Published in final edited form as:

Methods Mol Biol. 2018 ; 1766: 239–256. doi:10.1007/978-1-4939-7768-0_14.

Analysis of chromatin interactions mediated by specific architectural proteins in *Drosophila* cells

Masami Ando-Kuri[#], I. Sarahi M. Rivera[#], M. Jordan Rowley, and Victor G. Corces

Department of Biology, Emory University, 1510 Clifton Rd NE, Atlanta, GA 30322, USA

[#] These authors contributed equally to this work.

Abstract

Chromosome conformation capture assays have been established, modified, and enhanced for over a decade with the purpose of studying nuclear organization. A recently published method uses *in situ* Hi-C followed by chromatin immunoprecipitation (HiChIP) to enrich the overall yield of significant genome-wide interactions mediated by a specific protein. Here we applied a modified version of the HiChIP protocol to retrieve the significant contacts mediated by architectural protein CP190 in *D. melanogaster* cells.

Keywords

Chromatin Immunoprecipitation; Chromatin Architecture; *in situ* Hi-C; HiChIP; Epigenetics; Transcription

1 Introduction

Genome-wide architectural landscapes can be generated using Hi-C [1]; this approach shares template creation with its chromosome conformation capture assay predecessors. The major difference is the use of biotinylated nucleotides to fill in the overhangs of restriction fragments, which are then ligated and pulled down to retrieve all the interactions across the genome. The ligation step is performed after nuclear lysis under diluted conditions, and this has been shown to abate the number of meaningful ligation junctions [2]. A variant of the regular Hi-C method is *in situ* Hi-C, which has the advantage of reducing the number of false contacts arising from ligation proximity [3]. Several variations of the Hi-C technology have been developed to map interacting regions associated with a protein of interest with high resolution. These techniques include Chromatin Interaction Analysis with Paired-End Tag (ChIA-PET) [4] and, more recently, HiChIP [5]. HiChIP combines *in situ* Hi-C (crosslinking, digestion, ligation and sonication) and chromatin immunoprecipitation with antibodies against a protein of interest to enrich for interactions mediated by this protein. Here we have adapted the HiChIP method to characterize interactions mediated by the architectural protein CP190 in *D. melanogaster* Kc167 cells.

⁷The beads can be directly transferred to a PCR tube. For easier manipulation in this protocol we use 1.5 mL tubes.

The protocol consists of fixation of DNA-DNA-protein interactions, lysis and digestion of the DNA, biotinylation and proximity ligation of the restriction fragments followed by DNA shearing and ChIP. Afterwards, the crosslinks between DNA and proteins are reversed, DNA is precipitated with ethanol and the ligation junctions containing biotin are pulled-down with streptavidin beads. Once the DNA is on the streptavidin beads, the ends of sheared DNA are repaired and biotin is removed from unligated ends. Final steps involve the preparation of an Illumina sequencing library with adaptors for paired-end sequencing and size selection to sequence the DNA of interest. Subsequent computational analysis and visualization of the data was done with Juicer and Juicebox [6,7]. Significant DNA looping interactions were detected using Capture Hi-C Analysis of Genomic Organization (CHiCAGO) [8].

CHiCAGO is a tool developed to erase bias associated to Capture Hi-C experiments. However, both Capture Hi-C and HiChIP rely on the enrichment of interactions linked to specific regions of the genome. Therefore, the data from these two techniques share similar properties, making CHiCAGO a suitable tool for HiChIP data analysis. CHiCAGO performs larger numbers of tests at regions with smaller number of expected interactions and a background correction with a model that accounts for expected interactions and sequencing artifacts to detect significant interactions [8]. Specific software to account for biases in HiChIP data is not yet available, therefore tools developed for the analysis of ChIA-PET data, such as Mango [14] or MICC [15], may be just as effective as CHiCAGO at identifying significant interactions. A comparison of these algorithms and the development of new computational methods are essential for the advancement of the field.

2 Materials

Deionized water is the solvent for all solutions unless otherwise noted. Prepare and store all reagents at room temperature unless indicated otherwise.

2.1 Crosslinking

1. *D. melanogaster* Kc167 cells [10]
2. SFX-Insect cell culture media (HyClone)
3. Formaldehyde (37%). Store in dark conditions.
4. 2.5 M glycine solution.

2.2 Lysis and restriction digest

1. Hi-C lysis buffer: 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% Igepal CA-630 (NP-40). Add 500 µL of 1 M Tris-HCl pH 8.0 stock solution, 100 µL of 5 M NaCl stock solution and 500 µL of 20% Igepal CA-630 (NP-40) stock solution in a 50 mL tube. Add water to a volume of 50 mL. Store at 4°C.
2. 25X Protease Inhibitor stock. Store at 4°C.
3. 0.5% SDS solution.
4. 10% Triton X-100 Stock solution.
5. 10X DpnII Reaction Buffer

6. 1X DpnII restriction enzyme (10,000 U/mL)

2.3 End-repair and biotinylation

1. Fill-in master mix: Add per tube 67.5 µL of water, 45 µL of 1 mM biotin-16-dCTP, 4.5 µL of 10 mM dTTP, 4.5 µL of 10 mM dATP, 4.5 µL of 10 mM dGTP and 24 µL of 5 U/µL DNA polymerase I Large (Klenow) fragment.
2. Ligation Master mix: Add per tube 2007 µL of water, 360 µL of 10x NEB T4 DNA Ligase buffer, 300 µL of 10% Triton X-100, 18 µL of 20 mg/mL BSA and 15 µL 400 U/µL T4 DNA Ligase.

