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In vitro Chromatin Assembly - Strategies and Quality Control

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Abstract

Chromatin accessibility is modulated by structural transitions that provide timely access to the genetic and epigenetic information during many essential nuclear processes. These transitions are orchestrated by regulatory proteins that coordinate intricate structural modifications and signalling pathways. In vitro reconstituted chromatin samples from defined components are instrumental in defining the mechanistic details of such processes. The bottleneck to appropriate in vitro analysis is the production of high quality, and quality-controlled, chromatin substrates. In this chapter we describe methods for in vitro chromatin reconstitution and quality control. We highlight the strengths and weaknesses of various approaches, and emphasize quality control steps that ensure reconstitution of a bona fide homogenous chromatin preparation. This is essential for optimal reproducibility and reliability of ensuing experiments using chromatin substrates.

1. Introduction

In eukaryotic cells, chromatin refers to a hierarchical complex of DNA and proteins (histone and non-histone) that implements the proper structural and functional regulation of all genetic and epigenetic information. The minimal chromatin unit is the nucleosome, formed by a core histone octamer and 147 base pairs DNA wrapped around it (Figure 1A; Nuc147). Nucleosomes and their higher order assemblies hinder direct access to the DNA for the multitude of nuclear machineries that mediate DNA-related processes such as transcription, replication, and DNA damage repair. These machineries gain access to the packaged DNA, via tightly regulated structural transitions that expose the genetic and epigenetic information by controlling nucleosome dynamics, both spatially and temporally.

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Highly defined nucleosome and chromatin samples are essential for defining the detailed mechanisms that regulate the dynamics of chromatin. For these studies, defined chromatin templates are a pre-requisite. In vitro assembly can be performed at any level of complexity. We have previously described small- and large scale preparation of high quality nucleosomes, the minimal chromatin unit (Figure 1A; Nuc147) to be used for structural, biochemical, and biophysical applications (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004). We have also prepared nucleosomes containing histones from species other than *Xenopus laevis*, histone variants, histone modifications, different DNA sequences, and histone point mutations (Chatterjee, North, Dechassa, Manohar, Prasad, Luger, et al., 2015; Brehove, Matthew, Wang, North, Luo, Dreher, et al., 2015; Fierz, Beat, Kilic, Hieb, Luger, & Muir, 2012; Watanabe, Resch, Lilyestrom, Clark, Hansen, Peterson, et al., 2010; Lu, Simon, Chodaparambil, Hansen, Shokat, & Luger, 2008; Clapier, Chakravarthy, Petosa, Fernandez-Tornero, Luger, & Muller, 2008; Bao, White, & Luger, 2006; Chakravarthy & Luger, 2006; Chakravarthy, Bao, Roberts, Tremethick, & Luger, 2004; Black, Foltz, Chakravarthy, Luger, Woods, and Cleaveland, 2004; Bao, Konesky, Park, Rosu, Dyer, Rangasamy, et al., 2004; Park, Dyer, Tremethick, & Luger, 2004; Muthurajan, Bao, Forsberg, Edayathumangalam, Dyer, White, et al., 2004). Over the past few years, we have expanded our repertoire of chromatin templates assembled in vitro to include fluorescently labeled nucleosomes (either on the histones or DNA). Moreover, with the availability of highly positioned DNA sequences like the 601 DNA (Lowary & Widom, 1998), it is now possible to place linker arms of desirable lengths on either side of the minimal Nuc147 (Figure 1A). All these nucleosomes are valuable in studying dynamics of chromatin regulation in the context of mononucleosomes, where DNA extensions play a role in structure and dynamics (Clark, Kramer, Muthurajan, & Luger, 2012; Muthurajan, Hepler, Hieb, Clark, Kramer, Yao, et al., 2014; White, Hieb, & Luger, 2015).

Increasingly, chromatin interaction with its regulatory factors is studied in a poly-nucleosome context. To this end, we have developed minimal higher order chromatin templates containing three nucleosomes (Figure 1B; trinucleosome). We distinguish two types of trinucleosomes based on the availability of extra-nucleosomal DNA extension from the two outer nucleosomes. Trinucleosomes with extensions (Linker-Ended trinucleosomes – LE-Tri) provides a model for damaged chromatin with exposed DNA blunt ends required for binding proteins like PARP-1 which are known to regulate DNA repair dynamics. Trinucleosomes lacking such extensions (Non-Linker-Ended trinucleosomes - NLE-Tri) can therefore be used as a model for native chromatin (no exposed DNA ends).

These chromatin templates are not trivial to prepare and they require a number of quality control steps to ensure their proper assembly, homogeneity, and stability. In this chapter we revisit and update the salt gradient assembly method for assembling chromatin containing fluorescent labels, building on previously published articles (Luger, Rechsteiner, & Richmond, 1999; Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004). We describe the use of restriction enzyme and micrococcal nuclease (MNase) digestion, native gel electrophoresis (PAGE and agarose), bioanalyzer sequencing, Size Exclusion Chromatography coupled with Multi-Angle Light Scattering (SEC-MALS), Analytical Ultracentrifugation (AUC), Atomic Force Microscopy (AFM), and Small Angle

X-ray Scattering (SAXS) as tools to determine the quality and polydispersity of the chromatin templates.

2. Chromatin reconstitution strategies

An essential pre-requisite to assembling chromatin is the availability of pure recombinant histone proteins that are devoid of post-translational modifications, and defined DNA templates. All methods described here utilize *Xenopus laevis* histones expressed as inclusion bodies in *E. coli* and purified using previously published methods. The use of *Xenopus laevis* histones is mainly due to historical reasons; we have demonstrated that the same methods can be used to purify histones from many other species. Of all the species tested, only yeast histones are somewhat problematic, as their yields are generally lower. The purification of histones from bacteria has been described extensively (Luger, Mader, Richmond, Sargent, & Richmond, 1997; White, Suto, & Luger, 2001). In the preparation of histones, it is of utmost importance to avoid contamination with nucleic acids, as their presence will skew quantification and result in heterogeneous chromatin preparations. In our experience, this is the most prevalent reason for unsuccessful chromatin reconstitution experiments.

We generally use DNA comprised of a single or triple 601 sequence, due to their superior positioning properties. Other DNA sequences can be used with the same protocol, but the quality and stability of the final product may vary. Accuracy of positioning may also be variable, especially on longer DNA fragments. Details of the preparation of DNA for in vitro chromatin assembly can be found in (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004).

Histone proteins are refolded into octamer as a first step towards assembling chromatin. Octamer is assembled by mixing each unfolded histone at equimolar amounts and refolding in 2M salt. The refolded complex contains a mix of octamer, H3-H4 and H2A-H2B and excess histones. Histone octamer is purified by resolving the complexes on a size exclusion column (Superdex 200), as described (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004). The histone octamer is not stable at ionic strength lower than 2 M, and thus not a physiologically relevant entity, but it is a convenient starting point for assembling chromatin in vitro because it contains the correct stoichiometry of properly refolded histone complexes. Reconstitutions can also be performed with H2A-H2B dimer and (H3-H4)₂ tetramer, as described earlier (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004).

2.1 Fluorescent labeling of the histones and refolding into octamer

Comprehensive in vitro biophysical and structural characterization of chromatin complexes is greatly aided by the usage of labeled components. In particular, we have widely used fluorescently labeled histones to assemble nucleosomes that were used for solution-state binding affinity measurements (Chodaparambil, Pate, Hepler, Tsai, Muthurajan, Luger, et al., 2014; D'Arcy, Martin, Panchenko, Chen, Bergeron, Stargell, et al., 2013; Dechassa, Wyns, Li, Hall, Wang & Luger, 2011; Dechassa, Wyns, & Luger, 2014; Fierz, Kilic, Hieb, Luger, & Muir, 2012; Klein, Muthurajan, Lalonde, Gibson, Andrews, Hepler, et al., 2015;

Muthurajan, Hepler, Hieb, Clark, Kramer, Yao, et al., 2014; Park, Dyer, Tremethick, and Luger, 2004; White, Hieb and Luger, 2015; Winkler & Luger, 2011; Winkler, Luger, & Hieb, 2012; Chodaparambil, Pate, Hepler, Tsai, Muthurajan, Luger, et al., 2014). Only one of the four histones, H3, contains a cysteine, and this can be mutated without any adverse effect on nucleosome structure (Luger, Mader, Richmond, Sargent, & Richmond, 1997; White, Suto, and Luger, 2001). The absence of functional cysteine groups on histone proteins has enabled us to engineer single cysteine point mutations that can be used for the attachment of maleimide-based fluorophores in positions that do not affect the overall structure of nucleosomes.

