Genome-wide analysis of chromatin status using tiling microarrays

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Abstract

The eukaryotic genome is packaged into chromatin, and chromatin modification and remodeling play an important role in transcriptional regulation, DNA replication, recombination and repair. Recent findings have shown that various post-translational histone modifications cooperate to recruit different effector proteins that bring about mobilization of the nucleosomes and cause distinct downstream consequences. The combination of chromatin immunoprecipitation (ChIP) using antibodies directed against the core histones or specific histone modifications, with high-resolution tiling microarray analysis allows the examination of nucleosome occupancy and histone modification status genome-wide. Comparing genome-wide chromatin status with global gene expression patterns can reveal causal connections between specific patterns of histone modifications and the resulting gene expression. Here, we describe current methods based on recent advances in microarray technology to conduct such studies.

Keywords: Saccaromyces cerevisiae; Chromatin remodeling; Chromatin immunoprecipitation; Tiling microarray

1. Introduction

In eukaryotes, packaging of DNA into chromatin is essential for normal chromosomal structure, but it constitutes an impediment to protein factors that need to access specific DNA sequences. Therefore, various cellular processes such as gene transcription, DNA replication, recombination, and repair are accompanied by reorganization of the chromatin structure [1–9]. Chromatin structure changes dynamically, such that localized decondensation and remodeling facilitate the progress of the nuclear machinery. The reorganization of chromatin structure is brought about by ATP-dependent mobilization of nucleosomes and covalent modifications of the N-terminal tails of histones [4]. More than 20 residues within the four core histones are potential sites for post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, and sumoylation; and studies in the past decade have shown that multiple histone modifications act in a sequential or combinatorial manner to specify unique downstream functions [10]. This has been termed the “histone code hypothesis” [11]. However, the mechanisms by which these modifications regulate gene expression either directly or via other effector proteins has not been deciphered completely [12,13]. In addition to chromatin remodeling, alteration in nucleosome structure can also be brought about by the incorporation of histone variants [14–22]. Higher eukaryotes have many different histone variants for specific functions and regions of the genome, although some variants like H2AZ and H3.3 are conserved from yeasts to mammals [16,23,24].

Chromatin immunoprecipitation using antibodies directed against histone modifications and chromatin proteins provides a way to monitor the status of chromatin at any given locus. The combination of chromatin immunoprecipitation and DNA microarray technology (ChIP-chip) is a powerful tool that can be used to generate global maps of nucleosome occupancy and histone modifications [25–30]. For example, in yeast, such studies have shown that nucleosomes are depleted in active regulatory regions genome-wide, and that there is a partial loss of
histones H3 and H4 in the coding region of the genes that are highly transcribed [26,31]. High density tiling oligonucleotide arrays covering entire chromosomes or the entire genome allow such analysis to be performed at the resolution of individual nucleosomes [27,32]. Analysis using such high-resolution tiling arrays have shown, for example, that most genes transcribed by RNA polymerase II in yeast have a 200 bp region upstream of the start codon that is free of nucleosomes [27]. Also in yeast, histone acetylation and methylation are both associated with transcriptional activity but acetylation of histones occurs predominantly at the 5' region of genes, whereas methylation occurs throughout the transcribed region [19]. Comparison of genome-wide histone occupancy and modification maps with binding data for the different ATP-dependent remodelers and covalent histone modifiers have helped decipher functional specificities of individual remodelers [33,34]. Various studies have analyzed the interdependency of ATP-dependent remodelers and covalent histone modifiers, but few general principles have been established till now (reviewed in [12,35]).

This article describes the methods employed to study genome-wide chromatin modification states based on recent advances in chromatin immunoprecipitation and high-resolution tiling microarray technology, focusing on studies in yeast.

