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Integrated Analysis of Acetyl-CoA and Histone Modification via Mass Spectrometry to Investigate Metabolically Driven Acetylation

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Summary

Acetylation is a highly abundant and dynamic post-translational modification (PTM) on histone proteins which, when present on chromatin bound histones, facilitates the accessibility of DNA for gene transcription. The central metabolite, acetyl-CoA, is a substrate for acetyltransferases, which catalyze protein acetylation. Acetyl-CoA is an essential intermediate in diverse metabolic pathways, and cellular acetyl-CoA levels fluctuate according to extracellular nutrient availability and the metabolic state of the cell. The Michaelis constant (K_m) of most histone acetyltransferases (HATs), which specifically target histone proteins, fall within the range of cellular acetyl-CoA concentrations. As a consequence, global levels of histone acetylation are often restricted by availability of acetyl-CoA. Such metabolic regulation of histone acetylation is important for cell proliferation, differentiation and a variety of cellular functions. In cancer, numerous oncogenic signaling events hijack cellular metabolism, ultimately inducing an extensive rearrangement of the epigenetic state of the cell. Understanding metabolic control of the epigenome through histone acetylation is essential to illuminate the molecular mechanisms by which cells sense, adapt and occasionally disengage nutrient fluctuations and environmental cues from gene expression. In particular, targeting metabolic regulators or even dietary interventions to impact acetyl-CoA availability and histone acetylation is a promising target in cancer therapy. Liquid chromatography coupled to mass spectrometry (LC-MS) is the most accurate methodology to quantify protein PTMs and metabolites. In this chapter, we present state-of-the-art protocols to analyze histone acetylation and acetyl-CoA. Histones are extracted and digested into short peptides (4–20 aa) prior to LC-MS. Acetyl-CoA is extracted from cells and analyzed using an analogous mass spectrometry-based procedure. Model systems can be fed with isotopically labeled substrates to investigate the metabolic preference for acetyl-CoA production and the metabolic dependence and turnover of histone acetylation. We also present an example of data integration to correlate changes in acetyl-CoA production with histone acetylation.

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Keywords

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1. Introduction

Post-translational modifications (PTMs) of histone proteins are important epigenetic signals that change the physical accessibility of the genome. Histone PTMs affect DNA-protein interactions and, subsequently, gene transcription, without altering the underlying DNA sequence [1]. Lysine acetylation is a widespread histone PTM and is catalyzed by a class of enzymes known as histone acetyl-transferases (HATs). The core histone proteins (H2A, H2B, H3 and H4) form the nucleosome structure around which DNA is tightly wrapped. Core histone proteins can be acetylated on multiple residues; notably, histones have long unstructured tails that protrude from the nucleosome that can be abundantly acetylated. Histones are enriched in basic amino acid residues (Arg and Lys) making them positively charged. Acetylation of lysine residues neutralizes this positive charge, relaxing the interaction of the nucleosomes with the negatively charged DNA. Thus, histone acetylation is usually associated with chromatin openness (*euchromatin*), characterized by accessibility to transcription factors and other DNA-associated proteins, and active gene transcription. In addition to its physical impact on nuclear architecture, acetylated histone lysines are recognized by specific protein domains (*bromodomains*) usually found in potent gene activators [2]. A varied set of enzymes, known as histone deacetylases (HDACs), catalyze a rapid and energetically inexpensive removal of acetyl moieties from histones [3]. Altogether, histone acetylation is a dynamic and reversible chromatin modification that allows cells to rapidly and potently modulate gene expression in order to adapt to extracellular stimuli. However, it is now clear that levels of histone acetylation can also be influenced by availability of the universal acetyl donor, acetyl-CoA [4–6]. Acetyl-CoA is an essential intermediate in diverse metabolic pathways and cellular acetyl-CoA levels fluctuate according to extracellular nutrient availability and the metabolic state of the cell. Acetyl-CoA cannot cross the mitochondrial membrane, so only acetyl-CoA generated in the nucleocytoplasmic compartment is accessible for histone modification. In most cell settings, acetyl-CoA is produced in the nucleocytoplasmic compartment from citrate through the activity of ATP-Citrate Lyase (ACLY) (Figure 1A). Citrate generated in the mitochondrial TCA cycle is exported to fuel this process. As glucose is the primary substrate for TCA cycle activity in a number of settings, glucose limitation has been shown to restrict acetyl-CoA availability and decrease global levels of histone acetylation [7–9] (Figure 1B). Importantly, cancer cells, as well as highly specialized cell types can channel TCA carbon units into acetyl-CoA to sustain high levels of histone acetylation even when glucose is limiting or poorly utilized [10–12]. For example, activation of PI3K/Akt signaling in cancer cells leads to phosphorylation of ACLY on Serine 455, which increases enzymatic activity of approximately 6 fold and elevates acetyl-CoA and histone acetylation levels also in glucose limiting conditions [7]. Multiple groups have also shown that cells can utilize alternative carbon sources to produce acetyl-CoA, in particular acetate, which can be converted to acetyl-CoA by the acyl-coenzymeA synthetase short chain family member 2 (ACSS2). Although acetate-derived acetyl-CoA is less efficiently incorporated into histones,

contribution of acetate to histone acetylation is significantly enhanced under some conditions (e.g.: hypoxia) [10].

