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Isolation of Proteins on Nascent Chromatin and Characterization by Quantitative Mass Spectrometry

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Abstract

Replication-coupled chromatin assembly is a very dynamic process that involves not only the replication fork machinery but also chromatin-related factors such as histones, histone chaperones, histone-modifying enzymes, and chromatin remodelers which ensure not only that the genetic information is properly replicated but also that the epigenetic code is reestablished in the daughter cell. Of the histone modifications associated with chromatin assembly, acetylation is the most abundant. Determining how newly synthesized histones get acetylated and what factors affect this modification is vital to understanding how cells manage to properly duplicate the epigenome. Here we describe a combination of the iPOND, quantitative mass spectrometry, and SILAC methodologies to study the protein composition of newly assembled chromatin and the modification state of the associated histones.

Keywords

Nascent chromatin; Acetylation; SILAC; Mass spectrometry; Histone

1 Introduction

Accurate replication of the DNA and proper conservation of the chromatin structure are indispensable for cell function. Defects in this process are associated with cancer onset, premature aging, and other disease states [1, 2]. Chromatin structure is fundamental for the protection of the genetic material and for the orchestration of transcriptional programs. DNA replication-coupled chromatin assembly is a very well orchestrated process that involves precise and coordinated communication between the replication fork machinery and a variety of chromatin factors [3]. The main goal of this process is to faithfully transmit the epigenetic information to the daughter cell. Much of the epigenetic information is contained in the post-translational modifications that occur at the NH₂-terminal tail of the histones. A critical feature of epigenetic inheritance is the transfer of the parental patterns of histone

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modification to the newly synthesized histones that are deposited on the replicating DNA in an equal quantity. However, the newly synthesized histones are not a blank slate upon which specific patterns of modification can be reproduced. Rather, for histones H3 and H4, the newly synthesized molecules contain precise patterns of modification [4]. Therefore, proper epigenetic inheritance requires a precisely orchestrated process that involves the removal of the modifications associated with the new histones and the generation of histone modification patterns appropriate for the chromosomal location.

The iPOND (*Isolation of Proteins On Nascent DNA*) technique overcomes a number of the obstacles to the biochemical analysis of chromatin assembly [5, 6]. First, characterization of chromatin assembly is complicated by the fact that it is a moving target. The use of EdU labeling of replicating DNA allows for the marking of sites of chromatin assembly and their subsequent isolation following ligation of biotin to the EdU. Second, by using different pulse-chase strategies with EdU and thymidine, it is possible to isolate chromatin at different points in the assembly and maturation process. Third, the use of iPOND obviates the need to synchronize cell populations, as only the cells in S phase will incorporate significant amounts of EdU into their genomic DNA. Finally, this method overcomes difficulties associated with antibody specificity that can occur when using chromatin immunoprecipitation (ChIP) or immunofluorescence to monitor chromatin assembly.

When combined with mass spectrometry, iPOND becomes a very powerful and useful methodology to monitor the proteins and histone modifications occurring at sites of chromatin assembly. We have been interested in understanding the effect of the Hat1-mediated acetylation of newly synthesized histone H4 on lysines 5 and 12 on the chromatin assembly process [7]. Therefore, we integrated these two techniques with Stable isotope labeling with amino acids in cell culture (SILAC), a simple approach to label proteins in vivo for quantitative mass spectrometry. In a typical experiment, two cell populations (for example, Hat1 WT and Hat1 KO cells) are cultivated with one population grown in culture media containing normal amino acids while the other population is grown in culture media containing amino acids labeled with stable but nonradioactive heavy isotopes. In our case, we use media that contains lysine ($^{13}\text{C}_6$, $^{15}\text{N}_2$) and arginine ($^{13}\text{C}_6$, $^{15}\text{N}_4$). As the cells are grown in the labeled media, they incorporate the heavy amino acids into all their proteins. In this way, after protein digestion all the peptides coming from the cells grown in the labeled media will be heavier than the ones grown in the normal media. By comparison of the abundance of the light (normal) and the heavy labeled peptides, changes in the relative abundance of proteins on nascent chromatin between the two cell populations can be quantified (Fig. 1). This provides a powerful method for determining the influence of specific proteins on the outcome of replication-coupled chromatin assembly.

