Capture-C: a modular and flexible approach for high-resolution chromosome conformation capture

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Chromosome conformation capture (3C) methods measure the spatial proximity between DNA elements in the cell nucleus. Many methods have been developed to sample 3C material, including the Capture-C family of protocols. Capture-C methods use oligonucleotides to enrich for interactions of interest from sequencing-ready 3C libraries. This approach is modular and has been adapted and optimized to work for sampling of disperse DNA elements (NuTi Capture-C), including from low cell inputs (LI Capture-C), as well as to generate Hi-C like maps for specific regions of interest (Tiled-C) and to interrogate multiway interactions (Tri-C). We present the design, experimental protocol and analysis pipeline for NuTi Capture-C in addition to the variations for generation of LI Capture-C, Tiled-C and Tri-C data. The entire procedure can be performed in 3 weeks and requires standard molecular biology skills and equipment, access to a next-generation sequencing platform, and basic bioinformatic skills. Implemented with other sequencing technologies, these methods can be used to identify regulatory interactions and to compare the structural organization of the genome in different cell types and genetic models.

Introduction

Development of Capture-C methods

Chromosome conformation capture (3C) is a powerful method to measure the proximity of DNA elements within the three-dimensional confines of the nucleus. All 3C methods follow a general principle of chromatin digestion and re-ligation, with minimal disruption of nuclear structure achieved either by fixation or careful buffering to maintain native conditions. Chimeric ligation junctions are then assayed, with more frequent ligation between two distal fragments being a proxy for greater proximity. Originally, chimeric junctions were assayed directly in a low-throughput manner using PCR with specifically targeted primer pairs. The application of next-generation sequencing to assay ligation junctions has allowed high-throughput sampling of interactions in all-versus-all approaches, most commonly in situ Hi-C at relatively low resolution, and many-versus-all approaches at high resolution, commonly with the Capture-C or 4C-seq methods.

The first Capture-C method was established as a many-versus-all approach that used RNA oligonucleotide pull-down of restriction fragments of interest from in situ 3C material. Subsequent sequencing allowed detection of interacting fragments in an unbiased manner. This approach was later applied to Hi-C libraries to develop Capture Hi-C (CHi-C), often called Promoter Capture Hi-C, and most recently dubbed Enhancer Capture Hi-C. Capture-C was improved by the application of biotinylated single-stranded DNA (ssDNA) oligonucleotides for sequential ‘double capture’ of indexed and multiplexed 3C libraries. This improved method, Next Generation (NG) Capture-C, achieved 30–50% on-target sequencing efficiency, with 10,000–100,000+ unique reporters per viewpoint. Most importantly, as 3C libraries used in Capture-C methods are indexed after sonication, PCR duplicates can be distinguished and excluded from analysis. The Capture-C approach can be
divided into three distinct modules: 3C library generation, indexing and enrichment (Fig. 1a). By careful optimization of the library generation and indexing steps, the cell requirement was reduced from >1 million cells to as few as 10,000 cells for Low-Input Capture-C (LI Capture-C)\(^9\). Subsequent work to reduce protocol inefficiencies from the in situ 3C library generation module led to the development of Nuclear 3C, whereby intact nuclei are recovered after ligation, reducing the frequency of spurious ligation events between nuclei 3.3-fold\(^{10}\). Similarly, optimization of the enrichment step established Titrated Capture-C, which can achieve 30–50% on-target sequencing efficiency after a single capture enrichment step\(^{10}\). The combination of Nuclear 3C libraries, the indexing efficiency improvements of LI Capture-C, and the additive effects of Titrated Capture-C and double capture from NG Capture-C into a single method, Nuclear Titrated (NuTi) Capture-C, provides a highly sensitive approach for many-versus-all 3C experiments. NuTi Capture-C provides as high as 98% on-target sequencing and has been used to interrogate the regulatory interactions of 8,000 erythroid promoters simultaneously\(^{10}\).

In addition to allowing systematic optimization, the modular nature of the Capture-C method has enabled development of techniques for asking diverse and nuanced questions (Fig. 1b). By altering the size of fragments generated at the sonication step of indexing, Tri-C allows the interrogation of multiway interactions for inference of higher-order configurations and structures\(^{11,12}\). Similarly, using oligonucleotides targeting contiguous fragments in Mb-sized regions (rather than disperse fragments) allows Tiled-C to generate high-resolution many-versus-many contact matrices akin to Hi-C\(^{13}\). Tiled-C was also the first Capture-C method to implement double-stranded DNA (dsDNA)

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**Fig. 1 | Capture-C is modular and adaptable for characterizing chromatin folding.** a, The Capture-C family of methods involves three distinct modules. In the first module, a Nuclear 3C library is generated from 2% formaldehyde-fixed cells that are lysed, then permeabilized with SDS and digested with a frequent four-base cutter (DpnII or NlaIII). Proximity ligation rearranges the genome order to reflect spatial 3D organization. Finally, for this module, centrifugation is used to separate DNA in ruptured nuclei from DNA in intact nuclei, which contains more informative 3C material. Library indexing in module 2 is performed using standard next-generation sequencing kits with sonication providing unique ends for PCR duplicate filtering. For Tri-C, gentler sonication is used to generate longer fragments that contain multiple ligation junctions. The third module is the most diverse, with a unique oligonucleotide design for each method. NuTi Capture-C uses a pair of oligonucleotides from the same strand of DNA that overlap restriction digestion sites of disperse fragments. For Tiled-C, the same approach is used; however, contiguous fragments are targeted and double-stranded oligonucleotides have typically been used. In Tri-C, a single oligonucleotide in the center of a short restriction fragments enriches for sonication fragments with multiple ligation junctions. b, Schematic of results for a hypothetical locus, with one gene (red) and two enhancers (purple circles). NuTi Capture-C, or the low-cell variation LI Capture-C, from the promoter can be used to show direct interactions with both enhancers, Tiled-C produces a Hi-C-like interaction map showing the three elements are in a TAD-like regulatory domain, and Tri-C shows that the two enhancers can be found simultaneously interacting with each other and the promoter at single alleles.
oligonucleotides, although PCR-generated dsDNA oligonucleotides have also been used to target dispersed elements. Because of the inherent efficiency, Tiled-C can generate high-quality data in only a few thousand cells. The matrices generated with Tiled-C provide the ability to analyze topologically associated domains (TADs), without compromising on the high-resolution details provided by the other Capture-C methods.

**Applications of Capture-C methods**

Capture-C methods can be applied to analyze any fragment or region of the genome that is amenable to probe design. Therefore, Capture-C methods have been applied to numerous biological questions across a diverse range of organisms and species, including human, mouse, fly and chicken. This has allowed for exploration of the roles of enhancers, super-enhancers and promoter competition in the regulation of gene expression in numerous contexts. Similarly, insights into the dynamics of polycomb bodies, X-chromosome inactivation and CTCF boundaries have been achieved by targeting appropriate elements in a range of genetic models. Capture-C has also been applied to understand human disease, through mapping the interactions of regulatory polymorphisms associated with complex traits, and to help determine the effects of monogenic disease-causing mutations. Capture-C methods have also been applied in conjunction with other methods, to validate TADs predicted by the DeepC machine learning algorithm, to complement findings from high-resolution ‘resolution after single-strand exonuclease resection’ (RASER)-fluorescence in situ hybridization (FISH), and as input for and validation of polymer models of higher-order chromatin structure.

**Comparison with other methods**

**Disperse viewpoint targeting (many-versus-all)**

NuTi Capture-C is primarily applied in many-versus-all experiments to determine interactions for genomic elements of interest. Numerous approaches have been developed for this type of experiment. Early sequencing 3C methods, including the original Capture-C and 4C-seq, provided low depth of signal or lacked the ability to distinguish PCR duplicates. For this reason, some researchers have preferred 3C-qPCR, which was thought to be more quantitative, but it does not achieve many-versus-all data as primers need to be designed and optimized for all fragment pairs of interest, resulting in extremely low-resolution profiles. Improvements to the sequencing-based 3C approaches, NG Capture-C and UMI-4C, provided greater depth of signal and allowed PCR duplicates to be filtered by use of unique sonication ends (this was possible with the original Capture-C as well), overcoming previous limitations and allowing high-throughput analysis at tens to hundreds of targets, with NG Capture-C providing the greater number of unique reporters per viewpoint. The application of many-versus-all experiments to thousands of targets was initially limited to CHi-C. By performing pull-down in Hi-C libraries, 3C material is enriched for successful ligation junctions, at the expense of library complexity owing to the relative inefficiency of the molecular steps required to generate Hi-C libraries. Excluding these inefficient steps allows NuTi Capture-C to generate up to 1,000-fold greater depth of signal than Capture Hi-C. CHi-C experiments generally target in the region of 20,000 promoters or enhancers, but use infrequent-cutting enzymes in few replicates that result in low-resolution data (1–10 kb resolution with 100–1,000 interactions per viewpoint). However, with careful design and optimization, NuTi Capture-C has been applied to ~8,000 active erythroid promoters at high resolution in triplicate, indicating that genome-scale experiments are no longer limited to lower-resolution approaches.

3C resolution can be increased by using deoxyribonuclease (DNase I) or micrococcal nuclease (MNase) to digest chromatin instead of restriction endonucleases, as these enzymes have no specific cutting motif. MNase-digested 3C libraries were initially used in all-versus-all approaches. Recently, we have reported a new approach in which MNase digestion is combined with a targeted enrichment method, similar to NuTi Capture-C. Micro Capture-C (MCC) provides super-high-resolution 3C data for selected viewpoints, and even permits the footprinting of transcription factor binding at promoters and enhancers. Careful optimization of MNase levels is needed to achieve super-high-resolution data. Therefore, MCC requires tens of millions of cells and is currently not easily applied to low-abundance primary cell populations, in contrast to traditional Capture-C methods, although this will undoubtedly change as the MCC protocol is refined and optimized.

The Capture-C, Capture Hi-C and MCC methods use defined sequence specific oligonucleotides for enrichment. Other many-versus-all approaches use 3C combined with immunoprecipitation of proteins (ChIA-PET, PLAC-seq, ChIA-DROP, Hi-ChIP) or RNA (Hi-ChIRP) to achieve
enrichment. These methods enticingly allow the simultaneous identification of protein binding sites or enhancers and their interactions. In reality, the results are difficult to interpret because they are prone to bias caused by enrichment. This means they generally over-report that sites enriched for the targeted molecule contact other similarly enriched sites. Mathematical and experimental quantification of the bias induced between two simultaneously enriched distant sites (i.e., co-targeting) shows that it is incredibly difficult to correct accurately; as such, no method is generally applied in these hybrid technologies. In comparison, the defined nature of oligonucleotide pull-down in Capture-C, Capture Hi-C and MCC experiments allows the exclusion of biased fragments from analyses, providing more robust and interpretable findings.

Contiguous viewpoint targeting (many-versus-many)
Tiled-C was designed to combine the ability of all-versus-all 3C methods such as Hi-C to map large-scale chromatin structures including TADs, and the ability of one/many-versus-all 3C methods such as NuTi Capture-C to identify enhancer–promoter interactions within TADs at high resolution. While NuTi Capture-C targets disperse individual restriction fragments as viewpoints, Tiled-C uses a panel of capture oligonucleotides tiled across all contiguous restriction fragments within specified genomic regions. This allows for efficient enrichment for interactions within this region and thus for deep, targeted sequencing of these chromatin interactions. Although co-targeting of distal fragments induces enrichment bias, the contiguous nature of Tiled-C designs avoids this bias as targeted fragments are not enriched more than other fragments within the targeted region. Advantages compared with Hi-C are that Tiled-C can create high-resolution contact matrices of regions of interest at great depth in multiplexed samples for a fraction of the sequencing costs associated with genome-wide high-resolution Hi-C experiments. Other approaches that allow for many-versus-many analysis within regions of interest include methods such as Chromosome Conformation Capture Carbon Copy, Targeted Chromatin Capture, cHi-C, HYbrid Capture Hi-C and Tiled-MCC. An important advantage of Tiled-C compared with these methods is that it allows for high-quality data generation from as few as 2,000 cells.

