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iHi-C 2.0: A simple approach for mapping native spatial chromatin organisation from low cell numbers

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ABSTRACT

Genome organization is now understood to be tightly linked to all genomic functions. Thus, the high-resolution mapping of higher-order chromosomal structures via 3C-based approaches has become an integral tool for studying transcriptional and cell cycle regulation, signaling effects or disease onset. Nonetheless, 3C-based protocols are not without caveats, like dependencies on fixation conditions, restriction enzyme pervasiveness in crosslinked chromatin and ligation efficiency. To address some of these caveats, we describe here the streamlined iHi-C 2.0 protocol that allows for the genome-wide interrogation of native spatial chromatin contacts without a need for chemical fixation. This approach improves ligation efficiency and presents minimal material losses, and is thus suitable for analysing samples with limiting cell numbers. Following high throughput sequencing, iHi-C 2.0 generates high signal-to-noise and focal maps of the interactions within and between mammalian chromosomes under native conditions.

1. Introduction

The three-dimensional genome organization is strongly correlated to its function [1]. Chromosome conformation capture (3C) technology and its derivatives (e.g. 4C, Hi-C, ChIA-PET) developed over the past years have been established as powerful tools for mapping chromatin interactions at a large-scale [2] and, thus, for analysing genome architecture in physiological and in pathological contexts, during development or upon signalling cues.

Hi-C couples the 3C approach to high-throughput sequencing to generate whole-genome interaction maps at ever-increasing resolution [3–5]. In the typical protocol of *in situ* Hi-C [3], cells are fixed with formaldehyde to preserve interactions between loci, before chromatin is digested with a restriction enzyme and biotinylated nucleotides are incorporated into the 5′ overhangs generated. Subsequently, spatially-proximal DNA ends are ligated together to give rise to a library, in which ligation products are marked by the presence biotin in their junctions. These junctions should represent "true" interaction events, and are selectively pulled-down using streptavidin beads, before isolating DNA that will be subjected to massively parallel next-generation sequencing to obtain a catalogue of interacting loci. The use of Hi-C has added enormously to our understanding of distal interactions defining chromatin architecture across cell types and organisms [6–8]. However, this widely-used approach is not without limitations. To highlight three key limitations: (i) incorporation of biotinylated residues into DNA ends is not efficient [9], and ligation events were shown to be rare [10] potentially resulting in numerous proximity ligation junctions being lost; (ii) in order to increase detection rates of ligation products, large cell numbers are typically used, which could prove limiting for some tissue and patient samples [11]; (iii) formaldehyde fixation does alter the proteome on and around chromatin and may lead to differential crosslinking and accessibility in different nuclear compartments [12,13].

To at least in part address these limitations, and keeping in mind that any 3C-based approach will inevitably carry its own inherent biases, we recently introduced "intrinsic" 3C (i3C [14]) whereby native chromatin interactions are captured without a need for chemical fixation. This is achieved via the use of a custom "physiological" buffer (PB) closely resembling in-cell salt and ATP concentrations, thus retaining most transcriptional activity in isolated nuclei [15,16]. Under these conditions, i3C offers robust mapping of spatial interactions with improved signal-to-noise ratios. Importantly, the genome-wide variant of i3C, iHi-C, allowed the comprehensive mapping of pairwise interactions along human chromosomes. Critically, iHi-C confirmed, for the first time, the topological restrictions imposed by topologically-associated domains (TADs [4]) under native conditions [14], a finding that has

