

Video Article

Chromatin Immunoprecipitation from Dorsal Root Ganglia Tissue following Axonal Injury

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Abstract

Axons in the central nervous system (CNS) do not regenerate while those in the peripheral nervous system (PNS) do regenerate to a limited extent after injury (Teng *et al.*, 2006). It is recognized that transcriptional programs essential for neurite and axonal outgrowth are reactivated upon injury in the PNS (Makwana *et al.*, 2005). However the tools available to analyze neuronal gene regulation *in vivo* are limited and often challenging.

The dorsal root ganglia (DRG) offer an excellent injury model system because both the CNS and PNS are innervated by a bifurcated axon originating from the same soma. The ganglia represent a discrete collection of cell bodies where all transcriptional events occur, and thus provide a clearly defined region of transcriptional activity that can be easily and reproducibly removed from the animal. Injury of nerve fibers in the PNS (e.g. sciatic nerve), where axonal regeneration does occur, should reveal a set of transcriptional programs that are distinct from those responding to a similar injury in the CNS, where regeneration does not take place (e.g. spinal cord). Sites for transcription factor binding, histone and DNA modification resulting from injury to either PNS or CNS can be characterized using chromatin immunoprecipitation (ChIP).

Here, we describe a ChIP protocol using fixed mouse DRG tissue following axonal injury. This powerful combination provides a means for characterizing the pro-regeneration chromatin environment necessary for promoting axonal regeneration.

Protocol

1. Sciatic & Dorsal Column Nerve Injury

1. All animals are anesthetized for surgery with a continuous isoflurane/O₂ administration.
2. For sciatic injury, both hindquarters are carefully shaved, and depilation is completed with generic hair remover prior to cleansing the skin with alcohol spray/swipe.
3. The sciatic nerve is exposed by making a 4 mm incision posterior and parallel to the femur at mid-thigh through the skin and by spreading the muscles apart of the biceps femoris with fine forceps.
4. The exposed sciatic nerve is then injured, and the skin is closed with two suture clips.

NOTE: The same incision is made to expose the sciatic nerve, but the wound is sutured closed leaving the nerve intact for the sham injury.

5. For dorsal column injury, the back of the mouse is carefully shaved, and depilation is completed with generic hair remover prior to cleansing the skin with alcohol spray/swipe.
6. The spinal cord is exposed by making a 2.5 cm incision from about T7 to T13. The skin is spread apart, and the connective tissue is removed along the vertebra from T9 to T11.
7. A laminectomy is performed at T10 level

NOTE: For most experiments, the laminectomy is considered the Sham injury.

8. A few drops of Xylocain are added to the tissue to anaesthetize the cord, and the dura mater is removed, paying attention not to touch the spinal cord.
9. The dorsal columns are injured and the muscle is sutured close, again paying attention not to touch the spinal cord. Finally, the skin is sutured closed with suture clips.

2. Cross-linking

1. Dissect L4 and L5 DRG from injured mice. Collect a total of 16 DRG into ice-cold HBSS + protease inhibitors cocktail (2 injured and 2 sham DRG from each animal for a total of 4 animals).
2. Briefly centrifuge the DRG, then carefully remove the HBSS and add 500 µl of 1% formaldehyde in PBS + protease inhibitors cocktail and incubate the sample for 30 minutes at 37°C.

CRITICAL STEP. Use fresh, molecular biology grade formaldehyde.

3. Add 125 mM of glycine to stop the fixation and incubate for 5 minutes at room temperature.
4. Briefly centrifuge, aspirate off buffer, and wash the tissue twice with 500 µl ice-cold PBS + protease inhibitors cocktail.

3. Nuclei preparation and chromatin shearing

1. Aspirate off PBS, add 400 μ l of SDS lysis buffer, transfer to a pre-chilled microcentrifuge tube, and disrupt the tissue with approximately 30 strokes with the micropestle.

CRITICAL STEP. Lysis and disruption of the tissue are vital for a good yield of crosslinked chromatin.

2. Sonicate the sample with 8 pulses, 10 second each at 70% output (Bandelin, Sonoplus GM70).

CAUTION. The shearing of the chromatin must be optimized for your particular sonication set up and proper fragmentation of the chromatin should be checked before running the actual IP experiment (see section 3).

NOTE. Fragmentation of the chromatin could also be performed by micrococcal nuclease (MNase) digestion. As a rule of thumb, fragmentation by sonication is preferred for fixed tissue, while MNase digestion is favored for native tissue chromatin immunoprecipitation. Cross-linked, sheared chromatin can be stored at -80° C for up to 2 months, but avoid repeated freeze/thaw cycles.

4. Analysis of chromatin digestion (recommended)

1. Remove 10 μ l of your sheared chromatin sample for analysis. Add 200 mM of NaCl to the sample and reverse the cross-link by incubating at 65° C for 2h.
2. Purify DNA (see section 8) and run a 1% agarose gel. DNA should be fragmented to a length of approximately 200-1000 bp (Figure 1).

CAUTION. Over-fragmentation of chromatin may lead to decreased signal, while incomplete fragmentation will lead to diminished resolution and increased background.

