



Novel orthogonal methods to uncover the complexity and diversity of nuclear architecture

Sjoerd JD Tjalsma and Wouter de Laat

Recent years have seen a vast expansion of knowledge on three-dimensional (3D) genome organization. The majority of studies on chromosome topology consists of pairwise interaction data of bulk populations of cells and therefore conceals heterogenic and more complex folding patterns. Here, we discuss novel methodologies to study the variation in genome topologies between different cells and techniques that allow analysis of complex, multi-way interactions. These technologies will aid the interpretation of genome-wide chromosome conformation data and provide strategies to further dissect the interplay between genome architecture and transcription regulation.

Address

Oncode Institute, Hubrecht Institute-KNAW and University Medical Center Utrecht, 3584 CT Utrecht, The Netherlands

Corresponding author: de Laat, Wouter (w.delaat@hubrecht.eu)

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Introduction

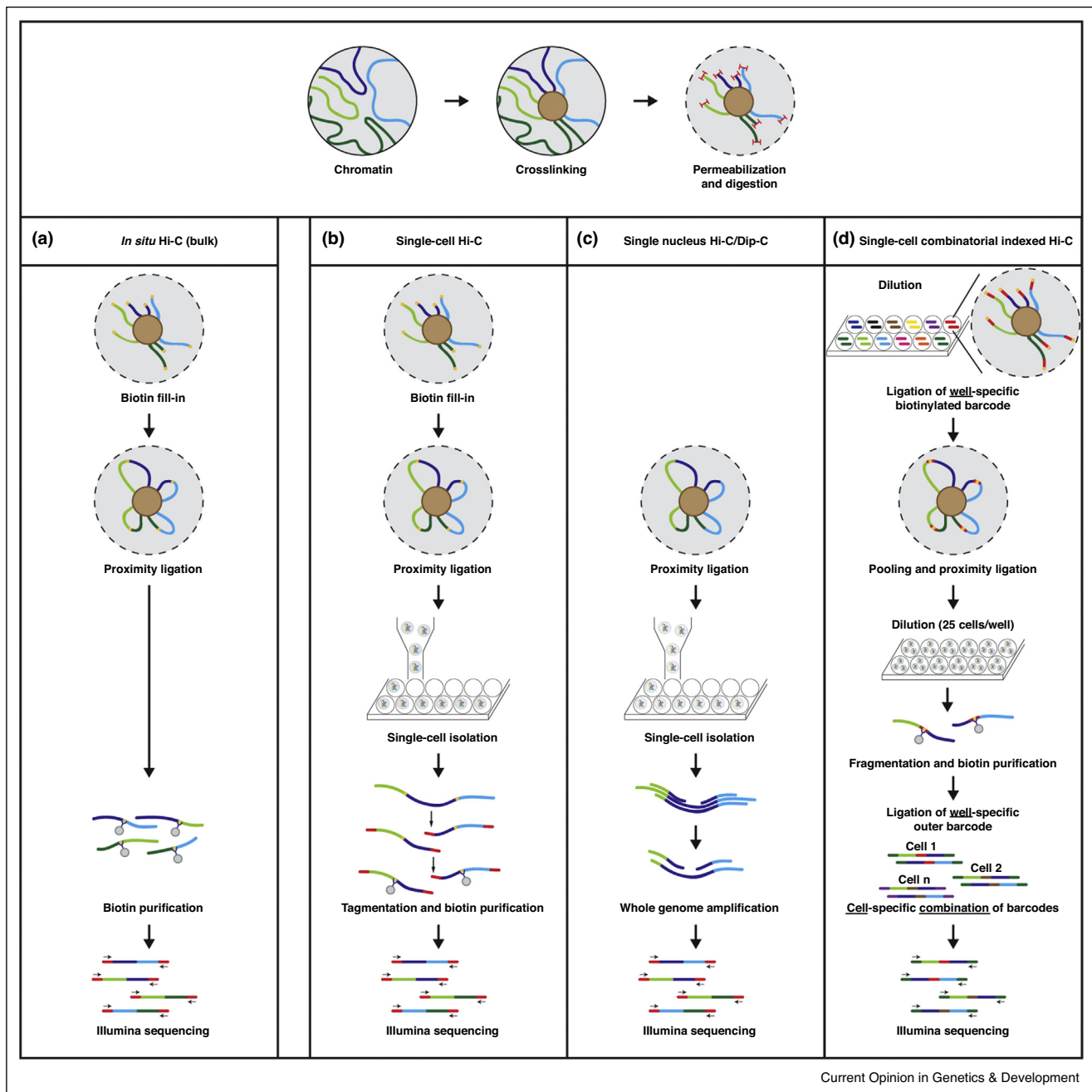
The structural organization of the genome influences transcription by providing a framework for communication between regulatory sequences and genes that are linearly separated in the genome [1]. Research on the structure-function relationship of chromosomes particularly benefitted from the development of the original chromosome conformation capture (3C) method [2] and its next-generation sequencing-based variants that enabled more systematic and higher resolution analysis of chromatin contacts across entire genomes [3]. 3C methodologies rely on the pairwise analysis of proximity ligated chromatin fragments. In these technologies, after crosslinking of nuclei with formaldehyde, chromatin is digested using a restriction enzyme. The ends of the resulting fragments are then ligated, and depending on the assay, the ligation junctions are analyzed in a targeted or genome-wide manner. The frequency of ligation of one