2.4 Bead preparation

1. Magnetic beads with recombinant Protein A and Protein G
2. Blocking buffer: 0.5% BSA/PBS. Store at 4°C.
3. Pre-immune rabbit serum or immunoglobulin
4. Anti-CP190 antibody
5. IP Dilution Buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA pH 8.0, 16.7 mM Tris-HCl pH 8.0, 16.7 mM NaCl. Add 50 µL of 10% SDS stock solution, 5.5 mL of 10% Triton X-100 stock solution, 120 µL of 0.5 M EDTA pH 8.0 stock solution, 835 µL of 1 M Tris-HCl pH 8.0 stock solution and 1.67 µL of 1 M NaCl stock solution in a 50 mL tube. Add water to a volume of 50 mL. Store at 4°C.

2.5 DNA shearing

1. Nuclei Lysis Buffer: 50 mM Tris-HCl pH 8, 10 mM EDTA pH 8.0, 1% SDS. Add 2.5 mL of 1 M Tris-HCl pH 8.0 stock solution, 1 mL of 0.5 M EDTA pH 8.0 stock solution and 5 mL of 10% SDS stock solution in a 50 mL tube. Add water to a volume of 50 mL. Store at 4°C.
2. 25X Protease Inhibitor stock. Store at 4°C.
3. RNase A 10 mg/mL aliquot. Store at -20°C.
4. Proteinase K (20 mg/mL). Store at -20°C.
5. Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated with 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0. Use only in the laminar flow hood. Store at 4°C.
6. Glycogen aliquot. Store at -20°C.
7. 3 M sodium acetate solution.
8. 100% ethanol solution. Store at 4 °C.
9. 70% ethanol solution. Store at 4 °C.
10. 2% Agarose Gel. Weigh 1 g of Agarose for 50 mL of 1X TAE buffer, dissolve until solution is translucent for 3–5 min in microwave.

11. 1 X TAE Buffer: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0.
12. 6X Gel loading dye, no SDS
13. 100 bp DNA ladder
14. Sonicator

2.6 Chromatin Immunoprecipitation

1. IP dilution buffer (previously described in 2.4 Bead preparation section)
2. IP Wash Buffer Low salt: 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0, 150 mM NaCl. Add 500 μ L of 10% SDS stock solution, 5 mL of 10% Triton X-100 stock solution, 200 μ L of 0.5 M EDTA pH 8.0 stock solution, 1 mL of 1 M Tris-HCl pH 8.0 stock solution and 1.5 mL of 5 M NaCl stock solution in a 50 mL tube. Add water to a volume of 50 mL.
3. IP Wash Buffer High salt: 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 20 mM, Tris-HCl pH 8.0, 500 mM NaCl. Add 500 μ L of 10% SDS stock solution, 5 mL of 10% Triton X-100 stock solution, 200 μ L of 0.5 M EDTA pH 8.0 stock solution, 1 mL of 1 M Tris-HCl pH 8.0 stock solution and 5 mL of 5 M NaCl stock solution in a 50 mL tube. Add water to a volume of 50 mL.
4. LiCl Buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.25 M LiCl, 1% Igepal CA-630 (NP-40), 1% DOC. Add 500 μ L of 1 M Tris-HCl pH 8.0 stock solution, 100 μ L of 0.5 M EDTA pH 8.0 stock solution, 3.125 mL of 4 M LiCl stock solution, 2.5 mL of 20% Igepal CA-630 (NP-40) and 5 mL of 10% DOC stock solution in a 50 mL tube. Add water to a volume of 50 mL.
5. IP Elution Buffer: 0.1 M NaHCO₃, 1% SDS. Prepare at the time of use. Add 100 μ L of 1 M NaHCO₃, and 100 μ L of 10% SDS. Add water to a total volume of 1.5 mL.
6. 5 M NaCl solution.
7. 0.5 M EDTA pH 8.0 stock solution
8. Elution Buffer: 10 mM Tris-Cl, pH 8.5
9. Proteinase K (20 mg/mL). Store at -20°C .

2.7 Biotin/Streptavidin pull-down and Preparation for Sequencing

1. Low-binding 1.5 mL tubes
2. Magnetic beads with recombinant streptavidin. Store at 4°C .
3. Tween Wash Buffer (TWB): 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1 M NaCl, 0.05% Tween 20. Add 250 μ L of 1 M Tris-HCl pH 7.5 stock solution, 50 μ L of 0.5 M EDTA pH 8.0 stock solution, 10 mL of 5 M NaCl stock solution, 250 μ L of 10% Tween 20 stock solution in a 50 mL tube. Add water to a volume of 50 mL.