2. Experimental strategy

Fig. 1 is a schematic representation of a ChIP-chip experiment used to detect the distribution of modified histones. Briefly, cells are treated with formaldehyde to crosslink histones to DNA [36]. The cells are then lysed, crosslinked chromatin is sheared, and modification-specific and histone-specific antibodies are used to immunoprecipitate (IP) the histone–DNA complexes. The complexes are isolated, the crosslinks are reversed, and the purified DNA is amplified by PCR. Although shearing by sonication is commonly used, precise mapping of individual nucleosomes requires digestion by micrococcal nuclease and isolation of the mononucleosomal sized DNA fractions [27]; these alterations to the basic procedure are outside the scope of this protocol. The amplified DNA is coupled to fluorescent dyes, and hybridized onto DNA microarrays along with labeled reference DNA [37,38].

When looking at histone occupancy, the antibody should ideally recognize a peptide corresponding to a region of the histone that is not post-translationally modified. Using such
an antibody is more appropriate for determining the overall nucleosome occupancy since it immunoprecipitates the histone irrespective of its post-translational modification status. However, if anti-peptide antibodies are not available, antibodies against native histones may be used. Alternatively, a strain with an epitope tagged histone of interest can be used [26]. The advantage of the latter approach is that high-affinity antibodies known to work in ChIP reactions are commercially available for a variety of epitope tags. When looking at a particular covalent histone modification, highly specific antibodies raised against peptides containing the desired modifications are used. Fig. 2 shows the post-translational histone modifications observed in vivo in Saccharomyces cerevisiae, and indicates the modifications for which ChIP grade antibodies are available commercially from Abcam and/or Upstate (now Millipore).

The reference DNA is selected depending on the experiment (Fig. 3A). For example, when comparing genome-wide histone occupancy under two different growth conditions 1 and 2, immunoprecipitated DNA from condition 1 and condition 2 can be labeled with two different fluorescent dyes and hybridized onto the same array (B’ in Fig. 3A). Alternatively, immunoprecipitated DNA from condition 1 and 2 can each be hybridized onto arrays using a common reference DNA sample which can either consist of amplified input DNA (DNA purified from sonicated cell extract prior to treatment with antibody, with crosslinks reversed) or amplified sheared genomic DNA (C’ and D’ in Fig. 3A). The input into the ChIP reaction and sonicated genomic DNA are essentially interchangeable as reference hybridization samples as they are usually virtually identical (Fig. 3B).

Since nucleosome occupancy is not uniform across a chromosome and near transcription start sites, it is important that any measurement of histone modifications be normalized to the underlying nucleosome occupancy, which is also dynamic. To compare the genome wide histone modification status under two different physiological conditions, the DNA immunoprecipitated using the modification specific antibody from condition 1 and from condition 2 can be labeled and hybridized onto arrays along with the DNA immunoprecipitated in parallel using an anti-histone antibody (A and A’ in Fig. 3A). This will
ensure that changes in the underlying nucleosome density do not confound the measurement of histone modification status [39]. The change in histone modification at different loci can then be calculated by dividing the modification level in condition 2 by modification level in condition 1 (A’/A in Fig. 3A).

