Chapter 24

Chromatin Immunoprecipitation in Mouse Hippocampal Cells and Tissues

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Abstract

Chromatin immunoprecipitation (ChIP) has been developed for studying protein–DNA interactions and has been extensively used for mapping the localization of posttranslationally modified histones, histone variants, transcription factors, or chromatin modifying enzymes at a given locus or on a genome-wide scale. ChIP methods have been modified and improved over the years to fit a variety of different cell types and tissues. Here, we present a detailed protocol for hippocampal ChIP, of both minced tissue and enzyme-separated hippocampal cells. This protocol enables to study chromatin–protein interactions in a specified population of hippocampal cells, allowing to study chromatin regulation in the central nervous system in a variety of conditions and disorders. Our assay has been developed for histone modifications but is suited for any chromatin binding protein for which specific ChIP-grade antibodies are available.

Key words: Chromatin immunoprecipitation, ChIP, Hippocampus, Histone modifications, Brain, Transcription, Neurons, Chromatin

1. Introduction

The eukaryotic genome is packaged as a highly compacted DNA–protein complex termed chromatin that forms primarily during DNA replication. The newly synthesized DNA is wrapped around a histone octamer particle, composed of two copies each of the core histones H2A, H2B, H3, and H4, which together with the DNA, form the core subunit of chromatin – the nucleosome. Two adjacent nucleo somes are connected through linker DNA, which is usually bound by the linker histone H1. Dynamic changes in chromatin structure, which are mainly manifested through chromatin modifications, mark an important contribution to the regulation of gene expression in dividing as well as nondividing cells, such as neurons (1–3).

Ales Vancura (ed.), *Transcriptional Regulation: Methods and Protocols*, Methods in Molecular Biology, vol. 809,
DOI 10.1007/978-1-61779-376-9_24,

In particular, acetylation/deacetylation and methylation/demethylation of specific lysine residues on nucleosomal histone proteins, as well as methylation/demethylation of cytosines in the DNA, are ways by which chromatin structure can influence ongoing transcription. Such chromatin-related changes may lead, in turn, to altered synaptic plasticity. Studying the interactions of nuclear proteins with DNA has been immensely improved by the advent of chromatin immunoprecipitation (ChIP) (4–6). ChIP provides a reliable method to monitor the presence of proteins and protein modifications (especially histones) on chromatin. ChIP coupled with real-time PCR (qPCR) has become the gold standard to determine chromatin–protein interactions (7) and has been continuously improved and modified to fit a variety of different cell types and tissues (8–12). In recent years, the combination of ChIP with DNA microarrays (ChIP-chip) or high-throughput sequencing (ChIP-Seq) technologies has enabled the profiling of histone modifications, histone variants, and transcription factor occupancy on a genome-wide scale (13). Here, we provide a refined ChIP protocol for hippocampal tissue and a modified version for hippocampal cells, which can be used as template for real-time PCR, tiling arrays, or high-throughput sequencing.

Hippocampal ChIP involves the surgical dissection and mincing of the hippocampus. ChIP can be performed on the entire hippocampus, on specific hippocampal cell types (i.e., neurons, cholinergic neurons, etc.) after FACS sorting, and also on cultured hippocampal neurons following single cell separation of the tissue (Fig. 1). In all cases, chromatin is purified and cross-linked. The isolated chromatin is then reduced to smaller fragments by sonication, and chromatin fragments in the form of protein–DNA complexes are precipitated using specific antibodies. The precipitated DNA is purified and can be used as a template for real-time PCR (ChIP), microarrays (ChIP-chip) or deep sequencing (ChIP-seq).