4. Binding buffer (2X): 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 2 M NaCl. Add 150 μ L of 1 M Tris-HCl pH 7.5 stock solution, 30 μ L of 0.5 M EDTA pH 8.0 stock solution, 6 mL of 5 M NaCl stock solution in a 15 mL tube. Add water to a volume of 15 mL.

2.8 Preparation of Illumina sequencing libraries

1. End-repair Master Mix: Add per tube 25 nM dNTPmix, 10 U/ μ L T4 Polynucleotide Kinase, 3 U/ μ L T4 DNA polymerase I, 5 U/ μ L DNA polymerase I large (Klenow) fragment
2. 1X NEB buffer 2. Store at -20°C
3. 10 nM dATP aliquot. Store at -20°C
4. Klenow Fragment ($3' \rightarrow 5'$ exo-) DNA Polymerase I (5,000 U/mL). Store at -20°C
5. 1X Quick ligase reaction buffer: Original stock is 2X concentration, dilute to reach 1X concentration.
6. Quick ligase enzyme
7. 2X KAPA SYBR FAST qPCR Kit aliquot. Store at -20°C
8. Refer to [11] for sequence information on adaptors and PCR primers.
9. Agencourt AMPure XP beads. Store at -20°C
10. 80% ethanol solution. Must be made before usage.

3 Methods

3.1 Crosslinking

1. Kc167 cells were grown to 80% confluence in SFX medium at 25°C . Use $\sim 100 \times 10^6$ as total number of cells.
2. In a 15 mL tube, pellet cells at 600 xg for 10 min at room temperature ($22-25^{\circ}\text{C}$). Discard supernatant.
3. Add 10 mL of SFX medium and pipette to resuspend cells.
4. Divide into two 15 mL tubes, 5 mL in each one.
5. Add formaldehyde to a final concentration of 1% (see Note 1).
6. Incubate 7 min at room temperature on a rocker.
7. Add glycine to a final concentration of 0.2 M.
8. Incubate 5 min at room temperature on a rocker.
9. Pellet cells at 300 xg for 5 min at 4°C .

¹ Handle in a fume hood. Commercial formaldehyde can be used up to 3 months from the date the bottle was opened.

10. Discard supernatant into an appropriate collection container (see Note 2).

3.2 Lysis and restriction digest

1. Add 1.5 mL of ice cold Hi-C lysis buffer. Mix by pipetting and move to a 2 mL tube.
2. Add 60 μ L of 25x Protease Inhibitor (PI).
3. Incubate on ice 20 minutes.
4. Pellet cells at 2500 xg for 5 min at 4°C and discard supernatant.
5. Add 1.5 mL of ice cold Hi-C lysis buffer. Mix by pipetting.
6. Pellet cells at 2500 xg for 5 min at 4°C and discard supernatant.
7. Add 150 μ L of 0.5% SDS and pipette to resuspend cells.
8. Incubate 5 min at 65°C.
9. Add 435 μ L of water and 75 μ L of 10% Triton X-100.
10. Incubate 15 min at 37°C.
11. Add 75 μ L of 10x DpnII buffer and 300 U of DpnII.
12. Digest overnight at 37°C.

3.3 End repair and biotinylation

1. Incubate digest reaction at 65°C for 10 min to inactivate DpnII.
2. Cool to room temperature.
3. Add 150 μ L of the Fill-in Master Mix.
4. Mix by pipetting and incubate at 37°C for 45 min.
5. Pass the sample to a 15 mL tube.
6. Add 2700 μ L of Ligation Master Mix to the sample.
7. Mix by inverting and incubate 4 hr at room temperature with gentle rocking.

3.4 Bead preparation

Prepare Pre-clear and Antibody beads during the 1.5 hr biotinylation incubation.

1. Pre-Clear beads: Add 10 μ L of protein A beads and 10 μ L of protein G beads in a 2 mL clean tube.

Antibody beads: Add 20 μ L of protein A beads and 20 μ L of protein G beads in a 2 mL clean tube.

2. Wash step:

².After freezing in liquid nitrogen either proceed to “Lysis and Digest” or store cell pellet at –80°C and flash freeze in liquid nitrogen

- 2.1 Add 1 mL of Blocking Buffer to each tube.
- 2.2 Rotate for 5 min at room temperature.
- 2.3 Collect on magnet stand.
- 2.4 Remove supernatant.
3. Repeat wash step 2 more times.
4. Add 500 μ L of Blocking Buffer to each tube.
5. Pre-Clear beads: Add 10 μ L of pre-immune rabbit serum (or IgG).

Antibody beads: The amount of antibody depends on its efficiency.

6. Incubate the beads at 4°C on rotator for at least 4 hr (see Note 3).
7. Collect on magnet stand and remove supernatant.
8. Wash with Blocking Buffer:
 - 8.1 Add 1 mL of Blocking Buffer to each tube.
 - 8.2 Rotate for 2 min at room temperature.
 - 8.3 Collect on magnet stand.
 - 8.4 Remove supernatant.
9. Wash with IP Dilution Buffer.
 - 9.1 Add 1 mL of ice cold IP Dilution Buffer.
 - 9.2 Rotate for 2 min at room temperature.
 - 9.3 Collect on magnet stand.
 - 9.4 Remove supernatant.
10. Repeat wash with IP Dilution Buffer.
11. Add 50 μ L of ice cold IP Dilution Buffer.