3. Protocol for chromatin immunoprecipitation (ChIP) in S. cerevisiae

Grow yeast cells to the desired O.D. (at 600 nm) at 30 °C. The volume and density of cells will depend on the strain background, media, growth conditions that are being tested and the number of immunoprecipitations (IPs) that need to be done. A healthy yeast strain growing in 200 ml of YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic complete media (yeast nitrogen base, 2% glucose and a complete mixture of amino acids and vitamins) yields enough material for about four IP reactions. Add 37% formaldehyde directly to the culture to get a final concentration of 1%. Incubate the cultures at room temperature for 15 to 30 min on an orbital shaker set at 100 rpm. The cross-linking time may need to be optimized for different DNA binding proteins. Add 2.5 M glycine to a final concentration of 125 mM to quench the cross-linking reaction. Continue shaking for 5 min at room temperature. Harvest cells by centrifugation and wash cells twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 1.7 mM KH₂PO₄, 10 mM Na₂HPO₄, pH 7.4). Re-suspend cells in lysis buffer (50 mM Hepes–KOH, pH 7.5, 150 mM KCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1× protease inhibitor cocktail (Roche)). Transfer 1 ml of re-suspended cell pellet into a 2 ml screw capped tube with a rubber O-ring. Add 1 ml of glass beads (500 µm diameter) per ml of lysate. Agitate the tubes in a mini bead-beater (Mini-BeadBeater-8™, BioSpec products Inc.) for four sessions of 1 min each, with 2 min incubations on ice between agitations to cool down the contents of the tube. Placing the tubes on ice in between agitations is important to prevent protein denaturation and a possible reversal of DNA–protein crosslinks due to increase in the temperature of the sample. Empty the contents of the tube into a 5 ml syringe (without the plunger) connected to a 25 G needle and placed in a 15 ml conical tube. Allow the cell lysate to flow through and then rinse the beads with 0.5 ml lysis buffer. Use the plunger to force the liquid out of the syringe. Shear the crosslinked DNA using a fine-tipped ultrasonicator at a power setting of 5 and duty cycle of 50%. Sonicate the lysate for 3 cycles of 30 s each with 2 min incubations on ice in between. This step randomly shears the DNA into 300–1000 bp fragments. Run 10 µl of the extract on a 1% agarose gel to confirm complete sonication. Transfer the sonicated cell lysate into microcentrifuge tubes and centrifuge at full speed for 10 min in a refrigerated tabletop centrifuge to pellet the cell debris. Transfer the supernatant into fresh tubes for use in IP reactions.

Pre-clear the extract by adding 50 µl of 50% suspension of protein A- or protein G-agarose beads (Roche) and incubating the mixture at 4 °C on a Nutator™ (Clay Adams Inc.) for 1 h. This step removes the DNA fragments that non-specifically bind to the protein A/G-agarose beads, and improves the signal to background ratio. The affinity with which protein A and protein G bind to different immunoglobulin classes vary, therefore, the choice is made depending on the primary antibody being used. Centrifuge the tube at 1000 g to pellet the beads, and transfer the pre-cleared extract into a fresh tube.

Add specific primary antibody to 500 µl of extract for each immunoprecipitation. The optimal antibody concen-

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Fig. 3. (A) A schematic representation of possible comparisons for a genome-wide histone occupancy and modified histone distribution experiment. The comparisons can be direct, i.e., applying amplified IP material from growth conditions 1 and 2 on the same array, or indirect i.e., applying amplified IP material from growth conditions 1 and 2 along with a different input genomic DNA on two different arrays. With the latter comparison, the actual change in histone occupancy or modification state is calculated by dividing the values obtained from one condition by the other. (B) Input DNA and genomic are virtually interchangeable. Amplified sheared genomic DNA labeled with Cy3 and amplified input DNA labeled with Cy5 were hybridized to microarrays representing ORF and intergenic sequences in the genome of S. cerevisiae. The graph shows the average intensities on a log (base2) scale from 11,029 spots for both the Cy5 and Cy3 channels obtained from four independent experiments.
tation should be determined empirically for different proteins and histone modifications. A series of immunoprecipitations with different antibody concentrations followed by quantitative PCR assays of known target loci can help determine the optimal antibody dilution. Most antibodies work well at 1:50 or 1:100 dilutions. Incubate the tubes on a Nutator™ at 4°C for 4 h to overnight. Add 50 µl of 50% (v/v) suspension of protein A- or protein G-agarose beads (selected based on the above-mentioned criteria) equilibrated in lysis buffer. Incubate the suspension for 2 h at 4 °C with slow rotation. Centrifuge at 1000g for 15 s to pellet the beads, and aspirate the supernatant. Wash the beads twice with wash buffer I (50 mM Heps–KOH, pH 7.5, 150 mM KCl, 1 mM EDTA, 0.1% sodium deoxycholate and 1% Triton X-100), once with wash buffer II (50 mM Heps–KOH, pH 7.5, 500 mM KCl, 1 mM EDTA, 0.1% sodium deoxycholate and 1% Triton X-100), and once with wash buffer III (10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate and 0.5% NP40), 1 ml each time for 5 min at room temperature. Wash beads with 1 ml of TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% SDS) and incubating the beads at 65 °C for 20 min. Spin down the beads at full speed for 2 min in a microcentrifuge and transfer the supernatant into a fresh tube. Add 50 µl of elution buffer to the beads and repeat the elution step. Pool the eluates and reverse the crosslinks by heating for at least 6 h at 65 °C. Spin down the contents, and add an equal volume of TE to the supernatant. Add 1 µl of glycogen (20 mg/ml) and proteinase K to a final concentration of 100 µg/ml. Incubate at 37 °C for 2 h. Add an equal volume of phenol–chloroform (1:1) and extract the aqueous phase. Repeat the extraction with equal volume of chloroform: the direct and the indirect labeling method. The indirect method involves the incorporation of modified nucleotides coupled to fluorescent dyes during the round B amplification reaction. The indirect method involves the incorporation of 5-(3-aminoallyl)-dUTP during the round B reaction, and coupling the purified round B product with mono-reactive N-hydroxysuccinimidyl (NHS) esters of fluorescent dyes. The procedure for round B amplification followed by indirect dye labeling is described below.