2. Materials

2.1. Chemicals

1. 36.5% Formaldehyde: Molecular biology grade formaldehyde solution is used. Dilute with PBS to make it 1% (add 270 µl of formaldehyde to 10 ml PBS). Formaldehyde is hazardous and should be handled inside a chemical hood.
2. Glycine: 2.5 M glycine stock is prepared by dissolving 18.76 g of tissue culture grade glycine in 100 ml of deionized water. Dilute in 10 ml of deionized water by adding 500 µl of 2.5 M glycine to make a final concentration of 0.125 M.
3. Protein A/G Agarose beads: Use protein A Agarose beads or magnetic beads, which can reduce some background and

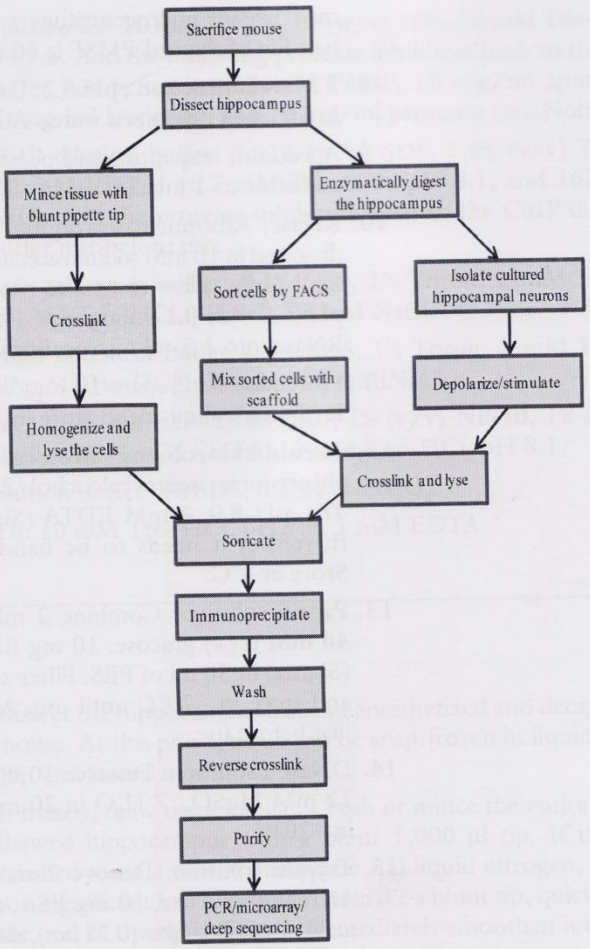

Fig. 1. A flowchart of the three ChIP protocols on hippocampal cells and tissue.

loss of sample. For magnetic beads, use Dynabeads Protein G (Invitrogen) for rabbit polyclonal primary antibodies and Dynabeads M-280 anti-Mouse IgG for mouse monoclonal antibodies.

4. If using magnetic beads, use DynaMag-2 magnet (Invitrogen).
5. Dounce homogenizer (Kontes): Use pestle B for hippocampal tissue.
6. PBS (phosphate buffered saline): Dissolve 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic in 1 l of deionized water to make it 1× PBS and adjust pH to 7.4 with HCl or NaOH.
7. PMSF: 200 mM PMSF stock is prepared by dissolving 0.348 g of PMSF in 10 ml of absolute ethanol or methanol. Aliquot

into 1.5 ml microcentrifuge tubes and store at −20°C. The half-life of thawed PMSF is 30 min, so add before use.