Acetyl-CoA is highly unstable, so the appropriate use of isotope labeled internal standard is recommended for accurate quantitation. Mass spectrometry (MS) is the most reliable approach for the quantification of acetyl-CoA. Enzyme based assays for acetyl-CoA quantitation suffer from sensitivity and specificity issues and cannot incorporate appropriate internal standards to account for sample degradation [13]. Internal standards added in equal amounts are used to normalize every sample accounting for sample loss and degradation. Exact quantitation can be achieved by comparing sample ratios to a standard curve generated by a serial dilution of known amounts of acetyl-CoA standards. Internal standard can be generated cheaply in yeast through stable isotope labeling of essential nutrients in cell culture (SILEC) where cells are fed with heavy labeled [$^{13}\text{C}_3^{15}\text{N}_1$]-vitamin B5, which is incorporated into the CoA backbone of acetyl-CoA [14]. The characteristic fragmentation pattern of Acetyl-CoA with MS/MS detection, ensures highly specific quantitation [13].

Antibody-based techniques such as Western blotting have been extensively adopted to characterize histone modifications, including acetylation. However, this approach is limited in throughput and sometimes specificity, as antibodies often cross-react with similar histone marks and multiple PTMs. A recent assessment of commercial antibodies found that more than 25% fail specificity tests in dot blot and Western blot experiments, and about 20% of antibodies fail in ChIP-seq experiments due to non-specific antibody binding [15]. In contrast, MS platforms can achieve high specificity and sensitivity, with automation facilitating high-throughput analyses. MS has thus become the most suitable analytical tool to study both Acetyl-CoA and histone modifications in general (reviewed in [16,17]).

In this chapter, we describe an integrated workflow to probe acetyl-CoA fluctuations and changes in histone acetylation. The protocol is designed for the analysis of multiple histone acetyl marks. Schematic representation of our approach is showed in Figure 2. Even though the protocol is primarily prepared for cell culture studies, adaptability to *in vivo* analysis of histone modifications and acetyl-CoA availability will be discussed.

2. Materials

2.1. Reagents and abbreviations

1. Acetonitrile (ACN)
2. Ammonium hydroxide (NH_4OH), 28% NH_3 in water
3. D-Glucose- $^{13}\text{C}_6$ and Acetate- $^{13}\text{C}_6$ (heavy labeled)
4. Propionic anhydride and acetonitrile for propionylation mixture
5. Sodium acetate- $^{13}\text{C}_2$ (heavy labeled)
6. Trichloroacetic acid (TCA)
7. Trifluoroacetic acid (TFA)
8. Trypsin (sequencing grade)

9. Hydrochloric Acid (HCl), 32%
10. Acetone, residue grade
11. Coomassie® (Bradford) Protein Assay kit
12. Coomassie® solution
13. Ammonium bicarbonate (NH₄HCO₃)
14. Glacial acetic acid

2.2. Buffers

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄
2. Ammonium bicarbonate (NH₄HCO₃): 50 mM NH₄HCO₃, pH 8.0
3. Nuclei isolation buffer (NIB-250): 15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 250 mM sucrose
4. Stage-tip loading and wash buffer: 0.1% TFA
5. Stage-tip elution buffer: 75% acetonitrile, 0.025% TFA
6. nanoLC buffer A (for histone peptide analysis): 0.1% formic acid in HPLC grade water
7. nanoLC buffer B (for histone peptide analysis): 0.1% formic acid, 95% HPLC grade ACN, in HPLC grade water
8. Extraction buffer A (for acetyl-CoA analysis): 10% (w/v) trichloroacetic acid in HPLC grade water
9. Extraction buffer B (for acetyl-CoA analysis): HPLC grade methanol containing 25mM ammonium acetate
10. Extraction buffer B (for acetyl-CoA analysis): 5% (w/v) 5-sulfosalicylic acid in HPLC grade water
11. HPLC buffer A (for acetyl-CoA analysis): 5 mM ammonium acetate in HPLC grade water
12. HPLC buffer B (for acetyl-CoA analysis): 5 mM ammonium acetate in HPLC grade acetonitrile/water (95:5, v/v)
13. HPLC buffer C (for acetyl-CoA analysis): 0.1% formic acid, 80% HPLC grade ACN, in HPLC grade water

2.3. Solutions

1. Protease inhibitors (add fresh to buffers prior to use): 1 M dithiothreitol (DTT) in ddH₂O (1000 ×); 200 mM AEBSF in ddH₂O (400 ×)
2. Phosphatase inhibitor (add fresh to buffers prior to use): 2.5 μM microcystin in 100% ethanol (500 ×)

3. HDAC inhibitor (add fresh to buffers prior to use): 5 M sodium butyrate, made by titration of 5 M butyric acid using NaOH to pH 7.0 (500 ×)
4. 10% (v/v) NP-40 Alternative in ddH₂O
5. 0.2 M H₂SO₄ in ddH₂O
6. 100% TCA (w/v) in ddH₂O

2.4. Equipment

1. Tissue and cell homogenizers (optional)
2. pH indicator strips (pH 0–14)
3. Liquid nitrogen
4. 1.5 mL microcentrifuge tubes
5. 15 and 50 mL conical tubes
6. Pipettes from P10 to P1000 range with respective tips
7. – 80 °C refrigerator
8. SpeedVac
9. Heat blocks or water baths
10. 3M Empore™ Solid Phase Extraction Disks C₁₈
11. 75 and 100 µm internal diameter fused silica tubings
12. Micro-stir magnets
13. C₁₈-AQ 3 µm bulk resin with 200–300 Å pore size for trap column and analytical column for nanoHPLC
14. Pressure cell for capillary column packing with respective compressed gas bomb (either helium, nitrogen or air)
15. Oasis HLB 1cc (30 mg) solid phase extraction columns (Waters).
16. Commercial 2.1 mm ID C₁₈ column