fragment to another fragment is then taken as a proxy for their pairwise contact frequency. Derivatives of 3C include 4C (one locus versus all others) [4,5], 5C (covering interactions within a selected region) [6,7], and Hi-C (high-throughput, genome-wide) [8–10] (Figure 1a). Numerous capture methods exist designed to enrich 3C libraries for targeted analysis of selected loci, including Capture-C [11,12] and capture Hi-C [13]. Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET) [14], Hi-ChIP [15], and PLAC-seq [16] are extensions of Hi-C which combine a chromatin immunoprecipitation (ChIP) step with proximity ligation, thereby enriching for contacts between genomic fragments bound by proteins such as transcription factors or architectural proteins. Although often proposed as cheaper, targeted, alternatives to Hi-C, it is important to realize that these methods introduce biases because protein occupancy and epitope availability differ between genomic sites. They are designed to find ligation products between sequences bound by the investigated protein, but do not allow assessing the significance of these interactions, nor investigating whether the interactions rely on the protein. In a different strategy, the CAPTURE system utilizes biotinylated dCas9 to pull down fragments of interest, thereby in parallel identifying interacting DNA elements and isolating proteins binding the locus [17,18]. The latter, distinguishing proteins bound to a given 1 kb locus from those associated with the rest of the genome, is a formidable task in human cells, given that the noise (rest of the genome) is in 3×10^6 excess to the signal (the locus under investigation) [19].

The rapid expansion of 3C-based technologies and their application to many biological systems has revealed important insights into the role of chromatin conformation on multiple scales. Hi-C and 5C datasets were critical in showing the organization of the genome in chromosome territories [9], A and B compartments (representing active and inactive chromatin, respectively) [9] and Topologically Associating Domains (TADs) [20–22]. More fine-scale interactions such as enhancer-promoter loops, first revealed by 3C and 4C studies [23], are corroborated by Hi-C: in many contexts, these interactions are observed [10,24,25].

Although numerous studies have now revealed principles of genome-wide organization at the chromosome and compartment level as well as more detailed topologies of locus-specific folding, limitations of classical 3C-based

Figure 1



Strategies for bulk and single-cell Hi-C.

Chromatin is crosslinked in the nucleus (*in situ*). After permeabilization of the nucleus and digestion using a restriction enzyme, chromatin is processed depending on the method. **(a)** *In situ* Hi-C [10]. **(b)** Single-cell Hi-C [28]. Tagmentation: combined fragmentation of chromatin fragments and adapter ligation. **(c)** Single-nucleus Hi-C [92] and Dip-C [32]. **(d)** Single-cell combinatorial indexed Hi-C [33].

assays are the population-based study of cells and the analysis of pairwise contacts. This conceals two crucial factors for the interpretation of the biological relevance of chromatin folding: the heterogeneity of 3D chromatin structure between different cells, and the complex folding patterns of individual loci. Data obtained from

3C-based methods represents contact frequencies from cells in a population and does not reveal the conformation in a specific cell, or high-resolution time scales of these interactions. Furthermore, pairwise data does not reveal whether higher-order structures exist and whether multiple sites compete for or cooperate in such structures. Still,

these issues are vital for understanding the functional implications of chromatin folding and therefore novel technologies have been developed to dissect the more complex nature of 3D chromatin structure.

