Methods 184 (2020) 19–28

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Enrichment of histones from patient samples for mass spectrometry-based analysis of post-translational modifications

Roberta Noberini*, Camilla Restellini, Evelyn Oliva Savoia, Tiziana Bonaldi*

Department of Experimental Oncology, IEO, European Institute of Oncology IRCCS, Milan, Italy

ARTICLE INFO

Keywords:
Histone post-translational modifications
Mass spectrometry
Epigenetics
Formalin-fixed paraffin-embedded tissues
Frozen tissues

ABSTRACT

Aberrations in histone post-translational modifications (PTMs) have been implicated with the development of numerous pathologies, including cancer. Therefore, profiling histone PTMs in patient samples could provide information useful for the identification of epigenetic biomarkers, as well as for the discovery of potential novel targets. While antibody-based methods have been traditionally employed to analyze histone PTM in clinical samples, mass spectrometry (MS) can provide a more comprehensive, unbiased and quantitative view on histones and their PTMs. To combine the power of MS-based methods and the potential offered by histone PTM profiling of clinical samples, we have recently developed a series of methods for the extraction and enrichment of histones from different types of patient samples, including formalin-fixed paraffin-embedded tissues, fresh- and optimal cutting temperature-frozen tissues, and primary cells. Here, we provide a detailed description of these protocols, together with indications on the expected results and the most suitable workflow to be used downstream of each procedure.

1. Introduction

Histones are small hydrophilic proteins responsible for DNA compaction in the nucleus and for the transcriptional regulation of underlying genes, a function that is exerted through numerous combinatorial modifications present mostly at their N-terminal tails. Histones comprise histone H3, H4, H2A and H2B, which assemble to form the basic unit of chromatin, the nucleosome, and linker histone H1, which is involved in the stabilization of higher-order chromatin structures. PTMs have been detected on amino acid residues in over 60 different sites on histone sequences, and many different types of modifications have been described [1], among which acetylations and methylations are the most studied and well characterized. Alterations in the levels of many histone PTMs, as wells as aberrant expression or mutations in the enzymes involved in their deposition and removal, have been linked with different diseases, particularly cancer [2,3]. The potential offered by the analysis of histone PTM for the diagnosis, prognosis and prediction of outcome of patients has been demonstrated in a number of studies, which mostly relied on immunohistochemistry analysis [4–6]. Although it is a sensitive and well-established method to profile proteins in a clinical setting, immunohistochemistry suffers from various limitations that make it less appropriate in a discovery setting. Such limitations include the restricted number of modifications that can be analyzed in one experiment, the availability of reliable antibodies and their cross-reactivity, the often-poor linearity of the signal of antibodies, and the difficulty in detecting adjacent modifications. All these issues can be overcome by mass spectrometry (MS), which in the last decade has become the method of choice to profile histone PTMs in a systematic manner. Indeed, MS analysis does not require any *a priori* knowledge of the modification site, offers a comprehensive view of multiple histone PTMs and their combinations, and provides an accurate quantification of even mild relative changes among samples.

To date, the majority of the studies exploiting MS to investigate histone PTMs has been performed on cultured cell lines or fresh animal tissues, using classical histone isolation protocols that take advantage of their hydrophilic nature and solubility in strong acids [7]. Typically, cell nuclei are first purified and then subjected to acidic extraction to isolate histones. The acid is then eliminated by dialysis or precipitation with acetone or trichloroacetic acid [8,9]. MS has been exploited to study histone PTMs in a number of different contexts, but only a few

Abbreviations: PAT-H-MS, PAThology tissue analysis of Histones by Mass Spectrometry; FFPE, Formalin-Fixed Paraffin Embedded; PTM, Post-Translational Modifications; OCT, Optimal Cutting Temperature; LMD, Laser Micro-Dissection; MS/MS, tandem mass spectrometry; SDS-PAGE, Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis

* Corresponding authors.
E-mail addresses: roberta.noberini@ieo.it (R. Noberini), camilla.restellini@ieo.it (C. Restellini), evelynoliva.savoia@ieo.it (E.O. Savoia), tiziana.bonaldi@ieo.it (T. Bonaldi).

https://doi.org/10.1016/j.ymeth.2019.10.001
Received 6 June 2019; Received in revised form 30 September 2019; Accepted 4 October 2019
Available online 09 October 2019

studies employed MS to study histone PTMs in patient-derived samples [10–12].

To couple the power of MS-based methods with the translational potential offered by histone PTM profiling of clinical samples, in the last few years we have adapted and developed a series of methods to extract and enrich histones from different types of clinical samples, including formalin-fixed paraffin-embedded (FFPE) tissues, frozen tissues, and primary cells [13–16]. Starting from well-established protocols for the purification of histones from cells, each protocol has been optimized to cope with limitations associated with clinical samples, such as the low starting amounts, or the presence of molecules that can interfere with the MS acquisition. We employed these protocols to profile for the first time histone PTMs in FFPE patient tissues, identifying markers distinguishing different breast cancer subtypes [13,15,16] and potential novel general hallmarks of cancer [17]. In addition, we used protocols to extract histones from FFPE, frozen and primary cells to investigate the epigenetic rewiring occurring when moving from primary tissues to culture models [14]. Here, we provide a detailed description of these protocols, together with indications on the expected results and the most suitable workflow to be used downstream of each procedure.

2. Overview of the methods

Fig. 1 summarizes the different methods that we have developed for the isolation/enrichment of histones from different types of primary samples, which include whole tissue sections [15], and manually macrodissected and laser-microdissected areas from FFPE tissues [13], fresh- and optimal cutting temperature (OTC)-frozen samples [15], and primary cells, which may be available in a wide range of starting amounts [14]. Each protocol was optimized for a specific type/amount of starting material, but all share some basic principles. First, paraffin or OCT, when present, must be removed prior to homogenizing the tissues. Then, nuclei isolation is performed for all samples, exception for FFPE tissues, where it is not feasible. Finally, if it is required and the amount of material allows it, histones can be further enriched through acidic extraction.

Each type of starting material and the specific protocol to isolate and enrich histones for MS-based PTM analysis is described and discussed in detail below. We also provide examples of the appearance on a SDS-PAGE gel of various cancer samples processed with the different protocols (Fig. 2A-B), and an indicative range of histone recovery from different types/amounts of starting material (Table 1). Alternatives procedures found in the literature are included in the *"Alternative protocol(s)"* notes.

3. Histone enrichment from FFPE tissues (PAT-H-MS approach)

Formalin fixation followed by embedding in paraffin is the most common form of tissue preservation to archive patient specimens. Tissues are first fixed in formalin for about 18–24 h to preserve the proteins and structures within the tissue, are then dehydrated and cleared using increasing concentrations of ethanol, and are finally embedded in a paraffin wax block that facilitates cutting slices for microscopy examination. FFPE samples are routinely generated for the diagnosis of the vast majority of diseases and for the storage of pathology specimens, therefore represent a precious source of clinical samples, particularly for retrospective studies. In addition, compared with frozen tissues, FFPE tissues can be conveniently stored at room temperature and they deteriorate more slowly. Historically, the analysis of FFPE tissues by proteomics methods has been limited by the extensive protein cross-linking generated by formaldehyde fixation, but has become possible in recent years thanks to extraction protocols based on heat-induced antigen retrieval techniques similar to those used in immunohistochemistry [18].