2 Materials

2.1 Cell Culture Media

1. DMEM supplemented with 10% FBS and penicillin/streptomycin.

2.2 EdU

1. Prepare a 10 mM stock solution in DMSO and store at -20°C in the dark.

2.3 Formaldehyde

1. Dilute formaldehyde to reach 1% v/v concentration in PBS. Prepare fresh every time.

2.4 Glycine

1. Prepare 1.25 M stock solution in distilled water, can be stored at room temperature.

2.5 Permeabilization buffer

1. Prepare a 20% Triton-X stock solution and keep at room temperature. Dilute to 0.25% in PBS and use it as permeabilization buffer. Keep the diluted solution at 4°C .

2.6 Click Reaction

This solution must be prepared fresh every time.

1. Biotin Azide: Prepare a stock solution of 1 mM in DMSO and keep it stored at -20°C protected from light.
2. CuSO_4 : Prepare a stock solution of 100 mM in distilled water; this solution can be stored at room temperature.
3. Sodium L-ascorbate: Prepare a solution of 20 mg/mL in distilled water. Prepare freshly every time and store on ice until needed. Protect from air and light.

2.7 Lysis Buffer

1. 1% SDS in 50 mM Tris-HCl, pH 8.0. Store at room temperature and prior to use complement with protease inhibitors.

2.8 In-Gel Digestion Buffers

1. These solutions must be prepared fresh every time and with HPLC grade reagents.
2. 100 mM Ammonium bicarbonate [8].
3. Wash Solution (WS): 50% Methanol:45% Water:5% Glacial acetic acid.
4. Extraction Solution (ES): 50% Acetonitrile:45% Water:5% Formic Acid.

2.9 Other Reagents Needed

1. PBS.
2. 1 M NaCl.
3. 2× Laemmli buffer.

4. DMSO.
5. 12% SDS-PAGE Gel.
6. 1× SDS Running Buffer (25 mM Tris–HCl base, 192 mM Glycine, 0.1% (w/v) SDS).
7. Bio-rad Bio-Safe Coomassie G-250 Gel Stain.
8. Iodoacetamide.
9. Dithiothreitol (DTT).
10. Promega Trypsin Gold, Mass Spectrometry Grade (after reconstitution in 50 mM Glacial Acetic Acid, diluted to 20 ng/μL with 100 mM ABC).
11. HPLC grade Water (H₂O).
12. HPLC grade Methanol (MeOH).
13. HPLC grade Acetonitrile (ACN).
14. HPLC grade Formic Acid (FA).
15. HPLC Buffer A: 0.1% HPLC grade FA in HPLC grade H₂O.
16. HPLC Buffer B: 0.1% HPLC grade FA in HPLC grade ACN.
17. Loading Buffer: 2% HPLC grade ACN, 0.1% HPLC grade FA in HPLC grade H₂O.

3 Methods

3.1 Cell Preparation

For mouse embryonic fibroblasts (MEFs) seed 10⁶ cells in 20 mL of complete media, light or heavy depending of the cell population, in a 150 mm² tissue culture dish (*see* Note 1).

You will use two 150 mm² dishes of each cell population per time point. Once the cells have been seeded, incubate them at 37 °C in a CO₂ incubator.

3.2 EdU Labeling

Add 20 μL of the 10 mM stock solution of EdU directly to the cells to achieve a final concentration of 10 μM. Immediately put the cells back into the incubator and incubate for the chosen labeling time (*see* Note 2).

