biological experiment is performed on both cells, using one of the samples as a control. At the end of the experiment, the cells are lysed and mixed in equal proportions. Typically, a protein of interest is immunoprecipitated and relative amounts of the protein are detected by mass spectrometry. The high accuracy of SILTmass in ion specific experiments suggests that SILTmass may enhance the accuracy of SILAC-style quantitative measurements, which are typically performed in data-dependent mode.

C3 complement protein and alpha-2 macroglobulin are produced in large quantities by HepG2 cells and are easily separated from other secreted proteins by SDS-PAGE alone. We tested the ability of SILT to detect small differences in relative amounts of unlabeled and highly-labeled C3 Complement protein and alpha-2 macroglobulin. In these experiments, 100% $^{13}\text{C}_6$, $^{15}\text{N}_2$ - L-lysine was used to label the cells, with a corresponding SIMD of +8. Thus, in the GUI, 'Lysine' was selected as the labeled amino acid and 8.0 was entered as the SIMD. For both proteins, SILT proved to be more accurate and precise than the precursor ion intensity method, when using only scans in which a peptide was identified (Figure 6). However, the accuracy and precision of the precursor ion intensity method was improved compared to the ApoE experiments, which had low labeling percentages. At the dilutions used in the SILAC-style experiment, both unlabeled and labeled ions likely had relatively high amounts of signal and thus were less likely to be corrupted by noise and unrelated ions. Additionally, quantitation using precursor ion intensities may be improved by further statistical analysis, such as in ASAPRatio.³⁶ We did not compare the SILT method to the state-of-the-art precursor ion intensity methods, because it is likely that the accuracy and precision of SILT would also be improved by additional statistical analysis. Nonetheless, these results suggest that SILT may enhance quantification of a variety of SILAC-style experiments.

Estimating kinetics of protein turnover in a proteomics experiment

The experiments with ApoE and the HepG2 proteins relied on a purification step. We wished to test the ability of SILTmass to quantify label incorporation into multiple proteins in a complex mixture of proteins. Thus, we labeled H4 APP695 Δ NL neuroglioma cells with $^{13}\text{C}_6$ -leucine for three days and analyzed the cell lysates without a purification step.³⁷ We were able to identify the following human proteins in most samples: vimentin (gi: 62414289); serine or cysteine proteinase inhibitor, clade H, member 1 precursor (gi: 32454741); annexin A2 isoform 2 (gi:4757756); heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) (gi:16507237); and H4 histone family, member A (gi:4504301). For quantification, we used both experiments A and B in Table 2. As expected, comparison of singly-labeled to doubly-labeled peptides using equation 5 yielded the percentage of labeled amino acid in the medium, because these peptides necessarily come from newly synthesized protein ('1->2 leucine', Figure 7). However, use of equations 1 or 9 showed that proteins in the cell lysate were labeled to lower extents than expected from the amount of labeled leucine in the medium. This likely reflected the kinetics of protein turnover as proteins with a long half-life would not reach steady state in three days of labeling. The ratio of the measured values to the expected values for the proteins in Figure 7 should yield the percentage of protein that is newly synthesized. The measured values were determined using: (1) peptides with one leucine (i.e. Experiment A from Table 2, with the SILAC/AQUA checkbox selected), or (2) peptides with 2 out of 2 leucines labeled (i.e. Experiment B from Table 2, using the correction given in Equation 8). SILT analysis showed that all of the cellular proteins were labeled to a lesser extent than A β , a protein secreted from these cells, which was labeled to $97.4 \pm 1.4\%$. Some of these differences were found to be significant (Figure 7B), although the replicates were only mass spectrometric replicates, not biological replicates. The measured fractions of newly synthesized histones and vimentin could be compared to known rates of turnover for these proteins,^{38,39} and were found to be high, but this may have simply reflected the presence of proliferating cells. The current results suggest that SILTmass has the precision required for both *in vitro* and *in vivo* measurement of protein turnover kinetics.

It was apparent that H4 APP695 Δ NL samples with lower amounts of labeling (<5%) yielded values that were higher than expected for most proteins (Figure 7A). This may also be seen in Figures 4 and 5, where completely unlabeled samples typically yielded labeling

percentages of about 0.5–1.25%. This suggests that additional analysis to account for noise may further enhance the accuracy of SILT at very low labeling levels.

