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Quantification of histone modifications using ^{15}N metabolic labeling

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Abstract

Mass spectrometry has made major contributions to recent discoveries in the field of epigenetics, particularly in the characterization of the myriad post-translational modifications (PTMs) of histones which are technically challenging to analyze. These new developments have further aroused great interest in development of robust, new mass spectrometric methods to quantitatively study the dynamics of histone modifications. This review covers quantitative analysis of histone PTMs and discusses an ^{15}N metabolic labeling procedure for quantifying histone PTMs applied to the analysis of methyltransferase knockouts in the model organism, *Tetrahymena thermophila*.

Keywords

Histone; Post-translational modification; Mass spectrometry; ^{15}N metabolic labeling

1. Introduction

Rapid developments in mass spectrometry (MS)-based proteomics over the past decade have eclipsed traditional methods like Edman sequencing as the predominant tool for protein identification. Its ability to both identify and quantify thousands of components in a single step with great sensitivity when coupled with liquid chromatography gives it a number of advantages compared with other analytical techniques[1–3]. Mass spectrometry can be used for either relative or absolute quantification depending on whether internal standards with known quantities are employed[4–6]. Most quantification methods are based on the intensities or peak areas of peptide precursors measured at the MS1 level[3,4,6], while protocols, such as Isobaric Tags for Relative and Absolute Quantification (iTRAQ), Selected Reaction Monitoring (SRM) and Multiple Reaction Monitoring (MRM), provide direct comparisons of different samples based on the relative intensities of fragment ions at the MS2 level[4,6–9]. Direct measurement of protein abundance by the MS signal or by the number of tandem

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spectra without using heavy isotopes in the sample preparation is usually referred to as label-free quantification[10,11]. In contrast to most label-free methods, analyses based on stable isotopic labeling (either by metabolic labeling *in vivo* or by chemical reactions *in vitro*) provide more accurate measurements of the changes in peptide/protein abundances between two or more physiological states[4].

Histones are relatively small proteins but are heavily modified with numerous epigenetic marks including methylation, acetylation, phosphorylation, biotinylation, citrullination, ADP-ribosylation, and ubiquitination[12]. The excess of basic residues, the large number and extent of post-translational modifications, and the relatively low-level of some of these modifications in their natural states all contribute to the technical challenges of studying histone PTMs by mass spectrometry[13]. These challenges emphasize the need for development of robust quantitative proteomic strategies for histone PTMs using mass spectrometry. Here, we briefly review label-free and isotope enrichment methods and discuss their applications in the field of epigenetics.

1.1 Label-free quantification

Mass spectral peak intensities and spectral counting are two major widely used strategies for label-free quantification of the relative levels of proteins in complex biological samples[10,11]. The basic concept behind this technique arises from the observation that both MS signal intensity, measured as peak area under the curve (AUC) or extracted ion chromatography (XIC), and spectral counts for a selected analyte molecule (e.g., tryptic peptide) correlate well with the corresponding protein abundances present in a sample[10,11,14]. The spectral counting approach quantifies proteins by comparing the number of MS/MS spectra from the same protein identified across multiple LC-MS/MS experiments[10,11]. It is worth noting that quantification by spectral counting is applied for a protein not for each peptide as the ionization efficiencies for individual peptides from a single protein may vary greatly. In contrast to the spectral counting approach, label-free quantification by peak intensities is technically somewhat more complicated. Mass to charge ratios, chromatographic peak areas and retention times for each ion across all experiments need to be precisely determined and aligned. For this reason, high reproducibility in sample preparation and LC-MS/MS runs is a key factor for successful quantification[10,11,14]. Good linearity ($r^2 > 0.95$) and wide linear dynamic range (>100-fold) measured by AUC or spectral counting were obtained when standard proteins were spiked into different fractions, representing different chemical backgrounds[15]. A typical coefficient of variance (CV) approximating 30% for protein ratios has been reported in two studies[11,15]. Although both spectral counting and label-free quantification exhibit good reproducibility and sensitivity for protein quantification, spectral counting is not as useful for peptide quantification, while AUC was found to be more accurate[14]. For this reason the application of spectral counting to quantification of protein PTMs has been largely limited.

Several studies have used label-free methods to quantify protein PTMs. In a study of phosphorylation stoichiometry by the label-free approach, Steen *et al.* showed that precise quantification of protein phosphorylation was possible if an appropriate normalization procedure was applied[16]. For histone studies, Beck, *et al.* successfully demonstrated an ion intensity-based LC-MS/MS label-free method for quantitative analysis of PTMs from four human core histones[17]. Unmodified tryptic peptides with small statistical variations (CV<30%) were selected for data normalization in this study, while internal standards were used to correct dataset variations between LC-MS runs. In their study of the global impact of histone deacetylase inhibitors (HDACi) on histone acetylation in H3 and H4, a relative standard deviation between 17% and 24% was observed in three replicate LC-MS/MS runs[18]. Fold-change and statistical analysis showed that the majority of peptides (~90%) were unaffected in the HDACi-treated group. A label-free approach, which combined mass

profiling analysis of intact histones with tryptic peptides generated from charge isoforms, was employed to study yeast histone PTM fold-changes in wild-type and histone acetyltransferase-deficient mutants[19]. The results showed that most PTMs were not affected in knockout cells but acetylation of H3K56 was found to be significantly down-regulated as investigators anticipated from previous studies. The label-free quantification method has also been used to determine the percentage of intact individual H4 isoforms in human embryonic stem cells based on their peak areas[20].

