Chapter 19

Chromatin Immunoprecipitation for Studying Chromosomal Localization of Meiotic Proteins in Maize

Yan He, Gaganpreet Sidhu, and Wojciech P. Pawlowski

Abstract

Chromatin immunoprecipitation (ChIP) is a method that allows identification of chromosomal sites occupied by specific proteins. In this technique, chromatin is extracted from cells, sheared, and, using a specific antibody, enriched in fragments that contain a protein of interest. Genomic location of the protein can then be identified by hybridization of the resulting DNA to tiling microarrays or by sequencing. Thanks to advances in high-throughput sequencing methods, studying protein localization using ChIP has become possible even in species with relatively large genomes. Here, we describe a ChIP protocol that we developed to examine localization of meiotic proteins in maize.

Keywords Chromosomes, Chromatin, Immunoprecipitation, Antibody, Maize, Recombination

1 Introduction

Many proteins involved in meiosis form complexes that are visible as distinct foci on chromosomes during prophase I. Some of these proteins are components of the recombination pathway and mark sites of DNA double-strand breaks (DSBs), recombination pathway intermediates, such as single-end invasion (SEI) events, and crossovers (COs). Key recombination proteins, such as SPO11, RAD51 (Fig. 1), DMC1, and MLH1 (1–6), belong to this group. Specifically modified chromatin proteins that are implicated in meiotic prophase progression also occur at discrete chromosomal locations. These modifications include histone H3K4 tri-methylation, thought to mark sites of DSB hotspots in some species, and γ-H2AX phosphorylation, which marks chromatin regions adjacent to nascent DSBs (6–8). Finally, synaptonemal complex proteins, such as ZYP1, often show localization at distinct sites on chromosomes during early stages of their installation (5).

Protein distribution on meiotic chromosomes is often examined using immunolocalization microscopy (see Chapters 9–11).

Wojciech P. Pawlowski et al. (eds.), *Plant Meiosis: Methods and Protocols*, Methods in Molecular Biology, vol. 990,
DOI 10.1007/978-1-62703-333-6_19,

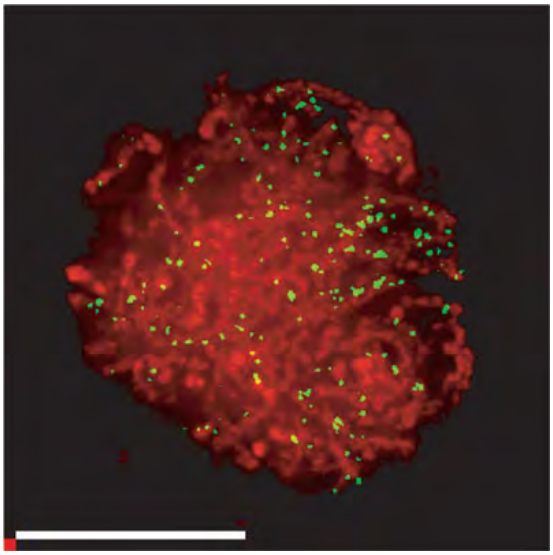

Fig. 1 The RAD51 protein localized to discrete sites on maize chromosomes during zygotene. Chromosome sites where RAD51 is located can be determined with high resolution using chromatin immunoprecipitation (ChIP). *Red* = chromatin. *Green* = RAD51. Bar = 10 m. Modified from Pawlowski et al. (3) copyright American Society of Plant Biologists (www.plantcell.org)

Another approach for studying patterns of protein distribution along chromosomes is chromatin immunoprecipitation (ChIP) (9, 10). In this method, chromosomal proteins are cross-linked and chromatin is extracted and enriched, using a specific antibody, in fragments containing the protein of interest. The antibody-enriched fragments can be identified using either whole-genome DNA tiling arrays or next-generation sequencing.