Our preferred fluorophores are Atto647N (acceptor) and Alexa 488 or Oregon Green 488 (donor), but other compatible Fluorescence Resonance Energy Transfer (FRET) pairs can be used (Winkler, Luger, & Hieb, 2012). Histones H4 E63C and/or H2B T112C (Figure 1C) in combination with H3 C110A are the most commonly used sites for labeling the histone octamer. Both H4 and H2B labeling mutants can be refolded together to make double labeled octamers containing both acceptor and donor fluorophores. The fluorescently labeled components can then be incorporated into nucleosomes or nucleosomal arrays via one of the several assembly protocols discussed previously (Chakravarthy & Luger, 2006; Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004).

2.1.1 Equipment

- Dialysis tubing (6- to 8-kDa cutoff, 2.3-cm flat width)
- HiLoad 16/60 Superdex 200 HR preparation-grade gel-filtration column and HPLC, equipped with UV detector and fraction collector
- Nanospec or any spectrophotometer
- Standard SDS-PAGE equipment, 15% SDS-PAGE gels
- Typhoon Scanner (GE Healthcare)
- ImageQuant TL for visualization and analysis

2.1.2 Buffers and Reagents

- Unfolding buffer for labeled histones: 20 mM Tris (hydroxymethyl)-aminomethane hydrochloride (Tris-HCl; pH 7.5), 6 M Guanidinium hydrochloride, 20 mM Sodium chloride (NaCl), 0.01 mM Tris (2-carboxyethyl)phosphine (TCEP); pH adjusted to 7 prior to addition – made fresh on the day of unfolding
- Unfolding buffer for unlabeled histones: 6 M Guanidinium HCl, 20 mM Tris-HCl (pH 7.5), 5 mM Dithiothreitol (DTT) – made fresh on the day of unfolding
- Refolding buffer: 2 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM β -mercaptoethanol (BME)

- Maleimide reactive dyes (Alexa 488, Atto647N, Alexa 532, etc.) – 10 mM stock in dimethyl sulfoxide (DMSO) or dimethyl fluoride (DMF) (Thermofisher) – freshly made or frozen stocks; thaw only once
- 10 mM Glutathione or DTT

2.1.3 Procedure

1. Add 2.0 mL of unfolding buffer to the lyophilized histone/s (2 mg/ mL final) to be labeled, wrap in aluminium foil (TCEP is light sensitive) and dissolve gently for 45 minutes at room temperature using a rotator.
2. Measure histone concentration at 276 nm and add an equimolar amount of fluorophore (up to 2-fold excess of fluorophore will work). Incubate overnight with rotation at 4°C in the dark.
3. Quench the reaction by adding reduced 10 mM (final) glutathione or DTT. Incubate 1 hour at 4°C.
4. Resuspend the other histone(s) with 2.0 mL of unfolding buffer and dissolve gently for 45 minutes at room temperature.
5. Determine the concentration of the unlabeled histone(s).
6. Mix the four histones at equimolar ratio, then bring the final protein concentration to 1 mg/mL using unfolding buffer.
7. Place the sample into dialysis bag and dialyze against cold refolding buffer at 4°C. It is essential to ensure that the dialysis bag is spinning freely, otherwise, precipitation of histones might occur. Use a volume of refolding buffer at least 100-fold greater than your sample for a thorough buffer exchange and change buffer at least three times total (4 hours each with at least one overnight).
8. Concentrate dialyzed sample to 1–2 mL or 0.5–2% of the column volume to be used for purification.
9. Spin sample, and inject into a Superdex 200 gel-filtration column (10/30 or 16/60) equilibrated in refolding buffer. Collect 1 mL fractions.
10. Run 15% SDS gel of eluted fractions, image gel on Typhoon to confirm labeling, then stain for protein. Determine fractions containing octamer, pool conservatively and concentrate.
11. Determine concentration of octamer and efficiency of labeling by determining moles dye per mole protein on the nanospec and freeze in liquid nitrogen by adding 0–20% (v/v) glycerol and store in –80°C.

2.1.4 Notes

1. Unfolding buffer used to unfold labeling mutants should not contain DTT or BME, however, once the labeling is quenched, BME can be used in the refolding buffer.

2. We typically dedicate columns to each fluorophore used to prevent cross-contamination. If this is not feasible, then the column will need stringent cleaning (per manufacturer's protocol) to remove the fluorophore.
3. We typically achieve 50–60% labeling efficiency.
4. If the octamer is going to be used immediately, it can be stored at 4°C where it is usually stable for several weeks, at least for non-variant octamer.

2.2 End-labeling of DNA

In a scenario when histone labeling is not desired (for e.g. if labeling of histones disrupts functional interactions, or to confirm the presence of DNA in a complex with increased sensitivity), the DNA can also be labeled. Fluorescently labeled primers are used to amplify double stranded labeled DNA by PCR amplification. DNA can also be labeled internally, by using mutated bases at strategic positions. We will not discuss the method for internal DNA labeling here but more information can be obtained from (Luo, North, & Poirier, 2014).

2.2.1 Equipment

- Polymerase Chain Reaction (PCR) machine
- Agarose/native acrylamide gel apparatus
- GE Healthcare Typhoon for fluorescence imaging
- ImageQuant TL for visualization and analysis

2.2.2 Buffers and Reagents

- Fluorescently-labeled primers (e.g. IDT)
- DNA polymerase
- PCR buffer
- dNTP stock solutions
- PCR purification kit (Zymo Research)

2.2.3 Procedure

1. Set up PCR using labeled primer(s) and analyze the product on a native gel. 5–10% acrylamide can be used for smaller DNA fragments, 1% agarose is suggested for DNA larger than 500 bp.
2. Purify DNA using a commercially available kit, and verify size by PAGE.
3. Verify labeling efficiency by determining moles of dye per mole DNA. Typically almost 100% of the amplified DNA should be fluorescently-labeled.

4. Scan the gel on Typhoon prior to staining with Sybr gold or ethidium bromide. Store in amber tubes or in a light protected environment to prevent photo-bleaching of the fluorophore.

2.2.4 Notes

1. The PCR reactions should be set up in amber tubes to keep the samples light-protected.

2.3 Nucleosome assembly

Nucleosome assembly via salt gradient is traditionally employed to obtain large quantities of nucleosomes for crystallization and other biochemical as well as biophysical assays. With strongly positioning DNA sequences like the 601 DNA this method results in high yields of homogenous mononucleosomes. We have previously described the method for reconstituting nucleosomes in detail (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004). Here we assume that the reader is familiar with these approaches, and focus on the generation of high quality nucleosomes containing one or two fluorescent labels.

2.3.1 Procedure—The method for preparing fluorescently labeled nucleosomes is identical to that for unlabeled nucleosomes (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004), except for the following variations:

1. The beaker containing the DNA: octamer mixtures during salt gradient reconstitution should be wrapped in aluminium foil to protect the fluorescent label from photo-bleaching.
2. Native PAGE analysis of fluorescently labeled nucleosomes should be carried out in the dark as well and the gel should be scanned on a Typhoon imager at the fluorophore-specific wavelength prior to staining with ethidium bromide or SyBr Gold.
3. To determine nucleosome concentration, use 0.1 M NaOH to make a 1:10 dilution of the nucleosomes, with the same dilution of TCS in 0.1 M NaOH as blank. Read absorbance at 260 nm and calculate concentration using epsilon derived from the molecular weight of the DNA used in the reconstitution. Accurate DNA epsilons and molecular weight can be obtained by using the DNA sequence online at <http://www.basic.northwestern.edu/biotools/oligocalc.html> (Kibbe, 2007).
4. Nucleosomes can be concentrated and stored in TCS buffer at 4 °C for several months.