3. Methods

All procedures should be carried out at room temperature, unless specified otherwise. Samples can be frozen and stored in –80 °C at the end of each section, best if previously dried in a SpeedVac concentrator centrifuge. The scheme of the full workflow is illustrated in Figure 2. For simplicity, we will be discussing the use of either [¹³C]-glucose or [¹³C]-acetate to refer to the ubiquitously labeled forms (every carbon atoms substituted with heavy [¹³C] isotope). Both are environmental sources for the generation of nucleo-cytoplasmic acetyl-CoA. Glucose tracing is preferred to assess the contribution of ACLY to the existing acetyl-CoA pool. Acetate is a carbon source that does not require ACLY activity, and thus

gauges the contribution of ACSS2 to acetyl-CoA production. The use of one is alternative to the other, so informative data can be obtained only tracing the two carbon sources separately.

3.1. Labeling of biological samples using stable isotopes of glucose or acetate

3.1.1. Cell culture—Glucose limitation restricts acetyl-CoA availability in various cell lines, in a way that ultimately impacts global levels of histone acetylation. We recommend using this feature as positive control for the experiment. However, keep in mind that some cell types adapt to glucose limitation by using alternative substrates such as acetate for acetyl-CoA generation.

1. Plate an appropriate number of cells in a 10 cm dish (see *Note 1*)
2. Culture cells in standard medium for 12–36 hours
3. Replace culture medium with medium (glucose-free) supplemented with 10 mM [^{13}C]-glucose or 100 μM [^{13}C]-acetate. Use medium supplemented with dialyzed serum. Regular serum contains traceable amount of glucose or acetate, which might affect analysis. Remember to add equivalent cold counterpart (e.g.: when tracing [^{13}C]-glucose, add 100 μM [^{12}C]-acetate to the medium, and *vice versa*). For optimization, see *Note 2*
4. As positive control, replace culture medium with medium supplemented with 1 mM [^{13}C]-glucose and 100 μM acetate (unlabeled)
5. Incubate for 2–24 hours at 37°C, depending on the metabolic activity of the system adopted (may need optimization)

3.1.2 Organisms—Carbon tracing into metabolites (and potentially histone proteins) has been performed to determine the metabolic fate of glucose, acetate or glutamine *in vivo*. This approach has generated extremely valuable results, especially when applied in humans [18]. Nonetheless, *in vivo* tracing of acylated metabolites is technically demanding and presents numerous challenges. Here, we briefly outline a simplified protocol for the quantification of labeled acetyl-CoA from either [^{13}C]-glucose or [^{13}C]-acetate in mice. Note that a “stress-free” protocol for efficient and reliable tracing of metabolites *in vivo* has very recently been proposed [19]. Also, glucose can more conveniently administered by IP injection or gavage [20]. In addition, authors recommend performing all the experiments described hereafter upon approval of a proper IACUC protocol (or equivalent approval from dedicated ethical committee).

1. Prepare or treat animal according to a pre-optimized study design. Remember to tag animals.
2. Restrain mouse movements (typically, use a mouse restrainer).
3. Prepare a 25% (w/v) [^{13}C]-glucose solution and/or 3 mM [^{13}C]-acetate solution in PBS. Filter the solution(s) with a 0.2 μm sterile filter
4. Inject 80 μl of stock solution into the tail vein. Repeat the injection 3 times at 15 min intervals

Sections 3.2–3.9 are for histone extraction and analysis. For extraction and analysis of Acetyl-CoA, proceed to step 3.10. The sample can be divided into two aliquots, and the two sample preparations can be performed in parallel.

3.2. Sample harvesting

3.2.1 Harvesting of adherent cells

1. Remove plate(s) from the incubator and place on ice.
2. Scrape cells using a cell lifter and place them in a new tube.
3. Centrifuge cells at 300 x g for 5–10 min.
4. Remove supernatant.
5. Resuspend cells in ice-cold PBS and repeat Steps 3–4.
6. Estimate the volume of cell pellets (approximate) and keep them frozen until use (*go to Section 3.3*).

3.2.2 Harvesting of suspension growing cells

1. Remove plate(s) from the incubator and put it on ice
2. Aspirate cells and transfer them in a new tube
3. Centrifuge cells at 300 x g for 5–10 min.
4. Remove supernatant.
5. Resuspend cells in ice-cold PBS and repeat Steps 3–4.
6. Estimate the volume of cell pellets (approximate) and keep them frozen until use (*go to Section 3.3*).

3.2.2 Harvesting of tissues

1. Sacrifice animals according to an IACUC-approved protocol.
2. Rapidly expose organ of interest using clean surgical tools.
3. Cut approximately 100 mg of tissue with sharp scissors and quickly rinse with ice-cold PBS.
4. Homogenize the tissue explant with a dounce homogenizer in 1 mL PBS.
5. Transfer tissue homogenate into a new tube.
6. Centrifuge tissue homogenate at 300 x g for 5–10 min
7. Remove supernatant.
8. Resuspend pellet in ice-cold PBS and repeat Steps 6–8.
9. Estimate the volume of pellets (approximate) and proceed directly to histone extraction without freezing (*go to Section 3.3*).

3.3. Isolation of cell nuclei

This section describes how to separate intact nuclei from the cell cytoplasm, membrane and other organelles. This reduces the presence of background proteins when histones are purified. Notably, protocols bypassing nuclei isolation have been published [21].