¹-To guarantee that the cells fully incorporate the isotopically labeled amino acids, start growing them in the heavy media for a minimum of four passages before seeding them for the experiment. In our case, we grow the Hat1 WT cells in normal DMEM media supplemented with 10% FBS and antibiotics (light population), and the Hat1 KO cells are grown in DMEM for SILAC supplemented with 50 mg of lysine (¹³C6, ¹⁵N2) and 50 mg of arginine (¹³C6, ¹⁵N4), 10% dialyzed FBS for SILAC and antibiotics; this will correspond to the heavy population. For the experiment to work best the cells should be seeded the day before the experiment. For cell types other than MEFs, the cell number needs to be determined. Keep in mind that the cells must be in the logarithmic phase of growth and cannot be over-confluent as they will stop replicating. Changes in this parameter will affect the rate of EdU incorporation. Seeding the exact same number of cells from both populations (light and heavy) is critical to guarantee accurate SILAC ratios during the mass spectrometry analysis. Include one extra plate per cell population as a control, the day of the experiment count the number of cells in both populations, they should be equivalent if not the cells should be seeded again.

3.3 Fixation

1. As soon as the EdU labeling time is finished, remove the media from the plates and replace with 10 mL of 1% Formaldehyde solution in PBS.
2. Incubate at room temperature for 20 min.
3. Add 1 mL of 1.25 M glycine to each plate to reach a final concentration of 0.125 M of glycine.
4. Collect the cells in a 50 mL conical tube (see Note 3).
5. Centrifuge the cells at $2000 \times g$ for 5 min at 4 °C.
6. Wash the cells with PBS and centrifuge the cells at $2000 \times g$ for 5 min at 4 °C.
7. Repeat **step 6** two more times.

At this point, cell pellets can be stored at –80 °C until further use.

3.4 Cell Permeabilization

1. Resuspend the cell pellets in 10 mL of permeabilization buffer by vortexing.
2. Incubate at room temperature for 30 min.
3. Centrifuge the cells at $2000 \times g$ for 5 min at 4 °C.
4. Wash the cells with 10 mL of PBS.
5. Centrifuge the cells at $2000 \times g$ for 5 min at 4 °C.
6. Remove supernatant and keep pellets on ice.

3.5 Click Reaction

1. Prepare the click reaction before every use.
For each samples you will need:
 - a. 10 μ M Biotin-azide.
 - b. 10 mM Sodium ascorbate.
 - c. 2uM Cupric Sulfate.
 - d. Complete to 5 mL with PBS.
2. Resuspend the pellet in 5 mL of click reaction by vortexing.
3. Incubate samples with rotation and protected from light at room temperature for 1 h.

²The experiment works best when the cells are labeled for a short period of time. For example, 10–30 min should be sufficient labeling time.

³During this step, the light and heavy population will be combined, in this way for the following steps both populations will be contained in the same sample. Immediately after quenching the fixation step collect the cells in a 50 mL conical tube using a clean cell scraper, in the same tube you will collect the light and the heavy population. To guarantee that you are combining the same number of cells from both populations, you must plate the exact same number for both, remember to include one extra plate as a control and count the cell number for each cell type. Stop the experiment if the cell number from both samples doesn't correspond.

4. Centrifuge the cells at $2000 \times g$ for 5 min at 4 °C.
5. Wash cell with 5 mL of PBS.
6. Centrifuge the cells at $2000 \times g$ for 5 min at 4 °C.
7. Remove supernatant, make sure that there is no PBS left in the tube.

At this point, cell pellets can be stored at -80°C until further use.

