2020

Quick and facile preparation of histone proteins from the green microalga Chlamydomonas reinhardtii and other photosynthetic organisms

Amanda L. Wong
Saint Mary's College of California

Nicholas N. Totah
Saint Mary's College of California

Anthony T. Iavarone
University of California - Berkeley

James J. Pesavento
Saint Mary's College of California, jjp6@stmarys-ca.edu

https://doi.org/10.1016/j.ymeth.2020.01.019

Quick and facile preparation of histone proteins from the green microalga *Chlamydomonas reinhardtii* and other photosynthetic organisms

Amanda L. Wong, Nicholas N. Totah, Anthony T. Iavarone, James J. Pesavento

Highlights

- Broadly applicable method for algal and plant tissue histone extraction.
- Histone extracts are suitable for top-down mass spectrometric analysis.
- Significantly different histone H4 proteoforms were observed in algae versus corn.

Abstract

The development of universal, broadly applicable methods for histone extraction from animal cells and tissues has unlocked the ability to compare these epigenetic-influencing proteins across tissue types, healthy and diseased states, and cancerous versus normal cells. However, for plants and green algae, a quick and easily implemented histone extraction method has yet to be developed. Here, we report an optimized method that provides a unified approach to extract histones for the green microalgal species *Chlamydomonas reinhardtii* and *Scenedesmus dimorphus* as well as for maize (corn) leaf tissue. Histone extraction methods include treatment with high salt concentrations and acidification. Preparations of nuclei can be made in ~3.5 h and histones extracted in ~3.5 h either immediately or nuclei may be frozen and histone proteins can be later extracted without a change in histone PTM patterns. To examine the efficiency of the new methods provided, we performed both qualitative and quantitative analysis of salt and acid-extracted whole histone proteins (SAEWH) via SDS-PAGE gel electrophoresis and intact protein mass spectrometry. SDS-PAGE analysis indicated that histone yields decrease when using walled Chlamydomonas strains relative to cell-wall-less mutants.

Using top-down mass spectrometry (TDMS) for intact protein analysis, we confirmed the presence of H4K79me1 in multiple algal species; however, this unique modification was not identified in corn leaf tissue and has not been reported elsewhere. TDMS measurements of SAEWH extracts also revealed that oxidation which occurs during the histone extraction process does not increase with exposure of harvested algal cells, their nuclei and the extracted histone samples to light.

Keywords

Histone extraction; Nuclei isolation; Post-translational modifications; Microalgae; Top-down mass spectrometry

1. Introduction

Beyond the sequence information embedded in DNA - both genetic and *cis*-acting regulatory elements - the histone family of proteins serves as the next level of genomic control through DNA compaction and gene regulation across the eukaryotic kingdom. This family of proteins contains extensive post-translational modifications (PTMs) such as methylation, acetylation, and phosphorylation, which provide real-time, reversible control of many processes that influence the compaction/expression of their associated DNA sequences. In order to faithfully characterize the type(s) and location(s) of individual and, most importantly, combinations of PTMs that make up this 'histone code', it is essential to perform intact protein analysis of histones. The best suited analytical approach for intact histone protein characterization is top-down mass spectrometry (TDMS) using high-resolution Fourier-transform mass spectrometers such as an Orbitrap or high magnetic field ion cyclotron resonance instruments [1], [2]. Our recent TDMS investigation of the histone code in *Chlamydomonas reinhardtii* (referred to as Chlamydomonas) revealed remarkably different histone variants and PTM profiles relative to those found in other organisms [2]. Even when the same histone protein (*e.g.*, H3), modified amino acid location (*e.g.*, K4), and PTM type (*e.g.*, methylation) occurs, there have been reports of different gene expression outcomes that are species-dependent. For example, histone H3 K4 methylation is associated with gene *activation* in yeast [3] and *Tetrahymena* [4] while the same modification in green algae is associated with gene *silencing* [5]. This suggests that the histone code is not universal across all eukaryotes and that specific codes may have emerged during evolution of the major lineages of multicellular organisms. As intact histone protein analysis in green algae and plants is currently understudied, a method that efficiently extracts histones from these organisms would provide a means to accelerate the elucidation of their histone codes and their role in epigenetic regulation.

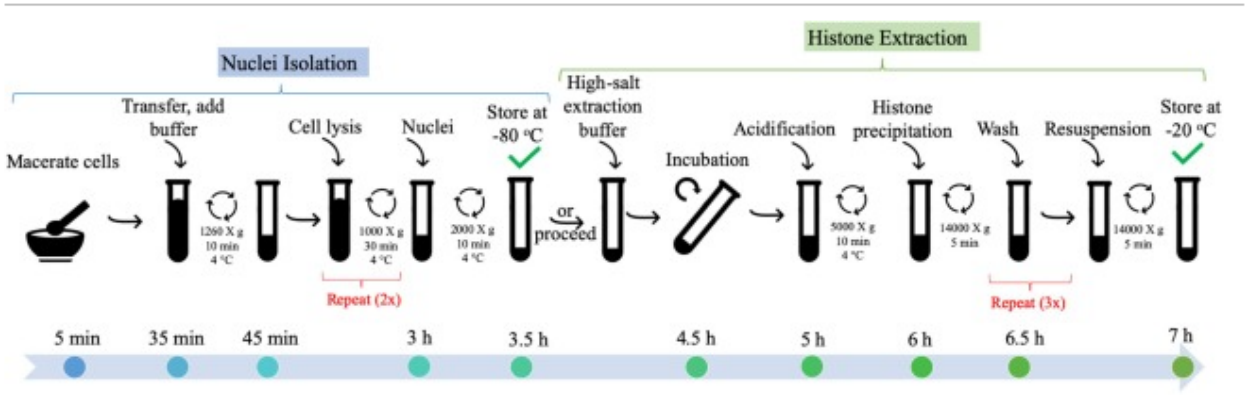
Before histone proteins can be analyzed, researchers must first extract these proteins from the organism, tissue or cell under investigation. For those working with algae and plants, their thick, resilient outer cell wall poses a particular problem for nuclei isolation. However, in some cases cell wall-deficient strains are available to significantly increase the speed and yield of nuclei. In fact, classic approaches to isolating nuclei from the green microalga Chlamydomonas involve use of cell wall-deficient strains (*e.g.,* cc503, cw-92, cw-15, etc.). A more general procedure that works on walled strains of Chlamydomonas would broaden the potential of studying histones in a much larger variety of strains, while alleviating concerns about additional phenotypes that may be associated with wall-less mutants such as osmotic sensitivity and low efficiency of colony formation on agar plates. Furthermore, depending on the procedure, a deflagellation step involving a 20-minute pH shock is sometimes included prior to nuclei isolation [6]. Both the use of cell-wall-less strains and/or prolonged exposure to non-physiological pH may cause alteration to histone PTMs associated with stress-related changes in gene expression. Indeed, because stress-related responses can potentially alter the epigenetic landscape and, in particular, histone PTMs, there is reason to use alternative nuclei isolation methods for histone extraction from cell-walled Chlamydomonas, when possible.

For both cell-walled and cell-wall-less eukaryotic cells, nuclei isolation buffer composition has been optimized to obtain high yields of intact nuclei. These buffers are composed of HEPES, membrane stabilization agents (sucrose, glycerol), and inorganic salts ($MgCl_2$, KCl) to maintain pH, to preserve nuclear membrane integrity, and to facilitate cell lysis, respectively [7]. Because buffer hypotonicity would render nuclear membranes susceptible to lysis, sucrose is added to increase the buffer's osmotic strength, thus preventing the diffusion of water through organelle membranes [8]. This maintains isotonicity of nuclei and other organelles, preserving them for subsequent isolation. Nuclei, packed with chromatin material, are the densest organelle and thus have the highest sedimentation rate, requiring low centrifugal force. This property further helps isolate and purify nuclei away from other contaminating organelles. Cell lysis is performed by mechanical force coupled with a plasma membrane-disrupting detergent. For both Chlamydomonas and land plant tissue, this step is accomplished by the addition of low amounts (<1%) of the nonionic detergent Triton X-100 to nuclei isolation buffers [6], [9], [10]. High concentrations of, or prolonged exposure to, detergent can disrupt intact nuclear membranes. Additionally, inclusion of Triton X-100 lyses chloroplasts and other organelles, but leaves the nuclei intact although somewhat distorted [11]. Reducing agents such as dithiothreitol (DTT) and beta-mercaptoethanol (BME) are added to the buffer to protect proteins from unwanted oxidation, especially at cysteine and methionine residues, and to ensure that cysteine residues remain in their reduced form. Once cells are lysed, nuclei are then separated from cytoplasmic proteins and cellular debris by centrifugation. Nuclei are washed to ensure further removal of

detergent and cytoplasmic proteins, which inherently enriches the (nuclear) histone protein fraction upon their subsequent extraction.