We have recently adapted these protocols to histone PTM MS-based analysis, through the Pathology Tissue analysis of Histones by Mass Spectrometry (PAT-H-MS) approach [15]. By comparing matched FFPE and frozen tissues, we showed that histone extracted from FFPE tissues

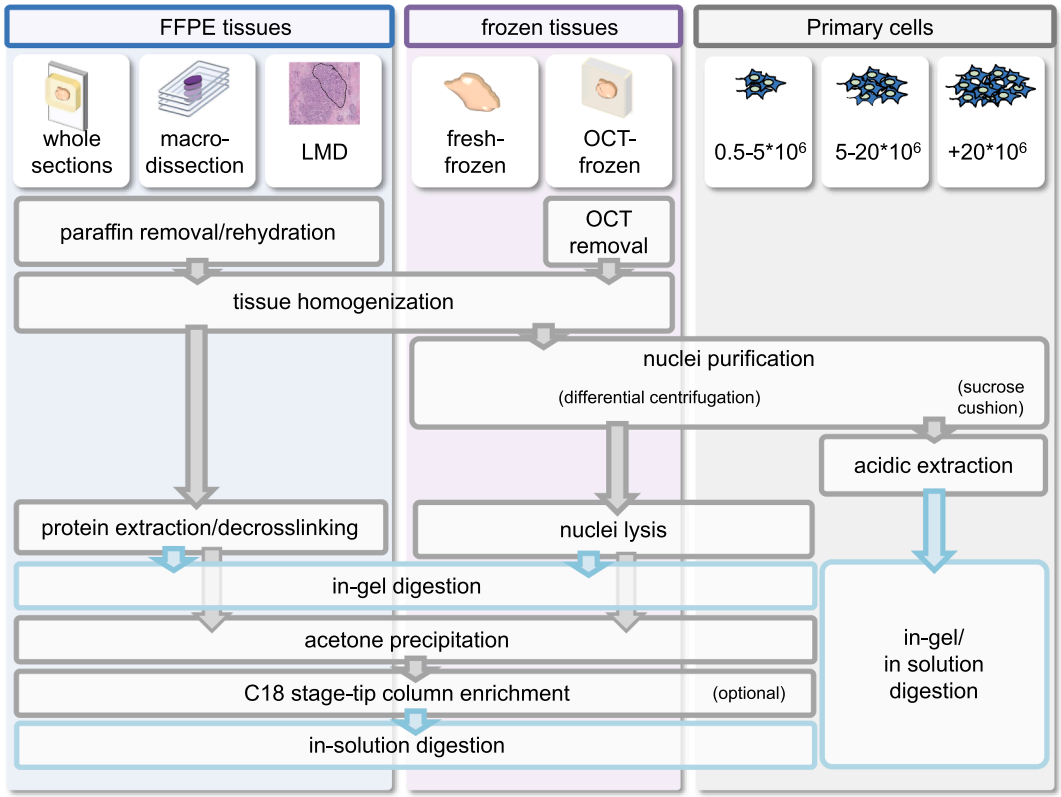

Fig. 1. Overview of protocols for the isolation and enrichment of histones from primary samples prior to MS analysis.

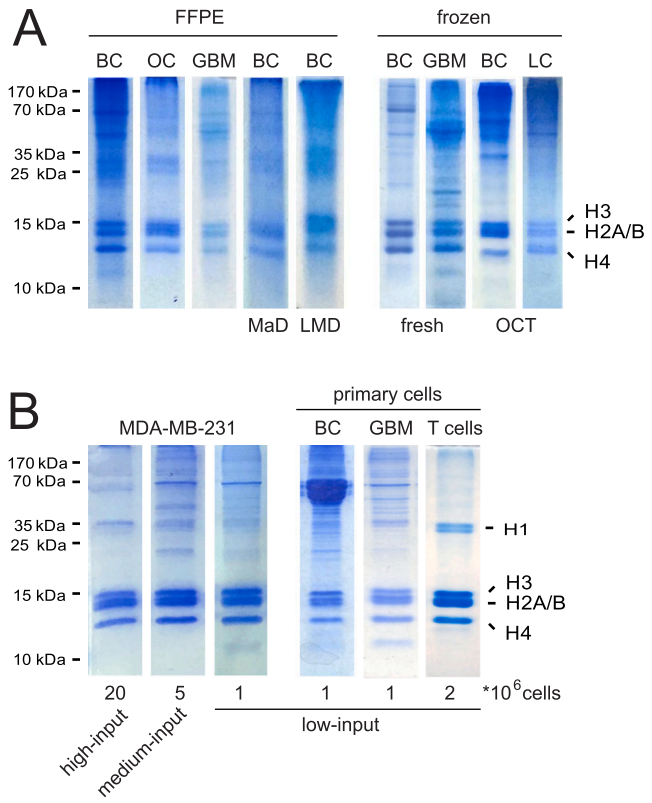

Fig. 2. Histones isolated from primary samples through different protocols. Appearance on a 17% SDS-PAGE gels of histones enriched from tissues through the protocols described in paragraph Sections 3 and 4 (A), and from cells through the protocols described in paragraph 5 (B). BC: breast cancer, OC: ovarian cancer; GBM: glioblastoma; LC: lung cancer; MaD: macrodissection; LMD: laser microdissection. All the samples were human, with the exception of T cells, which were of mouse origin.

have patterns very similar to frozen samples, defining a list of 52 differentially modified histone H3 and H4 peptides that can be identified and quantified in FFPE samples up to 10 years old [15,16] (Fig. 3). Importantly, a few well-characterized methylations (H3K18me1 and K79me1/me2) showed significantly and systematically increased levels in FFPE tissues, likely due to FFPE storage, and as a consequence, they should be excluded from the analysis. We also found the appearance in FFPE tissues of methylations and formylations in additional sites, which however represent a very minor fraction of the total (< 1%). Another study, which employed a different protocol for histone extraction, also reported a higher frequency of specific lysine histone modifications in FFPE compared with fresh-frozen pancreatic cancer xenograft tissues. However, these changes were not evaluated in a quantitative manner [10]. We report below as an "alternative protocol" the method which was used in this study, although it must be noted that it was not thoroughly tested and validated.

Like for frozen tissues, a major limitation of the PAT-H-MS method is related to tissue heterogeneity, which includes the presence of different histological structures or mixed cell populations within the same section. For instance, in a cancer specimen, non-tumoral cells (e.g. normal or immune cells), as well as different tumor cell populations can be found. To overcome this problem, we coupled the PAT-H-MS method with manual macrodissection or laser microdissection (LMD) [13], to isolate gross tissues areas, or specific cell populations, respectively.

The different versions of the PAT-H-MS approach consists of few straightforward steps (Fig. 1): (1) manual macrodissection or LMD of the tissue portion/cell population of interest (optional); (2) de-paraffinization and rehydration through standard techniques; (3) lysis and homogenization of the tissue by sonication, (4) protein extraction and reversion of crosslinking through incubation at high temperatures in the presence of high concentrations of SDS; (5a) SDS-PAGE followed by in-gel digestion, or (5b) acetone precipitation of proteins and histone enrichment by StageTip C18-column enrichment, followed by an in-solution digestion.

In-gel digestions can be performed directly downstream of these protocols. Instead, because these samples contain high percentages of detergent (2% SDS), and are whole protein extracts, the removal of detergents and the enrichment of histones through acetone precipitation followed by StageTip microcolumn enrichment (described in paragraph 6) is essential to be able to perform in-solution digestions.

3.1. Manual macro-dissection and laser microdissection

1. Place 10-μm-thick tissue sections on glass slides (for manual macrodissection) or LMD glass slides (for LMD).

NOTE 1: The number of sections to be used depends on the size of the area of interest. The smallest amount of material that allows profiling all the most common modifications shown in Fig. 3 based on our experience is an area of 8 mm^2 or approximately 450,000 cells. Using lower starting amounts is feasible, but some modified peptides may be undetectable (the modified forms of histone H3 peptide 27–40 are typically lost for lower amounts).

