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Mapping three-dimensional genome architecture through *in situ* DNase Hi-C

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Abstract

With the advent of massively parallel sequencing, considerable work has gone into adapting chromosome conformation capture (3C) techniques to study chromosomal architecture at genome-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, during *in situ* DNase Hi-C chromatin is chemically crosslinked, 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, making them valuable in cases where high sequencing depth is allowed for, or when hybrid capture technologies are expected to be used. The protocol described here, which involves approximately four days of bench work, is optimized for the study of mammalian cells but can be broadly applicable to any cell or tissue of interest given experimental parameter optimization.

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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 research. V.R., J.S., and Z.D. wrote the manuscript, with input from all authors.

Competing Financial Interests

The authors declare no competing financial interests.

Introduction

The manner in which an incredibly long DNA polymer toplogically organizes itself within a cell or nucleus is crucially linked to higher-order cellular function[1,2]. This form-function relationship, first realized through early light microscopic studies of higher-order structures like 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 resolution. Techniques like DNA adenine methyltransferase identication (DamID)[11,12], typically used to map protein-DNA interactions[13–15], have been modified to map genome-wide associations between primary sequence 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 chromosome conformation capture (3C)[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 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], which 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 in 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 (CIDs)[27,37], genomic domains that predominantly self-associate in three-dimensional space. Although the ultimate significance of these domains remains unknown, strong correlations between one-dimensional epigenomic features (*e.g.*, histone marks, DNA methylation, transcription factor binding) and sequence both within and at the borders of these domains suggest that they may play a gene regulatory role.

Although current Hi-C techniques generally allow us to visualize genome-scale chromosome architecture at the resolution of 100 kb to 1 Mb, methodological resolution limitations imposed by incomplete sequencing depth and genome-wide restriction site density have typically precluded identification of topological units at smaller scales, in which local interactions may play 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, one-dimensional 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 that 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 towards completely cataloguing functional DNA looping interactions. In the realm of proximity ligation protocol development, considerable work has gone towards improving the resolution of the Hi-C protocol to the scale of kilobases, where specific regulatory contacts (*i.e.* enhancer-promoter interactions, CCCTC-binding factor (CTCF)-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 towards 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 fashion[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 (lincRNA) 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 consequent improvements to traditional RE Hi-C using 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 illustrated in Figure 1. Anywhere from $5x10^5$ to $1x10^7$ cells are fixed in formaldehyde to reversibly crosslink *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 dA-tailed, facilitating the ligation of an exogenous, dT-tailed "bridge" adaptor containing a single biotinylated thymidine, half BamHI restriction site, and 4-base overhang. After clearing out excess adaptors, the free ends of 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 (SPRI) 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.

Following proximity ligation, nuclei are lysed and crosslinks 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 adapter 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. Prior to sequencing, libraries may be treated with a simple BamHI digestion to assess the efficiency of proximity ligation.

Traditional Hi-C vs. *in situ* DNase Hi-C

In situ DNase Hi-C can be used in any situation where 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 more efficiently generates *cis* (*i.e.* intrachromosomal) ligation products compared to *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 where loci may have particularly low restriction site density). In cases where high-resolution (*i.e.* 1 kb resolution) maps are desired, however, 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, 5C), albeit at the price of only interrogating interactions among a set number of loci.

In situ DNase Hi-C is broadly applicable to any situation where 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 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 $5x10^5$ to $1x10^7$ cells to generate sequenceable libraries. Thus, in cases where input might be particularly limited, or where small populations of cells sorted by fluorescence activated cell sorting (FACS), *in situ* DNase Hi-C may not be appropriate. Second, it is also important to note that while the DNase enzyme is nonspecific when compared to 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 considerations

The *in situ* DNase Hi-C protocol described here is relatively straightforward, and can be completed over four days, allotting 3 – 6 hours 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 of libraries, thanks to the integration of half BamH1 sites in the bridge adapter. 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 crosslinking, 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 crosslinking can lead to an underrepresentation 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% formaldehyde for 10 min at room temperature (RT, 25°C), can be employed. 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 crosslinking. For example, both human and mouse ESCs often aggregate to form large clumps in culture. As such, higher concentrations of formaldehyde are generally used in these situations[48,54].

Cell lysis and DNase I digestion

After crosslinking 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 employs a relative mild condition (0.3–0.5% SDS treatment for 45 min. at 37°C). During this step, it is crucial to avoid overly lysing 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. We recommend carrying out a DNase I and SDS optimization experiment using varying units of DNase I and varying concentrations of SDS when attempting the protocol on new cell types, and examining the DNase I fragmentation pattern following digestion. An example fragmentation pattern is shown in Figure 3a.

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 employ 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.

BamH1 Digestion Control

A BamH1 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 in our previous study[51].