Single-allele multiway analyses
Most 3C methods, including Capture-C, 4C and Hi-C, focus on the analysis of pairwise interactions in cell populations. These methods therefore do not provide information about the higher-order assembly of chromatin structures and their dynamics in individual cells. The long ligation products in 3C libraries contain many ligation junctions between multiple DNA elements in a concatemer. These elements were in close proximity in the cell nucleus at the time of fixation. Therefore, analysis of sequencing reads with multiple junctions allows for the investigation of multiway chromatin interactions between DNA elements in individual nuclei. Tri-C was developed to identify such multiway interactions with viewpoints of interest with high sensitivity and at high resolution. By using a restriction enzyme to create small restriction fragments at the viewpoints of interest—usually \( 	ext{NlaIII} \)—and creating longer sonication fragments, multiple interacting fragments can be analyzed efficiently using high-quality Illumina sequencing. Compared with other recently developed approaches to detect multiway chromatin interactions, such as chromosomal walks, three-way 4C, and multi-contact 4C, Tri-C offers advantages in throughput, sensitivity and resolution, as well as careful quantification of interaction frequencies due to robust PCR duplicate filtering. Other recent innovative techniques, such as genome architecture mapping (GAM) and Multiplex-GAM, split-pool recognition of interactions by tag extension, DNA seqFISH, and single-cell Hi-C, also allow for investigation of chromosomal organization in single cells. Since the resolution of these techniques at the moment is limited, these methods have predominantly contributed to our understanding of chromosomal structures in single cells at relatively large scale, rather than at the level of individual regulatory DNA elements.

Experimental design
Enzyme selection for resolution
While theoretically any restriction enzyme could be used in 3C, only a few enzymes digest chromatin efficiently, especially when it is heavily crosslinked. The choice of restriction enzyme for generation of 3C material is the largest determinant of experimental resolution; Capture-C libraries use four-base cutters (\( \text{NlaIII}, \text{DpnII} \)), which cut \(~16\) times more frequently than six-base cutters (\( \text{HindIII} \)). While the higher resolution provided by four-base cutters allows interactions of nearby elements to be
**Fig. 2 | Capture-C design considerations.** a, Plot of the number of viewpoints that can be sequenced at sufficient depth with the most common Illumina sequencing platforms. Calculations are performed for a NuTi Capture-C experiment with six multiplexed 3C libraries and two oligonucleotides per viewpoint. The range of the bar indicates the number of viewpoints that can be sequenced on the indicated Illumina platforms, with the lower number of viewpoints corresponding to ≥250,000 reads per viewpoint and the higher number of viewpoints to ~100,000 reads per viewpoint. Since capture oligonucleotides can be ordered in pools with a fixed price, a larger number of viewpoints corresponds to substantially reduced costs per oligonucleotide. b, When designing pools of oligonucleotides, it is important to consider the composition. For many-versus-all approaches, including NuTi Capture-C, pairs of elements that may have interactions of interest (e.g., defining enhancer-promoter interactions) should not be captured simultaneously due to co-capture bias. Instead, two pools, targeting only promoters and only enhancers, should be used in two separate hybridization reactions. For contiguous many-versus-many approaches, such as Tiled-C, low-resolution Hi-C can be used to guide selection of the area of interest and ensure domain context (e.g., boundaries and flanking domains) is included.

**Viewpoint selection for NuTi Capture-C**

Several tools exist for oligonucleotide design for selected viewpoint fragments, including Capsequm2 (ref. 75) (http://apps.molbiol.ox.ac.uk/CaptureC/cgi-bin/CapSequm.cgi), HiCapTools (https://github.com/sahlenlab/HiCapTools), GOPHER77 (https://gopher.readthedocs.io) and Oligo13 (https://oligo.readthedocs.io/en/latest/index.html). The targeted fragment should be either overlapping with or very close (<2 kb) to the genomic element of interest and be large enough to accommodate binding of enrichment oligonucleotides (70–120 bp), but not so large that probes are a long way from the element of interest. NuTi Capture-C with a single oligonucleotide per viewpoint is possible; however, this results in lower data depth than with two oligonucleotides, one targeting each end of the fragment. While still providing informative profiles, fragments shorter than 250 bp have been shown to have higher levels of trans interactions than longer fragments within the same 3C library12; therefore, optimal fragment length is 250–1,000 bp. The sequence underlying the oligonucleotides is also an important consideration. Duplication or high sequence similarity results in data that are harder to interpret, a limitation common to most sequencing-based 3C methods. Oligonucleotides likely to result in off-target pull-down can generally be avoided by selecting an adjacent fragment, or changing restriction enzyme.

**Oligonucleotide pool complexity**

Several important factors should be considered for combining multiple viewpoints into a single pull-down design. Although it is possible to work with very few oligonucleotides/viewpoints, it can be as cost-efficient to buy pairs of oligonucleotides targeting 25 viewpoints as it is to target only 2 viewpoints (Fig. 2a). These additional oligonucleotides also increase the total DNA recovered after titrated capture and help to avoid working with very small amounts of DNA. However, increasing the number of viewpoints does increase the total depth of sequencing required. Although it varies
depending on library quality and enrichment strategy, a sequencing depth of 100,000–500,000 read pairs per viewpoint per 3C library should be sufficient to identify 20,000 unique reporters for NuTi Capture-C\textsuperscript{10}. A single MiSeq run generating 20 million paired-end reads should therefore provide sufficient sequencing coverage for 5–25 viewpoints in six 3C libraries. Some analytical tools for calling interactions, such as peaky\textsuperscript{80} and peakC\textsuperscript{81}, also benefit from having numerous viewpoints, as this allows for generation of an accurate background model of nonspecific polymer interactions.

For targeting of specific disperse elements (NuTi Capture-C, Tri-C), it is important not to simultaneously enrich at two sites whose direct interactions you are interested in, for example co-targeting of a promoter and its cognate enhancers (Fig. 2b), or targeting two promoters that may interact. As all 3C enrichment methods are not 100% efficient, co-targeting is a substantial source of bias that results in increased observed interaction between targeted sites; see Downes et al. (2021)\textsuperscript{10} for an experimental and mathematical description of this phenomenon. To avoid this bias, separate enrichments can be performed on aliquots of the same 3C material, targeting, for example, only enhancers and only promoters.

**Comparative samples**

Capture-C enrichment can be performed on multiplexed samples in a single tube. This approach minimizes the technical variation in enrichment, generating highly reproducible profiles for statistical analysis. All of the Capture-C methods are usually performed in triplicate (at least) and can therefore be used to compare different genetic models or cell types in a single experiment. By performing experiments with triplicates, simple statistical tests (e.g., Student’s t-test) can be used to compare interactions with specific regions, or more advanced approaches (e.g., DESeq2 (ref. \textsuperscript{82})) can be used across entire domains of interaction. 3C interaction profiles from highly related cell types or throughout differentiation can be remarkably similar. Therefore, it is often beneficial to compare samples with a highly unrelated cell type where elements of interest (e.g., enhancer or promoters) are inactive. It is important to note that there can be considerable technical variability between different cell types in the 3C procedure, which can result in differing levels of background noise (i.e., trans interactions) across cell types. Care should be taken to ensure comparative samples have similar noise levels. This can partially be controlled for by normalization of interaction counts in cis rather than to total interactions, as different levels of trans interactions can alter observed proximal interaction frequencies\textsuperscript{10} after normalization.

**Tri-C design considerations**

Tri-C viewpoints should be located on small (~150–250 bp) restriction fragments generated by the restriction enzyme used for chromatin digestion, which is usually NlaIII, since it has a smaller median fragment size compared with DpnII\textsuperscript{11}. The ~120 bp capture oligonucleotides should be designed to the middle of the restriction fragments on which the viewpoints of interest are located, and repetitive sequences should be avoided.

**Tiled-C design considerations**

Similar considerations as for NuTi Capture-C apply to the design of capture oligonucleotides for Tiled-C. Probes for adjacent restriction fragments in regions of interest can be designed and filtered for repetitive sequences using Oligo\textsuperscript{13} (https://oligo.readthedocs.io/en/latest/index.html). When determining the extent of regions of interest captured, it is useful to use low-resolution Hi-C as a guide for the existence and location of regulatory domains and their boundaries. Both the 3D Genome Browser\textsuperscript{83} (http://3dgenome.fsm.northwestern.edu/view.php) and HiGlass\textsuperscript{84} (https://higlass.io/) provide rich resources of easily accessible Hi-C data in a range of cell types for this purpose. It is best to be generous in extending the tiled region beyond predicted boundaries for regions of interest to provide an informative regulatory context (Fig. 2b).

**Data analysis**

Multiple software packages exist for processing of Capture-C sequencing files. Reads from NuTi Capture-C and LI Capture-C experiments are compatible with HiC-Pro\textsuperscript{85} (https://github.com/nservant/HiC-Pro/releases), capC-MAP\textsuperscript{86} (https://github.com/cbrackley/capC-MAP) and CCseqBasic\textsuperscript{75} (https://github.com/Hughes-Genome-Group/CCseqBasic). Tri-C data can be analyzed using CCseqBasic or TriC\textsuperscript{11} (https://github.com/oudelaar/TriC) scripts, and Tiled-C data can be analyzed using the HiC-Pro pipeline\textsuperscript{85} (with the options for Capture Hi-C analysis) or Tiled-C\textsuperscript{13} https://github.com/oudelaar/TiledC) scripts. Interaction counts can be further processed in a range of 3C-specific tools, including
CHiCAGO\textsuperscript{87} (http://functionalogenecontrol.group/chicago), peakC\textsuperscript{81} (https://github.com/deWitLab/peakC), r3Cseq\textsuperscript{89} (http://r3cseq.genereg.net/Site/index.html), FourCSeq\textsuperscript{89} (http://bioconductor.org/packages/release/bioc/html/FourCSeq.html), peaky\textsuperscript{80} (https://github.com/cqgd/pky), CaptureCompare\textsuperscript{75} (https://github.com/djdownes/CaptureCompare) and CaptureSee\textsuperscript{75} (https://capturesee.molbiol.ox.ac.uk/). We find application of peaky, after processing with either CaptureSee\textsuperscript{10} or CHiCAGO\textsuperscript{90}, gives highly specific interaction calls.

To facilitate consistent data processing, analysis and interpretation of NuTi Capture-C, Tri-C and Tiled-C data, we developed a computational tool called CapCruncher\textsuperscript{91} (https://github.com/sims-lab/CapCruncher/releases) to analyze all three experiment types. This pipeline uses Python and is easy to both install and run. CapCruncher processes raw fastq files, removes PCR duplicates, identifies reporter reads and generates a UCSC Genome browser hub with depth-normalized tracks for individual replicates and for the mean of replicates. When multiple samples are provided simultaneously, CapCruncher also generates comparative tracks by subtracting sample means. For Tri-C and Tiled-C, CapCruncher generates visualization matrices over targeted regions. The CapCruncher pipeline is available on GitHub and Bioconda, and, for testing, a small NuTi Capture-C test dataset can be found in the Gene Expression Ontology database (GSE129378). It should be noted that CapCruncher is under continuous development to add and enhance functionalities. We recommend that users read the corresponding manual (https://capcruncher.readthedocs.io/en/latest/) provided online (the current manual is provided as the Supplementary Manual). Below we describe the basic requirements and implementation of CapCruncher.