Variance in the measurement of labeling in individual MS/MS scans

In Figure 8, the labeling percentages for individual scans are shown, as opposed to the averaged values reported earlier. The results in Figure 8A show comparisons made between singly-labeled and doubly-labeled versions of peptides with two leucines. The variances for individual scans are large, but the unweighted means and medians are actually quite representative of the expected labeling percentage. The large variance and the presence of outliers highlights the importance of maximizing the number of usable scans per protein. In Figure 8B, the raw data underlying Figure 4A is shown. The unweighted average and median values are good estimators of the expected values, but some outliers are observed, particularly at low labeling percentages. Understanding the sources of errors in the SILT method, particularly at low labeling percentages, is desirable.

Identifying potential sources of error in SILT measurements

To better understand the increasing relative errors in the samples with low amounts of labeling, we examined a number of individual MS/MS spectra from the ApoE dataset. The MS/MS spectra for one peptide, ApoE_{62–72}, with sequence ALMDETMKELK, are presented in Supplementary Figures 2–4. The unlabeled peptide has a molecular weight of 1307.6 Da and the doubly-charged, unlabeled ion has an m/z of 654.8. In Supplementary Figure 2, a scan identified as ApoE_{62–72} in a sample that was 20% labeled is shown, with the highest intensity peaks labeled. All of the high intensity peaks in the +0 spectrum could be matched to b and y ions from ApoE_{62–72} (either singly or doubly charged). The corresponding $+1/2 \times \text{SIMD}$ MS/MS scan is also shown (precursor m/z of 657.8). The peaks are less intense than in the +0 scan and only 9 of the 16 of the most prominent peaks correspond to b and y ions expected for the singly-labeled ApoE_{62–72} peptide.

The ApoE_{62–72} peptide, ALMDETMKELK, contains two leucines and the molecular weight cutoff of the MS/MS scan corresponds to incorporation of exactly one $^{13}\text{C}_6$ -leucine into the peptide. The $+1/2 \times \text{SIMD}$ scan is expected to have peptides with either Leu63 or Leu71 labeled and the two forms should be present in the ion trap in equal amounts. The molecular weights of the predicted fragment ions depend on which leucine is labeled and both versions of the labeled peptide must be compared to the MS/MS scan. In Supplementary Table 1, intensities of the b and y ions found in the spectra shown in Supplementary Figure 2 are listed, with fragment ions that contain a $^{13}\text{C}_6$ -leucine bolded. For fragment ions that either contain no leucines (y_1 and b_1) or both leucines (y_{10} and b_{10}), the location of the labeled leucine does not affect the molecular weight of the fragment. For the other fragment ions, the expected ratio of Leu63-labeled peptide to Leu71 labeled peptide is 1:1. Although the measured ratios of Leu63 to Leu71 fragment ion intensities vary widely, the ratio calculated after summing the intensities for all fragments is 0.9:1. Additionally, in the spectra for the $+1/2 \times \text{SIMD}$ peptide in Supplementary Figure 2, the presence of doubled peaks separated by 6 amu is apparent for ions y_2 , y_7 , y_8 , y_9 and b_9 , while a singlet is found for the b_{10} peak, which is expected. While b_1 , y_1 , b_{10} and y_{10} are expected to be double the size of other b and y peaks, peaks in the spectra are by default never double counted and thus no correction is required within SILTmass.

Samples of ApoE were also produced without added $^{13}\text{C}_6$ -leucine (0% labeled, Supplementary Figure 3). Ideally, the $+1/2 \times \text{SIMD}$ scan would contain no b and y ion peaks from the peptide identified in the +0 scan. However, we have noted the presence of some related fragment ions, which may result from imperfect gating of the ion trap. Supplementary Figure 3 illustrates that peaks corresponding to unlabeled b and y fragments

are observed, but some are shifted +3 amu from the expected value. These fragment ions should have a +1 charge and thus are inconsistent with the shift of +6 amu expected for incorporation of $^{13}\text{C}_6$ -leucine. Given the natural abundance of ^{13}C isotopes, some unlabeled peptides containing three or more ^{13}C atoms could be present in the ion trap. Although the performance of the ion trap may be less than ideal, the majority of the prominent peaks in the $+1/2 \times \text{SIMD}$ scan are not matched to expected ions and thus do not contribute to the calculation of the labeling percentage.