Beads are ready for steps in **ChIP** section.

3.5 DNA shearing

1. Pellet nuclei from ligation reaction at 1503 xg for 8 min at 4°C and discard supernatant.
2. Add 1.5 mL of ice cold Nuclei Lysis Buffer. Mix by pipetting and move to a 15 mL tube.
3. Add 60 μ L of 25x PI.
4. Incubate in ice for 15 min.

³Incubate “Pre-Clear beads” 15 min before the end of DNA shearing. Incubate “Antibody beads” 15 min before the end of “Chromatin and Pre-Clear incubation” in section 3.6.

5. Shear DNA to obtain DNA fragments between 250–350 bp (see Note 4 and 5).
6. Pellet cell debris at 21130 xg for 10 min at 4°C.
7. Transfer supernatant (chromatin) from 15 mL tube to a 1.5 mL clean tube.
8. Verify sonication quality (see Note 6):
 - 8.1 Add 15 µL of post-shear supernatant in a 2 mL clean tube.
 - 8.2 Add 185 µL of IP Dilution Buffer
 - 8.3 Add 0.5 µL of 10 mg/mL RNaseA
 - 8.4 Reverse cross link by heating to 95°C for 5 min.
 - 8.5 Add 4 µL of 10 mg/mL proteinase K.
 - 8.6 Incubate at 50°C for 2 hr.
 - 8.7 Extract DNA with Phenol:Chloroform:Isoamyl alcohol (P:C:I, 25:24:1)
 - 8.7.1 Add 1 volume of P:C:I (~205 µL) and vortex well.
 - 8.7.2 Spin at 21130 xg for 5 min at 4°C.
 - 8.7.3 Transfer aqueous (top) layer to a 2 mL clean tube.
 - 8.8 Add 1 µL of 20 mg/mL glycogen.
 - 8.9 Add 1/10 volume of 3 M Sodium Acetate.
 - 8.10 Add 2.5 volume of ice cold 100% Ethanol.
 - 8.11 Incubate 30 min at –80°C.
 - 8.12 Pellet DNA at 21130 xg for 10 min at 4°C and discard supernatant.
 - 8.13 Add 1 mL of 70% Ethanol.
 - 8.14 Pellet DNA at 21130 xg for 10 min at 4°C and discard supernatant.
 - 8.15 Let pellet air dry (5–10 min).
 - 8.16 Add 10 µL of water and resuspend the pellet.
 - 8.17 Run on a 2% agarose gel. Target is 250–300 bp.

3.6 Chromatin Immunoprecipitation

1. Add the sample from step 7 in DNA Shearing section to pre-clear beads.
2. Incubate 2 hr at 4°C with rotation.

⁴. We perform DNA shearing in a Diagenode Bioruptor 300 with the following parameters: Intensity: High; ON: 30 sec; OFF: 60 sec; Cycles: 28–30; Temperature: 4°C water bath.

⁵. Tn5 transposase can be used to construct the library [5]. The shearing step must be modified to obtain larger fragments (400–1000 bp), otherwise fragmentation will decrease size of fragments limiting the amplification of the library (described in 3.8 step of this protocol). End-repair, addition of dATP and ligation of the adaptor (1–16 steps in section 3.8 Preparation of Illumina sequencing libraries) will be skipped in order to construct the library using Tn5 transposase. Next steps should be performed normally.

⁶. Either proceed to verify sonication quality or store 15 µL of post-shearing supernatant from each 2 mL tube at –4°C overnight for later verification.

3. Take 75 μ L out to use as “input” reference. Can store at 4°C overnight.
4. Place the remaining solution on a magnet stand.
5. Move supernatant to tube with antibody coated beads.
6. Incubate at 4°C overnight with rotation.
7. Place on magnet stand and remove supernatant.
8. Add 1 mL of Low Salt Wash Buffer with PI freshly added. Mix by pipetting and move onto a 1.5 mL tube.
9. Incubate 5 min at room temperature with rotation.
10. Washes: Place on magnet stand and remove supernatant; add 1 mL of Wash Buffer with PI to a final concentration of 1x; mix by inverting.
- 10.1 Wash with Low Salt Wash Buffer 2 more times.
- 10.2 Wash with High Salt Wash Buffer 2 times.
- 10.3 Wash with LiCl Buffer 2 times.
- 10.4 Wash with TE buffer 1 time.
11. Place on magnet stand and remove supernatant.
12. Elution step:
 - 12.1 Add 400 μ L of fresh IP elution buffer.
 - 12.2 Vortex for 2 seconds.
 - 12.3 Incubate 15 min at 68 °C and vortex 2 seconds each 2 minutes.
 - 12.4 Centrifuge at 21130 xg for 1 min at room temperature.
 - 12.5 Place on magnet stand.
13. Move supernatant to a clean tube.
14. Add 20 μ L of 5 M NaCl, 8 μ L of 0.5 M EDTA pH 8.0 and 16 μ L of 1 M Tris-HCl pH 8.0.
15. Incubate for 6 hr at 68°C to reverse cross-link.
16. Add 8 μ L of proteinase K.
17. Incubate at 50°C for 1 hr.
18. Cool to room temperature.
19. Extract DNA with Phenol:Chloroform:Isoamyl alcohol (P:C:I, 25:24:1)
 - 19.1 Add 400 μ L volume of P:C:I and vortex well for one minute.
 - 19.2 Spin at 21130 xg for 5 min at 4°C.
 - 19.3 Transfer aqueous (top) layer to a 1.5 mL clean tube.
20. Precipitate the DNA:

- 20.1 Add 1 μ L of 20 mg/mL glycogen.
- 20.2 Add 40 μ L volume of 3 M Sodium Acetate.
- 20.3 Add 1 mL of ice cold 100% Ethanol.
- 20.4 Incubate 20 min at -80°C . Can go overnight.
22. Pellet DNA at 21130 xg for 20 min at 4°C and discard supernatant.
23. Add 1 mL of 70% ethanol.
24. Pellet DNA at 21130 g for 5 minutes at 4°C and discard supernatant.
25. Let pellet air dry (5–10 min).
26. Add 21 μ L of Elution Buffer and resuspend the pellet.
27. Incubate at 37°C for 5 min to make sure pellet is properly dissolved.
28. Quantify the amount of DNA on a spectrophotometer.
29. Bring volume up to 100 μ L with Elution Buffer.

3.7 Biotin/Streptavidin pull-down and Preparation for Sequencing

Perform remaining steps in 1.5 mL low-binding tubes

1. Add 400 μ L of Tween Wash Buffer (TWB) to a clean tube.
2. Add 20 μ L of MyOne Streptavidin T1 beads for every 5 μ g of DNA.
3. Mix by inverting.
4. Separate on magnet stand and discard supernatant.
5. Add 400 μ L of TWB.
6. Mix by inverting.
7. Separate on magnet stand and discard supernatant.
8. Add 100 μ L of 2x Binding Buffer to resuspend the beads and add to the sample from step 29 in section 3.6 Chromatin Immunoprecipitation.
9. Incubate at room temperature for 20 min with rotation.
10. Separate on magnet stand and discard supernatant.
11. Wash with TWB:
 - 11.1 Add 600 μ L of TWB
 - 11.2 Mix by inverting.
 - 11.3 Separate on magnet stand and discard supernatant.
12. Repeat wash with TWB one more time.

3.8 Preparation of Illumina sequencing libraries

1. Add 100 μ L of 1x T4 ligase buffer and resuspend beads by pipetting. (see Note 5)
2. Separate on magnet stand and discard supernatant.
3. Resuspend beads in 100 μ L of End-repair Master Mix.
4. Pipette gently to mix.
5. Incubate 30 min at room temperature with rotation.
6. Spin. Separate on magnet stand and discard supernatant.
7. Wash beads two times with TWB as before.
8. Add 100 μ L of 1x NEB Buffer 2 and resuspend beads.
9. Separate on magnet stand and discard supernatant.
10. Add 100 μ L of dATP Master Mix and resuspend by pipetting.
11. Incubate for 30 min at 37°C with rotation.
12. Spin. Separate on magnet stand and discard supernatant.
13. Wash beads two times with TWB as before.
14. Add 100 μ L of 1x Quick ligase buffer and resuspend beads.
15. Separate on magnet stand and discard supernatant.
16. Add 50 μ L of 1x Quick Ligase Buffer and resuspend beads.
17. Add 2 μ L of Quick Ligase and 2 μ L of the Illumina indexed adapter at 10 mM.
18. Incubate at room temperature for 15 min with rotation.
19. Spin. Separate on magnet stand and discard supernatant.
20. Wash beads two times with TWB as before.
21. Add 100 μ L of Elution Buffer and resuspend beads.
22. Separate on magnet stand and discard supernatant.
23. Add 50 μ L of Elution Buffer and resuspend beads.
24. Separate on magnet stand and discard supernatant.
25. Add 23 μ L of Elution Buffer and resuspend beads. Amplify the library directly on the beads
26. Transfer the beads to a PCR tube.
27. Add 25 μ L of Kapa SYBR FAST qPCR Kit Master Mix (2x), 2 μ L of Illumina primers.
28. PCR parameters:
 - 28.1 Initial Denaturation 3 min at 98°C.