**Expertise needed to implement the protocol**

The experimental processes associated with Capture-C methods are common modern molecular techniques, including: restriction enzyme digestion, proximity ligation, phenol–chloroform DNA extraction, quantitative and standard PCR, gel electrophoresis, next-generation sequencing library preparation (including AMPure XP SPRI Bead cleanups), streptavidin bead pull-down/washes and next-generation sequencing. The equipment for all of these processes (perhaps with exception of a sequencing platform) should be readily available in most research institutes, but where possible, alternatives are suggested. Following sequencing, analysis with CapCruncher requires basic-level unix command line operation, which can be easily learned. However, system administrator rights may be required to install tools, and advanced bioinformatics skills will aid in more complex analyses, such as interaction calling with peaky and other packages.

**Configuration files**

Three files are required for successful implementation of the Capture-C methods. For designing oligonucleotide probes, a bed format file (chromosome, start, stop, name) giving single base-pair coordinates to sites of interest is required for Capsequm2. For running of CapCruncher, a bed format file specifying the enriched regions (single fragments for NuTi Capture-C and Tri-C, and extended regions for Tiled-C) and a configuration file specifying the genome, mapping parameters, experimental method and output directories are required. Examples of all three files are included as Supplementary Data 1.

**Limitations**

Due to the extremely high efficiency of on-target sequencing afforded by oligonucleotide pull-down, no selection is performed for successful digestion events (unlike Hi-C). Although excluding these relatively inefficient steps reduces the number of cells required for high-resolution data, it does mean that quality control for a high-efficiency digestion is paramount to ensuring sequence reads are not wasted. Quality control can be performed either by agarose gel, or more accurately with quantitative real-time PCR (Box 1). Using both methods is optimal. Based on analysis with the latter, 3C libraries should have a minimum 70% digestion for use.

Capture-C methods provide a temporal, population-based snapshot of active chromatin folding processes. To develop a more granular or dynamic perspective of interactions, Capture-C can be complemented with imaging approaches, particularly high-resolution FISH or live-cell imaging\textsuperscript{92–97}. The requirement for single-cell suspensions also limits the application of Capture-C methods, since they are not suitable for complex tissues where mixed cell types cannot be easily separated into pure populations, or for formalin-fixed paraffin-embedded samples, such as biopsies and tissue sections.
In these cases, GAM\textsuperscript{5,6} provides a superior ability to separate cell types of interest and determine interaction dynamics.

3C provides information on chromatin folding; however, it is most informative when it is presented in conjunction with open-chromatin assays (e.g., ATAC-seq\textsuperscript{98}, DNase I-seq\textsuperscript{99}), and ChIP-seq for epigenetic markers, e.g., promoters (H3K4me3), enhancers (H3K4me1), active-transcription (H3K27ac), polycomb repression (H3K27me3), and boundary and insulator sites (CTCF).

Materials

**Biological materials**

- **Cells.** Capture-C methods are possible in any eukaryotic species or cell type where a single-cell suspension containing as few as 10,000–20,000 cells can be generated\textsuperscript{9,13}. However, if available, using >100,000 will result in data of higher depth and resolution. Successful experiments have been performed previously in fly and chicken, as well as in numerous mouse cell types, including embryonic stem cells (ESCs), ter119+ erythroid cells, ESC-derived mesoderm, definitive endoderm, neural progenitor cells, mouse embryonic fibroblasts (RRID: CVCL_4240), 416B myeloid progenitor cells (RRID: CVCL_3983) and J558L B myeloma cells (RRID: CVCL_3949). Capture-C has been widely used in primary human samples, including CD4\textsuperscript{+} T cells, CD14\textsuperscript{+} monocytes, HUVEC and CD71\textsuperscript{+}CD235\textsuperscript{+} erythroid cells, as well as human cell lines, including lymphoblastoid cell lines (GM12878 (RRID: CVCL_7526)), human ESCs (H1-hESC (RRID: CVCL_9771)), lung fibroblasts (IMR-90 (RRID: CVCL_0347)) and lung epithelial cells (NCI-H441 (RRID: CVCL_1561)), induced pluripotent stem cells and induced pluripotent stem cell–derived cardiomyocytes, pancreatic beta cells (EndoC-\beta1 (RRID: CVCL_L909)), cervical and breast cancer cell lines (MCF-7 (RRID: CVCL_0031), MDA-MB-231 (RRID: CVCL_0062), HeLa (RRID: CVCL_0058)), and leukemia-derived cell lines (K562 (RRID: CVCL_0004), SEM (RRID: CVCL_0093), K562 (RRID: CVCL_0004), SEM (RRID: CVCL_0093), THP1 (RRID: CVCL_0006)).

**CAUTION** Cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

**Reagents**

**Common reagents**

- PCR-grade water (Ambion, cat. no. AM9932)
- Absolute ethanol (VWR, cat. no. 20821.330)
- Agencourt AMPure XP SPRI beads (Beckman Coulter, cat. no. A63881)
- Qubit dsDNA BR Assay Kit (Invitrogen, cat. no. Q32850)
- D1000 reagents (Agilent, cat. no. 50675583)
- Formaldehyde, 37% (vol/vol) (Sigma, cat. no. 47608-250ML). **CAUTION** Formaldehyde is toxic; wear gloves, and avoid contact with skin.
- Glycine, 1 M (Sigma, cat. no. G7126)
- PBS (Invitrogen, cat. no. 10010031)
- Tris pH 8, 1 M (Invitrogen, cat. no. AM9760G)
- Igepal CA-630 (Sigma, cat. no. I8896)
- cOmplete Protease Inhibitor Cocktail (Sigma, cat. no. 11873580001)
- SDS, 20% (vol/vol) (Invitrogen, cat. no. AM9820)
- Triton-X 100 (Sigma, cat. no. T8787)
- DpnII HC (NEB, cat. no. R0543M) or NlaIII (NEB, cat. no. R0125L)
- T4 DNA HC Ligase (Life Tech, cat. no. E10013)
- Tris–EDTA (TE) Buffer Solution (Sigma, cat. no. 93302)
- Proteinase K (Thermo Fisher, cat. no. EO0491)
- RNase (Roche, cat. no. 1119915)
- Polyethylene glycol (PEG) 300 (Sigma, cat. no. 90878-250ML-F)
- Phenol–chloroform–isoamylalcohol (PCI) 25:24:1 (Sigma, cat. no. 77617). **CAUTION** Phenol is toxic; avoid skin contact, consider use in a fume hood, dispose of waste appropriately and have PEG 300 easily accessible to treat burns.
- NaOAc pH 5.6, 3 M (Invitrogen, cat. no. AM9740)
- GlycoBlue (Thermo Fisher, cat. no. AM9515)
Table 1 | 3C digestion efficiency qPCR primers

| Assay set   | Sequence                        | Site                           | DpnII  | NlaIII |
|-------------|---------------------------------|--------------------------------|--------|--------|
| **Homo sapiens (hg38)** |                                  |                                |        |        |
| Hs 1 forward | 5′-GTCAGAATAACAGGAACCACAAA-3′   | chr22:46,257,116-46,257,137   | Cut site| Cut site|
| Hs 1 reverse | 5′-TTACTTGGCAACCCAGGAAC-3′      | chr22:46,257,190-46,257,212   | Cut site| Cut site|
| Hs 2 forward | 5′-GAGAATGGCCACATAAAGTGA-3′     | chr22:46,257,407-46,257,429   | Cut site| Cut site|
| Hs 2 reverse | 5′-GGAGTTGCAACACAGACATATC-3′    | chr22:46,257,480-46,257,502   | Cut site| Cut site|
| **Mus musculus (mm9)** |                                  |                                |        |        |
| Mm 1 forward | 5′-GGAGAAAGAGGGCTGGTTAT-3′      | chr15:85,650,603-85,650,624   | Cut site| Fragment|
| Mm 1 reverse | 5′-TATCTGAGTTGGACACCTTG-3′      | chr15:85,650,686-85,650,707   | Cut site| Cut site|
| Mm 2 forward | 5′-TTACCTGCACTGCCAATCGT-3′      | chr15:85,650,801-85,650,822   | Fragment| Cut site|
| Mm 2 reverse | 5′-TGCGCTGCCCTGAATAG-3′         | chr15:85,650,880-85,650,900   | Fragment| Cut site|
| **Drosophila melanogaster (dm6)** |                                |                                |        |        |
| Dm 1 forward | 5′-CAGGCCCAACACATGATAC-3′       | chr3R:23,023,063-23,023,083   | Cut site| NA     |
| Dm 1 reverse | 5′-CGGCAAGGAAATCGAATAA-3′       | chr3R:23,023,146-23,023,165   | Cut site| NA     |
| Dm 2 forward | 5′-TGTTAGCCTTGGCTTGTA-3′        | chr3R:23,023,278-23,023,297   | Fragment| NA     |
| Dm 2 reverse | 5′-AAGTAACAGCAGCTGGAATAG-3′     | chr3R:23,023,358-23,023,379   | Fragment| NA     |

- Tris acetate–EDTA buffer (Sigma, cat. no. T9650)
- Agarose (Sigma, cat. no. A4718)
- Ethidium bromide, or equivalent (Invitrogen, cat. no. 15585011)
- Gel loading dye (NEB, cat. no. B7024S)
- 1 kb DNA ladder (NEB, cat. no. N0468S)
- Genomic DNA ScreenTape, if required (Agilent, cat. no. 50675365)
- Genomic DNA Reagents, if required (Agilent, cat. no. 50675366)
- Real-time PCR primers (see Table 1 and Box 1)
- KAPA Sybr Fast Universal (KAPA, cat. no. KK4602)

Library indexing
- 3C library (generated in earlier stage)
- NEBNext Ultra II (New England Biolabs, cat. no. E7645S/L)
- NEBNext Multiplex Oligos for Illumina Primer set 1 (New England Biolabs, cat. no. E7335S/L)
- NEBNext Multiplex Oligos for Illumina Primer set 2 (New England Biolabs, cat. no. E7500S/L)
- Herculase II Fusion Polymerase Kit (Agilent, cat. no. 600677)

Oligonucleotide pull-down: general reagents
- 1–2 µg of each of six indexed 3C libraries (generated in earlier stage)
- Qubit dsDNA HS Assay Kit (Invitrogen, cat. no. Q32851)
- High Sensitivity D1000 Reagents (Agilent, cat. no. 5067 5585)
- High Sensitivity D1000 ScreenTape (Agilent, cat. no. 5067-5584)
- KAPA Library Quantification Complete Kit, Universal (Sigma, cat. no. KK4824)
- Mouse COT DNA, if required (Invitrogen, cat. no. 18440016)
- Chicken COT DNA, if required (Applied Genetics, cat. no. 0907582801)
- KAPA Hybrid Enhancer Reagent, if required (Roche, cat. no. 09075763001) ▲ CRITICAL Cot-1 DNA inhibits nonspecific probe binding. Only Cot-1 DNA specific to the organism of interest is required. For human cells, use the Cot-1 DNA included in either the HyperCapture kit or the Twist Universal blockers. Hypoblock reagents are available for several additional organisms; when no species-specific Cot-1 DNA is available, the KAPA Hybrid Enhancer Reagent may be used.