There are two methods of labeling the immunoprecipitated DNA: the direct and the indirect labeling method. Direct labeling involves the incorporation of modified nucleotides coupled to fluorescent dyes during the round B amplification reaction. The indirect method involves the incorporation of 5-(3-aminoallyl)-dUTP during the round B reaction, and coupling the purified round B product with mono-reactive NHS esters of fluorescent dyes. The procedure for round B amplification followed by indirect dye labeling is described below.

Aliquot 15 µl of the round A product into a 0.2 ml PCR tube. Add 85 µl of round B reaction mix (20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2 mM MgCl₂, 0.25 mM dATP, dGTP, dCTP, 0.1 mM dTTP and 0.15 mM 5-(3-aminoallyl)-dUTP, 1.25 nanomoles of round B primer [5'-GTTTCCAGTC ACGATC-3']) to it. Carry out 32 cycles of PCR with denaturation at 92 °C for 30 s, two consecutive annealing steps at 40 °C and 50 °C for 30 s each, followed by extension at 72 °C for 60 s. Run 5 µl of the round B product alongside a 1 kb ladder on a 1% agarose gel and look for a smear from 300 to 1000 bp. Purify the PCR product using a MinElute PCR purification kit (Qiagen) and elute DNA in 10 µl of water. The eluate will be about 9 µl.

4.1. Round A extension reaction

Mix 8 µl of immunoprecipitated DNA with 1 µl of 10× Klenow reaction buffer and 1 µl of 40 µM stock of Round A primer (5'-GTTCACGATCAGATCNNTNNNNNNNTT-3') in a 0.2 ml PCR tube. Carry out all the steps in a thermocycler. Incubate the round A setup at 95°C for 5 min followed by cooling the tubes down to 8°C. Pause the PCR machine and add 5 µl of the reaction mix (1× Klenow reaction buffer, 1.5 mM dNTP mix and 1 U exo- Klenow) into the tubes. Allow the primer to anneal by slowly increasing the temperature from 8 to 37°C over an 8 min period. Incubate at 37°C for 40 min for primer extension. Repeat all the steps (denaturation, annealing and extension) except add 1 U exo- Klenow instead of adding the reaction mix. At the end of these two rounds, allow extension reactions to complete by incubating the tube for an additional 10 min at 37°C. Add 85 µl of water to bring the final volume of the round A product to 100 µl. This product can be stored at −20°C and used for about six round B reactions.

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5. Fluorescent dye coupling and array hybridization

The fluorescent dyes generally used in microarray reactions are Cyanine dyes or Alexa dyes. Both are available commercially as mono-reactive NHS esters over a wide range of emission/excitation wavelengths. The dye pairs that are commonly used for microarray analysis are Cy5/
Cy3 (GE Healthcare) and Alexa Fluor 647 and 555 (Molecular probes).

