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Chromatin Immunoprecipitation in Adult Zebrafish Red Cells

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Abstract

Zebrafish has been used for many years as a model to study development and disease. The ability of zebrafish to produce thousand of embryos in a synchronous manner has made zebrafish an invaluable tool for genetic and chemical screens. Since its emergence as an important model organism the molecular tools for studying zebrafish have been limited. In this chapter, we describe a simple method to identify DNA binding sites and chromatin architecture in erythrocytes from adult zebrafish using chromatin immunoprecipitation coupled with next generation sequencing. This technique has been used extensively and successfully in other systems and it will be a useful tool for studying epigenetics in zebrafish.

I. Introduction

The zebrafish, *Danio rerio*, is a well-established vertebrate model for studying developmental hematopoiesis (Paik and Zon, 2010). As a model organism it offers a number of unique advantages for studying development such as the transparency of the embryos. A major breakthrough that has extended the advantages of transparency to adult fish is the creation of the "casper" fish (White *et al*., 2008) that lacks melanocytes and iridophores and is transparent through adulthood. In addition, production of large amounts of embryos allows for large-scale phenotype-based forward genetics and chemical genetics screens. These classic advantages of zebrafish make it ideal for developmental and genetic studies, but up to now many molecular tools remain largely under-utilized in zebrafish studies. For example, despite the relative abundance of gene expression data on zebrafish development, only recently has chromatin immunoprecipitation (ChIP) been used to examine the changes in histone marks that occur during embryogenesis (Lindeman *et al*., 2009; Vastenhouw *et al*., 2010). Examination of histone modifications permits a better molecular understanding of the alterations to DNA packaging that are needed to elicit gene expression changes. Additionally, ChIP can be employed to define the specific DNA binding sites of transcription factors to delineate the genes that they directly versus indirectly regulate. Here we describe a protocol for ChIP coupled with next-generation sequencing in adult zebrafish erythrocytes that can be used for studying not only histone modifications but also the specific binding of transcription factors to DNA in these cells.

VIII. Conflicts of Interest L.I.Z. is a founder and stockholder of Fate, Inc. and a scientific advisor for Stemgent.

A. Rationale

Chromatin immunoprecipitation has been used extensively for studying the chromosome landscape, including histone modifications such as methylation and acetylation as well as the binding of a variety of transcription and chromatin factors to the DNA. In brief, the method describes the isolation of the cell type of interest, the chromatin immunoprecipitation, and the sequence analysis. To start, the cells are crosslinked in order to maintain the chromatin architecture and the factors bound to chromatin. Specific antibodies are used to precipitate a modified histone or a chromatin factor of interest. After the immunoprecipitation, the crosslink is reversed and the bound DNA fragments can be detected by quantitive PCR, to assess binding to specific sites of interest or all the immunoprecipitated sites can be identified in a genome-wide manner by massive sequencing. The sequenced fragments are then mapped to the genome and bioinformatics can provide additional information, including the identification of specific enriched genomic locations and motifs.

II. Adult Zebrafish Exsanguination

For each ChIP, a minimum of 10^7 cells should be used without involving an amplification step post ChIP. Using the following exsanguination protocol, approximately 10^6 red blood cells can be acquired from one adult fish, thus at least 10 adult zebrafish should be processed per ChIP. Fish are first euthanized in an overdose of Tricaine-S (MS-222, TMS, tricaine methanesulfonate 200–300 mg/L) and bled quickly using a heparinized 200 μL tip attached to a Pipette. To isolate the blood cells, puncture the zebrafish through the gills deep enough to penetrate the heart. To aid bleeding and to prevent clotting, 200 μL of heparin solution should be placed near the puncture site before the initial puncture. With the pipette tip, extract the blood by repeated pipetting and transfer isolated blood to an Eppendorf tube containing red cell isolation buffer (0.9% PBS, 5% fetal bovine serum, and 0.3–1 U heparin/mL). Samples should be kept at room temperature, since zebrafish erythrocytes are fragile and will get lysed at lower temperatures.