Oligonucleotide pull-down: for ssDNA oligonucleotides
- Biotinylated probes (e.g., Sigma HPLC purified oligonucleotides, IDT xGen Custom Hybridisation Capture Panel)
- HyperCapture Target Enrichment Kit, includes Human COT DNA (Roche, cat. no. 9075828001)
- M-270 Streptavidin Dynabeads (Invitrogen, cat. no. 65305)
Oligonucleotide pull-down: for dsDNA oligonucleotides

- Twist NGS Target Enrichment Oligonucleotide Panel (Twist, cat. no. 100533)
- Twist Hybridization and Wash Kit (Twist, cat. no. 101025/101026)
- Twist Universal Blockers (Twist, cat. no. 100767)
- KAPA HiFi HotStart ReadyMix (Roche, cat. no. KK2601)
- MyOne Streptavidin C1 Dynabeads (Thermo Fisher, cat. no. 65001)

Equipment

- Thermomixer C (Eppendorf, cat. no. 223000049), or equivalent
- Electrophoresis tank and power pack
- Qubit 4 Fluorometer (Thermo Fisher, cat. no. Q33238), or equivalent
- Sonicator, e.g., Covaris M220 or S220 Focused-ultrasonicator, or equivalent
- Quantitative thermocycler
- Thermocycler
- DynaMag-2 (Invitrogen, cat. no. 13221D), or equivalent
- 4200 TapeStation (Agilent, cat. no. G2991AA), or equivalent
- Speedy-Vac vacuum centrifuge, or equivalent but not essential
- Light PhaseLock gel tubes (5Prime, cat. no. 733-2477)
- 96-well optical PCR plate
- Covaris microTUBE AFA Fiber pre-split snap-cap 6 × 16 mm (Covaris, cat. no. 520045), or equivalent
- D1000 loading tips (Agilent, cat. no. 50675153)
- D1000 ScreenTape (Agilent, cat. no. 50675582)
- High-quality, non-sticky 1.5 mL microcentrifuge tubes (e.g., Sorenson BioScience, cat. no. 39640T)

**CRITICAL** Use these tubes for hybridization steps with streptavidin beads (Steps 86–109, 138–151).

Reagent setup

**Igepal CA-630, 10% (vol/vol)**
Mix 1 mL of Igepal CA-630 with 9 mL of PCR-grade water. Store at room temperature (RT, 20–22 °C) long term.

**Triton-X, 20% (vol/vol)**
Mix 2 mL of Triton-X with 8 mL of PCR-grade water. Store at RT long term.

**Fresh lysis buffer**
Mix reagents on the day of use. Cool to 4 °C on ice or on a roller in a cold room.

**TROUBLESHOOTING**

| Reagent                          | Stock concentration | Volume   | Work concentration |
|----------------------------------|---------------------|----------|--------------------|
| PCR-grade water                  | -                   | 48.4 mL  | -                  |
| Tris pH 8                        | 1 M                 | 500 µL   | 10 mM              |
| NaCl                             | 4 M                 | 125 µL   | 10 mM              |
| Igepal CA-630                    | 10% (vol/vol)       | 1 mL     | 0.2% (vol/vol)     |
| complete Protease Inhibitor Cocktail | -                 | 1 tablet | 1x                 |

**Ethanol, 70% (vol/vol)**
Mix 7 mL of absolute ethanol with 3 mL of PCR-grade water. Store at RT.

**Ethanol, 80% (vol/vol)**
Prepare fresh on day of use. Mix 8 mL of absolute ethanol with 2 mL of PCR-grade water.

Procedure

**CRITICAL** The following protocol describes the generation of a single Nuclear 3C library (for use in any Capture-C method) with either DpnII or NlaIII, followed by indexing, with appropriate information for Tri-C and low-input sample modifications. Prior to oligonucleotide pull-down, uniquely indexed 3C libraries can be pooled for multiplexed capture. The volumes in this section describe a six-library
experiment (i.e., triplicates for two cell types/genetic models) but can be scaled as necessary. Oligonucleotide pull-down can be carried out with either ssDNA oligonucleotides (first described for NG Capture-C<sup>5</sup>) or with dsDNA oligonucleotides (first described for Tiled-C<sup>13</sup>), and descriptions for both protocols are provided. A host of tools are available to analyze Capture-C experiments. Instructions are provided for processing of replicate samples with a portable Python script, CapCruncher, which can process all three experiment types.

**Viewpoint preparation**

**Oligonucleotide probe design**

- **Timing 3 h**

1. Use Capsequm2, Oligo or an equivalent tool to design appropriate probes for NuTi Capture-C, Tiled-C or Tri-C (see ‘Experimental design’ and Fig. 1). For Capsequm2, generate a bed file of single base pair regions under the genomic element of interest: tab-separated chromosome, start, stop, and viewpoint name (Supplementary Data 1).
2. Load bed file into Capsequm2 (http://apps.molbiol.ox.ac.uk/CaptureC/cgi-bin/CapSequm.cgi); select probe length (70–120 bp) and genome.
3. Proceed with filtering after fragment extraction error check.
4. Use AltSort to select probes passing filtering and download oligonucleotide sequences.

| Parameter      | Setting |
|----------------|---------|
| Duplicates     | ≤2a     |
| Blat density   | ≤40     |
| G/C content (%)| ≤60     |
| Repeats        | False   |

*Often, interactions at duplicated genes, e.g., HBA1, HBA2, can still be understood.

5. Order biotinylated oligonucleotides (either ssDNA or dsDNA) either in individual tubes for custom pooling, or as premixed pools.

**CRITICAL STEP** Unless performing Tiled-C, it is important not to mix two viewpoints that you wish to directly compare interactions for; co-targeting of distal fragments introduces significant bias for interactions between viewpoints compared with adjacent untargeted fragments. For an explanation of this effect, see the Supplementary Note associated with Downes et al. To avoid cross-contamination during production, it can be prudent to order on different days or from different suppliers.

**CRITICAL STEP** LI capture-C, NG Capture-C, NuTi Capture-C and Tri-C have traditionally been performed with ssDNA oligonucleotides, whereas Tiled-C has been performed with dsDNA oligonucleotides. However, there is no reason why a specific method could not be performed with either ssDNA or dsDNA oligonucleotides; therefore, both protocols are described. Follow the appropriate instructions for enrichment using either ssDNA oligonucleotides (Step 75–124) or dsDNA oligonucleotides (Steps 125–162).

**Oligonucleotide stock preparation**

- **Timing 1 h**

6. Reconstitute individual or pools of oligonucleotides following the manufacturer’s instructions or to a stock concentration so that each unique oligonucleotide is stored at ≥1 μM.
7. If oligonucleotides were ordered individually, generate pools of oligonucleotides by mixing in exact 1:1 stoichiometric ratio and store at −20 °C until required at Step 81 (ssDNA probes) or Step 129 (dsDNA probes).

**3C library generation**

**Formaldehyde fixation**

- **Timing 3 h**

8. Precool large centrifuge to 4 °C. Chill glycine, PBS and fresh lysis buffer.
9. Collect cells from whole tissue or culture, and make single-cell suspensions of 5 × 10<sup>6</sup> cells in 5 mL of growth medium.

**TROUBLESHOOTING**

10. Add 270 μL 37% (vol/vol) formaldehyde (2% (vol/vol) final concentration), and incubate for 10 min at RT while tumbling or rotating.

**CAUTION** Formaldehyde is toxic; avoid skin contact, consider use in a fume hood and dispose of waste appropriately.
**CRITICAL STEP** Varying levels of formaldehyde fixation can affect digestion efficiency and levels of trans ligation. Use bottles within 3 months of opening or use single-use ampules.

11 Quench formaldehyde by adding 750 µL 1 M cold glycine (125 mM final concentration).
12 Centrifuge for 10 min at 500g (4 °C), and wash pellet by gently resuspending in 5 mL ice-cold PBS.
13 Centrifuge for 10 min at 500g (4 °C), gently remove supernatant without disturbing pellet and resuspend in 5 mL ice-cold lysis buffer.
14 Incubate for 20 min on ice, then centrifuge for 15 min at 500g (4 °C), gently remove supernatant without disturbing pellet and wash by gently resuspending in 5 mL cold PBS.
15 Centrifuge for 15 min at 500g (4 °C), and gently remove supernatant without disturbing pellet, then resuspend pellet in 215 µL 1× DpnII buffer (DpnII libraries) or 215 µL 1× CutSmart buffer (NlaIII libraries) and transfer to microcentrifuge tube.

**PAUSE POINT** Either snap freeze and store at −80 °C or proceed to digestion.

### Digestion • **Timing 24 h**

16 Set a thermomixer to 37 °C, and warm nuclei.
17 Set up Digest and Control 1 (undigested DNA) as per the table below in Safe-Lock microcentrifuge tube.

| Reagent | Digest | Control 1 |
|---------|--------|-----------|
| DpnII digested 3C library | 200 µL | 15 µL |
| Nuclei in 1× DpnII buffer | | |
| PCR-grade water | 434 µL | 227.5 µL |
| 10× DpnII buffer | 60 µL | 28.5 µL |
| 20% (vol/vol) SDS (0.28% final concentration) | 10 µL | 4 µL |
| NlaIII digested 3C library | 200 µL | 15 µL |
| Nuclei in 1× CutSmart buffer | | |
| PCR-grade water | 393.5 µL | 227.5 µL |
| 10× CutSmart buffer | 60 µL | 28.5 µL |
| 20% (vol/vol) SDS (0.28% final concentration) | 9.5 µL | 4 µL |

**CRITICAL STEP** Add the SDS last to ensure a maximum concentration of 0.28% (vol/vol); prewarming the nuclei avoids SDS precipitation.
18 Shake horizontally at 500 rpm (intermittent shaking: 30 s on/30 s off) for 1 h at 37 °C on a thermomixer.
19 Add 20% (vol/vol) Triton X 100 at a final concentration of 1.67% (vol/vol). Add 66 µL to DpnII digests, 62 µL to NlaIII digests and 25 µL to Control 1.

**CRITICAL STEP** Triton-X quenches SDS by forming micelles and is vital to allow restriction enzyme function.
20 Shake horizontally at 500 rpm (intermittent shaking: 30 s on/30 s off) for 1 h at 37 °C on a thermomixer.
21 Add 10 µL DpnII (500 U) or 25 µL NlaIII (250 U) to digestion reactions, and incubate at 37 °C for several hours.
22 Add a further 10 µL DpnII (500 U) or 25 µL NlaIII (250 U) to digestion reactions, and incubate overnight at 37 °C.
23 Add a further 10 µL DpnII (500 U) or 25 µL NlaIII (250 U), and incubate for another 5–6 h.

### Ligation • **Timing 24 h**

**CRITICAL** For low-input samples (≤150,000 cells), skip Steps 25–27 and do not make a Control 2. Digestion efficiency can be directly determined using the 3C library.
24 Place the digest at 65 °C for 20 min to heat-inactivate restriction endonuclease, then cool on ice.

**CRITICAL STEP** Move directly to a preheated 65 °C thermomixer, and then immediately cool digests on ice to avoid de-crosslinking.
25 Take 100 µL from the digest reaction to make Control 2 (digested, unligated control).
26 Add 200 µL PCR-grade water to Control 2 to make up to 300 µL.
27 Store Control 1 and Control 2 at −20 °C to 4 °C until DNA extraction (Step 33).

