Mapping 3D genome architecture through *in situ* DNase Hi-C

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With the advent of massively parallel sequencing, considerable work has gone into adapting chromosome conformation capture (3C) techniques to study chromosomal architecture at a genome-wide scale. We recently demonstrated that the inactive murine X chromosome adopts a bipartite structure using a novel 3C protocol, termed *in situ* DNase Hi-C. Like traditional Hi-C protocols, *in situ* DNase Hi-C requires that chromatin be chemically cross-linked, digested, end-repaired, and proximity-ligated with a biotinylated bridge adaptor. The resulting ligation products are optionally sheared, affinity-purified via streptavidin bead immobilization, and subjected to traditional next-generation library preparation for Illumina paired-end sequencing. Importantly, *in situ* DNase Hi-C obviates the dependence on a restriction enzyme to digest chromatin, instead relying on the endonuclease DNase I. Libraries generated by *in situ* DNase Hi-C have a higher effective resolution than traditional Hi-C libraries, which makes them valuable in cases in which high sequencing depth is allowed for, or when hybrid capture technologies are expected to be used. The protocol described here, which involves ~4 d of bench work, is optimized for the study of mammalian cells, but it can be broadly applicable to any cell or tissue of interest, given experimental parameter optimization.

INTRODUCTION

The manner in which an incredibly long DNA polymer topologically organizes itself within a cell or nucleus is crucially linked to higher-order cellular function[1,2]. This form–function relationship, first discovered through early light microscopic studies of higher-order structures such as mitotic chromosomes[3], the inactive X Barr body[4], and polytene chromosomes[5], has only become clearer in the face of advancing technologies. Techniques such as fluorescence *in situ* hybridization (FISH) of chromatin[6–8] have provided clear evidence that chromosomes occupy compartments within the nucleus, ultimately leading to the development of correlative models associating biological function (i.e., transcription, splicing, silencing) with particular nuclear locales[9,10].

With the advent of genome-scale technologies, high-throughput assays have been developed to characterize nuclear architecture at both increasing scale and increasing resolution. Techniques such as DNA adenine methyltransferase identication[11,12], typically used to map protein–DNA interactions[13–15], have been modified to map genome-wide associations between primary sequences and the nuclear lamina[16] (i.e., lamina-associated domains or LADs), where silenced domains typically reside. Methods involving the 'proximity ligation' of chromatin, now termed 3C (ref. 17), have also gained popularity. 3C techniques represent matured versions of early methods that used T4 DNA ligase to quantify the physical proximity of DNA sequences brought together by proteins[18,19], and all share a common paradigm: fixation of chromatin within the nucleus via formaldehyde, endonucleolytic digestion of chromatin (normally via restriction enzyme (RE) digestion), and re-ligation of physically proximal fragments. The first 3C variants (e.g., 4C, 5C) used specific primers or sets of primers to determine contact frequencies between predefined sites in the genome[20,21]. Later, massively parallel versions of 3C, generally termed 'Hi-C', were developed[22–24] that leverage paired-end sequencing to generate contact-frequency estimates between sequence windows across entire genomes.

Since the advent of 3C techniques, much work has gone into characterizing 3D genome architecture in a wide variety of biological contexts[25–29], including mitotic cell division[30], the life cycle of a parasite[31], and mammalian dosage compensation[32–35]. The vast amount of available Hi-C data has also enabled the discovery of novel 'units' of genome topology, including topologically associating domains (TADs)[33,36] and chromosomal interacting domains[27,37], genomic domains that predominantly self-associate in 3D space. Although the ultimate significance of these domains remains unknown, strong correlations between 1D epigenomic features (e.g., histone marks, DNA methylation and transcription factor binding) and sequence, both within and at the borders of these domains, suggest that they may have a gene regulatory role.

Although current Hi-C techniques generally allow us to visualize genome-scale chromosome architecture at a resolution of 100 kb to 1 Mb, methodological resolution limitations imposed by incomplete sequencing depth and genome-wide restriction site density have typically precluded the identification of topological units at smaller scales, in which local interactions may have crucial gene regulatory roles. The need for fine-scale resolution of these higher-order interactions has only become clearer in the wake of the immense amount of high-resolution, 1D epigenomic data generated by consortia such as ENCODE[38] and Roadmap Epigenomics[39].

Given the availability of such data, one crucial interest of the gene regulatory field is the potential link between complex gene regulatory programs and dynamic long-range 'looping' interactions between distal regulatory elements, features at a scale even smaller than those of TADs and LADs[40]. Since the earliest realizations that long-range interactions are effectors of gene expression[41,42], the gene regulatory field has worked toward completely cataloging functional DNA looping interactions. In the realm of proximity ligation protocol development, considerable work has gone toward improving the resolution of the Hi-C protocol to the scale of kilobases, at which specific regulatory contacts (i.e., enhancer–promoter interactions, CCCTC-binding factor–mediated loops) might be identified.

The protocol presented here complements existing high-resolution Hi-C approaches[37,43] by providing another flexible, convenient, and scalable methodology that eschews the use of restriction enzymes. Our approach therefore avoids the theoretical limit in resolution of the standard Hi-C protocol imposed by the occurrence of restriction sites in the genome, given enough sequencing depth and library complexity.

Moving toward fine-scale resolution of 3D contacts

Core methodological improvements to the Hi-C protocol to improve resolution have broadly spanned three primary areas: deeper sequencing[36], simplified library preparation protocols[43,44], and the use of hybridization capture to enrich for sets of desired loci in a massively parallel manner[45–47]. We recently developed a method that unites many of these improvements with additional empirical changes to further increase the effective resolution of Hi-C libraries[48]. Our method, termed DNase Hi-C, eliminates the reliance on restriction enzymes associated with Hi-C by digesting fixed chromatin with the endonuclease DNase I in the presence of divalent manganese. We demonstrated that DNase Hi-C libraries mitigate many of the biases associated with traditional Hi-C, reducing the effective distance between fragments imposed by 4- and 6-cutter restriction enzymes while improving robustness with respect to G–C content, mappability, and genomic coverage. Furthermore, we also showed that DNase Hi-C may be paired with commercially available hybridization capture kits to visualize long intergenic noncoding RNA promoters at a previously unprecedented scale of 1 kb without the gross sequencing depth requirements typically associated with high-resolution contact maps.

Motivated by the observation that the vast majority of proximity ligations occur in insoluble chromatin[49], and the consequent improvements to traditional RE Hi-C based on this fact[43,44,50], we recently published an improved version of our previously published DNase Hi-C termed *in situ* DNase Hi-C[51]. We applied this simplified and robust Hi-C protocol to study the inactive X chromosome in primary mouse brain tissue and an immortalized mouse embryonic kidney cell line, demonstrating for the first time that the murine inactive X chromosome adopts a bipartite conformation. *In situ* DNase Hi-C represents a considerable improvement over its parent protocol, requiring considerably less hands-on time and lower cellular input requirements[51].

Overview of *in situ* DNase Hi-C

A schematic of the *in situ* DNase Hi-C protocol is shown in **Figure 1**. Anywhere from 5 × 10^5 to 1 × 10^7 cells are fixed in formaldehyde to reversibly cross-link *in vivo* protein–DNA interactions. Fixed cells are then lysed to liberate nuclei, which are treated with the endonuclease DNase I to digest chromatin. Digested chromatin ends are end-repaired and dATP-tailed, facilitating the ligation of an exogenous, dTTP-tailed 'bridge' adaptor containing a single biotinylated thymidine, a half BamHI restriction site, and a four-base overhang. After clearing out excess adaptors, the free ends of the chromatin (now capped with bridge adaptors) are phosphorylated with T4 polynucleotide kinase (T4 PNK) and proximity-ligated *in situ* with T4 DNA ligase I. During all of these steps, nuclei are immobilized against carboxylated paramagnetic beads (commonly referred to as solid-phase reversible immobilization beads[18]), both providing a scaffold to prevent loss of nuclei during enzymatic reactions and allowing for the simple removal of free DNA and excess bridge adaptor, which adversely affect downstream library preparation.