- 28.2 PCR parameters: Denaturation 15 sec at 98°C; Annealing 30 sec at 60°C; Extension 45 sec at 72°C.
29. Run a total of 5 PCR cycles on a thermocycler.
30. Separate on a magnet stand and move reaction (supernatant) to a clean real-time PCR (qPCR) tube.
- 30.1 Add more SYBR Green to a final concentration of 1x
31. qPCR parameters:
 - 31.1 Initial Denaturation 30 sec at 98°C.
 - 31.2 Cycle parameters: Denaturation 15 sec at 98°C; Annealing 30 sec at 60°C; Extension 45 sec at 72°C.
32. Run 4–6 cycles while monitoring on qPCR (see Note 8).
33. Move liquid from qPCR tube to a 1.5 mL clean tube.
34. Add 1.1x volumes of AMPure XP beads.
35. Incubate 5 min at room temperature on a rocker.
36. Separate on magnet stand and discard supernatant.
37. Without removing from magnetic stand, add 200 µL of 80% ethanol.
38. Wait for 30 seconds and discard ethanol.
39. Repeat last two steps.
40. Let pellet air dry (5–10 minutes).
41. Add 15 µL of Elution Buffer and resuspend the DNA.
42. Incubate 1 min at room temperature.
43. Separate on magnet stand and move the supernatant to a clean tube.
44. Quantify the amount of DNA on a spectrophotometer.
45. Send the library to sequencing by service provider for quality assessment and high throughput sequencing.

3.9 Data analysis

Most of the pipelines were ran through the terminal in a Linux-based system.

1. Set up
 - 1.1 Install Juicer software from <http://aidenlab.org/juicer/>.
 - 1.2 Install Juicebox software from <http://aidenlab.org/juicebox/>.

⁸.Stop after “Cycle Extension” and before “Cycle Denaturation”. Continue with cycles until a plateau is reached. Do not exceed 4–6 cycles (for a total of 10–12 PCR plus qPCR cycles).

- 1.3 Install R, download source file from <https://cran.r-project.org/mirrors.html> and choose the closest mirror to your location.
- 1.4 Download CHiCAGO from <https://bitbucket.org/chicagoTeam/chicago/downloads>.
2. Read mapping and read-pair level filtering: Juicer (see Note 9)
 - 2.1 Create a directory called “fastq” and move sequence fastq files to this directory.
 - 2.3 Change the name of the files for each of the two mates obtained from paired-end sequencing. Each mate should have the name of the sample followed by either “R1” or “R2” to distinguish between the two mates. For example, sample_R1.fastq and sample_R2.fastq (see Note 10).
 - 2.4 Go to the directory that contains the Juicer program “juicer.sh”
 - 2.5 Set the following parameters for Juicer and hit enter (see Note 11).
 - g genome: e.g. dm6
 - d path for “fastq” directory of the sample: e.g. /home/user/exp/
 - s restriction enzyme used: e.g. DpnII
 - p path for chromosome sizes file. This file should have two columns, the first is the name of the chromosome and the second one the length of the chromosome.
 - y enter path for restriction site file (locations of restriction sites in genome). This contains each chromosome and the positions of the cutting sites by the previously designated restriction enzyme in a row separated by a space, the last position of each row should be the end of that chromosome.
 - z enter path for sequence of the reference genome file in fasta format; the BWA index file must be in the same directory.

We use the following command:

```
$ juicer.sh -g dm6 -d /home/user/exp/ -s DpnII -p /home/user/exp/chromosome-sizes.txt -y /home/user/exp/restriction-sites.txt -z /home/user/exp/reference-sequence
```

- 2.6 To verify the success of the alignment, consult that the “aligned” directory from the Juicer output contains the “inter.hic” file (see Note 12).

⁹. Paired-end sequencing is performed on the sample; thus, the sequencing data is obtained in two fastq files.

¹⁰. Steps 1–2 can be done in any order. Also, it is possible to compress the fastq files, Juicer can work with both, compressed and uncompressed fastq files.

¹¹. Use the flags described below followed by the option chosen. Additional options are available, use ./juicer.sh -h to see additional information. The output from Juicer will contain the following directories: “aligned”, “errors”, “fastq” and “splits”.)

¹². For deeper sequencing it is advisable to check the duplication rate and determine the number of usable reads. This information can be consulted in the inter.txt file located inside of the directory called “aligned” in the output files. We recommend verifying that the “PCR Duplicates” is below 7% and that the “Hi-C Contacts” is above 40%

3. Normalization to account for experimental bias and extracting significant contacts: Juicebox and CHiCAGO
 - 3.1 Use the “juicebox dump” tool to retrieve a contact map. Set the following parameters and hit enter. (see Note 13 and 14)
 - Type of normalization: NONE (see Note 15).
 - Path of the .hic file: e.g. sample/aligned/inter.hic
 - Name of the first chromosome: either 2L, 2R, 3L, 3R, 4, X, Y.
 - Name of the second chromosome: either 2L, 2R, 3L, 3R, 4, X, Y.
 - Resolution desired: e.g. BP 5000 (see Note 16)
 - Name of output file: e.g. sample_firstchr_secondchr_obst.txt
 - 3.2 Create design files for CHiCAGO using dumped files from Juicer (see Note 17).
 - 3.2.1 Create a directory called “designDir”
 - 3.2.2 Create the .rmap file. This is a BED file with the positions of the bin with the columns chr, start, end, and fragmentID (see Note 18 and 19).
 - 3.2.3 Create the .baitmap file. This is also a BED file containing the bin with a bait, defined as the protein that is immunoprecipitated. It contains 5 columns: chr, start, end, fragmentID, baitAnnotation (see Note 20).
 - 3.2.4 Run `Chicago/chicagoTools/makeDesignFiles.py` to create .nbpb .poe and .npb files. Set the following parameters and hit enter (see Note 21).
 - designDir= path to the “designDir” directory
 - rmapfile= path to the .rmap file
 - baitmapfile=path to the .baitmap file
 - 3.3 Create .chinput file based on the juicebox dumped files. This 5-column file contains the contact frequency. The format is: baitID, otherendID, N, otherEndLen and distSign; where otherendID is the ID of the fragment or bin that is in the interacting pair of the bait, N is the number of reads detected for