2. Incubate the sections in histolemon twice for 5 min to deparaffinize them.
3. Rehydrate the sections in decreasing concentrations of ethanol (100%, 95%, and 80%, two 3 min incubations for each ethanol concentration) and rinse in distilled water for 30 sec.
4. Stain slides in Harris hematoxylin solution for 2 min and wash in running tap water for 5 min.
5. Counterstain in eosin Y-solution for 10 sec and wash in running tap water for 5 min.
6. Dehydrate in 95% and 100% ethanol for 5 min at room temperature.
7. Morphologically evaluate under a microscope the tissue areas corresponding to the area of interest.

NOTE 2: Although collecting the samples from hematoxylin and eosin-stained sections allows a more precise selection of the areas of interest, for manual macro-dissection staining can be avoided if a reference stained section is available. The reference section can be superimposed to the

Table 1
Histone octamer yield from different enrichment protocols.

Sample type	Protocol	Indicative starting amount	Histone Octamer yield	Text paragraph
FFPE	Classical PAT-H-MS	~4 10 μm-thick sections (20–70 mg)	10–120 μg	3.2
	Macrodissection + PAT-H-MS	8 mm2 of tissue	8–60 μg	3.1
	LMD-PAT-H-MS	at least 450,000 cells	4–40 μg	3.1
Frozen	Fresh-frozen protocol	20–70 mg	10–300 μg	4.2
	OCT-frozen protocol	~8 10 μm-thick sections (40–120 mg)	5–120 μg	4.1
Cells	High-input protocol	20–40*10^6 cells	300–800 μg	5.1
	Medium-input protocol	5–20*10^6 cells	100–400 μg (5*10^6 cells) 400–1000 μg (20*10^6 cells)	5.2
	Low-input protocol	0.5–5*10^6 cells	3–10 μg (0.5*10^6 cells) 10–100 μg (2*10^6 cells)	5.3

	Peptide		FFPE
Histone H3	3-8 unmod		✓
	K4me1		✓
	K4me2		✓
	K4me3		✓
	K4ac		✓
	9-17unmod		✓
	K9me1		✓
	K9me2		✓
	K9me3		✓
	K14ac		✓
	K9me1/K14ac		✓
	K9me2/K14ac		✓
	K9me3/K14ac		✓
	K9ac/K14ac		✓
	18-23 unmod		✓
	K18me1		X
	K18(23)ac		✓
	K18ac/K23ac		✓
	27-40 unmod		✓
	27-40 mono-me	K_{27}me1	✓
		K36me1	✓
	27-40 di-me	K_{27}me2	✓
		K36me2	✓
		K_{27}me1/K_{36}me1	✓
	27-40 tri-me	K_{27}me3	✓
		K_{36}me3	✓
		K_{27}me2/K_{36}me1	✓
		K_{27}me1/K_{36}me2	✓
	27-40 tetra-me	K_{27}me2/K_{36}me2	✓
		K_{27}me3/K_{36}me1	✓
		K_{36}me3/K_{27}me1	✓
	27-40 penta-me	K_{36}me2/K_{27}me3	✓
	K79 unmod		X
	K79me1		X
	K79me2		X
	K79ac		X

	Peptide		FFPE
Histone H4	4-17 unmod		✓
	4-17 mono-ac	K16ac	✓
		K8ac	✓
		K5ac	✓
		K12ac	✓
	4-17 di-ac	K5ac/K16ac	✓
		K8ac/K16ac	✓
		K12ac/K16ac	✓
		K5ac/K12ac	✓
		K8ac/K12ac	✓
	4-17 tri-ac	K5ac/K8ac/K16ac	✓
		K8ac/K12ac/K16ac	✓
		K5ac/K12ac/K16ac	✓
		K8ac/K12ac/K16ac	✓
		K5ac/12ac/K16ac	✓
		K5ac/K8ac/K12ac	✓
	4-17 tetra-ac		✓
	20-35 unmod		✓
	K20me1		✓
	K20me2		✓
	K20me3		✓

Fig. 3. List of differentially modified histone peptides that can be quantified from FFPE tissues. Peptides marked by a red cross showed artefactual levels in FFPE samples compared with matched frozen tissues, and cannot be reliably quantified [15,16]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unstained sections and used as a guide.

8a. MANUAL MACRODISSECTION: scrape the area of interest off the slide with a scalpel into a 1.5 ml Eppendorf tube.

8b. LASER MICRODISSECTION: Collect the areas of interest in 0.5 ml Eppendorf tubes by laser microdissection. Although different laser microdissectors can be used, in [13] we employed a Leica LMD 7000 instrument in the "draw and cut" mode with the following laser settings: wavelength 349 nm; pulse energy: 2 µJ; numerical aperture: 55; speed: 15; specimen balance: 35; head current: 100%; pulse frequency: 5000 Hz, focus offset: 65.

9. Transfer the tissue pieces at the bottom of the tubes through a 3-minute centrifugation at maximum speed. Open tubes carefully to avoid losing tissue pieces.
10. Add 1 ml of histolemon to remove any remaining paraffin and centrifuge at 16,000g for 3 min. In the case of LMD, transfer the tissue pieces with histolemon to a 1.5 ml Eppendorf tube, which is more convenient to use for the following steps. Make sure to collect and transfer all the pieces.
11. Proceed to step 5 of the classical PAT-H-MS approach below.

3.2. Classical PAT-H-MS approach

1. Place four 10 µm-thick FFPE sections in 1.5 ml Eppendorf tubes.

NOTE 3: The same considerations of NOTE 1 apply here. Depending on the tissue block under consideration, less material may be used. In average, four 10 µm-thick FFPE correspond to ~20–60 mg of tissue, as measured by weighting the tissue after paraffin removal, and are an excess amount for most experiments.

NOTE 4: To reduce tissue heterogeneity, for tumors we usually analyze tissues with a tumor cell content of at least 50%, without large necrosis areas or massive flogistic infiltrate.

2. Add 1 ml of histolemon and vortex vigorously for 20 s, making sure that all paraffin fragments are dissolved and do not remain on the

tube cap.

ALTERNATIVE PROTOCOL: In [10] FFPE pancreatic cancer xenograft tissues (eight 10 μm-thick tissue sections with an area up to 80 mm^2) were deparaffinized and extracted by incubating them twice for 10 min at 97 °C in 1 ml EnVision FLEX retrieval solution (pH 8) (Daco Denmark A/S, Glostrup, Denmark).

3. Centrifuge at 16,000g for 3 min. Carefully aspire the supernatant and replace it with 1 ml of fresh histolemon.
4. Repeat steps 2 and 3 for three additional times.
5. Resuspend the deparaffinized tissue in 1 ml of 95% ethanol solution and incubate for 3 min at RT.
6. Centrifuge at 16,000g for 3 min. Carefully discard the supernatant and resuspend the pellet in 1 ml of 70% ethanol. Incubate for 3 min at RT.
7. Repeat Step 6, progressively increasing the percentage of water, by using 50% and 20% ethanol solutions and Milli-Q water.
8. Resuspend the sections in 200 μl of Extraction Buffer (2% SDS, 20 mM Tris pH 7.4). If rather large pieces of tissues are present, it is useful to cut them in smaller pieces with scissors.
9. Homogenize the tissue by sonication in a Branson Digital Sonifier 250 with a 3-mm microtip. Sonicate the samples at room temperature until the tissue pieces have dissolved.

NOTE 5: If the pellet at point 8 appears particularly small, a lower volume of Extraction buffer may be used. For volumes below 100 μl, the sonication step can be performed in a Bioruptor sonication device (30 sec on, 30 sec off, 10 cycles, power: high).

ALTERNATIVE PROTOCOL: In [10] the deparaffinized tissues were sonicated with a probe in extraction buffer (150 μl of 500 mM Tris-HCl (pH 8) and 150 μl of 6 M guanidine-HCl in 50 mM ammonium bicarbonate) for 20 min on ice, followed by centrifugation for 1 min at 14,000g at 4 °C, to remove debris.