5.1. Coupling reaction

To 9 µl of eluate from the MinElute cleanup reaction, add 1 µl of freshly prepared 1 M sodium bicarbonate buffer, pH 9.0 (the pH of the buffer is very important as the coupling reaction occurs only at pH 9.0). Dissolve one vial of Cyanine (40,000 pmol) or Alexa dyes in 3.6 µl of DMSO and add 1.2 µl of the dye per 9 µl of eluate. Incubate the tubes at room temperature in the dark for 1 h for the reaction to proceed. After 1 h, add 70 µl of water to the tube and clean up the labeled DNA using the MinElute PCR purification kit (Qiagen). Elute the DNA in 10 µl of elution buffer. Combine the Cy3 and Cy5 or Alexa Fluor 555 and 647 labeled DNA, add 5 µg tRNA, 10 µg poly(A), 20 × SSC to a final concentration of 3.5 × and SDS to a final concentration of 0.25%. Denature the mixture at 100 °C for 2 min, cool down to room temperature and hybridize onto microarrays for 6 h to overnight at 65 °C. Wash arrays once each in wash solution I (0.57 × SSC, 0.028% SDS) and wash solution II (0.057 × SSC), dry arrays by centrifuging at 600 rpm for 5 min at room temperature in a table top centrifuge and scan arrays.

6. Microarray analysis

Two broad categories of microarrays are widely used for ChIP-chip studies. In one kind, promoter regions from the genome are selected to be represented on the array, generally through the use of PCR amplicons covering either the transcription start site (TSS) of genes, or CpG islands that are enriched for promoters and regulatory regions [41,42]. Such promoter arrays can also be made by using oligonucleotides to represent the selected promoter regions. The other kind of array is the tiling array, where either the entire genome or a large contiguous region such as an entire chromosome is represented on the array without any bias or preconception about where binding sites may be located. The latter kind of array can either be low resolution, made using PCR amplicons [37,38,43] or high resolution, made using oligonucleotides [27,44,45]. A major drawback of PCR-based microarrays in genome-wide studies to determine nucleosome positions and modification states is the low resolution of the measurements with respect to the size of the nucleosome, which is approximately 146 base pairs of DNA. With such low-resolution arrays, the measurement at a single spot on the array will be an average reading of two to eight nucleosomes. Modifications that affect individual nucleosomes will be very difficult, if not impossible to detect. The low-resolution obviously also means that the position of individual nucleosomes with respect to features such as transcription start sites and transcription factor binding sites cannot be determined, limiting any information gained about remodeling. For finer resolution mapping of nucleosomes, Yuan et al. [27] have used controlled micrococcal nuclease digestion followed by gel isolation of mono-nucleosomal DNA and microarray hybridization to identify nucleosome positions on chromosome 3. A slightly modified protocol, with an immunoprecipitation step after micrococcal nuclease digestion was used by Liu et al. [19] to map histone modifications at single nucleosome resolution.

The high-resolution oligonucleotide platforms are thus technically better for most ChIP-chip studies, their main limitation being their expense and availability. Such arrays are manufactured either by photolithography using physical masks [46–48] (Affymetrix arrays, currently limited to an oligonucleotide length of about 25 bases), maskless photolithography using dynamic micromirror devices [49] (NimbleGen arrays) or inkjet devices [50] (Agilent arrays). The latter platforms can have oligonucleotides that are 50–70 bases in length. These arrays can be designed such that the probes overlap; the extent of the overlap determines the effective resolution of mapping of desired binding sites or nucleosome positions, and can be as high as single nucleotide resolution.