8. 3 M sodium acetate, pH 5.2: Dissolve 20.4 g of sodium acetate in 50 ml of deionized water. Adjust pH to 5.2 with HCl.
9. Aprotinin, leupeptin, and pepstatin: Prepare 1 mg/ml stocks and store as 1 ml aliquots in −20°C.
10. RNase: Ribonuclease (Sigma) 10 mg/ml stock solution is dissolved in 10 mM sodium acetate buffer, pH 5.2. Heat to 100°C for 15 min, allow to cool to room temperature, then adjust to pH 7.4 with 0.1 volumes of 1 M Tris–HCl, pH 7.4. Aliquot RNase into 1.5-ml microcentrifuge tubes and store at −20°C.
11. Proteinase K: Prepare 10 mg/ml stock solution of Proteinase K (Sigma), aliquot and store in −20°C.
12. Phenol:chloroform: Molecular biology grade phenol–chloroform–isoamyl alcochol (25:24:1) saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma) is used. It is hazardous; therefore, it needs to be handled inside a chemical hood. Store at 4°C.
13. Papain solution: Combine 2 mM DL-cysteine hydrochloride, 46 mM D (+) glucose, 10 mg BSA, and 15,000 U of DNaseI (Sigma) in 30 ml of PBS. Filter-sterilize, prepare 3 ml aliquots, and store in −20°C until use. Add 100 µl of papain (Sigma) just before use.
14. DNase I solution: Dissolve 10,000 U of DNase I (Sigma) and 12 mM $MgSO_4$. 7 H_2O in 20 ml of HBSS. Aliquot and store in −20°C.
15. Trypsin solution: Dissove 2 mM DL-cysteine hydrochloride, 46 mM D-glucose, 10 mg BSA, and 15,000 DNase I (Sigma) in 30 ml of PBS. Filter (0.22 µm), aliquot (3 ml), and store in −20°C. Add 0.1% (v/v) trypsin EDTA solution just before use.
16. Plating medium: Use alpha MEM medium (Invitrogen) supplemented with 10% FBS and antibiotic–antimycotic mix (Invitrogen) 1:100.
17. Maintenance medium: Use Neurobasal medium (Invitrogen) supplemented with B-27 serum-free supplement 1:50 (Invitrogen), GlutaMax diluted 1:100 (Invitrogen) and antibiotic–antimycotic mix diluted 1:100.
18. Poly-L-lysine (PLL) coated dishes: Dissolve 1 mg/ml PLL hydrobromide (Sigma) into borate buffer (1.24 g boric acid, 1.9 g borax, 400 ml of deionized water), aliquot, and store at −20°C until use. Add 10 µg/ml PLL hydrobromide (diluted 1:100 in borate buffer) onto culture dishes and keep overnight at room temperature. Wash three times with deionized water and dry completely.

2.2. Buffers

1. Lysis buffer: 10 mM EDTA, 1% (w/v) SDS, 50 mM Tris–HCl, pH 7.5. Add the following protease inhibitors fresh to the lysis buffer just before use: 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml pepstatin (see Note 1).
2. ChIP dilution buffer: 0.01% (w/v) SDS, 1.1% (v/v) Triton, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, and 167 mM NaCl. Add the protease inhibitors fresh to the ChIP dilution buffer just before use.
3. Low salt wash buffer: 0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 15 mM NaCl.
4. High salt wash buffer: 0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl.
5. LiCl wash buffer: 0.25 M LiCl, 1% (v/v) NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1.
6. Elution buffer: 1% SDS, 0.1 M $NaHCO_3$.
7. TE: 10 mM Tris–HCl, pH 7.5, 1 mM EDTA.

3. Methods

3.1. ChIP on Mouse Hippocampus

3.1.1. Cross-Linking, Lysis, and Sonication

1. Dissect the hippocampus from an anesthetized and decapitated mouse. At this point, tissue can be snap-frozen in liquid nitrogen and stored in −80°C.
2. If frozen, thaw tissue on ice. Crush or mince the entire fresh/thawed hippocampus with a blunt 1,000 µl tip. If tissue is crushed using a mortar and pestle and liquid nitrogen, it leads to a significant loss of sample. To make a blunt tip, quickly heat the end of the tip in fire and immediately smoothen it inside a 1.5-ml microcentrifuge tube (see Note 2).
3. Fix the crushed tissue with 1% formaldehyde and incubate with gentle agitation for 15 min at room temperature to cross-link DNA to bound proteins (see Note 3). Centrifuge the sample at 440 × *g* for 5 min at room temperature in a swing-out rotor.
4. Add 0.125 M glycine from 2.5 M stock solution to terminate cross-linking. Incubate the sample in the centrifuge tube for 5 min at room temperature and centrifuge at 440 × *g* for 5 min at room temperature in a swing-out rotor.
5. Wash with cold PBS (2–3 times). Add 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin just before use and centrifuge the cells at 440 × *g* for 4 min at 4°C in a swing-out rotor.
6. Aspirate the supernatant with the pipette and resuspend the pellet in 1 ml of ice-cold lysis buffer with freshly added protease inhibitors and PMSF for 15 min on ice and homogenize the samples

