In situ* Hi-C in *Arabidopsis thaliana

Materials, reagents and equipment

Prepare all solutions with double-distilled water or milliQ water and store at room temperature (RT) unless otherwise specified. Use freshly prepared solutions. Handling of the sample along the protocol needs to be done precisely as described.

Plant material

Use approximately 2 gr of freshly harvested tissue.

Cross linking of plant tissue

- Nuclei isolation buffer (NIB-FA) with formaldehyde (FA): 20 mM HEPES pH 8.0, 250 mM sucrose, 1 mM Magnesium Chloride ($MgCl_2$), 5 mM Potassium Chloride (KCl), 40% (v/v) glycerol, 0.25% (v/v) Triton X-100, 2% (v/v) formaldehyde, 0.1 mM Phenylmethylsulfonyl Fluoride (PMSF), and 0.1% (v/v) 2-mercaptoethanol (2-ME). Add PMSF, 2-ME and formaldehyde under the fume hood prior to use.
- During the preparation of NIB-FA, once all the components are added, vortex thoroughly, and chill the solution on ice. PMSF is unstable in water. Due to the short half-life of 35 min at pH 8.0, PMSF must be added just prior to use. Formaldehyde slowly oxidizes to formic acid under normal atmospheric oxygen concentrations, therefore rather use fresh high-quality ampule-sealed formaldehyde solution. Poor-quality formaldehyde will adversely affect the experiment. Store formaldehyde at room temperature, since a precipitate of triozymethylene may form at low temperatures. Glycerol is viscous, it sticks to the pipette tips leading to inaccurate measurement of quantities. Alternatively, 75.6 g of glycerol (60ml x 1.26 g/cm^3) can be weighted using a balance scale instead of measuring 60 ml.
- 2 M glycine: store protected from light in a 50 ml tube at 4 ^{o}C. Filter and sterilize.
- Desiccator connected to vacuum pump with manometer and condensation trap.
- 50 ml tubes.
- Self-made 0.5-1.0 cm thick polystyrene plugs that fit 50 ml tubes.
- 10 ml pipettes.
- Paper towels.
- Fume hood: all steps involving NIB-FA buffer must be performed under a fume hood.

Nuclei isolation

- Liquid nitrogen.
- Vessel for transport and storage of liquid nitrogen.
- Mortar with pestle.
- Metal spoon.
- Deep freezer (-80 ^{o}C)
- 50 ml tubes.
- Funnels fitting into 50 ml tubes.
- Miracloth (Merck Millipore, 22 – 25 um).
- 10 ml pipettes.
- cOmplete EDTA-free Inhibitor Cocktail (Roche), prepare a 25X stock solution by dissolving one tablet in 2 ml water, can be stored up to 12 weeks at -20 ^{o}C.
- Nuclei isolation buffer with protease inhibitor (NIB-P): 20 mM HEPES pH 8.0, 250 mM sucrose, 1 mM $MgCl_2$, 5 mM KCl, 40 % (v/v) glycerol, 0.25% (v/v) Triton X-100, 0.1 mM PMSF, 0.1 % (v/v) 2-ME. NIB should be supplemented with 1 ml of 25X stock solution of cOmplete EDTA-free Inhibitor Cocktail (Roche) for each 25 ml of NIB. Add PMSF, 2-ME under the fume hood prior to use.
- Prepare NIB-P adding the reagents in the order listed above, and add PMSF, 2-ME and the cOmplete Inhibitor Cocktail prior to use. Mix the solution thoroughly using a magnetic stirrer and chill on ice before use. Glycerol is viscous, it sticks to the pipette tips leading to inaccurate measurement of quantities. Alternatively, 75.6 g of glycerol (60ml x 1.26 g/cm^3) can be weighted using a balance scale instead of measuring 60 ml.
- Refrigerated centrifuge for 50 ml tubes.
- 1.5 and 2.0 ml microtubes.
- Refrigerated centrifuge for microtubes.

Restriction enzyme digestion, overhang fill-in and ligation

- DpnII, 50 U/ul (NEB).
- 10X RE buffer: 1 M NaCl, 500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM 1,4-Dithiothreitol (DTT).
- Thermomixer.
- dNTPs: 10 mM dCTP, 10 mM dTTP, and 10 mM dGTP. Diluted from 100 mM stocks and stored at -20 ^{o}C.
- 0.4 mM biotin-14-dATP (Life Technologies). Stored at -20 ^{o}C.
- DNA Polymerase I Large (Klenow) Fragment (NEB).
- Tube rotator.
- 0.5% Sodium dodecyl sulfate (SDS).