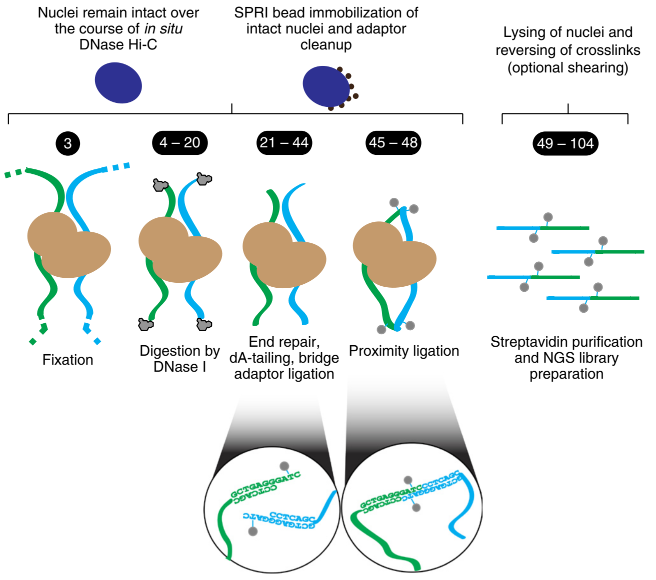


Figure 1 | A schematic overview of *in situ* DNase Hi-C. First, fixed cells are lysed and digested with the endonuclease DNase I in the presence of divalent manganese—yielding double-stranded breaks. Nuclei are then immobilized on carboxylated paramagnetic beads (i.e., 'AMPure' beads) to purify intact nuclei and to remove free digested DNA fragments. Chromatin is then end-repaired and dA-tailed *in situ*, and a biotinylated 'bridge adaptor' containing a half BamHI site is ligated onto free chromatin ends. Nuclei are then subjected to phosphorylation and *in situ* proximity ligation, after which DNA is purified and fragments containing ligation junctions are enriched via streptavidin beads for on-bead Illumina library preparation (optionally following sonication).

After proximity ligation, nuclei are lysed and cross-links are reversed with proteinase K treatment. DNA is then isolated with an isopropanol precipitation, after which fragments are optionally sheared. Ligated DNA fragments harboring the biotinylated bridge adaptor are then affinity purified using streptavidin beads, end-repaired, dA-tailed, and ligated to standard Illumina sequencing adaptors. Finally, ligation products are PCR-amplified to generate sequencing libraries. Before sequencing, libraries may be treated with a simple BamHI digestion to assess the efficiency of proximity ligation.

Traditional Hi-C versus *in situ* DNase Hi-C

In situ DNase Hi-C can be used in any situation in which traditional Hi-C would be used. Thanks to a reliance on the endonuclease DNase I, *in situ* DNase Hi-C eliminates the characteristic restriction enzyme biases that limit resolution in traditional Hi-C libraries while lowering the input cell requirements for library construction. Unlike other Hi-C protocols, *in situ* DNase Hi-C is the only protocol, to our knowledge, to use paramagnetic carboxylated beads as a tool to immobilize nuclei during *in situ* enzymatic treatments. This immobilization step not only reduces nuclei loss during the protocol, aiding low-input experiments, but also facilitates the removal of contaminating adaptors and free DNA. Finally, like traditional *in situ* Hi-C, *in situ* DNase Hi-C requires considerably less hands-on time for library prep, and it more efficiently generates *cis* (i.e., intrachromosomal) ligation products as compared with *trans* (i.e., interchromosomal) ligation products.

Considering the high sequencing depth required to generate high-resolution genome-wide contact maps, we note that, at low resolution, maps generated using *in situ* DNase Hi-C are practically very similar to those generated using other Hi-C protocols (except in cases in which loci may have particularly low restriction site density). However, in cases in which high-resolution (i.e., 1-kb resolution) maps are desired, we strongly believe that the relatively unbiased ligation junctions generated through DNase Hi-C present an important alternative to existing methods. This point is particularly relevant when hybrid capture techniques may be applied, as high-resolution, RE-independent maps can be generated for a fraction of the cost of genome-scale library sequencing.

Still, we acknowledge that in many cases cost may preclude the use of deep sequencing or hybrid capture. In cases such as these, we suggest more cost-effective solutions using more focused techniques (e.g., 3C, 4C and 5C), albeit at the price of interrogating interactions among only a set number of loci.

In situ DNase Hi-C is broadly applicable to any situation in which high-resolution chromatin conformation data or 3D maps are required. We have successfully carried out *in situ* DNase Hi-C in several immortalized cell lines and primary tissues, generating libraries for the human cell lines K562 and GM12878, as well as for mouse embryonic kidney cells and homogenized mouse brain tissue[51].

Limitations of the protocol

In situ DNase Hi-C is subject to the same limitations as any bulk Hi-C protocol. First, the protocol requires 5×10^5 to 1×10^7 cells to generate sequenceable libraries. Thus, in cases in which input might be particularly limited, or in which small populations of cells are sorted by FACS, *in situ* DNase Hi-C may not be appropriate. Second, it is also important to note that although the DNase enzyme is nonspecific when compared with restriction enzymes, it has been shown to exhibit mild sequence bias at cleavage sites[52]. This must be considered when applying *in situ* DNase Hi-C to organisms with radical nucleotide content (i.e., low GC content) and when considering the inherent biases within *in situ* DNase Hi-C maps (as would be done with any Hi-C contact map[53]).

Experimental design

The *in situ* DNase Hi-C protocol described here is relatively straightforward, and it can be completed over 4 d, allotting 3–6 h of bench work per day. Still, there are several experimental design parameters that should be considered before applying *in situ* DNase Hi-C to a new cell type of interest. These considerations primarily concern maintaining intact nuclei during the various *in situ* enzymatic treatments in the protocol. The *in situ* DNase Hi-C protocol also allows for sequencing-free quality control (QC) of libraries, due to the integration of half BamHI sites in the bridge adaptor. As discussed below, this allows for easy quantification of the efficiency of proximity ligation in the final *in situ* DNase Hi-C library.

Although the protocol presented here is robust to many different cell types, different immortalized cell lines may require optimization of formaldehyde cross-linking, DNase I digestion, and SDS concentration during digestion. Below we detail our process for optimizing these various parameters.

Formaldehyde concentration. As with other 3C methods and ChIP-seq protocols, formaldehyde fixation is an important component of the *in situ* DNase Hi-C protocol, promoting proximity ligation of long-range genomic contacts while maintaining the integrity of nuclei during *in situ* enzymatic steps. Incomplete cross-linking can lead to an under-representation of proximity ligation products in Hi-C libraries, and excessive breakage of nuclei can lead to considerable decreases in the ultimate molecular complexity of libraries and, at worst, can increase the degree of 'spurious' ligations formed. The guidelines for formaldehyde fixation of cells for *in situ* DNase Hi-C are the same as those for the other 3C-based techniques and ChIP-seq methods. In general, for single-cell suspension cultures (e.g., GM12878 and K562 cells) and monolayer adherent cells (e.g., HeLa cells), a standard condition of cross-linking, such as 1% (vol/vol) formaldehyde for 10 min at room temperature (RT, 25 °C), can be used. For other cell cultures (e.g., mouse and human embryonic stem cells (ESCs)) and primary-tissue cells (e.g., mouse brain cells and plant leaves), for which single-cell suspensions are difficult to obtain, increased formaldehyde concentrations or longer fixation times may be required to ensure efficient cross-linking. For example, both human and mouse ESCs often aggregate to form

Box 1 | Assessment of nuclear lysis at various steps ● TIMING ~3 d

To ascertain whether nuclei remain intact during the protocol, perform the following control experiment. After each enzymatic treatment step (Steps 15, 24, 29, 35 and 49), remove the supernatant and add 10 µl of proteinase K to it. Treat the supernatant overnight at 65 °C; then precipitate the DNA by adding 0.1 volumes of 3M sodium acetate, 3 µl of GlycoBlue, and 1 volume of 100% isopropanol, mixing, and then incubating the mixture at –80 °C for 1 h. Pellet the mixture at 4 °C at 16,000*g* for 30 min, carefully remove the supernatant, and resuspend the pellet in 100 µl of ddH_20. Add 10 µl of RNase A to each sample, incubate at 37 °C for 10 min, and then purify the DNA using 1.2 volumes of AMPure XP beads. Resuspend the beads in 15 µl of ddH_20, and run this out on a 6% TBE gel.