28 On ice, add 500 µL PCR-grade water and 134 µL 10× ligation buffer to the digest. Mix by pipetting.

▲ CRITICAL STEP For low-input samples (≤150,000 cells), add 400 µL PCR-grade water and 134 µL 10× ligation buffer.

29 Add 8 µL T4 Ligase (240 U), and incubate on a 16 °C thermomixer at 500 rpm (intermittent: 30 s on/30 s off) for ~18 h.

30 Centrifuge ligation reaction at 500g for 15 min (RT).

31 Gently remove all of the supernatant without disturbing nuclear pellet.

▲ CRITICAL STEP It is important to remove the majority of the ligation buffer as high levels of DTT will interfere with DNA quantification. However, take care not to disrupt the pelleted nuclei.

32 Resuspend the nuclear pellet in 300 µL of TE buffer.

▲ CRITICAL STEP If using column-based extraction rather than PCI DNA extraction (see Troubleshooting for Step 37), follow kit instruction rather than resuspending in TE buffer.

D N A e x t r a c t i o n  ●  T i m i n g  1 8  h

33 Remove Control 1 and Control 2 from storage.

34 Add 5 µL Proteinase K (3 U) to the library and the controls, and incubate at 65 °C for 4 h.

35 Cool reactions to 37 °C, and add 5 µL RNase (7.5 mU) to the library and the controls. Incubate for 30 min at 37 °C on a thermomixer (500 rpm, intermittent: 30 s on/30 s off).

36 Prepare three PhaseLock tubes by spinning at 5,000g for 2 min (RT).

37 Add 310 µL PCI to each tube, close tightly and vortex thoroughly to mix.

! CAUTION Phenol is toxic; avoid skin contact, consider use in a fume hood, dispose of waste appropriately and have PEG 300 easily accessible to treat burns.

? TROUBLESHOOTING

38 Transfer DNA/PCI mix to a prespun PhaseLock tube, and separate by centrifuging for 10 min at 12,600g (RT).

39 Transfer the upper layer to a new microcentrifuge tube, avoiding the viscous interface, and then add 30 µL of 3 M sodium acetate and 1 µL of glycoblu; mix by inversion.

40 Add 900 µL of ice-cold absolute ethanol (75% (vol/vol) final concentration), and mix thoroughly by inversion. Freeze at −20 °C for at least 2 h; overnight precipitation can improve yield.

■ PAUSE POINT DNA precipitation may be stored at −20 °C for several days.

41 During the incubation, cool a centrifuge to 4 °C and chill 70% (vol/vol) ethanol on ice.

42 Pellet DNA by centrifuging at 21,000g for 30 min at 4 °C, and discard supernatant. The pellet should be pale blue due to the dye in the glycoblu.

43 Wash the DNA pellet by adding 1 mL of ice-cold 70% (vol/vol) ethanol, and pellet by centrifuging at 21,000g for 30 min at 4 °C. Remove the ethanol, and repeat ethanol wash for a total of two washes.

44 After the supernatant from the second ethanol wash is discarded, use a benchtop centrifuge to collect residual ethanol. Discard this using a pipette.

45 Dry the DNA pellet at RT with the lid open (~15–20 min). When the pellet goes clear, resuspend by adding 30 µL TE buffer to controls and 140 µL to the 3C library (digestion reaction).

■ PAUSE POINT The 3C library can be stored at −20 °C for several years.

Q u a l i t y c o n t r o l  ( s e e  B o x  1 )  ●  T i m i n g  3  h

▲ CRITICAL Unless cell samples are extremely precious or difficult to isolate, only proceed with 3C libraries with >70% digestion efficiency. Unlike Hi-C methods, no enrichment for successful digestion–ligation events is carried out and low digestion efficiency leads to a high proportion of uninformative reads.

46 Make a 1% (wt/vol) agarose gel using 1× Tris acetate–EDTA buffer, and run at a moderate speed (~70 mA) using 1 µL of 1 kb DNA ladder, 15 µL of each control and 5 µL of 3C library.

? TROUBLESHOOTING

47 Use 2 µL of 3C library in a Qubit BR assay to determine DNA concentration. The DNA yield from a normal diploid mouse or human cell is ~6 pg. A standard yield of 60–75% of input DNA is expected, generally >15 µg from 5 × 10⁶ cells.

48 Perform quantitative real-time PCR to determine digestion efficiency using Control 1 and Control 2 with both cut-site and fragment primer sets (Table 1). Using triplicates for each reaction, combine
the reagents in a 96-well optical PCR plate as below. A master mix excluding the DNA can be made prior to adding to the plate.

| Sample | Assay set | Avg. CT | ΔCT (cut-site-fragment) | ΔΔCT (C1-C2) | Digestion efficiencyb |
|--------|-----------|---------|-------------------------|--------------|-----------------------|
| C1: Control 1 (undigested) | Fragment | 21.211 | −0.168 | −2.706 | 84.76% |
| | Cut site | 21.043 | | | |
| C2: Control 2 (unligated) | Fragment | 20.884 | 2.538 | | |
| | Cut site | 23.422 | | | |

*For low-input samples, genomic DNA can be used instead of Control 1, and the 3C library in place of Control 2. Due to re-ligation, a lower digestion efficiency is expected. bEfficiency = 100 × (1 − 2^ΔΔCT).*

? TROUBLESHOOTING

49 Perform quantitative PCR using the following conditions, and calculate digestion efficiency (Table 2).

| Step | Temperature | Duration |
|------|-------------|----------|
| 1    | 95 °C       | 20 s     |
| 2    | 95 °C       | 3 s      |
| 3    | 60 °C       | 30 s     |
| 4    | Go to Step 1 (×40) | |

? TROUBLESHOOTING

Library indexing

▲ CRITICAL Sequencing adaptors are added by ligation after sonication. Where sonication is not possible, tagmentation can be used for indexing9,10; however, custom blocking oligonucleotides may be required for capture.

Sonication ● Timing 2 h

50 Bring 235 µL of AMPure XP SPRI beads to RT in a microcentrifuge tube (set aside).
51 Add 130 µL of 3C library to a Covaris microtube, avoiding making bubbles.
52 Shear DNA to 200 bp with appropriate settings on the available sonicator.

| S220 | M220 |
|------|------|
| Duty cycle | 10% | Duty factor | 20% |
| Intensity | 5 | Peak power | 70 |
| Cycles per burst | 200 | Cycles per burst | 1,000 |
| Time | 360 s | Average power | 14.0 |
| Mode | Freq. sweeping | Time | 130 s |

▲ CRITICAL STEP These settings are a suggested starting point for optimization. Different sonicators of the same model can have differing resulting size fragments. It is essential to optimize settings with high molecular weight DNA when using a new machine for the first time. This optimization can be performed with genomic DNA.

▲ CRITICAL STEP To perform Tri-C, sonicate DNA to a mean fragment size of 450 bp.
### Box 1 | 3C quality control

The success of Capture-C methods relies on the generation of high-quality 3C material, with a high degree of digestion. To ensure the quality of 3C material, two controls are prepared (Steps 17 and 25). Control 1 contains undigested material and ensures DNA was not degraded prior to digestion. Control 2 contains digested, but unligated, chromatin. These two controls, along with the ligated 3C library, are assessed both qualitatively, using gel electrophoresis, and quantitatively, using real-time PCR.

#### Qualitative analysis

To visually inspect 3C library and control DNA, separate using a 1% (wt/vol) agarose gel with a moderate speed, -70 mA, or using a Genomic DNA ScreenTape in a TapeStation instrument (Fig. 3a). Because of the low DNA requirements, a TapeStation is preferable when working with low-input samples. Control 1 should contain a single band of high molecular weight DNA that is not degraded. Control 2 should contain a smear of low molecular weight fragments. Low digestion efficiency can be associated with a faint band of high molecular weight DNA. The ligated 3C library should have increased in molecular weight because of concatenation, and resemble Control 1. Although complete ligation will result in a greater number of informative junctions being formed, libraries with partial ligation can still be used for Capture-C. Where DNA is limiting, most commonly when working with low cell numbers, it is possible to assess both controls and 3C material using a genomic ScreenTape in a TapeStation, looking for a similar pattern of DNA distribution for each of the three samples. Qualitative assessment, using TapeStation profiles with small amounts of DNA can also be used to ensure indexing reactions proceed as expected (Fig. 3b).

#### Quantitative analysis

Real-time PCR is performed with primers that amplify across a restriction cut site, cut-site primers, or within a restriction fragment, fragment primers (Fig. 3c, Table 1). Fragment primers will amplify to the same extent in both Control 1 and Control 2, providing a loading control for quantitative PCR (Fig. 3c). The cut-site primers will readily amplify in Control 1, but due to digestion, have reduced amplification in Control 2. This difference in amplification allows the quantification of digestion efficiency (Table 2). We recommend that libraries have at least a 70% digestion efficiency for use. When working with low-input samples, it is possible to determine cutting efficiency using the re-ligated 3C library and a genomic DNA control; however, this will result in a lower calculated digestion efficiency due to re-ligation of some DNA into its original configuration.

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53 Perform an AMPure XP SPRI bead cleanup. Transfer 130 µL of sonicated DNA to 235 µL SPRI beads (1.8×), and mix by pipetting ten times; incubate at RT for 5 min.

54 Place on magnetic stand, discard liquid when clear (~2 min) and add 800 µL of fresh 80% (vol/vol) ethanol without removing from magnetic stand. Incubate for 30 s, and then remove the ethanol.

**▲ CRITICAL STEP** Avoid disturbing beads by running the ethanol down the front of tube.

55 Add a further 800 µL of fresh 80% (vol/vol) ethanol without removing from magnetic stand. Incubate for 30 s, and then remove the ethanol.

56 Spin down tube on a microcentrifuge, and replace on magnetic stand. Remove residual ethanol with a low-volume pipette, taking care not to remove any beads.

57 Air-dry SPRI beads at RT on magnetic stand until matt in appearance.

**▲ CRITICAL STEP** Take care not to overdry the beads as this will result in increased DNA losses. Beads will look damp but not glossy when they are ready, overdried beads will develop cracks.

58 Remove from magnetic stand, and resuspend beads in 55 µL of PCR-grade water.

59 Incubate at RT for 2 min to elute. Replace on magnetic stand, and once clear (~2 min) recover 53 µL.

60 Assess 1 µL of sonicated material using D1000 TapeStation (Fig. 3b).

61 Use 2 µL of sonicated 3C library in a Qubit BR assay to determine DNA concentration.

**■ PAUSE POINT** Sonicated DNA can be stored at −20 °C for several months.

**? TROUBLESHOOTING**

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**End prep and adaptor ligation ● Timing 3 h**

**▲ CRITICAL** It is important to maintain library complexity by maximizing input DNA and minimizing DNA losses with the bead cleanups. For this reason, we use a modified protocol for the NEBNext Ultra II Kit that only requires a single bead cleanup before indexing. Using 2 µg of human DNA is equivalent to ~5 × 10⁶ cells, which can each provide four interactions per viewpoint (two per fragment per allele). The same amount of *Drosophila* DNA is equivalent to ~7 × 10⁶ cells. When ≤1 µg is available for indexing, end prep and adaptor ligation can be performed as described in the product manual.

**▲ CRITICAL** This protocol is a modified version of the NEB Ultra II indexing protocol. Be aware of changes to composition of kit reagents and workflow.

62 In a PCR tube, dilute up to 2 µg of DNA to 50 µL in PCR-grade water and add 7 µL 10× End Prep Buffer, and 3 µL End Prep Enzyme, then mix by pipetting.