An increasingly detailed view of genome-wide chromatin organization

In order to study heterogeneity in chromatin organization, multiple strategies for the generation of three-dimensional contact maps of individual cells have been described. The first single-cell Hi-C strategies rely on crosslinking and proximity ligation of chromatin in bulk, followed by isolation of single cells and *in situ* Hi-C in individual reactions [26–28] (Figure 1b), or alternatively perform the entire procedure on isolated nuclei [29]. To increase resolution, single-nucleus Hi-C [30,31] and Dip-C [32[•]] employ whole genome amplification to prevent loss of material (Figure 1c), which allows high-resolution three-dimensional reconstruction of haplotype-phased human genomes [32[•]]. In a different strategy, single-cell combinatorial indexed Hi-C (sci-Hi-C) circumvents the need to physically isolate individual cells by barcoding individual cells in consecutive dilution steps, allowing very high-throughput generation of single-cell Hi-C libraries [33,34] (Figure 1d).

With single-cell Hi-C, several principles of chromatin folding were further refined. Confirming bulk Hi-C studies, the genome was found to be organized into chromosome territories in single cells [26,30], and active chromatin domains colocalize to the interfaces of these territories [26]. Previously observed in cell populations purified by cell cycle phase [35], phasing of single-cell chromatin structures revealed massive rewiring of chromatin architecture during the cell cycle [27], a phenomenon which is often underappreciated in bulk 3C assays. Intriguingly, various levels of chromatin folding, including compartmentalization, insulation, and looping, display distinct dynamics during the cell cycle, suggesting these processes rely on independent mechanisms [27]. From a biological perspective, the use of single-cell Hi-C also opens the way for the study of low-cell systems, such as early mammalian embryos, and can reveal features that were previously concealed in bulk Hi-C strategies [30,36,37].

The benefits of single-cell conformational information come at a cost: because of low efficiency in ligation and purification of already limited amount of material, single-cell Hi-C maps contain a lower density of chromatin contacts than comparable population-based assays. Topological variation observed between cells may therefore not only have a biological (e.g. cell cycle stage differences) but also a technical cause. Therefore, it is required to compare single-cell Hi-C maps to structures inferred from bulk Hi-C. For TADs, this indeed shows an enrichment for intradomain versus interdomain interactions in

single cells [26]. Still, many interactions occur across TAD borders, suggesting that within single cells, both intra-TAD interactions as well as TAD borders itself are highly dynamic structures and do not form fixed entities and loops in the DNA [26,30]. This suggests that TADs are properties that emerge in population Hi-C by the averaging of all possible contacts, which have a higher chance of existing within the TAD region [38–40].

Although single-cell Hi-C studies have revealed heterogeneity in chromatin folding in individual cells, due to the limited retrieval of contacts and resolution, it remains difficult to study complex folding patterns and quantify finer-scaled topologies. Recently, the development of Micro-C has increased resolution of chromosome conformation maps to the nucleosome scale, by replacing restriction enzyme digestion of crosslinked chromatin with MNase digestion [41,42[•],43[•]]. Indeed, Micro-C is more sensitive for ultra-fine scale interactions (up to 200 bp) than conventional Hi-C. These highly refined maps reveal a prevalence of enhancer-promoter interactions and gene promoter-associated stripes, which possibly result from dynamic processes associated with transcription and cohesin-mediated loop extrusion [44,45]. Although the Micro-C protocol has not yet been adapted for single cell analysis, and a higher theoretical resolution does not necessarily circumvent the technical problem of limited retrieval of contacts, this technology offers a high potential for further refining the global fine-scale picture of chromatin organization.

Beyond pair-wise interactions

3C assays, including single-cell Hi-C, use counting of pairwise contacts to quantify for each specific locus the contact frequency with other genomic fragments. In the cell nucleus, every genomic fragment is surrounded by many other fragments, but in pairwise 3C-based assays a given fragment in a given cell can only be detected to interact with one other fragment. This competitive nature of proximity ligation implies that results of 3C assays are relative: the ligation events of a given fragment reflect the competition for ligation between all fragments, with *in situ* physical distance as a strong, but not total, determinant [3]. Therefore, in a population data set, it cannot be concluded whether multiple interactions centered on a single genomic site are neutral with respect to each pairwise interaction, whether they are occurring in a cooperative hub, or whether they are present in a mutually exclusive manner in different cells.