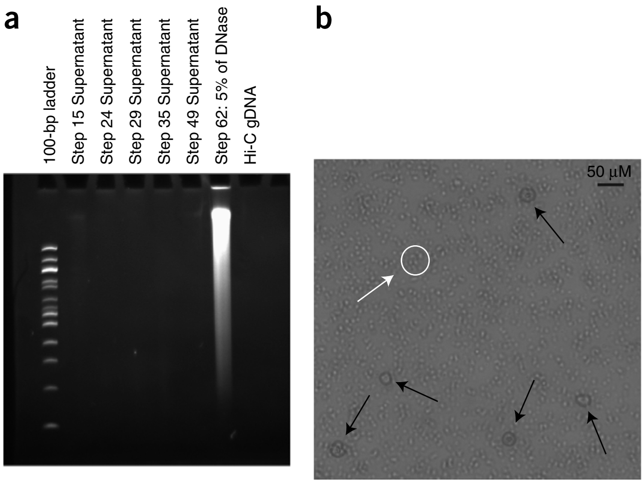


Figure 2 | Nuclei remain intact during the *in situ* DNase Hi-C protocol. (**a**) Purified supernatant DNA (**Box 1**) from six different steps of the DNase Hi-C protocol. Minimal DNA is purified after each enzymatic purification, as compared with the large amount of DNA from Step 62, purified from 5% of the total gDNA yield following nuclear lysis. (**b**) Phase-contrast micrograph (20× magnification) of GM12878 nuclei bound to beads following proximity ligation (Step 46). Nuclei are highlighted with black arrows, and an example of a clump of carboxylated beads, which are found scattered across the image, is shown circled in white, with an accompanying white arrow.

large clumps in culture. Higher concentrations of formaldehyde are generally used in these situations[48,54].

Cell lysis and DNase I digestion. After cross-linking chromatin interactions with formaldehyde, one must render fixed chromatin accessible to enable chromatin fragmentation and other downstream enzymatic reactions. As with restriction–digestion-based 3C methods, cell lysis in *in situ* DNase Hi-C is achieved primarily through SDS treatment. To ensure that nuclei remain intact throughout the multiple enzymatic reactions through the end of nuclear ligation (Step 48), the *in situ* DNase Hi-C protocol uses a relatively mild condition (0.3–0.5% (wt/vol) SDS treatment for 45 min at 37 °C). During this step, it is crucial to avoid overlysing nuclei. A simple experiment to determine the extent of nuclear lysis is detailed in **Box 1**, with expected results shown in **Figure 2a**. We also note that overly lysed nuclei become apparent during any of the many centrifugation steps in the *in situ* DNase Hi-C protocol, as no pellet forms. Nuclei should remain intact through proximity ligation, as shown in **Figure 2b**.

We stress that the required SDS concentration for cell lysis and the amount of DNase I used during the DNase I digestion step can vary depending on the cell type being studied and the number of nuclei being processed. When attempting the protocol on new cell types, we recommend carrying out a DNase I and SDS optimization experiment using varying amounts of DNase I and varying concentrations of SDS, and then examining the DNase I fragmentation pattern following digestion. An example fragmentation pattern is shown in **Figure 3a**.

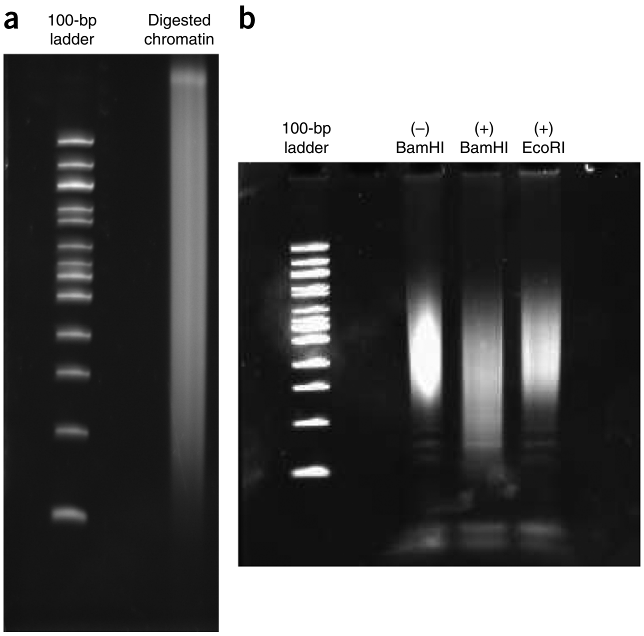


Figure 3 | Digestion QC throughout the *in situ* DNase Hi-C protocol. (**a**) A typical digestion pattern for DNase I-digested fixed chromatin before proximity ligation, run on a 6% TBE-PAGE gel. (**b**) Example of the BamHI quality-control experiment performed on GM12878 *in situ* DNase Hi-C libraries; in this example, BamHI shifts the *in situ* DNase Hi-C library by digesting the reconstituted BamHI site that forms following proximity ligation of the biotinylated bridge adaptors. Crucially, digestion with another 6-cutter (EcoR1) does not recapitulate this pattern, proving that the BamHI digestion is specific to proximity-ligated fragments. All reactions were run on one 6% TBE-PAGE gel.

The role of paramagnetic carboxylated beads. Paramagnetic carboxylated beads (i.e., AMPure XP beads) have been used in both our standard and *in situ* DNase Hi-C protocols. As demonstrated in **Figure 2**, these beads appear to bind to intact nuclei and serve as carriers to pellet the nuclei by low-speed centrifugation. Here, we use these beads to efficiently remove DNase I and low-molecular-weight DNA that might escape the nucleus following chromatin digestion, as well as free unligated internal bridge adaptor following bridge adaptor ligation. Furthermore, the beads also aid with visualization of the nuclei pellet throughout the protocol when starting the protocol with fewer than a million cells.

Nuclei treatment. It is crucial that the fixed nuclei remain intact over the course of the DNase Hi-C protocol. To this end, pipetting should be carried out gently to minimize shear forces that may burst nuclei.

BamHI digestion control. A BamHI digestion test on the final PCR-amplified library can be used to quantify ligation efficiency of the reaction. Lack of a library 'shift' (properly digested products shown in **Figure 3b**) suggests inefficiency in the formation of proximity ligation products and can be indicative of suboptimal fixation conditions or defective reagents.