1. Add protease inhibitors and other inhibitors to NIB-250 buffer. For 1 mL of cell pellet, approximately 50 mL of NIB-250 buffer is prepared. Add to 50 mL NIB-250 buffer 50 μ L of 1 M DTT, 125 μ L of 200 mM AEBSF, 100 μ L of 2.5 μ M microcystin and 100 μ L of 5 M sodium butyrate
2. Lyse the cell pellet with 10 times the volume of NIB-250 including inhibitors and 0.2% NP-40 Alternative
3. Properly homogenize the cell suspension. Normally, gentle pipetting is sufficient, but tissue samples might need the use of a dounce homogenizer.
4. Incubate the suspension on ice for 5–10 min; the outer cell membranes will lyse and release nuclei.
5. Centrifuge at 1000 x g for 5–10 min at 4 °C. Cell nuclei are pelleted, while the supernatant contains mostly cytoplasmic components.
6. Resuspend the nuclei pellet using 10 volumes of NIB-250 + inhibitors without the NP-40 alternative.
7. Centrifuge at 1000 x g for 5 min at 4 °C and remove supernatant.
8. Repeat Step 6–7 for complete removal of residual NP-40 Alternative.

3.4. Extraction and purification of histones

Histones are extracted exploiting their solubility in acid (H_2SO_4). Alternatively, salt extraction can be performed (see *Note 3*)

1. Resuspend cell nuclei with 5 volumes of 0.2 M H_2SO_4 by gentle pipetting.
2. Incubate the sample with gentle rotation or shaking for 2–4 h at 4 °C. Use the longer time frame in case of low abundance material, i.e. <200 μ L cell pellet.
3. Centrifuge at 3400 x g for 5 min.
4. Transfer the supernatant to a new tube.
5. Repeat Steps 3–4 with the supernatant to ensure complete cleanup from pellet residuals.
6. Add 100% TCA to the sample solution with a ratio of 1:3 (v/v), to obtain a final TCA concentration of 33%. This step will precipitate histones.
7. Let the mixture precipitate on ice for at least 1 hour or overnight.
8. Centrifuge at 3400 x g for 5 min. Remove the supernatant by aspiration without touching the precipitate. Histones are the white layer condensed around the bottom of the tube. The pellet in the very bottom of the tube normally contains other acid biomolecules, such as DNA.

9. By using a glass Pasteur pipette, rinse the tube with acetone + 0.1% HCl.
10. Centrifuge at 3400 x g for 2 minutes and discard the supernatant.
11. Repeat Steps 9–10 using acetone without 0.1% HCl.
12. Leave the tubes open on the bench for a few minutes to dry them completely.
13. Resuspend histone proteins in 30–50 μ L of ddH₂O. Rinse the borders of the tubes as best as possible, especially the white layer on the side of the tube.
14. Estimate the amount of histone proteins in the sample using either BCA, Bradford protein assay or amino acid analysis (AAA).
15. Verify the purity of extracted histones with SDS gel and coomassie staining (optional).
16. Histone isotypes can be separated and differentially purified using HPLC-UV equipped with a C₁₈ column (*Note 4*) (optional).

3.5. Propionic anhydride derivatization prior histone digestion for bottom-up analysis

In proteomics, proteins are digested into short (6–30 aa) peptides prior analysis. This approach is called “bottom-up” or “shotgun”. Histones are also digested into short peptides. The canonical protein digestion protocol uses trypsin as digestion enzyme, which cleaves at the C-termini of basic amino acid residues, i.e. lysine (K) and arginine (R). Histones are highly enriched of KR residues, and thus they require either the use of alternative enzymes [22] or derivatization strategies to reduce trypsin targets on the protein sequence [23–26]. Here, we discuss the most widely adopted histone digestion protocol, adopting propionic anhydride derivatization prior and after trypsin digestion [27,28]. Such derivatization blocks the ϵ -amino groups of unmodified and monomethyl lysine residues, allowing trypsin to perform proteolysis only at the C-terminal of arginine residues. Derivatization after digestion increases peptide hydrophobicity, which enhances efficient HPLC column retention for HPLC-MS.

1. Resuspend at least 20 μ g of histones in 30 μ L of 50 mM NH₄HCO₃, pH 8.0. Recommended amount is 50–100 μ g (see *Note 5* for estimated histone yield from cell counts).
2. Assess the pH using a P10 pipette tip; dip it into the sample and touch a pH indicator strip. NH₄OH and glacial acetic acid can be used to adjust the pH (see *Note 6* for safety instructions).
3. For three samples, prepare propionylation reagent by mixing propionic anhydride with acetonitrile (ACN) in the ratio 1:3 (v/v); i.e. mix 15 μ L of propionic anhydride and 45 μ L of ACN (see *Note 7* on alternative procedure in presence of a large number of samples).
4. Add rapidly the propionylation reaction to the histone sample with a ratio of 1:2 (v/v); i.e. 15 μ L propionylation reaction for 30 μ L sample.
5. Add rapidly ~7 μ L of NH₄OH to re-establish pH 8.0 to the solution (see *Note 8* for elucidations on issues related to not optimal pH).

6. Pipette up and down for a few seconds.
7. Assess pH as described in Step 2.
8. Incubate samples at room temperature for 15–20 min.
9. Dry samples down to 5–10 μL in a SpeedVac centrifuge.
10. Resuspend or dilute samples with 50 mM NH_4HCO_3 until achieving 30 μL of final volume.
11. Repeat Steps 3–10 to double the propionylation reaction to ensure complete derivatization.