Decades of research on histones have led to the development of several methods for the extraction, purification and separation of whole histone proteins. Because histone proteins are highly enriched in basic amino acid residues, histone extraction from animal cells is commonly based on their solubility in acid, although salt-only methods are employed when analysis of acid-labile PTMs (such as phosphorylation) is desired [12]. The low pH precipitates many non-histone proteins as well as genomic DNA while leaving the histone proteins in the soluble phase. However, in algae and land plants samples must be treated with high salt concentrations prior to acidification in order to efficiently extract histones from isolated nuclei [13]. As for the composition of histone extraction buffer, it is still unclear whether *slight* differences between extraction buffers (*e.g.,* salt type and ionic strength) affect the amount, quality, and purity of histone proteins, although recent studies suggest that they do not [14]. On the other hand, it is known that *significant* differences in buffer composition, especially ionic strength, have dramatic effects on the extraction of both histone and chromatin-associated proteins [15]. Once extracted, histone proteins are typically concentrated by precipitation using either 5% perchloric acid, at which point core histones precipitate and H1 remains soluble, or 20% trichloroacetic acid, which precipitates all histone family members [13]. After several acetone washes, the pellet is resuspended with water, urea, or guanidinium HCl, depending on the procedure used and downstream applications.

While many nuclei isolation and histone extraction methods have been developed, a general approach which works on plants and algae that is suitable for downstream mass spectrometry has not been previously described. In this study, we describe a detailed method for the extraction of histone proteins from the green microalga *Chlamydomonas reinhardtii, Scenedesmus dimorphus* (referred to as Scenedesmus), and Chlorella vulgaris (referred to as Chlorella) and also for leaves from the crop plant *Zea mays* (corn). We address the efficiency of histone extraction from both cell-walled and cell-wall-less algae, the persistence of post-lysis oxidation and the use of top-down mass spectrometry (TDMS) to profile histone H4 proteoforms present in these species. The nuclei isolation and histone extraction procedures can be completed in less than 7 h, as they each require around 3.5 h, and can be performed separately from each other (Scheme 1). Furthermore, this method is broadly applicable and does not require any specialized equipment or unusual reagents.



Scheme 1. The above workflow highlights each major step, and its approximate time in the process, during the nuclei and histone extraction method.

2. Materials and methods

Create all solutions, buffers and dilutions using the highest purity water (*i.e.*, water purified to a resistivity of 18.2 MΩ cm at 25 °C; referred to in the text as ddH_2O). Purchase all chemicals with the highest purity, preferably HPLC or mass-spectrometry grade if mass spectrometry is to be performed post-purification.

2.1. Cell culture

Keep microalgal stocks either on agar slants (TAP or Proteose medium with 2% agar) or in 25 cm_2 flasks. Use aseptic technique for the procedures described below. Sterilize all materials, reagents and media by filtration or autoclaving before use and perform all algal cell culture work in a sterile environment (*e.g.*, in a laminar flow hood) until the final harvest of cultures.

2.1.1. Materials and reagents

1. Organisms (a-d, green microalgae):
    a. *Chlamydomonas reinhardtii* strain cc-503 (cell-wall-less; Chlamydomonas Resource Center)

    b. *Chlamydomonas reinhardtii* strain nzp 70 D10+ (cell wall intact; gift from Jae-Hyeok Lee)

    c. *Scenedesmus dimorphus* (aka *Tetradesmus dimorphus*, UTEX #417)

    d. *Chlorella vulgaris* (UTEX #30)

e. *Zea mays* (corn, cultivar Kandy Korn; Lake Valley Seed Co., Boulder, CO)

2. Media:

a.

Tris-Acetate-Phosphate (TAP) Medium (1 L solution; adjust final pH to 7.0 with HCl)

i. 20 mL 1 M Tris base

ii. 1.0 mL Phosphate Buffer II

1. For 100 mL: 10.8 g K_2HPO_4, 5.6 g KH_2PO_4

iii. 10.0 mL Solution A

1. For 500 mL: 20 g NH_4Cl, 5 g $MgSO_4 \cdot 7H_2O$, 2.5 g $CaCl_2 \cdot 2H_2O$

iv. 1.0 mL Hutner's trace elements[16] (Chlamydomonas Resource Center)

v. 1.0 mL glacial acetic acid

b. Proteose Medium (1 L solution; pH should be ~6.8 after dissolving all components)

i. 1 g proteose peptone
ii. 2.94 mM $NaNO_3$
iii. 0.17 mM $CaCl_2 \cdot 2H_2O$
iv. 0.3 mM $MgSO_4 \cdot 7H_2O$
v. 0.43 mM K_2HPO_4
vi. 1.29 mM KH_2PO_4
vii. 0.43 mM NaCl

3. 10× Phosphate Buffered Saline (pH 7.4)

a. 1.37 M NaCl

b. 27 mM KCl

c. 100 mM Na_2HPO_4

d. 18 mM K_2HPO_4

4. 25 cm_2 Tissue culture flasks

2.1.2. Microalga cell culture and growth

1. Create subcultures by inoculating ~100 mL of TAP medium (for Chlamydomonas) or Proteose medium (for Scenedesmus and Chlorella) in a 250-mL Erlenmeyer flask.

2. Allow cells to grow by agitation on an orbital shaker (~120 rpm) at room temperature (~22 °C) under fluorescent lighting (~2.2 W/m_2).

3. Transfer the entire culture to a 2-L Erlenmeyer flask containing 1 L of either TAP or Proteose medium depending on the cell type once the cell cultures appear light to medium green (~$5 \times 10_5$ cells/mL).

4. Continue growing the cells on an orbital shaker at room temperature under fluorescent lighting for about a week until the cells reach ~$5 \times 10_6$ cells/mL (hand count using a hemocytometer).

5. Harvest the cells by centrifugation at 3000 × *g* for 5 min in a fixed-angle or swinging-bucket rotor.

6. Remove the media and pool cell pellets by resuspending with 1 × PBS.

7. Transfer to a 50-mL conical tube and add PBS up to 50 mL to wash the cells.

8. Centrifuge at 3000 × *g* for 5 min, remove supernatant and store flash-frozen pellet at −80 °C.

2.1.3. Corn growth

1. Plant corn in potting soil, 5 seeds per cell of a 72-cell seedling starter tray and allow to germinate for 7 days.

2. Continue to grow for 13 additional days with light exposure from a northeast-facing window.

3. Cut ~15 leaves (~5 g) at the base of the stalk, place in a 50-mL conical tube and immediately store at −80 °C.

2.2. Preparation of nuclei

The preparation of nuclei begins with maceration of frozen cells by chilled mortar and pestle.

2.2.1. Materials and reagents

Store reagents and buffers listed below at 4 °C and or keep on ice during the nuclei isolation procedure. Nuclei were isolated from Chlamydomonas cell pellets by a modified protocol based off the method of Winck *et al.*[11]. Note that the volumes below are adjusted for 1 L of cells (at ~10_7/mL = ~10_{10} cells total). The amounts of buffers used should be scaled accordingly if cell pellets are significantly larger or smaller.

1. 1 M HEPES, pH 7.5 (adjusted with KOH)

2. 100 mM phenylmethylsulfonyl fluoride (PMSF) using 200 proof ethanol (stock)

3. 500 mM sodium butyrate (stock)

4. 25× Protease inhibitor cocktail (PIC) cOmplete without EDTA (Roche #11873580001) – *make fresh*

5. 1 M dithiothreitol (DTT) – *make fresh*

6. 2× Nuclei Isolation Buffer (NIB) Stock
    a. 50 mM HEPES, pH 7.5
    b. 40 mM KCl
    c. 40 mM $MgCl_2$
    d. 1.2 M sucrose
    e. 20% glycerol

7. NIBA (Nuclei Isolation Buffer with Anti-protease/HDAC/etc. prepared immediately before use).

a. 1× NIB with:
b. 1 mM PMSF
c. 10 mM sodium butyrate (this is a HDAC inhibitor)
d. 0.5× PIC
e. 5 mM DTT
f. Other inhibitors, depending on need (*e.g.,* microcystin to inhibit phosphatases)

8. Pre-cooled mortar and pestle (−80 °C for at least two hours), preferably a larger mortar that is deep to prevent sample loss when macerating. Alternatively, the mortar and pestle can be cooled by using liquid nitrogen. Use appropriate personal protective equipment when handling liquid nitrogen.

9. 20% Triton X-100 (filter sterilized)

2.2.2. Cell lysis and isolation of nuclei

Perform all steps at 4 °C to minimize any enzymatic activities that can potentially alter endogenous histone PTMs. Because cell wall composition differs dramatically between algae and plants, one might want to gradually increase Triton-X percentage to increase lysis efficiency if nuclei and histone yields are low.