MATERIALS

REAGENTS

- Cell lines of interest (adherent, suspension or primary tissue): for example, we have used the human cell line GM12878 (Coriell GM12878) and the Patski cell line (provided by C. Disteche) in our previous study[51] **! CAUTION** Cell lines should be regularly checked to ensure that they are authentic and that they are not infected with *Mycoplasma*.
- Penicillin–Streptomycin (Thermo Fisher Scientific, cat. no. 15140122)
- FBS (Thermo Fisher Scientific, cat. no. 10437-010)
- RPMI 1640 (Thermo Fisher Scientific, cat. no. 11875-093)
- DMEM (Thermo Fisher Scientific, cat. no. 11965118)
- Biotinylated bridge adaptor 5′: /5Phos/GCTGAGGGA/iBiodT/C (Integrated DNA Technologies)
- Bridge adaptor 3′T: CCTCAGCT (Integrated DNA Technologies)
- Bridge adaptor 5′: GCTGAGGGAC (Integrated DNA Technologies)
- Blunt bridge adaptor 3′: CCTCAGC (Integrated DNA Technologies)
- SeqAdapt_F: ACACTCTTTCCCTACACGACGCTCTTCCGATC*T (Integrated DNA Technologies)
- SeqAdapt_R: /5Phos/GATCGGAAGAGCACACGTCTGAACTCCA GTCAC (Integrated DNA Technologies)
- SeqPrimer_F: AATGATACGGCGACCACCGAGATCTACACTCTT TCCCTACACGACGCTCTTCCGATCT (Integrated DNA Technologies)
- SeqPrimer_R: CAAGCAGAAGACGGCATACGAGAT[8 bp barcode]G TGACTGGAGTTCAGACGTGTGCT (Integrated DNA Technologies)
- 37% (vol/vol) Formaldehyde (Sigma-Aldrich, cat. no. F8775) **! CAUTION** Formaldehyde is flammable, can cause skin burns, and is toxic if inhaled. Formaldehyde should be handled in a chemical fume hood while using appropriate protective equipment. **▲ CRITICAL** Formaldehyde has a limited shelf life; discard the solution if it is older than 1 year.
- Glycine (Sigma-Aldrich, cat. no. 50046)
- NEBuffer 2 (NEB B7002S)
- 10% (wt/vol) UltraPure SDS (Life Technologies, cat. no. 15553-027)
- DNase I, RNase-free (supplied with $MnCl_2$ and 10x DNase I digestion buffer; 1 U/µl; Thermo Fisher Scientific, cat. no. EN0525)
- RNase A, DNase and protease-free (10 mg/ml; Thermo Fisher Scientific, cat. no. EN0531)
- Klenow fragment (10 U/µl; Thermo Fisher Scientific, cat. no. EP0052)
- Klenow fragment (exo^{-}) (5 U/µl; Thermo Fisher Scientific, cat. no. EP0422)
- T4 DNA polymerase (5 U/µl; Thermo Fisher Scientific, cat. no. EP0062)
- T4 DNA Ligase (5 U/µl) provided with 50% PEG-4000 (Thermo Fisher Scientific, cat. no. EL0012)
- T4 Polynucleotide kinase (10 U/µl; Thermo Fisher Scientific, cat. no. EK0032)
- 10× T4 DNA Ligase Reaction Buffer (with ATP; NEB, cat. no. B0202S)
- Agencourt AMPure XP (Beckman Coulter, cat. no. A63880)
- 2× HotStart PCR ReadyMix (KAPA, cat. no. KK2601)
- Fast DNA End Repair Kit (Thermo Fisher Scientific, cat. no. K0771)
- Proteinase K (Thermo Fisher Scientific, cat. no. EO0492)
- FastDigest BamHI (Thermo Fisher Scientific, cat. no. FD0054)
- Dynabeads MyOne Streptavidin C1 (Life Technologies, cat. no. 65001)
- dNTP Set (100 mM 4× 0.25 ml; Thermo Fisher Scientific, cat. no. FERR0181)
- 100-bp DNA ladder (Thermo Fisher Scientific, cat. no. SM0243)
- GlycoBlue (Ambion, cat. no. AM9516)
- 3 M Sodium acetate (pH 5.2; Cellgro, cat. no. 46-033-CI)
- Ethanol (Decon Labs, cat. no. 2716) **! CAUTION** Ethanol is flammable, and it should be stored and handled under appropriate conditions.
- Isopropanol (Sigma-Aldrich, cat. no. 437522) **! CAUTION** Isopropanol is flammable, and it should be stored and handled under appropriate conditions.
- 1 M UltraPure Tris-HCl (pH 8.0; Life Technologies, cat. no. 15568-025)
- 1 M UltraPure Tris-HCl (pH 7.5; Life Technologies, cat. no. 15567-027)
- Buffer EB (Qiagen, cat. no. 19086)
- PEG-8000 (Sigma-Aldrich, cat. no. 89510)
- 0.5 M EDTA (Cellgro, cat. no. 46-034-CI)
- Qubit dsDNA HS kit (Life Technologies, cat. no. Q32851)
- IGEPAL CA-630 (Sigma-Aldrich, cat. no. I8896-50ML)
- Triton X-100 (Sigma-Aldrich, cat. no. X100-5ML)
- 1× DPBS (Life Technologies, cat. no. 14190-250)
- Protease Inhibitor Tablets (Roche, cat. no. 04693116001)
- QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
- Rapid DNA Ligation Kit (Thermo Fisher Scientific, cat. no. K1422)
- 5 M Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5150-1L)
- 0.25% Trypsin-EDTA (Thermo Fisher Scientific, cat. no. 25200056)

EQUIPMENT

- Water bath (set to 60 °C; VWR 89501-464 or similar)
- Thermocycler (Eppendorf 6321000019 or similar)
- DynaMag Magnetic Rack (Life Technologies, cat. no. 12321D)
- DynaMag Spin Magnet (Thermo Fisher Scientific, cat. no.12320D)
- Qubit Fluorometer (Life Technologies, cat. no. Q33216)
- 0.2-ml PCR tubes (Fisher, cat. no. 14-230-212)
- 1.5-ml Microcentrifuge tubes (Fisher, cat. no. 05408129)
- 6% TBE-PAGE gels (Life Technologies, cat. no. EC6265BOX)
- Cell scraper (Fisher, cat. no. 08-100-241)
- 50-ml Tube (Fisher, cat. no. 14-432-22)
- Cell culture plates (Sigma-Aldrich, cat. no. CLS430167-100EA)
- Microcentrifuge
- Millipore Steriflip Filters (Millipore, cat. no. SCGP00525)
- Covaris Sonicator (Covaris, S220)
- Covaris Microtube (Covaris, cat. no. 520045)
- A computer running Unix/Linux distribution

Software

- Python 2.7 (http://www.python.org/)
- FastQC version 0.11.3 or higher (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
- BWA version 0.5.9 or higher (http://bio-bwa.sourceforge.net/)
- SAMtools version 0.1.18 or higher (http://samtools.sourceforge.net/)
- hiclib library (http://mirnylab.bitbucket.org/hiclib/)
- matplotlib library (http://matplotlib.org/)

REAGENT SETUP

Cell culture medium Dilute RPMI 1640 with 15% (vol/vol) FBS (for GM12878 cells) or dilute DMEM with 10% (vol/vol) FBS (for Patski cells).

2.5 M Glycine Adjust the volume of 9.35 g of glycine to 50 ml with ddH$_2$O, and filter-sterilize the solution using a Steriflip filter. Store the solution at RT for up to 6 months.

10 mM dATP Dilute 20 µl of 100 mM dATP from the dNTP Set with 80 µl of ddH2O.

80% (vol/vol) Ethanol Mix 8 ml of 100% (vol/vol) ethanol with 2 ml of ddH$_2$O. Freshly prepare 80% (vol/vol) ethanol on the day of the experiment.

10% (vol/vol) Triton X-100 Mix 1 ml of 100% (vol/vol) Triton X-100 with 9 ml of ddH$_2$O. Store the solution at RT for up to 6 months.

1× Cell lysis buffer Mix 500 µl of 1M Tris-HCl, pH 8.0, 100 µl of 5 M NaCl, and 1 ml of 10% IGEPAL CA-630 and bring the final volume to 50 ml with ddH$_2$O. Store the buffer at 4 °C for up to 6 months.

1× TE lysis buffer Mix 2.5 ml of 1 M Tris-HCl, pH 7.0, 100 µl of 0.5 M EDTA, and 5 ml of 10% (wt/vol) SDS and bring the final volume to 50 ml with ddH$_2$O. Store the buffer at RT for up to 6 months.

2× B&W buffer Mix 500 µl of 1 M Tris-HCl, pH 8.0, 100 µl of 0.5 M EDTA, and 20 ml of 5 M NaCl and bring the final volume to 50 ml with ddH$_2$O. Store the buffer at RT for up to 6 months.

1x B&W buffer Dilute 25 ml of 2x B&W buffer with 25 ml of ddH2O.

0.5x B&W buffer Dilute 25 ml of 1x B&W buffer with 25 ml of ddH2O.

0.5× DNase digestion buffer Mix 25 µl of 10× DNase digestion buffer with 25 µl of 10 mM $MnCl_2$ and 450 µl of ddH$_2$O. Use the buffer immediately.

0.5× DNase digestion buffer with 2% (vol/vol) Triton X-100 and RNase A Mix 25 µl of 10× DNase digestion buffer with 25 µl of 10 mM $MnCl_2$, 4 µl of RNase A, 100 µl of 10% (vol/vol) TritonX-100, and 346 µl of ddH$_2$O. Use the buffer immediately.

0.5× DNase I digestion buffer with 0.2% (wt/vol) SDS Mix 25 µl of 10× DNase I digestion buffer with 25 µl of 10 mM MnCl2, 10 µl of 10% (wt/vol) SDS and 440 µl of ddH2O. Use the buffer immediately.

6× Stop solution Mix 12.5 ml of 0.5 M EDTA with 12.5 ml of 10% (wt/vol) SDS and bring the final volume to 50 ml with ddH$_2$O. Store the solution at RT for up to 6 months.

AMPure buffer Mix 10 g of PEG-8000 in 25 ml of 5 M NaCl and bring the final volume to 50 ml with ddH$_2$O. Shake the solution vigorously to mix until PEG-8000 has completely dissolved. Store the buffer at 4 °C for up to 6 months.

PROCEDURE

Adaptor annealing and cross-linking of cells ● TIMING 3 h plus overnight incubation

1| Set up the reactions shown in **Tables 1–3**.

TABLE 1 | Blunt bridge adaptor (40 μM final).