3.6 Cell Lysis and Biotin Capture

1. Supplement the lysis buffer with protease inhibitors.
2. Resuspend cell pellets in 600 μL of lysis buffer.
3. Transfer the samples from the 50 to a 1.5 mL tube, make sure that all the cells are transferred and that no material is left behind.
4. Sonicate the samples using a bioruptor waterbath sonicator, using the high setting. Pulse the cells for 20 s and pause for 40 s, for a total of 6 cycles. Make sure to keep the samples on ice slurry.
5. Make sure that your lysates are clear and not cloudy.
6. Centrifuge your samples for 10 min at $16,000 \times g$ at room temperature.
7. During the centrifugation step supplement PBS with protease inhibitors and keep on ice.
8. Dilute your samples 1:1 (v/v) with PBS supplemented with protease inhibitors and set on ice (see Note 4).
9. For each sample you will need 100 μL of the 50% streptavidin-agarose beads slurry.
10. Wash beads for all the samples together, centrifuging the bead slurry at $500 \times g$ for 1 min at room temperature.
11. Remove supernatant and resuspend beads in 1 mL of lysis buffer.
12. Repeat **step 10**.
13. Repeat **step 11**.
14. Repeat **step 10**.
15. Remove supernatant and resuspend beads in 1 mL of PBS containing protease inhibitors.
16. Centrifuge at $5000 \times g$ for 1 min at room temperature.
17. Resuspend the beads 1:1 with PBS supplemented with protease inhibitors.
18. Add equal amount of beads to each sample (100 μL of the PBS bead slurry).

⁴Samples need to be diluted to 1% SDS and 25 mM Tris-HCl, since biotin capture is not efficient in lysates containing 1% SDS.

19. Rotate samples at 4 °C for 18 h.

3.7 Elution

1. Centrifuge the beads at $500 \times g$ for 3 min at room temperature.
2. Remove the supernatant and resuspend in 1 mL of cold lysis buffer.
3. Rotate samples at room temperature for 5 min.
4. Repeat **step 1**.
5. Wash beads with 1 mL of 1 M NaCl.
6. Rotate samples at room temperature for 5 min.
7. Repeat the lysis buffer two more times.
8. Add 50 μ L of 2 $\times$ Laemmli buffer and mix.
9. Boil samples for 25 min at 95 °C.
10. Quick spin the samples and transfer supernatant to a new clean tube.

At this point, eluted proteins can be stored at -80 °C until further use.

3.8 Sample Preparation and In-Gel Digestion (See Note 5)

1. Prepare 12% SDS-PAGE gel using fresh reagents. Load one gel lane with 25 μ L of iPOND elution, loading remaining sample into subsequent lane.
2. Run gel at 60 V (60–90 min) until sample has entered 2 cm into the resolving layer.
3. Stain gel with Coomassie overnight.
4. Destain gel with Milli-Q water until protein bands are visible.
5. Cut small gel bands (~ 1 mm $\times$ 1 mm) as close as possible to stained protein bands to lower background for downstream LC-MS/MS analysis and place in 1.5 mL tube.
6. Soak gel pieces in 200 μ L of WS at 4 °C for a minimum of 60 min (*see* Note 6).
7. Remove WS and replace with a new 200 μ L aliquot. Incubate at 4 °C for 60 min.
8. Remove WS and dehydrate gel pieces with 200 μ L ACN at room temperature for 5 min.
9. Remove ACN and dry samples in speedvac concentrator for 3–5 min.
10. Add 75 μ L of DTT (32 mM in 100 mM ABC) and incubate at 55 °C for 30 min to reduce disulfide bonds.

⁵ Volumes listed in subsequent steps can be adjusted as needed; it is important to cover gel pieces completely.

⁶ This is a good interim stopping point and overnight incubation at 4 °C aids in removing SDS and Coomassie dye to lower background prior to downstream mass spectrometry analysis.

11. Spin samples briefly in tabletop microcentrifuge and remove supernatant. Add 75 μ L of iodoacetamide (81 mM in 100 mM ABC) and incubate at room temperature in the dark for 30 min to alkylate the reduced cysteines.
12. Spin samples briefly in tabletop microcentrifuge and remove supernatant.
13. Wash samples with 200 μ L of 100 mM ABC. Incubate at room temperature for 5 min.
14. Remove ABC. Dehydrate gel pieces with addition of 200 μ L ACN at room temperature for 5 min.
15. Remove ACN and repeat **step 13**.
16. Repeat **step 14**.
17. Remove ACN and dry samples in speedvac concentrator for 10 min.
18. Add 30 μ L of trypsin (20 ng/ μ L in 100 mM ABC) to each tube and incubate on ice for 10 min.
19. Add additional 100 mM ABC to completely cover gel, taking into account for expansion during this incubation step. Check pH prior to incubation to ensure within the range for optimal activity (7.0–9.0).
20. Incubate at 37 °C for up to 16 h with agitation (600–1000 rpm).
21. Add 100 μ L of ES and vortex to mix. Incubate at room temperature for 10 min; spin tubes briefly in tabletop microcentrifuge.
22. Transfer supernatant to 2 mL tube.
23. Repeat **steps 21** and **22** three more times for a total of four extractions.
24. Place samples in speedvac concentrator to dryness.