1. Thaw cell pellet (10^8 to 5×10^9 cells) on ice or, if using corn leaves (~5.5 g), proceed to **Step 4** below.

2. While thawing, prepare 25 mL of NIBA.

3. Resuspend the thawed pellet with 1–2 mL NIBA then transfer, one drop at a time, around the bottom of the pre-cooled mortar (cells will re-freeze).
   a. **Technical note: add a small pool of liquid nitrogen to the mortar and slowly pipette the resuspended cells, drop-by-drop, so that the cells are refrozen as tiny beads. Wait until the last bit of liquid nitrogen bubbles away before maceration.**

4. Macerate cells with a pre-cooled pestle to a fine powder for ~5 min, adding liquid nitrogen to keep the cells frozen as needed.
   a. **Caution: It is important to use a pre-chilled mortar and pestle: do not mechanically stress the cellular membrane by other means (e.g., blender or bead-beater), as these methods will also lyse nuclei.**

b. **Technical note: use a spatula to help dislodge any embedded cell clumps from the mortar during maceration process. Macerate slowly, as the cellular material may eject from the extremely cold mortar.**

5. Transfer the frozen sample to a 50-mL conical tube using a spatula, add NIBA up to 20 mL, mix by inverting, and place on ice for 10 min.

a. **Technical note: To increase the purity of nuclei, filter this preparation through miracloth [17] or, in the case of leaf tissue, use an additional Percoll density gradient [10]. However, nuclei loss may occur resulting in a lower yield of extracted histones.**

6. Using a swinging-bucket rotor, centrifuge the cells at 1260× *g* for 10 min at 4 °C and remove the supernatant by aspiration.

7. Resuspend the pellet in 20 mL NIBA containing 1% Triton X-100 (v/v), mechanically lyse by passing through a long narrow-bore glass Pasteur pipet ~20–30 times and leave on ice for 10 min.

a. **Technical note: Increasing the Triton X-100 in NIBA (up to 5% v/v) may increase cell lysis and histone extraction efficiency.**

8. Using a swinging-bucket rotor, centrifuge at 1000× *g* for 30 min at 4 °C to collect nuclei.

9. Remove the supernatant by aspiration and repeat steps 7 and 8 above to ensure complete lysis of the plasma membrane.

10. Remove the supernatant by aspiration and resuspend nuclei with 1 mL NIBA to remove detergent, and then transfer the nuclei solution to an Eppendorf microcentrifuge tube.

11. Centrifuge at 2000× *g* for 10 min at 4 °C, and then store the nuclei pellet at −80 °C.

2.2.3. Purification of nuclei from algal species and plant leaves

The major steps in the isolation of nuclei from cell-wall-less and cell-walled algae and plant tissue are shown in Fig. 1. After ~5 min of maceration, the frozen cells should appear as a fine green powder. It is very important to macerate for this period of time and ensure there are no clumps, as the presence of clumps will lead to a decrease in nuclei yield. As such, a darker green ‘nuclei’ pellet will be observed as well as a significant decrease in the extraction of histones from such a nuclei preparation. Liquid

nitrogen may be added if the algal “powder” begins to thaw to the point of having a paste-like consistency. Anecdotal evidence provided in other studies concluded that mortar and pestle maceration yielded more nuclei relative to intact cells when compared to homogenization with a tissue grinder, bead milling, or bio-nebulization [17]. Our group has also tried optimizing bead milling for isolating Chlamydomonas nuclei, without success. Another study on nuclei extraction from tomato leaves revealed that homogenization with a blender yielded 57% intact nuclei while maceration with a mortar and pestle yielded 95% intact nuclei [18]. Taken together, there is a delicate balance required to disrupt the cell wall and plasma membrane to lyse the cells while retaining the structure and integrity of the nuclei. We believe that the maceration mechanically compromises the cell wall and plasma membrane integrity, but preserves the nuclei, leaving them susceptible to complete disruption and cellular lysis once the non-ionic detergent Triton X-100 is introduced. Indeed, for both cell-walled and cell-wall-less Chlamydomonas, the final nuclei pellets were observed to be lighter in color compared to the original cell pellets as the chloroplasts, which contain the green pigment chlorophyll, were lysed in NIBA containing Triton X-100. The nuclei isolated from corn leaf tissue appeared more ‘fluffy’ and less dense than nuclei prepared from algae (data not shown). The persistent white layer at the very bottom of the tube after cell lysis is precipitated starch and remains insoluble in subsequent histone extraction steps.

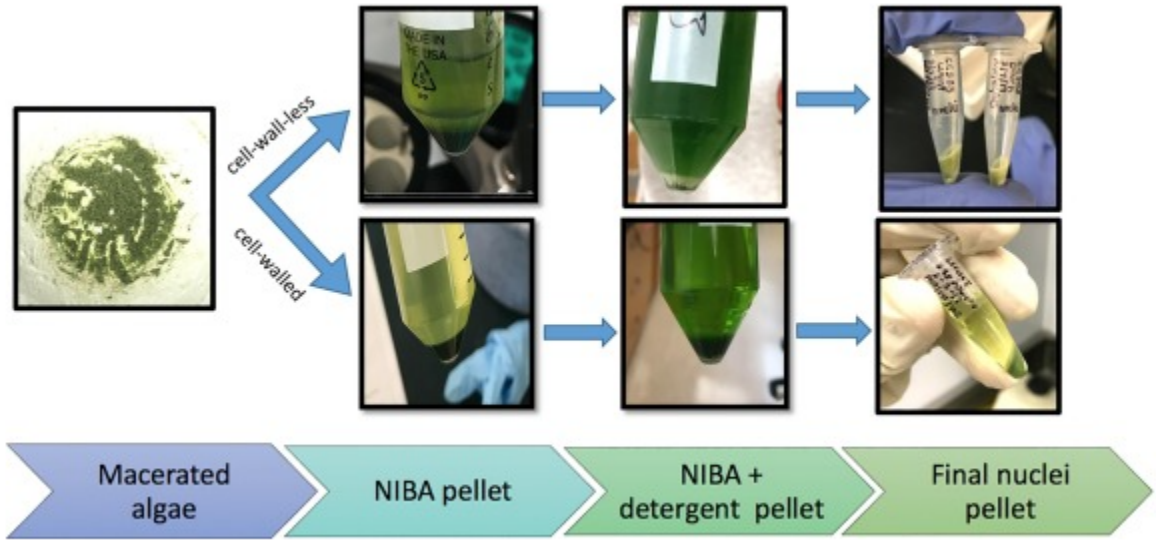


Fig. 1. Representative images of the steps involved in Chlamydomonas nuclei isolation. The far left image shows finely ground algae after maceration with a mortar and pestle. Macerated cell-walled (*nzp70 D10* +) and cell-wall-less (*cc503*) Chlamydomonas are first washed (NIBA pellet), then lysed with detergent (NIBA + detergent pellet) and the resulting nuclei are transferred to microcentrifuge tubes prior to histone extraction

(Final nuclei pellet). The final nuclei pellets show a faint green color, which is generally darker for cell-walled (bottom) than cell-wall-less Chlamydomonas (top). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Not surprisingly, we observed that nuclei pellets are slightly darker for cell-walled strains of Chlamydomonas, Scenedesmus and Chlorella (data not shown) compared to cell-wall-less strains of Chlamydomonas (Fig. 1). These data indicate that more intact cells remained for cell-walled alga post-lysis, which negatively affected histone extraction efficacy (*vide infra* and Fig. 2). While beyond the scope and purpose of this paper, improved lysis of cell-walled algae may be achieved through supplementation with cell wall-digesting enzymes or cryogenic milling (*e.g.,* Retsch MM400). Gamete wall lytic enzyme (autolysin [19], [20]) can be used for this purpose in vegetative Chlamydomonas cells with a protocol for its preparation available online (www.chlamycollection.org). Alternatively, pectinase/cellulase/hemicellulose [21] are other cell-wall digesting enzymes that could be used and are available from commercial sources.