- 10% Triton X-100. Prepare fresh.
- Blunt-end ligation buffer (10x): 400 mM Tris–HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, and 5 mM ATP.
- T4 DNA ligase (Thermo Fisher Scientific). Stored at -20 ^{o}C.
- Cabinet incubator at 16 ^{o}C.

Reversal of cross-linking and DNA extraction

- SDS lysis buffer: 50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, and 1% (v/v) SDS.
- 5 M NaCl: Dissolve 29.22 g NaCl in 80 ml water. Then make up the, then adjust volume to 100 ml.
- 20 mg/ml RNAse A..
- Thermomixer.
- 20 mg/ml Proteinase K. Stored at -20 ^{o}C.
- Phenol-chloroform-isoamyl alcohol (25:24:1). Stored at 4 ^{o}C.
- Chloroform.
- 5 mg/ml glycogen or glycoblue. Dilute 10 ul 20 mg/ml glycogen in 30 ul water, and store at -20 ^{o}C.
- Ice-cold 100% ethanol.
- 70% (v/v) ethanol.
- 3 M sodium acetate pH 5.2.
- EB buffer: 10 mM Tris-HCl pH 8.0. Dilute 500 ul 1 M Tris-HCl pH 8.0 in 49.5 ml water.
- NanoDrop spectrophotometer.
- 6X loading dye.
- 1 kb plus DNA ladder.
- Agarose.
- Gel electrophoresis chamber and accessories.

Methods

Plant growing and harvesting

1. Wild type seeds are stratified on MS plates in the dark at 4 ^{o}C for at least 2 days.
2. For seedling tissue (also for roots and leaves), transfer plates to a growth cabinet with daily cycles of 16 h light at 21 ^{o}C and 18 h darkness at 18 ^{o}C. After germination plants are grown for 8 days and subsequently harvested.
3. Collect 2 to 3 g of freshly harvested tissue.

Plant tissue fixation (Day 1)

- Prepare 15 ml of NIB without formaldehyde (FA).
- Prepare 15 ml of NIB-FA.
- Ice-cold four 50 ml tubes, per sample.

1. Prepare NIB without FA (15 ml per sample), prior to use add PMSF and 2-ME. Mix by vortex and store on ice.
2. Prepare NIB-FA (15 ml per sample), prior to use add PMSF, 2-ME and 4% (v/v) FA.
3. Harvest samples and transfer to a new pre-cooled 50 ml tube, store on ice.
4. Add 15 ml ice-cold NIB. Mix the contents gently by swirling with a pipette.
5. Add 15 ml of NIB-FA to the sample.
6. Plug the tube containing tissue NIB/NIB-FA solution with self-made polystyrene plugs.
7. Vacuum infiltrate the tissue for 1 h in vacuum desiccator. Cut the vacuum every 15 min to facilitate the entering of fixative into the tissue.
8. During the first 15 min of vacuum infiltration, take out 2 M glycine from 4 ^{o} C, and store it on ice.
9. At the end of 1h vacuum infiltration, remove polystyrene plug. Add 2 ml 2 M glycine to stop the cross-linking reaction. Mix carefully by pipetting up and down few times with 10 ml pipette.
10. Insert polystyrene plugs and vacuum infiltrate for 5 min at RT. Chlorophyll rich tissues change color from light green to dark green. This is a *control* step to check for efficient cross-linking.

    Fixed chlorophyll rich tissues appear dark green in color compared to the initial tissue used for fixation. If darkening is not observed, the fixative might not have entered completely, thus indicating incomplete cross-linking. Optimize the infiltration conditions, for example, by increasing the vacuum or cutting shorter leaf segments.
11. Decant liquid and wash the leaves three times with plenty of double-distilled water.

    Dry leaves well with a paper towel (if working with seedlings wrap with Miracloth and dry). Transfer the dried leaves into a fresh 50 ml tube. Close the tube and freeze in liquid nitrogen. Proceed to nuclei isolation or store at -80 ^{o}C.

    Properly fixed leaf tissues are either processed immediately or can be stored at -80 ºC for 2–3 days before proceeding to nuclei isolation.

Nuclei isolation (Day 2)

- Prepare NIB-P
- Pre-cool mortar and pestle using liquid nitrogen.
- Set a Thermomixer at 37 ^{o}C and another at 62 ^{o}C.
- Nuclei isolation done in the cold room yields better results.

1. Grind cross-linked tissues to fine powder using pre-cooled pestle and mortar. Transfer the powder into an ice-cold 50 ml tube.