2.3.2 Notes

1. There are certain histone variants that do not refold readily to a histone octamer (e.g. macroH2A; (Chakravarthy, Gundimella, Caron, Perche, Pehrson, Khockbin, et al., 2005); in such cases the histones need to be refolded as (H3–H4)₂ tetramer and H2A–H2B dimer and a two-step

reconstitution method is employed (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004).

2. For native PAGE, it is advisable to run a DNA ladder and an unlabeled nucleosome sample (if available), for visualization by ethidium bromide staining.

Figure 1D shows mononucleosomes assembled on 147 bp 5S DNA using Atto647N labeled histone octamer (H4E63C). Note that at lower ratios of octamer, there is free DNA present along with multiple nucleosome-like bands that can easily be mistaken for proper nucleosomes (lanes 1–4). At near-optimal ratios of octamer there is one discrete band of Nuc147 (Lanes 5–8). As more octamer is titrated, the complex aggregates and no nucleosomes appear in the gel (lanes 9 and 10).

Figure 1E shows mononucleosomes assembled on 207, 178, 165, and 147 bp DNA using Atto647N labeled octamer (at H4 E63C). There is a single discrete band in each case indicating homogenous assembly. The same gel was stained with Ethidium Bromide (Figure 1F) and shows <1% free DNA in each case. These nucleosomes were successfully utilized in HIFI-FRET assays (e.g. (Muthurajan, Hepler, Hieb, Clark, Kramer, Yao, et al., 2014)).

Figure 1G shows nucleosomes assembled on 165 bp DNA using double-labeled octamer (Atto647N on H2B T112C; Alexa488 on H4 E63C). These nucleosomes were used in an Electrophoretic Mobility Shift Assay (EMSA) to test binding of PARP-1. They are particularly useful to ascertain the presence of histones H4 (and H3), and H2B (with H2A). As seen in the figure, the nucleosomes form a single band of slower mobility upon PARP-1 binding (lanes 2, 4, 6 and 8-Cmplx). Note that fluorescence persists in both fluorophore channels (Atto647N and Alexa488), indicating that nucleosome integrity is preserved upon PARP-1 binding. Furthermore, the presence of both labels allows us to conclude that H2B and H4 are present in the nucleosomes as well as in the supershifted complex.

2.4. Trinucleosome assembly

In the past, the typical higher order chromatin templates used in vitro was composed of an array of 12 nucleosomes, assembled on a ~2400 bp DNA with 12 repeats of 601 DNA (Muthurajan, McBryant, Lu, Hansen, & Luger, 2011; Rogge, Kalashnikova, Muthurajan, Porter-Goff, Luger & Hansen, 2013; Ghosh, Nikitina, Horowitz-Scherer, Gierasch, Uversky, Hite, et al., 2010). These substrates are useful for a variety of biophysical assays, but not ideal for binding studies due to the fact that they can engage in multiple binding events.

Trinucleosomes are a minimalistic model for higher order chromatin that are more amenable to biochemical and biophysical studies (Figure 1H). The central nucleosome represents a nucleosome in a chromatin context, as it is flanked on either side by a nucleosome. Trinucleosomes are assembled using the same protocol as for mononucleosomes, however additional steps are needed to assess their quality and homogeneity. To exclude the possibility of under-saturation of the array, a DNA restriction digestion is carried out targeting the EcoRI sites located in the inter-nucleosomal DNA (Figure 1B). Moreover, to control for over-saturation, ideally analytical ultracentrifugation (AUC) needs to be performed.

2.4.1 Equipment

- Reconstitution set up described above
- Standard agarose gel running setup

2.4.2 Buffers and reagents

- As for mononucleosomes
- 1× Tris Acetic acid EDTA (TAE) buffer: 40 mM Tris, 20 mM Sodium acetate, 1 mM EDTA pH 8
- 50% sucrose (w/v)
- Heat block or water bath set at 37°C
- 10× NEB EcoRI buffer
- EcoRI (NEB)
- Low-EEO/Multi-Purpose/Molecular Biology Grade Agarose (Fisher BP160-100)
- Histone octamer and DNA

2.4.3 Procedure—The method for reconstituting trinucleosomes is exactly the same as that for mononucleosomes, with the following differences:

1. At every ratio of octamer titration, the number should be multiplied by 3 to account for 3 octamers on each DNA repeat. The final volume of the reconstitution can be increased to 50 µL.
2. The final concentration of DNA in the reconstitution can be in the of range 1–5 µM.
3. After the completion of TCS dialysis, set up EcoRI digestion for each DNA: histone octamer ratio using 1 µg of trinucleosome in a final volume of 10 µL of 1× EcoRI buffer. Incubate at 37°C for one hour.
4. While waiting for the restriction digestion, prepare a 1% agarose gel in 1× TAE buffer.
5. After one hour of EcoRI digestion, add 50% sucrose to EcoRI digestion to obtain a final concentration of 5% (v/v) to each tube and load on the agarose gel, run at 50 V for 2 hours. Load a ladder and a 207 bp DNA marker.
6. Stain the gel in ethidium bromide for ~15 minutes and determine if any of the DNA: octamer ratios have 207 bp free DNA in the digested lanes. Samples with free 207 bp DNA after digestion are under-saturated, that is, they contain less than three nucleosomes.
7. The DNA: octamer ratios that don't have any free DNA, as determined in the EcoRI digestion step, should now be checked by Analytical

Ultracentrifugation (AUC) before moving onto large scale reconstitutions (AUC procedure described below).

2.4.4 Notes

1. The quality of the agarose used for analyzing trinucleosomes and higher molecular weight arrays is extremely important because the arrays tend to smear in poor quality agarose.
2. It is important to run the larger complexes at 50 V, because at higher voltage the complexes might be unstable due to the heat generated.
3. Consider adding DNA-only uncut and cut samples as controls. A DNA ladder and 207 bp DNA should be included. A Nuc207 sample can also be loaded on the gel as a reference.
4. Trinucleosomes can also be assembled with fluorophore labeled octamer as reported before (Muthurajan, Hepler, Hieb, Clark, Kramer, Yao, et al., 2014; Winkler & Luger, 2011).
5. It is possible to over-assemble (have more histones than the three positioning repeats will allow), therefore it is critical to perform both EcoRI digestion as well as AUC for a good quality control. EcoRI digestion will look identical for correctly assembled and over-assembled trinucleosomes, and the difference will be visible only in their S_{avg} values via AUC.

A representative EcoRI digestion of a trinucleosome sample is shown in Figure 1H. Since the EcoRI digestion sites are present in the inter-nucleosomal linker DNA, the optimal assembly ratio will contain only mononucleosome bands upon digestion (lane 7). Note that at lower octamer ratios there is 207 bp DNA, indicative of under-saturated samples (lanes 3 and 5). Additionally, in the under-saturated trinucleosome samples both the undigested as well as the digested samples are heterogeneous with multiple bands (lanes 2–5) due to the presence of a mix of mono-, di- and trinucleosomes assembled on the DNA.

2.5. “Micro-scale” or step dilution method for nucleosome reconstitution

We have previously described a quicker method for the small-scale reconstitution of nucleosomes using radioactively labeled DNA by simply assembling the octamer and DNA in 2 M salt and then diluting it with a no salt buffer. This technique can be used for unlabeled DNA or labeled DNA, or labeled histones.

Figure 2 demonstrates mononucleosome assembly via microscale/step dilution method, note that at lower octamer ratios, multiple bands are visible when free DNA is present potentially due to the presence of non-native distribution of histones on DNA (lanes 1 and 2). At higher ratios of octamer, there is a single discrete band representing Nuc207 (lanes 3 and 4) that runs similar to the salt gradient reconstituted nucleosomes (lane 5) on a native gel. Details for this method are found in (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004). These nucleosomes are usually not suitable for experiments that require high quality chromatin.