  CAUTION: Cell lines should be regularly checked to ensure that they are authentic and not infected with *Mycoplasma*.
- Pen/Strep Cocktail (Thermo Scientific 15140122)
- Fetal Bovine Serum (Thermo Scientific 10437-010)
- Cell culture medium: RPMI-1760 w/15% FBS (for GM12878; Thermo Scientific 11875-093) or DMEM w/10% FBS (for Patski; Thermo Scientific 11965118)
- Biotinylated Bridge Adaptor 5′: /5Phos/GCTGAGGGA/iBiodT/C (IDT)
- Bridge Adaptor 3′T: CCTCAGCT (IDT)
- Bridge Adaptor 5′: GCTGAGGGAC (IDT)
- Blunt Bridge Adaptor 3′: CCTCAGC (IDT)
- SeqAdapt_F: ACACTCTTTCCCTACACGACGCTCTTCCGATC*T (IDT)
- SeqAdapt_R: /5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCAC (IDT)
- SeqPrimer_F: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT (IDT)
- SeqPrimer_R: CAAGCAGAAGACGGCATACGAGAT[8 bp barcode] GTGACTGGAGTTCAGACGTGTGCT (IDT)

- 37% (vol/vol) Formaldehyde (Sigma Aldrich F8775)

  CAUTION!: Formaldehyde is flammable, can cause skin burns, and is toxic by inhalation. Formaldehyde should be handled using appropriate protective equipment, and be handled in a chemical fume hood.

  CRITICAL: Formaldehyde has a limited shelf life; discard solution if it is older than 1 year.
- Glycine (Sigma-Aldrich 50046)
- NEBuffer 2 (NEB B7002S)
- 10% (wt/vol) UltraPure SDS (Life Tech 15553-027)
- DNase I, RNase-free (supplied with $MnCl_2$ and reaction buffer) (1 U/μL) (Thermo Scientific EN0525)
- RNase A, DNase and protease-free (10 mg/ml) (Thermo Scientific EN0531)
- Klenow Fragment (10 U/μL) (Thermo Scientific EP0052)
- Klenow Fragment (exo^-) (5 U/μL) (Thermo Scientific EP0422)
- T4 DNA Polymerase (5 U/μL) (Thermo Scientific EP0062)
- T4 DNA Ligase (5 U/μL) provided with 50% PEG-4000 (Thermo Scientific EL0012)
- T4 Polynucleotide Kinase (10 U/μL) (Thermo Scientific EK0032)
- 10X T4 DNA Ligase Buffer w/ATP (NEB B0202S)
- Agencourt AMPure XP (Beckman Coulter A63880)
- 2X HotStart PCR ReadyMix (KAPA KK2601)
- Fast DNA End Repair Kit (Thermo Scientific K0771)
- Proteinase K (Thermo Scientific EO0492)
- FastDigest® BamHI (Thermo Scientific FERFD0504)
- Dynabeads® MyOne™ Streptavidin C1 (Life Tech 65001)
- dNTP Set (100 mM 4x 0.25ml) (Thermo Scientific FERR0181)
- 100 bp DNA ladder (Thermo Scientific SM0243)
- GlycoBlue (Ambion AM9516)
- 3 M Sodium acetate (pH 5.2) (Cellgro 46-033-CI)
- Ethanol (Decon Labs Inc. 2716) **CAUTION!** Ethanol is flammable, and should be stored and handled under appropriate conditions
- Isopropanol (Sigma-Aldrich 437522) **CAUTION!** Isopropanol is flammable, and should be stored and handled under appropriate conditions
- 1M UltraPure Tris-HCl pH 8.0 (Life Tech 15568-025)

- 1M UltraPure Tris-HCl pH 7.5 (Life Tech 15567-027)
- Buffer EB (Qiagen 19086)
- PEG-8000 (Sigma-Aldrich 89510)
- 0.5 M EDTA (Cellgro 46-034-CI)
- Qubit dsDNA HS kit (Life Tech Q32851)
- IGEPAL CA-630 (Sigma-Aldrich I8896-50ML)
- Triton X-100 (Sigma-Aldrich X100-5ML)
- 1X DPBS (Life Tech 14190-250)
- Protease Inhibitor Tablets (*e.g.* Roche 04693116001)
- QIAquick PCR Purification Kit (Qiagen 28104)
- Rapid DNA Ligation Kit (Thermo Scientific K1422)
- 5M Sodium Chloride (NaCl) (S5150-1L)
- 0.25% Trypsin-EDTA (Thermo Scientific 25200056)
- Millipore Steriflip Filters (Millipore SCGP00525)

Equipment

- Water bath (set to 60°C)
- Thermocycler
- DynaMag Magnetic Rack (*e.g.* Life Tech 12321D)
- Qubit Fluorometer (*e.g.* Life Tech Q33216)
- 0.2 mL PCR tubes (*e.g.* Fisher 14-230-212)
- 1.5 mL microcentrifuge tubes (*e.g.* Fisher 05408129)
- 6% TBE-PAGE gels (*e.g.* Life Tech EC6265BOX)
- Cell scraper (*e.g.* Fisher 08-100-241)
- 50 ml tube (*e.g.* Fisher 14-432-22)
- Cell culture plates (*e.g.* Sigma CLS430167-100EA)
- Microcentrifuge
- A computer running Unix/Linux distribution with the software listed below installed.

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

2.5 M Glycine: Bring 9.35 g glycine to 50 mL in ddH_2O and filter sterilize using Steriflip column. Store at RT for up to 6 months.

80% (vol/vol) Ethanol: Mix 8 mL 100% ethanol with 2 mL ddH_2O. Make fresh for every day of experiments.

10% (vol/vol) Triton X-100: Mix 1 mL 100% Triton X-100 with 9 mL ddH_2O. Store at RT for up to 6 months.