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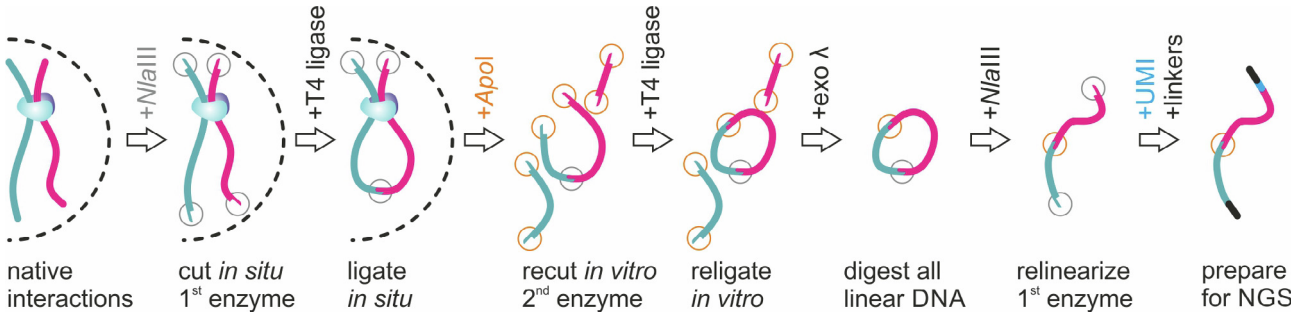

Fig. 1. Overview of the iHi-C 2.0 approach. Depiction of the key steps in the iHi-C 2.0 experimental protocol from *in situ* chromatin digestion in intact nuclei to the preparation of barcoded (UMI) DNA libraries for next-generation sequencing. The cut/ligated ends created by *Nla*III (*grey circles*) or *Apo*I (*orange circles*) are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

now been corroborated by an orthogonal, restriction digest- and ligation-free approach, DamC [17].

The iHi-C 2.0 version of the protocol described here (overview in Fig. 1), addresses the other two outstanding limitations. On one hand, iHi-C 2.0 does away with the incorporation of biotinylated nucleotides into ligation junctions for the most efficient ligation between cohesive ends, like that performed in 4C [18]. This, much like in 4C, is then followed by a second endonuclease digestion and re-ligation to induce the formation of circular DNA molecules, while linear contaminants are removed via treatment with λ exonuclease. Finally, products are re-linearized via digestion with the first restriction enzyme and the resulting linear DNA is used for library preparation for next-generation sequencing. This modified iHi-C 2.0 protocol was essentially developed on the basis of the "easy Hi-C" approach [19], results in minimal loss of DNA interactions with as few as few hundred thousand cells, and allows for the direct identification of native interactions in mammalian nuclei.

2. Experimental procedure

2.1. Reagent setup

2.1.1. Physiological buffer (PB) pH 7.4

CH_3COOK 100 mM, KCl 30 mM, Na_2HPO_4 10 mM, $MgCl_2$ 1 mM, Na_2ATP 1 mM, DTT 1 mM, β-glycorophosphate 10 mM, NaF 10 mM, Na_3VO_4 0.2 mM, RiboLock RNase Inhibitor 25 Units/ml, Complete protease inhibitor 1x, milliQ-water. Adjust the pH to 7.4 by using 100 mM KH_2PO4. CRITICAL: Prepare PB fresh just before the experiment since protease and RNA inhibitors are easily degraded.

2.1.2. Cell lysis buffer

PB with 0.6% IGEPAL CA-630.

2.1.3. Digestion buffer

PB with 0.6% IGEPAL CA-630 and 4% PEG. Note that not all restriction endonucleases are working well in that buffer [13]. One should test the efficiency of the digestion reaction for their specific cell type to confirm digestion efficiency. For the data from the hPSC-derived cardiomyocytes used, *Nla*III (a four-cutter enzyme) was chosen for the first digestion and *Apo*I (a six-cutter enzyme) for the second.

2.1.4. Ligation buffer

PB with 0.1 mg/ml BSA.

2.2. Cell culture

Cardiomyocytes (CMs) were derived from human pluripotent stem cells (hPSCs) as previously described [18]. As starting material for iHi-C 2.0, we tested aliquots of 10^6 CMs, as well as of 2×10^5 CMs. For the latter, where cell numbers are limiting, the volume of all reactions and reagents described below was reduced to 25% of that stated.