3. Quality control strategies for in vitro reconstituted chromatin

A variety of quality control techniques are used in our lab to assess in vitro assembled chromatin. Quality control measures are geared to ascertain the contents in the reconstituted nucleosomes either visually (gel electrophoresis, AFM) or by analyzing the properties of assembled chromatin in solution (AUC, SEC-MALS, and SAXS). Traditionally, gel electrophoresis is one of the time tested methods and is very easy to perform in any lab. Native PAGE is sensitive to shape and charge, and works particularly well with mono-nucleosomes. Indeed, it has been utilized in the past to distinguish different positions of the histone octamer on 146 base pairs of DNA (Dyer, Edayathumangalam, White, Bao, Chakravarthy, Muthurajan, et al., 2004). It is particularly powerful in conjunction with fluorescently labeled histones, and can be combined with MNase digestion to quantify the amount of completely assembled nucleosomes that protect the requisite 147 bp of DNA. In recent years, more advanced techniques like SEC-MALS, AUC, SAXS, and AFM have been employed. While these require access to more advanced equipment, they are uniquely positioned to answer specific questions, as detailed below. As an easy guide to the sample requirements and the type of information obtainable from each of the quality control methods and also to find training resources refer to Table 1.

3.1 Micrococcal Nuclease digestion

Micrococcal Nuclease (MNase) is an enzyme that digests DNA in regions that are not stably bound by proteins (Cuatrecasas, Edelhoch, & Anfinsen, 1967). Once the nucleosome is assembled, the DNA wrapped around the histones is protected from MNase digestion, while the linker arms are digested. This will yield a distinct protected band of 147 bp in length. This method can be used to assess the integrity and stability of nucleosomes in solution, and is also used to assess the end products of salt dialysis and chaperone-mediated assembly reactions (as shown below using Nucleosome Assembly Protein 1 – Nap1).

For MNase digestion of any sample, a pilot scale experiment needs to be performed to optimize the amount of enzyme and time of digestion. Optimally, the combination is such that the entire range of digestion products up to the end-point can be observed. Additionally, quantification of MNase digestion products can be performed if a reference DNA is added to all the samples. This can serve as a reference relative to which the recovery of the products can be normalized, and also serves as an internal control for any extraction steps.

3.1.1 Equipment

- Standard Native PAGE equipment, 5 or 10% acrylamide native gels

3.1.2 Buffers and Reagents

- MNase (NEB)
- Proteinase K (NEB)
- Mini-elute PCR cleanup kit (Qiagen)
- Nucleosome assembly buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM DTT

- 10× MNase reaction buffer: 500 mM Tris-HCl pH 7.9, 50 mM CaCl₂
- 6× DNA loading buffer (LB): 0.25% Bromophenol blue, 0.25% Xylene cynol, 15% (w/v) ficoll in water

3.1.3 Procedure—Nucleosomes assembled via any method and trinucleosomes can be tested by MNase digestion.

1. Mix 20 µL sample (200 nM DNA or nucleosomes) with 10 µL 10× MNase buffer, 1 µL 100× BSA, 30 U MNase (or appropriate amount as determined in pilot experiments; test a wide range of enzyme 0–100 U MNase) and bring the volume to 100 µL with nuclease-free water.
2. Incubate at room temperature for 10 minutes.
3. Quench the reaction with 25 µL of 0.5 M EDTA and then add 25 µg of Proteinase K.
4. Incubate for 30 minutes at 50°C.
5. Add a reference DNA to all samples (1 nM final concentration).
6. Purify the DNA using a MiElute Qiagen commercial kit that allows elution of DNA in a small volume (10 µl) of buffer.
7. Load 10 µL onto 5% or 10% Native PAGE and run in 0.2× TBE at 150 V for 60 minutes.

3.1.4 Notes

1. Always include samples with no MNase, or a “zero” time point along with DNA only controls.
2. Standard phenol: chloroform precipitation and ethanol extraction of the DNA can also be used to purify the final DNA products, but this step also leads to higher DNA losses and variability, and precludes size analysis and quantification by bioanalyzer (see below).
3. Note that different lengths of DNA might be protected for nucleosomes containing histone variants (Bao, Konesky, Park, Rosu, Dyer, Rangasamy, et al., 2004; Dechassa, Wyns, Li, Hall, Wang, & Luger, 2011)

Figure 3A depicts a gel representing MNase digestion of mononucleosomes assembled via salt gradient reconstitution on 207 bp DNA. Note that unassembled 207 bp DNA is completely digested (lanes 2 and 3), while the DNA-histone mix without reconstitution results in a smear of bands indicative of formation of non-nucleosomal species (lanes 4 and 5). For salt gradient assembled Nuc207, upon MNase treatment a discrete 147 bp protected band is observed (lanes 6 and 7).

Figure 3B is a representative MNase digestion of nucleosomes assembled on 147 bp and 207 bp DNA, mediated by Nap1 (chaperone-mediated assembly). A frequent concern regarding chaperone-assembled nucleosomes is whether the assembled nucleosomes are identical to

salt gradient reconstituted nucleosomes. MNase digestion shows that Nap1 assembled nucleosomes protect 147 bp of DNA for both Nuc147 (lanes 7 and 8) as well as Nuc207 (lanes 10 and 11). Note that DNA and histones mixed together in the absence of Nap1 (lanes 4 and 5) show a smear of protected bands due to absence of positioned nucleosomes.

Figure 3C represents a typical MNase digestion pattern of trinucleosomes. The samples show three discreet digestion products, corresponding to the tri-, di-, and mononucleosome species (561 bp, ~400 bp, and ~147 bp, respectively, obtained by random cuts in the linker DNA). As the digestion proceeds for longer time, the mononucleosome protection band (147 bp) becomes more prominent while the higher species (tri- and dinucleosomes) slowly disappear.

3.1.5 Analysis of MNase digested products via Bioanalyzer 2100—Gel

electrophoresis is a low resolution method of analyzing MNase digested products, since it may not resolve small base pair differences effectively. Additionally, quantification of relative amounts of different bands is difficult. The Agilent 2100 Bioanalyzer provides an alternative method that is fast, efficient, highly-sensitive, quantitative, accurate, and reproducible with minimal sample volume requirements (Falk, Guo, Sekulic, Smoak, Mani, Logsdon, et al., 2015). Bioanalyzers have several applications including protein analysis, here we have used it as a tool to analyze products resulting from MNase digestion.

3.1.6 Equipment

- Agilent Bioanalyzer 2100
- DNA 1000 Chip

3.1.7 Buffers and reagents

- Purified DNA sample (MiniElute PCR cleanup kit - Qiagen)
- DNA 100 Reagents (5067-1505 Agilent)

3.1.8 Procedure

1. Subsequent to MNase digestion, add 1–5 nM of a reference DNA (ideally somewhat longer than the products to be analysed) that will function as a loading control in each lane as described above.
2. Purify the samples using a MiniElute PCR kit, and elute DNA in 10 µL.
3. 1 µL of each sample is loaded on DNA 1000 chip and analyzed on Bioanalyzer 2100. The suggested quantitative range for this chip is 0.1–50 ng/µL.
4. Data can be visualized and analyzed on the free software 2100 Expert from Agilent.
5. Quantification of the data for recovered DNA can be done using Microsoft Excel. Here we used the calculated molarity value output from the '2100 Expert' software. The reference DNA is used to normalize the amount of

DNA fragment in each lane (normalized value = molarity of the DNA fragments of interest/molarity of the reference DNA). The normalized values are used for comparison between different lanes and to determine percent fragment recovered.

3.1.9 Notes

1. Use filter tips and gloves to lower the level of contaminants and DNA degradation.
2. Always use DNA only controls to compare with nucleosome samples, and internal standards to confirm recovery.
3. DNA 1000 chips have a separation range between 25–1000 bp.

Figure 3D shows a 10% native acrylamide gel of extracted DNA after MNase digestion of 207 bp DNA (lanes 2 and 3) or Nuc207 (lanes 4 and 5). 207 bp DNA is almost completely digested while Nuc207 has several protected bands.

Figures 3E and F show the Bioanalyzer results for 207 bp DNA (Figure 3E) and Nuc207 (Figure 3F). The undigested (gray trace) and digested (black trace) samples are overlaid in each panel. While 207 bp DNA has no defined protection pattern, Nuc207 clearly shows a distinct protection around 150 bp, (gray trace) and inset. This method allows for a more accurate analysis of the degradation pattern at lower levels of MNase, compared to gel electrophoresis (Figure 3D).