1.2 Isotope enrichment methods: ^{15}N metabolic labeling vs SILAC vs Chemical tags

The use of non-radioactive isotopes introduced by metabolic incorporation *in vivo* or by isobaric tags *in vitro* for quantitative proteomics is referred to as stable isotope labeling[4]. The most common selected stable isotopes are ^2H , ^{13}C , ^{18}O , and ^{15}N [4,24,25]. Because the stable isotope-labeled peptides are chemically identical to their unlabeled counterparts, they behave almost identically in response to chromatography (retention time, losses due to adsorption, etc.) and mass spectrometry (ionization) leading to a more accurate determination compared with label-free quantification[22]. In some cases ^2H -containing compounds exhibit significant changes in chromatographic retention times which makes integration more challenging but several of the major data analysis packages for LC-MS correct for these differences and most methods avoid the use of ^2H . The lower variance of isotope enrichment methods makes them more sensitive to quantitative changes and they are currently more generally used for quantifying PTMs than label-free approaches. ^{15}N metabolic labeling reported for proteome analysis by Oda *et al.*[23] and stable isotope labeling by amino acids in cell culture (SILAC) introduced by Ong *et al.*[24] are two of the widely used metabolic labeling approaches for quantitative proteomics. The major difference between the two approaches is that all nitrogen atoms throughout the whole proteome are uniformly replaced by heavy isotopes in ^{15}N metabolic labeling while in SILAC only specific amino-acid residues (e.g Lys or Arg) are selectively labeled. The mass offsets of paired peptides using SILAC are simple and well-defined[21]. Both strategies have been realized in a variety of species[21]. In contrast to metabolic labeling, incorporation of isotope tags *in vitro* is achieved by labeling proteins or peptides chemically or enzymatically after cell lysis or protein preparation. Chemical reagents, including ICAT, iTRAQ, tandem mass tags (TMT), and ^{18}O water, have been developed for quantitative proteomics[22]. Both metabolic labeling and chemical labeling strategies have been implemented in epigenetic studies and a few representative examples are described here.

A SILAC-based quantitative proteomics study conducted by Jung *et al.*[25] investigated the effects of the polycomb protein SUZ12 on H3.2 and H3.3 modifications in wild type and mouse embryonic stem cells. A significant reduction in H3K27 di/tri-methylation and an increase in H3K27 acetylation were observed in *Suz12* knockout cells. Another SILAC study performed by Cuomo, *et al.* revealed cancer-specific PTM patterns for histones H3 and H4 by comparing normal breast cells with breast cancer cell lines[26] and some of these modifications have been implicated in human cancers. Metabolic labeling using ^{15}N by Oda *et al.* also proved to be promising for accurate quantification of changes in protein phosphorylation[23]. Quantifying histone PTMs using chemical tags is an alternative strategy when *in vivo* labeling is not feasible. Lysyl residues and peptide N-termini have been labeled by differential chemical derivatization with d0/d10-propionic anhydride to map PTM changes in histone methyltransferase knockout cells[27,28]. A chemical method using differential labeling with deuterated methyl ester (D4-methanol) has also been developed to study histone PTMs[29]. Although the isobaric tag, iTRAQ, has not been previously used to quantify histone PTMs, we recently demonstrated feasibility by labeling both intact histones and tryptic peptides (Data not shown). The use of iTRAQ for intact histones was quite effective in most respects but we chose to use the ^{15}N metabolic labeling approach to reduce

long-term costs and because iTRAQ does not resolve the low retention time issues for histone peptides as well as propionylation does. The use of hydrophilic interaction chromatography (HILIC) may prove to be effective for iTRAQ-tagged histone peptides[30,31] but has not yet been widely used. Other examples of the application of quantitative proteomics to chromatin biology have been discussed in recent reviews[12,32,33].

1.3 Compare and contrast

Label-free quantification is a simple, sensitive, and cost effective way to compare up to hundreds of biological samples, but may require a significant number of biological replicates to reduce the p values for lower level PTMs[10,11]. The most obvious challenge of this technique is that quantification accuracy is not comparable with isotope enrichment techniques and this higher variance limits detectable changes. It may also yield poor results if LC-MS/MS runs are not reproducible[14]. Isotope enrichment methods provide more accurate quantification than label-free approaches because the comparison of two or more samples is made within a single LC-MS/MS run and ion suppression is not as great an issue. Thus variations derived from LC and sample handling are minimized. The use of stable isotopes by metabolic labeling has some advantages over chemical labeling: 1) Metabolic labeling minimizes experimental errors by introducing heavy isotopes into the entire cellular proteome before sample is mixed which reduces variance due to sample handling[33]. 2) Variations in labeling efficiency is typically low as incorporation of isotopes is generally highly efficient[24]. Unlike label-free approaches, metabolic labeling and chemical labeling only allow a limited number of samples to be compared in a single multiplex experiment and the reagents required for SILAC and iTRAQ add to the expense over label-free approaches. For SILAC, the auxotroph cells or organisms are preferred in many cases. The conversion of the labeled precursors to certain other amino acids (e.g arginine to proline) in certain organisms and slow growth cell lines, further limiting SILAC[34]. In contrast, metabolic labeling using an ^{15}N -labeled sample as the internal standard is a relatively cost effective alternative and also allows us to compare many biological samples. The complex mass offsets in ^{15}N labeling adds complexity to peptide identification and quantification but commercial software packages are available which deal effectively with ^{15}N -labeled data sets. The remainder of this manuscript describes the application of ^{15}N metabolic labeling to quantification of histone PTMs in *Tetrahymena* but variations of this method can be applied to many other organisms.