¹³Each parameter should be separated by a space; no use of flags is required.

¹⁴The Juicebox dumped file is done per chromosome.

¹⁵As we will normalize the data with CHiCAGO, retrieve raw data. Vanilla Coverage (VC), square root of vanilla coverage (VC_SQRT) and Knight-Ruiz/Balanced normalization (KR) are available normalization options, but are not recommended to be used with this method.

¹⁶To choose the resolution of the contact map you can select the size of the bin determining the base pair resolution with “BP” and designate it from these options: 2500000, 1000000, 500000, 250000, 100000, 50000, 25000, 10000, 5000; or you can specify the number of fragments by using “FRAG” followed by any of these options: 500, 200, 100, 50, 20, 5, 2, 1.

¹⁷Prior to proceeding, it is highly recommended to read the Vignette explaining the files required to run CHiCAGO: http://regulatorygenomicsgroup.org/wp-content/uploads/Chicago_vignette.html

¹⁸We created a 1 kb fragment resolution file and assigned sequential numbers as fragmentIDs (e.g. 2R 1 1000 1).

¹⁹The dumped data from juicebox can also include restriction fragment resolution which can be used to create the input files for CHiCAGO.

²⁰To obtain the baits, we overlapped the positions from a CP190 ChIP-seq to the .rmap previously described. The baitAnnotation was assigned as the protein name with sequential numbers (e.g. 2L 65000 66000 66 CP190_1).

²¹Use the flags described below followed by the option chosen. Make sure .nbpb .poe and .npb are inside the “designDir” directory. To see additional parameters, use: `python makeDesignFiles.py`.

ligation products between the “bait” and “other end”, otherEndLen is the length of the “other-end” bin and distSign is the linear distance between the bait and other-end fragments, respectively [9] (see Note 22).

- 3.4 Run CHiCAGO following the commands in http://regulatorygenomicsgroup.org/wp-content/uploads/Chicago_vignette.html
4. Visualization: Juicebox
 - 4.1 Raw data (see Note 23).
 - 4.1.1 The input file for visualization in Juicebox is inter.hic located inside the “aligned” directory.
 - 4.1.2 Open Juicebox
 - 4.1.3 Click on File>Open>Local and then choose the .hic file.
 - 4.2 CHiCAGO’s significant interactions: There are two ways to visualize CHiCAGO interactions. To visualize the data as a .hic matrix in juicebox go to 4.23, For an annotation track go to 4.27.
 - 4.2.1 CHiCAGO’s output in the designated directory contains the following directories: “data”, “diag_plots”, “enrichment_data examples”
 - 4.2.2 Enter “data” directory and find CP190_CHICAGO_washU_text.txt.
 - 4.2.3 Create .hic file using “juicebox pre” tool from CP190_CHICAGO_washU_text.txt file. to create a proper “juicebox pre” input file. It should be an 11-column file with the following format: readname, str1, chr1, pos1, frag1, str2, chr2, pos2, frag2, mapq1, mapq2; where str can be set to 0 for forward, anything else for reverse [7] (see Note 24) (e.g. 1 0 2L 73000 74000 0 2L 84000 85000 50 50)
 - 4.2.4 Set the parameters of “juicebox pre” tool and hit enter (see Note 25).
 - Pairwise Interaction BED file: CP190_CHICAGO_washU_text_5.txt
 - Output name: cp190_chicago.hic
 - Chromosome size file: path for chrom.sizes file. This file should have two columns, the first one is the name of the chromosome and the second one is the length of the chromosome.
 - Restriction map of *D.melanogaster* genome: enter path for restriction site file (locations of restriction sites in genome). This contains each

²²-To create the .chinput file, one must parse dumped files from Juicebox and use a script to generate it. We used a python script for this. The “otherEndLen” column is always 1000 because we set 1 kb resolution for the bin size fragment file. Additionally, CHiCAGO is capable of using several .chinput files as replicates. However, only one replicate for CP190 was processed in this analysis.

²³-Juicebox is capable of doing the normalization described in Lieberman-Aiden *et al.* 2009 [1] and KR matrix balancing [13], as well as other statistics. However, the normalization methods implemented do not account for HiChIP specific biases. For this reason, Juicebox is only used to visualize the data.

²⁴-We did not filter by MAPQ; 50 was used as a default number for mapq1, mapq2. Bash pipelines were used to generate this file.