10. Extract and de-crosslink the proteins with a 45-min incubation at 95 °C followed by a 4 h incubation at 65 °C, opening the lid several times to allow the formaldehyde to evaporate.
11. Centrifuge the sample at 16,000g for 1 min and store the supernatant at −20 °C.
12. To evaluate the purity of the histones and estimate their amount, load 1/10 of the preparation on a 17% polyacrylamide gel, together with known amounts of recombinant histone H3 (typically 2, 1, 0.5 and 0.25 μg). Prior to loading, add to the samples the gel loading buffer (e.g. Laemmli Buffer 5X: 10% SDS, 50% glycerol, 0.02% bromophenol blue, 0.3 M Tris-Cl, pH 6.8) and dithiothreitol (DTT, final concentration 10 mM). Stain the gel with colloidal Coomassie staining.

NOTE 6: Prior to loading on the gel, the protein concentration in the extracts can be evaluated by using assays that are not impaired by the presence of high concentrations of detergents, such as the bicinchoninic acid assay (BCA protein assay). However, given the extreme variability in the amount of histones present in different types of FFPE extracts, evaluating histone amount through gel visualization is mandatory.

4. Histone enrichment from frozen tissues

Frozen patient specimens can be stored either as fresh-frozen tissues, by immersion in a liquid, such as isopentane or liquid nitrogen, or embedded in the OCT compound. Fresh-freezing is generally considered the gold standard storage method for proteomics, since it avoids potential artifacts that can be caused by other preservation techniques. Fresh-frozen tissues are extremely versatile and allow different types of analysis of proteins, RNA and DNA, which can be performed from the same tissue piece, and do not contain contaminants that interfere with the MS acquisition, such as paraffin. However, fresh-frozen sample must be stored at –80 °C, requiring costly and space-consuming equipment, and the tissue slices obtained from this type of samples often do not completely preserve the morphology of the original tissue. On the contrary, OCT embedding generates a matrix around the samples that favors the morphological preservation of the tissue during section cutting. However, because OCT is a water-soluble blend of glycols and resins, it is a strong MS contaminant that must be carefully removed prior to MS analysis.

Our protocol for histone enrichment from frozen samples involves OCT removal (when required), tissue homogenization, nuclei purification through differential centrifugation, and nuclei lysis (Fig. 1). Histone purity varies greatly for different types of samples (Fig. 2A). Generally, acetone precipitation and StageTip microcolumn enrichment are required prior to performing in-solution digestions (see paragraph 6). Of note, fresh tissue may be processed as frozen tissue. Below, we report as “alternative protocols” procedures employing tissues, although some of them were used for animal tissues and not for patient ones.

4.1. Histone enrichment from OCT-frozen samples

1. Place eight 10-μm thick tissue sections in 1.5 ml Eppendorf tubes

NOTE 7: The same considerations of NOTE 1 apply here. We found the yield of histones obtained from OCT-frozen tissues to be lower compared with FFPE tissues, therefore more sections should be used.

2. Wash the sections by adding 1 ml of an ice-cold 70% ethanol solution and incubating for 2 min on a rotating wheel at 4 °C.
3. Centrifuge at 16,000g for 2 min at 4 °C.
4. Repeat step 3 twice with 70% ethanol.
5. Repeat step 3 with water.
6. Repeat step 3 twice with Phosphate buffered saline (PBS).
7. Proceed to step 2 of the histone enrichment protocol from frozen samples below.

4.2. Histone enrichment from fresh-frozen samples

1. Thaw 20–70 mg of frozen tissues on ice.
2. Add 1 ml of Nuclei Isolation Buffer (PBS, 0.1% Triton X-100, protease inhibitors: 0.5 mM PMSF, 5 μM Leupeptin, 5 μM Aprotinin, 5 mM Na-butyrate).

ALTERNATIVE PROTOCOL: Other isolation buffers can be used. For instance: 1) 10 mM Tris–Cl pH 8.0, 1 mM KCl, 1.5 mM MgCl2 and 1 mM DTT (used for brain [12]); 2) 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100 (used for mouse brain [11]); 3) PBS containing 0.5% Triton X-100 (used for rat brain [19]); 4) 15 mM Tris–HCl (pH 7.5), 60 mM KCl, 11 mM $CaCl_2$, 5 mM NaCl, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, and 0.3% NP-40 (used for different mouse tissues [20]). All buffer are additioned with protease/phosphatase inhibitors and Na-butyrate.

3. Mince the tissues in pieces as small as possible with scissors.
4. Homogenize the samples in a 1 ml Dounce homogenizer.

NOTE 8: If needed, the samples may be further homogenized by sonication in a Bioruptor sonication device (30 sec on, 30 sec off, 10 cycles, power: high, at 4 °C).

5. Remove tissue debris by filtering the homogenate through a 100 μm cell strainer and pipette vigorously through a 200 μl pipette tip several times to break the plasma membrane.
6. Isolate nuclei with a 15-min centrifugation at 2,300g at 4 °C.

ALTERNATIVE PROTOCOL: The centrifugation speed reported in published protocols varies, ranging between 1300 and 2300g.

ALTERNATIVE PROTOCOL: As described in [19] and [11] for fresh-frozen mouse brain, in [21] for fresh mouse liver, and in [20] for various mouse tissues, an acidic extraction step (see step 4–5 of Section 5.2 below) can be performed at this point to increase histone purity. Prior to acidic extraction, nuclear pellets must be washed with a buffer that does not contain sucrose or detergents. As acidic extraction involves loss of material, this step should be performed if an excess amount of material is available (such as when using animal models). This is often not the case when using human samples.

7. Resuspend the nuclear pellet in 100–200 µl of Nuclei Isolation Buffer supplemented with 0.1% SDS and 250 U benzonase, to digest nucleic acids and incubate few minutes at 37 °C.
8. Measure the protein concentration of nuclear extract using the Bradford protein assay or BCA protein assay.
9. To evaluate the purity of the histones and estimate their amount, load 5–10 µg of nuclear extract on a 17% polyacrylamide gel, together with known amounts of recombinants histone H3 (typically 2, 1, 0.5 and 0.25 µg). Prior to loading, add to the samples the loading buffer (e.g. Laemmli Buffer 5X: 10% SDS, 50% glycerol, 0.02% bromophenol blue, 0.3 M Tris-Cl, pH 6.8) and DTT (final concentration 10 mM). Stain the gel with colloidal Coomassie staining. Gel visualization is important to estimate the amount of histones relative to other proteins present in the nuclear extract, as such amount can be highly variable depending on the tissue type.

5. Histone enrichment from primary cells

Although profiling patient tissues is ideal to discover potential biomarkers and uncover disease mechanisms, disease models that can be easily manipulated become indispensable to validate such mechanisms and test potential treatments. Cultured cells, which include cell lines and primary cells, represent the most widely used model to study disease mechanisms. Primary cell cultures are derived directly from patient tissues, and can be grown adherent to cell culture plates or as three-dimensional (3D) cultures, which better mimic the cell growth conditions found *in vivo*. Compared with cell lines, both 2D and 3D primary cultures resemble much more their tissue of origin, also from the epigenetic point of view [14], although displaying some differences [22]. However, primary cells also have several drawbacks, including a poor ability to grow in culture conditions and a limited life-span, which often hinder obtaining high amounts of cells.

The protocols that we use to enrich histones from cells (and that can be applied either to primary cells or cells lines) vary based on the amount of starting material: we use a “high-input” protocol (similar to the traditional protocol described in [7]) if more than 20 $*10^6$ cells are available, a “medium-input” protocol for an amount of cells ranging between 5 and 20 $*10^6$, and a “low-input” protocol, for a starting cell amount between 0.5 and 5 $*10^6$ cells. All cell numbers are indicative, and protocols should be tested and adjusted based on the specific cell type used. Because from primary cells the amount of material is often limited, the method that we most commonly employ for this type of sample is the low-input protocol.