Peptides can be stored at –80 °C until further use.

3.9 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) (See Note 7)

1. Resuspend peptides in loading buffer and quantify via Nanodrop to estimate injection prior to mass spectrometry analysis. We analyze three biological replicates, each with two technical replicates at 1000 ng/sample per injection.
2. Program the HPLC method as follows: 2% Buffer B for 0–5 min (serves to desalt peptides on trap column prior to elution and mass spectrometry analysis), 2–35% Buffer B for 180 min, 35–55% Buffer B for 25 min, 55–90% Buffer B for 10 min, 90% Buffer B for 5 min and a column equilibration before loading the next sample: from 90% Buffer B to 2% Buffer B in 5 min with a flow of 2% Buffer B for 10 min.
3. Load peptides onto a PepMap100 C18 trap column (5 μ m, 100 Å, 0.3 $\times$ 50 mm).

⁷The following protocol is optimized for a ThermoFisher Scientific Orbitrap Fusion coupled to a Dionex UltiMate 3000 RSLCnano HPLC system with an EASYSpray PepMap C18 column. Methods should be adjusted for other proteomic systems.

4. Separate peptides on a Dionex UltiMate 3000 RSLCnano HPLC system coupled to an EASYSpray PepMap C18 column (3 μ m, 100 Å, 0.75 $\times$ 150 mm) operated at 275 °C and a spray voltage of 1.7 kV.
5. MS/MS data are collected in a ThermoFisher Scientific Orbitrap Fusion operated in top-speed mode with a cycle time of 3 s.
6. Program the instrument method as follows: isolate precursor ions in the quadrupole (width 1.6 m/z) and detect in the Orbitrap (120K resolution), CID fragmentation 35% (fragment ions are detected in the linear ion trap), AGC targets of 2E5 ions or 50 ms max time for MS1 and 3E3 ions or 250 ms max inject time for MS2 and exclude ions within ± 10 ppm from repeat

3.10 Data Analysis

1. RAW files are uploaded to MaxQuant and searched against complete, reviewed Uniprot Swiss-Prot database for appropriate species containing common contaminants (<ftp://ftp.thegpm.org/fasta/cRAP>) using Andromeda search engine.
2. Perform database searching with the following parameters: peptide mass tolerance 20 ppm and fragment mass tolerance 0.5 Da. Peptide minimum length of seven residues with maximum of three amino acids labeled with Arg10/Lys8. Up to two missed cleavages allowed with trypsin/P specificity. Variable modifications of oxidation of M and acetylation of N-termini and fixed modification includes carbamidomethylation of C. Set protein and PSM FDR to 0.05 and enable match between runs feature.
3. Quantitation is performed for proteins with at least two unique or razor peptides and any combination of the variable/fixed modifications mentioned in **step 2**.
4. Heavy/light SILAC ratios are calculated in Perseus using intensity values from MaxQuant proteinGroups output file.
5. Proteins with three valid SILAC ratios (proteins quantified on both channels in at least one technical replicate across all three biological replicates) are kept for further analysis.
6. Significance is determined by a two-tailed t -test and defined by passing a multi-test corrected q -value threshold of 0.05.

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Fig. 1.
Schematic representation of the iPOND/SILAC/quantitative mass spectrometry approach used to characterize histone modifications and other protein associated with nascent chromatin