Video Article

Quantification of Site-specific Protein Lysine Acetylation and Succinylation Stoichiometry Using Data-independent Acquisition Mass Spectrometry

Lei Wei[1], Jesse G. Meyer[1], Birgit Schilling[1]

[1]Buck Institute for Research on Aging

Correspondence to: Birgit Schilling at bschilling@buckinstitute.org

URL: https://www.jove.com/video/57209
DOI: doi:10.3791/57209

Keywords: Biology, Issue 134, Stoichiometry, acetylation, succinylation, data-independent acquisition, mass spectrometry, skyline

Date Published: 4/4/2018

Citation: Wei, L., Meyer, J.G., Schilling, B. Quantification of Site-specific Protein Lysine Acetylation and Succinylation Stoichiometry Using Data-independent Acquisition Mass Spectrometry. *J. Vis. Exp.* (134), e57209, doi:10.3791/57209 (2018).

Abstract

Post-translational modification (PTM) of protein lysine residues by N_ε-acylation induces structural changes that can dynamically regulate protein functions, for example, by changing enzymatic activity or by mediating interactions. Precise quantification of site-specific protein acylation occupancy, or stoichiometry, is essential for understanding the functional consequences of both global low-level stoichiometry and individual high-level acylation stoichiometry of specific lysine residues. Other groups have reported measurement of lysine acetylation stoichiometry by comparing the ratio of peptide precursor isotopes from endogenous, natural abundance acylation and exogenous, heavy isotope-labeled acylation introduced after quantitative chemical acetylation of proteins using stable isotope-labeled acetic anhydride. This protocol describes an optimized approach featuring several improvements, including: (1) increased chemical acylation efficiency, (2) the ability to measure protein succinylation in addition to acetylation, and (3) improved quantitative accuracy due to reduced interferences using fragment ion quantification from data-independent acquisitions (DIA) instead of precursor ion signal from data-dependent acquisition (DDA). The use of extracted peak areas from fragment ions for quantification also uniquely enables differentiation of site-level acylation stoichiometry from proteolytic peptides containing more than one lysine residue, which is not possible using precursor ion signals for quantification. Data visualization in Skyline, an open source quantitative proteomics environment, allows for convenient data inspection and review. Together, this workflow offers unbiased, precise, and accurate quantification of site-specific lysine acetylation and succinylation occupancy of an entire proteome, which may reveal and prioritize biologically relevant acylation sites.

Video Link

The video component of this article can be found at https://www.jove.com/video/57209/

Introduction

N_ε-acylation of protein lysine residues is an important regulator of protein function. Lysine acetylation and other acylations, such as succinylation, malonylation, and glutarylation, are thought to regulate metabolism, cell signaling, and other cellular processes[1,2,3,4], and have implications in metabolic disorders[5]. Numerous studies have found that lysine acyl modifications undergo large fold-changes under different conditions in mammalian tissues and cell lines[5,6,7,8] as well as bacteria[9,10,11], however, these relative fold changes do not provide insights into the proportion of the total protein modified. Studies reporting measurements of acylation site occupancy are scarce[12,13,14], despite the relevance and need for such studies as they provide more detail than relative fold change. For example, a 10-fold change could represent a site occupancy increase from 0.01 to 0.1%, 1 to 10%, or even 10 to 100%. Accurate stoichiometry measurements are required to interpret biological significance of acylation and to predict impact regarding the magnitude of protein structural, and possibly, functional changes.

One previous method to quantify site occupancy utilizes heavy stable-isotope chemical labeling of unmodified lysine residues followed by mass spectrometry to measure the ratio between endogenous "light" acetylation in comparison to the exogenous, chemically-labeled "heavy" acetyl-lysine using precursor ion intensities[12]. Another recent study by Zhou *et al.*[13] described a similar approach to assess lysine acetylation stoichiometry that also employed a complete chemical acetylation of all unmodified lysine residues in proteins with stable isotope labeling, but used fragment ion intensities as measured by DDA. Nakayasu *et al.*[14] used a similar DDA approach, but instead used the ratio of light and heavy acetyl-lysine immonium ions for quantification. Quantification based on fragment ions, immonium or sequence ions (MS2), in most cases results in less signal interferences compared to processing intact peptide precursor ions (MS1). However, quantification of fragment ions from DDA-generated MS/MS spectra can suffer from stochastic sampling deficiencies, where the high-abundance precursor ions are more likely to be selected for MS/MS and thus, lead to a biased and incomplete sampling of precursor ions.

This novel workflow[4] (**Figure 1**) uses conceptually a stable isotope chemical labeling approach originally developed by Baeza *et al.*[12], however the workflow is subsequently coupled with DIA to collect both precursor and multiple fragment ion abundances over the detectable mass range[15,16] providing accurate stoichiometry calculations.

Peak areas from fragment ions that contain the acylation site of interest, or 'differentiating fragment ions', are used to quantify acylation site occupancy (**Figure 2**). Fragment ions that do not contain the modification have identical light and heavy m/z values, and are used for peptide

identification but not for quantification. Skyline software[17] is used to extract precursor and fragment peak areas. The presence of multiple fragment ions containing a given acylation site provides flexibility if interferences are detected in some of the fragment ions. Data visualization in Skyline allows for critical inspection of light-to-heavy fragment ion ratios. In addition to manual data analysis, an open-source R package written in-house, StiochiolyzeR (https://github.com/GibsonLab/StoichiolyzeR), was developed, which uses the precursor ion and fragment ion data collected through DIA and quantifies site-specific acylation stoichiometry from peptides containing multiple lysine residues, a feature not possible with precursor ion-only intensity measurements[4].