ApoE that was isotope-labeled with 1.25% $^{13}\text{C}_6$ -leucine illustrates that molecules unrelated to ALMDETMKELK may sometimes be present in the $+1/2 \times \text{SIMD}$ scan (Supplementary Figure 4). Although the total ion intensity of the $+1/2 \times \text{SIMD}$ scan is low compared to the +0 scan, many distinct peaks are present. However, few of the peaks seem to bear any relation to the peptide in the +0 scan. In the case of an unrelated compound in the trap, there may by chance be peaks that correspond to expected b and y ion. This will lead to positive deviations from expected values in the SILT method. However, this type of error is expected to be worse with precursor ion intensities, since all of the artifact ion current from isobaric compounds is added to the measurement, while SILT excludes the majority of isobaric artifacts.

Conclusions

The SILT method was automated in SILTmass such that it could be easily applied to data-dependent scans typically used in proteomics experiments (e.g. top 5). The high accuracy and precision of the SILT method was retained. Additionally, SILTmass provides automated analysis of the isotopic labeling of multiple proteins at speeds much faster than manual quantitation. We demonstrated the applicability of SILTmass to SILAC-style experiments and a proteomics experiment with unpurified cell lysates. However, we believe that SILTmass will be most useful for studying the kinetics of protein turnover applied to multiple proteins simultaneously.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SILAC/AQUA experiments are distinct from the measurement of protein turnover kinetics. In SILAC/AQUA, labeling is typically near 100% and Experiment A from Table 2 can only be used with peptides that contain a single labeled amino acid (assuming +2 charge on the peptide). For measuring protein turnover kinetics, peptides with statistical distributions of labeling are present, mixed with older, unlabeled protein (see text for discussion). L = unlabeled leucine; L* = labeled leucine.

Figure 2. Criteria for applying the SILT method to a peptide identified by SILTmass. The equations listed in this diagram are relevant to Experiment A in Table 2.

Figure 3.

(A) Correlation between SILTmass and manual application of SILT in Excel, for A β protein analyzed in an ion-specific mass spectrometric experiment (A β 17–28 peptide, alternating MS/MS scans between the 663.5 and 666.5 Da). (B) Error as a function of total ion current in the first scan in an MS/MS series, for A β protein in an ion-specific experiment. The highest intensity peaks were associated with the elution peak of the peptide. Open squares = 20% labeled protein; solid circles = 10% labeled protein, open triangles = 5% labeled protein. (C) Error analysis from 479 data-dependent scans from the ApoE dataset. The absolute error was divided by the measured percent labeling to highlight results with values that were near zero and that had large errors. Three peptides were found to have large errors due to a near absence of signal in the $+1/2 \times$ SIMD scan (ApoE_{199–207} LGPLVEQGR,

ApoE₁₇₇₋₁₈₅ LAVYQAGAR and ApoE₂₉₃₋₃₀₀ QWAGLVEK). The elution times of these peptides coincided with the start of the gradient and was likely a chromatography artifact.

Figure 4. Measurement of the incorporation of ^{13}C -leucine into ApoE using SILTmass or precursor ion intensities. For both methods of analysis, only scans in which peptides were identified were used in the calculation (i.e. elution peaks were not reconstructed). A) Solid squares = SILTmass, Open triangles = precursor intensity. Error bars are standard deviations. Data collected were from one biological sample and four LC/MS/MS2 runs. B) The relative percent errors for the results in part A. Black bars = SILT, White bars = precursor intensity.

Figure 5.

Calculation of the percent labeling for other proteins identified in the data-dependent analysis of ApoE. Errors bars are standard deviations. Calculation by: A) SILTmass, B) precursor ion intensity. Solid squares = mouse fibronectin; open squares = IgG. Fibronectin apparently co-immunoprecipitated with ApoE and was a product of the cultured cells, as evidenced by its labeling. IgG was most likely from the immunoprecipitation itself and presumably would not be $^{13}\text{C}_6$ -leucine labeled. For both the SILT and precursor intensity methods, only scans containing peptides identified from fibronectin or IgG were used in the calculation of labeling percentages (i.e. elution peaks were not reconstructed).

Figure 6.

