Supplementary Protocol 1

In situ Hi-C protocol

This protocol was adapted from Rao et al, 2014 (ref[100]).

Crosslinking

1) Two to five million mammalian cells were grown under recommended culture conditions to about 80% confluence.
2) Wash cells one time with sterile PBS, then trypsinize cells as normal cell culture methods.
3) Take an aliquot for counting, then centrifuge cells at 1200rpm (300XG) for 5 min at room temp.
4) Resuspend cells in fresh medium (No FBS) at a concentration of 1x106 cells per 1ml media. In a fume hood, add freshly made formaldehyde solution to a final concentration of 1%, v/v. Incubate at room temperature for 10 minutes with end over end mixing.
5) Add 2.5M glycine solution to a final concentration of 0.2M to quench the reaction. Incubate at room temperature for 5 minutes on rocker.
6) Centrifuge for 5 minutes at 300xG at 4°C. Discard supernatant into an appropriate collection container.
7) Resuspend cells in 1ml of ice cold 1X PBS and spin for 5 minutes at 300xG at 4°C. Discard supernatant and flash-freeze cell pellets in liquid nitrogen or dry ice/ ethanol.
8) Either proceed to the rest of the protocol or store cell pellets at -80°C.

Lysis and Restriction Digest

9) Combine 10 mL of freshly made ice-cold Hi-C lysis buffer (10mM Tris-HCl pH8.0, 10mM NaCl, 0.2% Igepal CA630) with one protease inhibitor cocktail tablet from Roche (Cat#: 11836170001). Add 500 uL to one cross linked pellet of cells and resuspend pellet by pipetting slowly.
10) Incubate cell suspension on ice for 30 minutes with tube inversion to mix every 5-10min. Centrifuge at 2500xG for 5 minutes at 4°C. Discard the supernatant.
11) Resuspend pelleted nuclei once with 500µl of ice-cold Hi-C lysis buffer, centrifuge at 2500XG for 5 min at 4°C, discard supernatant.
12) Gently resuspend pellet in 50µl of 0.5% sodium dodecyl sulfate (SDS) (do not want nuclei clumps) and incubate at 62°C for 10 minutes + 400 rpm on thermomixer.
13) After heating is over, add 145µl of water and 25µl of 10% Triton X-100 (Sigma, 93443) to quench the SDS. Mix well by inversion or slow pipetting to break up nuclei clumping, avoiding excessive foaming. Incubate at 37°C for 15 minutes, with gentle shaking at 400rpm on thermomixer.
14) Add 25µl of DpnII Buffer and 10uL (100U) of DpnII restriction enzyme (NEB, R0147). Resuspend well by pipetting or inversion. Do not want clumping - make sure there is a nice milky white nuclei suspension. Digest chromatin at 37°C with gentle shaking at 400rpm using the thermomixer for 1 hour, with intermittent tube inversion to keep nuclei in suspension.
15) Add another 10uL of DpnII, make sure nuclei are resuspended well, and proceed with digestion overnight at 37°C with gentle shaking at 400rpm using the thermomixer.

Marking of DNA Ends, Proximity Ligation and Crosslink Reversal

16) Incubate at 62°C for 20 minutes to inactivate DpnII, then cool to room temperature.
17) To exchange the buffer so is compatible with DNA Pol I, centrifuge nuclei at 2500XG for 5min and discard the supernatant.
18) Resuspend nuclei in 25 uL 10X NEB Buffer 2 and 225 uL sterile H2O This seems to take out DpnII buffer and increase ligation efficiency.
19) To fill in the restriction fragment overhangs and mark the DNA ends with biotin, add 50µl of fill-in master mix:

37.5µl of 0.4mM biotin-14-dATP (Life Technologies, 19524-016)
1.5µl of 10mM dCTP
1.5µl of 10mM dGTP
1.5µl of 10mM dTTP
8µl of 5U/µl DNA Polymerase I, Large (Klenow) Fragment (NEB, M0210

20) Add 50uL of mastermix, mix by pipetting and incubate at ~25ºC for 20min (use waterbath), then 37°C for 70 minutes with 400rpm mixing using the thermomixer. Keep on ice until set up ligation late afternoon for o/n.
21) Add 900µl of ligation master mix:

663µl of water
120µl of 10X NEB T4 DNA ligase buffer (NEB, B0202)
100µl of 10% Triton X-100
12µl of 10mg/ml Bovine Serum Albumin (100X BSA)
5µl of 400 U/ µl T4 DNA Ligase (NEB, M0202)

Mix by inverting and incubate at 16ºC overnight in circulating waterbath. Mix by inversion as often as possible.
22) Pellet nuclei by centrifugation for 5 min at 3000g.
23) Wash with 100 uL of 10Mm Tris buffer Ph 8.0. Resuspend pellets in 900 µL of 10 mM Tris buffer with 50 µL of 20mg/ml proteinase K (NEB, P8102) and 120µl of 10% SDS and incubate at 55°C for 30 minutes.
24) Add 130µl of 5M sodium chloride and incubate at 68°C for 3 hours. Intermittent inversion to mix sample at least once an hour.