This protocol also demonstrates the use of endoproteinase Glu-C, which is specific for C-terminal cleavage at glutamic and aspartic acid residues, instead of using trypsin or Arg-C protease, as the latter often generate very large and potentially multiply acylated proteolytic peptides resulting from blocked trypsin cleavage at acylated lysine residues. The chemical labeling procedure was also extended to include use of succinic anhydride (**Figure 1**), thereby enabling the quantification of succinylation stoichiometry succinylation. In addition to improvements to the sample preparation, the implementation of peptide fractionation by offline, basic pH, reversed-phase (bRP) separation of peptides further decreased quantification interferences, allowing better de-convolution of acylation stoichiometry in whole proteome samples. Together, this method features and highlights several advantages: (1) increased efficiency of chemical acylation; (2) measurement of protein succinylation stoichiometry in addition to acetylation; and (3) improved quantification accuracy. The improved quantification is due to decreased interferences in fragment ion signals from DIA compared to DDA precursor signal, as well as the implementation of off-line peptide pre-fractionation by bRP.

Protocol

1. Quantitatively Acetylate and/or Succinylate Proteins Using Isotope-labeled Acetic or Succinic Anhydride

1. Prepare 3 replicates of 100 µg of bovine serum albumin (BSA) protein sample in parallel, at a concentration of 1 µg/µL (total of 100 µL), using urea and triethylammonium bicarbonate (TEAB) solution (8 M urea, 200 mM TEAB, pH 8).
   NOTE: It is important to use amine-free buffer. The protein samples used here in the representative results section are BSA, quantitatively succinylated BSA, and mixtures thereof yielding BSA samples with defined succinylation occupancy at 0%, 1%, 10%, 50%, and 100%, respectively (each sample will be prepared in 3 replicates). This protocol has also been performed using protein lysates from *Escherichia coli* and mouse liver[4]. The protocol can also be applied to other cell or tissue lysates.
2. Reduce 100 µL of BSA sample (100 µg) with 8 µL of 250 mM dithiothreitol (DTT) to reach a final concentration of 20 mM DTT and incubate the sample at 37 °C for 30 min with agitation at 1,400 rpm.
3. Alkylate the reduced BSA sample with 21.6 µL of 200 mM iodoacetamide to reach a final concentration of 40 mM iodoacetamide and incubate the sample in the dark at room temperature for 30 min.
   NOTE: It is important to have the iodoacetamide concentration slightly more than 2x of the DTT concentration to ensure that all reduced protein thiols are alkylated.
4. **Proceed with preparing either acetic anhydride-d_6 (step 1.4.1) or succinic anhydride-d_4 (step 1.4.2) needed for chemical (quantitative) acetylation or succinylation of the samples.**
   1. Determine the molarity (M) of the 1 g aliquot of aqueous acetic anhydride-d_6 solution from the molecular weight (108.13 g/mol) and density (1.143 g/mL at 25 °C).
      NOTE: The 1 g package contains 9,248 µmol of acetic anhydride-d_6 in 875 µL volume, yielding a 10.57 M solution used for per-acetylation of the samples.
   2. Prepare a 5 M solution of succinic anhydride-d_4 by dissolving the powder in anhydrous DMSO immediately prior to the reaction to avoid hydrolysis of the reagent. Dissolve 0.24 g of succinic anhydride-d_4 in 461 µL of anhydrous DMSO to obtain a 5 M solution.
5. Perform quantitative chemical acetylation or succinylation by adding 60 µmol of acetic anhydride-d_6 (6 µL of the 10.57 M solution) or succinic anhydride-d_4 (12 µL of the 5 M solution) respectively, and incubate the sample at 4 °C for 20 min on a vortex mixer.
   NOTE: Due to the acidity of anhydrides, proteins may precipitate. Take note and adjust as described in step 1.6.
6. Add 10 µL of 7.25 M NaOH after the incubation to increase the pH to ~8 to eliminate any O-acylation side-products. Vortex briefly and check the sample pH by spotting 1 µL on pH paper.
   NOTE: It may be necessary to add more than 10 µL of 7.25 M NaOH to reach pH 8. In addition, potentially precipitated proteins should re-dissolve.
7. Repeat the per-acylation and pH adjustment steps 1.5 and 1.6 for a total of three times. Check the pH values and note the volume of basic solution added per repetition. Work swiftly during the above chemical acylation steps because the anhydrides will hydrolyze and lose reactivity.
8. After the final labeling reaction and pH adjustment, add 10 µL of 50% hydroxylamine solution to revert O-acylation side reactions.

2. Digest Reacted Protein Samples Using Glu-C Endoproteinase

1. Dilute the urea concentration to 0.8 M using 50 mM TEAB, pH 8 and normalize the sample volumes.
2. Digest the protein samples by adding endoproteinase Glu-C at a 1:50 protease to substrate protein ratio (w/w) and incubate the samples overnight at 37 °C with agitation at 1,400 rpm.
3. Acidify and quench the protein digestion samples by adding formic acid to 1% by volume.
4. **Desalt the samples using hydrophilic-lipophilic balance solid-phase extraction cartridges. Use one 30 mg sorbent cartridge per sample, maximum 5 mg material per cartridge[4,18].**
   NOTE: It is important to keep the sorbent inside the cartridge wet throughout this desalting process. When necessary, turn off the vacuum pump to slow down the passage of solvent or sample through the cartridge.
   1. Insert the cartridge(s) into the port(s) on the 24-port glass block vacuum manifold and turn on the vacuum pump. Leave unused port(s) capped. Leave one or two ports un-capped to maintain lower pressure on the vacuum gauge if necessary.