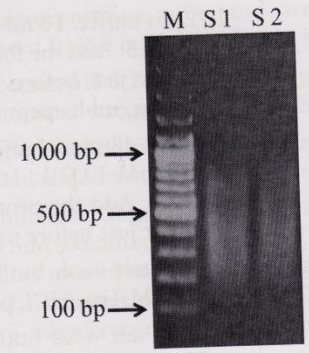

Fig. 2. Chromatin shearing of hippocampus. Chromatin is sonicated to fragments of 200–1,000 bp, which are electrophoresed on 1.2% agarose gels. M denotes a 100 bp ladder; S1 and S2 denote the sonicated samples.

slowly (10 strokes on ice) with dounce homogenizer and tight pestle. After homogenization, transfer the sample to the 1.5-ml microcentrifuge tube carefully to avoid sample loss in this critical step and centrifuge at 2,260×*g* for 5 min in a swing-out rotor.

7. Aspirate the supernatant and resuspend the pellet in ice-cold lysis buffer (250–600 µl).
8. Sonicate for 20 min using Bioruptor sonicator with 30 s on/off pulses twice for 10 min each while replenishing the ice and water. Alternatively, sonicate 3× for 7 min each (21 min total). Centrifuge at 20,780×*g* for 30 min at 4°C on fixed-angle rotor (see Note 4).
9. Collect the supernatant carefully and transfer to another 1.5-ml microcentrifuge tube. Chromatin can be snap-frozen in liquid nitrogen and kept in –80°C
10. To evaluate shearing, add 75 µl of deionized water and 4 µl of 5 M NaCl to 25 µl of supernatant and reverse cross-link at 65°C (4 h to overnight). Run the sheared chromatin on 1% agarose gel (Fig. 2).

3.1.2. Immunoprecipitation and DNA Purification

1. The chromatin should be thawed on ice, if previously frozen. Save 20% of pre- immunoprecipitated lysate as input for later normalization (step 7). The rest of the chromatin should be divided into three microcentrifuge tubes (for IgG, a positive control and the antibody of choice) for immunoprecipitation, each containing 10–60 µg of chromatin. Add ChIP dilution buffer to a final volume of 1.5 ml.
2. To reduce non specific background, pre-clear the 1.5 ml diluted cell supernatant with 40 µl of Protein A-Agarose beads for 45 min at 4°C with agitation (add beads with a pre-cut tip) and

pellet beads by centrifugation at 4,420×*g* for 1 min and collect the supernatant fraction (see Note 5). In case of magnetic beads, use magnet to collect the supernatant instead of centrifuge.

3. Add antibody (1–10 µg) to the 1.5 ml of supernatant and incubate overnight at 4°C with rotation to collect the antibody complex (see Note 6). Pellet beads by gentle centrifugation at 110×*g* for 1 min at 4°C. Carefully remove the supernatant that contains the unbound DNA.
4. Add 60 µl of salmon sperm DNA/Protein A-Agarose slurry with a precut tip and incubate for 1 h at 4°C with rotation to collect the antibody–histone complex and pellet beads by gentle centrifugation at 110×*g* for 1 min at 4°C. Carefully remove the supernatant that contains the unbound DNA.
5. The beads should be washed for 2 min on rotating platform with 1 ml of each buffer listed.
   (a) Low salt wash buffer
   (b) High salt buffer
   (c) LiCl buffer
   (d) 1× TE