2. General procedures for ^{15}N metabolic labeling

2.1 Overview of metabolic labeling using ^{15}N -labeled histones as an internal reference

In a typical heavy isotope labeling experiment, one of the samples is metabolically labeled with ^{15}N stable isotopes while all other samples incorporate ^{14}N and thus are unlabeled. Samples to be labeled may include mammalian tissues[35–37], multicellular organisms[38], plants[39,40], yeast[23], etc. The advantage of applying this method to histones is that the labeled histones can be selectively extracted from cells and readily purified to provide heavy isotope references. In our study, the model organism is *Tetrahymena thermophila*, a ciliated protozoan[41]. In the first step, bacteria (*E.Coli*) are grown in M9 minimal medium with [^{15}N] ammonium sulfate as the sole nitrogen source. To achieve ^{15}N metabolic labeling of *Tetrahymena*, wild-type *Tetrahymena* cells are then added to a fully grown bacteria population to convert the *Tetrahymena* cellular proteome from light to heavy forms by feeding on ^{15}N -labeled bacteria. Details of the labeling procedure are shown in Materials and Methods. Meanwhile, samples to be compared between wild-type and knockout cells are cultured in a standard cell culture medium that is not ^{15}N -enriched. Both labeled and unlabeled core histones are isolated, purified by reversed-phase HPLC, and equally mixed

before the experiment. Because a single preparation of ^{15}N -labeled histones serves as a global standard across all experiments, source variations are minimized. The isolated histones are propionylated to increase hydrophobicity and the size of peptides prior to trypsin digestion[29]. Variations from propionylation and trypsinization are also minimized, as both light and heavy forms are treated under identical conditions. The ratios of the unlabeled samples to the ^{15}N -labeled histone internal controls spiked into each sample are first determined and the final ratios between the unlabeled experiments can be calculated from the relative ratios of light vs heavy forms in each group divided by those from the control group. The general procedure of the metabolic labeling using ^{15}N -labeled histones as an internal standard is illustrated in Figure 1.

2.2 Materials and Methods

2.2.1 Labeling protocols, source of isotopes, and ^{15}N labeling of *Tetrahymena* cells—A variety of approaches can be used to incorporate ^{15}N into histones under cell culture conditions. For *Tetrahymena*, ^{15}N -enriched ammonium sulfate (~99%) was purchased from Cambridge Isotope Laboratories and used as the sole nitrogen source for *E.Coli* growth. Prepare M9 minimal medium supplemented with ^{15}N Bioexpress cell growth media (Cat No. CGM-1000-N-S, Cambridge Isotope Laboratories) for the differential labeling of bacteria as follows:

Na_2HPO_4	4.24g
KH_2PO_4	2g
NaCl	0.5g
Na_2SO_4 (0.3M)	1 ml
Biotin (1 mg/ml in 100% ethanol)	1 ml
Thiamine (1 mg/ml)	1 ml
MgSO_4 (1M)	1 ml
CaCl_2 (0.5M)	0.6 ml
$(\text{NH}_4)_2\text{SO}_4$ (^{15}N)	1g
Glucose (Dextrose)	10g

Add deionized water to a final volume of 1L.

Prepare 1 × SPP medium for growing *Tetrahymena* cells as follows:

Proteose peptone	10g
Yeast extract	1g
Glucose (Dextrose)	2g
Sequestrene	0.03g

Dissolve the above in deionized water to a final volume of 1L. Store solution at -20°C .

Day 1: Thaw *E.Coli* BL21 cells on ice and inoculate the bacteria directly to 1 ml LB medium as a starter culture. Grow *E.coli* at 37°C until it reaches $\sim\text{OD}_{600}=0.5$. Inoculate that into 500 ml [^{15}N] M9 medium and grow at 37°C overnight to reach saturation. At the same time, inoculate *Tetrahymena thermophila* wild-type strains 428 from soybean to a 25 ml *Tetrahymena* culture (1 × SPP growth media) and shake gently at 30°C , overnight.

Day 2: Collect the ^{15}N -labeled *E. coli* cells by centrifugation and resuspend the pellet in 500 ml $1 \times$ phosphate buffer (KCl 0.2g, Na_2HPO_4 1.15g, KH_2PO_4 0.2g in 1 L). To start labeling *Tetrahymena* cells, collect 5 ml of log-phase cells ($\sim 1 \times 10^5/\text{ml}$) growing in the $1 \times$ SPP medium by centrifugation ($300g \times 2$ min). Add the *Tetrahymena* cells to *E. coli* BL21 suspended in phosphate buffer and shake gently at 30°C for roughly 48 hours. The ^{15}N labeled *E. coli* represent the major nitrogen source and serve as food for the *Tetrahymena* cells.

Day 3: Monitor the growth of the *Tetrahymena* cells by checking the ratio of *Tetrahymena* and bacteria cells to confirm that *Tetrahymena* cells are feeding on bacteria. 2-day growth of *Tetrahymena* in bacteria should allow maximal *Tetrahymena* growth ($1 \times 10^5/\text{ml}$) and minimal bacteria retention.

Day 4: *Tetrahymena* cells are collected by centrifugation and proceed to nuclear preparation (see the protocol below) once they have sufficiently grown (at least $10 \times$ doubling time to reach the stationary phase, *Tetrahymena* cells typically have an average doubling time approximately to 2.5-3h in $1 \times$ SPP medium, and 4-5h in bacteria). Except for growing ^{15}N wild-type cells, all other wild-type cells or mutants are grown in $1 \times$ SPP medium without ^{15}N .

2.2.2 Nuclear Preparation and Histone Acid Extraction—Make 1L medium A buffer as follows:

34.2 g	sucrose	0.1 M
0.41 g (2 ml)	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1M MgCl_2)	2 mM
200 ml	20% gum arabic	4%
10 ml	1 M Tris, pH7.5	10 mM
920 ul	butyric acid	10 mM
10 ml	100 mM iodoacetamide	1 mM
10 ml	100 mM PMSF	1 mM

Adjust pH to 6.75 with 1N NaOH. Stored at -20°C .