In addition to analyses designed to extract sparse multi-way interactions from 4C-seq data [46] or Hi-C obtained using high-frequency cutting restriction enzymes [47], several technologies have been specifically developed to study such events. Chromosome-walks (C-walks) is based on dilution and shotgun sequencing of high-molecular weight products obtained after proximity ligation [48]

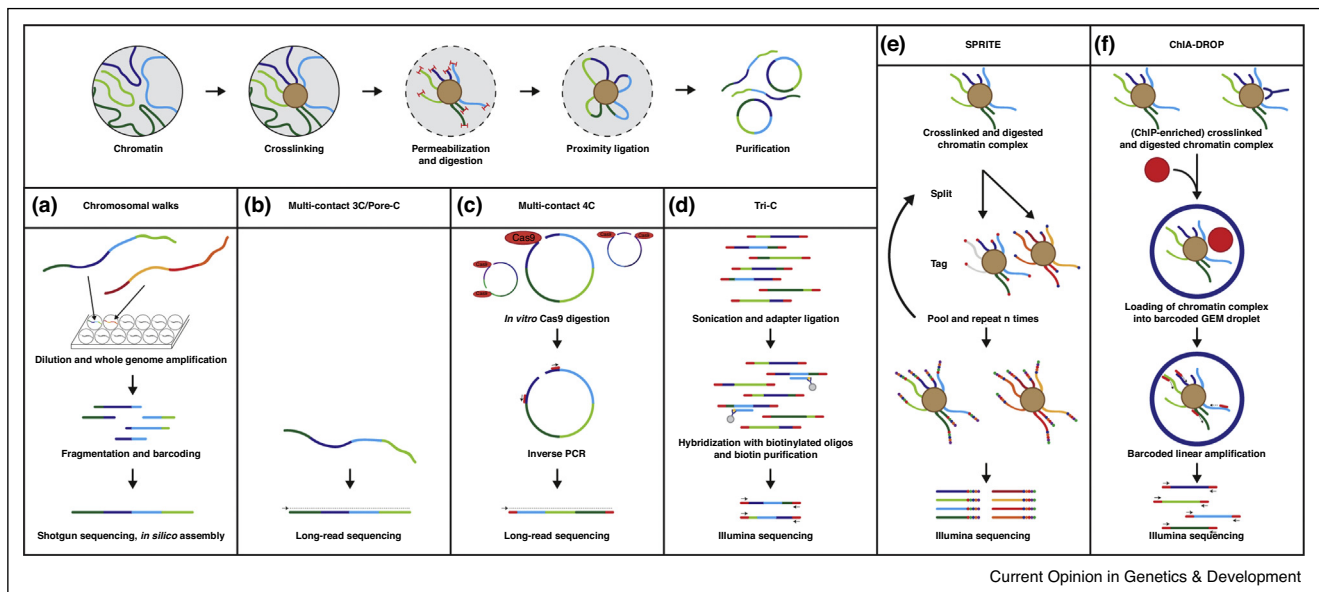
(Figure 2a). This strategy identified synergistic hub formation, but also suggested that many three-way interactions are neutral and predictable from pairwise contact frequencies. Other methods introduce long-read sequencing to map genome-wide multi-contact chromatin structure, by applying 3C-based template to PacBio sequencing (MC-3C) [49] or Nanopore sequencing (Pore-C) [50^{*}] (Figure 2c). Intriguingly, MC-3C revealed that the order of fragments of a ligation string is non-random and can be used to infer interaction surfaces of genomic domains, confirming that mixing of chromosome territory and compartment interfaces is relatively rare [49].

Although genome-wide multi-contact technologies give insights into complex higher-order chromatin topologies, these methods do not yield data of sufficient resolution to study functionally relevant structures at individual loci, such as enhancer-promoter interactions. Two locus-specific multi-way interaction mapping technologies are MC-4C, which applies Nanopore sequencing to viewpoint-specific PCR-amplified 4C template [51^{*},52] (Figure 2c), and Tri-C, which enriches for multi-contact ligations present in 3C concatemers using sonication and capture of viewpoints selected for having small DNA fragments [53^{*},54] (Figure 2d). While finding mutually exclusive contact pairs is relatively easy, distinguishing random from preferred 3-way interactions by these methods is not trivial and requires carefully defining pairwise background contact frequencies. Both approaches show

evidence for synergistic chromatin hubs containing multiple promoters or enhancers. As these methods provide single-allele topologies, they enable the study of topologies that occur in a low percentage of cells, which might be overlooked by population based 3C assays.