All the protocols include a nuclei purification step, through either a sucrose cushion (which allows obtaining cleaner nuclei, but is associated with a higher loss of material) or a mild detergent lysis of the plasma membrane, followed by centrifugation. Then, if enough material is available, nuclei can be subjected to acidic extraction to isolate histone proteins, which are soluble in strong acids such as HCl or H_2SO_4 [7]. The choice of the protocol also depends on the histone purity required. Although results vary by cells, typically the high-input protocol generates purer histones then the medium-input protocol, which -in turn- produces histones cleaner than the low-input protocol (Fig. 2B). A higher purity is usually associated with lower yields of histone recovery (Table 1). After acidic extraction, histones can undergo both in-gel and in-solution digestions, while in the case of the low-input protocol, the detergents present in the sample must be removed by acetone precipitation (see paragraph 6) prior to in-solution digestions. Further enrichment of histones by StageTip microcolumn (paragraph 6) is optional, and can be evaluated case-by-case, if higher histone purity is required, and enough material is available (at least 10 µg of histones are necessary).

5.1. High-input protocol (20–30*10^6 cells)

1. Wash cells with ice-cold PBS and pellet them. Either proceed to step 2, or freeze the cells and store them at −80 °C until ready to use them.

NOTE 9: It has been shown that freezing the cells before extraction is not harmful, and it might result in a higher histone purity [23].

2. Resuspend the cells in 7 ml of hypotonic Sucrose Nuclei Isolation Buffer (10% sucrose; 0.5 mM EGTA pH 8.0; 15 mM NaCl; 60 mM KCl; 15 mM HEPES; 0.5% Triton X-100; 1 mM DTT; 5 mM Na-butyrate; protease inhibitors: 0.5 mM PMSF, 5 µM Leupeptin, 5 µM Aprotinin).

NOTE 10: Initially resuspend the cell pellet in 0.5 ml of buffer and pipette up and down several times through a 200 µl pipette tip, then add lysis buffer to the final volume.

NOTE 11: An hypotonic lysis has been employed to study histone PTMs in human monocyte-derived macrophages [24] *and human male germ cells* [25].

3. Roll the tubes for 10 min at 4 °C to break the plasma membrane.
4. Pour carefully each lysate on a 20-mL sucrose cushion (obtained by adding an additional 10% sucrose to the Sucrose Nuclei Isolation Buffer), making sure to avoid mixing the two phases.

*NOTE 12: The maximum amount of cells that we recommend using per sucrose cushion is 30*10^6. *Increasing the number of cells per sucrose cushion results in lower sample purity.*

5. Proceed to step 3 of the Medium-input protocol below.

5.2. Medium-input protocol (5–20*10^6 cells)

1. Resuspend 5–20*10^6 cells in 12 ml of Nuclei Isolation Buffer (see paragraph 4.2).

NOTE 12: Initially resuspend the cell pellet in 0.5 ml of buffer and pipette up and down several times through a 200 µl pipette tip, then add lysis buffer to the final volume.

ALTERNATIVE PROTOCOL: In [26] *a nuclei extraction buffer made of PBS containing 0.5% Triton X-100, and 0.02% NaN3 was used to study human monocyte-derived macrophages. The reported yield of histones was* 12 µg *from 12 *10^6 *cells.*

2. Place the tubes in a rotator for 10 min at 4 °C to solubilise the plasma membrane.
3. Pellet the nuclei in a swing-out rotor at 3,250*g* for 30 min at 4 °C and remove the supernatant. The nuclear pellets can be more or less visible and brilliant-white, according to their purity.
4. Re-suspend pellets in 8 ml of ice-cold PBS and spin down nuclei at 3,250g for 20 min at 4 °C. This step is crucial to remove detergents, which would interfere with acidic extraction.

NOTE 13: Initially re-suspend the cell pellet in 0.5 ml of PBS. If needed, nuclei resuspension can be facilitated by a brief sonication in a Bioruptor

sonication device (30 sec on, 30 sec off, 10 cycles, power: high, at 4 °C).

5. Remove the supernatant and resuspend in approximately 1–2 ml of HCl 0.4 N or H_2SO_4 0.4 N and roll for 4 h at 4 °C to extract histones.

NOTE 14: The volume might vary based on the amount of nuclei pellet.
NOTE 15: In our experience, H_2SO_4 performs better in terms of histone yield than HCl for lower amounts of cells.
NOTE 16: Acidic extraction causes the loss of labile modifications, such as phosphorylation. Alternatively, one can avoid this purification step, or use methods that overcome this issue, such as high salt-based extraction [27] or hydroxyapatite chromatography [28].

6. Centrifuge at 16,000g for 10 min and collect the supernatant, which is enriched in soluble core histones, linker histones and high-mobility group box (HMGB) proteins.
7. Optional: to maximize the recovery of histones, resuspend the pellet obtained at step 7 in 1 ml of 0.4 N HCl or H_2SO_4 0.4 N, incubate it for 1–2 h and centrifuge it at 16,000g for 10 min. Pool the resulting supernatant with the one obtained in step 7.
8. Precipitate histones from the pooled supernatant in four volumes of acetone, overnight at −20 °C, and centrifuge at 16,000g for 25 min at 4 °C to pellet proteins.

ALTERNATIVE PROTOCOLS: As an alternative to acetone precipitation, histones can be precipitated by adding TCA to a final concentration of 33% [9]. HCl/ H_2SO_4 can also be eliminated by dialyzing the histones overnight against 100–200 volumes of 100 mM CH3COOH, using dialysis membranes with a 6–8 KDa cutoff. The dialyzed histones are then lyophilized in a Vacuum centrifuge and stored at −80 °C [8].

9. Resuspend histones in water and proceed to step 4 of the Low-input protocol below.

5.3. Low-input protocol (0.5–5*10^6 cells)

1. Resuspend 0.5–2*10^6 cells in 0.5–1 ml of Nuclei Isolation Buffer (see paragraph 4.2) to disrupt cellular membranes. Facilitate membranes rupture by pipetting vigorously through a 200 µl pipette tip several times.
2. Isolate nuclei with a 15-min centrifugation at 2,300g at 4 °C.
3. Resuspend the nuclear pellet in 100–200 µl of the Nuclei Isolation Buffer supplemented with 0.1% SDS and 250 U of benzonase to digest nucleic acids, and incubate few minutes at 37 °C.
4. Measure the protein concentration of the nuclear extract using the Bradford or BCA protein assays.
5. To confirm the measured concentration and assess the purity of the histone preparation, load 4–5 µg of histone preparation on a 17% gel to separate H3, H2A, H2B and H4, using known amounts of recombinant histone H3.1 as a standard (typically, 2, 1, 0.5 and 0.25 µg). Prior to loading, add to the samples the loading buffer (e.g. Laemmli Buffer 5X: 10% SDS, 50% glycerol, 0.02% bromophenol blue, 0.3 M Tris-Cl, pH 6.8) and DTT (final concentration 10 mM). Stain the gel with colloidal Coomassie staining. Gel visualization is particularly important to estimate the amount of histones relative to other proteins present in the nuclear extract in the low-input protocol, as such amount can be highly variable in primary cells, depending on the cell type (Fig. 2).