Tetrahymena cells have two types of nuclei: macronuclei and micronuclei. They can be separated by differential centrifugation, as previously described[42]. The whole protocol used for labeled and unlabeled cells is exactly same. Only a very limited number of bacteria are included in ^{15}N -labeled *Tetrahymena* cells if they are not completely consumed in the final stage. Briefly, collect *Tetrahymena* cells ($\sim 2 \times 10^8$) by centrifugation with $350g$ for 5 min. Resuspend cells in 100 ml medium A and break open cells by blending in a kitchen blender with 0.7 ml of 1-octanol. Nuclei are pelleted by centrifugation ($2,000g \times 10$ min). Pour the supernatant with the octanol skin into the blender and collect the nuclei from the bottom of centrifuge tubes by adding 1 ml medium A. Repeat the blending and centrifugation, with increasing centrifugal force, by gradually ramping up speed to 3,000, 4,000, and 5,000g. The quality of nuclear preparation from each centrifugation can be checked under a microscope after methyl green staining. Pool fractions containing mostly macronuclei for histone extraction[42,43].

Acid extraction is performed as previously described[43]. Briefly, extract the nuclear pellet with 1 ml of 0.4 N sulfuric acid (4°C , overnight rotation). Histones in the supernatant are precipitated by TCA (final concentration, 20% TCA). After washing first with acidified

acetone (0.2% HCl) and then pure acetone, the bulk histones are solubilized in 0.5 ml of water.

2.2.3 Isolation and purification of core histones by HPLC—The extracted bulk histones can be isolated and purified into the various histone isoforms by C8 reversed-phase HPLC (We use a Vydac C8 RP column with Part No. 208TP54 and Dimension: 250mm × 4.6mm). The HPLC running conditions are:

Step	Solvent A (%)	Solvent B (%)	Time (min)
Equilibrium	100	0	0-5
Ramp-up	65	35	5-10
Histone separation	35	65	10-70
Ramp-up	0	100	70-75
Column wash	0	100	75-80
Column wash	100	0	80-85
Re-equilibrium	100	0	85-100

Solvent A: 5% Acetonitrile in 0.1% TFA; Solvent B: 90% Acetonitrile in 0.1% TFA; Set UV wavelength at 214 nm.

Fractions collected from HPLC are vacuum-dried and suspended in water. Histone purity is evaluated by 15% SDS gels with Coomassie Brilliant blue staining. Individual histone fractions are then combined and protein concentrations are determined by Bradford assay. A typical HPLC chromatogram and core histones separated on 15% SDS-PAGE gels are shown in Figure 2. Typically, the yield for each core histone prepared from 2×10^8 cells following above steps is about 50 µg.

2.2.4 Histone propionylation, enzyme digestion, and mass spectrometry analysis—Because histones are very basic proteins, they have relatively short retention times on reversed-phase columns and many of their tryptic peptides are too short to be analyzed effectively. This effect can be partially alleviated by taking advantage of derivatization methods such as propionylation as one example. Chemical derivatization of histones with propionic anhydride increases the size and hydrophobicity of tryptic peptides by converting primary amines of N-termini and the epsilon amino groups of lysyl residues to propionyl amides which increases peptide retention times and prevents cleavage by trypsin[29]. Although Arg-C digestion will generate very similar peptides in this particular case, peptides without propionylation are more hydrophilic and thus are less likely to be detected and identified with PTMs from a C18 capillary column. Compared to Arg-C digestion, trypsin digestion is also more efficient and cost-effective. Propionylation allows us to identify many more PTMs in core histones (Data not shown). Prior to propionylation, labeled and unlabeled histone samples are equally mixed and vacuum-dried. They are then treated with 20 µl of propionylation reagent (anhydrous methanol: propionic anhydride, 3:1 (v/v)), immediately followed by addition of ammonium hydroxide (~15 µl) to increase the pH above 8.0. Reactions are maintained at room temperature for 30 min and then dried in a SpeedVac concentrator. Propionylated histones are in-solution digested with sequencing-grade trypsin in 100 mM ammonium bicarbonate buffer (enzyme:substrate ratio, 1:20) at 37°C for 6 hours. A second round of propionylation is repeated after enzymatic digestion to completely derivatize the newly generated N-termini. Sample aliquots are again vacuum-dried and reconstituted in 0.1% formic acid prior to LC-MS/MS analysis.

Tryptic peptides are resolved with a lab-packed (C18, 3 µm, 200 Å) or commercial C18 capillary column and analyzed on an Eksigent capillary HPLC coupled to a Thermo Fisher

Orbitrap XL mass spectrometer with a 90 min gradient for peptide separation from 5% to 40% acetonitrile in 0.1% formic acid with a flow rate at 200 nl/min. The mass spectrometer is operated with a positive nano-ESI source in the data-dependent mode with resolution of 30,000 and isolation width of 2.5 Dalton on MS1. In this way, 8 CID scans with 30% normalized collision energy are acquired after each full-scan. For precursor ions, dynamic exclusion is implemented for 120 seconds. Mass lock using polydimethylcyclsiloxane (PCM) peaks ($m/z=429.088735$ and 445.12002) are applied for internal mass calibration.

2.3 Database searching and software for data analysis

Mascot Distiller (Matrix Sciences, Version 2.4) was used to process raw data, perform database searching, and quantify peptides using the ^{15}N metabolic option with correlation and standard error thresholds set at 0.9 and 0.1, respectively. The NCBI *Tetrahymena* sequence database was searched and its decoy database was used to estimate the false discovery rate. Mass error tolerances for the precursor ions and the fragment ions were set at 10ppm and 0.8 Da, respectively. The following modifications were considered: Acetylation on N-terminal and K, Methylation on K and R, Propionylation on N-terminal and K, Monomethylation & Propionylation on K (+70 Da), and Phosphorylation on S, T, and Y. Five missed cleavages are allowed for trypsin digestion as lysine residues are blocked after propionylation. Several normalization options are available in Mascot Distiller. We chose data normalization by histone proteins as we assume that light and heavy histones are equally loaded. Statistical analysis should be performed separately in a third-party software such as Microsoft Excel, SPSS or R since Mascot distiller does not include a statistical module.