   This material can be stored at -80 ^{o}C or used immediately.
2. Add 10 ml NIB-P slowly to the ground leaf powder and thaw the powder.
3. Mix carefully by swirling the contents with the pipette tips until no clogs are visible and keep on ice.
4. Filter contents twice through a double-layered. Collect the filtrate in a new ice-cold 50 ml tube. It is important not to squeeze the filter to accelerate the process as it leads to contamination with cell debris.
5. For optimal recovery of nuclei, an additional 10 ml NIB-P was added to the leftover material residing in the Miracloth.
6. Centrifuge filtrate at 3000 x g for 15 min at 4 ^{o}C.
7. Carefully remove and discard the supernatant using a 10 ml pipette.
8. Suspend the pellet in 1 ml ice-cold NIB-P by gentle mixing with a pipette tip or a plastic inoculation loop. Transfer the suspended nuclei by pipetting slowly using wide-bore tips into an ice-cold 1.5 ml tube.
9. Centrifuge at 1900 x g for 5 min at 4 ^{o}C and discard supernatant.

   Here the pellet is composed of layers: the nuclei together with fibers and starch granules form the bottom layers (gray). The upper layer of the pellet appears as dark green slurry, which can be easily collected by pipetting. Discard such green slurry as well.
10. Re-suspend the pellet in 1 ml NIB-P. Centrifuge at 1900 x g for 5 min at 4 ^{o}C and discard supernatant.
11. Repeat step 10 to perform one more wash.
12. Re-suspend the pellet in 100 ul ice-cold NIB-P. Mix gently by swirling the contents with a pipette tip.

    To check for quality of nuclei, stain 7 ul nuclei suspension with 7 ul Vectashield containing DAPI for the integrity examination. Mount the stained sample onto a microscopic slide and add the cover slip. Analyze the nuclei using the epifluorescence microscope (100X objective, 1000X magnification, DAPI-filter, absorption 358 nm, emission 461 nm). Intact nuclei appear round or oval and show sharp contours as shown in Hovel *et al*. To check for quantity of nuclei, stain another aliquot of the nuclei suspension with Vectashield containing DAPI. Pipette mixture onto the counting chamber, and count it using the epifluorescence microscope using a 20X objective lens at 200 magnification using the DAPI-filter. Determine the concentration of the nuclei and continue the Hi-C library preparation with approximately 10^{7} nuclei.
13. Collect 10 ul of nuclei suspension as an intermediate nuclei preparation *control* before proceeding to next step and store it at -20 ^{o}C until further processing.
14. Centrifuge the nuclei suspension at 1900 x g for 5 min at 4 ^{o}C and discard supernatant.
15. Gently and slowly suspend the pellet in 300 ul of 1.2X RE buffer using wide-bore tips.
16. Centrifuge at 1900 x g for 5 min at 4 ^{o}C and discard the supernatant.
17. To remove traces of NIB-P repeat step 16.

18. Gently and slowly suspend the pellet in 50 ul of 0.5% SDS using wide bore-tips. Incubate for 15 min at 62 °C while shaking at 900 rpm. Prepare 10% Triton X-100.

    Addition of SDS and incubation at 62 °C for 15 min help with accessibility of chromatin for restriction digestion, inactivation of endogenous nucleases, and removal of non-cross-linked proteins from DNA. The duration of incubation at this step has to be optimized in a sample-dependent manner. Shorter incubation time leads to inefficient or partial digestion due to inaccessibility of chromatin to restriction enzyme. Longer incubation time leads to destruction of clear chromatin territories and may even lead to reversion of cross-links.

19. Add 145 ul of water and 25 ul of 10% (v/v) Triton X-100. Mix the contents gently by inverting the tube and incubate for 15 min at 37 °C while shaking at 450 rpm.

    Triton X-100 is used at this step to quench the SDS from solution, which inhibits the enzymatic activities of restriction enzymes, DNA Polymerase I, and T4 DNA ligase. Freshly prepared Triton X-100 yields better results.

Restriction enzyme digestion, overhang fill-in and ligation (Day 2)

- Set Thermomixer at 37 °C.
- Set chamber incubator at 16 °C.
- Prepare fresh 10X RE buffer.
- Prepare fresh 10X blunt-end ligation buffer.

1. Add 25 ul of 10X RE buffer and 100 U of DpnII. Mix gently by inverting the tube 8-10 times, and incubate overnight (or a minimum of 3 h) at 37 °C while shaking at 350 rpm.
2. Incubate for 20 min at 62 °C. Cool to RT (~5 min). Transfer to ice.

   This step is used for inactivation of the restriction enzyme. The conditions given here are for inactivation of DpnII. Inactivation of EcoRI or XhoI can be performed by addition of 1.6% SDS and heating at 65 °C for 20 min.
3. Take 10 ul of digested chromatin before proceeding to next step as an intermediate digested chromatin *control*, store at -20 °C until further processing.
4. Add 1 ul 10 mM dTTP (final concentration 0.033 mM), 1 ul 10 mM dCTP (final concentration 0.033 mM), 1 ul 10 mM dGTP (final concentration 0.033 mM), 25 ul 0.4 mM biotin-14-dATP (final concentration 0.033 mM), 34 ul water, and 50U DNA Polymerase I Large (Klenow) Fragment. Mix gently by inverting the tube and incubate for 2 h at 37 °C while shaking at 450 rpm.