2. Wet each cartridge two times with 800 µL of 80% acetonitrile (ACN)/0.2% formic acid/19.8% water. Ensure solvent level remains 2–3 mm above the sorbent level. Ensure the vacuum gauge on the manifold reads 16.9–67.7 kPa (5–20 in Hg).
3. Equilibrate each cartridge three times with 800 µL of 0.2% formic acid in water. Ensure the vacuum gauge on the manifold reads 16.9–67.7 kPa (5–20 in Hg).
4. Load the peptide samples on to the cartridge. Ensure the vacuum gauge on the manifold reads 6.77–8.47 kPa (2–2.5 in Hg), and flow rate does not exceed 1 mL/min.
5. Wash each cartridge three times with 800 µL 0.2% formic acid in water. Ensure the vacuum gauge on the manifold reads 16.9–67.7 kPa (5–20 in Hg).
6. Remove the cartridge from the vacuum manifold and settle into a 1.5 mL microtube.
7. Elute peptides one time with 800 µL of 80% ACN/0.2% formic acid/19.8% water and allow the solvent to incubate with sorbent for 2–3 min.
8. Use a pipette to push solvent through the sorbent layer inside the cartridge. Repeat for the second elution of 400 µL 80% ACN/0.2% formic acid/19.8% water into the same 1.5 mL microtube.

5. Dry the eluate by vacuum centrifugation (fixed centrifugation rate at 268 x *g*), typically for 3 h.
NOTE: If needed, dried eluate samples can be stored frozen at -20 °C until ready to proceed with fractionation.

3. Fractionate Peptides Using Offline Basic-pH Reversed Phase HPLC

1. Re-suspend the per-acylated and Glu-C digested samples (prepared as described in Sections 1 and 2) in 200 µL of Buffer A (10 mM ammonium formate in water, pH 10), mix the sample for 10 min on a vortex mixer at 4 °C, and centrifuge at 17,500 x *g* for 10 min at room temperature.
NOTE: The resulting peptide sample is ready to be offline fractionated by high pH separation[19,20].
2. Program the offline HPLC fractionation method depending on the instrument and software used. The following method is specific for a binary HPLC pump system including a dual wavelength UV detector, an autosampler, a fraction collector, and a C18 column (4.6 mm x 250 mm, 5 µm particle size) that tolerates pH 10.
3. Collect 40 fractions each for 2.1 min per fraction and combine every fourth fraction, resulting in four final pooled fractions[4]. A greater number of pooled fractions can be used to further reduce sample complexity at the expense of mass spectrometry data collection time.
4. Dry the pooled fractions in a lyophilizer, re-suspend the dried peptide samples in 1 mL of 50% ACN and 1% formic acid in water, and transfer to a smaller sample container. Dry the transferred sample via vacuum centrifugation (fixed centrifugation rate at 268 x *g*), typically for 1 h.
NOTE: The low vapor pressure of ammonium formate can make complete drying difficult. If significant amounts of ammonium formate salt remain as salt precipitate after drying, repeat the solid phase extraction.
5. Re-suspend the samples in 20 µL of 0.2% formic acid in water with indexed retention time (iRT) peptide standard mixture.
NOTE: Samples are now ready for analysis by mass spectrometry.

4. DIA Analysis of Fractionated Peptide Samples

1. **Analyze the samples using a DIA LC-MS/MS method, which can be adjusted according to the mass spectrometric instruments available. The analysis was performed using a nano-LC 2D HPLC system online connected to an orthogonal quadrupole time-of-flight (QqTOF) mass spectrometer.**
    1. Program the instrument software so that peptide mixtures are transferred onto a C18 pre-column chip (200 µm x 6 mm C18-CL chip, 3 µm, 300 Å) and wash at 2 µL/min for 10 min with the loading solvent (H_2O/0.1% formic acid) for desalting.
    2. Separate the peptides chromatographically on a 75 µm x 15 cm C18-CL chip (3 µm, 300 Å) at a flow rate of 300 nL/min with a 2 h gradient using typical (and LC-system specific) aqueous and ACN solvent buffers[4].
    3. Generate a variable window DIA method, where the mass range windows of variable width (5 to 90 *m/z*) are passed from the Q1 quadrupole into the collision cell in incremental steps over the full mass range (*m/z* 400-1,250).
    NOTE: The cycle time of 3.2 s includes a 250 ms precursor ion scan followed by 45 ms accumulation time for each of the 64 SWATH segments[21,22].

2. Identify peptides using a parallel analysis of samples by DDA MS and building of spectral libraries, or alternatively, peptides can be identified directly from DIA data using PIQED software[23], which handles all technical steps of data processing, identification, and import into Skyline for subsequent quantification.

5. Example Data Analysis Tutorial

1. **Download the Skyline software (http://proteome.gs.washington.edu/software/skyline) and create a PanoramaWeb account (https://panoramaweb.org).**
NOTE: For detailed tutorials describing use of Skyline with data, please see (https://skyline.ms/wiki/home/software/Skyline/page.view?name=tutorials). Other software algorithms may be used instead to extract light and heavy fragment ion peak areas from the DIA acquisitions, such as Open SWATH[24], SWATH 2.0 plugin into PeakView[25], and Spectronaut[26].
    1. Download the Skyline data file for the succinylated BSA representative results (**Figure 3**) from Panorama Public: https://panoramaweb.org/labkey/JoVEsuccBSA.url. On the Panorama webpage, go to the "Targeted MS Runs" table, and download the sky.zip file choosing the DOWNLOAD link on the right side. Extract the downloaded .zip folder and double click on the skyline.sky file to open it in Skyline. Check the peptide fragment ion target tree (left panel) and the four chromatograms from defined percentages of light succinylation (right panels).
    2. Select the "View" menu, navigate to "Peak Areas | Replicate Comparison". The "Peak Areas – Replicate Comparison" panel containing bar graphs will appear on the right side of the window. In the Peak Areas panel, right-click and select "Transitions | Single", which

shows the fragment ion peak area for the selected fragment ion. Finally, right-click the "Peak Areas" panel and select "Order | Document".