Another easier method for exsanguination is *via* decapitation. Euthanized fish are placed in a petri dish containing several milliliters of heparin. Zebrafish are then decapitated with a sharp razor blade. This procedure needs to be approved by the IC Institutional Animal Care and Use Committee (Fan and Yazulla, 1997) at every institution. The blood will flow freely out of the animal into the heparin solution. To collect the sample, use a 200 μL heparinized pipette tip and transfer cells to a 50-mL canonical tube containing red cell isolation buffer.

III. Chromatin Immunoprecipitation (Lee *et al*., 2006)

A. Erythrocyte Crosslinking

After isolation the cells should be immediately crosslinked by addition of 1/10 of the volume formaldehyde solution (50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 11% formaldehyde). For example, 1 mL of formaldehyde solution will be added to 10^7 cells in 10 mL of isolation buffer. Samples should then be placed on a shaker at room temperature for 5 min. Immediately after the 5 min incubation, 1/20 volume of 2.5 M glycine should be added to quench the formaldehyde. The cells should be

incubated for 5 min at room temperature and pelleted by centrifugation at 1100*g* for 5 min at 4 °C. The cells should be washed twice with excess amounts of PBS (for example 50 mL). This procedure should be done fast to prevent the lysis of the erythrocytes as much as possible. The researcher will notice the supernatant turning red indicating free hemoglobin from the lysed cells when significant cell lysis occurs. After the washes, cell pellets can be used immediately or flash frozen in liquid nitrogen and kept in −80 °C for months.

Crosslinking is crucial for an effective ChIP since inefficient crosslinking can lead to loss of interactions whereas overcrosslinking can result in false positive results. Crosslinking times may need to be experimentally determined for a specific antibody and/or a particular cell type.

In previously published protocols on ChIP analysis of mouse embryonic stem cells, a limited amount of cells of interest were mixed with a large number of cells from other species, such as drosophila cells, in order to minimize material loss in the process due to the small amount of starting material (carrier ChIP or CChIP) (O'Neill *et al*., 2006). For this protocol, this addition is unnecessary as an adequate number of adult zebrafish erythrocytes can be isolated for the performance of ChIP but in case the cell type of interest can be isolated in limited numbers that could be a good alternative.

B. Sonication

The sonication conditions are crucial for the success of the ChIP. Sonication permits the solubilization and fragmentation of chromatin. The size of the fragments determines the precision of the identification of transcription factor binding regions. Smaller fragments will allow more precise localization, but oversonication can lead to nosier results. The ideal fragment size should be between 300 and 1000 bp.

Prior to sonication, cells need to be lysed. For 10^7 erythrocytes, the cells are resuspended in 10 mL of lysis buffer 1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 1× protease inhibitors) and incubated for 10 min at 4 °C. Cells are centrifuged for 5 min at 1350*g* at 4 °C. Pellets are then resuspended in 10 mL of lysis buffer 2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mMEDTA, 0.5 mM EGTA, 1× protease inhibitors) and incubated for 10 min at room temperature. Cells are pelleted for 5 min at 1350*g* at 4 °C and then resuspended in 1 mL lysis buffer 3/sonication buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% *N*-lauroylsarcosine, 1× protease inhibitors). Samples are then sonicated in a Bioruptor sonicator for 24 cycles of 30-s sonication followed by a 1 min-resting interval after each cycle. After sonication, Triton X is added to sonicated lysates at 1% final concentration, which are then centrifuged for 10 min at 18,000*g* to pellet cell debris. After centrifugation, 50 μL of input should be set aside as a control. Approximately 10^7 cells are used for each ChIP.

Prior to the addition of the Triton X, the sonicated material should be checked for the appropriate fragmentation sizes before proceeding to the next step. To prepare the chromatin, combine 10 μL of sample with 20 μL TE containing 0.1% SDS and boil for 15 min. Add loading dye and run the samples on a 1.5% agarose gel to check the size of the

fragments. If the fragment size is larger than 1000 bp, additional cycles of sonication should be performed in 2–6 cycle increments. Repeat this procedure until the desired fragmentation size has been achieved. The boiling method of visualizing sonicated sample is a quick method for checking the result of sonication. To test the real sonication efficiency reversal of crosslinking is necessary (see below).