The ChIP technique offers several advantages over microscopical immunolocalization studies. First, chromosomal locations of the target protein can be determined at a very high resolution of a few hundred base pairs. Second, a very large number of meiotic cells, typically millions of them, are surveyed in a single experiment. Finally, relative frequencies of target protein occupancy can be determined for specific chromosome locations. These features can provide deep insights into how meiotic processes operate on a genome-wide scale. Good examples of how using the ChIP technique can further understanding of meiosis are studies on mapping the sites of meiotic DSBs in yeast and mouse using immunoprecipitation of chromatin fragments associated with the SPO11 and RAD51 proteins (11, 12). SPO11, a protein belonging to the topoisomerase family (13), is responsible for generating DSBs in chromosomal DNA that initiate meiotic recombination. The DSBs are subsequently resected to form single-stranded DNA ends that become coated by a complex containing two recombination proteins, RAD51 and DMC1 (14–16). Consequently, SPO11-associated chromosome fragments represent the sites of

DSBs while fragments associated with RAD51 are immediately adjacent to the DSB sites.

A key requirement for ChIP experiments is availability of antibodies with high specificity and affinity to native proteins. Performance of an antibody in immunolocalization and/or western blot experiments may not always be a sufficient predictor of its suitability for ChIP. Actual ChIP experiments, with appropriate negative controls, need to be carried out to determine antibody's performance. While a large number of antibodies against meiotic proteins are commercially available for mouse and humans, many of them do not show high enough affinity to plant proteins to be useful for ChIP. Consequently, many ChIP experiments in plant may require rising custom antibodies.

In this chapter, we describe a ChIP protocol to study distribution of chromosomal proteins in male meiosis in maize. We developed and optimized this protocol using an antibody against H3K4 tri-methylation and we utilize it to determine locations of meiotic DSB sites using an antibody against RAD51.

2 Materials

2.1 Plants

Starting with high-quality plant material is critical for success of ChIP experiments. Since temperature is known to affect the progression of meiosis (17), grow plants in a controlled-environment growth chamber. To grow maize plants, use a 12 h day/12 h night photoperiod, temperature of 31°C during the day and 22°C at night, and light intensity of about 600 mol/m^2/s.

2.2 Reagents

2.2.1 Staging Meiotic Flowers

1. Farmer's fixative: 3 volumes of 100% ethanol, 1 volume of glacial acetic acid.
2. Acetocarmine: 2% acetocarmine powder in 45% acetic acid. Boil for 6–8 h in a flask with an attached reflux column and then filter through filter paper when the solution is still warm. Store in a dark bottle at room temperature.

2.2.2 Cross-linking and Chromatin Preparation

1. Cross-linking buffer: 10 mM Tris–HCl (pH 8.0), 0.4 M sucrose, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1% formaldehyde.
2. 2 M glycine in water.
3. Distilled water.
4. Chromatin extraction buffer A: 10 mM Tris–HCl (pH 8.0), 0.4 M sucrose, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol. Before use, add 1 tablet of Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN, USA) per 50 ml of buffer.

5. Chromatin extraction buffer B: 10 mM Tris–HCl (pH 8.0), 0.25 M sucrose, 10 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol, 1 g/ml Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA).
6. Chromatin extraction buffer C: 10 mM Tris–HCl (pH 8.0), 1.7 M sucrose, 2 mM $MgCl_2$, 0.15% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol, 1 g/ml Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA).
7. Nuclei lysis buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% (w/v) SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 g/ml Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA).

2.2.3 Chromatin Immunoprecipitation

1. ChIP dilution buffer: 16.7 mM Tris–HCl (pH 8.0), 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 g/ml Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA).
2. Blocking buffer: 16.7 mM Tris–HCl (pH 8.0), 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 g/ml Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO, USA), 5 mg/ml bovine serum albumin (BSA).
3. Low salt wash buffer: 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100.
4. High salt wash buffer: 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X-100.
5. LiCl wash buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 1% NP-40, 1% sodium deoxycholate.
6. TE buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA.
7. Elution buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 200 mM NaCl, 1% SDS.
8. 10 mg/ml RNase.
9. 20 mg/ml Proteinase K.
10. MinElute PCR Purification Kit (Qiagen, Hilden, Germany).
11. Quant-IT dsDNA HS Assay Kit (Invitrogen, Grand Island, NY).