Component	Amount (μl)	Final concentration
100 μM bridge adaptor 5′	80	40 μM
100 μM blunt bridge adaptor 3′	80	40 μM
10× NEBuffer 2	20	1×
ddH$_2$O	20	
Total volume	200	

TABLE 2 | Biotinylated bridge adaptor (40 μM final).

Component	Amount (μl)	Final concentration
100 μM biotinylated bridge adaptor 5′	80	40 μM
100 μM bridge adaptor 3′T	80	40 μM
10× NEBuffer 2	20	1×
ddH$_2$O	20	
Total volume	200	

TABLE 3 | Y adaptor (25 μM final).

Component	Amount (μl)	Final concentration
100 μM SeqAdapt_F	50	25 μM
100 μM SeqAdapt_R	50	25 μM
10× NEBuffer 2	20	1×
ddH$_2$O	80	
Total volume	200	

2| Anneal the mixtures by heating them to 98 °C for 6 min, and then allow the tubes to naturally cool to RT overnight.
■ **PAUSE POINT** Annealed adaptors can be kept at –20 °C indefinitely.

3| Cells should be grown in appropriate culture medium. 2–5 × 10^6 cells are sufficient for making one DNase Hi-C library. However, we suggest growing, cross-linking, and aliquotting many cells (i.e., 1–5 × 10^7 cells) to provide replicates, if necessary. Below are protocols for handling adherent monolayer cells (option A) or suspension cells (option B):

(A) Cross-linking of adherent monolayer cells

- (i) Aspirate out the medium and add 10 ml of serum-free medium per 10-cm plate.
- (ii) Cross-link the cells by adding 280 μl of 37% (vol/vol) formaldehyde to obtain a 1% final concentration. Mix gently, immediately after the addition of formaldehyde.
- (iii) Incubate the cells at RT for exactly 10 min, gently rocking the plates every 2 min.
- (iv) Quench the reaction by adding 0.5 ml of 2.5 M glycine and mixing the solution well.
- (v) Incubate the cells for 5 min at RT, and then on ice for 15 min to stop cross-linking completely.
- (vi) Wash the cells once with cold 1× PBS.

(vii) Treat the cells with 3–5 ml of 0.25% trypsin per dish at 37 °C for 5 min.
(viii) Add 5 ml of fresh medium with serum.
(ix) Scrape the cells from the plates with a cell scraper and transfer them to a 50-ml tube (combine all the cells from all the dishes in one tube).
(x) Centrifuge the cross-linked cells at 800*g* for 10 min at RT.
(xi) Discard the supernatant by aspiration, and wash the cross-linked cells with 1 × PBS once.
(xii) Divide the cells into aliquots in 1.5-ml microtubes (2.5 million cells per tube).

■ **PAUSE POINT** The cells can be snap-frozen in liquid nitrogen and stored for at least one year at −80 °C, or one can continue with cell lysis.

(B) Cross-linking of suspension cells

(i) Gently pellet the cells by spinning them at 300*g* for 10 min at RT.
(ii) Discard the supernatant.
(iii) Resuspend the pellet in 10 ml of fresh culture medium without serum. Break the cell clumps by pipetting up and down.
(iv) Cross-link the cells by adding 280 µl of 37% (vol/vol) formaldehyde (1% final concentration). Mix quickly by inverting the tube several times.
(v) Incubate the cells at RT for exactly 10 min. Gently invert the tube every 2 min.
(vi) Add 0.5 ml of 2.5 M glycine to quench the cross-linking reaction, and mix well.
(vii) Incubate the mixture for 5 min at RT and then on ice for 15 min to stop cross-linking completely.
(viii) Centrifuge the cross-linked cells at 800*g* for 10 min at 4 °C.
(ix) Aspirate and discard the supernatant, and wash the cross-linked cells with 1× PBS once.
(x) Split the cross-linked cell suspension into aliquots of 2.5×10^6 cells (in 1.5-ml microtubes).
(xi) Centrifuge the cross-linked cells at 800*g* for 10 min at RT.
(xii) Aspirate and discard the supernatant.

■ **PAUSE POINT** Cells can be snap-frozen in liquid nitrogen and stored for up to 1.5 years at −80 °C, or one can continue with cell lysis.

Cell lysis and chromatin digestion with DNase I ● TIMING 1.5 h

4| Resuspend one cross-linked cell aliquot ($0.5–2.5 \times 10^6$ cells) in 0.4 ml of ice-cold cell lysis buffer containing protease inhibitor.
▲ **CRITICAL STEP** Add 1 tablet of protease inhibitor to 10 ml of ice-cold lysis buffer immediately before lysis. We recommend using lysis buffer with freshly added protease inhibitor for all experiments.

5| Incubate the mixture on ice for 10 min.

6| Centrifuge the mixture for 60 s at 2,500*g* at RT.

7| Discard the supernatant and resuspend the pellet in 100 µl of 0.5× DNase I digestion buffer containing 0.2% (wt/vol) SDS.
▲ **CRITICAL STEP** For larger cell inputs (i.e., $3–5 \times 10^6$), we recommend using 200 µl of 0.5× DNase I digestion buffer instead.

8| Incubate the mixture at 37 °C for 30 min.

9| Add 100 µl of 0.5× DNase I digestion buffer containing 2% (vol/vol) Triton X-100 and 4 µl of RNase A, and mix the solution well.

10| Incubate the mixture at 37 °C for 10 min.

11| Add 1.5 units of DNase I and mix well.

12| Incubate the mixture at RT for 4 min.

13| Add 40 µl of 6× stop solution, and mix well.

14| (Optional) To determine the efficacy of DNase I digestion, take 20 µl of lysed cells from the previous step and add them to a new tube. Add 70 µl of 1× TE lysis buffer and 10 µl of proteinase K (20 mg/ml). Incubate for 30 min at 65 °C. Purify the DNA using a Qiaquick PCR purification kit. Check the quality of chromatin digestion by running the samples out on a 6% TBE-PAGE gel. The sample is properly digested if one sees a large smear of DNA fragments between ~100 bp and 1 kb (**Fig. 3a**). We recommend characterizing DNase I digestion efficiency when performing the protocol on a new cell type.

In the event of overdigestion or underdigestion of chromatin, we recommend optimizing the concentration of SDS in the digestion reaction, the amount of DNase I used or the digestion time.
? TROUBLESHOOTING

15| Centrifuge the cells for 60 s at 2,500*g* at RT.

16| Discard the supernatant and resuspend the pellet in 150 µl of water.
? TROUBLESHOOTING

17| Add 300 µl of AMPure XP beads; mix them thoroughly by pipetting up and down.

18| Incubate the mixture at RT for 5 min and place the tube in a DynaMag magnet for 2 min.

19| Discard the supernatant and wash the beads twice with 1 ml of freshly prepared 80% (vol/vol) ethanol. Spin down the beads at 500*g* for 10 s to remove the residual ethanol.

20| Resuspend the beads in 169 µl of water, and proceed immediately to the next step.

Chromatin end repair and dA-tailing ● TIMING 2.5 h

21| Prepare the end-repair reaction as follows:

Reagents (add in this order)	Volume (µl)	Final concentration
Nuclei w/beads	169	
10× T4 ligase buffer with ATP	20	1×
10 mM dNTPs	5	0.25 mM
T4 DNA Polymerase (3 U/µl)	3	0.045 U/µl
Klenow fragment (10 U/µl)	3	0.15 U/µl
Total volume	200	

22| Incubate the mixture at RT for 1 h.

23| Add 5 µl of 10% (wt/vol) SDS to stop the reaction.

24| Centrifuge the mixture for 60 s at 2,500*g* at RT.

25| Aspirate and resuspend the pellet in 135 µl of water.

26| Prepare the dA-Tailing reaction as follows:

Reagents (add in this order)	Volume (µl)	Final concentration
Nuclei with beads	135	
10× NEBuffer 2	20	1×
10 mM dATP	10	0.5 mM
10% Triton X-100	20	1%
Klenow fragment (exo⁻) (5 U/µl)	15	0.375 U/µl
Total volume	200	

27| Incubate the resulting mixture at 37 °C for 1 h.

28| Add 5 µl of 10% (wt/vol) SDS to stop the reaction.

29| Centrifuge the mixture for 60 s at 2,500*g* at RT.

30| Aspirate and resuspend the pellet in 30 µl of nuclease-free water.