In addition, the output data from the Bioanalyzer allows for quantification of the DNA fragments. As shown in figure 3G, the molarity of individual fragments calculated by the Software can be used to measure the relative amount of DNA protected during MNase digestion of Nuc207. It is apparent that while 207 bp DNA is completely digested, a variety of digestion products were recovered for Nuc207 including some that were only a few base pairs apart. This allows us to estimate the amount of nucleosomes that are formed in each sample. Importantly, this method can be used to compare the efficiency of different assembly conditions including various chaperones or reconstitution methods in that it allows us to monitor only properly formed nucleosomes. Optimization of MNase digestion conditions is an essential pre-requisite. In fact, over digestion could result in an under-estimation of the amount of nucleosome in the samples.

3.2 Size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS)

Multi-angle light scattering (MALS) uses laser light scattering to provide information about shape, size and polydispersity of protein samples in solution and is widely used for determining homogeneity, stoichiometry, and molecular weight of the complexes. When connected in line with a size exclusion chromatography (SEC) column, MALS can provide information about the different species as they separate on the column. This provides a wealth of information about homogeneity, stability, and stoichiometry of the injected sample under the conditions tested. However, differences less than two-fold in molecular weight or shape cannot be detected. Nevertheless, this technique is powerful for analyzing complexes of nucleosome and chromatin regulatory proteins (Yang, van der Woerd, Muthurajan,

Hansen, & Luger, 2011; Clark, Kramer, Muthurajan, & Luger, 2012). A size exclusion column (run by an HPLC system) is connected to a MALS and Refractive Index (RI) detector. The SEC should be selected depending on the size of the complexes that need to be resolved.

3.2.1 Equipment

- Wyatt Instruments HELEOS and rEX detectors
- GE Healthcare HPLC and SEC column

3.2.2 Buffer and reagents

- 25 mM Tris pH 7.5, 200 mM NaCl, 2 mM arginine

3.2.3 Procedure

1. Prepare 120 μ L of nucleosome solution at about 0.2–0.5 mg/ml (use higher concentrations for smaller proteins).
2. Equilibrate the column with buffer pre-filtered via 0.1 μ m membrane.
3. Spin the sample at 14,000 rpm for 10 minutes and inject 100 μ L into the SEC column.
4. Unicorn software is used to run the SEC, while the ASTRA software from Wyatt records the MALS and RI data. Refer to the manufacturer manual for details.

3.2.4 Notes

1. Unstable reducing agents such as DTT or BME, or other unstable buffer components like detergents should be avoided. TCEP should be used as the reducing agent.
2. Thoroughly dialyze or buffer exchange samples in the running buffer to avoid high background due to buffer differences.
3. It is recommended that a BSA standard (2 mg/mL) be run as a control prior to testing samples.
4. Resolution power and lack of contaminations on the SEC column are essential to minimize light scattering background signal. Refer to the manufacturer's instruction for cleaning procedures.

Nuc165 injected onto Superdex 200 elutes in a single symmetric peak and the calculated molecular weight matches the theoretical value (Figure 4). This technique was applied to analyze nucleosome-McCP2 and nucleosome-PARP-1 complexes (Yang, van der Woerd, Muthurajan, Hansen and Luger, 2011; Clark, Kramer, Muthurajan & Luger, 2012).

3.3 Analytical Ultracentrifugation (AUC)

Analytical ultracentrifugation is a powerful technique that allows for particle analysis in solution, especially in conjunction with newly developed data analysis software. The rate of

sedimentation of a molecule depends on both its shape and size, therefore information pertaining to both can be obtained using this method. AUC can be used to analyze chromatin of any size (mono-, tri- or 12mer arrays) and their complexes, but is particularly useful for larger samples where native PAGE either is not applicable, or has insufficient resolution. Data should be collected in intensity mode rather than absorbance to reduce noise levels (Kar, Kingsbury, Lewis, Laue, & Schuck, 2000), where intensity refers to the transmitted light incident on the sample. The intensity data can easily be converted to pseudo-absorbance data using the software Ultrascan III. The partial specific volume is a key parameter required by the software to determine molecular weight, and is affected by the buffer components. AUC can be combined with fluorescence detector to obtain enhanced detection sensitivity, but this special application is not discussed here.

A list of institutes that have access to this instrument worldwide is available at <http://www.uslims3.uthscsa.edu/index.php>. A good starting point for a novice would be to locate the nearest facility that has the equipment and expertise required to operate AUCs. Additionally, several institutes also provide paid services and can run the experiment and data analysis for the users. However, some rudimentary guidelines are provided here for the experimenters to follow. These, in combination with a previous publication (Rogge, Kalashnikova, Muthurajan, Porter-Goff, Luger, & Hansen, 2013), would be a good starting point for researchers who wish to try AUC.

3.3.1 Equipment

- AUC cells with quartz windows
- Beckman XLA or XLI analytical ultracentrifuge
- Computer with Ultrascan III installed for data analysis and access to Laboratory Information management system (LIMS) for 2 dimensional spectrum analysis (2DSA) and genetic algorithm coupled with Monte-Carlo analysis (GA-MC) calculations

3.3.2 Buffers and reagents

- AUC buffer: 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, or any other buffer (see notes).

3.3.3 Procedure

1. Clean AUC cell components and assemble the cell (Figure 5A and B).
2. To perform an AUC run, sample absorbance at 260 or 280 nm of ~0.3–0.4 is required for high-quality data. Prepare 400 μ L of tri-nucleosomes (or other complexes) in the final dialysis buffer. Collect data in intensity mode, use water in reference channels (Figure 5 C–F).
3. The size of the particles to be analysed will influence the rotor speed during data collection, generally, the smaller the size of the particle, the higher the speed needed to sediment it (e.g. for mononucleosome samples

use 40,000 rpm, for a trinucleosome sample use 30,000 rpm) (Figure 5 G–J).

4. Data is collected as number of scans representing the sedimentation of the sample from the top to the bottom of the cell. The number of scans should be decided such that the sample fully sediments. This value depends on the number of samples in the rotor, the molecular weight of the complexes, the speed, etc. Generally, 150 scans could be collected per sample.
5. AUC data analysis can be performed using the Ultrascan 3 software, following the user manual details: <http://www.ultrascan2.uthscsa.edu/manual2/index.html> (Demeler, 2006; Cao, Weiming, & Demeler, 2005; Demeler, n.d.; Demeler, 2005; Demeler & Van Holde, 2004).

3.3.3 Procedure

1. Any buffer is compatible with AUC, but DTT above 1 mM should be avoided. BME and detergents should also be avoided due to background scatter.

Figure 6A shows an example of a nucleosome reconstituted on 165 bp of DNA (Nuc165), analyzed by sedimentation velocity. This particle sediments with an S_{avg} of ~11.2S and with a frictional ratio (f/f_0) of about 1.4 (Figure 6A). Fully saturated trinucleosomes sediment with an S_{avg} of ~17S (Figure 6B) and a frictional ratio of ~3 as would be expected for an extended array. As a case study, the trinucleosomes in complex with the chromatin architectural protein methyl-CpG-binding protein 2 (MeCP2) was analysed via AUC. The MeCP2-bound trinucleosomes have a mid-point S value of ~20S in accordance with an increase in the molecular weight of the complex (Figure 4C). In addition, for the MeCP2 bound trinucleosomes, the f/f_0 value dropped to ~1.9 indicating that the extended trinucleosomes form a more condensed structure upon MeCP2 binding, consistent with electron microscopy data of longer arrays (Ghosh, Nikitina, Horowitz-Scherer, Gierasch, Uversky, Hite, et al., 2010).

Monodispersity of the sample can be assessed by inspecting the trend of the sedimentation curve. A drift of the curve to higher s values is indicative of some degree of polydispersity. For example, the nucleosome sample shown in Figure 6A runs as a largely monodisperse sample, while the MeCP2 complex shown in figure 6B suggests some heterogeneity within the sample. This is also revealed in the analysis of the solutes performed by the software Ultrascan III.

3.4 Atomic Force Microscopy (AFM) analysis of trinucleosomes

AFM allows measurement of height profiles, volume, and physical properties of macromolecules based on their capacity to deflect laser light off of a cantilever as it scans the sample surface (Figure 7 A–E). A large number of images can be generated and should be analysed for statistical purposes. Sample requirements are minimal for this method. We have described AFM data collection in air (dry) which is a rapid and convenient method for visualizing complexes. AFM is quite versatile and can be used to collect images in liquid either statically or in a flow cell. The greatest advantage of this technique is the direct

visualization of chromatin and its complexes, it is particularly useful for chromatin arrays and larger complexes (Lohr, Bash, Wang, Yodh, & Lindsay, 2007; Lyubchenko & Shlyakhtenko, 2015; Nazarov, Chekliarova, Rychkov, Ilatovskiy, Crane-Robinson, & Tomilin, 2015).