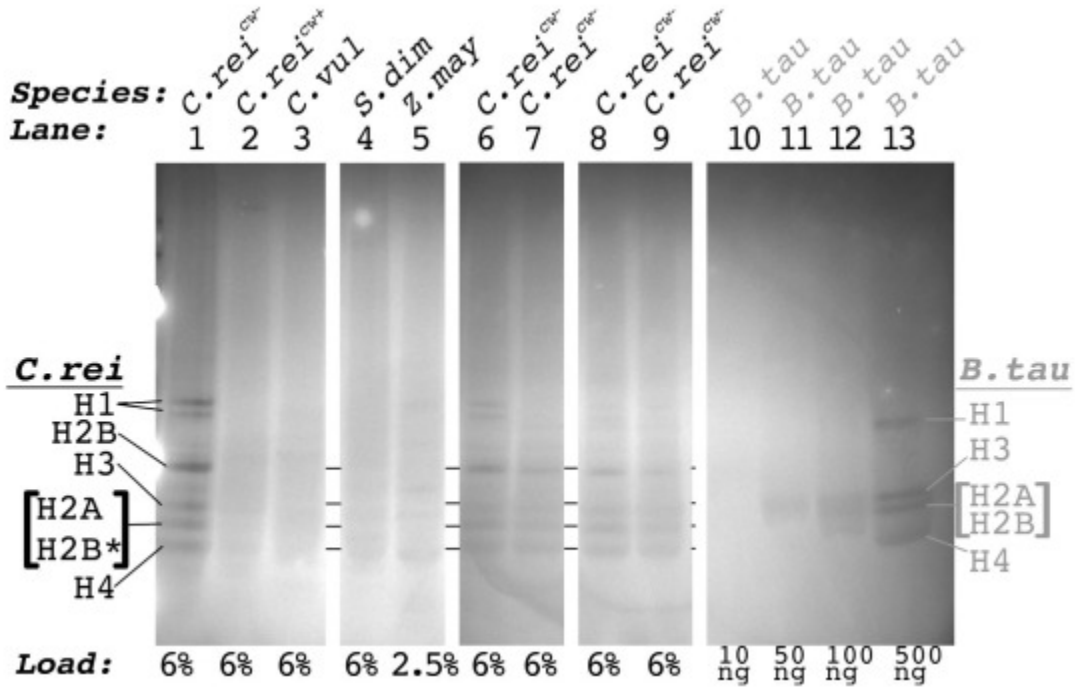


Fig. 2. SDS-PAGE separation of algal and corn SAEWH extracts visualized by zinc/imidazole staining. Lanes 1–5 show SAEWH extracts from (1) cell-wall-less Chlamydomonas, (2) cell-walled Chlamydomonas, (3) Chlorella, (4) Scenedesmus, and (5) corn leaf tissue. Lanes 6–9 show SAEWH extracts and reproducibility from cell-wall-less Chlamydomonas pellets exposed to light (6, 7) or dark (8,9) during nuclei

isolation. The amount of sample loaded is indicated under each lane. Black lines are drawn to orient the histone bands relative to Chlamydomonas histones (far left). Lanes 10–13 represent 10, 50, 100 and 500 ng of *B. taurus* (calf thymus) histone standards, respectively. Grey lines (far right) mark the migration of the core and linker calf thymus histone standards.

Centrifugation speed is also an important consideration: too fast and the nuclei become distorted and clumped, presumably due to leakage and aggregation of chromatin. The final nuclei centrifugation (*2.2.2*, Step 11) is faster and longer than others have reported previously because in our hands slower speeds led to incomplete sedimentation and loss of nuclei.

2.3. Histone extraction

2.3.1. Materials and reagents

1. Trichloroacetic acid (TCA)
    a. 100% w/v in ddH_2O (stored at 4 °C) - *do not dilute until required*

2. $CaCl_2 \cdot 2H_2O$

3. 1 M HEPES, pH 7.5

4. 500 mM Sodium butyrate

5. 100 mM PSMF in ethanol

6. 1 M DTT – *make fresh*

7. 25× Protease inhibitor cocktail (PIC) cOmplete without EDTA – *make fresh*

8. 2.0 mL low-binding Eppendorf tubes

9. 100% acetone (stored in glass at −20 °C)

10. 99% acetone with 1% v/v 12.2 M HCl (stored in glass at −20 °C)

11. 12.2 M HCl

12. long glass narrow-bore Pasteur pipets

13. (optional) polypropylene pestle (Sigma #Z359947)

2.3.2. Salt and acid extraction of whole histones (SAEWH) from nuclei

Unless noted, store all samples, buffers, and perform centrifugations at 4 °C.

1. For each nuclei sample (from ~10_9 cells), prepare 1.5 mL of the histone extraction buffer (HEB) comprised of:

    a. 10 mM HEPES pH 7.5 (adjusted with KOH)

    b. 10 mM sodium butyrate

    c. 1 mM PMSF

    d. 0.5× PIC

    e. 5 mM DTT

    f. 2 M $CaCl_2$

2. Add 1 mL of HEB to each nuclei pellet, pipet up and down several times, and vortex to ensure that the nuclei are completely resuspended.

3. Rotate the samples end-over-end at 4 °C for 1 h. Longer incubation times are unnecessary.

4. Working in a fume hood with appropriate personal protective equipment (PPE), add 12.2 M HCl to a final concentration of 0.3 M to each sample and vortex to mix.

5. Incubate the acidified samples on ice for 20 min.

6. Centrifuge at 5,000× *g* for 10 min at 4 °C to remove insoluble material.

7. To the supernatant containing salt- and acid-soluble histones, add concentrated 100% TCA to a final concentration of 20% (1 volume TCA to 4 volumes sample).

8. Incubate on ice for 30–60 min to precipitate proteins (sample should appear turbid).

9. Centrifuge at 14,000× *g* for 5 min to pellet precipitated protein and aspirate the supernatant.

10. Wash once with 1 mL ice-cold 20% TCA to remove residual salt, and again pellet proteins by centrifugation at 14,000× *g* for 5 min.

11. Repeat Step 10 two more times, but in place of TCA, use 100% acetone + HCl (once) and then 100% acetone (once), sequentially. Make sure to transfer the acetone using a glass Pasteur pipet.

a. **Technical note: Storing and transferring acetone must be done using glass containers and pipets as acetone may partially dissolve plastic. This is critical especially if mass spectrometry is used in subsequent protein analysis (*e.g.*, residual polyethylene glycol (PEG) extracted from plastic pipet tips may contaminate MS measurements, especially for low protein abundance extracts).**

12. Air dry the pellet for 10 min to remove the acetone.

13. Extract histones from the pellet by adding 30 µL ddH_2O to the dried pellet and then resolublize histones by mashing the pellet into small chunks using either:

a. A glass pestle made by using a flame to melt the tip of a glass Pasteur pipette into a tiny pestle.

b. A polypropylene pestle.

14. Centrifuge the sample at 14,000× *g* for 5 min and transfer the histone-containing supernatant to a new tube.

15. Repeat Steps 13 and 14 for a second 30-µL extraction.

16. Pool the two 30-µL extracts, centrifuge 14,000× *g* for 5 min to pellet any debris.

17. Transfer the supernatant to a fresh 1.5-mL Eppendorf tube and store at −20 °C.

2.4. Analysis of salt and acid extracted whole histones by SDS-PAGE and zinc staining

The following electrophoresis and staining protocols are geared for low-abundance samples. Care should be taken when handling the samples and the gel. Zinc staining has the combined benefit of low nanogram detection limits as well as being compatible with downstream proteomic experiments (*e.g.,* membrane blotting, in-gel protein digest followed by bottom-up mass spectrometry, etc.). It is also important to include calf thymus histone standards with the SAEWH samples to approximate extraction yields and suitability for downstream applications such as TDMS. If using homemade materials to pour SDS-PAGE gels, we recommend the final acrylamide concentration in the resolving gel to be 15% so that the histones migrate more slowly than the dye front.

2.4.1. Materials and methods

1. TruPAGE™ Pre-cast Gels (4–20%) (Sigma #PCG2012)

2. 4X TruPAGE™ LDS Sample Buffer (Sigma #PCG3009)

3. 1x TruPAGE™ Tris-MOPS-SDS Running Buffer

4. Invitrogen Novex XCell SureLock™ electrophoresis chamber

5. Bio-Rad Precision Plus Protein™ Dual Color Standards

6. Bio-Rad ChemiDoc XRS+

7. calf thymus histones (Sigma #H9250)

8. methanol

9. glacial acetic acid

10. Imidazole

11. sodium dodecyl sulfate (SDS)

12. 100 mM $ZnCl_2$ (pH 2–3 using HCl).

2.4.2. SDS-PAGE gel electrophoresis

1. Place a precast TruPAGE 4–20% polyacrylamide gel inside the electrophoresis chamber with an adequate amount of 1X TruPAGE Tris-MOPS Running Buffer

a. **Technical note: If hand-casting SDS-PAGE gels, use 15% polyacrylamide so that the histone proteins are retained on the gel during electrophoresis.**

2. Make a 1:10 dilution of the Precision Plus Protein Dual Color Standard using water and load 10 µL into the first well of the gel.

3. Typically, 1 to 10 µL from the ~60 µL SAEWH (see 2.3.2) is used for SDS-PAGE. Depending on the volume, add the LDS sample buffer to these samples to a 1x final concentration.

4. From a 5 µg/µL calf thymus histone (CTH) working stock, dilute the samples such that they contain either 10, 50, 100, or 500 ng of CTH along with 1X LDS sample buffer.