Alternatively, a single buffer can be used for lysing the cells and sonication so as to avoid loss of material. Collected cell pellets should be resuspended in a SDS ChIP buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH 8.1, 150 mM NaCl, and 1X protease inhibitors) (Kim *et al*., 2009). Sonication conditions will be different using this buffer, so fragmented chromatin should be checked on an agarose gel as described above to determine optimal number of sonication cycles.

C. Pretreatment of Beads and Antibodies

Prior to immunoprecipitation, the selected antibody should be attached to magnetic protein A or protein G Dynabeads. Fifty microliter of beads are used for each immunoprecipitation. To prepare the beads, wash twice with 1 mL blocking buffer (PBS with 0.5% BSA), then resuspend in 250 μL blocking buffer plus 5 μg of antibody. The amount of antibody can be adjusted. All washes are performed using the Dynal MPC magnet for bead/antibody collection. Beads are collected by 15–30 s incubation on the magnet, the supernatant is removed, and then the next buffer is added. The beads are rotated with the antibody at 4 °C for 6 h to overnight. After the incubation with the antibody, the beads are washed twice with 1 mL blocking buffer to remove the excess antibody. It is crucial that the beads do not get dry during the washes. The beads are now ready to be added to the lysate for immunoprecipitation.

D. Immunoprecipitation

The sonicated material from step B (after the addition of Triton X) can be directly added to the antibody-conjugated beads from step C. If the alternative buffer for sonication, which does not require nuclear extraction, is used, then the sonicated material can be used as it is in that buffer. The beads and lysates are incubated together with agitation at 4 °C for 4 h to overnight.

It is important to perform a control immunoprecipitation in parallel. Two standard controls used are an IgG isotype control that is the same isotype as the antibody used for the ChIP or beads alone (Sandmann *et al*., 2006). These controls will help eliminate false positive results stemming from the non-specific binding to the antibody or the beads. In addition to non-specific binding of chromatin to the beads, the chromatin may also adhere to the plastic tubes. To minimize this problem, siliconized tubes can be used, especially when the amount of starting material is less than optimal.

E. ChIP washes

Following the immunoprecipitation, several washes are employed to remove the non-bound chromatin from the bead/antibody mixture. Beads are washed 1× with wash buffer 1 (20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), 1× with

wash buffer 2 (20 mM Tris-HCl pH 8, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1%Triton X-100), 1× with wash buffer 3 (10 mM Tris-HCl pH 8, 250 nM LiCl, 2 mM EDTA, 1% NP40), and 1× with TE. All washes are performed at 4 °C using the Dynal magnet as described in part C. For each wash, the supernatant is removed and 1 mL of the next solution is added. The tubes are shaken vigorously until all the beads are resuspended, and then they are placed on a rocker at 4 °C for a 3 min incubation. If siliconized tubes are not used, the beads are transferred to new tubes after each wash to minimize non-specific binding of chromatin fragments to the plastic tubes. After the last wash with TE, the supernatant is removed and the tubes are centrifuged at 960*g* for 3 min at 4 °C for removal of residual TE.

F. Elution and Reversal of Crosslinking

The beads from step E are resuspended in 200 μL elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS) by heating at 65 °C for 30 min in a shaking heat block. The supernatant is then transferred to a new tube for crosslink reversal that is done at 65 °C for 6 h to overnight. Longer incubations may result in nosier data. It is important at this point to also perform crosslink reversal on the total input samples saved after step B. To prepare the total input control, add 150 μL of elution buffer to the 50 μL of whole cell extracts then incubate at 65 °C along with the immunoprecipitated samples.

G. DNA Recovery and Quality Control

After the reversal of crosslinking the samples are treated with RNase (0.2 mg/ml final concentration) for 2 h at 37 °C, and then proteinase K (0.2 μg/ml final concentration) at 55 °C for 2 h to overnight. Next the DNA is extracted using phenol/chloroform/isoamyl alcohol followed by an ethanol precipitation. ChIP material is then resuspended in 30 μL TE and the whole cell extracts (total input controls) in 150 μL TE. The concentration of the ChIP DNA samples should be at least 5 ng/μL.