3. In the target tree panel on the left side of the window, right-click on the peptide "E.LCKVASLRE.T | Ratios To |Oneheavy", which calculates the ratio of light ion signal over heavy ion signal, Light/Heavy.
   NOTE: This is not the same as site occupancy stoichiometry ratio of Light/(Heavy+Light).
4. Notice that the target tree now reports the ratios L/H to the right of the light fragment ions.

2. **Determine the "differentiation fragment ions" that contain the acylation site of interest.**
   NOTE: As shown in **Figure 3**, featuring this exact example, the differentiating ions were determined as y_7 (highest ranked), y_8, b_3, and b_4. Notice that the non-differentiating fragment ions show a ratio of 1 in the target tree.
   1. In the left panel target tree, under the 588.30++ sub node of peptide E.LCKVASLRE.T, click on the y7 differentiating fragment ion **K [y_7] – 902.49+ (rank1) [5]**
      Notice the increasing peak height and area.
   2. In the left panel target tree, under the 588.30++ sub node of peptide E.LCKVASLRE.T, click on the y_5 non-differentiating fragment ion **A[y_5] – 575.31+ (rank 6) [1]**. Observe that the y_5 fragment ion chromatograms and peak areas remain in the same range for all samples.
   3. In the target tree panel, browse through other differentiating (ratio other than 1) and non-differentiating (ratio of 1) fragment ions and notice the changes and patterns in the peak area bar graph and chromatograms.
   4. Determine the exact peak areas for the light and heavy pairs of the differentiating ions in order to calculate the stoichiometry (for additional details also see Meyer *et al.*[4]). Click on the "View | Document Grid" and the document grid will pop up. In the top left of the document grid, click on "Views | Transition Results" to see a multi-column table with fragment ion information, such as Peptide Sequence, Precursor mz, Replicate Name, *etc.*
   5. Change the report format to "pivot" by again clicking on "Views" in the document grid and selecting "Edit View" to open the Customize View window. At the bottom left of the "Customize View" window, check the "Pivot Replicate Name" and select "Ok" to close the Customize View window. Observe that now the "Document Grid: Transition Results" table window has re-arranged to group the Replicate Name, Retention Time, Area, Background, Peak Rank columns by Replicate (1%, 10%, 50%, 100% succinylation).
   6. For the peptide "E.LCKVASLRE.T" in the "Document Grid: Transition Result" table, find the "Precursor Mz" column, the "Fragment Ion" column, and the 1pct_light_sw1 Area (1% succinylation) column. Observe that there are two precursor ions for this peptide, light (Precursor Mz of 588.30 in the first 8 rows), and heavy (Precursor Mz of 590.32 in the last 8 rows).
   7. In the "Fragment Ion" column, find the two rows for the y8 fragment ion corresponding to the light and the heavy precursor ions. From the same rows in the 1pct_light_sw1 Area column, record the y8 areas for the light (15,820), and the heavy (1,426,461).

3. Using these peak area values, compute the succinylation stoichiometry, or the proportion modified, according to the formula below and obtain a stoichiometry ratio of 0.01, or 1% succinylation, which matches the proportion of light succinylation in this defined mixture:

$$\frac{light\ area}{(light\ area) + (heavy\ area)} = \frac{L}{L+H} = \frac{15{,}820}{15{,}820 + 1{,}426{,}461} = 0.01$$

4. Calculate the stoichiometry ratio for 10% succinylation using the y8 differentiating fragment ion peak area values from the 10pct_light_sw1 Area column, 144,953 (light) and 1,188,041 (heavy), to obtain a ratio of 0.10, or 10% succinylation.
5. Calculate the stoichiometry ratio for 100% succinylation using the y8 differentiating fragment ion peak area values from the 100pct_light_sw1 Area column, 954,513 (light) and 16,407 (heavy), to obtain a ratio of 0.98, or 98% succinylation.
6. Similarly, calculate the stoichiometry ratio for 1% succinylation using the b3 differentiating fragment ion peak area values from the 1pct_light_sw1 Area column, 55,697 (light) and 3,149,119 (heavy), to obtain a ratio of 0.01, or 1% succinylation.
7. Continue the stoichiometry ratio calculations using the formula for all the differentiating fragment ions, both y and b ions, to obtain the lysine succinylation stoichiometry values for 1, 10, 50, and 100% succinylation mixtures.

Representative Results

After data acquisition, differentiating MS2 fragment ions were determined from the proteolytic acylated peptides, subsequently extracted ion chromatograms (XIC) were processed in Skyline, and corresponding light and heavy peak areas were exported which were finally used to calculate site occupancy. **Figure 2A** shows a conceptual illustration of how the precursor-ion XIC may appear featuring both light and heavy peptide signals, and **Figure 2B** presents an example of the corresponding fragment ion XICs with color coding (red = light; blue = heavy) for differentiating fragment ions containing the lysine acylation modifications.

Figure 3 shows data resulting from an occupancy experiment, such as described above analyzing pre-defined ratios of light/heavy succinylated BSA generated in-house at 1, 10, 50, or 100%, and the determination of succinylation occupancy thereof. Importing DIA occupancy data into Skyline enables easy visualization of the target tree, displaying peptide sequences, and fragment ions both for the light and heavy precursor ions (**Figure 3A**). Data from DIA-MS of each defined succinylated BSA ratio revealed immanently the relative differences in ratio of light-to-heavy y_7 ion, the highest-ranked differentiating fragment ion from the identified peptide-spectra match (indicated in red, **Figure 3B**). **Figure 4** shows an overview of the processed results from the MS2 occupancy calculation that confirmed the succinylation percentages occupancy of input protein, here consisting of the pre-succinylated BSA. Measured lysine succinylation occupancy for 20 proteolytic succinylated peptides obtained from commercial pre-succinylated BSA, succinylated at defined percentages (*e.g.*, at 0, 1, 10, 50, and 100%) are shown in **Table 2**. For each of the 20 proteolytic BSA peptides, the highest ranked, differentiating MS2 fragment ion was used to calculate lysine succinylation (Ksucc) occupancy, as L/(L+H) in %. Overall, the MS2-based quantification showed very good accuracy in determining acylation occupancy even at low stoichiometries.