3. Intrinsic Hi-C 2.0

3.1. Isolation of cell nuclei

1. Remove growth medium and harvest cells in 1 ml of PB by gentle scraping. Transfer into a 2-ml round-bottom/low-retention microcentrifuge tube.
2. Spin down cells at 600 × *g* for 5 min and remove PB.
3. Resuspend cell pellet in 2 ml Cell Lysis Buffer to lyse the cells and release nuclei.
4. Incubate for 30 min on ice.
5. Spin down at 600 × *g* for 5 min.
6. Resuspend nuclei in 1 ml Cell Lysis Buffer
7. Incubate for 30 min on ice.
8. Spin down at 600 × *g* for 5 min.
9. Repeat steps 6–7 for two more times and check under an inverted light microscope for cytoplasm-free intact nuclei.
10. Spin down at 600 × *g* for 5 min.

TROUBLESHOOTING[1]: It is very important to confirm cell lysis and integrity of nuclei under the microscope. Different cell lines require diverse lysis conditions. In this protocol, nuclei isolation steps were adjusted for hPSC-CMs. Final detergent concentration, incubation time on ice, and numbers of washes may vary among different cells. For example, for human umbilical vein endothelial cells (HUVECs) 0.4% IGEPAL CA-630, 20 min incubation on ice and without repetition of steps 6–8 suffice, while for human primary lung fibroblasts (IMR90) 0.4% IGEPAL CA-630, 10 min incubation on ice with one repeat of steps 6–8 yields satisfying quality of isolated nuclei.

3.2. Chromatin digestion and in situ ligation

11. Gently resuspend pellet in 500 µl of digestion buffer.
12. Add stepwise 500 U of the first restriction enzyme (in this case *Nla*III, 10 U/µl). Before adding the enzyme, keep a 10-µl aliquot to use as an "undigested" control.
13. Digest at 33 °C for 45 min without shaking. Once digestion concludes, keep another 10-µl aliquot to use as a "digestion efficiency" control.

Add 200 µl of 1x PBS to the aliquots kept in steps 11 and 12 (undigested and digested nuclei). Incubate with Proteinase K (250 µg/ml) at 65 °C for 2 h. Add RNase A (250 µg/ml) and incubate at 37 °C for 30 min. *Purify genomic DNA from control samples using phenol/chloroform extraction and EtOH precipitation as described for steps 17–22. Resuspend DNA in 5 µl of 10 mM Tris-HCl (pH 7.5) and assess digestion efficiency by agarose or capillary gel electrophoresis* (see Fig. 2A).

14. Spin down at 600 × *g* for 5 min and discard all supernatant, which contains the restriction enzyme and any chromatin released from