To validate the ChIP, quantitative PCR is used. The primers are designed based on known information on binding sites of the transcription factor. Standard QPCR conditions are utilized. If available, positive and negative primer sets should be used. If a 384-well QPCR platform is available, less than 0.5 μL of the ChIP sample can be used in a 5 μL total reaction volume. Primer sets for a housekeeping gene are included to normalize different ChIP samples since there is always residual non-specific binding. The enrichment is quantified in comparison to the whole cell extract (total input control) samples and the negative controls ChIP (IgG isotype or beads alone) using the ΔΔCt method. Alternatively equal amounts of input and ChIP material can be used and the enrichment can be measured in comparison to the whole cell extract sample.

IV. ChIP-seq Sample Preparation (Guenther *et al*., 2008)

The samples can be prepared with the Illumina/Solexa Genomic DNA kit (Illumina-IP-102-1001) according to the manufacturer's instructions. Briefly 200 ng of input DNA and 50 ng of ChIP DNA are used. DNA overhangs are turned into phosphorylated blunt ends and the samples are purified with a Qiagen PCR purification kit. Sample preparation continues by the addition of a single A in the 3′ end to allow for directional ligation.

Samples are purified again and Illumina adapter oligonucleotides (1/100 dilution) are added to the samples followed by purification of the samples with the PCR purification kit. The samples are amplified by PCR (limited amplification to 18 cycles) that adds additional linker sequence to the fragments to prepare them for annealing to binding sites in the Genome Analyzer flow cell. The amplified samples are separated on a 2% agarose gel and products between 150 and 350 bp are selected and extracted from the gel (the products include fragments of 50–250 bp plus approximately 100 bp of primer sequence). All protocols for Illumina/Solexa sequence preparation, sequencing, and quality control are provided by Illumina (http://www.illumina.com/pages.ilmn?ID=203). Many other protocols are now available and the main steps are the same.

Different platforms can also be used such as the SOLID, or the Roche platform (Harismendy *et al*., 2009; Kircher and Kelso, 2010). Distinct experiments may require diverse sequencing platforms and thus unique sample preparations.

V. ChIP-seq Data Analysis

The analysis of ChIP-seq data includes five major steps: quality control, tag mapping, peak finding, motif finding, and annotation. This brief overview describes each step using Galaxy NGS Toolbox as a basis, and provides links to freely available software tools; although not detailed here, users can easily create and store a Galaxy workflow online. A more comprehensive summary of free analysis software is provided in the *Online Tools* box at the end of the chapter.

The first step, quality control, trims reads using QC scores and read lengths (e.g., Galaxy NGS Toolbox > QC & Manipulation). The second step maps short reads (aka tags) to an assembly of choice (e.g., Galaxy NGS Toolbox > Mapping & SAM Tools). For the zebrafish, consider setting input parameters to keep unique tags with no more than two mapped locations to account for gene duplications in the zebrafish genome. Third, peak finding (e.g., Galaxy NGS Toolbox > Peak Calling > MACS (Zhang *et al*., 2008)) is performed on the mapped tags for both background and ChIP lanes resulting in two tracks of genome-wide data – peak region boundaries (aka peaks) which appear as colored blocks on a browser, and a detailed signal of the pile-ups of mapped reads (aka wiggle). Manual inspection of biologically relevant regions is required at this point to assess overall quality of the results. Genomic sequences of statistically significant peaks are mapped to nearby genomic features, such as genes, promoter region, introns, and exons (e.g., Galaxy Operate on Genomic Intervals > Profile Annotations) to create a list of bound genes. Since proper annotation is of great importance, several other annotation tools should be considered (see *Online Tools* below). The peak locations output from peak finding are used to extract genomic sequence from the assembly of interest. A BED file is created by specifying a symmetric window around each peak (typically ±100 bp), and input to a sequence extraction algorithm (e.g., Galaxy Fetch Sequences > Extract Genomic DNA). The BED file is then input to the fourth step, motif finding (e.g., MEME Suite (Bailey and Elkan, 1994), GimmeMotifs (van Heeringen and Veenstra, 2011)). The raw output from motif finding algorithms needs to be validated using controls from published references. The motif information together with gene list are very useful in understanding the functional outcome