An experimental protocol similar to SILAC was followed using proteins secreted by HepG2 cells, mixing a heavily-labeled protein with an unlabeled protein in different ratios. C3 complement protein and alpha-2 macroglobulin are secreted in large amounts by HepG2 cells and are readily purified using SDS-PAGE alone. HepG2 cells were incubated with unlabeled medium or medium containing $^{13}\text{C}_6$, $^{15}\text{N}_2$ -lysine. Cell lysates were mixed and purified by SDS-PAGE. The bands were excised and analyzed with data-dependent scans. Error bars (standard deviations) are present on all datapoints, with $n = 3$. One biological sample was used to produce three processing replicates (i.e. samples were separated after mixing of cell lysates).

Figure 7.

SILTmass may be useful to determine the kinetics of protein turnover for multiple proteins simultaneously. The proteins listed in the legend were identified in most data-dependent scans of H4 APP695ΔNL neuroglioma cell lysates labeled with ^{13}C -leucine. A. Lower than expected values were observed for all non-secreted proteins. Peptides with two leucines were used to compare singly-labeled peptide to doubly-labeled peptide ('1->2 leucine'), yielding measurements much closer to the expected value. These peptides only come from newly synthesized proteins, which should be labeled at close to the expected value. Linear regressions are shown for Aβ (solid black line), S/C protease inhibitor (dashed black line), Annexin A2 (solid grey line) and vimentin (dashed grey line). Error bars are not present for clarity. B. Slopes measured from linear regression of data in part A, presumably indicating the fraction of each protein that is newly synthesized. Error bars represent 95% confidence intervals. *Significance determined from confidence intervals with Bonferroni correction for multiple comparisons. Only comparisons between nearest neighbors are shown for clarity.

Figure 8.

Variance in the measurement of labeling for individual MS/MS scans can be high, thus averaging multiple scans is important. The measured values from individual MS/MS scans that met the criteria in Figure 2 are shown (open circles). The data are presented as a box plot, but with all of the datapoints present on the plot. A. Comparison of singly-labeled to doubly-labeled peptides from proteins in H4 APP695 Δ NL neuroglioma cell lysates. B. Individual scans from the ApoE dataset in Fig. 4A. The medians and the unweighted averages tended to fall near the expected values, with some outliers. Averaging over multiple scans thus should lead to better approximations of the expected value.

Table 1

Types of comparisons made in SILTmass. Unlabeled leucine (L) or labeled leucine (L*) are shown with other residues in the peptide represented by dashes

Experiment (see Table 2)	First scan contains:	Second scan contains:	Analyzed using equation:	Notes
A	---L-----	---L*-----	1	<i>a</i>
	---L---L---	---L---L*--- & ---L*---L---	3, 9	<i>a,b</i>
	---L---L*---	---L*---L*---	5	<i>b,c</i>
	---L*---L---	---L*---L*---	5	<i>b,c</i>
B	---L---L---	---L*---L*---	7, 8	<i>a</i>

^aReflects the ratio of newly-synthesized to older, unlabeled protein.

^bIf newly synthesized protein is abundant and highly-labeled, the intensity of ions in one of the scans will be very low.

^cDirectly yields the percent incorporation of label into newly synthesized proteins.

Table 2

Mass spectrometric experiments amenable to SILT analysis

Experiment	1 st scan	2 nd scan	3 rd scan	4 th scan	5 th scan	Notes
A	+0 ^a	+1/2 SIMD	-	-		1
B	+0	+1 SIMD				2
C	+0	+1/2 SIMD	- 1/2 SIMD	-		1, 3, 4, 5
D	-1/2 SIMD	+0	+1/2 SIMD	-		1, 3, 4, 5
E	+0	+1/2 SIMD	+1 SIMD	-		2, 3, 5
F	+0	+1/2 SIMD	+1 SIMD	+1 1/2 SIMD		2, 3, 5
G	-1 SIMD	-1/2 SIMD	+0	+1/2 SIMD	+1 SIMD	2, 3, 4, 5

^aThe logic behind mass tags on Thermo Finnigan instruments is complex (see text). ‘+0’ typically refers to the precursor ion m/z, which may actually be a labeled peptide.

1 Notes: Assumes doubly-charged precursor ion

2 Useful for singly-charged precursor ions

3 May be useful for determining kinetics of label incorporation by analyzing peptides containing two or more residues that can be isotope-labeled

4 May be useful if singly- or doubly-labeled peptides are more common than unlabeled peptides

5 Not readily implemented on Thermo Finnigan instruments using mass tags