   Centrifuge at 110×*g* at 4°C for 1 min and remove the supernatant between each wash (see Note 7).
6. Add 125 µl of freshly prepared elution buffer to the beads, vortex briefly, and incubate at room temperature for 15 min with rotation. Spin down beads at 440 g at room temperature for 1 min and transfer the eluate to another 1.5-ml microcentrifuge tube. Repeat elution to make a final volume of 250 µl.
7. Add 20 µl of 5 M NaCl to the combined eluates and reverse histone–DNA cross-links by heating at 65°C for 2–16 h. Include input material from step 1 here (add 12 µl of 5 M NaCl to 150 µl of input). Add 5 µl of 0.5 M EDTA (3 µl to input), 10 µl of 1 M Tris–HCl, pH 6.5 (6 µl to the input), 1 µl of 10 mg/ml RNase and 2 µl of 10 mg/ml proteinase K to the combined eluates and incubate for 1 h at 45°C.
8. Recover DNA by adding equal volumes of phenol–chloroform (see Note 8). Add 1/10 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of isopropanol and keep in −80°C for 1 h to overnight. Wash pellets with 70% ethanol and air-dry. Resuspend the pellet in deionized water or TE (60–100 µl), and measure the concentration of the purified DNA. Alternatively, use DNA cleanup kit (Qiagen). The purified DNA can now be used as a template for real-time PCR or for high-throughput sequencing/hybridization on tiling arrays.

3.2. ChIP on Mouse Hippocampal Neurons by Enzymatic Hippocampal Cell Dissociation

In this method, the hippocampus is subjected to enzymatic digestion with trypsin or papain. To perform ChIP on a specified population of neurons, cell sorting can be performed using FACS ARIA sorter (or equivalent). Sorted cells should be mixed with scaffold cells of a different organism (with unrelated DNA), such as insect cells.

3.2.1. Preparation of Cells: Papain/Trypsin Digestion

1. Dissect hippocampi and wash with HBSS. Add papain solution (or trypsin solution) and incubate at 37°C for 15 min. Add 5 ml of plating medium to stop the digestion and centrifuge at 110 × *g* for 5 min and discard supernatant.
2. Add DNase I solution with glass pipette (5 ml). Use a 200-µl tip at the tip of a glass pipette and triturate until no visible chunk is seen. Add 5 ml of plating medium and pass through 70 µm filter to a 50 ml falcon tube. Centrifuge at 110 × *g* for 5 min.
3. Collect supernatant very carefully. Suspend the cells in 5 ml of plating medium and centrifuge at 110 × *g* for 5 min. Collect supernatant very carefully.
4. Suspend the cells in 1× PBS and wash with PBS. Indirect immunostaining protocol of FACS can be followed according to Abcam's protocol and sorting of the cells can be done with the specific antibodies using FACS ARIA sorter.

3.2.2. Cross-Linking, Cell Lysis and Sonication

1. After cell sorting, mix with proper scaffold and start with one to five million total cells.
2. Cross-link proteins to DNA by adding 1% formaldehyde (270 µl of 37% formaldehyde into 10 ml of medium). Incubate for 10 min at room temperature with rotation and centrifuge at 440 × *g* for 5 min at room temperature on a swing-out rotor.
3. Add 0.125 M glycine (500 µl of 2.5 M glycine into 10 ml of medium). Incubate for 10 min at room temperature with rotation and centrifuge at 440 × *g* for 5 min at room temperature on a swing-out rotor.
4. Wash cells twice with ice-cold PBS. Add 5 ml of ice-cold PBS containing protease inhibitor cocktail (1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). Add PMSF just prior to use. Centrifuge for 5 min, 110 × *g* at 4°C.
5. Warm lysis buffer to room temperature and add protease inhibitors just before use. Resuspend the pellet in ice-cold lysis buffer (250–600 µl) (250 µl to two million cells).
6. Proceed to the sonication step 8 of Subheading 3.1.1.