1X Cell Lysis buffer: Mix 500 μL 1M Tris-HCl pH 8.0, 100 μL 5M NaCl, and 1 mL 10% Igepal CA-630 and bring up to 50 mL in ddH_2O. Store at 4°C for up to 6 months.

1X TE Lysis buffer: Mix 2.5 mL 1M Tris-HCl pH 7.0, 100 μL 0.5M EDTA, and 5 mL 10% SDS and bring up to 50 mL in ddH_2O. Store at RT for up to 6 months.

2X B&W buffer: Mix 500 μL 1M Tris-HCl pH 8.0, 100 μL 0.5M EDTA, and 20 mL 5M NaCl and bring up to 50 mL in ddH_2O. Store at RT for up to 6 months.

0.5X DNase Digestion Buffer: Mix 25 μL 10X DNase digestion buffer with 25 μL 10 mM $MnCl_2$ and 450 μL ddH_2O. Use immediately.

6X Stop Solution: Mix 12.5 mL 0.5M EDTA with 12.5 mL 10% SDS and bring to 50 mL in ddH_2O. Store at RT for up to 6 months.

AMPure Buffer: Mix 10 g PEG-8000 in 25 mL 5M NaCl and bring to 50 mL with ddH_2O. Shake vigorously to mix until PEG-8000 has completely gone into solution. Store at 4°C for up to 6 months

Procedure

Steps 1–2: Adaptor Annealing (Timing: 1h + overnight incubation)

1) Set up the following reactions:

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
10X NEBuffer 2	20	1X
ddH_2O	20	

Blunt Bridge Adaptor (40 μM final)		
Component	**Amount (μL)**	**Final Concentration**
Total Volume	200	

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
10X NEBuffer 2	20	1X
ddH_2O	20	
Total Volume	200	

Y-Adaptor (25 μM final)		
Component	**Amount (μL)**	**Final Concentration**
100 μM SeqAdapt_F	50	25 μM
100 μM SeqAdapt_R	50	25 μM
10X NEBuffer 2	20	1X
ddH_2O	80	
Total Volume	200	

2) Anneal mixtures by heating to 98°C for 6 minutes, then allow the tubes to naturally cool to RT overnight.

PAUSE POINT Annealed adapters can be kept at −20°C indefinitely.

Step 3: Cross-linking of cells (Timing: 2h)

[TROUBLESHOOTING 3]

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

a) Adherent monolayer cells

i. Aspirate out media and add 10 ml of serum-free media per 10 cm plate.

ii. Crosslink the cells by adding 280 μl of 37% formaldehyde to obtain 1% final concentration. Mix gently, immediately after addition of formaldehyde.

iii. Incubate cells at RT for exactly 10 min, gently rocking the plates every 2 min.

iv. Quench reaction by adding 0.5 ml of 2.5 M glycine and mixing well.

v. Incubate for 5 min at RT, then on ice for 15 min to stop cross-linking completely.

vi. Wash cells once with cold 1X PBS.

vii. Treat the cells with 3–5 ml per dish trypsin (0.25%) at 37°C for 5 min.

viii. Add 5 ml fresh medium with serum.

ix. Scrape the cells from the plates with a cell scraper and transfer to a 50 ml tube (combine all the cells from all the dishes to one tube).

x. Centrifuge the cross-linked cells at 800x***g*** for 10 min.

xi. Discard the supernatant by aspiration and wash the cross-linked cells with 1 x PBS once.

xii. Aliquot the cells into 1.5 ml microtubes (2.5 million cells per tube).

PAUSE POINT: 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) Suspension cells

i. Gently pellet the cells by spinning at 300x***g*** for 10 min at RT.

ii. Discard the supernatant.

iii. Resuspend the pellet in 10 ml of fresh culture medium without serum. Break cell clumps by pipetting up and down.

iv. Crosslink the cells by adding 280 µl of 37% formaldehyde (1% final concentration). Mix quickly by inverting the tube several times.

v. Incubate 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, mix well.

vii. Incubate for 5 min at RT, then on ice for 15 min to stop cross-linking completely.

viii. Centrifuge the cross-linked cells at 800x***g*** for 10 min at 4°C.

ix. Discard the supernatant by aspiration and wash the cross-linked cells with 1X PBS once.

x. Split the cross-linked cell suspension into aliquots of 2.5 x 10^6 cells (in 1.5 ml microtubes).

xi. Centrifuge the cross-linked cells at 800x***g*** for 10 min at RT.

xii. Discard the supernatant by aspiration.

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.

Steps 4 – 20: Cell lysis and chromatin digestion with DNase I (Timing: 1.5 h)

4) Resuspend one cross-linked cell aliquot (0.5–2.5 x 10^6 cells) in 0.4 ml of ice-cold cell lysis buffer containing protease inhibitor.

CRITICAL STEP: Add 1 tablet protease inhibitor to 10 mL of ice-cold lysis buffer immediately prior to lysis. We recommend using lysis buffer with freshly added protease inhibitor for all experiments.

5) Incubate on ice for 10 min.

6) Centrifuge for 60 seconds at 2,500x***g*** at RT.

[TROUBLESHOOTING 7]

7) Discard the supernatant and resuspend the pellet in 100 µl of 0.5X DNase I digestion buffer containing 0.2% SDS.