²⁵-Separate each parameter by one space only; no use of flags required.

chromosome and its restriction positions by the previously designated enzyme in a row separated by a space.

4.2.5 Open Juicebox

4.2.6 Click on File>Open>Local and then choose the CP190_chicago.hic file.

4.2.7 Modify CP190_CHICAGO_washU_text.txt file to create a proper 2D annotation input file. It should be a 4-column file with the following format: readname, chr1, pos1, pos2, chr2, pos3, pos4, score (e.g. 2L 73000 74000 2L 2569000 2570000 35.6913535863312)

4.2.8 Open Juicebox

4.2.9 Open .hic file of the raw data. Click on File>Open>Local and then choose the CP190_raw.hic file.

4.2.10 Load the significant interactions called by CHiCAGO using the modified version of the CP190_CHICAGO_washU_text.txt file. Click on Annotations>Load Basic Annotations>Dataset-specific 2D-features>Add 2D and select local file.

Acknowledgements

This work was supported by U.S. Public Health Service Award R01 GM035463 from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

- [1]. Lieberman-Aiden E, Van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, et al. (2009). Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *science*, 326(5950), 289–293. [PubMed: 19815776]
- [2]. Gavrilov AA, Golov AK, & Razin SV (2013). Actual ligation frequencies in the chromosome conformation capture procedure. *PLoS One*, 8(3), e60403. [PubMed: 23555968]
- [3]. Rao SS, Huntley MH, Durand NC, Stamenova EK, Bochkov ID, Robinson JT, et al. (2014). A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. *Cell*, 159(7), 1665–1680. [PubMed: 25497547]
- [4]. Li G, Cai L, Chang H, Hong P, Zhou Q, Kulakova EV, et al. (2014). Chromatin interaction analysis with paired-end tag (ChIA-PET) sequencing technology and application. *BMC genomics*, 15(Suppl 12), S11.
- [5]. Mumbach MR, Rubin AJ, Flynn RA, Dai C, Khavari PA, Greenleaf WJ, & Chang HY (2016). HiChIP: Efficient and sensitive analysis of protein-directed genome architecture. *Nature Methods*.
- [6]. Durand NC, Shamim MS, Machol I, Rao SS, Huntley MH, Lander ES, & Aiden EL (2016). Juicer provides a one-click system for analyzing loop-resolution Hi-C experiments. *Cell Systems*, 3(1), 95–98. [PubMed: 27467249]
- [7]. Durand NC, Robinson JT, Shamim MS, Machol I, Mesirov JP, Lander ES, & Aiden EL (2016). Juicebox provides a visualization system for Hi-C contact maps with unlimited zoom. *Cell Systems*, 3(1), 99–101. [PubMed: 27467250]
- [8]. Cairns J, Freire-Pritchett P, Wingett SW, Várnai C, Dimond A, Plagnol V, Zerbino D, et al. (2015). CHiCAGO: robust detection of DNA looping interactions in Capture Hi-C data. *Genome biology*, 17(1), 127

- [9]. Cairns J, Freire-Pritchett P, Wingett SW, Várnai C, Dimond A, Plagnol V, Zerbino D, et al. (2015). CHiCAGO: robust detection of DNA looping interactions in Capture Hi-C data. *Genome biology*, 17(1), 127
- [10]. Cherbas P, Cherbas L, Lee SS, Nakanishi K (1988). 26-¹²⁵I-iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc. Natl. Acad. Sci. U.S.A.* 85(): 2096–2100. [PubMed: 3127825]
- [11]. Bowman SK, Simon MD, Deaton AM, Tolstorukov M, Borowsky ML, Kingston RE (2013) Multiplexed Illumina sequencing libraries from picogram quantities of DNA. *BMC Genomics* 14:466. doi: 10.1186/1471-2164-14-466 [PubMed: 23837789]
- [12]. Cairns Jonathan, Freire-Pritchett Paula, Spivakov Mikhail (2016) CHiCAGO Vignette regulatorygenomicsgroup.org/wp-content/uploads/Chicago_vignette.html#example-workflow
- [13]. Knight PA, & Ruiz D (2012). A fast algorithm for matrix balancing. *IMA Journal of Numerical Analysis*, drs019.
- [14]. Phanstiel DH, Boyle AP, Heidari N, & Snyder MP (2015). Mango: a bias-correcting ChIA-PET analysis pipeline. *Bioinformatics*, 31(19), 3092–3098. [PubMed: 26034063]
- [15]. He C, Zhang MQ, & Wang X (2015). MICC: an R package for identifying chromatin interactions from ChIA-PET data. *Bioinformatics*, 31(23), 3832–3834. [PubMed: 26231426]

Figure 1.

Significant interactions associated with CP190 retrieved by CHiCAGO.

The upper and the lower triangle represent Hi-C and HiChIP raw data respectively with a 5 kb bin resolution. The black dots depict significant interactions obtained from CHiCAGO. The axes show the annotated genes for *D. melanogaster* and CP190 ChIP-seq peaks in this region. Figure was created using Juicebox v1.5.