Other software packages supporting ^{15}N quantitation include: MSQuant and Census. Both are open-source software developed and maintained by two separate groups: the CEBI research group at University of Southern Denmark (<http://msquant.alwaysdata.net/msq/>), and the Yates group at The Scripps Research Institute (<http://fields.scripps.edu/>).

2.4 Applications to analysis of histone PTMs in *Tetrahymena thermophila*

2.4.1 Strategy and statement of research problem—Post-translational modifications of histones play a key role in gene regulation and nuclear organization and to date, more than 60 different residues on histones have been found with covalent modifications[44]. The recent identification of several new types of histone PTMs further expanded the growing pool of known PTMs in the cell[45–47]. Most histone PTMs exhibit dynamic changes in cells in response to cell cycle, differentiation, environmental challenges, etc[48,49]. Modifications may affect highly organized chromatin structure by changing the contacts between different histones in nucleosomes, with other proteins involved in transcription, or the interaction of positively charged histones with negatively charged DNA[44].

Edman degradation and immunoblotting using site-specific antibodies have been widely used for characterizing histone PTMs. However, identification of histone PTMs by Edman microsequencing are costly and time consuming. It is also a low-sensitivity method since a relatively large amount of sample is needed[50,51]. Sequencing may also be ineffective if the N-terminus of the protein is modified[51]. This is a particular problem since most histones are N-terminally acetylated. A growing number of site-specific antibodies to modified histones are now commercially available but although antibody analyses are sensitive, they can not identify unknown PTMs and also do not provide accurate quantitative information in most cases due to the cross-reactivity and non-specific binding[13,50–53]. Mass spectrometry-based proteomics provides a high-throughput, precise, and sensitive way to characterize protein PTMs. However, precise identification and quantification of protein

PTMs can still be extremely challenging especially when a protein to be investigated is heavily post-translationally modified as is the case for histone proteins.

We recently developed this metabolic labeling procedure for studying histone PTMs in *Tetrahymena thermophila*, a eukaryotic model organism. Details and general strategy were recently published[54]. Based on this approach, we successfully identified Txr1p and Ezl2p as histone methyltransferases specific for H3K27. The method we developed and tested is relatively robust and highly reproducible.

2.4.2 Exploring the effects of histone methyltransferases specific for H3K27-A case study—Histone modifying enzymes are the histone code writers and identification of new histone modifying enzymes and their specificities is a first step to understanding the code. A greater importance has been attached to histone methylation since the first histone methyltransferase (HMT), SUV39, was identified in mammals[55]. The recent discovery of histone demethylases (HDMs), the code erasers, has reversed our view of histone methylation as a permanent, heritable mark[56]. The number of HMTs identified is continuously increasing and many have been linked with human cancers[57].

We recently have explored the roles of two putative histone H3 HMTs identified in *Tetrahymena thermophila* whose specificities were undetermined[54]. We developed this method to map the global PTM changes of histones in response to the deletion of methyl histone modifying genes. Generally, the metabolic labeling is very efficient with ~99% isotope incorporation observed (Table 1, Figure 3). Only a very few potentially unlabeled peptides were found during database searching. About 80% sequence coverage was achieved in H3 and a total of 32 PTMs were identified with more than 60 H3 forms quantified with ~13% average coefficient of variation (CV) observed in 3 biological replicates[54].

We successfully identified the sites being affected as consequences of methyl transferase knockouts, observing a significant reduction in di- and trimethylation at H3K27 in $\Delta EZL2$ cells (Figure 4), as expected from its homology to *Drosophila E(z)*[58]. We also observed significant decreases in mono-methylation, and to a less degree, dimethylation at H3K27 in $\Delta TXR1$ cells, as expected from its homology to *Aradidopsis ATXR5* and *ATXR6*[59]. The results are validated by the modification-specific antibodies. More details are discussed in our recent publication[54].

2.4.3 Strengths and limitations—Generally, stable isotope labeling using ^{15}N metabolically labeled histone as a global standard is a robust quantitative proteomics method for histone PTMs. The chemical derivatization significantly increases the protein sequence coverage and the number of PTMs identified in histone H3. The average CV for the quantified peptides and PTMs is approximately 13%, which is comparable with SILAC-based quantification methods. However, missing data limited the number of PTMs that could be quantified which is common to most bottom-up MS-based methods. Only 18 out of 32 PTMs are statistically quantified largely due to the low levels of PTMs in the natural states (Note that statistically quantified means a peptide must be detected in more than one biological replicate to calculate a p-value). Therefore, a specific enrichment method may be considered when a particular type of PTM is targeted.

3. Concluding remarks

Stable isotope labeling using ^{15}N metabolically labeled histones as an internal standard provides more opportunities for monitoring the dynamics of histone modifications and elucidating more details of the histone code in future studies. This is a general approach that

can be expanded to study histones and other proteins that undergo extensive chemical modifications in many other organisms. Myelin basic protein is a good example of a small highly basic protein having a high density and range of modification, similarly to histones, which would be amenable to analysis using this method. Protein quantification by ^{15}N metabolic labeling can be performed in hundreds of physiological conditions, similarly to label-free methods but with variances similar to other isotope enrichment methods. The use of a heavy-labeled sample as an internal control in SILAC or the use of one reagent as a common standard in iTRAQ/TMT also allows multiple comparisons across large sample numbers, however, they add significantly to project costs. Therefore, the use of ^{15}N labeling becomes more competitive particularly when the use of other isotope labeling techniques is not applicable due to availability or cost. Similar to SILAC, ^{15}N labeling is only feasible in certain species where metabolic labeling is possible and highly efficient. Compared to SILAC, it can be a more general metabolic labeling technique used in comparative proteomics due to reasons discussed in this review. Metabolic labeling using ^{15}N has been applied to a wide range of species[34], which suggests the feasibilities of our method to study histone PTMs on a large-scale in a broad range of other model organisms. Although multiple methodologies are available for researchers in this field, there is no universal quantitative strategy that can deal with all analytical problems due to limitations inherent to each method. The choice of a particular method for quantitative analysis of biological systems must be case-dependent. Fortunately, a large number of methods are available which may be adapted to most systems.