3.6. Histone digestion and propionylation of peptide N-termini

1. Resuspend histones in 30 μL of 50 mM NH_4HCO_3 .
2. Assess the pH to be around 8.0 by using pH strips.
3. Add trypsin (sequencing grade) at a 1:20 ratio (w/w); e.g. 5 μg of trypsin for 100 μg of histones.
4. Incubate at room temperature for 6 hours or overnight.
5. Repeat Steps 3–12 of section 3.5, double round of propionylation included. At the second round, stop at the Step 10 (included).
6. Resuspend the histone peptide samples in 50 μL of ddH_2O + 0.1% trifluoroacetic acid (TFA).

3.7. Stage tipping for sample desalting

To remove residuals of salt, propionylation reagent and other debris leftover of the histone extraction, samples are passed through a tip packed with reversed-phase material for cleanup. Not doing so might result in column clogging and/or instrument contamination during HPLC-MS analysis.

1. Take a 3M Empore™ Solid Phase Extraction Disk C_{18} and cut a disc of ~2–3 mm diameter, e.g. by using the tip of a P1000 pipette.
2. Push this disc to the bottom of a P100/200 pipette tip, e.g. by using a fused silica capillary.
3. Repeat Steps 1–2 in the same P100/200 tip if you are desalting more than 10 μg of sample. This will increase the capacity of the Stage tip.
4. Wash the Stage tip by flushing 50 μL of 75% ACN and 0.025% TFA with air pressure, e.g. using a syringe (see *Note 9* for higher throughput procedure using centrifugation).
5. Equilibrate the Stage tip by flushing 50 μL of 0.1% TFA by air pressure. Do not dry completely the disc(s).
6. Load the sample onto the Stage tip by air pressure. Do not dry completely the disc(s).

7. Wash the sample by flushing 50 μ L of 0.1% TFA by air pressure. Do not dry completely the disc(s).
8. Elute the sample by flushing 50 μ L of 75% ACN and 0.025% TFA by air pressure. Collect the sample in a 1.5 mL tube.
9. Dry samples in a SpeedVac centrifuge.

3.8. Histone peptide analysis via nano liquid chromatography coupled to mass spectrometry (nanoLC-MS)

NanoLC is now the separation technique most preferred for proteomics, due to its high sensitivity and the possibility of online coupling to MS. Here, we describe how to prepare columns for nanoLC (Steps 1–8 can be omitted if using commercial columns) and how to configure the nanoLC-MS analysis. This procedure described how to prepare a picofrit column, i.e. a nano column integrated with the tip. For alternative procedure, see *Note 10*.

1. Cut ~30 cm of fused silica capillary with 75 or 100 μ m internal diameter (ID).
2. Tape one end of the capillary on a solid surface, e.g. the bench (Figure 3A).
3. By using a torch, fire close to the end of the capillary while gently pulling from the other end (Figure 3A). After a few seconds, the capillary will elongate and detach from the surface creating a tip at the end (Figure 3B). This tip might look different every time; its end might be very long, like a “hair” (Figure 3C).
4. Gently remove the “hair” part of the tip, e.g. with the finger, until it looks like a tip for nano columns (Figure 3D). This procedure might take a few attempts (see *Note 11* to overview possible consequences of failure).
5. Prepare in a clean HPLC glass vial the resin slurry for column packing. This includes (i) C₁₈-AQ reversed-phase 3 μ m particles; (ii) 100% methanol, (iii) a micro-stir magnet. The ratio between particles and methanol are flexible, and should be optimized for a rapid and efficient column packing.
6. Place the resin slurry in a pressure bomb and turn on magnetic stirring.
7. Place the capillary in the pressure bomb, secure it and open the gas tank (containing helium, nitrogen or air).
8. Leave the column on the bomb until it is packed for at least ~25 cm.
9. Remove the column from the pressure bomb, and connect it to the nanoLC.
10. Program the HPLC method as follows: from 2 to 28% buffer B in 45 min, from 28 to 80% B in 5 min and 10 min at isocratic 80% B. Buffer A and B composition are described in Section 2.2 (#3 and #4, respectively).
11. Program the MS acquisition method to perform data-independent acquisition (DIA) (references to set up the method using SWATH™, using low resolution instrumentation and using multiplexed DIA, respectively [29–31]). The instrument will alternate a Full MS scan with MS/MS scans of the entire mass

range using acquisition windows of 50 m/z . All other settings are in common to standard proteomic experiments.

12. Load ~1 μ g of sample onto the HPLC column.
13. Run the nanoLC-MS/MS method as programmed.

3.9. Extracted ion chromatography (XIC) of histone peptides

Raw files obtained from the LC-MS runs are now ready to be processed. In our lab, we developed EpiProfile [32], a software tool that performs extracted ion chromatography (XIC) of histone peptides (Figure 4).

1. Group the raw files into the same folder.
2. Run EpiProfile using Matlab or GNU Octave. The software will provide a table containing the XIC of the desired analytes. In the output, peptides are already normalized to obtain the relative abundance of each post-translational modification (PTM). The relative abundance is calculated by dividing the XIC of a given peptide modified form by the sum of all XIC of peptides sharing the same sequence. The software automatically discriminates isobaric species, i.e. differentially modified peptides with the same intact mass, by using the MS/MS events acquired with DIA.
3. Light and heavy labeled acetylations are considered independently by the software. The turnover of a given acetylation is calculated by dividing the area of the XIC of the peptide with the heavy acetyl group by the peptide with the light acetyl group.
4. Alternatively to Steps 1–3, the XIC can be performed with other software tools such as Skyline [33]. However, EpiProfile is currently the only available software trained for this purpose. Other software tools require extensive manual integration and post-processing to discriminate isobaric forms.