CRITICAL STEP: For larger cell inputs (*i.e.* 3–5 x 10^6), we recommend using 200 µl 0.5X DNase I digestion buffer instead.

8) Incubate at 37°C for 30 min.

9) Add 100 µl of 0.5X DNase I digestion buffer containing 2% Triton X-100 and 4 µl RNase A, mix well.

10) Incubate at 37°C for 10 min.

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

12) Incubate at RT for 4 min.

13) Add 40 µl of 6X Stop Solution, mix well.

[**TROUBLESHOOTING 14**]

14) (Optional) To determine the efficacy of DNase I digestion, take 20 µl of lysed cells from the previous step and add to a new tube. Add 70 µl 1X TE lysis buffer and 10 µl Proteinase K (20 mg/ml). Incubate for 30 minutes at 65°C. Purify 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 (see Figure 3a). We recommend characterizing DNase I digestion efficiency when performing the protocol on a new cell type. In the event of over-digestion or under-digestion of chromatin, we recommend optimizing the concentration of SDS in the digestion reaction, amount of DNase I used, or digestion time.

15) Centrifuge for 60 seconds at 2,500x***g*** at RT.

16) Discard the supernatant and resuspend the pellet in 150 µl water.

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

18) Incubate 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% ethanol. Briefly spin down the beads and remove the residual ethanol.

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

Steps 21 – 30: 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	
10X T4 ligase buffer w/ ATP	20	1X
10 mM dNTPs	5	0.25 mM
T4 DNA Polymerase (3U/ µl)	3	0.045 U / µL
Klenow (10U/ µl)	3	0.15U / µL
Total Volume	200	

22) Incubate at RT for 1 h.

23) Add 5 µl 10% SDS to stop the reaction.

24) Centrifuge for 60 seconds at 2,500x***g*** at RT.

25) Aspirate and resuspend the pellet in 135 µl water.

26) Prepare the dA-Tailing reaction as follows:

Reagents (add in this order)	Volume (µL)	Final Concentration
Nuclei w/ beads	135	
10X NEBuffer 2	20	1X
10 mM dATP	10	0.5 mM
10% Triton X-100	20	1%
Klenow (exo$^-$) (5U/ µl)	15	0.375U / µL
Total Volume	200	

27) Incubate the resulting mixture at 37°C for 1 hr.

28) Add 5 µl 10% SDS to stop reaction.

29) Centrifuge for 60 seconds at 2,500x***g*** at RT.

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

Steps 31 – 44: Ligation of Biotin-labeled Bridge adaptors (Timing: Overnight, followed by 0.5 h)

31) Prepare the adaptor ligation reaction as follows:

Reagents (add in this order)	Volume (µl)	Final Concentration
Nuclei w/ beads	30	

Reagents (add in this order)	Volume (μl)	Final Concentration
Annealed Bridge Adaptor w/ Biotin (40 μM)	20	8 μM
Annealed Blunt Adaptor w/o Biotin (40 μM)	20	8 μM
10X T4 ligase buffer w/ ATP	10	1X
PEG-4000 (50%)	10	5%
10% Triton X-100	5	0.5%
T4 DNA Ligase (5 U/ μl)	5	0.25U / μL
Total Volume	100	

32) Incubate at 16°C overnight.

PAUSE POINT: Reaction should be allowed to incubate overnight.

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

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

After incubation at 16°C for overnight, add 70 μl 1X TE lysis buffer and 10 μl Proteinase K (20 mg/ml). Incubate 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 genomic DNA as template. We recommend this quality control 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 the amount of DNase I digestion used.

34) Add 5 μl of 10% SDS to stop the reaction.

35) Centrifuge for 60 seconds at 2,500x***g*** at RT.

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

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

38) Incubate 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% ethanol. Briefly spin down the beads and remove the residual ethanol.

CRITICAL STEP: We recommend diluting fresh 80% ethanol before every experiment.

40) Resuspend the pellet in 200 µl water.

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

42) Incubate mixture at RT for 5 min, then place tube in DynaMag magnet for 2 min.

43) Discard the supernatant and wash the beads twice with 500 µl of 80% ethanol. Briefly spin down the beads and remove residual ethanol as completely as possible, then air-dry the beads for no more than 2 min.

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

Steps 45 – 46: *In situ* phosphorylation (Timing: 1.25 h)

45) Prepare the PNK reaction as follows:

Reagents (add in this order)	Volume (µL)	Final Concentration
Nuclei w/ beads	80	
10X T4 ligase buffer w/ ATP	10	1X
PNK (10 U/ µl)	10	1U / µL
Total Volume	100	

46) Incubate at 37°C for 1 hr.

Steps 47 – 48: *In situ* ligation (Timing: 4.25 h)

47) Add the following reaction to the above tube:

Reagents (add in this order)	Volume (µl)	Final Concentration
H_2O	794	
10X T4 ligase buffer	100	1X
T4 DNA ligase (5 U/ µl)	6	0.03U / µL
Total Volume	1 mL	

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

Steps 49 – 62: Cross-linking reversal, isopropanol precipitation and DNA purification (Timing: Overnight, followed by 2.5 h)

49) Centrifuge for 60 seconds at 2,500x***g*** at RT.