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Abbreviations

Ac	Acetylation
Pr	Propionylation
CV	Coefficient of variation
WT	Wild-type
KO	Knockout
PTM	Post-translational modification
HMT	Histone methyltransferase
AUC	peak area under the curve
XIC	extracted ion chromatography

References

1. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422:198–207. [PubMed: 12634793]
2. Cox J, Mann M. Quantitative, high-resolution proteomics for data-driven systems biology. *Annu. Rev. Biochem.* 2011; 80:273–99. [PubMed: 21548781]
3. Schulze WX, Usadel B. Quantitation in mass-spectrometry-based proteomics. *Annu. Rev. Plant Biol.* 2010; 61:491–516. [PubMed: 20192741]
4. Ong S-E, Mann M. Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol.* 2005; 1:252–62. [PubMed: 16408053]

5. Kirkpatrick DS, Gerber SA, Gygi SP. The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. *Methods*. 2005; 35:265–73. [PubMed: 15722223]
6. Xie F, Liu T, Qian W-J, Petyuk V. a, Smith RD. Liquid chromatography-mass spectrometry-based quantitative proteomics. *J. Biol. Chem.* 2011; 286:25443–9. [PubMed: 21632532]
7. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics*. 2006; 5:573–88. [PubMed: 16332733]
8. Carr SA, Anderson L. Protein quantitation through targeted mass spectrometry: the way out of biomarker purgatory? *Clin. Chem.* 2008; 54:1749–52. [PubMed: 18957555]
9. Pan S, Aebersold R, Chen R, Rush J, Goodlett DR, McIntosh MW, Zhang J, Brentnall TA. Mass Spectrometry Based Targeted Protein Quantification : Methods and Applications reviews. *J. Proteome Res.* 2009; 8:787–97. [PubMed: 19105742]
10. Zhu W, Smith JW, Huang C-M. Mass spectrometry-based label-free quantitative proteomics. *J. Biomed. Biotechnol.* 2010;840518. 2010. [PubMed: 19911078]
11. Wang M, You J, Bemis KG, Tegeler TJ, Brown DPG. Label-free mass spectrometry-based protein quantification technologies in proteomic analysis. *Brief. Funct. Genomic. Proteomic.* 2008; 7:329–39. [PubMed: 18579615]
12. Sidoli S, Cheng L, Jensen ON. Proteomics in chromatin biology and epigenetics: Elucidation of post-translational modifications of histone proteins by mass spectrometry. *J. Proteomics*. 2012; 75:3419–33. [PubMed: 22234360]
13. Britton L-MP, Gonzales-Cope M, Zee BM, Garcia BA. Breaking the histone code with quantitative mass spectrometry. *Expert Rev. Proteomics*. 2011; 8:631–43. [PubMed: 21999833]
14. Neilson KA, Ali N. a, Muralidharan S, Mirzaei M, Mariani M, Assadourian G, Lee A, van Sluyter SC, Haynes P. a. Less label, more free: approaches in label-free quantitative mass spectrometry. *Proteomics*. 2011; 11:535–53. [PubMed: 21243637]
15. Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevensky JR, Resing K. a, Ahn NG. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol. Cell. Proteomics*. 2005; 4:1487–502. [PubMed: 15979981]
16. Steen H, Jebaranathirajah JA, Springer M, Kirschner MW. Stable isotope-free relative and absolute quantitation of protein phosphorylation stoichiometry by MS. *Proc. Natl. Acad. Sci. USA*. 2005; 102:3948–53. [PubMed: 15741271]
17. Beck HC, Nielsen EC, Matthiesen R, Jensen LH, Sehested M, Finn P, Grauslund M, Hansen AM, Jensen ON. Quantitative proteomic analysis of post-translational modifications of human histones. *Mol. Cell. Proteomics*. 2006; 5:1314–25. [PubMed: 16627869]
18. Drogaris P, Villeneuve V, Pomiès C, Lee E-H, Bourdeau V, Bonneil E, Ferbeyre G, Verreault A, Thibault P. Histone deacetylase inhibitors globally enhance h3/h4 tail acetylation without affecting h3 lysine 56 acetylation. *Sci. Rep.* 2012; 2:220. [PubMed: 22355734]
19. Drogaris P, Wurtele H, Masumoto H, Verreault A, Thibault P. Comprehensive profiling of histone modifications using a label-free approach and its applications in determining structure-function relationships. *Anal. Chem.* 2008; 80:6698–707. [PubMed: 18671409]
20. Phanstiel D, Brumbaugh J, Berggren WT, Conard K, Feng X, Levenstein ME, McAlister GC, Thomson J. a, Coon JJ. Mass spectrometry identifies and quantifies 74 unique histone H4 isoforms in differentiating human embryonic stem cells. *Proc. Natl. Acad. Sci. USA*. 2008; 105:4093–8. [PubMed: 18326628]
21. Beynon RJ, Pratt JM. Metabolic labeling of proteins for proteomics. *Mol. Cell. Proteomics*. 2005; 4:857–72. [PubMed: 15849272]
22. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: a critical review. *Anal. Bioanal. Chem.* 2007; 389:1017–31. [PubMed: 17668192]
23. Oda Y, Huang K, Cross FR, Cowburn D, Chait BT. Accurate quantitation of protein expression and site-specific phosphorylation. *Proc. Natl. Acad. Sci. USA*. 1999; 96:6591–6. [PubMed: 10359756]
24. Ong S-E. Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. *Mol. Cell. Proteomics*. 2002; 1:376–86. [PubMed: 12118079]