3.10. Extraction of acetyl-CoA from cells

Here, we describe the method for the extraction, purification and quantification of labeled acetyl-CoA from cells. Final data will be expressed as the ratio between heavy (labeled) and light (unlabeled). The use of trichloroacetic acid as extraction buffer helps both to stabilize the unstable thioester bond and slow acetyl-CoA degradation, and precipitates protein from the samples. As acetyl-CoA is highly unstable, extraction from cells should be carried out as quickly as possible and samples should be kept cold on ice at all times. For accurate quantitation of total levels of acetyl-CoA, we recommend the use of stable isotope labeled internal standards added to samples as early as possible in the sample processing. With high resolution MS (>10,000), the use of [$^{13}\text{C}_3^{15}\text{N}_1$]-labeled internal standard for accurate quantitation can be used to generate labeling and quantitation data simultaneously [34]. Use a replicate dish for cell counting / cell volume measurement using a BD Coulter Counter or analogous instruments. See *Note 12* for extraction of Acetyl-CoA from whole tissues.

1. Take cell dishes out of the incubator and place them on ice (for suspension cells, spin down at 400 x g at 4°C and place on ice).

2. Aspirate tissue culture media thoroughly (for adherent cells, tilt dishes on a slope on ice after initial aspiration to allow residual media to drain down and remove by repeating aspiration). See *Note 13* on why cells are not washed.
3. Add 1 mL of ice-cold 10% TCA directly to the cell plate and scrape cells with a cell lifter (for cell pellets from suspension cells, add 1 mL of ice-cold 10% TCA and mix briefly by vortexing).
4. Transfer cell suspension to a new 1.5 mL Eppendorf tube. You may store the samples at -80°C .
5. Sonicate samples with a probe tip sonicator (12×0.5 sec pulses).
6. Centrifuge dismembranated cells at $13,000\text{--}17,000 \times g$ for 10 minutes at 4°C . The cleared supernatant contains Acetyl-CoA extract, the protein pellet can be used for Western blotting and/or protein determination.
7. Take 1 ml capacity solid phase extraction columns with strongly hydrophilic, reversed-phase chemistry.
8. Wash columns with 1 mL of methanol.
9. Equilibrate columns with 1 mL of water.
10. Load columns with the supernatant from samples.
11. Desalt columns with 1 mL of water.
12. Elute columns with 1 mL of methanol containing 25 mM ammonium acetate and recover the eluted fraction.
13. Evaporate to dryness under nitrogen flow.

3.11. HPLC-MS analysis of acetyl-CoA

1. Resuspend the sample purified in Section 3.10 in $50 \mu\text{l}$ 5% (w/v) 5-sulfosalicylic acid dissolved in HPLC grade water and transfer to HPLC compatible vials or 96-well plate for analysis. Store at 4°C . See *Note 14* for details on why the quick extraction.
2. Connect a C_{18} reversed-phase chromatographic column to an HPLC system capable of running flows at 0.2 ml/min. See *Note 15* for recommended commercial columns.
3. Program the HPLC gradient as follows: isocratic 2% buffer B (98% buffer A) for 2 min, from 2–25% B for 3.5 min, from 25–100% B in 0.5 min and isocratic 100% B for 8.5 min, washed with 100% buffer C for 5 min followed by equilibration to 2% buffer B for 5 min. The composition of buffer A, B and C is described in Section 2.2 (#11–13). Set flow rate to 0.2 ml/min.
4. Set up the MS acquisition method as DIA or targeted. The m/z acquisition window should include the masses for precursors and fragments as outlined in Table 1. Use the instrument in positive mode.

5. HPLC-MS/MS method as programmed.

3.12. Extracted ion chromatography (XIC) of acetyl-CoA

1. Import data into a peak detection software compatible with your MS platform. An example of universal software for peak area extraction is Skyline [33].
2. Perform extracted ion chromatography (XIC) for each acetyl-CoA species to obtain area under the curve values.
3. To confirm the specificity of the peak, check that the retention time of parent acetyl-CoA isotopologue peaks align with their corresponding product ion peaks.
4. Calculate percent isotopic enrichment from ^{13}C -labeled substrate by entering AUC data from labeled and unlabeled control samples into the FluxFix web tool (www.fluxfix.science) [35]. See *Note 16* on how to computationally correct biases due to isotopic enrichment.

3.13. Data integration and interpretation

EpiProfile [32] provides a result table with approximately 300 peptide isoforms. Because the list can be overwhelming for manual inspection, it is very helpful to process the data using the proper statistics, in order to detect the most significant and reliable changes. When using a limited number of replicates (<5), we recommend the use of t-test to estimate the significant differences between conditions. Non-parametric statistics is generally more appreciated, as it can be applied also if replicates do not have a Gaussian distribution; however, it is not sufficiently powerful to deal with such small number of data points. In case an overall trend of acetylation increase/decrease is observed, we recommend to correlate the observation with the levels of acetyl-CoA before jumping to conclusions like “we observe an overall higher activity of enzymes catalyzing histone acetylation”.