   For workshop purposes 1 ul 10 mM dATP is going to be used instead of biotinylated nucleotide, so please adjust the volume of water accordingly.

5. To filled-in products, add 120 ul 10X blunt end-ligation buffer, 100 ul 10% (v/v) Triton X-100, 50 U T4 DNA ligase and fill up to 1200 ul with water. Mix gently and incubate overnight at 16 ^{o}C placing tube in tube rotator.

Reversal of cross-linking (Day 3)

- Prepare fresh SDS lysis buffer, store at RT.
- Set a Thermomixer at 55 ^{o}C and another at 65 ^{o}C.

1. Centrifuge the ligation products at 3000 x g for 10 min at RT. Discard the supernatant carefully and re-suspend the pellet in 380 ul SDS lysis buffer.
2. Add 20 ul 20 mg/ml Proteinase K. Mix gently by inverting the tube and incubate for 30 min at 55 ^{o}C while shaking at 450 rpm.
3. Add 100 ul of 5 M NaCl. Mix gently by inverting the tube and incubate overnight at 65 ^{o}C.

DNA extraction (Day 4)

- Pre-cool the centrifuge at 4 ^{o}C; ice-chill 1.5 ml tubes.
- Set Thermomixer at 37 ^{o}C.
- Thaw glycogen and store it on ice.
- Prepare EB buffer.

1. Thaw nuclei preparation *control* sample and digested chromatin *control* sample, and adjust volume to 200 ul using EB buffer.
2. To tube containing reverse cross-linked DNA, add 500 ul phenol-chloroform-isoamyl alcohol (25:24:1) solution. To nuclei preparation *control* sample and digested chromatin *control* sample, add 200 ul phenol-chloroform-isoamyl alcohol (25:24:1) solution. Mix all tubes shaking by hand vigorously for 2 min. Centrifuge at 13,000 x g for 5 min at RT. Transfer upper phase to a new 1.5 ml tube.
3. To tube containing reverse cross-linked DNA, add 500 ul chloroform. To nuclei preparation *control* sample and digested chromatin *control* sample, add 200 ul chloroform. Mix all tubes shaking by hand vigorously for 2 min. Centrifuge at 13,000 x g for 5 min at RT. Transfer upper phase to a new 1.5 ml tube.
4. Add 1/10 volume of 3 M sodium acetate pH 5.2, add 2 ul of glycogen (5 mg/ml) and 1250 ul ice-cold 100% ethanol.
5. Mix by inverting 10 times and incubate the tubes at -80 ^{o}C for 1 h.
6. Centrifuge at 13,000 x g for 30 min at 4 ^{o}C. Carefully remove the supernatant.
7. Wash the pellet twice with 800 ul 70% (v/v) ethanol. Centrifuge at 13,000 x g for 5 min at RT. Carefully remove the supernatant.
8. Air dry the pellet (5-10 min) and dissolve the Hi-C sample in 50 ul EB buffer and the *control* samples and in 20 ul EB buffer.

9. Add 1 ul 20 mg/ml RNAse A to all tubes, mix by pipetting and incubate at 37 ^{o}C for 20 min while shaking at 900 rpm.
10. Measure DNA concentrations using a NanoDrop spectrophotometer.
11. Run ~500 ng diluted in 15ul (+ 3 ul 6X loading dye) of the Hi-C and *control* samples, together with 1 ul of 1 kb DNA ladder on a 1.8% agarose gel for 40 min at 90 V.

    DNA from nuclear preparation runs as a band of >40 kb, digested DNA runs as a smear of low molecular weight fragments, and the ligated DNA runs as a smear of intermediate sizes between DNA from nuclear preparation and digested DNA. This shows an efficient digestion and blunt-end ligation.

References

Hövel, I., Louwers, M., Stam, M., 2012. 3C Technologies in plants. Methods 58, 204–211. https://doi.org/10.1016/j.ymeth.2012.06.010

Padmarasu, S., Himmelbach, A., Mascher, M., Stein, N., 2019. In Situ Hi-C for Plants: An Improved Method to Detect Long-Range Chromatin Interactions. In: Checkanova J.A., Wang HL:V. (eds) Plant Long Non-Coding RNAs. Methods in Molecular Biology, vol 1933. Humana Press, New York, NY, pp. 441–472. https://doi.org/10.1007/978-1-4939-9045-0_28