3.2.3. Immunoprecipitaion and DNA Purification

Refer to Subheading 3.1.2.

3.3. ChIP on Cultured Primary Hippocampal Neurons

3.3.1. Culture Isolated Hippocampal Neurons (see Note 9)

1. After triturating hippocampal neurons (follow steps 1 and 2 of Subheading 3.2.1), suspend the cells in plating medium and determine the cell density. The total yield should be around $0.8–1.0 \times 10^6$ cells per embryo. Plate cells onto Poly-L-lysine (PLL) coated dishes. Our standard density is $1.6–1.8 \times 10^6$ cells per 100-mm dish. We usually use three 100-mm dishes for one histone antibody but the number should be optimized for each antibody (Fig. 3).
2. After 3–4 h, change the medium to maintenance medium.
3. On the third day of culture, replace one half of the volume with fresh maintenance medium containing 10 μM of cytosine arabinoside (1-β-D- arabinofuranosylcytosine).
4. Replace one half of the medium every 3 days. After more than 10 days in culture, intertwined network of dendrites and axons is developed. Cells are ready to be used for ChIP. Neurons can be depolarized with KCl or a GABA-A receptor antagonist, bicuculline, or stimulated with a variety of ligands. The culture has the advantage of being easy to manipulate for studying neuronal responses to various stimuli.

3.3.2. Cross-Linking, Cell Lysis, and Sonication

1. Cross-link cells by directly adding 1% formaldehyde. Incubate at room temperature for 10 min.

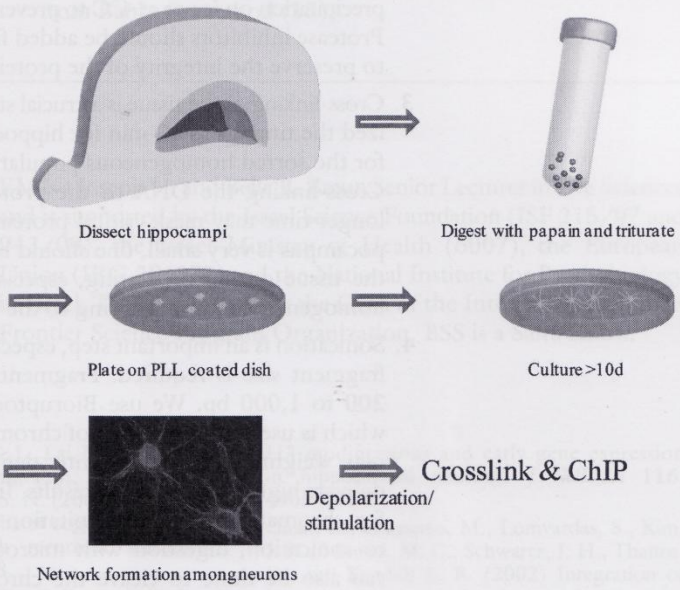

Fig. 3. Culture of hippocampal neurons. Hippocampi are digested and cell suspension is plated on dishes. After culturing for more than 10 days, neurons develop intricated connections and can be depolarized or stimulated.

2. Add 0.125 M glycine and incubate for 10 min at room temperature.
3. Wash the cells with ice-cold PBS twice and then add PBS (2 ml per 100 mm-dish) containing protease inhibitor cocktail. Collect the cells into a 15-ml tube by scraping off with cell lifter.
4. Spin down the cells by centrifugation for 10 min, 110×*g* at 4°C.
5. Proceed to step 5 of Subheading 3.2.2.