6. Histone enrichment through StageTip microcolumns

Samples deriving from FFPE tissues, frozen tissues, and primary cells (low input protocol) contain detergents at different concentrations (0.1–2%), which must be removed prior to performing an in-solution digestion. In addition, histones must be further enriched from some of these sample, particularly those for FFPE tissues, which consist in total protein exctract. To this aim, we have recently developed a procedure [16] that includes two main steps: 1) protein precipitation in acetone, to remove the detergents; and 2) intact histone enrichment through handmade "StageTip" reversed phase C18 microcolumns. Step 2 exploits the small size and hydrophilicity of histones, which can be eluted from C18 microclumns at concentrations of organic solvents lower than the majority of the other proteins. The yield of histone recovery ranges between 50% and 100% of the initial histone amount loaded when using histones obtained from cells, while it is approximately 50% when starting from tissues. Histone loss occurrs partly during the precipitation step, and partly during the C18 enrichment step [16]. Such loss of material is comparable to that observed during an in-gel digestion and is compatible with histone PTM MS-analysis, when starting from −10 µg of histones.

Detailed procedure:

1. Dilute 10 µg of histones (based on the comparison of recombinant histone H3.1 performed at the end of each of the protocols described above) in 100–150 µl of water and precipitate proteins in four volumes of acetone, overnight at −20 °C.

NOTE 17. This amount of histones results in reproducible results from all the types of starting material tested. A lower starting amount may be used, but can result in a higher loss of material and less reproducible results [16].

2. Centrifuge the samples at 16,000g for 25 min at 4 °C to pellet proteins.
3. Discard the supernatant, add the same amount of acetone of step 1, and repeat step 2.
4. Discard acetone and let the samples dry at room temperature. Do not over-dry samples.
5. Re-suspend the dried proteins in 20–30 µl of 10% acetonitrile (ACN) in water.

NOTE 17: If needed, protein resuspension can be facilitated by a brief sonication (30 sec on, 30 sec off, 10 cycles, power: high, at 4 °C).

6. Dilute the samples in 100 µl of solvent A (0.1% TFA, 0.5% acetic acid) and load them on C18 StageTip microcolumns by centrifugation at 4,300g for 15 min.

NOTE 18: StageTips [29] are reversed phase chromatography microcolumns manually assembled by placing three 14-gauge StageTip plugs of Empore material in an ordinary 200 µl pipette tip. Prior to being used, StageTips must be activated with 70 µl 100% methanol, washed with 70 µl solvent B (80% ACN, 0.5% acetic acid) and equilibrated twice with 70 µl solvent A, by centrifuging at 4,300g for 5 min.
NOTE 19: The C18 resin was selected as the best performing among several resins that rely on different chromatography principles, including C8 and Strong Anion Exchange resins [16].

7. Wash StageTips with 100 µl of solvent A to eliminate unspecific binders and elute histones sequentially with 40 µl of solvent B containing 40% and 45% of ACN, respectively, through a 10-min centrifugation at 4,300g.

NOTE 20: The presence of adipose tissues (e.g. in breast/breast cancer tissues) will interfere and sometimes impair loading on the C18 StageTip microcolumn, particularly for OCT frozen samples.

8. Pool the two eluates and concentrate samples in vacuum concentrator (SpeedVac) to 10 µl.

7. Histone derivatization and digestion

Following extraction and enrichment from primary samples,

histones are typically digested into peptides prior to MS analysis. An in-gel digestion -which is performed on histone bands cut from samples separated by SDS-PAGE- can be performed downstream of any of the protocols described above, as the gel both eliminates contaminants possibly interfering with MS acquisition, and separates the histones from the other proteins present in the preparation. Instead, an in-solution digestion requires samples that are relatively pure and void of MS contaminants. Therefore, it can be performed only following acidic extraction, or acetone precipitation combined with the C18 StageTip microcolumn enrichment protocol [16] (Fig. 1).

The most common protocols involve the analysis of relatively short peptides (4–20 amino-acid in length), using a so-called “bottom up” approach. While trypsin is the most widely used protease for global proteomics studies, it is unfit for the analysis of PTMs on core histones, because of their high content of lysines and arginines. Besides generating peptides that are too short for MS analysis, trypsin does not cut efficiently next to modified residues, generating peptides of inconsistent length that are impossible to quantify accurately. Two options to overcome this problem exist. The first involves using the Arg-C protease, which cuts only at the C-terminus of arginines, generating peptides of suitable length for MS analysis. It has been shown that an Arg-C digestion is particularly useful to dissect all the differentially acetylated forms of the histone H4 tail [16,30], but the major drawback of the Arg-C protease is its inability to work in gel. As an alternative, a widely used approach involves the derivatization of lysines with acylating agents, such as deuterated acetic or propionic anhydride [8,9], which block trypsin cutting and generate an “Arg-C-like” digestion. Because these approaches involve the use of trypsin, they can be performed both in-solution and in-gel. In addition, because the derivatization occurs only on unmodified and mono-methylated lysines, it causes shifts in the retention times of isobaric peptides, which has proved to be useful for the quantifications of the differentially modified forms of the H3 27–40 peptide [16,30]. Following digestion, a second round of derivatization can be performed to increase the hydrophobicity of the peptides and thus their reversed-phase chromatographic retention. In a traditional protocol, such second step is performed with propionic anhydride [9], but other hydrophobic anhydrides can also be employed [31]. Among the alternatives, the derivatization with phenyl isocyanate (PIC) is particularly useful to increase the retention and the detectability of short and hydrophilic peptides, such as the histone H3 3–8 peptide, as well as to increase the signal-to-noise ratio for low-abundance acetylations, such as H3K27ac and H3K36ac [32]. We refer to other readings for detailed protocols on the Arg-C and the Arg-C like digestions [8,9].

As an alternative to Arg-C and Arg-C-like digestions, Glu-C or Asp-N, which cleave at less frequently-occurring residues within histone sequences, can be used to obtain longer peptides (50–60 amino acids) and investigate combinatorial PTMs, using a “middle-down” approach (reviewed in [33]). Because Glu-C and Asp-N do not work efficiently in-gel, they can only be used in-solution (Section 6). Although to our knowledge a middle-down approach has never been applied to the analysis of FFPE or frozen tissues, it is has been recently used to study ageing in mouse tissues and primary adult human hepatocytes [20].

All these considerations apply to core histones H3, H4, H2A and H2B. A special case is represented by linker histone H1, whose PTMs and isoforms can be best analyzed using trypsin, as Arg-C generates peptides that are too long for MS acquisition.

8. Notes on histone PTM quantification from patient samples

Histone PTM quantification can be performed using various approaches (reviewed in [34]), some of which are suitable for patient-derived samples obtained from any of the protocols described above. A label free quantification does not involve the use of any tags/labels, and compares histones deriving from different samples that are acquired independently. This approach is straightforward and cost-effective, but suffers from experimental variability during the sample preparation or MS acquisition steps. Such variability can become a particularly relevant issue in the case of large cohorts of patient samples, which may be run over long periods of time. In our experience, it is much preferable to employ an internal standard that can be used as a reference to compare all the samples to. The internal standard can be represented by histones isolated from a single cell line [8] or multiple cell lines [35] labelled with isotope-encoded amino acids, or by a library of synthetic peptides [36]. We have shown that the presence of an internal standard greatly increase the reproducibility of peptide quantification, improving the detection of small but significant differences among patient samples –particularly for low-abundance modifications- compared with a label-free strategy [35].

In a discovery setting, a data dependent acquisition (DDA) MS mode is typically used, which allows detecting known as well as novel and less characterized modifications, whose levels are compared across samples in a relative manner. Although we have focused so far only on well-characterized histone H3 and H4 lysine modifications, a number of other modifications can be potentially analyzed in clinical samples. These include low-abundance PTMs, PTMs on histone H2A, H2B and linker histone H1, and modifications other than acetylation and methylation, and on residues other than lysines. It is important to note that the quantification of modifications that are not on the list reported in Fig. 3 from FFPE tissues should be preceded by tests to exclude that they are artefactual modifications caused by storage. Previously acquired datasets containing matched FFPE/frozen tissues may be helpful in this regard (e.g. [37]). Histone PTMs can also be quantitated through MS targeted approaches [38], which allow analyzing with higher sensitivity and throughput a set of peptides known *a priori*. When coupled with the use of a library of synthetic isotope-labeled peptides, MS targeted approaches can provide an absolute quantitation of histones and their PTMs [39], which may be particularly useful during the validation phase of a potential biomarker.