50) Resuspend the pellet in 400 µl 1X NEBuffer 2.

51) Add 40 µl 10% SDS.

52) Add 40 µl of 20 mg/ml Proteinase K.

53) Incubate overnight at 60°C.

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

55) Incubate mixture at −80°C for 2 hours.

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

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

58) Add 100 µl AMPure XP beads, mix well.

59) Incubate 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% ethanol. Briefly spin down the beads and remove residual ethanol as completely as possible, then air-dry the beads for no more than 2 min.

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

62) Incubate beads at RT for 1 min. Collect beads via DynaMag magnet and transfer eluent to 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 starting with $2.5x10^6$ cells.

PAUSE POINT: Purified DNA can be stored indefinitely at −20°C.

Steps 63–65: DNA Sonication (Timing: 0.5 h)

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

63) Transfer DNA to Covaris microtube.

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

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 sonicated DNA to a 1.5 ml tube.

PAUSE POINT: Eluted DNA may be stored indefinitely at −20°C.

Steps 66 – 74: Biotin pull-down (Timing: 0.5 h)

66) Wash 30 µl of MyOne C1 beads twice with 100 µl 1X B&W buffer, once with 100 µl 2X B&W buffer, then resuspend in 100 µl 2X B&W buffer.

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

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

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

70) Wash beads once with 300 µl 0.5X TE Lysis Buffer plus 300 µl 0.5X B&W buffer.

71) Wash beads twice with 600 µl 1X B&W buffer.

72) Wash beads once with 600 µl 1X NEBuffer 2.

73) Wash beads once with 600 µl EB buffer.

74) Resuspend beads in 170 µl of EB buffer.

PAUSE POINT: Resuspended beads may be stored at −20°C indefinitely or 4°C for short-term storage.

Steps 75 – 84: 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	
10X Reaction buffer	20	1X
End-repair enzyme mix	10	
Total Volume	200	

76) Incubate at 18°C for 10 min.

77) Add 200 µl of Ampure buffer, mix thoroughly by pipetting up and down.

78) Incubate at RT for 5 min and place the tube in a DynaMag-Spin magnet for 2 min.

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

80) Resuspend beads in 21.5 µl water.

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

Reagents (add in this order)	Volume (µl)	Final Concentration
End-repaired DNA w/ beads	21.5	
10X NEBuffer 2	3	1X
10 mM dATP	3	1 mM
Klenow (exo$^-$) (5 U/ µl)	2.5	0.42U / µL
Total Volume	30	

82) Incubate at 37°C for 30 min.

83) Wash beads twice with 400 µl 1X B&W buffer.

84) Wash beads twice with 400 µl EB buffer and resuspend in 30 µl EB buffer.

CRITICAL STEP: Proceed immediately to adaptor ligation.

Steps 85 – 95: Ligation of sequencing adaptors (Timing: 1 h)

85) Immediately resuspend beads in the following reaction mixture:

Reagents (add in this order)	Volume (µl)	Final Concentration
dA-tailed DNA w/ beads	30	
5X Thermo Rapid Ligation Buffer	10	1X
Y-Adaptor (2.5 µM)	6	0.3 µM
T4 DNA ligase (5 U/ µl)	4	0.4U / µL
Total Volume	50	

86) Incubate 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_2O to bring up 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 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% ethanol. Briefly spin down the beads and remove residual ethanol as completely as possible, then air-dry the beads for no more than 2 min.

91) Resuspend beads in 200 µl ddH_2O and add 165 µl of AMPure buffer

92) Mix thoroughly by pipetting up and down.

93) Incubate 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% ethanol. Briefly spin down the beads and remove the residual ethanol as completely as possible, then air dry the beads for 5 min.

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

Steps 96 – 104: 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 necessary to generate ample PCR products for sequencing—importantly, without over-amplification—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 w/ beads	2.5 / 5	
2X HotStart ReadyMix	10	1X
10 µM SeqPrimer_F	1	1 µM
10 µM SeqPrimer_R	1	1 µM
ddH_2O	up to 20	
Total Volume	20	

Using the following PCR program:

Cycle number	Denature	Anneal	Extend
1	95°C, 3 min.		
2–6	98°C, 20 sec.	60°C, 20 sec.	72°C, 1 min.
7–17*	98°C, 20 sec.	65°C, 20 sec.	72°C, 1 min.

*Use optimized cycle number

97) Run 2 µl of 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 ~300 bp to ~1 kbp, with the vast majority of the fragments with a size of 300bp–600bp, as shown in Figure 3b. Presence of products much larger than 1 kbp (*i.e.* will not migrate on a 6% TBE-PAGE gel)

indicates overamplification, and should be avoided by reducing PCR cycle number or volume of beads used.

98) Aliquot remaining beads into 20 μl PCR reaction and amplify the remaining beads 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 library by adding 0.8X volumes of AMPure XP beads.

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

102) Discard the supernatant and wash the beads twice with 1 ml of 80% ethanol. Briefly spin down the beads and remove residual ethanol as completely as possible, then air-dry the beads for no more than 2 min.

103) Resuspend beads in 25 μl EB buffer and incubate at RT for 1 min.

104) Place resuspended beads on DynaMag magnet and transfer supernatant containing eluted DNA to fresh 1.5 mL tube.