5. Heat all the samples at 70 °C for 5 min, cool and then briefly centrifuge to collect liquid.

6. Perform electrophoresis at 170 V for ~45 min, or until the dye front reaches the bottom of the gel.

2.4.3. Zinc/Imidazole negative staining

1. After electrophoresis, place the gel in a container with 100 mL 1x TruPAGE™ Tris-MOPS-SDS Running Buffer and gently shake for 20 min.

2. Discard the running buffer and add 100 mL imidazole-SDS solution containing 0.2 M imidazole and 0.01% SDS with gentle shaking for 20 min.

3. Discard the imidazole-SDS solution and briefly wash twice with 100 mL of ddH_2O.

4. Add 100 mL of 100 mM $ZnCl_2$ to the gel and agitate until desired opaqueness is achieved, typically 10 s to 2 min.

5.Once staining is sufficient, remove the zinc solution and wash gel twice with 50 mL ddH_2O.

6. Image the gel using a ChemiDoc XRS + Imaging System or equivalent, taking advantage of the dark background and white light illumination as the gel will be negatively stained.

2.4.4. Histone proteins from algal and plant tissue nuclei are successfully extracted and enriched following salt and acid treatment

Purity and quantity of the histone extracts were evaluated by SDS-PAGE followed by zinc-imidazole negative staining [22], [23], which is more sensitive than coomassie staining and less toxic than silver staining. Subjecting plant and algal nuclei to a high salt buffer (2 M $CaCl_2$) followed by acidification (pH <1) successfully extracted histone proteins (Fig. 2). The washed TCA-precipitated histone pellets were resuspended in ddH_2O (2 × 30 μL) and were mashed with a homemade glass pestle or a polypropylene pestle. To identify residual histone proteins remaining in the pellets, the remaining twice-water-extracted pellets were completely solubilized with concentrated 8 M guanidinium. SDS-PAGE analysis of these samples revealed little, if any, residual Chlamydomonas histones, indicating that the majority of histone proteins were successfully extracted from isolated algal and corn nuclei preparations with only water as the solvent (data not shown). The migration of histone proteins of previously uncharacterized Scenedesmus, Chlorella and corn samples were compared against characterized Chlamydomonas histones[9] for identification (Fig. 2, lane 1) and *B. taurus* (calf thymus) histone standard for quantitation (Fig. 2, lanes 10–13). Extracted histone protein bands, both core (H2A, H2B, H3 and H4) and linker histone (H1) standards, migrated to positions appropriate for their molecular weights when compared to the Chlamydomonas histones. In addition, there were very few non-histone protein bands present on the gel, indicating that our SAEWH extracts were specifically enriched in histone proteins. Not surprisingly, extraction of histones from similar starting numbers of cells appeared to have greater yields for cell-wall-less Chlamydomonas than for cell-walled Chlamydomonas. (Compare lane 2 (cell-walled) to lane 6 (cell-wall-less) in Fig. 2.) Indeed, histones extracted from cell-walled Chlamydomonas, Chlorella, Scenedesmus and corn tissue had dramatically lower abundance relative to cell-wall-less Chlamydomonas. This is most likely due to persistence of cell walls in these samples as evidenced by the darker green nuclei preparations, which reduces histone extraction efficiency. Nevertheless, enough histones were extracted for informative nanoLC-MS measurements (*vide infra*). Extraction of histones from algal pellets harvested on different days (compare lanes 6–7 and 8–9) or having cells/nuclei exposed to light or kept in the dark (compare lanes 6–8 and 7–9) neither significantly affected the histone extraction efficiency nor the types of histone proteins extracted. This suggests that our method is reproducible and is not heavily influenced by the consequences of light exposure. Longer salt extraction incubations (up to 16 h) were also investigated and shown to have no impact on the yield

of extracted histones (data not shown). Based on the calf thymus histone standards, the concentration of histone samples analyzed in Fig. 2 ranged from ~ 20 to 500 ng/μL and total amounts from ~1,200 to 30,000 ng (analysis done using Bio-Rad's ImageLab software). Table 1 provides additional information on the samples analyzed by SDS-PAGE and zinc staining in Fig. 2. Interestingly, not all of the histone family members were negatively stained to the same extent: histone H4 and H1 are not readily detectable until 100 ng or 500 ng of total calf histones were loaded, respectively (Fig. 2, lanes 10–13).

Table 1. Information on Chlamydomonas, Chlorella, Scenedesmus and Corn Samples Represented in Fig. 2.

Species (strain)	**Lane Number (Fig. 2)**	**Harvest Date**	**Harvested Total Cell Count or Weight**
C. reinhardtii (cc503)	1	3-26-19	5.1 × 10_9 cells
C. reinhardtii (nzp70d10 +)	2	3-28-19	1.6 × 10_9 cells
C. vulgaris	3	3-14-19	1.7 × 10_9 cells
S. dimorphus	4	3-13-19	1.7 × 10_9 cells
Z. mays	5	3-28-19	5.5 g
*C. reinhardtii (cc503)*L	6	3-07-19	0.8 × 10_9 cells
*C. reinhardtii (cc503)*L	7	3-14-19	2.0 × 10_9 cells
*C. reinhardtii (cc503)*D	8	3-07-19	0.8 × 10_9 cells
*C. reinhardtii (cc503)*D	9	3-14-19	2.0 × 10_9 cells

L = light-exposed
D = kept in the dark.

2.5. Analysis of salt and acid-extracted whole histones by nanoflow-liquid chromatography/top-down mass spectrometry

The following section describes MS analysis of SAEWH in basic terms. For a more detailed protocol on liquid chromatography and TDMS analysis of histones see Zhou *et al.*[24]. Reverse-phase separation of histone proteins is usually performed using a column packed with C18 stationary phase, a particle size of 3 μm, and a pore size of 100 Å. If nano LC is to be done, use a column with a length of at least 25 cm and an inner diameter of 50–75 μm.

2.5.1. Materials and methods

1. 0.22-μm Millex-GV Durapore (PVDF) syringe filters (Millipore-Sigma, #SLGVR04NL)

2. UltiMate 3000 RSLCnano liquid chromatography system (Thermo Fisher Scientific)

3. LTQ-Orbitrap-XL mass spectrometer (Thermo Fisher Scientific)

4. Acclaim PepMap 100 C18 column (75 μm × 25 cm, nanoViper, #164569)

5. Optima LC-MS grade acetonitrile (Thermo Fisher Scientific)

6. Optima LC-MS grade formic acid (Thermo Fisher Scientific)

7. 0.30 mL polypropylene LC Vial (Wheaton, #225180)

8. LC Vial Snap Cap (Wheaton, #242786)

2.5.2. Sample preparation

1. Filter each water-dissolved SAEWH sample through a 0.22-μm PVDF membrane to remove any particulates.
    a. **Technical note: The Millex-GV filters have a female Luer-Lok fitting and can be used with Luer-Lok syringes. Because of the small volumes used, we recommend puncturing the middle of a 1.5-mL Eppendorf tube cap with a spatula and placing the filter**

in the hole created. The sample then can be easily filtered by using a bench-top centrifuge set at 2,000 × *g* for 5 min.

2. To the filtered sample, add formic acid such that the final concentration is 0.1% (v/v). Keep the volume to a minimum to prevent sample dilution.

3. Transfer the acidified sample to an LC Vial and cap it to prevent evaporation.

2.5.3. Nanoflow-LC parameters prior to top-down analysis

Below we describe a fast gradient geared towards rapid elution of histones, often resulting in the co-elution of histone family members and variants (along with their oxidized forms), which provides quality MS profiles and shorter MS run times. To achieve better chromatographic separation and if data-dependent acquisition (DDA) is desired, longer elution gradients, and additional dimensions of chromatography, can be used [24]. General instrument settings for an LTQ-Orbitrap-XL have been provided below, however providing detailed mass spectrometry parameters was deemed beyond the scope of this paper. If you are new to top-down mass spectrometry analysis of histones, we recommend reviewing the literature to familiarize yourself with the general process[24], explore online resources (*e.g.,* https://www.topdownproteomics.org/), and discuss your project with the staff scientists at the facility with which you plan to work.

1. Equilibrate the C18 column for 30 min with 1% buffer B (acetonitrile with 0.1% formic acid) and 99% buffer A (ddH_2O with 0.1% formic acid) at a flow rate of 300 nL / min.

2. Inject 1 µL of the sample.

3. Separate the histone proteins by using the following gradient:
    a. 1% buffer B for 6 min
    b. 1 to 60% buffer B in 13 min
    c. 60 to 95% buffer B in 6 min
    d. 95% buffer B for 6 min
    e. 1% buffer B for 15 min

4. LTQ-Orbitrap-XL MS parameters:
    a. General: Capillary temperature = 250 °C, source voltage = 2.2 kV, tube lens = 150 V, FTMS full micro scans = 1 @ 500 ms, FTMS full AGC target = 10^6, resolution = 100,000.
    b. Collision-induced dissociation (CID): Isolation window = 6 m/z, CE = 28%, Q = 0.250, time = 30 ms, resolution = 100,000.