Ligation of biotin-labeled bridge adaptors ● TIMING overnight, followed by 0.5 h

31| Prepare the adaptor ligation reaction as follows, using the annealed adaptors from Step 2:

Reagents (add in this order)	Volume (µl)	Final concentration
Nuclei with beads	30	
Annealed bridge adaptor with biotin (40 µM)	20	8 µM
Annealed blunt adaptor w/o biotin (40 µM)	20	8 µM
10× T4 ligase buffer with ATP	10	1×
PEG-4000 (50%)	10	5%
10% Triton X-100	5	0.5%
T4 DNA Ligase (5 U/µl)	5	0.25 U/µl
Total volume	100	

32| Incubate the mixture at 16 °C overnight.

33| (Optional) To examine the efficacy of the above end-repair, dA-tailing, and adaptor ligation reactions, take 3 µl of nuclei from Step 30 to perform a control ligation reaction with the Illumina Y adaptor from Step 2 as given below:

Reagents (add in this order)	Volume (µl)	Final concentration
Nuclei with beads	3	
Illumina Y adaptor (50 µM)	1	2.5 µM
Water	10	
10× T4 ligase buffer with ATP	2	1×
PEG-4000 (50%)	2	5%
10% Triton X-100	1	0.5%
T4 DNA Ligase (5 U/µl)	1	0.25 U/µl
Total volume	20	

After incubation at 16 °C overnight, add 70 µl of 1× TE lysis buffer and 10 µl of proteinase K (20 mg/ml). Incubate the mixture for 30–60 min at 65 °C. Purify genomic DNA using a QiaQuick PCR purification kit. Check the ligation efficiency by carrying out qPCR with Illumina PCR primers. If upstream end-repair and dA-tailing steps are efficient, one should see amplification before 10 PCR cycles using 10 ng of genomic DNA as a template. We recommend this QC step when performing the protocol on a new cell type. In the event of inefficiency of these steps, we recommend optimizing the concentration of SDS in the cell lysis step or optimizing the amount of DNase I used for digestion.

34| Add 5 µl of 10% (wt/vol) SDS to stop the reaction.

35| Centrifuge the mixture for 60 s at 2,500*g* at RT.

36| Resuspend the pellet in 200 µl of nuclease-free water.

37| Add 165 µl of AMPure buffer; mix thoroughly by pipetting up and down.

38| Incubate the mixture at RT for 5 min, and place the tube in a DynaMag magnet for 2 min.

39| Discard the supernatant and wash the beads once with 1 ml of freshly prepared 80% (vol/vol) ethanol. Spin down the beads at 500*g* for 10 s to remove the residual ethanol.
▲ **CRITICAL STEP** We recommend diluting fresh 80% (vol/vol) ethanol before each experiment.

40| Resuspend the pellet in 200 µl of water.

41| Add 165 µl of AMPure bead buffer; mix the solution thoroughly by pipetting up and down.

42| Incubate the mixture at RT for 5 min, and then place the tube in a DynaMag magnet for 2 min.

43| Discard the supernatant and wash the beads twice with 500 µl of 80% (vol/vol) ethanol. Spin down the beads at 500*g* for 10 s to remove the residual ethanol as completely as possible, and then air-dry the beads for no more than 2 min.

44| Resuspend the nuclei–bead mixture in 80 µl of nuclease-free water.

In situ phosphorylation ● TIMING 1.25 h

45| Prepare the PNK reaction as follows:

Reagents (add in this order)	Volume (µl)	Final concentration
Nuclei with beads	80	
10× T4 ligase buffer w/ATP	10	1×
PNK (10 U/µl)	10	1 U/µl
Total volume	100	

46| Incubate the mixture at 37 °C for 1 h.

In situ ligation ● TIMING 4.25 h

47| Add the following to the PNK mixture after incubation in Step 46:

Reagents (add in this order)	Volume (µl)	Final concentration
H_2O	794	
10× T4 ligase buffer	100	1×
T4 DNA Ligase (5 U/µl)	6	0.03 U/µl
Total volume	1 ml	

48| Incubate the mixture at RT for 4 hr. For a micrograph of nuclei after this stage, see **Figure 2b**.

Cross-linking reversal, isopropanol precipitation, and DNA purification ● TIMING overnight, followed by 2.5 h

49| Centrifuge the mixture for 60 s at 2,500*g* at RT.

50| Resuspend the pellet in 400 µl of 1× NEBuffer 2.

51| Add 40 µl of 10% (wt/vol) SDS.

52| Add 40 µl of 20 mg/ml proteinase K.

53| Incubate the mixture overnight at 60 °C.

54| Add 3 µl of GlycoBlue, 50 µl of 3 M sodium acetate, pH 5.2, and 550 µl of isopropanol.

55| Incubate the mixture at –80 °C for 2 h.

56| Centrifuge the mixture for 30 min at 4 °C at maximum speed in a microcentrifuge.

57| Resuspend the DNA pellets in each tube with 100 µl of nuclease-free water.

58| Add 100 µl of AMPure XP beads, and mix well.

59| Incubate the mixture at RT for 5 min, and place the tube in a DynaMag magnet for 2 min.

60| Discard the supernatant and wash the beads twice with 1 ml of 80% (vol/vol) ethanol. Spin down the beads at 500*g* for 10 s to remove the residual ethanol as completely as possible, and then air-dry the beads for no more than 2 min.

61| Resuspend the beads in 130 µl of nuclease-free water.

62| Incubate the beads at RT for 1 min. Collect the beads via DynaMag magnet, and transfer the eluent to a fresh 1.5-ml tube. At this point, determine the concentration of the recovered DNA with a spectrophotometer. A typical yield is 3–5 µg if one is starting with 2.5 × 10^6 cells.
■ **PAUSE POINT** Purified DNA can be stored indefinitely at −20 °C.

DNA sonication ● TIMING 0.5 h

▲ **CRITICAL** At this point, purified DNA may be sonicated to shear large fragments to the 100- to 500-bp range or one may proceed directly to sequencing library preparation. Sonication promotes a less biased representation of fragment ends at the cost of additional preparation time and loss of material. The protocol here is suitable for Covaris sonicators. If sonication is not desired, skip to Step 66.

63| Transfer the DNA to a Covaris microtube.

64| Shear the DNA to a size of 100–500 bp using a sonicator. For a Covaris instrument, use the following parameters:

Parameter	Setting
Duty cycle	15%
Peak incident power	450
Cycles per burst	200
Set mode	Frequency sweeping
Continuous degassing	
Process time	80 s
Number of cycles	5

65| Transfer 130 µl of sonicated DNA to a 1.5-ml tube.
■ **PAUSE POINT** Eluted DNA may be stored indefinitely at −20 °C.

Biotin pull-down ● TIMING 0.5 h

66| Wash 30 µl of MyOne C1 beads twice with 100 µl of 1× B&W buffer, wash once with 100 µl of 2× B&W buffer, and then resuspend in 100 µl of 2× B&W buffer.

67| Add 100 µl of eluted DNA to resuspended streptavidin beads and mix well.

68| Incubate the sample for 20 min at RT on a rotator.

69| Place the tube in DynaMag magnet for 1 min, and discard the supernatant.

70| Wash the beads once with 300 µl of 0.5× TE lysis buffer plus 300 µl of 0.5× B&W buffer.

71| Wash the beads twice with 600 µl of 1× B&W buffer.

72| Wash the beads once with 600 µl of 1× NEBuffer 2.

73| Wash the beads once with 600 µl of Buffer EB.

74| Resuspend the beads in 170 µl of Buffer EB.
■ **PAUSE POINT** Resuspended beads may be stored at −20 °C indefinitely or at 4 °C for short-term storage.

End repair and dA-tailing ● TIMING 1.5 h

75| Set up the end-repair reaction with the Fast DNA End Repair Kit as follows:

Reagents (add in this order)	Volume (µl)	Final concentration
Purified DNA	170	
10× Reaction buffer	20	1×
End-repair enzyme mix	10	
Total volume	200	

76| Incubate the mixture at 18 °C for 10 min.

77| Add 200 µl of AMPure buffer, and mix it thoroughly by pipetting up and down.

78| Incubate the mixture at RT for 5 min, and place the tube in a DynaMag. Spin magnet at 500g for 10s.

79| Discard the supernatant and wash the beads twice with 500 µl of 80% (vol/vol) ethanol. Briefly spin down the beads, remove the residual ethanol as completely as possible, and air-dry the beads for 5 min.

80| Resuspend the beads in 21.5 µl of water.