5. Immunoprecipitation

1. Determine the number of immunoprecipitation reactions (see NOTE below), and divide samples equally into new tubes. Bring the volume of each up to 500 μ l with ChIP buffer + protease inhibitors cocktail.
2. Remove 5 μ l of your diluted sample and transfer into a new tube. This is your 1% input sample and it will be stored at -20° C until needed (Section 7).
3. For each immunoprecipitation, add appropriate antibody or normal IgG control and incubate at 4° C overnight with rotation.

NOTE. When determining the number of immunoprecipitation, a positive control (e.g. Histone H3 antibody) and a negative control (Normal IgG) should be considered. The quantity of antibody used for each IP varies between antibodies and must be empirically determined. However, it is usually within a range of 2-10 μ g/immunoprecipitation.

4. To immunoprecipitate your antibody/protein/DNA complex, add 30 μ l of ChIP Grade Protein G Magnetic Beads and incubate for 2 h at 4° C with rotation.

6. Washing

1. To pull-down bound chromatin-bead complex, place the tube on the magnetic rack. Wait until the solution is clear and then carefully remove your supernatant.
2. Add 1 ml of low salt wash (ChIP buffer) to the beads and incubate at 4° C for 3-5 minutes with rotation, then pull-down beads and aspirate off wash as in step 6.1. Repeat this step for a total of 3 washes.
3. Add 1 ml of high salt wash buffer (ChIP buffer+ 350 mM NaCl) to the beads and incubate for 3-5 minutes with rotation, then pull-down beads and aspirate off wash as in step 6.1.

7. Elution and reversal of cross-linking

1. Take your input samples out of the -20° C, add 150 μ l of ChIP Elution buffer and set aside at room temperature until Step 7.5.
2. Add 150 μ l of 1x ChIP Elution buffer for each IP sample from Step 6.3.
3. Place the tubes in a thermomixer and elute the chromatin from the beads by incubating samples at 65° C for 30 minutes with gentle vortexing.
4. Pull-down beads on the magnetic rack, and carefully transfer the eluted chromatin (supernatant) into new tubes.
5. To all tubes, including your input samples, add 200mM of NaCl and 40 μ g/reaction of Proteinase K and incubate at 65° C for 2 hours.

NOTE. The elution step can be performed at room temperature, too, but it might be not as efficient.

8. DNA recovery

1. Add 1 volume of Phenol/Chloroform to your samples from Step 7.5 and vortex for 30 seconds.
2. Centrifuge at maximum speed in a microcentrifuge at room-temperature for 5 minutes.
3. Carefully recover the aqueous (upper) phase into new tubes, add 1 volume of chloroform to the aqueous phase, and vortex for 30 seconds.
4. Centrifuge at maximum speed in a microcentrifuge at room-temperature for 5 minutes.
5. Carefully recover the aqueous (upper) phase into new tubes, then add 300mM of NaOAc, 20 μ g/reaction of glycogen, and 2.5 volumes of ice-cold 100% ethanol.

CAUTION. The addition of a carrier, such as the glycogen, is needed to enhance the precipitation of relatively small sized DNA fragments.

6. Incubate at -80° C for 2 hours to precipitate DNA.

NOTE. The precipitation step can also be performed overnight at -20° C.

1. Centrifuge the samples at maximum speed in a microcentrifuge for 20 minutes at 4°C.
2. Carefully remove the supernatant and wash the precipitated DNA pellets with 300 µl of ice-cold 70% ethanol.
3. Centrifuge the samples at maximum speed in a microcentrifuge for 10 minutes at 4°C.
4. Carefully remove as much supernatant as possible and let the pellets air-dry.
5. Resuspend in 20 µl of sterile H₂O. Samples are now ready for PCR, or the DNA can be stored at -20°C.

9. Representative Results

A representative result of a ChIP experiment in DRGs following sciatic lesion is shown (Figures 1 and 2). First, we show fragmented DNA to a length of approximately 200-1000 bp (Figure 1). Second, we demonstrate a PCR signal following ChIP from the proximal promoter of the growth associated protein-43 (GAP-43) only upon sciatic nerve injury (lane 4, Figure 2, 5'). No PCR signal is present when the animal receives a sham injury only (lane 3), and when using normal IgG serum for the IP (lane 5 and 6). To test for the specificity of the antibody, we analyzed the same DNA samples, but used a control primer set which detects a region in the 3'-UTR of our gene of interest where no occupancy is expected. PCR signals are absent from all lanes (lanes 3-6, Figure 2, 3'), except for the input signal, representing non-IP DNA samples as standard PCR controls (lane 1-2, Figure 2). This ChIP procedure can be performed following either sciatic or spinal dorsal column injury as summarized in a schematic (Figure 3).