2.5.4. Procedure-based oxidation of histone proteins is not enhanced by exposure to light during nuclei isolation and histone extraction

Our previous work on MS-profiling of Chlamydomonas core histones revealed significant procedure-based oxidation of the methionine- and/or cysteine-containing family members (*i.e.,* H4, H2B and H3) [2]. In that study, the source(s) of histone oxidation during their preparation from Chlamydomonas cells were not further investigated. The quick and facile method described here enabled us to easily investigate whether reactive oxygen species (ROS) caused by light exposure contributed to the degree of histone oxidation detected in those TDMS measurements. We postulated that thawed cells and/or nuclei exposed to light during histone purification may contribute to oxidation, given that the histone extraction protocol involves the lysis of chloroplasts and release of light-harvesting complexes and photosynthetic reaction centers (and their by-products) into solution, potentially increasing the levels of ROS. To discern whether oxidation of histone proteins is increased by light exposure during their extraction, a thawed cell pellet was split in half (in the dark) and one half was kept in the dark during the histone extraction procedure while the other half was exposed to light. With this approach, any *in vivo* oxidation should be the same for each histone preparation and any increase in oxidation between samples can be directly ascribed to the exposure to light. Additionally, in order to ensure that enough antioxidants were present during the purification protocols, freshly prepared dithiothreitol (DTT) was added to both nuclei isolation and histone extraction buffers for both conditions. NanoLC-MS/MS analysis was performed on these samples and intact histone H4 (*HFO4*) and H2B (*HTB4*) mass spectral profiles were acquired (Fig. 3). Histone H4 and H2B were chosen because their amino acid sequences contain one and two methionine residues, respectively, and are known to be oxidation-sensitive [2]. Histone H3, which has one methionine and one cysteine, was not selected for analysis due to poor signal-to-noise ratios stemming from the combination of its complex PTM profile with partial oxidation. The MS profiles from histone H4 (top) and histone H2B (bottom) show no significant profile changes between light or dark exposure (blue/green vs. yellow/lavender) or between replicate sample preparations (blue/lavender vs. green/yellow), suggesting exposure to light did little or no additional oxidative damage to these proteins. Indeed, the Nα-ac/K79me1/M84ox proteoform of histone H4 did not increase in abundance for light-exposed samples and ranged between 10 and 25% abundance [25] in all four samples (Fig. 3, top, dotted-line). These data are in agreement with the results of the SDS-PAGE gel (Fig. 2), which showed little qualitative difference in the abundance or quality of the SAEWH bands. Some of the samples were dried and concentrated by vacuum centrifugation (*i.e.,* speed

vac) and nanoLC-MS was repeated. These samples exhibited expected increases in the signal-to-noise ratio; however, there were no noticeable increases in oxidation relative to measurements acquired pre-concentration (data not shown), suggesting the exposure to low heat, vacuum, and water evaporation do not significantly contribute to methionine oxidation.

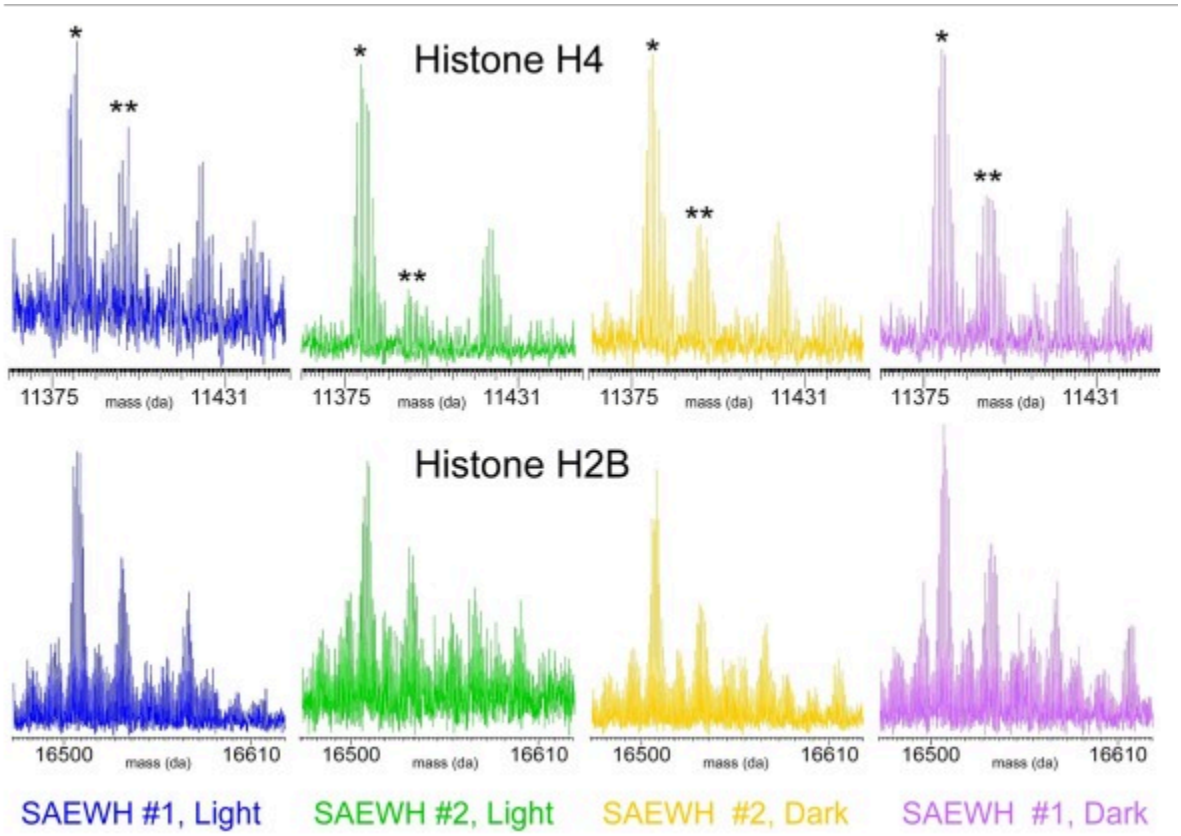


Fig. 3. Exposure to light during nuclei isolation does not increase procedure-based oxidation of extracted histone H2B and H4. Harvested cell-wall-less Chlamydomonas were exposed to light (blue, green) or kept in the dark (yellow, lavender) during nuclei isolation prior to preparing salt and acid-extracted whole histones (SAEWH) and nanoLC TDMS analysis. The experiment was duplicated, from cell growth to histone extraction, and also analyzed by TDMS (blue/lavender (SAEWH #1) vs. green/yellow (SAEWH #2)). A histone H4 proteoform (Nα-ac, K79me1) that is unoxidized is marked with an asterisk (*), while the oxidized proteoform (Nα-ac, K79me1, M84ox) is marked with a double asterisk (**). These samples were also analyzed by SDS-PAGE: SAEWH #1, light (blue) = Fig. 2, lane 6; SAEWH #2, light (green) = Fig. 2, lane 7; SAEWH #1, dark (yellow) = Fig. 2, lane 8; SAEWH #2, dark (lavender) = Fig. 2, lane 9. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

These results, while informative, do not identify the source of histone oxidation within our sample preparation process. Based on previous studies utilizing TDMS on RPLC-purified human histone H4 proteins, we have ruled out the possibility that lysing cells and acid-extracting histones results in high levels of oxidation [26]. Other sources could be from compounds generated through photosynthetic processes that have a lower reduction potential than methionine (and cysteine) and thus are more likely to oxidize them. As the cells harvested in this study were grown and harvested mid-day, the concentration of photosynthesis-generated oxidative species should be high. We speculate that cells harvested during the mid- to late-dark phase of their diurnal cycle would have lower concentrations of reactive species and thus would be expected to exhibit lower levels of histone oxidation after extraction.