63 Incubate End Prep reaction in a thermocycler for 45 min at 20 °C, followed by 30 min at 65 °C (lid set to 75 °C).

64 Add 30 µL Ultra II Ligation Master Mix, 7 µL NEBNext Adaptor and 1 µL Ligation Enhancer, then mix by pipetting and incubate in a thermocycler for 30 min at 20 °C (lid off).
65 Add 3 µL of USER enzyme, mix by pipetting and incubate in a thermocycler for 30 min at 37 °C (lid 47 °C).
66 During the final incubation, bring 180 µL of AMPure XP SPRI beads to RT.
67 Perform an SPRI bead cleanup as described at Steps 53–59 with 180 µL of AMPure XP SPRI beads.

Elute in 59 µL of PCR-grade water, and recover 28.5 µL into two PCR tubes.

PCR addition of indices  

**Timing 2 h**

68 Bring 180 µL of AMPure XP SPRI beads to RT (set aside until Step 71).
69 To each PCR tube with 28.5 µL of adaptor ligated DNA, add indexing reagents with index-specific primers to allow pooling with other samples of interest.

| Component          | Quantity |
|--------------------|----------|
| Adaptor Ligated library | 28.5 µL |
| NEB Universal primer | 5 µL     |
| NEB Index primer    | 5 µL     |
| Herculase II 5× buffer | 10 µL   |
| dNTP               | 0.5 µL   |
| Herculase II polymerase | 1 µL   |

70 Mix by pipetting, and amplify DNA using the settings below for a total of six cycles of amplification.

| Step | Temperature | Time |
|------|-------------|------|
| 1    | 98 °C       | 30 s |
| 2    | 98 °C       | 10 s |
| 3    | 65 °C       | 30 s |

Table continued
(continued)

| Step   | Temp | Time |
|--------|------|------|
| 4      | 72 °C| 30 s |
| 5      | Go to Step 2 |
| 6      | 72 °C| 5 min |
| 7      | 4 °C | Hold |

71 Combine PCR reactions, and perform an AMPure XP SPRI bead cleanup as described at Steps 53–59 using 180 µL of AMPure XP SPRI beads. Elute in 55 µL of PCR-grade water, and recover 53 µL into a new microcentrifuge tube.

72 Assess 1 µL of indexed material using D1000 TapeStation to ensure increase in fragment size (Fig. 3b).

73 Quantify 2 µL of indexed library using Qubit dsDNA BR assay kit.

**PAUSE POINT** Indexed 3C DNA can be stored at −20 °C for several years.

Capture enrichment

74 Perform oligonucleotide pull-down of target fragments using either single-stranded oligonucleotides (ssDNA probes, Steps 75–124) or double-stranded oligonucleotides (dsDNA probes, Steps 125–162).

Hybridization (ssDNA probes) ● Timing 4 d

**CRITICAL** Capture-C methods are highly adaptable for multiplexing any number of libraries of interest, and triplicates of each sample (e.g., cell type, genetic model, treatment, timepoint) are highly recommended. The instructions here are for a standard three-versus-three experiment that permits statistical comparison of interactions. For HyperCapture reagents (ssDNA probes), the maximum number of libraries per tube is 6. For larger designs, pool all libraries, then split equivalent amounts of DNA across multiple tubes and scale reaction volumes accordingly.

**CRITICAL** This protocol is a modified version of the Roche HyperCapture streptavidin pull-down protocol. Be aware of changes to composition of kit reagents and workflow.

75 Heat vacuum centrifuge to 40–50 °C.

76 In a PCR tube, combine 1–2 µg from each of six uniquely indexed samples (from Step 71) 1:1 by mass, then add 30 µg of species-specific Cot-1 DNA (30 µL of 1 mg/mL stock, 5 µL per library).

**CRITICAL STEP** Cot-1 DNA blocks repetitive elements and is species specific; when a species-specific product is not available, KAPA Hybrid Enhancer Reagent (Roche) can be used.

77 Desiccate in a vacuum centrifuge with tube lids open until sample is completely dry.

? TROUBLESHOOTING

78 To the DNA, add 40.2 µL of Universal Enhancing Oligonucleotides (6.7 µL per library), and mix by pipetting.

**CRITICAL STEP** DNA is at a very high concentration and may be sticky, so take care to eject all liquid from the pipette tip.

79 Add 84 µL hybridization buffer (14 µL per library) and 36 µL of Hybridization Component H (6 µL per library); mix carefully by pipetting and briefly centrifuge, then incubate at RT for 2 min.

80 Replace all buffers and blocking reagents in the freezer to avoid contamination with hybridization oligonucleotides.

81 Defrost oligonucleotide stocks (from Step 7); make at least 32 µL of working concentration oligonucleotides by diluting pools to at an optimal titrated concentration (see Box 2 and Fig. 4).

82 Add 27 µL of diluted oligonucleotides to hybridization mixture (4.5 µL per library), and mix carefully by pipetting. Briefly centrifuge to collect at bottom of tube. Store remaining oligonucleotides at −20 °C until used in double capture (Step 118).

83 Program a thermocycler to incubate at 95 °C for 5 min then hold at 47 °C indefinitely (lid 105 °C). Add hybridization mixture.

84 Label PCR machine to prevent it being inadvertently turned off, and incubate capture reaction at 47 °C for 18–22 h.

Streptavidin bead binding (ssDNA probes) ● Timing 2 h

85 Heat a thermostirrer to 47 °C.

86 Bring 300 µL M-270 streptavidin Dynabeads to RT (50 µL per library) in a low-affinity tube.

? TROUBLESHOOTING
Prepare wash buffers; unless stated, leave at RT until required:

| Buffer                             | Buffer volume | Water volume |
|------------------------------------|---------------|--------------|
| 2.5× Bead Wash buffer              | 600 µL        | 900 µL       |
| 10× Stringent Wash buffer          | 120 µL        | 1,080 µL     |
| 10× Wash buffer I                  | 93 µL         | 837 µL       |
| 10× Wash buffer II                 | 60 µL         | 540 µL       |
| 10× Wash buffer III                | 60 µL         | 540 µL       |

**CRITICAL STEP** If any precipitate is seen in concentrated wash buffers, heat to 37 °C and ensure complete resuspension prior to making 1× mixtures.

Place 1× Stringent Wash buffer at 47 °C.

Aliquot 330 µL of 1× Wash buffer I (55 µL per library) in a fresh tube, and place at 47 °C.

Place beads on a magnetic stand; remove liquid once clear (30 s).

Add 600 µL of 1× Bead Wash buffer (100 µL per library), and vortex to resuspend the beads; spin briefly. Replace on magnetic stand; remove liquid once clear (30 s).
92 Repeat Step 91 for a total of two washes.
93 Remove tube from the magnetic stand, and resuspend the beads in 300 µL of 1× Bead Wash buffer (50 µL per library).
94 Replace beads on magnetic stand.
95 Working quickly, remove Bead Wash buffer from streptavidin beads, transfer the entire ~185 µL hybridization reaction from Step 84 (31.2 µL per library) to the streptavidin beads and mix by pipetting.
96 Place on the 47 °C thermomixer (600 rpm), and incubate for 45 min to allow probes to bind to the beads.

▲ CRITICAL STEP The beads may settle out during hybridization; resuspend by pipetting after 5 min, but take care not to lose too many beads in the tip owing to their affinity for plastic.
97 Add 300 µL of heated 1× Wash buffer I (50 µL per library) to the bead-bound DNA, and mix by vortexing for 10 s.
98 Perform a quick spin, then place in magnetic stand and discard all the liquid when clear. Remove from magnetic stand, add 600 µL of heated 1× Stringent Wash buffer (100 µL per library) and mix by vortexing.
99 Incubate on a thermostoquer for 5 min at 47 °C (600 rpm), then briefly centrifuge to remove any liquid from lid.
100 Place in magnetic stand, and discard all the liquid when clear (30 s). Remove from magnetic stand, and perform a second stringent wash with 600 µL of heated 1× Stringent Wash buffer (100 µL per library).
101 Incubate on a thermostoquer for 5 min at 47 °C (600 rpm), then briefly centrifuge to remove any liquid from lid.
102 Place in magnetic stand, and discard all the liquid when clear (30 s). Remove from magnet, and add 600 µL of RT 1× Wash Buffer I (100 µL per library).
103 Mix by vortexing for 10 s, and briefly spin in benchtop microcentrifuge to remove any liquid from lid, then incubate at RT for 1 min.
104 Place in magnetic stand, and discard all the liquid when clear (30 s). Remove from magnet, and add 600 µL of RT 1× Wash Buffer II (100 µL per library).
105 Mix by vortexing for 10 s, and briefly spin in benchtop microcentrifuge to remove any liquid from lid, then incubate at RT for 1 min.
106 Place in magnetic stand, and discard all the liquid when clear (30 s). Remove from magnet, and add 600 µL of RT 1× Wash Buffer III (100 µL per library).
107 Mix by vortexing for 10 s, and briefly spin in benchtop microcentrifuge to remove any liquid from lid, then incubate at RT for 1 min.
108 Place in magnetic stand, and discard all the liquid when clear (30 s).
109 Remove from the magnetic stand, and resuspend beads in 240 µL PCR-grade water (40 µL per library).

■ PAUSE POINT DNA is not eluted but amplified off the beads; either store the bead-bound DNA at −20 °C or proceed to amplification.

PCR amplification (ssDNA probes) ● Timing 2 h
110 Bring 540 µL of AMPure XP beads to RT (90 µL per library), and set aside for Step 114.
111 To 120 µL of bead-bound DNA in water, add 150 µL of 2× KAPA HiFi HotStart ReadyMix (25 µL per library) and 30 µL of Post-Capture PCR Oligos (5 µL per library), and mix by pipetting.
112 Aliquot 50 µL of PCR mix into each of six PCR tubes, and perform PCR using the following settings with a total of 10–14 cycles of amplification.

| Step  | Temp  | Time |
|-------|-------|------|
| 1     | 98 °C | 45 s |
| 2     | 98 °C | 15 s |
| 3     | 60 °C | 30 s |
| 4     | 72 °C | 30 s |
| 5     | Go to Step 2 |
| 6     | 72 °C | 60 s |
| 7     | 4 °C  | Hold |

113 Pool six reactions in a microcentrifuge tube, and place on a magnetic stand.
When clear (30 s), transfer supernatant to a new microcentrifuge tube containing 540 µL of AMPure XP beads (90 µL per library) and perform bead cleanup as per Steps 53–59 using 540 µL of AMPure XP SPRI beads. Elute into 56 µL of PCR-grade water, recovering 53 µL.

(Optional) Confirm size of amplified DNA using a high-sensitivity D1000 TapeStation.

Use 2 µL of amplified material in a Qubit dsDNA HS assay kit to quantify the DNA.

**TROUBLESHOOTING**

Repeat amplification (Steps 112–114) on the remaining volume of DNA-bound streptavidin beads, and combine DNA from both amplifications.

**Double capture (ssDNA probes) ● Timing 2 d**

▲ CRITICAL When using optimally titrated probes, double capture increases the on-target sequencing efficiency by two- to threefold over single capture. The amount of DNA recovered after single capture is generally <2 µg, so capture is performed as described for a single library using all of the recovered material. For Tiled-C, the high density of probes leads to an extremely high efficiency enrichment, and a second capture is not required. If performing Tiled-C, proceed to ‘Sequencing and analysis’ (Step 163).

Some users have also found that single round of capture at 55 °C rather than 47 °C can provide high specificity; however, this has not been robustly tested.

Use up to 2 µg of DNA from Step 117 for double capture.