81| Set up the dA-tailing reaction as follows:

Reagents (add in this order)	Volume (µl)	Final concentration
End-repaired DNA with beads	21.5	
10× NEBuffer 2	3	1×
10 mM dATP	3	1 mM
Klenow fragment (exo$^-$) (5 U/µl)	2.5	0.42 U/µl
Total volume	30	

82| Incubate the mixture at 37 °C for 30 min.

83| Wash the beads twice with 400 µl of 1× B&W buffer.

84| Wash the beads twice with 400 µl of Buffer EB and resuspend them in 30 µl of Buffer EB.
▲ **CRITICAL STEP** Proceed immediately to adaptor ligation.

Ligation of sequencing adaptors ● TIMING 1 h

85| Immediately resuspend the beads in the following reaction mixture:

Reagents (add in this order)	Volume (µl)	Final concentration
dA-tailed DNA with beads	30	
5× Rapid Ligation Buffer (from Rapid DNA Ligation Kit)	10	1×
Y adapter (2.5 µM)	6	0.3 µM
T4 DNA Ligase (5 U/µl)	4	0.4 U/µl
Total volume	50	

86| Incubate the mixture at RT for 30 min.
■ **PAUSE POINT** The ligation reaction in Step 86 can also be performed at 16 °C overnight.

87| Add 5 µl of 0.5 M EDTA to stop the reaction. Add 145 µl of ddH$_2$0 to bring the volume to 200 µl, and mix thoroughly by pipetting up and down.

88| Add 200 µl of AMPure buffer to each tube and mix thoroughly by pipetting up and down.

89| Incubate the mixture at RT for 5 min and then place the tubes in a DynaMag magnet for 2 min.

90| Discard the supernatant and wash the beads twice with 500 µl of 80% (vol/vol) ethanol. Spin down the beads at 500*g* for 10 s to remove the residual ethanol as completely as possible, and then air-dry the beads for no more than 2 min.

91| Resuspend the beads in 200 µl of ddH$_2$O and add 165 µl of AMPure buffer

92| Mix thoroughly by pipetting up and down.

93| Incubate the mixture at RT for 5 min, and place the three tubes in a DynaMag magnet for 2 min.

94| Discard the supernatant and wash the beads twice with 0.5 ml of 80% (vol/vol) ethanol. Spin down the beads at 500*g* for 10 s to remove the residual ethanol as completely as possible, and then air-dry the beads for 5 min.

95| Resuspend the beads in each tube with 50 µl of EB.

Library amplification ● TIMING 2.5 h

▲ **CRITICAL** Optimization of input amount and PCR cycle number is integral to obtaining a sufficiently diverse *in situ* DNase Hi-C library. We recommend running several 'pilot' PCR reactions with various bead input amounts and various cycle numbers and running these 'pilot' libraries on a 6% TBE-PAGE gel to ensure that library overamplification is not occurring.

96| To determine the number of PCR cycles that are necessary to generate ample PCR products for sequencing—importantly, without overamplification—set up trial PCR reactions with 10, 12, or 14 cycles, and 2.5 or 5 µl of DNA-bound streptavidin beads as follows:

Reagents (add in this order)	Volume (µl)	
End-repaired DNA with beads	2.5/5	
2× HotStart ReadyMix	10	1×
10 µM SeqPrimer_F	1	1 µM
10 µM SeqPrimer_R	1	1 µM
ddH$_2$O	Up to 20	
Total volume	20	

Use the following PCR program:

Cycle number	Denature	Anneal	Extend
1	95 °C, 3 min		
2–6	98 °C, 20 s	60 °C, 20 s	72 °C, 1 min
7–17[a]	98 °C, 20 s	65 °C, 20 s	72 °C, 1 min

[a] Use optimized cycle number.

97| Run 2 µl from each PCR reaction on a 6% TBE-PAGE gel to determine the appropriate number of cycles and amount of input beads for each PCR reaction. PCR products should run from ~200 bp to ~1 kbp, with the majority of product running from 300–600 bp, as shown in **Figure 3b**. The presence of products much larger than 1 kbp (i.e., those that do not migrate on a 6% TBE-PAGE gel) indicates overamplification, and this should be avoided by reducing PCR cycle number or the volume of beads used.

98| Divide the remaining beads into 20-µl aliquots, and amplify the DNA using multiple PCR reactions at the optimized cycle and input parameters.

99| Pool all PCR reactions into one 1.5-ml microcentrifuge tube.

100| Purify the library by adding 0.8× volumes of AMPure XP beads.

101| Incubate the mixture at RT for 5 min and place the tube in a DynaMag magnet for 2 min.

102| Discard the supernatant and wash the beads twice with 1 ml of 80% (vol/vol) ethanol. Spin down the beads at 500*g* for 10 s to remove the residual ethanol as completely as possible, and then air-dry the beads for no more than 2 min.

103| Resuspend the beads in 25 µl of Buffer EB and incubate the mixture at RT for 1 min.

104| Place the resuspended beads on DynaMag magnet, and transfer the supernatant containing the eluted DNA to a fresh 1.5-ml tube.

QC of DNase Hi-C library by BamHI digestion ● TIMING 1.25 h

105| Quantify the amount of dsDNA in the library using the Qubit dsDNA HS kit per the manufacturer's protocols.

106| Digest a small aliquot of the final DNase Hi-C library (50–100 ng) with BamHI to estimate the portion of molecules with valid biotinylated junctions as follows:

Reagents (add in this order)	Digest	(–) Control
10× Fast digestion buffer	1 µl	1 µl
DNase Hi-C product	1–2 µl (50–100 ng)	1–2 µl (50–100 ng)
FastDigest BamHI	1 µl	0 µl
Water	To 10 µl	to 10 µl

107| Incubate the mixture at 37 °C for 30 min.

108| Run the entire volume of the mixture on a 6% TBE-PAGE gel. Digested libraries should demonstrate a marked shift in library size distribution, as shown in **Figure 3b**. If the libraries pass this QC metric, proceed to Illumina sequencing.
? TROUBLESHOOTING

109| (Optional) Hybrid capture experiments may be carried out according to the manufacturer's protocols provided with the Agilent SureSelect system.
■ **PAUSE POINT** Data can be analyzed at any point after sequencing.

Mapping, normalization, and visualization of Hi-C contact maps ● TIMING variable, dependent on the volume of data

110| Copy the output fastq sequencing files generated by the Illumina sequencer to the storage on the Linux computer.

111| Open a terminal on the computer and enter after the $ sign the commands described in the following steps. First, run FastQC to investigate the sequencing qualities, in which 'L1_1' and 'L1_2' correspond to the fastq sequence files for read 1 and read 2, respectively.

```
$ fastqc --extract -f fastq L1_1.fq L1_2.fq
```

? TROUBLESHOOTING

112| Obtain reference genome sequences. For instance, the mouse mm9 reference sequences can be downloaded from the UCSC Genome Browser using the command below.

```
$ wget "http://hgdownload.cse.ucsc.edu/goldenPath/mm9/bigZips/chromFa.tar.gz"
$ tar -xzvf chromFa.tar.gz
$ gunzip -c chr*.fa.gz > mm9.fa
```

▲ **CRITICAL STEP** If the *in situ* DNase Hi-C data are from female cells, do not include chrY (delete the chrY.fa.gz file prior to generating the mm9.fa file).

113| Run BWA to generate index files for the reference genome.

```
$ bwa index -a bwtsw -p mm9 mm9.fa
```

114| Run BWA to map each end of the pair-ended reads to the reference genome separately.

```
$ bwa aln mm9 L1_1.fq > L1_1.sai
$ bwa samse mm9 L1_1.sai mm9.fa > L1_1.sam
$ bwa aln mm9 L1_2.fq > L1_2.sai
$ bwa samse mm9 L1_2.sai mm9.fa > L1_2.sam
```

▲ **CRITICAL STEP** The two ends of the reads should be mapped separately.

115| Run SAMtools to exact high-quality (mapping quality (MAPQ) score ≥30) and uniquely mapped reads.

```
$ samtools view -S -F 4 L1_1.sam | awk ’$5 ≥30 &&
$ 12=="XT:A:U"' | cut -f 1-4 | sort -k1,1 > L1_1.mapped
$ samtools view -S -F 4 L1_2.sam | awk ’$5 ≥30 &&
$ 12=="XT:A:U"' | cut -f 1-4 | sort -k1,1 > L1_2.mapped
```

116| Join mapped loci pairs if both ends are successfully mapped.

```
$join L1_1.mapped L1_2.mapped > L1.mapped
```

117| Remove PCR duplicates.

```
$ cut -f 2-7 L1.mapped | awk 'BEGIN{OFS="\t";}
{if($2<$5){print $0;} else if($2>$5){print
$4,$5,$6,$1,$2,$3;} else if($3<=$6){print $0;} else{print
$1,$2,$6,$2,$5,$3;}}' | sort -u > L1.unique
```

118| Parse the mapped contacts loci pairs to generate the Hi-C contact map at a given resolution.