2.3 Supplies and Equipment

2.3.1 Staging and Collecting Meiotic Flowers

1. Glass scintillation vials or 15 ml plastic tubes to collect flowers for staging.
2. Razor blade.
3. Tweezers with fine tips.
4. Dissecting needle.
5. Rusty nail (see Note 1).

6. Glass microscope slides and coverslips.
7. Alcohol lamp.
8. Masking tape.
9. Plastic tray with wet paper towels.
10. Dissecting stereoscope.
11. Bright-field microscope.

2.3.2 Cross-linking and Chromatin Preparation

1. 50 ml conical tubes.
2. Miracloth.
3. Small ceramic mortar and pestle.
4. Plastic funnel.
5. Vacuum desiccator
6. Probe sonicator.
7. Microcentrifuge.
8. Refrigerated centrifuge.
9. Tabletop shaker.
10. 4°C refrigerator.

2.3.3 Chromatin Immunoprecipitation

1. Dynabeads (Invitrogen, Grand Island, NY).
2. Magnetic Separation Stand (Invitrogen, Grand Island, NY).
3. Tube rotator.

3 Methods

3.1 Staging Meiotic Flowers

1. At the time of meiosis, the immature tassel in maize is still inside the stalk. The presence of the tassel can be felt just below the top node of the plant by gently squeezing the leaf whorl.
2. After establishing that the tassel is large enough to be felt, make a small longitudinal incision with a razor blade through the leaves to the tassel, just below the top node.
3. Remove several flowers with needle-nosed forceps into a glass scintillation vial or tube containing Farmer's fixative.
4. Dissect anthers from the collected flowers on a microscope slide under a stereo dissecting microscope.
5. Add a drop of acetocarmine solution for staining. Mix anthers with the stain using a dissecting needle or, preferably, a rusty nail (see Note 1) over gentle heat until the color of the stain turns from deep red to purple. Place a coverslip over the anthers and gently press on to break the anthers and release meiocytes. Determine the stage of meiosis under a bright-field compound microscope.

6. If the anthers are not yet at the desired meiosis stage, tape over the incision with masking tape and repeat the staging procedure in a day or two.
7. Cut plants with anthers at the desired stage of meiosis at several nodes below the tassel.
8. Gently remove leaves surrounding the tassel. To prevent the tassel from drying out during dissection, place it on wet paper towels in a tray and put more wet paper towels on top of it.

3.2 Chromatin Cross-linking

1. Harvest 1.5 g of healthy-looking flower tissue (see Note 2) and transfer it into a 50 ml conical tube.
2. Gently submerge the tissue in 37 ml of cross-linking buffer (see Note 3). Cap the tube with Miracloth to prevent the tissue from floating on the surface.
3. Vacuum infiltrate for 10 min.
4. Release vacuum slowly and remove Miracloth. Stop the cross-linking reaction by adding 2.5 ml of 2 M glycine. Vacuum infiltrate for 5 min.
5. Decant supernatant and wash the tissue three times with 40 ml of distilled water. After the third wash, remove as much water as possible by putting the tissue between dry paper towels.
6. Transfer dry tissue into a new 50 ml conical tube. Snap-freeze in liquid nitrogen and store at –80°C.

3.3 Chromatin Preparation

1. Grind the tissue to a fine power with a precooled mortar and pestle.
2. Resuspend the powder in 40 ml of ice-cold chromatin extraction buffer A. Incubate for 20 min at 4°C with gentle shaking.
3. Filter solution into a new 50 ml conical tube through 2 layers of Miracloth placed in a plastic funnel.
4. Centrifuge at 1,250 $\times g$ for 20 min at 4°C.
5. Pour out the supernatant and resuspend the pellet in 1 ml of ice-cold extraction buffer B by gently pipetting up and down with a 1,000 l automatic pipette. Transfer the suspension to a 1.5 ml microcentrifuge tube. Incubate on ice for 15 min with occasional agitation.
6. Centrifuge at 18,500 $\times g$ in a microcentrifuge for 10 min at 4°C. Discard the supernatant and resuspend the pellet in 500 l of ice-cold extraction buffer C by gently pipetting up and down with a 1,000 l automatic pipette (see Note 4).
7. In a clean 1.5 ml microcentrifuge tube, add 500 l of extraction buffer C. Layer the resuspended pellet from step 6 on top of this "cushion."