Acknowledgments

Funding: This research was supported by the Italian Association for Cancer Research, grant number IG-2018-21834 (to T.B.), by EPIC-XS, project number 823839, funded by the Horizon 2020 programme of the European Union (to T.B.), and by the Italian Ministry of Health, grant number GR-2016-02361522 (to R.N.).

Appendix A

Reagents

100 μm cell strainer (Falcon 352360)
Absolute ethanol (VWR Life Science 20821.321)
Acetic acid glacial (Carlo Erba 401422)
Acetone (VWR 20066.296)
Acetonitrile (Carlo Erba 412341)
Aprotinin (Sigma-Aldrich A1153)
BCA protein assay (Pierce, 23225)

Benzonase (1.01654.0001, Merk)
Dialysis membranes (Spectrum Laboratories 9201639)
DTT - Dithiothreitol (VWR 441496P)
EGTA - Ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (Sigma-Aldrich E3889)
Empore Octadecyl C18 47 mm Extraction Disks (Supelco 66883-U)
Eosin Y solution (Sigma-Aldrich HT110380)
Ethanol absolute (Carlo Erba 414608)
Harris Hematoxylin (Diapath cod. 0305)
HCl (Sigma-Aldrich H1758)
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma H3375)
Histolemon- Erba (Carlo Erba 454912)
KCl (Sigma-Aldrich P9333)
Leupeptin (Sigma-Aldrich L8511)
LMD glass slides (Leica Microsystems-11505158)
M-butyric Acid Sodium Salt (Sigma-Aldrich B5887-5G)
Mounting media (Eukitt RE.5541.93)
NaCl (Sigma-Aldrich S3014)
PBS - Phosphate buffered saline (MicroGem, TL1006-500ML)
PMSF - Phenylmethylsulfonyl fluoride (Sigma-Aldrich P7626)
SDS - Sodium dodecyl sulfate (Sigma-Aldrich L3771)
Sucrose (VWR Life Science, 27480.294)
Trifluoroacetic acid (Sigma-Aldrich T6508-25ML)
Tris hydrochloride (Sigma-Aldrich T5941)
Triton X-100 (Sigma-Aldrich X100)

Buffers/Solvents

Sucrose nuclei isolation buffer: water, 10% sucrose, 0.5 mM EGTA pH 8.0, 15 mM NaCl, 60 mM KCl, 15 mM HEPES, 0.5% Triton, 1 mM DTT, 5 mM Na-butyrate, 0.5 mM PMSF, 5 μg/ml Leupeptin, 5 μg/ml Aprotinin

Nuclei isolation buffer: PBS supplemented with 0.5 mM PMSF, 5 μg/ml Leupeptin, 5 μg/ml Aprotinin, 5 mM Na-butyrate, 0.1% Triton X-100

Extraction buffer FFPE: 2% SDS, 20 mM Tris pH 7.4

StageTip solvent A: 0.1% TFA, 0.5% acetic acid

StageTip solvent B: 80% ACN, 0.5% acetic acid

Equipment

- Benchtop centrifuges (e.g. Biofuge, Heraeus)
- Centrifuge (e.g. Allegra X-15R, Beckman coulter)
- Electrophoresis power supply (e.g. PowerPack HC, Bio-Rad) and electrophoresis cell (e.g. Criterion Vertical Electrophoresis Cell, Bio-Rad)
- Dounce Homogenizer (e.g 1 ml Tissue Grinder, Dounce, Wheaton)
- Vacuum concentrator (e.g Concentrator 5301, Eppendorf)
- Rotator (e.g SB3, Stuart)
- Branson Digital Sonifier 250 with a 3 mm microtip
- Incubator 37° C (e.g M120-TB, PID Systems)
- Vortex (e.g Vibromix, pbi)
- Thermomixer (e.g thermomixer compact, Eppendorf)
- Laser microdissector (e.g. Leica LMD 7000 instrument, Leica Microsystems, Wetzlar, Germany)

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2019.10.001.

References:

[1] Y. Zhao, B.A. Garcia, Comprehensive Catalog of Currently Documented Histone Modifications, Cold Spring Harb. Perspect. Biol. 7 (9) (2015).
[2] J.E. Audia, R.M. Campbell, Histone Modifications and Cancer, Cold Spring Harb. Perspect. Biol. 8 (4) (2016) a019521.
[3] A. Portela, M. Esteller, Epigenetic modifications and human disease, Nat. Biotechnol. 28 (10) (2010) 1057–1068.
[4] S.E. Elsheikh, A.R. Green, E.A. Rakha, D.G. Powe, R.A. Ahmed, H.M. Collins, D. Soria, J.M. Garibaldi, C.E. Paish, A.A. Ammar, M.J. Grainge, G.R. Ball, M.K. Abdelghany, L. Martinez-Pomares, D.M. Heery, I.O. Ellis, Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome, Cancer Res. 69 (9) (2009) 3802–3809.
[5] D.B. Seligson, S. Horvath, M.A. McBrian, V. Mah, H. Yu, S. Tze, Q. Wang, D. Chia, L. Goodglick, S.K. Kurdistani, Global levels of histone modifications predict prognosis in different cancers, Am. J. Pathol. 174 (5) (2009) 1619–1628.
[6] D.B. Seligson, S. Horvath, T. Shi, H. Yu, S. Tze, M. Grunstein, S.K. Kurdistani, Global histone modification patterns predict risk of prostate cancer recurrence, Nature 435 (7046) (2005) 1262–1266.
[7] D. Shechter, H.L. Dormann, C.D. Allis, S.B. Hake, Extraction, purification and analysis of histones, Nat. Protoc. 2 (6) (2007) 1445–1457.
[8] A. Cuomo, M. Soldi, T. Bonaldi, SILAC-based quantitative strategies for accurate histone posttranslational modification profiling across multiple biological samples, Methods Mol. Biol. 1528 (2017) 97–119.
[9] S. Sidoli, N.V. Bhanu, K.R. Karch, X. Wang, B.A. Garcia, Complete workflow for analysis of histone post-translational modifications using bottom-up mass spectrometry: from histone extraction to data analysis, J. Vis. Exp. 111 (2016).
[10] M. Bauden, T. Kristl, R. Andersson, G. Marko-Varga, D. Ansari, Characterization of histone-related chemical modifications in formalin-fixed paraffin-embedded and fresh-frozen human pancreatic cancer xenografts using LC-MS/MS, Lab. Invest. 97 (3) (2017) 279–288.
[11] L.A. Farrelly, B.D. Dill, H. Molina, M.R. Birtwistle, I. Maze, Current proteomic methods to investigate the dynamics of histone turnover in the central nervous system, Methods Enzymol. 574 (2016) 331–354.
[12] Q. Zhang, P. Xue, H.L. Li, Y.H. Bao, L.H. Wu, S.Y. Chang, B. Niu, F.Q. Yang, T. Zhang, Histone modification mapping in human brain reveals aberrant expression of histone H3 lysine 79 dimethylation in neural tube defects, Neurobiol. Dis. 54 (2013) 404–413.
[13] R. Noberini, R. Longuespee, C. Richichi, G. Pruneri, M. Kriegsmann, G. Pelicci, T. Bonaldi, PAT-H-MS coupled with laser microdissection to study histone post-translational modifications in selected cell populations from pathology samples,