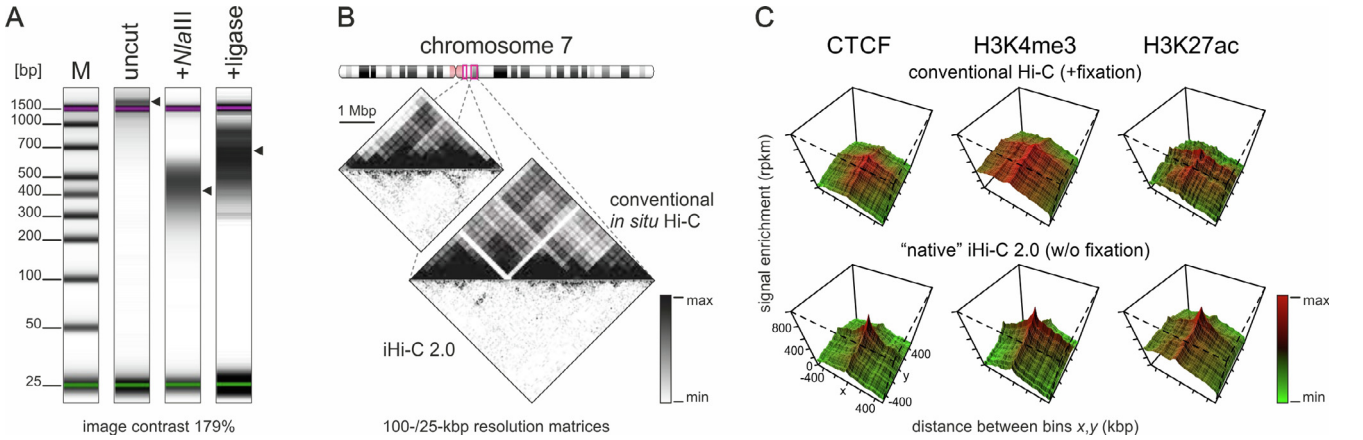

Fig. 2. Features of the iHi-C 2.0 protocol. (A) Capillary electrophoresis profiles of uncut, *Nla*III-digested, and ligated chromatin from CM nuclei. The sizes (in bp) of bands in the molecular marker (M) are indicated (*left*). (B) Exemplary conventional and "native" iHi-C 2.0 interaction matrices of two Mbp-long subregions from human chromosome 7 at 100- and 25-kbp resolution, respectively, generated from hPSC-derived CMs. (C) Three-dimensional enrichment plots generated using ChIP-seq data for CTCF, H3K4me3, and H3K27ac from CMs and conventional Hi-C (*top row*) or iHi-C 2.0 (*bottom row*) data. All plots show stronger and more focal ChIP-seq signal enrichment when iHi-C 2.0 contacts are used as input.

nuclei that was not associated to any nuclear substructure.

15. Repeat step 14 once by resuspending in 400 µl PB to wash any residual restriction enzyme away.
16. Resuspend the nuclei pellet in 1 ml Ligation Buffer, add 100 U T4 DNA Ligase (5 U/µl) and incubate at 16 °C for at least 6 h (and no more than overnight).

TROUBLESHOOTING[2]: The digestion control should present a smear migrating faster than the tight undigested control band in the agarose gel. If the digestion product still consists of a heavy band similar to the undigested, then the nuclei obtained in Steps 1–10 were not accessible to restriction endonuclease. Check nuclei purity and integrity (see Troubleshooting[1]) and/or use different restriction enzymes that work efficiently in PB, such as *Apo*I, *Dpn*II, *Nla*III or *Cvi*QI (see also Reagent Setup).

3.3. DNA purification

17. Add SDS to a final concentration of 2% and Proteinase K to a final concentration of 300 µg/ml to the nuclei, and incubate overnight at 65 °C.
18. Next day, add 300 µg/ml RNase A and incubate at 37 °C for 30 min.
19. Dilute ligation samples with an equal volume of milliQ water before proceeding with DNA purification to deal with the high salt concentrations contained in PB.
20. Purify DNA by adding an equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1, pH 8.0) and mixing vigorously. Spin down at 12.000 × *g* for 15 min at room temperature and then transfer the aqueous phase into a fresh tube.
21. Precipitate DNA by adding 1/10 vol of 3 M sodium acetate (pH5.2) and 2.5 volumes of pure ethanol. Use 20 µg/ml glycogen as a carrier and to visualise the DNA pellet. Mix by inverting the tube 6–8 times and freeze first for 30 min at −80 °C, then for 60 min at −20 °C.
22. Spin down at 12.000 × *g* for 20 min at 4 °C and discard the supernatant.
23. Wash pellet with 500 µl 70% ethanol and spin down again at 12.000 × *g* for 10 min at 4 °C.
24. Air-dry pellet for 20 min at room temperature and resuspend in 100 µl of 10 mM Tris-HCl (pH 7.5) by incubating for 30 min at 37 °C. This material constitutes the "i3C library" and can be stored at −20 °C for several months.
25. To confirm ligation efficiency, electrophorese 1 µl of the i3C library in an agarose gel. A successful ligation should result in a single band (or tight smear) migrating at a similar or slightly smaller position as the "undigested" control from step 12.

3.4. Second digestion and self-ligation

26. Digest with 20 U of the second restriction enzyme (*Apo*I, 10 U/µl) pre each µg DNA of the i3C library by incubating for at least 2 h at 37 °C (and up to overnight).
27. Heat inactivate the enzyme according to the manufacturer's instructions (for *Apo*I at 80 °C for 20 min).
28. Purify DNA using 1.8x volumes of Ampure XP PCR magnetic beads according to the manufacturer's instructions, and elute DNA in 300 µl milliQ water.
29. Add the supplied T4 DNA ligase buffer, 5U of the ligase enzyme, and milliQ water to the purified DNA to achieve a final volume of 1 ml. Ligate overnight at 16 °C.