3.4.1 Equipment

- Asylum Research MFP-3D Atomic Force Microscope or comparable instrument
- Olympus 240TS tips, or comparable brand
- Freshly cleaved mica discs (SPI Supplies) glued to glass slides (VWR) (Figure 7 F–G)
- 3-aminopropyltriethoxysilane (APTES) (Sigma)
- Syringe filters and Syringes or Steriflips

3.4.2 Buffers and reagents

- Sample buffer – 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, filtered through a 0.2 μ m filter

3.4.3 Procedure

1. Cleave mica using a piece of scotch tape by pressing it down on the mica disc and peeling it off repeatedly until a smooth mica surface is obtained (Fig. 7I). Modify with APTES: 1:1000 dilution in water, add 30 μ L on to the mica disc, incubate covered at room temperature for 30 minutes, gently rinse with filtered water and dry in a gentle stream of pure nitrogen) (Figure 7 H–J).
2. Dilute trinucleosome or nucleosomal array samples to ~ 1.5 ng/ μ L, however the dilution range might vary depending on the sample (Figure 7J).
3. Add ~ 30 μ L of the diluted sample on the APTES-mica surface and wait for 15 minutes.
4. Rinse off surface with buffer, and dry in a gentle stream of air or pure nitrogen.
5. Wipe off buffer droplets from around the sample area, place slide on stage, secure with magnets.
6. Start scanning 5×5 μ m areas. Identify areas with well-spread chromatin specimens and collect 2×2 μ m scans. Follow these up with 1×1 μ m scans and go to smaller areas if the instrument capability allows. At higher resolution scans (smaller area) are more sensitive and therefore likely to contain noise (Figure 7 K–L).

3.4.4 AFM data processing—Images are flattened and analyzed using the MFP-3D software provided by Asylum, or by a comparable software. In this example, each image was divided into four quadrants and zoomed in digitally to obtain a clearer view of well separated trinucleosomes. Multiple free hand lines were drawn through the trinucleosomes, and height profiles were obtained. For statistical analysis and large number of images (at least a few dozens) should be analysed. Results can be analysed by Microsoft Excel for easy visualization and relative comparison.

3.4.5 Notes

1. Mononucleosomes are too small to be analyzed by AFM and thus this method is not recommended for these samples.

In the 200×200 nm images obtained with trinucleosomes, the three nucleosomes held together by linker arms were clearly visible and the height profile was ~ 1.5 – 2 nm (Figure 8A and B; right panel in B for height profile). Complexes of trinucleosomes bound to chromatin interacting proteins, such as MeCP2 can be visualized (Figure 8C and D). MeCP2 compacts trinucleosomes as previously shown for nucleosomal arrays (Hansen, Ghosh, & Woodcock, 2010; Ghosh, Nikitina, Horowitz-Scherer, Gierasch, Uversky, Hite, et al., 2010). Some clustering of nucleosomes (more than one trinucleosome) can be clearly seen (Figure 8D). The contrast in the amplitude and phase traces (Figure 8C and D, middle and right panels) allows us to better visualize the change in structure. We have successfully employed AFM to analyse magnesium-dependent and PARP-1 induced chromatin compaction of macro-H2A containing 12-mer arrays and trinucleosomes respectively (Muthurajan, McBryant, Lu, Hansen, & Luger, 2011; Muthurajan, Hepler, Hieb, Clark, Kramer, Yao, et al., 2014).

3.5 Small Angle X-ray Scattering (SAXS)

Small angle X-ray scattering of a purified sample in solution can be analysed to infer size and shape information. SAXS data is collected at synchrotron sources equipped with a SAXS beamline (e.g. ALS – SYBILS BL 12.3.1, Berkeley; APS Chicago – BioCAT, Sector 18ID; SSRL BL4-2 Stanford University, Stanford). For larger complexes such as mono- and trinucleosomes, the beamline additionally should be equipped with a mobile detector for collecting lower angle scattering. Data collection can be done either statically or continuously as the sample is being eluted off of a SEC (SEC-SAXS) (Malaby, Kathuria, Nobrega, Bilsel, Muthurajan, Luger, et al., 2013).

In the static mode, data is collected at multiple concentrations in replicates. Concentration and buffer-dependent sample aggregation is more apparent in data collected statically. In line SEC-SAXS is free of these pitfalls. A high flux beam and advent of noiseless high speed detectors allow short exposure times (thereby reducing radiation damage of samples) and virtually real time data reduction and analysis. SAXS data can provide information about the structure, shape and size of chromatin and its complexes with interacting proteins. A new development, time-resolved SAXS can potentially be utilized to monitor real-time conformational changes induced by chromatin binding proteins (Graceffa, Nobrega, Barrea,

Kathuria, Chakravarthy, Bilsel, et al., 2013; Kathuria, Kayatekin, Barrea, Kondrashkina, Graceffa, Guo, et al., 2014).

3.5.1 Equipment

- Synchrotron beamline with SAXS data collection capabilities preferably with in-line SEC. All data in the present work was collected at Sector 18ID (BioCAT) located at Advanced Photon Source located in Argonne National Laboratory, Chicago.
- Size exclusion column (Superose 6 or Superdex 200)
- GE Healthcare FPLC system

3.5.2 Buffers and reagents

- Sample buffer: 25 mM Tris-HCl pH 7.5, 2 mM Arginine, 50 mM NaCl and 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT– filtered via 0.2 μ m membrane.

3.5.3 Procedure

1. Write a proposal requesting beam time and wait for approval. Proposal submission procedures and deadlines can be found on beamline web sites.
2. Concentrate samples to ~2 mg/mL in ~150 μ L. Save an aliquot for examining the complexes by other methods (such as gel electrophoresis and AUC).
3. Prepare samples and ship them to the beamline scientist a day or two in advance. For protein stocks that are stored in -80°C , ship on dry ice. For chromatin samples, prepare a makeshift ice pack by filling Ziploc bags with ice and place sample tubes sealed with parafilm inside a 50 mL falcon tubes.
4. Arrive at the beamline the night before to do the wet lab set up like preparation of buffers and any complexes (e.g. trinucleosome-MeCP2).
5. Clean the quartz sample chamber after each run using a Hamilton Syringe Pump sequentially with water, bleach, water, isopropanol, and water to remove cross contamination between runs.
6. Data-reduction (radial averaging of the scattering intensities to generate I (q) vs q plots), usually involves software indigenous to the beam line and should be done at the beamline. Subtract buffer from sample data.
7. All further data processing can be done with the ATSAS suite. R_g and D_{max} can be determined by Primus in ATSAS (Petoukhov, Franke, Shkumatov, Tria, Kikhney, Gajda, et al., 2012).
8. Ab initio modeling is done using Dammin (Svergun, D.i., 1999).

9. Images of the envelopes can be generated in VMD by first generating a shell in pdb2vol (Humphrey, Dalke, & Schulten, 1996; Wriggers & Chacón, 2001; Wriggers, Milligan, & Mccammon, n.d.).

3.4.4 Notes

1. Inline SEC-SAXS is better for SAXS data collection on chromatin due to fractionation and removal of aggregates prior to SAXS data collection. Even though the samples undergo dilution, it is still possible to calculate the exact concentration if the scattering plots and the UV chromatogram are synchronized.
2. It is important to keep track of sample amounts loaded onto SEC, and to compare it with the relative UV signal of the eluate to determine if any aggregates were retained in the column.
3. Collect data for individual components as well as complexes.

Mononucleosomes (Nuc165 - ~2.5 mg/mL) prepared by salt gradient reconstitution were subjected to SAXS data collection combined with in-line SEC. An R_g of ~46.9 Å and a D_{max} of ~145 Å was determined (Figure 9A left and middle panels). Ab initio modeling produced a low resolution model that matched our previously published data (Figure 9A right panel; (Yang, van der Woerd, Muthurajan, Hansen, & Luger, 2011).