Steps 105 – 109: Quality control of DNase Hi-C library by BamHI digestion (Timing: 1.25 h)

105) Quantitate amount of dsDNA in library using Qubit dsDNA HS kit as per 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
10X Fast digestion buffer	1 μl	1 μl
DNase Hi-C product	1–2 μl (50–100 ng)	1–2 μl (50–100 ng)
Fast digestion BamHI	1 μl	0 μl
Water	to 10 μl	to 10 μl

107) Incubate at 37°C for 30 min.

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

109) (Optional) Hybrid capture experiments may be carried out according to manufacturer's protocols provided with the Agilent SureSelect system.

Steps 110 – 120: Mapping, Normalization, and Visualization of Hi-C Contact Maps. TIMING dependent on volume of data)

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

[TROUBLESHOOTING 111]

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
```

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.

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 (MAPQ>=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[53] to normalize the contact matrix using the Mirny lab's hiclib library (https://bitbucket.org/mirnylab/hiclib).

120) Visualize the contact map.

Troubleshooting

Step	Problem	Possible reasons	Solution
3	Low percentage of long-range contacts in sequencing library or BamHI digest does not shift library	Inefficient or incomplete formaldehyde crosslinking	For new cell types, optimizing the amount of formaldehyde used for crosslinking may be necessary.
7	Nuclear pellet disappears during *in situ* enzymatic treatments	Overtreatment of fixed nuclei with SDS	Reduce the amount of SDS used in the cell lysis.
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.* 1U – 8U).
111	FastQC metrics are poor	High duplication rate in library (*e.g.* Fewer than 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

Day 1: Steps 1 – 32: Fixation; cell lysis; chromatin digestion, end repair, and adaptor ligation; ~6 h

Day 2: Steps 33 – 53: Adapter cleanup; *in situ* phosphorylation and ligation; crosslink reversal; ~6.5 h

Day 3: Steps 54 – 65: DNA purification and sonication; 2.5 – 3.5 h

Day 4: Steps 66 – 86: Biotin pulldown, end repair/dA tailing, and adaptor ligation of Hi-C fragments; ~3 h