3.3.3. Immunoprecipitation and DNA Purification Refer to Subheading 3.1.2.

4. Notes

1. ChIP assays have been extensively used to study histone acetylation and methylation patterns at a variety of genomic loci. To analyze histone acetylation, add 10 mM sodium butyrate to the lysis buffer, dilution and elution buffers. Sodium butyrate prevents the loss of histone acetylation during sample preparation by inhibition of histone deacetylase activity.
2. Mincing of tissue is done by the pipette blunt tip to mince the tissue before cross-linking. Tissue can also be cut into small pieces with a scalpel. Perform all steps of cell lysis and immunoprecipitation on ice or at 4°C to prevent chromatin degradation. Protease inhibitors should be added fresh wherever mentioned to preserve the integrity of the proteins.
3. Cross-linking of the tissue is a crucial step, and we have standardized the timings as 15 min for hippocampal tissue and 10 min for the sorted homogeneous population of hippocampal cells. Cross-linking the DNA to the protein by formaldehyde for longer time may mask critical protein isotopes. Since the hippocampus is very small, one should be very careful not to lose the tissue during processing, especially when douncing with homogenizer and transferring to the microcentrifuge tube.
4. Sonication is an important step, especially when a specific DNA fragment size is required. Fragment size usually varies from 200 to 1,000 bp. We use Bioruptor sonicator (Diagenode), which is used for sonication of chromatin (or any high molecular weight nucleic acid) into defined size fragments and delivers highly reproducible results. It is particularly well suited for chromatin immunoprecipitation assays. As an alternative to sonication, digestion with micrococcal nuclease (MNase) can also be done to cleave the chromatin into oligonucleosomes or mononucleosomes. This method is normally used for native chromatin immunoprecipitation (14) when chromatin is not fixed. However, this technique is less favorable with

formaldehyde cross-linking, since cross-linked chromatin is inefficiently cleaved by nucleases.

5. We find that the best beads to use are protein A-Agarose beads from Millipore. ChIP assays can be performed with protein A, protein G, or protein A/G beads. The choice of beads depends on the isotype of the antibody to be used for the immunoprecipitation. If unknown, use a mixture of protein A/G beads.
6. Standardization of antibodies needs to be done prior to the experiment. Good quality and proper amount of antibodies should be used. Usually try lower amounts such as 1–2 μg and proceed to higher amounts if necessary (5–10 μg).
7. Washing steps are more crucial after the immunoprecipitation reactions. The washes should be efficient and stringent to reduce nonspecific background. The number of washes can be increased depending on the antibody. After washing, one should be extremely careful while handling the supernatant.
8. Phenol and chloroform are volatile, toxic, and inflammable and should be used with caution inside a chemical hood. During phenol–chloroform (P/C) precipitation, add an equal volume of P/C to sample, mix well but do not vortex. Use aqueous (upper) layer. If the sample appears dirty, repeat P/C step again until the interface is fairly clean.
9. The protocol for culturing hippocampal neurons is adapted from Banker's method (15).

Acknowledgments

EM is a Joseph H. and Belle R. Braun Senior Lecturer in Life Sciences and is supported by the Israel Science Foundation (ISF 215/07 and 943/09), the Israel Ministry of Health (6007), the European Union (IRG-206872) and the National Institute for Psychobiology in Israel. TT is supported by the CDA of the International Human Frontier Science Program Organization. BSS is a Safra fellow.

References

1. Huang, L. T., Holmes, G. L., Lai, M. C., Hung, P. L., Wang, C. L., Wang, T. J., Yang, C. H., Liou, C. W., and Yang, S. N. (2002) Maternal deprivation stress exacerbates cognitive deficits in immature rats with recurrent seizures. *Epilepsia* **43**, 1141–1148.
2. Crosio, C., Heitz, E., Allis, C. D., Borrelli, E., and Sassone-Corsi, P. (2003) Chromatin remodeling and neuronal response: multiple signaling pathways induce specific histone H3 modifications and early gene expression in hippocampal neurons. *J Cell Sci* **116**, 4905–4914.
3. Guan, Z., Giustetto, M., Lomvardas, S., Kim, J. H., Miniaci, M. C., Schwartz, J. H., Thanos, D., and Kandel, E. R. (2002) Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell* **111**, 483–493.