of DNA–protein interactions and in designing the next stage experiment to examine functions of specific protein–DNA interactions. Using the BED and WIG track data output from peak calling, genome browser tracks are then annotated and rendered (e.g., UCSC tools) on the browser of choice (e.g., they can be uploaded as custom tracks). Further, pathway- and network-level analysis can be performed (e.g., Cytoscape, Gene Ontology) to identify known and novel higher-level relationships among annotated bound entities. ChIP-seq tracks can also be compared to other HTseq experimental data in the rapidly expanding public repositories (e.g., 1000 genomes (Durbin *et al*., 2010), ENCODE (Birney *et al*., 2007)) using track comparison tools (e.g., ChromaSig (Hon *et al*., 2008), cisGenome (Ji *et al*., 2008)). Track comparison tools typically require loading your track data into a local database. The derived feature lists can then be used to perform genome-wide cross-species comparisons, and to integrate gene expression and other protein–DNA interaction data using established workflows (Hawkins *et al*., 2010; Won *et al*., 2009, 2010). These publicly available data can be further integrated to discover evolutionary conservations and help to narrow in specific DNA–protein interactions for further gene function studies. Molecular mechanisms underlying specific gene expression regulation and biological processes can then be derived and tested using genetics and molecular biology tools.

VI. Summary and Conclusions

This chapter describes a simple method for identifying transcription factor binding sites in a genome-wide manner by coupling chromatin immunoprecipitation to next generation sequencing. This method can be easily extended to other cell types besides adult erythrocytes. It is essential though, for results consistency, that the cell population tested will be as homogenous as possible. It should be also noted that the choice of antibody is crucial and that not all antibodies can be used successfully in ChIP although they may be used in other methods such as western blotting or immunofluoresence. The conditions of the experiment may need to be adjusted depending on the cell number, the abundance of the factor of interest and the available antibodies. In conclusion, this method can reveal the role of certain factors in a particular chromatin environment. In depth, bioinformatic analysis is essential for extracting the most information from this kind of experiments.

VII. Online tools

Quality control	
SolexaQA	http://solexaqa.sourceforge.net/
FastQC	http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/
Tag Mapping	
Bowtie	http://bowtie-bio.sourceforge.net/index.shtml
SOAP2	http://soap.genomics.org.cn/
Peak Finding	
DESQ	http://www-huber.embl.de/users/anders/DESeq/
USEQ	http://useq.sourceforge.net/
MACS	http://liulab.dfci.harvard.edu/MACS
Motif Finding	
GimmeMotifs	http://www.ncmls.eu/bioinfo/gimmemotifs/

The MEME Suite	http://meme.sdsc.edu/meme
Annotation.	
Affymetrix & NimbleGen gene expression microarray human-zebrafish orthologs	http://zfblast1.danio.tchlab.org/zgMap/ZGMAP.htm
Anno-J	http://www.annoj.org
DAVID	http://david.abcc.ncifcrf.gov
Gene Ontology	http://www.geneontology.org
GREAT	http://great.stanford.edu/public/cgi-bin/great.php
STRING	http://string-db.org
General	
Bioconductor / R	http://www.bioconductor.org/
BioMart	www.biomart.org
The Cancer Genome Atlas	http://tcga.cancer.gov
ChromaSig	http://bioinformatics-renlab.ucsd.edu/rentrac/wiki/ChromaSig
CEAS	http://ceas.cbi.pku.edu.cn
CisGenome	http://www.biostat.jhsph.edu/~hji/cisgenome
Cytoscape	http://www.cytoscape.org
ENCODE Project	http://www.genome.gov/10005107
Entrez Genome	http://www.ncbi.nlm.nih.gov/sites/genome
Ensembl	http://www.ensembl.org
EpiGRAPH	http://epigraph.mpi-inf.mpg.de/WebGRAPH/
Galaxy	http://galaxy.psu.edu
Gene Set Enrichment Analysis	http://www.broadinstitute.org/gsea
NCBI	http://www.ncbi.nlm.nih.gov
NextBio	http://www.nextbio.com
NPG	http://www.sanger.ac.uk/resources/software/npg/
Peak Analyzer	http://www.bioinformatics.org/peakanalyzer/wiki/
Roadmap Epigenomics Project	http://www.roadmapepigenomics.org
SEQanswers	http://seqanswers.com/wiki/Software/list
UCSC Genome Browser	http://genome.ucsc.edu

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