Perform hybridization (Steps 75–84) as described using volumes for a single library.

Perform streptavidin bead binding (Steps 85–109) as described using volumes for a single library and preparing buffers as below. Unless stated otherwise, leave at RT until required.

| Buffer                  | Buffer volume | Water volume |
|-------------------------|---------------|--------------|
| 2.5× Bead Wash buffer   | 100 µL        | 150 µL       |
| 10× Stringent Wash buffer | 20 µL       | 180 µL       |
| 10× Wash buffer I       | 16 µL         | 144 µL       |
| 10× Wash buffer II      | 10 µL         | 90 µL        |
| 10× Wash buffer III     | 10 µL         | 90 µL        |

Place 1× Stringent Wash buffer at 47 °C.

Aliquot 60 µL of 1× Wash buffer I in a fresh tube, and place at 47 °C.

Following the washes, resuspend the streptavidin beads in 20 µL PCR-grade water.

Perform a single PCR amplification (Steps 110–117) as described using volumes for a single library with the following adjustments. Elute DNA off beads in 26 µL of PCR-grade water, and recover 23 µL. Post-amplification size evaluation is not optional and should be performed with standard-sensitivity reagents. We recommend using a D1000 TapeStation. Perform DNA quantification with standard-sensitivity reagents. We recommend using 2 µL of library in the Qubit dsDNA BR assay kit.

▲ PAUSE POINT Captured DNA may be stored at −20 °C for several months until sequencing (Step 163).

**Hybridization (dsDNA probes) ● Timing 1.5 d**

▲ CRITICAL Capture-C methods are highly adaptable for multiplexing any number of libraries of interest, and triplicates of each sample (e.g., cell type, genetic model, treatment, timepoint) are highly recommended. The instructions here are for a standard three-versus-three experiment that permits statistical comparison of interactions. For Twist reagents (dsDNA probes), the maximum number of libraries per tube is 8. For larger designs, pool all libraries, then split equivalent amounts of DNA across multiple tubes and scale reaction volumes accordingly.

▲ CRITICAL This protocol is a modified version of the Twist target enrichment protocol. Be aware of changes to composition of kit reagents and workflow.

Heat a vacuum centrifuge to 40–50 °C.

In a PCR tube, combine 375–500 µg of six uniquely indexed 3C libraries (from Step 71) 1:1 by mass (1,500 µg from up to four libraries per reaction; two reactions can be performed in a single tube or split over two tubes).

▲ CRITICAL STEP Use high-quality PCR tubes to avoid loss through evaporation during hybridization.
Desiccate in a vacuum centrifuge at 40–50 °C with tube lids open until sample is completely dry. **PAUSE POINT** Dried DNA may be stored at −20 °C.

Thaw Hybridization Mix, Hybridization Enhancer, Blocker Solution and Universal Blockers on ice. Once reagents are thawed, vortex briefly to mix components and spin in a microcentrifuge. If precipitate is observed, heat buffers until it is dissolved.

Defrost oligonucleotide stocks (from Step 7); make at least 15 µL of working concentration oligonucleotides by diluting pools to an optimal titrated concentration (see Box 2, Fig. 4 and Table 2). If oligonucleotides are ordered from Twist, follow manufacturer’s recommendations. Store excess probes at −20 °C for double capture.

In a PCR tube, combine 40 µL Hybridization Mix (20 µL per reaction), 8 µL of capture oligonucleotide and 8 µL of PCR-grade water (4 µL of each per reaction), and mix by pipetting. **CRITICAL STEP** The hybridization buffer is very viscous, so pipette slowly to ensure accuracy.

Resuspend the dried indexed 3C libraries by adding 10 µL Blocker Solution, or species-specific Cot-1 DNA, (5 µL per reaction) and 14 µL Universal Blocker (7 µL per reaction), then carefully mix with a pipette.

Denature dsDNA capture Probe Mix by heating to 95 °C for 2 min in a thermocycler (lid 105 °C), then immediately cool on ice for 5 min.

While the Probe Mix is cooling on ice, heat the tube containing the resuspended indexed 3C library pool at 95 °C for 5 min in a thermal cycler with the lid at 105 °C.

Equilibrate both the probe solution and resuspended indexed 3C library pool to RT on the benchtop for 3 min.

Carefully mix the RT Probe Mix, transfer all 56 µL (28 µL per reaction) to the RT indexed 3C Library/Blocker Mix and mix carefully by pipetting.

Add 60 µL of Hybridization Enhancer (30 µL per reaction). Briefly centrifuge to ensure all solution is collected at the bottom of the PCR tube.

Incubate hybridization reaction at 70 °C for 16 h with lid at 85 °C.

**Streptavidin bead binding (dsDNA probes) ● Timing 2 h**

Heat a thermomixer to 48 °C. Bring 200 µL MyOne Streptavidin C1 Dynabeads to RT (100 µL per reaction) in a low-affinity tube.

**TROUBLESHOOTING**

Bring 1.6 mL Binding buffer (800 µL per reaction) and 400 µL Wash buffer 1 (200 µL per reaction) to RT, and heat 1.4 mL Wash buffer 2 to 48 °C (700 µL per reaction).

**CRITICAL STEP** If any precipitate is seen in Binding buffer, Wash buffer 1 or Wash buffer 2, heat to 48 °C until dissolved.

Add 400 µL of Binding buffer (200 µL per reaction) to streptavidin beads, and mix thoroughly by pipetting, place in a magnetic stand until clear (1 min), and discard the supernatant without disturbing beads.

Repeat Binding buffer wash of streptavidin beads (Step 140) two times for a total of three washes.

After the third and final wash, remove from stand and resuspend in 400 µL of Binding buffer (200 µL per reaction).

Remove hybridization reaction from 70 °C thermocycler, and quickly transfer all 140 µL (70 µL per reaction) to streptavidin beads in Binding buffer. Incubate at RT for 30 min with gentle mixing (on a mixer, shaker, rocker or rotator) to ensure solution stays homogenized.

Briefly centrifuge the binding reaction to collect the material at the bottom of the tube, and place on magnetic stand. When solution is clear (1 min), discard the entire supernatant without disturbing the pellet.

Remove from rack, add 400 µL of Wash buffer 1 (200 µL per reaction) and mix by pipetting.

Briefly centrifuge to collect the material at the bottom of the tube, and transfer the entire volume to a new tube. Place on magnetic stand, and when the solution is clear (1 min) discard the entire supernatant without disturbing the pellet.

Remove from rack, add 400 µL of 48 °C Wash buffer 2 (200 µL per reaction), mix by pipetting and incubate at 48 °C for 5 min.

Briefly centrifuge to collect the material to the bottom of tube, and place on magnetic stand. When solution is clear (1 min), discard the entire supernatant without disturbing the pellet.

Repeat heated wash buffer 2 wash (Steps 147–148) two times for a total of three washes.

After the third and final wash, collect residual buffer with a low-volume pipette. Proceed immediately to the next step, and do not allow the beads to dry.
151 Remove from the magnetic stand, and resuspend in 90 µL of PCR-grade water (45 µL per reaction). Store on ice in preparation for PCR amplification.

**PCR amplification (dsDNA probes) ● **Timing 2 h

152 Bring 360 µL of AMPure XP beads to RT (180 µL per reaction); set aside for Step 157.

153 Thaw KAPA HiFi HotStart ReadyMix and Amplification Primers on ice, and mix.

154 To the streptavidin-bead-bound DNA, add 100 µL of KAPA HiFi HotStart ReadyMix (50 µL per hybridization reaction) and 10 µL of Amplification Primers (5 µL hybridization reaction), and mix by pipetting.

155 Aliquot 50 µL of PCR mix into each of four PCR tubes (two per hybridization reaction), and perform PCR with a total of 10–14 cycles of amplification.

| Step   | Temperature | Time |
|--------|-------------|------|
| Step 1 | 98 °C       | 45 s |
| Step 2 | 98 °C       | 15 s |
| Step 3 | 60 °C       | 30 s |
| Step 4 | 72 °C       | 30 s |
| Step 5 | Go to Step 2|      |
| Step 6 | 72 °C       | 60 s |
| Step 7 | 4 °C        | Hold |

156 Pool four reactions in a microcentrifuge tube, and place on a magnetic stand.

157 When clear (30 s), transfer supernatant to a new microcentrifuge tube containing 360 µL of AMPure XP beads (180 µL per hybridization reaction) and perform bead cleanup as per Steps 53–59 using 360 µL of AMPure XP SPRI beads. Elute into 56 µL of PCR-grade water, and recover 53 µL.

158 (Optional) Confirm size of amplified DNA using a high-sensitivity D1000 TapeStation.

159 Use 2 µL of amplified material in a Qubit dsDNA HS assay kit to quantify the DNA.

**TROUBLESHOOTING**

**Double capture (dsDNA probes) ● **Timing 2 d

▲ CRITICAL When using optimally titrated probes, double capture increases the on-target sequencing efficiency by two- to three-fold over single capture. The amount of DNA recovered after single capture is generally <2 µg, so capture is performed as described for a single library using all of the recovered material. For Tiled-C, the high density of probes leads to an extremely high efficiency enrichment and a second capture is not required. If performing Tiled-C, proceed to ‘Sequencing and analysis’ (Step 163).

160 Perform hybridization (Steps 125–137) as described using volumes for a single reaction.

161 Perform streptavidin bead binding (Steps 138–151) as described using volumes for a single reaction.

162 Perform PCR amplification (Steps 152–159) as described using volumes for a single reaction.

▲ PAUSE POINT Captured DNA may be stored at −20 °C for several months until sequencing (Step 163)

**Sequencing and analysis**

**Sequencing ● **Timing 2 d

163 Using the measured DNA concentration, make a 10 nM dilution of amplified captured DNA from Step 124 or 162.

164 Perform accurate library quantification of the 10 nM dilution using quantitative PCR with size correction. We recommend using KAPA Library Quantification Kit with 1:10,000 and 1:20,000 dilutions.

165 Dilute DNA to appropriate concentration for sequencing (generally 4 nM), and sequence with paired-end reads. Libraries should be sequenced to a depth of 1–5 × 10^5 reads per viewpoint per sample for NuTi Capture-C, 1–10 × 10^6 reads per viewpoint per sample for Tri-C, and 3–5 × 10^6 reads per Mb per sample for Tiled-C, which is sufficient for 5 kb resolution.

▲ CRITICAL STEP Using long reads (150 bp) allows the reconstruction of sequencing fragments. From these fragments, it is possible to detect restriction digestion sites and in silico digest the chimeric reads generated by 3C. This step is essential for Tri-C experiments where multiway interactions are detected, but not for Tiled-C and NuTi Capture-C where using shorter reads (40–75 bp) can reduce sequencing costs.
CapCruncher analysis ● Timing ~1 d (will vary depending on viewpoint number and sequencing depth)

▲ CRITICAL In this section, we provide a step-by-step description of how to use the CapCruncher pipeline using triplicate many-versus-all capture of the HBA1, HBA2, HBB, HBD, MYC and SLC25A37 genes in human erythroid and ES cells (GSE129378). Installation (Step 166) needs only be implemented once. In this walk-through, we assume that a Conda environment on a Linux operating system is in use. Full descriptions for using the software can be found on the GitHub page (https://github.com/sims-lab/CapCruncher/). Modifications may be required in the commands below when using different operating systems. Key differences for analyzing Tiled-C and Tri-C data are highlighted; please refer to the software manuals and relevant GitHub pages for full documentation. Commands starting with ‘>’ are executed in the command line.