3.5. Exonuclease digestion and DNA relinearization

30. Purify self-ligated DNA by phenol extraction and EtOH precipitation (steps 17–23).
31. Digest DNA with 6 U of λ exonuclease in a final reaction volume of 200 µl at 37 °C for 30 min to remove all linear (unligated) DNA molecules.
32. Inactivate the exonuclease at 65 °C for 20 min.
33. Purify DNA using 1.8x volumes of Ampure XP PCR magnetic beads according to the manufacturer's instructions, and elute in 100 µl milliQ water.
34. To relinearize DNA, digest with 20 U of the first restriction enzyme used in step 12 in a volume of 150 µl and in 1x of the supplied buffer at 37 °C for 2 h.
35. Heat inactivate the enzyme according to the manufacturer's instructions (for *Nla*III, at 65 °C for 20 min).
36. Purify DNA using 1.8x volumes of Ampure XP PCR magnetic beads according to the manufacturer's instructions, and elute in 50 µl milliQ water.

3.6. Library preparation

37. When staring with 2×10^6 CMs, 250–500 ng of DNA were obtained, while when staring with 2×10^5 cells, 50–100 ng DNA. Library preparation on this material began by the addition of a 10-nt unique molecular identifier (UMI) oligo provided in the Sure Select High Sensitivity kit (Agilent) according to the manufacturer's

instructions.

38. Next, sequencing adapter primers are added to the barcoded library using the TruSeq SR cluster kit (Illumina) according to the manufacturer's instructions to allow for paired-end sequencing.
39. iHi-C 2.0 libraries are amplified with the minimum number of PCR cycles (typically 6, and no more than 12 in total) using the 15 µl-reactions with the KAPA HiFi Hotstart poymerase (KAPA Biosystems) and aiming at a final library concentration of 20–40 nm. Library quantification can be done via an Illumina-specific kit (KAPA Biosystems).
40. Libraries are sequenced on a HiSeq4000 (or equivalent capacity) platform (Illumina) to at least 15×10^7 read pairs each; each read was of 75 nt in length.

3.7. iHi-C 2.0 data analysis

Raw sequencing reads (.fastq files) were mapped independently to the human reference genome (hg19) using BWA-MEM [20] after trimming of 5 nt from each end to remove identical *Nla*III sequence motifs. PCR duplicates were defined as those read pairs mapping to the same locations in the genome and carrying the same UMI, and were manually removed. Following this, reads were further filtered to remove any that did not precisely map next to genomic *Nla*II recognition sites. Similarly, read pairs with both ends mapping to the same *Nla*III fragment or with "dangling" ends were also removed (expected read processing statistics from iHi-C 2.0 experiments are included in Table 1). Ultimately, the library of filtered mapped read pairs were normalized and converted to .hic files and visualized via Juicebox [21] to give rise to interactive and zoomable iHi-C maps or manually printed using R (for an example see Fig. 2B). Focal enrichment for interactions between, for example, CTCF-bound sites or gene promoters (marked by H3K4me3) can be used to assess iHi-C map quality (see examples in Fig. 2C generated using code from Ref. [22]).