Day 5: Steps 87 – 109: Library amplification, Bam HI quality check, and sequencing; ~4 h for amplification and quality check; up to several days / weeks for sequencing, instrumentation depending.

Day 6 and beyond: Steps 110 – 120: Data analysis time depends on sequencing depth and available compute resources.

Anticipated Results

We recommend QCing all libraries that pass the BamHI digestion test (typical results, including a negative control EcoRI digest, shown in Figure 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 Figure 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 Figure 4b). Corrected matrices generated from valid *in situ* 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, consistent with results obtained using traditional Hi-C both in an analogous murine system[35] and human lymphoblastoid cells[43]. These results suggest that the *in situ* DHC protocol produces signal comparable to existing Hi-C protocols while ultimately providing a less-biased empirical method for generating higher-resolution 3D maps of chromatin structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BOX 1

Assessment of nuclear lysis at various steps

To ascertain whether nuclei remain intact during the protocol perform the following control experiment. Following each enzymatic treatment step (Steps 17, 23, 28, 33, and 47), remove the supernatant and add 10 uL Proteinase K to it. Treat the supernatant overnight at 65°C, and then isopropanol precipitate the DNA by adding 0.1 volumes of 3M sodium acetate, 3 uL GlycoBlue, and 1 volume of 100% isopropanol, mixing, and then incubating mixtures at −80°C for 1 hour. Pellet mixtures at 4°C at 16,000**xg**, carefully remove the supernatant, and resuspend the pellet in 100 uL ddH_2O. Add 10 uL RNase A to each sample and incubate at 37°C for 10 minute, then purify DNA using 1.2 volumes of AMPure XP beads. Resuspend beads in 15 uL ddH_2O, and run this out on a 6% TBE gel.

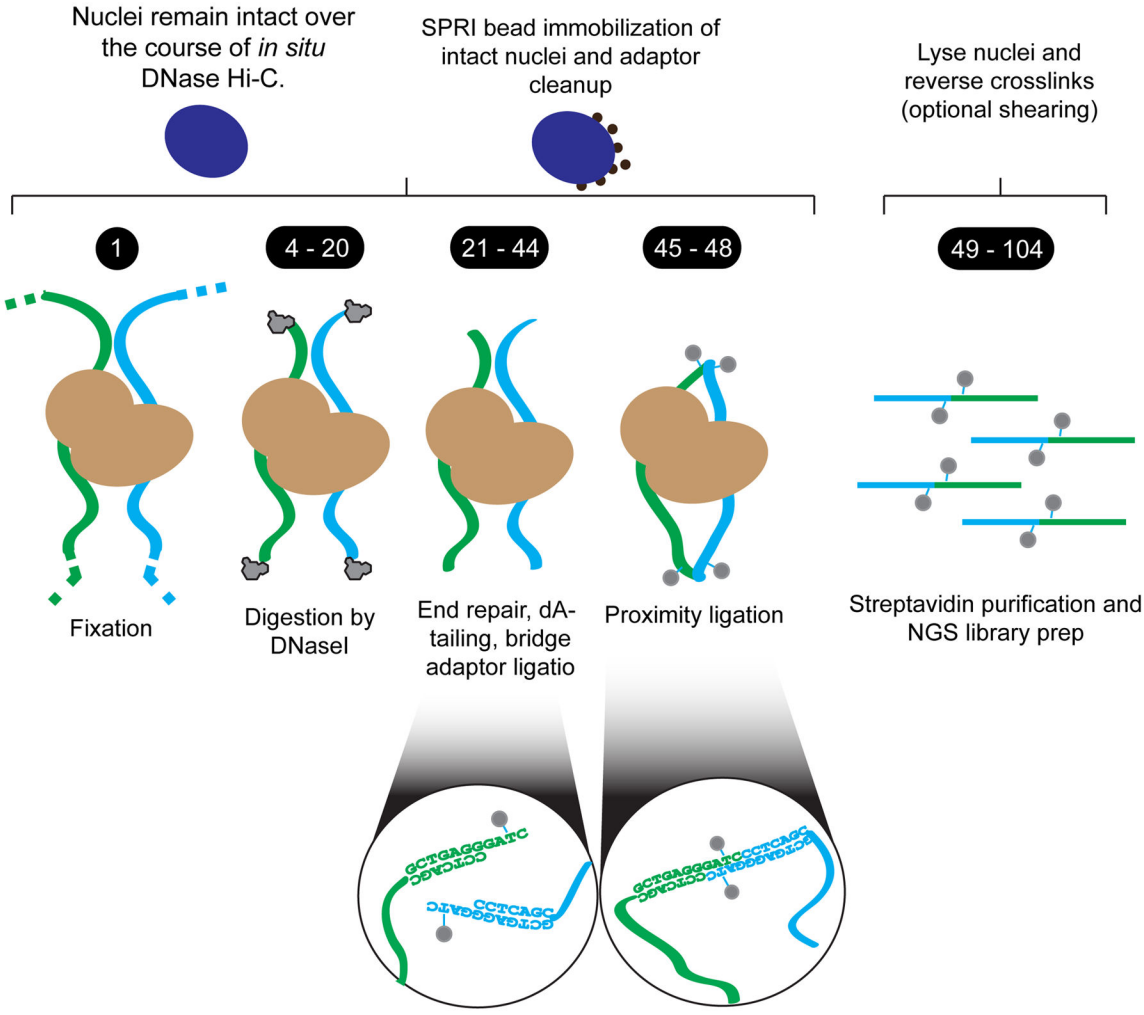


Figure 1. A schematic overview of *in situ* DNase Hi-C

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 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 for via streptavidin beads and on-bead Illumina library prep (optionally following sonication).