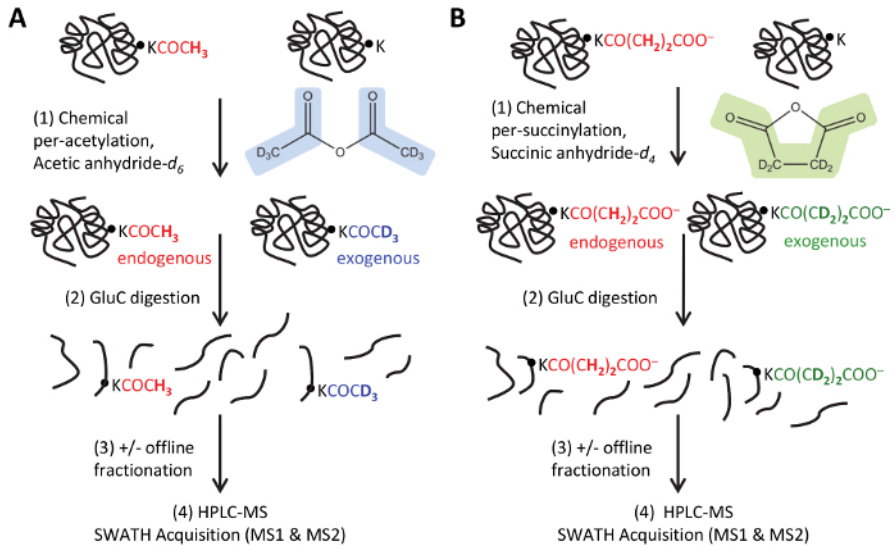

Figure 1: Stoichiometry workflow. (**A**) Proteins are incubated three times with acetic anhydride-d_6 to acetylate unmodified lysine residues. Next, acetylated proteins are digested with endoproteinase Glu-C, followed by HPLC fractionation of the proteolytic peptides using basic-pH reversed-phase chromatography. Finally, peptides are analyzed by LC-MS using DIA with variable precursor window widths. (**B**) The same workflow is used to determine the succinylation stoichiometry as described in (**A**), except the heavy acylation reagent is changed to succinic anhydride-d_4. Figure adapted from Meyer *et al.*[4] (J. Am. Soc. Mass Spectrom., Open Choice). Please click here to view a larger version of this figure.

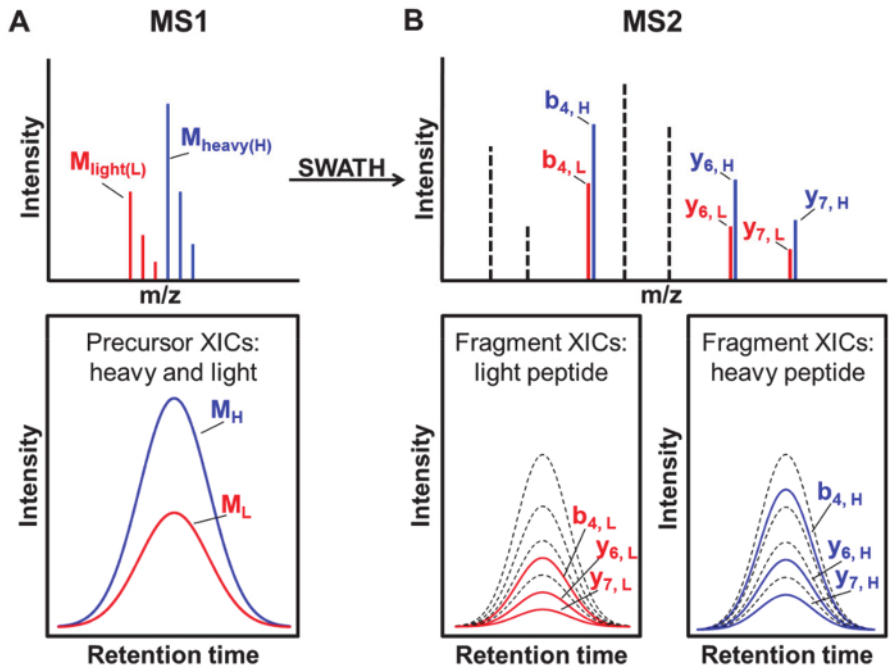

Figure 2: Example of possible data obtained and stoichiometry calculations. (**A**) Light (L) and heavy (H) MS1 precursor ions containing one acetylated lysine differ by 3 mass units. XICs are generated for both light and heavy isotopic envelopes indicated in red and blue, respectively. (**B**) Quantification from the MS2 fragment ion XICs from DIA acquisitions can be performed using 'differentiating' light and heavy fragment ions that contain the acetylation site indicated in red and blue. Common fragment ions are displayed in dotted black and do not contain the site of modification. Figure adapted from Meyer *et al.*[4] (J. Am. Soc. Mass Spectrom., Open Choice). Please click here to view a larger version of this figure.