119| Run ICE (iterative correction and eigenvector decomposition)[53] to normalize the contact matrix using the Mirny laboratory's hiclib library (https://bitbucket.org/mirnylab/hiclib).

120| Visualize the contact map.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Steps	Problem	Possible reasons	Solutions
14	gDNA digestion efficiency is poor	Undertreatment of fixed nuclei with SDS; inadequate amount of DNase I used for digestion	Optimization of the appropriate SDS and DNase I amounts may be necessary. We recommend performing the protocol through Step 38 for a variety of SDS concentrations (i.e., 0.1–0.5%) and DNase I amounts (i.e., 1–8U)
16	Nuclear pellet disappears during *in situ* enzymatic treatments	Overtreatment of fixed nuclei with SDS in Step 7	Reduce the amount of SDS used in the cell lysis
108, 120	Low percentage of long-range contacts in sequencing library or BamHI digest does not shift the library	Inefficient or incomplete formaldehyde cross-linking in Step 3	For new cell types, optimizing the amount of formaldehyde used for cross-linking may be necessary
111	FastQC metrics are poor	High duplication rate in library (e.g., <60% unique sequences); low-quality sequencing run (e.g., total percentage of bases with *q* > 30 is less than 85%)	To maximize library complexity, make sure to set up several PCR reactions in Step 114. Issues with sequencing runs themselves may be difficult to diagnose and may require outside help

● TIMING

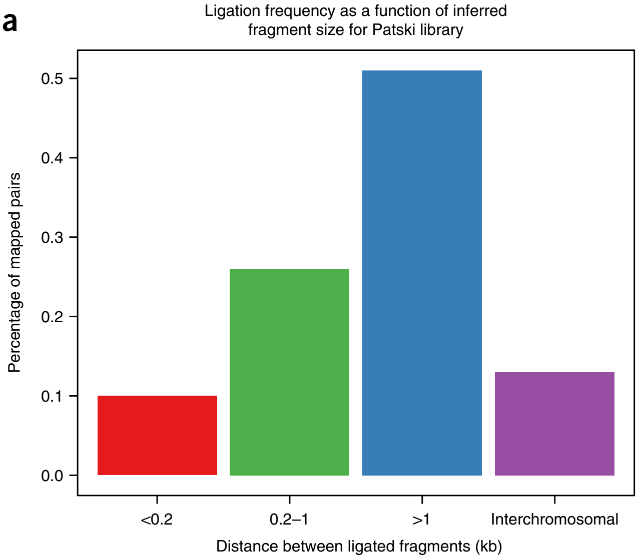
Steps 1 and 2 (day 1), adaptor annealing: 1 h plus overnight
Steps 3–32 (day 2), fixation, cell lysis, chromatin digestion, end repair, and adaptor ligation: ~6 h plus overnight
Steps 33–53 (day 3), adaptor cleanup; *in situ* phosphorylation and ligation; cross-link reversal: ~8 h plus overnight
Steps 54–65 (day 4), DNA purification and sonication: 2.5–3.5 h
Steps 66–86 (day 5), biotin pull-down, end repair/dA tailing, and adaptor ligation of Hi-C fragments: ~3 h
Steps 87–109 (day 6), library amplification, BamHI quality check, and sequencing: ~4 h for amplification and quality check; up to several days/weeks for sequencing, depending on the instrumentation
Steps 110–120 (day 7 and beyond), data analysis time is variable and depends on the sequencing depth and available computer resources
Box 1, assessment of nuclear lysis at various steps: ~3 d

ANTICIPATED RESULTS

We recommend QCing all libraries that pass the BamHI digestion test (typical results, including a negative control EcoRI digest, shown in **Fig. 3b**) by sequencing at low depth first to ensure that the libraries are sufficiently complex for your desired application. We also recommend quantifying the length classes of sequenced ligation pairs in libraries; *in situ* DNase Hi-C libraries should demonstrate an enrichment for pairs mapping with long-range (i.e., >1 kb) distances between them (example distributions shown in **Fig. 4a**). Furthermore, we recommend quantifying the relative numbers of different ligation pairs (i.e., 'in-facing,' 'out-facing,' 'left', and 'right') in libraries (a typical example is shown in **Fig. 4b**). Corrected matrices generated from valid *in situ* DNase Hi-C (DHC) libraries should be analogous to the example shown in **Figure 4c**, with large-scale structures (i.e., TADs) clearly visible even at 100-kb resolution.

We have observed that the relative fraction of interchromosomal ligation pairs in *in situ* DNase Hi-C libraries is largely cell type–specific but highly reproducible—in line with previously published *in situ* results[43,50]. This is evident in **Supplementary Figure 1**, which compares fractions of various ligation pairs between the Patski cell line and three replicates of the human lymphoblastoid cell line GM12878. When considering gold standards for *in situ* DNase Hi-C experiments, we typically look to the abundance of 'long-range' ligation pairs in our libraries, which typically make up >40% of uniquely mapped read pairs.

Using this modified DHC protocol, we have shown that the inactive murine X chromosome adopts a bipartite structure,



b

	Left–left	Right–right	Inner	Outer
Fragments >1 kb	120,252	120,010	121,724	120,262
Fragments ≤1 kb	13,259	13,298	293,999	21,621

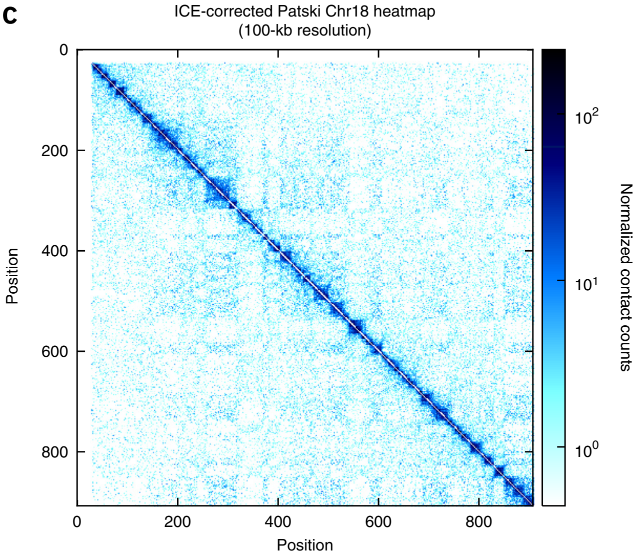


Figure 4 | *In situ* DNase Hi-C results for the mouse embryonic kidney Patski cell line. (**a**) *In situ* DNase Hi-C reads (950,206 downsampled reads from data published in Deng *et al.*[51] (using the mouse Patski cell line, rather than the GM12878 cell line) demonstrate an enrichment for long-range (i.e., >1 kb) intrachromosomal read pairs expected of Hi-C libraries. (**b**) Expected breakdown of orientations for read pairs in *in situ* DNase Hi-C data. For intrafragment distances >1 kb, a roughly 25% split should be observed for each orientation class. (**c**) Normalized heatmap generated from data published in Deng *et al.* (GEO accession no. GSE68992) for mouse chromosome 18- at 100-kb resolution. The data set used to generate this heatmap contained 60,666,200 uniquely mapped, high-quality read pairs.

consistent with results obtained using traditional Hi-C, in both an analogous murine system[35] and human lymphoblastoid cells[43]. These results suggest that the *in situ* DHC protocol produces a signal comparable to that of existing Hi-C protocols while ultimately providing a less-biased empirical method for generating higher-resolution 3D maps of chromatin structure.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS V.R. and Z.D. developed the protocol. V.R., D.A.C., R.J.H., R.Q., and Z.D. performed experiments and optimized the protocol. W.M. and W.S.N. devised the processing pipeline for *in situ* DNase Hi-C data. X.D., C.A.B., C.M.D., W.S.N., J.S., and Z.D. supervised the research. V.R., J.S., and Z.D. wrote the manuscript, with input from all authors.

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