SAXS analysis of trinucleosomes confirmed previous AUC and AFM data suggesting that in solution these are extended with an R_g of ~154 Å and a D_{max} of ~500 Å (Figure 9B, left and middle panels). Remarkably, the three nucleosome particles were visible as spheres attached via a string of linker DNA (Figure 9B, right panel).

Summary and Conclusion

For the preparation of highly defined chromatin, it is imperative to start with ingredients of highest purity. Both the DNA and the histone octamer need to be purified using stringent measures to prevent any contaminants to be carried over into the assembly reaction (such as plasmid DNA for DNA preparations and dimer/tetramer/aggregates/single histones, or nucleic acid contaminants for octamer). Determining the precise concentrations of the components is also essential. Contamination of histones with nucleic acids, or inaccurate stoichiometries of components, is the most frequent cause for failed chromatin reconstitutions. This is common for any method of nucleosome assembly used to make chromatin substrates.

As a general rule of thumb, nucleosomes stored in low salt buffer such as TCS are stable at 4 °C. However, such low salt concentrations may not be ideal when studying chromatin-binding proteins, which are often highly charged. In these experiments we suggest using buffers containing salt concentration in the range of 25–250 mM, where the nucleosome are also stable.

Assembly of nucleosomes by salt gradient dialysis, when done properly, is an efficient way for generating homogenous nucleosome species. In fact, combined with purification over a

preparatory gel electrophoresis setup these nucleosomes can be used directly for crystallization (Tan & Davey, 2011). In the cell, nucleosomes are assembled and disassembled by histone chaperones and ATP-remodelers (Hughes & Rando, 2014). It is plausible that these proteins may yield nucleosomes with subtly altered thermodynamic or structural properties.

Nucleosomes assembled by histone chaperones in vitro contain free DNA, nucleosomes, non-nucleosomal species and histone-DNA aggregates. The presence of these contaminants is a caveat when using these samples in functional assays with chromatin regulatory proteins, and likely have been the source of numerous discrepancies in the published literature. For example, many chromatin binding proteins bind free DNA in addition to nucleosomes. The free DNA present in such preparations will compete for binding of the chromatin interacting protein and this in turn will skew the binding data. Partially assembled nucleosomes may exhibit different binding properties compared to fully folded nucleosomes. These caveat exist for virtually every assembly method to different degrees. It is important to be aware of such issues to properly control for and then interpret the experimental data.

Rigorous quality control measures of chromatin substrates are essential to ensure reproducibility and validity of conclusions. Gel electrophoresis, while simple and therefore widely used in the past, only provides limited information and can instill a false sense of confidence in sample homogeneity. Current methods such as SEC-MALS and AUC allows one to determine the molecular weight and shape of the solutes. AFM allows visualization of the assembled chromatin, and a statistical analysis can be performed based on the height profile data if desired. SAXS performed in line with size exclusion chromatography allows us to collect data exclusively on a homogenous population of nucleosomes as they elute off of the SEC column and obtain shape and structural information. AUC and SAXS provide the added advantage of studying molecules in solution unhindered by any immobilization. Using a multitude of quality control methods allows us to obtain consistent high quality chromatin substrates successfully.

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Figure 1. Chromatin assembly via salt gradient reconstitution

A. Schematic of mononucleosomes with different extranucleosomal linker lengths.

B. Schematic of the trinucleosome templates. An EcoRI recognition sequence is engineered in the inner linker arms of the DNA (indicated by red arrows).

C. Location of histone residues H4 E63 and H2B T112 used for attaching fluorescent labels (pdb 1AOI).

D. Titration of Atto647N labeled octamer (H4E63C) onto 5S 147 bp DNA. Note free DNA and additional bands at lower octamer ratios (lanes 1–4). As more histones are titrated in, the

free DNA band disappears and a more homogenous nucleosome band is observed (lanes 5–9). Upon further addition of histones, yields decrease due to sample aggregation (lane 10). E. Fluorescently labeled nucleosomes: DNA fragments 207, 178, 165, and 147 base pairs in length, all containing the 601 positioning sequence, were assembled into nucleosomes with histone octamers labeled at H4E63C with Atto647N. Nucleosomes were analyzed by 5% native PAGE and scanned on a Typhoon imager at an emission wavelength of 670 nm. Lanes 2, 4, 6 and 8 are labeled nucleosomes assembled on 207, 178, 165 and 147 bp DNA, respectively.

F. The same gel as in D was stained with ethidium bromide. Lanes 2 and 3 are labeled and unlabeled Nuc207 respectively; Lanes 4 and 5 are labeled and unlabeled Nuc178; Lanes 6 and 7 are labeled and unlabeled Nuc165; Lanes 8 and 9 are labeled and unlabeled Nuc147. Lanes 11–14 are 207, 178, 165, and 147 bp DNA fragments, respectively. Note the absence of free DNA (<1%) in the nucleosome samples. Lanes marked M contain 50–2000 bp ladder. Note that figures E and F are reused from a previous publication (Clark et al., 2012).

G. EMSA using double labeled nucleosomes (Atto647N on H2B and Alexa488 on H4) on 165 bp DNA, with and without PARP-1. Note that the donor and acceptor signal colocalizes upon PARP-1 binding (lane 2 – cmplx) indicating that the nucleosomes remain intact.

H. EcoRI digestion of assembled trinucleosomes. Lane 1, 50-2k ladder; Lane 2, uncut NLE-Tri; Lane 3, EcoRI digested NLE-Tri; Lane 4, uncut NLE-Tri; Lane 5, EcoRI digested NLE-Tri; Lane 6, uncut NLE-Tris; Lane 7, EcoRI digested NLE-Tris. Note that EcoRI digestion of trinucleosomes results in Nuc207 and/or 207 bp DNA. Note that there is no free 207bp DNA in the EcoRI digested lane for fully saturated trinucleosomes (lane 7 whereas undersaturated trinucleosomes lanes 3 and 5 contain free 207 bp DNA) indicating that all three nucleosome positioning sites are occupied.

Figure 2. “Micro-scale”/step dilution method for nucleosome reconstitution

Lane 1–4: 207 bp DNA titrated with 1.2, 1.6, 2.0, 2.2 – fold excess octamer respectively;

Lane 5, Nuc207 control reconstituted via salt gradient; Lane 6, DNA ladder.

Figure 3. MNase digestion of mono- and trinucleosomes

A. Micrococcal nuclease digestion of Nuc207 assembled via salt gradient dialysis. Lane 1, 50- 2k ladder; Lane 2, undigested 207 bp DNA; Lane 3, MNase digested 207 bp DNA; Lane 4, DNA-histone mix (1:1), untreated; Lane 5, DNA-histone mix, MNase treated; Lane 6, salt gradient assembled Nuc207, untreated; Lane 7, Nuc207 MNase treated. Note that DNA is digested completely (lane 3). DNA-histone mix results in a smear of protected bands because histones do not get positioned into nucleosomes without reconstitution (lane 5).

Nuc207 upon MNase treatment gives a characteristic protected band of ~147 bp DNA (lane 7).

B. MNase digestion of chaperone-assembled nucleosomes. Lane 1, 147 bp DNA; Lane 2, 207 bp DNA; Lanes 3–5, 207 bp DNA + histones with 0, 0.5, and 1 U MNase; Lanes 6–8, Nap1 assembled Nuc147 with 0, 0.5, and 1 U MNase; Lanes 9–11, Nap1 assembled Nuc207 with 0, 0.5, and 1 U MNase. Digested samples were treated with Proteinase K and run on 5% native PAGE stained with Sybr Gold, followed by visualization on the Typhoon imager. Note that the Micrococcal nuclease protection assay indicates similar sized protected fragments (147bp) for both Nap1-assembled nucleosomes (Nuc147 and Nuc207).

C. Micrococcal nuclease digestion of trinucleosomes: NLE-Tri were incubated with MNase for 0, 2, 4, 6, and 8 minutes. Bands corresponding to 147, 414, and 561 bp were obtained indicating the presence of mono, di and trinucleosome lengths. Lane 1: 50-2k ladder; Lanes 2 through 6: 0–8 minutes of MNase treatment; Lane 7: 147 bp DNA; Lane 8: 561 bp DNA; Lane 9: 207 bp DNA.