166 Install CapCruncher using Bioconda.

> conda create -n cc capcruncher

167 If appropriate, prepare sequence fastq files by concatenating multiple lanes and then compress using gzip.

> zcat hESC_rep1_L001_R1.fastq.gz hESC_L002_R1.fastq.gz | gzip > hESC_rep1_R1.fastq.gz

168 Make a directory where the analysis will be carried out.

> mkdir captureC_experiment1
> cd captureC_experiment1

169 Copy or generate symbolic links for all samples for analysis.

> cp /path/to/fastq/hESC_rep1_R1.fastq.gz
OR
> ln -s /full/path/to/fastq/file/hESC_rep1_R1.fastq.gz

170 Prepare a tab-separated four-column bed file of viewpoints (viewpoints.hg38.bed, Supplementary Data 1) with chromosome, fragment start, fragment stop and viewpoint name. When analyzing Tiled-C data, provide the start and end coordinates for the targeted region.

> nano viewpoints.hg38.bed
chr16 226254 227156 HBA1
chr16 222450 223352 HBA2
chr8  128748253 128748439 MYC
chr8  23385780  23386686 SLC25A37
chr11 5247977 5248607 HBB
chr11 5255391  5256556 HBD

171 Prepare a config file (config.yml, Supplementary Data 1) specifying mapping genome, restriction enzyme, path to viewpoints file, and public file folder. Analysis method should be specified as ‘capture’. When analyzing Tiled-C data, use ‘tiled’, and for Tri-C data use ‘tri’.

> wget https://raw.githubusercontent.com/sims-lab/CapCruncher/master/config.yml
> nano config.yml

172 Run the pipeline.

> conda activate cc
> capcruncher pipeline make

*TROUBLESHOOTING*
Combine the http server URL with the public path specified in config.yml (e.g., http://userweb.molbiol.ox.ac.uk/datashare/project/fgeneomics/publications/Downes_2022_NuTi_Protocol/Downes_2022_NuTi_Protocol.hub.txt), and load this into the UCSC Genome Browser track hub ‘My hubs’ tab.

In the ‘My hubs’ tab, click on the hub description to visualize analysis statistics or go to Genome Browser to view data.

Troubleshooting

Troubleshooting advice can be found in Table 3.

| Step          | Problem                                      | Possible reason                                      | Solution                                                                 |
|---------------|----------------------------------------------|------------------------------------------------------|--------------------------------------------------------------------------|
| Reagent setup | Excess lysis buffer                          | Small number of samples                              | To make smaller volumes of lysis buffer, one Complete Protease Inhibitor Cocktail tablet can be dissolved in 2 mL of PCR-grade water to generate a 25× stock. This can be aliquoted and stored at −20 °C for several months |
| 9             | Fewer than 5 × 10⁶ cells                     | Working with a rare cell population or limited number of cells following cell sort | For fixation, PBS wash and lysis, the volumes can be scaled down to accommodate fewer cells (down to 2 × 10⁶ cells). Maintain cells at -1 × 10⁶ cells per 1 mL of growth medium except for ≤1 × 10⁶ cells where 1 mL of medium should be used and fixation and lysis performed in a 1.5 mL tube. Perform digestion reactions in 200 µL for between 2 × 10⁴ and 5 × 10⁵ cells |
| More than 5 × 10⁶ cells | Working with a cell line            |                                                      | For fixation, PBS wash and lysis, the volumes can be scaled up to accommodate more cells. Maintain cells at -1 × 10⁶ cells per 1 mL of growth medium. For greater numbers of cells, perform multiple, parallel digestions and combine material in 300 µL of TE buffer after nuclear isolation |
| 37            | Phenol use is undesirable or prohibited      | Phenol is a dangerous chemical                       | Use of column extraction is possible and is considerably faster, e.g., Qiagen DNeasy Mini Kit can used from the point where nuclei are pelleted, Step 32. However, also pellet Control 1 and Control 2, then increase Proteinase K treatment to 4 h at 65 °C, and elute the DNA from the columns using the volumes outlined at Step 45, before preceding to quality control |
| 46            | DNA not visible in agarose gel               | Low amount of DNA because of low cell constraints    | Run 1 µL of each sample on a genomic DNA ScreenTape                      |
| 47            | Low DNA yield                                | Loss of nuclear pellet                               | The nuclear pellet can be hard to see and may be disturbed accidentally. If suffering low DNA yields, retain the supernatant and perform PCI DNA extraction. A good Nuclear 3C library should have >90% of DNA within the nuclear pellet. The combined DNA from the nuclear pellet and the supernatant is equivalent to an in situ 3C library |
|               |                                              | Incomplete de-crosslinking                           | Perform de-crosslinking overnight                                       |
|               |                                              | Incomplete precipitation                             | Freezing at −80 °C overnight may be beneficial for DNA yield, particularly for low-input samples |
| 48            | No control DNA                               | Working with low cell numbers                        | For low-input samples (≤150,000 cells), where very little DNA is available for controls, digestion efficiency can be directly calculated from re-ligated 3C libraries against a genomic DNA input control. Note that, due to re-ligation into the original fragment configuration, lower values for digestion will be observed than for a true digestion control |
| 49            | Low digestion efficiency                     | Short digestion period or suboptimal enzyme activity  | The total digest time should be 20–24 h. Additional restriction enzyme can be added at each of the three timepoints (Steps 21–23) for cells generating low digestion efficiency |
|               | Non-exponential amplification                | Reaction conditions for primers not optimized to thermocycler | Perform a dilution series analysis with genomic DNA, and include a melt curve to ensure no primer dimers are being produced |
| 61            | DNA not at correct size                      | Sonicator settings not optimized                     | Each sonicator may vary and should be set accordingly. Settings for sonication should be first determined by testing with high molecular weight genomic DNA rather than wasting 3C library. It is important to take into account the mass of DNA being sheared |
| 77            | Vacuum centrifuge is not available           | Specific equipment may not be available              | DNA may be purified by AMPure XP SPRI bead cleanup (e.g., Steps 53–59) with elution into 40.2 µL of Universal Enhancing Oligonucleotides (6.7 µL per library) |

Table continued
Timing

**Viewpoint preparation**
Steps 1–5, oligonucleotide probe design: 3 h
Steps 6–7, oligonucleotide stock preparation: 1 h

**3C library generation**
Days 1–5
Steps 8–15, formaldehyde fixation: 3 h
Steps 16–23, digestion: 24 h
Steps 24–32, ligation: 24 h
Steps 33–45, DNA extraction: 24 h
Steps 46–49, quality control: 3 h

**Library indexing**
Day 6
Steps 50–61, sonication: 2 h
Steps 62–67, end prep and adaptor ligation: 3 h
Steps 68–73, PCR addition of indices: 2 h

**Capture enrichment—ssDNA probes**
Days 7–12
Steps 75–84, hybridization: 4 d
Steps 85–109, streptavidin bead binding: 2 h
Steps 110–117, PCR amplification: 2 h
Steps 118–124, double capture: 2 d

**Capture enrichment—dsDNA probes**
Days 7–12
Steps 125–137, hybridization: 1.5 d
Steps 138–151, streptavidin bead binding: 2 h

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| 86   | Beads stick to plastic | High affinity of streptavidin beads for plastic tubes | Streptavidin Dynabeads tend to stick to plastics. We find this effect is minimized by using high-quality, non-sticky tubes, from Sorenson BioScience (39640T) |
| 116  | Loss of DNA after capture | Failed PCR reaction, user error during DNA bead cleanup | Captured material is amplified off the beads in two batches. Although these reactions can be performed simultaneously, it is prudent to do each individually to protect against error or misfortune and to ensure adequate amplification has occurred |
| 138  | Beads stick to plastic | High affinity of streptavidin beads for plastic tubes | Streptavidin beads tend to stick to plastics. We find this effect is minimized by using high-quality, non-sticky tubes, from Sorenson BioScience (39640T) |
| 159  | Loss of DNA after capture | Failed PCR reaction, user error during DNA bead cleanup | Captured material is amplified off the beads in four PCR reactions (two per hybridization reaction). Here, these reactions are performed simultaneously, although it is possible to do these in two batches to protect against error or misfortune and to determine if adequate amplification has occurred |
| 172  | Tiled-C matrix not generated | Using coordinates for a single viewpoint not a region | Change the bed file coordinates to match the Tiled-C targeted region including the start of the first targeted fragment and the end of the and last targeted fragment |
| 172  | Interaction matrix not generated | Using Capture-C configuration settings | Set analysis method in config.xml to either ‘tiled’ for Tiled-C or ‘tri’ for Tri-C |
Steps 152–159, PCR amplification: 2 h  
Steps 160–162, double capture: 2 d

**Sequencing and analysis**  
**Day 13–16**  
Steps 163–165, sequencing: 2 d  
Steps 166–174, CapCruncher processing: 2–48 h

**Anticipated results**

A successful NuTi Capture-C profile will provide a near-continuous distribution of reported interactions around the central viewpoint, with >20,000 unique cis-interaction events (Fig. 5a). When comparing cell types or genetic models, a CapCruncher run will also generate comparison tracks for identification of tissue-specific interactions. These interaction profiles can be further processed with a range of tools to identify statistically significant interactions.

**Quality control**

The CapCruncher output provides comprehensive quality control metrics as an html webpage to allow users to judge the success, or shortcomings, of a given experiment or 3C library. This report includes fastq PCR duplicate content, adapter trimming, fast length alignment of short reads (FLASh) percentage, in silico digestion statistics, alignment statistics and capture statistics (Fig. 5). Generally, Capture-C libraries are deeply sequenced to ensure maximum detection of all possible ligation events. Deep sequencing results in a high number of duplicate reads, generally 25–50%, although if very deeply sequenced up to 90% may be observed. Sequencing is preferably, although not essentially, carried out with long reads to facilitate FLAShing for identification of restriction enzyme cut sites, with 150 bp paired-end reads generating ~90% flashed reads, of which ~70% will contain DpnII sites, although this will vary with different sonication conditions and oligonucleotide probe length.

The key metrics of a Capture-C experiment are the alignment filtering statistics, where capture efficiency and reporter content are measured (Fig. 5b). Titration of capture oligonucleotides will result in 80–98% of mapped reads containing a target capture fragment. Lower percentages may...
Data availability
Example results were generated by analyzing GSE129378 (ref. 10).

Code availability
CapCruncher can be used following direct installation from Bioconda or accessed via GitHub91 (https://github.com/sims-lab/CapCruncher/releases).

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Author contributions

J.O.J.D. and J.R.H. designed the original protocol. D.J.D., M.A.K., T.V. and A.M.O. performed optimization experiments and developed the protocol. D.J.D., A.L.S., J.O.J.D., K.R., D.S. and A.M.O. designed and created the data analysis scripts. D.S., T.A.M., A.M.O. and J.R.H. acquired funding and oversaw the work. D.J.D. and A.M.O. wrote the manuscript and generated the figures. All authors critically evaluated and edited the manuscript.

Competing interests

J.R.H. and J.O.J.D. are founders and shareholders of Nucleome Therapeutics. J.R.H., J.O.J.D. and D.J.D. are paid consultants for Nucleome Therapeutics. J.R.H. and J.O.J.D. hold patents for Capture-C (WO2017068379A1, EP3365464B1, US10934578B2) and have a patent application for MCC. T.A.M. is a founding shareholder of OxStem Oncology (a subsidiary company of OxStem Ltd.) and a founding shareholder and paid consultant for Sandymount Therapeutics (a subsidiary company of Dark Blue Therapeutics). The other authors have no competing interests.

Additional information

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