Clin. Epigenetics 9 (2017) 69.
[14] R. Noberini, D. Osti, C. Miccolo, C. Richichi, M. Lupia, G. Corleone, S.P. Hong, P. Colombo, B. Pollo, L. Fornasari, G. Pruneri, L. Magnani, U. Cavallaro, S. Chiocca, S. Minucci, G. Pelicci, T. Bonaldi, Extensive and systematic rewiring of histone post-translational modifications in cancer model systems, Nucleic Acids Res. (2018).
[15] R. Noberini, A. Uggetti, G. Pruneri, S. Minucci, T. Bonaldi, Pathology tissue-quantitative mass spectrometry analysis to profile histone post-translational modification patterns in patient samples, Mol. Cell. Proteomics 15 (3) (2016) 866–877.
[16] C. Restellini, A. Cuomo, M. Lupia, M. Giordano, T. Bonaldi, R. Noberini, Alternative digestion approaches improve histone modification mapping by mass spectrometry in clinical samples, Proteomics Clin. Appl. 13 (1) (2019) e1700166.
[17] R. Noberini, C. Restellini, E.O. Savoia, F. Raimondi, L. Ghiani, M.G. Jodice, G. Bertalot, G. Bonizzi, M. Capra, F.A. Maffini, M. Tagliabue, M. Ansarin, M. Lupia, M. Giordano, D. Osti, G. Pelicci, S. Chiocca, T. Bonaldi, Profiling of epigenetic features in clinical samples reveals novel widespread changes in cancer, Cancers (Basel) 11 (5) (2019).
[18] C.B. Fowler, T.J. O'Leary, J.T. Mason, Protein mass spectrometry applications on FFPE tissue sections, Methods Mol. Biol. 724 (2011) 281–295.
[19] N. Rachdaoui, L. Li, B. Willard, T. Kasumov, S. Previs, D. Sarkar, Turnover of histones and histone variants in postnatal rat brain: effects of alcohol exposure, Clin. Epigenetics 9 (2017) 117.
[20] A. Tvardovskiy, V. Schwammle, S.J. Kempf, A. Rogowska-Wrzesinska, O.N. Jensen, Accumulation of histone variant H3.3 with age is associated with profound changes in the histone methylation landscape, Nucleic Acids Res. 45 (16) (2017) 9272–9289.
[21] P.A. Gruppuso, J.M. Boylan, V. Zabala, N. Neretti, N.A. Abshiru, J.W. Sikora, E.H. Doud, J.M. Camarillo, P.M. Thomas, N.L. Kelleher, J.A. Sanders, Stability of histone post-translational modifications in samples derived from liver tissue and primary hepatic cells, PLoS ONE 13 (9) (2018) e0203351.
[22] C.E. Nestor, R. Ottaviano, D. Reinhardt, H.A. Cruickshanks, H.K. Mjoseng, R.C. McPherson, A. Lentini, J.P. Thomson, D.S. Dunican, S. Pennings, S.M. Anderton, M. Benson, R.R. Meehan, Rapid reprogramming of epigenetic and transcriptional profiles in mammalian culture systems, Genome Biol. 16 (2015) 11.
[23] E. Govaert, K. Van Steendam, E. Scheerlinck, L. Vossaert, P. Meert, M. Stella, S. Willems, L. De Clerck, M. Dhaenens, D. Deforce, Extracting histones for the specific purpose of label-free MS, Proteomics 16 (23) (2016) 2937–2944.
[24] T.C. Minshull, J. Cole, D.H. Dockrell, R.C. Read, M.J. Dickman, Analysis of histone post translational modifications in primary monocyte derived macrophages using reverse phasexreverse phase chromatography in conjunction with porous graphitic carbon stationary phase, J. Chromatogr. A 1453 (2016) 43–53.
[25] L.J. Luense, X. Wang, S.B. Schon, A.H. Weller, E. Lin Shiao, J.M. Bryant, M.S. Bartolomei, C. Coutifaris, B.A. Garcia, S.L. Berger, Comprehensive analysis of histone post-translational modifications in mouse and human male germ cells, Epigenetics Chromatin 9 (2016) 24.
[26] P. Olszowy, M.R. Donnelly, C. Lee, P. Ciborowski, Profiling post-translational modifications of histones in human monocyte-derived macrophages, Proteome Sci. 13 (2015) 24.
[27] C. von Holt, W.F. Brandt, H.J. Greyling, G.G. Lindsey, J.D. Retief, J.D. Rodrigues, S. Schwager, B.T. Sewell, Isolation and characterization of histones, Methods Enzymol. 170 (1989) 431–523.
[28] R.H. Simon, G. Felsenfeld, A new procedure for purifying histone pairs H2A + H2B and H3 + H4 from chromatin using hydroxylapatite, Nucleic Acids Res. 6 (2) (1979) 689–696.
[29] J. Rappsilber, M. Mann, Y. Ishihama, Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, Nat. Protoc. 2 (8) (2007) 1896–1906.
[30] M. Soldi, A. Cuomo, T. Bonaldi, Improved bottom-up strategy to efficiently separate hypermodified histone peptides through ultra-HPLC separation on a bench top Orbitrap instrument, Proteomics (2014).
[31] S. Sidoli, Z.F. Yuan, S. Lin, K. Karch, X. Wang, N. Bhanu, A.M. Arnaudo, L.M. Britton, X.J. Cao, M. Gonzales-Cope, Y. Han, S. Liu, R.C. Molden, S. Wein, L. Afjehi-Sadat, B.A. Garcia, Drawbacks in the use of unconventional hydrophobic anhydrides for histone derivatization in bottom-up proteomics PTM analysis, Proteomics 15 (9) (2015) 1459–1469.
[32] T.M. Maile, A. Izrael-Tomasevic, T. Cheung, G.D. Guler, C. Tindell, A. Masselot, J. Liang, F. Zhao, P. Trojer, M. Classon, D. Arnott, Mass spectrometric quantification of histone post-translational modifications by a hybrid chemical labeling method, Mol. Cell. Proteomics 14 (4) (2015) 1148–1158.
[33] S. Sidoli, B.A. Garcia, Middle-down proteomics: a still unexploited resource for chromatin biology, Expert Rev. Proteomics 14 (7) (2017) 617–626.
[34] H. Huang, S. Lin, B.A. Garcia, Y. Zhao, Quantitative proteomic analysis of histone modifications, Chem. Rev. 115 (6) (2015) 2376–2418.
[35] R. Noberini, T. Bonaldi, A super-SILAC strategy for the accurate and multiplexed profiling of histone posttranslational modifications, Methods Enzymol. 586 (2017) 311–332.
[36] S. Lin, S. Wein, M. Gonzales-Cope, G.L. Otte, Z.F. Yuan, L. Afjehi-Sadat, T. Maile, S.L. Berger, J. Rush, J.R. Lill, D. Arnott, B.A. Garcia, Stable-isotope-labeled histone peptide library for histone post-translational modification and variant quantification by mass spectrometry, Mol. Cell. Proteomics 13 (9) (2014) 2450–2466.
[37] R. Noberini, G. Pruneri, S. Minucci, T. Bonaldi, Mass-spectrometry analysis of histone post-translational modifications in pathology tissue using the PAT-H-MS approach, Data Brief 7 (2016) 188–194.
[38] P. Picotti, R. Aebersold, Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions, Nat. Methods 9 (6) (2012) 555–566.
[39] A. Darwanto, M.P. Curtis, M. Schrag, W. Kirsch, P. Liu, G. Xu, J.W. Neidigh, K. Zhang, A modified "cross-talk" between histone H2B Lys-120 ubiquitination and H3 Lys-79 methylation, J. Biol. Chem. 285 (28) (2010) 21868–21876.