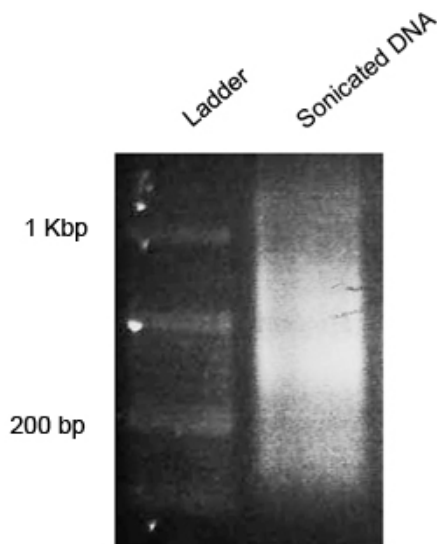


Figure 1. Agarose gel of reversed cross-linked DNA that has been sheared by sonication to the proper range of fragment lengths.

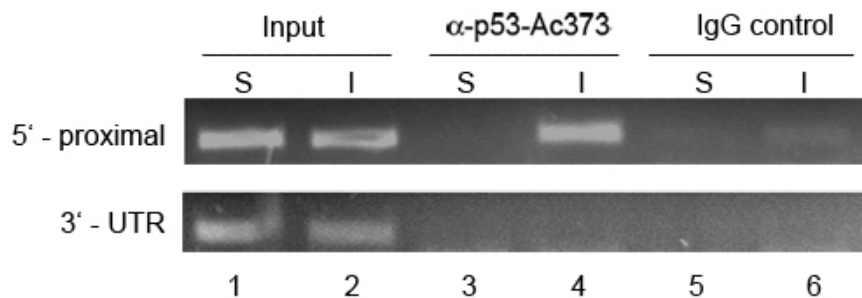


Figure 2. Semi-quantitative PCR results from DRG tissue following sciatic nerve lesion showing that acetylated p53 binds to the GAP-43 proximal promoter region 48 hours after injury to the sciatic nerve. Control PCR from the same DRG tissue show that acetylated p53 does not bind to a control region of DNA located in the 3'-untranslated region (UTR) of the GAP-43 gene (S = Sham, I = Injured).

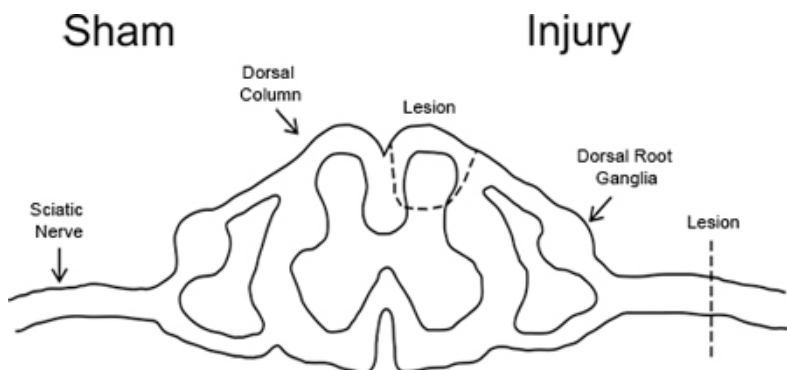


Figure 3. A general diagram showing the location of the lesion sites on the sciatic nerve, and the dorsal column.

Disclosures

No conflicts of interest declared.

Discussion

This protocol provides a method to directly ask about the chromatin environment during axonal regeneration in the adult nervous system following axonal injury. It incorporates the DRG injury model with chromatin immunoprecipitation to probe the transcriptional and epigenetic environment subsequent to injury to either the PNS or CNS. It is particularly useful for investigators who would like to characterize putative binding sites for their favorite transcription factor, and to determine whether the occupancy of these sites occurs in response to injury. Epigenetic modification of histones and DNA at these sites can also be simultaneously monitored. A similar protocol can be performed following facial nerve lesion, where the facial nerve nuclei can be dissected from the brainstem and processed by ChIP as we previously reported (Tedeschi *et al.*, 2009).

As typical for chromatin immunoprecipitation assays, two critical steps in the protocol are, (1) efficient fragmentation and solubilization of the DNA-protein complex, and (2) the availability of a good immunoprecipitation antibody for your protein of interest. One limitation that might confound these two steps is the low level of starting material. Compared to other structures such as the brain or spinal cord, DRG provide a relatively small amount of tissue. In addition, DRG consists of a mix population of neurons as well as glial cells, all of which might have different chromatin environments. This may lead to heterogeneous IP signals; however this caveat is present in most samples taken from the nervous system. A potential solution is to perform ChIP after fluorescent activated cell sorting of transgenically fluorescently labeled DRG neurons, (see for example YFP-H mice, Bogdan *et al.*, 2004), or immuno-isolation of neurons via magnetic beads (Lee *et al.*, 2005). However, these two approaches need to be validated for ChIP in DRGs and will likely need an increased amount of starting material.

We have successfully used this method to identify binding sites for several transcription factors and coactivators at the promoter of known regeneration associated genes, and we have used both semi-quantitative and quantitative PCR methods to detect the immunoprecipitated DNA. One future addition to this protocol could be the use of tiled microarrays following ChIP (ChIP-on-chip), as means to detect the final DNA signal. ChIP-on-chip would greatly increase the number of identified transcription sites via a high throughput unbiased approach. It could also allow for the study of how two or more transcription factor might cooperatively interact on a genomic level.

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