4. Materials

- Agarose (Sigma-Aldrich, A9539)
- AMPure XP beads (Beckman Coulter Life Sciences, A63880)
- *Apo*I 10 U/µL (NEB, R0566L)
- ATP disodium salt (Sigma-Aldrich, A2383)
- B-Glycerophosphate Disodium Salt (Sigma-Aldrich, G9422)
- BSA (Bovine Serum Albumin), non-acetylated (Sigma-Aldrich, B6917)
- cOmplete ULTRA Tablets, EDTA-free, glass vials (ROCHE, 06538282001)
- Ethanol ca. 96% (Fisher Scientific, 24102)
- Glycogen 20 mg/ml (ROCHE, 10901393001)
- IGEPAL® CA-630 - for molecular biology (Sigma-Aldrich, I8896)
- Lambda Exonuclease (NEB, M0262S)
- Magnesium chloride hexahydrate (Sigma-Aldrich, M2670)
- *Nla*III 10 U/µL (NEB, R0125L)
- Phenol:Chloroform:Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris, 1 mM EDTA, pH 8.0 (Sigma-Aldrich, P2069)
- Polyethylene glycol 8000 (Sigma-Aldrich, 89510)
- Potassium acetate (Sigma-Aldrich, P1190)
- Potassium chloride (Sigma-Aldrich, P9541)
- Potassium phosphate monobasic (Sigma-Aldrich P9791)
- Proteinase K 10 mg/ml (Sigma-Aldrich, P2308)
- RIBOLOCK RNASE INHIBITOR (Life Technologies GmbH, EO0384)
- RNAse A 10 mg/ml (Sigma-Aldrich, R6513)
- Sodium acetate, anhydrous (Sigma-Aldrich, S2889)
- Sodium dodecyl sulfate (Sigma-Aldrich, 74255)
- Sodium fluoride (Sigma-Aldrich, 30105)
- Sodium orthovanadate (Sigma-Aldrich, S6508)
- Sodium phosphate dibasic (Sigma-Aldrich, S3264)
- T4 DNA Ligase 5 U/µL (ThermoFisher, 15224041)

Table 1
Metrics of iHi-C 2.0 read processing steps compared to conventional *in situ* and "easy" Hi-C.

Type of dataset	Number of cells used	Sequenced read pairs	Mapped (hg19)	Properly paired	After UMI filtering	After RE filtering	*Cis*- to *trans*-contact ratio
iHi-C 2.0 (hCMs)	$\sim 2 \times 10^6$	353,318,748 (100%)	261,198,402 (73.93%)	231,038,364 (65.39%)	196,967,136 (55.75%)	59,117,708 (16.73%)	1.84
	$\sim 10^5$	184,298,681 (100%)	137,071,712 (74.37%)	122,493,514 (66.46%)	104,744,696 (56.83)	28,464,153 (15.44%)	1.92
in situ Hi-C (hCMs)	$\sim 20 \times 10^6$	389,138,689 (100%)	377,683,900 (97.06%)	358,436,903 (92.11%)	N/A	119,566,816 (30.73%)	4.12
"easy" Hi-C (MEFs)	$\sim 3 \times 10^6$	211,067,038 (100%)	183,948,967 (87.15%)	174,937,916 (82.88%)	148,038,561 (70.14%)	63,008,452 (29.85%)	2.39

- Trizma® base, BioPerformance (Sigma-Aldrich, T6066-500G)
- TruSeq SBS kit v3-HS (Illumina, FC-401-3002)
- TruSeq SR cluster kit v3-cBot-HS (Illumina, GD-401-3001)
- KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, 07958927001)
- KAPA Library Quantification kit for Illumina platforms (KAPA Biosystems, 07960140001)
- Sure Select High Sensitivity DNA kit (Agilent, 5067-4626)

5. Equipment

- Agarose gel electrophoresis equipment, Biometra Compact (Jena Analytik, 846-025-199)
- Cell Scrapers (Sarstedt, 83.1830)
- DynaMag™-2 Magnet (ThermoFisher, 12321D)
- Fresco 17 Heraeus Refrigerated microcentrifuge 24 × 1.5 ml (Thermofisher, 75002425)
- Inverted microscope Primovert (Carl Zeiss, 491206-0003-000)
- Microcentrifuge tubes, 1.5 ml (StarLab, S1615-5500)
- Low-retention microcentrifuge tubes, 2.0 ml (Kisker, G018)
- Neubauer-hematocytometer (Hartenstein, ZK06)
- Spectrophotometer NanoDrop™ 2000 (ThermoFisher, ND-2000)
- ThermoMixer® C (Eppendorf, 5382000015).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2019.07.003.

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