1. Open the table containing the raw intensities (Figure 5A) of the identified and quantified histone peptides.
2. Normalize each modified histone peptide by the sum intensity of all peptides sharing the same sequence (Figure 5B). For instance, the peptide containing H3K4me3 has the sequence TKQTAR in human (and most other eukaryotes). The relative abundance of the peptide modified as $\text{TK}_{\text{me3}}\text{QTAR}$ is calculated as:

$$\text{intensity } \text{TK}_{\text{me3}}\text{QTAR} / (\text{intensity } \text{TKQTAR} + \text{intensity } \text{TK}_{\text{me1}}\text{QTAR} + \text{intensity } \text{TK}_{\text{me2}}\text{QTAR} + \text{intensity } \text{TK}_{\text{me3}}\text{QTAR})$$

We strongly recommend to perform the extracted ion chromatography of histone peptides using EpiProfile, as this calculation is already performed, and the software automatically deals with isobaric peptides.

3. Perform the t-test when comparing two conditions, or ANOVA when comparing more than two conditions. Data can be displayed as a volcano plot, using for the x-axis the log2 fold change between the two conditions, and as y-axis the $-\log_2$

of the t-test p-value (Figure 5C). Conventionally, the significance threshold is set at a p-value <0.05 , which when transformed as $-\log_2$ becomes >4.32 .

4. Sum the relative abundance of all acetylated peptides, and compare them with the relative changes of acetyl-CoA (Figure 5D). If the correlation is linear and positive, a possible biological interpretation of the data is that the acetyl-CoA levels change affecting the abundance of histone acetylation (Figure 5F). An example of this analysis is illustrated in [36]
5. For validation of the findings, we recommend performing western blotting (Figure 5E) [37]. Ensure that the used antibody is specific for the acetylation site investigated.
6. If using metabolically labeled acetylations, it is also possible to estimate their turnover rate by dividing the relative abundance of the acetylated peptide with heavy labeled acetylation from the one with light acetylation. IMPORTANT: do not confuse the turnover rate with the relative abundance. The turnover rate indicates how frequently an acetylation is recycled with a new one, while the relative abundance indicates how much of that site is acetylated. An acetylation might change in abundance between two conditions, but maintain its turnover rate, or vice versa.
7. Once identified the PTMs that are significantly regulated between the analyzed conditions, a potential next step can be determining which histone writer is potentially responsible for this regulation. This enzyme is a potential target for complementary treatment using either inhibitors or other post-transcriptional regulation (e.g. knock-down). A comprehensive list of known histone modifications and their respective writers has been recently published in [38].

4. Notes

1. Number of cells plated for every single experiment should be optimized. It is important to avoid confluency at the moment of harvesting. Factors to take into consideration include, but not limited to:
 - Growth rate
 - Length of the experiment
 - Viability upon treatment
2. Nutrient availability can be optimized for any specific cell line/condition. We found that the above-described concentrations mimic well a physiological situation in most cell lines we tested. Nutrient sensing and impact of acetyl-CoA availability on histone acetylation can be well appreciated under these culture conditions. However, some primary well-differentiated cell types display minimal nutrient consumption. We recommend lower nutrient availability when metabolic activity is low.

3. The high-salt extraction protocol [39] is alternative to acid extraction for histone purification. High-salt is a milder procedure, and it preserves acid-labile PTMs. However, it produces samples with very high concentrated salt, preventing an effective LC-MS analysis. Desalting can be performed as described in Section 3.7, but it is not 100% effective.
4. Fractionation of intact histone isotypes can be performed by using HPLC-UV. It requires at least 100 µg of starting material (if 2.1 mm ID column is used), or 300 µg (if 4.6 mm ID column is used). Given the optimized nanoLC-MS and the EpiProfile software for the analysis of the runs, histone fractionation is not recommended. It might be convenient for scarcely pure histone extractions and if interested in very low abundance PTMs.
5. In case estimating the yield of extracted histones is prohibitive, it is reasonable to assume that standard procedures extract about 1 µg of histone every 1 µL of cell pellet. Unpublished data from our lab demonstrate that histone analysis can be performed with as low as 50,000 cells as starting material.
6. NH₄OH, glacial acetic acid and propionic anhydride should be used in the fume-hood. The bottle of propionic anhydride must be filled with argon after its use to preserve the reagent.
7. In presence of multiple samples (>3–4), consider re-preparing the propionylation mix every 3–4 samples. This mix is very reactive, and using it for a long list of samples can prevent its efficacy. In case of preparation of a large number of samples (>20), consider performing the reaction in a 96-well plate using a multi-channel pipette.
8. If the pH of the propionylation reaction is acidic, no reaction will occur. In case the pH is > 10.0, other amino acid residues with higher pK_a might be labeled as well, generating issues in the proper identification and quantification of histone peptides by LC-MS.
9. Buffers can be pushed through Stage tips by using centrifugation instead of air pressure. Use appropriate holders on the top of the tube to place the Stage tip, or drill a hole on the cap. This approach is not recommended, as it is harder to prevent complete drying of the Stage tip during the procedure.
10. When preparing a nano column, the top can be capped using a “frit” instead of pulling a tip. Frits are prepared by mixing 88 µL Kasil® into a 0.5 mL tube with 16 µL formamide. One end of the capillary is dipped into this solution and it is left for polymerization in a heater at ~110 °C for 3–4 hours. Fritted columns require a connection with a tip for spraying sample into MS.
11. If the tip of the nano column was not prepared properly, two issues can be encountered. If the tip has a too small orifice, columns will be plugged and they cannot be utilized. This issue can be solved by carefully cutting the very top of the tip, or by torching for a few seconds the tip while the column is packing. If the tip has been cut with a too large orifice, the column will not retain the

particles and they will geyser out from the tip while packing. In this case, immediately close the pressure bomb and discard the capillary.