8. Centrifuge at 14,000 rpm in a microcentrifuge for 1 h at 4°C.
9. Discard the supernatant and resuspend the nuclei pellet in 500 l of ice-cold nuclei lysis buffer.

3.4 Chromatin Sonication

1. Sonicate chromatin on ice using several pulses lasting for 5 s each to shear DNA into fragments of desirable size (see Notes 5 and 6).
2. After sonication, centrifuge the chromatin solution at 14,000 rpm in a microcentrifuge for 5 min at 4°C to pellet tissue debris. Transfer the supernatant to a new tube.
3. Take a 10 l aliquot of the sonicated chromatin sample to check sonication efficiency and use as an input control sample for ChIP product sequencing (see Note 7).

3.5 Blocking Protein A Beads (see Notes 8 and 9)

1. For each ChIP sample, take 100 l of Dynabeads (Invitrogen, Grand Island, NY) slurry into a 1.5 ml microcentrifuge tube.
2. Separate the beads on a Magnetic Separation Stand (Invitrogen, Grand Island, NY) for 1 min. Without disturbing the beads, pipette out the supernatant.
3. Wash beads twice with 1 ml of ChIP dilution buffer. For each wash, add the buffer and vortex the beads briefly to break coagulates. Then, remove the buffer as described in step 2.
4. Resuspend the beads in 1 ml of blocking buffer. Incubate at 4°C with gentle shaking for at least 2 h.
5. Wash the beads three times with 1 ml of ChIP dilution buffer as described in step 3.
6. Add ChIP dilution buffer back to the original bead volume from step 1.

3.6 Chromatin Immunoprecipitation

1. Split the chromatin sample from Subheading 3.4, step 3 (approx. 450 l) into three 1.5 ml tubes of equal volume (150 l in each tube) and dilute the chromatin sample in each tube tenfold by adding 1,350 l of ChIP dilution buffer (see Note 10).
2. Preclear each chromatin sample by mixing with 40 l of protein A-coated beads for 3 h with gentle rotation of the tubes on a tube rotator at 4°C.
3. Separate the beads on the Magnetic Separation Stand.
4. Transfer the supernatant from each tube into a new tube. The first tube will serve as a "no-antibody" control. Add 10 g of normal rabbit IgG to the second tube to use as an "IgG control." Add 10 g of your target antibody to the third tube (see Note 11).
5. Incubate the chromatin samples overnight with rotating at 4°C.

6. The next morning, capture the protein–DNA complexes by adding 40 μl of coated-beads and rotating the tubes on a tube rotator for 2.5 h at 4°C. Separate beads on the Magnetic Separation Step and remove the supernatant.
7. Wash the beads with 1 ml of each of low salt wash buffer, high salt wash buffer, LiCl wash buffer, and TE by rotating the tubes for 5 min at 4°C. Conduct each wash as described in Subheading 3.5, step 2. After the final wash, make sure to remove all TE.

3.7 Eluting Immunoprecipitated Complexes (See Note 12)

1. Add 200 μl of freshly prepared elution buffer. Resuspend beads by vortexing and incubate at 65°C for 30 min with occasional agitation.
2. Centrifuge at 2,000 ×*g* for 1 min and collect the supernatant into a new tube.

3.8 De-Cross-linking

1. Add 4 μl of 10 mg/ml RNase to the supernatants from Subheading 3.7, step 2, and incubate at 37°C for 1.5 h.
2. Add 4 μl of 20 mg/ml Proteinase K and incubate at 45°C for 2 h.
3. Reverse cross-link at 65°C for 8 h or overnight.