DNA Shearing and Size Selection

25) Cool tubes at room temperature.
26) Split into two 750µl aliquots in 2ml LoBind tubes. Make sure to note volumes and add 1.6X volumes (1200uL) of pure ethanol and 0.1X volumes (75uL) of 3M sodium acetate, pH 5.2, to each tube. Mix by inverting and incubate at -80°C for 30minutes. Take out Ampure beads and warm to room temperature.
27) Centrifuge at maximum speed, 2°C for 15 minutes. Keep the tubes on ice after spinning and carefully remove the supernatant by pipetting, and discard.
28) Resuspend, combining the two aliquots, in 800µl of 70% ethanol. Centrifuge at max speed for 5 minutes at 2°C.
29) Remove all supernatant and wash the pellet once more with 800µl of 70% ethanol.
30) Dissolve the pellet in 130µl of 1X Tris buffer (10 mM Tris-HCl, pH 8) and incubate at 37°C for 15 minutes to fully dissolve the DNA. When you are transferring the sample to a covaris tube, be careful not to make bubbles as they will interfere with shearing.
31) To make the biotinylated DNA suitable for high-throughput sequencing using Illumina sequencers, shear to a size of 300-500bp using the following parameters:

Instrument: Covaris LE220 (Covaris, Woburn, MA)
Volume of Library: 130µl in a Covaris microTUBE
Fill Level: 10
Duty Cycle: 15
PIP: 500
Cycles/Burst: 200
Time: 58 seconds

32) Transfer sheared DNA to a fresh 1.5ml tube using a 20 µL tip. Wash the Covaris vial with 70µl of water and add to the sample, bringing the total reaction volume to 200µl.

33) Keep a 1:5 dilution of DNA to be run on a 2% agarose gel to verify successful shearing. For libraries containing fewer than 2x106 cells, the size selection using AMPure XP beads described in the next steps could be performed on final amplicons rather than before biotin pull-down.
34) Warm a bottle of AMPure XP beads (Beckman Coulter, A63881) to room temperature (at least 30min prior to use) SEE STEP 27. To increase yield, AMPure XP beads can be concentrated by removing some of the clear solution before the beads are mixed for use in the next steps.
35) Add exactly 120µl (0.6X volumes) of beads to the reaction. Mix well by pipetting and incubate at room temperature for 5 minutes.
36) Separate on a magnet. Transfer the clear solution to a fresh tube, avoiding any beads. The supernatant will contain fragments shorter than 500bp.
37) Add exactly 40µl of fresh AMPure XP beads to the solution. Mix by pipetting and incubate at room temperature for 5 minutes.
38) Separate on a magnet and keep the beads. Fragments in the range of 300-500bp will be retained on the beads. Discard the supernatant containing degraded RNA and short DNA fragments.
38) Keeping the beads on the magnet, wash twice with 700µl of 70% ethanol.
39) Leave the beads on the magnet for 5 minutes to allow remaining ethanol to evaporate.
40) To elute DNA, add 300µl of 1X Tris buffer, gently mix by pipetting,incubate at room temperature for 5 minutes, separate on a magnet, and transfer the solution to a fresh 2.0ml tube.
Keep a 1:5 dilution of DNA to be run on a 2% agarose gel to verify successful shearing
41) Quantify DNA by Qubit dsDNA High Sensitivity Assay (Life Technologies, Q32854) and run undiluted DNA and 1:5 diluted post-shearing DNA on a 2% agarose gel to verify successful size selection.

Biotin Pull-Down and Preparation for Illumina Sequencing

Perform all the following steps in low-bind tubes.

42) Prepare for biotin pull-down by washing 150µl of 10mg/ml Dynabeads MyOne Streptavidin T1 beads (Life technologies, 65602) with 400µl of 1X Tween Washing Buffer (1X TWB: 5mM Tris-HCl (pH 7.5); 0.5mM EDTA; 1M NaCl; 0.05% Tween 20). Separate on a magnet and discard the solution.
43) Resuspend the beads in 300µl of 2X Binding Buffer (2X BB: 10mM Tris-HCl (pH 7.5); 1mM EDTA; 2M NaCl) and add to the reaction. Incubate at room temperature for 15 minutes with rotation to bind biotinylated DNA to the streptavidin beads.
44) Wash the beads by adding 600µl of 1X TWB. Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Discard supernatant.
45) Repeat wash.
46) Resuspend beads in 100ul 1X NEB T4 DNA ligase buffer (NEB, B0202). Reclaim beads and discard the buffer.
47) To repair ends of sheared DNA and remove biotin from unligated ends, resuspend beads in 100µl of master mix:

88µl of 1X NEB T4 DNA ligase buffer with 10mM ATP
2µl of 25mM dNTP mix
5µl of 10U/µl NEB T4 PNK (NEB, M0201)
4µl of 3U/µl NEB T4 DNA polymerase I (NEB, M0203)
1µl of 5U/µl NEB DNA polymerase I, Large (Klenow) Fragment (NEB, M0210)

48) Incubate at room temperature for 30 minutes. Separate on a magnet and discard the solution.
49) Wash the beads by adding 600µl of 1X TWB. Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Discard supernatant.
50) Repeat wash.
51) Resuspend beads in 100µl 1X NEBuffer 2. Reclaim beads and discard the buffer.
52) Resuspend beads in 100µl of dATP attachment master mix:

90µl of 1X NEBuffer 2
5µl of 10mM dATP
5µl of 5U/µl NEB Klenow exo minus (NEB, M0212)

53) Incubate at 37°C for 30 minutes. Separate on a magnet and discard the solution.
54) Wash the beads by adding 600µl of 1X TWB. Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Discard supernatant.
55) Repeat wash.
56) Resuspend beads in 100µl 1X Quick ligation reaction buffer (NEB, B6058). Reclaim beads and discard the buffer.
57) Resuspend in 50µl of 1X NEB Quick ligation reaction buffer.
58) Add 2µl of NEB T4 DNA ligase. Add 3µl of an Illumina indexed adapter (1.5 µM final, 10 times diluted from NEB E7645S) (dilute Indexes in 1X Tris Buffer to 1.5µM and add 3 µL). Record the sample-index combination. Mix thoroughly.
59) Incubate at room temperature for 1 hour. Mix every 20~30 minutes. Separate on a magnet and discard the solution.
60) Wash the beads by adding 600µl of 1X TWB. Heat the tubes on a Thermomixer at 55°C for 2 min with mixing. Reclaim the beads using a magnet. Remove supernatant.
61) Repeat wash.
62) Resuspend beads in 100µl 1X Tris buffer. Reclaim beads and discard the buffer.

Final Amplification and Purification

63) Resuspend in 200µl of PCR amplification mastermix:

80 uL 1X Tris buffer
100 uL KAPA HiFi Hotstart ReadyMix (Kapa Biosystems, Cat # KR0370)
20 uL KAPA Library Amp Primer Mix 10x (Kapa Biosystems, Cat # KR0370)

64) Divide the 200 uL sample to 4 X 50uL and aliquot each into a PCR tube
65) Amplify the Hi-C library directly off of the T1 beads with 8-12 cycles of PCR, using Illumina adapter and primers using the following cycling conditions:

98 ºC – 45 sec
98 ºC – 15 sec
60 ºC – 30 sec
65 ºC -45 sec
Go to step 2 for 10 cycles (8-12 cycles)
72ºC for 5 min
4ºC Hold

66) After amplification is complete, pool samples and bring the total library volume to 250µl with 1X TRIS buffer
67) Separate on a magnet. Transfer the solution (250uL) to a fresh tube and discard the beads.
68) Warm a bottle of AMPure XP beads to room temperature. Gently shake to resuspend the magnetic beads. Add 175 µL of beads to the PCR reaction (0.7X volumes). Mix by pipetting and incubate at room temperature for 5 minutes.
69) Separate on a magnet and remove the clear solution.
70) Keeping the beads on the magnet, wash once with 700µl of 70% ethanol.
71) Remove ethanol completely .Leave beads on magnet for 5min to allow ethanol to evaporate. To remove traces of short products, resuspend in 100µl of 1X Tris buffer and add another 70µl of AMPure XP beads. Mix by pipetting and incubate at room temperature for 5 minutes.
72) Separate on a magnet and remove the clear solution.

73) Keeping the beads on the magnet, wash twice with 700µl of 70% ethanol without mixing.
74) Leave the beads on the magnet for 5 minutes to allow the remaining ethanol to evaporate.
75) Add 30 µl of 1X Tris buffer to elute DNA. Mix by pipetting, incubate at room temperature for 5 minutes, separate on a magnet, and transfer the solution to a freshly labeled tube. The result is a final in situ Hi-C library ready to be quantified and sequenced using an Illumina sequencing platform.
76) Take 1uL for High sensitivity Qubit quantitation, and 1uL for assessing final size on a DNA High Sensitivity BioAnalyzer chip.