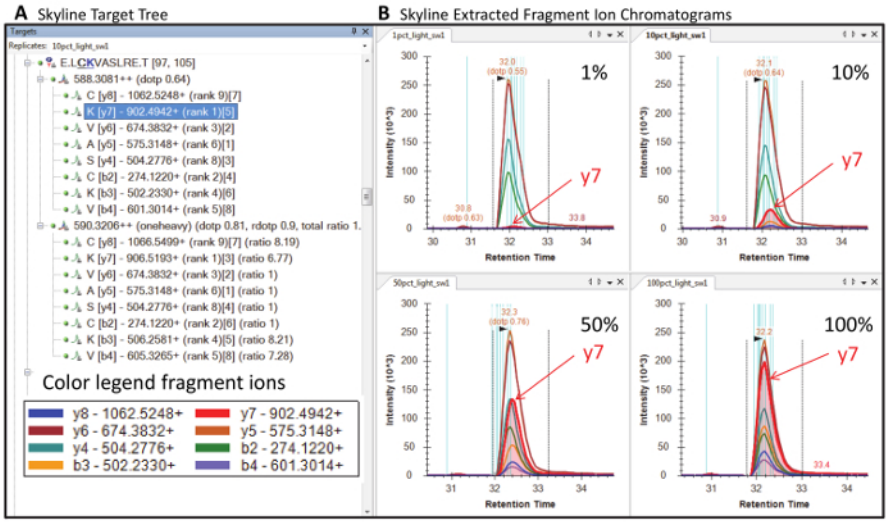

Figure 3: Skyline visualization of succinylated proteolytic BSA peptide at different occupancy levels. (**A**) Skyline Target Tree showing the proteolytic peptide LCKsuccVASLRE and its resulting fragment ions. (**B**) Skyline extracted fragment ion chromatograms at varying levels of succinylation occupancy. The highest ranked differentiating ion, y_7, increases in peak area 1, 10, 50, and 100% correlating to the input percentage of pre-succinylated BSA (generated in-house). Please click here to view a larger version of this figure.

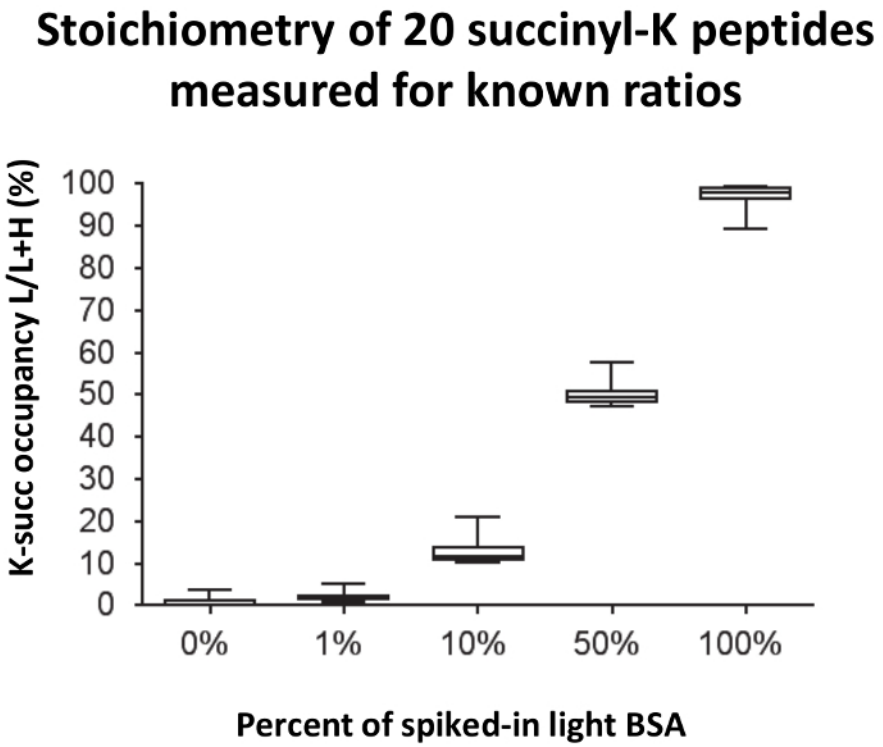

Figure 4: Assessing BSA acylation stoichiometry of pre-succinylated BSA. Five different BSA samples at defined percentages of heavy modification (*e.g.*, at 0, 1, 10, 50, and 100%) were subjected to MS2 occupancy determination. Site occupancy for 20 succinylated BSA peptides were determined from the highest ranked differentiating fragment ions for five different BSA samples at defined percentages of light modification (*e.g.*, at 0, 1, 10, 50, and 100%). The Box Whisker plot displays the distribution of the 20 succinyl peptides, values from 50% of peptides are in the 'box' (25% percentile to 75% percentile), the upper whisker indicates the values from 75% percentile to maximum (100%), and the lower whisker indicates the values from 25% percentile to minimum (0% percentile). (J. Am. Soc. Mass Spectrom., Open Choice). Quantification of measurements can be found in **Table 2**. Please click here to view a larger version of this figure.

Time (minute)	% A	% B
0.00	100	0
7.27	92	8
45.27	73	27
49.27	69	31
65.27	61	39
72.27	40	60
80.00	10	90
85.00	10	90
86.00	100	0
120.00	100	0
Flow rate: 0.7 mL/min		
Buffer A: 10 mM ammonium formate in water, pH 10		
Buffer B: 10 mM ammonium formate in 90% ACN and 10% water, pH 10		
Note: The pH of both mobile phases adjusted to 10 with neat ammonia		

Table 1: Gradient for offline basic-pH reversed-phase HPLC fractionation. Gradient length: 120 min. Buffer A: 10 mM ammonium formate in water, pH 10. Buffer B: 10 mM ammonium formate in 90% ACN and 10% water, pH 10.