12. For metabolite analysis *in vivo*, freeze clamping of tissue of interest in alive, deeply anesthetize is often recommended, but rarely feasible. We propose to rapidly sacrifice the animal (cervical dislocation for rodents), and quickly expose the tissue of interest. Everything must take less than one minute. Pre-chill a tissue clamp in liquid nitrogen. Various models are commercially available and equivalent; alternatively, a toothed forceps/scissor with large, flat extremities can be used. Snap freeze a chunk of tissue then cut approximately 50 mg of tissue in a superchilled ceramic tile on dry ice. Weight the tissue with a precision scale.
13. Washing cells is not necessary as acetyl-CoA is not present in the cell medium and washing can skew metabolite quantitation. It is, however, important to minimize cell medium in the sample so as to prevent excessive salt and matrix effects from media components in extraction and MS acquisition.
14. Acyl-CoA analysis should be performed as quickly as possible after extraction to avoid sample degradation. Acetyl-CoA is relatively stable for several days at 4 °C in 5-sulfosalicylic acid, with ~50% sample loss after 15 days [40]. Low pH helps to minimize hydrolysis of the acyl-CoA thioester bond [41]. 5-sulfosalicylic acid is also an antimicrobial agent.
15. For analysis of metabolites like acetyl-CoA, high flow HPLC (flow-rate of 100–200 µL/min) is currently preferred to nanoLC due to robustness. High flow HPLC requires columns of larger diameter than nanoLC, thus not packed in-house. We personally tested and considered reliable the following commercial columns: Acquity HSS T3 column (2.1 × 150 mm, 1.7 µm particle size); Phenomenex HPLC Luna C18 (2.0 × 150 mm, 5 µm particle size); Waters XBridge C18 (2.1 × 150 mm, 3.5-µm particle-size).
16. Calculation of % isotopic enrichment involves applying a correction matrix that compensates for the non-linearity of isotopic enrichment [42].

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Figure 1: pathways leading to histone acetylation.

(A) Acetyl-CoA is commonly produced by processing of either glucose or acetate. Citrate is converted into acetyl-CoA by the ATP-Citrate Lyase (ACLY), while acetate is processed into acetyl-CoA by the enzyme Acetate Synthetase 2 (ACSS2). (B) Increased availability of acetyl-CoA correlates with elevated histone acetylation.

Figure 2: proposed workflow.

Cell cultures can be grown in the presence of either normal (represented by blue medium) or labeled (green medium) nutrients. Labeled histones and metabolites can be analyzed in parallel, according to different protocols. Quantification of either is performed by LC-MS analysis. Data can then be integrated for correlation analysis.

Figure 3: tip pulling for nanocolumns.

(A) Capillaries should be secured on a rigid surface, e.g. by using tape. (B) Heating combined with gentle pulling elongates the capillary into a smaller and smaller internal diameter, until it detaches from the surface as a pulled needle. (C) This needle is frequently a non-rigid end, similar to a hair. This end has no internal diameter, preventing any liquid to pass through it. (D) By gently breaking the non-rigid part of the tip (e.g. with a finger), a normal size tip is created. This capillary is ready to be packed and become a nanocolumn for LC-MS.

Figure 4: example of histone peptide and acetyl-CoA spectra before and after metabolic labeling. (A) Left: acetyl-CoA chemical structure. The acetyl group (R – 2 carbon atoms) is attached to the Coenzyme A backbone through a thioester bond. Right: glucose, acetate and other nutrient provide the carbon atoms to the acetyl group. Using isotope labeling, ^{13}C atoms can be incorporated into acetyl-CoA (carbons highlighted in red in the structure; each +1 Da). Rate of incorporation and carbon source depends on the metabolic activity of the cell and may be studied with the approach presented here. (B) The spectra display how the isotopic pattern of histone peptides (top) and acetyl-CoA (bottom) change after growing fast replicating cells into a media containing isotopically heavy glucose (+6 Da). A histone peptide carrying a single acetylation increments its third isotope (+2 Da), as only two ^{13}C carbon atoms are incorporated into the acetyl group. With time, the relative abundance of the heavy isotope increase until the unlabeled acetylation disappears from the signal. Acetyl-CoA gets labeled in multiple carbon residues, as the CoA group is also synthesized using glucose. Therefore, the isotopes increasing their relative abundance are multiple.

Figure 5: representative workflow for data analysis.

(A) Raw data produced by EpiProfile are the area of the extracted ion chromatogram of selected histone peptides (currently ~300) and acetyl-CoA. EpiProfile can also extract ion chromatograms of histone peptides carrying isotopically labeled acetylations. (B) The raw intensity is automatically converted into a relative abundance by dividing the intensity of each peptide by the sum of all peptides (both unmodified and labeled with heavy carbons) sharing the same amino acid sequence. (C) Plotting and analysis of differentially-modified histone species and/or metabolite isotopomers (recommended statistics is based on t-test or ANOVA). (D) The relative changes of histone acetylation can be compared with relative changes of acetyl-CoA when comparing samples A and B, so that a correlation can be performed to predict causes of global regulation of histone acetylation. (E) Validation can be performed by, e.g. western blotting. (F) Data-driven hypotheses are easier to formulate when both histone and acetyl-CoA data are acquired.

Table 1:

Mass transitions for relevant acetyl-CoA isotopologues in MS positive mode.

Species	Isotopologue	Parent m/z	Product m/z
Acetyl-CoA	M0	810.1	303.1
Acetyl-CoA	M1	811.1	304.1
Acetyl-CoA	M2	812.1	305.1
Acetyl-CoA	M3	814.1	306.1
Acetyl-CoA	M4	814.1	307.2
Acetyl-CoA	M5	815.1	308.2