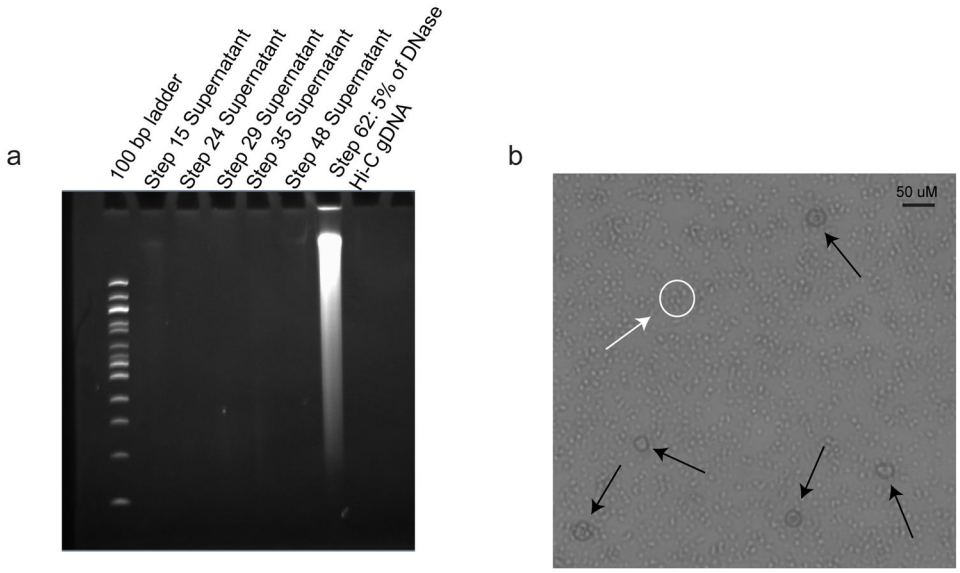


Figure 2. Nuclei remain intact during the *in situ* DNase Hi-C protocol

a.) Purified supernatant DNA (see Box 1) from 6 different steps of the DNase Hi-C protocol. Minimal DNA is purified after each enzymatic purification, compared to a large amount of DNA, taken from 5% of the total gDNA yield following nuclear lysis. b.) Phase contrast micrograph (20X magnification) of GM12878 nuclei bound to beads, following proximity ligation (Step 46). Nuclei are highlighted using 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.

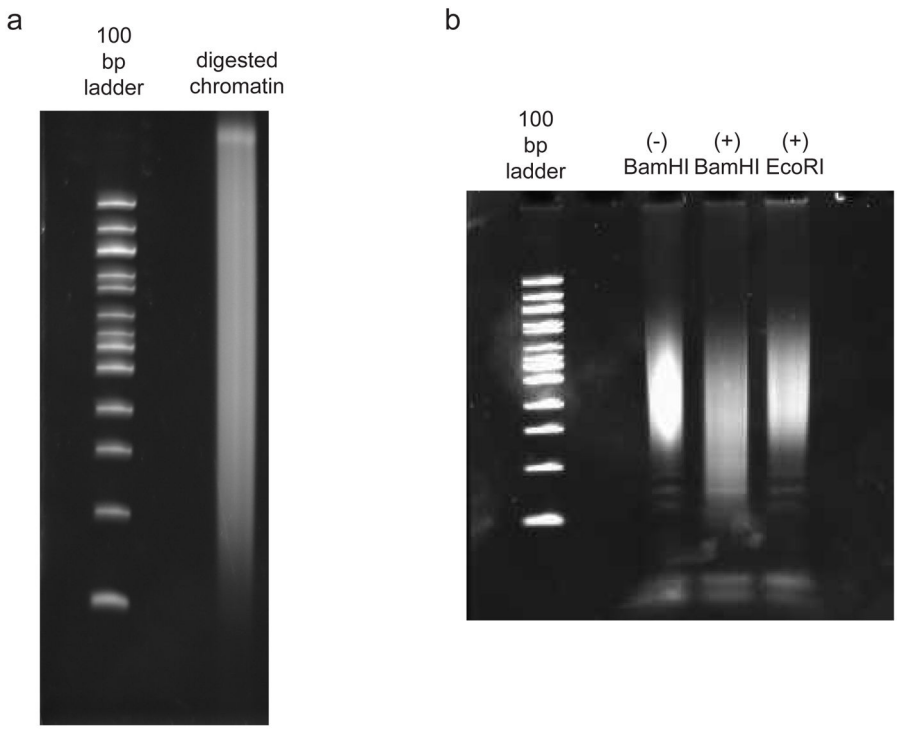
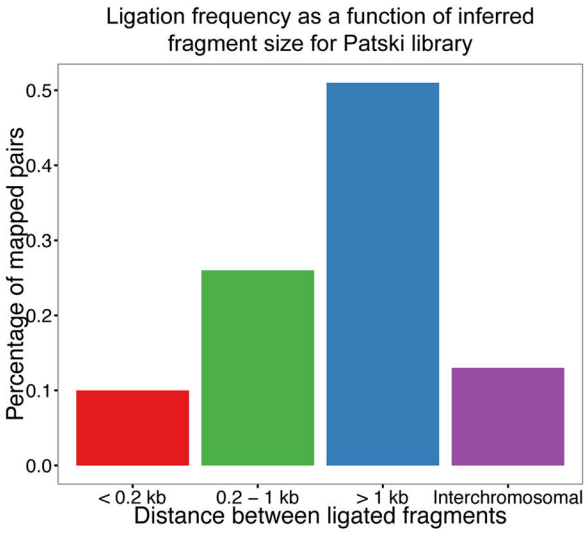


Figure 3. Digestion quality controls throughout the *in situ* DNase Hi-C protocol

a.) A typical digestion pattern for DNase I-digested fixed chromatin prior to proximity ligation, run on a 6% TBE-PAGE gel. b.) Example of the BamH1 quality control experiment performed on GM12878 *in situ* DNase Hi-C libraries; in this example, BamH1 shifts the *in situ* DNase Hi-C library by digesting the reconstituted BamH1 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 BamH1 digestion is specific to proximity ligated fragments. All reactions were run on one 6% TBE-PAGE gel.

a



b

	Left - Left	Right - Right	Inner	Outer
Fragments > 1 kb	120252	120010	121724	120262
Fragments <= 1 kb	13259	13298	293999	21621

c

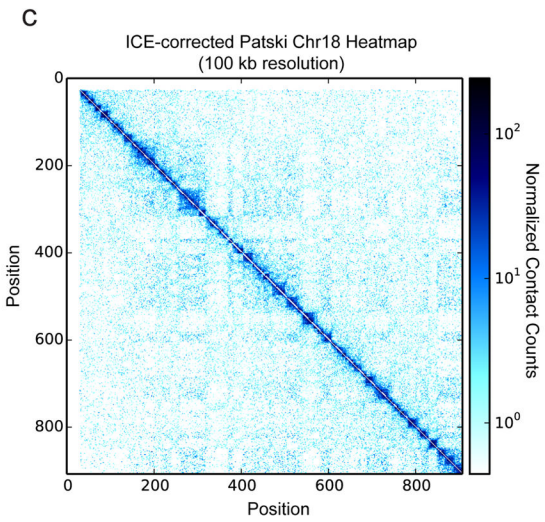


Figure 4. *In situ* DNase Hi-C results for the mouse embryonic kidney Patski cell line

a.) *In situ* DNase Hi-C reads (950,206 downsampled reads from data published in Deng, Ma *et al*[51] (using the mouse Patski cell line, rather than GM12878) demonstrate an enrichment for long-range (*i.e.* > 1 kb) intrachromosomal read pairs expected of Hi-C libraries. b.) Expected breakdown of mate 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 heat map generated from data published in Deng, Ma *et al* (GEO Accession: GSE68992) for mouse chromosome 18 at 100 kb resolution. The dataset used to generate this heatmap contained 60,666,200 uniquely mapped, high-quality read pairs.