2.5.5. Top down mass spectrometric analysis of salt and acid-extracted histones reveals H4K79me1 in algae but not corn leaf tissue

We recently reported a novel, nearly complete monomethylation at lysine 79 in Chlamydomonas histone H4[2]. As this modification had not been reported previously for other green algae or land plants (evolved from charophycean green algae ~400 million years ago [27]), we applied our histone extraction method to Chlamydomonas, Chlorella, Scenedesmus and corn leaf tissue and profiled histone H4 by nanoLC-MS (Fig. 4). The most abundant H4 mass observed for Chlamydomonas, Scenedesmus and corn was 11391.46 Da (Δm = +72 Da), 11329.47 (Δm = +86 Da), and 11329.46 (Δm = +58 Da), respectively (Fig. 4A). These mass differences suggest that all three of these H4 proteoforms have an N-terminal acetylation (+42 Da) and a single oxidation (+16 Da). Indeed, tandem MS (MS/MS) measurements confirmed the presence of an N-terminal acetylation and M84ox for all three proteoforms. It was striking to detect such high degrees of histone H4 oxidation in cell-walled Scenedesmus (>60%) and corn leaf tissue (>80%), further illustrating the intrinsically oxidative nature of histone extraction from algae and plants, and showing that oxidation is not Chlamydomonas-specific. Furthermore, the total levels of histone H4 oxidation for cell-wall-less Chlamydomonas harvested on 3-26-19 (Fig. 4A top MS; Fig. 2, lane 1) were approximately 75%, while the levels for cell-wall-less Chlamydomonas harvested 3-07-19 (Fig. 3, blue MS; Fig. 2, lane 6) were approximately 40%. Such a wide range of oxidation levels for samples that were grown and processed very similarly suggests that the source of oxidation may be multifactored. While the histone H4 sequences of the three species differ slightly, the very C-terminal sequences are identical and can be monitored for PTMs such as K79me1 and M84ox by the y_{28} reporter ion (Fig. 4B, top). As expected based on our previous studies, analysis of Chlamydomonas H4 shows methylation (+14 Da) and methylation + oxidation (+30 Da), at fragment ion abundance ratios that approximate the intact precursor ion ratios[28] (compare the $y_{28}{}_{4+}$ ion in Fig. 4B top MS/MS with bracketed precursor ions in Fig. 4A top MS). However, the y_{28}

ion from Scenedesmus was found to be methylated and oxidized (+30 Da) or, for corn, only oxidized (+16 Da) (Fig. 4B middle and bottom MS/MS, respectively). Interestingly, the Scenedesmus histone H4 has an additional + 14-Da mass difference that is most likely due to a monomethylation between residues T54 and E63 (about 50% abundance based on the fragment ion abundance ratios). However, an amino acid substitution, such as G56 to A56, accounting for the observed mass difference, while unlikely, cannot be ruled out (data not shown). The nanoLC-MS data obtained from Chlorella histone extraction revealed histone-like masses and RPLC retention times, but histone H4 was not conclusively identified after MS/MS measurements. Taken together, these data show the presence of H4K79me1 in other species of algae, but not corn leaf tissue, suggesting the histone methyltransferase responsible was either acquired after the land plant lineage diverged from algae or the enzyme was lost during land plant evolution. Another explanation for the absence of H4K79me1 in corn is that only young leaf tissue cells were investigated here and the expression of the enzyme, and concomitant formation of H4K79me1, may occur in other tissue or at a later developmental stage.

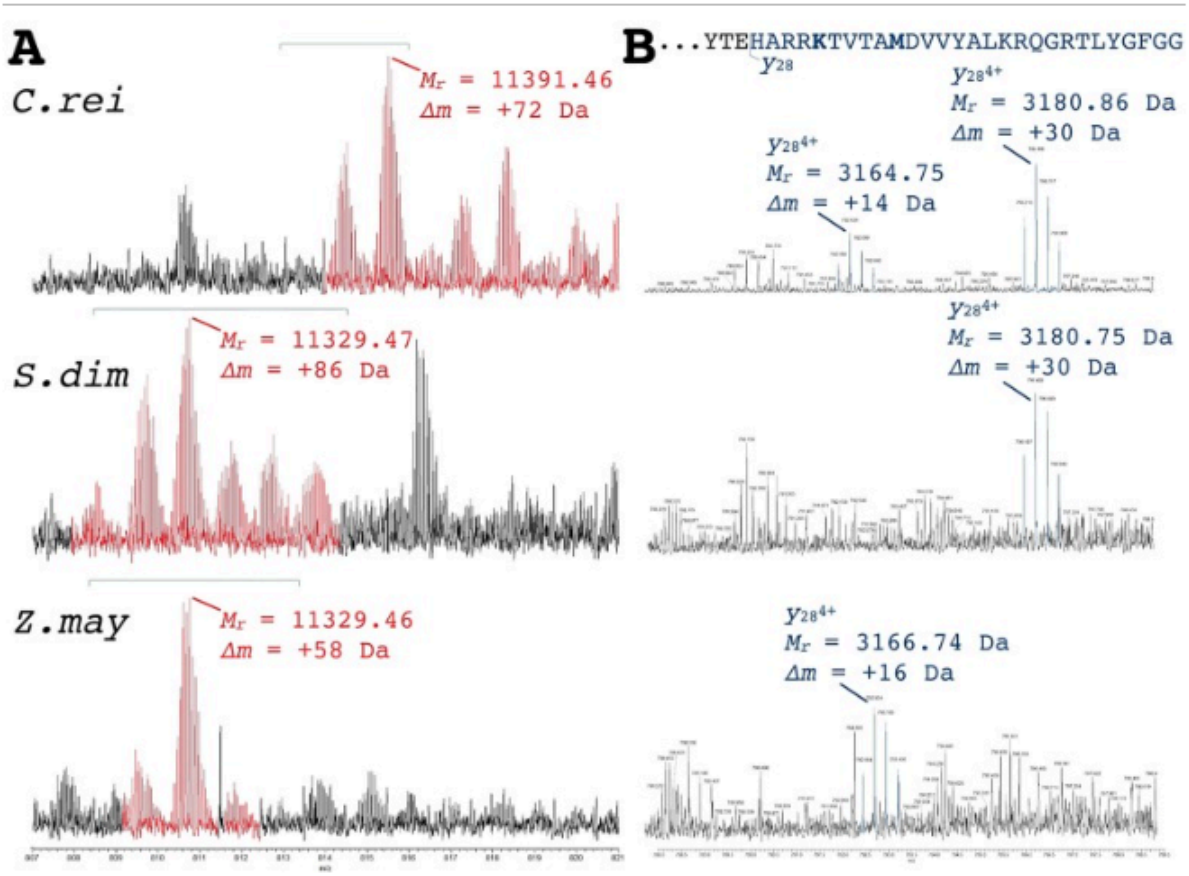


Fig. 4. NanoLC-MS and MS/MS analysis of SAEWH samples from Chlamydomonas, Scenedesmus, and corn reveals histone H4 PTM profiles and sites of modification. (A)

Intact histone H4 MS profiles show multiple posttranslationally modified proteoforms (14 + charge state shown in red). The mass-to-charge (m/z) isolation windows used for MS/MS are represented by the blue bars. (B) Collision-induced dissociation (CID) of the selected precursor ions in (A) reports on the oxidation state of M84 and the methylation state of K79 through the y_{28}^{4+} ion (blue). The change in mass (Δm) from the theoretical unmodified y_{28} mass reports methylation (+14 Da), oxidation (+16 Da), or a combination of both (+30 Da). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5.6. Additional sample-handling and preparation considerations may increase the quality of TDMS measurements of salt and acid-extracted histones

Crude histones prepared by trichloroacetic acid (TCA) and/or perchloric acid (PCA) precipitation followed by TCA/PCA washing and acetone washing were presumed to be sufficiently desalted based on previous accounts of their unadducted TDMS profiles acquired without any desalting steps (*i.e.,* no RPLC separation or ziptip cleanup) [29]. With this in mind, we prepared samples for MS analysis without desalting by ziptips or any other means, which mitigated any sample losses associated with sample cleanup.

Partial oxidation of proteins, especially highly modified ones like histones, dilutes ion signal in mass spectrometry by spreading ion current across multiple mass-to-charge ratio (m/z) channels, and becomes even more detrimental as the number of oxidizable residues increases. Thus, intentional and complete oxidation of histone samples prior to MS can both reduce mass spectral complexity and improve signal-to-noise ratios. To accomplish this, one can either treat the SAEWH with mild performic acid [26] either (1) after the salt and acid extraction, but before TCA precipitation (*i.e.*, the supernatant generated in section *2.3.2* Step 6) or (2) after water extraction, just prior to nanoLC-MS analysis.

The nanoLC-MS profiles shown in Fig. 4 were generated from injecting 1 μL (1.7%) of the total extracted sample onto a C18 column (estimated protein load of 200 ng). We recommend loading no less than 200 ng and no greater than 600 ng of SAEWH on a 75 μm × 25 cm C18 nanoLC column. Post-PVDF membrane filtered SAEWH samples can be concentrated via speed vac to a concentration that yields 200–600 ng per μL. Additionally, we recommend the final volume to be no less than 10 μL, so that multiple 1-μL injections can be run. This has the benefit of first screening the MS to obtain histone PTM profiles and retention times, a very important first step when working with uncharacterized histones (or histones from organisms with no sequenced genomes). Subsequent runs can then be performed to target specific histone proteoforms for MS/MS.

3. Concluding remarks

The histone extraction method described here provides researchers interested in algal and plant histone variants and PTMs with a quick and easily implementable approach to prepare samples that are suitable for experimental study. The resulting SAEWH extracts are specifically enriched in histone proteins and are suitable for downstream SDS-PAGE and TDMS analysis. We hope that this accessible method will expedite the characterization of algal and plant histones and expand the tool set for researchers studying the epigenomes of algae and plants.