D. MNase digested and undigested 207 bp DNA and Nuc207 were analyzed by 10% native PAGE after DNA extraction. Lane 1: DNA ladder; Lanes 2–3: Undigested and MNase digested 207 bp DNA respectively; Lanes 4–5: Undigested and MNase digested Nuc207 respectively.

E. Analysis of MNase digested Nuc207 via Bioanalyzer 2100. Undigested 207 bp DNA (gray trace) overlaid with MNase digested DNA (black trace). Note that 207 bp DNA is almost completely digested (samples loaded in lanes 2 and 3 in D.).

F. Overlay of MNase digestion profile for undigested (blue trace) and MNase treated Nuc207 (red trace). For nucleosomes, several protected bands are visible around 150 bp (samples loaded in lane 4 and 5 in DNA; see inset).

G. Bar graph of quantification data obtained from Bioanalyzer 2100. The added advantage of analyzing MNase digestion products on a Bioanalyzer is that the molarity of each DNA fragment is calculated based on the DNA markers loaded along with the samples. Hardly any fragment is recovered upon digestion of free DNA, while digestion of Nuc207 reveals several fragments in the range of 130–200 bp. The lines on top of the undigested DNA and Nuc207 indicate continuity to 100% on Y-axis.

Figure 4. Size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) A profile for mono-nucleosomes assembled on 165 bp DNA (Nuc165). The experimentally determined molecular weight (2.24×10^5 Da) matches closely with the theoretical molecular weight (2.1×10^5) for Nuc165.

Figure 5. Overview of an AUC experiment and equipment

A. Components of an AUC cell

B. An assembled AUC cell.

C. Two sample loading apertures on the side of the cell housing.

D. The reference channel is loaded with 400 μL of water and the sample channel has 400 μL of the sample.

E and F. The channels are sealed with a neoprene gasket and a brass housing plug.

G. The correct weight is inserted into the counterbalance and placed in the rotor.

H. The sample AUC cell is placed across from the counterbalance.

I. The rotor is placed inside the centrifuge chamber and the monochromator is inserted in its slot.

J. An initial radial calibration is done at 3000 rpm to ascertain that the cells are not leaking and to calibrate the rotor. Subsequently the centrifuge is allowed to equilibrate for an hour to allow the temperature and vacuum to equilibrate prior to starting the run. Data is collected until the sample has completely sedimented to the bottom of the cell. Data analysis is done using Ultrascan III and the associated LIMS.

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Figure 6. Analysis of chromatin by AUC

A. Sedimentation velocity profile for Nuc165 indicates an S_{avg} value of $\sim 11.2S$. The vertical nature of the $G(s)$ plot indicates homogeneity.

B. The sedimentation profile for trinucleosomes was overlaid with trinucleosomes in complex with a 3-fold excess of MeCP2. The latter showed a forward shift of S_{avg} from $\sim 17S$ to $\sim 20S$ indicative of MeCP2 binding to and compacting trinucleosomes.

Figure 7. Overview of an AFM experiment and equipment

A. Asylum MFP-3D AFM.

B. Controller box and computers for data collection.

C. AFM head inverted to show cantilever holder.

D. Close up of the cantilever holder.

E. Close up of a 240TS cantilever with a tip (black arrow).

F. Mica discs are purchased separately and glued on to standard glass slides (**G and H**).

- I.** Mica is cleaved using a piece of scotch tape to obtain a smooth surface which is then modified using APTES (**J**).
- K.** The sample is then incubated on modified mica and placed on the stage.
- L.** The cantilever holding the tip is lowered on to the sample and images are collected.

Figure 8. Atomic force microscopy (AFM) to visualize trinucleosomes

A. Trinucleosomes assembled by salt gradient reconstitution were imaged in air using AFM.

A 250×250 nm scan was imaged (left panel)

B. A free hand line drawn through a representative trinucleosome and the height profile of the nucleosomes (right panel) indicates a height of 1.5–2 nm.

C. A 200×200 nm scan of trinucleosomes with 0.5-fold of MeCP2 show both uncompacted and compacted trinucleosomes (height trace: left panel; amplitude trace: middle panel; phase trace, right panel).

D. A 200×200 nm scan of trinucleosomes with a 3-fold excess of MeCP2 show mostly compacted trinucleosomes (height trace, left panel), (amplitude trace, middle panel), (phase trace, right panel). Note that while linker DNA was visible in the uncompacted and partially

compacted trinucleosomes (A and B) it is completely invisible in fully compacted trinucleosomes in C.

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A. Nuc165 **B. Trinucleosomes**

Guinier Plot

 $P(R)$ function Plot

Molecular Envelope

Figure 9. Small angle X-ray scattering (SAXS) data for in vitro assembled chromatin

A. Left panel - Guinier plot for data acquired for Nuc165 indicates a radius of gyration (R_g) of $46.9 \text{ \AA}$. Middle panel - $P(R)$ function plot for Nuc165 indicates a maximum dimension ($D_{\max}$) of $\sim 145 \text{ \AA}$. Right panel - Ab initio model calculated from SAXS data for Nuc165, note the globular area for the nucleosome core. Image was generated using VMD.

B. SAXS as quality control for trinucleosomes. Left panel - Guinier plot for trinucleosomes indicates an R_g of $\sim 154 \text{ \AA}$. Middle panel - $P(R)$ function plot for trinucleosomes indicates a $D_{\max}$ of $\sim 510 \text{ \AA}$. Right panel - Ab initio model for trinucleosomes calculated from SAXS data.

Table 1
Quality control methods listed in increasing order of complexity, with their advantages and disadvantages at a glance

Sample volume and concentration is listed for nucleosomes and chromatin, and is meant only as a rough guideline.

Method for QC	Sample volume, concentration	Information obtained	Caveats	Useful resources
Native gel electrophoresis	2–3 μ L of 5 μ M reconstitutions	- Homogeneity - Fluorescently labeled histones and/or DNA confirm the presence of components (scanning at appropriate wavelengths)	Any complex with similar mobility can be mistaken for nucleosomes	N/A
Restriction enzyme digestion	1 μ g	- Saturation of nucleosome arrays	Saturated and over-saturated nucleosome arrays appear the same after digestion with restriction enzyme	NEB website
Micrococcal nuclease digestion	0.5–1 μ g	- Size of protected fragment Quantify amount of properly assembled nucleosomes	Biased to AT sequences	NEB website
Bioanalyzer 2100	1 μ L of 0.2 mg/mL DNA	- Base pair resolution of MNase digested products - Efficiency of nucleosome assembly	There might be small variations in how the DNA fragment sizes are determined based on the DNA ladder and/or chip	Agilent website
SEC-MALS	120 μ L of 0.2–0.3 mg/mL	- Homogeneity - Stability and molecular weight	Small changes in molecular weight cannot be detected	http://www.wyatt.com/training/light-scattering-university.html
AUC	400 μ L of 25–30 ng/ μ L	- Homogeneity - Shape of molecules - Small molecular weight changes can be detected - For a mix of species the composition can be quantified by advanced data analysis	Molecular weights can be determined only if the samples are rather homogenous	AUC theory and operation: https://www.beckmancoulter.com/wsrportal/wsr/support/training-education/teomelab-xl-a-xl-i/index.htm AUC workshop for data analysis: http://www.ultrascan.uthscsa.edu/workshop.php

Method for QC	Sample volume, concentration	Information obtained	Caveats	Useful resources
AFM	30 μ L of 1.5 ng/ μ L	<ul style="list-style-type: none"> - Visualizing the complexes - Height profiles of nucleosomes and complexes 	Best for nucleosomal arrays and larger molecular weight complexes	http://www.asylumresearch.com/Events/Classes/
SAXS	150 μ L of ~2 mg/mL	<ul style="list-style-type: none"> - Overall shape of complexes - Maximum dimension and radius of gyration. 	No information about which domains are interacting or other molecular details, only a shape of the complex is obtained	<p>SAXS data collection and processing at ALS Berkeley: http://bl1231.als.lbl.gov/2013/08/2013_als_UM_workshop.php At APS Chicago: http://small-angle.aps.anl.gov/</p>