L/L+H in %	L/L+H in %	L/L+H in %	L/L+H in %	L/L+H in %
Ksucc 0%	Ksucc 1%	Ksucc 10%	Ksucc 50%	Ksucc 100%
0.2	1.7	11.1	50.9	99.2
1.2	2.3	12.3	49.4	97.6
2.9	3.8	14	48.6	99.5
0.5	1.8	11.7	50.8	99.5
0.2	1.7	11.1	48.3	98.2
0.2	1.2	11	47.5	96.1
0.3	1.6	12.8	51	99.2
3.7	5.2	14.7	51.9	89.5
1.5	1.8	12.5	47.2	91.1
0.8	1.5	11.3	48.4	96.8
0.2	1.6	13.9	49.7	98.9
0.1	1.1	10.7	48.2	98.6
0.2	0.9	10.3	49.4	99.4
0.5	2.5	17.2	52.3	97.5
0.1	3.5	20.8	51	99
0.3	1.9	10.7	49.6	98.2
2	1.7	10.9	47.7	96.3
0.3	1.2	10	53	98
1.1	1.8	12.9	57.9	94.1
0.2	1.4	11.6	48.6	98.8

Table 2: Quantification of measured BSA lysine succinylation occupancy for 20 proteolytic succinylated peptides. Succinylated peptides obtained from commercial pre-succinylated BSA, succinylated at defined percentages (*e.g.*, at 0, 1, 10, 50, and 100%). For each of the 20 proteolytic BSA peptides, the highest ranked, differentiating MS2 fragment ion was used to calculate lysine succinylation (Ksucc) occupancy, as L/(L+H) in %.

Discussion

This protocol provides a novel and accurate method to quantify site-specific lysine acetylation and succinylation occupancy that can be applied to an entire proteome. This method relies on measurement of endogenous light peptides and exogenous heavy peptides, the latter of which

are generated *in vitro* using quantitative chemical acylation of proteins with deuterated acetic anhydride-d_6 or succinic anhydride-d_4 (**Figure 1**). Similar methods have used stable isotopic labeling of native unmodified lysine residues and performed site occupancy quantification based on either precursor ion-only[12] or fragment ions from DDA acquisitions[13,14]. This protocol applies several steps during sample preparation to improve acylation efficiency and extends the chemical labeling to succinylation. Data collection using DIA acquisitions obtains both precursor ion and MS2 fragment ion intensities over the entire detectable mass range, thus reducing the fragment ion interferences. Analysis and quantification via Skyline and custom scripts developed in-house allow the site occupancy calculation for peptides containing more than one lysine residue and more than one acylation at one site.

There are several critical steps during the sample preparation stage of this protocol that should be followed closely. Since the entire protocol relies on efficient chemical modification of all lysine residues, this step is of utmost importance. Anhydrides react with free amines, so amine-containing buffer or contaminant molecules in the protein lysate must be avoided. Also during the chemical per-acylation step, ensure that the pH of the reaction mixture is adjusted back to pH 8 after each of the three incubations with anhydride reagent as this reaction acidifies the mixture, and O-acylation side-reactions may form. Furthermore, dilution of the 8 M urea-containing reaction mixture to around 0.8 M urea prior to digestion and checking that the pH is within the optimal range is important for the optimum activity of the endoproteinase Glu-C. Another key component of our protocol is the data collection step and the introduction of a DIA workflow, which overcomes any DDA data sampling inconsistencies and which allows for quantification at the MS2 fragment ion level. One main advantage of the DIA methodology is the decrease of interferences which are typically much more prone and problematic when quantifying the MS1 precursor ion signal (as some of the previous publications have suggested).

Several modifications to the workflow can be made when preparing highly complex samples. The number of fractions pooled after the offline basic-pH reversed-phase HPLC fractionation can be decreased to lower the complexity of the pooled samples that will be acquired by LC/MS. Additionally, longer chromatographic gradients can also be used during acquisition to allow for better peptide separation. Alternatively, multiple proteases may be used to digest samples to produce a larger variety of cleaved peptides and increase coverage of protein lysine residues. Trial runs and optimization can be conducted using commercial BSA containing specific percentages of acetylation or succinylation.

One limitation to this protocol is potentially slight site occupancy overestimation due to unaccounted other lysine modifications, such as methylation or ubiquitination, *etc.*, at the same site[4]. Calculated from the peak areas of light and heavy acyl modifications, L/(L+H), the site occupancy is based on the assumption that the total level of modification at a lysine site consists of only endogenous 'light' acylation (L) and chemically labeled 'heavy' acylation (H). However, the actual total acylation occupancy at a site *in vivo* may include other modifications beyond acetylation and/or succinylation. Additionally, the reported isotopic purity of the purchased reagents acetic anhydride-d_6 (99%) and succinic anhydride-d_4 (>98%) may contribute to a small overestimation of up to 2% (succinyl) or 1% (acetyl) of the endogenous acylation level[4]. Together, these slight overestimations add to the difficulty in quantifying sites with less than 1% occupancy. Large abundance differences between the complete chemically acylated peptides and the native acylated peptides also contribute to potential site occupancy quantification errors, especially for low endogenous acylated peptides[27]. A recent study by Weinert *et al.*[27] found that decreased abundance differences between complete per-acetylated peptides can reduce the quantification error[27].

Stoichiometry quantifications gathered using this protocol can pinpoint important acylation hotspots in a particular protein and form hypotheses for biological follow-up experiments, such as site-directed mutagenesis of certain sites of interest. This protocol could also reveal combined effects of low acylation stoichiometry sites that could exert indirect or subtle influences on protein structural stability and localized cellular environments. Additionally, implementation of this protocol to measure acetylation and succinylation and other possible lysine acylation modifications beyond acetylation would uniquely offer insights into the dynamic acylation effects on proteins under different biological conditions.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by the NIH National Institute of Diabetes and Digestive and Kidney Diseases NIH-NIDDK grant R24 DK085610 (PI: E. Verdin). JGM was supported by a NIH T32 fellowship (T32 AG000266, PI: J. Campisi). The authors acknowledge support from the NIH shared instrumentation grant for the TripleTOF 6600 system at the Buck Institute (1S10 OD016281, PI: B.W. Gibson).