25. Jung HR, Pasini D, Helin K, Jensen ON. Quantitative mass spectrometry of histones H3.2 and H3.3 in Suz12-deficient mouse embryonic stem cells reveals distinct, dynamic post-translational modifications at Lys-27 and Lys-36. *Mol. Cell. Proteomics*. 2010; 9:838–50. [PubMed: 20150217]
26. Cuomo A, Moretti S, Minucci S, Bonaldi T. SILAC-based proteomic analysis to dissect the “histone modification signature” of human breast cancer cells. *Amino Acids*. 2011; 41:387–99. [PubMed: 20617350]
27. Plazas-Mayorca MD, Zee BM, Young NL, Fingerhman IM, LeRoy G, Briggs SD, Garcia B. a. One-Pot Shotgun Quantitative Mass Spectrometry Characterization of Histones. *J. Proteome Res*. 2009; 8:5367–74. [PubMed: 19764812]
28. Plazas-Mayorca MD, Bloom JS, Zeissler U, Leroy G, Young NL, DiMaggio PA, Kruglyak L, Schneider R, Garcia BA. Quantitative proteomics reveals direct and indirect alterations in the histone code following methyltransferase knockdown. *Mol. Biosyst*. 2010; 6:1719–29. [PubMed: 20577673]
29. Garcia BA, Mollah S, Ueberheide BM, Busby S. a, Muratore TL, Shabanowitz J, Hunt DF. Chemical derivatization of histones for facilitated analysis by mass spectrometry. *Nat. Protoc*. 2007; 2:933–8. [PubMed: 17446892]
30. Young NL, DiMaggio PA, Plazas-Mayorca MD, Baliban RC, Floudas CA, Garcia BA. High throughput characterization of combinatorial histone codes. *Mol. Cell. Proteomics*. 2009; 8:2266–84. [PubMed: 19654425]
31. Boersema PJ, Mohammed S, Heck AJR. Hydrophilic interaction liquid chromatography (HILIC) in proteomics. *Anal. Bioanal. Chem*. 2008; 391:151–9. [PubMed: 18264818]
32. Arnaudo AM, Molden RC, Garcia B. a. Revealing histone variant induced changes via quantitative proteomics. *Crit. Rev. Biochem. Mol. Biol*. 2011; 46:284–94. [PubMed: 21526979]
33. Eberl HC, Mann M, Vermeulen M. Quantitative proteomics for epigenetics. *Chembiochem*. 2011; 12:224–34. [PubMed: 21243711]
34. Gouw JW, Krijgsveld J, Heck AJR. Quantitative proteomics by metabolic labeling of model organisms. *Mol. Cell. Proteomics*. 2010; 9:11–24. [PubMed: 19955089]
35. Ishihama Y, Sato T, Tabata T, Miyamoto N, Sagane K, Nagasu T, Oda Y. Quantitative mouse brain proteomics using culture-derived isotope tags as internal standards. *Nat Biotech*. 2005; 23:617–21.
36. Mcclatchy DB, Dong M.-qiu, Wu CC, Venable JD, Yates JR III. ¹⁵N metabolic labeling of mammalian tissue with slow protein turnover. *J. Proteome Res*. 2007; 6:2005–10. [PubMed: 17375949]
37. Wu CC, MacCoss MJ, Howell KE, Matthews DE, Yates JR III. Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. *Anal. Chem*. 2004; 76:4951–9. [PubMed: 15373428]
38. Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-sanz M, Verrijzer CP, Plasterk RHA, Heck AJR. Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics. *Nat. Biotech*. 2003; 21:927–31.
39. Engelsberger WR, Erban A, Kopka J, Schulze WX. Metabolic labeling of plant cell cultures with K(15)NO₃ as a tool for quantitative analysis of proteins and metabolites. *Plant Methods*. 2006; 2:14. [PubMed: 16948866]
40. Lanquar V, Kuhn L, Lelièvre F, Khafif M, Espagne C, Bruley C, Barbier-Brygoo H, Garin J, Thomine S. ¹⁵N-metabolic labeling for comparative plasma membrane proteomics in *Arabidopsis* cells. *Proteomics*. 2007; 7:750–4. [PubMed: 17285564]
41. Orias E. Mapping the Germ-Line and Somatic Genomes of a Ciliated Protozoan , *Tetrahymena thermophila*. *Genome Res*. 1998; 8:91–9. [PubMed: 9477337]
42. Gorovsky MA. Studies on nuclear structure and function in *Tetrahymena pyriformis*. II. Isolation of macro- and micronuclei. *J. Cell Biol*. 1970; 47:619–30. [PubMed: 5497543]
43. Gorovsky MA. Studies on nuclear structure and function in *Tetrahymena pyriformis*. 3. Comparison of the histones of macro- and micronuclei by quantitative polyacrylamide gel electrophoresis. *J. Cell Biol*. 1970; 47:631–6. [PubMed: 5497544]
44. Kouzarides T. Chromatin modifications and their function. *Cell*. 2007; 128:693–705. [PubMed: 17320507]