CRediT authorship contribution statement

Amanda L. Wong: Investigation, Writing - original draft, Visualization. Nicholas N. Totah: Investigation, Writing - original draft, Visualization. Anthony T. Iavarone: Writing - review & editing, Methodology. James J. Pesavento: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition.

Acknowledgements

This material is based partially upon work supported by the National Science Foundation under Grant No. MCB-1943439. J.J.P., A.L.W., and N.N.T were further supported through Faculty Development Awards provided by Saint Mary's College of California. A.L.W. was also supported through the Saint Mary's College Undergraduate Research Award. This work was also funded by the Department of Education HSI STEM Award (PR Award #P031C160168). A.T.I. acknowledges National Institutes of Health support (grant 1S10OD020062-01). We thank Henna Shaghasi for her help with preparation of nuclear extracts.

References

[1] M.V. Holt, T. Wang, N.L. Young
One-pot quantitative top- and middle-down analysis of GluC-digested histone H4
J. Am. Soc. Mass. Spectrom. (2019), 10.1007/s13361-019-02219-1

[2] A. Khan, C.K. Eikani, H. Khan, A.T. Iavarone, J.J. Pesavento
Characterization of chlamydomonas reinhardtii core histones by top-down mass spectrometry reveals unique algae-specific variants and post-translational modifications
J. Proteome. Res., 17 (1) (2018), pp. 23-32, 10.1021/acs.jproteome.7b00780

[3] H. Santos-Rosa, R. Schneider, A.J. Bannister, et al.
Active genes are tri-methylated at K4 of histone H3
Nature, 419 (6905) (2002), pp. 407-411, 10.1038/nature01080

[4] B.D. Strahl, R. Ohba, R.G. Cook, C.D. Allis
Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena
Proc. Natl. Acad. Sci., 96 (26) (1999), pp. 14967-14972, 10.1073/pnas.96.26.14967

[5] K. van Dijk, K.E. Marley, B. Jeong, et al.
Monomethyl histone H3 lysine 4 as an epigenetic mark for silenced euchromatin in Chlamydomonas
Plant Cell., 17 (9) (2005), pp. 2439-2453, 10.1105/tpc.105.034165

[6] L. Keller, J.A. Schloss, C.D. Silflow, J.L. Rosenbaum
Transcription of a- and b-Tubuline genes in vitro in isolated Chlamydomonas reinhardtii nuclei
J. Cell Biol., 98 (1984), pp. 1138-1143

[7] J.D. Dignam, R.M. Lebovitz, R.G. Roeder
Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei
Nucl. Acids Res., 11 (5) (1983), pp. 1475-1489, 10.1093/nar/11.5.1475

[8] A. Nabbi, K. Riabowol
Isolation of pure nuclei using a sucrose method
Cold Spring Harb. Protoc., 2015 (8) (2015), 10.1101/pdb.prot083741
pdb.prot083741

[9] J.H. Waterborg, A.J. Robertson, D.L. Tatar, C.M. Borza, J.R. Davie
Histones of Chlamydomonas reinhardtii. Synthesis, acetylation, and methylation
Plant Physiol., 109 (2) (1995), pp. 393-407

[10] S. Sikorskaite, M.-L. Rajamäki, D. Baniulis, V. Stanys, J.P.T. Valkonen
Protocol: Optimised methodology for isolation of nuclei from leaves of species in the Solanaceae and Rosaceae families
Plant Methods, 9 (1) (2013), p. 31, 10.1186/1746-4811-9-31

[11] T.J. Guilfoyle

Isolation and characterization of plant nuclei
Methods Cell Biol., 50 (4) (1995), pp. 101-112, 10.1042/bst0051128

[12] D. Shechter, H.L. Dormann, C.D. Allis, S.B. Hake
Extraction, purification and analysis of histones
Nat. Protoc., 2 (6) (2007), pp. 1445-1457, 10.1038/nprot.2007.202

[13] R.L. Morris, L.R. Keller, A. Zweidler, P.J. Rizzo
Analysis of chlamydomonas reinhardtii histones and chromatin
J. Protozool., 37 (2) (1990), pp. 117-123, 10.1111/j.1550-7408.1990.tb05880.x

[14] K.O. Kizer, T. Xiao, B.D. Strahl
Accelerated nuclei preparation and methods for analysis of histone modifications in yeast
Methods, 40 (4) (2006), pp. 296-302, 10.1016/j.ymeth.2006.06.022

[15] C. Herrmann, D.C. Avgousti, M.D. Weitzman
Differential salt fractionation of nuclei to analyze chromatin-associated proteins from cultured mammalian cells
Bio-protocol, 7 (6) (2017), 10.1016/j.jaac.2013.12.025
417 24.e5

[16] S.H. Hutner, L. Provasoli, A. Schatz, C.P. Haskins
Some approaches to the study of the role of metals in the metabolism of microorganisms
Proc. Am. Philos. Soc., 94 (2) (1950), pp. 152-170

[17] F.V. Winck, M. Kwasniewski, S. Wienkoop, B. Mueller-Roeber
An optimized method for the isolation of nuclei from chlamydomonas reinhardtii (chlorophyceae)
J. Phycol., 47 (2) (2011), pp. 333-340, 10.1111/j.1529-8817.2011.00967.x

[18] H.-B. Zhang, X. Zhao, X. Ding, A.H. Paterson, R.A. Wing
Preparation of megabase-size DNA from plant nuclei
Plant J., 7 (1) (1995), pp. 175-184, 10.1046/j.1365-313X.1995.07010175.x

[19] T. Kubo, S. Kaida, J. Abe, T. Saito, H. Fukuzawa, Y. Matsuda
The chlamydomonas hatching enzyme, sporangin, is expressed in specific phases of the cell cycle and is localized to the flagella of daughter cells within the sporangial cell wall
Plant Cell Physiol., 50 (3) (2009), pp. 572-583, 10.1093/pcp/pcp016

[20] W.J. Snell
Cell-Cell Interactions in chlamydomonas
Annu. Rev. Plant. Physiol., 36 (1) (1985), pp. 287-315, 10.1146/annurev. pp. 36.060185.001443

[21] G. D'Alessio, A.R. Trim
A Method for the Isolation of nuclei from leaves
J. Exp. Bot., 19 (4) (1968), pp. 831-839, 10.1093/jxb/19.4.831

[22] C. Fernandez-Patron, E. Hardy, A. Sosa, J. Seoane, L. Castellanos
Double staining of coomassie blue-stained polyacrylamide gels by imidazole-sodium dodecyl sulfate-zinc reverse staining: sensitive detection of coomassie blue-undetected proteins
Anal. Biochem., 224 (1) (1995), pp. 263-269
http://www.ncbi.nlm.nih.gov/pubmed/7535985

[23] **Chen H. Revisit of Imidazole-Zinc Reverse Stain for Protein Polyacrylamide Gel Electrophoresis. In: Vol 1853. ; 2018:139-147. doi:10.1007/978-1-4939-8745-0_17.**

[24] **Zhou M, Wu S, Stenoien DL, et al. Profiling Changes in Histone Post-translational Modifications by Top-Down Mass Spectrometry. In: Wajapeyee N, Gupta R, eds. Vol 1507. Methods in Molecular Biology. New York, NY: Springer New York; 2017:153-168. doi:10.1007/978-1-4939-6518-2_12.**

[25] J.J. Pesavento, C.A. Mizzen, N.L. Kelleher
Quantitative analysis of modified proteins and their positional isomers by tandem mass spectrometry: human histone H4
Anal. Chem., 78 (13) (2006), pp. 4271-4280, 10.1021/ac0600050

[26] J.J. Pesavento, B.A. Garcia, J.A. Streeky, N.L. Kelleher, C.A. Mizzen
Mild performic acid oxidation enhances chromatographic and top down mass spectrometric analyses of histones
Mol. Cell. Proteomics., 6 (9) (2007), pp. 1510-1526, 10.1074/mcp.M600404-MCP200

[27] L.E. Graham
Green algae to land plants: an evolutionary transition
J. Plant. Res., 109 (3) (1996), pp. 241-251, 10.1007/BF02344471

[28] J.J. Pesavento, C.R. Bullock, R.D. LeDuc, C.A. Mizzen, N.L. Kelleher
Combinatorial modification of human histone H4 quantitated by two-dimensional liquid chromatography coupled with top down mass spectrometry
J. Biol. Chem., 283 (22) (2008), pp. 14927-14937,

[29] Y. Zheng, S. John, J.J. Pesavento, et al.
Histone H1 phosphorylation is associated with transcription by RNA polymerases I and II
J. Cell Biol., 189 (3) (2010), pp. 407-415, 10.1083/jcb.201001148