3.9 DNA Recovery

1. Purify DNA from each sample Subheading 3.8, step 3 using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany), eluting in 30 μl of H_2O.
2. Measure the DNA concentration with the Quant-IT dsDNA HS assay kit (Invitrogen, Grand Island, NY) following manufacturer's instructions.

4 Notes

1. Iron oxide that leaches from the rusty nail enhances the staining reaction. Too little iron oxide will result in weaker staining.
2. This protocol is primarily designed for immunoprecipitating proteins that are specific to meiosis and are not present, or not associated with chromatin, in somatic cells. Targeting proteins that accumulate on chromatin in somatic tissues will lead to confounded results. In such cases, isolated meiocytes (see Chapter 20) can be used instead of whole flowers. This modification will require a scaled-down ChIP protocol to accommodate a much smaller amount of input material.
3. The length of cross-linking and the formaldehyde concentration need to be optimized for each type of tissue. Insufficient cross-linking may result in decreased binding of the ChIP antibody, while excessive cross-linking may lead to nonspecific binding of the antibody.

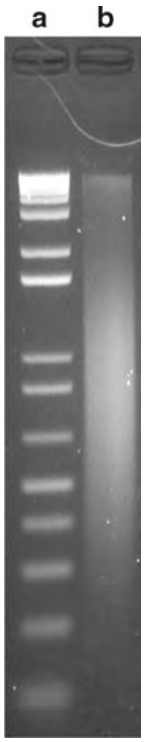

Fig. 2 Analysis of chromatin sonication efficiency. (**a**) 200 ng of sonicated chromatin de-cross-linked and run in a 2% agarose gel. (**b**) 1 kb DNA molecular size ladder (Invitrogen, Grand Island, NY)

4. Avoid introducing air bubbles or forming froth on the surface as this may lead to protein degradation in subsequent steps.
5. In most experiments, chromatin is fragmented to sizes of 200–500 bp. However, in our RAD51 ChIP, we shear DNA into 500–1,000 bp fragments (using 8 sonicator pulses of 5 s each). To produce desired fragment sizes, sonication conditions need to be optimized for every sonicator type, type of tissue, and, in some cases, also for each specific target protein. Over-sonication will lead to DNA degradation, whereas insufficient sonication will lead to nonspecific antibody binding and decreased ChIP yield. See also Note 7.
6. Keep sample on ice to avoid generating excessive heat during sonication, since it will cause protein degradation. Leave samples on ice for at least 30 s between each sonicator pulse to allow them to cool down.
7. The sonicated chromatin sample needs to be de-cross-linked before DNA extraction. To do this, add 140 l of TE buffer, 5 l of 5 M NaCl, and 10 l of 10% SDS to a 10 l aliquot of sonicated chromatin. Reverse-cross-link overnight at 65°C. Purify DNA using the Qiagen MinElute PCR Purification Kit (Qiagen, Hilden, Germany). To check sonication efficiency, electrophorese an aliquot of the extracted DNA in 2% agarose gel (Fig. 2).

8. Blocking the beads decreases nonspecific binding of the antibody. We strongly recommend including this step, even though it is not always suggested in published ChIP protocols. Do not use DNA as a blocking reagent if the ChIP DNA product will be analyzed by sequencing. Otherwise, most of the sequence reads will represent carrier DNA.
9. The bead blocking step can be carried out before starting the ChIP experiment. After blocking, beads can be stored at 4°C.
10. From this step on, use low-retention microcentrifuge tubes.
11. The amount of antibody used may need to be optimized for each antibody. This amount is dependent on the affinity between the antibody and the antigen that varies from one antibody to another.
12. In some cases, a second immunoprecipitation may be desired to increase specificity. In this case, chromatin elution after the first immunoprecipitation round should be performed at room temperature so that protein–DNA complexes are not denatured. The second immunoprecipitation round should be performed in the same way as the first immunoprecipitation, except the elution step, which should be carried out at 65°C.

Acknowledgment

Research to develop this protocol was supported by a grant from National Science Foundation (IOS-1025881) to W.P.P.

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