45. Zhang K, Chen Y, Zhang Z, Zhao Y. Identification and Verification of Lysine Propionylation and Butyrylation in Yeast Core Histones Using PTMap Software research articles. *J. Proteome Res.* 2009; 8:900–6. [PubMed: 19113941]
46. Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N, Lu Z, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao Y. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell.* 2011; 146:1016–28. [PubMed: 21925322]
47. Zhang Z, Tan M, Xie Z, Dai L, Chen Y, Zhao Y. Identification of lysine succinylation as a new post-translational modification. *Nat. Chem. Biol.* 2010; 7:58–63. [PubMed: 21151122]
48. Krebs JE. Moving marks: Dynamic histone modifications in yeast. *Mol. Biosyst.* 2007; 3:8–12.
49. Zee BM, Levin RS, Xu B, LeRoy G, Wingreen NS, Garcia B. a. In vivo residue-specific histone methylation dynamics. *J. Biol. Chem.* 2010; 285:3341–50. [PubMed: 19940157]
50. Su X, Ren C, Freitas MA. Mass spectrometry-based strategies for characterization of histones and their post-translational modifications. *Expert Rev. Proteomics.* 2007; 4:211–25. [PubMed: 17425457]
51. Ueberheide B, Mollah S. Deciphering the histone code using mass spectrometry. *Int. J. Mass Spectrom.* 2007; 259:46–56.
52. Zhang K, Tang H. Analysis of core histones by liquid chromatography-mass spectrometry and peptide mapping. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2003; 783:173–9.
53. Garcia BA, Shabanowitz J, Hunt DF. Characterization of histones and their post-translational modifications by mass spectrometry. *Curr. Opin. Chem. Biol.* 2007; 11:66–73. [PubMed: 17157550]
54. Zhang C, Molascon AJ, Gao S, Liu Y, Andrews PC. Quantitative proteomics reveals that the specific methyltransferases Txr1p and Ezl2p differentially affect the mono-, di- and tri-methylation states of histone H3 lysine 27. *Mol. Cell. Proteomics.* Epub ahead of print.
55. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, Jenuwein T. Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature.* 2000; 406:593–9. [PubMed: 10949293]
56. Mosammaparast N, Shi Y. Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. *Annu. Rev. Biochem.* 2010; 79:155–79. [PubMed: 20373914]
57. Zhang K, Dent SYR. Histone modifying enzymes and cancer : going beyond histones. *J. Cell. Biochem.* 2005; 1148:1137–48. [PubMed: 16173079]
58. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell.* 2002; 111:185–96. [PubMed: 12408863]
59. Jacob Y, Feng S, LeBlanc C. a, Bernatavichute YV, Stroud H, Cokus S, Johnson LM, Pellegrini M, Jacobsen SE, Michaels SD. ATXR5 and ATXR6 are H3K27 monomethyltransferases required for chromatin structure and gene silencing. *Nat. Struct. Mol. Biol.* 2009; 16:763–8. [PubMed: 19503079]

Figure 1. Schematic of the general procedure for ^{15}N metabolic labeling

One culture of WT *Tetrahymena* cells was ^{15}N metabolically labeled as a global standard, while WT and HMT KO cell were normally grown in the SPP medium. Histones were acid extracted and separated by reversed-phase HPLC. The light forms were equally mixed with ^{15}N -histones before propionylation and trypsinization. The digests were analyzed by nano-LC-MS/MS. PTMs were identified by CID tandem mass spectra and quantified by AUC of light vs heavy forms. A final ratio was calculated from the individual ratios of light vs heavy forms.

Figure 2. HPLC and SDS-PAGE separation of core histones

The general protocol for separation and identification of core histones by HPLC and SDS-PAGE gel electrophoresis.

Figure 3. H/L ratios of 3 tryptic peptides from histone H3

Histones typically are labeled with high efficiency using ^{15}N as indicated by tryptic peptides.

Figure 4. Down-regulation of H3K27Me2 in the HMT mutant

Based on the strategy described in Figure 1, we identified a peptide dimethylated at H3K27 which was significantly down-regulated in the HMT KO cells.

Table 1
Analysis of labeling efficiency of ^{15}N H3 purified from HPLC

Mascot distiller analysis of tryptic peptides generated from ^{15}N histone H3 showed that most peptides incorporated ^{15}N over 99%. The overall labeling efficiency of protein was greater than 99%.

Sequence	Modifications	Charge	L/H Ratio
PGTVALR		1	0.002688
DMQLAR		1	0.04009
IRGERF		1	0.002353
SAPATGGIK		1	0.01163
STGAKAPR	Acetyl (K5)	2	0.000574
DIAHEFK		1	0.001722
DIAHEFK		2	0.007891
QLASKAAR	Acetyl (K5)	2	0.02226
KSAPATGGIK	Methyl (K1)	1	0.000026
KSAPATGGIK	Methyl (K1)	2	0.001268
KSAPATGGIK	Dimethyl (K1)	2	0.01466
KSTGAKAPR	Acetyl (K6)	2	0.05549
KSAPATGGIK	Acetyl (K1)	1	0.000019
KSAPATGGIK	Trimethyl (K1)	2	0.008467
KQLASKAAR	Dimethyl (K6)	2	0.000026
KQLASKAAR	Acetyl (K6)	2	0.005969
KQLASKAAR	Trimethyl (K6)	2	0.001694
KQLASKAAR	Acetyl (K1, K6)	1	0.007236
DIAHEFKAELR		1	0.000028
DIAHEFKAELR		2	0.006066
SAPATGGIKKPHR	Methyl (K9)	2	0.001383
SAPATGGIKKPHR	Methyl (K9)	3	0.01701
SAPATGGIKKPHR	Dimethyl (K9)	2	0.008501
KSAPATGGIKKPHR	Methyl (K10)	2	0.000027
KSAPATGGIKKPHR	Methyl (K1, K10)	2	0.004502
KSAPATGGIKKPHR	Methyl (K1, K10)	3	0.01104
KSAPATGGIKKPHR	Methyl (K1); Dimethyl (K10)	2	0.003767
KSAPATGGIKKPHR	Dimethyl (K1); Methyl (K10)	2	0.003767
KSAPATGGIKKPHR	Acetyl (K1); Methyl (K10)	2	0.002172
KSAPATGGIKKPHR	Acetyl (K1); Methyl (K10)	3	0.01525
KSAPATGGIKKPHR	Acetyl (K1); Dimethyl (K10)	2	0.007509
LVRDIAHEFKAELR		2	0.0114
LVRDIAHEFKAELR		3	0.00302

Note: Ratios are corrected with the purity of ammonia sulfate (98%).