References

1. Choudhary, C., Weinert, B. T., Nishida, Y., Verdin, E., & Mann, M. The growing landscape of lysine acetylation links metabolism and cell signalling. *Nat Rev Mol Cell Biol.* **15** (8), 536-550 (2014).
2. Verdin, E., & Ott, M. 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat Rev Mol Cell Biol.* **16** (4), 258-264 (2015).
3. Choudhary, C. *et al.* Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science.* **325** (5942), 834-840 (2009).
4. Meyer, J. G. *et al.* Quantification of Lysine Acetylation and Succinylation Stoichiometry in Proteins Using Mass Spectrometric Data-Independent Acquisitions (SWATH). *J Am Soc Mass Spectrom.* **27** (11), 1758-1771 (2016).
5. Hirschey, M. D., & Zhao, Y. Metabolic Regulation by Lysine Malonylation, Succinylation, and Glutarylation. *Mol Cell Proteomics.* **14** (9), 2308-2315 (2015).
6. Still, A. J. *et al.* Quantification of mitochondrial acetylation dynamics highlights prominent sites of metabolic regulation. *J Biol Chem.* **288** (36), 26209-26219 (2013).
7. Wagner, G. R., & Payne, R. M. Widespread and enzyme-independent Nε-acetylation and Nε-succinylation of proteins in the chemical conditions of the mitochondrial matrix. *J Biol Chem.* **288** (40), 29036-29045 (2013).

8. He, W., Newman, J. C., Wang, M. Z., Ho, L., & Verdin, E. Mitochondrial sirtuins: regulators of protein acylation and metabolism. *Trends Endocrinol Metab.* **23** (9), 467-476 (2012).
9. Castano-Cerezo, S., Bernal, V., Rohrig, T., Termeer, S., & Canovas, M. Regulation of acetate metabolism in Escherichia coli BL21 by protein N(epsilon)-lysine acetylation. *Appl Microbiol Biotechnol.* **99** (8), 3533-3545 (2015).
10. Zhang, J. *et al.* Lysine Acetylation is a Highly Abundant and Evolutionarily Conserved Modification in Escherichia coli. *Mol Cell Proteomics.* **8** (2), 215-225 (2009).
11. Kuhn, M. L. *et al.* Structural, kinetic and proteomic characterization of acetyl phosphate-dependent bacterial protein acetylation. *PLoS One.* **9** (4), e94816 (2014).
12. Baeza, J. *et al.* Stoichiometry of site-specific lysine acetylation in an entire proteome. *J Biol Chem.* **289** (31), 21326-21338 (2014).
13. Zhou, T., Chung, Y. H., Chen, J., & Chen, Y. Site-Specific Identification of Lysine Acetylation Stoichiometries in Mammalian Cells. *J Proteome Res.* **15** (3), 1103-1113 (2016).
14. Nakayasu, E. S. *et al.* A method to determine lysine acetylation stoichiometries. *Int J Proteomics.* **2014** 730725 (2014).
15. Rardin, M. J. *et al.* MS1 Peptide Ion Intensity Chromatograms in MS2 (SWATH) Data Independent Acquisitions. Improving Post Acquisition Analysis of Proteomic Experiments. *Mol Cell Proteomics.* **14** (9), 2405-2419 (2015).
16. Gillet, L. C. *et al.* Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics.* **11** (6), O111 016717 (2012).
17. MacLean, B. *et al.* Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics.* **26** (7), 966-968 (2010).
18. Addona, T. A. *et al.* Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol.* **27** (7), 633-641 (2009).
19. Svinkina, T. *et al.* Quantitative Coverage of the Lysine Acetylome Using Novel Anti-acetyl-lysine Antibodies and an Optimized Proteomics Workflow. *Mol Cell Proteomics.* **14** (9), 2429-2440 (2015).
20. Wang, Y. *et al.* Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. *Proteomics.* **11** (10), 2019-2026 (2011).
21. Schilling, B. *et al.* Protein acetylation dynamics in response to carbon overflow in Escherichia coli. *Mol Microbiol.* **98** (5), 847-863 (2015).
22. Schilling, B., Gibson, B. W., & Hunter, C. L. Generation of High-Quality SWATH Acquisition Data for Label-free Quantitative Proteomics Studies Using TripleTOF Mass Spectrometers. *Methods Mol Biol.* **1550** 223-233 (2017).
23. Meyer, J. G. *et al.* PIQED: automated identification and quantification of protein modifications from DIA-MS data. *Nat Methods.* **14** (7), 646-647 (2017).
24. Rost, H. L. *et al.* OpenSWATH enables automated, targeted analysis of data-independent acquisition MS data. *Nat Biotechnol.* **32** (3), 219-223 (2014).
25. Lambert, J. P. *et al.* Mapping differential interactomes by affinity purification coupled with data-independent mass spectrometry acquisition. *Nat Methods.* **10** (12), 1239-1245 (2013).
26. Bruderer, R. *et al.* Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. *Mol Cell Proteomics.* **14** (5), 1400-1410 (2015).
27. Weinert, B. T. *et al.* Accurate Quantification of Site-specific Acetylation Stoichiometry Reveals the Impact of Sirtuin Deacetylase CobB on the E. coli Acetylome. *Mol Cell Proteomics.* **16** (5), 759-769 (2017).