6.1. Analysis of ChIP-chip data

In many respects, ChIP-chip data look superficially like gene expression data from microarrays, but many considerations set ChIP-chip data apart. The main difference is that the distribution of ratios is expected to be asymmetric, because targets will have high ratios relative to the ratios of the background set of spots on the array. Because of this, some of the assumptions that are made for normalization of gene expression microarray data may not be valid and caution must be exercised when processing the data. For example, ratio based normalization works by assuming that all ratios are symmetrically distributed about a median log ratio of zero and sets the median to zero. In ChIP-chip data, target spots with high ratios are also expected to have higher total intensities than non-target background spots with low ratios. Thus, lowess normalization, which assumes that there is no relationship between ratio and intensity, can also be risky because it will effectively reduce the ratios of target spots. Several approaches can be used to address the limitations of normalization methods that make inappropriate assumptions. One option is to use a simple percentile ranking of the ratios, which is not affected by normalization, and set a cut-off for targets based on the distribution of the ranks [37,51]. Another option is to use spiked-in controls [52]. Some methods originally developed for gene expression analysis as the Single Array Error Model [53] and SAM (Significance Analysis of Microarrays) [54] can be used to identify significant target spots in ChIP-chip data from low-resolution spotted arrays. The main consideration with the application of such methods is that one wants to identify only spots with significant (high) ratios in one direction, because spots with very low ratios are not of interest in a ChIP-chip experiment.
Analysis of high-resolution tiling array data is more straightforward in some ways because the signal from each target locus is distributed as a peak over several adjacent spots along the chromosome. Using a moving window averaging approach is usually a very effective way of identifying significant peaks. A window covering a given number of features on the array or a genomic size is moved incrementally over each chromosomal region. In each window, the average signal is calculated, and if the average is greater than some threshold, it is called a significant peak. The threshold is determined based on the overall distribution of values on the array or the chromosomal region. This approach has been implemented in programs like ChIP-OE-Tle [55], Chipper [56], Mpeak [45] and also some Affymetrix data analysis suites [44].

7. Conclusions

Different patterns of post-translational modifications, the presence of histone variants, and ATP-dependent mobilization of nucleosomes, allow partitioning of chromatin into “open” and “closed” structures to regulate gene expression. The use of chromatin immunoprecipitation with antibodies to histone modifications or variants, combined with high-resolution tiling microarrays can provide a detailed and global view of chromatin status and how it changes in relation to changes in gene expression. There are several different chromatin remodelers in eukaryotes and many of them have redundant functions. It is believed that the interactions of bromo, chromo, and SANT domains with modified histone tails recruit chromatin remodeling complexes to nucleosomal templates, to bring about short-term changes in transcriptional states [57]. However, it is not well established how remodelers contribute to and maintain long-term transcriptional states in higher eukaryotes. To decipher this, it will be necessary to integrate studies on global histone modification states with DNA methylation and other possible modes of epigenetic control such as silencer RNAs [58,59].

There are a few important considerations to keep in mind while designing a whole genome ChIP-chip experiment. Perhaps the most important is the availability of protein-specific ChIP grade antibodies. When such antibodies are not available, the histone proteins can be tagged with an epitope tag, such as Myc, hemagglutinin (HA), or TAP tag [26,60]. This approach is relatively easy in yeast cells, but in higher eukaryotes, where it is difficult to generate epitope tagged fusions at the exact chromosomal location, one has to rely on targeted proteins expressed ectopically on a plasmid, which could affect the results. Another important consideration is the ability to crosslink proteins to DNA. When examining histone occupancy, formaldehyde sufficiently preserves nuclear structure and crosslinks histones to DNA. However, when looking at the occupancy of chromatin remodelers and covalent histone modifiers that do not interact with DNA directly, but are recruited via other DNA binding proteins, the use of a bifunctional protein crosslinker, like dimethyl adipimidate (DMA) and dimethyl pimelimidate, in addition to formaldehyde has been reported to increase the probability of successful cross-links [61,62].

Although the experiments described in this article are targeted towards yeast cells, they are easily adaptable to other cells with minor modifications. The emergence of high-density tiling microarrays as a preferred platform for gene expression and protein–DNA interaction studies will continue to shed light on the complex dynamics of gene regulation in eukaryotes.

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References