Several ligation-free methods have been developed which are also informative of multi-way chromatin interactions. SPRITE [55^{*}] and ChIA-DROP [56^{*}] are both based on barcoding of crosslinked chromatin complexes. For SPRITE, barcoding is established after crosslinking and fragmentation by repeated rounds of splitting of chromatin complexes, tagging with barcodes, and pooling [55^{*}] (Figure 2e). SPRITE identifies active and inactive gene hubs, which can be linked to nuclear speckles and the nucleolus, respectively. Refinements of the SPRITE protocol additionally enable mapping of RNA localization [57] or uncover single-cell multi-contact structural maps [58]. ChIA-DROP employs a microfluidics platform to load crosslinked chromatin complexes into unique barcode-containing droplets, finding high conformational heterogeneity within TADs [56^{*}] (Figure 2f). The addition of a ChIP step, similarly to ChIA-PET, makes ChIA-DROP a promising method to study transcription factor-based regulation of transcription-associated chromatin hubs. Finally, Genome Architecture Mapping (GAM), which is based on co-segregation of genomic loci in laser-dissected nuclear slices, also detects simultaneous interactions of more than two genomic loci, which are

Figure 2



Strategies for detection of multi-way chromatin interactions.

(a)–(d): After crosslinking of chromatin, permeabilization and restriction enzyme digestion, fragment ends are ligated. DNA is purified and further processed. (a) Chromosomal walks [48]. (b) MC-3C [49] and Pore-C [50^{*}]. (c) MC-4C [51^{*}]. (d) Tri-C [53^{*}]. (e) SPRITE [55^{*}]. (f) ChIA-DROP [56^{*}]. GEM: gel-bead-in-emulsion.

especially prevalent for highly active loci including strong enhancer (clusters) [59].

Visualizing single-allele topologies using microscopy

Orthogonal to sequencing studies, microscopy-based assays have the potential to bridge the gap between single-cell and high-resolution multi-loci interaction studies. Historically, microscopy studies have been highly important in revealing nuclear biology. For example, DNA FISH has been used extensively to study localization of loci in single cells and to validate 3C-based observations [21,60,61]. However, microscopy experiments typically allow analysis of a limited number of loci. Recently, high-throughput multiplexed sequential DNA FISH approaches have succeeded in observing the folding of stretches of chromatin at increasing resolutions [62,63,64*,65–67]. By applying this, genomic regions are covered at resolutions of up to two kilobases allowing detailed reconstruction of chromatin folding in thousands of single cells. For TADs, these methods provide evidence that cell-type specific TAD-like globular domain structures exist in single cells [63,66]. Additionally, refinements of sequential FISH approaches such as optical reconstruction of chromatin architecture (ORCA) [64*] and Hi-M [68] allow the tracing of 3D chromatin folding with simultaneous detection of nascent mRNA. With ORCA, enhancer-promoter interactions were shown to be predictive of gene activation, although interactions might be too heterogeneous to suggest stable loops, and rather point to a model in which enhancers scan topological domains to stochastically activate target genes [64*]. As these microscopy-based assays have recently been adapted for genome-scale analyses [69], these tools will be useful to probe the complex folding pattern of loci of interest in thousands of individual cells, thereby enabling the study of linking genome structure to transcriptional output in single cells.

Discussion

Recent years have shown the development of tools increasingly capable at dissecting the complex nature of chromatin conformation capture libraries. Both in single-cell genomic mapping and microscopy studies, it has been revealed that chromatin folding in single cells is more heterogeneous and dynamic than immediately appreciable from population-based analyses. Multi-contact chromatin conformation assays have started to shed light on chromatin interactions beyond pairwise contacts. Furthermore, ligation-free approaches have been developed and in general have shown high similarity to 3C-based methods [55*,59,70,71]. The introduction of Micro-C technologies leads to a further enhancement of resolution of genome-wide 3D chromatin structure [42*,43*].

Although it has now become clearer that 3D chromosome architecture is flexible in nature, to fully understand how

this intersects with gene expression, the next step will be to study the dynamics of these processes. Systematically combining technologies such as ORCA with high-resolution multi-contact chromatin conformation maps will shed light on how complex folding patterns are organized in single cells. Furthermore, the increased possibilities for live-cell imaging of genomic loci and associated transcription [72–79] will enable the assessment of the direct impact of interactions between architectural (CTCF-bound) sites on TAD formation, and between enhancer and promoter elements on transcription activation. The combination of these tools with the application of selected depletion of architectural and transcription-associated factors provides approaches to functionally validate and refine the picture of the direct impact of chromatin conformation on gene expression, such as used to study the role of the cohesin and mediator complexes [80–84]. Finally, it will be revealing to integrate the information 3D genome folding with other hallmarks of nuclear biology, such as the association of genomic domains with the nuclear lamina [85,86], speckles [87,88] and RNA [89], radial organization [90], and methylation [91]. Integration of these technologies will further enable the delineation of the impact of topological structures on nuclear biology and gene expression.

Conflict of interest statement

Sjoerd Tjalsma declares no conflict of interest. Wouter de Laat is founder and shareholder of Cergentis BV.

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